Selective permeabilisation of the blood-brain barrier during brain metastasis via TNFR1



Christina Simoglou Karali
New College
University of Oxford

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In the early 1950's, my great grandmother, a tiny and proud farmer, accompanied my grandfather on foot on his way to the school in order to encourage him to move on. This thesis is devoted to him, who constantly encouraged me to continue what he started more than 60 years ago.

This moment that I have been dreaming of for years, or even for moths has arrived. My DPhil thesis is ready for printing. This wouldn't have happened without the extreme support, patience, and passion for science of my supervisors Prof. Niki Sibson and Prof. Daniel Anthony. I am extremely grateful for the endless discussions, the motivation, but most of all for the life lessons I was given.

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Abstract

The presence of an intact, impermeable blood-brain barrier (BBB) during the early stages of metastatic colonisation significantly limits early detection and effective treatment of brain metastasis. Systemic tumour necrosis factor (TNF) administration transiently permeabilises the metastasis-associated vasculature, whist retaining the integrity of the surrounding the BBB. This approach enables both the enhanced detection of metastatic colonies with contrast-enhanced MRI and the improved delivery of therapeutically relevant molecules to the tumour site. The aim of this thesis was to unravel the mechanism underlying the TNF-induced BBB breakdown.

TNFR1, but not TNFR2, is upregulated on tumour-associated vasculature in experimental models, human brain metastasis resections and endothelial cells co-cultured with metastatic tumour cells (breast, lung or melanoma) or stimulated with relevant tumour cell conditioned media.

Treatment of an endothelial monolayer with either TNF or a TNR1-selective mutein increased permeability, which was eliminated in TNFR1-silenced endothelial cells stimulated with TNF. TNFR2 silencing did not alter TNF-induced endothelial permeability.

In vitro studies revealed that the blockade of the Ras/PI3K/Akt/mTOR signalling cascade, alongside components of the RhoGTPase family and NFκB pathway prevented the TNF-induced permeabilisation of the brain endothelium. On the contrary, targeting of the MAPK pathways had no effect on TNF-induced permeabilisation.

The results of this study suggest that TNF-induced BBB permeabilisation is TNFR1-mediated and involves activation of the Ras/PI3K, the Ras/ RhoGTPases and NFκB pathways. These findings support the concept that TNFR1-targeted agents may provide a sensitive and specific means to selectively and transiently modulate the BBB at sites of brain micrometastasis.

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List of abbreviations

231Br: MDA-MB-231Br
231Par: MDA-MB-231
4E-BP1: eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
ADAM: a disintegrin and metalloproteinase
ADAM17: metallopeptidase domain 17
ADF: actin depolymerising factor
ALCAM: activated leukocyte cell adhesion molecule
AJ: adherens junction
Ang-1/Tie2: angiopoietin-1/tyrosine kinase with immunoglobulin- and EGF-like domains-2
ANOVA: analysis of variance
Apaf-1: apoptotic protease-activating factor 1
BBB: blood-brain barrier
BCRP: breast cancer resistance protein
BCSFB: blood-cerebrospinal fluid cerebrospinal fluid barrier
BEC: brain endothelial cell
BTB: blood-tumour barrier
CAM: cellular adhesion molecule
cAMP: cyclic adenosine monophosphate
CCL2: chemokine (C-C motif) ligand 2
CD31: cluster of differentiation
CEC: cerebral endothelial cell
CNS: central nervous system
CSF: cerebrospinal fluid
ctDNA: circulating tumour DNA
CVO: circumventricular organ
CXCL5: C-X-C motif chemokine 5
DAB: 3,3'-diaminobenzidine
DAMP: damage-associated molecular pattern
DD: death domain
DEPTOR: DEP domain containing mTOR-interacting protein
DISC: death-inducing signaling complex
DMEM: Dulbecco's Modified Eagle's Medium
DUB: deubiquitylase
ECM: extracellular matrix
EDTA: ethylene diamine tetraacetic acid
EGFR: epidermal growth factor receptor
ERK: extracellular-signal-regulated kinase
EMT: epithelial-mesenchymal transition
eNOS: endothelial nitric-oxide synthase
ESAM: endothelial cell-selective adhesion molecule
FA: focal adhesion
F-actin: filamentous actin

FADD: Fas-associated death domain
FAK: focal adhesion kinase
FAN: factor-associated-with-nSMase
FasL: factor associated with suicide ligand
FBS: foetal bovine serum
FGFR: fibroblast growth factor receptor
FITC: fluorescein isothiocyanate
FKBP: FK506- and rapamycin-binding protein
FTI-II: farnesyltransferase inhibitor II
G-actin: globular actin
GAP: GTPase-activating protein
GDI: GDP dissociation inhibitor
GFP: green fluorescent protein
GJ: gap junction
GPCR: G-protein-coupled receptors
GSK-3β: glycogen synthase kinase-3
GEF: guanine nucleotide exchange factor
GFP: green fluorescent protein
GLUT-1: glucose transporter-1
HAEC: human aortic endothelial cell
HBEC: human brain endothelial cell
HBSS: Hanks' balanced salt solution
hCMEC/D3: human cerebral microvascular endothelial cells, clone D3
HDMEC: human dermal microvascular endothelial cell
HIMEC: human intestinal microvascular endothelial cell
HRP: horseradish peroxidase
huTNF: human TNF
HUVEC: human umbilical cord endothelial cell
IBD: inflammatory bowel disease
ICAM-1: intercellular adhesion molecule-1
IFNα: interferon-α
IκB: inhibitor of κB
IKK: IκB kinase
IKKβ: IκB kinase β
IGF: Insulin-like growth factor
ISF: interstitial fluid
JAM: junctional adhesion molecule
JNK: c-Jun NH2-terminal kinase/ Jun N-terminal kinase
KLC: kinesin light chain
L1CAM: L1 Cell Adhesion Molecule
LFA-1: lymphocyte function-associated antigen-1
LIMK: LIM kinase
LPS: lipopolysaccharide
Lt: lymphotoxin

LUBAC: linear ubiquitin chain assembly complex
MAPK: mitogen-activated protein kinase
MAPKK: MAPK kinase
MAPKKK: upstream MAPK kinase
MART1: Melanoma Antigen Recognized by T cells 1
MEKK: mitogen-activated protein kinase kinase
MelA: Melanoma Antigen
MKK: MAPK kinase
MLC: myosin light chain
MLCK: myosin light chain kinase
mLST8: lethal with sec-13 protein 8
MMP: matrix-metalloproteinase
MRI: magnetic resonance imaging
MRLC: myosin regulatory light chain
mSin1: mammalian stress-activated map kinase-interacting protein 1
MTD: maximum tolerated dose
mTNF: mouse TNF
mTOR: mechanistic Target of Rapamycin, formerly named mammalian TOR
mTORC1: mechanistic Target of Rapamycin complex 1
mTORC2: mammalian Target of Rapamycin complex 2
MYPT: myosin pohsphatase target subunit
NEAA: non-essential amino acids
NEMO: NF-κB essential modifier
NF- κB: nuclear factor κB
NGF: nerve growth factor
NIK: NF-κB-inducing kinase
NO: nitric oxide
ns: non-significant
NSCLC: non-small cell lung cancer
NVU: neurovascular unit
PAK: p21-activated kinase
PAM: PI3K/AKT/mTOR
PAMP: pathogen-associated molecular patterns
PBS: phosphate-buffered saline
PDGFR: platelet-derived growth factor receptor
PDK1: Phosphoinositide-dependent kinase-1
PD-L1: Programmed death-ligand 1
PECAM-1: platelet endothelial cell adhesion molecule
PFA: paraformaldehyde
PI3K: phosphoinositide 3-kinase-related kinase
PIKK: phosphatidylinositol 3-kinase-related kinase
PKB: protein kinase B
PIP3: phosphatidylinositol,3,4,5-triphosphate
PKB: protein kinase B

PMA: phorbol -myristate -acetate
PP2A: protein phosphatase 2A
PRAS40: proline-rich Akt substrate 40 kDa
P-Rex: phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1
PRK: protein kinase C-related kinase
PRR: pattern recognition receptor
Protor1/2: protein observed with rictor 1 and 2
PTEN: phosphatase and tensin homolog
R3- IGF-1: R3-insulin-like growth factor-1
RAC: Ras-related C3 botulimum toxin substrate
RacGEF: Rac-specific guanine nucleotide exchange factor
Raptor: regulatory-associated protein of mechanistic target of rapamycin
Rheb: Ras homolog enriched in brain
rhEGF: recombinant human epidermal growth factor
rhFGFF-b: recombinant human fibroblast growth factor-beta
RIPK: receptor interacting serine/threonine kinase
RTK: receptor tyrosine kinase
rt-PA: recombinant tissue-type plasminogen activator
Rictor: rapamycin-insensitive companion of mTOR
ROCK: Rho-associated coiled-coil-forming kinase
ROS: reactive oxygen species
RPL4: Ribosomal protein L4
RSK1: ribosomal S6 kinase
S6K1: S6 kinase 1
SCID: severe combined immunodeficiency
SEM: standard error of the mean
SMI: small-molecule inhibitor
SRS: stereotactic radiosurgery
STI: signal transduction inhibitor
SOS: Son-of-Sevenless
sTNF: soluble TNF
TAB: TAK1 binding protein
TACE: TNF-α -converting enzyme
TAK1: TGFβ- activated kinase 1
TAM: tumour-associated macrophages
TBI: traumatic brain injury
TBK1: TANK-binding kinase 1
TCM: tumour- conditioned media
TEER: transendothelial electrical resistance
TEM: transendothelial migration
TGFβ: transforming growth factor β
TJ: tight junction
TNF: tumour necrosis factor, formerly named TNF-α (Grimstad, 2016)
TNFR: tumour necrosis factor receptor

TNFRSF: tumour necrosis factor receptor superfamily
TRADD: TNF receptor associated via death domain
TRAF: tumour necrosis factor receptor- associated factor
TRAIL: TNF related apoptosis inducing ligand
TRAPS: TNFR1-associated periodic syndrome
TSC: tuberous sclerosis complex
TTF-1: Thyroid transcription factor 1
TWEAK: TNF-like weak inducer of apoptosis
VASP: vasodilator-stimulated phosphoprotein
VCAM-1: vascular cell adhesion molecule-1
VE: vascular endothelial
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
VLA-4: very late antigen-4
WBRT: whole-brain radiation therapy
ZO: zonula occludens

Chapter 1| Introduction

1.1 Cancer

Cancer is a class of diseases comprising more than 200 distinct disease entities that are characterised by genomic instability and mutability endowing aberrant cellular proliferative dynamic to neoplastic cells. This uncontrolled cell division leads to the formation of an abnormal mass of tissue, coined as neoplasm or tumour. Such malignancies can arise throughout the body, with the lung and colon cancer being most common amongst both sexes, and breast and prostate cancer being more common in women and men, respectively (Siegel et al., 2017).

The various types of cancer differ in the site of malignant transformations, the load of somatic mutations acquired, the transcriptional networks altered and the influences from the local tissue microenvironments. Two seminal reviews in 2000 and 2011 by Hanahan and Weinberg have attempted to distill this complexity by proposing that all neoplasms share some common denominators that drive tumourigenesis: genomic instability and mutability that resort to unlimited replicative potential, self-sufficiency in growth signals, reprogramming of energy metabolism, resistance to cell death, evasion from growth suppression, enhanced invasiveness and metastatic potential, induction of pro-angiogenic signalling, evasion from immunological destruction and enhanced response to tumour-promoting inflammation (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

The following thesis focuses on a strategy aiming to harness the innate immune response in the brain metastasis context.

1.2 Brain metastasis

Metastasis describes the dissociation of cancer cells from a primary site and their spread to distal organs. This process entails the local invasion of these cells into surrounding tissues, their dissemination through the bloodstream or the lymphatic system, their survival during the hematogenous transit, their subsequent arrest and extravasation through vascular walls into the parenchyma of secondary organs, the establishment of micrometastatic colonies, and the following propagation into overt, clinically detectable macrometastatic lesions. Patients with advanced secondary tumours confront a terminal illness that is largely incurable by the current treatment modalities. Approximately 90% of the cancer-associated deaths is related to metastatic disease (Gupta & Massague, 2006).

Brain metastasis involves the spread of systemic cancer to the brain and results in neurologic disability and death.

1.2.1 Clinical incidence

Brain metastasis is the most common intracranial tumour in adults, affecting 10-30% of cancer patients (Brastianos, Curry, & Oh, 2013; Louis et al., 2016). The

incidence of brain metastases ranges from 8.3-14.3 per 100.000, according to population-based studies (Nayak et al., 2012). Local epidemiological estimates suggest that 16.000 new cases are diagnosed annually in the UK (Cancer Research UK, 2016). Despite the improved intracranial and extracranial disease control, and the use of targeted agents in clinical practice, the central nervous system (CNS) remains a common site of progression. Moreover, the recent advances in brain imaging have facilitated the detection of asymptomatic brain metastases (de Jong et al., 2014; Heye et al., 2014). The accelerated detection of secondary brain tumour and/or the improved systemic therapies have contributed to the rising incidence of brain metastases. A 30-year follow-up study including more than 33.000 patients reported the development of metastasis at distal sites, including brain, for 60% of breast cancer patients (van den Hurk et al., 2011). Another study monitoring a cohort of more than 1.000 breast cancer patients demonstrated diagnosis of single brain metastasis for at least 15% of the participants (Steeg & Theodorescu, 2008). Moreover, the risk of admission with brain metastasis for cancer patients has increased dramatically by 44% for patients diagnosed with breast cancer, as documented in reports from the Swedish National Cancer registry comparing the records between 1998-2000 and 2004-2006 (Bachmann et al., 2015). This is also mirrored by retrospective studies illustrating a 4-fold increase in the cost of brain metastasis treatment (Guerin et al., 2016).

Parenchymal metastases are the most frequently encountered secondary brain tumours. Leptomeningeal metastases involving the spread of tumour cells within the subarachnoid space, occur in up to 10% of cancer patients and may arise from any

malignancy (Brower et al., 2016). The distribution of metastases is associated with the vascularisation and the tissue volume (Delattre et al., 1988; Lu et al., 2007), with 80% of metastatic deposits detected in cerebral hemispheres; 15% in the cerebellum; and 5% in the brainstem (Eichler et al., 2011). The incidence proportions of brain metastases vary by primary site, stage, and subtype of primary cancer. Approximately, 50-60% of lung cancer (Rangachari et al., 2015), 10-30% of breast cancer (Ramakrishna et al., 2014), 50% of melanoma, 5% of colorectal and 4% of renal cancer patients (Spagnolo et al., 2016) will present brain metastasis either at diagnosis or during their disease course (Brastianos, Cahill, & Brastianos, 2015; Cagney et al., 2017).

Upon diagnosis, brain metastasis patients face dismal prognosis associated with higher morbidity and mortality. Unfortunately, the median survival after diagnosis ranges from 3 to 27 months depending on disease entities (Berghoff & Preusser, 2018; Brastianos et al., 2013).

1.2.2 Diagnosis

The clinical gold-standard technique is contrast-enhanced magnetic resonance imaging (MRI). The detection is dependent on BBB breakdown, enabling the extravasation of contrast agents such as gadolinium, into the parenchyma. Novel imaging modalities (Heyn et al., 2006; Sundstrøm et al., 2013) targeting adhesion molecules that are upregulated within the brain endothelium soon after the metastatic colonisation enable the early detection of metastatic disease (Sibson et al., 2011; Serres

et al., 2012). Other widely used imaging modalities include computerised tomography (CT), or positron emission tomography (PET).

The initial diagnosis is often followed by tissue resection and histological assessment. As serial sampling of brain metastases is not advisable, longitudinal analysis of less-invasive molecular diagnostic platforms, such as serum sampling or the acquisition of liquid biopsies enabling the characterisation of cell-free DNA isolated from the cerebrospinal fluid (CSF) is considered as a promising supplementary approach to be incorporated into clinical practice (Pentsova et al., 2016). Recently, the diagnostic potential of CSF-derived circulating tumour DNA (ctDNA) has been explored in order to establish biomarkers of early stage metastatic progression (Siravegna et al., 2017). However, further prospective studies are required for the validation of these promising technologies (reviewed by Achrol et al., 2019).

Symptoms at the time of clinical manifestation include headache, seizures and neurological deficit (Zhang et al., 2012). These might occur due to brain tissue damage or displacement by the expanding malignancy, peritumoral oedema, vascular compromise and increased intracranial pressure.

1.2.3 Treatment

Current treatments are largely palliative in nature and the improved therapeutic approaches in place underscore an unmet clinical need. Local therapies aim to improve loco-regional control and include neurosurgical resection, stereotactic radiosurgery

(SRS) and whole-brain radiation therapy (WBRT) remain the mainstay treatments for symptomatic brain metastasis (Brastianos et al., 2013; Soffietti et al., 2017; Berghoff & Preusser, 2018).

The application of systemically successful chemotherapeutic compounds is limited due to the minimal BBB penetration and the consequent limited diffusion into the brain parenchyma (Conrad, 2001). However, even compounds with good brain diffusion characteristics present either heterogeneous drug concentration (Morikawa et al., 2015) or no clinically relevant therapeutic efficacy (Holohan et al., 2013; Zhu et al., 2014).

The combination of SRS with immunotherapy is an emerging treatment modality based on the notion that SRS leads to increased release of tumour antigens that could be targeted by tumour-infiltrating leukocytes (TIL), thus favourably altering the composition of the inflammatory microenvironment (Popp et al., 2016). Recently, the effects of immune checkpoint inhibitors targeting immunomodulatory molecules such as the Programmed death-ligand 1 (PD-L1), PD-L2 and cytokines present in brain metastases have been extensively studied and trialled (Wyler et al., 2014; Okada et al., 2015; Duchnowska et al., 2016). Although some of these immunotherapy approaches seem promising, their mechanism of action remains to be elucidated. Optimal management might include drug combinations targeting both the truncal alterations shared amongst all metastatic sites, and the CNS-specific alterations.

1.2.4 Prevention

Given the considerable difficulties associated with the treatment of established brain metastases, the development of preventive interventions holds great promise. Despite the demonstrated efficacy of prophylactic cranial irradiation in lung cancer patients, the neurocognitive side effects observed limit the potential for clinical application (Slotman et al., 2007). Moreover, the validation of predictive biomarkers indicative of brain-specific tumour cell–host interactions holds great promise (Valiente et al., 2018), since accumulating literature aims to decipher the gene specific signatures of brain metastases originating from different primary sites (Bos et al., 2009; Harrell et al., 2012; Brastianos et al., 2015; Hoshino et al., 2015; van der Weyden et al., 2017; Vareslija et al., 2018). Strategies focusing on the metastasis-initiating cells and the micrometastases at the perivascular niche have led to increased survival in preclinical models and could be beneficial in the clinical setting for the prevention of local relapse after neurosurgery (Winkler, 2015). Lastly, suppression of the angiogenic switch using anti-angiogenic drugs such as bevacizumab is currently trialled (Ilhan-Mutlu et al., 2016).

1.2.5 The invasion-metastasis cascade

Metastatic colonisation is an inherently inefficient, multistep process, where most of the circulating tumour cells will perish (Talmadge & Fidler, 2010; Labelle & Hynes, 2012). Evidence from experimental mouse models demonstrates that the majority of

the neoplastic cells that infiltrate the brain will die (Heyn et al., 2006; Kienast et al., 2010; Valiente et al., 2014).

The metastatic cascade initiates with the escape of cancer cells from the primary tumour site. The invasion of the tumour-surrounding stroma, functioning as a supportive cellular network, is promoted by cytoskeletal rearrangements and diminished adhesive forces between the tumour cells, and the secretion of matrix metalloproteinases (MMPs) and cathepsins that degrade the extracellular matrix (Perides et al., 2006; Mendes et al., 2007; Quail & Joyce, 2013). These steps are enabled by the epithelial-mesenchymal transition (EMT) program that involves the loss of epithelial properties and the acquisition of mesenchymal characteristics in their stead (Thiery, 2002). This reversible process enhances the motility and the invasiveness of the disseminating cells, alongside their ability to degrade components of the extracellular matrix (ECM) (Jeevan et al., 2016; Nieto et al., 2016). Neoplastic cells migrate either as single cells or in clusters (Friedl & Alexander, 2011), and upon intravasation into the circulatory system are exposed to shear forces, oxidative stress and the innate immune system. In order to overcome these obstacles during transit, they associate with platelets and undergo reversible metabolic changes (Labelle et al., 2011; Le Gal et al., 2015).

Metastasising cells reach the brain through the arterial blood supply (Preusser et al., 2012). The circulating tumour cells become mechanically entrapped within the capillary bed at vascular branch points, where the tortuous cerebral microvasculature limits the speed of blood flow and shear stress (Kienast et al., 2010). At this stage, the

tumour cells undergo cytoskeletal rearrangements and become elongated (Miles et al., 2008). This arrest step is considerably longer in the brain metastasis setting and might last for up to 5 days, whereas extravasation in other distant organs happens within 24 hours (Lorger & Felding-Habermann, 2010). This delay is also represented in studies employing the mouse 4T1-GFP breast carcinoma model, where brain metastases are very small at day 5 and exhibit rapid metastatic growth between days 5 and 10 (Serres et al., 2012b). In line with this, extravasation of lung cancer cells into the brain has been reported within approximately 48h (Paku et al., 2000), whereas melanoma cells might require up to 14 days to reach the brain parenchyma (Kienast et al., 2010).

The mechanisms underlying the traversal of metastatic tumour cells through the BBB are still unclear (Arshad et al., 2010; Hamilton & Sibson, 2013). The arrest of the tumour cells and their attachment to the endothelium is mediated by the interaction of surface receptors with adhesion molecules including integrins, selectins and chemokine receptors (Brayton et al., 1998; Carbonell et al., 2009; Chen et al., 2011; Lee et al. 2004; Yoshimasu et al., 2004; Salmaggi et al., 2009; Nguyen et al., 2009). This contact triggers the endothelial upregulation of a subset of cellular adhesion molecules (CAMs) including E-selectin, vascular cellular adhesion molecule-1 (VCAM-1) (Serres et al., 2012b), activated leukocyte cell adhesion molecule (ALCAM), intercellular adhesion molecule-1 (ICAM-1), very late antigen-4 (VLA-4) and β4 (Soto et al., 2014). Endothelial P-selectin levels are progressively increasing during metastatic colonisation, whereas other molecules, including E-selectin, the integrin β4,

ICAM-1 and lymphocyte function-associated antigen-1 (LFA-1), are overexpressed throughout tumour development.

Two routes, analogous to leukocyte trafficking, have been proposed for the migration of tumour cells through the brain endothelium: the paracellular and the transcellular. The first is supported by electron microscopy findings demonstrating the retraction of the endothelium upon the adhesion of cancer cells (Paku et al., 2000). Moreover, *in vitro* models of melanoma and breast cancer cell extravasation indicate the disruption of endothelial integrity upon anchorage of the tumour cells, as illustrated by transendothelial electrical resistance (TEER) measurements, dissasembly of endothelial tight junctions (TJs) and endothelial cell apoptosis induction (Fazakas et al., 2011; Molnar et al., 2016). In addition, metastatic cells "hijack" systemic immune cells via receptor-ligand interactions, thus facilitating paracellular extravasation (Jadhav et al., 2001). *In vitro* studies have proposed the transcellular extravasation of breast cancer cells through the endothelium, via a mechanism regulated by the activation of the endothelial non-muscular myosin light chain kinase (nm-MLCK) (Khuon et al., 2010). However, this hypothesis has been disproved *in vivo* (Wilhelm et al., 2013). Another scenario suggests the modification of the vascular wall by matrix-metalloproteinases (MMPs) expressed by arrested tumour cells (Mendes et al., 2005; Kienast et al., 2010; Lee et al., 2011; Rempe, Hartz, & Bauer, 2016).

The colonisation of the secondary organ is the most rate-limiting step of the metastatic cascade (Massague & Obenauf, 2016). Upon infiltration, the metastatic cells experience attrition (Chambers et al., 2002). The surviving metastatic cells remain

closely associated with the abluminal endothelial membrane and spread along the surrounding basal lamina (Eichler et al., 2011; Berghoff et al., 2013). This proliferative activity leads to the formation of a sheath that remodels the pre-existing vasculature, and is coined as vascular co-option (Carbonell et al., 2009). The establishment of a perivascular niche provides a source of nutrients, oxygen and access to angiocrine factors secreted by brain endothelial cells to the tumour cells (Rafii et al., 2016) and has been demonstrated in *in vivo* models of brain metastasis derived from breast cancer, lung cancer and melanoma. Upregulation of β1-integrin and L1CAM is required for vascular co-option (Loulier et al., 2009; Valiente et al., 2014). Furthermore, the LFA-1/ICAM-1 interactions play a crucial role in metastasis growth (Soto et al., 2016). The metastases expand in close association with the perivascular niche and spread into the brain parenchyma only upon breach of the glia limitans (macrometastatic stage) (Saito et al., 2007). Metastatic cells that bypass the BBB might enter a dormant state either as solitary dormant cells or us dormant micrometastasis. This phenomenon, explains the late presentation and/or relapse of asymptomatic minimal residual diseases observed in mammary adenocarcinomas, prostate and kidney cancers (Palmieri et al., 2007; Lambert et al., 2017). Inability of angiogenesis induction or active immune suppression might be underlying this latent, clinically inapparent phase of brain metastasis (Aguirre-Ghiso, 2007).

The freshly disseminated neoplastic cells are vulnerable to immune surveillance mechanisms involving the activation of astrocytes (O'Brien et al., 2014; Chen et al., 2016), endothelial cells (Serres et al., 2012; Soto et al., 2014), and microglial cells in

the brain (O'Brien et al., 2014; Andreou et al., 2017). Astrocytes release the serine protease plasminogen activator and reject the extravasated cancer cells by inducing Fas ligand (FasL)-mediated pro-apoptotic signalling. A mechanism of evasion for the brain metastatic cells from breast and lung adenocarcinomas involves the secretion of plasminogen activator inhibitors neuroserpin and serpin B2 (Valiente et al., 2014). Moreover, the assembly of carcinoma-astrocyte gap junctions leads to the inducible secretion of pro-inflammatory cytokines such as interferon-α (IFNα) and TNF by astrocytes, and the subsequent activation of pro-survival signalling that enhances tumour growth and confers chemoresistance (Chen et al., 2016).

Lastly, the metastatic cells might extravasate and seed either tumour-free secondary sites or reseed primary sites. Tumour self-seeding has been proposed as a mechanism of amplification for the most aggressive cancer-cell clones (Kim et al., 2009).

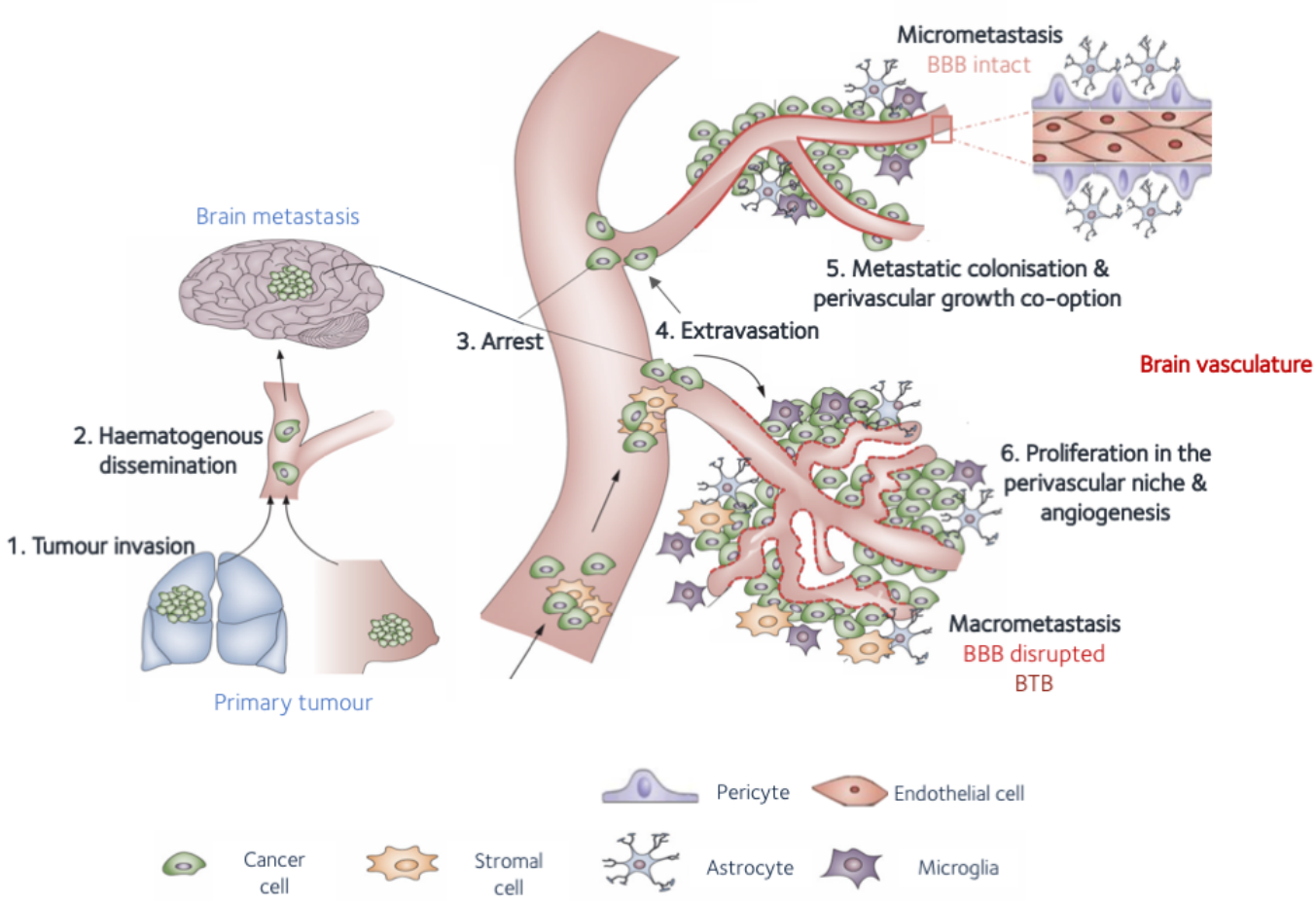


Fig.1 The metastatic cascade
Upon shedding from the primary site, the metastatic cells disseminate through the bloodstream or the lymphatic system, arrest in the capillary bed, extravasate across the BBB and enter the brain parenchyma. Upon metastatic colonisation, the tumour cells grow in a co-optive fashion within the perivascular niche. The BBB is intact during the early (micrometastatic) stage of tumour development and is disrupted once gross structural abnormalities have developed.
Modified from Eichler et al., 2011. Permission sought.

1.2.6 The role of the tumour microenvironment

The formation of solid brain metastases is believed to be relying on specific interactions between the disseminated metastatic cells and the microenvironment of the brain parenchyma. Different cancers demonstrate a predilection for metastatic spread in different organs. Stephen Paget hypothesised that the propensity of cancer

cells to form harbour metastases at distal organs is dependent on two factors: the cancer cell (the *"seed"*) and the microenvironment of the secondary tumour site (the *"soil"*) (Paget, 1889). The interpretation of this metaphor in the brain metastasis context implies that some distant soils are less lethal than others for the disseminating neoplastic cells. Metastasis initiating tumour cells are exposed to adverse microenvironmental conditions including low pH, hypoxia, nutrient deprivation and poor blood supply (Fidler, 2011). Moreover, increasing evidence supports that brain metastases arise due to secondary features of the tumour cells that favour the CNS (Rahmathulla, Toms, & Weil, 2012).

On a separate note, James Ewing proposed that the organo-tropism of metastases can be explained by the circulatory patterns between the primary tumour site and the secondary organs (Ewing, 1928). Although, this hypothesis is useful to explain the location of some metastatic deposits, it is failing to explain the incidence of secondary tumours to certain organs (Fidler, 2003). Recently, it has been proposed that the disseminating tumour cells bring *"their own soil"*: stromal compartments encompassing activated fibroblasts, from the primary tumour to the secondary site (Duda et al., 2010).

The CNS, by the virtue of its relative isolation compared to other metastatic sites, is not only an inhospitable site for the incoming cancer cells, but also acts as a sanctuary site upon metastatic colonisation, supporting the branched evolution of infiltrating tumour cells (Winkler, 2015). This pattern denotes the divergent evolution of multiple subclonal populations arising from the same ancestor and is associated both

with the genetic discordance between the primary tumour and the metastatic site, and with the even greater intermetastatic heterogeneity (Dagogo-Jack et al., 2016; De Mattos-Arruda et al., 2018).

1.3 The BBB

The existence of an interstitial barrier between blood and brain parenchyma was first suggested by the pioneering work of Paul Ehrlich in 1885, when he reported the distribution of water-soluble dyes into the blood, in almost all organs apart from the brain (Ehrlich, 1885). Additional work from Edwin Goldmann demonstrated that the exclusion of trypan blue from the CNS tissue was not attributable to the reduced binding affinity of the dye, but was associated with the presence of a barrier (Goldmann, 1909, 1913). Further experiments (Biedl, A., Kraus, 1898; Lewandowski, 1900; Stern & Gautier, 1921) defined the structure as the blood-brain barrier (BBB)- *"barrière hémato-encéphalique"* and confirmed that it arises during the embryonal development and acquires increased tightness until birth.

More than 40 years later, the seminal work of Reese, Karnovsky and Brightman led to the anatomical localisation of the BBB, employing electron microscopy, in order to describe the distribution of the electron-dense marker horseradish peroxidase (HRP). Their collective observations associated the previously demonstrated limited uptake of peroxidase by the brain tissue (Straus, 1958) with two specialised features of the BBB: the localisation at the level of the non-fenestrated, highly polarised brain microvascular

endothelial cells, and the presence of highly expressed interendothelial tight junctions (TJs) that physically restrict the HRP flux between the brain and the blood (Reese & Karnovsky, 1967; Brightman & Reese, 1969).

1.3.1 Anatomy of the CNS vasculature

The CNS is a highly vascularised system comprising three different segments of endothelial cells that are anatomically, morphologically, biochemically and functionally distinct. The cerebral microvasculature includes; i) arterioles (10-100μm diameter) that penetrate the cerebral parenchyma, ii) capillaries (4-10μm diameter) that constitute the largest surface area for the exchange between the blood and brain, and exhibit the most highly organised TJs, iii) the venules (10-100μm diameter) that constitute the main area of leukocyte infiltration, while possessing loosely arranged TJs (Cardoso, Brites, & Brito, 2010).

The CNS is envisaged as an immune-privileged site due to the presence of the BBB that is controlling and modulating the impact of peripheral immune events. The BBB inhibits the efferent arm of the immune system through the blockade of immune-cell entry into the CNS. On the contrary, the absence of conventional lymphatic vessels in the CNS parenchyma supported the concept that the afferent arm of the immune system is hindered (Engelhardt, Vajkoczy, & Weller, 2017). However, this dogma has been revolutionised by the discovery of a "glymphatic system", involving the limited lymphatic drainage localised in the meningeal surface that provides an exchange

system between the cerebrospinal fluid (CSF) and the interstitial fluid (ISF) (Iliff et al., 2012; Louveau et al., 2015; Asgari et al., 2016; Plog & Nedergaard, 2018).

The formation of BBB between the periphery and brain relies on multiple cellular and acellular layers that interact, form the barrier, and regulate its tightness. The overarching BBB structure is the highly specialised brain endothelial vasculature, although there are other interfaces considered as branches of the BBB such as the choroid plexus epithelial cells of the blood-CSF barrier (BCSFB) and the hypothalamic tanycytes that are placed in the ventricular boundaries of circumventricular organs (CVOs) (Sisó et al., 2010). Moreover, specialised vascular CNS barriers that have distinct characteristics from the brain parenchymal vessels support CNS homeostasis. These include the blood-retinal barrier, blood-nerve barrier, blood-labyrinth barriers, and the blood-spinal cold barrier (Neuwelt et al., 2008).

The vascular bed of the CNS is composed of highly specialized capillary endothelial cells lining a basement membrane formed by the fusion of the endothelial and the glia limitans basement membranes. Pericytes are embedded in the endothelial basement membrane. The glia limitans that is surrounding CNS microvessels is composed of the parenchymal basement membrane and astrocytic end-feet. At the capillary BBB, the two basement membranes fuse at the level of postcapillary venules thus leaving a perivascular space where the extravasation of immune cells into the CNS parenchyma occurs (Engelhardt et al., 2017).

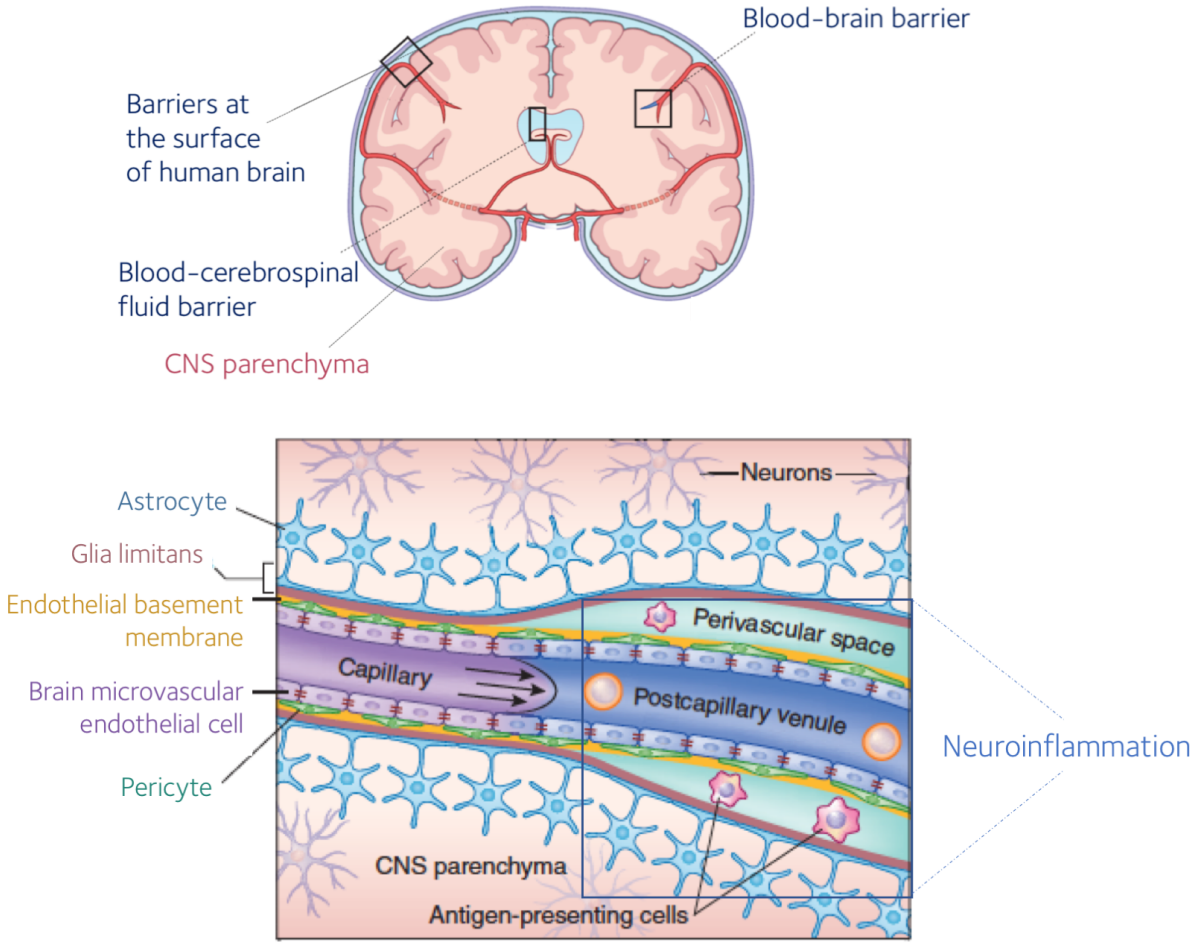


Fig.2 The blood-brain barrier

Modified from Engelhardt et al., 2017 . Permission sought.

1.3.2 Structure and components of the BBB

The capillary BBB that is situated at the interface between blood and brain parenchyma is the site of controlled transport of fluids and solutes into the CNS and restricts the bidirectional diffusion between the brain and the blood circulation.

The BBB is a dynamic interface of the brain, subject to coordinated and tightly regulated stimuli stemming from diverse cell types and signalling pathways. The barrier is formed by a tightly sealed monolayer of brain capillary endothelial cells, endowed with barrier properties, adjacent perivascular astrocytes and pericytes that

induce and maintain these characteristics, nerve terminals and perivascular microglia (Abbott et al., 2006; Weiss et al., 2009). This complex cellular structure has been also coined as neurovascular unit (NVU) due to the complexity of the coordinated responses required for the maintenance of the integrity of the BBB.

1.3.3 The neurovascular unit

The brain microvascular endothelial cells bear distinct molecular and cellular characteristics that differ from that in the periphery (Aird, 2003). They lack fenestrations but have few pinocytic vesicles and a high number of mitochondria, indicative of considerable metabolic activity. The brain inter-endothelial junctional complexes line and seal the brain and the spinal cord microvasculature, hence preserving CNS homeostasis. These complexes have a characteristic protein expression pattern and are comprised of tight, adherens and gap junctions. These highly elaborated structures serve as the primary determinants of the BBB restrictive angioarchitecture. They diminish paracellular diffusion of non-ionic hydrophilic and ionic molecules, and minimise vesicle trafficking, whilst reducing transcellular transport. On the contrary, the epithelial cells of the choroid plexus have vesicles residing at their apical membranes (Johanson et al., 2011).

Astrocytes are the most abundant cell type and have a key role in the induction and the preservation of the mature BBB phenotype (Abbott et al., 2006). They project their end-feet towards the brain microvascular endothelial cell and pericytes (Abbott, 2002; Hawkins & Davis, 2005), thereby promoting the phenotypic specialisation of the

brain endothelium. Reactive astrocytes exhibit a delayed pro-inflammatory phenotype, compared to that of microglia (Norden et al., 2016), thus affecting the stability of the BBB . Astrocytes secrete increased amounts of pro-inflammatory cytokines and chemokines in response to systemic inflammatory insults or inflammatory mediators (Bellaver et al., 2018; Norden et al., 2016). Astrocytic function can also result to the resolution of neuroinflammation (Vincent et al., 1997).

Pericytes wrap the abluminal capillary surface and provide physical support and stability to the BBB. They are directly contacting both the brain endothelial cells and the basement membrane. Pericytes are contributing to the neuroimmune response (Erickson & Banks, 2018) and they might be involved in the transport or pathogens into the brain or propagate neuroinflammation by activating both resident and recruited immune cells (Bechmann, Galea, & Perry, 2007). Pericytes contribute to the formation, maturation and maintenance of the microvasculature through the modification of endothelial gene expression, the inhibition of the endothelial cell proliferation. Moreover, pericytes confer structure and stability to the cerebrovascular endothelial cells, thus providing mechanical support to the brain endothelium (Lai & Kuo, 2005; Lindahl et al., 1997). In addition, pericytes might be enhancing BBB leakiness either by secreting factors that disrupt the endothelial monolayer or through physical disassociation with the endothelium (Hori et al., 2004; Nishioku et al., 2009; Daneman et al., 2010). Pericytes, also induce the polarisation of astrocytic end-feet towards the basement membrane (Armulik et al., 2010).

The brain is extensively vascularised, with the mean distance of a neuronal cell body from a capillary being approximately 15μm in mice (Tsai et al., 2009). Neurons induce vasodilation in response to glutamate release through their communication with astrocytes (Zonta et al., 2003). Furthermore, neurons contribute to the cytoarchitecture of the NVU through promotion of neurovascular density and branching during adulthood (Lacoste et al., 2014).

Microglia associate with the brain vasculature and promote angiogenesis during development (Arnold & Betsholtz, 2013). They modulate BBB integrity through the production of pro-inflammatory mediators including cytokines, chemokines, MMPs, prostaglandins, nitric oxide and reactive oxygen species (da Fonseca et al., 2014). After brain injury, they not only migrate rapidly at the sites affected and prevent the diffusion of neurotoxic solutes into the brain parenchyma (Roth et al., 2014), but also contribute to the resolution of inflammation (Cherry et al., 2014).

The basement membrane, also termed as basal lamina, is an acellular, negatively charged layer comprised of collagen type IV, laminins, fibronectin and perlecan. This layer is generated and maintained by brain endothelial cells and astrocytes and serves as an interface for the anchorage of endothelial cells and pericytes (Del Zoppo et al., 2006). The basement membrane provides considerable mechanical support to the BBB as its interactions with cell surface molecules on the cerebrovascular endothelium induces the expression of inter-endothelial tight junctions (TJs) (Owens et al., 2008).

The glycocalyx is a hydrated structure, of approximately 5μm thickness, that lines the luminal surface of all endothelial cells and constitutes a barrier to the passage of

proteins macromolecules across the endothelium (Singh et al., 2007). Glycocalyx functions as a mechanosensor that relays signals of shear stress to the endothelium (Tarbell, 2010). Dysruption of the glycocalyx during inflammatory conditions leads to the passage of solutes across the endothelial barrier, and the enhanced leukocyte adhesion to the endothelium (Varatharaj & Galea, 2017).

1.3.4 The function of the BBB

The BBB and its cellular components accomplish a plethora of pivotal tasks, thus preserving the normal function of the brain. The highly regulated BBB acts as a *physical barrier* providing stringent control of the influx of gases such as O_2, nutrients, water and electrolytes required for the maintenance of cells in the parenchyma. In addition, the BBB shields the brain from *ca.* 98 % of blood-borne components, and from the infiltration of immune cells and pathogens, thereby maintaining a CNS milieu that preserves essential brain functions, preventing the disruption of neuronal activity, and restoring homeostasis during disease and inflammation (Davies, 2002; Abbott et al., 2006).

Moreover, BBB functions as a *selective gatekeeper*. The high transendothelial electrical resistance (TEER), averaging over 8000 Ω/cm^2 in parenchymal vessels, confers exceptionally low ion permeabilities to the brain vasculature (Erickson & Banks, 2018). Small, lipophilic molecules with molecular mass below a threshold of 400-500 Da, traverse the BBB through a non-saturable mechanism, the passive transmembrane diffusion. Alternatively, the passage of solutes is assisted by solute

carrier proteins that permit the directional diffusion of aqueous molecules across the cell membranes. The transport of larger macromolecules can be facilitated by the receptor-mediated transcytosis, an energy-dependent, saturable mechanism that is relying on the activation of vesicular pathways. Moreover, efflux transporters at brain barriers facilitate the passage of solutes both in the brain-to-blood and in the CSF-to-blood direction. A subclass, the ABC transporters limit the uptake of endogenous macromolecules and xenobiotics. In addition, adsorptive endocytosis is a receptor-independent mode of vesicular transport involving interactions of cationic proteins with the anionic glycocalyx.

The BBB *contributes to immune surveillance* since it hinders the extensive penetration of immune cells into the blood parenchyma. Immune cells move along the vessel walls but not frequently penetrate into the parenchyma. However, some leukocytes, mainly T lymphocytes traverse the vessel walls and patrol the CNS.

In addition, BBB functions as a *metabolic barrier* since the brain microvascular endothelial cells contain several catalytic transmembrane and cytosolic enzymes that regulate the metabolism of both endogenous and xenobiotic compounds. BBB is also *responsive to metabolic demands* as augmented neuronal activity fires rapid, regional increases in blood supply, aiming to compensate for the glucose and oxygen requirements. Additionally, the cerebrovascular endothelium constricts or dilates as a response to secretion of vasoactive mediators by other cellular components of the NVU.

1.4 The BBB in neuroinflammatory conditions

Neuroinflammation can induce the loss of the protective, minimal inherent BBB permeability and permit the infiltration of immune cells that patrol the CNS into the parenchyma. The interaction of recruited immune cells with the endothelium can lead to further BBB compromise and reinforce the continuous passage of blood-derived immune cells into the brain.

The increased concentration of immune mediators such as pro-inflammatory cytokines and chemokines is the driving force for the uncoupling of the NVU and the dysruption of neuronal homeostasis (Neuwelt et al., 2008). These factors potentiate the expression of adhesion molecules that are causally implicated in the extravasation of circulating leukocytes through the endothelium, and in the concomitant BBB dysfunction (Vestweber, 2012).

Microvascular hyperpermeability is a hallmark of inflammation and oedematous tissue injury in various acute and chronic pathological conditions, such as sepsis, shock, acute respiratory distress syndrome and ischemia-reperfusion injury. Inflammatory mediators, including thrombin, serotonin, bradykinin, histamine, and the vascular endothelial growth factor (VEGF) (Breslin et al., 2003) increase microvascular leakiness (Alexander, 2000). Extensive research has focused on the vaso-modulating effects of pro-inflammatory cytokines including TNF (discussed in chapters 3 and 4), IL-1β (Ni et al., 2017), IFN-γ (Lopez-Ramirez et al., 2012), LPS (Banks et al., 2015),

TWEAK (Polavarapu et al., 2005), and chemokines such as CCL2 (Dimitrijevic et al., 2006).

1.4.1 The molecular mechanisms of BBB compromise

Pathophysiological conditions that involve the secretion of pro-inflammatory cytokines activate the brain endothelium and elicit cytoskeletal remodelling. This comprises the formation of actin stress fibres and the generation of minute intercellular gaps. This endothelial stimulation induces hyperpermeability to small molecules, and the upregulation of receptors mediating endothelial adherence to immune cells. The excessive activation of the endothelium leads to aberrant vascular leakage and brain oedema (Papadopoulos et al., 2001). Vasogenic oedema results from elevated BBB permeability and cell death leading to vascular collapse. Perturbations in the autoregulation of brain perfusion and the paracellular transport result to TJ and AJ disassembly (Marmarou et al., 2000).

The mechanisms underlying the regulation of BBB compromise at the junctional level include the endocytosis of transmembrane TJ proteins, their subsequent cleavage at the plasma membrane, alterations in cytoskeletal dynamics, and modification of protein expression. These changes are usually transient and their reversal requires the reannealing of the intercellular junctions, and the restoration of the equilibrium between competing contractile and adhesive forces produced by junctional and cytoskeletal proteins, leading to the resealing of paracellular clefts and the enhanced anchorage of the endothelial cells to the ECM (Dejana et al., 2009). Moreover, the

molecular machinery underlying the disruption of the cerebrovascular endothelium might involve endothelial cell death yielding gross but punctual permeability changes.

The implication of the aforementioned mechanisms on the TNF-induced brain endothelial permeabilisation will be discussed on the following chapters 5 and 6.

1.4.2 Pharmacological modulators of BBB integrity

Glucocorticoids have wide clinical application as anti-oedema drugs and ameliorate BBB recovery. Dexamethasone, a paradigmatic member of this group, prevents TNF-mediated alterations in endothelial integrity by triggering the trans-activation of the glucocorticoid receptor and the subsequent repression of NF-ϰB and VEGF signalling (Aveleira et al., 2010; Wang et al., 2008). Another potent vasomodulator, the angiopoietin-1/ tyrosine kinase with Immunoglobulin-like and EGF-like domains 2 (Tie2) system (Ang-1/ Tie2) reinforces the BBB close-up by modulating the RhoA-mediated reorganisation of the actin cytoskeleton, by activating the Akt serine kinase, and by phosphorylating the endothelial nitic oxide synthase (eNOS) (Mammoto et al., 2007; Saharinen et al., 2008). Statins are another paradigm suggesting the pharmacological inhibition of RhoA-activation and the downstream MLC phosphorylation as a successful therapeutic strategy after traumatic brain injury onset (Shivanna et al., 2010). Other barrier-stabilising factors include prostaglandins (PG)E_2 and PGI_2, the metabolic homeostasis-regulating hormone insulin, the cyclic adenosine monophosphate (cAMP), the actin-binding vasodilator-stimulated phosphoprotein (VASP), and the recently reported TGF-β (Ronaldson et al., 2009; Huang, 2012).

These clinically successful treatment paradigms have set the foundation stone our attempt to uncover the molecular machinery underlying TNF-induced endothelial hyperpermeability, discussed in the following chapters.

1.5 The BBB in brain metastasis

The BBB is intact during the early (micrometastatic) stages of metastatic colonisation and is compromised and heterogeneously leaky at later stages of secondary brain tumour growth and proliferation (Gril et al., 2008; Taskar et al., 2012; Murrell et al., 2015). The impermeable BBB excludes diagnostic imaging agents (e.g. gadolinium-DTPA [Gd-DTPA]) and therapeutic compounds from the CNS (Puhalla et al., 2015). The limitations on brain uptake imposed by the BBB not only hinder the early detection of micrometastases, but also diminish the bioavailability of systemically successful therapeutic agents at metastatic colonies (Lampson, 2011), with only 15% of drug conjugates reaching therapeutic levels in the metastatic lesions (Lockman et al., 2010). Moreover, the brain uptake of imaging or chemotherapeutic agents is further impaired by the enhanced presence of efflux transporters such as the breast cancer resistance protein (BCRP) and P-gp at the BBB (de Vries et al., 2007; Lemos et al., 2008).

As a consequence, optimised, BBB-permeable compounds exhibit some degree of therapeutic efficacy (Osswald et al., 2016), and BBB-impermeable agents, clinically beneficial for other sites of non-CNS metastasis, reach clinically relevant does only

upon administration following severe BBB impairment (Margolin et al., 2012). Currently, the detection of metastatic colonies employing the clinical gold-standard method of gadolinium-enhanced MRI is only feasible at later stages of metastatic growth, once gross structural abnormalities have developed, and the BBB is breached (Zhang et al., 1992). Apart from the concept of mechanical disruption of BBB due to extensive tumour outgrowth, BBB integrity has recently been associated also with the growth speed of individual metastatic colonies (Osswald et al., 2016).

By the time brain metastases come to clinical attention, the secondary brain tumour deposits have acquired increased aggressiveness, rendering their clinical management challenging. It remains still unclear whether the properties of the BBB are altered upon metastatic colonisation or whether the modifications ongoing are better reflected by a barrier with disorganised structure and poor structural integrity, the blood-tumour barrier (BTB) (Jain et al., 2007). At this stage, the barrier is characterised by abnormal basement membrane abnormalities, dysfunctional TJs (Kodack et al., 2012; Thorsen et al., 2013), and highly fenestrated cerebrovascular endothelium that is associated with higher efflux and peritumoral brain oedema presentation (Percy et al., 2011). Despite these functional and structural impairments, the barrier seems to retain its inherent impermeable nature that confers chemoresistance. Indeed, altered pericytic subpopulations form a perivascular sheath, thus imposing additional constraints limiting the passage of macromolecular structures through the BTB (Lyle et al., 2016). However, at later stages of advanced metastatic outgrowth, the necrotic core of brain metastases exhibits optimal drug penetration due to BTB impairment, whereas the

invasive tumour edge is exposed to sub-optimal concentrations of therapeutic agents suggesting that BTB remains a significant impediment to treatment at these sites (Lockman et al., 2010; Morikawa et al., 2015).

1.5.1 BBB permeabilisation strategies for early diagnosis and therapy

The transient permeabilisation of the BBB has attracted increasing interest as a strategy aiming to facilitate or improve the passage of imaging or therapeutic agents through the BBB. Intravenous administration of hyperosmotic agents such as mannitol has been the first strategy aiming to transiently open up the BBB, through induction Ca^{2+}-dependent cell contraction and tight junction disassembly (Rapoport et al., 1972; Haluska & Anthony, 2004). However, the clinical translation of this approach for the delivery of chemotherapeutic agents has been limited due to neurotoxicity (Neuwelt et al., 1983), lack of specificity for tumour sites, and limited therapeutic efficacy due to the short working window provided (Hersh et al., 2016).

An alternative concept involved the chemical-mediated disruption of the BBB. This relies on a transient inflammatory stimulation of the brain endothelium, leading to destabilisation of the endothelial cell membrane (Warren et al., 2001). In line with this, administration of vasoactive substances such as bradykinin has attracted considerable interest. Such compounds stimulate receptors preferentially expressed in the tumour associated vasculature, but not in the normal brain tissue, and induce reversible TJs disassembly (Kemper et al., 2004). Pharmacological targeting of bradykinin B2 receptor (Liu et al., 2010), employing the synthetic bradykinin analog

cereport (RMP-7), has been shown to transiently modulate the BBB and increase drug uptake at rat and human gliomas in a TNF-dependent manner (Matsukado et al., 1996; Borlongan & Emerich, 2003). However, this approach was discontinued from clinical application due to limited efficacy on a randomized, placebo-controlled trial (Prados et al., 2003).

Other strategies facilitate the drug delivery through administration at routes bypassing the BBB (Bellavance et al., 2008). These include intrathecal; intraventricular; intranasal (da Fonseca et al., 2008), and interstitial delivery employing convection enhanced administration via stereotactically placed catheters (Bobo et al., 1994). Furthermore, administration of chemotherapeutic agents can be facilitated using biodegradable wafer polymers that are implanted into the brain (Ewend et al., 2007; Dolghih & Jacobson, 2013). This approach has been trialled in the treatment of malignant glioma (Judy et al., 1995). However, the minimal and often inhomogeneous diffusion of the compounds into the CNS parenchyma, alongside tissue-specific toxicities are serious confounders limiting the application of this approach into clinical practice.

Moreover, the MRI-guided, ultrasound-mediated focal BBB disruption and the exploitation of tumour treating fields are two techniques that bear limited applicability due to the requirement for prior detection of the metastatic sites (Hynynen et al., 2001; Treat et al., 2007; Hottinger et al., 2016).

Emerging approaches involve the production of chimeric peptides with lipophilicity, suitable for enhanced transcellular transport via receptor-mediated transcytosis, coined

as “molecular Trojan horses” (Drappatz et al., 2013; Papademetriou & Porter, 2015) or the stem cell-mediated antibody delivery (Frank et al., 2011). Another promising strategy involves the use of magnetic nanoparticles that can be encapsulated in liposomes for the targeted administration of therapeutic agents (Thomsen et al., 2015). These delivery strategies benefit from limited CNS toxicity and are currently trialed. Moreover, administration of efflux-pump inhibitors that modulate P-glycoprotein function at the BBB enhances CNS drug delivery, but is lacking selectivity towards tumour sites (Miller et al., 2008). Lastly, the radiation-induced BBB disruption (van Vulpen et al., 2002) is being under question, as it fails to increase BBB permeability in the brain metastasis setting (Murrell et al., 2016).

The limitations of the aforementioned strategies include the neutoxic off-target effects associated with either limited selectivity or permanent compromise of the BBB, the absence of targeting for undetected or early stage metastases, the requirement for prior detection of the tumour site and the limited applicability associated either with increased invasiveness or with interventions compromising the quality of life of the patients.

1.5.2 Selective permeabilisation of the BBB during brain metastasis

Preclinical studies aiming to achieve enhanced drug delivery to systemic tumours have illustrated the ability of intravenous bolus dose of recombinant human tumour necrosis factor (TNF) to disrupt the TJs of tumour-associated endothelium through the activation of the RhoA/ Rho kinase (ROCK) pathway (Seki et al., 2011). The

concomitant enhanced permeability of the tumour vasculature facilitated the virus particle delivery to a solid subcutaneous xenograft EL4 lymphoma model in mice.

Interaction of TNF with its endogenous receptors tumour necrosis factor receptor 1 (TNFR1) and TNFR2 leads to endothelial cytoskeletal remodelling and destabilisation of the endothelial adhesion complexes (Ferrero et al., 2001; Förster, 2008). Although the activation of the TNFRs is associated with pathophysiological processes, induction of their signalling by controlled, low dosages of TNF might be beneficial. The normal adult brain vasculature, unlike peripheral blood vessels, exhibits resistance to the permeabilising effects of cytokines (Schnell et al., 1999). However, this might be reversed in the neuroinflammation setting. Microinjection of TNF into the rat brain has been demonstrated to induce focal, but delayed BBB circumvention, alongside focal inflammatory response (Sibson et al., 2002).

Intravenous administration of TNF or Lt in experimental models of brain metastasis from breast, has been shown to induce a transient permeabilisation of the BBB, specifically and selectively at sites of micrometastasis in the brain, whilst leaving the surrounding cerebrovascular BBB unaffected. This approach facilitates both the detection of micrometastasis through the conduction of contrast-enhanced MRI, but also increases the uptake of the otherwise inaccessible cancer therapeutic trastuzumab at micrometastatic colonies (Connell et al., 2013; Connell et al., 2014). Preliminary evidence in this work suggested that the permeabilisation observed is associated with the activation of TNFR1, since the receptor was detected at selectively on vessels closely associated with brain metastases both in mouse models and in human biopsy

tissue of brain metastasis from breast cancer. Furthermore, this strategy provides a critical window of opportunity (24-72h) that enables the access of drugs at tumour sites and renders the BBB permissive to the entry of a wide range of molecules, spanning from gadolinium-DTPA (590 Da) to monoclonal antibodies, such as trastuzumab (~148kDa), whilst targeting brain metastatic colonies via the systemic route of TNF delivery. The transient therapeutic opening of the BBB is devoid of chronic neuropathologic changes and toxicities reported for permanent BBB disruption strategies (Salahuddin et al., 1988; Pardridge, 2012).

1.6 Aims of this thesis

The transient, cytokine-enhanced modulation of the BBB selectively at sites of micrometastasis holds great promise both for the earlier detection of the metastatic sites, and for the successful delivery of drugs at the sites of brain metastasis. However, our understanding on the role of endothelial activation in the context of brain metastasis is evolving. Moreover, the understanding of the molecular underpinnings of this cytokine-induced response is a requisite for the clinical translation of this strategy. We hypothesised that the previously demonstrated (Connell et al., 2013) TNF-induced permeability at brain metastasis sites is mediated through the activation of TNF receptors within the tumour-associated brain endothelium.

Therefore, the aims of this study were to:

1. Determine the impact of the metastatic foci on the susceptibility of the tumour-associated endothelium to TNF-induced BBB permeabilisation
2. Address the role of TNF receptors expression within the brain endothelium on the TNF-induced BBB permeabilisation
3. Elucidate the mechanism underlying the TNF-induced BBB permeabilisation

Addressing these questions is critical for the development of targeted TNF analogues that could be used for the selective and transient opening of the BBB at brain early brain metastasis sites, thus providing a window of opportunity for the early detection and the successful treatment of brain metastases.

Chapter 2| Materials and Methods

The following chapter describes the materials and experimental methods used throughout this thesis. The methods are broken down into their corresponding chapters and in addition, a brief overview of the relevant techniques will be given at the beginning of each experimental chapter.

2.1 The spatial profile of endothelial activation

Four different mouse models of brain metastasis were used throughout the thesis, induced via different routes – either intracardially for hematogenous dissemination or intracerebrally for direct cerebral induction of a tumour. In both cases, 6-7 week-old female BALB/c mice, or 6-7 week-old female SCID mice (Charles River, Kent, UK) were housed under a standard 12 hour light/dark cycle and provided with food and water *ad libitum*. All procedures were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986, and animals were scored for behavioural and health changes throughout.

2.1.1 Cell lines and primary cultures

2.1.1.1 Cell line culture

A mouse breast cancer cell line, 4T1, expressing green fluorescent protein (GFP, kind gift from the Muschel lab, University of Oxford), the human breast cancer cell lines, MDA-MB-231 (parental) and MDA-MB-231-Brain, expressing enhanced GFP (eGFP, kind gift of the Palmieiri lab) were each cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma-Aldrich, St Louis, USA) supplemented with 1% (v/v) 200mM glutamine (Life Technologies, Carlsbad, USA) and 10% (v/v) heat inactivated fetal bovine serum (FBS, Labtech International, Heathfield, UK). The human lung adenocarcinoma cell line, SEBTA-001 (kind gift from the Pilkington lab) was cultured in DMEM supplemented with 2% human serum (Sigma-Aldrich, St Louis, USA). The human melanoma cell line, H1DL2 (kind gift from the Thorsen lab) was cultured in ALT-DMEM supplemented with 8% (v/v) non-essential amino acids (NEAA, Life Technologies, Carlsbad, USA) as **described by** . Cultures were seeded in T25 flasks (Greiner Bio-One, Kremsmünster, Austria), typically grown in un-coated polystyrene T75 flasks (Greiner Bio-One, Kremsmünster, Austria), unless stated otherwise, and incubated at 37°C in a water-saturated 5% CO2 atmosphere in a HeraCell™ 150 incubator (Thermo Fisher Scientific, Waltham, USA). In cases where tumour conditioned media was required, media was replaced when cells were approximately 70% confluent and then harvested after 48 hours.

2.1.1.2 Generation of tumour-conditioned medium

MDA-MB-231-Br-eGFP cells ($1x10^6$ cells/ ml) were plated in 175 cm^2 Sarstedt tissue culture flasks and incubated at 37°C in growth medium until reaching subconfluence (3 days) followed by medium replacement. TCM was collected at 90% confluence and

processed using Millex^{TM} syringe filter polyethersulfone membranes (0.22 μm pore- Merck Millipore, USA), yielding a cell-free medium (TCM_{filt}). TCM_{cent} was collected after further processing by centrifugation (1500rpm, 5min) serving to remove any cellular debris. TCM_{son}, EV- cleared medium was derived from additional sonication for 5x 10s on/off cycles using a Sonic Dismembrator 60 (Fisher Scientific, Hampton, USA). Filtered cell-free medium was stored at -20°C until use.

2.1.1.3 Cell preparation for injection

Medium was aspirated and cells were washed with sterile Dulbecco's phosphate-buffered saline (PBS) devoid of Ca^{2+} and Mg^{2}, then incubated with 5 ml 0.05% trypsin with EDTA (Life Technologies, Carlsbad, USA) for 10 minutes at 37°C. Trypsin activity was neutralised by adding 10 ml of culture medium. Cells were collected by sedimentation (250x*g*, 5 minutes, 4°C) in a Jouan CR4i centrifuge (Thermo Fisher Scientific, Waltham, USA). The resulting pellet was re-suspended in 1 ml of sterile PBS and cell number determined using a NucleoCounter® (Chemometec, Copenhagen, Denmark), as per the manufacturer's instructions.

2.1.1.4 hCMEC/D3 culture

The immortalised human cerebral microvascular endothelial cell line (hCMEC/D3) were obtained under license from Professor Ignacio Romero (The Open University, Milton Keynes, UK). The cell line was isolated from brain tissue

surgically excised from the temporal lobe of an adult female epileptic patient and transduced immortalised by lentiviral transduction employing vectors containing the catalytic subunit of human telomerase or SV40-T antigen. The clone selected was expressing normal endothelial markers, including the platelet endothelial cell adhesion molecule (PECAM-1), also named cluster of differentiation (CD31), VE-cadherin, and von Willebrand factor (Weksler et al., 2005).

The hCMEC/D3 cells were cultured at passages 25-30 in the endothelial growth medium (EGM-2) BulletKit (CC-3162, Lonza , UK) supplemented with the following components obtained from the manufacturer: 0.025 % (v/v) recombinant human epidermal growth factor (rhEGF), 0.025 % (v/v) vascular endothelial growth factor (VEGF), 0.025 % (v/v) R3-insulin-like growth factor-1 (R3- IGF-1), 0.1 % (v/v) recombinant human fibroblast growth factor-beta (rhFGF-b), 0.1 % (v/v) gentamicin and amphotericin-B cocktail, 0.1 % (v/v) ascorbic acid, 0.04 % (v/v) hydrocortisone and 2.5 % (v/v) foetal bovine serum (FBS). When required, the aforementioned components were substituted by the following reagents: 10ng/ml rhEGF (Thermo Fisher Scientific, Waltham, USA), 1ng/ml rhFGF (Thermo Fisher Scientific, Waltham, USA), 75µg/ml L-ascorbic acid (Sigma-Aldrich, St Louis, USA), 50µg/ml gentamicin (Sigma-Aldrich, St Louis, USA), 1.4µM hydrocortisone (Sigma-Aldrich, St Louis, USA).

Tissue culture flasks were pre-coated for 1-3 hours with collagen type I solutions serving as basement membrane substitutes (Li et al., 2010). Cells were initially seeded at T25 flasks until reaching a density of 4 x 10^5 cells/ cm^2 and being

expanded in T75 flasks. Cells were maintained in a humidified cell culture incubator at 37°C in 95% air and 5% CO_2 until confluence.

2.1.1.5 Growth surface preparation

Collagen type I (Merck Millipore, USA or Enzo Life Sciences, USA)stock solution (in 0.02N acetic acid, pH=3.67) was diluted (1:20) in Hanks' balanced salt solution (HBSS) to prepare a final concentration of 150μg/ml (Life Technologies, Carlsbad, USA) and left to dry for 1-3h at room temperature (RT) on the plastic surface. The flasks were then rinsed with HBSS and filled with EGM-2 in preparation for cell culture.

Transwells were coated with collagen as previously described and then coated with fibronectin (Sigma-Aldrich, St Louis, USA) stock solution (0.1% (w/v) in 0.5M NaCl, 0.05M Tris pH=7.5) diluted 1:100 in HBSS and left to dry for 1-3 hours at RT on the collagen-coated surfaces. The fibronectin coating solution was removed, and cells were seeded.

hCMEC/D3 cells were grown until subconfluent (~$1x10^5$ cells/cm^2) and culture medium was renewed every 2-3 days. To split subconfluent monolayers, hCMEC/D3 cells were washed in HBSS devoid of Ca^{2+} and Mg^{2+} at 37°C for 1-5min, followed by incubation with 0.25% (w/v) porcine trypsin and 0.2% (w/v) ethylene diamine tetraacetic acid (EDTA) solution (Sigma-Aldrich, St Louis, USA) at 37°C until detached. The enzymatic activity of trypsin was neutralised

upon addition of EGM-2 medium and cells were split at a ratio of 1:5 in collagen-coated tissue culture flasks (Greiner Bio-One, Kremsmünster, Austria)

2.1.2 Experimental models of brain metastasis

2.1.2.1 Intracardiac model of brain metastasis

BALB/c mice were anaesthetised with 2% (v/v) isoflurane (Abbot, UK) in oxygen. Shaving and hair removal cream was used to ensure a clean ultrasound image removed hair over the thorax. Mice were maintained at 1.8% (v/v) isoflurane and placed on a heated stage. Ultrasound (Vevo770 system, VisualSonics, Toronto, Canada) was used to visualise the heart and guide intracardiac injection in the left ventricle with 100µL PBS containing 10^4 4T1-GFP cells, using a 27 gauge needle (Balathasan et al., 2013). At days 14 or 21 post injection, animals (n=5-6 per time point) were transcardially perfusion-fixed under terminal anaesthesia with 0.9% heparinised saline, followed by 200ml of periodate lysine paraformaldehyde (PLP) containing 0.025% (w/v) glutaraldehyde (Sigma-Aldrich, St Louis, USA) (PLP_{light}). Brains were removed and post-fixed for 4 h in PLP_{light} prior to cryo-protection in 30% (w/v) sucrose in water for ~24 hours. Tissue was embedded and frozen in Tissue-Tek (Bayer PLC, UK) for sectioning. 10µm-thick serial coronal sections were taken throughout the brain at a 1 in 5 sampling rate, using a cryostat (Leica, UK). Sections were

mounted on Superfrost® Plus slides (Thermo Fisher Scientific, Waltham, USA) and allowed to dry overnight at room temperature.

2.1.2.2 Intracerebral model of brain metastasis

BALB/c mice were anaesthetised with 2% (v/v) isoflurane in a gas mix of oxygen and N_2O (30:70), and placed on ear bars in a small animal stereotactic frame (Stoetling Co., USA) under maintenance anaesthesia (1.5% (v/v) isoflurane). The skull was exposed and a small hole drilled at the injection site. Animals were injected in the left striatum (+0.5 mm, +1.5 mm laterally and -2.5 mm vertically, from Bregma). Either 0.5 µl saline containing 10^7 cells/ml 4T1-GFP cells or 0.5 µl saline alone were injected using a glass micro-capillary (Clark Electromedical Instruments, UK) pulled with a single stage glass microelectrode puller (Narishige, model PP-830, Japan), heated to 72°C and cut such that the bore diameter was 75µm, as determined using a graticule. Following surgery, the skin incision was sutured using Mersilk sutures (Ethicon, Dülmen Germany). At days 10, 14, 21 and 28 animals (n=5 per time point) were perfusion-fixed, and tissue processed as in section 2.1.2.1. SCID mice (n=4) were also injected with 5 x 10^3 MDA-Br-231-EGFP cells in 0.5 µl, and perfusion-fixed at day 14 post injection, with tissue processed as in section 2.1.2.1. In the case of the LLC model, C57 mice (female, n=3) were injected intrastriatally with 5 x 10^3 LLC cells in 0.5 µl, and perfusion-fixed at day 14.

2.1.2.3. Immunohistochemistry

Throughout, antigens were revealed using an avidin-biotin-pexoxidase system (Warnke & Levy, 1980). All incubations were performed at room temperature unless otherwise specified. Sections were washed in phosphate buffered saline (PBS) and quenched in methanol supplemented with 1% (v/v) H_2O_2 (30% w/w) for 20 minutes. Slides were further washed and placed in Shandon Sequenza® clips (Thermo Electron Cooperation, UK) under PBS. Sections were blocked with 10% serum derived from the same species that the secondary antibody is raised in (see Table 1) in PBS, washed (3 x 5 minutes) with PBS or PBS supplemented with 0.05% Tween20 v/v (PBS-T) if the antigen is intracellular and incubated with primary antibody overnight at 4°C as detailed in Table 1. Primary antibody binding was detected using the relevant biotinylated secondary antibody for 1 hour (See Table 1) and an ABC kit (Vector Laboratories, Peterborough, UK), 1:100, 45 min. Immunoreactivity was revealed using 3,3'-diaminobenzidine HCl (DAB) (0.016% w/v, Sigma-Aldrich, St Louis, USA) and (0.05% v/v) H_2O_2 (30% w/v) in sodium phosphate buffer (0.1M, pH7.4) to yield an insoluble brown precipitate. Sections were counter-stained with 0.5% (w/v) cresyl violet in water, dehydrated through a series of ethanol baths at 70%, 95%, 100% and 100% (v/v)

in water) and cleared in xylene (Fisher Scientific, UK). Slides were mounted using DPX mounting medium (Fisher Scientific, UK)

Table 2.1: Immunohistochemistry antibody concentrations

	Target	Host	Clonality	Isotype	Species reactivity	Suggested dilution
ab19139	**TNFR1**	rabbit	polyclonal	IgG	Mouse, Rat, Human	16h, 4oC, 1 :100
ab90121	**TNFR1**	rabbit	polyclonal	IgG	Human, (Mouse, Rat)	10µg/ml, **1:50**
ab90436	**TNFR1**	**rabbit**	**polyclonal**	**IgG**	Mouse, Rat, Sheep, Rabbit, Human	16h, 4oC, 1 :100
ab15563	**TNFR2**	rabbit	polyclonal	IgG	Human, Mouse	1µg/ml, overnight, 4oC, 1:100
ab8161	TNFR2	mouse	monoclonal	IgG1	Human	1:100, overnight, 4oC
	Colocalization					
AF3628	**CD31**	goat	polyclonal	IgG	Mouse, Rat	**1:100**, overnight, 4oC
ab8158	**CD34**	rat	monoclonal	IgG2a	Mouse	**1:100**, overnight, 4oC
ab54263	**Glut1**	sheep	polyclonal	IgG	Mouse, Rat, Rabbit, Human	**1:100**, overnight, 4oC
P1-35926-0.1	**Glut1** (SPM498)	mouse	monoclonal	IgG2a	Human, Mouse, Rat	Immunohistochemistry (Paraffin), 1:20
ab13970	**GFP**	chicken	polyclonal	IgY	Mouse	**1:100**, overnight, 4oC
ab187369	**MelanA**	mouse	monoclonal	IgG2b	Mouse, Rat, Human	
ab72876	**TTF1**	mouse	monoclonal	IgG	Mouse, Rat, Human	**1:50**, overnight, 4oC
ab51061	**MelanA**	rabbit	monoclonal	IgG	Human	1:100, overnight, 4oC
345779	**Cytokeratin (CAM5.2)**	mouse	monoclonal	IgG2a	Mouse	**1:2**, overnight, 4oC
ab16911	**F4/80**	rat	monoclonal	IgG2a	Mouse	**1:50**, overnight, 4oC

2.1.2.4 Tumour quantification

Immunohistochemical slides were scanned at 200x magnification using the ScanScope CS system (Aperio, Leica Biosystems, Wetzlar, Germany). Tumour area and the associated area of endothelial activation were demarcated manually, and areas calculated using ImageScope software (Aperio, Leica Biosystems, Wetzlar, Germany).

2.1.2.5 Immunocytochemistry

hCMEC/D3 cells were passaged by washing with PBS and incubating with trypsin, as described in section 2.1.1.2. Trypsin activity was blocked with glial culture medium, and then the cells were collected by sedimentation (800x*g*, 5 minutes, 4°C). In order to confirm the presence of endothelial cells in the culture, the pellet was re-suspended in endothelial growth medium and plated in a 6 well plate, containing cover slips, previously sterilised with UV light (15min each side in a CL-1000 Ultraviolet Crosslinker, UVP). After 48 hours growth, cells were removed from the incubator for fixation. All subsequent steps were performed at room temperature. Wells were washed with PBS and fixed with 4% w/v paraformaldehyde (PFA) in phosphate buffer for 10 minutes. Cells were permeabilised with PBS supplemented with 0.025% v/v Triton X-100 (10 minutes) and then washed with PBS supplemented with PBS-T (3 x 5 minutes), blocked with streptavidin solution (20 minutes) followed by biotin solution (20 minutes, Vector Laboratories, Peterborough, UK). Non-specific antibody binding was blocked with TNB (Tris-NaCl blocking buffer; 0.1M Tris-HCl pH7.5, 0.15M NaCl,

0.5% w/v Blocking Reagent, Perkin Elmer, Waltham, USA) for 30 minutes, prior to incubation with rabbit anti-mouse GFAP (1:500, Dako) for 2 hours. Primary antibody binding was detected using a goat anti-rabbit biotinylated antibody (1:100, 30 minutes, Vector Laboratories) and streptavidin-Cy3 (1:300, 30 minutes, Invitrogen). Cover slips were mounted on Superfrost® Plus slides (Thermo Fisher Scientific, Waltham, USA) using DAPI mounting medium for nuclei detection (Vector Laboratories, UK). Fluorescence was visualised using a Leica DM IRBA epifluorescent microscope.

2.1.2.6 Histological Assessment of BBB Permeabilization

At 13 days post metastasis induction in the 4T1-GFP model, mice were intravenously injected with 100 μL saline containing doses 3 μg per mouse of either recombinant mouse TNF (PeproTech, London, UK) or recombinant human TNF (PeproTech, London, UK) or saline as control. Two hours later, mice were intravenously injected with of 100 μL of type II horseradish peroxidase (44kDa) (HRP; 300 units; Sigma-Aldrich, St Louis, USA) and killed by transcardial perfusion fixation. Brains were excised and prepared for histology. Slides were stained using a modified Hanker–Yates method and counterstained using cresyl violet. Brown staining indicating permeation of HRP was assessed blindly, and a percentage of positive metastases for each group was calculated.

2.1.3 Endothelial proliferation assays

2.1.3.1 *In vitro* MTT assay for endothelial cell proliferation

Endothelial cells were isolated as previously described and plated in a 6 well plate. When cells had reached approximately 50% confluence, the medium was aspirated and replaced with either tumour conditioned medium (harvested as previously described) or endothelial culture medium. After 24 hours incubation at 37°C, 5% CO_2 the effect on cell proliferation was determined, using a colorimetric MTT dye reduction assay. Assay medium was prepared by mixing 2 volumes of culture medium with 1 volume of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5mg/mL). Each well had 2 mL assay medium added and was incubated for 4 hours at 37°C. The medium was then removed and replaced with 1 ml DMSO in order to dissolve the purple formazan crystals that had formed. Absorbance was measured by a photometric microplate reader (Tecan, Männedorf, Switzerland) at 570 nm within 30 minutes of dissolving crystals.

2.1.3.2 *In vitro* viability, cytotoxicity and caspase activity assays

Determination of intact, viable cells was based on the detection of fluorescent signal emitted by proteolytic cleavage of GF-AFC, whereas cells devoid of membrane integrity were distinguished through measurement of a cell-impermeant peptide substrate (bis-AAF-R110).

Confirmation of caspase-3/7 activity in hCMEC/D3 cells was facilitated based on the measurement of a luminogenic caspase-3/7 substrate. Samples were handled according to the manufacturer's protocol for the ApoTox-Glo Triplex Assay (Promega, Madison, USA).

2.1.3.3 PCR quantification of TNF receptors expression in hCMEC/D3 cells

hCMEC/D3 cells were grown and treated on collagen-coated T25 flasks. Cells were washed once with pre-warmed HBSS. Total RNA was isolated from cell pellets using the ReliaPrep RNA Cell Miniprep System (Promega, Madison, USA), resuspended in RNase-free water, measured by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and reverse transcribed using the Precision nanoScript 2 Reverse Transcription Kit (Primerdesign, Southampton, USA).

Real-time quantitative PCR was conducted using the PrecisionPLUS Mastermix (Primerdesign Ltd, Eastleigh, UK) and custom designed probes for human TNFRSF1A (NM_001065), TNFRSF1B (NM_001066) and mouse TNFRSF1A (NM_0011609), TNFRSF1B (NM_0011610), from Primerdesign gene expression assays in a Mx3000P QPCR System (Stratagene, San Diego, USA). The mRNA expression levels have been recorded in the relevant MxPro QPCR software (Agilent, Santa Clara, USA).

hCMEC/D3 were screened using the geNorm-Reference Gene Selection Kit (Primerdesign), leading to the selection of RPL4 and GAPDH as the relevant optimal reference genes for the normalization. The $\Delta\Delta$ cycle threshold (CT) method has been used for the calculation of the relative gene expression levels. All samples were assayed

as three independent biological and three technical replicates.

For each brain region analysed, tissue was dissected into three pieces and processed separately so as to maximize RNA extraction. RNA was then pooled at the end of the extraction protocol. RNA was extracted from mouse tissue using a Qiagen® RNeasy® Mini Kit (Qiagen, UK). β-mercaptoethanol (BME) was added to the RLT buffer (1% v/v), to inhibit endogenous ribonucleases which may be present in the sample, in order to maintain RNA intergrity. Tissue samples were homogenised in RLT + BME. Buffer RLT is a lysis buffer which also enhances binding of RNA to the silica membrane in the RNeasy Mini spin column, due its guanidine isothiocycanate component. Homogenised samples were transferred to a QIAshredder column (Qiagen, Venlo, Netherlands) and centrifuged at 10000xgfor 3 minutes in a Biofuge™ Pico Heraeus™ centrifuge (Thermo Fisher Scientific, Waltham, USA), in order to remove cell and extra-cellular matrix debris. The flow through was added to 70% v/v ethanol in water and inverted several times. Subsequently, the sample was transferred to an RNeasy Mini spin column in a 2ml collection tube. In the presence of ethanol, RNA precipitates, whilst protein and salts remain soluble, and hence flow through the column. The column was eluted by centrifugation (10,000xg, 15 s) and the flow-through discarded. 700µl of Buffer RW1 was subsequently added to the RNeasy spin column, and centrifuged at 10,000 RPM for 15 seconds. RW1 is a wash buffer which removes lipids, carbohydrates and protein that may have non-specifically bound to the silica membrane. Subsequently, 500 µl RPE (containing ethanol) was added. RPE is a wash buffer that removes salts from the column. The column was spun at 10000

RPM for 15 seconds, and flow through discarded. This was repeated, before changing collection tube and spinning the column spun for 2 minutes at 10000 RPM, removing ethanol contamination. The column was transferred to an Eppendorf, and 25 µl of RNase-free water was added to the membrane. After leaving to stand for 1 minute, the column was spun at 10000 RPM for 1 minute, eluting the RNA.

Contaminating genomic DNA was removed using a DNase treatment as per the manufacturer's instructions (Primerdesign Ltd, Eastleigh, UK). 5µl of *Precision*™ DNase reaction buffer was added per 50 µl of RNA solution, along with 1 µl of *Precision*™ DNase enzyme. The solution was incubated for 10 minutes at 30°C and subsequently the enzyme was inactivated by heating the mixture to 55°C for 5 minutes. RNA yield was calculated using a NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, USA), and samples stored at -80°C until further use.

2.1.3.4 Histological confirmation of gene of interest expression

Immunofluoresence was performed on tissue from intracardiac and intracerebral animals perfused at day 14 after tumour cell injection. Sections were washed with PBS-T and endogenous peroxidase activity blocked with PBS + 1% (v/v) H2O2 for 20 minutes. Streptavidin/Biotin activity was blocked with streptavidin solution (20 minutes) followed by biotin solution (20 minutes, Vector Laboratories, Peterborough, UK). After washing (3xPBS-T), a signal enhancer was used (Image iT FX signal enhancer, Invitrogen, Carlsbad, USA), incubating sections for 30 minutes. After

washing (3xPBS-T), non-specific antibody binding was blocked with TNB (Tris-NaCl blocking buffer; 0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% w/v Blocking Reagent, Perkin Elmer, Waltham, USA) for 40 minutes, prior to incubation with primary antibodies (anti-CD31 and anti-TNFR1, anti-TNFR2, anti-GFP, anti-TTF-1, anti-MelanA for 2 hours or overnight at 4°C, as detailed in Table 1. In the cases of TNFR1, TNFR2, primary antibody binding was detected using a goat anti-rabbit biotinylated antibody (1:100, 30 minutes, Vector Laboratories), prior to streptavidin-HRP (Perkin Elmer, Waltham, USA) incubation (1:200, 30 minutes) and TSA amplification (Biotinyl tyramide reagent, Perkin Elmer, Waltham, USA, 1:100, 7 minutes). Amplification was visulaised with streptavidin-AMCA (1:100, 30 minutes). CD31 reactivity was revealed with Texas-red fluorophore (1:100, 30 minutes). Sections were mounted with fluouresence mounting medium (Vector Laborotories, Peterborough, USA) and staining visualised with a Leica epifluoresence microscope.

2.1.4 Statistical analysis

All data were presented as mean ± standard error of the mean. All the samples were assayed in triplicates. Two-sided one-way ANOVA with Bonferroni correction was employed for multiple groups where appropriate. The statistical significance was accepted at $p<0.05$ as compared to vehicle-treated controls. All statistical analyses were determined using GraphPad Prism6 (GraphPad Software, La Jolla, USA).

Chapter 3| The impact of tumour microenvironment on TNFR1-associated endothelial activation

3.1 Introduction

The following chapter will focus on the assessment of the impact of the tumour microenvironment on the brain endothelium during brain metastasis with particular focus i) on the expression of TNF receptors (TNFR1 and TNFR2) in the tumour-associated brain endothelium, and ii) on understanding the role of the crosstalk between metastatic tumour cells and the brain endothelium particularly in the early brain metastasis (micrometastatic) setting. In order to answer these questions experimental mouse models of brain metastasis, human biopsies from patient diagnosed with secondary brain tumours, and relevant *in vitro* co-culture models were employed.

3.1.1 TNF and TNF receptors

In 1975, the prototypic pro-inflammatory cytokine tumour necrosis factor (TNF), initially termed *"tumour-necrotising factor"* (O'Malley et al., 1962) or cachectin, was identified in the human serum as a molecule responsible for necrosis associated with vascular collapse in tumour-bearing mice (Carswell et al., 1975). Lymphotoxin (Lt), an endogenous analogue of TNF, was discovered as a cytotoxic factor, functionally and sequentially homologous to TNF (Pennica et al., 1984). TNF was renamed thereafter as TNF-α, and lymphotoxin as TNF-β. Lympthotoxin-β, a close homologue of

lymphotoxin was discovered in 1993 (Browning et al., 1993). The characterisation of these primordial cytokines laid the foundation for the isolation and identification of the TNF superfamily. In 1998, the term TNF-β has been replaced by lymphotoxin-α and TNF-α became an orphan term. To date, the official symbol of the cytokine according to the HUGO Gene Nomenclature Committee is TNF (HUGO Gene Nomenclature Committee, n.d.). The resulting protein is coined as TNF by the Universal Protein Resource (UniProt) (UniProt, n.d.).

Tumour necrosis factor (TNF) is a pleiotropic, pro-inflammatory cytokine that functions primarily as an innate immune defence molecule, regulating homeostasis at the cellular tissue and organism levels. Consistent with this, TNF expression at low levels exerts beneficial homeostatic functions, being a requisite for host defence responses against pathogens, and for the initiation of healing processes (Pasparakis et al., 1996). At higher concentrations, TNF can have deleterious effects through the exacerbation of inflammatory insults leading to organ failure. TNF is also regulating cellular transformation (Komori et al., 1993), tumourigenesis (Aggarwal et al., 1996; Giri & Aggarwal, 1998; Suganuma et al., 1999), and metastatic colonisation (Orosz et al., 1993; Hafner et al., 1996; Takeda et al., 2001; Ham et al., 2016). This archetypal cytokine is rapidly secreted mainly by activated macrophages, monocytes, T cells, natural killer (NK) cells, neutrophils and microglia, but also by other cell types including endothelial cells, as a response to diverse stimulatory environmental cues. The potent pro-inflammatory properties of TNF dictate a tight spatiotemporal expression pattern and upregulated secretion, during acute responses only. Elaborate

control mechanisms include the modulation of *Tnf* expression at the transcriptional and translational levels, and the shedding of TNF or its cognate receptors in response to various agonists.

TNF is expressed as a homotrimer, either as a transmembrane protein (tmTNF, monomer: 26kDa), or as a soluble TNF protein (sTNF, monomer: 17kDa) that can be released after cleavage of tmTNF by metalloproteinase TNF-α -converting enzyme (TACE) or disintegrin and metallopeptidase domain 17 (ADAM17) (Kriegler et al., 1988; Idriss & Naismith, 2000). Both tmTNF and sTNF are biologically active. TNF binds two homotrimeric transmembrane receptors: the 55 kDa TNF receptor 1 (TNFR1/CD120a/ p55), encoded by the *TNFRSF1A* gene and the 75 kDa TNF receptor 2 (TNFR2/CD120b/p75), encoded by *TNFRSF1B.* TNFR1 is activated by both sTNF and tmTNF (Grell et al, 1998; Lewis et al., 1991a), whereas tmTNF is the main TNFR2 ligand (Grell et al., 1995). TNFR1 is constitutively expressed on almost every nucleated cell type and tissue, whereas the expression of TNFR2 is highly regulated with prominent expression in cells of the immune system and endothelium, including brain microvascular endothelial cells (Lucas et al., 1998; Lombardi et al., 2009).

The trimerisation of the receptors is a requisite for the interaction with TNF. The extracellular domains of both receptors are conserved and consist of a pre-ligand assembly domain, a ligand-binding domain, and a TACE substrate domain. On the contrary, their intracellular domains are structurally unrelated and modulate diverse signalling cascades (Aggarwal, 2003). TNFR1 is death domain (DD)-containing

receptor amenable to protein-protein interactions with adaptor-DD containing proteins (TNFR1-associated death domain; TRADD or Fas-associated death domain; FADD) (Tartaglia et al.,1993). TNFR2 recruits TNF associated factor 2 (TRAF2) and other TRAF2- associated proteins (Wajant et al., 2003).

Most cellular responses to sTNF, including two diametrically opposed outcomes; pro-inflammatory signalling and cell death induction, are regulated by TNFR1 (Ferrero et al., 2001; Grell et al., 1998). The activation of this receptor triggers pathways that seem to be hierarchically layered to regulate cell fate upon TNF-binding. TNF alone or synergistically with interferons induces the TNFR1-mediated expression of chemokines and adhesion molecules (VCAM-1 and ICAM-1), thereby indirectly contributing to the recruitment of immune cells to the site of antigen presentation (Mackay et al., 1993).

TNF-induced activation of the NF-κB pathway surpasses apoptosis induction and promotes gene transcription and pro-survival, pro-inflammatory signalling, while FADD-mediated activation of caspases triggers TNF-induced cell death (Faustman & Davis, 2010). Notably, TNFR1 can also induce caspase-independent, pro-inflammatory cell death, coined as necroptosis (Kalliolias & Ivashkiv, 2016). The relevant signalling cascades are further discussed in **chapter 5**. On the other hand, TNFR2 controls TNF-induced endothelial cell migration and angiogenesis, alongside various immune regulatory and anti-inflammatory functions (Pan et al., 2002; Faustman & Davis, 2010; Faustman & Davis, 2013). TNFR2 exerts its functions mainly through the AKT and NF-κB signalling nodes (Medvedev et al., 1994; Rao et al., 1995). TNFR2 activation

elicits poor pro-apoptotic signalling (Liu et al., 1996), but rather promotes cellular proliferation or potentiates TNFR1 signalling (Medvedev et al., 1994; Tartaglia et al., 1993). The cross-regulation of TNFRs might involve additive, synergistic or even antagonistic signalling (Wajant et al., 2003).

Aberrations in the control mechanisms governing TNF signalling result in pathological conditions (Probert, 2015; Vassalli, 1992). TNFR1 mutations that impinge on receptor cleavage and the concomitant enhancement of TNF activity, generate TNFR1-associated periodic syndromes (TRAPS) and systemic autoimmune syndromes with CNS involvement (Minden et al., 2004). Aberrant TNF production is associated with a wide spectrum of inflammatory pathologies such inflammatory bowel disease, rheumatoid arthritis, psoriasis, insulitis. Moreover, TNF secretion is a key component of various peripheral and CNS inflammatory pathologies involving inflammation such as chronic immune and autoimmune disorders, sepsis, neurodegenerative diseases and cancer. Anti-TNF biologics such as adalimumab, etanercept and infliximab have been clinically successful in the treatment of such inflammatory disorders due to their selective binding to TNF. However, their clinical application is undermined by serious side effects converging on immune suppression.

On the contrary, administration of TNF is practiced clinically in the isolated limb perfusion setting for the treatment of melanoma or sarcoma, and for the regression bulky hepatic neoplasms confined to the liver (Lejeune et al., 2006; Eggermont et al., 1996; Alexander et al., 1998). However, the therapeutic applications of TNF are undermined by dose-limiting off-target effects of systemic administration, with the

minimum effective dose being significantly higher than the maximum tolerated dose. This is reflected by the discontinuation of all monotherapy phase I and II clinical trials conducted employing human TNF (huTNF) as an anti-cancer agent due to dose-dependent toxicities including fever, hypotension and tachycardia (Roberts et al., 2011; Steeland et al., 2018).

3.1.1.1 The role of TNF in the CNS

TNF is constitutively expressed at low levels in the adult brain under physiological conditions (Boulanger, 2009), functions as a gliotransmitter and is secreted by neurons, microglia, astrocytes and ependymal cells of the choroid plexus. TNF regulates the synaptic communication amongst neurons, whilst orchestrating learning and memory processes (Albensi & Mattson, 2000; Stellwagen & Malenka, 2006). Elevated TNF levels are detected in affected brain tissue in many neurological disorders including multiple sclerosis, Alzheimer's disease, Parkinson's disease and in acute injuries such as cerebral stroke and traumatic brain injury (TBI) (Probert, 2015). In this context, TNF release has been demonstrated to be both toxic through the promotion of cell death, but also tropic through the stimulation of focal immune responses (Hofman et al., 1989; Mogi et al., 1994). Genetic studies in mouse models of acute brain injury have demonstrated the neuroprotective role of TNFR1 (Probert, 2015).

Importantly, systemic inflammation leads to elevated TNF levels in the brain (Holmes et al., 2009). Moreover, TNF can cross the BBB by the virtue of a saturable transport system (Gutierrez et al., 1993). Microglial activation is followed by the establishment of a positive TNFR1-mediated feedback loop, leading to augmented TNF release (Kuno et al., 2005). Astrocytic activation and the concomitant secretion of TNF (Chen et al., 2016) is controlling the TNFR1-induced endothelial activation and the BBB compromise (Akassoglou et al, 1997; Akassoglou et al., 1998; Probert, 2015). On the other hand, TNFR2 expression is minimal under physiological conditions and is primarily driven by microglia and astrocytes in neurological conditions (H. Gao et al., 2017). The functional pleiotropic repertoire of TNFRs signalling is reflected by the dichotomy between TNFR2 activation in peripheral macrophages and in microglia. The first elicits T cell activation and leukocyte transmigration across the BBB, whereas microglial TNFR2 signalling exerts anti-inflammatory signalling (H. Gao et al., 2017; Veroni et al., 2010).

3.1.2 Endothelial activation during brain metastasis

The extravasation of metastasising tumour cells through the brain endothelium is often considered as a rate-limiting step during metastatic progression. It is consensual that pro-inflammatory cytokines, including TNF, dictate endothelial activation and modulate the integrity of interendothelial junctions, thus potentiating transendothelial migration (TEM) (Miles et al., 2008; Jassam et al., 2017).

Paracellular TEM involves the disassembly of interendothelial junctional complexes, and the concomitant passage of tumour cells through the minute clefts formed. This is dictated by the secretion of vaso-modulating factors such as transforming growth factor β (TGFβ) and vascular endothelial growth factor (VEGF) that compromise the VE-cadherin – β-catenin complex and induce the local disruption of endothelial cell junctions (Lee et al., 2003; Reymond et al., 2013). Moreover, metastasising cells might also secrete pro-apoptotic factors, thus inducing permanent damage to the cerebrovascular endothelium (Reymond et al., 2013). Additionally, TEM can be potentiated by TNF-secreting tumour-associated macrophages (TAMs), and concomitant relaxation of the interendothelial junctional complexes (Reymond et al., 2013; van Zijl, Krupitza, & Mikulits, 2011). Transendothelial TEM encompasses also the direct traversal of tumour cells through the endothelial body. The latter migratory mechanism seems to be uncommon (Leong et al., 2014). It is speculated that the mechanism of intravasation is dependent not only on structural features of the local microvasculature, but also on the origin of the disseminating cells (Reymond et al., 2013). Furthermore, extracellular vesicles derived from mammary carcinoma, brain-seeking 231Br cells have been reported contain miRNA-181c that impairs the integrity of the brain endothelium through promotion of alterations in cytoskeletal dynamics (Tominaga et al., 2015).

The extravasation step relies on the expression of cognate ligands and receptors by both tumour and endothelial cells. These comprise a broad spectrum of selectins, integrins, cadherins and immunoglobulin superfamily receptors that act synergistically.

The panel of tumour cell- endothelial interactions reported seems to be dependent on the origin of the disseminating cancer cells.

Angiopoietin-2, cathepsin S and αB-crystallin have been associated with the extravasation of breast cancer cells through the BBB (Avraham et al., 2014; Sevenich et al., 2014; Malin et al., 2014). Endothelial upregulation E-selectin, VCAM-1, ALCAM, ICAM-1, VLA-4 and β4 integrin, alongside ALCAM/ALCAM and VLA-4/ VCAM-1 interactions potentiate the metastatic seeding of breast cancer cells (Serres et al., 2012; Soto et al., 2014). Importantly, depletion of LFA-1 in metastasising breast cancer cells yields a significant reduction (>70%) in tumour burden through disruption of the LFA-1/ ICAM-1 interaction (Soto et al., 2016).

Moreover, α3β1/laminin interaction has been reported as a crucial driver of the extravasation of lung cancer cells (Yoshimasu et al., 2004). Interestingly, CD62E/CD15s interaction enhanced the TEM of non-small cell lung cancer (NSCLC) cells in a TNF-dependent fashion (Jassam et al., 2016, 2017).

Additionally, the extravasation of melanoma cells triggers the endothelial upregulation of E-selectin, VCAM-1, ICAM-1 and involves the subsequent VLA-4/VCAM-1 (Klemke et al., 2007) and LFA-1/ICAM-1 interactions. TEM of melanoma cells is also facilitated by αVβ3 and α4β1 integrins (Voura et al., 2001). αVβ3 binding to endothelial platelet endothelial cell adhesion molecule (PECAM1/CD31) is linked to endothelial junction disruption (Reymond et al., 2013).

3.2 Methods

3.2.1 Mouse models of brain metastasis

Preclinical mouse models of brain metastasis are providing insight into the molecular mechanisms involved in the metastatic cascade and have assisted the development of novel therapies and approaches aiming to treat the disease (Daphu et al., 2013; Soto & Sibson, 2016).

Spontaneous metastatic models established involve genetically engineered mice bearing downregulated or upregulated oncogenes that drive tumourigenesis. These models exhibit short latency time between tumour implantation and metastatic spread. In addition, these experimental metastasis models allow for greater control over the disease induction, since the number of cells inoculated, and the implantation site are targeted. These models enable the protracted study of disease progression in absence of terminal illness by restricting or eliminating the systemic dissemination of neoplastic cells (Francia et al., 2011).

Rodent syngeneic models involve tumour growth in inbred animals bearing the same genetic background as the tumour cells inoculated, whereas xenogeneic (human-rodent xenotransplantation) models employ cell lines derived from human cancers are transplanted into immunocompromised mice.

There are multiple routes established for the induction of brain metastasis *in vivo*. The injection of tumour cells via the carotid artery has been frequently used in experimental models of brain metastasis (Kienast et al., 2010; Lorger & Felding-Habermann, 2010). This route yields high numbers of metastatic colonies in the brain, with limited induction of systemic disease. However, this approach is highly invasive as it requires extensive surgical procedures that may induce systemic inflammation. This is a crucial confounder in the brain metastasis setting, as inflammation in the periphery has been linked to CNS inflammation (Combrinck et al., 2002; Cunningham et al., 2009), and subsequent enhanced potential for metastatic seeding (Solinas et al., 2010). On the contrary, intravenous injection of tumour cells is a minimally invasive technique that is undermined by the high systemic burden yielded (Daphu et al., 2013).

Inoculation of tumour cells into the arterial blood stream is more advantageous, as it avoids their passage in the pulmonary circulation and the subsequent frequent formation of lung metastases. Intracardiac injection into the left ventricle of immunocompromised mice facilitates the systemic distribution of cancer cells to most organs, including brain. Accurate positioning of needle tip into a beating heart is necessary throughout the injection process. This route of injection might result in tumour formation within the ventricular walls, whilst administration of cells in the

right ventricle leads to subsequent dissemination to the lungs (Zhang & Yu, 2011). Ultrasound-guided intracardiac inoculation has been proven to yield increased CNS burden, whilst benefiting from reduced systemic disease (Balathasan et al., 2013).

Moreover, local growth models established involve the direct implantation of metastatic cells into the brain using stereotactic guidance (Lockshin et al., 1986; Price et al., 1986). The site of inoculation, the striatum is a self-contained brain region, displaying inflammatory responses that have been extensively studied (Blond et al., 2002; Sibson et al., 2004). Nevertheless, this intracerebral model omits the extravasation process, whilst facilitating the study of the parenchymal response to metastatic growth. The mode of delivery of tumour cells into the brain is critical in order to minimise complications associated with inflammatory responses elicited by the injection process itself. Injection volumes not higher than 0.5-1μl, at a slow and constant rate, employing finely-drawm microcannulas (tip *ca.* 75μm) is critical for the avoidance of physical disruption of the brain tissue (Soto & Sibson, 2016).

3.2.2 Tumour cell lines

The 4T1 mouse mammary carcinoma cell line is thioguanine-resistant variant of the 410.4 cell line, transfected with green fluorescent protein (GFP). 4T1 cells were harvested from a mammary tumour that spontaneously arose in BALB/cfC3H breeding female mouse (Miller, 1983; Yamashina & Heppner, 1985). This cell line is highly tumourigenic, with the potential to form spontaneous metastases in distal sites,

including lymph nodes, blood, bone, lung and brain (Pulaski & Ostrand-Rosenberg, 1998; Lelekakis et al., 1999). Importantly, the syngeneic model of brain metastasis created employing this cell line benefits from a intact immune system; a crucial component for the creation of a clinical relevant model that recapitulates the paracrine signalling between endothelial cells and immune effector cells.

The metastatic human estrogen-independent breast carcinoma cell line MDA-MB-231 was isolated from a pleural effusion of a patient with stage III breast carcinoma (Higuchi et al., 1980). This cell line has been extensively used for the study of the initial steps of metastatic dissemination and leads mostly to the formation of bone, brain, adrenal and ovarian metastasis (Tester et al., 2004; Lorger & Felding-Habermann, 2010). The subclone MDA-MB-231Br (231Br) has been established by serial *in vivo* and *in vitro* passages (Yoneda et al., 2001), and has been transfected with GFP. 231Br cells metastasise preferentially to the brain, whilst no other sites of distal metastasis have been detected upon injection into severe combined immunodeficiency (SCID) mice. This strain is employed in order to avoid acute phase responses that would elicit tumour cell destruction.

Both 4T1 and 231Br cells have been captured to extravasate through the BBB at days 3-5 post intracarotid artery injection (Heyn et al., 2006; Lorger & Felding-Haberman, 2010). Their metastatic seeding has been demonstrated to evoke an immediate astrocytic and microglial activation (O'Brien et al., 2014; Andreou et al., 2017).

The H1DL2 cell line was isolated from a human melanoma brain metastasis resection and transduced with lentiviral vectors encoding Dentra (a GFP variant) and luciferase. Intracardiac inoculation led to arrest in the brain of approximately 0.05% of the injected cells within 24 hours, and to the formation of contrast-enhancing metastatic colonies by 3.5% of the injected cells at later timepoints, suggesting that this model effectively recapitulates the metastatic dissemination process (Sundstrøm et al., 2013).

The SEBTA-001 cell line was isolated from a human non-small cell lung cancer brain metastasis biopsy. *In vitro* studies confirmed the AJs driven- interaction of these tumour cells with brain endothelial monolayers (Jassam et al., 2016).

3.2.3 Immunohistochemical and immunofluorescent analysis of human tissue

Human brain metastasis resection samples, from breast carcinoma (n=6), lung adenocarcinoma (n=6), melanoma (n=4) patients, were obtained from the leading edge of brain metastases, as previously described (Zakaria & Jenkinson, 2014). All samples came from an approved Biobank (Oxford Brain Bank; OBB, South Central- Oxford C Research Ethics Committee, 15/SC/0639). All samples were stored and processed according to the HTA Codes of Practice and the relevant regulations of the University of Oxford.

3.2.3.1 Autofluorescence quenching for human brain metastasis biopsies

Inherent autofluorescence in the human brain tissue restricts the conduction of immunofluorescence and the parallel detection of multiple markers. The sources of autofluorescent signal are variable and required specialised treatment steps prior the use of established staining protocols.

Elastin and collagen are common components of blood vessels that emit fluorescent signal. Amongst other fluorophores, elastin contains a naturally occurring cross-linking tricarboxylic amino acid with a pyridinium ring (Deyl et al., 1980). Collagen possesses a similar fluorophore. Cowen *et al.* (Cowen et al., 1985) used a dye called pontamine/ Chicago sky blue to successfully quench autofluorescence in carotid arteries and mesenteric vessels using a fluorescence resonance energy transfer method. This method shifts the fluorescent emission of elastin and collagen from green to red.

Additionally, the naturally fluorescent lipofuscin pigment accumulates with age in the cytoplasm of numerous cell types, including those of the CNS (Yin, 1996; Schnell et al., 1999). Commonly referred to as ageing pigment, it is yellow-brown in appearance, accumulates within lysosomes due to peroxidation of lipids (Huang et al., 1976), and has an excitation and emission spectrum similar to that of FITC. TrueBlack signal quencher was employed in order to minimise lipofuscin-derived signal.

Moreover, the tissue fixation process might result in fluorescent signal (Davis et al., 2014). Autofluorescent compounds are formed between aldehyde fixatives and proteins or amines, with glutaraldehyde fixation giving a greater degree of autofluorescence than formaldehyde due to extensive cross-linking. Sodium borodydrate quenches aldehyde-derived autofluorescence in human brain sections (Clancy & Cauller, 1998).

Sodium borohydrate reduces aldehyde-induced autofluorescence by reducing Schiff bases ($R_1HC{=}NR_2$) that are formed from the reaction between the aldehyde and NH_2 groups.

Lastly, application of Evan's Blue and trypan blue decrease the signal of direct immuno-fluorescence or auto-antibodies. Each was used on tissues/cells at 0.001% to 0.1% in water or buffer. They are soluble in aqueous mounting media and diffuse out quickly. Both reduce background significantly; both unwanted fluorescence due to the conjugate and native fluorescence due to structural elements in the tissues/cells (Huang et al., 1976).

3.3 Results

3.3.1 Endothelial TNFRs expression in response to brain metastasis; intracardiac model

The intracardiac route of tumour inoculation, yields disseminated brain metastases, dispersed throughout the brain. Based on previous work in the lab (Serres et al., 2012), 10^5 4T1-GFP or 10^5 231Br cells were inoculated into the left cardiac ventricle of BALB/c or SCID mice, respectively. This level of tumour burden enables the study of most steps of the metastatic cascade including haematogenous dissemination, arrest at vascular bed, extravasation through the BBB, metastatic colonisation, and co-optive growth of micrometastases in the perivascular niche, without observation of animal sickness. At day 14 (for the syngeneic BALB/c- 4T1-GFP model), and at day 21 (for

the xenogeneic SCID-231Br model), when the mice were terminally anaesthetised and transcardially perfusion-fixed, the BBB was usually uncompromised, as evidenced by absence of contrast enhancement in Gd-enhanced MRI previously conducted (Connell et al., 2013), but also by the absence of histological detection of HRP extravasation in *ex vivo* brain tissue slices employing Hanker -Yates staining **(section 4)**. The different time points used are indicative of diverse patterns of tumour development and propagation between the syngeneic and the xenogeneic models, but also of the pronounced aggressiveness of the 4T1-GFP cells (Gao et al., 2011).

Moreover, two recently established experimental models of brain metastasis from lung adenocarcinoma or melanoma were used, involving the inoculation of 10^5 SEBTA-001 and 10^5 H1DL2 cells, respectively (Cheng et al., 2019). These models are characterised by extended tumour cell arrest at the vascular walls and delayed outgrowth of metastatic colonies. Mice were followed-up until day 28 for the experimental model of lung cancer brain metastasis, and up to day 21 for the melanoma brain metastasis model. BBB integrity has been previously confirmed employing Gd-enhanced MRI (absence of hypointensities detection) for both models at relevant timepoints.

As evidenced in **figure 3.1**, micrometastases appear to grow in the perivascular space, in close association with the local microvasculature. These structures are indicative of vascular co-option. The cerebrovascular endothelium can be determined by increased expression of the well-studied, endothelial-specific marker CD31 (PECAM-1; platelet endothelial cell adhesion molecule) (Goncharov et al., 2017). The absence of any sites

of discontinuity in the endothelial staining is indicative of BBB integrity at sites of metastasis for the observed time frames.

GFP, thyroid transcription factor 1/thyroid specific enhancer binding protein (TTF-1), and Melanoma Antigen/Melanoma Antigen Recognized by T-cells 1 (MelanA/MART1) were employed as histological markers for metastatic breast cancer, lung adenocarcinoma, and melanoma cells, respectively. MelanA/MART1 is a well-characterised histological marker of melanoma cells (Coulie et al., 1994; Kawakami et al., 1994; Orosz, 1999). TTF-1 is a clinically relevant histological marker of primary lung carcinoma (Fabbro et al., 1996; Pelosi et al., 2001).

Upregulated TNFR1 expression in the tumour-surrounding vasculature was confirmed through colocalization of TNFR1 with CD31 by immunofluorescence for all the animal models assessed. This pattern was also evident for vessels associated with micrometastases of less than 50μm in diameter **(Figure)**. On the contrary, diffuse TNFR2 expression was detected in the peritumoural area in all models. Positive TNFR2 immunoreactivity was demonstrated for non-vascular, tumour-infiltrating structures with a morphology similar to that of microglia/macrophages and for intravascular leukocytes. Moreover, no endothelial TNFR1 or TNFR2 staining was found on normal appearing brain tissue.

**Experiments conducted in collaboration with Dr. Vinton Cheng and Niloufar Zarghami*

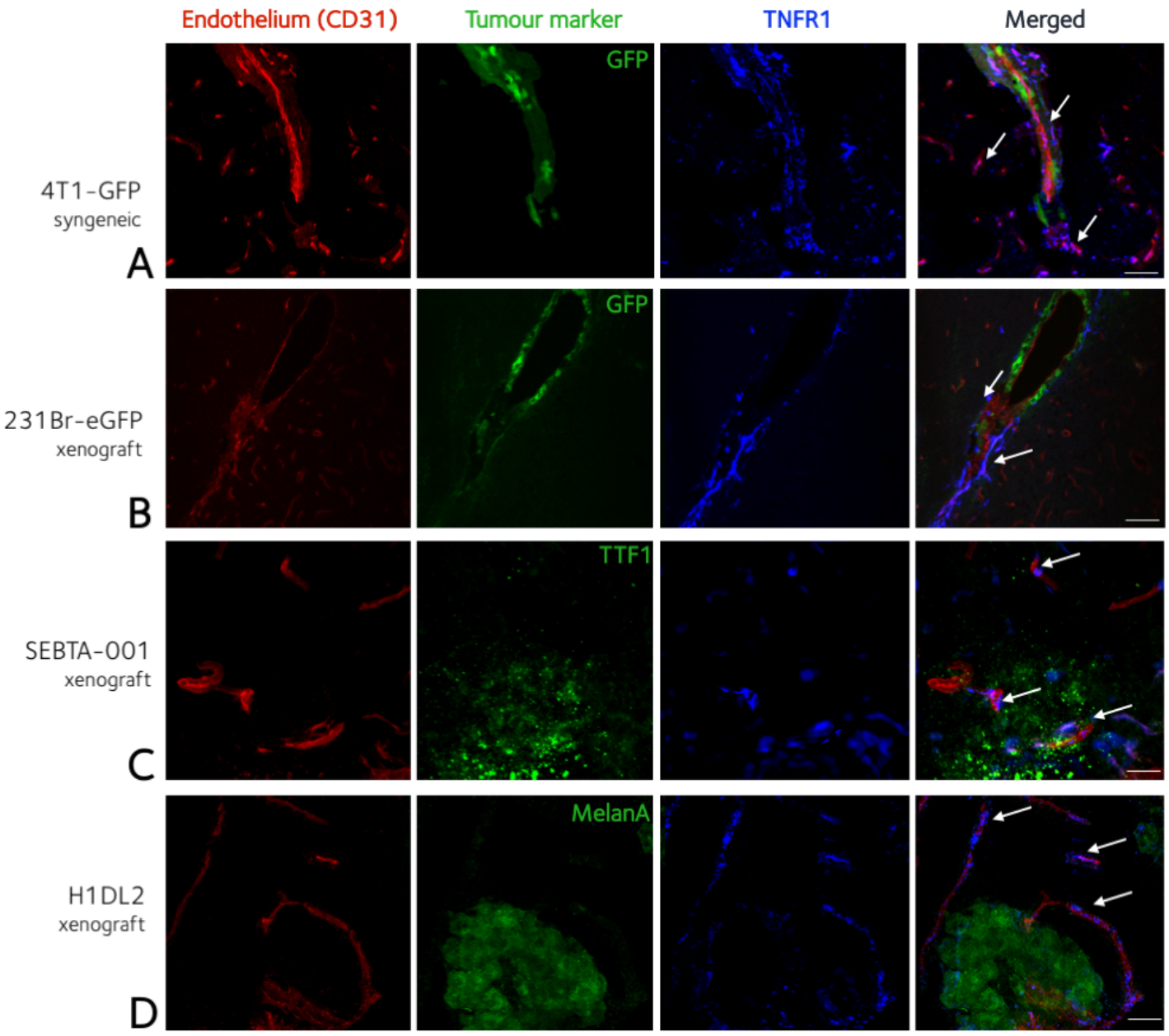


Fig.1 TNF receptor 1 (TNFR1) upregulation in the tumour-associated vasculature

Triple-colour fluorescence images showing co-localisation (arrows) between TNFR1 and CD31 in a syngeneic (4T1-GFP) mouse model of brain metastasis from breast **(A)**, and in xenogeneic mouse models of brain metastasis from breast cancer (231Br-eGFP) **(B)**, lung adenocarcinoma (SEBTA-001) **(C)**, and melanoma (H1DL2) **(D)**. All cell lines were injected intracardially.

Histological markers: endothelial cells: CD31 (red), tumour cells (green); GFP (for 4T1 and 231Br-eGFP), TTF1 (for SEBTA-001), MelanA (for H1DL2), TNF receptor 1 (blue). Scale bars= 30μm.

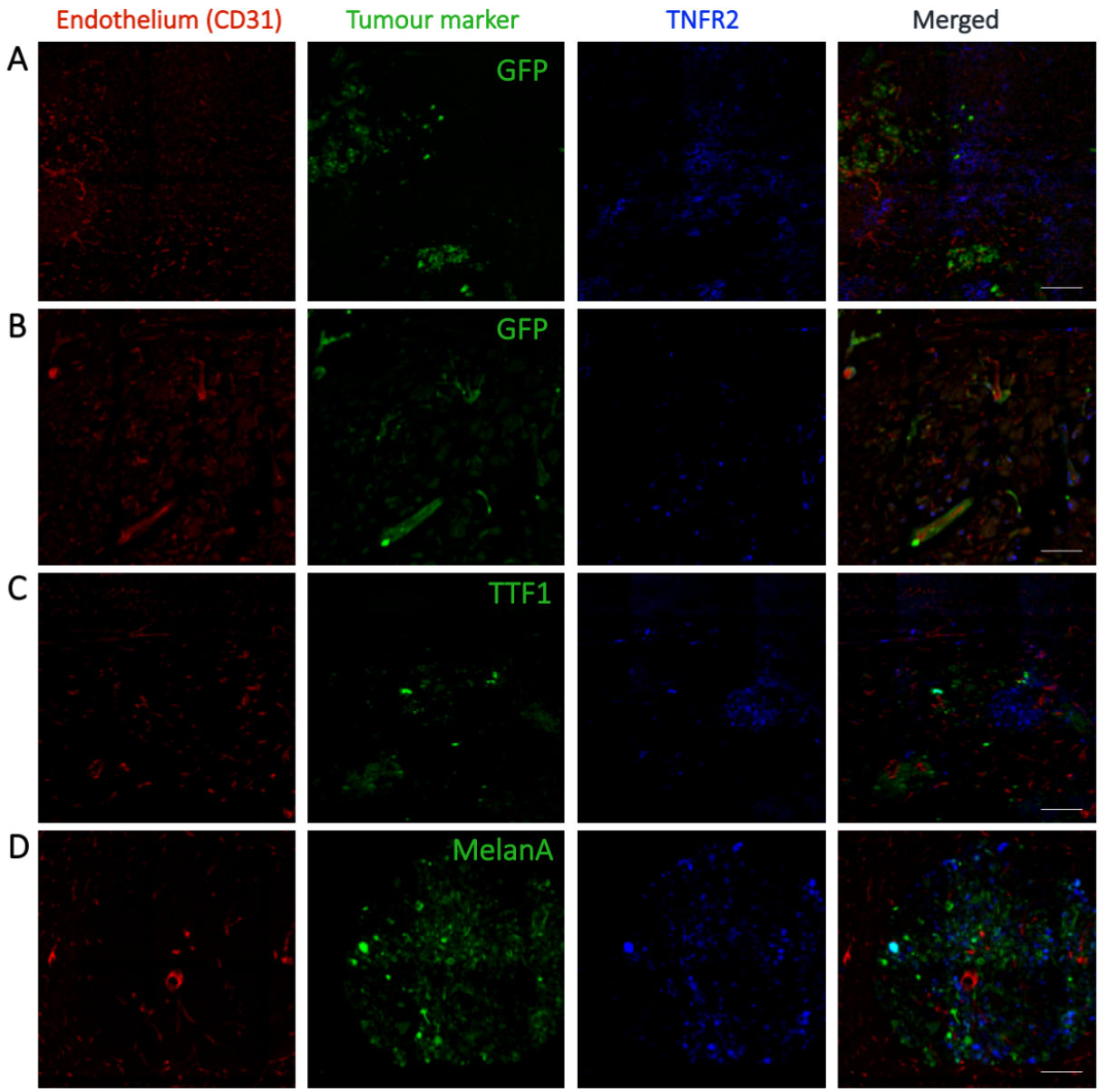


Fig.3.2 TNF receptor 2 (TNFR2) expression in tumour-surrounding brain parenchyma

Triple-colour fluorescence images showing TNFR2-localisation in non-vascular structures; in parenchymal microglia and intravascular leukocytes in a syngeneic (4T1-GFP) mouse model of brain metastasis from breast **(A)**, and in xenogeneic mouse models of brain metastasis from breast cancer (231Br-eGFP) **(B)**, lung adenocarcinoma (SEBTA-001) **(C)**, and melanoma (H1DL2) **(D)**. All cell lines were injected intracardially.

Histological markers: endothelial cells: CD31 (red), tumour cells (green); GFP (for 4T1 and 231Br-eGFP), TTF1 (for SEBTA-001), MelanA (for H1DL2), TNF receptor 2 (blue). Scale bars= 50µm.

3.3.2 Spatial profile of TNFRs expression in response to brain metastasis

To uncover the spatial distribution patterns of TNFR1 and TNFR2 expression in the perivascular niche, we used a local growth model employing intracerebral inoculation of tumour cells at a known site (striatum).

Immunofluorescent staining for the relevant tumour markers (GFP, TTF-1 and MelanA) revealed the clearly circumscribed tumour margins. The diameters of these foci were measured in order to determine the tumour core and the margin for each of them. Circles of increasing radii (100μm, 300μm, 1000μm) were generated with the origin allocated at the tumour edge. The radii were set in order to be able to delineate patterns of vascular TNFR1 positivity within the perivascular niche.

Immunofluorescent staining for TNFR1/TNFR2 and CD31 revealed the sites of endothelial TNFR1/TNFR2 expression within the tumour and the tumour-surrounding stroma. The points of TNFR1/CD31 and TNFR2/CD31 co-localisation were marked and allocated to groups, depending on their distance from the tumour boundary. Analysis of Z-stack images and 3D reconstruction was conducted in order to distinguish between the signal arising from adjacent microvessels or cross-sectional representations of bigger vessels. Multiple positive co-localisation sites arising from identical vessels were considered as single points for the following analysis.

The percentage of TNFR1-positive vessels in healthy, uninflamed brains was 10.3%±2.7% for BALB/c (n=6), immunocompetent mice and 8.4%±3.9% for SCID, immunocompromised mice (n=6). For the syngeneic 4T1-GFP model, 76.3%+9.5% ($p<0.001$, compared to TNFR1-vascular positivity in naïve BALB/c brains) were

positive within the tumour boundary, and 62.7%±9.8% ($p<0.001$) of vessels within 1-99μM from the tumour edge, respectively. For the xenogeneic models, the percentages for these areas were: 85%±3.9% ($p<0.001$, compared to TNFR1-vascular positivity in naïve SCID brains) and 65%±5.4% ($p<0.001$) for 231Br, 62.3%±8.5% ($p<0.001$) and 51%±5.2%($p<0.001$) for SEBTA-001, 54.6%±8.9% ($p<0.001$) and 50.4%±7.9% ($p<0.001$) for H1DL2, respectively. TNFR1-expressing vessels reached 14.8%±6.6% (ns; non-significant, compared to TNFR1-vascular positivity in naïve BALB/c brains) within 100-299μm, and 11.8%±6.6% (ns) within 300-999μm from tumour margins, respectively. Additionally, the relevant percentages in the xenogeneic models were 14.3%±4.8% (ns, compared to TNFR1-vascular positivity in naïve SCID brains) and 11.5%±4.2% (ns) for 231Br-injected mice, 13.9%±5.1% (ns) and 4.5%±2.1% (ns) for SEBTA-001-injected mice, 10.3%±5% (ns) and 6.9%±3%(ns) for H1DL2-injected mice.

On the other side, the percentage of TNFR2-positive vessels in healthy, uninflamed brains was 4.3%±2.6% for BALB/c mice (n=6), and 2.7%±2.1% for SCID mice (n=6). The percentage of TNFR2-positive vessels ranged from 4.4%±1.1% (ns, compared to TNFR2-vascular positivity in naïve BALB/c brains) to 7.3%±1.8% (ns) for the 4T1-GFP model. Similar trends were reported for the syngeneic models; 4.9%±1.3% (ns, compared to TNFR2-vascular positivity in naïve SCID brains) to 7.5%±2.8% (ns) for the 231Br model, 5.9%±2.2% (ns) to 7.4%±3% (ns) for the SEBTA-001 model, 8%±1.9% (ns) to 10.7%±4% (ns) for the H1DL2 model.

Since we aimed to focus on the early steps of the metastatic progression in our models, the observed time frames reflected micrometastatic tumour growth rather than later steps of tumour development. Micrometastases are defined as colonies comprised of 3-50 tumour cells with a diameter less than 500μm, whilst macrometastases are tumour deposits including more than 50 cells and having a diameter of at least 1mm (Osswald et al., 2016). Of the 472 metastatic colonies analysed, 431 (91%) were of diameters spanning from 10μm to 150μm. Indeed, 13% of the metastatic colonies detected in the sections derived from animals inoculated with the aggressive 4T1-GFP cells (n=6) were classified as macrometastases. The xenogeneic models were characterised by a slightly delayed tumour growth, with 7%, 5% and 8% detected in the 231Br (n=6), the SEBTA-001 (n=6), and the H1DL2 (n=6) models, respectively.

**Experiments conducted in collaboration with Dr. Vinton Cheng*

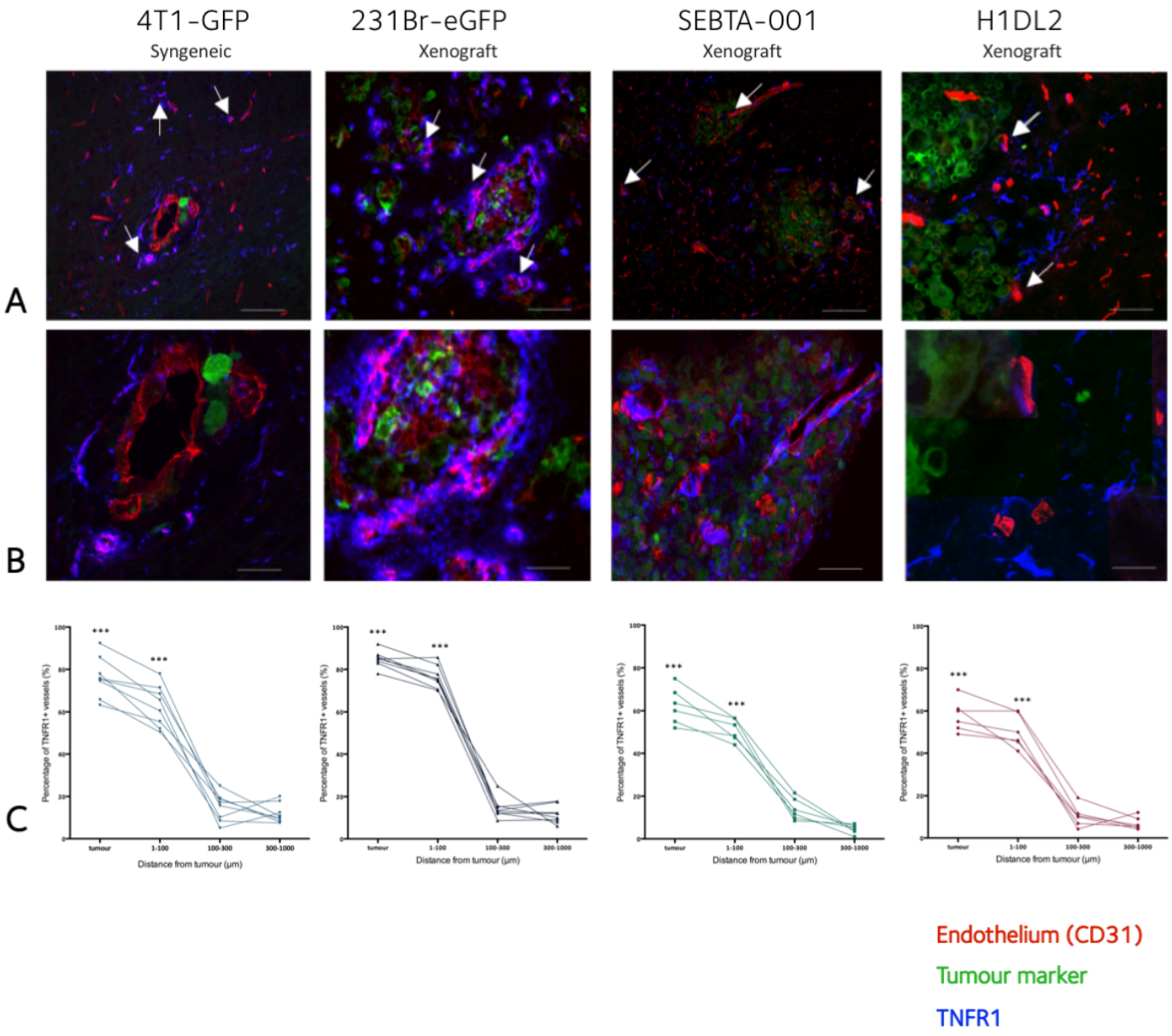


Fig.3.3 A-C: Spatial distribution of endothelial TNF receptor 1 (TNFR1) expression within the tumour area

Triple-colour fluorescence images showing co-localisation (arrows) between TNFR1 and CD31 in a syngeneic (4T1-GFP) mouse model of brain metastasis from breast, and in xenogeneic mouse models of brain metastasis from breast cancer (231Br-eGFP), lung adenocarcinoma (SEBTA-001), and melanoma (H1DL2). All cell lines were injected intracerebrally. **(A)** 20x magnification, **(B)** 63x magnification of areas presented in A

Histological markers: endothelial cells: CD31 (red), tumour cells (green); GFP (for 4T1 and 231Br-eGFP), TTF1 (for SEBTA-001), MelanA (for H1DL2), TNF receptor 1 (blue). Scale bars= 30µm.

(C) Percentage $TNFR1^+$ vessels at a distance of 0-1000µm from tumour boundary

A minimum of 8 sections per animal (n=6), evenly distributed throughout the striatum, were used for the quantification. Statistical analysis: one-way ANOVA, with Tukey post-hoc tests. ***p<0.001. Comparison vs % of $TNFR1^+$ vessels in healthy brain tissue. Data are presented as mean values ff SEM.

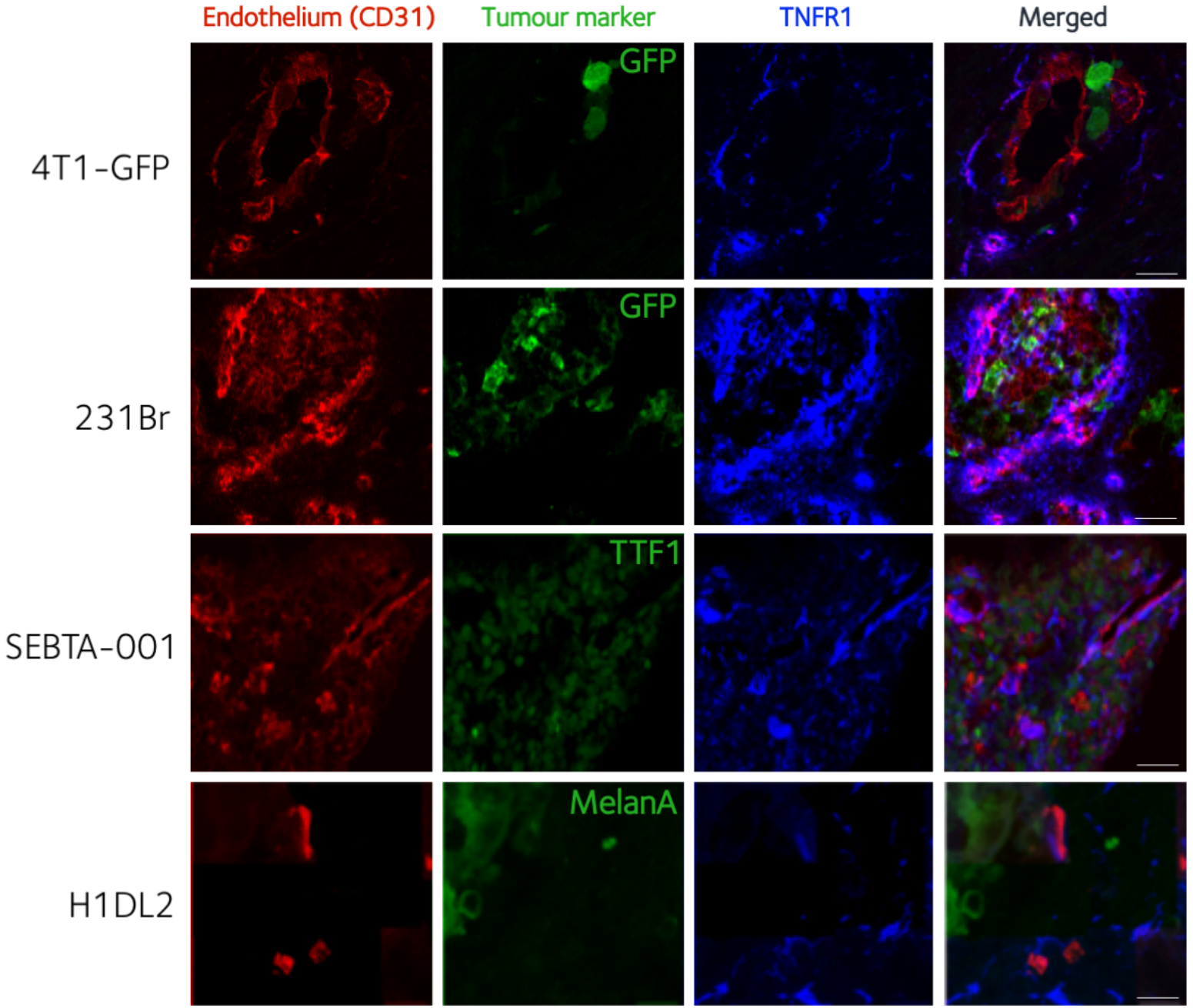


Fig.3.3D Spatial distribution of endothelial TNF receptor 1 (TNFR1) expression within the tumour area

Scale bars= 30μm

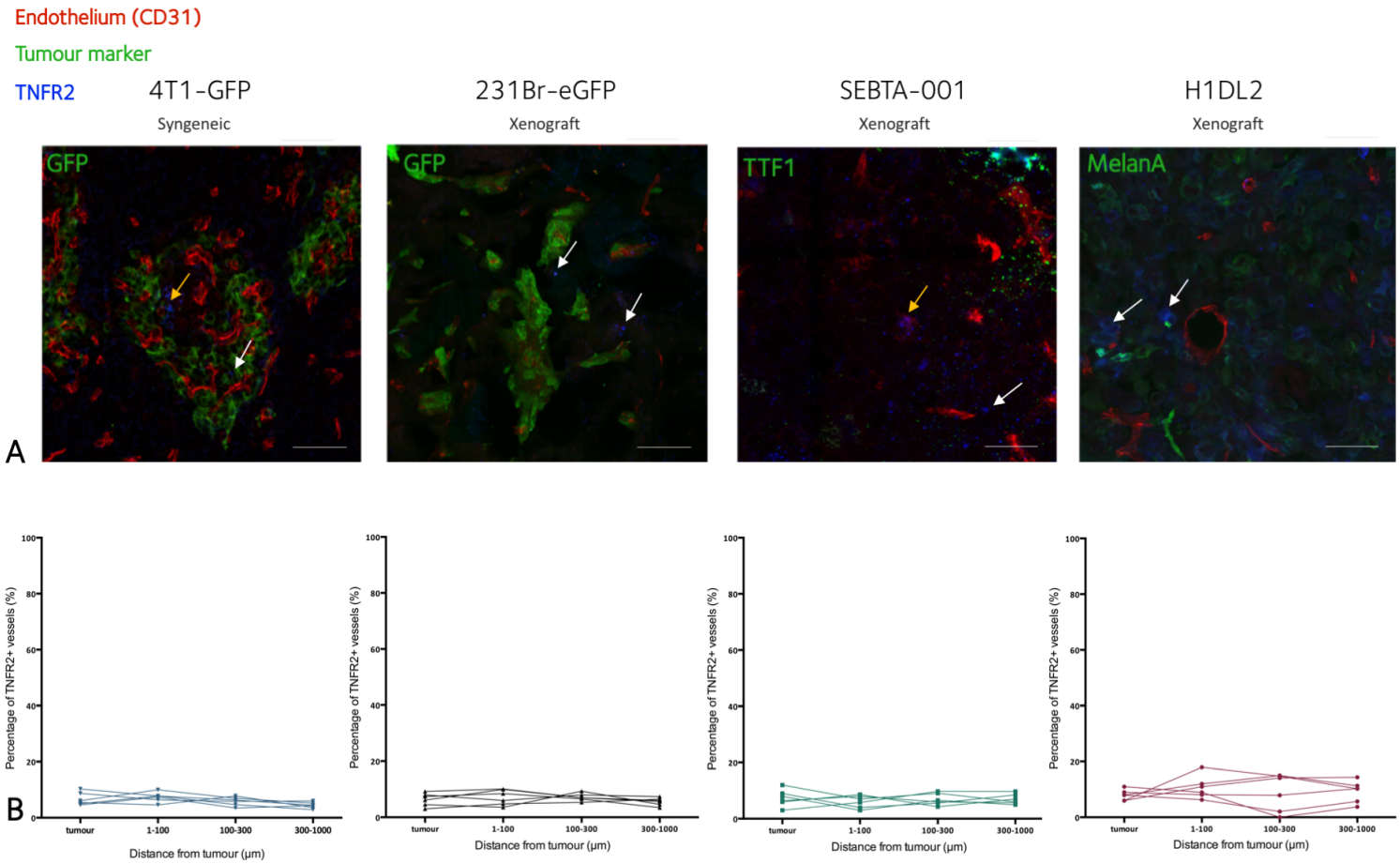


Fig.3.4 Spatial distribution of endothelial TNF receptor 2 (TNFR2) expression within the tumour area

Triple-colour fluorescence images showing TNFR2-localisation in non-vascular structures; in parenchymal microglia (white arrows) and intravascular leukocytes (orange arrows) in a syngeneic (4T1-GFP) mouse model of brain metastasis from breast, and in xenogeneic mouse models of brain metastasis from breast cancer (231Br), lung adenocarcinoma (SEBTA-001), and melanoma (H1DL2). All cell lines were injected intracerebrally.

Histological markers: endothelial cells: CD31 (red), tumour cells (green); GFP (for 4T1 and 231Br-eGFP), TTF1 (for SEBTA-001), MelanA (for H1DL2), TNF receptor 2 (blue). Scale bars= 30µm.

(B) Percentage $TNFR2^+$ vessels at a distance of 0-1000µm from tumour boundary

A minimum of 8 sections per animal (n=6), evenly distributed throughout the striatum, were used for the quantification. Statistical analysis: one-way ANOVA, with Tukey post-hoc tests. ***p<0.001. Comparison vs % of $TNFR2^+$ vessels in healthy brain tissue. Data are presented as mean values ff SEM.

3.3.3 TNFRs expression in human brain metastasis tissue

To determine whether the local, TNF-mediated BBB permeabilisation strategy in brain metastasis bears the potential for clinical translation, human biopsy tissue of brain metastasis from breast cancer (6 samples), lung adenocarcinoma (6 samples), and melanoma (4 samples) was assessed for TNFR1 and TNFR2 immunostaining.

For immunofluorescent analysis, consecutive tissue sections, matched with the ones used for the immunohistochemical staining were employed. This allowed us to focus on previously detected tumour sites, without using a third histological marker to label metastatic foci. Triple labelling immunofluorescence could not be conducted due to the the extensive autofluorescence of the human brain tissue. Moreover, the spatial patterns detected by immunohistochemistry, served as maps assisting the conduction of targeted confocal microscopy and minimising fluorescent signal losses due to prolonged exposure and concomitant signal quenching.

Endothelial expression of both receptors was confirmed in human brain samples with non-specific inflammation. Assessment of control, unaffected brain tissue revealed negative TNFR1 immunoreactivity, whereas minimal TNFR2 staining was detected.

Positive TNFR1 immunoreactivity was detected on the endothelium lining the tumour-associated vessels in all biopsies analysed. To confirm the endothelial upregulation of TNFR1, immunofluorescent staining was conducted employing the glucose transporter-1 (GLUT1), a hallmark of brain capillary endothelial cells (Cornford et al., 1994).

TNFR2 expression was restricted to tumour-infiltrating immune cells, mainly structures reminiscent of intravascular neutrophils and recruited macrophages. No endothelial TNFR2 expression was observed in the samples analysed (**Figure 3.8**).

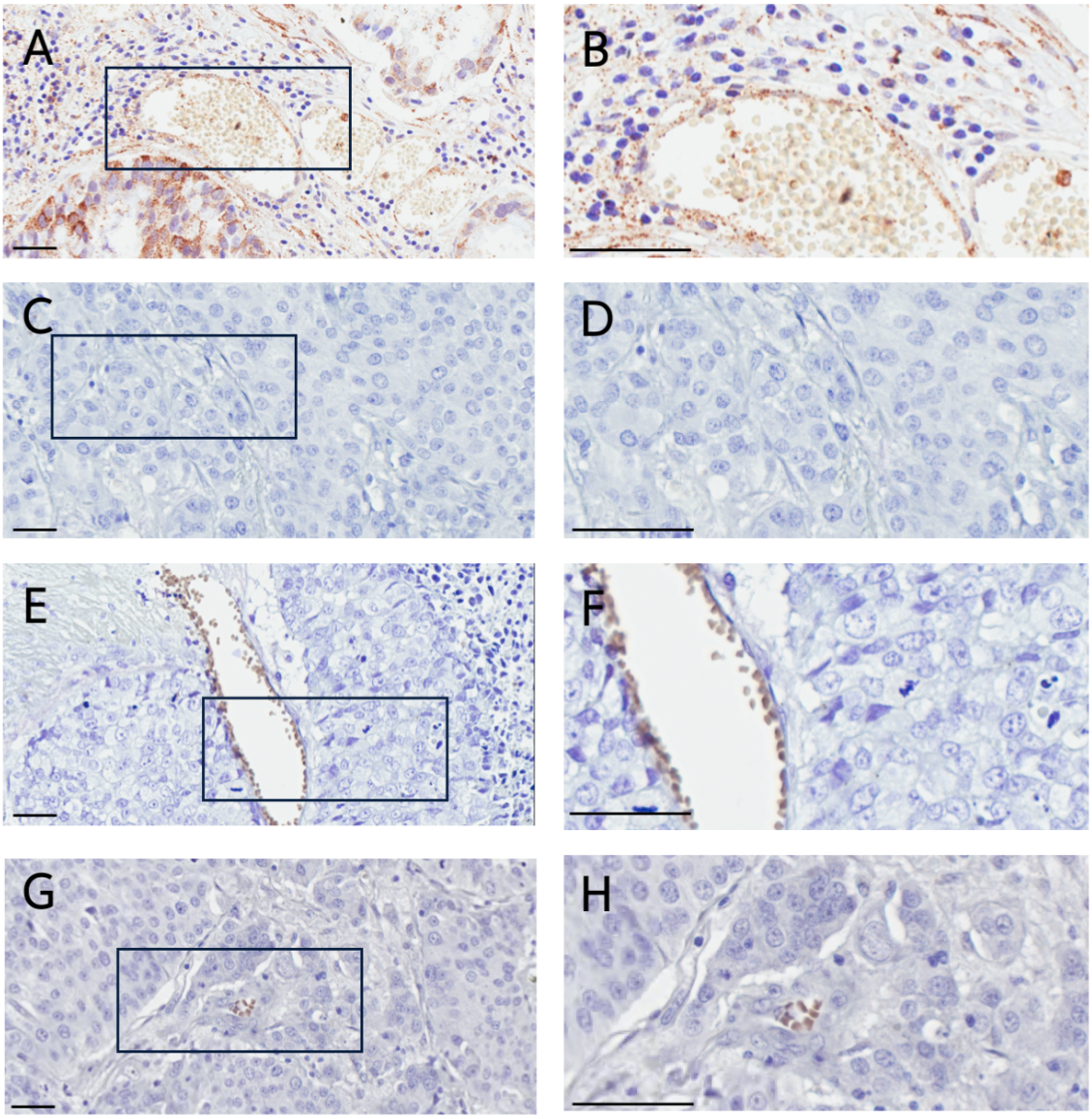


Fig.3.5 TNFR1 and TNFR2 expression in human brain tissue

(A,B) Positive control section showing TNFR1 expression (brown) on a brain vessel adjacent to nonspecific inflammation. **(C,D)** Normal brain tissue showing minimal TNFR1 immunostaining.

(E,F) Positive control section showing non-vascular TNFR2 expression (brown) on section with nonspecific inflammation. **(G,H)** Normal brain tissue showing TNFR2 immunostaining.

B,D,F, H: higher magnification images of boxed areas shown in **A,C,E,G**

Scale bars=50**µ**m.

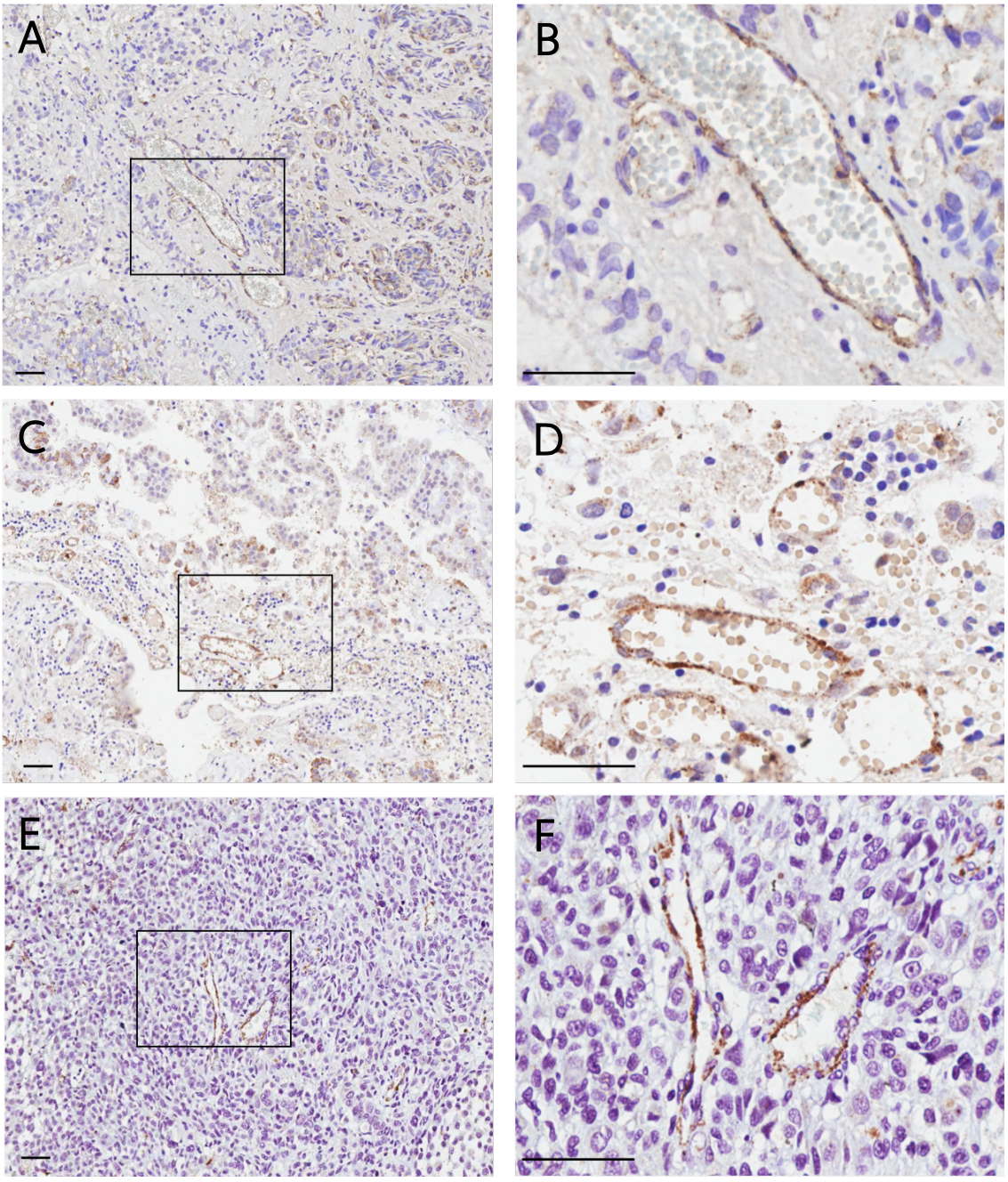


Fig.3.6 Endothelial TNFR1 expression in human brain metastasis

Photomicrographs of human breast carcinoma **(A,B)**, lung adenocarcinoma **(C,D)**, and melanoma **(E,F)** brain metastasis resections stained immunohistochemically against TNFR1 (brown).

B,D,F: higher magnification images of boxed areas shown in **A,C,E**

Scale bars=50**µ**m.

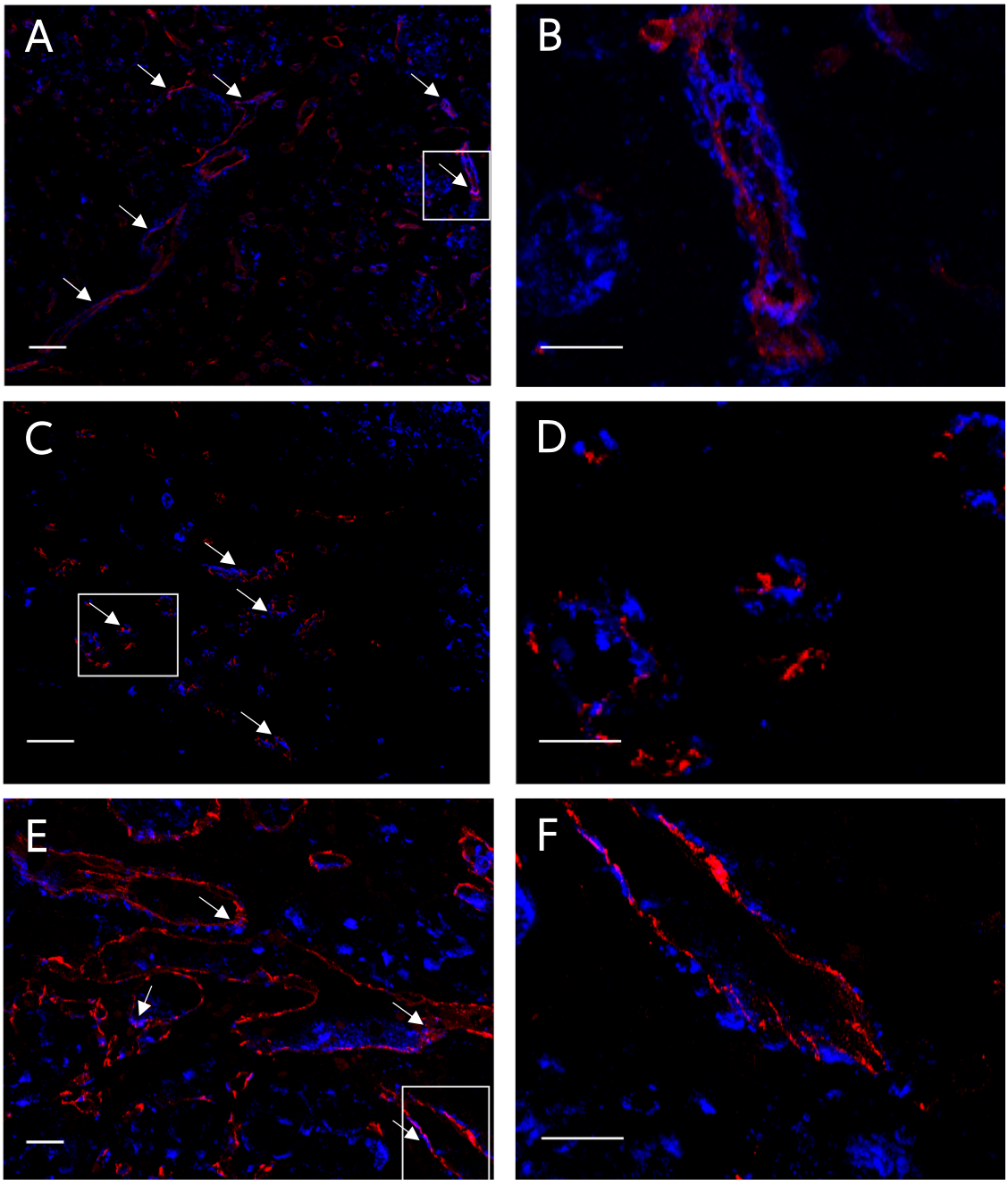


Fig.3.7 Endothelial TNFR1 expression in human brain metastasis

Photomicrographs of human breast carcinoma **(A,B)**, lung adenocarcinoma **(C,D)**, and melanoma **(E,F)** brain metastasis resections stained immunofluorescently against TNFR1 (blue), and the endothelial-specific marker GLUT1(red). Arrows show sites of co-localisation between TNFR1 and GLUT1.

B,D,F: higher magnification images of boxed areas shown in **A,C,E**

Scale bars=50μm.

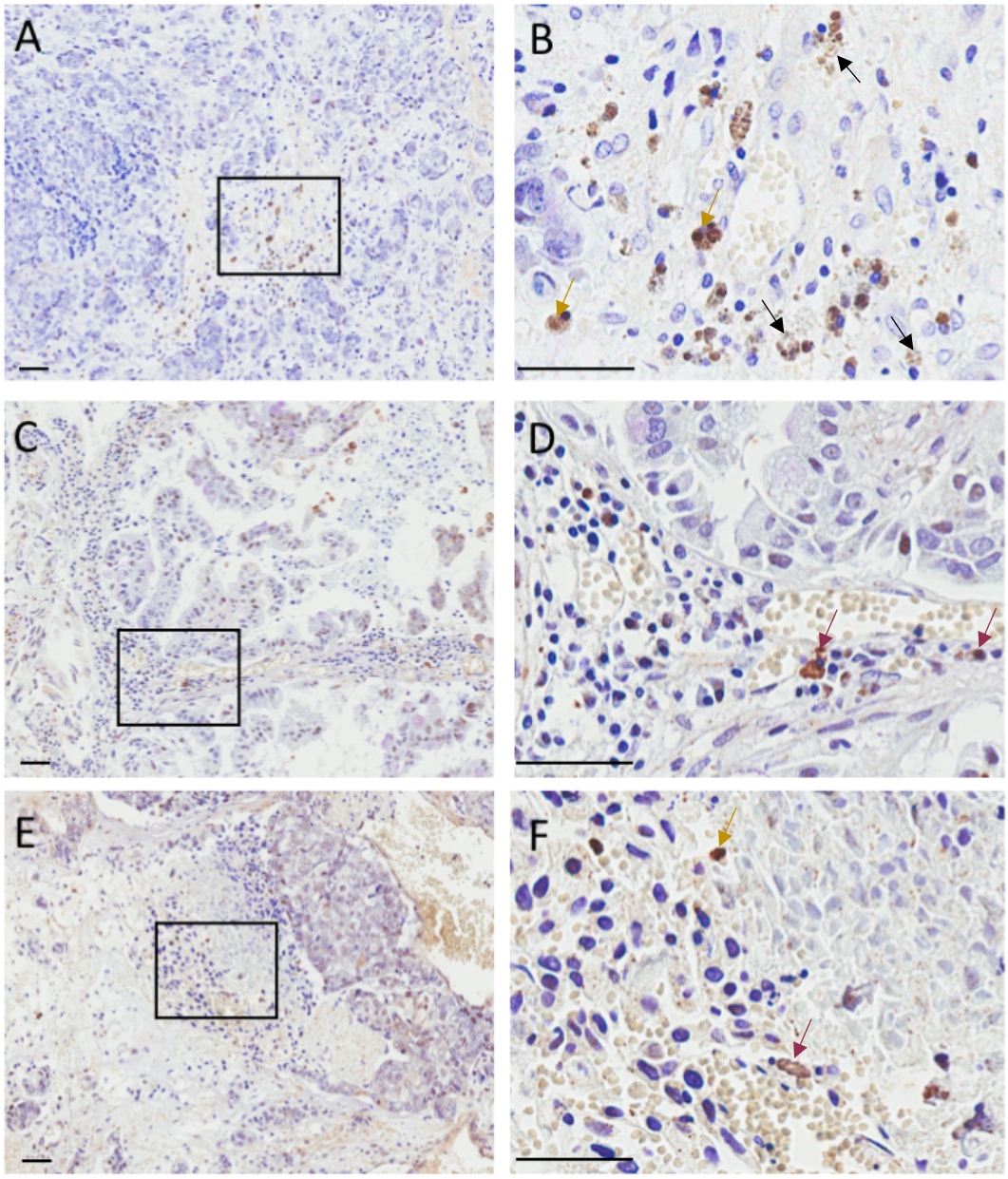


Fig.3.8 Endothelial TNFR2 expression in human brain metastasis

Photomicrographs of human breast carcinoma **(A, B)**, lung adenocarcinoma **(C,D)**, and melanoma **(E,F)** brain metastasis resections stained immunohistochemically against TNFR2 (brown). Black arrows indicate TNFR2 staining on neurons near metastatic tissue. Red arrows indicate TNFR2 staining on recruited leukocytes, and yellow arrows on intravascular neutrophils, respectively.

B,D,F higher magnification images of boxed areas shown in **A,C,E**

Scale bars=50**µ**m.

3.3.4 The role of metastatic tumour cells on vascular endothelial TNFRs expression

3.3.4.1 Validation of the *in vitro* system

In order to elucidate the molecular underpinnings of the specific TNFR1 upregulation in the tumour-associated vasculature, we exposed the human brain microvascular endothelial cell line (hCMEC/D3) to either direct (contact) or distant (indirect) co-culture employing metastatic tumour cells derived from breast cancer, lung adenocarcinoma or melanoma **(Figure 3.9)**. In addition, relevant tumour- conditioned media (TCM) were harvested and processed as described in section 2.1.1.2 for the stimulation of the hCMEC/D3 monolayers. For that purpose, we used the human cancer cell lines previously inoculated in the xenogeneic experimental models discussed; 231Br (breast cancer), SEBTA-001 (lung adenocarcinoma), H1DL2 (melanoma). These tumour cell lines metastasise preferentially to the brain. We also wished to assess the effect of tumour cells with high metastatic potential but no cerebrotropism using the parental clone of the mammary carcinoma cell line MDA-MB-231 (231Par).

TNFR1 and TNFR2 mRNA expression levels in hCMEC/D3 cells were determined using qPCR. Ribosomal protein L4 (*RPL4*) was determined as the optimal housekeeping gene for normalisation. Additionally, TNFRs expression at protein level was illustrated employing immunohistochemical staining.

In order to confirm the biological relevance of the *in vitro* system used, we assessed the proliferation status of the brain endothelial monolayer, and the potential induction

of cytotoxic or pro-apoptotic signalling upon co-culture with metastatic cancer cells or exposure to TCM harvested thereof.

Analysis of endothelial cellular proliferation upon co-culture with tumour cells or TCM exposure was based on the detection of altered cellular DNA content via fluorescent label binding. No significant increase in hCMECs proliferation was reported for any of the stimulations tested. Assessment of cytotoxicity was based on measurement of fluorescence, indicative of the release of substrate-modulating proteases by affected endothelial cells and the resulting presence of cell-impermeant, fluorogenic peptide substrates. None of the treatments assessed resulted in cytotoxicity induction in the endothelial monolayers. Apoptosis induction was detected using the same system and luminogenic signal increases as a readout, indicative of endothelial caspase -3/-7 activation and loss of cell membrane integrity. Only direct co-culture with 231Br cells lead to increased pro-apoptotic signalling ($p<0.05$).

To further explore the effect of both indirect and direct co-cultures on hCMEC/D3 monolayer stability, fluorescent labelling approaches detecting condensed chromatin (Hoechst/ Propidium iodide counterstain) or DNA damage (TUNEL assay) were used. Nuclear counterstain employing the cell-permeant Hoechst 33342 revealed the presence of viable endothelial cells. Double labelling using both Hoechst 33324 and the cellular membrane impermeant dye propidium iodide demonstrated the presence of cells undergoing apoptosis (pink) and dead cells (purple). Evidence of pro-apoptotic signalling was detected for 3% of hCMECs indirectly co-cultured with 231Br cells, and

for 4% of hCMECs directly co-cultured with 231Br cells. No indications of cell death were present for the co-cultures involving SEBTA-001 or H1DL2.

TUNEL assay was conducted in order to detect DNA damage levels in hCMECs directly co-cultured with metastatic cancer cells. None of the treatments led to significant increase of DNA strand breaks compared to the positive control ionomycin (ns, for all comparisons).

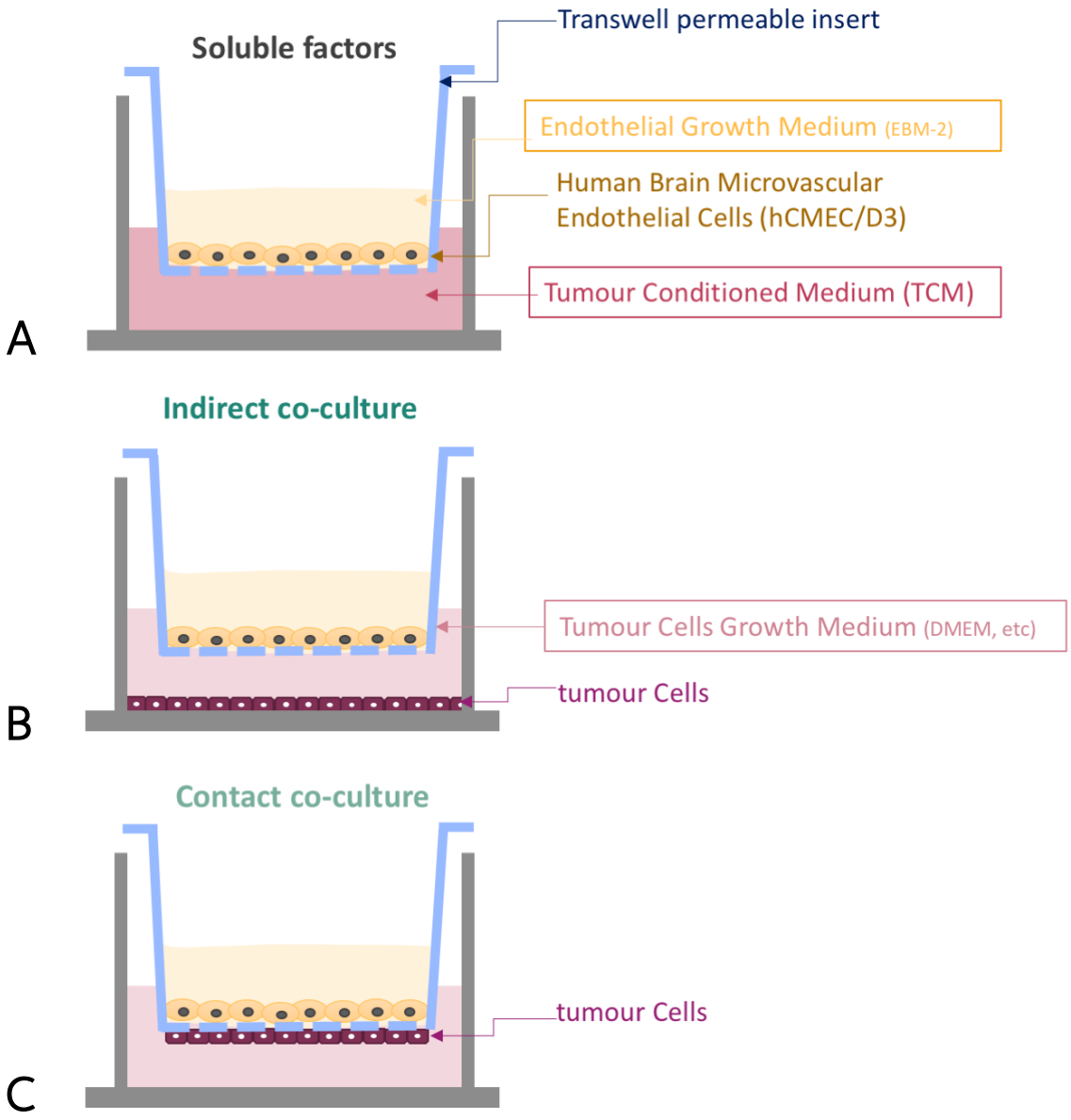


Fig.3.9 Schematic of the *in vitro* system established for the study of the impact of tumour microenvironment on endothelial TNF receptors expression

Human brain microvascular endothelial cells (hCMEC/D3) were seeded on permeable transwell inserts pre-coated with extracellular matrix proteins (collagen IV and fibronectin).

hCMEC/D3 cells were exposed to either tumour-conditioned media (TCM) harvested from cancer cell lines **(A)**, or co-cultured indirectly **(B)** or directly **(C)** with cancer cells.

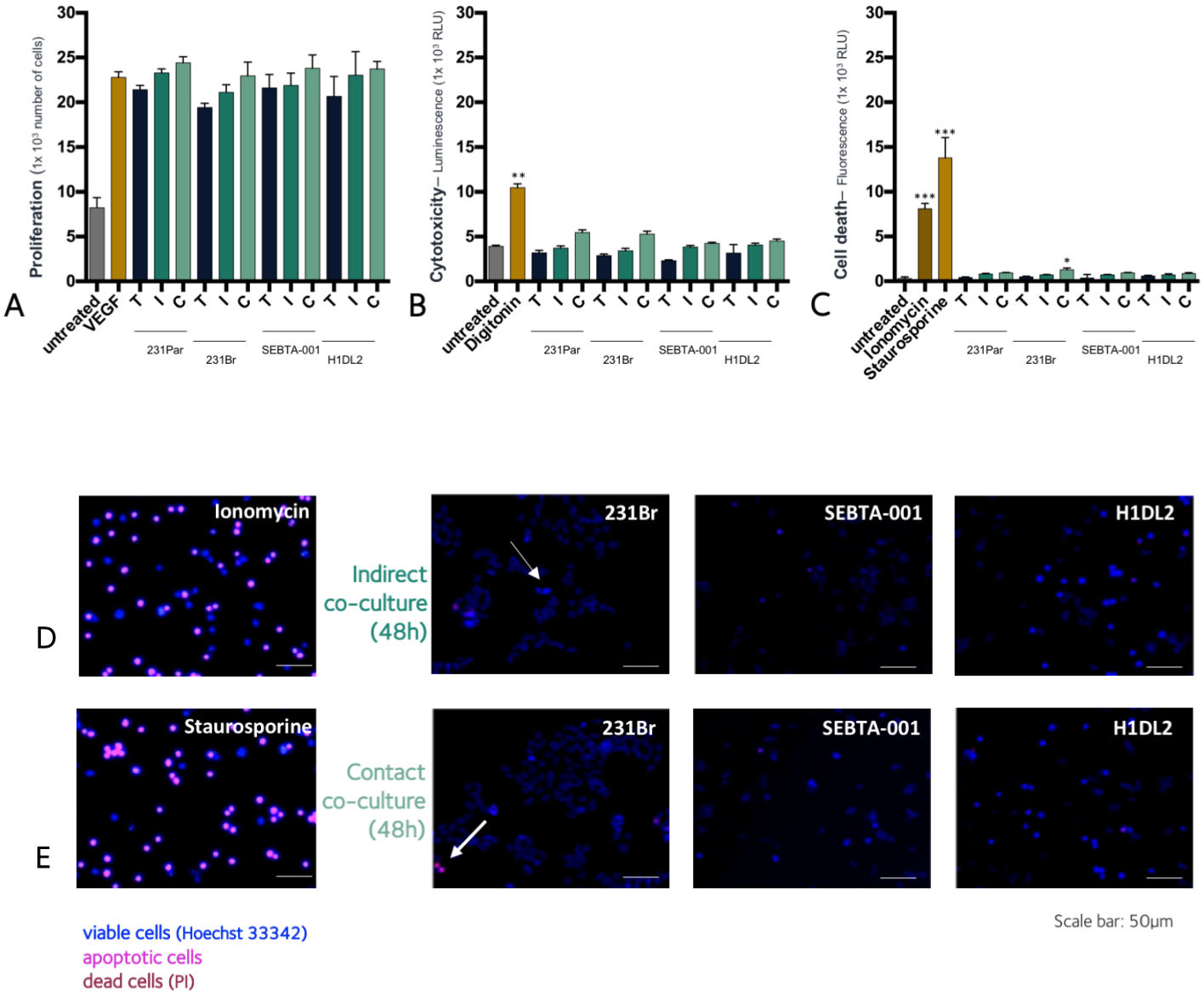
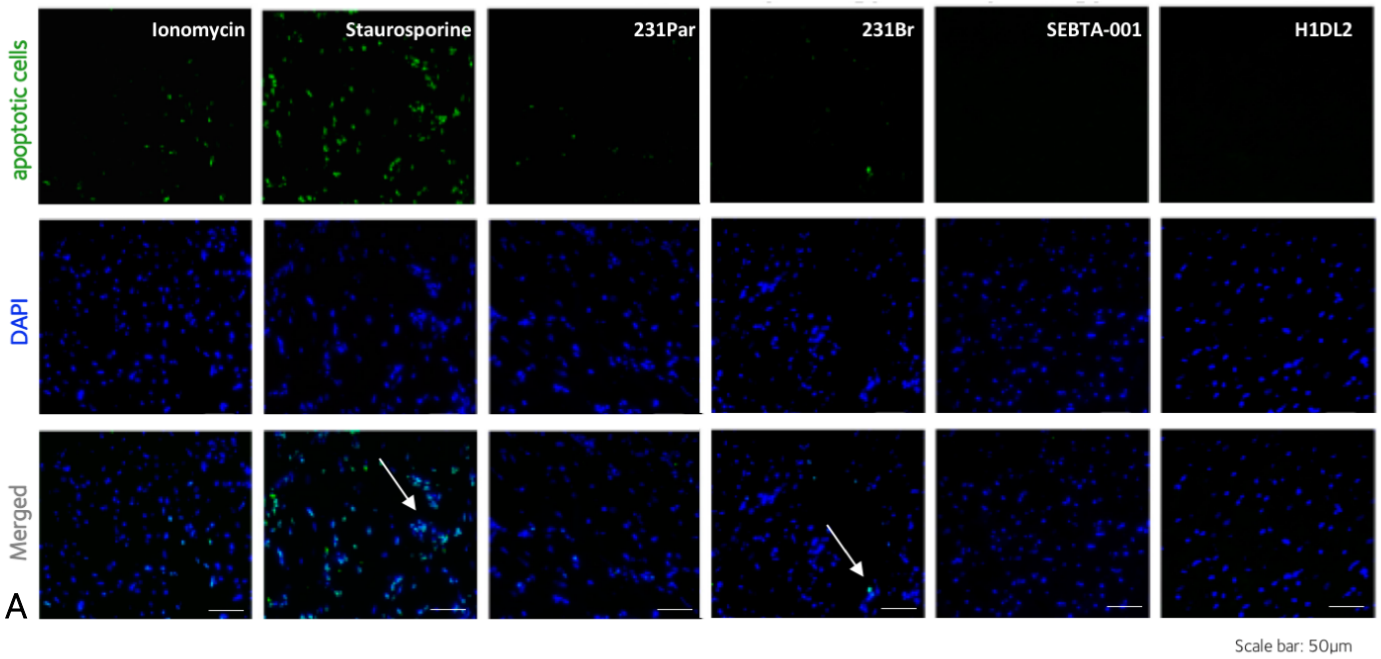


Fig.3.10 The effect of tumour microenvironment on the induction of proliferative or pro-apoptotic signalling in an *in vitro* endothelial monolayer model

A-C: *In vitro* assays assessing cellular proliferation **(A)**, cytotoxicity **(B)**, and apoptotic cell death induction **(C)** in hCMEC/D3 cells exposed to tumour-conditioned media (TCM), or co-culture (indirect; I) or (contact; C) with brain-seeking tumour cells for 48h.
Vascular endothelial growth factor (VEGF, 10ng/ml for 48h): positive control for endothelial proliferation. Digitonin (30mg/ml,15min): positive control for cytotoxicity induction
One way-ANOVA. **p<0.01, ***p<0.001, comparisons vs untreated cells.
Data are presented as mean values ff SEM
D,E: Hoechst 33342/ propidium iodide counterstaining for the detection of condensed chromatin (pink) indicative of apoptosis (arrows) in hCMEC/D3 cells treated with the DNA synthesis inhibitor staurosporine (3µM-18h), or the pro-apoptotic agent ionomycin (10µM-18h) or co-cultured with brain-seeking tumour cells. Scale bars=50µm



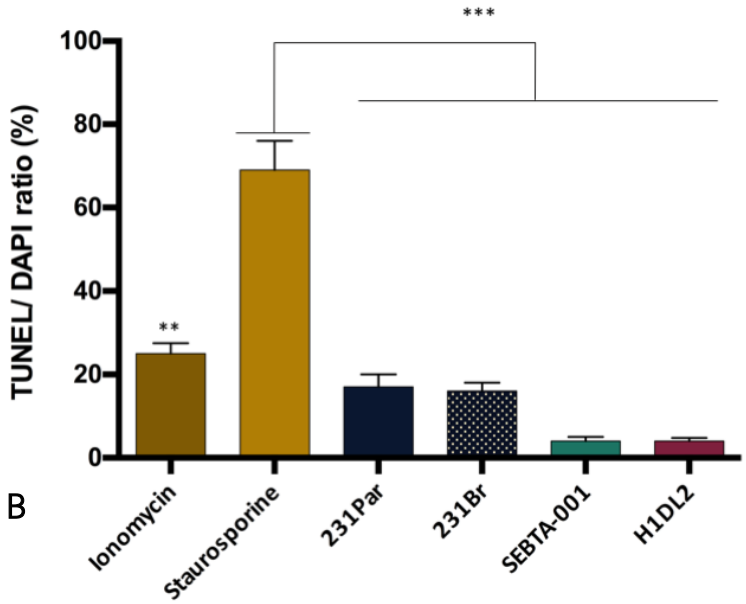


Fig.3.11 Contact co-culture of hCMEC/D3 cells with metastatic cancer cells does not induce apoptosis

A: *In vitro* TUNEL assay detecting DNA double strand breaks (green) indicative of pro-apoptotic signalling. Nuclear counterstain with DAPI (blue). Scale bars=50µm

B: TUNEL/DAPI ratio for the confirmation of cell death induction using the positive controls (ionomycin, staurosporine) in hCMEC/D3 monolayers.

One way-ANOVA. **p<0.01, ***p<0.001, comparisons vs staurosporine.

Data are presented as mean values ff SEM

3.3.4.2 Endothelial activation induced by contact with tumour cells

Exposure of endothelial monolayers to either distant co-culture (indirect) or contact co-culture (direct) elicited remarkable endothelial TNFR1 upregulation, compared to baseline levels, for all brain-seeking metastatic cancer cell lines assessed (231Br, SEBTA-001, H1DL2) **(Figure 3.12)**. On the contrary, co-culture with the non-brain specific clone 231Par did not elicit significant alterations in TNFR1 expression (ns for all comparisons with baseline TNFR1 expression levels). Indirect co-culture yielded an 8-fold increase ($p<0.001$) for 231Br, 17-fold ($p<0.001$) for SEBTA-001, and 18-fold ($p<0.001$) for H1DL2, compared to baseline TNFR1 expression. Lastly, direct co-culture lead to 8-fold increase ($p<0.001$) for 231Br, 18-fold ($p<0.001$) for SEBTA-001, and 18-fold ($p<0.001$) for H1DL2, in comparison to TNFR1 expression in unstimulated hCMEC/D3 cells.

Assessment of alterations in TNFR2 expression **(Figure 3.13)** demonstrated significant increases only upon co-culture with H1DL2, melanoma cells. Indirect and direct co-culture led to a 7-fold increase in endothelial TNFR2 levels ($p<0.001$ compared to TNFR2 expression in untreated hCMEC/D3 cells, in both cases).

3.3.4.3 Endothelial activation induced by soluble factors secreted by tumour cells

Exposure of endothelial monolayers to TCM elicited similar alterations in TNFR1 and TNFR2 expression as the co-culture with tumour cells. Stimulation with TCM led to significant increases in endothelial TNFR1 expression: 7-fold upregulation ($p<0.001$)

for 231Br TCM, 18-fold ($p<0.001$) for SEBTA-001, and 16-fold ($p<0.001$) for H1DL2, compared to baseline endothelial TNFR1 expression levels. However, treatment with TCM derived from the non-brain specific clone 231Par did not increase in TNFR1 expression (ns, compared to baseline endothelial TNFR1 expression levels).

Only stimulation with TCM from the melanoma cell line H1DL2 induced significant upregulation of endothelial TNFR2 expression (6-fold increase, $p<0.001$ compared to TNFR2 levels in untreated hCMEC/D3 cells) **(Figure 3.13)**.

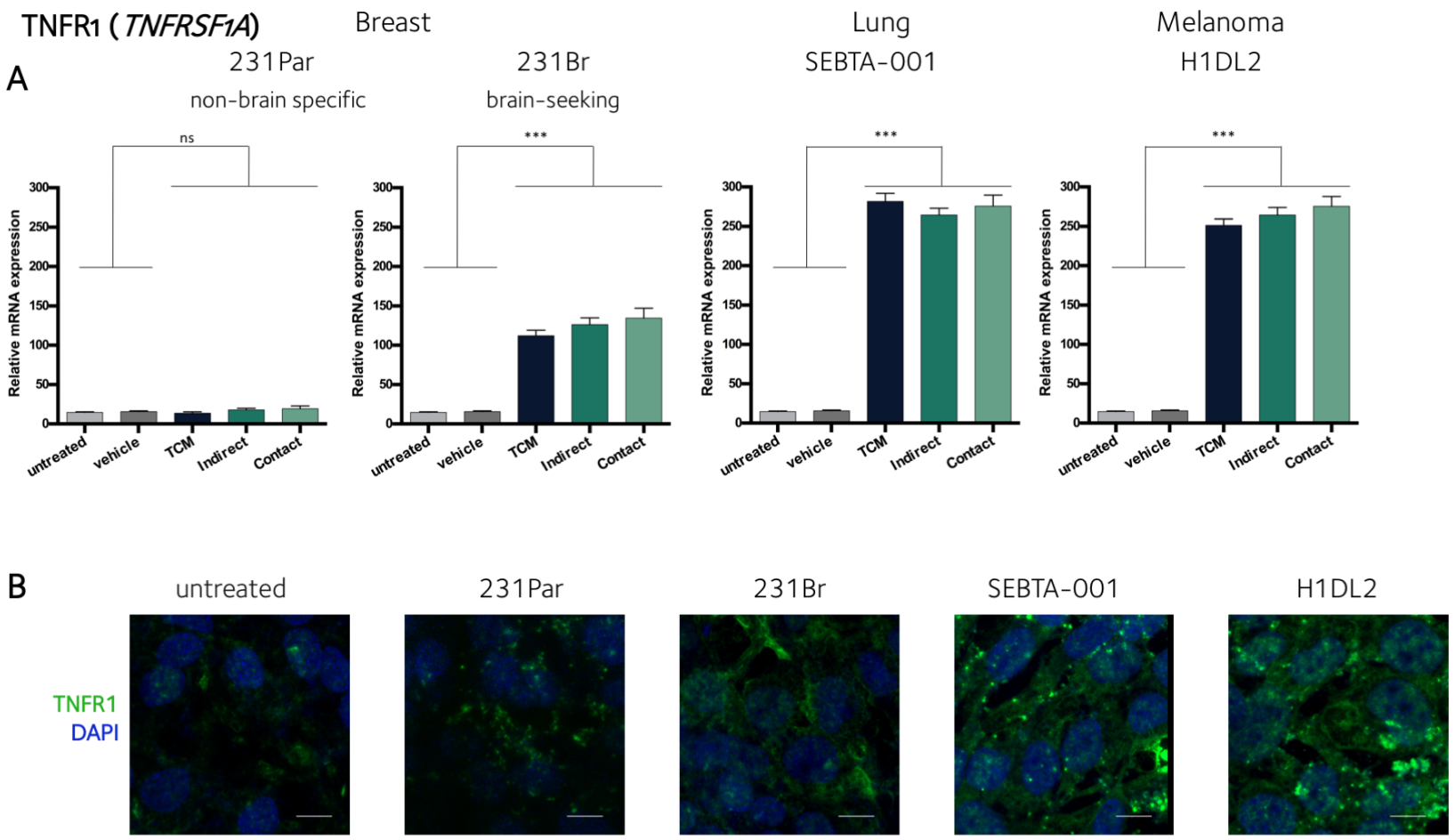


Fig.3.12 Endothelial TNFR1 upregulation upon exposure to brain-seeking tumour cells or soluble factors secreted thereof

A,B: TNFR1 expression at mRNA **(A)** or protein **(B)** level in hCMEC/D3 cells exposed to growth medium (vehicle), tumour-conditioned media (TCM), or co-culture with tumour cells (indirect/contact) for 48h.

A: relative *Tnsfr1a* mRNA expression determined using qPCR. Expression levels were compared to the housekeeping gene RPL4 and normalized to unstimulated hCMEC/D3 cells. One way-ANOVA. ***p<0.001 Data are presented as mean values ff SEM

B: Immunofluorescent staining illustrating TNFR1 expression (green) and localisation in unstimulated hCMEC/D3 cells or hCMECs exposed to TCM derived from breast cancer cells (231Par; non brain-seeking or 231Br; brain-seeking), lung adenocarcinoma cells (SEBTA-001; brain-seeking), and melanoma cells (H1DL2; brain-seeking). Nuclear counterstaining employing DAPI (blue). Scale bars=10μm

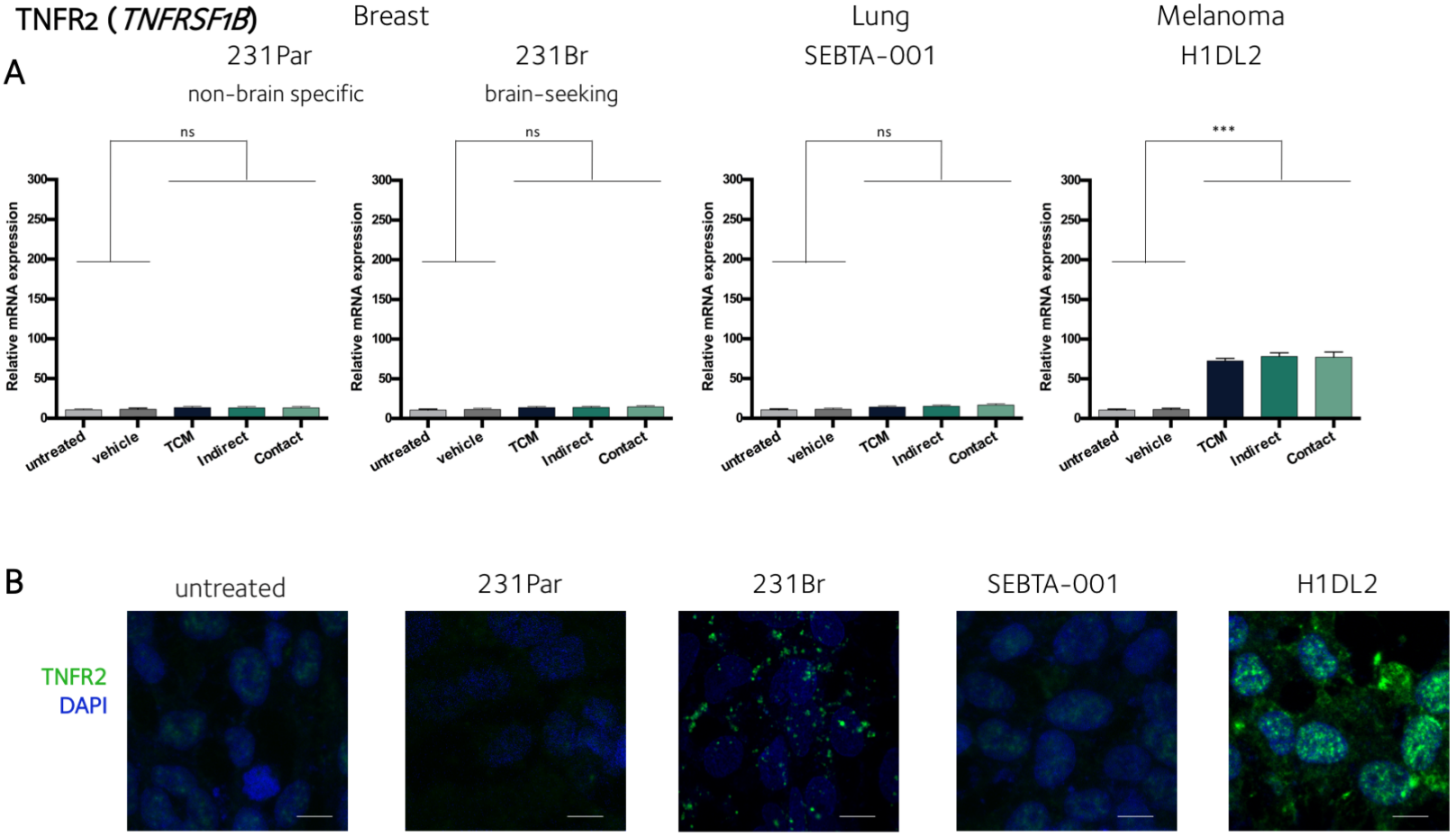


Fig.3.13 Endothelial TNFR2 upregulation upon exposure to brain-seeking tumour cells or soluble factors secreted thereof

A,B: TNFR2 expression at mRNA **(A)** or protein **(B)** level in hCMEC/D3 cells exposed to growth medium (vehicle), tumour-conditioned media (TCM), or co-culture with tumour cells (indirect/contact) for 48h.
A: relative *Tnsfr1b* mRNA expression determined using qPCR. Expression levels were compared to the housekeeping gene RPL4 and normalized to unstimulated hCMEC/D3 cells. One way-ANOVA. ***p<0.001 Data are presented as mean values ff SEM
B: Immunofluorescent staining illustrating TNFR2 expression (green) and localisation in unstimulated hCMEC/D3 cells or hCMECs exposed to TCM derived from breast cancer cells (231Par; non brain-seeking or 231Br; brain-seeking), lung adenocarcinoma cells (SEBTA-001; brain-seeking), and melanoma cells (H1DL2; brain-seeking). Nuclear counterstaining employing DAPI (blue). Scale bars=10μm

3.4 Discussion

3.4.1 Characterisation of the impact of tumour microenvironment on the TNFR1-associated endothelial activation

The studies presented in this chapter involve the syngeneic BALB/c-4T1-GFP model of breast-to-brain metastasis and the xenogeneic models of brain metastasis from breast cancer, lung adenocarcinoma and melanoma. Both haematogenous dissemination of tumour cells and direct introduction into the brain were employed for the models established.

The observed pattern of metastatic growth in both the intracardiac, and the intracerebral models was indicative of tumour establishment and proliferation along the pre-existing cerebral vasculature, suggesting that co-optive growth rather than neovascularisation (indicative of neoangiogenesis) was ongoing. This is reminiscent of the early stages of secondary tumour development at the brain parenchyma in humans with metastatic disease from breast, lung and melanoma, with analysis of early stage biopsies demonstrating that micrometastases develop along blood vessels (Carbonell et al., 2009).

The similarities between the BALB/c-4T1-GFP mice and the SCID mice inoculated with the human cell line MDA-MB-231-eGFP suggest that the syngeneic model is relevant to the clinical situation. Moreover, the BALB/c mice are immunocompetent and enable the study of the entire immune response of the host and, of the paracrine

signaling with the brain endothelium. Taken together, the *in vivo* models used, provide a powerful tool recapitulating the interactions between the tumour infiltrating cells and the resident immune cells (activated brain endothelial cells, reactive astrocytes, microglia). Immunohistochemical analysis is a well-established approach for the determination of protein targets specifically expressed either in the tumour colonies or in the brain parenchyma during metastatic progression (Müller et al., 2001; Soto et al., 2014).

TNFR1 upregulation was detected selectively in the cerebrovascular endothelium associated with metastatic colonies. TNFR1 expression was characterised by a consistent spatial pattern of endothelial upregulation, regardless of the primary site of origin for the tumour cells inoculated. Vessels present within the tumour core and the tumour boundary exhibited the highest percentage of TNFR1 positivity. Additionally, vessels within 100μm from the tumour margin were TNFR1 positive to a significant extent, compared to TNFR1 expression in the brain endothelium of naïve mice. This spatial pattern suggests the implication of dynamic interactions within the perivascular niche that are associated with the transcriptional regulation of endothelial TNFR1 expression.

TNFR1 upregulation is indicative of endothelial activation in response to the presence of metastatic tumour cells in the brain parenchyma. Since the models assessed focused on the study of early brain metastasis, it is consensual that the observed TNFR1 upregulation is an event preceding macrometastatic growth and neovascularisation. It is plausible that the vascular endothelium is being primed by factors secreted by

disseminating tumour cells during the arrest step at the vascular walls. Moreover, endothelial activation and concomitant increased TNFR1 expression could be triggered during the extravasation step, due to dynamic interaction of the vascular endothelium with the metastasising cells. This hypothesis is supported by the TNFR1-stimulatory effect of direct co-culture of hCMEC cells with brain-seeking cancer cells. Alternatively, TNFR1 expression could be dictated at a later point, following the metastatic seeding step, since exposure of brain endothelial monolayers to soluble factors secreted by tumour cells is sufficient for the induction of TNFR1 expression. In line with the above scenarios, TNFR1 expression could be dictated by sustained signalling that is induced during the extravasation step and is further enhanced during the following metastatic colonisation and tumour growth stages. Nevertheless, the detection of extensive TNFR1 expression in vessels associated with micrometastases of less than 50μm in diameter, is suggestive of the early onset of TNFR1 upregulation, possibly as part of the co-optive growth of freshly established micrometastases.

The specificity of the TNFR1 upregulation within the vicinity of metastatic deposits, but also the temporal profile of the underlying endothelial activation, point to the direction of the tumour-associated neuroinflammatory responses. Activation of astrocytes and microglia are hallmarks of the local innate immune response occurring during the early stages of metastatic seeding in the brain. Both reactive astrocytes (Akassoglou et al., 1998; Chen et al., 2016), and microglia (Kuno et al., 2005) have been shown to release TNF, following their stimulation during the early stages of brain metastasis formation. In this context, endothelial TNFR1 expression could be triggered

either directly through the secretion of its endogenous ligand, or indirectly by other vaso-modulating agents secreted as a response to TNF-induced pro-inflammatory signalling.

On the contrary, TNFR2 was detected primarily in peritumoural, non-vascular structures suggestive of tumour-infiltrating immune cells; mainly intravascular neutrophils and recruited microglia/ macrophages and neurons. Microglia, the CNS resident macrophages are major components of the immune response to brain metastatic growth and their clustering in tumour-surrounding tissue has been reported both for experimental and human brain metastases (Lorger & Felding-Habermann, 2010; Berghoff et al., 2013; O'Brien et al., 2014; Andreou et al., 2017). Peritumoural accumulation of HLA-DR+ microglia/ macrophages was evidenced via histological analysis of intracranial lesions established upon implantation of breast, melanoma, small cell lung, and NSCLC cell lines (Berghoff et al., 2013). Additionally, other immune cell types including neutrophils and monocytes are known to preferentially cross the vascular basement membrane at sites with low levels of junctional proteins, thus harnessing the paracellular clefts transiently formed during the tumour cells extravasation process. The passage of leukocytes through the BBB is potentiated by endothelial expression of CAMs such as PECAM-1, ICAM-1, VCAM-1 (Ley et al., 2007). As previously discussed these CAMs constitute hallmarks of endothelial activation during early brain metastasis, and are overexpressed owing to the intercellular contact with the disseminating tumour cells (Serres et al., 2012; Soto et

al., 2014). In line with these findings, TNFR2 expression has been previously confirmed in tumour-infiltrating immune cells (Chen et al., 2013).

Importantly, the observed TNFR1 and TNFR2 expression patterns in the experimental models of brain metastasis corroborate the findings arising from the histological assessment of human brain metastasis resections. This homogeneity of TNFR1 and TNFR2 distribution not only confirms the physiological relevance of the *in vivo* models employed, but also holds promise for the translation of endothelial TNFR1-targetting strategies into the human condition.

Furthermore, the discussed TNFR1 and TNFR2 expression patterns within the tumour-surrounding stroma were consistent for secondary brain tumours arising from breast carcinoma or lung adenocarcinoma or melanoma, both in the experimental models used and human biopsies tissue. The molecular underpinnings underlying the metastatic dissemination from these primary sites have been reported to differ in many stages of the metastatic cascade. However, despite the distinct genetic and metabolic profiles of these metastasising tumour cells, their impact on the brain endothelium remains unique. It is plausible that common soluble factors, dictating endothelial activation and concomitant TNFR1 upregulation, are secreted by all brain-seeking cell lines assessed. Moreover, the differential effects of the non-brain seeking breast carcinoma parental clone (231Par), and its variant that metastasises preferentially to the brain (231Br) endothelial TNFR1 expression, suggest that molecular determinant of TNFR1 upregulation might also be associated with increased cerebrotropism. Preliminary evidence from *in vitro* proliferation and cytotoxicity screening employing

relevant TCM suggests that the soluble factors contained do not enhance endothelial proliferation or induce pro-apoptotic signalling within the brain microvascular endothelium. Furthermore, the absence of cytotoxicity and pro-apoptotic signalling upon either prolonged exposure of endothelial monolayers to TCM or direct co-culture with metastatic cancer cell lines reflects in the *in vitro* setting, the existence of an intact, functional BBB during the early stages of the metastatic outgrowth. These findings suggest that the *in vitro* model established recapitulates the reciprocal cross-talk ongoing between tumour cells and the brain endothelium following the metastatic seeding step.

Ongoing proteomic screening of these factors aims to elucidate the molecular drivers of endothelial activation and TNFR1 upregulation during early brain metastasis. Finally, characterisation of the distinct secretomes of metastatic breast and lung cancer, and melanoma cells will delineate points of convergence, but also factors associated with differential responses such as the melanoma-induced upregulation of endothelial TNFR2 expression upon prolonged stimulation. The diverse effects elicited by the various TCM might be a reflection of different molecular contents also involved in the diverse temporal patterns of arrest during extravasation (Lorger & Felding-Habermann, 2010; Paku et al., 2000; Kienast et al., 2010). The survival of the metastasising cells during the arrest step is dependent on their interactions with the components of the vascular wall (JuanYin et al., 2009).

3.4.2 Clinical implications

Evidence from the human biopsies analysed suggests that the endothelial TNFR1 expression is triggered by the presence of metastatic tumour cells from breast cancer, lung adenocarcinoma and melanoma. Since these primary tumour sites are the most common sources of synchronous or metachronous metastatic progression to the CNS, the potential for applicability of endothelial TNFR1 upregulation as a biomarker of early brain metastasis is enhanced.

The upregulation of TNFR1 selectively at sites of brain metastasis, and not in the vasculature of unaffected brains, provides a possible link for the observed selectivity of the TNF-induced BBB permeabilisation. Moreover, the increased expression of the receptor in the luminal side of tumour-associated vasculature renders the endothelium susceptible to the vasomodulating effects of circulating TNF. The causal implication of endothelial TNFR1 activation on the observed TNF-induced BBB breakdown remained to be further assessed through approaches targeting TNFR1-selectively (Chapter 4).

Advanced metastatic lesions often present necrotic tumour cores, devoid of intact BBB/BTB that are accessible by systemically administered chemotherapeutic agents. However, the invasive tumour edge of such colonies is covered by a usually heterogeneously leaky BBB/BTB that impedes or limits the uptake of systemic therapies to clinically relevant doses (Lockman et al., 2010; Morikawa et al., 2015). The specific presence and the spread of TNFR1 expressing vessels throughout the tumour boundary and within the tumour-surrounding vasculature allows for the

potential use of the vaso-modulating TNF as a surrogate that could ameliorate the access of diagnostic imaging agents and targeted therapeutic agents at metastasis sites. Moreover, the presence of TNFR1 in the cerebrovascular endothelium associated with both micrometastases and established macrometastases potentiates the detection of asymptomatic, currently undetectable micrometastases that lie below the current detection limit in the clinic. In addition, the delivery of chemotherapeutics at smaller and less aggressive metastases has been shown to be more therapeutically efficacious than the targeting of macrometastases (Osswald et al., 2016).

Chapter 4| TNFR1-mediated permeabilisation of the blood-brain barrier at sites of metastasis

4.1 Introduction

The following chapter describes the studies conducted, focusing i) on the impact of the endothelial TNFR1 upregulation in the tumour-associated vasculature in the context of TNF-induced permeabilisation, and ii) on the effect of TNF muteins targeting TNFR1 selectively in order to develop novel constructs that could facilitate a selective and transient BBB breakdown devoid of the systemic toxicities associated with the activation of TNFR2, or the activation of both receptors. This strategy could provide a window of opportunity for early diagnosis and intervention.

4.1.1 TNF-induced endothelial activation

TNF is crucial for the onset and the termination of prolonged inflammatory responses (Madge & Pober, 2001) and acts as a reversible stimulator of endothelial activation. This master pro-inflammatory cytokine regulates a diverse array of cellular responses in the endothelial cells that lead to compromised endothelial integrity (Friedl et al., 2002; Kerkar et al., 2006) and to concomitant increased paracellular permeability (Megyeri et al., 1992; Saija et al., 1995). The molecular underpinnings of this TNF-induced endothelial dysfunction span from interendothelial junctional disassembly (de Vries et al., 1996), dissociation of intercellular adhesion molecules (Lampugnani, M.G.

et al., 1992; Romer et al., 1995), F-actin depolymerisation (Goldblum et al., 1993); and altered expression of cytoskeletal proteins (Stolpen et al., 1986; Blum et al., 1997; Petrache et al., 2003), to transcriptional upregulation of numerous inflammatory genes. The latter, is driven by the transcription factor NF-κB and by AP-1, a downstream target of JNK (Baud & Karin, 2001; Chen & Goeddel, 2002; Wajant, Pfizenmaier, & Scheurich, 2003). The ligation of TNF receptors elicits the acute activation of NF-κB, the subsequent increased expression of adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin (de Vries et al., 1996), and of inflammatory mediators (cytokines; IL-6 and IL-8, and chemokines; CCL-5, CXCL8, VEGF, FGF) (O'Carroll et al., 2015). Moreover, the glycocalyx that is surrounding the brain endothelium is prone to TNF-dependent disruption involving the activation of MMPs (Henry & Duling, 2000; Ramnath et al., 2014).

The molecular mechanisms underlying the TNF-induced endothelial activation remain elusive. TNF simultaneously activates pro-apoptotic pathways (e.g. caspase activation), alongside pro-survival pathways (e.g. NF-κB pathway). The crosstalk between the major TNF-induced pathways is the ultimate determinant of cellular fate (Gratton et al., 2001; Aggarwal, 2003). Activation of the p38 MAPK pathway is required for TNF-mediated apoptosis in endothelial cells. However, the endothelium eludes apoptosis as TNF stimulates the upregulation of the type 2A protein phosphatases (PP2A) within 8h (Janzen et al., 2011). PP2A catalyses the dephosphorylation of both p38 (Grethe et al.,2004) and 4E-BP1 (Elia et al., 2008), thus terminating the transient activation of p38. Abrogation of PP2A activity through

exposure to inhibitors of protein synthesis (cycloheximide, etc.) triggers the TNF-induced apoptosis associated with the preservation of p38 activity and 4E degradation. Consistent with these findings, caspase-3 activity, indicative of activation of the pro-apoptotic cascade, is increased soon after TNF treatment (4h), but does not further promote cell death, as this expression pattern is reversed at 8h post stimulation (Janzen et al., 2011). *In vitro* studies have revealed the resistance of brain microvascular endothelial monolayers to TNF-induced pro-apoptotic signalling (Wosik et al., 2007).

4.1.2 The role of the cytoskeleton in BBB integrity

The cytoskeleton is a dynamic scaffold comprising protein filaments that pervade the cellular cytoplasm. This multiprotein complex confers structure and rigidity to the cell, whilst dictating its subcellular organisation, the intracellular transport of molecules, cell division and motility. The composition of the cytoskeleton involves three classes of protein filaments: microfilaments, microtubules, and intermediate filaments. Each class is assembled from non-covalently bound monomers. Actin filaments consist of parallel protofilaments that are composed of linear homopolymers of actin monomers, termed globular actin (G-actin). Myosin is a hexameric protein, member of the actin-specific molecular motors superfamily. Each myosin monomer includes pairs of long heavy chains (MHC), regulatory light chains (MRLC), and essential light chains (ELC).

The polarised movement of anti-parallel myosin bundles across actin filaments is the main driving force of the cytoskeleton (Vicente-Manzanares et al., 2009). Filamentous actin (F-actin) and non-muscle myosin II motor proteins (collectively termed actomyosin) are the main cytoskeletal components governing mechanotransduction (Schnittler et al., 1990). Actomyosin forms a contractile and cross-linked network that underlies the cell cortex that is attached to the plasma membrane (Salbreux et al., 2012). The actomyosin machinery can generate both protrusive and contractile, polarised forces that facilitate cell motility.

Upon activation, G-actin shifts to F-actin and assembles into contractile actin bundles, the stress fibres (Adyshev et al., 2013). The latter, anchor to the membranes of neighbouring endothelial cells. The remodelling of the perijunctional F-actin is accompanied by alterations in its subcellular distribution and/or its expression (Ivanov et al., 2005). These alterations in the intercellular contacts lead to the disruption of the BBB, followed by increased vascular leakiness (Fischer et al., 2002), and by subsequent vasogenic oedema (Manaenko et al., 2018; Rosenberg & Yang, 2007; Stamatovic et al., 2006).

The BBB integrates opposing signals from the intercellular tethering forces generated by the interendothelial junctional complexes and the contractile state of the cytoskeleton. The continuous belt of tight junctions (TJs) and adherens junctions (AJs) that seals adjacent brain capillary endothelial cells has been demonstrated to play an integral structural and functional role in the maintenance of the BBB integrity *in vivo* (Hawkins & Davis, 2005; Dejana et al., 2008;; Millan et al., 2010).

The AJs are multiprotein junctional complexes located at the basal membrane that have a characteristic jagged pattern. The AJs are not only holding the neighbouring endothelial cells together, but also restrain the TJs (Dejana, 2004). They are formed by cytosolic catenins and transmembrane vascular endothelial cadherins that associate with each other in the extracellular space (Dejana, 2004). The AJs establish intercellular interactions linked to actin filaments that are anchored to the plasma membrane and the ECM via focal adhesions (FA) (Huveneers et al., 2012; Millan et al., 2010).

The continuous brain endothelial TJ complexes are contributing to the integrity of the BBB since they have both structural and signalling roles, and also occlude the paracellular cleft. These complexes endow the brain endothelium with two features. The *"gate function"* involves the restriction of paracellular permeability. The *"fence function"* refers to the limited transcellular diffusion of circulating substances and the lateral diffusion of membrane proteins, alongside the anchorage of proteins between the apical and basolateral membrane (van Meer & Simons, 1986; Gonzalez-Mariscal et al., 2003; Abbott et al., 2010; Schlunk & Greenberg, 2015). These TJ complexes are parallel, anastomosing, membrane-spanning protein strands that are organised as series of multiple barriers and are situated at the lateral membrane. The building blocks of TJs are transcellular adhesion proteins, subdivided in integral membrane proteins (occludin and claudins), and members of the immunoglobulin family; the junctional adhesion molecules (JAM-A, -B, and -C); and the endothelial cell-selective adhesion molecule (ESAM) that anchor to the cytoskeleton via cytoplasmic adaptor proteins,

such as the zonula occludens (ZO)-1, -2, -3 proteins and cingulin that are the PDZ containing molecules. These scaffolding proteins serve as signal integrators that bridge the transmembrane proteins with the actin cytoskeleton (Afonso et al., 2007). Moreover, the function of polarity proteins (also named PDZ lacking proteins) such as the atypical protein kinase C (PKCζ and λ), initiates junctional formation and defines cell polarity (Stamatovic et al., 2016). The last class of TJ-associated proteins are the heterotrimeric G-proteins (Gαi), such as RhoA, that interact with ZO-1 and play an important role in the biogenesis and regulation of the TJ complex (Tornavaca et al., 2015). The RhoGTPases (RhoA, Cdc42 and Rac) regulate actin organisation and trafficking and will be further discussed in section 5.1.1. These proteins are interacting with the TJ complex using their exchange factors as scaffolding proteins (Stamatovic et al., 2016).

Brain endothelial cells also possess gap junctions (GJs) formed amongst adjacent endothelial cells or between multiple cell types of the NVU. GJs are formed by connexins and regulate intercellular communication (Johnson et al., 2018). AJs, TJs and GJs are often intermingled in order to form the complex aforementioned zonular system.

4.1.2.1 Molecular organisation of tight junctions and adherens junctions at the BBB

Claudin-5 is the major member of the family of scaffolding proteins that is constitutively expressed at the BBB, followed by claudin-3 that is regulating the embryonic and the postnatal development of the BBB, and is at least 1000-fold less

abundant in adult brain endothelial cells (Ohtsuki et al., 2008). Claudin-1 and -12 expression is inducible in the brain endothelium (Pfeiffer et al., 2011). Occludin is predominantly interacting with claudin-based strands and binds to scaffolding proteins such as ZO-1, ZO-2 and F-actin, thus forming a TJ platform for signal transduction. Therefore, occludin exerts a regulatory role rather than acting as an adhesion molecule by itself (Cording et al., 2013; Li et al., 2005). ESAM has recently emerged as a mediator of neutrophils extravasation during the onset of neuroinflammation (Wegmann et al.,2004).

The redistribution of TJ proteins is often preceded by transcriptional regulation or by post-translational modifications including phosphorylation, methylation, glycosylation and palmitoylation status (Cui et al., 2013; Stamatovic et al., 2016). Phosphorylation is regulating the functions of occludin, claudin-5, the large phosphoprotein ZO-1, the AJ proteins VE-cadherin and β-catenin, and actin binding proteins (Stamatovic et al., 2006; Yamamoto et al., 2008). Interestingly, pro-inflammatory factors (TNF, IL-6, LPS, etc.) promote ZO-1 phosphorylation and its concomitant downregulation, thereby inducing the activation of signalling pathways including PKC, ROCK, p38/ JNK that elicit BBB dysfunction (Rochfort & Cummins, 2015; Stamatovic et al., 2016). Palmitoylation, the covalent attachment of fatty acids, controls the localisation of transmembrane TJs within the membrane. The methylation status of TJ proteins is associated with the regulation of phosphorylation, acetylation and protein-protein interactions. Moreover, glycosylation controls the activation of critical signalling kinases. Aberrations in the expression or the localisation of TJs,

alongside post-translational modifications lead to increased paracellular permeability of solutes across the BBB (Deli et al., 2005).

Genetic or pharmacologic inhibition of VE-cadherin activity and of its tight junctional counterpart, claudin-5, compromises the vascular integrity and contributes to increased BBB compromise and transmigration of leukocytes (Gotsch et al., 1997; Liebner et al., 2008; Taddei et al., 2008). In addition, diminished claudin-3, and -5 expression is associated with BBB hyperpermeability *in vivo* (Wolburg et al., 2003; Liu et al., 2015).

To sum up, the execution of these convoluted processes requires the polymerisation of actin dynamics, the organisation of actin networks and myosin contractility. This fine-tuned cytoskeletal organisation integrates multiple parallel signalling cascades governed by dynamic protein-protein interactions and spatiotemporal alterations in the expression patterns of second messengers.

4.1.2.2 The role of junctional proteins on BBB dysfunction

The relocation and redistribution of junction proteins under pathological conditions might potentiate BBB dysfunction through the reduction of the adhesive properties of transmembrane molecules or the acquisition of new functions. The redistribution of claudin-5, occludin and VE-cadherin is causally implicated in the loosening of the adhesion at the TJ complex and the formation of paracellular gaps (Lopez-Ramirez et al., 2012; Park et al., 2013; Stamatovic et al., 2006). On the contrary, the redistribution

of junction proteins may not only alter the physical properties of the barrier but also interfere with signalling cascades enhancing BBB disruption.

The proteolytic degradation of junctional proteins that is involved in increased BBB compromise is executed either by matrix- metalloproteinases (MMPs) or by the lysosomes and the proteasome. The fate of the proteins undergoing cleavage depends on the context, the duration and the severity of the inflammatory stimulation. Under mild conditions the proteins processed might be recycled to the cell membrane and become counterparts of functional complexes. On the contrary, severe or prolonged BBB dysfunction is associated with lysosomal degradation of the targets.

4.1.2.3 The role of non-muscular MLCK

The non-muscular myosin light-chain kinase (nmMLCK) is the most prominent regulator of myosin activation (Biro et al., 2014; Lee & Dominguez, 2010). Phosphorylation of MLC by nmMLCK is a key determinant of endothelial barrier integrity (Edelman et al., 1992). Upregulated nmMLCK expression elicits nmMLCK-dependent contraction of endothelial cells and disruption of the TJ complex involving claudin-5 and occludin (Al-Sadi et al., 2011; Mirzapoiazova et al., 2011; Haorah et al., 2005). nmMLCK catalyses the Ca^{2+}- dependent MLC phosphorylation (Garcia et al., 1995; Goeckeler & Wysolmerski, 1995), the subsequent activation of the Mg^{2+}-myosin ATPase that fires the contraction of the perijunctional actomyosin filaments, and the formation of stress fibres (Suzuki & Takahashi, 2001). Fluctuations in Ca^{2+} gradients have been demonstrated in both rat and mouse brain capillary endothelial cells (Feine

et al., 2012; Paemeleire et al., 1999) and are more likely to represent priming events at a single cell level. Cellular contraction is considered to have minimal effect due to the passive, elastic compensation offered by neighbouring cells, thus supporting the concept that coordinated responses are required for the presentation of BBB dysfunction (Demer et al., 1993). nmMLCK expression peaks at brain endothelial cells at 24h post BSA treatment and stimulates the activation of the astrocytic nmMLCK isoforms, thereby perpetuating the cyclic disruption of the BBB (Rossi et al., 2011). Mechanistically, nmMLCK upregulation is characterised as a precipitating event leading to BBB disruption (Haorah et al., 2005). The latter, is accompanied by extravasation of determinants of neuroinflammation, peaking at 72h after traumatic brain injury (TBI) (Rossi et al., 2013).

As previously described, MLC phosphorylation is also under the regulation of numerous kinases, including ROCK and PKC and NIK, and regardless of its upstream signalling module has been well-established as a key regulator of BBB impairment (Srivastava et al., 2013; Kuhlmann et al., 2007; Yu et al., 2010). Moreover, increased phosphorylation of the accessory light chains of non-muscular myosin II leads to BBB compromise. Hyperphosphorylation of MRLC prompts diminished TEER and enhances monocyte migration (Clark et al., 2015; Haorah et al., 2007), whilst treatment with statins leads to MRLC dephosphorylation and negates these effects (Kuhlmann et al., 2006). The distribution of these myosin motor proteins throughout the cerebral vasculature of the mouse has been confirmed, suggesting the integral role of these components in the preservation of BBB stability (Ishmael et al., 2008).

To sum up, the integrity of the endothelial barrier is reflecting an equilibrium between its structural elements; the contractile forces produced by the cytoskeleton and the adhesive forces generated at the intracellular junctions and the cell-matrix contracts.

4.2 Methods

4.2.1 *In vitro* paracellular permeability assay

Paracellular permeability of hCMEC/D3 or b.End3 monolayers was assessed employing FITC-labeled different-sized (4kDa, 10kDa, 20kDa, 40kDa, 70kDa) dextrans as tracers. hCMEC/D3 cells were seeded onto collagen (1:20, Enzo, Exeter, UK) and fibronectin-coated (1:100- Sigma-Aldrich, St Louis, MO, USA) permeable polyester Transwell filter inserts (0.4-mm pore, 12-mm diameter - Corning Costar, Buckingham, UK), assembled into 12-well plates and maintained for 72h until the formation of confluent monolayers. Culture media were then changed to EGM-2 media excluding VEGF which has been reported to be associated with increased BBB leakiness (Boveri et al., 2005). Cells were maintained for 72h until stimulation with TCM and/or treatment with cytokines. Culture media were then removed from the apical chamber, and 500 µl of transport buffer (2% FBS in DMEM without phenol red) containing 1-2 mg/ml of the tracer was loaded on the apical compartment of the insert. Paracellular flux of the FITC-dextran to the basolateral side of the insert was evaluated through sequential measurements at 5-min intervals for 30 min (excitation, 480 nm; emission, 530 nm) on a TECAN plate reader (Ortenberg, Germany). The permeability coefficients (Pe, cm/min) of the endothelial monolayers were calculated based on the plots of the volume cleared plotted against time and the the slopes of

The cleared volume (in µl/ml) represents the cumulative volume of FITC-Dextran solution passing through the cell monolayer between subsequent sampling events (5min intervals for 30min):

$$Cleared\ volume = \frac{[C]_A \times V_A}{[C]_L}$$

where $[C]_L$ is the initial FITC-Dextran solution concentration in the upper chamber, $[C]_A$ is the concentration of the FITC-Dextran solution in the lower chamber, and V_A is the volume in the lower chamber.

The cleared volume is then plotted against time.

The permeability coefficient for the endothelial monolayer (Pe in cm/min) is calculated from the slopes of the linear part of the curves above using the following formulas:

$$1/PS = 1/PS_E - 1/PS_F \text{ and } Pe = PS/S$$

Where PSE and PSF correspond to the slope of the clearance curves (µl/min) for endothelial cells grown on filters or control filters without cells, respectively. PS represents the permeability of the endothelial monolayer (cm^3/min) and S is the surface area of the filter (cm^2) while Pe is the permeability coefficient for the endothelial monolayer (cm/min).

4.3 Results

We sought to establish an *in vitro* model of brain endothelial monolayer in order to

recapitulate the observed effect of systemically administered TNF on the cerebrovascular endothelium, and to determine whether TNFR1 is associated with the previously demonstrated BBB permeabilisation (Connell et al., 2013).

4.3.1 TNF-induced endothelial TNFR1 upregulation *in vitro*

In order to explore the sensitivity of TNFRs towards soluble TNF, human brain microvascular endothelial monolayers of hCMEC/D3 cells were exposed to prolonged treatment (6-72h) with intermediate dose (10ng/ml) of human TNF (huTNF). TNFR1 and TNFR2 expression at mRNA level was determined employing qPCR. Ribosomal protein L4 (RPL4) was used as housekeeping gene for normalisation in accordance with Urich, *et al.*, 2012.

Pro-inflammatory mediators IL-1β and LPS were used as positive and negative controls, respectively. IL-1β is a well-characterised inducer of brain endothelial TNFR1 and TNFR2 expression, exhibiting increased potency for both receptors compared to TNF (O'Carroll et al., 2015). On the contrary, only high dose (100ng/ml) of LPS has been reported to elevate TNFR1, and TNFR2 mRNA levels in brain microvascular endothelial cells within 48h (Pan et al., 2008). The 1ng/ml dose used here does not induce endothelial upregulation of TNFRs, as previously demonstrated (O'Carroll et al., 2015).

Administration of 10ng/ml of IL-1β for 48h, yielded a 5-fold increase ($p<0.001$) in TNFR1 expression compared to TNFR1 mRNA levels in unstimulated cells. On the

contrary, prolonged LPS treatment (1ng/ml for 48h) did not increase TNFR1 and TNFR2 expression (ns for both comparisons with baseline mRNA levels).

TNF stimulation for 6h-18h did not increase TNFR1 expression significantly (ns, compared to untreated cells for 6h, 12h, 18h comparisons). Stimulation for 24h led to a significant 2-fold increase in TNFR1 mRNA levels ($p<0.5$, compared to baseline levels in hCMEC/D3 cells). Prolonged exposure for 36h and 48h demonstrated linear increase in TNFR1 mRNA levels; 3-fold ($p<0.01$) and 4-fold ($p<0.001$), respectively. Extended incubation with TNF for 60h or 72h did not elicit further increases, suggesting that TNF-induced endothelial TNFR1 upregulation reached a plateau at 48h. Relevant treatments did not induce any significant alterations in endothelial TNFR2 expression (ns, for all comparisons vs TNFR2 expression in unstimulated hCMEC/D3 cells).

Further dose-response studies determined the minimal TNF dose eliciting TNFR1 upregulation within 24h of exposure. TNFRs expression at protein level was detected employing western blot, using β-tubulin as reference marker. Stimulation with 1ng/ml did not increase TNFR1 expression significantly (ns), whereas stimulation with 10ng/ml, 100ng/ml, 1000ng/ml led to 2-fold ($p<0.05$), 4-fold ($p<0.001$), 12-fold ($p<0.001$) increases, respectively. Only huTNF stimulation with 1000ng/ml elicited significant alterations in TNFR2 expression (2-fold increase, $p<0.05$, compared to baseline TNFR2 expression). These trends in TNFR1 and TNFR2 mRNA expression were in line with relevant protein expression levels **(Figure 4.1)**.

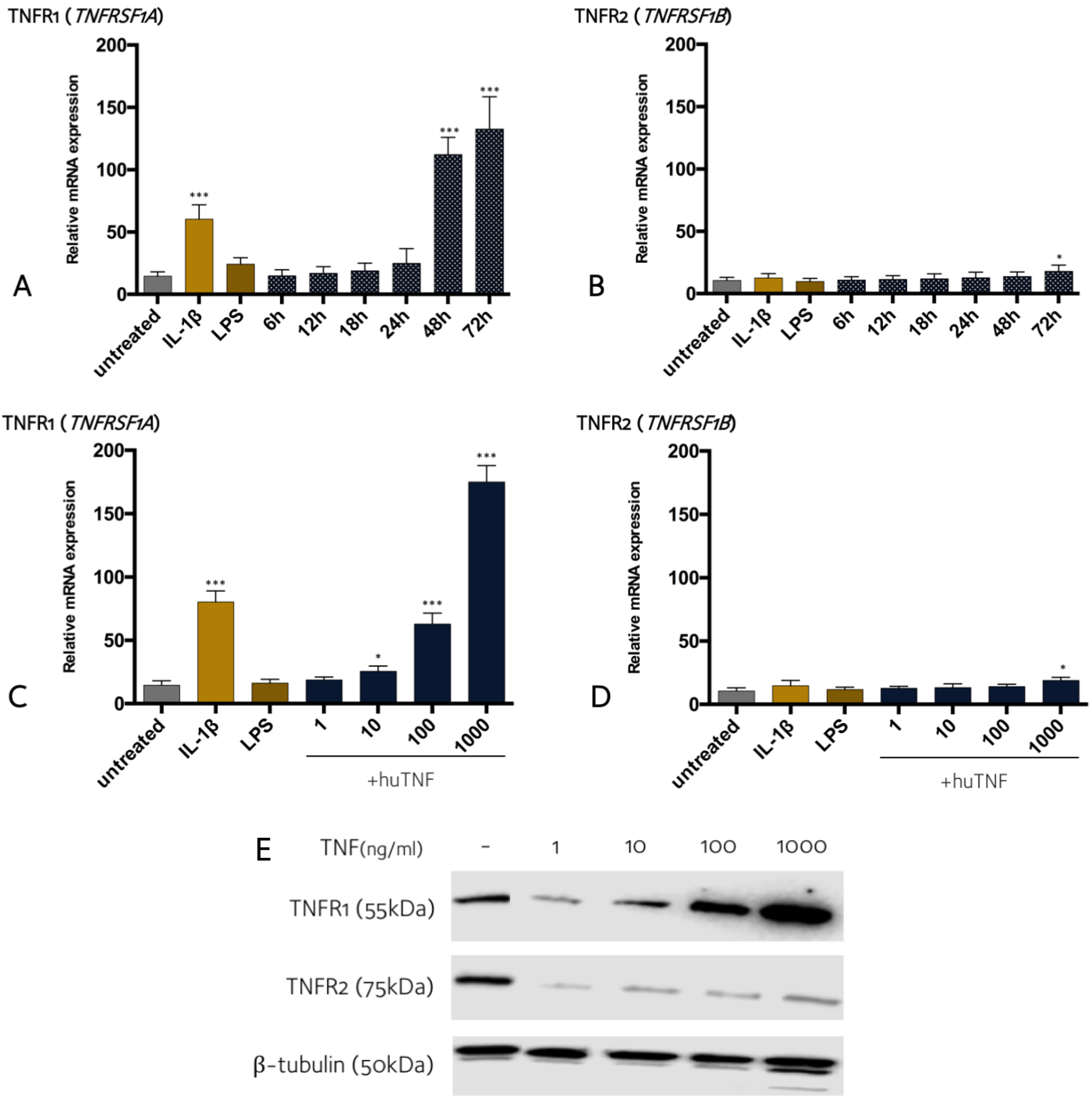


Fig.4.1 Endothelial TNFR1 and TNFR2 expression upon exposure to TNF

A,B: Temporal pattern of TNFR1 **(A)** and TNFR2 expression **(B)** at mRNA level in hCMEC/D3 cells exposed to pro-inflammatory cytokines; IL-1β (10ng/ml, 48h), LPS (1ng/ml, 48h), TNF (10ng/ml, for various timepoints; 6-72h).

C-E: TNFR1 **(A)** and TNFR2 **(B)** expression at mRNA level, or at protein level **(E)** in hCMEC/D3 cells exposed to increasing doses of TNF (1ng/ml, 10ng/ml, 100ng/ml, 1000ng/ml) for 24h.

A-D: relative *Tnsfr1a* **(A,C)** and *Tnsfr1b* **(B,D)** mRNA expression in hCMEC/D3 cells determined using qPCR. Expression levels were compared to the housekeeping gene RPL4 and normalized to unstimulated hCMEC/D3 cells. One way-ANOVA. *p<0.05, ***p<0.001 Data are presented as mean values ± SEM.

E: Western blot showing the levels of TNFR1 and TNFR2 protein expression in hCMEC/D3 cells upon exposure to increasing doses of TNF (1ng/ml, 10ng/ml, 100ng/ml, 1000ng/ml) for 24h. β-tubulin was employed as reference gene.

4.3.2 TNFR1-mediated endothelial permeabilisation *in vitro*

4.3.2.1 Assessment of TNF-induced endothelial permeability *in vitro*

In order to explore the molecular determinants of the TNF-induced BBB breakdown during brain metastasis, we established an *in vitro* paracellular permeability assay using the hCMEC/D3 human brain microvascular endothelial cell line.

Permeability changes were measured employing a hydrophilic tracer. The 70 kDa fluorescein isothiocyanate (FITC) -dextran was chosen in order to employ a tracer with similar molecular weight to that of the active, trimeric TNF (52 kDa) (Wingfield et al., 1987).

After treatment with intermediate dose of TNF (10 ng/ml), the permeability coefficient (Pe) in hCMEC/D3 monolayers grown on transwell permeable inserts increased by 7-fold at 24 h of treatment compared with control cells ($p<0.01$). The vasopermeant VEGF was used as a positive control and triggered a 10-fold increase ($p<0.001$) in the permeability of hCMEC/D3 cells. The pharmacological inhibitors of TNFR1 activation R-8507 and SPD-304 were used in order to confirm that TNF-TNFR1 ligation is a key event, controlling TNF-induced endothelial permeability changes. R-8507 is a small molecule antagonist of TNFR1 (Gururaja et al., 2007). Treatment with this compound abrogates the formation of the TNFR1 signalling complex and elicited a 77% decrease in TNF-induced endothelial permeability ($p<0.001$). Additionally, SPD-304 is an TNF-inhibitor that prevents the TNF-TNFR1 ligation (IC_{50}=22μM) (He et al., 2005).

Administration of SPD-304 led to a 79% decrease in TNF-induced endothelial permeability increase ($p<0.001$).

4.3.2.2 Effect of TNFR1 or TNFR2 depletion on endothelial monolayer integrity

To determine the contribution of TNFR1 and TNFR2 activation on TNF-induced endothelial permeability siRNA was used to diminish *TNFRSF1A* and *TNFRSF1B* expression. TNFRs expression at protein level was detected employing western blot, using β-tubulin as reference marker. Prolonged treatment (96h) with anti-TNFR1 siRNA (8μl/ml) achieved the maximum suppression (90%) of endothelial TNFR1 expression. In addition, administration of anti-TNFR2 siRNA (6μl/ml) diminished endothelial TNFR2 expression by 76%. Treatment with scrambled sequences (negative control) did not alter baseline TNFR1 or TNFR2 expression.

Exposure of hCMEC/D3 cells treated with anti-TNFR1 to TNF for 24h did not induce significant alterations in endothelial paracellular permeability (ns, compared with Pe for hCMEC/D3 treated with anti-TNFR1 siRNA only). On the contrary, TNF stimulation for 24h in hCMEC/D3 cells treated with anti-TNFR2 led to 6-fold permeability increase ($p<0.001$, compared with Pe for hCMEC/D3 treated with anti-TNFR2 siRNA only).

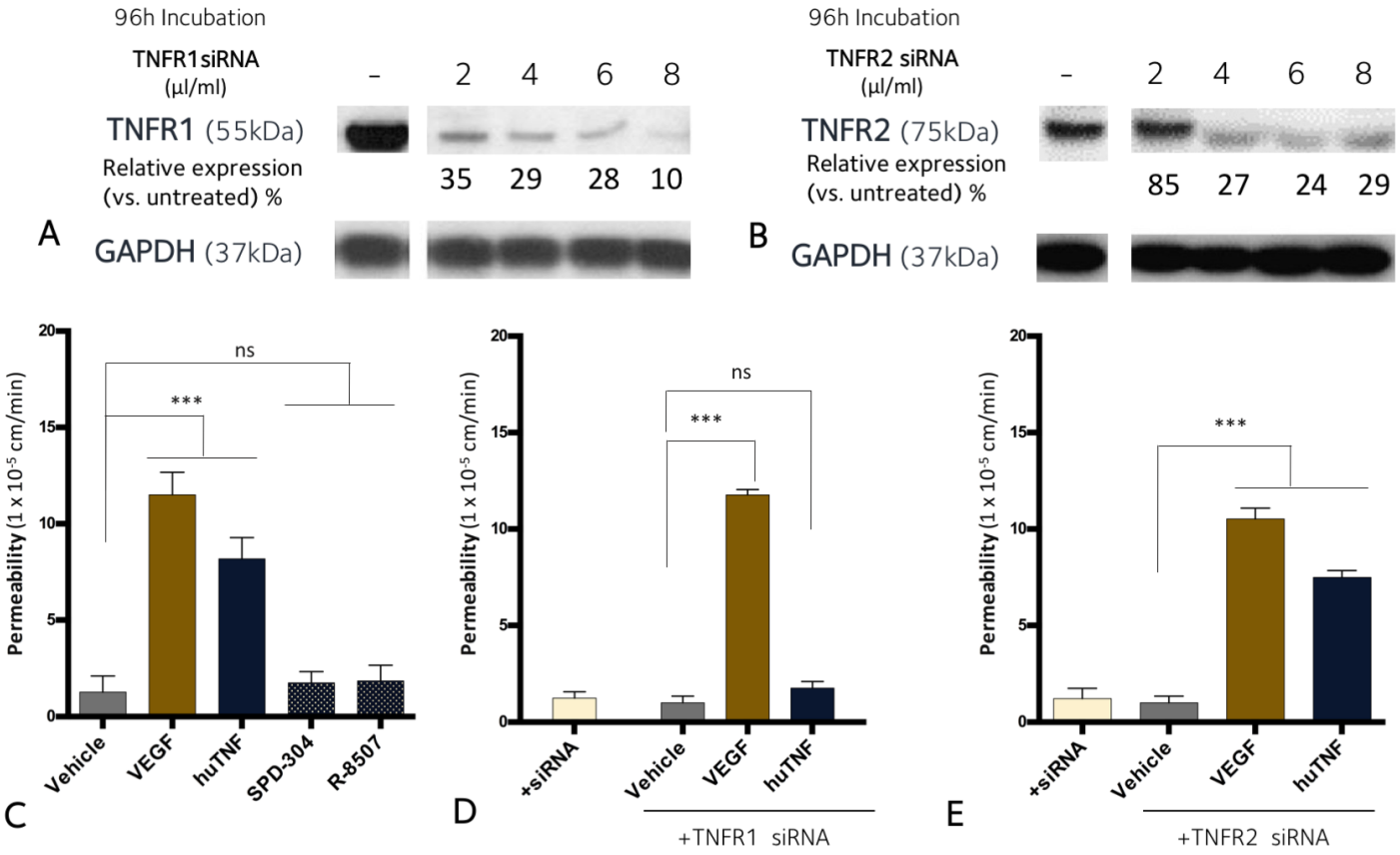


Fig.4.2 Assessment of TNFRs contribution to TNF-induced endothelial permeabilisation *in vitro*

A, B: TNFR1 **(A)** and TNFR2 **(B)** protein expression in hCMEC/D3 cells exposed to either growth medium (-) or increasing concentrations of anti-TNFR1/ -TNFR2 siRNA for 96h, as determined by western blot. Quantitation of the mean intensity of each band with respect to untreated hCMEC/D3 cells. Band densitometry was normalised to the intensity of GAPDH bands and quantified across three independent experiments.

C-E: Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control) **(C-E)**, or soluble human TNF (huTNF; 10ng/ml, 24h) **(C-E)**, or pharmacological inhibitors targeting the activation of the TNFR1 signalling complex; SPD-304 (10µM, 24h), R-8507 (10µM, 24h) **(C)**.

D, E: Assessment of paracellular permeability of hCMEC/D3 monolayers pre-treated with anti-TNFR1 siRNA (8µl/ml, 96h) **(D)**, or anti-TNFR2 siRNA (6µl, 96h) **(E)**, followed by stimulation with TNF (10ng/ml) for 24h.
Statistical analysis: Student *t* test, two-tailed and paired. ***p<0.001 (compared to vehicle). Data are presented as mean values ± SEM.

4.3.2.3 *In vitro* assessment of TNF muteins

In order to further assess the causal implication of TNFR1 activation on the induction of endothelial perpermeability, TNF muteins with variable potency for TNFR1 or TNFR2 were employed. These constructs have been generated through side-directed mutagenesis near the trimerisation sites of the receptors. The design of TNF-selective candidates is based on conformational changes rendering huTNF as the sole agonist for mouse TNFR1 (Bossen et al., 2006).

The constructs used for the following assays exhibited enhanced affinity for human TNFR1 as reflected by equilibrium dissociation constant (K_D) values obtained upon conduction of custom-designed binding assays by external collaborators of our group (Abzena, Cambridge, UK); B2: K_D:2.16 x 10^{-11}, F3: K_D:4.62 x 10^{-10}, G4: K_D:8.72 x 10^{-12} (Table 4.1). These muteins were employed for the conduction of *in vitro* paracellular permeability assays using the endothelial monolayer model established. A TNF analogue, coined as wild type (WT), has been constructed using the same protocol established for the production TNF muteins and was used instead of the native cytokine in the following assays.

Dose-response treatments revealed that all three constructs could mimic the vaso-permeabilising effect of WT and induce significant permeability changes upon prolonged (24h) treatment at doses ranging from 300pg/ml up to 10ng/ml. All constructs did elicit robust permeability increases for the highest doses (3 anD 10ng/ml), in an analogous manner to WT (ns, compared to equivalent doses of WT).

However, only G4 was a more potent inducer of endothelial permeability than WT for the lowest dose tested (100pg/ml, $p<0.01$ compared to equal dose of WT).

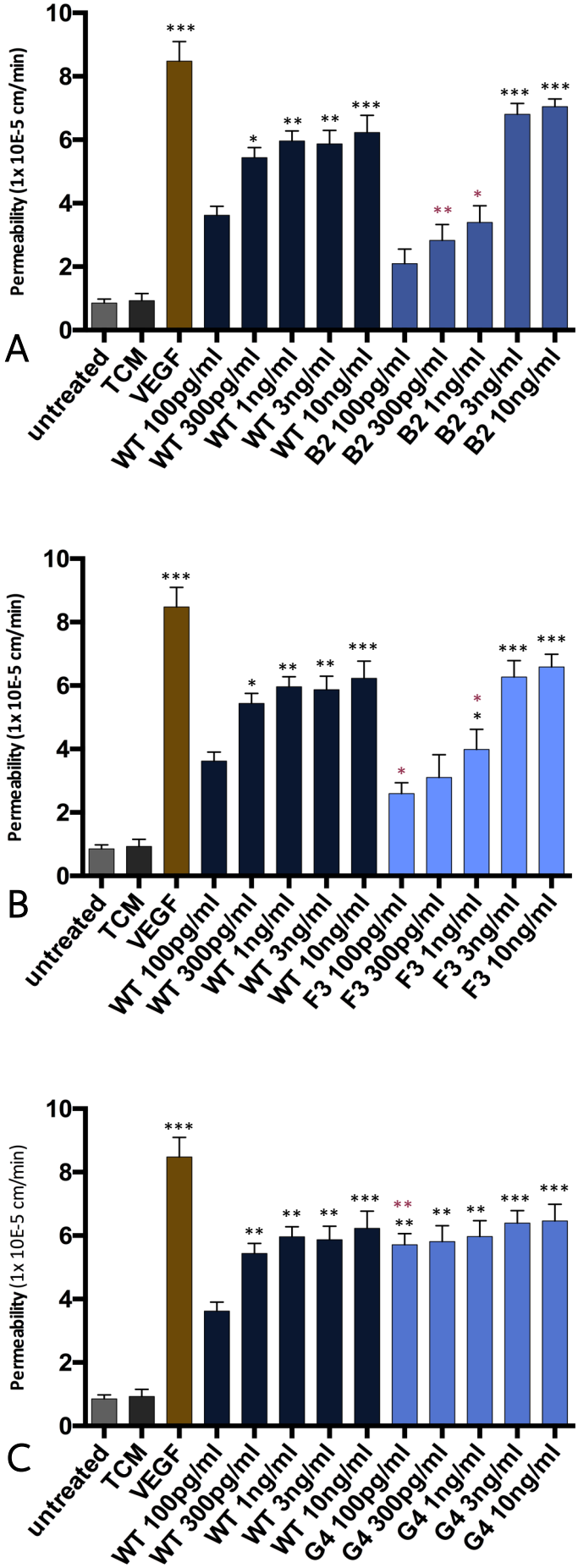


Fig.4.3 Assessment of the permeabilising effect of TNF muteins with enhanced bioaffinity for TNFR1 *in vitro*

A-C: Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant analogue of human TNF (WT), or TNF muteins (B2,F3,G4). Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared with untreated hCMEC/D3 cells). *p<0.05, **P<0.01, ***p<0.001 (compared with equal dose of WT). Data are presented as mean values ± SEM

4.3.2.4 Comparison of human and mouse TNF mouse endothelial monolayer

Before assessing the role of specific TNFR1 activation on the regulation of BBB integrity *in vivo*, we established an *in vitro* mouse endothelial monolayer model, employing the mouse brain endothelial cell line b.End3. For the conduction of *in vitro* paracellular permeability assays, cells were seeded in pre-coated permeable transwell inserts and maintained until reaching confluence, followed by enhanced expression of interendothelial junctional proteins.

Recombinant human TNF (huTNF) is a potent agonist for mouse TNFR1 alone (Bossen et al., 2006). Binding assays conducted by our external partners (Abzena, Cambridge, UK) confirmed the enhanced affinity of huTNF for mTNFR1; K_D:6.44 x 10^{-11}, whilst the affinity of mTNF for mTNFR1 is characterised by K_D:1.66 x 10^{-10}.

Extended (24h) treatment of b.End3 cells with intermediate dose of huTNF (10ng/ml) led to increased expression of TNFR1 only.

Dose-response treatment employing both huTNF and mTNF led to significant paracellular permeability increases. huTNF-induced activation of the mTNFR1 only, led to significant endothelial permeability increase ($p<0.5$ for the 10ng/ml treatment, and $p<0.01$ for the 30ng/ml and the 100ng/ml treatments, respectively; compared to untreated cells).

Moreover, pre-treatment with TNFR1 neutralising antibody (anti-TNFR1; 100ng/ml, 6h) followed by stimulation with either huTNF (10ng/ml, 24h) or mTNF (10ng/ml, 24h), protected the endothelium from TNF-induced permeability increases (ns, compared to untreated cells, for both comparisons).

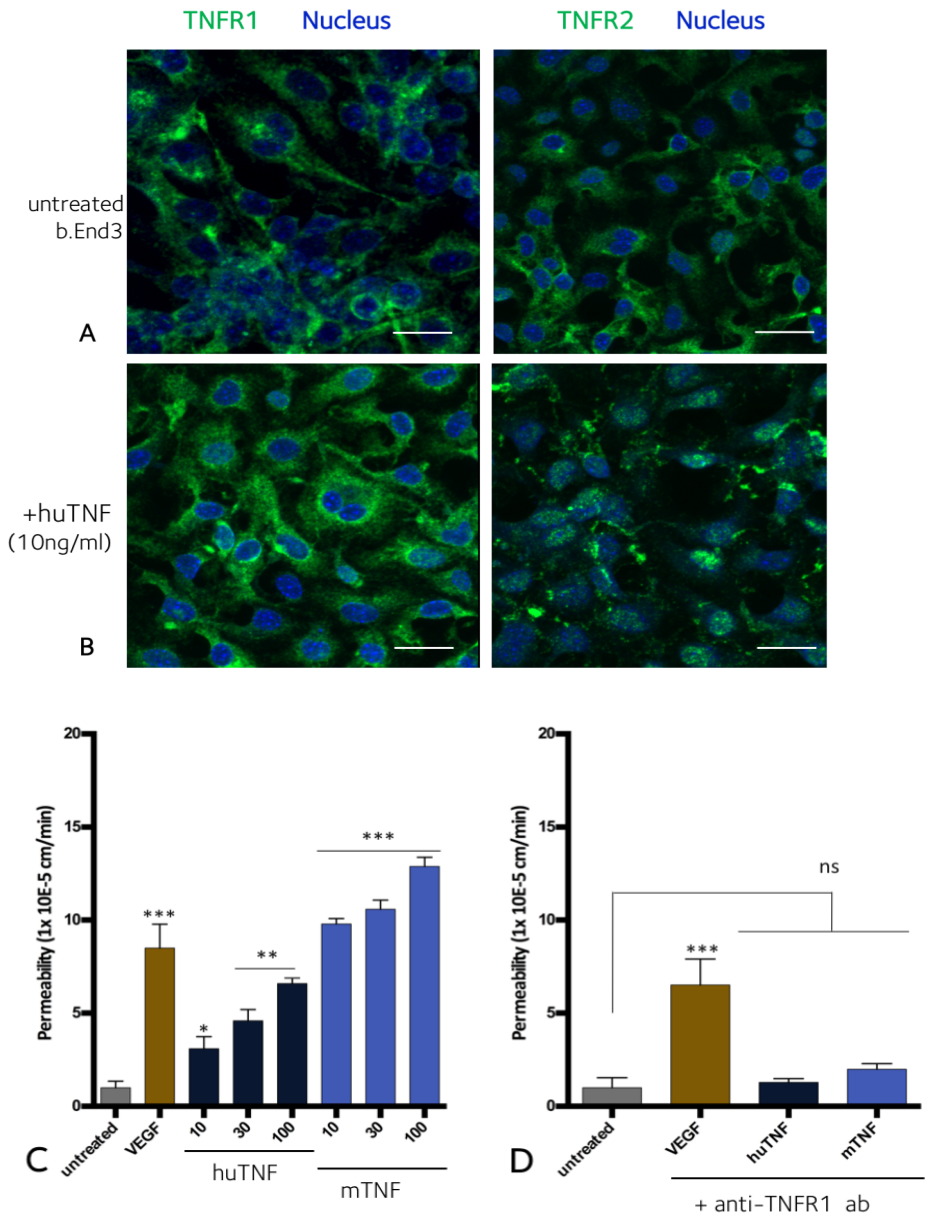


Fig.4.4 Comparison of vaso-permeabilising activity of huTNF and mTNF *in vitro*

A,B: Immunofluorescent staining of TNFR1 (green), or TNFR2 (green) in resting **(A)** mouse brain endothelial cells bEnd3, or **(B)** bEnd3 monolayer stimulated with huTNF (10ng/ml, 24h). Nuclear DAPI (blue) counterstain. Scale bars=10µm

C, D: Assessment of paracellular permeability to FITC dextran 70 kDa of bEnd3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control) **(C, D)**, or recombinant human TNF **(C, D)**, or recombinant mouse TNF (mTNF; 10ng/ml, 24h) **(C, D),** and TNFR1 neutralising antibody (anti-TNFR1) **(D)** .

D: Treatment with anti-TNFR1 neutralising antibody (100ng/ml) followed by stimulation with huTNF (10ng/ml, 24h) or mTN (10ng/ml, 24h)

Statistical analysis: Student t test, two-tailed and paired. **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells). Data are presented as mean values ± SEM.

4.3.2.5 TNFR1-mediated cytoskeletal remodelling

The b.End3 monolayer established was used in order to determine whether TNF-induced activation of TNFR1 leads to cytoskeletal remodelling and altered expression of intercellular junctional proteins.

Rhodamine-phalloidin staining was employed to visualize F-actin stress fibres formation upon exposure to huTNF. Prolonged exposure to huTNF (10ng/ml, 24h) induced extensive plasma membrane blebbing and increased actin-myosin contractility. Moreover, TNFR1 signalling elicited the disassembly of interendothelial adhesion junctions as reflected by downregulation and/or altered cellular localisation of the adhesion molecules VE-cadherin, and talin. In addition, the expression of the actin-binding protein ZO-1; and of the tight junctional proteins occludin, and claudin-5 was diminished as evidenced by immunofluorescent staining or western blot.

Table 4.1 Binding affinities of TNF muteins tested for TNF receptors

	huTNFR1	huTNFR2	mTNFR1	mTNFR2
WT	1.58×10^{-11}	3.53×10^{-11}	6.67×10^{-11}	-
B2	2.16×10^{-11}	4.07×10^{-10}	1.77×10^{-10}	-
F3	4.62×10^{-10}	-	5.08×10^{-9}	-
G4	8.72×10^{-12}	-	1.40×10^{-10}	-

Equilibrium dissociation constant (K_D) values obtained by Abzena (Cambridge, UK) employing custom-designed binding assays against human TNFR1 (huTNFR1), human TNFR2 (huTNFR2), mouse TNFR1 (mTNFR1) or mouse TNFR2 (mTNFR2). Dashes indicate absence of binding of TNF analogue (WT) or TNF muteins (B2, F3, or G4) to TNF receptors.

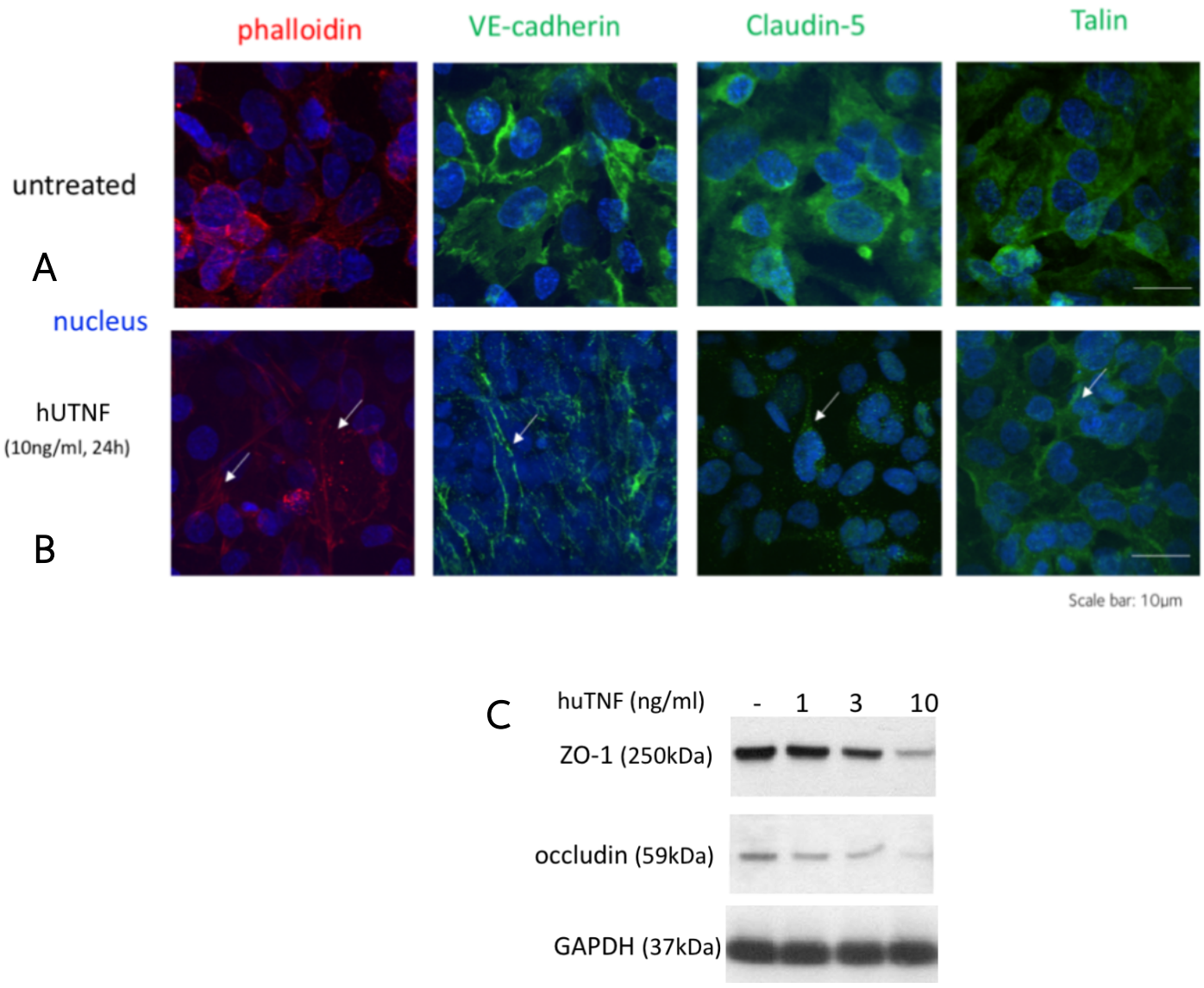


Fig.4.5 mTNFR1-mediated compromise of interendothelial integrity *in vitro*

A,B: Immunofluorescent staining for F-actin (red), or for adhesion molecules VE-cadherin (green) and Talin (green), or for the tight junctional protein claudin-5 (green) in **(A)** resting mouse brain endothelial cells b.End3, or **(B)** b.End3 monolayers stimulated with huTNF (10ng/ml, 24h).

Nuclear DAPI (blue) counterstain. Scale bars=10μm

Phalloidin staining: arrows represent F-actin stress fibres formation throughout the cytoplasm with cortical actin being less prominent.

VE-cadherin, claudin-5, talin stainings: arrows illustrate sites of abrogated protein expression

C: Western blot showing the levels of ZO-1 and occludin protein expression in b.End3 cells upon exposure to increasing doses of human TNF (1ng/ml, 3ng/ml, 10ng/ml) for 24h. GAPDH was employed as reference gene.

4.3.3 Histological detection of BBB permeabilisation

TNF-induced permeabilisation of the BBB was revealed *ex vivo*, employing intravenously injected HRP as a histological marker. Brain metastases were detected with cresyl violet staining.

Brown staining within Hanker-Yates- treated tissue sections (50μm thickness), indicative of HRP passage through the BBB, was defined as BBB permeabilisation site for metastases larger than 50μm in diameter. Consecutive sections were stained with cresyl violet for the detection of tumour sites. Metastatic colonies were blindly analysed to assess their permeabilisation status. No BBB compromise was observed at non-metastatic sites prior or upon cytokine administration.

Both mTNF and huTNF induced HRP staining (Hanker et al., 1977) that was generally limited to the metastatic colony, and minor spread in the surrounding tissue was only detected in some metastatic colonies. The observed BBB compromise was statistically significantly higher for both mTNF (60.3% ± 3.4% vs control: 29.6% ±3.1%, $p<0.001$), and huTNF (61.3% ± 1.3% vs control: 29.6% ±3.1%, $p<0.001$). The BBB breakdown was specific and local at sites of brain metastasis, whilst no BBB permeabilisation was evidenced for normal brain tissue. This pattern is reminiscent of the focal TNFR1-upregulation at the tumour-associated vasculature discussed in section 3.3.2. Taken together, these findings confirm that this specific, endothelial TNFR1 activation is sufficient for the permeabilisation of the tumour vasculature in response to TNF.

**Experiments conducted in collaboration with Dr. Mario Munoz Pinto*

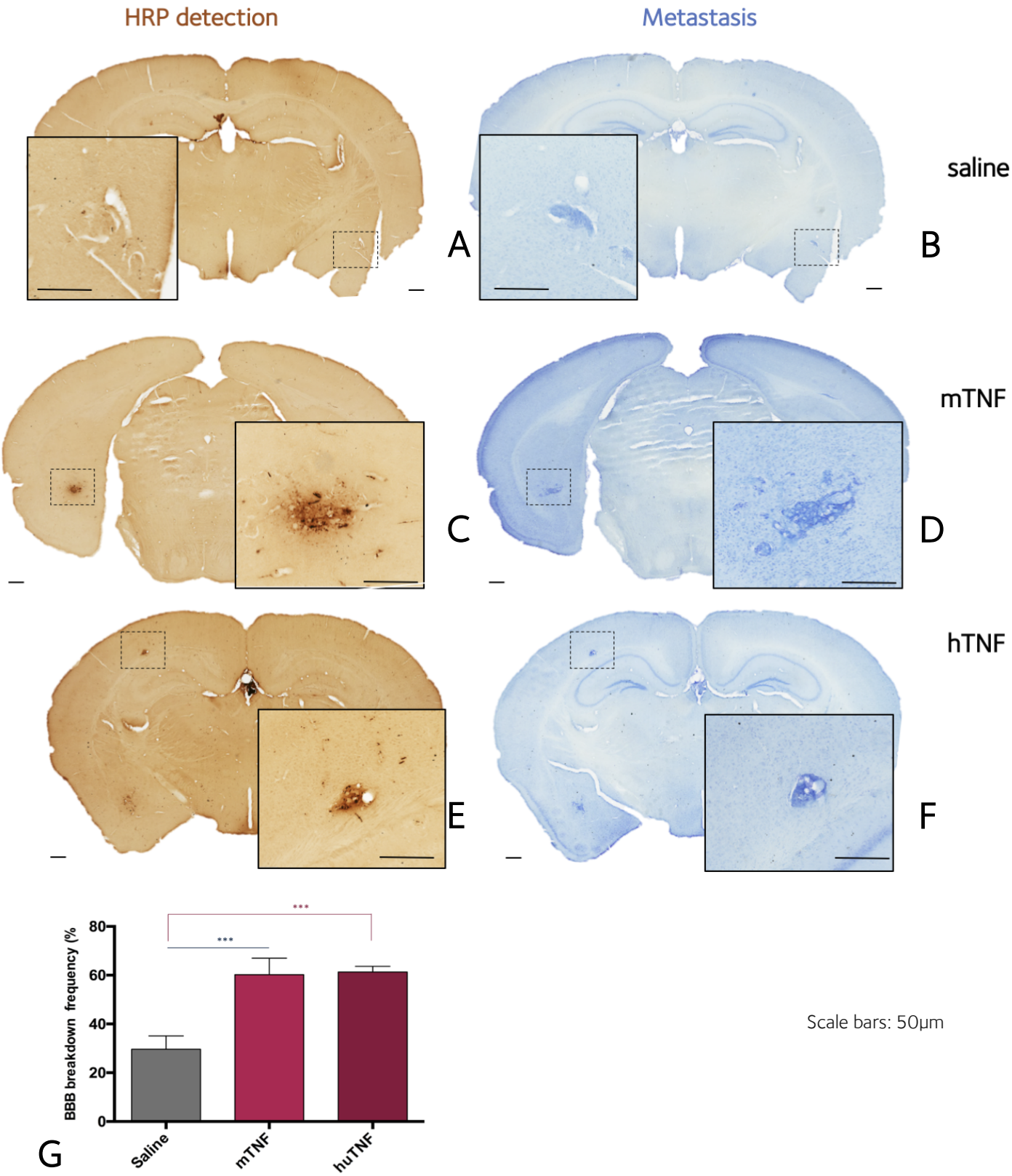


Fig.4.6 TNFR1-mediated blood-brain barrier (BBB) breakdown

Histological detection of brain metastases and cytokine- induced BBB breakdown. Photomicrographs of brain metastases from mice injected systemically with horseradish peroxidase (HRP) and saline **(A,B)**, 3μg of mouse TNF (mTNF) **(C,D)**, or 3μg of human TNF (huTNF) **(E,F)**. Hanker-Yates histology for HRP detection (brown) reveals areas of BBB breakdown. Cresyl violet stain (blue) was conducted in consecutive tissue sections for the detection of metastatic foci. Insets show higher magnification of the tumour colonies in boxed regions.
Data were analysed blind to the treatment group (n=6). Scale bars= 50μm
(G) Statistical analysis: Two-side, 1-way ANOVA with Dunnet post-hoc test vs saline group ***$p<0.01$. Data are presented as mean values ± SEM.

4.4 Discussion

The effects of endothelial stimulation employing the pro-inflammatory cytokine TNF have been extensively studied not only for the endothelium lining the vasculature in the periphery, but also for the cerebrovascular endothelium. *In vitro* systems employing either primary endothelial cells or immortalised endothelial cell lines have been utilised extensively for the elucidation of the molecular drivers of endothelial permeability. The vaso-permeabilising activity of TNF has been demonstrated using *in vitro* systems aiming to model the cerebrovascular endothelium more than 20 years ago (de Vries et al., 1996; Deli et al., 1995).

The immortalised human brain microvascular endothelial cell line hCMEC/D3 (Weksler et al., 2005) employed for the establishment of an *in vitro* model of endothelial monolayer has been well-studied as a valuable tool assisting the characterisation of the signalling underlying endothelial responses either in monoculture systems or in co-culture approaches employing astrocytes or pericytes (Weksler, Romero, & Couraud, 2013). We established an *in vitro* model of the cerebrovascular endothelium in order to decipher the molecular underpinnings of TNF-induced BBB breakdown previously observed *in vivo* (Connell et al., 2013).

Extended (24h) TNF-induced pro-inflammatory stimulation led to selective TNFR1 upregulation, but baseline, constitutive TNFR2 expression. The time frame of increased TNFR1 expression was consistent with the presentation of significant alterations in endothelial permeability. Given the fact that the soluble TNF employed in the relevant *in vitro* assays has increased affinity for TNFR1 (Grell et al., 1998), we

sought to determine whether TNFR1 activation is associated with increased leakiness in TNF-stimulated endothelial monolayers. TNFR1 and TNFR2 expression was diminished using siRNA.

hCMEC/D3 cells treated with anti-TNFR1 siRNA exhibited a residual 10% TNFR1 expression, whereas depletion of TNFR2 was weaker with a residual 29% of expression, compared to baseline levels. Extended exposure of siRNA treated hCMECs to TNF lead to permeability increase only for the TNF2-depleted population, suggesting that TNFR1 is a crucial component of TNF-induced endothelial permeabilisation. These results are in line with similar approaches involving the use of either neutralising anti-TNF antibodies or anti-TNFR1 antibodies in experimental mouse models of acute liver failure (Lv et al., 2010). Blockade of the TNF-TNFR1 ligation repressed the TNF-induced brain endothelial monolayer compromise.

Further extension of these studies in an *in vitro* mouse model of endothelial monolayer employing the microvascular endothelial cell line b.End3 allowed us to validate the role of TNFR1 activation benefiting from the interspecies cross-reactivity between huTNF and mTNFR1 (Bossen et al., 2006). Administration of huTNF led to significant permeability increases, thus confirming that activation of TNFR1 only is sufficient for the onset of the vaso-modulating activity of TNF on the cerebrovascular endothelium. Moreover, the reduced effect of equal doses of huTNF compared to mTNF on the induction of endothelial permeability increase is reflective of the weaker bioaffinity of huTNF for mTNFR1, compared to mTNF. Additionally, abrogation of TNFR1

activity using a neutralising antibody exerted a protective effect on the integrity of TNF-stimulated endothelial monolayer.

Assessment of the above findings employing a syngeneic experimental model of breast-to-brain metastasis (4T1-GFP), underscored the role of TNFR1 as the primary determinant of TNF-induced endothelial permeabilisation in the *in vivo* setting. Indeed, administration of huTNF led to selective BBB breakdown at sites of brain metastasis, as evidenced by histological detection of HRP, in analogous manner to mTNF.

4.4.5 TNF-induced systemic toxicity

Contradicting evidence posits that both TNFRs are causally implicated in the systemic toxicity of TNF. Activation of TNFR2, but not TNFR1, has been associated with sensitisation towards a sublethal dose of TNF *in vivo* (Douni & Kollias, 1998). TNFR1-targeted muteins exhibit minimal toxicity even in concentrations exceeding the maximum tolerated dose (MTD) of TNF (Kumaratilake et al., 1995), whereas a triple mutant mouse TNF (mTNF) with considerably diminished (50-fold) TNFR2-mediated bioactivity exhibited significantly reduced toxicity (Lucas et al., 1997). Given the finding that human TNF (huTNF) is a specific agonist of the mTNFR1 alone (Kramer et al., 1988; Lewis et al., 1991), and that the LD_{50} of mTNF is 50-fold lower than that of huTNF, it is plausible that TNFR2 activation is associated with TNF-induced toxicity (Van Ostade et al., 1993; Brouckaert et al., 1994).

Opposing outcomes support the concept of TNFR1-associated systemic toxicity. Administration of a TNFR1-specific mutein induced systemic toxicity in baboons (Van Zee et al., 1994), whilst suppression of TNFR1 activation in knockout mice (Van Hauwermeiren et al., 2013), or downregulation of TNFR1 employing antisense oligonucleotides supress systemic toxicity to mTNF (Van Hauwermeiren et al., 2014). Taken together, the mechanism of TNF-induced systemic toxicity remains elusive, since the reduced toxicity exhibited by huTNF administration in mice could be attributed to either diminished TNFR2 activation or absence of co-operative signalling between TNFRs.

Given the permeabilising activity of TNFR1 upon TNF stimulation, we used TNF muteins with increased bioaffinity for TNFR1 only, in our *in vitro* hCMEC/D3 model and demonstrated that they could mimic the vaso-modulating effect of wild type TNF. Indeed, the permeabilising effect of these constructs, especially for low dose treatments, was reflective of their potency for TNFR1, with the G4 mutein bearing the lowest K_D value, indicative of increased bioaffinity, exhibiting the highest efficacy and even exceeding the permeabilising effect of higher doses of wild type TNF. The above findings suggest that the use of such constructs allows for the improvement of the therapeutic index of TNF and provides a valuable tool for the potential exploitation of the permeabilising activity of TNF in the clinic.

4.4.6 Clinical implications of TNFR1-mediated BBB permeabilisation

Systemically successful chemotherapeutic agents such as doxorubicin and trastuzumab cannot reach the BBB by the virtue of their hydrophilic nature. However, recent administration of small-molecule inhibitors (SMIs) demonstrated reasonable activity in brain metastases (Ahluwalia & Winkler, 2015). Even when the accessibility constraints are diminished, classical chemotherapies with demonstrated efficacy on systemic tumours bear limited or no activity on brain metastases (Preusser et al., 2012), probably due to phenotypic and genomic alterations conferred by the metastatic dissemination process that lead to extensive chemoresistance (Sperduto et al., 2017). This is underlying the importance of early targeting of brain metastatic deposits. Indeed, treatment of micrometastases, using targeted formulations in an experimental model of melanoma brain metastasis, was proven to be considerably more effective than the management of established macrometastases (Osswald et al., 2016).

Nevertheless, early treatment requires accelerated diagnosis. Micrometastases with approximately 50μm diameter exhibited TNFR1 upregulation in the tumour-associated vasculature **(chapter 3)** and concomitant increased sensitivity to TNF-induced BBB permeabilisation **(chapter 4)**. This allows for the targeting of metastases *ca.*200-fold below the current detection limit (0.5-1 cm diameter) of clinical imaging modalities (Nomoto et al., 1994; Percy et al., 2011; Katakami et al., 2011; Gaudino et al., 2012). Moreover, the fact that the transient vaso-modulating effect of TNF is evident for a

few hours following its extravasation, provides a window of opportunity for the therapeutic modulation of the BBB.

Chapter 5| Mechanism of TNF-induced endothelial permeabilisation

5.1 Introduction

The role of TNFR1 as a key mediator of TNF-induced endothelial permeability has been demonstrated in Chapter 5. This chapter will focus on the downstream signalling networks implicated in the TNFR1-mediated BBB rupture.

The Rho-family of GTPases are key regulators of actin dynamics that have been previously associated with TNF-induced endothelial permeability. Indeed, systemic administration of TNF has been shown to enhance the leakiness of tumour-associated vasculature in systemic tumours through a RhoA/ ROCK dependent mechanism (Seki et al., 2011). Moreover, TNF is a well-studied, potent inducer of NFκB-mediated pro-inflammatory signalling, MAPK pathway -mediated transcriptional activity, and pro-apoptotic signalling leading to cell death. Further than assessing the potential impact of i) the RhoGTPases, ii) the NFκB, iii) MAPK, and iv) the pro-apoptotic signalling cascades on TNF-induced, TNFR1-mediated permeability increases, we sought to v) determine the signalling hierarchy of the downstream targets of TNFR1 activation in the brain endothelium, and vi) the possible interactions of the different signalling cascades implicated.

The first part of the chapter includes an overview of the roles of the individual signalling cascades assessed based on a literature review focusing primarily on endothelial systems (wherever applicable). Emphasis has been given on literature illustrating TNF-associated signalling. The results section refers to the assessment of

the effects of a panel of agonists and pharmacological inhibitors on TNF-induced endothelial permeability increase, employing the *in vitro* paracellular permeability model established (chapter 5). The inhibitory effects, alongside the off-target effects of the antagonists (inhibitors) and the agonists employed, are summarised in tables included in the Methods section.

Targeted inhibition of signalling cascades using kinase inhibitors

The protein kinase inhibitors are a class of therapeutic compounds that have been intensively studied for more than 40 years (Cohen, 1999) due to their critical role in the modulation of cellular signalling. The existence of a highly conserved ATP-binding pocket amongst most of the reported kinases renders them susceptiple to a wide range of synthetic chemical compounds.

The lack of specificity is recognised as a major problem for active-site SMIs that target kinases which have a high degree of conservation in the catalytic core (Hindie et al., 2009). Kinases closely related by sequence have overlapping substrate specificities. The AGC family of kinases that are serine/threonine kinases, members of the protein kinase A, G, and C families, including PDK1, PKCα, PKCζ, ROCK2, S6K1 tend to be inhibited by the same compounds (Anastassiadis et al., 2011a; Manning & Toker, 2017).

Recent technological advances have enabled the profiling of kinase target selectivity against fractions of human protein kinases. This has led to the establishment of databases listing collections of average phosphorylation scores, indicative of activation status, upon treatment with specific or multitargeted inhibitors (Anastassiadis et al.,

2011a; Anastassiadis et al., 2011b; Bain et al., 2003; Davies et al., 2000; MRC Phosphorylation and Ubiquitylation Unit, 2018h). These approaches provide essential information for the interpretation of studies SMIs.

5.1.1 The Rho GTPases family

The Rho family of small GTPases consists of more than 20 molecular switches that are linked to an increasing number of RhoGTPase-regulating molecules; including ~80 guanine nucleotide exchange factors (RhoGEFs), ~65 GTPase- activating proteins (RhoGAPs), 3 GDP dissociation inhibitors (RhoGDIs), and at least 21 Rho-associated proteins (Etienne-Manneville & Hall, 2002). The Rho GTPases share high sequence homology but are differentially expressed and are frequently functionally distinct. This functional pleiotropy is mirrored by their 76 effector proteins that have been reported to date.

Most RhoGTPases bind to cytosolic RhoGDIs. These molecular chaperones retain the inactive, GDP-bound state of Rho GTPases, thus preventing their association with the membrane and their proteasomal degradation (Boulter et al., 2010). RhoGEFs facilitate the local activation of RhoGTPases through the exchange of RhoGDP for RhoGTP (Vazquez-Prado et al., 2004; van Buul et al., 2014).

The RhoGTPase family is tightly controlled as a response to extracellular stimuli promoting cellular adhesion, and migration (Heasman & Ridley, 2008; Kozma et al., 1995; Nobes & Hall, 1995; Ridley & Hall, 1992; Ridley et al., 1992). Rho GTPases orchestrate both contractile and expansive forces driving cell polarisation and

translocation through the regulation of F-actin assembly and disassembly (Etienne-Manneville & Hall, 2002). The best characterised members of the family are Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3) and Cdc42 (Takai et al., 2001; Burridge & Wennerberg, 2004).

RhoA is a key driver of increased microvascular permeability (Spindler et al., 2010). It promotes the disruption of endothelial integrity through the induction of the actomyosin-mediated contractility of F-actin stress fibers and the resulting formation of intercellular gaps. Notably, RhoA is implicated in the formation of small gaps at the junctional protrusions (Szulcek et al., 2013), alongside the maintenance of interendothelial junctions (Gavard et al., 2008). Despite its pronounced role in the regulation of endothelial integrity, loss of RhoA does not directly affect the basal resistance of the endothelium (Amado-Azevedo et al., 2017), suggesting that the aforementioned signalling is triggered only in presence of vasomediators such as histamine and thrombin (van Nieuw Amerongen et al., 1998).

RhoA has also been established as a positive upstream regulator of NF-κB in endothelial cells, during inflammation through the activation of its downstream regulators ROCK and PKCζ (He et al., 2011; Huang et al., 2001). However, the cross-regulation of RhoA and NF-κB remains a controversial topic. NF-κB signalling is a crucial component of the TNF-mediated RhoA upregulation in bronchial smooth muscle and in intestinal epithelial cells (Goto et al., 2009; He et al., 2012).

5.1.1.1 ROCK

Rho-associated coiled-coil-forming kinase (ROCK) is a serine/threonine kinase that serves as a downstream effector of the small GTPase RhoA (Matsui et al., 1996). Two ROCK isoforms have been characterised: ROCK-1 (p160ROCK; ROKβ) and ROCK-2 (ROKα). ROCK-1 is ubiquitously expressed, but minimally present in brain and muscle, whereas ROCK-2 expression is most prominent in brain, muscle, heart, lung and placenta tissues. ROCK-2 is the most abundant isoform in the brain, particularly in neurons and reactive astrocytes (Iizuka et al., 2012; Jeon et al., 2013). Recently, it has been identified as the most predominant isoform expressed in the rodent NVU; mainly in endothelial cells (Niego et al., 2017).

Activation of the ROCK signalling under pro-inflammatory conditions elicits the upregulation of the matrix-metalloproteinases (MMP)-2, and -9 in brain endothelial cells (Ishiguro et al., 2012) and astrocytes (Sophia Wang et al., 2006). The ROCK inhibitors, Y-27632 and fasudil, restore endothelial integrity through the suppression of MMPs expression, thus reducing the resulting mortality and neurological deficit rates in *in vivo* acute ischemic stroke models (Fukuta et al., 2017; Ishiguro et al., 2012).

The regulatory repertoire of ROCK during endothelial dysfunction also involves the transcriptional modulation of eNOS (reviewed by Yao et al., 2010) and the response to vasoconstriction-dictated by acetylocholine (ACh) (Denniss et al., 2010). ROCK activation can also be facilitated via Rho-independent mechanisms. Both the

interaction of Rho with lipid messengers and the proteolytic cleavage of Rho by caspase-3 can trigger the ROCK signalling cascade (Loirand et al., 2006).

As discussed in section 4.1.2, elevated levels of soluble mediators induce actin binding that stabilises the intercellular junctions and controls AJs dynamics. ROCK activation is a requisite for the formation of discontinuous AJs that often interconnect stress fibre ends from adjacent endothelial cells (Millan et al., 2010). TNF-stimulated endothelial cells are characterised by discontinuous AJs that stabilise stress fibres that are tension-bearing and force-generating, independently of FAs.

Interestingly, inhibition of the Rho/ROCK pathway in cerebral endothelial cells (CECs) does not render the endothelial monolayer more susceptible to transmigration of melanoma cells. However, silencing of the Rho GTPases induces a shift of the latter towards the mesenchymal phenotype, underlying the cell type-specific roles of the Rho GTPase family (Wilhelm et al., 2014).

5.1.1.1.1 TNF-induced ROCK signalling

TNF induces the TNFR1-dependent activation of the Rho family small GTPases (Hunter & Nixon, 2006). Activation of the RhoA/ROCK axis is transient, cell type-specific and pronounced for macrovascular and microvascular endothelial cells (McKenzie & Ridley, 2007; Mong et al., 2008; Schlegel & Waschke, 2009; Fernandez-Martin et al., 2012). ROCK-2 is the major Rho kinase stimulating actomyosin during long-term cytokine treatment. ROCK suppression acutely diminishes TNF-driven endothelial compromise increases (Mong & Wang, 2009). Short-term inhibition of

ROCK, employing selective inhibitors, has confirmed the pivotal role of ROCK in stress fibre formation as a response to TNF stimulation (McKenzie & Ridley, 2007; Mong et al., 2008; Millan et al., 2010). However, pharmacological inhibition of members of the Rho GTPases family cannot always restore endothelial integrity, implying the involvement of additional interacting signalling cascades.

Surprisingly, dual targeting of both the RhoA/ROCK axis and of nmMLCK does not further enhance the vaso-modulating effect of TNF on microvascular and macrovascular endothelial barriers (Petrache et al., 2001; McKenzie & Ridley, 2007; Mong & Wang, 2009; Schlegel & Waschke, 2009; Fernandez-Martin et al., 2012). This is mirrored by the minimal myosin activity of stress fibres generated due to prolonged TNF exposure, suggesting the gradual stabilisation of these filaments and the generation of fractional tension in absence of additional inflammatory stimuli (McKenzie & Ridley, 2007). Interestingly, inhibition of RhoA activity restores TEER values upon short-term TNF (within 2h) stimulation. However, silencing of both ROCK-1 and ROCK-2 in HDMECs does not significantly rescue endothelial barrier integrity (Clark et al., 2015). Conversely, non-selective ROCK inhibitors such as Y-27632 enhance the function of the monolayer, suggesting that the key drivers of TNF-induced permeability increase might be tightly regulated by the same substrates.

5.1.1.2 Rac, Cdc42, PAK

Rac1, on the contrary, usually fires intracellular signalling that stabilises the endothelial barrier. This response dictates the onset of membrane protrusions and the

concomitant cell-cell contact. Similarly, Cdc42 acts as a stabilising mediator of the endothelial barrier integrity and is involved in cellular adhesion, polarity establishment, membrane ruffling, and intracellular trafficking. Moreover, Cdc42 signalling supports the formation of small membrane protrusions, coined as filopodia (Nobes & Hall, 1995). It is consensual that RhoA signalling counteracts Rac1, whereas Rac1 and Cdc42 govern endothelial cell function in an analogous way. The diverse array of functions controlled by particular RhoGTPases are dependent on the cell type, the localisation along the vasculature and tissues, and are reflective of a balance between redundancy and unique functionality.

The p21 proteins Rac and Cdc42 are also triggering the formation of both branched and unbranched actin networks. They positively regulate the p21-activated kinases (PAKs) that are predominantly involved in the establishment of cellular polarity, elongation and plasticity, the loss of focal adhesions and stress fibres, and the formation of cell protrusions (Manser et al., 1997; Szczepanowska, 2009).

The PAKs family is subdivided into two groups of serine/ threonine kinases: the group I PAKs (PAK1, PAK2, PAK3) are directly activated upon interaction with Rac and Cdc42, whereas the group II PAKs (PAK4, PAK5, PAK6) activity is not cross-regulated by other RhoGTPases (Chan & Manser, 2012). Interestingly, the kinase domains of both groups share approximately 50% identity and may govern a diverse array of functions by signalling to both overlapping substrates and distinct effectors (Ye & Field, 2012).

PAK1 expression is enriched in brain tissue, whereas PAK2 is highly expressed in endothelial cells and is associated with angiogenesis. PAK1 silencing has subtle impact on CNS function, and on immune regulation (Rudolph et al., 2015). Intriguingly, although Rac1 inactivation or depletion enhances vascular permeability during the embryonic development (Fiedler, 2009), activation of its downstream effector PAK1 promotes the deregulation of AJs, cell contractility and endothelial leakiness (Stockton et al., 2004; Orr et al., 2007).

The Rac- and Cdc42-induced activation of PAKs suppresses nmMLCK activity and induces LIM kinase (LIMK) signalling (Manser et al., 1994; Pertz, 2010). Rac and Cdc42 also control the mitogen-activated protein kinase (MAPK) homologs; Jun N-terminal kinase (JNK) and p38 (Minden et al., 1994; Bagrodia et al., 1995; Coso et al., 1995). Conversely, the Cdc42 and Rac-induced JNK activation is not associated with cytoskeletal regulation as this link is not amenable to treatment with cytochalasin D, a potent inhibitor of actin polymerisation (Coso et al., 1995).

Rac positively catalyses the emergence of cell protrusions, named lamellipodia, that are filled with newly polymerised F-actin. Rac signalling has also been associated with actin-independent functions. Impairment of either P-Rex or Rac function in human endothelial cells abrogates the TNF-induced ROS production (Naikawadi et al., 2012). On the contrary, hyperactivation of Rac yields elevated ROS levels and concomitant deregulation of cell signalling imposed by the oxidation of various residues from phosphatases and kinases involved in the mitochondrial respiratory chain (Corcoran & Cotter, 2013)

5.1.1.3 MYPT1, LIMK, nmMLCK

The enhanced phosphorylation of the downstream effectors of ROCK; myosin light chain (MLC-2), myosin phosphatase target subunit (MYPT1), and LIMK, is a hallmark of ROCK-mediated endothelial dysfunction (Cho et al., 2008; Kolozsvari et al., 2012; Yi, Xiao-Ping, & Hui, 2015).

The regulatory MLC-2 is phosphorylated by the non-muscular myosin light chain kinase (nmMLCK) (Takashima, 2009). nmMLCK is pivotal for the maintenance of physiological BBB permeability, whereas activation of the kinase under pathological conditions leads to microvascular endothelial barrier dysfunction (Shen et al., 2010). The activating phosphorylation of MLC-2 by nmNMMLCK induces an actomyosin-mediated cytoskeletal contraction that is followed by endothelial cell membrane retraction and potentiates the formation of intercellular gaps. This classical mechanism of endothelial barrier impairment can be assisted by the direct interaction of nmMLCK with junctional proteins and the concomitant alterations in their phosphorylation status (Coisne & Engelhardt, 2011).

Inflammatory mediators, such as thrombin, LPS and histamine increase microvascular leakiness by dictating an acute endothelial contraction. This is attributed in part to the RhoA-induced calcium sensitisation and the following attenuation of the Ca^{2+}/ calmodulin-dependent nmMLCK activity (Amano et al., 1996; Shasby et al., 1997; Wojciak-Stothard & Ridley, 2002; Birukova et al., 2004; van Nieuw Amerongen et al., 2007). However, other types of inflammatory stimuli such as activated neutrophils, have been reported to affect endothelial integrity in a nmMLCK-

independent fashion (Yuan et al., 2002), thus prompting the existence of diverse pathways regulating endothelial permeability increase.

ROCK regulates MLC activation not only through a direct activating phosphorylation (Amano et al., 1996), but also through an inactivating phosphorylation from MYPT-1. MYPT-1 is not only a major substrate of ROCK, but also a transducer of myosin light chain (MLC) phosphorylation (Wang et al. 2009). Suppression of MLC activity triggers myosin and actomyosin contractility (Chrzanowska-Wodnicka & Burridge, 1996) and may drive the disruption of TJs (Fanning et al., 2002), alongside compromising endothelial integrity (Kuhlmann et al., 2007; Yu et al., 2010). However, silencing of MYPT1 does not repress the TNF-induced MLC phosphorylation in HDMECs and does not diminish the endothelial TNF-induced leak (Clark et al., 2015).

The LIMKs (LIMK1 and LIMK2) are potent regulators of actin and microtubule dynamics, abundantly expressed in the CNS (Bernard, 2007; Yi et al., 2015). These serine/ threonine and tyrosine kinases are highly homologous, despite exhibiting distinct patterns of tissue distribution and variable cellular localisation. LIMK1 is the brain-specific isoform.

RhoA is directly interacting with LIMK, and triggers the accumulation of F-actin and the subsequent formation of stress fibres (Thirone et al., 2009), leading to BBB rupture (Fu et al., 2014; Y. Yu et al., 2014; Manaenko et al., 2018). Hyperactivation of the RhoA/ROCK axis by disturbed shear conditions induces phosphorylation of LIMK, cytoskeletal remodelling, degradation of BBB components and concomitant

disruption of endothelial integrity (Miyazaki et al., 2010). LIMKs modulate cell morphology and motility when localised in the cortical cytoplasm and in focal adhesions, while regulating cell cycle progression and cytokinesis when abundant in the centrosome and the nucleus (Prudnikova et al., 2015).

Members of the actin depolymerising factor (ADF)/ cofilin family are the major LIMK substrates (Galkin et al., 2011). Cofilin regulates the equilibrium between actin depolymerisation and polymerisation, depending on the cellular content of actin filaments. LIMKs phosphorylate and inactivate cofilin and govern actin microfilament dynamics. LIMKs are also regulated by PAKs, and nmMLCK (Prunier et al., 2017).

Moreover, LIMK1 colocalises with microtubules in endothelial cells (Gorovoy et al., 2005). Pharmacological inhibition of LIMK diminishes the levels of pLIMK-positive brain cells, alongside mitigating stress fibres formation and replenishing AJ integrity (Manaenko et al., 2018). Silencing of LIMK1 in human umbilical cord endothelial cells (HUVECs) represses the vasomodulating activity of thrombin by inhibiting actin polymerisation and microtubule disassembly (Gorovoy et al., 2005).

Adding another layer of complexity to the aforementioned signalling, TNF elicits the MMP-mediated degradation of the glycocalyx that is lining the endothelium, thereby regulating vascular integrity (Henry & Duling, 2000). Moreover, the TNF-mediated endothelial cytoskeletal and junctional reorganisation is regulated by Rho, ROCK, Rac and Cdc42. The concomitant alterations in endothelial barrier integrity occur as a delayed inflammatory response (Goldblum et al., 1989; Goldblum & Sun,

1990)that is characterised by advanced endothelial Rho and nmMLCK activity (McKenzie & Ridley, 2007; Wu et al., 2009; Xu et al., 2015).

These signalling nodes have been identified as key drivers of endothelial dysfunction as reflected by deteriorated transendothelial electrical resistance (TEER) values (De Vries et al., 1996) and increased endothelial permeability in numerous endothelial cell types (McKenzie & Ridley, 2007; Seki et al., 2011). In particular, the compromise of HUVEC monolayers is significantly increased at 8 hours and peaks at 24hours after the onset of the inflammatory stimulation (McKenzie & Ridley, 2007).

5.1.1.4 Other members of the RhoGTPase family

Interestingly, some RhoGTPases such as RhoE/Rnd antagonise RhoA cytoskeletal functions, eliciting both the disassembly of actin stress fibres and the disruption of focal adhesions, thus yielding the so-called *"round phenotype"*. RhoE is a paradigm of the interplay between the Rho GTPase family and mTOR. RhoE impinges on the translational repressor 4EBP-1 and impairs cell growth in response to extracellular stimuli (Villalonga et al., 2009).

5.1.1.5 FAK

Focal adhesion kinase (FAK) is an ubiquitously expressed member of the non-receptor tyrosine kinases. The Src family tyrosine kinases phosphorylate and activate this cytoplasmic kinase as a response to shear stress, growth factors, and costimulatory

integrins (Yuan 2003). FAK activity governs vascular morphogenesis, endothelial cell survival, motility and polarity (Lim et al., 2010; Mitra et al., 2005; Roy-Luzarraga & Hodivala-Dilke, 2016), alongside malignant transformation (McLean et al., 2004) and the execution of inflammatory responses (Walsh et al., 2010). Moreover, FAK is involved in the regulation of multiple signalling pathways including the activation of MAPKs, PI3K, Rac, and the phosphorylation of focal adhesion proteins such as paxillin (Schaller, 2010; Zhao et al., 2010). FAK has been suggested as a phospho-regulated suppressor of Rac-induced proliferative signalling in human endothelial cells (Bryant et al., 2012).

Moreover, FAK is a gatekeeper of endothelial barrier function in response to inflammatory mediators such as thrombin and VEGF (Chen et al., 2012; Mehta et al., 2002). Loss of FAK is associated with altered endothelial cell morphology, actin redistribution and enhanced transmembrane VE-cadherin localisation (Arnold et al., 2013). Disruption of FAK and paxillin phosphorylation, restores endothelial monolayer TEER values (Mehta et al., 2002). These vasomodulating effects are consistent with the inverse correlation between FAK and Rho in the regulation of FA turnover, with FAK being a suppressor of the GTPase (Ren et al., 2000).

FAK promotes cell contraction and locomotion through the regulation of FA assembly, and the modulation of integrin engagement with the extracellular matrix (ECM). Additionally, FAK inhibition restores TNF-induced redistribution of endothelial AJs, diminishes VE-cadherin internalisation, and stabilises the VE-cadherin- catenin linkage, thus diminishing vascular impairment (Schulte et al., 2011;

Jean et al., 2014). Moreover, suppression of FAK activity prevents the TNF-induced endothelial VCAM-1 upregulation, in a MAPK-dependent manner (Lim et al., 2012).

5.1.2 The PI3K pathway

5.1.2.1 PI3K

Phosphoinositide 3-kinase-related kinase (PI3K) is a lipid kinase, member of the family of Phosphatidylinositol 3-kinase-related kinases (PIKKs), that is associated with cellular transformation and insulin response. PI3K activation is triggered by various stimuli including the binding of ligands such as cytokines, growth factors and hormones to receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs). The autophosphorylation of RTKs leads to conformational changes that liberate the catalytic domain of PI3K. The activated PI3K generates the second messenger phosphatidylinositol,3,4,5-triphosphate (PIP3). PIP3 ensures the spatiotemporal distribution of molecules involved in the PI3K-dependent signalling (Vanhaesebroeck et al., 2010). PIP3 not only recruits phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane, but also anchors and relocates AKT, thus potentiating its PDK1-driven, priming phosphorylation (Guertin et al., 2006; Manning & Cantley, 2007). AKT is fully activated by a complementary phosphorylation driven by mechanistic Target of Rapamycin complex 2 (mTORC2) (Jacinto et al., 2006). The PI3K-dependent activation of AKT is also antagonised by the phosphatase and tensin homolog (PTEN). Moreover, PI3K is regulated by its interaction with members of the Ras family of small GTPases (Rodriguez-Viciana et al., 1994).

There is little evidence on the implication of PI3K on the deterioration of endothelial integrity. Targeted inhibition of this signalling node has been reported to block endocytosis and the formation of endosomes (Li et al., 1995; Niles & Malik, 1999).

5.1.2.2 AKT

AKT, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase (Coffer & Woodgett, 1991). AKT is a major signalling component downstream PI3K (Brazil et al., 2004). The involvement of the PI3K signalling cascade in AKT activation is considered to be obligate and rate limiting. The physiological roles of the AKT network include the governance of cellular growth, differentiation, proliferation, metabolism, apoptosis, and angiogenesis through the phosphorylation of several effectors. AKT-mediated signalling encompasses cell type-specific mechanisms and a highly branched downstream signalling network.

The exposure of vascular endothelial cells to mitogens, cytokines or growth factors triggers PI3K activation, PIP3 production, and the recruitment and activation of transmembrane PDK1 (Alessi et al., 1997). The latter is the main transducer of AKT, regulating a priming phosphorylation of the kinase. Maximal activation of AKT is achieved by an additional stabilising, activating, mTORC2-mediated phosphorylation (Alessi et al., 1996).

The AKT signalling cascade has prominent anti-apoptotic functions. These include both activating phosphorylations of substrates that hamper apoptosis initiation, and the stimulation of molecules controlling the activity of anti-apoptotic proteins.

FasL /TRAIL-driven stimulation of Fas resorts to the activation of AKT (Wosik et al., 2007). Moreover, AKT is an upstream regulator of NF-κB transcriptional activity (Sizemore et al., 1999; Sizemore et al., 2002). TNF-stimulation results in the activating phosphorylation of IKKα, but not IKKβ by AKT, and triggers the transactivation of the p65 component of the NF-κB pathway (Ozes et al., 1999; Madrid et al., 2000). The impact of AKT signalling on NF-κB induction is cell-type specific and is limited on HUVECs that favourably express IKKβ but not IKKα (Gustin et al., 2004).

AKT is a well-characterised major gatekeeper of the endothelial pro-survival signalling (Downward, 2004; Dormond et al., 2007; Fernandez-Hernando et al., 2007; Dada et al., 2008). This comprises the AKT-mediated direct phosphorylation of eNOS (Fulton et al., 1999), alongside the regulation of GSK-3β signalling and the inactivation of p21 (Cross et al., 1995). In addition, TNF-induced activation of the PI3K/AKT signalling cascade inhibits the pro-apoptotic cascades without manipulating NF-κB signalling in HUVECs (Madge & Pober, 2000). These findings align with the signalling ongoing in HBECs.

AKT also controls cell cycle progression (Liang & Slingerland, 2003). The relevant molecular machinery encompasses the negative regulation of GSK3β, alongside the phosphorylation of mTOR. The interrelationship between the AKT and mTOR

signalling nodes involves both positive and negative regulatory feedback loops (Hay, 2005).

Amongst the three members of the AKT family identified (Akt1, Akt2, Akt3), Akt1 is the most abundant isoform in endothelial cells under physiological conditions (Somanath, Razorenova, Chen, & Byzova, 2006), whereas Akt3 is more avidly expressed in brain tissue. Akt1 controls cellular survival and metabolism (Bhutani et al., 2013). VEGF-stimulated endothelial Akt1 activation promotes cell proliferation, survival and migration (Juhua Chen et al., 2005). Moreover, eNOS is an Akt1-specific substrate (Lee et al., 2014) and loss of that isoform is followed by disrupted NO release and impaired angiogenesis (Ackah et al., 2005). Endothelial Akt1 activity is also associated with vascular remodelling and exacerbated endothelial compromise (Phung et al., 2006).

5.1.2.3 GSK-3β

Glycogen synthase kinase-3 (GSK-3β) was identified as the final enzyme modulating the glycogen synthesis metabolic pathway. This ubiquitous serine/ threonine kinase (Embi et al., 1980) controls numerous and divergent biological functions such as glycogen and insulin metabolism, cell division and transcriptional activity, cell differentiation, apoptosis, stem cell renewal and the circadian rhythm (Jope et al., 2007; Maqbool et al., 2016). GSK-3β serves as a point of convergence for multiple signalling outputs and the complexity of its regulation is reflected in the multitude of its substrates, including signalling nodes (PTEN, TSC2, etc.); structural proteins

involved in cytoskeletal regulation and cell adhesion (paxillin, kinesin light chains; KLCs, etc.); and transcription factors (NF-κB, p65, c-Jun, etc.) (Kaidanovich-Beilin & Woodgett, 2011).

The paradoxical fine regulation of GSK-3β involves the constitutive activation of the kinase in resting cells, in absence of exogenous signals. AKT, PKC and PKA orchestrate the acute suppression of GSK-3β activity, through an inhibitory phosphorylation, as a response to various environmental cues, including inflammatory conditions. However, autophosphorylation, or phosphorylation by other kinases may enhance the activity of GSK-3β (Rayasam et al., 2009).

GSK-3β has been well-established as a crucial regulatory switch in the modulation of inflammation. The anti-inflammatory effects of GSK-3β inhibitors have garnered increasing attention and have been studied in several *in vitro* and *in vivo* models of acute and chronic inflammation. Pharmacological targeting of this abundant in the brain isoform (Kaidanovich-Beilin & Woodgett, 2011) successfully mitigates the activation of the vascular endothelium and ameliorates BBB function. GSK-3β inactivation triggers a gradual and sustained increase in TEER (Ramirez et al., 2013). Mechanistically, this intervention is maintaining BBB fidelity and is conferring junctional stability by significantly extending the half-life of occludin and claudin-5.

By contrast, TNF-challenge enhances GSK-3β activity and deteriorates the function of the brain endothelium, as mirrored by TEER decrease (Ramirez et al., 2010). Moreover, GSK-3β signalling is causally implicated in the presentation of increased BBB leakiness in an experimental model of autoimmune encephalomyelitis (Graesser

et al., 2002). Consistent with this, GSK-3β inactivation plays a neuroprotective role in experimental murine models of stroke and spinal cord injury (Cuzzocrea et al., 2006; Ren et al., 2003). Additionally, suppression of GSK-3β signalling diminishes the adhesion and the infiltration of recruited monocytes, and suppresses the expression of adhesion molecules and pro-inflammatory mediators (Eto et al., 2005; Gong et al., 2006; Ramirez et al., 2010).

Importantly, GSK-3β is also amenable to AKT-driven negative feedback (Cross et al., 1995). The AKT-mediated inactivating phosphorylation of GSK-3β stabilises the AJs and increases TEER values in lung endothelial cells (Liu et al., 2002). Conditions that result in decreased AKT activity, such as stroke, trigger the activation of GSK-3β. Moreover, emerging evidence from rat ischemic brain tissue supports the association of upregulated pAkt (active) and pGSK-3β (inactive), alongside the decreased nuclear accumulation of NF-ϰB with the avoidance of neurological impairment (Zhao et al., 2014). Combined, these data suggest the implication of the downregulation of the AKT/ GSK-3β stream and the enhancement of the GSK-3β/ NF-ϰB signalling in the exacerbation of BBB dysfunction.

In agreement with these findings, inhibition of GSK-3β activity promotes the remodelling of the brain endothelium after stroke in a PI3K/ GSK-3β -dependent manner (Guo et al., 2009; Zuo et al., 2017). The PI3K/AKT- driven attenuation of GSK-3β signalling not only potentiates BBB stability, but also suppresses neuroinflammation *in vivo* (Li et al., 2015). Finally, consistent with its BBB-enhancing

role, GSK-3β inhibition limits the release of the vasomodulator VEGF (Alhusban et al., 2017).

5.1.2.4 eNOS

The endothelial nitric-oxide synthase (eNOS) regulates vascular tone. TNF downregulates eNOS gene expression and triggers a decrease in bioactive NO production through transcriptional and post-transcriptional modifications (Anderson et al., 2004). The impairment of eNOS activity is resulting in reduced bioavailability of NO, a hallmark of endothelial dysfunction (Fischer et al., 1999).

Activation of RhoA/ROCK signalling directly diminishes endothelial nitric-oxide synthase (eNOS) mRNA stability (Laufs & Liao, 1998), but also indirectly affects cellular NO production through the induction of PTEN signalling and the concomitant suppression of AKT activation (Meili et al., 2005; Ming et al., 2002; Zhao et al., 2017). Mechanistically, TNF-mediated activation of RhoA induces elevated arginase activity, thus limiting the bioavailability of NO (Horowitz et al., 2007).

Pharmacological inhibition of ROCK, employing fasudil, rapidly triggers the PI3K/AKT pathway and enhances eNOS phosphorylation. Moreover, depletion of the co-factors Raptor and Rictor, interacting with mTORC1 and mTORC2 respectively suppresses eNOS phosphorylation (Wang et al., 2015). In line with this, long-term exposure to rapamycin that acts as a modulator of AKT, downregulates eNOS activity (Barilli et al., 2008).

Dual inhibition employing fasudil plus a PI3K or an eNOS inhibitor, LY294002 or L-NAME respectively, diminishes the protective effect of PI3K and eNOS on the endothelium, thus confirming the involvement of the ROCK/PTEN/AKT/eNOS axis on endothelial welfare (Wolfrum et al., 2004).

5.1.2.5 mTOR

The mechanistic target of rapamycin (mTOR) is a highly phylogenetically conserved, atypical serine/threonine kinase that belongs to the PI3K family. mTOR integrates extracellular growth signals and nutrients to control cellular growth and metabolism, survival, migration, immune responses and cytoskeletal reorganisation. Historically, mTOR was considered to be associated only with activities targeted by the immunosuppressant named rapamycin or its analogues, the rapalogues (Heitman et al., 1991). mTOR is the core of signalling pathways activated in response to nutrients and growth factors and a sensor of energy and oxidative status.

mTOR resides in two multiprotein signalling complexes that are functionally and structurally distinct; mechanistic Target of Rapamycin complex 1 (mTORC1) and mTORC2. These complexes have different sensitivities to rapamycin, alongside diverse upstream inputs and downstream outputs. The catalytic component of both multiprotein complexes is mTOR (Loewith et al., 2002). Rapamycin is an allosteric inhibitor of mTOR as it is binding to the intracellular receptor FK506- and to

rapamycin-binding protein-12 (FKBP12), thus selectively inhibiting mTORC1 by rendering the kinase domain of mTOR inaccessible.

mTORC1 and mTORC2 also share; mammalian lethal with sec-13 protein 8 (mLST8/ GβL), DEP domain containing mTOR-interacting protein (DEPTOR), and the Tti1/Tel2 complex. Regulatory-associated protein of mechanistic target of rapamycin (Raptor), and the proline-rich AKT substrate 40 kDa (PRAS40) are mTORC1-specific. Conversely, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protein 1 (mSin1), and protein observed with Rictor 1 and 2 (protor1/2) interact with mTORC2 only (Laplante & Sabatini, 2012). Moreover, multiple upstream inhibitory proteins repress the mTOR pathway; TSC1, TSC2, PRAS40, PTEN, REDD1, DEPTOR.

The molecular machinery underlying the activation of the two complexes relies on the fact that the binding of the specific co-factors Raptor and Rictor is mutually exclusive. The interplay between the constitutive elements or the dynamic regulatory partners of the complexes commands the spatiotemporal pattern of mTOR signalling. Notably, numerous cross-regulatory mechanisms of communication between mTORC1 and mTORC2 signalling have been documented, highlighting the biological importance of keeping these complexes tightly regulated.

5.1.2.5.1 mTORC1

mTORC1 serves a multitude of biological functions that promote cell growth. The complex regulates translation initiation, ribosome biogenesis, and autophagy in

response to various intracellular and extracellular cues; cytokines, mitogenic growth factors, and amino acids, energy status, oxygen and stress. Consistent with its role as a master regulator of cell growth, mTORC1 controls the G1 growth phase of the cell cycle, alongside protein and lipid synthesis, and VEGF secretion. These processes are dictated by the direct phosphorylation of the S6 kinase 1 (S6K1), the inactivation of the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), and the activity of the S6 ribosomal protein (Inoki et al., 2005). This mechanism is targeted by the cell-permeable, S6K1-specific inhibitor PF-4708671 (Bilanges & Vanhaesebroeck, 2010; Pearce et al., 2010). On the other hand, 4E-BP1 is a well-conserved metabolic brake, regulating the cap-dependent translation and maintaining cellular viability and energy homeostasis during stress conditions (Richter & Sonenberg, 2005). Moreover, the pathway has been associated with the cell-cycle dependent organisation of the cytoskeleton (Schmidt et al., 1996). Overexpression of components of the Rho pathway rescues mTOR mutations (Schmidt et al., 1997), and mTORC1 positively regulates ROCK activity. Moreover, mTORC1 hyperactivation increases actomyosin contractility, defective cytokinesis, and extensive plasma membrane blebbing, these being reminiscent of the effects of ROCK-2 overexpression. Elevated mTORC1 activation promotes Rho activity and phosphorylation of its downstream effectors; MLC2, Mypt1 and cofilin (Peterson et al., 2015).

The upstream regulators of mTORC1 include the heterodimer consisting of hamartin/ tuberous sclerosis complex 1 (TSC1) and tuberin/ TSC2. TSC1/2 interacts with the Ras homolog enriched in brain (Rheb) GTPase and functions as a GTPase-

activating protein (GAP). GTP-bound Rheb activates mTOR. TSC2 represses mTORC1 by converting Rheb into its inactive GDP-bound state (Inoki et al., 2006; Saxton & Sabatini, 2017). TSC1/2 transmits upstream signals mediating the inactivating phosphorylation of the heterodimer, followed by the activation of mTORC1. As previously discussed, growth factors stimulate the PI3K and Ras pathways and their effector kinases; protein kinase B (AKT/PKB), extracellular-signal-regulated kinase 1/2 (ERK1/2), and ribosomal S6 kinase (RSK1) that interact with TSC1/2 (Inoki et al., 2002; Ma et al., 2005; Manning et al., 2002; Roux et al., 2004). Additionally, the canonical Wnt pathway activates mTORC1 through inhibition of the glycogen synthase kinase 3β (GSK-3β) that normally promotes TSC2 activity (Inoki et al., 2006).

5.1.2.5.2 mTORC2

mTORC2 coordinates a diverse array of cellular processes spanning from cell migration and survival to regulation of the actin cytoskeleton via the activation of the RhoGTPases and the multifunctional ubiquitous serine/threonine kinases Protein Kinase Cα (PKCα) and AKT.

mTORC2 stimulation is under the direct control of the PI3K signalling pathway. mTORC2 signalling results in a stabilising phosphorylation of AKT in concert with PDK1 activation (Sarbassov et al., 2005). In addition, the binding of mTORC2 to the second messenger PIP3 leads to the relief of the autoinhibition of the kinase activity (Liu et al., 2015). This interaction not only facilitates the translocation of the complex

to cell membranes where AKT signals, but also mitigates the conformational constraints suppressing mTORC2 activation (Manning & Toker, 2017). However, PI3K is dispensable for mTORC2 transmembrane localisation (Ebner et al., 2017).

Taken together, AKT is a nodal point of mTOR signalling, being a downstream effector of mTORC2, and an upstream activator of mTORC1 (Gulati et al., 2009). However, the molecular and spatial nature of AKT activation does warrant further attention.

Additionally, loss of Rictor, the mTORC2 co-factor, hinders endothelial cell proliferation and assembly, whilst Raptor ablation does only have a modest impact on vascular endothelial monolayers (Dada et al., 2008; Wang et al., 2015). Consistent with these findings, mTORC2 does not signal to S6K (Jhanwar-Uniyal et al., 2017).

The mTORC2-associated proliferation defect has been linked to dysregulation of both PKC and -to a lesser extent- AKT. Interestingly, dissection of both Raptor and Rictor reduces VEGF-induced endothelial breaching and suppresses eNOS phosphorylation, whilst leaving PDK1, ERK1/2 and p38MAPK unaffected. Inducible expression of AKT or PKCα in Rictor-null endothelial cells restores eNOS activity, suggesting eNOS as a downstream effector of both AKT and PKCα (Wang et al., 2015).

The mTORC2-Rictor complex regulates actin polymerisation through modulation of the phosphorylation status of PKCα that is modulating cellular shape and motility (Nakashima, 2002; Sarbassov et al., 2004). Silencing of the complex prevents actin polymerisation and can be rescued by the activation of Rho and Rac (Jacinto et al.,

2004; Sarbassov et al., 2005). Notably, phosphatidylinositol 3,4,5-trisphosphate-1 (P-Rex1) and P-Rex2 are Rac-specific, PIP3-dependent GEFs (RacGEFs) downstream of mTOR, that establish links between the PI3K signalling cascade and Rac. Following their stimulatory interaction with constituents of the mTORC2 complex, they orchestrate Rac activation and cell migration, thus triggering a rapamycin-resistant mechanism (Hernandez-Negrete et al., 2007; Kim et al., 2011). The mTORC2/P-Rex-1/Rac regulatory loop has been identified in endothelial cells (Dada et al., 2008) and has been proven to be instrumental for cell migration and angiogenesis (Carretero-Ortega et al., 2010).

The mTORC2 complex is also controlling cell polarity, rear formation and chemotaxis. These processes converge on the polarisation of actin and myosin filament networks and are vital for the survival of eukaryotic cells during starvation conditions. The aforementioned mTORC2- mediated signalling involves the synthesis of cyclic AMP and the stimulation of the RhoA-mediated ROCK and nmMLCK activity (David, 2011; Liu et al., 2010).

Although mTORC2 was originally considered to be rapamycin insensitive (Jacinto et al., 2004; Sarbassov et al., 2004), prolonged rapamycin treatment can impede the *de novo* assembly of the mTORC2 signalling complex both in cancer cells (Sarbassov et al., 2006), and in endothelial cells (Barilli et al., 2008). It has been postulated that this is reflecting the reduced availability of mTOR for mTORC2 due to sequestration of mTOR in the rapamycin-bound mTORC1 and the allosteric effect of rapamycin on mTORC2 (Chen et al., 1995).

5.1.3 The NFκB pathway

Since its discovery in 1986 (Baltimore, 2011), the transcription factor nuclear factor-κB (NF-κB), is a topic of intense investigation due to its dynamically branching and looping signalling network. NF-κB is a key regulator of innate immunity, inflammation, cell survival, proliferation, and differentiation (Baltimore, 2011; Hayden & Ghosh, 2012; Oeckinghaus & Ghosh, 2009). The importance of this major signalling crossroad is underscored by its presence in almost every cell type (Wenzel & Schwaninger, 2016) and by the development of more than 780 substances affecting NF-κB activation (Gilmore & Herscovitch, 2006).

The NF-κB transcription complexes include a variety of homodimers and heterodimers of Rel family proteins (p65/RelA, RelB, c-Rel, p105/p50/NF-κB1, and p100/p52/NF-κB2) that bind to DNA regulatory sites, the κB sites, hence triggering specific target gene expression (Lernbecher et al., 1993). The cellular compartmentalisation of NF-κB is dependent on its activation status. The latent NF-κB transcription dimers are retained in the cytoplasm in unstimulated cells and interact intermolecularly with regulatory protein inhibitors of the κB (IκB) family. Pro-inflammatory stimulation by TNF triggers the TNFR-associated factor (TRAF) adaptor proteins and exerts the activating phosphorylation of IκB proteins by the IκB kinase (IKK) complex, followed by their proteasomal degradation. The translocation of the free NF-κB enables its binding to the target DNA sequence, thus promoting the transcriptional activation of pro-inflammatory genes including TNF, chemokine (C-C

motif) ligand 2 (CCL2) and C-X-C motif chemokine 5 (CXCL5) (Kaulmann & Bohn, 2014; Lawrence, 2009; Tak & Firestein, 2001).

The IKK complexes governing NF-κB activation comprise two kinases: IKKα (IKK1) and IKKβ (IKK2) plus a regulatory subunit IKKγ/ NF-κB essential modifier (NEMO) that directly interacts with the TNFRs. IKKγ is a scaffolding protein, devoid of any catalytic activity. However, its preferential interaction with IKKβ is indispensable for the activation of the IKK complex (Rothwarf et al., 1998). Although IKKα and IKKβ are homologous, they are functionally distinct. IKKβ is more effectively phosphorylating IκBα. The expression levels of both kinases are responsive to environmental cues, including TNF stimulation, thereby dictating the involvement of diverse regulatory signalling cascades (Madge & Pober, 2000; O'Mahony et al., 2000; Gustin et al., 2004).

The molecular armoury involved in the NF-κB signalling cascade is cell type-specific, and dependent on the stimulus and the context of its activation (Pahl, 1999; Tabruyn & Griffioen, 2008). This is highlighted by the non-redundant functions controlled by the two distinct NF-κB signalling cascades.

The canonical (or classical) NF-κB pathway is rapidly induced by IKKβ and NEMO as a response to pro-inflammatory stimuli including TNF, IL-1β and microbial factors (LPS, etc.) (Alain Israel, 2010; Lawrence, 2009; Vallabhapurapu & Karin, 2009) or due to physical stress (UV radiation, etc.). The cascade triggers a positive feedback loop that prompts the secretion of pro-inflammatory cytokines (Oeckinghaus & Ghosh, 2009) and is driven by the TGFβ- activated kinase 1 (TAK1). This signalling node

regulates the phosphorylation of IKKβ and the inaugural, IKKβ-mediated phosphorylation of IκBα. The induction of the pathway promotes the *de novo* synthesis of IκBα molecules (p65 and p50) that are sequestered in the nucleus, attenuates the single binding of active NF-κB subunits to its DNA targets and supports the cytoplasmic accumulation of the IκBα/NF-κB heterodimer (Arenzana-Seisdedos et al., 1995; Browning et al., 1993; Rodriguez et al., 1999). On the contrary, the delayed, non-canonical (or alternative) pathway is IKKα-dependent and is controlled by the activity of NF-κB-inducing kinase (NIK/ MAP3K14), AKT, and the degradation of TRAF proteins, but not NEMO or IKKβ (Hacker & Karin, 2006; Sizemore et al., 2002). The IκB protein of the alternative pathway is p100. Both streams of the NF-κB signalling pathway can be induced concurrently and are cross-regulated (Scheidereit, 2006; Hayden & Ghosh, 2012).

5.1.3.1 NF-κB and TNF

The implication of members of the TNF family on the activation of the NF-κB pathway has been well-established more than 30 years ago and is demonstrated for TNF (Israel et al., 1989; Osborn et al., 1989), Lt (Messer et al., 1990) and the TNF-like weak inducer of apoptosis (TWEAK) (Jin et al., 2004). The MAP3 kinases, NIK and mitogen-activated protein kinase kinase-1 (MEKK-1), and the PI3K/AKT pathway are well-recognised as upstream regulators of the activation of NF-κB that are recruited by TNF (Nakano et al., 1998; Nemoto et al., 1998; Sizemore et al., 2002). MEKK-1 phosphorylates and activates both IKKα and IKKβ (Lee et al., 1998),

whereas the non-canonical signalling is highly dependent on NIK (Sun, 2012). Under physiological conditions NIK is continuously degraded but pro-inflammatory stimulation leads to the stabilisation and the activation of this key transducer. Members of the tumour necrosis factor receptor superfamily (TNFRSF) are directly modulating the activation status of NIK via the sequential interaction of TNFR1 with TRADD, TRAF2, and NIK itself (Hsu et al., 1996; Simeonidis et al., 1999). Additionally, the TNFR-associated factors 2/3 (TRAF2/3) form a ligase complex that stimulates the dissociation of NIK and its accumulation at IKKα, followed by the nuclear localisation of NF-κB and the transcription of target genes (Razani et al., 2011; Zarnegar et al., 2008).

5.1.3.2 NF-κ B at the BBB

NF-κB activation is a hallmark of tissue damage and BBB compromise under conditions triggering inflammatory mechanisms, such as stroke, meningitis, etc. NF-κB is a key driver of neuronal damage, cell death, and of the activation of different cell types within the NVU. Astrocytic and pericytic NF-κB activation elicits MMPs secretion and concomitant degradation of the barrier (Bell et al., 2012; Coelho-Santos et al., 2015). Moreover, silencing of the most common subtype of IκB, IκBα, in astrocytes promotes neuroinflammation.

TNF triggers a handful of signalling pathways in brain microvascular endothelial cells that converge on the activation of NF-κB. Administration of intermediate doses of TNF, rapidly (within 5min) activates NF-κB (Chaturvedi et al., 1994). TNF

prompts the production of reactive oxygen species (ROS); the secretion of chemokines CCL2/MCP-1 and CXCL8/IL-8; and the activation of the ERK pathway, thus activating the NF-ϰB signalling cascade and promoting the expression of VCAM-1 and ICAM-1 (Wong et al., 1992; Wong et al., 1995; Tang et al., 2011; Zhong et al., 2012). Endothelial NF-ϰB activation is also linked to TJs disruption, to altered cytoskeletal dynamics, and to subsequent elevated barrier leakiness (Ma et al., 2004; Banan et al., 2007; Verstrepen et al., 2008; Aveleira et al., 2010; Aslam et al., 2012).

5.1.4 The MAPK pathway

The mitogen-activated protein kinases (MAPKs) represent a highly conserved family of Ser/Thr protein kinases that comprises four major subclasses: the extracellular signal-regulated kinases (ERK1/2), c-Jun NH2-terminal kinase (JNK), p38 MAPK (p38α, p38β, p38γ,p38δ), and the ERK5 pathways (Dinsmore & Soriano, 2018). All canonical MAPK pathways share a three-tiered kinase cascade consisting of an upstream MAPK kinase (MAPKKK), a MAPK kinase (MAPKK), and the MAPK itself. MAPKs coordinate a series of cellular processes such as proliferation, survival, apoptosis, migration, and cell differentiation. Although these signalling cascades partially overlap and modulate some common substrates, their divergent functions rely on the stimulation of unique ensembles of effectors, the involvement of feedback mechanisms and scaffolding proteins, and their distinct spatiotemporal and dynamic regulation. Endothelial activation of the MAPK pathways has been reported for several

neuropathological processes including ischemia, trauma and seizures (Nonaka et al., 1999; Zhang & Wood, 2005).

TNF is strongly inducing these pathways through signalling networks directly involving the TNFR1 complex (Mong et al., 2008). This cytokine prompts a time-dependent activation of the MAPK pathway in various cell types. An early and robust MAPK activation phase (5-30min) is succeeded by a moderate and prolonged activation phase that lasts for hours. These phases encompass diverse signalling cascades and are functionally distinct (Ventura et al., 2006). In agreement with this pattern, TNF stimulation triggers a rapid (within 15min) upregulation of phospho-p38 MAPK, phospho-ERK and phospho-JNK expression levels in hCMEC/D3 cells and in HUVECs (Li et al., 2002; Modur et al., 1996; Ni et al., 2017). These findings are complemented by the LPS-induced stimulation of the p38 MAPK and JNK pathways and the concomitant activation of MMP-2 in hCMECs (Qin et al., 2015). p38 MAPK signalling responds to various extracellular cues including growth factors, hormones, ligands for GPCRs inflammatory cytokines (IL-1, IL-8, TNF), and stresses (heat, osmotic shock).

The cytokine-induced activation of the MAPK pathways prompts the redistribution or the downregulation of the junctional proteins occludin and ZO-1 (Fischer et al., 2005; Krizbai et al., 2005; Qin et al., 2015). In support of this mechanism, blockade of these pathways partially restores occludin but not ZO-1 levels, suggesting that MAPK might be involved in occludin biosynthesis. Inhibition of the p42-44 MAPK pathway abolishes the TNF-mediated transcytosis (Miller et al., 2005).

5.1.4.1 JNK

Activation of TNFR1 might prompt JNK-regulated responses indirectly, by targeting signalling molecules of the Rho pathway, predominantly ROCK (Mong et al., 2008). This signalling involves a cascade with distinct activities from those associated with the ROCK-mediated actin polymerisation (Marinissen et al., 2004). Interestingly, JNK-mediated ROCK stimulation is RhoA-independent in pulmonary microvascular endothelial cells (Mong et al., 2008) and only the late phase of JNK activation is MAPK kinase 4 (MKK4)-dependent.

5.1.4.2 ERK

The ERK1/2 pathway is activated in response to various stimuli including receptor tyrosine kinases (RTKs) signalling, G-protein-coupled receptors (GPCRs), and cellular stress. The RTKs directly recruit the GEF Son-of-Sevenless (SOS) that is activating the membrane-associated GTP-binding protein Ras. The GTP-bound, activated Ras, in turn, initiates the ERK1/2 signalling cascade that comprises the MAPKKKs A-RAF, B-RAF, and RAF-1/C-RAF; the MAPKKs MEK1 and MEK2; and the MAPKs ERK1 and ERK2 (Casar & Crespo, 2016). Upon its activating phosphorylation by MEK, ERK1/2 acts on its cytoplasmic targets or regulates a panel of nuclear transcription factors, thereby controlling cellular proliferation and survival, and migration (Dinsmore & Soriano, 2018).

MEK and ERK1/2 phosphorylation is triggered by inflammatory agonists (FasL, TRAIL, TWEAK, VEGF) and by intracellular calcium-elevating agents, leading to a rapid increase in MMP-9 activity in HBECs (Wosik et al., 2007; Stephan et al., 2013). Moreover, the ERK pathway is a key regulator of the interplay between integrins and cytokine receptor signalling. Transmembrane integrins positively regulate the NF-κB pathway as a response to TNF-induced pro-survival signalling (Beg & Baltimore, 1996; Schwartz, 2001).

5.1.5 TNFR1-mediated pro-apoptotic signalling

Although TNF was discovered more than 40 years ago, the physiological and pathophysiological roles of TNF-induced cell death remain uncertain. TNFR1 blockade is detrimental for the transcriptional upregulation of pro-inflammatory genes, governed by the activation of MAPKs and the canonical NF-κB pathway (Kalliolias & Ivashkiv, 2016). Furthermore, TNF signalling can promote or exacerbate cell death, in the form of apoptosis or necroptosis (Pasparakis & Vandenabeele, 2015). This inflammatory response may involve pattern recognition receptors (PRRs) of damage-associated molecular patterns (DAMPs) revealed or secreted by dying cells. Alternatively, endothelial cellular death followed by barrier leakiness leads to sensing of pathogen-associated molecular patterns (PAMPs) present in microbes traversing the barrier.

The engagement of TNFR1 by TNF serves two divergent cellular responses; the sequential assembly of the membrane-bound primary signalling complex (complex I/

TNFR1-SC), followed by the formation of the secondary cytoplasmic complex (complex II) (Blackwell et al., 2013; Ting & Bertrand, 2016).

TNFR1 complex I is rapidly formed, within seconds of the TNFR1-TNF ligation, and is orchestrating the activation of the canonical NF-κB pathway, thus triggering the expression of pro-survival molecules. Activated TNFR1 does recruit TNF receptor associated via death domain (TRADD) and receptor interacting serine/threonine kinase (RIPK1) through homotypic death domain (DD) interactions. TRADD further attracts TNF receptor associated factor 2 (TRAF2) and/or TRAF5, which subsequently interact with cellular inhibitor of apoptosis1 (cIAP1) and cIAP2 to the receptor complex (Haas et al., 2009). These complexes generate ubiquitin chains that recruit the linear ubiquitin chain assembly complex (LUBAC). The ubiquitin chains serve as scaffolds for the recruitment, activation and retention of TAK1 binding protein 2/3 (TAB2/3) and the NEMO/IKKα or IKKβ kinase complexes (Ting & Bertrand, 2016). The TAK1-driven phosphorylation of IKKβ evokes IκBα phosphorylation and its subsequent ubiquitylation-dependent, proteasomal degradation. This digestion permits the nuclear translocation of the NF-κB heterodimer p50/p65 (Hayden & Ghosh, 2014). The ubiquitylation-dependent activation of NF-κB and the concomitant transcription of pro-inflammatory and pro-survival genes is tightly regulated by deubiquitylases (DUBs) that actively repress the TNF-induced response by dismantling the ubiquitin network linked to complex I (Gerlach et al., 2011).

The cytosolic complex II assembles slowly and transiently upon TNF stimulation and orchestrates pro-survival responses. The activation of the complex requires the

dissociation of TRADD, followed by its association with Fas associated via death domain (FADD), serving as a platform for the recruitment and the activation of caspase-8, thus inducing apoptosis.

This molecular machinery is regulated by a NF-κB-inducible gene product, cFLIP that antagonises caspase-8 for binding to FADD. Under NF-κB transcriptionally active conditions, cFLIP is upregulated and perturbs the transient formation of complex II. Conversely, NF-κB activation is abrogated when cFLIP expression is minimal.

The aforementioned TNF-induced apoptotic complex that requires the activation of the TRADD/FADD/caspase-8 cascade is coined as complex IIa. TNF-mediated apoptosis is regulated via the physical interaction of the TNFR1 signalling complex with caspase-8 (Guicciardi & Gores, 2009). This late, NF-κB-dependent checkpoint reflects a genetic reprogramming mechanism that allows the cell to evade cell death and achieve a more permanent death-resistant state.

Moreover, RIPK1 is also involved in the generation of pro-survival signalling in an NF-κB-independent manner. The early, cytosolic complex IIb requires signalling from the RIPK1/FADD/caspase-8 axis and functions as a brake on the RIPK1-associated pro-apoptotic signalling (Wilson et al., 2009). The cascade is also effective in refraining necroptotic signalling. Necroptosis is a regulated form of necrosis prevailing upon the suspension of caspase-8 activation and is involved in the pathobiology of numerous diseases (stroke, inflammatory bowel disease, systemic inflammatory response syndrome, etc.) (Linkermann & Green, 2014).

Notably, complex IIb exerts a critical inflammatory role since it serves as a sensor for the presence of pathogens encoding signals that disrupt its function. Pathogen-derived inhibitors that block IKKs in an attempt to hinder the NF-κB-driven pro-inflammatory gene expression succumb by RIPK1-depend cell death (Yatim et al., 2015).

Taken together, the TNF-induced cell death signalling is suppressed unless some of the cell death checkpoints are disrupted. The physiological conditions turning off these checkpoints are causally implicated in the cell-type specific resistance towards TNF-induced cell death. The late cell death checkpoint (complex I and complex IIa) is controlled by the activation of the NF-κB signalling cascade. On the contrary, the early NF-κB-independent checkpoint (complex IIb) is regulated post-translationally and represents a rapid way to determine the fate of TNF-stimulated cells. This machinery can be exemplified by the molecular underpinnings of the diverse cell fates governed by inflammatory mediators. Ligation of the tumour necrosis factor receptor superfamily (TNFRSF) members; TNFR2, NIK or TWEAK, suppresses the TNFR1-mediated response, through the degradation of a pool of TRAF2/cIAP1/2 (Boutaffala et al., 2015; Fotin-Mleczek et al., 2002; Li et al., 2002). By contrast, binding to TNFR1 leads to cell survival, whereas simultaneous ligation of both TNFR1 and TNFR2 promotes the RIPK1 kinase-dependent cell death signalling.

5.1.5.1 TWEAK

Emerging evidence supports the role of TWEAK in the modulation of the BBB. Brain endothelial cells and astrocytes are responsive to the interaction of members of the TNF ligand family with its cognate receptor Fn14 (Yepes, 2013). Intracerebral injection of TWEAK induces NF-κB dictated upregulation of MMP levels (Polavarapu et al., 2005). Moreover, injection of monoclonal antibodies targeting TWEAK during the recruitment phase of immune cells retains BBB integrity (Desplat-Jego et al., 2005). These findings are in agreement with the observation of increased endothelial permeability upon administration of soluble TWEAK *in vitro* (Stephan et al., 2013). The overarching mechanism of action of TWEAK involves the induction of proliferation and angiogenesis in the brain endothelium (Lynch et al., 1999). The molecular armoury of endothelial stimulation by TWEAK also comprises an inflammatory profile, as evidenced in hCMECs, through the secretion of cytokines, the modulation of the production and activity of MMPs, and the increased expression of CAMs (Stephan et al., 2013). Further to the regulation by the NF-κB pathway, the TWEAK-stimulated expression of MMPs encompasses MAPK signalling, particularly the activation of the MEK and ERK branches. Interestingly, pharmacological targeting of MEK and ERK suppresses MMPs expression in hCMEC/D3 but does not rescue the endothelial monolayer impairment (Stephan et al., 2013).

5.1.5.2 Fas/ FADD

The caspase cysteine protease family is well-recognised for its role in the execution of programmed cell death. However, caspase activation does not always trigger cell death (Kroemer & Martin, 2005). Notably, the maintenance of embryonic endothelial cells is a FADD-mediated, caspase-8- dependent process (Kang et al., 2004).

The tumour necrosis factor receptor superfamily (TNFRSF) comprises TNFR1, Fas (CD95/APO-1), and the decoy receptors DR4 and DR5. The cognate ligands for these receptors are TNF, factor associated with suicide ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL/ APO2L). TNFR1, Fas, and DR5 are expressed under basal conditions in primary hBECs (Wosik et al., 2007).

Fas is a prototypical death receptor, member of the TNF/ nerve growth factor (NGF) superfamily. Fas forms homotrimers, predominantly located at the cell surface and is particularly abundant in the brain tissue (Brint et al., 2013). The expression of its cognate ligand Fas (FasL/CD95L) is restricted to immune cells. Constitutive FasL expression serves as a mechanism to disrupt noxious inflammatory immune responses through the induction of apoptosis.

Fas- FasL ligation elicits conformational changes in the intercellular domain of the receptor and the recruitment of the death domain (DD)-containing adaptor molecule Fas-associated death domain (FADD). FADD recruits the initiator caspase, pro-caspase-8 and forms the death-inducing signalling complex (DISC) within seconds after FasL binding. Pro-caspase-8 is autoactivated through self-cleavage and the release of

active proteases. Depending on the signalling cascade activated upon Fas ligation, the extrinsic or the intrinsic apoptosis pathways are executed (Scaffidi et al., 1998). The extrinsic apoptosis pathway involves the direct caspace-8-mediated activation of its downstream effector caspases (caspase-3, -6, -7) and the resulting cleavage of a restricted set of target proteins, followed by apoptotic cell death. The intrinsic pathway is triggered in cells with reduced DISC formation and caspase-8 activation, where mitochondria function as signal amplifiers. This cascade involves the activation of caspase-9, its interaction with cytochrome c and the apoptotic protease-activating factor 1 (Apaf-1) in a multiprotein complex called apoptosome. Activation of caspase-9 is the key event towards the execution of apoptotic cell death.

The fate FasL/Fas- induced signalling is cell type-specific and depends on the stimulus eliciting the binding of FasL. Surprisingly, ligation of Fas can also induce proliferative signalling, stressing the role of Fas as a bidirectional regulator (Brint et al., 2013).

Ligation of TNFR1, Fas, and DR5 in human brain endothelial cells induces distinct signalling repertoires, devoid of pro-apoptotic or proliferative functions (Wosik et al., 2007). Unlike TNF (Wong et al., 1992, Wong et al.,1995), FasL and TRAIL do not trigger the NF-κB-regulated transcription of pro-inflammatory mediators, but induce an ERK1/2-mediated release of MMP-9. Additionally, FasL modulates the PI3K/AKT axis by conferring an activating phosphorylation on AKT.

Soluble FasL transmits anti-inflammatory signals in the brain endothelium through the activation of the cytosolic complex II. Low levels of sFasL induce proliferative

signalling involving the increase in autocrine VEGF and the induction of the FADD/FLIP/TRAF/NF-κB axis (Zhang et al., 2015). Moreover, endothelial cells are not susceptible to Fas/FasL-induced apoptosis (Richardson et al., 1994).

On the contrary, evidence from TNF-stimulated human and mouse brain microvascular endothelial cells (hCMEC/D3 and b.End3, respectively) suggests that they undergo apoptosis upon binding of TRAIL but not FasL (Chen & Easton, 2010; Richardson et al., 1994).

These opposing outcomes confirm that diverse members of the TNFRSF are inducing variable and even opposing outcomes in the brain endothelium, despite the activation of overlapping signalling nodes downstream of TNFRs. Notably, the intensity of the inflammatory stimulus and the length of the exposure to that are determinants of the unique combinations of effectors activated each time.

5.1.6 Tyrosine kinases

Tyrosine phosphorylation is well-established as a primitive event, indicative of signal transduction in multicellular organisms (Ullrich & Schlessinger, 1990). Protein tyrosine kinases encompass receptor tyrosine kinases (RTKs; vascular endothelial growth factor receptor -VEGFR, platelet-derived growth factor receptor- PDGFR, epidermal growth factor receptor -EGFR, etc.), and non-receptor tyrosine kinases such as FAK, c-Src, Csk, Yes, etc. Src has been proven to control TNF-induced endothelial dysfunction through tyrosine phosphorylation of VE-cadherin in HUVECs (Nwariaku et al., 2002).

Imatinib, the first signal transduction inhibitor (STI) used in a clinical setting, enhances the BBB either by alleviating the effect of PDGFR signalling or by suppressing MMP-2 expression (Chi et al., 2006; Schultz et al., 2011).

TNF drives tyrosine kinase activation in human lung endothelial cells. Execution of that cascade not only promotes tyrosine phosphorylation of numerous endothelial substrates, including zonula adherence proteins, VE-cadherin, β-, γ-, and p120-catenin, but also increases paracellular permeability (Angelini et al., 2006). However, pre-treatment of renal or lung endothelial cells with genistein does not avert the TNF-mediated rupture of the endothelium (Safdar et al., 2003; Xu et al., 2015).

	Permeability ↓ (%)	Significance	related signaling nodes					↓: downregulation				↑: upregulation			&: additive effect			+ to +++ : inhibition				✘: lack of inhibition (activity remaining)							treatment with agonist: (activity remaining)					
			TNF	TNFR1	Ras	MEK	ERK	STAT	JNK	p38 MAPK	PI3K	PDK1	GSK3β	Akt	mTORC1	mTORC2	S6K	4E-BP1	IKK	PKA	PKC	FAK	Cdc42	RAC	ROCK1	ROCK2	PAK	LIMK	actin	myosin/ mmLCK	RTKs (EGFR, PDGFR, CSF1R and FGFR)	Src	Ser/Thr	MMPs	iNos/ eNOS
SPD304	78.5	***	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R-8507	77.2	***	+++	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Necrostatin-1 (Nec-1)	65.6	***	+++	+++	-	✘ (100%)	✘ (92%)	-	✘ (79-100%)	✘ (88-100%)	-	✘ (92%)	✘ (100%)	✘ (68-91%)	-	-	✘ (99%)	-	✘ (86-96%)	✘ (93%)	✘ (96%)	-	-	-	-	✘ (97%)	✘ (94-99%)	-	-	-	✘ (88-100%)	✘ (100%)	-	↓	-
Nec-1i (inactive control)	2.1	ns	+	+	-	+ (2%)	✘ (82-88%)	-	✘ (95-100%)	✘ (82-98%)	-	✘ (92%)	✘ (92%)	✘ (90-97%)	-	-	✘ (100%)	-	✘ (100%)	✘ (95%)	✘ (100%)	-	-	-	-	✘ (96%)	✘ (98-100%)	-	-	-	✘ (80-100%)	✘ (99%)	-	-	-
NSC 47147 (Prodigiosin)	62.8	***	-	-	-	-	-	-	-	-	-	-	↑	+	-	-	-	-	++ (37%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
z-DEVD-fmk	3.3	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Boc-D-fmk	7.9	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	-	-	-	-	-	-	-	-	-	↓	-	-	-	-	-	-
PND1186	76.6	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ (50%)	-	-	-	-	-	-	↓ 38% MLC	-	✘	-	-	-
ZCL278	55.3	***	-	-	-	-	↓	-	↓	-	-	-	-	↓ (PIP2)	-	-	-	-	-			-	+++	-	-	-	-	-	↓	-	-	-	-	-	-
EHT1864	44.0	***	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	✘	+++	✘		✘	+	-	-	-	-	-	-	-
EHop-016	48.0	***	-	-	-	-	-	-	↓	+	-	-	-	↓	-	-	-	-	-	-	-	-	+ (72%)	++ (7-21%)	✘		+	-	-	-	-	-	-	-	-
Rho activator II (↑)	-4.0	ns	-	-	✘	-	-	-	-	-	✘	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑		-	-	-	-	-	-	-	-
Rho/Rac/Cdc42 activator (↑)	0.0	ns	-	-	✘	-	-	-	-	-	✘	-	-	-	-	-	-	-	-	-	-	-	-	↑ (120-150%)	↑ (>300 %)	↑ (>150%)		-	-	-	-	-	-	-	-
U-46619 (↑)	22.9	*	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	↑		-	-	-	-	-	-	-	+
Fasudil (HA1077)	54.9	***	-	-	-	✘ (63-91%)	✘ (84-92%)/↓	-	✘ (81-100%)	✘ (84-100%)	-	✘ (93%)	✘ (80%)	✘ (55-89%)	-	-	+ (33%)	-	✘ (92-94%)	+ (26%)	✘ (77%)	✘ (94%)	-	✘ (90 %)	+ (60%)	+ (12%)	✘ (83-99%)	✘ (100%)	-	✘ (100%)	✘ (60-100%)	✘ (93%)	-	↓	-
Y-27632	65.1	***	-	-	-	✘ (100%)	✘ (94%)	✘ (82-98%)	✘ (77-92%)	✘ (87-100%)	-	✘ (74%)	✘ (86%)	✘ (73-86%)	-	-	✘ (93%)	-	✘ (76-78%)	+ (72%)	+ (57%)	✘ (98%)	-	✘ (92 %)	++ (32%)	+ (7%)	✘ (88-89%)	✘ (86%)	-	++, ↓ 35% MLC	✘ (93-100%)	✘ (79%)	-	-	-
KD025 (SLx-2119)	76.0	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+++	-	-	✘	++	-	-	-	-	-
ML-7 (% activity remaining)	63.1	***	-	-	-	✘ (100%)	✘ (85%)	-	✘ (97%)	✘ (85%)	-	✘ (96%)	✘ (78%)	-	-	-	+(50%)	-	+++, (45%), ↓ 50% pMLC	+(52%)	✘ (76%)	-	-	-	-	+(54%)	✘ (95%)	-	-	+++, ↓ 10% pMLC	-	✘ (98%)	-	&	&
SR7826	77.2	***	-	-	-	-	-	-	✘	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	✘	✘	-	+++ (<20%)	-	-	-	-	-	-	-
FRAX597	67.5	***	-	-	-	↓	↓	-	-	↓	-	-	-	↓	↓	✘	↓	-	-	-	-	↓	-	-	-		+++ (7-18%)	-	-	-	++	✘ (81%)	-	-	✘
Latrunculin A	8.8	ns	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-
Latrunculin B	-12.3	ns	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	+	-	-	-	-	-	-

	Permeability ↓ (%)	Significance	related signaling nodes					↓: downregulation				↑: upregulation			&: additive effect		+ to +++ : inhibition					✘: lack of inhibition (activity remaining)							treatment with agonist: (activity remaining)					
			TNF	TNFR1	Ras	MEK	ERK	STAT	JNK	p38 MAPK	PI3K	PDK1	GSK3β	Akt	mTORC1	mTORC2	S6K	4E-BP1	IKK	PKA	PKC	FAK	Cdc42	RAC	ROCK1	ROCK2	PAK	LIMK	actin	myosin/ mmMLCK	RTKs (EGFR, PDGFR, CSF1R and FGFR)	Src	Ser/Thr	MMPs	iNos/ eNOS
Farnesyltransferase (Ftase) Inhibitor (FTI-II)	76.0	***	-	+++ not p65	+++	+	+	✘	✘	✘	+	-	-	✘	+	++	+++ (10-20%)	-	+++ not p65	-	↑	✘	✘	-	✘	✘	-	-	-	-	-	✘	-	↓	↑
Farnesylthiosalicylic Acid (FTS/salirasib)	79.8	***	-	-	+	↓	✘	-	-	-	↓	-	↓	↓	↓		↓	-	-	-	-	-	↓	↓	↓		-	✘	-	-	-	-	-	↓	↓
GW5074 (Raf1 Kinase Inhibitor I)	12.0	ns	-	-	+++	++ (73%)	++ (89-99%)	-	✘ (91-96%)	✘ (77-100%)	-	✘ (98%)	++ (54%)	✘ (82-90%)	-		-	-	✘ (87-93%)	✘ (100%)	✘ (92%)	-	-	-	-	✘ (66%)	✘ (95-98%)	-	-	-	-	✘ (74%)	-	-	-
PD98059	10.2	ns	-	-	-	-	+++	✘ 85%	✘ 100%	✘ 94-96%	-	✘ (86%)	✘ 100%	-	-		✘ 100%	-	-	✘ 100%	✘ 93%	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SB202190	7.4	ns	✘	-	-	✘ (93-100%)	✘ (90-100%)	+	✘ (42-83%)	+++ (3-5%)	-	✘ (93%)	+ (27%)	+	+		✘ (83%)	-	✘ (95%)	✘ (73%)	✘ (69-100%)	✘ (97%)	-	-		✘ (84%)	✘ (83-89%)	✘ (91%)	-	✘ (100%)	✘	✘ (79%)	+	-	-
SP600125	8.8	ns	+	✘	-	-	✘ (85-88%)	-	+++ (22-47%)	+ (82-100%)	-	✘ (56%)	✘ (81%)	✘ (92-97%)	-		✘ (85%)	-	✘ (49%)/ +	✘ (48-80%)	✘ (68-100%)	-	-	-	-	✘ (92%)	✘ (60-82%)	-	-	-	-	✘ (89%)	-	+	↓
740 Y-P (↑)	16.1	ns	-	-	-	-	-	-	-	-	↑	-	-	-	↑		-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	↑	-
LY294002	76.2	***	✘	✘	✘	✘ (86%)	✘ (85-88%)	✘	✘ (86-92%)	✘ (100%)	+++	↓	↓/+(37%)	+	↓/ ✘	↓/ ✘	↓/ ✘ (91%)	↓	✘ (100%)	✘ (97%)	✘ (77-100%)	✘	-	+	✘ (100%)	✘ (86%) /+	↓/ ✘ (67-100%)	✘ (99%)	↓	✘ (98%)	✘	✘ (100%)	-	↓	↓
PS48 (↑)	21.1	ns	-	-	-	-	-	-	-	-	-	↑	-	↑	-		-	-	-	-	✘	-	-	✘	✘		-	-	-	-	-	-	✘	-
GSK2334470	79.1	***	-	-	-	✘ (74-94%)	✘ (100%	-	-	✘ (97-100%)	-	+++	✘ (100%)	+ (36-56%)	++		+ (81%)	-	✘ (45-100%)	+ (84%)	+	-	-	-	-	✘ (27%)	✘ (77-100%)	-	-	-	-	✘ (99%)	PDK1	-	-
BX-795	71.0	***	-	-	-	✘ (33%)	✘ (81-88%)/ ++ [<5%]	↓	✘ (100%) /+ [<20%]	✘ (83-94%)/++	-	+++ (29%)	✘ (82%)	✘ (90-100%)	-		✘ (100%)	-	✘ (40-100%)	✘ (100%)	✘ (95-100%)	-	✘ (87-96%)	-	-	✘ (90%)	✘ (90-97%)	-	-	-	-	✘ (77%)	TBK1 +++	-	-
GSK3β Inhibitor VIII (AR-A014418)	51.8	***	↓	-	↑	✘ (77%)	✘ (94-100%)	-	✘ (72-91%)	✘ (80-100%)	-	✘ (74%)	+++ (3%)	✘ (85-95%)	-	↑	✘ (75%)	-	✘ (75-83%)	✘ (68%)	✘ (80%)	✘ (100%)	-	-	✘ (100%)	✘ (83%)	✘ (88-93%)	✘ (96%)	-	✘ (94%)	-	+ (10%)	-	↑	✘
GSK690693	79.0	***	+	-	-	-	↓	-	-	-	-	-	-	+++	-		-	-	-	++	++	-	-	-	-	+	++	-	-	-	-	-	-	-	-
MK-2206	80.0	***	-	-	-	✘ (90-100%)	✘ (75-93%)	-	✘ (84-97%)	✘ (76-100%)	-	-	✘ (72%)	+++	-		-	✘ (84%)	✘ (89%)	✘ (90%)	✘ (92-98%)	-	-	-	-	✘ (91%)	-	✘ (66-100%)	-	-	-	✘ (100%)	-	-	-
TIC10 (ONC201)	29.3	**	- TRAIL	-	-	-	++	↓	-	✘	-	-	↓	++	↓	-	-	-	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MHY1485 (↑)	5.9	ns	-	-	-	-	-		-	-	-	-	-	-	↑		-	↑	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rapamycin	77.5	***	-	-	-	✘ (93%)	✘ (94-100%)	-	✘ (81-90%)	✘ (75-84%)	-	✘ (85%)	✘ (83%)	✘ (84-91%)	+++	++	↓ 30-45%	↓	✘ (82%)	✘ (77%)	✘ (88-92%)	✘ (100%)	-	-	✘ (100%)	✘ (100%)	✘ (81-89%)	✘ (90%)	-	✘ (100%)	-	✘ (86%)	-	-	-
PF-4708671	85.9	***	-	-	-	✘ (96%)	✘ (71-86%)	-	✘ (95-98%)	✘ (86-100%)	-	✘ (95%)	✘ (92%)	✘ (52-60%)	-		++ (6%)	-	✘ (96-100%), ↑	✘ (34%)	✘ (62-70%)	-	-	-	-	✘ (58%)	✘ (86-88%)	-	-	✘ (87%)	✘ (37-100%)	✘ (92%)	-	-	-

	Permeability ↓ (%)	Significance	related signaling nodes					↓: downregulation				↑: upregulation			&: additive effect			+ to +++ : inhibition				✘: lack of inhibition (activity remaining)							treatment with agonist: (activity remaining)					
			TNF	TNFR1	Ras	MEK	ERK	STAT	JNK	p38 MAPK	PI3K	PDK1	GSK3β	Akt	mTORC1	mTORC2	S6K	4E-BP1	IKK	PKA	PKC	FAK	Cdc42	RAC	ROCK1	ROCK2	PAK	LIMK	actin	myosin/ nmMLCK	RTKs (EGFR, PDGFR, CSF1R and FGFR)	Src	Ser/Thr	MMPs	iNos/ eNOS
L-NAME	33.5	***	-	✘	-	-	-	↓	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++
Prostratin	2.6	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PMA	-8.4	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMD0354	58.9	***	↓ 11.58%	-	-	✘ (100%)	↑	-	✘ (100 %)	✘ (100%)	-	✘ (97%)	-	✘ (93-100%)	-	✘ (100%)	-	++ (90-99%)	✘ (99%)	✘ (95%)	-	-	-	-	✘ (100%)	✘ (80-100%)	-	↓	-	-	✘ (100%)	-	↓	-
Piceatannol	25.3	*	-	-	-	-	✘ (100%)	-	✘ (96-100%)	✘ (73-100%)	-	✘ (99%)	✘ (100%)	✘ (96-100%)	-	✘ (92%)	-	+	✘ (100%)	(100%)	-	-	-	-	✘ (66%)	✘ (86-100%)	-	-	-	-	✘ (81%)	+	-	-
BMS-345541 (IKK Inhibitor III)	67.5	***	-	-	-	✘ (66%)	✘ (87-92%)	✘	✘ (95-100%)	✘ (100%)	✘	✘ (98%)	✘ (82%)	✘ (92-100%)	-	✘ (83%)	-	+++ (29%)	✘ (79%)	✘ (84-88%)	-	-	-	-	✘ (80%)	✘ (77-81%)	-	-	-	-	✘ (90%)	✘	✘	✘
Wedelolactone (WEL/WDL)	33.3	***	↓	-	-	+	+/↑	+	↑	✘	-	-	-	✘	+	-	-	+	-	↓	-	-	-	-	-	-	-	-	-	-	-	+	-	+
BAY 11-7085	52.1	***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	✘
GM-6001	-13.6	ns	↓	-	-	-	↓	-	✘	✘	-	-	↑	↑	-	-	-	-	-	-	-	-	-	-	-	-	-	-	✘	✘	+	-	-	+++	-
SB-3CT	-21.4	**	✘	-	✘	✘	✘	-	-	-		-	-	-	-	-	-	-	-	-	✘	-	-	-	-	-	-	+++	-	EGFR	-	-	+++	-
PD173074	4.5	ns	-	-	-	✘	↓	-	-	↓	✘/+	-	-	↓/+	-	-	-	↓	-	✘	-	-	-	-	-	-	-	-	-	-	✘ to +++	✘	-	↓	-
Genistein	19.5	*	-	-	-	✘ (86-100%)	+	-	+	✘ (95-100%)	-	✘ (87%)	✘ (90%)	✘ (83-100%)	-	✘ (84%)	-	+	✘ (100%)	+ to +++ (81%)	-	-	-	-	✘ (95%)		-	-	-	✘	++ to +++ (70-96%)	-	-	-	-
Src kinase inhibitor I	27.2	**	-	✘	-	++ (58%)	✘ (75-100%)	✘	+ (81-94%)	+ (83-100%)	-	✘ (88%)	✘ (89%)	-	-	✘ (92%)	-	++ (79%)	✘ (95%)	✘ (95%)	✘ (98%)	-	-	-	-	✘ (84%)	+ (78%)	+	+	✘ (100%)	++ to +++	+++(12%)	-	-	-
Staurosporine	-57.2	***	-	-	-	+ (8-37%)	+ (22-33%)	-	+ (6-21%)	+ (6-72%)	-	+ (18%)	+++ (1%)	+++ (0-2%)	-	+++ (4%)	-	+ (11-14%)	+++ (2%)	+++ (4-7%)	-	-	-	-	-	+ (24%)	+++ (0-8%)	-	-	-	+++ (2-15%)	+++ (3%)	+++	-	+++

5.3 Results

5.3.1 Suppression of TNF-induced permeability increase by inhibition of the RhoGTPase pathway

5.3.1.1 The role of ROCK, PAK and their downstream effectors

Since the role of RhoGTPases on the regulation of cytoskeletal dynamics is a topic of intense investigation we sought to investigate the participation of this pathway on the TNF-mediated alterations in endothelial integrity.

Thrombin and forskolin were employed as positive controls for endothelial permeability increase. These well-studied inducer of endothelial dysfunction functions by abrogating the signalling of multiple Rho GTPases (Csortos et al., 2007).

We used U-46619 and the Rho activator II to stimulate the RhoA-governed cascade of the Rho GTPases in our system. U-46619 is a thromboxane A_2 mimetic that perturbs the normal quiescent phenotype of endothelial cells. Mechanistically, U-46619 enhances RhoA activation, abrogates the activity of AKT/eNOS signalling and promotes PTEN phosphorylation. Conversely, Rho activator II is small G-protein rendering the RhoGTPase constitutively active (Flatau et al., 1997). This compound has been reported to be targeting RhoA specifically, whilst increasing its activity in HUVECs by 80-150%.

Surprisingly, administration of U-46619 prior TNF-treatment yielded a permeability decrease by 22% ($p<0.05$), whereas Rho activator II surpassed the effect of TNF on endothelial monolayer permeability increase induction by 4% (ns).

The well-established ROCK inhibitors fasudil and Y-27632 provided an important starting point for uncovering the molecular basis of the implication of ROCK on the TNF-induced permeability increase. Fasudil (Dihydrochloride/HA1077) is the most extensively studied isoquinosulfonamide inhibiting ROCK (Uehata et al., 1997) through competitive interaction with the ATP-binding motif of ROCK (Asano et al., 1989; Nagumo et al., 2000). Fasudil is not isoform-specific, although displaying higher affinity for ROCK-2 and blocking more than 92% of the kinase activity. It was the first ROCK inhibitor trialled and approved for the treatment of cerebral vasospasms (Velat et al., 2011) and pulmonary hypertension (Kojonazarov et al., 2012). However, this compound is a broad-spectrum kinase inhibitor interfering with numerous kinases that control cytoskeletal remodelling. Fasudil is blocking at similar concentrations not only ROCK, but also S6K1 and PKC-related kinases (PRK) (Davies et al., 2000; Riento & Ridley, 2003; Anastassiadis et al., 2011a; MRC Phosphorylation and Ubiquitylation Unit, 2018d;). Administration of fasudil dose-dependently induces cell death (Li et al., 2002).

The more selective compound Y-27632 is an isoquinoline-based synthetic pyridine derivative that targets the ATP-binding pocket of ROCK in two distinct regions. Y-27632 is a potent inhibitor of both ROCK isoforms and is blocking at least 85% of

ROCK-2 activity (IC_{50}=800nM) (Davies et al., 2000; MRC Phosphorylation and Ubiquitylation Unit, 2018o).

Treatment with both non-selective ROCK inhibitors significantly reduced TNF-induced endothelial permeability by 55% ($p<0.001$) for fasudil and 65% for Y-27632 ($p<0.001$).

However, since ROCK-2 is the most predominantly expressed isoform in the brain, we compared the effect of these compounds with the selective ROCK-2 inhibitor KD025 (SLx-2119). This next generation inhibitor, selectively targets ROCK-2, exhibiting a 200-fold higher specificity for ROCK-2 than for ROCK-1 (IC_{50}: ROCK-1,-2=24μM, 105nM, respectively) (Lee et al., 2014). KD025 repressed TNF-induced permeability by 76% ($p<0.001$), being more successful than the non-targeted fasudil by more than 20%.

Moreover, pharmacological inhibition of the group I PAKs that are amongst RhoA direct downstream targets decreased permeability changes by 67% ($p<0.001$). FRAX597 is a pyridopyrimidinone that potently inhibits group I PAKs (IC_{50}=7.7-19.3nM). This selective inhibitor interacts with the back cavity of the ATP binding pocket, that is highly homologous amongst the group I PAKs, whilst having a minimal effect on the group II PAKs (IC_{50}>10μM) (Licciulli et al., 2013; Rudolph et al., 2015).

Next, we aimed to assess the role of nmMLCK and LIMK being regulated by both ROCK and PAKs that act as interrelated transducers. ML-7 is a reversible nmMLCK inhibitor (IC_{50}=300nM), targeting the ATP-binding pocket of nmMLCK (Saitoh et

al., 1987). This compound reversed the TNF-mediated endothelial leakage by 63% ($p<0.001$).

Finally, administration of the potent and highly-selective LIMK inhibitor SR7826 resorted to a pronounced permeability suppression by 77% ($p<0.001$). SR7826 is targeting LIMK1 with a 400-fold selectivity (IC_{50}=43nM), whilst having minimal effect on over a panel of 61 kinases, including ROCK and JNK (Yin et al., 2015). Administration of SR7826 reduces the p-cofilin levels, but not the total content of LIMK1, LIMK2, cofilin, and MYPT1, whilst mediating cytoskeletal remodelling (Yu et al., 2018).

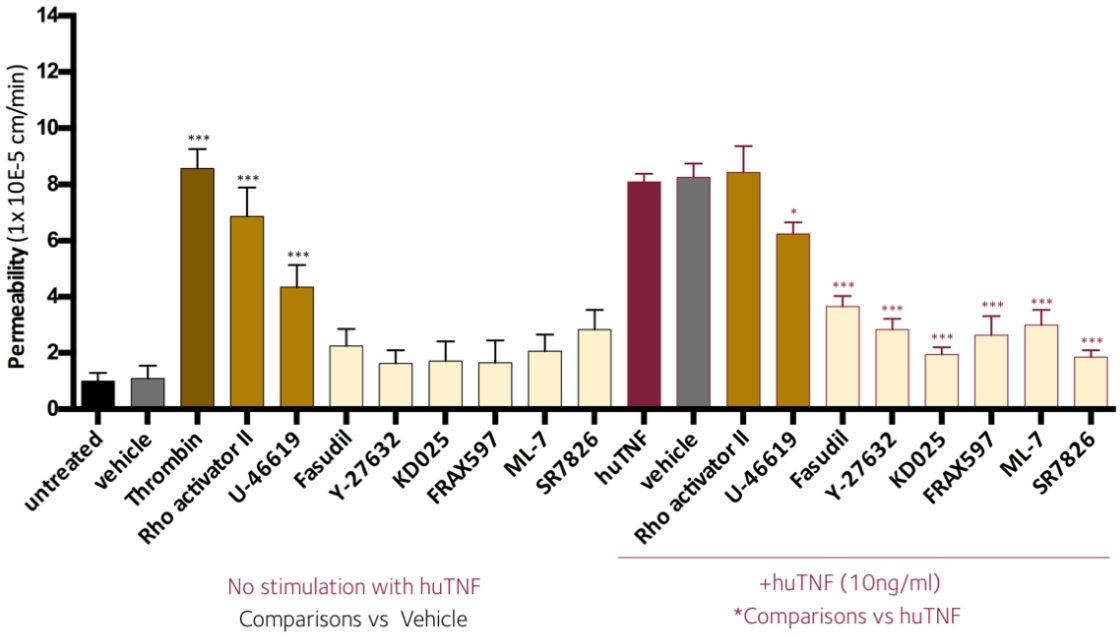
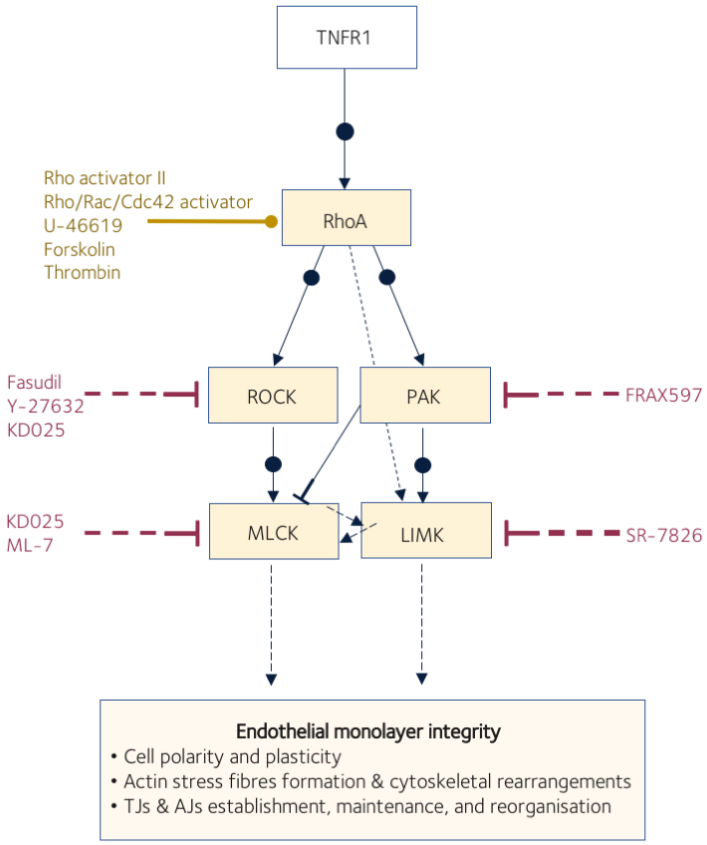


Fig.5.1 The role of ROCK, PAK and their downstream effectors

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.1.2 The role of Rac and Cdc42 and FAK

Furthermore, we wished to target the RhoGTPases Rac and Cdc42 to elucidate whether they annulate or enhance the RhoA-dictated signalling, hence improving or deteriorating the vascular integrity in the context of the TNF insult in brain microvascular endothelial cells.

As the commercially available Rac/Cdc42 activators are prone to cell death induction, we decided to employ the Rho/Rac/Cdc42 activator that preferentially stimulates Rac. This agent blocks the intrinsic and GAP-stimulated GTPase activities of the kinases, hence resulting in constitutively active RhoA, Rac and Cdc42. The treatment triggers upregulation of RhoA by more than 150%; Rac by more than 300%; and Cdc42 by 120-150%. Combined stimulation of the endothelium with both the activator and TNF does not confer any further permeability increase (ns).

In order to dissect the effect of the Rac-derived signalling, we tested both compounds as EHT-1864 is also benefiting from the lack of effect on PAK, whereas EHop-016 is also inhibiting PAK at concentrations higher than 2μM. Administration of the Rac inhibitors EHop-016 and EHT-1864 elicited a drop in the TNF-mediated permeability by 44% ($p<0.001$) and 48% ($p<0.001$), respectively.

EHop-016 is a potent SMI targeting Rac1, Rac3 (IC_{50}=1.1-3μM) and Cdc42, but not Rho (Dharmawardhane et al., 2013). It antagonises the relevant GEF-mediated activation and has been reported to successfully perturb the interaction between Rac and its GEF (Vav, a TNF-activated RhoGEF) (Biro et al., 2014).

EHT-1864 is inhibiting Rac1, Rac2, and Rac3 (IC_{50}=40, 60, 250nM, respectively) through direct interaction at the isolated GTPase- binding domain, thus placing Rac to an inert and inactive state (Desire et al., 2005). It is highly specific and does not affect RhoA signalling or the Cdc42/PAK interaction (Onesto et al., 2008).

Additionally, treatment with the novel, selective Cdc42 SMI ZCL278 led to an inhibition of TNF-associated permeability by 55% ($p<0.001$). ZCL278 perturbs Cdc42 activity as it is blocking directly the binding of its GEF (Cerione, 2004).

Lastly, we wanted to investigate the implication of the upstream regulator of Rac and Cdc42 in endothelial permeability. FAK is inhibited modestly by EHop-016, retaining 72% of its activity upon prolonged exposure. Administration an additional SMI PND1186 (VS-4718) that selectively and reversibly targets FAK (IC_{50}=1.5nM) yielded a 77% ($p<0.001$) reduction in the TNF-induced endothelial breach.

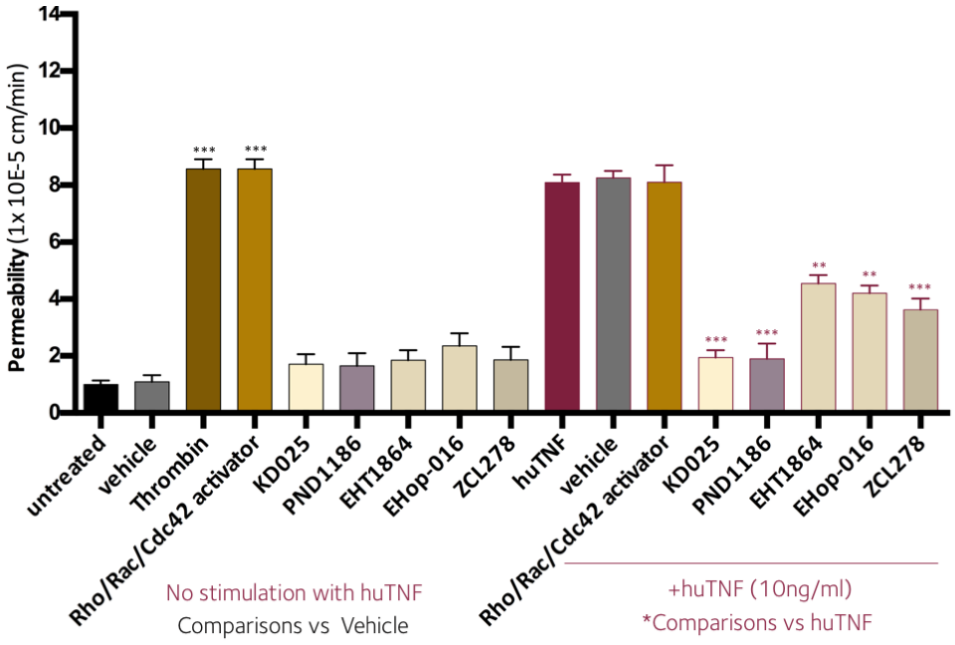
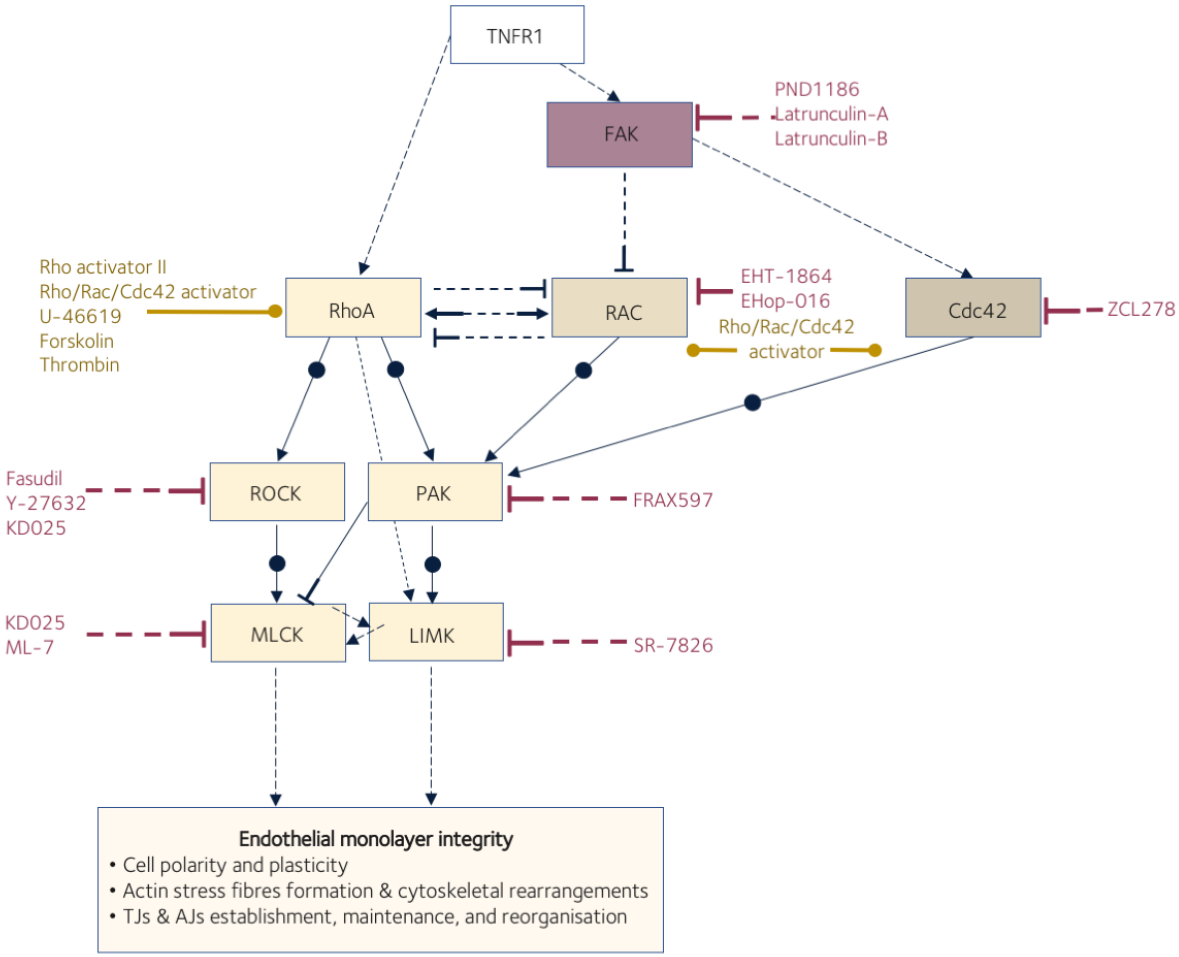


Fig.5.2 The role of Rac and Cdc42 and FAK

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability.
Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.2 Inhibition of NF-κB signalling enhances endothelial integrity

It is consensual that TNF is a potent inducer of the transcriptional activity of NF-κB. To assess the possibility of the causal implication of this pathway on the observed endothelial compromise in our system, we tested selective inhibitors impinging on one or more steps of the NF-κB signalling cascade. These include the abrogation of the incoming stimulating signal, the interference with the activation at the cytoplasmic step or the blockade of the NF-κB nuclear translocation.

First, we evaluated whether the classical NF-κB pathway is dictating alterations in endothelial stability. Blockade of IKKβ, a signature molecule of that route employing IMD0354, diminished TNF-induced permeability increase by 59% ($p<0.001$). IMD0354 is a selective IKKβ inhibitor (IC_{50}=~250nM), targeting the formation of the IKK complex (MRC Phosphorylation and Ubiquitylation Unit, 2018g). This agent blocks the activation of NF-κB by enhancing the stability of the IκB-NF-κB complex (Kobori et al., 2004; Inayama et al., 2006). Unlike other competitive kinase inhibitors, IMD0354 does not target ATP-binding and preserves vascular integrity through suppression of VEGF expression *in vivo* (Lennikov et al., 2014).

We then sought to compare the effect of single IKKβ targeting with dual inhibition of both IKKα and IKKβ in order to further investigate the role of the IKK complex. IKK is a prime pharmacological target due to its pivotal role in funnelling upstream signals into the two NF-κB signalling nodes. For that reason, we employed piceatannol, BMS-345541 and wedelolactone that abolish IKK signalling by targeting diverse events associated with the activation and the function of the complex.

Piceatannol is a hydroxystilbene, derivative of resveratrol that is well-recognised for its anti-inflammatory, anti-platelet, anti-proliferative and tumoricidal activities (Jang et al., 1997; Kimura et al., 1985; Rotondo et al., 1998). Piceatannol suppresses IKKα and IKKβ activation (EC_{50}=25μM), thus hindering IκB activation and degradation, and abolishing NF-κB activation (Islam et al., 2004; MRC Phosphorylation and Ubiquitylation Unit, 2018l). Furthermore, this phenolic antioxidant has been reported to interfere with the TNF-induced NF-κB activation through inhibition of the IκBα kinase and the NF-κB transcription complexes (Ashikawa et al., 2002).

Additionally, BMS-345541 (IKK Inhibitor III) is a cell-permeable inhibitor of IKKα and IKKβ (IC_{50}: IKKα=4μM, IKKβ=0.3μM) (Burke et al., 2003; MRC Phosphorylation and Ubiquitylation Unit, 2018b). This compound has allosteric effects on IKK structure, although having an approximately 10-fold enhanced affinity for IKKβ.

Moreover, wedelolactone is a natural anti-inflammatory agent (Deng & Fang, 2012) that directly inhibits IKKα and IKKβ kinase activity (IC_{50}=1-10μM) (Kobori et al., 2004). This selective and irreversible inhibitor impedes the phosphorylation and degradation of IκBα in epithelial cells (Maalouf et al., 2010).

All IKK inhibitors ameliorated the TNF-induced permeability increase, although to a variable extent. Pre-treatment with piceatannol resulted to a decrease by 25% ($p<0.05$), and administration of BMS-345541 and wedelolactone elicited a drop by 68% ($p<0.001$) and 33% ($p<0.001$), respectively.

We then wished to target the activation of IκBα that is preceding the nuclear translocation and the activation of the NF-κB transcription complex. BAY 11-7085 is an irreversible inhibitor of IκBα phosphorylation (IC_{50}=10μM) that abolishes the cytokine-induced activation of the canonical NF-κB pathway and blocks cell adhesion, inflammation and apoptosis (Scaife et al., 2002). Administration of that inhibitor, led to a pronounced repression of the TNF-induced vascular compromise (52%, $p<0.001$).

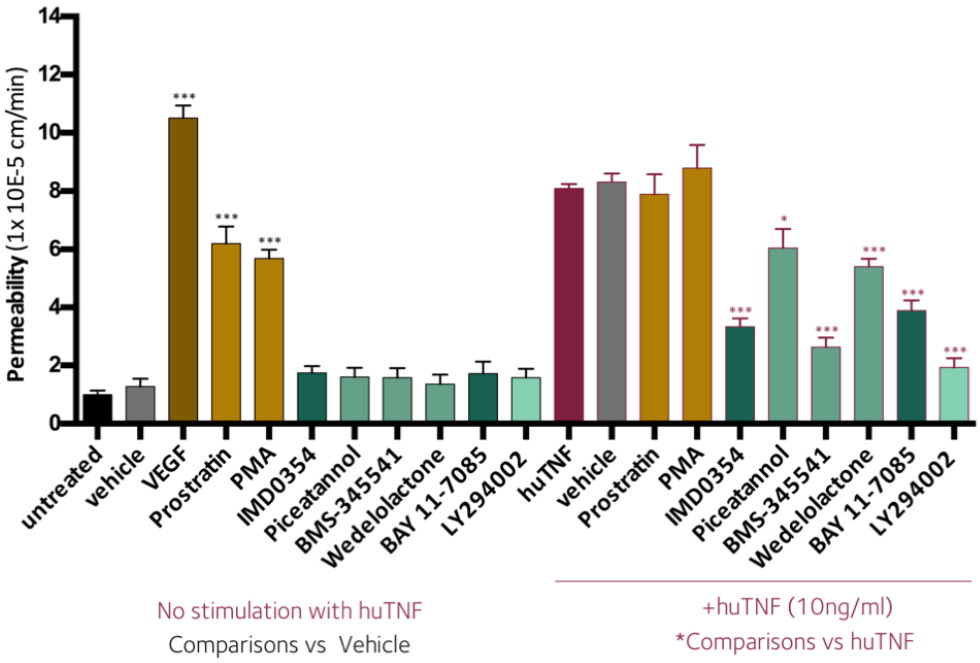
Lastly, we targeted the NF-κB-dependent transactivation employing LY294002, an inhibitor of the class I of PI3Ks. Suppression of the last step of the NF-κB signalling cascade, diminished the impact of TNF on endothelial integrity by 76% ($p<0.001$).

We employed two activators of the NF-κB signalling network with varying potency for the pro-inflammatory stimulation by TNF as positive controls for the aforementioned assays.

Prostratin is a PKC-activating agent serving as a NF-κB agonist (Williams et al., 2004). Prostratin negated the integrity of the endothelial barrier (6-fold increase, $p<0.001$ in unstimulated cells and 2% increase in TNF-treaded cells) in absence of cell death or cellular proliferation induction in hCMEC/D3 cells. Moreover, this specific compound elicits the secretion of pro-inflammatory mediators alongside disrupting the expression of occludin, claudin-5 and JAM-A; altering the cellular localisation of ZO-1; and promoting the expression of ICAM-1 (Dental et al., 2017)

In an analogous manner, the phorbol -myristate -acetate (PMA) increased paracellular permeability in both unstimulated and TNF-treated cells (6-fold increase, $p<0.001$ in unstimulated cells and 8% increase, respectively). This forbol ester is a

potent nanomolar activator of PKC, but also potently activates a wider signalling network stimulated by TNF by inducing NF-ϰB DNA binding (O'Carroll et al., 2015). Treatment with PMA induces endothelial dysfunction in HBECs as evidenced by decreases in TEER values (Shao & Bayraktutan, 2014).



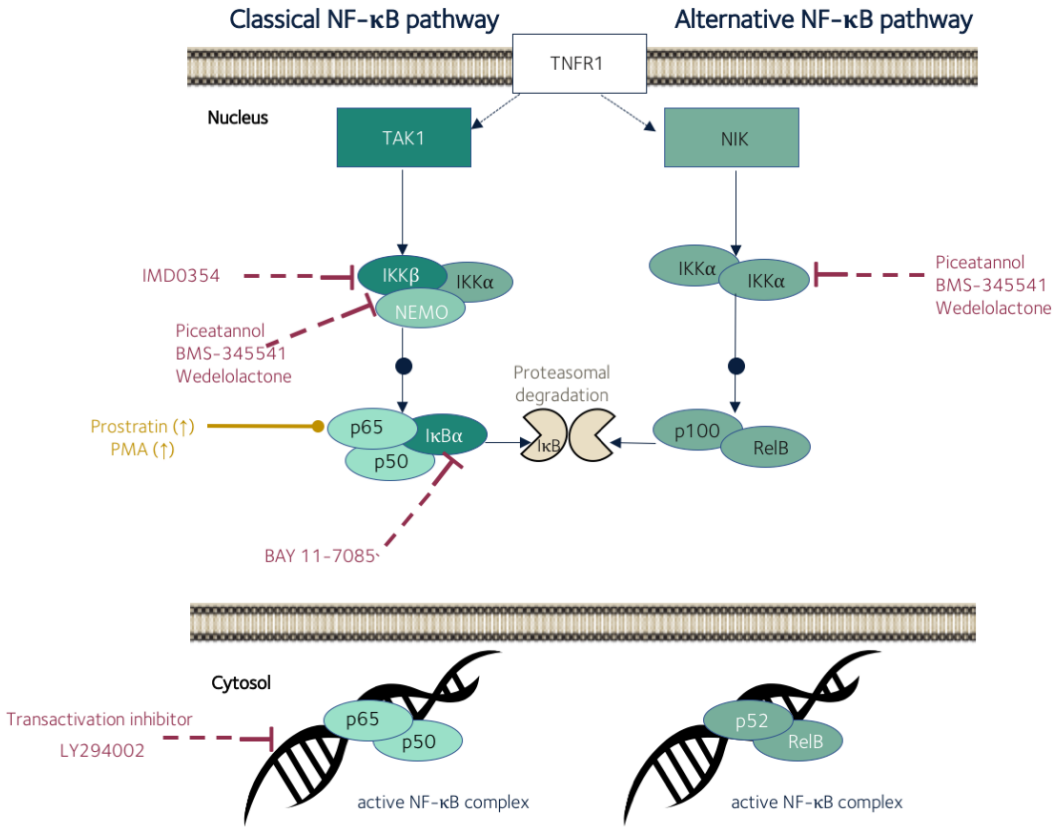


Fig.5.3 TNF-induced activation of the classical and alternative NF-κB signalling pathways contributes to brain endothelial hyperpermeability

The activation of the classical NF-κB pathway leads to the stimulation of the kinase activity of the IKK complex and the proteolytic degradation of IκB proteins. The induction of the alternative NF-κB pathway involves the NIK-mediated stimulation of IKKα and conversion of p100-containing NF- κ B complexes into p52-containing NF-κB complexes through the proteolysis of p100 to p52.

5.3.2.1 NF-κB is a downstream target of RhoA

The demonstrated implication of both the NF-κB signalling network and nmMLCK on the regulation of the function of the brain endothelium upon TNF-pro-inflammatory stimulation prompted us to investigate whether these signalling nodes are cross-regulated. The activation status of nmMLCK is a well-established determinant of NF-κB transcriptional activity in peripheral endothelial cells (Wadgaonkar et al., 2005; Wu et al., 2009).

We administered the potent inhibitors of NF-κB alongside the Rho activator II to address if RhoA is a transducer of NF-κB signalling. The RhoA agonist induced a 7-fold incrementation of endothelial permeability in unstimulated hCMECs. Concurrent blockade of NF-κB activation using IMD0354, BMS-345541 or BAY 11-7085 significantly decreased the permeabilising effect of the Rho activator by 59% ($p<0.001$), 74% ($p<0.001$) and 65% ($p<0.001$), respectively.

Further than that, we tested the effect of NF-κB activation followed by nmMLCK inhibition in order to preclude nmMLCK being a downstream target of NF-κB activation. The NF-κB agonists prostratin and PMA elicited a 6-fold and a relative 5-fold increase of paracellular permeability, under basal conditions. Co-administration of the NF-κB activators with the potent nmMLCK inhibitors KD025 or ML-7 resorted to sustained endothelial permeability (5-10% decrease, ns), suggesting that nmMLCK is an upstream regulator of NF-κB signalling in brain endothelial cells.

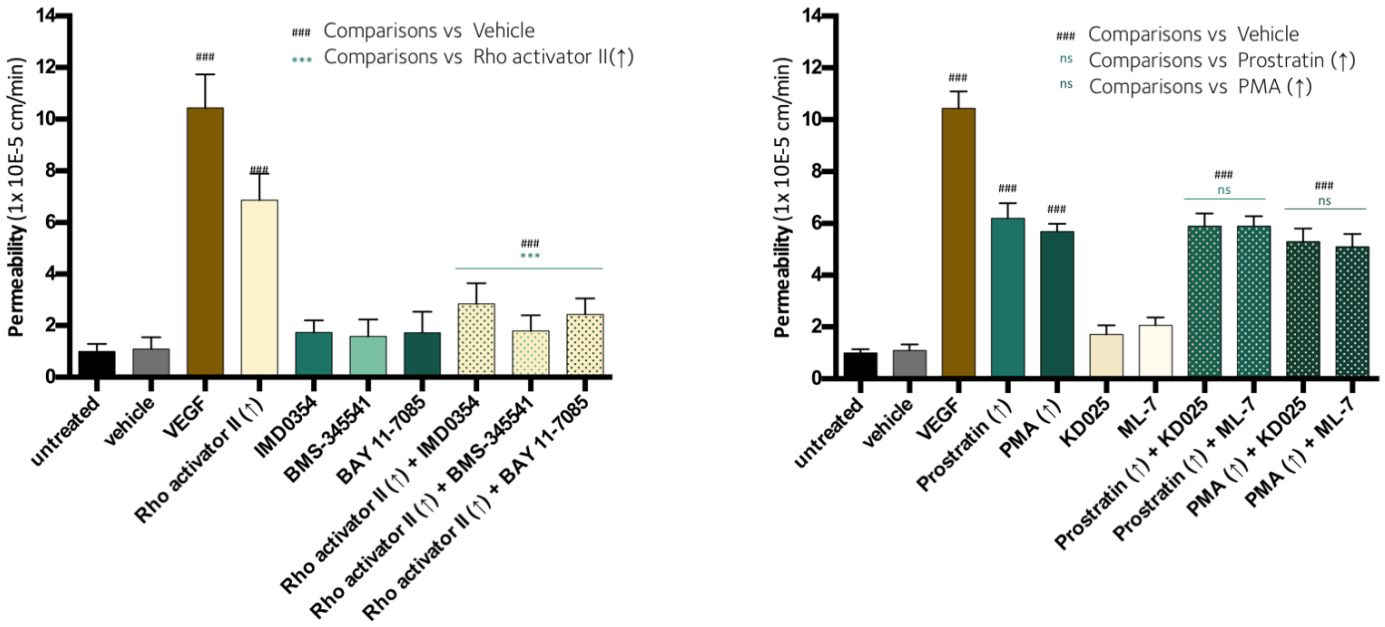
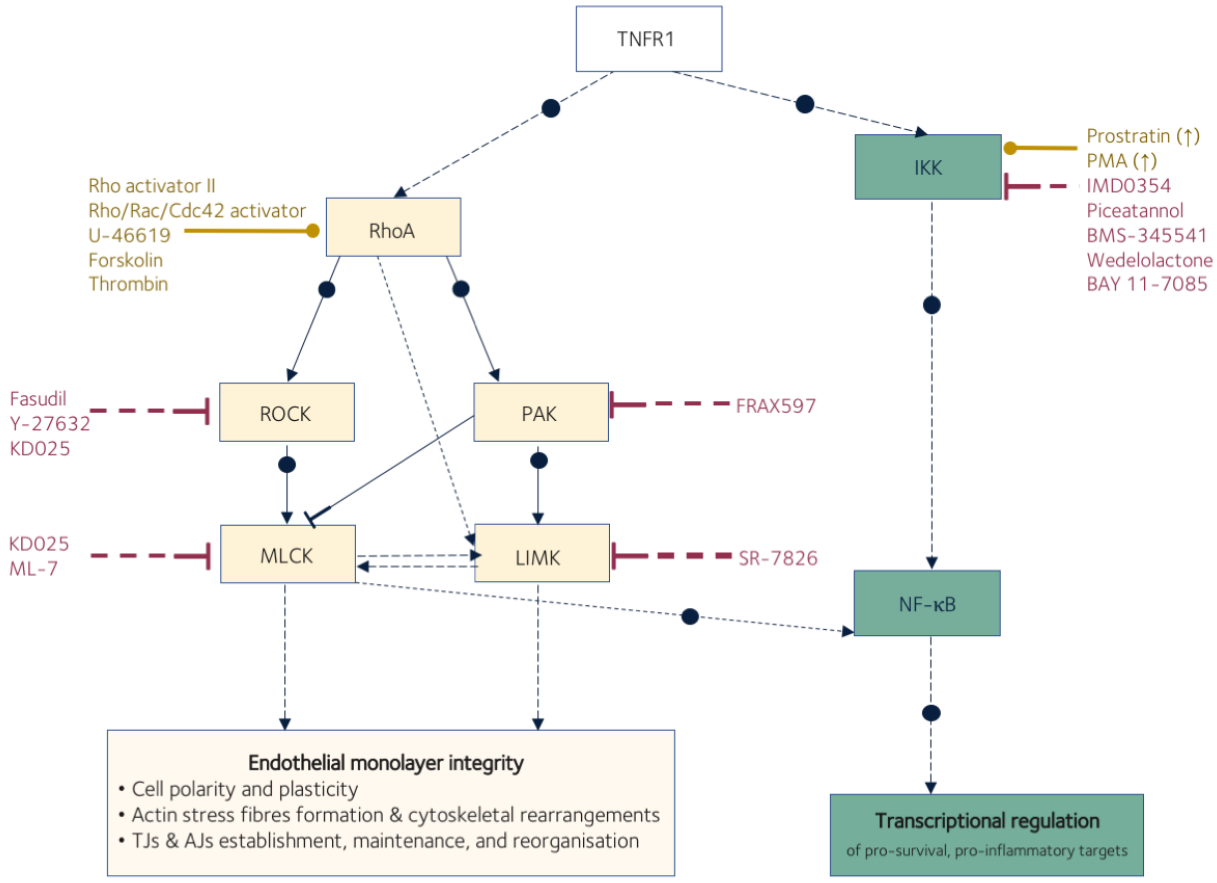


Figure 5.4 NF-κB is a downstream target of RhoA

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.3 MAPK signalling is not causally implicated in TNF-induced permeability

Given the strong link between the TNFR1 signalling and the activation of the MAPK pathways, we wished to determine if any of these pathways are determinants of the TNF-induced exacerbation of paracellular permeability.

VEGF was used as pathway-specific positive control by virtue of its stimulatory role on all the MAPK pathways assessed. VEGF is a strong vasopermeant, triggering a more than 10-fold increase ($p<0.001$) in the permeability of previously unstimulated cells.

To address the effects of the disruption of the p38 MAPK pathway we treated the endothelial monolayer with SB202190. This is a selective, potent, cell-permeable inhibitor of p38 MAPK (p38α: IC_{50}=50nM, p38β IC_{50}=100nM) (Jiang et al., 1996). This compound is a member of the pyridinyl imidazole inhibitors that target the ATP binding pocket of the kinase (Fox et al., 1998) and has negligible effects on ERK and JNK (ERK, JNK IC_{50}=~10μM) (Davies et al., 2000). Prolonged suppression of the p38 MAPK signalling did not alter the TNF-induced permeability significantly (7% decrease).

Assessment of the JNK inhibitor SP600125 yielded similar results, with a 9% (ns) permeability change only. SP600125 (JNK Inhibitor II, SAPK Inhibitor II) is a reversible, highly selective, ATP-competitive inhibitor of JNK (IC_{50}= 40-90nM) (Wang et al., 2007; MRC Phosphorylation and Ubiquitylation Unit, 2018n).

On the contrary, pharmacological inhibition of the activator of the ERK pathway Ras, using the potent inhibitors FTI-II and FTS triggered a pronounced restoration of endothelial integrity through the repression of the permeabilising effects of TNF by 76% ($p<0.001$) and 80% ($p<0.001$), respectively.

The farnesyltransferase (FTase) inhibitor II (FTI-II) is inhibiting the post-translational modification of farnesylation. This process requires an intermediate in the mevalonate pathway, the farnesyl pyrophosphate (FPP) to load the lipid farnesyl to certain proteins. Farnesylation not only accounts for the cellular localisation of the proteins targeted at the cell membrane, but also is essential for their functionality. FTI-II blocks FTase (IC_{50}=50-75nM), preventing farnesylation of Ras (Manne et al., 1995; Kloog & Cox, 2000).

On the other hand, farnesyl Thiosalicylic Acid (FTS/salirasib) is hindering Ras-mediated signalling by dislodging Ras from the inner surface of the cell membrane and rendering it susceptible to proteolytic degradation (Marom et al., 1995; Haklai et al., 1998; Kloog & Cox, 2000).

However, this effect was not coincident with minimal effects of blockade of the downstream targets of the cascade. Inhibition of Raf reduced TNF-mediated permeability by 12% (ns). GW5074 (Raf1 Kinase Inhibitor I) is a potent, selective and cell-permeable c-Raf-1 kinase inhibitor (IC_{50}=9nM) that blocks ERK1/2 phosphorylation and also diminishes GSK-3β activity (Lackey et al., 2000; MRC Phosphorylation and Ubiquitylation Unit, 2018f).

Additionally, blockade of MEK1/2 did not confer any significant alterations on the debilitating effect of TNF on brain endothelium (decrease by 10%, ns). PD98059 is a potent and selective inhibitor of the MAPKKKs (IC_{50}=2-7μM), MEK1 and MEK2 but does not inhibit Raf-activated MAPKK1 (Dudley, et al., 1995; Davies et al., 2000). Mechanistically, this agent targets the inactive form of MAPKK and blocks the activation by upstream activators such as c-Raf (Alessi et al., 1995).

Inhibition of the downstream target of cascade, ERK, using TIC10 that suppresses the activity of the ERK and Akt (IC_{50}: Akt=0.86-8.8μM) signalling pathways, decreased the TNF-induced endothelial breach significantly (29% decrease, $p<0.01$). TIC10 (ONC201) is a BBB-permeant, TRAIL agonist with a wide therapeutic index as a potent anti-cancer agent. Mechanistically, TIC10 suppresses the activity of the ERK and AKT (IC_{50}: AKT=0.86-8.8μM) signalling pathways, hence promoting TRAIL expression (Allen et al., 2013).

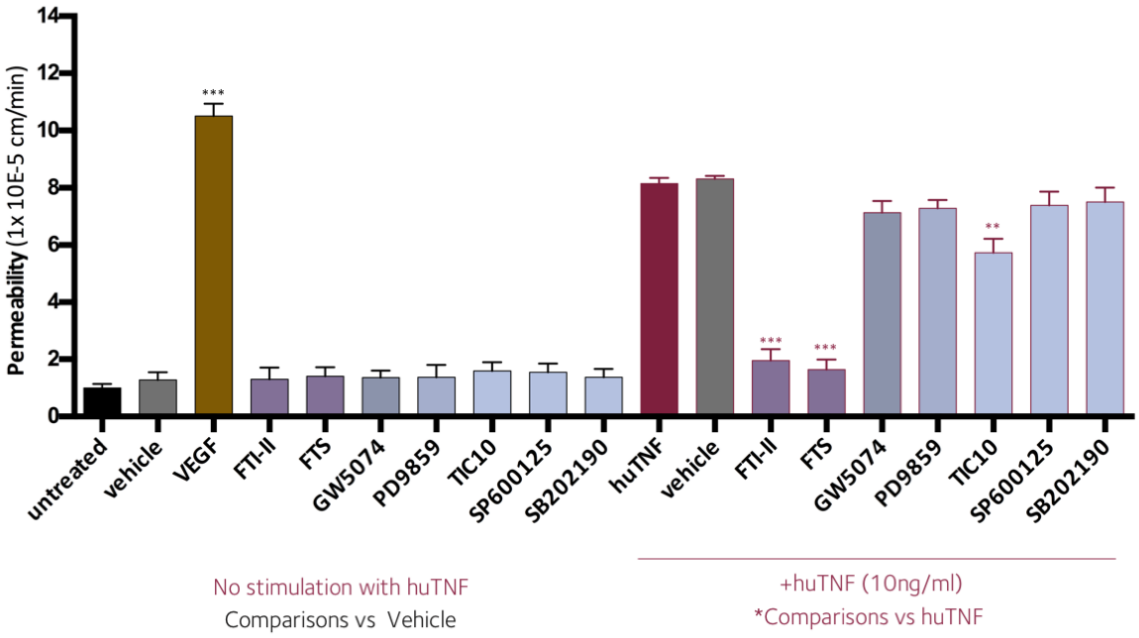
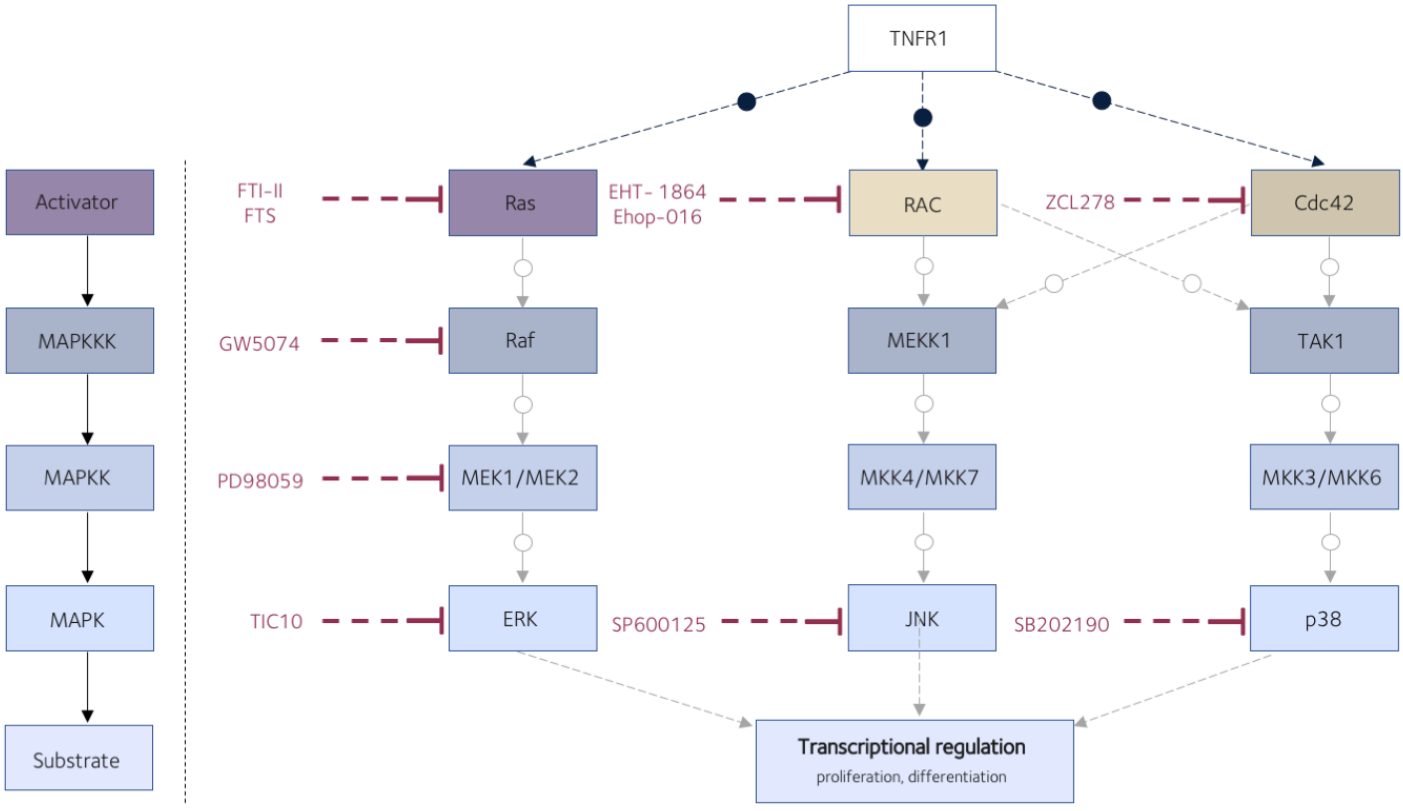


Figure 5.5 MAPK signalling is not causally implicated in TNF-induced permeability

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Grey arrows and white circles indicate lack of contribution to the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.4 Activation of MMPs are not involved in TNF-induced endothelial permeability increase

Given the absence of direct involvement of the MAPK pathway in the regulation of endothelial integrity upon pro-inflammatory stimulation with TNF, we wished to investigate their potential indirect involvement via the activation of MMPs.

For that assay, we compared the effect of MMPs inhibition to that of ROCK-2 or IKK suppression, since both have been reported to abrogate MMPs expression in the brain endothelium under neuroinflammatory conditions (Suzuki et al., 2013; Fu et al., 2014; Yu et al., 2014)

GM-6001 is a potent, reversible broad-spectrum inhibitor of zinc-containing proteases, such as MMPs (IC_{50}=0.1-10nM), and disintegrin and metalloproteinase domain-containing (ADAM) proteins (Auge et al., 2003; Niego et al., 2012). In addition, SB-3CT is a selective inhibitor of the gelatinases MMP-2 and MMP-9 that can cross the BBB (Gooyit et al., 2012). Administration of both antagonists did not suppress TNF-induced endothelial permeability (14% and 21% increase for GM-6001 and SB-3CT, respectively, both ns).

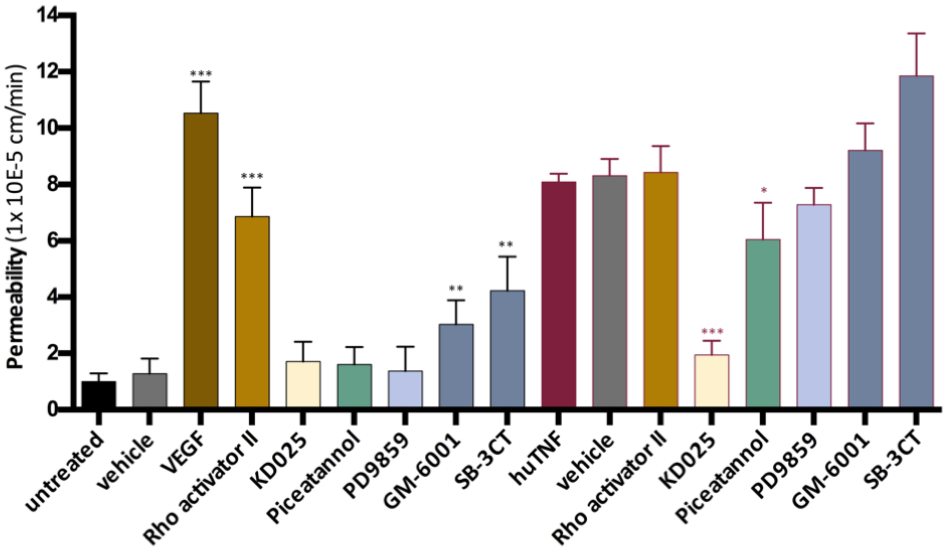
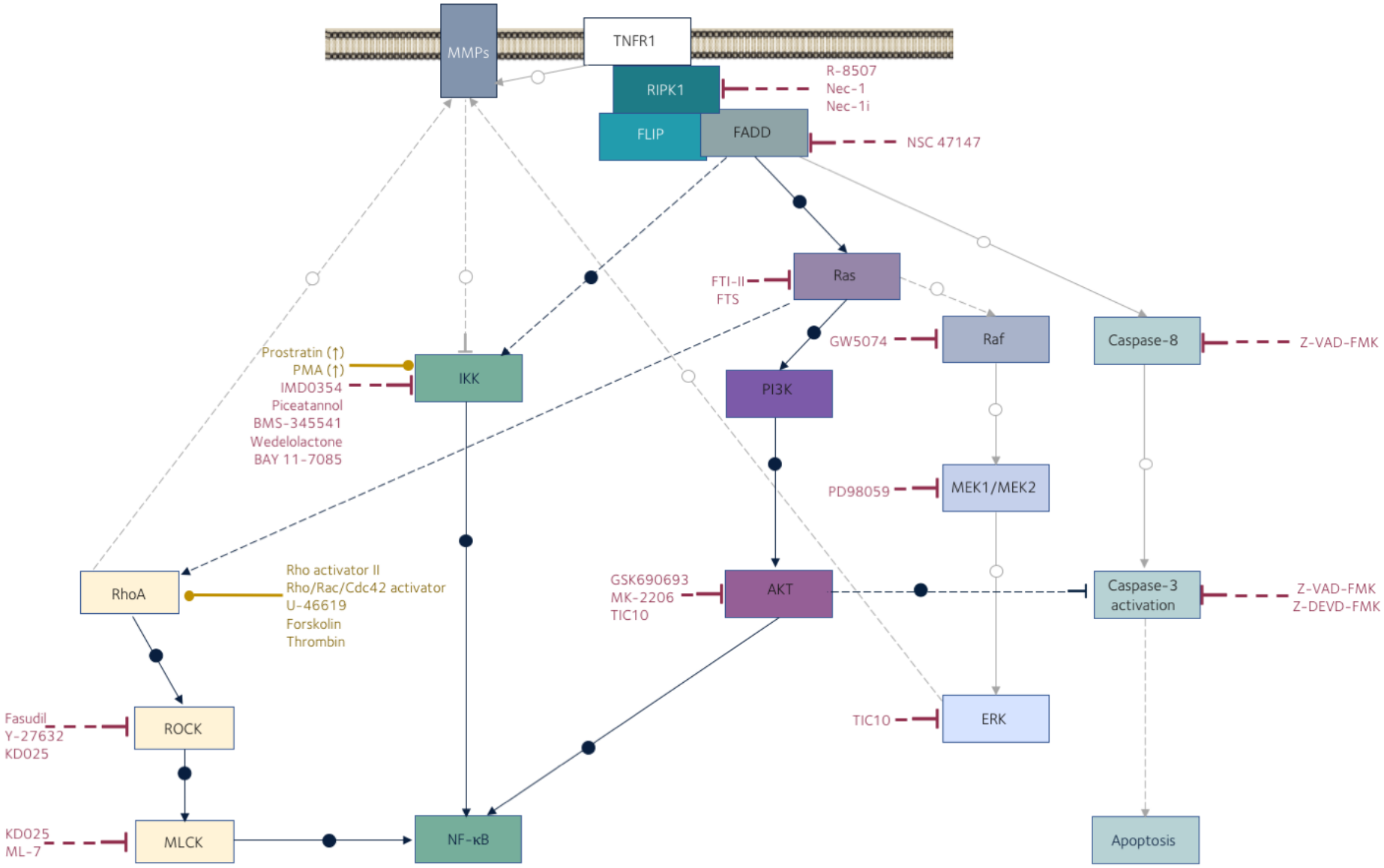


Figure 5.6 Activation of MMPs are not involved in TNF-induced endothelial hyperpermeability
Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Next page: Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.


MMPs
TNFR1
RIPK1
R-8507
Nec-1
Nec-1i
FLIP
FADD
NSC 47147
FTI-II
FTS
Ras
Prostratin (↑)
PMA (↑)
IMD0354
Piceatannol
BMS-345541
Wedelolactone
BAY 11-7085
IKK
GW5074
Raf
Caspase-8
Z-VAD-FMK
PI3K
PD98059
MEK1/MEK2
RhoA
Rho activator II
Rho/Rac/Cdc42 activator
U-46619
Forskolin
Thrombin
GSK690693
MK-2206
TIC10
AKT
Caspase-3
activation
Z-VAD-FMK
Z-DEVD-FMK
TIC10
ERK
Fasudil
Y-27632
KD025
ROCK
KD025
ML-7
MLCK
NF-κB
Apoptosis

5.3.5 Inhibition of pro-apoptotic signalling does not affect endothelial permeability

The demonstrated implication of TNFR1 on the regulation of endothelial permeability, suggests the activation of the TNFR signalling complex. Induction of RIPK1 and FADD could resort to permeability increase associated with cell death.

First, we tested the effect of RIPK1 inhibition employing R-8507 and Necrostatin-1. R-8507 is a TNFR1 antagonist, abrogating the interaction of TNFR1 with RIPK1 and TRADD (Gururaja et al., 2007). Necrostatin-1 (Nec-1) is a small-molecule inhibitor of RIPK1 (EC_{50}=490nM) (Degterev et al., 2013; MRC Phosphorylation and Ubiquitylation Unit, 2018k). Both inhibitors suppressed the TNF-induced permeability increase significantly (77% and 66% decrease, respectively $p<0.001$ for both comparisons vs huTNF treatment).

The necrostatin-1 inactive control (Nec-1i) was employed in order to exclude the non-specific off-target effects of Nec-1. Nec-1i is ~200 fold less effective than Nec-1 in blocking RIPK1 and 10-fold less potent inducer of necroptosis. Despite their diverse specificities, both Nec-1 and Nec-1i block the TNF-induced systemic inflammatory response syndrome *in vivo* (Takahashi et al., 2012). Nec-1i did not confer any significant alterations in TNF-associated endothelial leakiness (2% decrease, ns).

Next, we sought to explore the role of FADD. We employed NSC 47147 (prodigiosin), a natural red bacterial pigment bearing antibiotic and immunosuppressant properties (Kim et al., 2008). This compound prevents FADD phosphorylation (IC_{50}=2μM) through the blockade of FADD-kinase CK1α

(IC50=~6μM). Moreover, NSC 47147 supresses NF-κB activation by decreasing total IκBα and pIκBα levels (Schinske et al., 2011). Pre-conditioning with NSC 47147 averted the TNF-induced endothelial breach (63% decrease, <0.001).

Since we confirmed the implication of FADD signalling on the observed increased endothelial permeabilisation, we asked whether this effect is attributed to the activation of the pro-apoptotic cascade, comprising consecutive caspase-8 and caspase-3 activation. For that purpose, we employed the caspase inhibitors Z-DEVD-FMK and Boc-D-FMK. Z-DEVD-FMK is cell-permeable, irreversible inhibitor of caspase-3 (IC_{50}=130nM) that potently inhibits apoptosis (Brockstedt et al., 1998). Boc-D-FMK is a cell-permeable, irreversible pan-caspase inhibitor that blocks apoptosis in TNF-stimulated renal endothelial cells (Wu et al., 2009). Both antagonists did not confer significant changes in TNF-associated endothelial permeability increase (3% and 8%, respectively, ns).

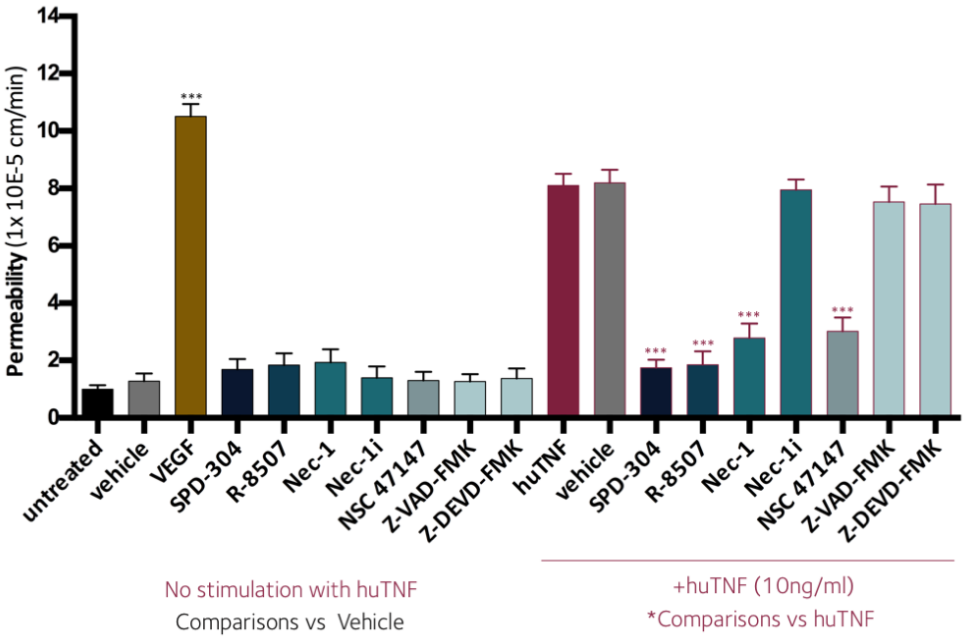
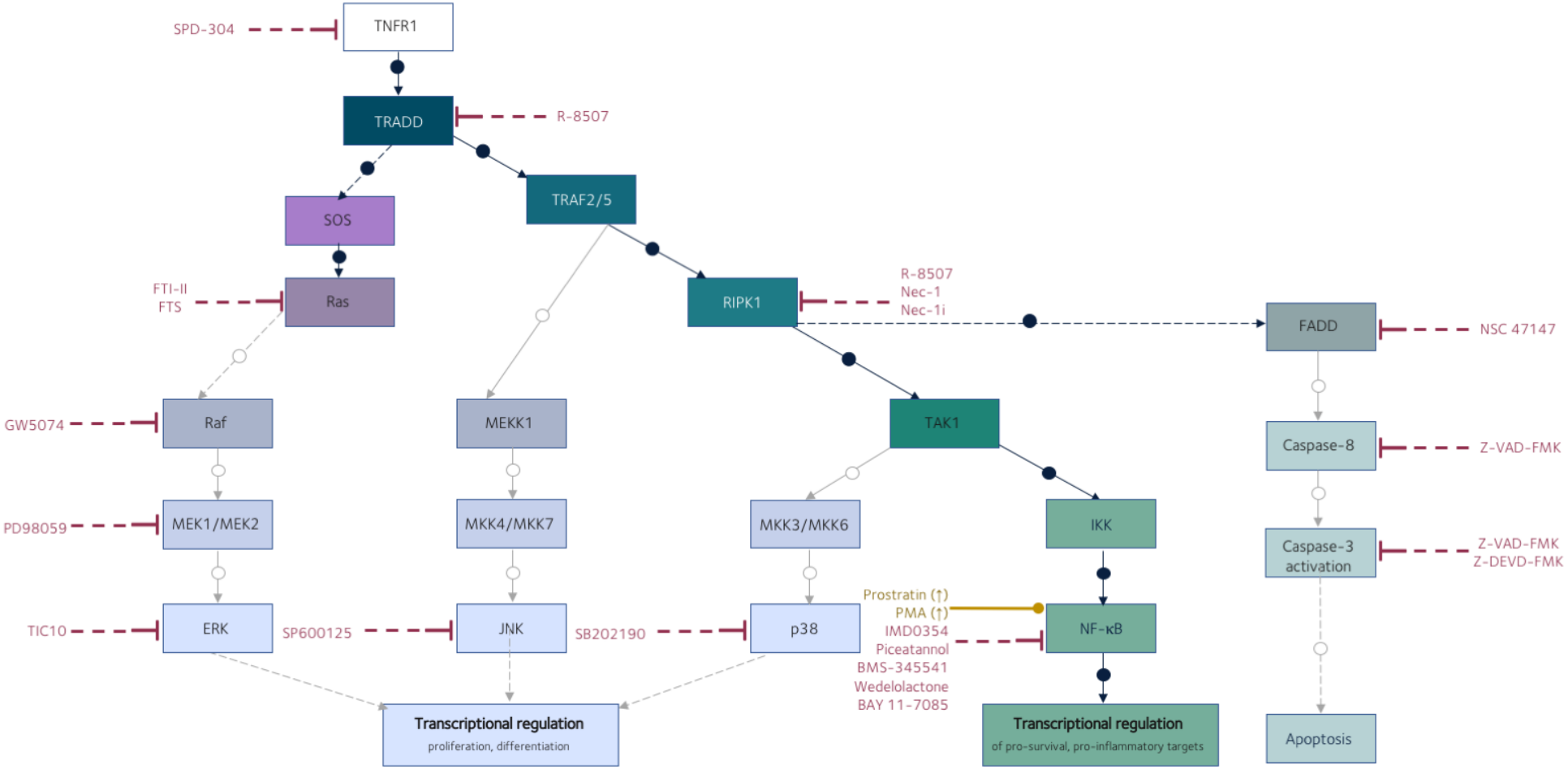


Figure 5.7 Inhibition of pro-apoptotic signalling does not affect endothelial permeability

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Next page: Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.


SPD-304
TNFR1
TRADD
R-8507
SOS
TRAF2/5
FTI-II
FTS
Ras
RIPK1
R-8507
Nec-1
Nec-1i
FADD
NSC 47147
GW5074
Raf
MEKK1
TAK1
Caspase-8
Z-VAD-FMK
PD98059
MEK1/MEK2
MKK4/MKK7
MKK3/MKK6
IKK
Caspase-3
activation
Z-VAD-FMK
Z-DEVD-FMK
Prostratin (↑)
PMA (↑)
IMD0354
Piceatannol
BMS-345541
Wedelolactone
BAY 11-7085
TIC10
ERK
SP600125
JNK
SB202190
p38
NF-κB
Transcriptional regulation
proliferation, differentiation
Transcriptional regulation
of pro-survival, pro-inflammatory targets
Apoptosis

5.3.6 The Ras/ PI3K/AKT axis regulates TNF-induced endothelial permeability increase

5.3.6.1 Suppression of PI3K/PDK1/AKT signalling reverses endothelial permeability increase

Our interest in the role PI3K signalling pathway emanated from the demonstrated suppression of the TNF-induced endothelial permeability increase through the pharmacological inhibition of Ras and the absence of involvement of its effector Raf. The PI3K/AKT/mTOR signalling cascade is one the best-established downstream targets of Ras activation under inflammatory conditions.

We have chosen the PI3K agonist 740 Y-P as a positive control in the assays aiming to delineate the role of PI3K and its downstream targets on the TNF-mediated endothelial leakiness. 740 Y-P is a cell-permeable phospho-peptide targeting the regulatory subunit of PI3K. The priming activity of the activator is amenable to suppression upon treatment with the PI3K-specific inhibitor wortmannin (Derossi et al., 1998). Combination treatment with 740 Y-P and TNF led to permeability increase, similar to that evoked by stimulation with TNF only (ns).

In order to address the role of PI3K on the permeabilising effect exerted by TNF, we employed the extensively characterised agonist LY294002. This SMI is a flavonoid inhibiting the class I of PI3Ks (IC_{50}=1.4μM) (Vlahos et al., 1994). The compound is targeting a complementary region of the ATP-binding pocket (Walker et al., 2000). The use of LY294002 is by its off-target effects as it is also partially blocking the

activity of the PI3K downstream effectors AKT and GSK3β, and the RhoGTPases ROCK-2 and Rac. In order to fully repress the activation of PI3K, this inhibitor is often applied at high concentrations, thus affecting unrelated targets including calcium and potassium channels, phosphodiesterases and the estrogen receptor (Knight, 2010). The dose chosen (5μM) is devoid of such off-target effects (MRC Phosphorylation and Ubiquitylation Unit, 2018i). Inhibition of TNF-induced PI3K activation restored endothelial integrity, yielding a 76% repression of the TNF-mediated permeability increase.

Next, we sought to explore the role of the PI3K downstream target PDK1. We tested PS48, an allosteric PDK1 activator targeting the phosphate binding site of the kinase, whilst leaving the ATP-binding pocket unaffected (Hindie et al., 2009). Like 740 Y-P, administration of PS48 didn't alter permeability significantly (ns). Mechanistically, PS48 induces the activation of the downstream effector AKT. Proteomic screening of porcine foetal fibroblasts treated with PS48 reveals the absence of targeting of PKC, Rho/Rac, MMPs and the sustained basal expression of junctional proteins such as claudin-5 (Mordhorst et al., 2018).

Moreover, inhibition of PDK1 using BX-795 repressed the vasomodulating effect of TNF by 70% ($p<0.001$). BX-795 is a N-Phenylpyrimidin-2-amine that serves as a selective ATP-competitive inhibitor (IC_{50}=6nM) (Peifer & Alessi, 2008; MRC Phosphorylation and Ubiquitylation Unit, 2018c) that suppresses more than 90% of PDK1 activity *in vitro* (Clark et al., 2009).

Since PDK1 is an archetypal member of the AGC family comprising kinases such as ROCK-2 and S6K1, we employed also GSK2334470 that is not interfering with the ATP-binding pocket of PDK1 to rule out any possible off-target effects of BX-795. GSK2334470 significantly ameliorated the permeabilising effect of TNF (permeability decline by 79%, $p<0.001$). GSK2334470 (GSK-470) is a highly potent and selective PDK1 inhibitor (IC_{50}=~10nM) that even at 100-fold higher concentrations does not exhibit any off-target effects on a panel of more than 90 other protein kinases (Najafov et al., 2011; MRC Phosphorylation and Ubiquitylation Unit, 2018e).

Since repression of both PI3K and PDK1 negated the permeabilising effect of TNF, we focused on the point of convergence of their signalling, the downstream effector AKT. For that purpose, two SMIs, GSK690693 and MK2206 that have been extensively studied as therapeutic targets in cancer have been utilised. Pre-treatment with these agents, prior TNF-stimulation, elicited a permeability reduction by 79% and 80% ($p<0.001$), respectively.

GSK690693 is an ATP-competitive pan-AKT kinase inhibitor of AKT (IC_{50}=2-13nM). Additionally, MK-2206 is a specific, potent, allosteric AKT inhibitor (IC_{50}=5-65nM) (MRC Phosphorylation and Ubiquitylation Unit, 2018j). MK2206 is targeting the inactive conformation of AKT and prevents the phosphorylation of the kinase (Barnett et al., 2005; Hirai et al., 2010; Bhutani et al., 2013; Ma et al., 2016).

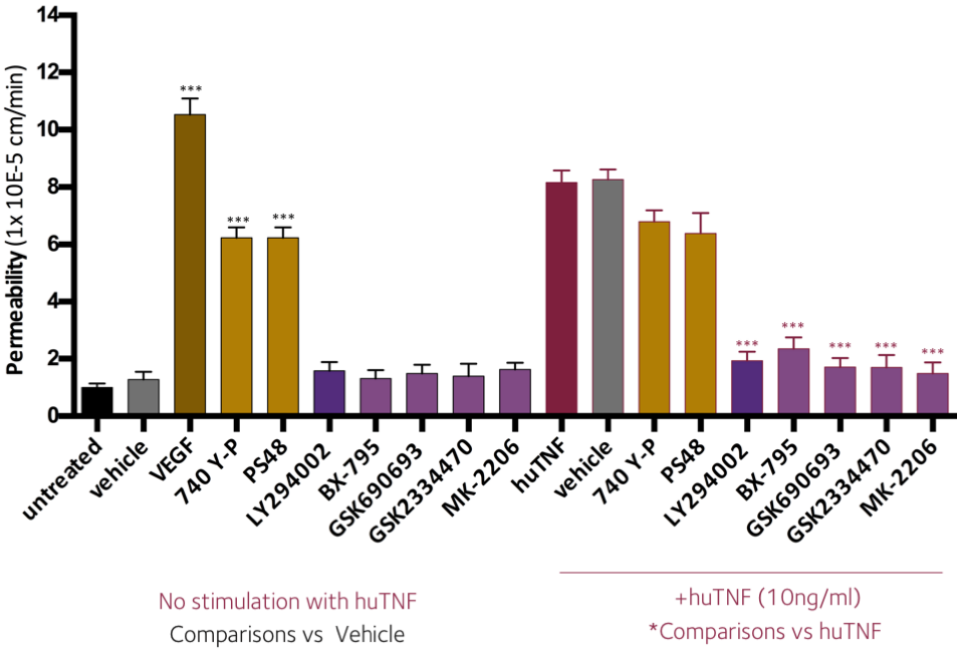


Figure 5.8 Suppression of PI3K/PDK1/AKT signalling reverses endothelial hyperpermeability

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest. Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

5.3.6.2 TNF elicits the activation of mTORC1 and mTORC2

As AKT was proven to be causally implicated in the presentation of TNF-mediated endothelial permeability increase, we asked to confirm that the pro-inflammatory stimulus activates the mTOR complexes in hCMECs. We assessed the expression levels of phospho-S6K1 (pS6K1) that is a hallmark of mTORC1 activation, and those of phospho-AKT (pAkt) being indicative of *de novo* assembly of mTORC2 complexes. The potent mTOR inhibitor rapamycin was employed in order to delineate the temporal pattern of mTORC1 activation and also to confirm the transition from mTORC1 to mTORC2 signalling.

Rapamycin is an extensively studied macrolide targeting mTOR (IC_{50}=5pM). Prolonged exposure to rapamycin (IC_{50}=5pM) affects endothelial viability, motility and cytoskeletal organisation (Kay et al., 1991; Barilli et al., 2008; MRC Phosphorylation and Ubiquitylation Unit, 2018m). Rapamycin potentiates apoptosis in endothelial cells at concentrations higher than 1μM. However, co-administration of lower doses (up to 200nM) with TNF does not induce cell death in hCMECs (appendix).

Pro-inflammatory stimulation with intermediate dose of TNF (10ng/ml) elicits the acute assembly of the mTORC1 complex, as confirmed by the increased presence of activated (phosphorylated) S6K1 at 2h (6-fold increase, $p<0.001$). Prolonged exposure to TNF for 6h, induced a further 1.6-fold upregulation of pS6K1 levels ($p<0.05$) and an additional 2-fold rise at 20h ($p<0.05$), suggesting the sustained activity of mTORC1.

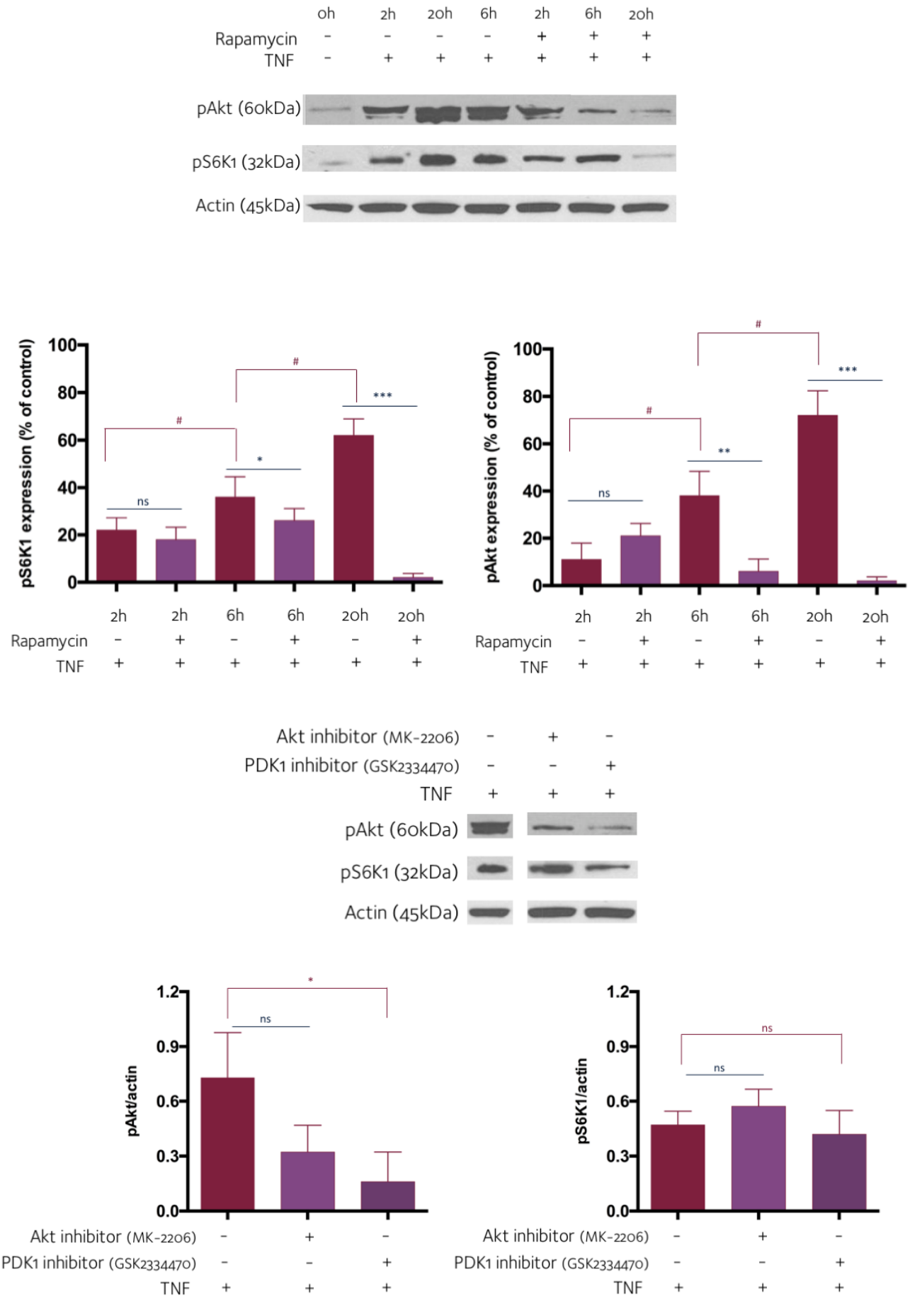
Combined administration of rapamycin with TNF did not alter pS6K1 expression significantly at 2h (ns, compared to 2h treatment with TNF only). However, pS6K1 expression was significantly affected by rapamycin treatment at 6h (1.4-fold decrease, $p<0.05$) and almost completely diminished at 20h (31-fold decrease, $p<0.001$).

TNF stimulation triggered upregulation of pAkt expression at 2h that progressively escalated at 6h (3.5-fold increase, $p<0.05$) and peaked at 20h (2-fold increase, $p<0.05$), indicating the gradually augmenting activation of mTORC2. Parallel treatment with TNF and rapamycin did not confer significant alterations at pAkt levels at 2h (ns). However, extended exposure to rapamycin led to a 6-fold ($p<0.01$) downregulation of pAkt at 6h, followed by a further 12-fold decline ($p<0.001$) at 20h.

Next page: Figure 5.9 Figure 5.9 TNF elicits the activation of mTORC1 and mTORC2

A-C: Western blot showing the levels of phospho-S6K1 (pS6K1) **(B)** and phospho-AKT (pAkt) **(C)** expression in hCMEC/D3 cells at 2h, 6h or 20h post exposure to TNF (10ng/ml) with/without Rapamycin treatment (50nM). Band densitometry was normalised to the intensity of Actin bands and quantified across three independent experiments.
Statistical analysis: Student t-test, two-tailed and paired. Comparisons in red demonstrating alterations across different timepoints. Comparisons in black demonstrating alterations across cells treated with TNF only or with both Rapamycin and TNF.
D-F: Western blot showing the levels of phospho-AKT (pAkt) **(E)** and phospho-S6K1 (pS6K1) **(F)** expression in hCMEC/D3 cells at 6h post exposure to TNF (10ng/ml) with/without PDK1 antagonist treatment (GSK2334470;5μM) or AKT antagonist treatment (MK-2206;10μM). Band densitometry was normalised to the intensity of Actin bands and quantified across three independent experiments.
Statistical analysis: Student t-test, two-tailed and paired. Comparisons in red demonstrating alterations across different timepoints. Comparisons in black demonstrating alterations across cells treated with TNF only or with both PDK1/AKT antagonist and TNF.

Figure 5.9 TNF elicits the activation of mTORC1 and mTORC2



5.3.6.2.1 Inhibition of mTORC1 and mTORC2 disrupts TNF-induced endothelial permeability increase

Having confirmed the responsiveness of both mTORC1 and mTORC2 to pro-inflammatory TNF stimulation and the implication of regulatory AKT signalling on the exacerbation of endothelial compromise, we wished to investigate whether mTOR is controlling endothelial permeability.

The mTOR agonists insulin-like growth factor (IGF) and leucine (Leu) (Gulati et al., 2009; Hernandez-Negrete et al., 2007), alongside the cell-permeable activator of the mTOR pathway MHY1485 (Choi et al., 2012) significantly increased paracellular permeability in unstimulated cells. Mechanistically, MHY1485 functions by increasing the levels of cellular mTOR and of its downstream effector 4E-BP1. Moreover, IGF is an agonist that induces proximal phosphorylation of S6K1 (Pearce et al., 2010). Additionally, branched chain amino acids like leucine execute mTORC1 activation, and S6K1 phosphorylation (Lynch et al., 2003).

Administration of intermediate dose of rapamycin (50nM) suppressed the TNF-induced endothelial leakiness. Incubation for 3h was not sufficient for the amelioration of endothelial integrity (9% decrease, ns). Extended treatment for 6h led to a steep decrease in paracellular permeability (40%, $p<0.001$), indicating the vasomodulating role of mTORC1. Prolonged incubation for 12h elicited a further decrease (69%, $p<0.001$) peaking at 24h (77% decrease, $p<0.001$). Rapamycin suppressed the TNF-

induced pAkt expression within 6h-24h of exposure, suggesting that the observed additional decrease is attributed to mTORC2 targeting.

Moreover, we tested the effect of S6K1 inhibition using the cell-permeable agonist PF-4708671. This compound potently targets S6K1 (IC_{50}=160nM) and does not promote the phosphorylation of Akt, being a valuable tool for the assessment of the effects of mTORC1 suppression. Treatment with that S6K1 antagonist induced a considerable decrease in TNF-induced permeability increase by 86% ($p<0.001$).

Interestingly, pre-treatment with the PDK1 inhibitor GSK2334470, followed by TNF exposure for 6h, completely suppressed pAkt levels, whereas direct inhibition of Akt employing MK-2206 did not alter pAkt expression significantly. Moreover, none of the inhibitors did affect p6K1 expression significantly.

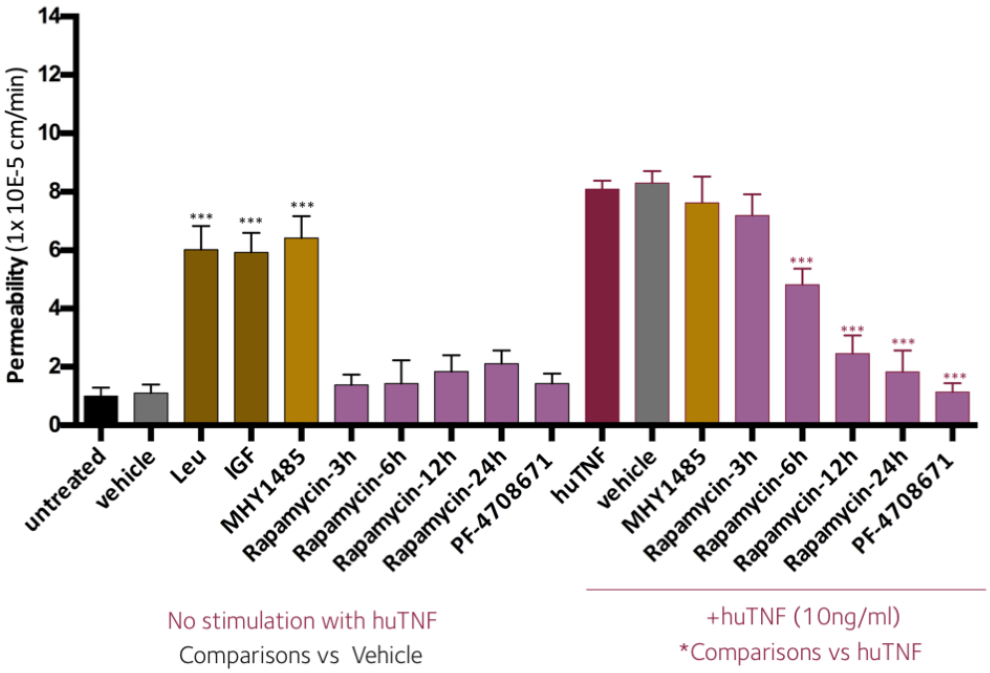
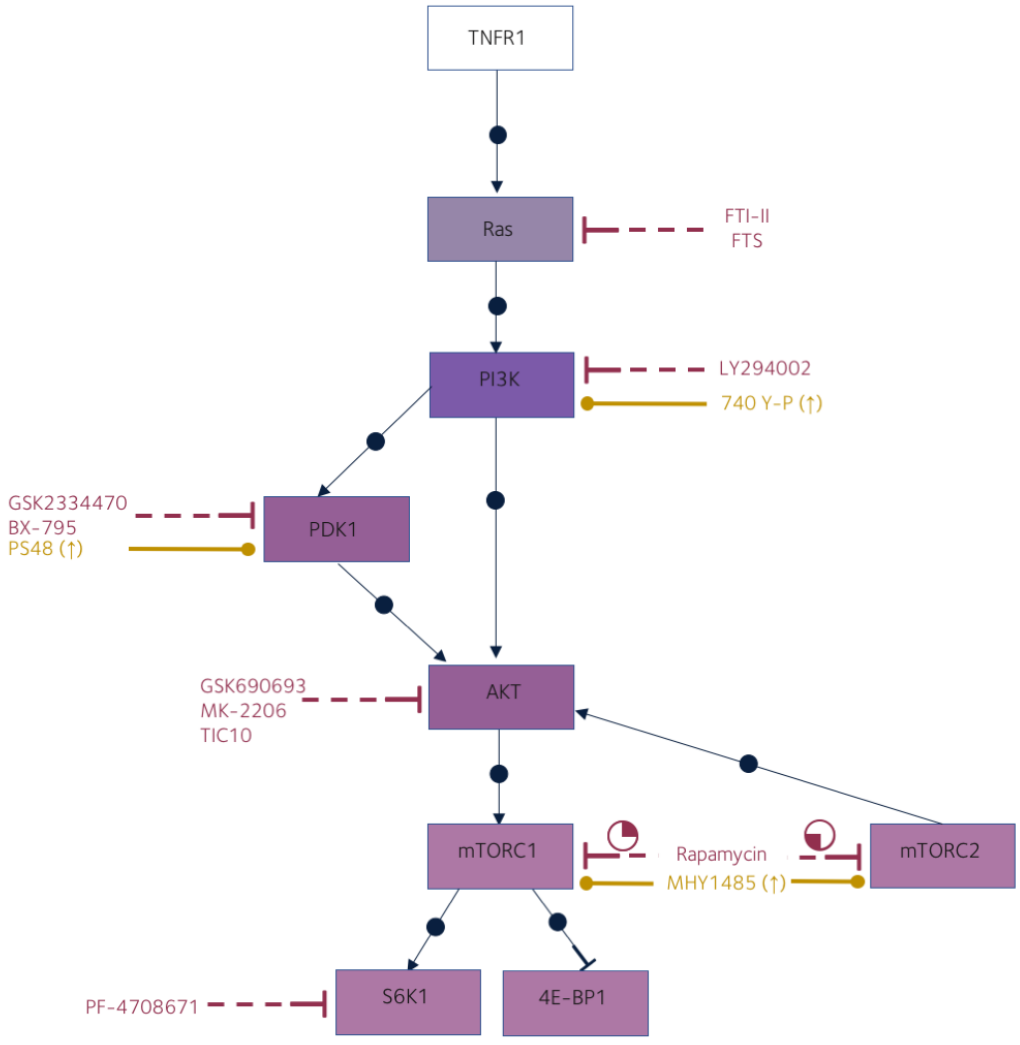


Figure 5.10 ***Inhibition of mTORC1 and mTORC2 disrupts TNF-induced endothelial hyperpermeability***
Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.

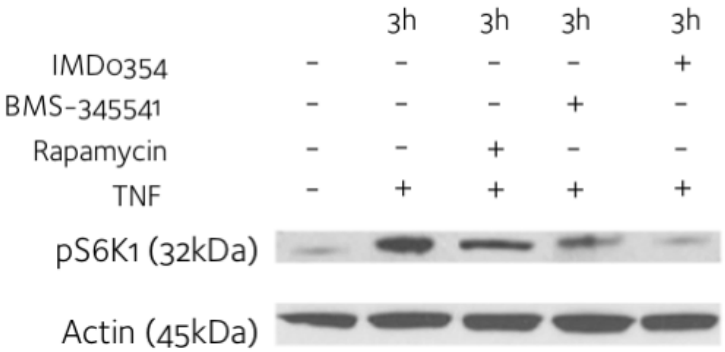


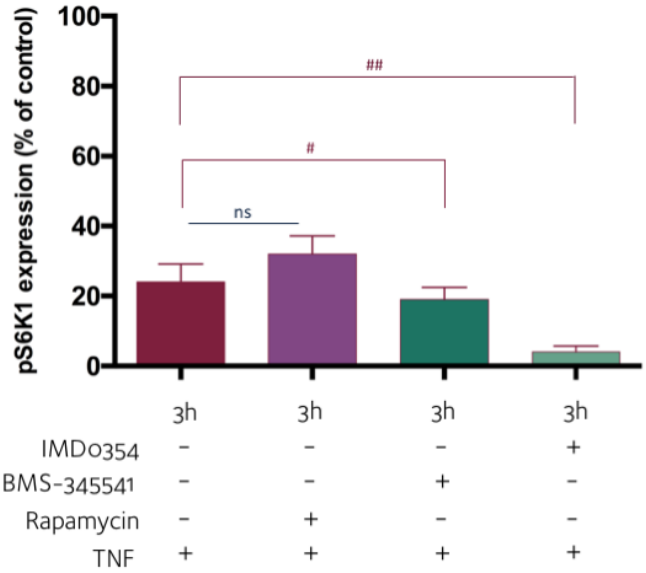
5.3.6.2.2 IKKβ regulates the TNF-mediated activation of mTORC1

In view of the reported physical interaction of IKKβ with the mTORC1 co-factor TSC1 (Lee et al., 2007), we wished to determine whether these signalling nodes modulating the TNF-associated endothelial dysfunction are interrelated.

Combined treatment with rapamycin and TNF for 3h did not alter p6K1 expression (ns). Dual targeting of IKKα and IKKβ, employing BMS-345541 conferred a 1.75-fold decrease ($p<0.05$) in pS6K1 levels, whereas the potent and selective IKKβ inhibitor IMD0354 elicited an 8-fold downregulation of the TNF-induced p6K1 activation ($p<0.01$). Of note, BMS-345541 has a 10-fold weaker affinity for IKKβ, compared to its potency for IKKα (IC_{50}: IKKα=4μM, IKKβ=0.3μM, respectively) (Burke et al., 2003).

Figure 5.11 IKKβ regulates the TNF-mediated activation of mTORC1





5.3.6.3 PI3K modulates the TNF-mediated transcriptional activation of NF-κB

The demonstrated transcriptional regulation of pS6K1 by IKKβ under the context of TNF-insult, and the reported physical interaction of the two signalling nodes, prompted us to investigate whether NF- κB is an effector of PI3K.

Combination treatment employing the PI3K agonist 740 Y-P or the PDK1 agonist PS48 led and NF-κB inhibitors (IMD0354, BMS-345541, BAY 11-7085) led to significant suppression of the permeabilising activity of the agonists ($p<0.01$ upon consecutive exposure to IMD0354 and $p<0.001$ after administration of BMS-345541 or BAY 11-7085). Notably, apart from initiating the activation of the PI3K signalling cascade, PS48 induces also mTOR phosphorylation (Jiang et al., 2015).

Furthermore, administration of the NF-κB activators prostratin and PMA advanced endothelial permeability that remained unaffected upon subsequent treatment with the PI3K inhibitor LY294002 or the PDK1 antagonist GSK2334470 (ns, for all pairs of comparisons).

Combined, these data suggest the positive, upstream regulation of the transcriptional activity of NF-κB by the PI3K pathway.

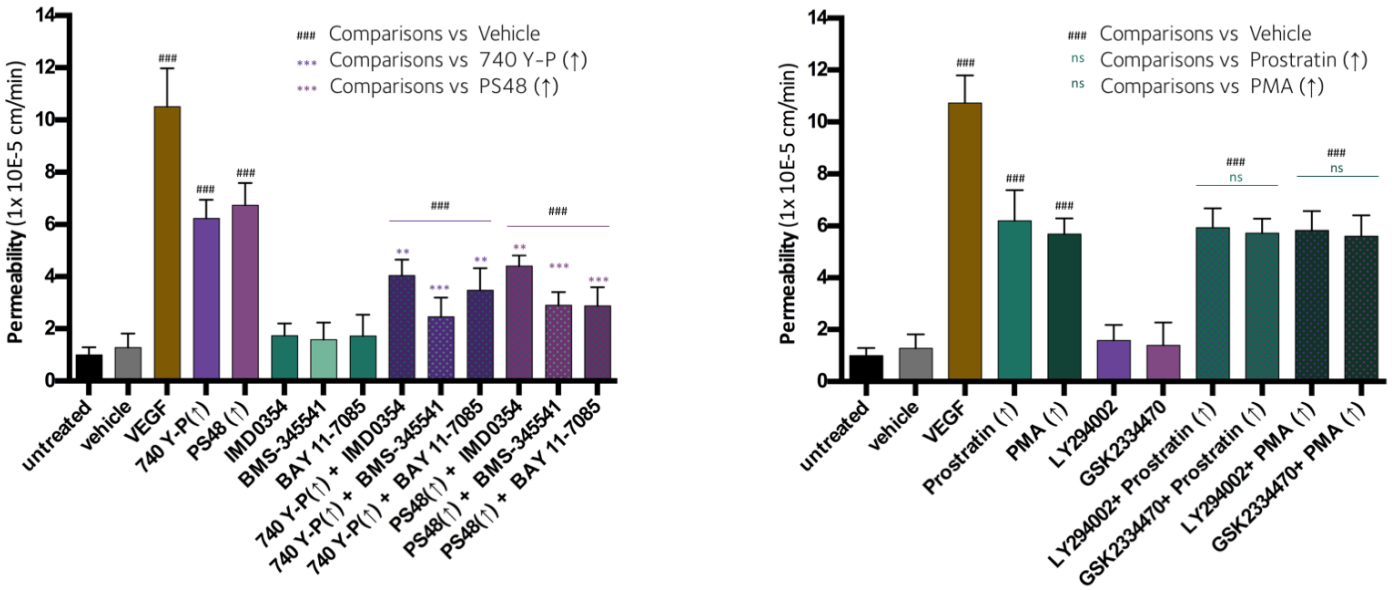
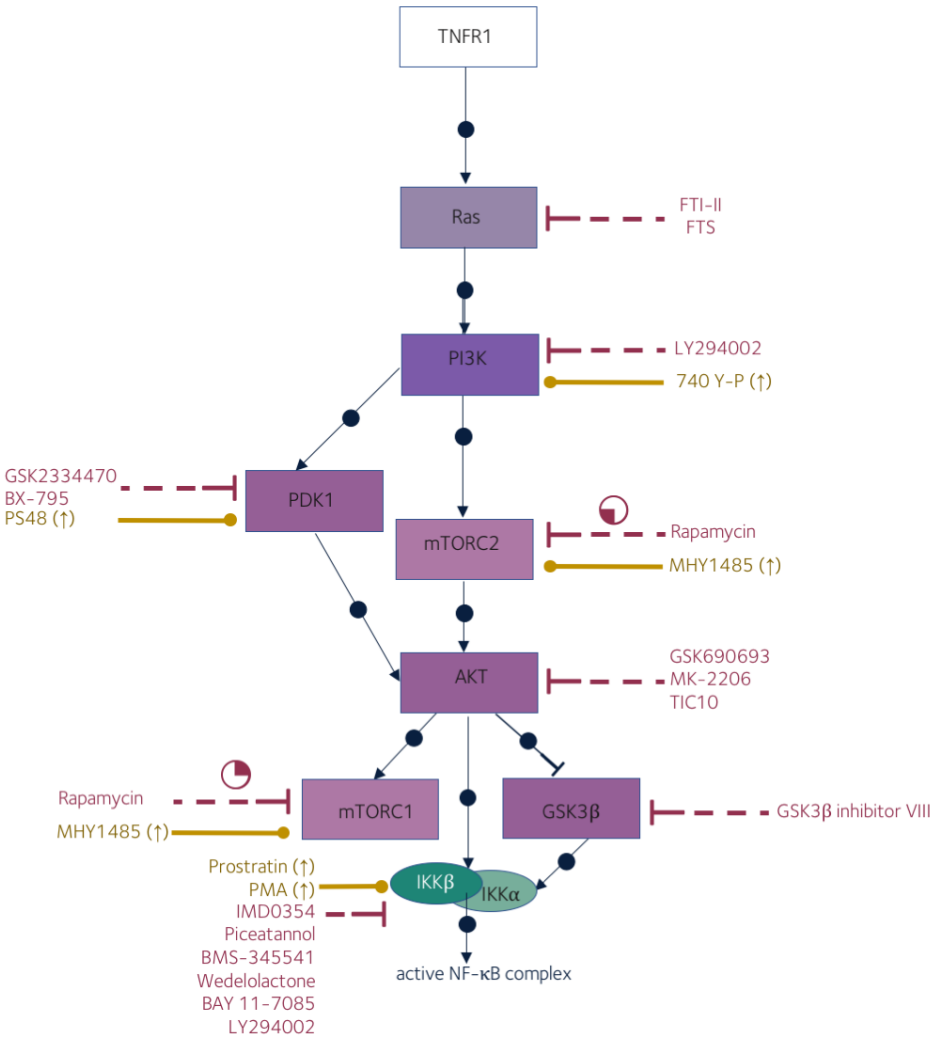


Fig.5.12 PI3K modulates the TNF-mediated transcriptional activation of NF-κB

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.6.4 Akt is a signalling cross-road modulating TNF-induced endothelial permeability increase

Next, we wished to address whether GSK-3β, a downstream target of Akt activation contributes to the observed TNF-mediated compromise of endothelial integrity.

Targeted perturbation of GSK3β activity enhanced the stability of the endothelial monolayer significantly (52% decrease, $p<0.001$). We employed the GSK-3β inhibitor VIII (AR-A014418), a potent antagonist (IC_{50}=104nM) (MRC Phosphorylation and Ubiquitylation Unit, 2018a) that interferes with the ATP binding pocket of the kinase (Bhat et al. 2003).

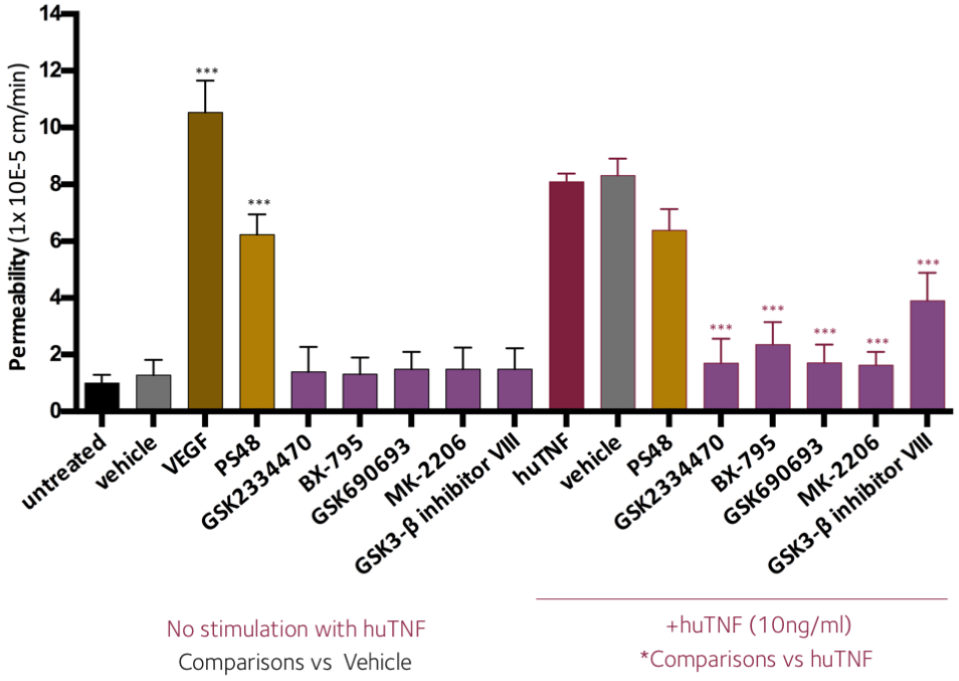
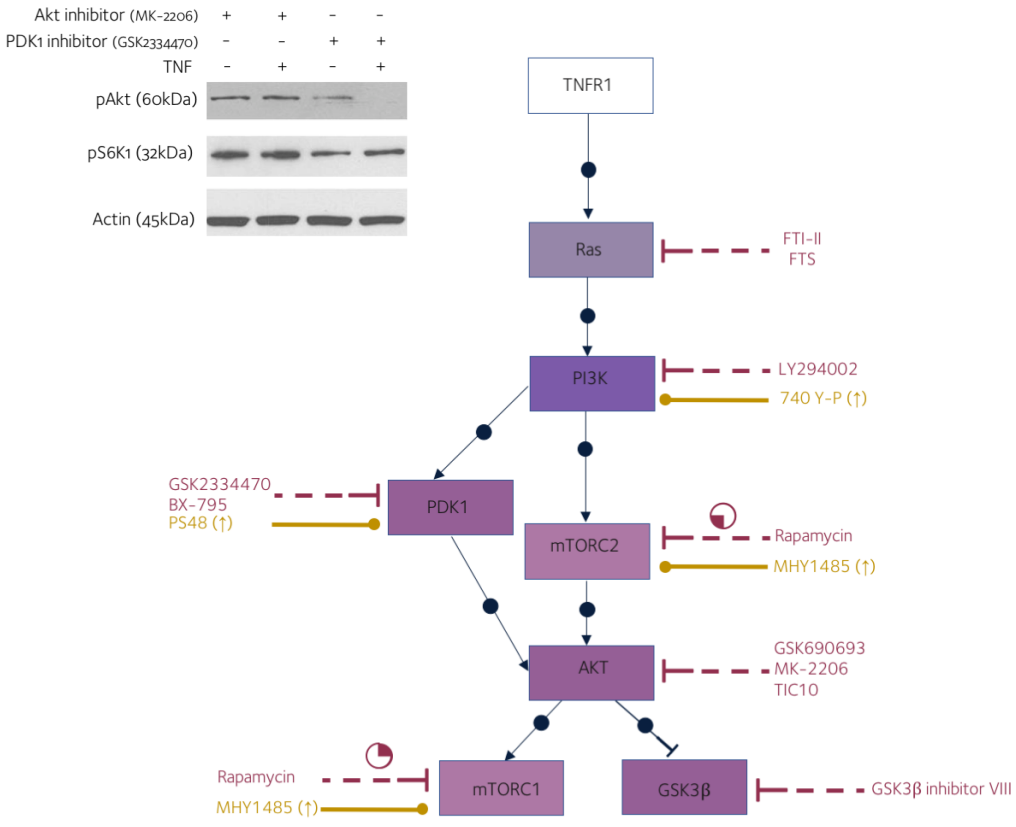


Fig.5.13 Akt is a signalling cross-road, modulating TNF-induced endothelial hyperpermeability

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.6.4.1 eNOS dysregulation contributes to TNF-induced endothelial permeability increase

The pronounced decline in TNF-induced permeability increase upon Akt inhibition and the well-established implication of the RhoA/PTEN/Akt/eNOS axis on endothelial dysfunction prompted us to explore the role of eNOS on the integrity of the brain endothelium.

For that purpose, we employed L-NAME an extensively studied, non-selective inhibitor of nitric oxide synthetases (NOS). This compound perturbs cGMP formation in endothelial cells (IC_{50}=3.1μM) (Rees et al., 1990). Inhibition of eNOS signalling utilising L-NAME conferred a significant decrease TNF-induced endothelial permeability increase (33%, $p<0.001$). Notably, this decline was significantly less pronounced than those yielded by ROCK or Akt inhibition (ROCK inhibition via KD025: 76% decline, $p<0.001$ compared to L-NAME, and for Akt inhibition using GSK2334470 or MK-2206: 79% and 80% decline, $p<0.001$ for both comparisons against inhibition of eNOS with L-NAME).

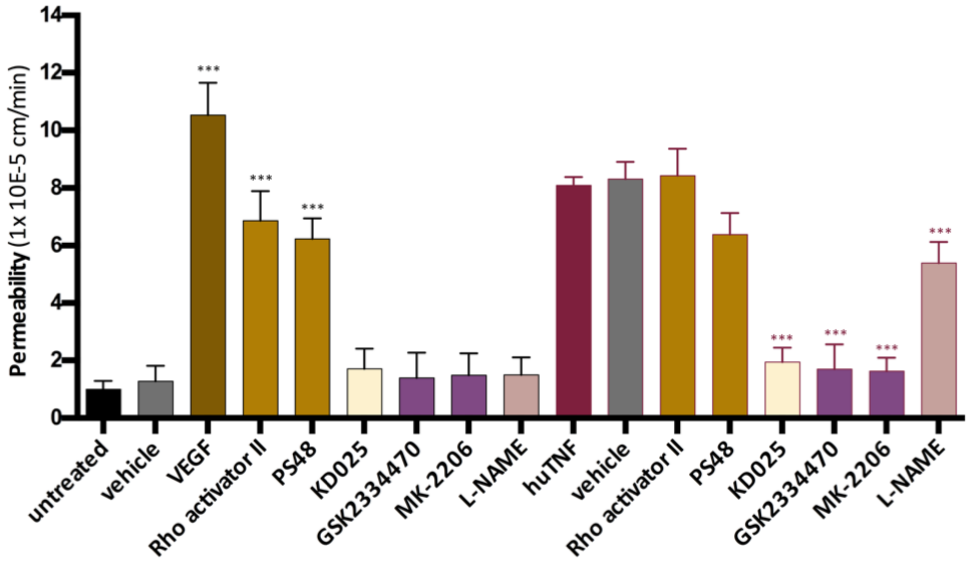
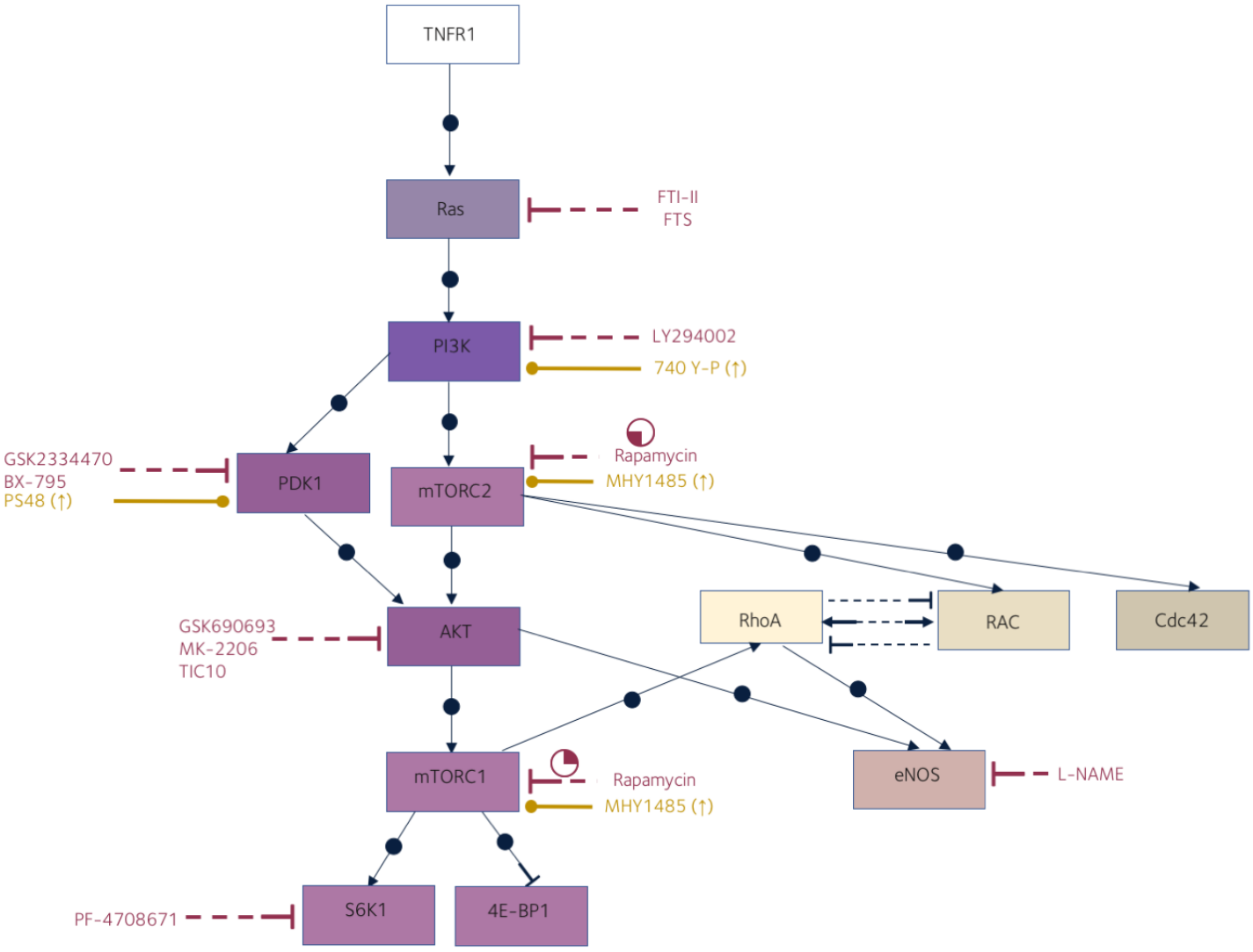


Fig.5.14 eNOS dysregulation contributes to TNF-induced endothelial hyperpermeability

Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.



5.3.6.5 The Ras/PI3K/PDK1 axis is an upstream regulator of the RhoGTPase pathway

Based on the existence of multiple layers of cross-regulation amongst the PI3K pathway and the RhoGTPase family, we wished to investigate the regulatory hierarchy of the two pathways.

First, we employed potent agonists of PI3K (740 Y-P), of PDK1 (PS48), and of mTOR (MHY1485). Combined administration of selective inhibitors of ROCK (KD025), Rac (EHT1864), and Cdc42 (ZCL278) with the aforementioned agonists, led to significant permeability suppression ($p<0.001$ for all comparisons against treatment with the agonists only).

Additionally, we assessed the effect of co-administration of activators of the RhoGTPase cascade, namely Rho activator II and Rho/Rac/Cdc42 activator with suppressors of PI3K (LY294002), PDK1 (GSK2334470) and mTOR (rapamycin for 24h) signalling. In that case, none of the treatments did yield significantly lower permeability than treatment with the agonists only ($p<0.001$). Combined these data suggest that the PI3K/PDK1/mTORC2 axis is signalling upstream the RhoGTPase cascade comprised by RhoA, Rac and Cdc42.

Finally, we sought to explore whether the RhoGTPase pathway is under the regulatory signalling of Ras. For that assay, we employed the PI3K agonist 740Y-P as a positive control, given the established allocation of the PI3K pathway as an effector cascade amenable to Ras signalling.

Combined treatment with the Ras inhibitors FTI-II or FTS, and the compounds 740 Y-P (PI3K agonist) or Rho activator II (RhoA agonist), did not suppress the permeability increase uptakes elicited by the agonists significantly ($p<0.001$, for all relative comparisons with the agonist only). These results confirm that the signalling of the RhoGTPases is controlled by the activity of Ras.

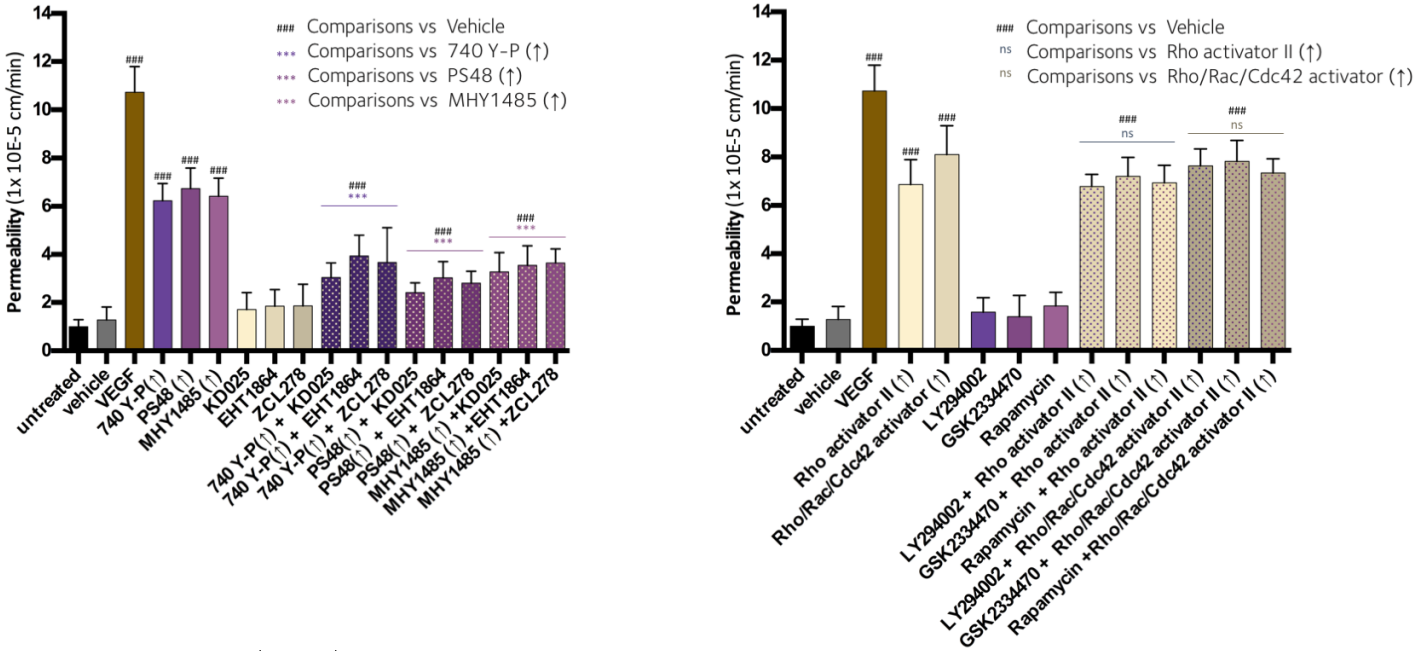
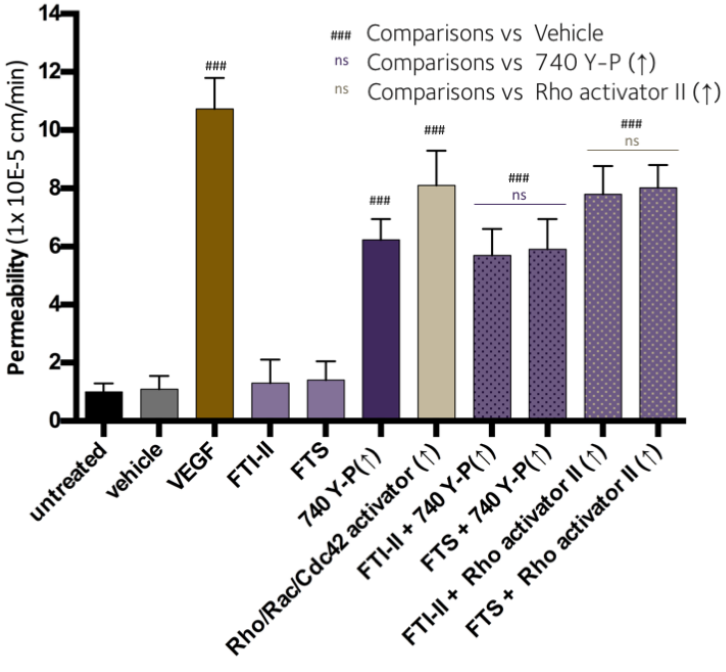
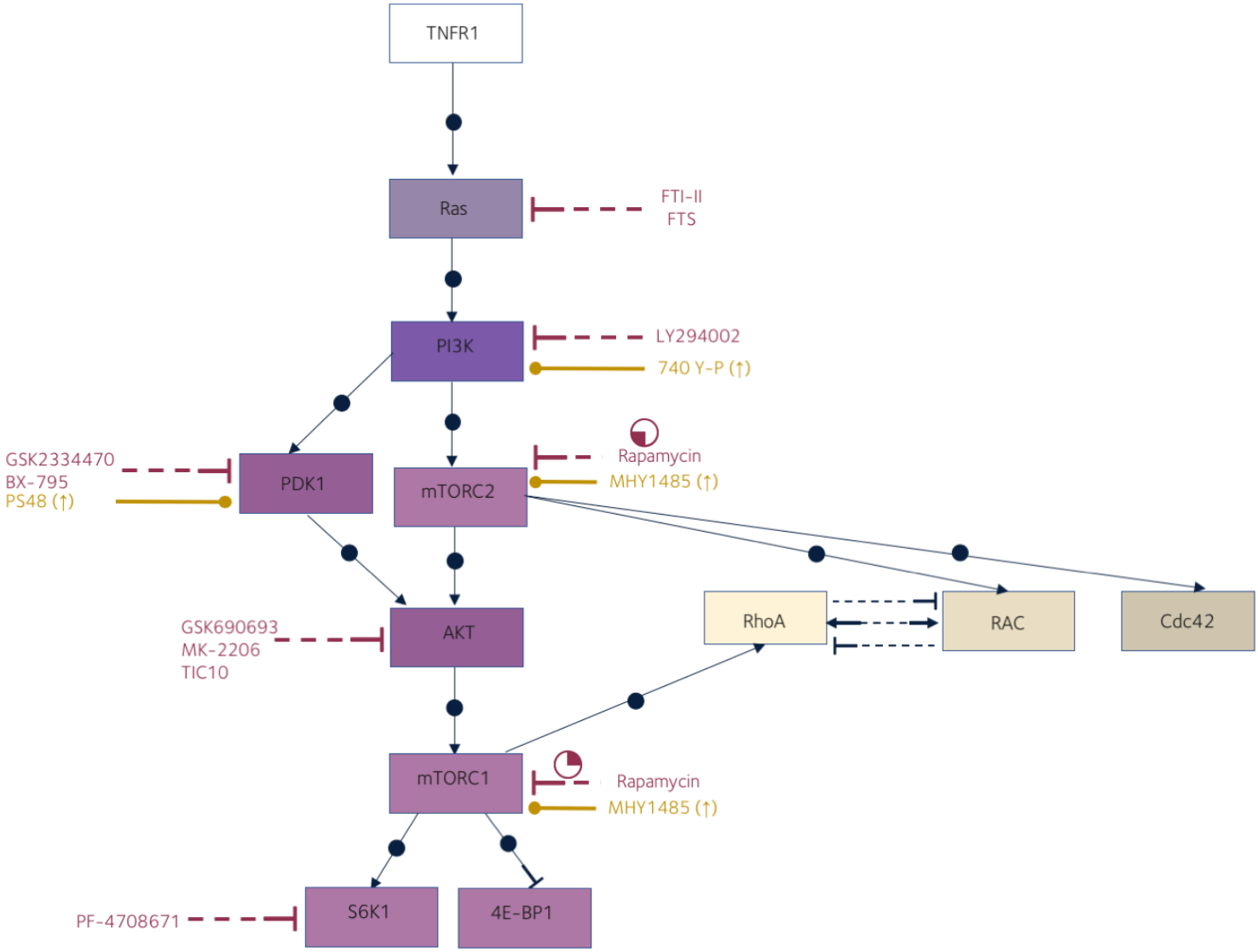


Fig.5.15 The Ras/PI3K/PDK1 axis is an upstream regulator of the RhoGTPase pathway

Next page: Schematic presenting the signalling interactions assessed. Blue arrows and circles indicate implication on the TNF-induced endothelial hyperpermeability. Compounds illustrated in yellow refer to pathway-specific agonists. Compounds illustrated in red refer to pathway-specific antagonists.




TNFR1
Ras
FTI-II
FTS
PI3K
LY294002
740 Y-P (↑)
GSK2334470
BX-795
PS48 (↑)
PDK1
mTORC2
Rapamycin
MHY1485 (↑)
RhoA
RAC
Cdc42
GSK690693
MK-2206
TIC10
AKT
mTORC1
Rapamycin
MHY1485 (↑)
PF-4708671
S6K1
4E-BP1

5.3.6.6 Inhibition of tyrosine kinases

Activation of tyrosine kinases has been reported to contribute to cytokine-induced endothelial permeability increase. Moreover, we have demonstrated that tyrosine kinases such as FAK are causally implicated in the TNF-mediated loss of endothelial integrity. Therefore, we aimed to address whether suppression of this target could exert a beneficial effect.

We employed inhibitors targeting a diverse array of tyrosine kinases. PD173074 is a potent and selective inhibitor of the fibroblast growth factor receptors (FGFRs) (IC_{50}=21.5nM)(Mohammadi et al., 1998). Genistein is a tyrosine-specific inhibitor of protein kinase activity, targeting the tyrosine phosphorylation of histone H2B (Akiyama et al., 1987). Genistein is potently inhibiting PKC (IC_{50}=1nM-40μM), EGFR (IC_{50}=1μM), JNK (IC_{50}=9μM), ERK (IC_{50}=5-57μM), IKKβ (IC_{50}=9μM) and to a lesser extent IKKα (C_{50}=34.8μM) (Kinexus Bioinformatics Corporation, 2017).

The Src kinase inhibitor I is a potent, selective inhibitor of a panel of non-receptor tyrosine kinases. This compound is targeting a wide range of kinases including LIMK (IC_{50}=1nM), MEKK1 (IC_{50}=1nM), RIPK1(IC_{50}=26nM), Src and related proteins (IC_{50}=50-180nM), EGFR(IC_{50}=250nM), VEGFR(IC_{50}= 0.32μM), PAK2 (IC_{50}=5μM), FAK (IC_{50}=10μM),p38 MAPK (IC_{50}=10μM) (Kinexus Bioinformatics Corporation, 2017).

In order to control for the off-target effects of the aforementioned agents we used the LIMK inhibitor SR-7826 to compare the effect of targetted LIMK inhibition with

that of genistein, Nec-1 to control for MEKK-1 inhibition by Src kinase inhibitor I, and IMD0354 to rule out the off-target effect of genistein on IKKα.

Pre-treatment with genistein or Src kinase inhibitor I decreased TNF-induced permeability increase significantly (19% decrease, $p<0.05$ and 27% decrease, $p<0.01$). It has to be noted that the protective effect of both the Src kinase inhibitor I and genistein is not due to the inhibition of an overarching target but is rather reflective of the cumulative effect of the decreased activity of a set of interacting kinases.

Moreover, the high potency of Src kinase I for LIMK and RIPK1 is suggestive of the mechanisms underlying the observed decrease in paracellular permeability. However, this effect might be also diluted by the targeting of kinases of the MAPK pathways that have been proven to have little or no effect on paracellular permeability (Tian et al., 2001). Administration of PD173074 that is targeting selective RTKs (4% permeability decline, ns), hits that this subset of the tyrosine kinase family is not contributing to the exacerbation of TNF-induced endothelial leakiness.

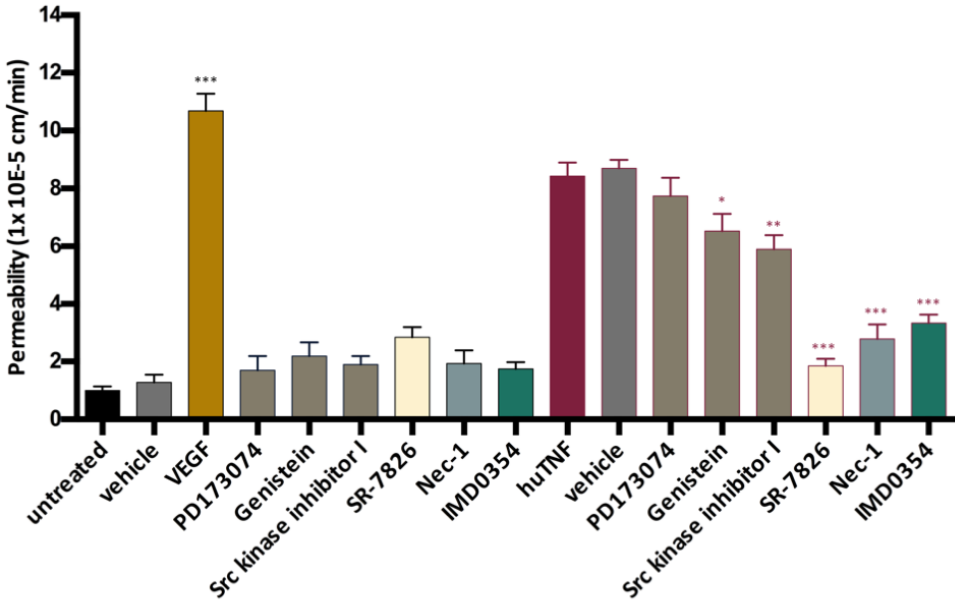


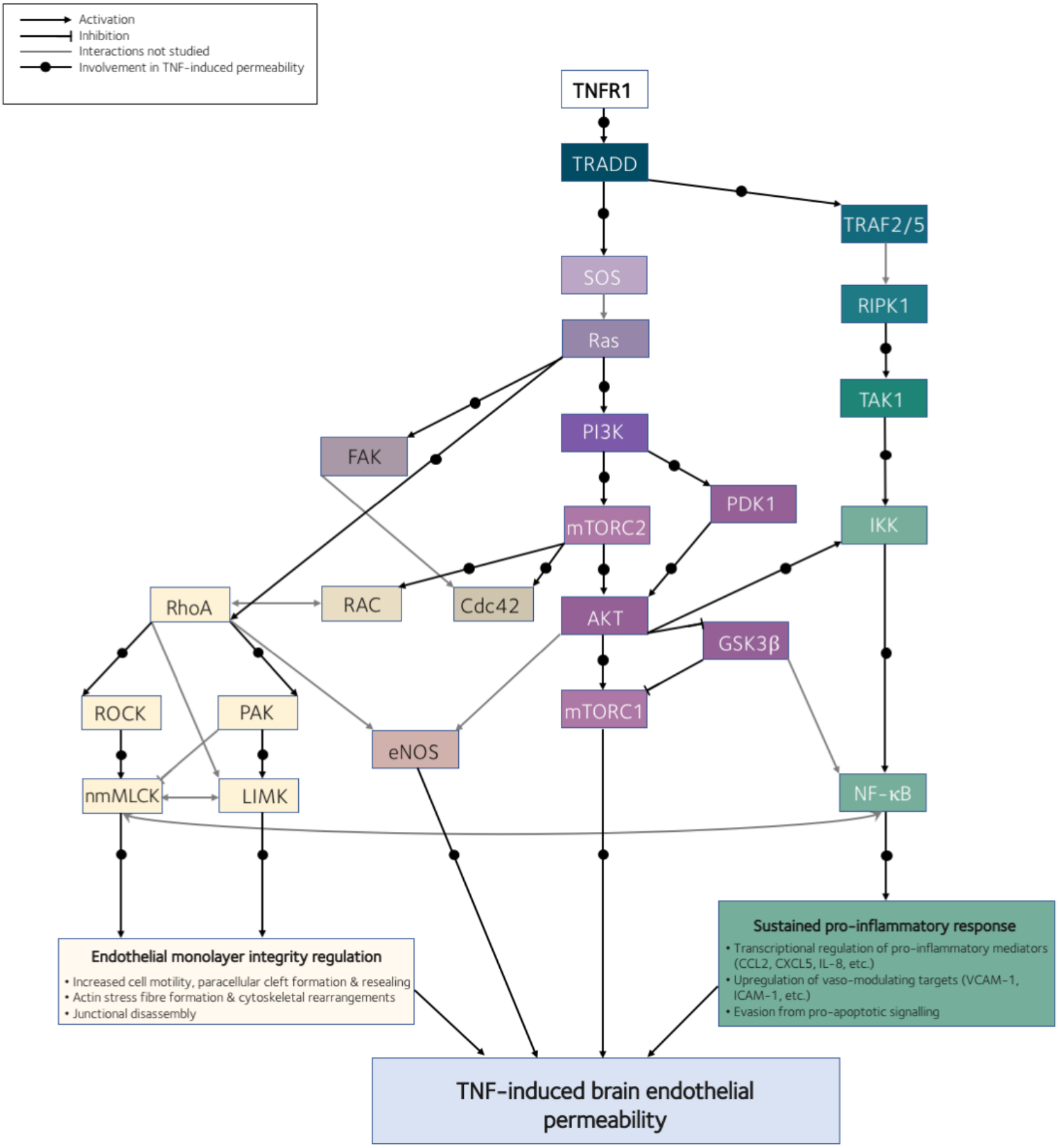
Fig.5.16 Inhibition of tyrosine kinases

Assessment of paracellular permeability to FITC dextran 70 kDa of hCMEC/D3 cells. Confluent endothelial monolayers were treated with the vasopermeant VEGF (10ng/ml, 24h; positive control), or recombinant human TNF (10ng/ml, 24h) with / without parallel treatment with selective agonists and antagonists targeting the signalling cascade of interest.

Statistical analysis: Student t test, two-tailed and paired. *p<0.05, **P<0.01, ***p<0.001 (compared to untreated hCMEC/D3 cells).*p<0.05, **P<0.01, ***p<0.001 (compared to huTNF stimulation). Data are presented as mean values ± SEM

Next pages: Schematics of the proposed mechanism underlying the TNF-induced endothelial hyperpermeability

Fig. 5.17A Mechanism of TNF-induced endothelial permeability



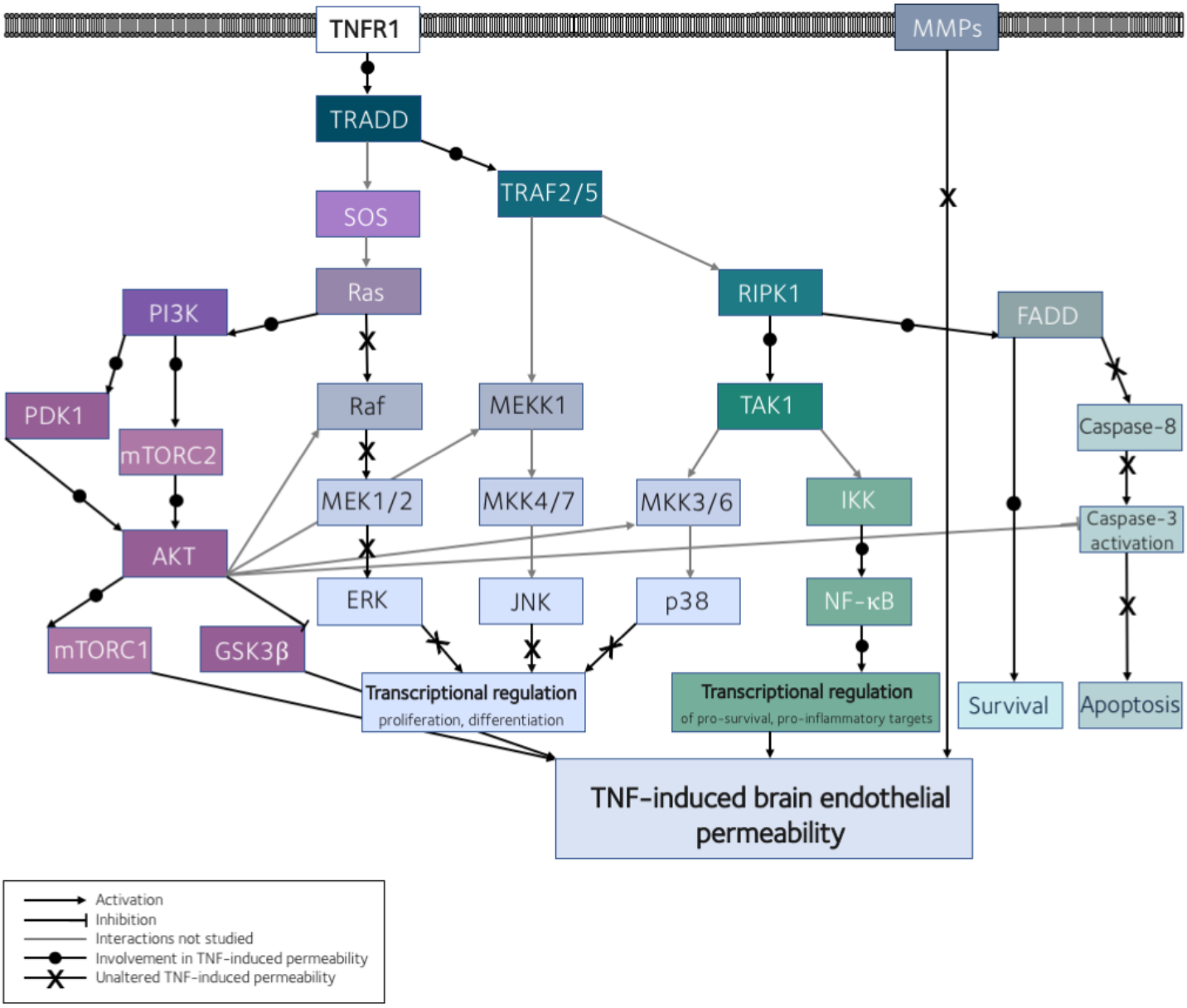


Fig. 5.17B Mechanism of TNF-induced endothelial permeability

5.4 Discussion

5.4.1 The RhoGTPase pathway is involved in the TNF-mediated loss of endothelial integrity

5.4.4.1 The role of RhoA and its effectors

Amongst the inducers of the RhoGTPase pathway tested, both Rho activator II and the Rho/Rac/Cdc42 activator potently elicit permeability increases in the endothelial monolayer. The U-46619 compound does not overcome the effect of TNF, probably due to interference with the Akt signalling pathway.

On the contrary, ROCK inhibitors significantly suppressed TNF-induced permeability increases, underscoring the implication of the signalling exerted on the regulation of endothelial cytoskeletal dynamics. The variable efficacy of the compounds assayed is reflective of their affinity for ROCK-2 specifically, alongside their effect on other Rho-associated upstream or downstream signalling nodes.

Apart from its pronounced selectivity over ROCK-2, compared to other ROCK inhibitors, KD025 benefits from its favourable safety profile. Administration of intermediate doses of KD025 (10-20μM) tightens the monolayer of brain endothelial cells (BECs) under basal conditions and, is indicating that selective inhibition of ROCK-2 is beneficial for the barrier function. Moreover, administration of the inhibitor in presence of the vasopermeant recombinant tissue-type plasminogen activator (rt-PA) reverts permeability changes by 79%, whilst treatment with the non-isoform-specific inhibitor fasudil achieves only a repression by 38% (Niego et al., 2012). The

protective effects of KD025 are accompanied by prevention of any morphological alterations and tight junctional disassembly (Niego et al., 2017). Our results corroborate these findings as treatment with low-dose KD025 (2μM) did not increase the inherent permeability of the monolayer but did repress the TNF-induced permeability by 76%.

The effects of Y-27632 on TNF-induced endothelial compromise confirm the pivotal role of ROCK on the governance of actin dynamics. Y-27632 is also affecting other cytoskeleton-associated kinases (PKA, PKC, Rac, nmMLCK, etc.), although to a lesser extent than fasudil (Anastassiadis et al., 2011a). Y-27632 has been successful in the suppression of ROCK-mediated endothelial cytoskeletal remodelling, as part of its broader anti-inflammatory actions (Tasaka et al., 2005). Pre-treatment with Y-27632 represses the debilitating effect of U-46619 on rat brain endothelial cells (Ruegg & Burgess, 1989; Z. Zhao et al., 2017). Like KD025, this agent is impinging on ROCK downstream signalling. This inhibitor is directly perturbing Rac-induced nmMLCK phosphorylation through downregulation of its downstream target MLC by 65% (Brzeska et al., 2004). Additionally, Y-27632 decreases the levels of phosphorylated LIMK and its downstream effector, cofilin (Ishibashi, 2008), alongside the expression of the proteolytic enzyme MMP-9 (Yu et al., 2014).

Fasudil is a broad-spectrum kinase inhibitor, targeting kinases that execute vaso-regulatory signalling. Apart from enhancing ROCK-2 activity by 12%, fasudil is also suppressing Rac activity by 60%, suggesting that its less pronounced effect on diminishing TNF-induced permeability increase might be due to the suppression of the

putative vaso-enhancing signalling of Rac. Despite its reduced specificity, this inhibitor is a useful tool in overcoming the inherent redundancies in cytoskeletal regulation.

Rho inhibitors directly affect several RhoA and RhoC substrates. Overexpression of inhibitors of RhoA mimics the effect of nmMLCK inhibitors, underscoring the crucial role of the Rho GTPases in the regulation of acto-myosin contractility (Dhawan & Helfman, 2004).

Notably, the enhanced efficacy of both KD025 and Y-27632 might also be associated with the blockade of the downstream effector nmMLCK. Nevertheless, suppression of ROCK is directly linked to downregulation of nmMLCK activity and administration of both Y-27632 and ML-7 does not exert a cumulative effect on the basal permeability of mouse renal endothelial cells, confirming the overlap between the signalling modulated by both agents (Xu et al., 2015).

Direct pharmacological targeting of nmMLCK using ML-7 had a beneficial effect on the enhancement of the stability of the TNF-stimulated endothelial monolayer. In support of this, pre-treatment with ML-7 reverses TEER decline in b.End3 cells and suppresses the IL-17-induced BBB breakdown in experimental autoimmune encephalomyelitis models (Huppert et al., 2010). Furthermore, ML-7 effectively reduces cerebral oedema upon cortical impact (Luh et al., 2010). Administration of ML-7, at a clinically relevant timepoint (4h after TBI), decreases nmMLCK expression, enhances BBB stability and ameliorates cognitive and motor outcomes (Kuhlmann et al., 2007; Rossi et al., 2013). Mechanistically, ML-7 interrupts the signalling of the

RhoA/ NF-κB axis by blocking the TNF-induced transactivation of NF-κB in endothelial cells (Wadgaonkar et al., 2005; Wu et al., 2009).

The decreased effect of ML-7, compared to KD025, on endothelial integrity restoration (63% and 76%, respectively), is reflective of the overarching role of ROCK as the orchestrator of the downstream events following RhoA activation, but also of the considerable contribution of the PAK/LIMK axis on the maintenance of endothelial integrity. Indeed, inhibition of LIMK achieves similar effects as ROCK-2 inhibition (77% and 76%, respectively), underlining that RhoA-associated signalling converges on LIMK both through the RhoA/ROCK/nmMLCK/LIMK, but also through the previously undermined RhoA/PAK/LIMK axis. Our findings are in line with, experiments targeting the only LIMK substrate identified to date, cofilin. Constitutive activation of mutant cofilin leads to reduced actin polymerisation and junctional protein disassembly, hence attenuating BBB impairment and yielding improved clinical outcomes (Shi et al., 2016).

5.4.4.2 The role of Rac and Cdc42

RhoA and Rac signalling are often antagonistic depending on the stimulatory conditions and the vascular cells examined. TNF elicits an acute Rac activation within minutes. However, the duration of the activation has been reported to be varying amongst endothelial cell lines. Peripheral microvascular endothelial cells (dermal and pulmonary) are reported to exhibit reduced Rac activation upon long-term TNF

stimulation (Schlegel & Waschke, 2009; Shao et al., 2013). Moreover, Rac has been proven to be an integral part of the resistance of the lung microvascular endothelial monolayer (Bandman et al., 2002).

On the contrary, the RacGEFs Vav, Tiam-1, Trio and P-Rex, signalling upstream of Rac, are also positively regulated by TNF and involved in endothelial barrier disturbance (Cain et al., 2010; Naikawadi et al., 2012). Rac retains its activity for up to 20 hours post-stimulation in HUVECs (Van Rijssel et al., 2013). Silencing of the kinase prior TNF-stimulation diminishes cell elongation, the redistribution of intercellular junctions and the upregulation of F-actin, whilst ameliorating endothelial barrier leakage (Cain et al., 2010).

In agreement with these findings, we demonstrated that inhibition of Rac significantly reduced endothelial permeability increase in hCMECs by 44-48%. Moreover, pro-longed administration of a targeted agonist that enhances Rac activity by more than 300% did not achieve any further paracellular permeability increases than TNF, reasoning that Rac remains on its activated state in that system for at least 24h.

Less is known about the role of Cdc42 on the modulation of endothelial integrity. The TNF-induced Cdc42 activation is reported to peak a few minutes after the onset of the treatment (Van Rijssel et al., 2013). The acute response to the pro-inflammatory stimulus evokes the recruitment of an adaptor protein linking the plasma membrane to the cytoskeleton, the factor-associated-with-nSMase (FAN) (Haubert et al., 2007). This cytokine-driven signalling is crucial for the maintenance of AJs (Vandenbroucke

et al., 2008), the induction of pro-survival signalling (Papakonstanti & Stournaras, 2004), and the regulation of cytoskeletal remodelling (Wojciak-Stothard et al., 1998). Our data posit that Cdc42 exhibits an active, vaso-regulatory role up to 24h after the onset of the pro-inflammatory stimulation.

The effect of both the Rac and the Cdc42 inhibitors tested might be also associated with their demonstrated ability to abrogate actin-based motility and impair lamellipodia formation (Montalvo-Ortiz et al., 2012; Shutes et al., 2007 Friesland et al., 2013).

5.4.4.3 The role of FAK

We also demonstrated that FAK is a critical regulator of TNF induced permeability in the brain endothelium. Consistent with its allocation upstream of Rac and Cdc42, suppression of this signalling node elicits a robust suppression of the debilitating effect of TNF, considerably increased compared to single targeting of Rac or Cdc42 (77% decrease, compared to 44-55%, respectively). Our results align with studies characterising FAK is a gatekeeper of endothelial barrier function in response to inflammatory mediators such as thrombin and VEGF (Chen et al., 2012; Mehta et al., 2002).

Moreover, administration of the FAK inhibitor PND-1186 mitigates TNF-induced secretion of cytokines (Schlaepfer et al., 2007), suggesting that the observed protective effect might be linked to repression of a positive feedback loop inducing the

exacerbation of the pro-inflammatory signalling. Additionally, FAK inhibition replenishes the TNF-induced redistribution of endothelial AJs, diminishes VE-cadherin internalisation, and stabilises the VE-cadherin - catenin linkage, thus diminishing vascular impairment (Jean et al., 2014; Schulte et al., 2011). Suppression of FAK activity also prevents the TNF-induced endothelial VCAM-1 upregulation (Lim et al., 2012).

Loss of FAK is associated with altered endothelial cell morphology, actin redistribution and cellular motility, and enhanced transmembrane VE-cadherin localisation (Arnold et al., 2013; Tanjoni et al., 2010). Disruption of the phosphorylation status of FAK and its effector paxillin restores endothelial monolayer TEER values (Mehta et al., 2002). These vasomodulating effects are consistent with the inverse correlation between FAK and Rho in the regulation of FA turnover, with FAK being a suppressor of the GTPase (Ren et al., 2000).

5.4.2 Inhibition of the MAPK pathway does not rescue TNF-induced endothelial permeability increase

Despite the well-established link between TNFR1 activation and MAPK signalling, there is no conclusive evidence supporting the effect of MAPKs on the regulation of endothelial leakiness. Our findings suggest that activation of the Cdc42/ p38 axis does not affect the integrity of the brain vascular endothelium and corroborates with studies

on TNF-stimulated hCMECs demonstrating that the inhibition of p38 MAPK cannot enhance endothelial integrity or avert morphological changes (Ni et al., 2017).

5.4.2.1 The role of the JNK pathway

The pro-inflammatory activity of JNK signalling is extensively characterised. Pharmacological targeting of JNK with the potent inhibitor SP600125 dose-dependently inhibits the phosphorylation of c-Jun, and suppresses the MAPK-dependent expression of inflammatory genes COX-2, IL-2, IFN-γ, TNF (Bennett et al., 2001). However, the implication of JNK signalling on the regulation of endothelial compromise remains unclear. Activation of the Cdc42/JNK or the Rac/JNK axes is not implicated in the regulation of the cytoskeleton (Coso et al., 1995). Moreover, JNK inhibition does not ameliorate the TNF-induced endothelial dysfunction or revert the TNF-mediated cytoskeletal changes in human pulmonary microvascular endothelial cells (Mong et al., 2008), and in mouse renal endothelial cells (Xu et al., 2015). In the first case, endothelial permeability relies on the JNK-induced production of pro-inflammatory IL-6 rather than the initiation of vascular remodelling (Mong et al., 2008). These results parallel our findings that support the notion that JNK inhibition is not sufficient for the restoration of endothelial integrity. On the contrary, silencing of c-Jun ameliorates vascular compromise in murine models of acute inflammation and rheumatoid arthritis (Fahmy et al., 2006), implying that the impact of the activation

of the MAPK pathways on endothelial disruption might rely on the context of the inflammatory insult.

It has to be noted that both the p38 MAPK inhibitor SP600125 and the JNK inhibitor SB202190 target a subset of the MAPKs present in mammalian cells, thus blocking only partially the activation of MAPK and JNK (Davies et al., 2000). However, both agents are extensively used in signalling studies due to their enhanced affinities for their targets and their limited off-target effects.

5.4.2.2 The role of the ERK pathway

There is no conclusive evidence supporting the effect of ERK1/2 signalling on the regulation of endothelial leakiness. Inhibition of Raf phosphorylation using GW5074 represses VEGF-induced permeability increase in HUVECs (Breslin et al., 2003), whilst having no effect on BBB integrity on TWEAK-treated hCMECs (Stephan et al., 2013).

Increased activation of ERK1/2 is not followed by elevated baseline permeability in porcine coronary venules and targeted inhibition of the pathway does not refrain the VEGF- or histamine-induced permeability increase (Wu et al., 2005). Moreover, blockade of mTORC1 signalling in HUVECs induces the activation of ERK (Dormond-Meuwly et al., 2011). However, Raptor and Rictor deficiency in VEGF-stimulated HUVECs does not alter ERK signalling, confirming that ERK is not causally implicated in the VEGF-induced endothelial breaching (Shan Wang et al., 2015). On

the contrary, treatment with the MEK inhibitor PD98059 attenuates VEGF-induced leakiness in HUVECs (Breslin et al., 2003).

The protective effect of the dual ERK and AKT inhibitor TIC10, should not be solely attributed to the abrogation of ERK signalling as our findings employing AKT inhibitors with enhanced potency and selectivity, suggest that AKT is a key regulator of the TNF-induced endothelial permeability increase. Moreover, the transducers Raf and MEK1/2 did not alter endothelial stability significantly, implying that the Raf/MEK/ ERK axis in not causally implicated in the TNF-mediated deterioration of endothelial permeability.

To sum up, there is sound evidence that the activation of MAPKs is an early event in the TNF-induced signalling network in the brain endothelium (Messmer et al., 2000; Shivanna et al., 2010). It is plausible that this point reflects a commitment regulating the cell fate, since prolonged p38 MAPK signalling triggers endothelial dysfunction linked to endothelial apoptosis (Messmer et al., 2000; Shivanna et al., 2010; Lopez-Ramirez et al., 2012). The absence of TNF-induced apoptotic signalling coincides with the lack of MAPK-mediated endothelial leakiness in our model. This notion is based not only on the study of a broad spectrum of signalling molecules of the MAPK pathway, but also on the decreased efficacy of NF-κB inhibitors such as piceatannol that signal through MAPKs. It is plausible that TNF-induced MAPK signalling might be implicated in other steps of the inflammatory cascade such as transcytosis. Taken together, our results support that the evasion from the attenuated MAPK signalling

resorts to signalling networks promoting cellular proliferation and differentiation rather than endothelial integrity.

5.4.2.3 MAPKs do not contribute to TNF-induced permeability increase through activation of MMPs

MMP inhibitors have a beneficial effect in animal models of ischemic stroke by preserving BBB integrity (Chen et al., 2009). Treatment with either the IKK inhibitor piceatannol in animal models of ischemic stroke-induced injury by suppresses the expression of MMPs and enhances the stabilisation of junctional proteins (Suzuki et al., 2013). Moreover, administration of the ROCK-2 inhibitor Y-27632 abrogates the expression of MMP-9 and replenishes the functionality of the brain endothelium (Fu et al., 2014; Yu et al., 2014).

TNF-induced impairment of the brain endothelium, accompanied by caspase-3 activation, can be ameliorated employing MMP-9 selective inhibitors (Wiggins-Dohlvik et al., 2014).

Despite these findings, the majority of the specific-MMP inhibitors or broad-spectrum MMP inhibitors cannot prevent endothelial permeability increase. A plausible explanation is that these agents can hinder only the activity of soluble endopeptidases. In line with this, robust endogenous inhibitors for MMP-9, such as TIMP-1, cannot suppress the activity of these peptidases upon their presentation at

the cell membrane (Owen et al., 2003). Another possible explanation is the requirement on concurrent blockade of multiple MMPs for the regulation of BBB integrity.

Our data posit that prolonged TNF-stimulation in brain endothelial monolayers did not resort to MAPK signalling or activation of pro-apoptotic signalling, as reflected by increased levels of caspases. Additionally, since targeted inhibition of MMPs did not avert TNF- induced permeability increase, it is conceivable that their activation is not heavily induced in brain endothelial cells in the given context.

Taken together, the demonstrated absence of MMPs implication on the regulation of TNF-induced permeability increase is reflective of the lack of signalling favoring cell death accompanied by vascular collapse. The opposing outcomes in other relevant studies might be representing conditions depending on more intense pro-inflammatory stimulation.

5.4.3 Prolonged TNF exposure does not induce cell death

Sustained exposure to TNF (intermediate dose; 10ng/ml) does not induce cell death. In agreement with our findings, the evaluation of prolonged TNF-induced signalling in human peripheral microvascular endothelial cells (HUVECs; HAECs; and HDMECs) , confirms the absence of pro-apoptotic signalling (Barilli et al., 2008; Clark et al., 2015; Janzen et al., 2011). TNF triggers apoptosis in endothelial cells only in presence of *de novo* protein synthesis inhibitors such as cycloheximide, actinomycin D, lovastatin (Li et al., 2002).

Even in systems modelling severe pro-inflammatory insults, the TNF-induced disruption of the endothelial monolayer is reversed within 12-18h in wound healing assays (Barilli et al., 2008; Clark et al., 2015). It is postulated that the endothelial barrier evades TNF-induced apoptosis through the clearance of apoptotic bodies from neighboring endothelial cells and the closure of the gaps formed between the activated cells (Petrache et al., 2001). The latter scenario can be explained by increased motility within the endothelial monolayer.

Administration of the broad-spectrum caspase inhibitors z-VAD-fmk and Boc-D-fmk does not prevent the TNF-induced endothelial disruption in renal endothelial cells (Xu et al., 2015), suggesting that the TNF leak is independent of cell death induction. On the contrary, studies in hCMECs stimulated with high cytokine concentrations (10ng/ml and 100ng/ml of TNF and/or IFN-γ) attribute the observed permeability increase and TJ disassembly to a JNK-mediated activation of caspase-3 and -9 (Lopez-Ramirez et al., 2012). There is growing literature that associates the TNF-induced activation of the MAPK pathways with the commitment to cell death, and concomitant vascular leakiness. Our findings contradict these studies, as neither targeted inhibition of caspase-3, nor inhibition of the pro-apoptotic cascade seemed to exert any protective effect on the endothelial integrity. Additionally, cytotoxicity assays conducted **(chapter 4)** did not detect significantly elevated levels of caspases in this setting.

It has to be noted that the interpretation of these findings varies considerably and is highly dependent on the context of the pro-inflammatory stimulation.

Our findings challenge the notion that prolonged low- or intermediate-dose TNF stimulation induces cell death in brain microvascular endothelial cells. In our models, TNF-induced cell death was evidenced only in presence of suppressors of DNA or proteins synthesis **(chapter 4)**, or upon prolonged treatment with high doses of TNF (100ng/ml or above). Moreover, similar treatments elevate severely the inherent permeability of these *in vitro* systems, thus annulating the physiological relevance of these models. In this regard, extensive induction of cell death is more likely to mirror severe inflammatory insults involving vascular collapse rather than milder TNF-induced fluctuations in endothelial permeability.

Our data suggest that the neither the MAPK pathways or the pro-apoptotic cascade are determinants of TNF-induced permeability increase. The activation of the TNFR1 signalling complex involves the activation of RIPK1, that signals through TAK-1 and FADD and activate the NF-κB and the Ras pathways, respectively.

5.4.4 NF-κB signalling is crucial for the exacerbation of TNF-induced endothelial permeability

The role of NF-κB in BBB rupture is still elusive. Activation of this signalling node could be detrimental for endothelial integrity, but also a parallel event (Wenzel & Schwaninger, 2016). It has been postulated that there is a bell-shaped association between the stimulation of the NF-κB pathway and endothelial impairment (Wenzel & Schwaninger, 2016). According to this theory, minimal induction of the pathway

leads to BBB dysfunction, whereas robust activation exerts permeability increase. An alternative hypothesis posits that BBB opening is the aftermath of acute NF-κB activation, whilst prolonged NF-κB signalling negates the initial response through the engagement of anti-inflammatory proteins and the subsequent resealing of the barrier.

Our data suggest the implication of both the classical and the alternative NF-κB pathway on the dictation of the TNF-induced abrogation of TNF integrity. Disruption of the activity of IKKβ, one of the inaugural regulators of classical NF-κB signalling, restores the stability of the endothelial monolayer. Deterioration of IKKβ signalling distorts NF-κB-driven amplification of pro-inflammatory TNF stimulus. Inhibition of that target has been associated with reduced severity of the inflammatory response through the suppression of the TNF-induced expression of CCL2 and CXCL5, whilst diminishing actin stress fibres and filopodia formation, and TNF-mediated NF-κB nuclear translocation in HUVECs. Additionally, administration of IMD0354 suppresses the secretion of TNF by endothelial cells *in vivo*, in experimental mouse models of corneal oedema (Lennikov et al., 2018).

Additionally, dual inhibition of both signalling transducers IKKα and IKKβ diminishes endothelial breach. Interestingly, the mechanism of action of the compounds employed uncover critical pathways possibly associated with the impact of NF-κB on signalling on the orchestration of the events following the TNF insult.

Piceatannol diminishes the signalling towards the Raf-1/MEKK-1 axis that drives the subsequent induction of the alternative NF-κB pathway, without activating the downstream targets of the pathway; Erk1/2, JNK and MAPK (Baumann et al., 2000).

Treatment with piceatannol has a protective role for the NVU in experimental models of ischemic stroke through the downregulation of MMPs expression and the upregulation and stabilisation of junctional proteins (Suzuki et al., 2013). The decreased efficacy of this inhibitor on the restoration of TNF-induced permeability increase in hCMECs, compared to the IKK inhibitor BMS-345541, might be attributed to the demonstrated minimal role of the MAPK pathway on the TNF-induced endothelial impairment.

The latter achieved a pronounced recovery of the endothelial monolayer. BMS-345541 averts the TNF or LPS-mediated activation of the RhoA/ NF-κB axis (Goto et al., 2009), and the subsequent exacerbation of endothelial leak in bEnd3 cells (He et al. 2011). Additionally, pharmacological targeting of RhoA in brain microvascular endothelial cells is more efficient than BMS-345541 in restoring endothelial integrity and in the recovery of TJs and actin dynamics. This holds true for selective ROCK-2 inhibitor KD025 and the LIMK inhibitor SR7826. These data stress that NF-κB activation serves to orchestrate an acute response to inflammatory challenge and is exerting its vasomodulating effects as a branch interrelated with RhoA activation. Moreover, BMS-345541 is having a 10-fold higher affinity for IKKα than IKKβ, providing a valuable tool for the inhibition of the alternative NF-κB pathway primarily. The remarkable restoration of endothelial integrity using that compound, prompts that extended TNF-stimulation triggers the alternative pathway.

Moreover, use of the IκBα inhibitor BAY 11-7085 underscores the implication of NF-κB signalling on the regulation of junctional proteins safeguarding the integrity of

the BBB. In agreement with our findings, the inhibitor suppresses the TNF-induced endothelial barrier impairment in primary BECs *in vitro* (Trickler et al., 2005). BAY 11-7085 diminishes the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in HUVECs (Pierce et al., 1997). Administration of the inhibitor restores BBB integrity *in vivo* (Coelho-Santos et al., 2015; Koedel et al., 2000).

Combined, our data suggest the predominant role of the NF-κB, as a downstream target of TNFR1 activation, on the modulation of brain endothelial integrity. The NF-κB signals as a downstream target of RhoA activation, thus impacting cytoskeletal dynamics. It is conceivable that the transcriptional activity of the pathway inflicts alterations on the expression levels and the phosphorylation status of key components of the AJs in presence of pro-inflammatory stimulation. Finally, NF-κB dictates the amplification of that stimulus through the permissive transcriptional regulation of pro-inflammatory mediators.

5.4.4.1 The Rho and NF-κB pathways interact and control endothelial integrity

A growing body of literature supports the role of nmMLCK on inflammatory processes. Inflammatory challenge with LPS triggers RhoA and NF-κB activation before the occurrence of F-actin rearrangement and the disruption of TJs in b.End3 cells. RhoA is proposed as a positive upstream regulator of NF-κB signalling in the

brain endothelium, since inhibition of the RhoGTPase activation impedes NF-κB activation (He et al., 2011).

MLC phosphorylation has been proposed as the signalling node linking the RhoGTPase activity and the NF-κB pathway, since the nmMLCK promoter bears NF-κB binding motifs (Ye & Ma, 2008), thus providing a scaffold for the physical interaction with NF-κB (Recoquillon et al., 2015).

However, the mechanism underlying the cross-regulation of nmMLCK and NF-κB remain elusive. Pro-inflammatory cytokines (TNF and IL-1β) advance the NF-κB-mediated nmMLCK-gene transcription (Mashukova et al., 2011; Ye et al., 2006). Moreover, studies on the impact of TNF on intestinal epithelial model systems demonstrate the implication of both the classical and the alternative NF-κB pathways on the increase of endothelial permeability.

Interestingly, silencing of NIK that is controlling the activation of the alternative pathway annulates the activation of both IKK catalytic subunits, abrogates the concomitant nmMLCK transcriptional activation, and supresses the TNF-induced TJ disassembly. Conversely, inhibition of MEKK-1, the regulator of the classical pathway, does not impact IKK and nmMLCK activation (Al-Sadi et al., 2016).

On the contrary, administration of the reversible nmMLCK inhibitor ML-7, attenuates the TNF-induced activation of the classical NF-κB pathway as evidenced by reduced IKKβ phosphorylation, decreased nuclear translocation of p65 and repression of the NF-κB transcriptional activity in peripheral endothelial cells (Wadgaonkar et al., 2005; X. Wu et al., 2009; Tauseef et al., 2012). Our results align

with these findings since inhibition of the classical NF-κB pathway decreases the permeabilising effect exerted by extended incubation with the Rho activator II. However, since none of the inhibitors employed targets the alternative NF-κB pathway selectively, the driving forces driving that delayed response remain to be elucidated.

Moreover, administration of NF-κB agonists followed by inhibition of nmMLCK does not tighten the endothelial monolayer. Moreover, inhibition of both ROCK-2 and nmMLCK with the selective inhibitor KD025 conferred a 76% decrease in TNF-mediated paracellular permeability, whereas administration of NF-κB inhibitors led to a drop by 25-67%. However, the latter comparison cannot be conclusive due to the variable affinities and off-target effects of the aforementioned compounds.

It is plausible that these conflicting results represent the compensatory regulatory mechanisms, differentially activated, depending on the extent of the pro-inflammatory stimulation. The prevailing model suggests that RhoA activation precedes the onset of NF-κB signalling. Activation of the acute, classical NF-κB cascade is under the regulation nmMLCK. The onset of the delayed response, implying the activation of the alternative NF-κB pathway, renders nmMLCK a downstream target of the pro-inflammatory transcriptional regulation exerted by NF-κB.

5.4.5 The PI3K/AKT and NF-κB pathways are interlinked and their interaction enhances brain endothelial permeability

5.4.5.1 The PI3K/PDK1/AKT/NF-κB axis

The transcriptional regulation of NF-κB signalling by the Ras/PI3K/AKT signalling network is another topic of intense investigation. Ras stimulates parallel pathways to facilitate both DNA binding and the transcriptional activity of NF-κB. Activation of PI3K by pro-inflammatory stimuli (TNF and IL-1β) promotes the transactivation of NF-κB (Sizemore et al., 1999; Wang & Baldwin, 1998) and increases the DNA binding capacity of the NF-κB complex (Koul et al., 2001). AKT, the main PI3K effector is predominantly associated with the NF-κB transactivation step and suppresses apoptosis (Madrid et al., 2000).

Despite these molecular underpinnings, the detailed mechanism of action of PI3K inhibitors on the transcriptional regulation of NF-κB remains elusive. Administration of the potent PI3K inhibitor LY294002, in hCMECs and in other cell types, abolishes the activation of NF-κB in an analogous manner to PTEN, a natural biological inhibitor of AKT (Mayo et al., 2002). Treatment with both the irreversible PI3K inhibitors LY294002 and wortmannin in TNF-stimulated HUVECs exhibits minimal or no effect on the NF-κB DNA binding (Gustin et al., 2004). The observed limited responsiveness led to the discovery of the prevalence of IKKβ over IKKα in vascular

endothelial cells, and the preferential association of the PI3K/AKT signalling with the less abundant IKKα only (Ozes et al., 1999; Madge & Pober, 2001).

Consistent with this, LY294002 abrogates TNF-induced NF-κB transcriptional activation in IKKβ -/- cells only, whereas the wild-type or IKKα -/- populations that are devoid of IKKα activity remain unaffected (Madge & Pober, 2000).

In this regard, the successful suppression of TNF-induced permeability increase using LY294002 implies the advanced presence of IKKα in hCMECs, at 24h post-stimulation. Given the implication of IKKα in both NF-κB pathways, either as a co-factor at the IKKβ/IKKα dimers formed as part of the classical pathway, or as a homodimer on the alternative NF-κB cascade, we cannot draw definitive conclusion on their temporal activation. However, the robust suppression of TNF-mediated permeability increase using the selective IKKβ inhibitor IMD0354, confirms the activity of IKKβ as part of the canonical pathway upon prolonged exposure (24h) to that pro-inflammatory stimulus.

Furthermore, we have provided evidence that the signalling of the PI3K cascade and the NF-κB pathway converse on the PDK1/AKT level, as the action of the classical NF-κB route is under the positive regulation of PI3K and PDK1. In line with our findings, GSK-3β has been proposed to be potentiating the pro-inflammatory, gene-specific transcriptional activation of NF-κB (Steinbrecher et al., 2005). This molecular machinery might be underlying the remarkable overexpression of pro-inflammatory agents in TNF-stimulated brain microvascular endothelial cells. Interestingly, these targets of the pro-inflammatory stimulation exhibit various degrees of sensitivity

towards GSK-3β inhibition (Ramirez et al., 2010), suggesting the implication of additional regulatory signalling circuits in their transcriptional modulation.

5.4.5.2 The bidirectional signalling between mTOR and NF-κB

Moreover, the downstream target of AKT activity, mTORC1 provides an additional scaffold for the cross-regulation of the permeabilising signalling driven by TNF. Pro-inflammatory stimulation (TNF, IL-1β, LPS) triggers IκB kinase β (IKKβ) that physically interacts with TSC1 and suppresses its inhibitory function, thus activating mTORC1 (Lee et al., 2007). TNF executes an acute IKKβ-driven activation of mTOR and S6K1 in hCMECs, as evidenced by the accumulation of pS6K1 at 3h post stimulation. The time-frame of the activation is wider for brain microvascular endothelial cells, compared to breast cancer cells and in mammary epithelial cells derived from normal tissues, where pS6K1 upregulation is evident at 10min. Interestingly, inhibitors of IKKβ activity suppress the TNF-induced activation of mTORC1, and the concomitant upregulation of pS6K1 within 3h, whereas rapamycin treatment becomes only effective at 6h. Our findings corroborate the mechanism reported in other non-endothelial systems, where the TNF-induced S6K1 activity is completely diminished by rapamycin or BAY 11-7082 treatment (Brown et al., 1995; Lee et al., 2007). Of note, the IKKβ/TSC1/mTOR signalling is not induced by other stimuli enhancing mTOR activity, such as mitogens.

This accelerated response of IKKβ inhibitors compared to the effect of rapamycin, might be indicative of the role of NF-κB as a signal enhancer for the AKT, pro-inflammatory signalling. TNF induces AKT-mediated, mTOR activation in different cell systems (Delhase et al., 2000; Osman Nidai Ozes et al., 2001; Ghosh et al., 2006), reasoning that synergistic TNF-induced activation of both IKKβ and AKT might be required in order to facilitate TSC1/2 inactivation and the resulting mTOR activation.

Alternatively, the rapid activation of NF-κB, upstream of mTORC1 could also serve as a molecular switch from mTORC1 to mTORC2 activation, thus prompting the execution of cytoskeletal remodelling, driven by the mTORC2/Rac and Cdc42 axis. mTORC2 has been proven to be positively regulate the Rho GTPase family (Jacinto et al. 2004; Sarbassov et al. 2005; Hernandez-Negrete et al. 2007). Moreover, TNF-treated HUVECs exhibit mTORC2 activity and repression of the Raf/MERK/ERK axis orchestrated by AKT. *In vivo*, rapamycin suppresses mTORC2 signalling and potentiates ERK1/2 hyperactivation that is inversely correlated with reduced VCAM-1 expression as a response to stimulation with TNF (Wang et al., 2014).

Notably, IKK is also directly regulating mTORC2 activity, hence adding more layers of complexity on the interplay of the mTOR and NF-κB signalling pathways (Xu et al., 2013). Both endogenously and ectopically expressed IKKα and IKKβ are physically interacting with the complex. Pharmacological targeting of IKK activity, alongside IKK knockdown represses the phosphorylation of mTORC2 downstream effectors AKT and PKCα, thus inducing cytoskeletal reorganisation.

To sum up, various effects of the pro-inflammatory signalling exerted by both the PI3K/PDK1/AKT/mTOR axis and the transcriptional factor NF-κB are reconciled in their interaction at the AKT signalling node. Their concerted signalling regulation promotes the permeabilising effect of TNF on the brain endothelium bidirectionally by driving the signal towards the activation of RhoGTPases through the mTORC2/Rac and Cdc42 axis, but also by prompting the negative transcriptional regulation of cell adhesion molecules and junctional proteins by NF-κB.

Moreover, since both AKT and NF-κB serve as repressors of the TNF-driven cell death, it is plausible that their commitment towards cell survival mirrors an alternative strategy to achieve sustained endothelial activation, rendering the endothelium even more prone to the vasomodulating activities of TNF.

5.4.5.3 AKT as a signalling crossroad regulating interrelated pathways

In agreement with the priming role of PDK1 in AKT activation, administration of GSK2334470 inhibits the AKT/mTOR axis, whilst further supressing the PI3K-driven signalling through interference with the PTEN expression levels. Interestingly, this mechanism of action involving growth inhibition and cell cycle arrest is rather cell-type specific, since HUVECs are considerably less prone to GSK2334470- induced cell death (Yang et al., 2017). These findings can also be attributed to the emerging roles of the mTORC2-mediated activation of AKT in the regulation of cytoskeletal remodelling in endothelial cells.

The beneficial effects of the blockade of AKT signalling are associated with the temporal regulation of the effector molecules suppressed. Although GSK3β is directly affiliated with AKT, the effects of GSK-3β inhibition are pronounced at 24h post- TNF treatment. This delay between pAkt upregulation at 6h and the enhancement of GSK-3β activity is in agreement with previous studies (Ramirez et al., 2010). The sustained effect of GSK-3β inhibition mirrors the requirement for transcriptional activity regulated by the NF-ϰB pathway in order for the anti-inflammatory effect of the antagonist to be enforced. Indeed, GSK-3β inhibitor VIII is a successful suppressor of the pro-inflammatory signalling orchestrated by TNF or IL-1β (Martins et al., 2011; Mazzardo-Martins et al., 2012). Blockade of GSK-3β downregulates the production of pro-inflammatory cytokines (TNF, IL-1β, IL-6, etc.), while increasing the secretion of the anti-inflammatory IL-10 (Jope et al., 2007). It also attenuates the emergence of perijunctional spikes and potentiates the resealing paracellular clefts (Czikora et al., 2011).

Moreover, the significantly weaker effect of eNOS inhibition on brain endothelial integrity, compared to targeting of ROCK or AKT could be suggestive of either a less pronounced role of NO abrogation in the pathogenesis of TNF-induced endothelial permeability increase, or of the implication of that mechanism as a later event, preceded by the acute activation of RhoA and the following induction of AKT signalling. The latter hypothesis implies eNOS dysregulation as part of the mechanisms in play once the inflammatory stimulus is enhanced.

Taken together, our data suggest that short-term TNF-induced AKT activation in brain microvascular endothelial cells is associated with signalling towards the RhoGTPase cascade, primarily driven by the activation of the mTORC2/Rac, Cdc42 axis. Prolonged TNF-induced AKT stimulation is part of a bidirectional positive feedback loop involving signal relay between the PI3K/PDK1/AKT/GSK3-β, mTORC1 axes and both streams of the NF-κB pathway. This cross-regulation supports the evasion from cell death and reinforces the pro-inflammatory signalling of TNF, through the transcriptional regulation of pro-inflammatory mediators and suppressors of endothelial integrity.

5.4.5.4 The protective role of mTORC1 and mTORC2 suppression

Prolonged exposure to rapamycin (IC_{50}=5pM) affects endothelial viability, motility and cytoskeletal organisation (Kay et al., 1991; Barilli et al., 2008; MRC Phosphorylation and Ubiquitylation Unit, 2018m). Rapamycin potentiates apoptosis in endothelial cells at concentrations higher than 1μM.

Prolonged exposure to rapamycin impairs endothelial motility and the cytoskeletal organisation. Treatment with high doses of this immunosuppressant is followed by an initial impairment of cell motility (0-12h), an ongoing alteration in cell shape (increased cell pleomorphism and shift from cobblestone to rounded morphology), alongside cytoskeletal rearrangement and formation of bundles of actin stress fibres. These responses are accompanied by diminished raptor and rictor expression, and a

progressive decrease in eNOS expression. This state is indicative of complete mTORC1 inhibition followed by S6K1 suppression, but also loss of mTORC2 signalling that triggers diminished pro-survival AKT-driven signalling (Madge & Pober, 2000; Dormond et al., 2007; Fernandez-Hernando et al., 2007), and aberrated cytoarchitecture.

Our findings support the notion that sustained rapamycin treatment progressively abrogates the TNF-induced activation of mTORC1, starting at 6h, as evidenced by the initial downregulation in pS6K1 levels, and peaking at 20h, with an almost complete repression of S6K1 activity. Additionally, the activity of mTORC2 is dramatically decreased in a similar fashion as mTORC1.

Initially, rapamycin interacts with mTORC1 only, and the gradual shift to the formation of mTORC2-rapamycin complexes takes place at a later point (Gulati et al., 2009). Since the alterations in both pS6K1 and pAkt levels are evident at 6h in hCMECs, we suspect a cell type-specific delay between the blockade of mTORC1 and the observation of a significant decline in pS6K1 expression. It is plausible that inhibition of mTORC1 should be occurring within 0-6h of exposure to rapamycin, followed by the disengagement of mTORC1-Raptor complex and the subsequent blockade of the mTORC2 complex lasting up to at least 20h.

Notably, the continuous decrease in pS6K1 levels up to 20h demonstrates the sustained effect of mTORC1 inhibition even at a point when rapamycin is preferentially interacting with mTORC2 complexes.

Extended treatment with rapamycin for at least 6h is required for the successful restoration of endothelial integrity, implying that mTORC1 is causally implicated in the observed TNF-induced permeability increase. These findings are also supported by the protective effect of the selective S6K1 inhibitor PF-4708671.

Moreover, chronic rapamycin treatment further enhances the stability of the brain endothelial monolayer. This is coinciding with partially inhibition of AKT signalling (Edinger et al., 2003).

Interestingly, these findings align with recent findings assessing alterations in the contractility of hCMEC/D3 monolayer upon preconditioning with and intermediate dose of TNF (5ng/ml) and consecutive administration of high doses of rapamycin (0.2μM-1μM). In this setting, the recovery in the monolayer function was evident at 10h post treatment with the immunosuppressant, and was progressively increasing up to 30h (van Vliet et al., 2016).

Lastly, the effect of PDK1 on pAkt levels confirmed its role as a master regulator of the AGC kinases. Moreover, the lack of pS6K1 decline following inhibition of both PDK1 and AKT confirmed the absence of mTORC1 regulation by the PDK1/AKT axis.

5.4.5.5 PI3K signalling regulates the activity of RhoGTPases

It is supported that PI3K signalling cascade and the family of RhoGTPases reside on a common signalling network. Our findings support the function of the RhoGTPases family downstream of the PI3K/PDK1/AKT/mTOR axis in the brain endothelium.

In line with our findings, PI3K inhibitors have been shown to interfere with the activity of Rac and Cd42. Blockade of the PI3K signalling prevents Rac GTP loading and membrane ruffling. This response can be reversed by microinjection of activated Rac reasoning that PI3K signalling locates upstream of the Rho family (Ridley & Hall, 1992; Nobes & Hall, 1995). Moreover, Cdc42 and Rac1, but not RhoA, stimulate S6K (Chou & Blenis, 1996; Fang et al., 2003). This activation can be blocked by the PI3K-specific inhibitors wortmannin and LY294002.

Furthermore, combination treatment with ROCK-2 and GSK-3β inhibitors recovers BBB function and plasticity in experimental murine spinal cord injury models, suggesting that these kinases are triggering either parallel or interrelated signalling cascades (Zhang et al., 2016). This interaction supports the notion that activation of the PI3K signalling cascade under pro-inflammatory conditions promotes the cytoskeletal reorganisation and the altered expression of junctional proteins dictated by RhoA, Rac and Cdc42 signalling.

5.4.5.6 The mechanism of TNF-induced brain endothelial permeabilisation

To sum up, the studies presented in this chapter employing paracellular permeability changes as an endpoint confirmed the implication of diverse patterns of the TNF-induced signalling.

To our understanding, the initial response (0-1h) to TNF is dominated by the acute activation of the TNFR1 complex that signals through members of the TNF receptor superfamily (TNFRSF) and the implication of the FADD/Ras axis. These stimuli lead to the activation of the Rho GTPase cascade through feedback loops involving the RhoA/ROCK complex, the Rac/ Cdc42 kinases, and their effectors PAK, nmMLCK LIMK, and eNOS that result to rapid cytoskeletal rearrangements followed by the deregulation of adherence junctions, the formation minute clefts at the junctional protrusions, and increased endothelial leakiness. In parallel, the activation of the TNFR1 complex initiates the acute (classical) NF-κB pathway. This signalling provides a pool of readily available transcription factors required for the resistance towards the TNF-induced pro-apoptotic signaling, but also supports the propagation of the pro-inflammatory stimulus. Surprisingly, the transcriptional machinery governed by the MAPK pathways is not involved in the observed TNFR1-mediated permeability increases.

The second phase of the endothelial response following stimulation with TNF (3-12h) might be dictated by the interplay of the PAM (PI3K/AKT/mTOR) and the NF-κB pathways that converge at the PDK1/AKT level. Our findings suggest that the mTORC2/AKT/GSK3β axis serves as a signalling crossroad that not only

orchestrates signalling towards both the Rac/ Cdc42 axis and the NF-κB pathway, but importantly suppresses completely the TNF-induced signalling that prompts necroptotic cell death, thus advancing the homeostasis of the brain endothelium. It is plausible that this response is governed by the de novo synthesis of factors bearing a protective role for the brain endothelium.

The later phase of the RhoGTPase activation (12-24h post TNF exposure) is characterised by the promotion of extensive alterations in cellular polarity, and junctional disassembly as evidenced by the loss of focal adhesions and stress fibres, complemented by the synthesis of pro-inflammatory mediators and vaso-permeants regulated by NF-κB. Extended exposure to TNF supports the enhanced involvement of the PAM/NFκB signalling crosstalk, as reflected by the accumulation of disruptive alterations that progressively hinder the integrity of the vascular endothelium of the brain.

Chapter 6| Discussion and Future Directions

Despite the recent advances facilitating earlier diagnosis of secondary brain tumours and application of targeted therapies, the prognosis of brain metastasis remains dismal. The presence of an intact BBB during the early (micrometastatic) stages of tumour growth impedes the access of diagnostic imaging agents and systemically successful therapeutic formulations at brain metastasis sites. The barrier is only disrupted upon development of overt lesions, allowing for their detection with the clinical gold-standard imaging technique; contrast-enhanced MRI. However, at this stage, the increased tumour burden and aggressiveness of metastatic foci renders the clinical management challenging and often palliative in nature. Therefore, the earlier diagnosis and the successful delivery of advanced therapeutic approaches represent an unmet clinical need.

Strategies aiming to transiently modulate the BBB at brain tumour sites have been increasingly popular within the last four decades (further discussed at section 1.5.1) . Intravenous administration of hyperosmotic agents has been a promising approach that was discontinued from clinical trials due to extensive off-target effects and neurotoxicity (Rapoport et al., 1972; Haluska & Anthony, 2004). The development of chimeric peptides with enhanced lipophilicity (Drappatz et al., 2013; Papademetriou & Porter, 2015), and of efflux-pump inhibitors aiming to foster bioavailability at the

CNS was hindered by their lack of selectivity (Miller et al., 2008). On the contrary, targeted approaches such as the MRI-guided, ultrasound-mediated focal BBB disruption (Hynynen et al., 2001; Treat et al., 2007) or the use of novel tumour treating fields (Hottinger et al., 2016) are dependent on the prior detection of the metastatic foci. Moreover, drug delivery through routes bypassing the BBB (intrathecal; intraventricular; intranasal, and interstitial administration) (da Fonseca et al., 2008; Bellavance et al., 2008) or through the implantation of biodegradable wafer polymers serving as carriers for chemotherapeutic agents (Ewend et al., 2007; Dolghih & Jacobson, 2013) is often limited by the complexity and the invasiveness of the surgical procedures required, and the inadequate diffusion of the agents at the CNS parenchyma.

An alternative approach was the therapeutic chemical-mediated BBB circumvention employing bradykinin analogues enabled the improved drug transport into rat and human gliomas (Matsukado et al., 1996; Borlongan & Emerich, 2003), in a TNF-dependent manner (Qin e al., 2009). A similar strategy has been applied for the treatment of systemic tumours in xenogeneic lymphoma models (Seki et al., 2011). Intravenous administration of recombinant TNF enhanced significantly the permeability of the relatively leaky, tumour-surrounding neo-vasculature. Importantly, this strategy facilitated the enhanced and selective delivery of oncolytic virus particles at tumour sites.

The vaso-modulating activity of TNF has been well-characterised *in vitro* systems modelling the brain endothelium (Deli et al., 1995; de Vries et al., 1996). Microinjection

of this pro-inflammatory cytokine into the rat brain elicited focal, but delayed BBB rupture and focal inflammatory response (Sibson et al., 2002).

Moreover, pronounced inflammatory response has been observed during the early stages of metastatic colonisation in the brain parenchyma. This encompasses astrocytic (O'Brien et al., 2014; Chen et al., 2016), endothelial (Serres et al., 2012; Soto et al., 2014), and microglial activation (O'Brien et al., 2014; Andreou et al., 2017). In this context, intravenous administration of TNF or its endogenous analogue Lt led to the transient permeabilisation of the BBB, selectively at tumour sites in experimental mouse models of brain metastasis from breast (Connell et al., 2013). This approach facilitated the accelerated detection of micrometastases employing Gd-enhanced MRI, and the targeted delivery of the systemically successful trastuzumab at metastatic colonies.

6.1 Selective TNFR1 upregulation within the tumour-associated vasculature

Endothelial activation at sites of brain metastasis is a result of transcriptional and post-transcriptional modifications occurring as part of the neuroinflammatory stimulation during metastatic seeding to the brain and the downstream proliferation to the brain parenchyma.

TNFR1-associated endothelial activation

TNFR1 is selectively upregulated within the tumour-associated vasculature at sites of brain metastasis. TNFR1 is only minimally, constitutively expressed in the endothelium of unaffected brains. Since the experimental mouse models employed in this thesis emphasised on the micrometastatic stages of brain tumour development, the colonies assessed spanned from 10-200μm in diameter. Evidence from experimental models of brain metastasis from breast cancer, lung adenocarcinoma and melanoma revealed the spatial distribution of endothelial TNFR1 within a diameter of 100μm from the tumour core. This uniform expression pattern implies the presence of shared molecular drivers within the tumour microenvironment that lead to endothelial activation and TNFR1 uptake, albeit the well-studied differences of secondary brain malignancies arising from these different primaries.

The above findings are indicative of dynamic interactions promoting TNFR1 expression within the perivascular niche. This could be arising from direct secretion of TNF by reactive astrocytes (Akassoglou et al., 1998; Chen et al., 2016) and/or microglia (Kuno et al., 2005) as part of their response during metastatic colonisation. It has been hypothesised that the TNFR1-associated endothelial activation could also be triggered by the attachment of disseminating cells at the cerebrovascular endothelium during the seeding stage. However, *in vitro* studies conducted revealed that the observed endothelial TNFR1 upregulation is dictated by soluble factors secreted by tumour cells metastasising preferentially to the brain rather than from the

direct contact of tumour cells with the brain endothelium. Furthermore, this pattern was conserved amongst the secretomes of brain-seeking neoplastic cells from breast, lung and melanoma, suggesting the presence of common denominators (single agents or superfamilies).

On a separate note, the selective TNFR1 upregulation allows for targeting not only the tumour core, but also the invasive tumour edge that usually presents inhomogeneous BBB/BTB leakiness and concomitant insufficient bioavailability of systemically successful targeted treatments (Lockman et al., 2010; Blecharz et al., 2015). The increased TNFR1 expression within the cerebrovascular endothelium was evident during the early stages of metastatic co-optive growth, as it was detected in the pre-existing vasculature that is associated with colonies comprising less than 10 tumour cells (50μm diameter). In this setting, intravenous administration of TNF facilitated the detection of metastatic foci of similar size, employing Gd-enhanced MRI (Connell et al., 2013), suggesting that selective targeting of the TNFR1-associated endothelial activation could enable the detection of asymptomatic micrometastases that are considerably below the current detection threshold. To date, only macrometastases of 0.5-1 cm in diameter are detectable with common imaging techniques (Percy et al., 2011; Gaudino et al., 2012). Importantly, administration of therapeutic regimens to micrometastases was more effective than therapy targeting established macrometastases in an experimental model of melanoma brain metastasis (Osswald et al., 2016), supporting the notion that micrometastases could be managed more effectively in the clinic.

On the other hand, the expression of TNFR2 is restricted in non-vascular structures and exhibits a diffuse pattern that is suggestive of microglial activation and/ or accumulation of recruited macrophages, intravascular neutrophils. These findings are corroborated by previous reports of TNFR2-positive tumour-infiltrating immune cells (Chen et al., 2013).

Notably, similar patterns of TNFR1 and TNFR2 expression have been detected in human biopsy tissue of brain metastasis from breast cancer, lung adenocarcinoma and melanoma, underlining the potential for clinical translation of the proposed therapeutic BBB permeabilisation strategy.

6.2 TNFR1-mediated permeabilisation of the blood-brain barrier at sites of metastasis

An *in vitro* model of the cerebrovascular endothelium was established in order to delineate the molecular determinants of the TNF-induced BBB permeabilisation observed *in vivo*. Prolonged pro-inflammatory stimulation employing TNF triggered the preferential upregulation of TNFR1, but not TNFR2, whilst diminishing the integrity of the brain endothelial monolayer *in vitro*. The reported permeability increase was not associated with TNF-induced endothelial cell death (chapter 5) but was in accord with abrogated expression of junctional proteins. TNF stimulation led to extensive cytoskeletal rearrangement and disassembly of the interendothelial junctions as reflected by downregulation and/or altered cellular localisation of the

adhesion molecules VE-cadherin, and talin; the actin-binding ZO-1; and junctional proteins occludin, and claudin-5.

Depletion of TNFR1 expression in brain microvascular endothelial cells abolished the responsiveness of the endothelial monolayer to TNF-mediated alterations in endothelial integrity. On the contrary, silencing of TNFR2 did not repress the TNF-induced endothelial permeabilisation, suggesting that TNFR1 activation is involved in the vaso-modulating effects of TNF.

Furthermore, administration of low doses TNF muteins with enhanced affinity for TNFR1 only, induced significant permeability increases in our *in vitro* system, thus providing a valuable platform for an induction of TNF-induced permeability devoid of TNFR2-associated systemic toxicities.

Extension of these studies to an additional mouse model of brain endothelial monolayer confirmed the causal implication of TNFR1 activation on TNF-associated endothelial dysfunction. Treatment with huTNF, a specific agonist for mTNF alone (Bossen et al., 2006), yielded dose-dependent endothelial permeability increases, in an analogous manner to the endogenous ligand mTNF that binds to both receptors. Administration of neutralising antibody against TNFR1 depleted that effect. These findings were corroborated by systemic administration of both huTNF and mTNF in a syngeneic mouse model of breast cancer brain metastasis. Both cytokines induced remarkable BBB permeabilisation as evidenced by histological detection of HRP extravasation in *ex vivo* brain tissue slices. Taken together, these findings suggest that TNFR1 is the primary determinant of TNF-induced BBB rupture.

The TNFR1-mediated BBB permeabilisation holds promise for the avoidance of TNFR2-related systemic toxicities and the improvement of the vaso-modulating index of TNF through the development of TNFR1-targeted TNF muteins with enhanced selectivity and efficacy. Importantly, the use of such constructs could afford potential dose escalations, since similar compounds exhibited minimal systemic toxicity even in concentrations exceeding the MTD of TNF (Kumaratilake et al., 1995).

6.3 Mechanism of TNF-induced endothelial permeabilisation

6.3.1 Spatial regulation of the TNF-induced endothelial permeability increase

The spatial compartmentalisation of TNFR1 is required for the generation of distinct signalling outcomes in response to RhoA. Indeed, early RhoA activation occurs proximal to TNFR1, suggesting that recruitment of adaptor molecules to the receptor is a requisite for the activation of the signalling network. These findings are in agreement with the observed TNF-induced activation of RhoA in airway smooth muscle cells, where both TNFR1 and RhoA were present in lipid rafts (Hunter & Nixon, 206). Furthermore, the late phase of RhoA activation (3h) is characterised by

its accumulation in focal areas along the endothelial cells edges, areas devoid of RhoGDIs (Mong, Petrulio, Kaufman, & Wang, 2008).

mTOR localisation may be nucleo-cytoplasmic (Bachmann et al., 2006). Upon exposure to growth factors, mTOR rapidly (30min) translocates to the nucleus. This subnuclear localisation has been associated with AKT phosphorylation (Salomoni & Pandolfi, 2002). In addition, many components of the mTOR pathway are expressed in the nucleus because of the critical role of mTORC1 in ribosome biogenesis and translation initiation. PI3K is expressed in the nucleus. Moreover, PDK1 and AKT shuttle between the cytoplasm and the nucleus and S6K1 is localised predominantly at the nucleus. It is plausible that the Rac- and Cdc42-mediated activation of S6K1 (Chou & Blenis, 1996; Fang et al., 2003) brings the latter to the proximity of signal transducers such as PDK1, thus facilitating the signal relay.

6.3.2 Temporal regulation of the TNF-induced endothelial permeability increase

Apart from the spatial organisation of the TNF-induced response, the temporal pattern of activation is a key determinant of the pathways involved. Interestingly, real-time analysis of fluctuation in endothelial integrity, employing TEER, underscored the involvement of two distinct phases, characterised by diverse Rho signalling. The TNF-induced disassembly of the endothelial junctional complexes was demonstrated to be executed in discrete steps, and not as a uniform process, as reported by continuous

TEER measurements in HDMECs (Clark et al., 2015). The stimulation exerted an initial 10% decrease in TEER levels within 2h of stimulation, followed by an interphase plateau and a subsequent major decline (<70%), indicative of vascular collapse by 8-10h. These two distinct leak phases are also characterised by a remarkable difference in EC_{50} values (~30pg/ml and ~150pg/ml, respectively). Although both phases reported were NF-κB-dependent, targeted inhibition of the RhoGTPase pathway was more effective during the early phase of TNF stimulation.

These diverse patterns of the TNF-induced signalling might be attributed to the requirement for transcription factors executing the initial response. To our understanding, the first phase is dominated by the activation of the TNFR1 complex that signals through the FADD/Ras axis to activate the Rho GTPase cascade that rapidly triggers cytoskeletal rearrangements. In parallel, activation of the TNFR1 complex stimulates TAK1 that initiates the acute (classical) NF-κB pathway, which serves possibly as a pool of readily available transcription factors required for the resistance towards the TNF-induced pro-apoptotic signaling, but also for the propagation of the pro-inflammatory stimulus.

The second phase of the endothelial response might be dictated by the interplay of the PI3K and NF-κB pathways. It has been suggested that prolonged TNF exposure might be governed by the *de novo* synthesis of factors bearing a protective role for the endothelium (Bach et al., 1997). Our findings support the crucial role of the mTORC2/AKT axis, as a signalling cross-road that not only orchestrates signalling towards both the Rac/ Cdc42 axis and the NF-κB pathway, but also suppresses

completely TNF-induced signalling prompting cell death. The later phase of the RhoGTPase activation is characterised by the promotion of junctional disassembly, complemented by the synthesis of pro-inflammatory mediators and vaso-permeants regulated by NF-κB. Extended exposure to TNF, supports the enhancement of the aforementioned signalling interplays, as reflected by the accumulation of disruptive alterations that progressively hinder the stability of the vascular endothelium of the brain.

6.3.3 Clinical translation paradigm

Our results have demonstrated the vaso-modulating effect of the TNF-induced activation of the PAM (PI3K/AKT/mTOR) pathway in brain endothelial cells. Importantly, this cascade is an increasingly recognised therapeutic target for adaptive, brain-specific metastatic outgrowth. Assessment of human brain metastasis biopsies demonstrated clinically actionable alterations enriched in the PAM pathway (Brastianos et al., 2015). Recent findings have underscored the importance of PI3K expression in the disseminating tumour cells during their brain-tropic metastatic seeding. Silencing of the PI3K suppressor PTEN is transiently induced by astrocyte-derived exosomes (Zhang et al., 2015). In line with these findings, enhanced activation of the PI3K/AKT axis has been reported in melanoma, breast and lung cancer cells that metastasise to the brain (Li et al., 2013; Chen et al., 2014; Cho et al., 2015; Molnar et al., 2016).

Moreover, extracellular vesicles derived from brain-seeking breast cancer cells contain miRNA-181c that promotes endothelial cytoskeletal remodelling and consequently impairs the integrity of endothelial monolayers *in vitro*, by targeting the PI3K pathway (Tominaga et al., 2015). However, it is well-established that neither the tumour cell extravasation in the brain parenchyma, nor the establishment of micrometastases lead to BBB disruption. Therefore, the interpretation of these *in vitro* findings might reflect an intact, activated cerebrovascular endothelium with increased amenability to vaso-modulating stimuli.

A recent study comparing the efficacy of both a BBB-optimised and a non-permeable PI3K/mTOR inhibitor administered systemically in an experimental mouse model of melanoma brain metastasis suggested that only the first, BBB-penetrating agent, could achieve shrinkage of micrometastases. As expected, the compound that could not penetrate the BBB did only affect metastases growing behind an intact BBB and did only have some efficacy on macrometastases associated with a disrupted BBB (Osswald et al., 2016).

The aforementioned paradigms illustrate the parallel activation of the PAM signalling pathways both in metastatic cells and in the cerebrovascular endothelium. Our *in vitro* findings have underscored the pivotal role of PI3K and its downstream targets in the regulation of paracellular permeability in the activated brain endothelium. Importantly, a multitude of pharmacological inhibitors of certain signalling pathways that have been predicted to bypass the BBB, do not achieve clinically relevant concentrations in the brain when trialled. It is plausible that systemically administered,

targeted inhibitors cannot access their ultimate targets, the parenchymal brain tumours, since they exhibit off-target effects at the level of the cerebrovascular endothelium. This could result in suppression of vaso-modulating signalling pathways and concomitant repression of the inherent permeability of the BBB. This hypothesis suggests that the molecular machinery governing BBB integrity should be considered when designing systemically administered therapeutic agents targeting parenchymal tumours.

6.4 Limitations

6.4.1 In vitro endothelial monolayer model

The work presented in chapter 6 is based on in vitro assays employing the human brain microvascular endothelial cell line hCMEC/D3. Although, this is one of the best *in vitro* models available for the study of the cerebrovascular endothelium, this model suffers from low TEER values, implying the tendency for increased inherent permeability.

The BBB exhibits an inherent permeability permitting the passive diffusion of 3 kDa dextran beads at sites of advanced brain metastasis (Lockman et al., 2010). Assessment of our *in vitro* model demonstrated an inherent permeability of the brain endothelial monolayer established for 4 kDa dextrans under basal conditions.

Treatment with 30 kDa yielded significant decrease in baseline paracellular leakage, suggesting that our model is reliable for the assessment of the passage of molecules above 30kDa.

Moreover, the model used did not employ co-culture with other cell types present in the NVU [Chou et al 2014] and did not assess the effect of shear stress on the induction of the barrier properties in the endothelium. Further work, employing more advanced systems benefiting from continuous flow would be beneficial for the development of more physiologically relevant models. In agreement with this notion, the use of human-derived pluripotent stem cells to produce endothelial cells, astrocytes and pericytes to develop in vitro NVU/BBB models holds great promise for the future (Lippmann et al., 2015).

6.4.2 Criticism regarding the transient BBB opening in the brain metastasis setting

Pro-inflammatory cytokines, including TNF, have been reported to promote endothelial activation during brain metastasis. TNF-induced endothelial upregulation of CAMs has been associated with enhanced adhesion of metastasising cells to the brain endothelium during TEM (Miles et al., 2008; Jassam et al., 2017). It is postulated that such heterophilic interactions potentiate the transient formation of paracellular clefts, facilitating the traversal of circulating tumour cells through the BBB. However, to our knowledge there is no evidence from *in vivo* models reporting increased

metastatic burden upon administration of pro-inflammatory cytokines. Moreover, the transient opening of the BBB has been demonstrated to occur selectively at metastasis sites within 2h post TNF stimulation, with the breakdown peaking at 6h (Connell et al., 2013). Notably, the approach proposed involves a short-term TNF stimulation, aiming to facilitate an enhanced and targeted delivery of therapeutic agents at metastatic colonies, as previously evidenced for the selective accumulation of radiolabelled trastuzumab selective at tumour sites (Connell et al., 2013). To conclude, further studies are required in order to assess whether TNF-enhanced delivery of chemotherapeutic agents can lead to reduced tumour burden at brain metastasis sites.

6.5 Future work

6.5.1 Ongoing studies

Proteomic screening of the factors secreted by both non-brain seeking and brain-seeking metastatic cancer cells from breast, lung and melanoma is ongoing (discussed in Chapter 3).

Assessment of TNFR1-specific muteins in an experimental model of brain metastasis from breast (discussed in Chapter 4).

Live cell imaging for the determination of the spatiotemporal patterns of cytoskeletal rearrangements, and of the alterations in cellular shape and motility associated with

TNF-induced endothelial permeability increase is ongoing. This approach is also used for the assessment of the transience of the alterations occurring in the interendothelial junctional complexes as a response to TNF-exposure (discussed in Chapters 4, 5).

6.5.2 Experimental models

The assessment of the role of systemic TNF administration on the regulation BBB integrity in experimental models of brain metastasis from melanoma and lung holds great promise, given the demonstrated endothelial TNFR1 upregulation in the tumour-associated vasculature in such models. Moreover, the assessment of the role of TNFR1 on the endothelial activation ongoing during establishment of primary brain tumours could be beneficial.

Preliminary findings from histological analysis of spontaneous leptomeningeal metastases detected in BALB/c mice intracardially inoculated with 4T1-GFP breast cancer cells, revealed pronounced TNFR1 expression within the surrounding vascular endothelium, with TNFR2 being minimally expressed in vascular structures. Although this thesis focused on the interplay between the presentation of parenchymal micrometastases and brain endothelial activation, the enhanced endothelial expression of TNFR1 suggests a potential overlap between the mechanisms underlying endothelial stimulation within the tumour-surrounding stroma. This hypothesis could be tested employing recently established experimental models of leptomeningeal metastasis induction (Valiente et al., 2018).

6.5.3 Clinical translation

Despite the well-established anti-cancer activity of TNF (Hagen & Eggermont, 2004), all relevant phase I and phase II clinical trials employing huTNF monotherapy have been discontinued. Dose-limiting off-target effects have hindered the widespread use of TNF as a systemically administered anti-cancer agent (Roberts et al., 2011; Steeland et al., 2018), with the minimum effective dose being considerably greater than the MTD. To date, TNF is employed in the clinic as a vasomodulating factor, enhancing the uptake of chemotherapeutic drugs during isolated limb perfusion for the treatment of melanoma or sarcoma, and in the treatment of advanced hepatic cancers (Lejeune et al., 2006; Eggermont et al., 1996; Alexander et al., 1998).

Selective administration of TNF muteins exhibiting enhanced efficacy for the signalling cascades implicated on the signalling downstream of TNFR1 activation, and limited systemic toxicity induction holds greats promise for the use of such compounds in order to facilitate both the earlier detection of micrometastases and the enhanced delivery of systemically successful therapeutic agents.

- Abbott, N. J. (2002). Astrocyte-endothelial interactions and blood-brain barrier permeability. *Journal of Anatomy*, *200*(6), 629–638. http://doi.org/10.1046/j.1469-7580.2002.00064.x
- Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood–brain barrier. *Neurobiology of Disease*, *37*(1), 13–25. http://doi.org/10.1016/j.nbd.2009.07.030
- Abbott, N. J., Rönnbäck, L., & Hansson, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. Nature Reviews Neuroscience, 7(1), 41–53. http://doi.org/10.1038/nrn1824
- Achrol, A. S., Rennert, R. C., Anders, C., Soffietti, R., Ahluwalia, M. S., Nayak, L., ... Chang, S. D. (2019). Brain metastases. Nature Reviews. Disease Primers, 5(1), 5. http://doi.org/10.1038/s41572-018-0055-y
- Ackah, E., Yu, J., Zoellner, S., Iwakiri, Y., Skurk, C., Shibata, R., ... Sessa, W. C. (2005). Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *The Journal of Clinical Investigation*, *115*(8), 2119–2127. http://doi.org/10.1172/JCI24726
- Adyshev, D. M., Dudek, S. M., Moldobaeva, N., Kim, K., Ma, S.-F., Kasa, A., ... Verin, A. D. (2013). Ezrin/radixin/moesin proteins differentially regulate endothelial hyperpermeability after thrombin. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *305*(3), L240-55. http://doi.org/10.1152/ajplung.00355.2012
- Afonso, P. V., Ozden, S., Prevost, M.-C., Schmitt, C., Seilhean, D., Weksler, B., ... Ceccaldi, P.-E. (2007). Human blood-brain barrier disruption by retroviral-infected lymphocytes: role of myosin light chain kinase in endothelial tight-junction disorganization. *Journal of Immunology (Baltimore, Md. : 1950)*, *179*(4), 2576–2583.
- Aggarwal, B. B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nature Reviews. Immunology*, *3*(9), 745–56. http://doi.org/10.1038/nri1184
- Aggarwal, B. B., Schwarz, L., Hogan, M. E., & Rando, R. F. (1996). Triple helix-forming oligodeoxyribonucleotides targeted to the human tumor necrosis factor (TNF) gene inhibit TNF production and block the TNF-dependent growth of human glioblastoma tumor cells. *Cancer Research*, *56*(22), 5156–5164.
- Aguirre-Ghiso, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews. Cancer*, *7*(11), 834–846. http://doi.org/10.1038/nrc2256
- Ahluwalia, M. S., & Winkler, F. (2015). Targeted and immunotherapeutic approaches in brain metastases. *American Society of Clinical Oncology Educational Book. American Society of Clinical Oncology. Annual Meeting*, 67–74. http://doi.org/10.14694/EdBook_AM.2015.35.67
- Aird, W. C. (2003). Endothelial cell heterogeneity. *Critical Care Medicine*, *31*(4 Suppl), S221-30. http://doi.org/10.1097/01.CCM.0000057847.32590.C1
- Akassoglou, K., Bauer, J., Kassiotis, G., Pasparakis, M., Lassmann, H., Kollias, G., & Probert, L. (1998). Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. *The American Journal of Pathology*, *153*(3), 801–813. http://doi.org/10.1016/S0002-9440(10)65622-2
- Akassoglou, K., Probert, L., Kontogeorgos, G., & Kollias, G. (1997). Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *Journal of Immunology (Baltimore, Md. : 1950)*, *158*(1), 438–445.
- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., ... Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *The Journal of Biological Chemistry*, *262*(12), 5592–5595.
- Al-Sadi, R., Guo, S., Ye, D., Rawat, M., & Ma, T. Y. (2016). TNF-alpha Modulation of Intestinal Tight Junction Permeability Is Mediated by NIK/IKK-alpha Axis Activation of the Canonical NF-kappaB Pathway. *The American Journal of Pathology*, *186*(5), 1151–1165. http://doi.org/10.1016/j.ajpath.2015.12.016
- Al-Sadi, R., Ye, D., Said, H. M., & Ma, T. Y. (2011). Cellular and molecular mechanism of interleukin-1beta modulation of Caco-2 intestinal epithelial tight junction barrier. *Journal of Cellular and Molecular Medicine*, *15*(4), 970–982. http://doi.org/10.1111/j.1582-4934.2010.01065.x
- Albensi, B. C., & Mattson, M. P. (2000). Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse (New York, N.Y.)*, *35*(2), 151–159. http://doi.org/10.1002/(SICI)1098-2396(200002)35:2<151::AID-SYN8>3.0.CO;2-P
- Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., & Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO Journal*, *15*(23),

6541–6551.
- Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., & Saltiel, A. R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *The Journal of Biological Chemistry*, *270*(46), 27489–27494.
- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current Biology : CB*, *7*(4), 261–269.
- Alexander, H. R., Brown, C. K., Bartlett, D. L., Libutti, S. K., Figg, W. D., Raje, S., & Turner, E. (1998). Augmented capillary leak during isolated hepatic perfusion (IHP) occurs via tumor necrosis factor-independent mechanisms. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *4*(10), 2357–2362.
- Alexander, J. S. (2000, August). Rho, tyrosine kinase, Ca(2+), and junctions in endothelial hyperpermeability. *Circulation Research*. United States.
- Alhusban, A., Alkhazaleh, E., & El-Elimat, T. (2017). Silymarin Ameliorates Diabetes-Induced Proangiogenic Response in Brain Endothelial Cells through a GSK-3beta Inhibition-Induced Reduction of VEGF Release. *Journal of Diabetes Research*, *2017*, 2537216. http://doi.org/10.1155/2017/2537216
- Allen, J. E., Krigsfeld, G., Mayes, P. A., Patel, L., Dicker, D. T., Patel, A. S., ... El-Deiry, W. S. (2013). Dual inactivation of Akt and ERK by TIC10 signals Foxo3a nuclear translocation, TRAIL gene induction, and potent antitumor effects. *Science Translational Medicine*, *5*(171), 171ra17. http://doi.org/10.1126/scitranslmed.3004828
- Amado-Azevedo, J., Reinhard, N. R., van Bezu, J., de Menezes, R. X., van Beusechem, V. W., van Nieuw Amerongen, G. P., ... Hordijk, P. L. (2017). A CDC42-centered signaling unit is a dominant positive regulator of endothelial integrity. *Scientific Reports*, *7*(1), 10132. http://doi.org/10.1038/s41598-017-10392-0
- Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., ... Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *The Journal of Biological Chemistry*, *271*(34), 20246–20249.
- Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H., & Peterson, J. R. (2011a). Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nature Biotechnology*, *29*(11), 1039–1045. http://doi.org/10.1038/nbt.2017
- Anastassiadis, T., Deacon, S. W., Devarajan, K., Ma, H., & Peterson, J. R. (2011b). Kinase Inhibitor Resource Databaset. *Nature Biotechnology*, *29*(11), 1039–1045. Retrieved from http://reactionbiology.com/webapps/largedata/Default.aspx
- Anderson, H. D. I., Rahmutula, D., & Gardner, D. G. (2004). Tumor necrosis factor-alpha inhibits endothelial nitric-oxide synthase gene promoter activity in bovine aortic endothelial cells. *The Journal of Biological Chemistry*, *279*(2), 963–969. http://doi.org/10.1074/jbc.M309552200
- Andreou, K. E., Soto, M. S., Allen, D., Economopoulos, V., de Bernardi, A., Larkin, J. R., & Sibson, N. R. (2017). Anti-inflammatory Microglia/Macrophages As a Potential Therapeutic Target in Brain Metastasis. *Frontiers in Oncology*, *7*, 251. http://doi.org/10.3389/fonc.2017.00251
- Angelini, D. J., Hyun, S.-W., Grigoryev, D. N., Garg, P., Gong, P., Singh, I. S., ... Goldblum, S. E. (2006). TNF-alpha increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *291*(6), L1232-45. http://doi.org/10.1152/ajplung.00109.2006
- Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., & Hay, R. T. (1995). Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. *Molecular and Cellular Biology*, *15*(5), 2689–2696.
- Armulik, A., Genové, G., Mäe, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., ... Betsholtz, C. (2010). Pericytes regulate the blood–brain barrier. *Nature*, *468*(7323), 557–561. http://doi.org/10.1038/nature09522
- Arnold, K. M., Goeckeler, Z. M., & Wysolmerski, R. B. (2013). Loss of focal adhesion kinase enhances endothelial barrier function and increases focal adhesions. *Microcirculation (New York, N.Y. : 1994)*, *20*(7), 637–649. http://doi.org/10.1111/micc.12063
- Arnold, T., & Betsholtz, C. (2013). The importance of microglia in the development of the vasculature in the central nervous system. *Vascular Cell*, *5*(1), 4. http://doi.org/10.1186/2045-824X-5-4
- Arshad, F., Wang, L., Sy, C., Avraham, S., & Avraham, H. K. (2010). Blood-brain barrier integrity and breast cancer metastasis to the brain. *Pathology Research International*, *2011*, 920509. http://doi.org/10.4061/2011/920509
- Asano, T., Suzuki, T., Tsuchiya, M., Satoh, S., Ikegaki, I., Shibuya, M., ... Hidaka, H. (1989).

Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. *British Journal of Pharmacology*, *98*(4), 1091–1100.

- Asgari, M., de Zelicourt, D., & Kurtcuoglu, V. (2016). Glymphatic solute transport does not require bulk flow. *Scientific Reports*, *6*, 38635. http://doi.org/10.1038/srep38635
- Ashikawa, K., Majumdar, S., Banerjee, S., Bharti, A. C., Shishodia, S., & Aggarwal, B. B. (2002). Piceatannol inhibits TNF-induced NF-kappaB activation and NF-kappaB-mediated gene expression through suppression of IkappaBalpha kinase and p65 phosphorylation. *Journal of Immunology (Baltimore, Md. : 1950)*, *169*(11), 6490–6497.
- Aslam, M., Ahmad, N., Srivastava, R., & Hemmer, B. (2012). TNF-alpha induced NFkappaB signaling and p65 (RelA) overexpression repress Cldn5 promoter in mouse brain endothelial cells. *Cytokine*, *57*(2), 269–275. http://doi.org/10.1016/j.cyto.2011.10.016
- Auge, F., Hornebeck, W., Decarme, M., & Laronze, J.-Y. (2003). Improved gelatinase a selectivity by novel zinc binding groups containing galardin derivatives. *Bioorganic & Medicinal Chemistry Letters*, *13*(10), 1783–1786.
- Aveleira, C. A., Lin, C.-M., Abcouwer, S. F., Ambrosio, A. F., & Antonetti, D. A. (2010). TNF-alpha signals through PKCzeta/NF-kappaB to alter the tight junction complex and increase retinal endothelial cell permeability. *Diabetes*, *59*(11), 2872–2882. http://doi.org/10.2337/db09-1606
- Avraham, H. K., Jiang, S., Fu, Y., Nakshatri, H., Ovadia, H., & Avraham, S. (2014). Angiopoietin-2 mediates blood-brain barrier impairment and colonization of triple-negative breast cancer cells in brain. *The Journal of Pathology*, *232*(3), 369–381. http://doi.org/10.1002/path.4304
- Bach, F. H., Hancock, W. W., & Ferran, C. (1997). Protective genes expressed in endothelial cells: a regulatory response to injury. *Immunology Today*, *18*(10), 483–486.
- Bachmann, C., Schmidt, S., Staebler, A., Fehm, T., Fend, F., Schittenhelm, J., ... Grischke, E. (2015). CNS metastases in breast cancer patients: prognostic implications of tumor subtype. *Medical Oncology (Northwood, London, England)*, *32*(1), 400. http://doi.org/10.1007/s12032-014-0400-2
- Bachmann, R. A., Kim, J.-H., Wu, A.-L., Park, I.-H., & Chen, J. (2006). A nuclear transport signal in mammalian target of rapamycin is critical for its cytoplasmic signaling to S6 kinase 1. *The Journal of Biological Chemistry*, *281*(11), 7357–7363. http://doi.org/10.1074/jbc.M512218200
- Bagrodia, S., Derijard, B., Davis, R. J., & Cerione, R. A. (1995). Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *The Journal of Biological Chemistry*, *270*(47), 27995–27998.
- Bain, J., McLauchlan, H., Elliott, M., & Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. *The Biochemical Journal*, *371*(Pt 1), 199–204. http://doi.org/10.1042/BJ20021535
- Balathasan, L., Beech, J. S., & Muschel, R. J. (2013). Ultrasonography-Guided Intracardiac Injection. *The American Journal of Pathology*, *183*(1), 26–34. http://doi.org/10.1016/j.ajpath.2013.03.003
- Baltimore, D. (2011). NF-kappaB is 25. *Nature Immunology*, *12*(8), 683–685. http://doi.org/10.1038/ni.2072
- Banan, A., Keshavarzian, A., Zhang, L., Shaikh, M., Forsyth, C. B., Tang, Y., & Fields, J. Z. (2007). NF-kappaB activation as a key mechanism in ethanol-induced disruption of the F-actin cytoskeleton and monolayer barrier integrity in intestinal epithelium. *Alcohol (Fayetteville, N.Y.)*, *41*(6), 447–460. http://doi.org/10.1016/j.alcohol.2007.07.003
- Bandman, O., Coleman, R. T., Loring, J. F., Seilhamer, J. J., & Cocks, B. G. (2002). Complexity of inflammatory responses in endothelial cells and vascular smooth muscle cells determined by microarray analysis. *Annals of the New York Academy of Sciences*, *975*, 77–90.
- Banks, W. A., Gray, A. M., Erickson, M. A., Salameh, T. S., Damodarasamy, M., Sheibani, N., ... Reed, M. J. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *Journal of Neuroinflammation*, *12*, 223. http://doi.org/10.1186/s12974-015-0434-1
- Barilli, A., Visigalli, R., Sala, R., Gazzola, G. C., Parolari, A., Tremoli, E., ... Bussolati, O. (2008). In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function. *Cardiovascular Research*, *78*(3), 563–571. http://doi.org/10.1093/cvr/cvn024
- Barnett, S. F., Defeo-Jones, D., Fu, S., Hancock, P. J., Haskell, K. M., Jones, R. E., ... Huber, H. E. (2005). Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *The Biochemical Journal*, *385*(Pt 2), 399–408. http://doi.org/10.1042/BJ20041140
- Baud, V., & Karin, M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology*, *11*(9), 372–377.
- Baumann, B., Weber, C. K., Troppmair, J., Whiteside, S., Israel, A., Rapp, U. R., & Wirth, T. (2000).

Raf induces NF-kappaB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(9), 4615–4620. http://doi.org/10.1073/pnas.080583397

- Bechmann, I., Galea, I., & Perry, V. H. (2007). What is the blood-brain barrier (not)? *Trends in Immunology*, *28*(1), 5–11. http://doi.org/10.1016/j.it.2006.11.007
- Beg, A. A., & Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science (New York, N.Y.)*, *274*(5288), 782–784.
- Bell, R. D., Winkler, E. A., Singh, I., Sagare, A. P., Deane, R., Wu, Z., ... Zlokovic, B. V. (2012). Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature*, *485*(7399), 512–516. http://doi.org/10.1038/nature11087
- Bellavance, M.-A., Blanchette, M., & Fortin, D. (2008). Recent Advances in Blood–Brain Barrier Disruption as a CNS Delivery Strategy. *The AAPS Journal*, *10*(1), 166–177. http://doi.org/10.1208/s12248-008-9018-7
- Bellaver, B., Dos Santos, J. P., Leffa, D. T., Bobermin, L. D., Roppa, P. H. A., da Silva Torres, I. L., ... Quincozes-Santos, A. (2018). Systemic Inflammation as a Driver of Brain Injury: the Astrocyte as an Emerging Player. *Molecular Neurobiology*, *55*(3), 2685–2695. http://doi.org/10.1007/s12035-017-0526-2
- Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., ... Anderson, D. W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 13681–13686. http://doi.org/10.1073/pnas.251194298
- Berghoff, A. S., Lassmann, H., Preusser, M., & Hoftberger, R. (2013). Characterization of the inflammatory response to solid cancer metastases in the human brain. *Clinical & Experimental Metastasis*, *30*(1), 69–81. http://doi.org/10.1007/s10585-012-9510-4
- Berghoff, A. S., & Preusser, M. (2018). New developments in brain metastases. *Therapeutic Advances in Neurological Disorders*, *11*, 1756286418785502. http://doi.org/10.1177/1756286418785502
- Berghoff, A. S., Rajky, O., Winkler, F., Bartsch, R., Furtner, J., Hainfellner, J. A., ... Preusser, M. (2013). Invasion patterns in brain metastases of solid cancers. *Neuro-Oncology*, *15*(12), 1664–1672. http://doi.org/10.1093/neuonc/not112
- Bernard, O. (2007). Lim kinases, regulators of actin dynamics. *The International Journal of Biochemistry & Cell Biology*, *39*(6), 1071–1076. http://doi.org/10.1016/j.biocel.2006.11.011
- Bhutani, J., Sheikh, A., & Niazi, A. K. (2013). Akt inhibitors: mechanism of action and implications for anticancer therapeutics. *Infectious Agents and Cancer*, *8*(1), 49. http://doi.org/10.1186/1750-9378-8-49
- Biedl, A., Kraus, R. (1898). Über eine bisher unbekannte toxische Wirkung der Gallensäuren auf das Zentralnervensystem. *Zentralblatt Inn Med*, *19*, 1185–1200.
- Bilanges, B., & Vanhaesebroeck, B. (2010). A new tool to dissect the function of p70 S6 kinase. *The Biochemical Journal*, *431*(2), e1-3. http://doi.org/10.1042/BJ20101445
- Biro, M., Munoz, M. A., & Weninger, W. (2014). Targeting Rho-GTPases in immune cell migration and inflammation. *British Journal of Pharmacology*, *171*(24), 5491–5506. http://doi.org/10.1111/bph.12658
- Birukova, A. A., Smurova, K., Birukov, K. G., Kaibuchi, K., Garcia, J. G. N., & Verin, A. D. (2004). Role of Rho GTPases in thrombin-induced lung vascular endothelial cells barrier dysfunction. *Microvascular Research*, *67*(1), 64–77.
- Blackwell, K., Zhang, L., Workman, L. M., Ting, A. T., Iwai, K., & Habelhah, H. (2013). Two coordinated mechanisms underlie tumor necrosis factor alpha-induced immediate and delayed IkappaB kinase activation. *Molecular and Cellular Biology*, *33*(10), 1901–1915. http://doi.org/10.1128/MCB.01416-12
- Blecharz, K. G., Colla, R., Rohde, V., & Vajkoczy, P. (2015). Control of the blood-brain barrier function in cancer cell metastasis. *Biology of the Cell*, n/a-n/a. http://doi.org/10.1111/boc.201500011
- Blond, D., Campbell, S. J., Butchart, A. G., Perry, V. H., & Anthony, D. C. (2002). Differential induction of interleukin-1beta and tumour necrosis factor-alpha may account for specific patterns of leukocyte recruitment in the brain. *Brain Research*, *958*(1), 89–99.
- Blum, M. S., Toninelli, E., Anderson, J. M., Balda, M. S., Zhou, J., O'Donnell, L., ... Bender, J. R. (1997). Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. *The American Journal of Physiology*, *273*(1 Pt 2), H286-94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9249502
- Bobo, R. H., Laske, D. W., Akbasak, A., Morrison, P. F., Dedrick, R. L., & Oldfield, E. H. (1994). Convection-enhanced delivery of macromolecules in the brain. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(6), 2076–2080.
- Borlongan, C. V, & Emerich, D. F. (2003). Facilitation of drug entry into the CNS via transient

permeation of blood brain barrier: laboratory and preliminary clinical evidence from bradykinin receptor agonist, Cereport. *Brain Research Bulletin*, *60*(3), 297–306.

- Bos, P. D., Zhang, X. H.-F., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., ... Massagué, J. (2009). Genes that mediate breast cancer metastasis to the brain. *Nature*, *459*(7249), 1005–1009. http://doi.org/10.1038/nature08021
- Bossen, C., Ingold, K., Tardivel, A., Bodmer, J.-L., Gaide, O., Hertig, S., ... Schneider, P. (2006). Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *The Journal of Biological Chemistry*, *281*(20), 13964–13971. http://doi.org/10.1074/jbc.M601553200
- Boulanger, L. M. (2009). Immune Proteins in Brain Development and Synaptic Plasticity. *Neuron*, *64*(1), 93–109. http://doi.org/10.1016/j.neuron.2009.09.001
- Boulter, E., Garcia-Mata, R., Guilluy, C., Dubash, A., Rossi, G., Brennwald, P. J., & Burridge, K. (2010). Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nature Cell Biology*, *12*(5), 477–483. http://doi.org/10.1038/ncb2049
- Boutaffala, L., Bertrand, M. J. M., Remouchamps, C., Seleznik, G., Reisinger, F., Janas, M., ... Dejardin, E. (2015). NIK promotes tissue destruction independently of the alternative NF-kappaB pathway through TNFR1/RIP1-induced apoptosis. *Cell Death and Differentiation*, *22*(12), 2020–2033. http://doi.org/10.1038/cdd.2015.69
- Brastianos, H. C., Cahill, D. P., & Brastianos, P. K. (2015). Systemic therapy of brain metastases. *Current Neurology and Neuroscience Reports*, *15*(2), 518. http://doi.org/10.1007/s11910-014-0518-9
- Brastianos, P. K., Curry, W. T., & Oh, K. S. (2013). Clinical discussion and review of the management of brain metastases. *Journal of the National Comprehensive Cancer Network : JNCCN*, *11*(9), 1153–1164.
- Brayton, J., Qing, Z., Hart, M. N., VanGilder, J. C., & Fabry, Z. (1998). Influence of adhesion molecule expression by human brain microvessel endothelium on cancer cell adhesion. *Journal of Neuroimmunology*, *89*(1–2), 104–112.
- Brazil, D. P., Yang, Z.-Z., & Hemmings, B. A. (2004). Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends in Biochemical Sciences*, *29*(5), 233–242. http://doi.org/10.1016/j.tibs.2004.03.006
- Breslin, J. W., Pappas, P. J., Cerveira, J. J., Hobson, R. W. 2nd, & Duran, W. N. (2003). VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *American Journal of Physiology. Heart and Circulatory Physiology*, *284*(1), H92–H100. http://doi.org/10.1152/ajpheart.00330.2002
- Brightbill, H. D., Suto, E., Blaquiere, N., Ramamoorthi, N., Sujatha-Bhaskar, S., Gogol, E. B., ... Ghilardi, N. (2018). NF-kappaB inducing kinase is a therapeutic target for systemic lupus erythematosus. *Nature Communications*, *9*(1), 179. http://doi.org/10.1038/s41467-017-02672-0
- Brint, E., O'Callaghan, G., & Houston, A. (2013). Life in the Fas lane: differential outcomes of Fas signaling. *Cellular and Molecular Life Sciences : CMLS*, *70*(21), 4085–4099. http://doi.org/10.1007/s00018-013-1327-z
- Brockstedt, E., Rickers, A., Kostka, S., Laubersheimer, A., Dorken, B., Wittmann-Liebold, B., ... Otto, A. (1998). Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line. Cleavage of heterogeneous nuclear ribonucleoprotein A1 by caspase 3. *The Journal of Biological Chemistry*, *273*(43), 28057–28064.
- Brouckaert, P., Ameloot, P., Cauwels, A., Everaerdt, B., Libert, C., Takahashi, N., ... Fiers, W. (1994). Receptor-selective mutants of tumour necrosis factor in the therapy of cancer: preclinical studies. *Circulatory Shock*, *43*(4), 185–190.
- Brower, J. V, Saha, S., Rosenberg, S. A., Hullett, C. R., & Ian Robins, H. (2016). Management of leptomeningeal metastases: Prognostic factors and associated outcomes. *Journal of Clinical Neuroscience : Official Journal of the Neurosurgical Society of Australasia*, *27*, 130–137. http://doi.org/10.1016/j.jocn.2015.11.012
- Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., & Schreiber, S. L. (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature*, *377*(6548), 441–446. http://doi.org/10.1038/377441a0
- Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., ... Ware, C. F. (1993). Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. *Cell*, *72*(6), 847–856.
- Bryant, P. W., Zheng, Q., & Pumiglia, K. M. (2012, August). Focal adhesion kinase is a phospho-regulated repressor of Rac and proliferation in human endothelial cells. *Biology Open*. Bidder Building,

140 Cowley Road, Cambridge, CB4 0DL, UK. http://doi.org/10.1242/bio.20121008
- Brzeska, H., Szczepanowska, J., Matsumura, F., & Korn, E. D. (2004). Rac-induced increase of phosphorylation of myosin regulatory light chain in HeLa cells. *Cell Motility and the Cytoskeleton*, *58*(3), 186–199. http://doi.org/10.1002/cm.20009
- Burke, J. R., Pattoli, M. A., Gregor, K. R., Brassil, P. J., MacMaster, J. F., McIntyre, K. W., ... Zusi, F. C. (2003). BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *The Journal of Biological Chemistry*, *278*(3), 1450–1456. http://doi.org/10.1074/jbc.M209677200
- Burridge, K., & Wennerberg, K. (2004). Rho and Rac take center stage. *Cell*, *116*(2), 167–179.
- Cagney, D. N., Martin, A. M., Catalano, P. J., Redig, A. J., Lin, N. U., Lee, E. Q., ... Aizer, A. A. (2017). Incidence and prognosis of patients with brain metastases at diagnosis of systemic malignancy: a population-based study. *Neuro-Oncology*, *19*(11), 1511–1521. http://doi.org/10.1093/neuonc/nox077
- Cain, R. J., Vanhaesebroeck, B., & Ridley, A. J. (2010). The PI3K p110alpha isoform regulates endothelial adherens junctions via Pyk2 and Rac1. *The Journal of Cell Biology*, *188*(6), 863–876. http://doi.org/10.1083/jcb.200907135
- Cancer Research UK. (2016). Brain, other CNS and intracranial tumours mortality statistics.
- Candelario-Jalil, E., Taheri, S., Yang, Y., Sood, R., Grossetete, M., Estrada, E. Y., ... Rosenberg, G. A. (2007). Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. *The Journal of Pharmacology and Experimental Therapeutics*, *323*(2), 488–498. http://doi.org/10.1124/jpet.107.127035
- Carbonell, W. S., Ansorge, O., Sibson, N., & Muschel, R. (2009). The vascular basement membrane as "soil" in brain metastasis. *PloS One*, *4*(6), e5857. http://doi.org/10.1371/journal.pone.0005857
- Cardoso, F. L., Brites, D., & Brito, M. A. (2010). Looking at the blood–brain barrier: Molecular anatomy and possible investigation approaches. *Brain Research Reviews*, *64*(2), 328–363. http://doi.org/10.1016/j.brainresrev.2010.05.003
- Carretero-Ortega, J., Walsh, C. T., Hernandez-Garcia, R., Reyes-Cruz, G., Brown, J. H., & Vazquez-Prado, J. (2010). Phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger 1 (P-Rex-1), a guanine nucleotide exchange factor for Rac, mediates angiogenic responses to stromal cell-derived factor-1/chemokine stromal cell derived factor-1 (SDF-1/CXCL-12) linked to Rac. *Molecular Pharmacology*, *77*(3), 435–442. http://doi.org/10.1124/mol.109.060400
- Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(9), 3666–3670.
- Casar, B., & Crespo, P. (2016). ERK Signals: Scaffolding Scaffolds? *Frontiers in Cell and Developmental Biology*, *4*, 49. http://doi.org/10.3389/fcell.2016.00049
- Cerione, R. A. (2004). Cdc42: new roads to travel. *Trends in Cell Biology*, *14*(3), 127–132. http://doi.org/10.1016/j.tcb.2004.01.008
- Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews. Cancer*, *2*(8), 563–572. http://doi.org/10.1038/nrc865
- Chan, P. M., & Manser, E. (2012). PAKs in human disease. *Progress in Molecular Biology and Translational Science*, *106*, 171–187. http://doi.org/10.1016/B978-0-12-396456-4.00011-0
- Chaturvedi, M. M., LaPushin, R., & Aggarwal, B. B. (1994). Tumor necrosis factor and lymphotoxin. Qualitative and quantitative differences in the mediation of early and late cellular response. *The Journal of Biological Chemistry*, *269*(20), 14575–14583.
- Chen, G., Chakravarti, N., Aardalen, K., Lazar, A. J., Tetzlaff, M. T., Wubbenhorst, B., ... Davies, M. A. (2014). Molecular profiling of patient-matched brain and extracranial melanoma metastases implicates the PI3K pathway as a therapeutic target. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *20*(21), 5537–5546. http://doi.org/10.1158/1078-0432.CCR-13-3003
- Chen, G., & Goeddel, D. V. (2002). TNF-R1 signaling: a beautiful pathway. *Science (New York, N.Y.)*, *296*(5573), 1634–1635. http://doi.org/10.1126/science.1071924
- Chen, G., Wang, Z., Liu, X., & Liu, F. (2011). High-level CXCR4 expression correlates with brain-specific metastasis of non-small cell lung cancer. *World Journal of Surgery*, *35*(1), 56–61. http://doi.org/10.1007/s00268-010-0784-x
- Chen, J., Somanath, P. R., Razorenova, O., Chen, W. S., Hay, N., Bornstein, P., & Byzova, T. V. (2005). Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nature Medicine*, *11*(11), 1188–1196. http://doi.org/10.1038/nm1307
- Chen, J., Zheng, X. F., Brown, E. J., & Schreiber, S. L. (1995). Identification of an 11-kDa FKBP12-

rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(11), 4947–4951.

- Chen, P.-L., & Easton, A. S. (2010). Evidence that tumor necrosis factor-related apoptosis inducing ligand (TRAIL) inhibits angiogenesis by inducing vascular endothelial cell apoptosis. *Biochemical and Biophysical Research Communications*, *391*(1), 936–41. http://doi.org/10.1016/j.bbrc.2009.11.168
- Chen, Q., Boire, A., Jin, X., Valiente, M., Er, E. E., Lopez-Soto, A., ... Massague, J. (2016). Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature*, *533*(7604), 493–498. http://doi.org/10.1038/nature18268
- Chen, W., Hartman, R., Ayer, R., Marcantonio, S., Kamper, J., Tang, J., & Zhang, J. H. (2009). Matrix metalloproteinases inhibition provides neuroprotection against hypoxia-ischemia in the developing brain. *Journal of Neurochemistry*, *111*(3), 726–736. http://doi.org/10.1111/j.1471-4159.2009.06362.x
- Chen, X. L., Nam, J.-O., Jean, C., Lawson, C., Walsh, C. T., Goka, E., ... Schlaepfer, D. D. (2012). VEGF-induced vascular permeability is mediated by FAK. *Developmental Cell*, *22*(1), 146–157. http://doi.org/10.1016/j.devcel.2011.11.002
- Chen, X., Wu, X., Zhou, Q., Howard, O. M. Z., Netea, M. G., & Oppenheim, J. J. (2013). TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T. cell phenotype in the inflammatory environment. Journal of Immunology (Baltimore, Md. : 1950), 190(3), 1076–1084. http://doi.org/10.4049/jimmunol.1202659
- Cheng, V. W. T., Soto, M. S., Khrapitchev, A. A., Perez-Balderas, F., Zakaria, R., Jenkinson, M. D., ... Sibson, N. R. (2019). VCAM-1-targeted MRI Enables Detection of Brain Micrometastases from Different Primary Tumors. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 25(2), 533–543. http://doi.org/10.1158/1078-0432.CCR-18-1889
- Cherry, J. D., Olschowka, J. A., & O'Banion, M. K. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *Journal of Neuroinflammation*, *11*, 98. http://doi.org/10.1186/1742-2094-11-98
- Chi, O. Z., Hunter, C., Liu, X., & Weiss, H. R. (2006). Effects of 17beta-estradiol on blood-brain barrier disruption during focal cerebral ischemia in younger and older rats. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones et Metabolisme*, *38*(6), 377–381. http://doi.org/10.1055/s-2006-944521
- Cho, J. H., Robinson, J. P., Arave, R. A., Burnett, W. J., Kircher, D. A., Chen, G., ... Holmen, S. L. (2015). AKT1 Activation Promotes Development of Melanoma Metastases. *Cell Reports*, *13*(5), 898–905. http://doi.org/10.1016/j.celrep.2015.09.057
- Cho, T., Jung, Y., & Koschinsky, M. L. (2008). Apolipoprotein(a), through its strong lysine-binding site in KIV(10'), mediates increased endothelial cell contraction and permeability via a Rho/Rho kinase/MYPT1-dependent pathway. *The Journal of Biological Chemistry*, *283*(45), 30503–30512. http://doi.org/10.1074/jbc.M802648200
- Choi, Y. J., Park, Y. J., Park, J. Y., Jeong, H. O., Kim, D. H., Ha, Y. M., ... Chung, H. Y. (2012). Inhibitory effect of mTOR activator MHY1485 on autophagy: suppression of lysosomal fusion. *PloS One*, *7*(8), e43418. http://doi.org/10.1371/journal.pone.0043418
- Chou, M. M., & Blenis, J. (1996). The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. *Cell*, *85*(4), 573–583.
- Chrzanowska-Wodnicka, M., & Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *The Journal of Cell Biology*, *133*(6), 1403–1415.
- Clancy, B., & Cauller, L. J. (1998). Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. *Journal of Neuroscience Methods*, *83*(2), 97–102.
- Clark, K., Plater, L., Peggie, M., & Cohen, P. (2009). Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and IkappaB kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. *The Journal of Biological Chemistry*, *284*(21), 14136–14146. http://doi.org/10.1074/jbc.M109.000414
- Clark, P. R., Kim, R. K., Pober, J. S., & Kluger, M. S. (2015). Tumor necrosis factor disrupts claudin-5 endothelial tight junction barriers in two distinct NF-kappaB-dependent phases. *PloS One*, *10*(3), e0120075. http://doi.org/10.1371/journal.pone.0120075
- Coelho-Santos, V., Leitão, R. A., Cardoso, F. L., Palmela, I., Rito, M., Barbosa, M., ... Silva, A. P. (2015). The TNF-α/Nf-κB Signaling Pathway has a Key Role in Methamphetamine–Induced Blood–Brain Barrier Dysfunction. *Journal of Cerebral Blood Flow & Metabolism*, *35*(8), 1260–1271. http://doi.org/10.1038/jcbfm.2015.59
- Coffer, P. J., & Woodgett, J. R. (1991). Molecular cloning and characterisation of a novel putative

protein-serine kinase related to the cAMP-dependent and protein kinase C families. *European Journal of Biochemistry*, *201*(2), 475–481.

- Cohen, P. (1999). The development and therapeutic potential of protein kinase inhibitors. *Current Opinion in Chemical Biology*, *3*(4), 459–465. http://doi.org/10.1016/S1367-5931(99)80067-2
- Coisne, C., & Engelhardt, B. (2011). Tight junctions in brain barriers during central nervous system inflammation. *Antioxidants & Redox Signaling*, *15*(5), 1285–1303. http://doi.org/10.1089/ars.2011.3929
- Combrinck, M. I., Perry, V. H., & Cunningham, C. (2002). Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease. *Neuroscience*, *112*(1), 7–11.
- Connell, J. J., Anthony, D. C., & Sibson, N. R. (2014). *Selective permeabilisation of the blood-brain barrier at sites of metastasis*. Oxford University, UK.
- Connell, J. J., Chatain, G., Cornelissen, B., Vallis, K. A., Hamilton, A., Seymour, L., ... Sibson, N. R. (2013). Selective permeabilization of the blood-brain barrier at sites of metastasis. *Journal of the National Cancer Institute*, *105*(21), 1634–1643. http://doi.org/10.1093/jnci/djt276
- Conrad, C. A. (2001). Chemotherapy for metastatic tumors to the central nervous system. *Current Oncology Reports*, *3*(6), 490–494.
- Corcoran, A., & Cotter, T. G. (2013). Redox regulation of protein kinases. *The FEBS Journal*, *280*(9), 1944–1965. http://doi.org/10.1111/febs.12224
- Cording, J., Berg, J., Kading, N., Bellmann, C., Tscheik, C., Westphal, J. K., ... Blasig, I. E. (2013). In tight junctions, claudins regulate the interactions between occludin, tricellulin and marvelD3, which, inversely, modulate claudin oligomerization. *Journal of Cell Science*, *126*(Pt 2), 554–564. http://doi.org/10.1242/jcs.114306
- Cornford, E. M., Hyman, S., & Swartz, B. E. (1994). The human brain GLUT1 glucose transporter: ultrastructural localization to the blood-brain barrier endothelia. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *14*(1), 106–112. http://doi.org/10.1038/jcbfm.1994.15
- Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., ... Gutkind, J. S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, *81*(7), 1137–1146.
- Coulie, P. G., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., ... Boon, T. (1994). A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *The Journal of Experimental Medicine*, *180*(1), 35–42.
- Cowen, T., Haven, A. J., & Burnstock, G. (1985). Pontamine sky blue: a counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry. *Histochemistry*, *82*(3), 205–208.
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., & Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, *378*(6559), 785–789. http://doi.org/10.1038/378785a0
- Csortos, C., Kolosova, I., & Verin, A. D. (2007). Regulation of vascular endothelial cell barrier function and cytoskeleton structure by protein phosphatases of the PPP family. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *293*(4), L843-54. http://doi.org/10.1152/ajplung.00120.2007
- Cui, W., Wang, Y., Ma, L., Wen, Y., & Liu, P. (2007). [The impact of tumor necrosis factor alpha on expression of intestinal epithelial tight junction protein occludin in fulminant hepatic failure mice model]. *Zhonghua nei ke za zhi*, *46*(6), 478–481.
- Cunningham, C., Campion, S., Lunnon, K., Murray, C. L., Woods, J. F. C., Deacon, R. M. J., ... Perry, V. H. (2009). Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease. *Biological Psychiatry*, *65*(4), 304–312. http://doi.org/10.1016/j.biopsych.2008.07.024
- Cuzzocrea, S., Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Muia, C., ... Thiemermann, C. (2006). Glycogen synthase kinase-3 beta inhibition reduces secondary damage in experimental spinal cord trauma. *The Journal of Pharmacology and Experimental Therapeutics*, *318*(1), 79–89. http://doi.org/10.1124/jpet.106.102863
- Czikora, I., Kim, K., Kasa, A., Becsi, B., Verin, A. D., Gergely, P., ... Csortos, C. (2011). Characterization of the effect of TIMAP phosphorylation on its interaction with protein phosphatase 1. *Biochimie*, *93*(7), 1139–1145. http://doi.org/10.1016/j.biochi.2011.03.011
- da Fonseca, A. C. C., Matias, D., Garcia, C., Amaral, R., Geraldo, L. H., Freitas, C., & Lima, F. R. S. (2014). The impact of microglial activation on blood-brain barrier in brain diseases. *Frontiers in Cellular Neuroscience*, *8*, 362. http://doi.org/10.3389/fncel.2014.00362
- da Fonseca, C. O., Schwartsmann, G., Fischer, J., Nagel, J., Futuro, D., Quirico-Santos, T., & Gattass, C. R. (2008). Preliminary results from a phase I/II study of perillyl alcohol intranasal administration in

adults with recurrent malignant gliomas. *Surgical Neurology*, *70*(3), 257–259. http://doi.org/10.1016/j.surneu.2007.07.040
- Dada, S., Demartines, N., & Dormond, O. (2008). mTORC2 regulates PGE2-mediated endothelial cell survival and migration. *Biochemical and Biophysical Research Communications*, *372*(4), 875–879. http://doi.org/10.1016/j.bbrc.2008.05.154
- Dagogo-Jack, I., Carter, S. L., & Brastianos, P. K. (2016). Brain Metastasis: Clinical Implications of Branched Evolution. *Trends in Cancer*, *2*(7), 332–337. http://doi.org/10.1016/j.trecan.2016.06.005
- Daneman, R., Zhou, L., Kebede, A. A., & Barres, B. A. (2010). Pericytes are required for blood–brain barrier integrity during embryogenesis. *Nature*, *468*(7323), 562–566. http://doi.org/10.1038/nature09513
- Daphu, I., Sundstrøm, T., Horn, S., Huszthy, P. C., Niclou, S. P., Sakariassen, P. Ø., ... Thorsen, F. (2013). In vivo animal models for studying brain metastasis: value and limitations. *Clinical & Experimental Metastasis*, *30*(5), 695–710. http://doi.org/10.1007/s10585-013-9566-9
- David, R. (2011). Cell migration: MTORC2 brings up the rear. *Nature Reviews. Molecular Cell Biology*, *12*(2), 74. http://doi.org/10.1038/nrm3046
- Davies, D. C. (2002). Blood-brain barrier breakdown in septic encephalopathy and brain tumours. *Journal of Anatomy*, *200*(6), 639–646.
- Davies, S. P., Reddy, H., Caivano, M., & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *The Biochemical Journal*, *351*(Pt 1), 95–105.
- Davis, A. S., Richter, A., Becker, S., Moyer, J. E., Sandouk, A., Skinner, J., & Taubenberger, J. K. (2014). Characterizing and Diminishing Autofluorescence in Formalin-fixed Paraffin-embedded Human Respiratory Tissue. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, *62*(6), 405–423. http://doi.org/10.1369/0022155414531549
- De Bock, M., Wang, N., Decrock, E., Bol, M., Gadicherla, A. K., Culot, M., ... Leybaert, L. (2013). Endothelial calcium dynamics, connexin channels and blood-brain barrier function. *Progress in Neurobiology*, *108*, 1–20. http://doi.org/10.1016/j.pneurobio.2013.06.001
- de Jong, M., Essers, J., & van Weerden, W. M. (2014). Imaging preclinical tumour models: improving translational power. *Nature Reviews Cancer*, *14*(7), 481–493. http://doi.org/10.1038/nrc3751
- De Mattos-Arruda, L., Ng, C. K. Y., Piscuoglio, S., Gonzalez-Cao, M., Lim, R. S., De Filippo, M. R., ... Reis-Filho, J. S. (2018). Genetic heterogeneity and actionable mutations in HER2-positive primary breast cancers and their brain metastases. *Oncotarget*, *9*(29), 20617–20630. http://doi.org/10.18632/oncotarget.25041
- de Vries, H. E., Blom-Roosemalen, M. C., van Oosten, M., de Boer, A. G., van Berkel, T. J., Breimer, D. D., & Kuiper, J. (1996). The influence of cytokines on the integrity of the blood-brain barrier in vitro. *Journal of Neuroimmunology*, *64*(1), 37–43.
- de Vries, N. A., Zhao, J., Kroon, E., Buckle, T., Beijnen, J. H., & van Tellingen, O. (2007). P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *13*(21), 6440–6449. http://doi.org/10.1158/1078-0432.CCR-07-1335
- Degterev, A., Maki, J. L., & Yuan, J. (2013, February). Activity and specificity of necrostatin-1, small-molecule inhibitor of RIP1 kinase. *Cell Death and Differentiation*. http://doi.org/10.1038/cdd.2012.133
- Dejana, E. (2004). Endothelial cell–cell junctions: happy together. *Nature Reviews Molecular Cell Biology*, *5*(4), 261–270. http://doi.org/10.1038/nrm1357
- Dejana, E., Orsenigo, F., & Lampugnani, M. G. (2008). The role of adherens junctions and VE-cadherin in the control of vascular permeability. *Journal of Cell Science*, *121*(Pt 13), 2115–2122. http://doi.org/10.1242/jcs.017897
- Dejana, E., Tournier-Lasserve, E., & Weinstein, B. M. (2009). The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Developmental Cell*, *16*(2), 209–221. http://doi.org/10.1016/j.devcel.2009.01.004
- Del Zoppo, G. J., Milner, R., Mabuchi, T., Hung, S., Wang, X., & Koziol, J. A. (2006). Vascular matrix adhesion and the blood-brain barrier. *Biochemical Society Transactions*, *34*(Pt 6), 1261–1266. http://doi.org/10.1042/BST0341261
- Delattre, J. Y., Krol, G., Thaler, H. T., & Posner, J. B. (1988). Distribution of brain metastases. *Archives of Neurology*, *45*(7), 741–744.
- Delhase, M., Li, N., & Karin, M. (2000). Kinase regulation in inflammatory response. *Nature*, *406*(6794), 367–368. http://doi.org/10.1038/35019154
- Deli, M. A., Abrahám, C. S., Kataoka, Y., & Niwa, M. (2005). Permeability studies on in vitro blood-brain barrier models: physiology, pathology, and pharmacology. *Cellular and Molecular Neurobiology*,

25(1), 59–127. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15962509

- Deli, M. A., Descamps, L., Dehouck, M. P., Cecchelli, R., Joo, F., Abraham, C. S., & Torpier, G. (1995). Exposure of tumor necrosis factor-alpha to luminal membrane of bovine brain capillary endothelial cells cocultured with astrocytes induces a delayed increase of permeability and cytoplasmic stress fiber formation of actin. *Journal of Neuroscience Research*, *41*(6), 717–726. http://doi.org/10.1002/jnr.490410602
- Demer, L. L., Wortham, C. M., Dirksen, E. R., & Sanderson, M. J. (1993). Mechanical stimulation induces intercellular calcium signaling in bovine aortic endothelial cells. *The American Journal of Physiology*, *264*(6 Pt 2), H2094-102. http://doi.org/10.1152/ajpheart.1993.264.6.H2094
- Deng, H., & Fang, Y. (2012). Anti-inflammatory gallic Acid and wedelolactone are G protein-coupled receptor-35 agonists. *Pharmacology*, *89*(3–4), 211–219. http://doi.org/10.1159/000337184
- Denniss, S. G., Jeffery, A. J., & Rush, J. W. E. (2010). RhoA-Rho kinase signaling mediates endothelium- and endoperoxide-dependent contractile activities characteristic of hypertensive vascular dysfunction. *American Journal of Physiology. Heart and Circulatory Physiology*, *298*(5), H1391-405. http://doi.org/10.1152/ajpheart.01233.2009
- Dental, C., Proust, A., Ouellet, M., Barat, C., & Tremblay, M. J. (2017). HIV-1 Latency-Reversing Agents Prostratin and Bryostatin-1 Induce Blood-Brain Barrier Disruption/Inflammation and Modulate Leukocyte Adhesion/Transmigration. *Journal of Immunology (Baltimore, Md. : 1950)*, *198*(3), 1229–1241. http://doi.org/10.4049/jimmunol.1600742
- Derossi, D., Williams, E. J., Green, P. J., Dunican, D. J., & Doherty, P. (1998). Stimulation of mitogenesis by a cell-permeable PI 3-kinase binding peptide. *Biochemical and Biophysical Research Communications*, *251*(1), 148–152. http://doi.org/10.1006/bbrc.1998.9444
- Desire, L., Bourdin, J., Loiseau, N., Peillon, H., Picard, V., De Oliveira, C., ... Schweighoffer, F. (2005). RAC1 inhibition targets amyloid precursor protein processing by gamma-secretase and decreases Abeta production in vitro and in vivo. *The Journal of Biological Chemistry*, *280*(45), 37516–37525. http://doi.org/10.1074/jbc.M507913200
- Desplat-Jego, S., Creidy, R., Varriale, S., Allaire, N., Luo, Y., Bernard, D., ... Boucraut, J. (2005). Anti-TWEAK monoclonal antibodies reduce immune cell infiltration in the central nervous system and severity of experimental autoimmune encephalomyelitis. *Clinical Immunology (Orlando, Fla.)*, *117*(1), 15–23. http://doi.org/10.1016/j.clim.2005.06.005
- Deyl, Z., Macek, K., Adam, M., & Vancikova, O. (1980). Studies on the chemical nature of elastin fluorescence. *Biochimica et Biophysica Acta*, *625*(2), 248–254.
- Dharmawardhane, S., Hernandez, E., & Vlaar, C. (2013). Development of EHop-016: a small molecule inhibitor of Rac. *The Enzymes*, *33 Pt A*, 117–146. http://doi.org/10.1016/B978-0-12-416749-0.00006-3
- Dhawan, J., & Helfman, D. M. (2004). Modulation of acto-myosin contractility in skeletal muscle myoblasts uncouples growth arrest from differentiation. *Journal of Cell Science*, *117*(Pt 17), 3735–3748. http://doi.org/10.1242/jcs.01197
- Dimitrijevic, O. B., Stamatovic, S. M., Keep, R. F., & Andjelkovic, A. V. (2006). Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *26*(6), 797–810. http://doi.org/10.1038/sj.jcbfm.9600229
- Dinsmore, C. J., & Soriano, P. (2018). MAPK and PI3K signaling: At the crossroads of neural crest development. *Developmental Biology*. http://doi.org/10.1016/j.ydbio.2018.02.003
- Dolghih, E., & Jacobson, M. P. (2013). Predicting efflux ratios and blood-brain barrier penetration from chemical structure: combining passive permeability with active efflux by P-glycoprotein. *ACS Chemical Neuroscience*, *4*(2), 361–367. http://doi.org/10.1021/cn3001922
- Dormond-Meuwly, A., Roulin, D., Dufour, M., Benoit, M., Demartines, N., & Dormond, O. (2011). The inhibition of MAPK potentiates the anti-angiogenic efficacy of mTOR inhibitors. *Biochemical and Biophysical Research Communications*, *407*(4), 714–719. http://doi.org/10.1016/j.bbrc.2011.03.086
- Dormond, O., Madsen, J. C., & Briscoe, D. M. (2007). The effects of mTOR-Akt interactions on anti-apoptotic signaling in vascular endothelial cells. *The Journal of Biological Chemistry*, *282*(32), 23679–23686. http://doi.org/10.1074/jbc.M700563200
- Douni, E., & Kollias, G. (1998). A critical role of the p75 tumor necrosis factor receptor (p75TNF-R) in organ inflammation independent of TNF, lymphotoxin alpha, or the p55TNF-R. *The Journal of Experimental Medicine*, *188*(7), 1343–1352.
- Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Seminars in Cell & Developmental Biology*, *15*(2), 177–182.
- Drappatz, J., Brenner, A., Wong, E. T., Eichler, A., Schiff, D., Groves, M. D., ... Wen, P. Y. (2013).

Phase I study of GRN1005 in recurrent malignant glioma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *19*(6), 1567–1576. http://doi.org/10.1158/1078-0432.CCR-12-2481

- Duchnowska, R., Peksa, R., Radecka, B., Mandat, T., Trojanowski, T., Jarosz, B., ... Jassem, J. (2016). Immune response in breast cancer brain metastases and their microenvironment: the role of the PD-1/PD-L axis. *Breast Cancer Research : BCR*, *18*(1), 43. http://doi.org/10.1186/s13058-016-0702-8
- Duda, D. G., Duyverman, A. M. M. J., Kohno, M., Snuderl, M., Steller, E. J. A., Fukumura, D., & Jain, R. K. (2010). Malignant cells facilitate lung metastasis by bringing their own soil. *Proceedings of the National Academy of Sciences*, *107*(50), 21677–21682. http://doi.org/10.1073/pnas.1016234107
- Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., & Saltiel, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(17), 7686–7689.
- Ebner, M., Sinkovics, B., Szczygiel, M., Ribeiro, D. W., & Yudushkin, I. (2017). Localization of mTORC2 activity inside cells. *The Journal of Cell Biology*, *216*(2), 343–353. http://doi.org/10.1083/jcb.201610060
- Edelman, A. M., Higgins, D. M., Bowman, C. L., Haber, S. N., Rabin, R. A., & Cho-Lee, J. (1992). Myosin light chain kinase is expressed in neurons and glia: immunoblotting and immunocytochemical studies. *Brain Research. Molecular Brain Research*, *14*(1–2), 27–34.
- Edinger, A. L., Linardic, C. M., Chiang, G. G., Thompson, C. B., & Abraham, R. T. (2003). Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. *Cancer Research*, *63*(23), 8451–8460.
- Eggermont, A. M., Schraffordt Koops, H., Lienard, D., Kroon, B. B., van Geel, A. N., Hoekstra, H. J., & Lejeune, F. J. (1996). Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *14*(10), 2653–2665. http://doi.org/10.1200/JCO.1996.14.10.2653
- Ehrlich, P. (1885). Das Sauerstoff-Bedurfnis des Organismus: Eine Farbenanalytische studie (The oxygen requirement of the organism: a color-analytical study). *Hirschwald, Berlin*, 85.
- Eichler, A. F., Chung, E., Kodack, D. P., Loeffler, J. S., Fukumura, D., & Jain, R. K. (2011). The biology of brain metastases—translation to new therapies. *Nature Reviews Clinical Oncology*, *8*(6), 344–356. http://doi.org/10.1038/nrclinonc.2011.58
- Elahy, M., Jackaman, C., Mamo, J. C., Lam, V., Dhaliwal, S. S., Giles, C., ... Takechi, R. (2015). Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immunity & Ageing : I & A*, *12*, 2. http://doi.org/10.1186/s12979-015-0029-9
- Elia, A., Constantinou, C., & Clemens, M. J. (2008). Effects of protein phosphorylation on ubiquitination and stability of the translational inhibitor protein 4E-BP1. *Oncogene*, *27*(6), 811–822. http://doi.org/10.1038/sj.onc.1210678
- Embi, N., Rylatt, D. B., & Cohen, P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European Journal of Biochemistry*, *107*(2), 519–527.
- Engelhardt, B., Vajkoczy, P., & Weller, R. O. (2017). The movers and shapers in immune privilege of the CNS. *Nature Immunology*, *18*(2), 123–131. http://doi.org/10.1038/ni.3666
- Erickson, M. A., & Banks, W. A. (2018, April). Neuroimmune Axes of the Blood–Brain Barriers and Blood–Brain Interfaces: Bases for Physiological Regulation, Disease States, and Pharmacological Interventions. (R. Dantzer, Ed.), *Pharmacological Reviews*. http://doi.org/10.1124/pr.117.014647
- Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature*, *420*(6916), 629–635. http://doi.org/10.1038/nature01148
- Eto, M., Kouroedov, A., Cosentino, F., & Luscher, T. F. (2005). Glycogen synthase kinase-3 mediates endothelial cell activation by tumor necrosis factor-alpha. *Circulation*, *112*(9), 1316–1322. http://doi.org/10.1161/CIRCULATIONAHA.105.564112
- Ewend, M. G., Brem, S., Gilbert, M., Goodkin, R., Penar, P. L., Varia, M., ... Carey, L. A. (2007). Treatment of single brain metastasis with resection, intracavity carmustine polymer wafers, and radiation therapy is safe and provides excellent local control. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *13*(12), 3637–3641. http://doi.org/10.1158/1078-0432.CCR-06-2095
- Ewing, J. (1928). Neoplastic diseases. a treatise on tumors. *Journal of the American Medical Association*, *90*(16), 1319. http://doi.org/10.1001/jama.1928.02690430057034

- Fabbro, D., Di Loreto, C., Stamerra, O., Beltrami, C. A., Lonigro, R., & Damante, G. (1996). TTF-1 gene expression in human lung tumours. *European Journal of Cancer (Oxford, England : 1990)*, *32A*(3), 512–517.
- Fahmy, R. G., Waldman, A., Zhang, G., Mitchell, A., Tedla, N., Cai, H., ... Khachigian, L. M. (2006). Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun. *Nature Biotechnology*, *24*(7), 856–863. http://doi.org/10.1038/nbt1225
- Fang, Y., Park, I.-H., Wu, A.-L., Du, G., Huang, P., Frohman, M. A., ... Chen, J. (2003). PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. *Current Biology : CB*, *13*(23), 2037–2044.
- Fanning, A. S., Ma, T. Y., & Anderson, J. M. (2002). Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *16*(13), 1835–1837. http://doi.org/10.1096/fj.02-0121fje
- Faustman, D., & Davis, M. (2010). TNF receptor 2 pathway: drug target for autoimmune diseases. *Nature Reviews. Drug Discovery*, *9*(6), 482–493. http://doi.org/10.1038/nrd3030
- Faustman, D. L., & Davis, M. (2013). TNF Receptor 2 and Disease: Autoimmunity and Regenerative Medicine. *Frontiers in Immunology*, *4*, 478. http://doi.org/10.3389/fimmu.2013.00478
- Fazakas, C., Wilhelm, I., Nagyoszi, P., Farkas, A. E., Hasko, J., Molnar, J., ... Krizbai, I. A. (2011). Transmigration of melanoma cells through the blood-brain barrier: role of endothelial tight junctions and melanoma-released serine proteases. *PloS One*, *6*(6), e20758. http://doi.org/10.1371/journal.pone.0020758
- Feine, I., Pinkas, I., Salomon, Y., & Scherz, A. (2012). Local oxidative stress expansion through endothelial cells--a key role for gap junction intercellular communication. *PloS One*, *7*(7), e41633. http://doi.org/10.1371/journal.pone.0041633
- Fernandez-Hernando, C., Ackah, E., Yu, J., Suarez, Y., Murata, T., Iwakiri, Y., ... Sessa, W. C. (2007). Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metabolism*, *6*(6), 446–457. http://doi.org/10.1016/j.cmet.2007.10.007
- Fernandez-Martin, L., Marcos-Ramiro, B., Bigarella, C. L., Graupera, M., Cain, R. J., Reglero-Real, N., ... Millan, J. (2012). Crosstalk between reticular adherens junctions and platelet endothelial cell adhesion molecule-1 regulates endothelial barrier function. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *32*(8), e90-102. http://doi.org/10.1161/ATVBAHA.112.252080
- Ferrero, E., Zocchi, M. R., Magni, E., Panzeri, M. C., Curnis, F., Rugarli, C., ... Corti, A. (2001). Roles of tumor necrosis factor p55 and p75 receptors in TNF-alpha-induced vascular permeability. *American Journal of Physiology. Cell Physiology*, *281*(4), C1173–C1179.
- Fidler, I. J. (2003, June). The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. *Nature Reviews. Cancer*. England. http://doi.org/10.1038/nrc1098
- Fidler, I. J. (2011). The role of the organ microenvironment in brain metastasis. *Seminars in Cancer Biology*, *21*(2), 107–112. http://doi.org/10.1016/j.semcancer.2010.12.009
- Fiedler, L. R. (2009). Rac1 regulates cardiovascular development and postnatal function of endothelium. *Cell Adhesion & Migration*, *3*(2), 143–145.
- Fischer, S., Clauss, M., Wiesnet, M., Renz, D., Schaper, W., & Karliczek, G. F. (1999). Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO. *The American Journal of Physiology*, *276*(4 Pt 1), C812-20.
- Fischer, S., Wiesnet, M., Renz, D., & Schaper, W. (2005). H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. *European Journal of Cell Biology*, *84*(7), 687–697. http://doi.org/10.1016/j.ejcb.2005.03.002
- Fischer, S., Wobben, M., Marti, H. H., Renz, D., & Schaper, W. (2002). Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvascular Research*, *63*(1), 70–80. http://doi.org/10.1006/mvre.2001.2367
- Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C., & Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature*, *387*(6634), 729–733. http://doi.org/10.1038/42743
- Förster, C. (2008). Tight junctions and the modulation of barrier function in disease. *Histochemistry and Cell Biology*, *130*(1), 55–70. http://doi.org/10.1007/s00418-008-0424-9
- Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., ... Wajant, H. (2002). Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *Journal of Cell Science*, *115*(Pt 13), 2757–

2770.
- Fox, T., Coll, J. T., Xie, X., Ford, P. J., Germann, U. A., Porter, M. D., ... Wilson, K. P. (1998). A single amino acid substitution makes ERK2 susceptible to pyridinyl imidazole inhibitors of p38 MAP kinase. *Protein Science : A Publication of the Protein Society*, *7*(11), 2249–2255. http://doi.org/10.1002/pro.5560071102
- Francia, G., Cruz-Munoz, W., Man, S., Xu, P., & Kerbel, R. S. (2011). Mouse models of advanced spontaneous metastasis for experimental therapeutics. *Nature Reviews. Cancer*, *11*(2), 135–141. http://doi.org/10.1038/nrc3001
- Frank, R. T., Aboody, K. S., & Najbauer, J. (2011). Strategies for enhancing antibody delivery to the brain. *Biochimica et Biophysica Acta*, *1816*(2), 191–198. http://doi.org/10.1016/j.bbcan.2011.07.002
- Friedl, J., Puhlmann, M., Bartlett, D. L., Libutti, S. K., Turner, E. N., Gnant, M. F. X., & Alexander, H. R. (2002). Induction of permeability across endothelial cell monolayers by tumor necrosis factor (TNF) occurs via a tissue factor-dependent mechanism: relationship between the procoagulant and permeability effects of TNF. *Blood*, *100*(4), 1334–1339.
- Friedl, P., & Alexander, S. (2011). Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell*, *147*(5), 992–1009. http://doi.org/10.1016/j.cell.2011.11.016
- Friesland, A., Zhao, Y., Chen, Y.-H., Wang, L., Zhou, H., & Lu, Q. (2013). Small molecule targeting Cdc42-intersectin interaction disrupts Golgi organization and suppresses cell motility. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(4), 1261–1266. http://doi.org/10.1073/pnas.1116051110
- Fu, Z., Chen, Y., Qin, F., Yang, S., Deng, X., Ding, R., ... Zhu, J. (2014). Increased activity of Rho kinase contributes to hemoglobin-induced early disruption of the blood-brain barrier in vivo after the occurrence of intracerebral hemorrhage. *International Journal of Clinical and Experimental Pathology*, *7*(11), 7844–7853.
- Fukuta, T., Asai, T., Yanagida, Y., Namba, M., Koide, H., Shimizu, K., & Oku, N. (2017). Combination therapy with liposomal neuroprotectants and tissue plasminogen activator for treatment of ischemic stroke. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *31*(5), 1879–1890. http://doi.org/10.1096/fj.201601209R
- Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., ... Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*, *399*(6736), 597–601. http://doi.org/10.1038/21218
- Galkin, V. E., Orlova, A., Kudryashov, D. S., Solodukhin, A., Reisler, E., Schroder, G. F., & Egelman, E. H. (2011). Remodeling of actin filaments by ADF/cofilin proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(51), 20568–20572. http://doi.org/10.1073/pnas.1110109108
- Gao, H., Danzi, M. C., Choi, C. S., Taherian, M., Dalby-Hansen, C., Ellman, D. G., ... Brambilla, R. (2017). Opposing Functions of Microglial and Macrophagic TNFR2 in the Pathogenesis of Experimental Autoimmune Encephalomyelitis. *Cell Reports*, *18*(1), 198–212. http://doi.org/10.1016/j.celrep.2016.11.083
- Gao, Z.-G., Tian, L., Hu, J., Park, I.-S., & Bae, Y. H. (2011). Prevention of metastasis in a 4T1 murine breast cancer model by doxorubicin carried by folate conjugated pH sensitive polymeric micelles. *Journal of Controlled Release : Official Journal of the Controlled Release Society*, *152*(1), 84–89. http://doi.org/10.1016/j.jconrel.2011.01.021
- Garcia, J. G., Davis, H. W., & Patterson, C. E. (1995). Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *Journal of Cellular Physiology*, *163*(3), 510–522. http://doi.org/10.1002/jcp.1041630311
- Gaudino, S., Di Lella, G. M., Russo, R., Lo Russo, V. S., Piludu, F., Quaglio, F. R., ... Colosimo, C. (2012). Magnetic resonance imaging of solitary brain metastases: main findings of nonmorphological sequences. *La Radiologia Medica*, *117*(7), 1225–1241. http://doi.org/10.1007/s11547-012-0846-2
- Gavard, J., Patel, V., & Gutkind, J. S. (2008). Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. *Developmental Cell*, *14*(1), 25–36. http://doi.org/10.1016/j.devcel.2007.10.019
- Gerlach, B., Cordier, S. M., Schmukle, A. C., Emmerich, C. H., Rieser, E., Haas, T. L., ... Walczak, H. (2011). Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature*, *471*(7340), 591–596. http://doi.org/10.1038/nature09816
- Ghosh, S., Tergaonkar, V., Rothlin, C. V, Correa, R. G., Bottero, V., Bist, P., ... Hunter, T. (2006). Essential role of tuberous sclerosis genes TSC1 and TSC2 in NF-kappaB activation and cell survival. *Cancer Cell*, *10*(3), 215–226. http://doi.org/10.1016/j.ccr.2006.08.007

- Gilmore, T. D., & Herscovitch, M. (2006). Inhibitors of NF-kappaB signaling: 785 and counting. *Oncogene*, *25*(51), 6887–6899. http://doi.org/10.1038/sj.onc.1209982
- Giri, D. K., & Aggarwal, B. B. (1998). Constitutive activation of NF-kappaB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. *The Journal of Biological Chemistry*, *273*(22), 14008–14014.
- Goeckeler, Z. M., & Wysolmerski, R. B. (1995). Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *The Journal of Cell Biology*, *130*(3), 613–627.
- Goldblum, S. E., Ding, X., & Campbell-Washington, J. (1993). TNF-alpha induces endothelial cell F-actin depolymerization, new actin synthesis, and barrier dysfunction. *The American Journal of Physiology*, *264*(4 Pt 1), C894-905. http://doi.org/10.1152/ajpcell.1993.264.4.C894
- Goldblum, S. E., Hennig, B., Jay, M., Yoneda, K., & McClain, C. J. (1989). Tumor necrosis factor alpha-induced pulmonary vascular endothelial injury. *Infection and Immunity*, *57*(4), 1218–1226.
- Goldblum, S. E., & Sun, W. L. (1990). Tumor necrosis factor-alpha augments pulmonary arterial transendothelial albumin flux in vitro. *The American Journal of Physiology*, *258*(2 Pt 1), L57-67. http://doi.org/10.1152/ajplung.1990.258.2.L57
- Goldmann, E. (1909). Die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der 'vitalen Färbung. *Beiträg Klinische Chirurgie*, *62*(192–265.).
- Goldmann, E. (1913). Vitalfarbung am Zentralnervensystem. *Abhandl Konigl Preuss Akad Wiss*, *1*, 1–60.
- Gong, R., Rifai, A., & Dworkin, L. D. (2006). Hepatocyte growth factor suppresses acute renal inflammation by inhibition of endothelial E-selectin. *Kidney International*, *69*(7), 1166–1174. http://doi.org/10.1038/sj.ki.5000246
- Gonzalez-Mariscal, L., Betanzos, A., Nava, P., & Jaramillo, B. E. (2003). Tight junction proteins. *Progress in Biophysics and Molecular Biology*, *81*(1), 1–44.
- Gooyit, M., Suckow, M. A., Schroeder, V. A., Wolter, W. R., Mobashery, S., & Chang, M. (2012). Selective gelatinase inhibitor neuroprotective agents cross the blood-brain barrier. *ACS Chemical Neuroscience*, *3*(10), 730–736. http://doi.org/10.1021/cn300062w
- Gorovoy, M., Niu, J., Bernard, O., Profirovic, J., Minshall, R., Neamu, R., & Voyno-Yasenetskaya, T. (2005). LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. *The Journal of Biological Chemistry*, *280*(28), 26533–26542. http://doi.org/10.1074/jbc.M502921200
- Goto, K., Chiba, Y., Sakai, H., & Misawa, M. (2009). Tumor necrosis factor-alpha (TNF-alpha) induces upregulation of RhoA via NF-kappaB activation in cultured human bronchial smooth muscle cells. *Journal of Pharmacological Sciences*, *110*(4), 437–444.
- Gotsch, U., Borges, E., Bosse, R., Boggemeyer, E., Simon, M., Mossmann, H., & Vestweber, D. (1997). VE-cadherin antibody accelerates neutrophil recruitment in vivo. *Journal of Cell Science*, *110 (Pt 5*, 583–588.
- Graesser, D., Solowiej, A., Bruckner, M., Osterweil, E., Juedes, A., Davis, S., ... Madri, J. A. (2002). Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. *The Journal of Clinical Investigation*, *109*(3), 383–392. http://doi.org/10.1172/JCI13595
- Gratton, J. P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., & Sessa, W. C. (2001). Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *The Journal of Biological Chemistry*, *276*(32), 30359–30365. http://doi.org/10.1074/jbc.M009698200
- Grell, M., Becke, F. M., Wajant, H., Mannel, D. N., & Scheurich, P. (1998). Tumor necrosis factor (TNF) receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1. *European Journal of Immunology*, *28*(1), 257–263. http://doi.org/10.1002/(SICI)1521-4141(199801)28:01<257::AID-IMMU257>3.0.CO;2-G
- Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., ... Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell*, *83*(5), 793–802.
- Grell, M., Wajant, H., Zimmermann, G., & Scheurich, P. (1998). The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(2), 570–575. http://doi.org/10.1073/pnas.95.2.570
- Grethe, S., Ares, M. P. S., Andersson, T., & Porn-Ares, M. I. (2004). p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). *Experimental Cell*

Research, *298*(2), 632–642. http://doi.org/10.1016/j.yexcr.2004.05.007

- Gril, B., Palmieri, D., Bronder, J. L., Herring, J. M., Vega-Valle, E., Feigenbaum, L., ... Steeg, P. S. (2008). Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *Journal of the National Cancer Institute*, *100*(15), 1092–1103. http://doi.org/10.1093/jnci/djn216
- Guerin, A., Sasane, M., Dea, K., Zhang, J., Culver, K., Nitulescu, R., ... Macalalad, A. R. (2016). The economic burden of brain metastasis among lung cancer patients in the United States. *Journal of Medical Economics*, *19*(5), 526–536. http://doi.org/10.3111/13696998.2016.1138962
- Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., ... Sabatini, D. M. (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Developmental Cell*, *11*(6), 859–871. http://doi.org/10.1016/j.devcel.2006.10.007
- Guicciardi, M. E., & Gores, G. J. (2009). Life and death by death receptors. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *23*(6), 1625–1637. http://doi.org/10.1096/fj.08-111005
- Gulati, N., Karsy, M., Albert, L., Murali, R., & Jhanwar-Uniyal, M. (2009). Involvement of mTORC1 and mTORC2 in regulation of glioblastoma multiforme growth and motility. *International Journal of Oncology*, *35*(4), 731–740.
- Guo, S., Arai, K., Stins, M. F., Chuang, D.-M., & Lo, E. H. (2009). Lithium upregulates vascular endothelial growth factor in brain endothelial cells and astrocytes. *Stroke*, *40*(2), 652–655. http://doi.org/10.1161/STROKEAHA.108.524504
- Gupta, G. P., & Massague, J. (2006). Cancer metastasis: building a framework. *Cell*, *127*(4), 679–695. http://doi.org/10.1016/j.cell.2006.11.001
- Gururaja, T. L., Yung, S., Ding, R., Huang, J., Zhou, X., McLaughlin, J., ... Kinoshita, T. (2007, October). A class of small molecules that inhibit TNFalpha-induced survival and death pathways via prevention of interactions between TNFalphaRI, TRADD, and RIP1. *Chemistry & Biology*. United States. http://doi.org/10.1016/j.chembiol.2007.08.012
- Gustin, J. A., Ozes, O. N., Akca, H., Pincheira, R., Mayo, L. D., Li, Q., ... Donner, D. B. (2004). Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. *The Journal of Biological Chemistry*, *279*(3), 1615–1620. http://doi.org/10.1074/jbc.M306976200
- Gutierrez, E. G., Banks, W. A., & Kastin, A. J. (1993). Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. *Journal of Neuroimmunology*, *47*(2), 169–176.
- Haas, T. L., Emmerich, C. H., Gerlach, B., Schmukle, A. C., Cordier, S. M., Rieser, E., ... Walczak, H. (2009). Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Molecular Cell*, *36*(5), 831–844. http://doi.org/10.1016/j.molcel.2009.10.013
- Hacker, H., & Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. *Science's STKE : Signal Transduction Knowledge Environment*, *2006*(357), re13. http://doi.org/10.1126/stke.3572006re13
- Hafner, M., Orosz, P., Kruger, A., & Mannel, D. N. (1996). TNF promotes metastasis by impairing natural killer cell activity. *International Journal of Cancer*, *66*(3), 388–392. http://doi.org/10.1002/(SICI)1097-0215(19960503)66:3<388::AID-IJC20>3.0.CO;2-6
- Hagen, T. L. M. ten, & Eggermont, A. M. M. (2004). Tumor vascular therapy with TNF: critical review on animal models. *Methods in Molecular Medicine*, *98*, 227–246. http://doi.org/10.1385/1-59259-771-8:227
- Haklai, R., Weisz, M. G., Elad, G., Paz, A., Marciano, D., Egozi, Y., ... Kloog, Y. (1998). Dislodgment and accelerated degradation of Ras. *Biochemistry*, *37*(5), 1306–1314. http://doi.org/10.1021/bi972032d
- Haluska, M., & Anthony, M. (2004). Osmotic blood-brain barrier modification for the treatment of malignant brain tumors. *Clinical Journal of Oncology Nursing*, 263–268. Retrieved from http://ons.metapress.com/index/20v83100xp7q4230.pdf
- Ham, B., Fernandez, M. C., D'Costa, Z., & Brodt, P. (2016, January). The diverse roles of the TNF axis in cancer progression and metastasis. *Trends in Cancer Research*.
- Hamilton, A., & Sibson, N. R. (2013). Role of the systemic immune system in brain metastasis. *Molecular and Cellular Neurosciences*, *53*, 42–51. http://doi.org/10.1016/j.mcn.2012.10.004
- Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70.
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646–74. http://doi.org/10.1016/j.cell.2011.02.013
- Hanker, J. S., Yates, P. E., Metz, C. B., & Rustioni, A. (1977, November). A new specific, sensitive and

non-carcinogenic reagent for the demonstration of horseradish peroxidase. *The Histochemical Journal*. Netherlands.

- Haorah, J., Heilman, D., Knipe, B., Chrastil, J., Leibhart, J., Ghorpade, A., ... Persidsky, Y. (2005). Ethanol-induced activation of myosin light chain kinase leads to dysfunction of tight junctions and blood-brain barrier compromise. *Alcoholism, Clinical and Experimental Research*, *29*(6), 999–1009.
- Haorah, J., Knipe, B., Gorantla, S., Zheng, J., & Persidsky, Y. (2007). Alcohol-induced blood-brain barrier dysfunction is mediated via inositol 1,4,5-triphosphate receptor (IP3R)-gated intracellular calcium release. *Journal of Neurochemistry*, *100*(2), 324–336. http://doi.org/10.1111/j.1471-4159.2006.04245.x
- Harrell, J. C., Prat, A., Parker, J. S., Fan, C., He, X., Carey, L., ... Perou, C. M. (2012). Genomic analysis identifies unique signatures predictive of brain, lung, and liver relapse. *Breast Cancer Research and Treatment*, *132*(2), 523–535. http://doi.org/10.1007/s10549-011-1619-7
- Haubert, D., Gharib, N., Rivero, F., Wiegmann, K., Hosel, M., Kronke, M., & Kashkar, H. (2007). PtdIns(4,5)P-restricted plasma membrane localization of FAN is involved in TNF-induced actin reorganization. *The EMBO Journal*, *26*(14), 3308–3321. http://doi.org/10.1038/sj.emboj.7601778
- Hawkins, B. T., & Davis, T. P. (2005). The Blood-Brain Barrier / Neurovascular Unit in Health and Disease. *Pharmacological Reviews*, *57*(2), 173–185. http://doi.org/10.1124/pr.57.2.4.173
- Hay, N. (2005). The Akt-mTOR tango and its relevance to cancer. *Cancer Cell*, *8*(3), 179–183. http://doi.org/10.1016/j.ccr.2005.08.008
- Hayden, M. S., & Ghosh, S. (2012). NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes & Development*, *26*(3), 203–234. http://doi.org/10.1101/gad.183434.111
- Hayden, M. S., & Ghosh, S. (2014). Regulation of NF-kappaB by TNF family cytokines. *Seminars in Immunology*, *26*(3), 253–266. http://doi.org/10.1016/j.smim.2014.05.004
- He, F., Peng, J., Deng, X.-L., Yang, L.-F., Wu, L.-W., Zhang, C.-L., & Yin, F. (2011). RhoA and NF-kappaB are involved in lipopolysaccharide-induced brain microvascular cell line hyperpermeability. *Neuroscience*, *188*, 35–47. http://doi.org/10.1016/j.neuroscience.2011.04.025
- He, F., Peng, J., Deng, X., Yang, L., Camara, A. D., Omran, A., ... Yin, F. (2012). Mechanisms of tumor necrosis factor-alpha-induced leaks in intestine epithelial barrier. *Cytokine*, *59*(2), 264–272. http://doi.org/10.1016/j.cyto.2012.04.008
- He, M. M., Smith, A. S., Oslob, J. D., Flanagan, W. M., Braisted, A. C., Whitty, A., ... Cunningham, B. C. (2005). Small-molecule inhibition of TNF-alpha. *Science (New York, N.Y.)*, *310*(5750), 1022–1025. http://doi.org/10.1126/science.1116304
- Heasman, S. J., & Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nature Reviews. Molecular Cell Biology*, *9*(9), 690–701. http://doi.org/10.1038/nrm2476
- Heitman, J., Movva, N. R., & Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science (New York, N.Y.)*, *253*(5022), 905–909.
- Henry, C. B., & Duling, B. R. (2000). TNF-alpha increases entry of macromolecules into luminal endothelial cell glycocalyx. *American Journal of Physiology. Heart and Circulatory Physiology*, *279*(6), H2815-23. http://doi.org/10.1152/ajpheart.2000.279.6.H2815
- Hernandez-Negrete, I., Carretero-Ortega, J., Rosenfeldt, H., Hernandez-Garcia, R., Calderon-Salinas, J. V., Reyes-Cruz, G., ... Vazquez-Prado, J. (2007). P-Rex1 links mammalian target of rapamycin signaling to Rac activation and cell migration. *The Journal of Biological Chemistry*, *282*(32), 23708–23715. http://doi.org/10.1074/jbc.M703771200
- Hersh, D. S., Wadajkar, A. S., Roberts, N., Perez, J. G., Connolly, N. P., Frenkel, V., ... Kim, A. J. (2016). Evolving Drug Delivery Strategies to Overcome the Blood Brain Barrier. *Current Pharmaceutical Design*, *22*(9), 1177–1193.
- Heye, A. K., Culling, R. D., Valdés Hernández, M. del C., Thrippleton, M. J., & Wardlaw, J. M. (2014). Assessment of blood–brain barrier disruption using dynamic contrast-enhanced MRI. A systematic review. *NeuroImage: Clinical*, *6*, 262–274. http://doi.org/10.1016/j.nicl.2014.09.002
- Heyn, C., Ronald, J. A., Ramadan, S. S., Snir, J. A., Barry, A. M., MacKenzie, L. T., ... Foster, P. J. (2006). In vivo MRI of cancer cell fate at the single-cell level in a mouse model of breast cancer metastasis to the brain. *Magnetic Resonance in Medicine*, *56*(5), 1001–1010. http://doi.org/10.1002/mrm.21029
- Higuchi, M., Robinson, D. S., Cailleau, R., Irie, R. F., & Morton, D. L. (1980). A serologic study of cultured breast cancer cell lines: lack of antibody response to tumour specific membrane antigens in patients. *Clinical and Experimental Immunology*, *39*(1), 90–96.
- Hindie, V., Stroba, A., Zhang, H., Lopez-Garcia, L. A., Idrissova, L., Zeuzem, S., ... Biondi, R. M.

(2009). Structure and allosteric effects of low-molecular-weight activators on the protein kinase PDK1. *Nature Chemical Biology*, *5*(10), 758–764. http://doi.org/10.1038/nchembio.208

- Hirai, H., Sootome, H., Nakatsuru, Y., Miyama, K., Taguchi, S., Tsujioka, K., ... Kotani, H. (2010). MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. *Molecular Cancer Therapeutics*, *9*(7), 1956–1967. http://doi.org/10.1158/1535-7163.MCT-09-1012
- Hofman, F. M., Hinton, D. R., Johnson, K., & Merrill, J. E. (1989). Tumor necrosis factor identified in multiple sclerosis brain. *The Journal of Experimental Medicine*, *170*(2), 607–612.
- Holmes, C., Cunningham, C., Zotova, E., Woolford, J., Dean, C., Kerr, S., ... Perry, V. H. (2009). Systemic inflammation and disease progression in Alzheimer disease. *Neurology*, *73*(10), 768–774. http://doi.org/10.1212/WNL.0b013e3181b6bb95
- Holohan, C., Van Schaeybroeck, S., Longley, D. B., & Johnston, P. G. (2013). Cancer drug resistance: an evolving paradigm. *Nature Reviews. Cancer*, *13*(10), 714–726. http://doi.org/10.1038/nrc3599
- Hori, S., Ohtsuki, S., Hosoya, K., Nakashima, E., & Terasaki, T. (2004). A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. *Journal of Neurochemistry*, *89*(2), 503–513. http://doi.org/10.1111/j.1471-4159.2004.02343.x
- Horowitz, S., Binion, D. G., Nelson, V. M., Kanaa, Y., Javadi, P., Lazarova, Z., ... Rafiee, P. (2007). Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *292*(5), G1323-36. http://doi.org/10.1152/ajpgi.00499.2006
- Hoshino, A., Costa-Silva, B., Shen, T.-L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., ... Lyden, D. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature*, *527*(7578), 329–335. http://doi.org/10.1038/nature15756
- Hottinger, A. F., Pacheco, P., & Stupp, R. (2016). Tumor treating fields: a novel treatment modality and its use in brain tumors. *Neuro-Oncology*, *18*(10), 1338–1349. http://doi.org/10.1093/neuonc/now182
- Hsu, H., Shu, H. B., Pan, M. G., & Goeddel, D. V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, *84*(2), 299–308.
- Huang, Q.-B. (2012). Barrier stabilizing mediators in regulation of microvascular endothelial permeability. *Chinese Journal of Traumatology = Zhonghua Chuang Shang Za Zhi*, *15*(2), 105–112.
- Huang, S., Chen, L. Y., Zuraw, B. L., Ye, R. D., & Pan, Z. K. (2001). Chemoattractant-stimulated NF-kappaB activation is dependent on the low molecular weight GTPase RhoA. *The Journal of Biological Chemistry*, *276*(44), 40977–40981. http://doi.org/10.1074/jbc.M105242200
- Huang, S. N., Minassian, H., & More, J. D. (1976). Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *35*(4), 383–390.
- HUGO Gene Nomenclature Committee. (n.d.). TNF Nomenclature, HGNC: 11892. Retrieved August 1, 2018, from https://beta.genenames.org/data/gene-symbol-report/#!/hgnc_id/11892
- Hunter, I., & Nixon, G. F. (2006). Spatial compartmentalization of tumor necrosis factor (TNF) receptor 1-dependent signaling pathways in human airway smooth muscle cells. Lipid rafts are essential for TNF-alpha-mediated activation of RhoA but dispensable for the activation of the NF-kapp. *The Journal of Biological Chemistry*, *281*(45), 34705–34715. http://doi.org/10.1074/jbc.M605738200
- Huppert, J., Closhen, D., Croxford, A., White, R., Kulig, P., Pietrowski, E., ... Kuhlmann, C. R. W. (2010). Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *24*(4), 1023–1034. http://doi.org/10.1096/fj.09-141978
- Huveneers, S., Oldenburg, J., Spanjaard, E., van der Krogt, G., Grigoriev, I., Akhmanova, A., ... de Rooij, J. (2012). Vinculin associates with endothelial VE-cadherin junctions to control force-dependent remodeling. *The Journal of Cell Biology*, *196*(5), 641–652. http://doi.org/10.1083/jcb.201108120
- Hynynen, K., McDannold, N., Vykhodtseva, N., & Jolesz, F. A. (2001). Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. *Radiology*, *220*(3), 640–646. http://doi.org/10.1148/radiol.2202001804
- Idriss, H. T., & Naismith, J. H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microscopy Research and Technique*, *50*(3), 184–195. http://doi.org/10.1002/1097-0029(20000801)50:3<184::AID-JEMT2>3.0.CO;2-H
- Iizuka, M., Kimura, K., Wang, S., Kato, K., Amano, M., Kaibuchi, K., & Mizoguchi, A. (2012). Distinct distribution and localization of Rho-kinase in mouse epithelial, muscle and neural tissues. *Cell Structure and Function*, *37*(2), 155–175.

- Ilhan-Mutlu, A., Osswald, M., Liao, Y., Gommel, M., Reck, M., Miles, D., ... Winkler, F. (2016). Bevacizumab Prevents Brain Metastases Formation in Lung Adenocarcinoma. *Molecular Cancer Therapeutics*, *15*(4), 702–710. http://doi.org/10.1158/1535-7163.MCT-15-0582
- Iliff, J. J., Wang, M., Liao, Y., Plogg, B. A., Peng, W., Gundersen, G. A., ... Nedergaard, M. (2012). A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Science Translational Medicine*, *4*(147), 147ra111. http://doi.org/10.1126/scitranslmed.3003748
- Inayama, M., Nishioka, Y., Azuma, M., Muto, S., Aono, Y., Makino, H., ... Sone, S. (2006). A novel IkappaB kinase-beta inhibitor ameliorates bleomycin-induced pulmonary fibrosis in mice. *American Journal of Respiratory and Critical Care Medicine*, *173*(9), 1016–1022. http://doi.org/10.1164/rccm.200506-947OC
- Inoki, K., Corradetti, M. N., & Guan, K.-L. (2005). Dysregulation of the TSC-mTOR pathway in human disease. *Nature Genetics*, *37*(1), 19–24. http://doi.org/10.1038/ng1494
- Inoki, K., Li, Y., Zhu, T., Wu, J., & Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature Cell Biology*, *4*(9), 648–657. http://doi.org/10.1038/ncb839
- Inoki, K., Ouyang, H., Zhu, T., Lindvall, C., Wang, Y., Zhang, X., ... Guan, K.-L. (2006). TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell*, *126*(5), 955–968. http://doi.org/10.1016/j.cell.2006.06.055
- Ishibashi, F. (2008). High glucose increases phosphocofilin via phosphorylation of LIM kinase due to Rho/Rho kinase activation in cultured pig proximal tubular epithelial cells. *Diabetes Research and Clinical Practice*, *80*(1), 24–33. http://doi.org/10.1016/j.diabres.2007.11.004
- Ishiguro, M., Kawasaki, K., Suzuki, Y., Ishizuka, F., Mishiro, K., Egashira, Y., ... Hara, H. (2012). A Rho kinase (ROCK) inhibitor, fasudil, prevents matrix metalloproteinase-9-related hemorrhagic transformation in mice treated with tissue plasminogen activator. *Neuroscience*, *220*, 302–312. http://doi.org/10.1016/j.neuroscience.2012.06.015
- Ishmael, J. E., Lohr, C. V, Fischer, K., & Kioussi, C. (2008). Localization of myosin II regulatory light chain in the cerebral vasculature. *Acta Histochemica*, *110*(2), 172–177. http://doi.org/10.1016/j.acthis.2007.08.005
- Islam, S., Hassan, F., Mu, M. M., Ito, H., Koide, N., Mori, I., ... Yokochi, T. (2004). Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-kappaB activation by inhibiting IkappaB kinase (IKK). *Microbiology and Immunology*, *48*(10), 729–736.
- Israel, A. (2010). The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harbor Perspectives in Biology*, *2*(3), a000158. http://doi.org/10.1101/cshperspect.a000158
- Israel, A., Le Bail, O., Hatat, D., Piette, J., Kieran, M., Logeat, F., ... Kourilsky, P. (1989). TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors. *The EMBO Journal*, *8*(12), 3793–3800.
- Ivanov, A. I., Hunt, D., Utech, M., Nusrat, A., & Parkos, C. A. (2005). Differential roles for actin polymerization and a myosin II motor in assembly of the epithelial apical junctional complex. *Molecular Biology of the Cell*, *16*(6), 2636–2650. http://doi.org/10.1091/mbc.e05-01-0043
- Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S. Y., ... Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*, *127*(1), 125–137. http://doi.org/10.1016/j.cell.2006.08.033
- Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A., & Hall, M. N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature Cell Biology*, *6*(11), 1122–1128. http://doi.org/10.1038/ncb1183
- Jadhav, S., Bochner, B. S., & Konstantopoulos, K. (2001). Hydrodynamic shear regulates the kinetics and receptor specificity of polymorphonuclear leukocyte-colon carcinoma cell adhesive interactions. *Journal of Immunology (Baltimore, Md. : 1950)*, *167*(10), 5986–5993.
- Jain, R. K., di Tomaso, E., Duda, D. G., Loeffler, J. S., Sorensen, A. G., & Batchelor, T. T. (2007). Angiogenesis in brain tumours. *Nature Reviews. Neuroscience*, *8*(8), 610–622. http://doi.org/10.1038/nrn2175
- Jandial, R., Neman, J., & Chen, M. Y. (2014). Therapeutic Disruption of the Blood Brain Barrier in Metastasis. *Neurosurgery*, *74*(4), N11–N12. http://doi.org/10.1227/01.neu.0000445333.56165.a0
- Jang, M., Cai, L., Udeani, G. O., Slowing, K. V, Thomas, C. F., Beecher, C. W., ... Pezzuto, J. M. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (New York, N.Y.)*, *275*(5297), 218–220.
- Janzen, C., Sen, S., Cuevas, J., Reddy, S. T., & Chaudhuri, G. (2011). Protein phosphatase 2A promotes endothelial survival via stabilization of translational inhibitor 4E-BP1 following exposure to

tumor necrosis factor-alpha. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(11), 2586–2594. http://doi.org/10.1161/ATVBAHA.111.230946

- Jassam, S. A., Maherally, Z., Smith, J. R., Ashkan, K., Roncaroli, F., Fillmore, H. L., & Pilkington, G. J. (2016). TNF-alpha enhancement of CD62E mediates adhesion of non-small cell lung cancer cells to brain endothelium via CD15 in lung-brain metastasis. *Neuro-Oncology*, *18*(5), 679–690. http://doi.org/10.1093/neuonc/nov248
- Jassam, S. A., Maherally, Z., Smith, J. R., Ashkan, K., Roncaroli, F., Fillmore, H. L., & Pilkington, G. J. (2017). CD15s/CD62E Interaction Mediates the Adhesion of Non-Small Cell Lung Cancer Cells on Brain Endothelial Cells: Implications for Cerebral Metastasis. *International Journal of Molecular Sciences*, *18*(7). http://doi.org/10.3390/ijms18071474
- Jean, C., Chen, X. L., Nam, J.-O., Tancioni, I., Uryu, S., Lawson, C., ... Schlaepfer, D. D. (2014). Inhibition of endothelial FAK activity prevents tumor metastasis by enhancing barrier function. *The Journal of Cell Biology*, *204*(2), 247–263. http://doi.org/10.1083/jcb.201307067
- Jeevan, D. S., Cooper, J. B., Braun, A., Murali, R., & Jhanwar-Uniyal, M. (2016). Molecular Pathways Mediating Metastases to the Brain via Epithelial-to-Mesenchymal Transition: Genes, Proteins, and Functional Analysis. *Anticancer Research*, *36*(2), 523–532.
- Jeon, B. T., Jeong, E. A., Park, S.-Y., Son, H., Shin, H. J., Lee, D. H., ... Roh, G. S. (2013). The Rho-kinase (ROCK) inhibitor Y-27632 protects against excitotoxicity-induced neuronal death in vivo and in vitro. *Neurotoxicity Research*, *23*(3), 238–248. http://doi.org/10.1007/s12640-012-9339-2
- Jhanwar-Uniyal, M., Amin, A. G., Cooper, J. B., Das, K., Schmidt, M. H., & Murali, R. (2017). Discrete signaling mechanisms of mTORC1 and mTORC2: Connected yet apart in cellular and molecular aspects. *Advances in Biological Regulation*, *64*, 39–48. http://doi.org/10.1016/j.jbior.2016.12.001
- Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S., & Han, J. (1996). Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *The Journal of Biological Chemistry*, *271*(30), 17920–17926.
- Jiang, Y., Zhang, Q., Bao, J., Du, C., Wang, J., Tong, Q., & Liu, C. (2015). Schisandrin B suppresses glioma cell metastasis mediated by inhibition of mTOR/MMP-9 signal pathway. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *74*, 77–82. http://doi.org/10.1016/j.biopha.2015.07.006
- Jin, L., Nakao, A., Nakayama, M., Yamaguchi, N., Kojima, Y., Nakano, N., ... Ogawa, H. (2004). Induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes. *The Journal of Investigative Dermatology*, *122*(5), 1175–1179. http://doi.org/10.1111/j.0022-202X.2004.22419.x
- Johanson, C. E., Stopa, E. G., & McMillan, P. N. (2011). The blood-cerebrospinal fluid barrier: structure and functional significance. *Methods in Molecular Biology (Clifton, N.J.)*, *686*, 101–131. http://doi.org/10.1007/978-1-60761-938-3_4
- Johnson, A. M., Roach, J. P., Hu, A., Stamatovic, S. M., Zochowski, M. R., Keep, R. F., & Andjelkovic, A. V. (2018). Connexin 43 gap junctions contribute to brain endothelial barrier hyperpermeability in familial cerebral cavernous malformations type III by modulating tight junction structure. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *32*(5), 2615–2629. http://doi.org/10.1096/fj.201700699R
- Jope, R. S., Yuskaitis, C. J., & Beurel, E. (2007). Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochemical Research*, *32*(4–5), 577–595. http://doi.org/10.1007/s11064-006-9128-5
- JuanYin, J., Tracy, K., Zhang, L., Munasinghe, J., Shapiro, E., Koretsky, A., & Kelly, K. (2009). Noninvasive imaging of the functional effects of anti-VEGF therapy on tumor cell extravasation and regional blood volume in an experimental brain metastasis model. *Clinical & Experimental Metastasis*, *26*(5), 403–414. http://doi.org/10.1007/s10585-009-9238-y
- Judy, K. D., Olivi, A., Buahin, K. G., Domb, A., Epstein, J. I., Colvin, O. M., & Brem, H. (1995). Effectiveness of controlled release of a cyclophosphamide derivative with polymers against rat gliomas. *Journal of Neurosurgery*, *82*(3), 481–486. http://doi.org/10.3171/jns.1995.82.3.0481
- Kaidanovich-Beilin, O., & Woodgett, J. R. (2011). GSK-3: Functional Insights from Cell Biology and Animal Models. *Frontiers in Molecular Neuroscience*, *4*, 40. http://doi.org/10.3389/fnmol.2011.00040
- Kalliolias, G. D., & Ivashkiv, L. B. (2016). TNF biology, pathogenic mechanisms and emerging therapeutic strategies. *Nature Reviews. Rheumatology*, *12*(1), 49–62. http://doi.org/10.1038/nrrheum.2015.169
- Kang, T.-B., Ben-Moshe, T., Varfolomeev, E. E., Pewzner-Jung, Y., Yogev, N., Jurewicz, A., ... Wallach, D. (2004). Caspase-8 serves both apoptotic and nonapoptotic roles. *Journal of Immunology (Baltimore, Md. : 1950)*, *173*(5), 2976–2984.

- Katakami, N., Inaba, Y., Sugata, S., Tsurusaki, M., Itoh, T., Machida, T., ... Aitoku, Y. (2011). Magnetic resonance evaluation of brain metastases from systemic malignances with two doses of gadobutrol 1.0 m compared with gadoteridol: a multicenter, phase ii/iii study in patients with known or suspected brain metastases. *Investigative Radiology*, *46*(7), 411–8. http://doi.org/10.1097/RLI.0b013e3182145a6c
- Kaulmann, A., & Bohn, T. (2014). Carotenoids, inflammation, and oxidative stress--implications of cellular signaling pathways and relation to chronic disease prevention. *Nutrition Research (New York, N.Y.)*, *34*(11), 907–929. http://doi.org/10.1016/j.nutres.2014.07.010
- Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Rivoltini, L., Topalian, S. L., ... Rosenberg, S. A. (1994). Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(9), 3515–3519.
- Kay, J. E., Kromwel, L., Doe, S. E., & Denyer, M. (1991). Inhibition of T and B lymphocyte proliferation by rapamycin. *Immunology*, *72*(4), 544–549.
- Kemper, E. M., Boogerd, W., Thuis, I., Beijnen, J. H., & van Tellingen, O. (2004). Modulation of the blood-brain barrier in oncology: therapeutic opportunities for the treatment of brain tumours? *Cancer Treatment Reviews*, *30*(5), 415–423. http://doi.org/10.1016/j.ctrv.2004.04.001
- Kerkar, S., Williams, M., Blocksom, J. M., Wilson, R. F., Tyburski, J. G., & Steffes, C. P. (2006). TNF-alpha and IL-1beta increase pericyte/endothelial cell co-culture permeability. *The Journal of Surgical Research*, *132*(1), 40–45. http://doi.org/10.1016/j.jss.2005.06.033
- Khuon, S., Liang, L., Dettman, R. W., Sporn, P. H. S., Wysolmerski, R. B., & Chew, T.-L. (2010). Myosin light chain kinase mediates transcellular intravasation of breast cancer cells through the underlying endothelial cells: a three-dimensional FRET study. *Journal of Cell Science*, *123*(Pt 3), 431–440. http://doi.org/10.1242/jcs.053793
- Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W. E. F., Goldbrunner, R., Herms, J., & Winkler, F. (2010). Real-time imaging reveals the single steps of brain metastasis formation. *Nature Medicine*, *16*(1), 116–122. http://doi.org/10.1038/nm.2072
- Kim, D., Kim, J. F., Yim, J. H., Kwon, S.-K., Lee, C. H., & Lee, H. K. (2008). Red to red - the marine bacterium Hahella chejuensis and its product prodigiosin for mitigation of harmful algal blooms. *Journal of Microbiology and Biotechnology*, *18*(10), 1621–1629.
- Kim, E. K., Yun, S. J., Ha, J. M., Kim, Y. W., Jin, I. H., Yun, J., ... Bae, S. S. (2011). Selective activation of Akt1 by mammalian target of rapamycin complex 2 regulates cancer cell migration, invasion, and metastasis. *Oncogene*, *30*(26), 2954–2963. http://doi.org/10.1038/onc.2011.22
- Kim, M.-Y., Oskarsson, T., Acharyya, S., Nguyen, D. X., Zhang, X. H.-F., Norton, L., & Massague, J. (2009). Tumor self-seeding by circulating cancer cells. *Cell*, *139*(7), 1315–1326. http://doi.org/10.1016/j.cell.2009.11.025
- Kimura, Y., Okuda, H., & Arichi, S. (1985). Effects of stilbenes on arachidonate metabolism in leukocytes. *Biochimica et Biophysica Acta*, *834*(2), 275–278.
- Kinexus Bioinformatics Corporation. (2017). Human Kinase Drug Interaction KnowledgeBase. Retrieved from http://www.drugkinet.ca/KinaseInhibitorResult.aspx
- Klemke, M., Weschenfelder, T., Konstandin, M. H., & Samstag, Y. (2007). High affinity interaction of integrin alpha4beta1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. *Journal of Cellular Physiology*, *212*(2), 368–374. http://doi.org/10.1002/jcp.21029
- Kloog, Y., & Cox, A. D. (2000). RAS inhibitors: potential for cancer therapeutics. *Molecular Medicine Today*, *6*(10), 398–402.
- Knight, Z. A. (2010). Small molecule inhibitors of the PI3-kinase family. *Current Topics in Microbiology and Immunology*, *347*, 263–278. http://doi.org/10.1007/82_2010_44
- Kobori, M., Yang, Z., Gong, D., Heissmeyer, V., Zhu, H., Jung, Y.-K., ... Yuan, J. (2004). Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. *Cell Death and Differentiation*, *11*(1), 123–130. http://doi.org/10.1038/sj.cdd.4401325
- Kodack, D. P., Chung, E., Yamashita, H., Incio, J., Duyverman, A. M. M. J., Song, Y., ... Jain, R. K. (2012). Combined targeting of HER2 and VEGFR2 for effective treatment of HER2-amplified breast cancer brain metastases. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(45), E3119-27. http://doi.org/10.1073/pnas.1216078109
- Koedel, U., Bayerlein, I., Paul, R., Sporer, B., & Pfister, H. W. (2000). Pharmacologic interference with NF-kappaB activation attenuates central nervous system complications in experimental Pneumococcal meningitis. *The Journal of Infectious Diseases*, *182*(5), 1437–1445. http://doi.org/10.1086/315877

- Kojonazarov, B., Myrzaakhmatova, A., Sooronbaev, T., Ishizaki, T., & Aldashev, A. (2012, February). Effects of fasudil in patients with high-altitude pulmonary hypertension. *The European Respiratory Journal*. England. http://doi.org/10.1183/09031936.00095211
- Kolozsvari, B., Bako, E., Becsi, B., Kiss, A., Czikora, A., Toth, A., ... Erdodi, F. (2012). Calcineurin regulates endothelial barrier function by interaction with and dephosphorylation of myosin phosphatase. *Cardiovascular Research*, *96*(3), 494–503. http://doi.org/10.1093/cvr/cvs255
- Komori, A., Yatsunami, J., Suganuma, M., Okabe, S., Abe, S., Sakai, A., ... Fujiki, H. (1993). Tumor necrosis factor acts as a tumor promoter in BALB/3T3 cell transformation. *Cancer Research*, *53*(9), 1982–1985.
- Koul, D., Yao, Y., Abbruzzese, J. L., Yung, W. K., & Reddy, S. A. (2001). Tumor suppressor MMAC/PTEN inhibits cytokine-induced NFkappaB activation without interfering with the IkappaB degradation pathway. *The Journal of Biological Chemistry*, *276*(14), 11402–11408. http://doi.org/10.1074/jbc.M007806200
- Kozma, R., Ahmed, S., Best, A., & Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Molecular and Cellular Biology*, *15*(4), 1942–1952.
- Kramer, S. M., Aggarwal, B. B., Eessalu, T. E., McCabe, S. M., Ferraiolo, B. L., Figari, I. S., & Palladino, M. A. J. (1988). Characterization of the in vitro and in vivo species preference of human and murine tumor necrosis factor-alpha. *Cancer Research*, *48*(4), 920–925.
- Kriegler, M., Perez, C., DeFay, K., Albert, I., & Lu, S. D. (1988). A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell*, *53*(1), 45–53.
- Krizbai, I. A., Bauer, H., Bresgen, N., Eckl, P. M., Farkas, A., Szatmari, E., ... Bauer, H.-C. (2005). Effect of oxidative stress on the junctional proteins of cultured cerebral endothelial cells. *Cellular and Molecular Neurobiology*, *25*(1), 129–139.
- Kroemer, G., & Martin, S. J. (2005). Caspase-independent cell death. *Nature Medicine*, *11*(7), 725–730. http://doi.org/10.1038/nm1263
- Kuhlmann, C. R. W., Lessmann, V., & Luhmann, H. J. (2006). Fluvastatin stabilizes the blood-brain barrier in vitro by nitric oxide-dependent dephosphorylation of myosin light chains. *Neuropharmacology*, *51*(4), 907–913. http://doi.org/10.1016/j.neuropharm.2006.06.004
- Kuhlmann, C. R. W., Tamaki, R., Gamerdinger, M., Lessmann, V., Behl, C., Kempski, O. S., & Luhmann, H. J. (2007). Inhibition of the myosin light chain kinase prevents hypoxia-induced blood-brain barrier disruption. *Journal of Neurochemistry*, *102*(2), 501–507. http://doi.org/10.1111/j.1471-4159.2007.04506.x
- Kumaratilake, L. M., Rathjen, D. A., Mack, P., Widmer, F., Prasertsiriroj, V., & Ferrante, A. (1995). A synthetic tumor necrosis factor-alpha agonist peptide enhances human polymorphonuclear leukocyte-mediated killing of Plasmodium falciparum in vitro and suppresses Plasmodium chabaudi infection in mice. *The Journal of Clinical Investigation*, *95*(5), 2315–2323. http://doi.org/10.1172/JCI117923
- Kuno, R., Wang, J., Kawanokuchi, J., Takeuchi, H., Mizuno, T., & Suzumura, A. (2005). Autocrine activation of microglia by tumor necrosis factor-alpha. *Journal of Neuroimmunology*, *162*(1–2), 89–96. http://doi.org/10.1016/j.jneuroim.2005.01.015
- Labelle, M., Begum, S., & Hynes, R. O. (2011). Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell*, *20*(5), 576–590. http://doi.org/10.1016/j.ccr.2011.09.009
- Labelle, M., & Hynes, R. O. (2012). The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. *Cancer Discovery*, *2*(12), 1091–1099. http://doi.org/10.1158/2159-8290.CD-12-0329
- Lackey, K., Cory, M., Davis, R., Frye, S. V, Harris, P. A., Hunter, R. N., ... Wood, E. R. (2000). The discovery of potent cRaf1 kinase inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *10*(3), 223–226.
- Lacoste, B., Comin, C. H., Ben-Zvi, A., Kaeser, P. S., Xu, X., Costa, L. da F., & Gu, C. (2014). Sensory-related neural activity regulates the structure of vascular networks in the cerebral cortex. *Neuron*, *83*(5), 1117–1130. http://doi.org/10.1016/j.neuron.2014.07.034
- Lai, C.-H., & Kuo, K.-H. (2005). The critical component to establish in vitro BBB model: Pericyte. *Brain Research. Brain Research Reviews*, *50*(2), 258–265. http://doi.org/10.1016/j.brainresrev.2005.07.004
- Lambert, A. W., Pattabiraman, D. R., & Weinberg, R. A. (2017). Emerging Biological Principles of Metastasis. *Cell*, *168*(4), 670–691. http://doi.org/10.1016/j.cell.2016.11.037
- Lampson, L. A. (2011). Monoclonal antibodies in neuro-oncology: Getting past the blood-brain barrier.

MAbs, *3*(2), 153–160.
- Lampugnani, M.G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L.P., Dejana, E. (1992). A Novel Endothelial-specific Membrane Protein Is a Marker of Call-Cell Contacts. *The Journal of Cell Biology*, *118*(6).
- Laplante, M., & Sabatini, D. M. (2012, April). mTOR signaling in growth control and disease. *Cell*. http://doi.org/10.1016/j.cell.2012.03.017
- Laufs, U., & Liao, J. K. (1998). Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *The Journal of Biological Chemistry*, *273*(37), 24266–24271.
- Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology*, *1*(6), a001651. http://doi.org/10.1101/cshperspect.a001651
- Le Gal, K., Ibrahim, M. X., Wiel, C., Sayin, V. I., Akula, M. K., Karlsson, C., ... Bergo, M. O. (2015). Antioxidants can increase melanoma metastasis in mice. *Science Translational Medicine*, *7*(308), 308re8. http://doi.org/10.1126/scitranslmed.aad3740
- Lee, B.-C., Lee, T.-H., Avraham, S., & Avraham, H. K. (2004). Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells. *Molecular Cancer Research : MCR*, *2*(6), 327–338.
- Lee, D.-F., Kuo, H.-P., Chen, C.-T., Hsu, J.-M., Chou, C.-K., Wei, Y., ... Hung, M.-C. (2007). IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell*, *130*(3), 440–455. http://doi.org/10.1016/j.cell.2007.05.058
- Lee, F. S., Peters, R. T., Dang, L. C., & Maniatis, T. (1998). MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(16), 9319–9324.
- Lee, J., Borboa, A. K., Chun, H. B., Baird, A., & Eliceiri, B. P. (2010). Conditional deletion of the focal adhesion kinase FAK alters remodeling of the blood-brain barrier in glioma. *Cancer Research*, *70*(24), 10131–10140. http://doi.org/10.1158/0008-5472.CAN-10-2740
- Lee, J. H., Zheng, Y., von Bornstadt, D., Wei, Y., Balcioglu, A., Daneshmand, A., ... Ayata, C. (2014). Selective ROCK2 Inhibition In Focal Cerebral Ischemia. *Annals of Clinical and Translational Neurology*, *1*(1), 2–14. http://doi.org/10.1002/acn3.19
- Lee, K. Y., Kim, Y.-J., Yoo, H., Lee, S. H., Park, J. B., & Kim, H. J. (2011). Human brain endothelial cell-derived COX-2 facilitates extravasation of breast cancer cells across the blood-brain barrier. *Anticancer Research*, *31*(12), 4307–4313.
- Lee, M. Y., Luciano, A. K., Ackah, E., Rodriguez-Vita, J., Bancroft, T. A., Eichmann, A., ... Sessa, W. C. (2014). Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(35), 12865–12870. http://doi.org/10.1073/pnas.1408472111
- Lee, S. H., & Dominguez, R. (2010). Regulation of actin cytoskeleton dynamics in cells. *Molecules and Cells*, *29*(4), 311–325. http://doi.org/10.1007/s10059-010-0053-8
- Lee, T.-H., Avraham, H. K., Jiang, S., & Avraham, S. (2003). Vascular endothelial growth factor modulates the transendothelial migration of MDA-MB-231 breast cancer cells through regulation of brain microvascular endothelial cell permeability. *The Journal of Biological Chemistry*, *278*(7), 5277–5284. http://doi.org/10.1074/jbc.M210063200
- Lejeune, F. J., Lienard, D., Matter, M., & Ruegg, C. (2006). Efficiency of recombinant human TNF in human cancer therapy. *Cancer Immunity*, *6*, 6.
- Lelekakis, M., Moseley, J. M., Martin, T. J., Hards, D., Williams, E., Ho, P., ... Anderson, R. L. (1999). A novel orthotopic model of breast cancer metastasis to bone. *Clinical & Experimental Metastasis*, *17*(2), 163–170.
- Lemos, C., Jansen, G., & Peters, G. J. (2008). Drug transporters: recent advances concerning BCRP and tyrosine kinase inhibitors. *British Journal of Cancer*, *98*(5), 857–862. http://doi.org/10.1038/sj.bjc.6604213
- Lennikov, A., Hiraoka, M., Abe, A., Ohno, S., Fujikawa, T., Itai, A., & Ohguro, H. (2014). IkappaB kinase-beta inhibitor IMD-0354 beneficially suppresses retinal vascular permeability in streptozotocin-induced diabetic mice. *Investigative Ophthalmology & Visual Science*, *55*(10), 6365–6373. http://doi.org/10.1167/iovs.14-14671
- Lennikov, A., Mirabelli, P., Mukwaya, A., Schaupper, M., Thangavelu, M., Lachota, M., ... Lagali, N. (2018). Selective IKK2 inhibitor IMD0354 disrupts NF-kappaB signaling to suppress corneal inflammation and angiogenesis. *Angiogenesis*, *21*(2), 267–285. http://doi.org/10.1007/s10456-018-9594-9
- Leong, H. S., Robertson, A. E., Stoletov, K., Leith, S. J., Chin, C. A., Chien, A. E., ... Lewis, J. D. (2014). Invadopodia are required for cancer cell extravasation and are a therapeutic target for

metastasis. *Cell Reports*, *8*(5), 1558–1570. http://doi.org/10.1016/j.celrep.2014.07.050
- Lernbecher, T., Muller, U., & Wirth, T. (1993). Distinct NF-kappa B/Rel transcription factors are responsible for tissue-specific and inducible gene activation. *Nature*, *365*(6448), 767–770. http://doi.org/10.1038/365767a0
- Lewandowski, M. (1900). Zur Lehre von der Cerebrospinalflüssigkeit. *Z Klin Forsch*, *40*, 480–494.
- Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H., ... Goeddel, D. V. (1991a). Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(7), 2830–2834.
- Lewis, M., Tartaglia, L. a, Lee, a, Bennett, G. L., Rice, G. C., Wong, G. H., ... Goeddel, D. V. (1991b). Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(7), 2830–2834. http://doi.org/10.1073/pnas.88.7.2830
- Ley, K., Laudanna, C., Cybulsky, M. I., & Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature Reviews. Immunology*, *7*(9), 678–689. http://doi.org/10.1038/nri2156
- Li, C.-H., Shyu, M.-K., Jhan, C., Cheng, Y.-W., Tsai, C.-H., Liu, C.-W., ... Kang, J.-J. (2015). Gold Nanoparticles Increase Endothelial Paracellular Permeability by Altering Components of Endothelial Tight Junctions, and Increase Blood-Brain Barrier Permeability in Mice. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *148*(1), 192–203. http://doi.org/10.1093/toxsci/kfv176
- Li, G., D'Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., Klippel, A., Williams, L. T., & Stahl, P. D. (1995). Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(22), 10207–10211.
- Li, Q., Yang, J., Yu, Q., Wu, H., Liu, B., Xiong, H., ... Liao, Z. (2013). Associations between single-nucleotide polymorphisms in the PI3K-PTEN-AKT-mTOR pathway and increased risk of brain metastasis in patients with non-small cell lung cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *19*(22), 6252–6260. http://doi.org/10.1158/1078-0432.CCR-13-1093
- Li, X., Liu, L., Tupper, J. C., Bannerman, D. D., Winn, R. K., Sebti, S. M., ... Harlan, J. M. (2002). Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. *The Journal of Biological Chemistry*, *277*(18), 15309–15316. http://doi.org/10.1074/jbc.M201253200
- Li, X., Yang, Y., & Ashwell, J. D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature*, *416*(6878), 345–347. http://doi.org/10.1038/416345a
- Li, Y., Fanning, A. S., Anderson, J. M., & Lavie, A. (2005). Structure of the conserved cytoplasmic C-terminal domain of occludin: identification of the ZO-1 binding surface. *Journal of Molecular Biology*, *352*(1), 151–164. http://doi.org/10.1016/j.jmb.2005.07.017
- Liang, J., & Slingerland, J. M. (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle (Georgetown, Tex.)*, *2*(4), 339–345.
- Licciulli, S., Maksimoska, J., Zhou, C., Troutman, S., Kota, S., Liu, Q., ... Kissil, J. L. (2013). FRAX597, a small molecule inhibitor of the p21-activated kinases, inhibits tumorigenesis of neurofibromatosis type 2 (NF2)-associated Schwannomas. *The Journal of Biological Chemistry*, *288*(40), 29105–29114. http://doi.org/10.1074/jbc.M113.510933
- Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C. J., ... Dejana, E. (2008). Wnt/beta-catenin signaling controls development of the blood-brain barrier. *The Journal of Cell Biology*, *183*(3), 409–417. http://doi.org/10.1083/jcb.200806024
- Lim, S.-T., Chen, X. L., Tomar, A., Miller, N. L. G., Yoo, J., & Schlaepfer, D. D. (2010). Knock-in mutation reveals an essential role for focal adhesion kinase activity in blood vessel morphogenesis and cell motility-polarity but not cell proliferation. *The Journal of Biological Chemistry*, *285*(28), 21526–21536. http://doi.org/10.1074/jbc.M110.129999
- Lim, S.-T., Miller, N. L. G., Chen, X. L., Tancioni, I., Walsh, C. T., Lawson, C., ... Schlaepfer, D. D. (2012). Nuclear-localized focal adhesion kinase regulates inflammatory VCAM-1 expression. *The Journal of Cell Biology*, *197*(7), 907–919. http://doi.org/10.1083/jcb.201109067
- Lindahl, P., Johansson, B. R., Leveen, P., & Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science (New York, N.Y.)*, *277*(5323), 242–245.
- Linkermann, A., & Green, D. R. (2014). Necroptosis. *The New England Journal of Medicine*, *370*(5), 455–465. http://doi.org/10.1056/NEJMra1310050
- Lippmann, E. S., Al-Ahmad, A., Azarin, S. M., Palecek, S. P., & Shusta, E. V. (2014). A retinoic acid-

enhanced, multicellular human blood-brain barrier model derived from stem cell sources. *Scientific Reports*, *4*, 4160. http://doi.org/10.1038/srep04160

- Liu, F., Schaphorst, K. L., Verin, A. D., Jacobs, K., Birukova, A., Day, R. M., ... Garcia, J. G. N. (2002). Hepatocyte growth factor enhances endothelial cell barrier function and cortical cytoskeletal rearrangement: potential role of glycogen synthase kinase-3beta. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *16*(9), 950–962. http://doi.org/10.1096/fj.01-0870com
- Liu, L., Das, S., Losert, W., & Parent, C. A. (2010). mTORC2 regulates neutrophil chemotaxis in a cAMP- and RhoA-dependent fashion. *Developmental Cell*, *19*(6), 845–857. http://doi.org/10.1016/j.devcel.2010.11.004
- Liu, L., Xue, Y., & Liu, Y. (2010). Bradykinin increases the permeability of the blood-tumor barrier by the caveolae-mediated transcellular pathway. *Journal of Neuro-Oncology*, *99*(2), 187–194. http://doi.org/10.1007/s11060-010-0124-x
- Liu, P., Gan, W., Chin, Y. R., Ogura, K., Guo, J., Zhang, J., ... Wei, W. (2015). PtdIns(3,4,5)P3-Dependent Activation of the mTORC2 Kinase Complex. *Cancer Discovery*, *5*(11), 1194–1209. http://doi.org/10.1158/2159-8290.CD-15-0460
- Liu, W.-Y., Wang, Z.-B., Wang, Y., Tong, L.-C., Li, Y., Wei, X., ... Li, L. (2015). Increasing the Permeability of the Blood-brain Barrier in Three Different Models in vivo. *CNS Neuroscience & Therapeutics*, *21*(7), 568–574. http://doi.org/10.1111/cns.12405
- Liu, Z. G., Hsu, H., Goeddel, D. V, & Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell*, *87*(3), 565–576.
- Lockman, P. R., Mittapalli, R. K., Taskar, K. S., Rudraraju, V., Gril, B., Bohn, K. A., ... Smith, Q. R. (2010). Heterogeneous blood-tumor barrier permeability determines drug efficacy in experimental brain metastases of breast cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *16*(23), 5664–5678. http://doi.org/10.1158/1078-0432.CCR-10-1564
- Lockshin, A., Giovanella, B. C., & Stehlin, J. S. J. (1986). Quantitative evaluation of anticancer agents against human melanoma cells implanted in nude mice. *Experimental Cell Biology*, *54*(3), 149–154.
- Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., ... Hall, M. N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Molecular Cell*, *10*(3), 457–468.
- Loirand, G., Guerin, P., & Pacaud, P. (2006). Rho kinases in cardiovascular physiology and pathophysiology. *Circulation Research*, *98*(3), 322–334. http://doi.org/10.1161/01.RES.0000201960.04223.3c
- Lombardi, A., Cantini, G., Mello, T., Francalanci, M., Gelmini, S., Cosmi, L., ... Luconi, M. (2009). Molecular mechanisms underlying the pro-inflammatory synergistic effect of tumor necrosis factor ?? and interferon ?? in human microvascular endothelium. *European Journal of Cell Biology*, *88*(12), 731–742. http://doi.org/10.1016/j.ejcb.2009.07.004
- Lopez-Ramirez, M. A., Fischer, R., Claudia, C., Davies, H. A., Logan, K., Male, D. K., ... Romero, I. A. (2012). Role of Caspases in Cytokine-Induced Barrier Breakdown in Human Brain Endothelial Cells. http://doi.org/10.4049/jimmunol.1103460
- Lorger, M., & Felding-Habermann, B. (2010). Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *The American Journal of Pathology*, *176*(6), 2958–2971. http://doi.org/10.2353/ajpath.2010.090838
- Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., ... Ellison, D. W. (2016). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathologica*, *131*(6), 803–820. http://doi.org/10.1007/s00401-016-1545-1
- Loulier, K., Lathia, J. D., Marthiens, V., Relucio, J., Mughal, M. R., Tang, S.-C., ... Ffrench-Constant, C. (2009). beta1 integrin maintains integrity of the embryonic neocortical stem cell niche. *PLoS Biology*, *7*(8), e1000176. http://doi.org/10.1371/journal.pbio.1000176
- Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., ... Kipnis, J. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature*, *523*(7560), 337–341. http://doi.org/10.1038/nature14432
- Lu, W., Bucana, C. D., & Schroit, A. J. (2007). Pathogenesis and vascular integrity of breast cancer brain metastasis. *International Journal of Cancer*, *120*(5), 1023–1026. http://doi.org/10.1002/ijc.22388
- Lucas, R., Echtenacher, B., Sablon, E., Juillard, P., Magez, S., Lou, J., ... de Baetselier, P. (1997). Generation of a mouse tumor necrosis factor mutant with antiperitonitis and desensitization activities

comparable to those of the wild type but with reduced systemic toxicity. *Infection and Immunity*, *65*(6), 2006–2010.

- Lucas, R., Garcia, I., Donati, Y. R. A., Hribar, M., Stefano, J., Giroud, C., ... Grau, G. E. (1998). Both TNF receptors are required for direct TNF- mediated cytotoxicity in microvascular endothelial cells, (April 2016), 3577–3586. http://doi.org/10.1002/(SICI)1521-4141(199811)28
- Luh, C., Kuhlmann, C. R., Ackermann, B., Timaru-Kast, R., Luhmann, H. J., Behl, C., ... Thal, S. C. (2010). Inhibition of myosin light chain kinase reduces brain edema formation after traumatic brain injury. *Journal of Neurochemistry*, *112*(4), 1015–1025. http://doi.org/10.1111/j.1471-4159.2009.06514.x
- Lv, S., Song, H.-L., Zhou, Y., Li, L.-X., Cui, W., Wang, W., & Liu, P. (2010). Tumour necrosis factor-alpha affects blood-brain barrier permeability and tight junction-associated occludin in acute liver failure. *Liver International : Official Journal of the International Association for the Study of the Liver*, *30*(8), 1198–1210. http://doi.org/10.1111/j.1478-3231.2010.02211.x
- Lyle, T., Lockman,P., Adkins, C., Steeg,P., B. G. (2016). Alterations in Pericyte Subpopulations are Associated with Elevated Blood-Tumor Barrier Permeability in Experimental Brain Metastasis of Breast Cancer. L. Tiffany Lyle. *Clinical Cancer Research*. http://doi.org/10.1158/1078-0432.CCR-15-1836
- Lynch, C. J., Halle, B., Fujii, H., Vary, T. C., Wallin, R., Damuni, Z., & Hutson, S. M. (2003). Potential role of leucine metabolism in the leucine-signaling pathway involving mTOR. *American Journal of Physiology. Endocrinology and Metabolism*, *285*(4), E854-63. http://doi.org/10.1152/ajpendo.00153.2003
- Lynch, C. N., Wang, Y. C., Lund, J. K., Chen, Y. W., Leal, J. A., & Wiley, S. R. (1999). TWEAK induces angiogenesis and proliferation of endothelial cells. *The Journal of Biological Chemistry*, *274*(13), 8455–8459.
- Ma, C. X., Sanchez, C., Gao, F., Crowder, R., Naughton, M., Pluard, T., ... Ellis, M. J. (2016). A Phase I Study of the AKT Inhibitor MK-2206 in Combination with Hormonal Therapy in Postmenopausal Women with Estrogen Receptor-Positive Metastatic Breast Cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *22*(11), 2650–2658. http://doi.org/10.1158/1078-0432.CCR-15-2160
- Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., & Pandolfi, P. P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell*, *121*(2), 179–193. http://doi.org/10.1016/j.cell.2005.02.031
- Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A., & Said, H. M. (2004). TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *286*(3), G367-76. http://doi.org/10.1152/ajpgi.00173.2003
- Maalouf, S. W., Talhouk, R. S., & Schanbacher, F. L. (2010). Inflammatory responses in epithelia: endotoxin-induced IL-6 secretion and iNOS/NO production are differentially regulated in mouse mammary epithelial cells. *Journal of Inflammation (London, England)*, *7*, 58. http://doi.org/10.1186/1476-9255-7-58
- Mackay, F., Loetscher, H., Stueber, D., Gehr, G., & Lesslauer, W. (1993). Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. *The Journal of Experimental Medicine*, *177*(5), 1277–1286.
- Madge, L. A., & Pober, J. S. (2000). A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells. *The Journal of Biological Chemistry*, *275*(20), 15458–15465. http://doi.org/10.1074/jbc.M001237200
- Madge, L. A., & Pober, J. S. (2001). TNF signaling in vascular endothelial cells. *Experimental and Molecular Pathology*, *70*(3), 317–325. http://doi.org/10.1006/exmp.2001.2368
- Madrid, L. V, Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S. J., & Mayo, M. W. (2000). Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Molecular and Cellular Biology*, *20*(5), 1626–1638.
- Malin, D., Strekalova, E., Petrovic, V., Deal, A. M., Al Ahmad, A., Adamo, B., ... Cryns, V. L. (2014). alphaB-crystallin: a novel regulator of breast cancer metastasis to the brain. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *20*(1), 56–67. http://doi.org/10.1158/1078-0432.CCR-13-1255
- Mammoto, T., Parikh, S. M., Mammoto, A., Gallagher, D., Chan, B., Mostoslavsky, G., ... Sukhatme, V. P. (2007). Angiopoietin-1 requires p190 RhoGAP to protect against vascular leakage in vivo. *The Journal of Biological Chemistry*, *282*(33), 23910–23918. http://doi.org/10.1074/jbc.M702169200
- Manaenko, A., Yang, P., Nowrangi, D., Budbazar, E., Hartman, R. E., Obenaus, A., ... Tang, J. (2018).

Inhibition of stress fiber formation preserves blood-brain barrier after intracerebral hemorrhage in mice. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *38*(1), 87–102. http://doi.org/10.1177/0271678X16679169

- Manne, V., Ricca, C. S., Brown, J. G., Tuomari, A. V, Yan, N., Patel, D., ... Biller, S. A. (1995). Ras farnesylation as a target for novel antitumor agents: Potent and selective farnesyl diphosphate analog inhibitors of farnesyltransferase. *Drug Development Research*, *34*(2), 121–137. http://doi.org/10.1002/ddr.430340205
- Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell*, *129*(7), 1261–1274. http://doi.org/10.1016/j.cell.2007.06.009
- Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., & Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Molecular Cell*, *10*(1), 151–162.
- Manning, B. D., & Toker, A. (2017). AKT/PKB Signaling: Navigating the Network. *Cell*, *169*(3), 381–405. http://doi.org/10.1016/j.cell.2017.04.001
- Manser, E., Huang, H. Y., Loo, T. H., Chen, X. Q., Dong, J. M., Leung, T., & Lim, L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Molecular and Cellular Biology*, *17*(3), 1129–1143.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., & Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*, *367*(6458), 40–46. http://doi.org/10.1038/367040a0
- Maqbool, M., Mobashir, M., & Hoda, N. (2016). Pivotal role of glycogen synthase kinase-3: A therapeutic target for Alzheimer's disease. *European Journal of Medicinal Chemistry*, *107*, 63–81. http://doi.org/10.1016/j.ejmech.2015.10.018
- Margolin, K., Ernstoff, M. S., Hamid, O., Lawrence, D., McDermott, D., Puzanov, I., ... Hodi, F. S. (2012). Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *The Lancet. Oncology*, *13*(5), 459–465. http://doi.org/10.1016/S1470-2045(12)70090-6
- Marinissen, M. J., Chiariello, M., Tanos, T., Bernard, O., Narumiya, S., & Gutkind, J. S. (2004). The small GTP-binding protein RhoA regulates c-jun by a ROCK-JNK signaling axis. *Molecular Cell*, *14*(1), 29–41.
- Mark, K. S., & Davis, T. P. (2002). Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. *American Journal of Physiology. Heart and Circulatory Physiology*, *282*(4), H1485-94. http://doi.org/10.1152/ajpheart.00645.2001
- Marmarou, A., Portella, G., Barzo, P., Signoretti, S., Fatouros, P., Beaumont, A., ... Bullock, R. (2000). Distinguishing between cellular and vasogenic edema in head injured patients with focal lesions using magnetic resonance imaging. *Acta Neurochirurgica. Supplement*, *76*, 349–351.
- Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y., & Kloog, Y. (1995). Selective inhibition of Ras-dependent cell growth by farnesylthiosalisylic acid. *The Journal of Biological Chemistry*, *270*(38), 22263–22270.
- Martins, D. F., Rosa, A. O., Gadotti, V. M., Mazzardo-Martins, L., Nascimento, F. P., Egea, J., ... Santos, A. R. S. (2011). The antinociceptive effects of AR-A014418, a selective inhibitor of glycogen synthase kinase-3 beta, in mice. *The Journal of Pain : Official Journal of the American Pain Society*, *12*(3), 315–322. http://doi.org/10.1016/j.jpain.2010.06.007
- Mashukova, A., Wald, F. A., & Salas, P. J. (2011). Tumor necrosis factor alpha and inflammation disrupt the polarity complex in intestinal epithelial cells by a posttranslational mechanism. *Molecular and Cellular Biology*, *31*(4), 756–765. http://doi.org/10.1128/MCB.00811-10
- Massague, J., & Obenauf, A. C. (2016). Metastatic colonization by circulating tumour cells. *Nature*, *529*(7586), 298–306. http://doi.org/10.1038/nature17038
- Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., ... Kaibuchi, K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *The EMBO Journal*, *15*(9), 2208–2216.
- Matsukado, K., Inamura, T., Nakano, S., Fukui, M., Bartus, R. T., & Black, K. L. (1996). Enhanced tumor uptake of carboplatin and survival in glioma-bearing rats by intracarotid infusion of bradykinin analog, RMP-7. *Neurosurgery*, *39*(1), 124–125.
- Mayo, M. W., Madrid, L. V, Westerheide, S. D., Jones, D. R., Yuan, X.-J., Baldwin, A. S. J., & Whang, Y. E. (2002). PTEN blocks tumor necrosis factor-induced NF-kappa B-dependent transcription by inhibiting the transactivation potential of the p65 subunit. *The Journal of Biological Chemistry*, *277*(13), 11116–11125. http://doi.org/10.1074/jbc.M108670200
- Mazzardo-Martins, L., Martins, D. F., Stramosk, J., Cidral-Filho, F. J., & Santos, A. R. S. (2012). Glycogen synthase kinase 3-specific inhibitor AR-A014418 decreases neuropathic pain in mice: evidence

for the mechanisms of action. *Neuroscience*, *226*, 411–420. http://doi.org/10.1016/j.neuroscience.2012.09.020

- McKenzie, J. A. G., & Ridley, A. J. (2007). Roles of Rho/ROCK and MLCK in TNF-alpha-induced changes in endothelial morphology and permeability. *Journal of Cellular Physiology*, *213*(1), 221–228. http://doi.org/10.1002/jcp.21114
- McLean, G. W., Komiyama, N. H., Serrels, B., Asano, H., Reynolds, L., Conti, F., ... Frame, M. C. (2004). Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes & Development*, *18*(24), 2998–3003. http://doi.org/10.1101/gad.316304
- Medvedev, A. E., Sundan, A., & Espevik, T. (1994). Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. *European Journal of Immunology*, *24*(11), 2842–2849. http://doi.org/10.1002/eji.1830241139
- Megyeri, P., Abraham, C. S., Temesvari, P., Kovacs, J., Vas, T., & Speer, C. P. (1992). Recombinant human tumor necrosis factor alpha constricts pial arterioles and increases blood-brain barrier permeability in newborn piglets. *Neuroscience Letters*, *148*(1–2), 137–140.
- Mehta, D., Tiruppathi, C., Sandoval, R., Minshall, R. D., Holinstat, M., & Malik, A. B. (2002, March). Modulatory role of focal adhesion kinase in regulating human pulmonary arterial endothelial barrier function. *The Journal of Physiology*. http://doi.org/10.1113/jphysiol.2001.013289
- Meili, R., Sasaki, A. T., & Firtel, R. A. (2005, April). Rho Rocks PTEN. *Nature Cell Biology*. England. http://doi.org/10.1038/ncb0405-334
- Mendes, O., Kim, H.-T., Lungu, G., & Stoica, G. (2007). MMP2 role in breast cancer brain metastasis development and its regulation by TIMP2 and ERK1/2. *Clinical & Experimental Metastasis*, *24*(5), 341–351. http://doi.org/10.1007/s10585-007-9071-0
- Mendes, O., Kim, H.-T., & Stoica, G. (2005). Expression of MMP2, MMP9 and MMP3 in breast cancer brain metastasis in a rat model. *Clinical & Experimental Metastasis*, *22*(3), 237–246. http://doi.org/10.1007/s10585-005-8115-6
- Messer, G., Weiss, E. H., & Baeuerle, P. A. (1990). Tumor necrosis factor beta (TNF-beta) induces binding of the NF-kappa B transcription factor to a high-affinity kappa B element in the TNF-beta promoter. *Cytokine*, *2*(6), 389–397.
- Messmer, U. K., Briner, V. A., & Pfeilschifter, J. (2000). Basic fibroblast growth factor selectively enhances TNF-alpha-induced apoptotic cell death in glomerular endothelial cells: effects on apoptotic signaling pathways. *Journal of the American Society of Nephrology : JASN*, *11*(12), 2199–2211.
- Miles, F. L., Pruitt, F. L., van Golen, K. L., & Cooper, C. R. (2008). Stepping out of the flow: capillary extravasation in cancer metastasis. *Clinical & Experimental Metastasis*, *25*(4), 305–324. http://doi.org/10.1007/s10585-007-9098-2
- Millan, J., Cain, R. J., Reglero-Real, N., Bigarella, C., Marcos-Ramiro, B., Fernandez-Martin, L., ... Ridley, A. J. (2010). Adherens junctions connect stress fibres between adjacent endothelial cells. *BMC Biology*, *8*, 11. http://doi.org/10.1186/1741-7007-8-11
- Miller, D. S., Bauer, B., & Hartz, A. M. S. (2008). Modulation of P-glycoprotein at the blood-brain barrier: opportunities to improve central nervous system pharmacotherapy. *Pharmacological Reviews*, *60*(2), 196–209. http://doi.org/10.1124/pr.107.07109
- Miller, F., Fenart, L., Landry, V., Coisne, C., Cecchelli, R., Dehouck, M.-P., & Buee-Scherrer, V. (2005). The MAP kinase pathway mediates transcytosis induced by TNF-alpha in an in vitro blood-brain barrier model. *The European Journal of Neuroscience*, *22*(4), 835–844. http://doi.org/10.1111/j.1460-9568.2005.04273.x
- Miller, F. R. (1983). Tumor subpopulation interactions in metastasis. *Invasion & Metastasis*, *3*(4), 234–242.
- Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., ... Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science (New York, N.Y.)*, *266*(5191), 1719–1723.
- Minden, K., Aganna, E., McDermott, M. F., & Zink, A. (2004, October). Tumour necrosis factor receptor associated periodic syndrome (TRAPS) with central nervous system involvement. *Annals of the Rheumatic Diseases*. England. http://doi.org/10.1136/ard.2003.016006
- Ming, X.-F., Viswambharan, H., Barandier, C., Ruffieux, J., Kaibuchi, K., Rusconi, S., & Yang, Z. (2002). Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Molecular and Cellular Biology*, *22*(24), 8467–8477.
- Mirzapoiazova, T., Moitra, J., Moreno-Vinasco, L., Sammani, S., Turner, J. R., Chiang, E. T., ... Garcia, J. G. N. (2011). Non-muscle myosin light chain kinase isoform is a viable molecular target in

acute inflammatory lung injury. *American Journal of Respiratory Cell and Molecular Biology*, *44*(1), 40–52. http://doi.org/10.1165/rcmb.2009-0197OC

- Mitra, S. K., Hanson, D. A., & Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nature Reviews. Molecular Cell Biology*, *6*(1), 56–68. http://doi.org/10.1038/nrm1549
- Miyazaki, T., Honda, K., & Ohata, H. (2010). m-Calpain antagonizes RhoA overactivation and endothelial barrier dysfunction under disturbed shear conditions. *Cardiovascular Research*, *85*(3), 530–541. http://doi.org/10.1093/cvr/cvp311
- Modur, V., Zimmerman, G. A., Prescott, S. M., & McIntyre, T. M. (1996). Endothelial cell inflammatory responses to tumor necrosis factor alpha. Ceramide-dependent and -independent mitogen-activated protein kinase cascades. *The Journal of Biological Chemistry*, *271*(22), 13094–13102.
- Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., & Nagatsu, T. (1994). Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neuroscience Letters*, *165*(1–2), 208–210.
- Mohammadi, M., Froum, S., Hamby, J. M., Schroeder, M. C., Panek, R. L., Lu, G. H., ... Hubbard, S. R. (1998). Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. *The EMBO Journal*, *17*(20), 5896–5904. http://doi.org/10.1093/emboj/17.20.5896
- Molnar, J., Fazakas, C., Hasko, J., Sipos, O., Nagy, K., Nyul-Toth, A., ... Wilhelm, I. (2016). Transmigration characteristics of breast cancer and melanoma cells through the brain endothelium: Role of Rac and PI3K. *Cell Adhesion & Migration*, *10*(3), 269–281. http://doi.org/10.1080/19336918.2015.1122156
- Mong, P. Y., Petrulio, C., Kaufman, H. L., & Wang, Q. (2008). Activation of Rho kinase by TNF-alpha is required for JNK activation in human pulmonary microvascular endothelial cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(1), 550–558.
- Mong, P. Y., & Wang, Q. (2009). Activation of Rho kinase isoforms in lung endothelial cells during inflammation. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(4), 2385–2394. http://doi.org/10.4049/jimmunol.0802811
- Montalvo-Ortiz, B. L., Castillo-Pichardo, L., Hernandez, E., Humphries-Bickley, T., De la Mota-Peynado, A., Cubano, L. A., ... Dharmawardhane, S. (2012). Characterization of EHop-016, novel small molecule inhibitor of Rac GTPase. *The Journal of Biological Chemistry*, *287*(16), 13228–13238. http://doi.org/10.1074/jbc.M111.334524
- Mordhorst, B. R., Murphy, S. L., Ross, R. M., Samuel, M. S., Salazar, S. R., Ji, T., ... Prather, R. S. (2018). Pharmacologic Reprogramming Designed to Induce a Warburg Effect in Porcine Fetal Fibroblasts Alters Gene Expression and Quantities of Metabolites from Conditioned Media Without Increased Cell Proliferation. *Cellular Reprogramming*, *20*(1), 38–48. http://doi.org/10.1089/cell.2017.0040
- Morikawa, A., Peereboom, D. M., Thorsheim, H. R., Samala, R., Balyan, R., Murphy, C. G., ... Seidman, A. D. (2015). Capecitabine and lapatinib uptake in surgically resected brain metastases from metastatic breast cancer patients: a prospective study. *Neuro-Oncology*, *17*(2), 289–295. http://doi.org/10.1093/neuonc/nou141
- MRC Phosphorylation and Ubiquitylation Unit. (2018a). AR-A014418: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018b). BMS345541: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018c). BX-795: Kinase Profiling Inhibitor Database. Retrieved from http://www.kinase-screen.mrc.ac.uk/screening-compounds/341044
- MRC Phosphorylation and Ubiquitylation Unit. (2018d). Fasudil: Kinase Profiling Inhibitor Database. Retrieved from http://www.kinase-screen.mrc.ac.uk/screening-compounds/341069
- MRC Phosphorylation and Ubiquitylation Unit. (2018e). GSK2334470: Kinase Profiling Inhibitor Database. Retrieved from http://www.kinase-screen.mrc.ac.uk/screening-compounds/348819
- MRC Phosphorylation and Ubiquitylation Unit. (2018f). GW5074: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018g). IMD-0354: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018h). Kinase Profiling Inhibitor Database. Retrieved from http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors
- MRC Phosphorylation and Ubiquitylation Unit. (2018i). LY294002: Kinase Profiling Inhibitor Database. Retrieved from http://www.kinase-screen.mrc.ac.uk/screening-compounds/345899
- MRC Phosphorylation and Ubiquitylation Unit. (2018j). MK-2206: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018k). Necrostatin-1: Kinase Profiling Inhibitor

Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018l). Piceatannol: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018m). Rapamycin: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018n). SP600125: Kinase Profiling Inhibitor Database.
- MRC Phosphorylation and Ubiquitylation Unit. (2018o). Y-27632: Kinase Profiling Inhibitor Database.
- Müller, W., Burgart, L. J., Krause-Paulus, R., Thibodeau, S. N., Almeida, M., Edmonston, T. B., ... Moslein, G. (2001). The reliability of immunohistochemistry as a prescreening method for the diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC)--results of an international collaborative study. *Familial Cancer*, *1*(2), 87–92.
- Murrell, D. H., Hamilton, A. M., Mallett, C. L., van Gorkum, R., Chambers, A. F., & Foster, P. J. (2015). Understanding Heterogeneity and Permeability of Brain Metastases in Murine Models of HER2-Positive Breast Cancer Through Magnetic Resonance Imaging: Implications for Detection and Therapy. *Translational Oncology*, *8*(3), 176–184. http://doi.org/10.1016/j.tranon.2015.03.009
- Murrell, D. H., Zarghami, N., Jensen, M. D., Chambers, A. F., Wong, E., & Foster, P. J. (2016). Evaluating Changes to Blood-Brain Barrier Integrity in Brain Metastasis over Time and after Radiation Treatment. *Translational Oncology*, *9*(3), 219–227. http://doi.org/10.1016/j.tranon.2016.04.006
- Nagumo, H., Sasaki, Y., Ono, Y., Okamoto, H., Seto, M., & Takuwa, Y. (2000). Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. *American Journal of Physiology. Cell Physiology*, *278*(1), C57-65. http://doi.org/10.1152/ajpcell.2000.278.1.C57
- Naikawadi, R. P., Cheng, N., Vogel, S. M., Qian, F., Wu, D., Malik, A. B., & Ye, R. D. (2012). A critical role for phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 1 in endothelial junction disruption and vascular hyperpermeability. *Circulation Research*, *111*(12), 1517–1527. http://doi.org/10.1161/CIRCRESAHA.112.273078
- Najafov, A., Sommer, E. M., Axten, J. M., Deyoung, M. P., & Alessi, D. R. (2011). Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. *The Biochemical Journal*, *433*(2), 357–369. http://doi.org/10.1042/BJ20101732
- Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., & Okumura, K. (1998). Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(7), 3537–3542.
- Nakashima, S. (2002). Protein kinase C alpha (PKC alpha): regulation and biological function. *Journal of Biochemistry*, *132*(5), 669–675.
- Nayak, L., Lee, E. Q., & Wen, P. Y. (2012). Epidemiology of brain metastases. *Current Oncology Reports*, *14*(1), 48–54. http://doi.org/10.1007/s11912-011-0203-y
- Nemoto, S., DiDonato, J. A., & Lin, A. (1998). Coordinate regulation of IkappaB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-kappaB-inducing kinase. *Molecular and Cellular Biology*, *18*(12), 7336–7343.
- Neuwelt, E. A., Glasberg, M., Frenkel, E., & Barnett, P. (1983). Neurotoxicity of chemotherapeutic agents after blood-brain barrier modification: neuropathological studies. *Annals of Neurology*, *14*(3), 316–324. http://doi.org/10.1002/ana.410140310
- Neuwelt, E., Abbott, N. J., Abrey, L., Banks, W. a., Blakley, B., Davis, T., ... Drewes, L. R. (2008). Strategies to advance translational research into brain barriers. *The Lancet Neurology*, *7*(1), 84–96. http://doi.org/10.1016/S1474-4422(07)70326-5
- Nguyen, D. X., Bos, P. D., & Massague, J. (2009). Metastasis: from dissemination to organ-specific colonization. *Nature Reviews. Cancer*, *9*(4), 274–284. http://doi.org/10.1038/nrc2622
- Ni, Y., Teng, T., Li, R., Simonyi, A., Sun, G. Y., & Lee, J. C. (2017). TNFalpha alters occludin and cerebral endothelial permeability: Role of p38MAPK. *PloS One*, *12*(2), e0170346. http://doi.org/10.1371/journal.pone.0170346
- Niego, B., Freeman, R., Puschmann, T. B., Turnley, A. M., & Medcalf, R. L. (2012). t-PA-specific modulation of a human blood-brain barrier model involves plasmin-mediated activation of the Rho kinase pathway in astrocytes. *Blood*, *119*(20), 4752–4761. http://doi.org/10.1182/blood-2011-07-369512
- Niego, B., Lee, N., Larsson, P., De Silva, T. M., Au, A. E.-L., McCutcheon, F., & Medcalf, R. L. (2017). Selective inhibition of brain endothelial Rho-kinase-2 provides optimal protection of an in vitro blood-brain barrier from tissue-type plasminogen activator and plasmin. *PloS One*, *12*(5), e0177332. http://doi.org/10.1371/journal.pone.0177332
- Nieto, M. A., Huang, R. Y.-J., Jackson, R. A., & Thiery, J. P. (2016). EMT: 2016. *Cell*, *166*(1), 21–45.

http://doi.org/10.1016/j.cell.2016.06.028
- Niles, W. D., & Malik, A. B. (1999). Endocytosis and exocytosis events regulate vesicle traffic in endothelial cells. *The Journal of Membrane Biology*, *167*(1), 85–101.
- Nishioku, T., Dohgu, S., Takata, F., Eto, T., Ishikawa, N., Kodama, K. B., ... Kataoka, Y. (2009). Detachment of brain pericytes from the basal lamina is involved in disruption of the blood-brain barrier caused by lipopolysaccharide-induced sepsis in mice. *Cellular and Molecular Neurobiology*, *29*(3), 309–316. http://doi.org/10.1007/s10571-008-9322-x
- Nobes, C. D., & Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, *81*(1), 53–62.
- Nomoto, Y., Miyamoto, T., & Yamaguchi, Y. (1994). Brain metastasis of small cell lung carcinoma: comparison of Gd-DTPA enhanced magnetic resonance imaging and enhanced computerized tomography. *Japanese Journal of Clinical Oncology*, *24*(5), 258–262.
- Nonaka, M., Chen, X. H., Pierce, J. E., Leoni, M. J., McIntosh, T. K., Wolf, J. A., & Smith, D. H. (1999). Prolonged activation of NF-kappaB following traumatic brain injury in rats. *Journal of Neurotrauma*, *16*(11), 1023–1034. http://doi.org/10.1089/neu.1999.16.1023
- Norden, D. M., Trojanowski, P. J., Villanueva, E., Navarro, E., & Godbout, J. P. (2016). Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge. *Glia*, *64*(2), 300–316. http://doi.org/10.1002/glia.22930
- Nwariaku, F. E., Liu, Z., Zhu, X., Turnage, R. H., Sarosi, G. A., & Terada, L. S. (2002). Tyrosine phosphorylation of vascular endothelial cadherin and the regulation of microvascular permeability. *Surgery*, *132*(2), 180–185.
- O'Brien, E. R., Kersemans, V., Tredwell, M., Checa, B., Serres, S., Soto, M. S., ... Sibson, N. R. (2014). Glial activation in the early stages of brain metastasis: TSPO as a diagnostic biomarker. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine*, *55*(2), 275–280. http://doi.org/10.2967/jnumed.113.127449
- O'Carroll, S. J., Kho, D. T., Wiltshire, R., Nelson, V., Rotimi, O., Johnson, R., ... Graham, E. S. (2015). Pro-inflammatory TNFalpha and IL-1beta differentially regulate the inflammatory phenotype of brain microvascular endothelial cells. *Journal of Neuroinflammation*, *12*, 131. http://doi.org/10.1186/s12974-015-0346-0
- O'Mahony, A., Lin, X., Geleziunas, R., & Greene, W. C. (2000). Activation of the heterodimeric IkappaB kinase alpha (IKKalpha)-IKKbeta complex is directional: IKKalpha regulates IKKbeta under both basal and stimulated conditions. *Molecular and Cellular Biology*, *20*(4), 1170–1178.
- O'Malley, W. E., Achinstein, B., & Shear, M. J. (1962). Action of Bacterial Polysaccharide on Tumors. II. Damage of Sarcoma 37 by Serum of Mice Treated With Serratia Marcescens Polysaccharide, and Induced Tolerance. *JNCI: Journal of the National Cancer Institute*, *29*(6), 1169–1175. http://doi.org/10.1093/jnci/29.6.1169
- Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, *1*(4), a000034. http://doi.org/10.1101/cshperspect.a000034
- Ohtsuki, S., Yamaguchi, H., Katsukura, Y., Asashima, T., & Terasaki, T. (2008). mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. *Journal of Neurochemistry*, *104*(1), 147–154. http://doi.org/10.1111/j.1471-4159.2007.05008.x
- Okada, H., Weller, M., Huang, R., Finocchiaro, G., Gilbert, M. R., Wick, W., ... Reardon, D. A. (2015). Immunotherapy response assessment in neuro-oncology: a report of the RANO working group. *The Lancet. Oncology*, *16*(15), e534–e542. http://doi.org/10.1016/S1470-2045(15)00088-1
- Onesto, C., Shutes, A., Picard, V., Schweighoffer, F., & Der, C. J. (2008). Characterization of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *Methods in Enzymology*, *439*, 111–129. http://doi.org/10.1016/S0076-6879(07)00409-0
- Orosz, P., Echtenacher, B., Falk, W., Ruschoff, J., Weber, D., & Mannel, D. N. (1993). Enhancement of experimental metastasis by tumor necrosis factor. *The Journal of Experimental Medicine*, *177*(5), 1391–1398.
- Orosz, Z. (1999). Melan-A/Mart-1 expression in various melanocytic lesions and in non-melanocytic soft tissue tumours. *Histopathology*, *34*(6), 517–525.
- Orr, A. W., Stockton, R., Simmers, M. B., Sanders, J. M., Sarembock, I. J., Blackman, B. R., & Schwartz, M. A. (2007). Matrix-specific p21-activated kinase activation regulates vascular permeability in atherogenesis. *The Journal of Cell Biology*, *176*(5), 719–727. http://doi.org/10.1083/jcb.200609008
- Osborn, L., Kunkel, S., & Nabel, G. J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate

the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(7), 2336–2340.

- Osswald, M., Blaes, J., Liao, Y., Solecki, G., Gommel, M., Berghoff, A. S., ... Winkler, F. (2016). Impact of Blood-Brain Barrier Integrity on Tumor Growth and Therapy Response in Brain Metastases. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *22*(24), 6078–6087. http://doi.org/10.1158/1078-0432.CCR-16-1327
- Owen, C. A., Hu, Z., Barrick, B., & Shapiro, S. D. (2003). Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *American Journal of Respiratory Cell and Molecular Biology*, *29*(3 Pt 1), 283–294. http://doi.org/10.1165/rcmb.2003-0034OC
- Owens, T., Bechmann, I., & Engelhardt, B. (2008). Perivascular spaces and the two steps to neuroinflammation. *Journal of Neuropathology and Experimental Neurology*, *67*(12), 1113–1121. http://doi.org/10.1097/NEN.0b013e31818f9ca8
- Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E., & Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proceedings of the National Academy of Sciences*, *98*(8), 4640 LP-4645. Retrieved from http://www.pnas.org/content/98/8/4640.abstract
- Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., & Donner, D. B. (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature*, *401*(6748), 82–85. http://doi.org/10.1038/43466
- Paemeleire, K., de Hemptinne, A., & Leybaert, L. (1999). Chemically, mechanically, and hyperosmolarity-induced calcium responses of rat cortical capillary endothelial cells in culture. *Experimental Brain Research*, *126*(4), 473–481.
- Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *The Lancet*, *133*(3421), 571–573. http://doi.org/https://doi.org/10.1016/S0140-6736(00)49915-0
- Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*, *18*(49), 6853–6866. http://doi.org/10.1038/sj.onc.1203239
- Paku, S., Dome, B., Toth, R., & Timar, J. (2000). Organ-specificity of the extravasation process: an ultrastructural study. *Clinical & Experimental Metastasis*, *18*(6), 481–492.
- Palmieri, D., Chambers, A. F., Felding-Habermann, B., Huang, S., & Steeg, P. S. (2007). The biology of metastasis to a sanctuary site. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *13*(6), 1656–1662. http://doi.org/10.1158/1078-0432.CCR-06-2659
- Pan, S., An, P., Zhang, R., He, X., Yin, G., & Min, W. (2002). Etk/Bmx as a tumor necrosis factor receptor type 2-specific kinase: role in endothelial cell migration and angiogenesis. *Molecular and Cellular Biology*, *22*(21), 7512–7523.
- Pan, W., Yu, C., Hsuchou, H., Zhang, Y., & Kastin, A. J. (2008). Neuroinflammation facilitates LIF entry into brain: role of TNF. *American Journal of Physiology. Cell Physiology*, *294*(6), C1436-42. http://doi.org/10.1152/ajpcell.00489.2007
- Papademetriou, I. T., & Porter, T. (2015). Promising approaches to circumvent the blood-brain barrier: progress, pitfalls and clinical prospects in brain cancer. *Therapeutic Delivery*, *6*(8), 989–1016. http://doi.org/10.4155/tde.15.48
- Papadopoulos, M. C., Saadoun, S., Davies, D. C., & Bell, B. A. (2001). Emerging molecular mechanisms of brain tumour oedema. *British Journal of Neurosurgery*, *15*(2), 101–108.
- Papakonstanti, E. A., & Stournaras, C. (2004). Tumor necrosis factor-alpha promotes survival of opossum kidney cells via Cdc42-induced phospholipase C-gamma1 activation and actin filament redistribution. *Molecular Biology of the Cell*, *15*(3), 1273–1286. http://doi.org/10.1091/mbc.e03-07-0491
- Pardridge, W. M. (2012). Drug transport across the blood–brain barrier. *Journal of Cerebral Blood Flow & Metabolism*, *32*(11), 1959–1972. http://doi.org/10.1038/jcbfm.2012.126
- Park, M., Kim, H.-J., Lim, B., Wylegala, A., & Toborek, M. (2013). Methamphetamine-induced occludin endocytosis is mediated by the Arp2/3 complex-regulated actin rearrangement. *The Journal of Biological Chemistry*, *288*(46), 33324–33334. http://doi.org/10.1074/jbc.M113.483487
- Pasparakis, M., Alexopoulou, L., Episkopou, V., & Kollias, G. (1996). Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *The Journal of Experimental Medicine*, *184*(4), 1397–1411.
- Pasparakis, M., & Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature*, *517*(7534), 311–320. http://doi.org/10.1038/nature14191

- Pearce, L. R., Alton, G. R., Richter, D. T., Kath, J. C., Lingardo, L., Chapman, J., ... Alessi, D. R. (2010). Characterization of PF-4708671, a novel and highly specific inhibitor of p70 ribosomal S6 kinase (S6K1). *The Biochemical Journal*, *431*(2), 245–255. http://doi.org/10.1042/BJ20101024
- Peifer, C., & Alessi, D. R. (2008). Small-molecule inhibitors of PDK1. *ChemMedChem*, *3*(12), 1810–1838. http://doi.org/10.1002/cmdc.200800195
- Pelosi, G., Fraggetta, F., Pasini, F., Maisonneuve, P., Sonzogni, A., Iannucci, A., ... Viale, G. (2001). Immunoreactivity for thyroid transcription factor-1 in stage I non-small cell carcinomas of the lung. *The American Journal of Surgical Pathology*, *25*(3), 363–372.
- Pennica, D., Nedwin, G. E., Hayflick, J. S., Seeburg, P. H., Derynck, R., Palladino, M. A., ... Goeddel, D. V. (1984). Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature*, *312*(5996), 724–729.
- Pentsova, E. I., Shah, R. H., Tang, J., Boire, A., You, D., Briggs, S., ... Berger, M. F. (2016). Evaluating Cancer of the Central Nervous System Through Next-Generation Sequencing of Cerebrospinal Fluid. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *34*(20), 2404–2415. http://doi.org/10.1200/JCO.2016.66.6487
- Percy, D. B., Ribot, E. J., Chen, Y., McFadden, C., Simedrea, C., Steeg, P. S., ... Foster, P. J. (2011). In vivo characterization of changing blood-tumor barrier permeability in a mouse model of breast cancer metastasis: a complementary magnetic resonance imaging approach. *Investigative Radiology*, *46*(11), 718–725. http://doi.org/10.1097/RLI.0b013e318226c427
- Perides, G., Zhuge, Y., Lin, T., Stins, M. F., Bronson, R. T., & Wu, J. K. (2006). The fibrinolytic system facilitates tumor cell migration across the blood-brain barrier in experimental melanoma brain metastasis. *BMC Cancer*, *6*, 56. http://doi.org/10.1186/1471-2407-6-56
- Pertz, O. (2010). Spatio-temporal Rho GTPase signaling - where are we now? *Journal of Cell Science*, *123*(Pt 11), 1841–1850. http://doi.org/10.1242/jcs.064345
- Peterson, T. R., Laplante, M., Van Veen, E., Van Vugt, M., Thoreen, C. C., & Sabatini, D. M. (2015). mTORC1 regulates cytokinesis through activation of Rho-ROCK signaling. *ARXIV*. Retrieved from http://arxiv.org/abs/1506.04437
- Petrache, I., Birukova, A., Ramirez, S. I., Garcia, J. G. N., & Verin, A. D. (2003). The role of the microtubules in tumor necrosis factor-??-induced endothelial cell permeability. *American Journal of Respiratory Cell and Molecular Biology*, *28*(5), 574–581. http://doi.org/10.1165/rcmb.2002-0075OC
- Petrache, I., Verin, A. D., Crow, M. T., Birukova, A., Liu, F., & Garcia, J. G. (2001). Differential effect of MLC kinase in TNF-alpha-induced endothelial cell apoptosis and barrier dysfunction. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *280*(6), L1168-78. http://doi.org/10.1152/ajplung.2001.280.6.L1168
- Pfeiffer, F., Schäfer, J., Lyck, R., Makrides, V., Brunner, S., Schaeren-Wiemers, N., ... Engelhardt, B. (2011). Claudin-1 induced sealing of blood-brain barrier tight junctions ameliorates chronic experimental autoimmune encephalomyelitis. *Acta Neuropathologica*, *122*(5), 601–14. http://doi.org/10.1007/s00401-011-0883-2
- Phung, T. L., Ziv, K., Dabydeen, D., Eyiah-Mensah, G., Riveros, M., Perruzzi, C., ... Benjamin, L. E. (2006). Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell*, *10*(2), 159–170. http://doi.org/10.1016/j.ccr.2006.07.003
- Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T., & Gerritsen, M. E. (1997). Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *The Journal of Biological Chemistry*, *272*(34), 21096–21103.
- Plog, B. A., & Nedergaard, M. (2018). The Glymphatic System in Central Nervous System Health and Disease: Past, Present, and Future. *Annual Review of Pathology*, *13*, 379–394. http://doi.org/10.1146/annurev-pathol-051217-111018
- Polavarapu, R., Gongora, M. C., Winkles, J. A., & Yepes, M. (2005). Tumor necrosis factor-like weak inducer of apoptosis increases the permeability of the neurovascular unit through nuclear factor-kappa B pathway activation. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(44), 10094–10100. http://doi.org/10.1523/JNEUROSCI.3382-05.2005
- Popp, I., Grosu, A. L., Niedermann, G., & Duda, D. G. (2016). Immune modulation by hypofractionated stereotactic radiation therapy: Therapeutic implications. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *120*(2), 185–194. http://doi.org/10.1016/j.radonc.2016.07.013
- Prados, M. D., Schold, S. C. J., Fine, H. A., Jaeckle, K., Hochberg, F., Mechtler, L., ... Graney, W. (2003). A randomized, double-blind, placebo-controlled, phase 2 study of RMP-7 in combination with

carboplatin administered intravenously for the treatment of recurrent malignant glioma. *Neuro-Oncology*, *5*(2), 96–103.

- Preusser, M., Capper, D., Ilhan-Mutlu, A., Berghoff, A. S., Birner, P., Bartsch, R., ... von Deimling, A. (2012). Brain metastases: pathobiology and emerging targeted therapies. *Acta Neuropathologica*, *123*(2), 205–222. http://doi.org/10.1007/s00401-011-0933-9
- Price, J. E., Aukerman, S. L., & Fidler, I. J. (1986). Evidence that the process of murine melanoma metastasis is sequential and selective and contains stochastic elements. *Cancer Research*, *46*(10), 5172–5178.
- Probert, L. (2015). TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. *Neuroscience*, *302*, 2–22. http://doi.org/10.1016/j.neuroscience.2015.06.038
- Prudnikova, T. Y., Rawat, S. J., & Chernoff, J. (2015). Molecular pathways: targeting the kinase effectors of RHO-family GTPases. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *21*(1), 24–29. http://doi.org/10.1158/1078-0432.CCR-14-0827
- Prunier, C., Prudent, R., Kapur, R., Sadoul, K., & Lafanechère, L. (2017, June). LIM kinases: cofilin and beyond. *Oncotarget*. http://doi.org/10.18632/oncotarget.16978
- Puhalla, S., Elmquist, W., Freyer, D., Kleinberg, L., Adkins, C., Lockman, P., ... Neuwelt, E. (2015). Unsanctifying the sanctuary: challenges and opportunities with brain metastases. *Neuro-Oncology*, *17*(5), 639–651. http://doi.org/10.1093/neuonc/nov023
- Pulaski, B. A., & Ostrand-Rosenberg, S. (1998). Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. *Cancer Research*, *58*(7), 1486–1493.
- Qin, L.-J., Gu, Y.-T., Zhang, H., & Xue, Y.-X. (2009). Bradykinin-induced blood-tumor barrier opening is mediated by tumor necrosis factor-alpha. *Neuroscience Letters*, *450*(2), 172–175. http://doi.org/10.1016/j.neulet.2008.10.080
- Qin, L., Huang, W., Mo, X., Chen, Y., & Wu, X. (2015). LPS Induces Occludin Dysregulation in Cerebral Microvascular Endothelial Cells via MAPK Signaling and Augmenting MMP-2 Levels. *Oxidative Medicine and Cellular Longevity*, *2015*, 120641. http://doi.org/10.1155/2015/120641
- Quail, D. F., & Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature Medicine*, *19*(11), 1423–1437. http://doi.org/10.1038/nm.3394
- Rafii, S., Butler, J. M., & Ding, B.-S. (2016). Angiocrine functions of organ-specific endothelial cells. *Nature*, *529*(7586), 316–325. http://doi.org/10.1038/nature17040
- Rahmathulla, G., Toms, S. A., & Weil, R. J. (2012). The molecular biology of brain metastasis. *Journal of Oncology*, *2012*, 723541. http://doi.org/10.1155/2012/723541
- Ramakrishna, N., Temin, S., Chandarlapaty, S., Crews, J. R., Davidson, N. E., Esteva, F. J., ... Lin, N. U. (2014). Recommendations on disease management for patients with advanced human epidermal growth factor receptor 2-positive breast cancer and brain metastases: American Society of Clinical Oncology clinical practice guideline. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology*, *32*(19), 2100–2108. http://doi.org/10.1200/JCO.2013.54.0955
- Ramirez, S. H., Fan, S., Dykstra, H., Rom, S., Mercer, A., Reichenbach, N. L., ... Persidsky, Y. (2013). Inhibition of glycogen synthase kinase 3beta promotes tight junction stability in brain endothelial cells by half-life extension of occludin and claudin-5. *PloS One*, *8*(2), e55972. http://doi.org/10.1371/journal.pone.0055972
- Ramirez, S. H., Fan, S., Zhang, M., Papugani, A., Reichenbach, N., Dykstra, H., ... Persidsky, Y. (2010). Inhibition of glycogen synthase kinase 3beta (GSK3beta) decreases inflammatory responses in brain endothelial cells. *The American Journal of Pathology*, *176*(2), 881–892. http://doi.org/10.2353/ajpath.2010.090671
- Ramnath, R., Foster, R. R., Qiu, Y., Cope, G., Butler, M. J., Salmon, A. H., ... Satchell, S. C. (2014). Matrix metalloproteinase 9-mediated shedding of syndecan 4 in response to tumor necrosis factor alpha: a contributor to endothelial cell glycocalyx dysfunction. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *28*(11), 4686–4699. http://doi.org/10.1096/fj.14-252221
- Rangachari, D., Yamaguchi, N., VanderLaan, P. A., Folch, E., Mahadevan, A., Floyd, S. R., ... Costa, D. B. (2015). Brain metastases in patients with EGFR-mutated or ALK-rearranged non-small-cell lung cancers. *Lung Cancer (Amsterdam, Netherlands)*, *88*(1), 108–111. http://doi.org/10.1016/j.lungcan.2015.01.020
- Rao, P., Hsu, K. C., & Chao, M. V. (1995). Upregulation of NF-kappa B-dependent gene expression mediated by the p75 tumor necrosis factor receptor. *Journal of Interferon & Cytokine Research : The Official Journal of the International Society for Interferon and Cytokine Research*, *15*(2), 171–177.

http://doi.org/10.1089/jir.1995.15.171
- Rapoport, S. I., Hori, M., & Klatzo, I. (1972). Testing of a hypothesis for osmotic opening of the blood-brain barrier. *The American Journal of Physiology*, *223*(2), 323–331. http://doi.org/10.1152/ajplegacy.1972.223.2.323
- Rayasam, G. V., Tulasi, V. K., Sodhi, R., Davis, J. A., & Ray, A. (2009). Glycogen synthase kinase 3: more than a namesake. *British Journal of Pharmacology*, *156*(6), 885–898. http://doi.org/10.1111/j.1476-5381.2008.00085.x
- Razani, B., Reichardt, A. D., & Cheng, G. (2011). Non-canonical NF-kappaB signaling activation and regulation: principles and perspectives. *Immunological Reviews*, *244*(1), 44–54. http://doi.org/10.1111/j.1600-065X.2011.01059.x
- Recoquillon, S., Carusio, N., Lagrue-Lakhal, A.-H., Tual-Chalot, S., Filippelli, A., Andriantsitohaina, R., & Martinez, M. C. (2015). Interaction in endothelium of non-muscular myosin light-chain kinase and the NF-kappaB pathway is critical to lipopolysaccharide-induced vascular hyporeactivity. *Clinical Science (London, England : 1979)*, *129*(8), 687–698. http://doi.org/10.1042/CS20140625
- Rees, D. D., Palmer, R. M., Schulz, R., Hodson, H. F., & Moncada, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *British Journal of Pharmacology*, *101*(3), 746–752.
- Rempe, R. G., Hartz, A. M. S., & Bauer, B. (2016). Matrix metalloproteinases in the brain and blood-brain barrier: Versatile breakers and makers. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *36*(9), 1481–1507. http://doi.org/10.1177/0271678X16655551
- Ren, M., Senatorov, V. V, Chen, R.-W., & Chuang, D.-M. (2003). Postinsult treatment with lithium reduces brain damage and facilitates neurological recovery in a rat ischemia/reperfusion model. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(10), 6210–6215. http://doi.org/10.1073/pnas.0937423100
- Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D., & Schwartz, M. A. (2000). Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *Journal of Cell Science*, *113 (Pt 2*, 3673–3678.
- Reymond, N., d'Agua, B. B., & Ridley, A. J. (2013). Crossing the endothelial barrier during metastasis. *Nature Reviews. Cancer*, *13*(12), 858–870. http://doi.org/10.1038/nrc3628
- Richardson, B. C., Lalwani, N. D., Johnson, K. J., & Marks, R. M. (1994). Fas ligation triggers apoptosis in macrophages but not endothelial cells. *European Journal of Immunology*, *24*(11), 2640–2645. http://doi.org/10.1002/eji.1830241111
- Richter, J. D., & Sonenberg, N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature*, *433*(7025), 477–480. http://doi.org/10.1038/nature03205
- Ridley, A. J., & Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*, *70*(3), 389–399.
- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., & Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, *70*(3), 401–410.
- Riento, K., & Ridley, A. J. (2003). Rocks: multifunctional kinases in cell behaviour. *Nature Reviews. Molecular Cell Biology*, *4*(6), 446–456. http://doi.org/10.1038/nrm1128
- Roberts, N. J., Zhou, S., Diaz, L. A. J., & Holdhoff, M. (2011). Systemic use of tumor necrosis factor alpha as an anticancer agent. *Oncotarget*, *2*(10), 739–751. http://doi.org/10.18632/oncotarget.344
- Rochfort, K. D., & Cummins, P. M. (2015). Cytokine-mediated dysregulation of zonula occludens-1 properties in human brain microvascular endothelium. *Microvascular Research*, *100*, 48–53. http://doi.org/10.1016/j.mvr.2015.04.010
- Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., ... Downward, J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*, *370*(6490), 527–532. http://doi.org/10.1038/370527a0
- Rodriguez, M. S., Thompson, J., Hay, R. T., & Dargemont, C. (1999). Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. *The Journal of Biological Chemistry*, *274*(13), 9108–9115.
- Romer, L. H., McLean, N. V, Yan, H. C., Daise, M., Sun, J., & DeLisser, H. M. (1995). IFN-gamma and TNF-alpha induce redistribution of PECAM-1 (CD31) on human endothelial cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *154*(12), 6582–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7759892
- Ronaldson, P. T., Demarco, K. M., Sanchez-Covarrubias, L., Solinsky, C. M., & Davis, T. P. (2009). Transforming growth factor-beta signaling alters substrate permeability and tight junction protein

expression at the blood-brain barrier during inflammatory pain. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *29*(6), 1084–1098. http://doi.org/10.1038/jcbfm.2009.32

- Rosenberg, G. A., & Yang, Y. (2007). Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia. *Neurosurgical Focus*, *22*(5), E4.
- Rossi, J. L., Ralay Ranaivo, H., Patel, F., Chrzaszcz, M., Venkatesan, C., & Wainwright, M. S. (2011). Albumin causes increased myosin light chain kinase expression in astrocytes via p38 mitogen-activated protein kinase. *Journal of Neuroscience Research*, *89*(6), 852–861. http://doi.org/10.1002/jnr.22600
- Rossi, J. L., Todd, T., Bazan, N. G., & Belayev, L. (2013). Inhibition of Myosin light-chain kinase attenuates cerebral edema after traumatic brain injury in postnatal mice. *Journal of Neurotrauma*, *30*(19), 1672–1679. http://doi.org/10.1089/neu.2013.2898
- Roth, T. L., Nayak, D., Atanasijevic, T., Koretsky, A. P., Latour, L. L., & McGavern, D. B. (2014). Transcranial amelioration of inflammation and cell death after brain injury. *Nature*, *505*(7482), 223–228. http://doi.org/10.1038/nature12808
- Rothwarf, D. M., Zandi, E., Natoli, G., & Karin, M. (1998). IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature*, *395*(6699), 297–300. http://doi.org/10.1038/26261
- Rotondo, S., Rajtar, G., Manarini, S., Celardo, A., Rotillo, D., de Gaetano, G., ... Cerletti, C. (1998). Effect of trans-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function. *British Journal of Pharmacology*, *123*(8), 1691–1699. http://doi.org/10.1038/sj.bjp.0701784
- Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P., & Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(37), 13489–13494. http://doi.org/10.1073/pnas.0405659101
- Roy-Luzarraga, M., & Hodivala-Dilke, K. (2016). Molecular Pathways: Endothelial Cell FAK-A Target for Cancer Treatment. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *22*(15), 3718–3724. http://doi.org/10.1158/1078-0432.CCR-14-2021
- Rudolph, J., Crawford, J. J., Hoeflich, K. P., & Wang, W. (2015). Inhibitors of p21-activated kinases (PAKs). *Journal of Medicinal Chemistry*, *58*(1), 111–129. http://doi.org/10.1021/jm501613q
- Ruegg, U. T., & Burgess, G. M. (1989). Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends in Pharmacological Sciences*, *10*(6), 218–220.
- Safdar, Z., Wang, P., Ichimura, H., Issekutz, A. C., Quadri, S., & Bhattacharya, J. (2003). Hyperosmolarity enhances the lung capillary barrier. *The Journal of Clinical Investigation*, *112*(10), 1541–1549. http://doi.org/10.1172/JCI18370
- Saharinen, P., Eklund, L., Miettinen, J., Wirkkala, R., Anisimov, A., Winderlich, M., ... Alitalo, K. (2008). Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. *Nature Cell Biology*, *10*(5), 527–537. http://doi.org/10.1038/ncb1715
- Saija, A., Princi, P., Lanza, M., Scalese, M., Aramnejad, E., & Sarro, A. De. (1995). Systemic cytokine administration can affect blood-brain barrier permeability in the rat. *Life Sciences*, *56*(10), 775–784. http://doi.org/10.1016/0024-3205(95)00008-T
- Saito, N., Hatori, T., Murata, N., Zhang, Z.-A., Ishikawa, F., Nonaka, H., ... Samejima, H. (2007). A double three-step theory of brain metastasis in mice: the role of the pia mater and matrix metalloproteinases. *Neuropathology and Applied Neurobiology*, *33*(3), 288–298. http://doi.org/10.1111/j.1365-2990.2007.00799.x
- Saitoh, M., Ishikawa, T., Matsushima, S., Naka, M., & Hidaka, H. (1987). Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *The Journal of Biological Chemistry*, *262*(16), 7796–7801.
- Salahuddin, T. S., Johansson, B. B., Kalimo, H., & Olsson, Y. (1988). Structural changes in the rat brain after carotid infusions of hyperosmolar solutions. An electron microscopic study. *Acta Neuropathologica*, *77*(1), 5–13.
- Salbreux, G., Charras, G., & Paluch, E. (2012). Actin cortex mechanics and cellular morphogenesis. *Trends in Cell Biology*, *22*(10), 536–545. http://doi.org/10.1016/j.tcb.2012.07.001
- Salmaggi, A., Maderna, E., Calatozzolo, C., Gaviani, P., Canazza, A., Milanesi, I., ... Pollo, B. (2009). CXCL12, CXCR4 and CXCR7 expression in brain metastases. *Cancer Biology & Therapy*, *8*(17), 1608–1614.
- Salomoni, P., & Pandolfi, P. P. (2002). The role of PML in tumor suppression. *Cell*, *108*(2), 165–170.
- Sarbassov, D. D., Ali, S. M., Kim, D.-H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., ... Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Current Biology : CB*, *14*(14), 1296–1302.

http://doi.org/10.1016/j.cub.2004.06.054
- Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J.-H., Hsu, P. P., Bagley, A. F., ... Sabatini, D. M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular Cell*, *22*(2), 159–168. http://doi.org/10.1016/j.molcel.2006.03.029
- Sarbassov, D. D., Guertin, D. A., Ali, S. M., & Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science (New York, N.Y.)*, *307*(5712), 1098–1101. http://doi.org/10.1126/science.1106148
- Saxton, R. A., & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, *168*(6), 960–976. http://doi.org/10.1016/j.cell.2017.02.004
- Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., ... Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *The EMBO Journal*, *17*(6), 1675–1687. http://doi.org/10.1093/emboj/17.6.1675
- Scaife, C. L., Kuang, J., Wills, J. C., Trowbridge, D. B., Gray, P., Manning, B. M., ... Kuwada, S. K. (2002). Nuclear factor kappaB inhibitors induce adhesion-dependent colon cancer apoptosis: implications for metastasis. *Cancer Research*, *62*(23), 6870–6878.
- Schaller, M. D. (2010). Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *Journal of Cell Science*, *123*(Pt 7), 1007–1013. http://doi.org/10.1242/jcs.045112
- Scheidereit, C. (2006). IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene*, *25*(51), 6685–6705. http://doi.org/10.1038/sj.onc.1209934
- Schinske, K. A., Nyati, S., Khan, A. P., Williams, T. M., Johnson, T. D., Ross, B. D., ... Rehemtulla, A. (2011). A novel kinase inhibitor of FADD phosphorylation chemosensitizes through the inhibition of NF-kappaB. *Molecular Cancer Therapeutics*, *10*(10), 1807–1817. http://doi.org/10.1158/1535-7163.MCT-11-0362
- Schlaepfer, D. D., Hou, S., Lim, S.-T., Tomar, A., Yu, H., Lim, Y., ... Mitra, S. K. (2007). Tumor necrosis factor-alpha stimulates focal adhesion kinase activity required for mitogen-activated kinase-associated interleukin 6 expression. *The Journal of Biological Chemistry*, *282*(24), 17450–17459. http://doi.org/10.1074/jbc.M610672200
- Schlegel, N., & Waschke, J. (2009). Impaired cAMP and Rac 1 signaling contribute to TNF-alpha-induced endothelial barrier breakdown in microvascular endothelium. *Microcirculation (New York, N.Y.: 1994)*, *16*(6), 521–533. http://doi.org/10.1080/10739680902967427
- Schlunk, F., & Greenberg, S. M. (2015). The Pathophysiology of Intracerebral Hemorrhage Formation and Expansion. *Translational Stroke Research*, *6*(4), 257–263. http://doi.org/10.1007/s12975-015-0410-1
- Schmidt, A., Bickle, M., Beck, T., & Hall, M. N. (1997). The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell*, *88*(4), 531–542.
- Schmidt, A., Kunz, J., & Hall, M. N. (1996). TOR2 is required for orfile:///Users/xtinaks/Google Drive/Thesis writing/References/Results-Chapter 3/Schmidt et al -1997- Cellganization of the actin cytoskeleton in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(24), 13780–13785.
- Schnell, L., Fearn, S., Schwab, M. E., Perry, V. H., & Anthony, D. C. (1999). Cytokine-induced acute inflammation in the brain and spinal cord. *Journal of Neuropathology and Experimental Neurology*, *58*(3), 245–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10197816
- Schnell, S. A., Staines, W. A., & Wessendorf, M. W. (1999). Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, *47*(6), 719–730. http://doi.org/10.1177/002215549904700601
- Schnittler, H. J., Wilke, A., Gress, T., Suttorp, N., & Drenckhahn, D. (1990). Role of actin and myosin in the control of paracellular permeability in pig, rat and human vascular endothelium. *The Journal of Physiology*, *431*, 379–401. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1181779&tool=pmcentrez&rendertype=abstract
- Schulte, D., Kuppers, V., Dartsch, N., Broermann, A., Li, H., Zarbock, A., ... Vestweber, D. (2011). Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. *The EMBO Journal*, *30*(20), 4157–4170. http://doi.org/10.1038/emboj.2011.304
- Schultz, J. D., Rotunno, S., Erben, P., Sommer, J. U., Anders, C., Stern-Straeter, J., ... Sauter, A. (2011). Down-regulation of MMP-2 expression due to inhibition of receptor tyrosine kinases by imatinib and carboplatin in HNSCC. *Oncology Reports*, *25*(4), 1145–1151. http://doi.org/10.3892/or.2011.1153
- Schwartz, M. A. (2001). Integrin signaling revisited. *Trends in Cell Biology*, *11*(12), 466–470.
- Seki, T., Carroll, F., Illingworth, S., Green, N., Cawood, R., Bachtarzi, H., ... Seymour, L. W. (2011).

Tumour necrosis factor-alpha increases extravasation of virus particles into tumour tissue by activating the Rho A/Rho kinase pathway. *Journal of Controlled Release : Official Journal of the Controlled Release Society*, *156*(3), 381–389. http://doi.org/10.1016/j.jconrel.2011.08.022

- Serres, S., Soto, M. S., Hamilton, a., McAteer, M. a., Carbonell, W. S., Robson, M. D., ... Sibson, N. R. (2012). Molecular MRI enables early and sensitive detection of brain metastases. *Proceedings of the National Academy of Sciences*, *109*, 6674–6679. http://doi.org/10.1073/pnas.1117412109
- Sevenich, L., Bowman, R. L., Mason, S. D., Quail, D. F., Rapaport, F., Elie, B. T., ... Joyce, J. A. (2014). Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. *Nature Cell Biology*, *16*(9), 876–888. http://doi.org/10.1038/ncb3011
- Shao, B., & Bayraktutan, U. (2014). Hyperglycaemia promotes human brain microvascular endothelial cell apoptosis via induction of protein kinase C-ssI and prooxidant enzyme NADPH oxidase. *Redox Biology*, *2*, 694–701. http://doi.org/10.1016/j.redox.2014.05.005
- Shao, M., Yue, Y., Sun, G.-Y., You, Q.-H., Wang, N., & Zhang, D. (2013). Caveolin-1 regulates Rac1 activation and rat pulmonary microvascular endothelial hyperpermeability induced by TNF-alpha. *PloS One*, *8*(1), e55213. http://doi.org/10.1371/journal.pone.0055213
- Shasby, D. M., Stevens, T., Ries, D., Moy, A. B., Kamath, J. M., Kamath, A. M., & Shasby, S. S. (1997). Thrombin inhibits myosin light chain dephosphorylation in endothelial cells. *The American Journal of Physiology*, *272*(2 Pt 1), L311-9. http://doi.org/10.1152/ajplung.1997.272.2.L311
- Shen, Q., Rigor, R. R., Pivetti, C. D., Wu, M. H., & Yuan, S. Y. (2010). Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovascular Research*, *87*(2), 272–280. http://doi.org/10.1093/cvr/cvq144
- Shi, Y., Zhang, L., Pu, H., Mao, L., Hu, X., Jiang, X., ... Chen, J. (2016). Rapid endothelial cytoskeletal reorganization enables early blood-brain barrier disruption and long-term ischaemic reperfusion brain injury. *Nature Communications*, *7*, 10523. http://doi.org/10.1038/ncomms10523
- Shivanna, M., Jalimarada, S. S., & Srinivas, S. P. (2010). Lovastatin inhibits the thrombin-induced loss of barrier integrity in bovine corneal endothelium. *Journal of Ocular Pharmacology and Therapeutics : The Official Journal of the Association for Ocular Pharmacology and Therapeutics*, *26*(1), 1–10. http://doi.org/10.1089/jop.2009.0025
- Shivanna, M., Rajashekhar, G., & Srinivas, S. P. (2010). Barrier dysfunction of the corneal endothelium in response to TNF-alpha: role of p38 MAP kinase. *Investigative Ophthalmology & Visual Science*, *51*(3), 1575–1582. http://doi.org/10.1167/iovs.09-4343
- Shutes, A., Onesto, C., Picard, V., Leblond, B., Schweighoffer, F., & Der, C. J. (2007). Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *The Journal of Biological Chemistry*, *282*(49), 35666–35678. http://doi.org/10.1074/jbc.M703571200
- Sibson, N. R., Anthony, D. C., van Kasteren, S., Dickens, A., Perez-Balderas, F., McAteer, M. A., ... Davis, B. G. (2011). Molecular MRI approaches to the detection of CNS inflammation. *Methods in Molecular Biology (Clifton, N.J.)*, *711*, 379–396. http://doi.org/10.1007/978-1-61737-992-5_19
- Sibson, N. R., Blamire, A. M., Bernades-Silva, M., Laurent, S., Boutry, S., Muller, R. N., ... Anthony, D. C. (2004). MRI detection of early endothelial activation in brain inflammation. *Magnetic Resonance in Medicine*, *51*(2), 248–252. http://doi.org/10.1002/mrm.10723
- Sibson, N. R., Blamire, A. M., Perry, V. H., Gauldie, J., Styles, P., & Anthony, D. C. (2002). TNF-alpha reduces cerebral blood volume and disrupts tissue homeostasis via an endothelin- and TNFR2-dependent pathway. *Brain*, *125*(Pt 11), 2446–59. http://doi.org/10.1093/brain/awf256
- Siegel, R. L., Miller, K. D., & Jemal, A. (2017). Cancer Statistics, 2017. *CA: A Cancer Journal for Clinicians*, *67*(1), 7–30. http://doi.org/10.3322/caac.21387
- Simeonidis, S., Stauber, D., Chen, G., Hendrickson, W. A., & Thanos, D. (1999, January). Mechanisms by which IκB proteins control NF-κB activity. *Proceedings of the National Academy of Sciences of the United States of America*.
- Singh, A., Satchell, S. C., Neal, C. R., McKenzie, E. A., Tooke, J. E., & Mathieson, P. W. (2007). Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. Journal of the American Society of Nephrology : JASN, 18(11), 2885–2893. http://doi.org/10.1681/ASN.2007010119
- Siravegna, G., Geuna, E., Mussolin, B., Crisafulli, G., Bartolini, A., Galizia, D., ... Montemurro, F. (2017). Genotyping tumour DNA in cerebrospinal fluid and plasma of a HER2-positive breast cancer patient with brain metastases. ESMO Open, 2(4), e000253. http://doi.org/10.1136/esmoopen-2017-000253
- Sisó, S., Jeffrey, M., & González, L. (2010). Sensory circumventricular organs in health and disease. Acta Neuropathologica, 120(6), 689–705. http://doi.org/10.1007/s00401-010-0743-5
- Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., & Stark, G. R. (2002). Distinct roles of the

Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. The Journal of Biological Chemistry, 277(6), 3863–3869. http://doi.org/10.1074/jbc.M110572200

- Sizemore, N., Leung, S., & Stark, G. R. (1999). Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Molecular and Cellular Biology, 19(7), 4798–4805.
- Slotman, B., Faivre-Finn, C., Kramer, G., Rankin, E., Snee, M., Hatton, M., ... Senan, S. (2007). Prophylactic cranial irradiation in extensive small-cell lung cancer. *The New England Journal of Medicine*, *357*(7), 664–672. http://doi.org/10.1056/NEJMoa071780
- Soffietti, R., Abacioglu, U., Baumert, B., Combs, S. E., Kinhult, S., Kros, J. M., ... Weller, M. (2017). Diagnosis and treatment of brain metastases from solid tumors: guidelines from the European Association of Neuro-Oncology (EANO). *Neuro-Oncology*, *19*(2), 162–174. http://doi.org/10.1093/neuonc/now241
- Solinas, G., Marchesi, F., Garlanda, C., Mantovani, A., & Allavena, P. (2010). Inflammation-mediated promotion of invasion and metastasis. *Cancer Metastasis Reviews*, *29*(2), 243–248. http://doi.org/10.1007/s10555-010-9227-2
- Somanath, P. R., Razorenova, O. V, Chen, J., & Byzova, T. V. (2006). Akt1 in endothelial cell and angiogenesis. *Cell Cycle (Georgetown, Tex.)*, *5*(5), 512–518. http://doi.org/10.4161/cc.5.5.2538
- Soto, M. S., O'Brien, E. R., Andreou, K., Scrace, S. F., Zakaria, R., Jenkinson, M. D., ... Sibson, N. R. (2016). Disruption of tumour-host communication by downregulation of LFA-1 reduces COX-2 and e-NOS expression and inhibits brain metastasis growth. *Oncotarget*, *7*(32), 52375–52391. http://doi.org/10.18632/oncotarget.10737
- Soto, M. S., Serres, S., Anthony, D. C., & Sibson, N. R. (2014). Functional role of endothelial adhesion molecules in the early stages of brain metastasis. *Neuro-Oncology*, *16*(4), 540–551. http://doi.org/10.1093/neuonc/not222
- Soto, M. S., & Sibson, N. R. (2016). Mouse Models of Brain Metastasis for Unravelling Tumour Progression. *Advances in Experimental Medicine and Biology*, *899*, 231–244. http://doi.org/10.1007/978-3-319-26666-4_13
- Spagnolo, F., Picasso, V., Lambertini, M., Ottaviano, V., Dozin, B., & Queirolo, P. (2016). Survival of patients with metastatic melanoma and brain metastases in the era of MAP-kinase inhibitors and immunologic checkpoint blockade antibodies: A systematic review. *Cancer Treatment Reviews*, *45*, 38–45. http://doi.org/10.1016/j.ctrv.2016.03.003
- Sperduto, P. W., Yang, T. J., Beal, K., Pan, H., Brown, P. D., Bangdiwala, A., ... Mehta, M. P. (2017). Estimating Survival in Patients With Lung Cancer and Brain Metastases: An Update of the Graded Prognostic Assessment for Lung Cancer Using Molecular Markers (Lung-molGPA). JAMA Oncology, 3(6), 827–831. http://doi.org/10.1001/jamaoncol.2016.3834
- Spindler, V., Schlegel, N., & Waschke, J. (2010). Role of GTPases in control of microvascular permeability. *Cardiovascular Research*, *87*(2), 243–253. http://doi.org/10.1093/cvr/cvq086
- Srivastava, K., Shao, B., & Bayraktutan, U. (2013). PKC-beta exacerbates in vitro brain barrier damage in hyperglycemic settings via regulation of RhoA/Rho-kinase/MLC2 pathway. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *33*(12), 1928–1936. http://doi.org/10.1038/jcbfm.2013.151
- Stamatovic, S. M., Dimitrijevic, O. B., Keep, R. F., & Andjelkovic, A. V. (2006). Inflammation and brain edema: new insights into the role of chemokines and their receptors. *Acta Neurochirurgica. Supplement*, *96*, 444–450.
- Stamatovic, S. M., Dimitrijevic, O. B., Keep, R. F., & Andjelkovic, A. V. (2006). Protein kinase Calpha-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability. *The Journal of Biological Chemistry*, *281*(13), 8379–8388. http://doi.org/10.1074/jbc.M513122200
- Stamatovic, S. M., Johnson, A. M., Keep, R. F., & Andjelkovic, A. V. (2016). Junctional proteins of the blood-brain barrier: New insights into function and dysfunction. *Tissue Barriers*, *4*(1), e1154641. http://doi.org/10.1080/21688370.2016.1154641
- Steeg, P. S., & Theodorescu, D. (2008). Metastasis: a therapeutic target for cancer. *Nature Clinical Practice. Oncology*, *5*(4), 206–219. http://doi.org/10.1038/ncponc1066
- Steeland, S., Libert, C., & Vandenbroucke, R. E. (2018). A New Venue of TNF Targeting. *International Journal of Molecular Sciences*, *19*(5). http://doi.org/10.3390/ijms19051442
- Steinbrecher, K. A., Wilson, W. 3rd, Cogswell, P. C., & Baldwin, A. S. (2005). Glycogen synthase kinase 3beta functions to specify gene-specific, NF-kappaB-dependent transcription. *Molecular and Cellular Biology*, *25*(19), 8444–8455. http://doi.org/10.1128/MCB.25.19.8444-8455.2005

- Stellwagen, D., & Malenka, R. C. (2006). Synaptic scaling mediated by glial TNF-alpha. *Nature*, *440*(7087), 1054–1059. http://doi.org/10.1038/nature04671
- Stephan, D., Sbai, O., Wen, J., Couraud, P.-O., Putterman, C., Khrestchatisky, M., & Desplat-Jego, S. (2013). TWEAK/Fn14 pathway modulates properties of a human microvascular endothelial cell model of blood brain barrier. *Journal of Neuroinflammation*, *10*, 9. http://doi.org/10.1186/1742-2094-10-9
- Stern, L., & Gautier, R. (1921). Recherches Sur Le Liquide CÉphalo-Rachidien: I.–Les Rapports Entre Le Liquide CÉphalo-Rachidien et la Circulation Sanguine. *Archives Internationales de Physiologie*, *17*(2), 138–192. http://doi.org/10.3109/13813452109146211
- Stockton, R. A., Schaefer, E., & Schwartz, M. A. (2004). p21-activated kinase regulates endothelial permeability through modulation of contractility. *The Journal of Biological Chemistry*, *279*(45), 46621–46630. http://doi.org/10.1074/jbc.M408877200
- Stolpen, A. H., Guinan, E. C., Fiers, W., & Pober, J. S. (1986). Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. *The American Journal of Pathology*, *123*(1), 16–24.
- Straus, W. (1958). Colorimetric analysis with N, N-dimethyl-p-phenylenediamine of the uptake of intravenously injected horseradish peroxidase by various tissues of the rat. *The Journal of Biophysical and Biochemical Cytology*, *4*(5), 541–550.
- Suganuma, M., Okabe, S., Marino, M. W., Sakai, A., Sueoka, E., & Fujiki, H. (1999). Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Research*, *59*(18), 4516–4518.
- Sun, S.-C. (2012). The noncanonical NF-kappaB pathway. *Immunological Reviews*, *246*(1), 125–140. http://doi.org/10.1111/j.1600-065X.2011.01088.x
- Sundstrøm, T., Daphu, I., Wendelbo, I., Hodneland, E., Lundervold, A., Immervoll, H., ... Thorsen, F. (2013). Automated tracking of nanoparticle-labeled melanoma cells improves the predictive power of a brain metastasis model. *Cancer Research*, *73*(8), 2445–2456. http://doi.org/10.1158/0008-5472.CAN-12-3514
- Suzuki, K., & Takahashi, K. (2001). Actin filament assembly and actin-myosin contractility are necessary for anchorage- and EGF-dependent activation of phospholipase Cgamma. *Journal of Cellular Physiology*, *189*(1), 64–71. http://doi.org/10.1002/jcp.1134
- Suzuki, Y., Nakano, Y., Mishiro, K., Takagi, T., Tsuruma, K., Nakamura, M., ... Hara, H. (2013). Involvement of Mincle and Syk in the changes to innate immunity after ischemic stroke. *Scientific Reports*, *3*, 3177. http://doi.org/10.1038/srep03177
- Szczepanowska, J. (2009). Involvement of Rac/Cdc42/PAK pathway in cytoskeletal rearrangements. *Acta Biochimica Polonica*, *56*(2), 225–234.
- Szulcek, R., Beckers, C. M. L., Hodzic, J., de Wit, J., Chen, Z., Grob, T., ... van Nieuw Amerongen, G. P. (2013). Localized RhoA GTPase activity regulates dynamics of endothelial monolayer integrity. *Cardiovascular Research*, *99*(3), 471–482. http://doi.org/10.1093/cvr/cvt075
- Tabruyn, S. P., & Griffioen, A. W. (2008). NF-kappa B: a new player in angiostatic therapy. *Angiogenesis*, *11*(1), 101–106. http://doi.org/10.1007/s10456-008-9094-4
- Taddei, A., Giampietro, C., Conti, A., Orsenigo, F., Breviario, F., Pirazzoli, V., ... Dejana, E. (2008). Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nature Cell Biology*, *10*(8), 923–34. http://doi.org/10.1038/ncb1752
- Tak, P. P., & Firestein, G. S. (2001). NF-kappaB: a key role in inflammatory diseases. *The Journal of Clinical Investigation*, *107*(1), 7–11. http://doi.org/10.1172/JCI11830
- Takahashi, N., Duprez, L., Grootjans, S., Cauwels, A., Nerinckx, W., DuHadaway, J. B., ... Vandenabeele, P. (2012). Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death & Disease*, *3*, e437. http://doi.org/10.1038/cddis.2012.176
- Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiological Reviews*, *81*(1), 153–208. http://doi.org/10.1152/physrev.2001.81.1.153
- Takashima, S. (2009). Phosphorylation of myosin regulatory light chain by myosin light chain kinase, and muscle contraction. *Circulation Journal : Official Journal of the Japanese Circulation Society*, *73*(2), 208–213.
- Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., ... Okumura, K. (2001). Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nature Medicine*, *7*(1), 94–100. http://doi.org/10.1038/83416
- Talmadge, J. E., & Fidler, I. J. (2010). AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Research*, *70*(14), 5649–5669. http://doi.org/10.1158/0008-5472.CAN-10-

1040
- Tang, C., Xue, H.-L., Bai, C.-L., & Fu, R. (2011). Regulation of adhesion molecules expression in TNF-alpha-stimulated brain microvascular endothelial cells by tanshinone IIA: involvement of NF-kappaB and ROS generation. *Phytotherapy Research : PTR*, *25*(3), 376–380. http://doi.org/10.1002/ptr.3278
- Tanjoni, I., Walsh, C., Uryu, S., Tomar, A., Nam, J.-O., Mielgo, A., ... Schlaepfer, D. D. (2010, May). PND-1186 FAK inhibitor selectively promotes tumor cell apoptosis in three-dimensional environments. *Cancer Biology & Therapy*.
- Tarbell, J. M. (2010). Shear stress and the endothelial transport barrier. *Cardiovascular Research*, *87*(2), 320–330. http://doi.org/10.1093/cvr/cvq146
- Tartaglia, L. A., Ayres, T. M., Wong, G. H., & Goeddel, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. *Cell*, *74*(5), 845–853.
- Tasaka, S., Koh, H., Yamada, W., Shimizu, M., Ogawa, Y., Hasegawa, N., ... Ishizaka, A. (2005). Attenuation of endotoxin-induced acute lung injury by the Rho-associated kinase inhibitor, Y-27632. *American Journal of Respiratory Cell and Molecular Biology*, *32*(6), 504–510. http://doi.org/10.1165/rcmb.2004-0009OC
- Taskar, K. S., Rudraraju, V., Mittapalli, R. K., Samala, R., Thorsheim, H. R., Lockman, J., ... Smith, Q. R. (2012). Lapatinib Distribution in HER2 Overexpressing Experimental Brain Metastases of Breast Cancer. *Pharmaceutical Research*, *29*(3), 770–781. http://doi.org/10.1007/s11095-011-0601-8
- Tauseef, M., Knezevic, N., Chava, K. R., Smith, M., Sukriti, S., Gianaris, N., ... Mehta, D. (2012). TLR4 activation of TRPC6-dependent calcium signaling mediates endotoxin-induced lung vascular permeability and inflammation. *The Journal of Experimental Medicine*, *209*(11), 1953–1968. http://doi.org/10.1084/jem.20111355
- Tester, A. M., Waltham, M., Oh, S.-J., Bae, S.-N., Bills, M. M., Walker, E. C., ... Thompson, E. W. (2004). Pro-matrix metalloproteinase-2 transfection increases orthotopic primary growth and experimental metastasis of MDA-MB-231 human breast cancer cells in nude mice. *Cancer Research*, *64*(2), 652–658.
- Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nature Reviews. Cancer*, *2*(6), 442–454. http://doi.org/10.1038/nrc822
- Thirone, A. C. P., Speight, P., Zulys, M., Rotstein, O. D., Szaszi, K., Pedersen, S. F., & Kapus, A. (2009). Hyperosmotic stress induces Rho/Rho kinase/LIM kinase-mediated cofilin phosphorylation in tubular cells: key role in the osmotically triggered F-actin response. *American Journal of Physiology. Cell Physiology*, *296*(3), C463-75. http://doi.org/10.1152/ajpcell.00467.2008
- Thomsen, L. B., Thomsen, M. S., & Moos, T. (2015). Targeted drug delivery to the brain using magnetic nanoparticles. *Therapeutic Delivery*, *6*(10), 1145–1155. http://doi.org/10.4155/tde.15.56
- Thorsen, F., Fite, B., Mahakian, L. M., Seo, J. W., Qin, S., Harrison, V., ... Ferrara, K. W. (2013). Multimodal imaging enables early detection and characterization of changes in tumor permeability of brain metastases. *Journal of Controlled Release : Official Journal of the Controlled Release Society*, *172*(3), 812–822. http://doi.org/10.1016/j.jconrel.2013.10.019
- Tian, G., Cory, M., Smith, A. A., & Knight, W. B. (2001). Structural determinants for potent, selective dual site inhibition of human pp60c-src by 4-anilinoquinazolines. *Biochemistry*, *40*(24), 7084–7091.
- Ting, A. T., & Bertrand, M. J. M. (2016). More to Life than NF-kappaB in TNFR1 Signaling. *Trends in Immunology*, *37*(8), 535–545. http://doi.org/10.1016/j.it.2016.06.002
- Tominaga, N., Kosaka, N., Ono, M., Katsuda, T., Yoshioka, Y., Tamura, K., ... Ochiya, T. (2015). Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. *Nature Communications*, *6*, 6716. http://doi.org/10.1038/ncomms7716
- Tornavaca, O., Chia, M., Dufton, N., Almagro, L. O., Conway, D. E., Randi, A. M., ... Balda, M. S. (2015). ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. *The Journal of Cell Biology*, *208*(6), 821–838. http://doi.org/10.1083/jcb.201404140
- Treat, L. H., McDannold, N., Vykhodtseva, N., Zhang, Y., Tam, K., & Hynynen, K. (2007). Targeted delivery of doxorubicin to the rat brain at therapeutic levels using MRI-guided focused ultrasound. *International Journal of Cancer*, *121*(4), 901–907. http://doi.org/10.1002/ijc.22732
- Trickler, W. J., Mayhan, W. G., & Miller, D. W. (2005). Brain microvessel endothelial cell responses to tumor necrosis factor-alpha involve a nuclear factor kappa B (NF-kappaB) signal transduction pathway. *Brain Research*, *1048*(1–2), 24–31. http://doi.org/10.1016/j.brainres.2005.04.028
- Tsai, P. S., Kaufhold, J. P., Blinder, P., Friedman, B., Drew, P. J., Karten, H. J., ... Kleinfeld, D. (2009). Correlations of neuronal and microvascular densities in murine cortex revealed by direct counting and colocalization of nuclei and vessels. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(46), 14553–14570. http://doi.org/10.1523/JNEUROSCI.3287-09.2009

- Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., ... Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*, *389*(6654), 990–994. http://doi.org/10.1038/40187
- Ullrich, A., & Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell*, *61*(2), 203–212.
- UniProt. (n.d.). TNFA_HUMAN, P01375. Retrieved August 1, 2018, from https://www.uniprot.org/uniprot/P01375
- Valiente, M., Ahluwalia, M. S., Boire, A., Brastianos, P. K., Goldberg, S. B., Lee, E. Q., ... Soffietti, R. (2018). The Evolving Landscape of Brain Metastasis. *Trends in Cancer*, *4*(3), 176–196. http://doi.org/10.1016/j.trecan.2018.01.003
- Valiente, M., Obenauf, A. C., Jin, X., Chen, Q., Zhang, X. H.-F., Lee, D. J., ... Massague, J. (2014). Serpins promote cancer cell survival and vascular co-option in brain metastasis. *Cell*, *156*(5), 1002–1016. http://doi.org/10.1016/j.cell.2014.01.040
- Vallabhapurapu, S., & Karin, M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. *Annual Review of Immunology*, *27*, 693–733. http://doi.org/10.1146/annurev.immunol.021908.132641
- van Buul, J. D., Geerts, D., & Huveneers, S. (2014). Rho GAPs and GEFs: controling switches in endothelial cell adhesion. *Cell Adhesion & Migration*, *8*(2), 108–124.
- van den Hurk, C. J. G., Eckel, R., van de Poll-Franse, L. V, Coebergh, J. W. W., Nortier, J. W. R., Holzel, D., ... Engel, J. (2011). Unfavourable pattern of metastases in M0 breast cancer patients during 1978-2008: a population-based analysis of the Munich Cancer Registry. *Breast Cancer Research and Treatment*, *128*(3), 795–805. http://doi.org/10.1007/s10549-011-1372-y
- van der Weyden, L., Arends, M. J., Campbell, A. D., Bald, T., Wardle-Jones, H., Griggs, N., ... Adams, D. J. (2017). Genome-wide in vivo screen identifies novel host regulators of metastatic colonization. *Nature*, *541*(7636), 233–236. http://doi.org/10.1038/nature20792
- Van Hauwermeiren, F., Armaka, M., Karagianni, N., Kranidioti, K., Vandenbroucke, R. E., Loges, S., ... Kollias, G. (2013). Safe TNF-based antitumor therapy following p55TNFR reduction in intestinal epithelium. *The Journal of Clinical Investigation*, *123*(6), 2590–2603. http://doi.org/10.1172/JCI65624
- Van Hauwermeiren, F., Vandenbroucke, R. E., Grine, L., Puimege, L., Van Wonterghem, E., Zhang, H., & Libert, C. (2014). Antisense oligonucleotides against TNFR1 prevent toxicity of TNF/IFNgamma treatment in mouse tumor models. *International Journal of Cancer*, *135*(3), 742–750. http://doi.org/10.1002/ijc.28704
- van Meer, G., & Simons, K. (1986). The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *The EMBO Journal*, *5*(7), 1455–1464.
- van Nieuw Amerongen, G. P., Beckers, C. M. L., Achekar, I. D., Zeeman, S., Musters, R. J. P., & van Hinsbergh, V. W. M. (2007). Involvement of Rho kinase in endothelial barrier maintenance. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*(11), 2332–2339. http://doi.org/10.1161/ATVBAHA.107.152322
- van Nieuw Amerongen, G. P., Draijer, R., Vermeer, M. A., & van Hinsbergh, V. W. (1998). Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. *Circulation Research*, *83*(11), 1115–1123.
- Van Ostade, X., Vandenabeele, P., Everaerdt, B., Loetscher, H., Gentz, R., Brockhaus, M., ... Fiers, W. (1993). Human TNF mutants with selective activity on the p55 receptor. *Nature*, *361*(6409), 266–9. http://doi.org/10.1038/361266a0
- Van Rijssel, J., Timmerman, I., Van Alphen, F. P. J., Hoogenboezem, M., Korchynskyi, O., Geerts, D., ... Van Buul, J. D. (2013). The Rho-GEF Trio regulates a novel pro-inflammatory pathway through the transcription factor Ets2. *Biology Open*, *2*(6), 569–579. http://doi.org/10.1242/bio.20134382
- van Vliet, E. A., Otte, W. M., Wadman, W. J., Aronica, E., Kooij, G., de Vries, H. E., ... Gorter, J. A. (2016). Blood-brain barrier leakage after status epilepticus in rapamycin-treated rats II: Potential mechanisms. *Epilepsia*, *57*(1), 70–78. http://doi.org/10.1111/epi.13245
- van Vulpen, M., Kal, H. B., Taphoorn, M. J. B., & El-Sharouni, S. Y. (2002). Changes in blood-brain barrier permeability induced by radiotherapy: implications for timing of chemotherapy? (Review). *Oncology Reports*, *9*(4), 683–688.
- Van Zee, K. J., Stackpole, S. A., Montegut, W. J., Rogy, M. A., Calvano, S. E., Hsu, K. C., ... Stuber, D. (1994). A human tumor necrosis factor (TNF) alpha mutant that binds exclusively to the p55 TNF receptor produces toxicity in the baboon. *The Journal of Experimental Medicine*, *179*(4), 1185–1191.
- van Zijl, F., Krupitza, G., & Mikulits, W. (2011). Initial steps of metastasis: cell invasion and

endothelial transmigration. *Mutation Research*, *728*(1–2), 23–34. http://doi.org/10.1016/j.mrrev.2011.05.002

- Vandenbroucke, E., Mehta, D., Minshall, R., & Malik, A. B. (2008). Regulation of endothelial junctional permeability. *Annals of the New York Academy of Sciences*, *1123*, 134–145. http://doi.org/10.1196/annals.1420.016
- Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., & Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nature Reviews. Molecular Cell Biology*, *11*(5), 329–341. http://doi.org/10.1038/nrm2882
- Varatharaj, A., & Galea, I. (2017). The blood-brain barrier in systemic inflammation. *Brain, Behavior, and Immunity*, *60*, 1–12. http://doi.org/10.1016/j.bbi.2016.03.010
- Vareslija, D., Priedigkeit, N., Fagan, A., Purcell, S., Cosgrove, N., O'Halloran, P. J., ... Young, L. S. (2018). Transcriptome Characterization of Matched Primary Breast and Brain Metastatic Tumors to Detect Novel Actionable Targets. *Journal of the National Cancer Institute*. http://doi.org/10.1093/jnci/djy110
- Vassalli, P. (1992). The pathophysiology of tumor necrosis factors. *Annual Review of Immunology*, *10*, 411–452. http://doi.org/10.1146/annurev.iy.10.040192.002211
- Vazquez-Prado, J., Basile, J., & Gutkind, J. S. (2004). Modular architecture and novel protein-protein interactions regulating the RGS-containing Rho guanine nucleotide exchange factors. *Methods in Enzymology*, *390*, 259–285. http://doi.org/10.1016/S0076-6879(04)90017-1
- Velat, G. J., Kimball, M. M., Mocco, J. D., & Hoh, B. L. (2011). Vasospasm after aneurysmal subarachnoid hemorrhage: review of randomized controlled trials and meta-analyses in the literature. *World Neurosurgery*, *76*(5), 446–454. http://doi.org/10.1016/j.wneu.2011.02.030
- Ventura, J.-J., Hubner, A., Zhang, C., Flavell, R. A., Shokat, K. M., & Davis, R. J. (2006). Chemical genetic analysis of the time course of signal transduction by JNK. *Molecular Cell*, *21*(5), 701–710. http://doi.org/10.1016/j.molcel.2006.01.018
- Veroni, C., Gabriele, L., Canini, I., Castiello, L., Coccia, E., Remoli, M. E., ... Agresti, C. (2010). Activation of TNF receptor 2 in microglia promotes induction of anti-inflammatory pathways. *Molecular and Cellular Neurosciences*, *45*(3), 234–244. http://doi.org/10.1016/j.mcn.2010.06.014
- Verstrepen, L., Bekaert, T., Chau, T.-L., Tavernier, J., Chariot, A., & Beyaert, R. (2008). TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. *Cellular and Molecular Life Sciences : CMLS*, *65*(19), 2964–2978. http://doi.org/10.1007/s00018-008-8064-8
- Vestweber, D. (2012). Relevance of endothelial junctions in leukocyte extravasation and vascular permeability. *Annals of the New York Academy of Sciences*, *1257*, 184–192. http://doi.org/10.1111/j.1749-6632.2012.06558.x
- Vicente-Manzanares, M., Ma, X., Adelstein, R. S., & Horwitz, A. R. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature Reviews. Molecular Cell Biology*, *10*(11), 778–790. http://doi.org/10.1038/nrm2786
- Villalonga, P., Fernandez de Mattos, S., & Ridley, A. J. (2009). RhoE inhibits 4E-BP1 phosphorylation and eIF4E function impairing cap-dependent translation. *The Journal of Biological Chemistry*, *284*(51), 35287–35296. http://doi.org/10.1074/jbc.M109.050120
- Vincent, V. A., Tilders, F. J., & Van Dam, A. M. (1997). Inhibition of endotoxin-induced nitric oxide synthase production in microglial cells by the presence of astroglial cells: a role for transforming growth factor beta. *Glia*, *19*(3), 190–198.
- Vlahos, C. J., Matter, W. F., Hui, K. Y., & Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *The Journal of Biological Chemistry*, *269*(7), 5241–5248.
- Voura, E. B., Ramjeesingh, R. A., Montgomery, A. M., & Siu, C. H. (2001). Involvement of integrin alpha(v)beta(3) and cell adhesion molecule L1 in transendothelial migration of melanoma cells. *Molecular Biology of the Cell*, *12*(9), 2699–2710. http://doi.org/10.1091/mbc.12.9.2699
- Wadgaonkar, R., Linz-McGillem, L., Zaiman, A. L., & Garcia, J. G. N. (2005). Endothelial cell myosin light chain kinase (MLCK) regulates TNFalpha-induced NFkappaB activity. *Journal of Cellular Biochemistry*, *94*(2), 351–364. http://doi.org/10.1002/jcb.20250
- Wajant, H., Pfizenmaier, K., & Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death.Differ.*, *10*(1350–9047 (Print)), 45–65. http://doi.org/10.1038/sj.cdd.4401189
- Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., & Williams, R. L. (2000). Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular Cell*, *6*(4), 909–919.
- Walsh, C., Tanjoni, I., Uryu, S., Tomar, A., Nam, J.-O., Luo, H., ... Schlaepfer, D. D. (2010, May). Oral

delivery of PND-1186 FAK inhibitor decreases tumor growth and spontaneous breast to lung metastasis in pre-clinical models. *Cancer Biology & Therapy*.

- Wang, B., Parobchak, N., Martin, A., Rosen, M., Yu, L. J., Nguyen, M., ... Rosen, T. (2018). Screening a small molecule library to identify inhibitors of NF-kappaB inducing kinase and pro-labor genes in human placenta. *Scientific Reports*, *8*(1), 1657. http://doi.org/10.1038/s41598-018-20147-0
- Wang, C., Qin, L., Manes, T. D., Kirkiles-Smith, N. C., Tellides, G., & Pober, J. S. (2014). Rapamycin antagonizes TNF induction of VCAM-1 on endothelial cells by inhibiting mTORC2. *The Journal of Experimental Medicine*, *211*(3), 395–404. http://doi.org/10.1084/jem.20131125
- Wang, D., & Baldwin, A. S. J. (1998). Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. *The Journal of Biological Chemistry*, *273*(45), 29411–29416.
- Wang, K., Wang, Y., Gao, L., Li, X., Li, M., & Guo, J. (2008). Dexamethasone inhibits leukocyte accumulation and vascular permeability in retina of streptozotocin-induced diabetic rats via reducing vascular endothelial growth factor and intercellular adhesion molecule-1 expression. *Biological & Pharmaceutical Bulletin*, *31*(8), 1541–1546.
- Wang, S., Amato, K. R., Song, W., Youngblood, V., Lee, K., Boothby, M., ... Chen, J. (2015). Regulation of endothelial cell proliferation and vascular assembly through distinct mTORC2 signaling pathways. *Molecular and Cellular Biology*, *35*(7), 1299–1313. http://doi.org/10.1128/MCB.00306-14
- Wang, S., Lee, S.-R., Guo, S.-Z., Kim, W. J., Montaner, J., Wang, X., & Lo, E. H. (2006). Reduction of tissue plasminogen activator-induced matrix metalloproteinase-9 by simvastatin in astrocytes. *Stroke*, *37*(7), 1910–1912. http://doi.org/10.1161/01.STR.0000226923.48905.39
- Wang, Y., Ji, H.-X., Xing, S.-H., Pei, D.-S., & Guan, Q.-H. (2007). SP600125, a selective JNK inhibitor, protects ischemic renal injury via suppressing the extrinsic pathways of apoptosis. *Life Sciences*, *80*(22), 2067–2075. http://doi.org/10.1016/j.lfs.2007.03.010
- Warren, K. E., Patel, M. C., Aikin, A. A., Widemann, B., Libucha, M., Adamson, P. C., ... Balis, F. M. (2001). Phase I trial of lobradimil (RMP-7) and carboplatin in children with brain tumors. *Cancer Chemotherapy and Pharmacology*, *48*(4), 275–282.
- Wassmer, S. C., Moxon, C. A., Taylor, T., Grau, G. E., Molyneux, M. E., & Craig, A. G. (2011). Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. *Cellular Microbiology*, *13*(2), 198–209. http://doi.org/10.1111/j.1462-5822.2010.01528.x
- Wegmann, F., Ebnet, K., Du Pasquier, L., Vestweber, D., & Butz, S. (2004). Endothelial adhesion molecule ESAM binds directly to the multidomain adaptor MAGI-1 and recruits it to cell contacts. *Experimental Cell Research*, *300*(1), 121–133. http://doi.org/10.1016/j.yexcr.2004.07.010
- Weiss, N., Miller, F., Cazaubon, S., & Couraud, P.-O. (2009). The blood-brain barrier in brain homeostasis and neurological diseases. *Biochimica et Biophysica Acta*, *1788*(4), 842–857. http://doi.org/10.1016/j.bbamem.2008.10.022
- Wenzel, J., & Schwaninger, M. (2016). The blood--brain barrier and its regulation by NF-$\varkappa$B. *E-Neuroforum*, *7*(2), 23–30. http://doi.org/10.1007/s13295-016-0022-6
- Wiggins-Dohlvik, K., Merriman, M., Shaji, C. A., Alluri, H., Grimsley, M., Davis, M. L., ... Tharakan, B. (2014). Tumor necrosis factor-α disruption of brain endothelial cell barrier is mediated through matrix metalloproteinase-9. *The American Journal of Surgery*, *208*(6), 954–960. http://doi.org/10.1016/j.amjsurg.2014.08.014
- Wilhelm, I., Fazakas, C., Molnar, J., Hasko, J., Vegh, A. G., Cervenak, L., ... Krizbai, I. A. (2014). Role of Rho/ROCK signaling in the interaction of melanoma cells with the blood-brain barrier. *Pigment Cell & Melanoma Research*, *27*(1), 113–123. http://doi.org/10.1111/pcmr.12169
- Wilhelm, I., Molnar, J., Fazakas, C., Hasko, J., & Krizbai, I. A. (2013). Role of the blood-brain barrier in the formation of brain metastases. *International Journal of Molecular Sciences*, *14*(1), 1383–1411. http://doi.org/10.3390/ijms14011383
- Williams, S. A., Chen, L.-F., Kwon, H., Fenard, D., Bisgrove, D., Verdin, E., & Greene, W. C. (2004). Prostratin antagonizes HIV latency by activating NF-kappaB. *The Journal of Biological Chemistry*, *279*(40), 42008–42017. http://doi.org/10.1074/jbc.M402124200
- Wilson, H. K., Canfield, S. G., Hjortness, M. K., Palecek, S. P., & Shusta, E. V. (2015). Exploring the effects of cell seeding density on the differentiation of human pluripotent stem cells to brain microvascular endothelial cells. *Fluids and Barriers of the CNS*, *12*, 13. http://doi.org/10.1186/s12987-015-0007-9
- Wilson, N. S., Dixit, V., & Ashkenazi, A. (2009). Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nature Immunology*, *10*(4), 348–355.

http://doi.org/10.1038/ni.1714
- Wingfield, P., Pain, R. H., & Craig, S. (1987). Tumour necrosis factor is a compact trimer. *FEBS Letters*, *211*(2), 179–184.
- Winkler, F. (2015). The brain metastatic niche. *Journal of Molecular Medicine (Berlin, Germany)*, *93*(11), 1213–1220. http://doi.org/10.1007/s00109-015-1357-0
- Wojciak-Stothard, B., Entwistle, A., Garg, R., & Ridley, A. J. (1998). Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. *Journal of Cellular Physiology*, *176*(1), 150–165. http://doi.org/10.1002/(SICI)1097-4652(199807)176:1<150::AID-JCP17>3.0.CO;2-B
- Wojciak-Stothard, B., & Ridley, A. J. (2002). Rho GTPases and the regulation of endothelial permeability. *Vascular Pharmacology*, *39*(4–5), 187–199.
- Wolburg, H., Wolburg-Buchholz, K., Kraus, J., Rascher-Eggstein, G., Liebner, S., Hamm, S., ... Engelhardt, B. (2003). Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathologica*, *105*(6), 586–92. http://doi.org/10.1007/s00401-003-0688-z
- Wolfrum, S., Dendorfer, A., Rikitake, Y., Stalker, T. J., Gong, Y., Scalia, R., ... Liao, J. K. (2004). Inhibition of Rho-kinase leads to rapid activation of phosphatidylinositol 3-kinase/protein kinase Akt and cardiovascular protection. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*(10), 1842–1847. http://doi.org/10.1161/01.ATV.0000142813.33538.82
- Wong, D., & Dorovini-Zis, K. (1992). Upregulation of intercellular adhesion molecule-1 (ICAM-1) expression in primary cultures of human brain microvessel endothelial cells by cytokines and lipopolysaccharide. *Journal of Neuroimmunology*, *39*(1–2), 11–21.
- Wong, D., & Dorovini-Zis, K. (1995). Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture. *Microvascular Research*, *49*(3), 325–339. http://doi.org/10.1006/mvre.1995.1028
- Wosik, K., Biernacki, K., Khouzam, M.-P., & Prat, A. (2007). Death receptor expression and function at the human blood brain barrier. *Journal of the Neurological Sciences*, *259*(1–2), 53–60. http://doi.org/10.1016/j.jns.2006.08.018
- Wu, M. H., Yuan, S. Y., & Granger, H. J. (2005). The protein kinase MEK1/2 mediate vascular endothelial growth factor- and histamine-induced hyperpermeability in porcine coronary venules. *The Journal of Physiology*, *563*(Pt 1), 95–104. http://doi.org/10.1113/jphysiol.2004.076075
- Wu, X., Guo, R., Chen, P., Wang, Q., & Cunningham, P. N. (2009). TNF induces caspase-dependent inflammation in renal endothelial cells through a Rho- and myosin light chain kinase-dependent mechanism. *American Journal of Physiology. Renal Physiology*, *297*(2), F316-26. http://doi.org/10.1152/ajprenal.00089.2009
- Wyler, L., Napoli, C. U., Ingold, B., Sulser, T., Heikenwalder, M., Schraml, P., & Moch, H. (2014). Brain metastasis in renal cancer patients: metastatic pattern, tumour-associated macrophages and chemokine/chemoreceptor expression. *British Journal of Cancer*, *110*(3), 686–694. http://doi.org/10.1038/bjc.2013.755
- Xu, C., Wu, X., Hack, B. K., Bao, L., & Cunningham, P. N. (2015). TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports*, *3*(12). http://doi.org/10.14814/phy2.12636
- Xu, Y., Lai, E., Liu, J., Lin, J., Yang, C., Jia, C., ... Li, M. (2013). IKK interacts with rictor and regulates mTORC2. *Cellular Signalling*, *25*(11), 2239–2245. http://doi.org/10.1016/j.cellsig.2013.07.008
- Yamamoto, M., Ramirez, S. H., Sato, S., Kiyota, T., Cerny, R. L., Kaibuchi, K., ... Ikezu, T. (2008). Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. *The American Journal of Pathology*, *172*(2), 521–533. http://doi.org/10.2353/ajpath.2008.070076
- Yamashina, K., & Heppner, G. H. (1985). Correlation of frequency of induced mutation and metastatic potential in tumor cell lines from a single mouse mammary tumor. *Cancer Research*, *45*(9), 4015–4019.
- Yang, C., Huang, X., Liu, H., Xiao, F., Wei, J., You, L., & Qian, W. (2017). PDK1 inhibitor GSK2334470 exerts antitumor activity in multiple myeloma and forms a novel multitargeted combination with dual mTORC1/C2 inhibitor PP242. *Oncotarget*, *8*(24), 39185–39197. http://doi.org/10.18632/oncotarget.16642
- Yao, L., Romero, M. J., Toque, H. A., Yang, G., Caldwell, R. B., & Caldwell, R. W. (2010). The role of RhoA/Rho kinase pathway in endothelial dysfunction. *Journal of Cardiovascular Disease Research*, *1*(4), 165–170. http://doi.org/10.4103/0975-3583.74258
- Yatim, N., Jusforgues-Saklani, H., Orozco, S., Schulz, O., Barreira da Silva, R., Reis e Sousa, C., ... Albert, M. L. (2015). RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of

CD8(+) T cells. *Science (New York, N.Y.)*, *350*(6258), 328–334. http://doi.org/10.1126/science.aad0395
- Ye, D., Ma, I., & Ma, T. Y. (2006). Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *290*(3), G496-504. http://doi.org/10.1152/ajpgi.00318.2005
- Ye, D., & Ma, T. Y. (2008). Cellular and molecular mechanisms that mediate basal and tumour necrosis factor-alpha-induced regulation of myosin light chain kinase gene activity. *Journal of Cellular and Molecular Medicine*, *12*(4), 1331–1346. http://doi.org/10.1111/j.1582-4934.2008.00302.x
- Ye, D. Z., & Field, J. (2012). PAK signaling in cancer. *Cellular Logistics*, *2*(2), 105–116. http://doi.org/10.4161/cl.21882
- Yepes, M. (2013). TWEAK and Fn14 in the Neurovascular Unit. *Frontiers in Immunology*, *4*, 367. http://doi.org/10.3389/fimmu.2013.00367
- Yi, R., Xiao-Ping, G., & Hui, L. (2015). Atorvastatin prevents angiotensin II-induced high permeability of human arterial endothelial cell monolayers via ROCK signaling pathway. *Biochemical and Biophysical Research Communications*, *459*(1), 94–99. http://doi.org/10.1016/j.bbrc.2015.02.076
- Yin, D. (1996). Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. *Free Radical Biology & Medicine*, *21*(6), 871–888.
- Yin, Y., Zheng, K., Eid, N., Howard, S., Jeong, J.-H., Yi, F., ... Feng, Y. (2015). Bis-aryl urea derivatives as potent and selective LIM kinase (Limk) inhibitors. *Journal of Medicinal Chemistry*, *58*(4), 1846–1861. http://doi.org/10.1021/jm501680m
- Yoneda, T., Williams, P. J., Hiraga, T., Niewolna, M., & Nishimura, R. (2001). A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research*, *16*(8), 1486–1495. http://doi.org/10.1359/jbmr.2001.16.8.1486
- Yoshimasu, T., Sakurai, T., Oura, S., Hirai, I., Tanino, H., Kokawa, Y., ... Matsuura, N. (2004). Increased expression of integrin alpha3beta1 in highly brain metastatic subclone of a human non-small cell lung cancer cell line. *Cancer Science*, *95*(2), 142–148.
- Yu, D., Marchiando, A. M., Weber, C. R., Raleigh, D. R., Wang, Y., Shen, L., & Turner, J. R. (2010). MLCK-dependent exchange and actin binding region-dependent anchoring of ZO-1 regulate tight junction barrier function. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(18), 8237–8241. http://doi.org/10.1073/pnas.0908869107
- Yu, Q., Gratzke, C., Wang, Y., Herlemann, A., Sterr, C. M., Rutz, B., ... Hennenberg, M. (2018). Inhibition of human prostate smooth muscle contraction by the LIM kinase inhibitors, SR7826 and LIMKi3. *British Journal of Pharmacology*, *175*(11), 2077–2096. http://doi.org/10.1111/bph.14201
- Yu, Y., Qin, J., Liu, M., Ruan, Q., Li, Y., & Zhang, Z. (2014). Role of Rho kinase in lysophosphatidic acid-induced altering of blood-brain barrier permeability. *International Journal of Molecular Medicine*, *33*(3), 661–669. http://doi.org/10.3892/ijmm.2014.1618
- Yuan, S. Y., Wu, M. H., Ustinova, E. E., Guo, M., Tinsley, J. H., De Lanerolle, P., & Xu, W. (2002). Myosin light chain phosphorylation in neutrophil-stimulated coronary microvascular leakage. *Circulation Research*, *90*(11), 1214–1221.
- Zakaria, R., & Jenkinson, M. D. (2014). Using ADC Maps with Structural Scans to Improve Intraoperative Biopsy Specimens in Brain Metastases. *The Neuroradiology Journal*, *27*(4), 422–424. http://doi.org/10.15274/NRJ-2014-10075
- Zarnegar, B. J., Wang, Y., Mahoney, D. J., Dempsey, P. W., Cheung, H. H., He, J., ... Cheng, G. (2008). Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nature Immunology*, *9*(12), 1371–1378. http://doi.org/10.1038/ni.1676
- Zhang, C., Gao, F., Teng, F., & Zhang, M. (2015). Fas/FasL Complex Promotes Proliferation and Migration of Brain Endothelial Cells Via FADD-FLIP-TRAF-NF-kappaB Pathway. *Cell Biochemistry and Biophysics*, *71*(3), 1319–1323. http://doi.org/10.1007/s12013-014-0351-4
- Zhang, C., & Yu, D. (2011). Microenvironment determinants of brain metastasis. *Cell & Bioscience*, *1*(1), 8. http://doi.org/10.1186/2045-3701-1-8
- Zhang, D., & Wood, C. E. (2005). Neuronal prostaglandin endoperoxide synthase 2 responses to oxygen and glucose deprivation are mediated by mitogen-activated protein kinase ERK1/2. *Brain Research*, *1060*(1–2), 100–107. http://doi.org/10.1016/j.brainres.2005.08.033
- Zhang, G., Lei, F., Zhou, Q., Feng, D., & Bai, Y. (2016). Combined application of Rho-ROCKII and GSK-3beta inhibitors exerts an improved protective effect on axonal regeneration in rats with spinal cord injury. *Molecular Medicine Reports*, *14*(6), 5180–5188. http://doi.org/10.3892/mmr.2016.5918

- Zhang, L., Zhang, S., Yao, J., Lowery, F. J., Zhang, Q., Huang, W.-C., ... Yu, D. (2015). Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature*, *527*(7576), 100–104. http://doi.org/10.1038/nature15376
- Zhang, R. D., Price, J. E., Fujimaki, T., Bucana, C. D., & Fidler, I. J. (1992). Differential permeability of the blood-brain barrier in experimental brain metastases produced by human neoplasms implanted into nude mice. *The American Journal of Pathology*, *141*(5), 1115–1124.
- Zhang, X., Zhang, W., Cao, W.-D., Cheng, G., Liu, B., & Cheng, J. (2012). A review of current management of brain metastases. *Annals of Surgical Oncology*, *19*(3), 1043–1050. http://doi.org/10.1245/s10434-011-2019-2
- Zhao, T., Zhang, X., Zhao, Y., Zhang, L., Bai, X., Zhang, J., ... Cui, L. (2014). Pretreatment by evodiamine is neuroprotective in cerebral ischemia: up-regulated pAkt, pGSK3beta, down-regulated NF-kappaB expression, and ameliorated BBB permeability. *Neurochemical Research*, *39*(8), 1612–1620. http://doi.org/10.1007/s11064-014-1356-5
- Zhao, X., Peng, X., Sun, S., Park, A. Y. J., & Guan, J.-L. (2010). Role of kinase-independent and -dependent functions of FAK in endothelial cell survival and barrier function during embryonic development. *The Journal of Cell Biology*, *189*(6), 955–965. http://doi.org/10.1083/jcb.200912094
- Zhao, Z., Hu, J., Gao, X., Liang, H., Yu, H., Liu, S., & Liu, Z. (2017). Hyperglycemia via activation of thromboxane A2 receptor impairs the integrity and function of blood-brain barrier in microvascular endothelial cells. *Oncotarget*, *8*(18), 30030–30038. http://doi.org/10.18632/oncotarget.16273
- Zhong, X., Li, X., Liu, F., Tan, H., & Shang, D. (2012). Omentin inhibits TNF-alpha-induced expression of adhesion molecules in endothelial cells via ERK/NF-kappaB pathway. *Biochemical and Biophysical Research Communications*, *425*(2), 401–406. http://doi.org/10.1016/j.bbrc.2012.07.110
- Zhu, W., Zhou, L., Qian, J.-Q., Qiu, T.-Z., Shu, Y.-Q., & Liu, P. (2014). Temozolomide for treatment of brain metastases: A review of 21 clinical trials. *World Journal of Clinical Oncology*, *5*(1), 19–27. http://doi.org/10.5306/wjco.v5.i1.19
- Zonta, M., Angulo, M. C., Gobbo, S., Rosengarten, B., Hossmann, K.-A., Pozzan, T., & Carmignoto, G. (2003). Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nature Neuroscience*, *6*(1), 43–50. http://doi.org/10.1038/nn980
- Zuo, S., Ge, H., Li, Q., Zhang, X., Hu, R., Hu, S., ... Feng, H. (2017). Artesunate Protected Blood-Brain Barrier via Sphingosine 1 Phosphate Receptor 1/Phosphatidylinositol 3 Kinase Pathway After Subarachnoid Hemorrhage in Rats. *Molecular Neurobiology*, *54*(2), 1213–1228. http://doi.org/10.1007/s12035-016-9732-6

Presentations, awards, conference publication and collaborations relevant to this thesis

Christina Simoglou Karali, Mario Munoz Pinto, Vinton Cheng, Niloufar Zarghami, Daniel Beard, Daniel C.Anthony and Nicola R. Sibson "Towards TNFR1-selective permeabilisation of the blood-brain barrier". *Oral presentation* 8th Annual Brain Metastases Research and Emerging Therapy Conference, 21-22/09/2018, Marseille, France

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Manuel Sarmiento Soto, Yvonne Couch, Daniel C.Anthony, Nicola R. Sibson. "The impact of brain metastasis on endothelial cell function". *Poster presentation.* CRUK Brain Tumour Conference, 01-03/05/2018, London, UK

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Manuel Sarmiento Soto, Yvonne Couch, Daniel C.Anthony, Nicola R. Sibson. "The impact of brain metastasis on endothelial cell function". *Poster presentation.* 12th International Conference on Cerebral Vascular Biology (CVB 2017), 28/11/2017, Melbourne, Australia

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Manuel Sarmiento Soto, Yvonne Couch, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Poster presentation & Best poster award.* 7th Annual Brain Metastases Research and Emerging Therapy Conference, 06-07/10/2017, Marseille, France

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Manuel Sarmiento Soto, Yvonne Couch, Daniel C.Anthony, Nicola R. Sibson. "The impact of brain metastasis on endothelial cell function". *Oral presentation & Conference publication.* 20th International Symposium on Signal Transduction at the Blood-Brain Barriers, 13-15/09/2017, Krakow, Poland

Christina Simoglou Karali, Mario Munoz Pinto, Vinton Cheng, Niloufar Zarghami, Manuel Sarmiento Soto, Yvonne Couch, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Poster presentation* CRUK/MRC Oxford Institute for Radiation Oncology Symposium, 07-08/09/2017

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Kleopatra Andreou, Manuel Sarmiento Soto, Daniel C.Anthony, Nicola R. Sibson. "The impact of brain metastasis on endothelial cell function". *Oral presentation.* 7th UK& Ireland Early Career BBB Symposium, 01/09/2017, Portsmouth, UK

Christina Simoglou Karali, Vinton Cheng, Niloufar Zarghami, Kleopatra Andreou, Manuel Sarmiento Soto, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Oral presentation.* 6th UK& Ireland Early Career BBB Symposium, 25/11/2016, Dublin, Ireland

Christina Simoglou Karali, Manuel Sarmiento Soto, Vasiliki Economopoulos, Kleopatra Andreou, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Poster presentation & Conference publication* BNOS 2016 Conference, 29/06-01/07/2016, Leeds, UK

Christina Simoglou Karali, Manuel Sarmiento Soto, Vasiliki Economopoulos, Kleopatra Andreou, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Oral presentation* 2016 Oncology Student Symposium 23/06/2015, Oxford, UK

Christina Simoglou Karali, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier". *Oral presentation.* 5th UK& Ireland Early Career BBB Symposium, 01/09/2017, Southampton, UK

Christina Simoglou Karali, Stavros Melemenidis, John Connell, James Larkin, Manuel Sarmiento-Soto, Kleopatra Andreou, Len Seymour, Daniel C.Anthony, Nicola R. Sibson. "Towards TNFR1-specific permeabilisation of the blood-brain barrier in brain metastasis" *Poster presentation & Best poster award.*
2015 Oncology Student Symposium 03/07/2015, Oxford, UK

Grants

CRUK studentship (£32,000/4 years)
Clarendon scholarship (£24,421/ 3 years) & New college scholarship (£20,750/4 years)
A.G. Leventis foundation studentship (£7000/ 2 years)
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European Association of Neuro-Oncology poster award (300€)
New College travel awards (£900)

Collaborations

Prof Alastair Buchan, Dr Daniel Beard, Dr Yvonne Couch, Dr Paul Holloway, University of Oxford, Laboratory of Cerebral Ischemia
Prof Olaf Ansorge, University of Oxford, Clinical Neuropathology Group
Prof Ignacio Romero, The Open University, Neurovascular Research Group
Prof Pierre-Olivier Couraud, INSERM, Institut Cochin, Paris
Prof Geoff Pilkington, University of Portsmouth, Brain Tumour Research Centre of Excellence
Dr Roman Fischer, Dr Iolanda Vendrell, University of Oxford, Targeted Discovery Institute
Prof Eleanor Stride, Dr Miles Aron, University of Oxford, Institute of Biomedical Engineering
Dr Graham Brown, Dr Rhodri Wilson, University of Oxford, Department of Oncology, Microscopy core