Endothelial Activation in Experimental Metastasis Models

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ABSTRACT

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The majority of cancer related deaths occur due to the invasive growth of metastatic lesions. In the early stages of metastasis, circulating cell interact with the endothelial cells to establish at a distant site. In inflammation endothelial activation results in induction of adhesion molecules on the endothelium that participate in the homing of leukocytes. Because of the interactions of metastatic cells with the endothelium, the question was whether some of the characteristic molecules of endothelial activation were induced during metastasis.

In vivo pulmonary metastatic models were used to characterize the expression profile of endothelial activation. Immunohistochemistry identified VCAM-1 to be induced on the pulmonary endothelium following tumour cell arrest. VCAM-1 upregulation was not observed prior to tumour cells arrest or within the first hours. In contrast, tumour cell arrest appeared to be required for endothelial activation, arguing against a mechanism analogous to leukocyte homing. The upregulation of VCAM-1 upon tumour cell arrest corresponded with the initiation of platelet clot formation around the tumour cell and recruitment of leukocytes to the site, both previously shown to be essential for metastasis. Disruption of both phenomena, either through genetic or pharmacological manipulation, demonstrated that in contrast to the recruited leukocytes, platelets were involved in inducing endothelial activation.

Another protein investigated was VAP-1. In contrast to VCAM-1, central to VAP-1 adhesive function is its enzymatic activity. Blocking the functions of either molecule highlighted their role in facilitating the recruitment of the leukocyte population to the tumour cell. Disruption of which led to a significant attenuation of metastasis. While VCAM-1 and VAP-1 function appears critical in the early steps of metastasis, their inhibition had no effect at later stages of pulmonary colonization.

To my family.

To my parents, for their support, patience and encouragement.
Without them I would not be here.

To my grandfather, the best role-model one could ever wish for
and my grandmother for her caring support.

To my sisters, for always being there for me.

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LIST OF ABBREVIATIONS

AP – alkaline phosphatase

CAMs – cell adhesion molecules

CSC – cancer stem cell

CSF1R-GFP - FVB-CSF-1R-GFP mouse (FVB.*(tg(Csd1r-EGFP)1Jwp)*)

CX3CR1-GFP - $CX_3CR1^{GFP/+}$ mouse (B6.129P(Cg)-Ptprc^{a} Cx3cr1^{tm1Litt}/LittJ)

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DTT – dithiothreitol

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EGFP – enhanced green fluorescent protein

EMT – epithelial-mesenchymal transition

FBS – foetal bovine serum

GPIbα – glycoprotein Ib alpha chain

HEV – high endothelial venules

HRP – horseradish peroxidase

i.p. – intraperitoneal

i.v. – intravenous

ICAM-1 – Inter-cellular adhesion molecule-1

IHC – immunohistochemistry

K.R.B. – Krebs-Ringer Bicarbonate Buffer

KO – gene knockout

LLC – Lewis lung carcinoma

MDSCs – myeloid derived suppressor cells

NK cells – natural killer cells

NO – nitric oxide

PAR – poly(ADP-ribose)

PBS – phosphate buffered saline

PECAM-1 – platelet endothelial cell adhesion molecule-1

PSGL-1– P-selectin glycoprotein ligand-1

PVDF – polyvinylidene fluoride

ROS – reactive oxygen species

RPMI - Roswell Park Memorial Institute medium

s.c. – subcutaneous

SCID – Severe combined immune deficiency

SD – standard deviation

SEM – standard error of the mean

TF – tissue factor

TFPI – tissue factor pathway inhibitor

TM – thrombomodulin

VAP-1 – vascular adhesion protein -1

VCAM-1 – vascular cell adhesion molecule -1

vWF- von Willebrand factor

INTRODUCTION

METASTASIS

The term metastasis was formulated by Jean Claude Recamier in 1829.[1] It can be defined as the development and growth of secondary tumours at sites distant from the primary tumour[2]. Cancer metastasis represents the most formidable aspect of cancer, as despite significant advances in diagnosis, local and systemic therapies and surgical techniques[3], it is responsible for over 90% of deaths from solid tumours and so represents the main clinical challenge.[1,2,4-7] However, research in the past has largely been focused on the mechanisms of oncogenic transformation and primary tumour establishment rather than metastatic disease.[2-4,7]

Since essential regulatory genes were identified and better experimental models became available with technological advances, the study of the metastatic process has expanded dramatically in the last decade.[2-4,8] While many unanswered questions remain, significant progress has been made and is summarized below.

The metastatic process consists of a series of sequential steps.[1,3,7] The process begins with the acquisition of cell motility, followed by local invasion of the surrounding tissue at the site of the primary tumour. This is followed by intravasation into the haematogenous circulation or lymphatics, survival in the vascular environment and finally translocation to the secondary site. Here tumour cells generally extravasate across the endothelial cell layer into the tissue parenchyma. However for the metastatic process to be successful tumour cell survival and proliferation are required. This last stage is termed colonization. [5,7,9,10] Each of these steps in the cascade has been shown to be extremely inefficient,[1,7,9,11-17] as demonstrated by Fidler *et al* in the 1970s. They used the melanoma murine melanoma cancer cells (B16), where the cells were labelled via incorporation of radioactive

deoxyuridine into their DNA. Following tumour cell injection and metastasis establishment, the organs were harvested, minced and radioactivity was measured. They noted that within 24h of entry into the circulation less than 0.1% of cells remained viable and only 0.01% produced metastasis.[1,3,9,12] Furthermore, it has been shown that large tumours can shed millions of cells into the vasculature every day, while only few macroscopic colonies develop.[11,17-19] Furthermore in the work of Yang *et al*[20], cell clones were isolated through serial passage of a cell line. Each clone was deficient in one step in the metastatic cascade and thereby unable to form metastatic foci.[2,3,20,21] These data suggest that each of the steps in the metastatic cascade can be rate-limiting,[3,22] as failure at any point will lead to metastatic failure.

An intriguing aspect of cancer cell dissemination is the variability in the tissue tropism that is displayed by different types of cancer.[6,14,23] This was noted as long ago as 1889 by an English surgeon, Stephen Paget.[24] He analysed autopsies of 735 women, who died of breast cancer and noted a discrepancy between blood supply to the metastatic organs and the frequency of metastasis; more specifically, the high incidence of metastasis observed in the liver, ovary and bone and lack of metastatic prevalence in the spleen. He then postulated the "Seed and soil hypothesis", which states that the development of metastasis depends on the intrinsic properties of the tumour cells and on the host response. [1,3,5,24,25] His hypothesis was challenged in 1929 by James Ewing, who proposed that metastatic dissemination is governed purely by mechanical factors resulting from the anatomy of the vasculature and direction of circulation. [1,3,26]

In the 1970s the selective nature of metastasis was documented by Fidler *et al*, showing that while common local regional metastases (e.g. lymph nodes) could be attributed to mechanical arrest, interactions with the local microenvironment are required for subsequent colonization events.[1,3,25,27,28] They injected B16 murine melanoma cells i.v. and observed that metastases only developed in the lungs and in fragments of pulmonary and ovarian tissue implanted intramuscularly, while failing to

grow in implanted renal tissue or at the site of surgery.[1 28]

Additional evidence has also been provided in human ovarian cancer. Ovarian cancer cells spread through the peritoneal cavity and attach to the surface of peritoneal organs or grow in ascites fluid but do not metastasize to visceral organs. Palliative surgery, generally practiced, involves the introduction of venous shunts to drain the ascites fluid. Inadvertently this also allows entry of ovarian cancer cells into the haematogenous circulation. Tarin *et al* demonstrated that this has no effect on the risk for developing metastases, thereby again demonstrating the crucial role of other microenvironmental factors.[1,3,19,29] It is now generally accepted that anatomic architecture plays a role in the homing of tumour cells to distant organs but is not sufficient to fully account for the pattern of metastatic spread.[1,3,27,28,30-32]

In order to chart the pathways of metastasis, mathematical analysis was performed to construct models analysing the progression dynamics of different types of cancer based on their organ of origin. Using the Medicare forms of two million American patients, the researchers were able to predict the sites of metastatic spread based on the primary tumour and vice versa.[2,33] Such patterns of metastatic spread that have emerged through autopsies and clinical examinations over the past decade have been extended to attempt to define precise molecular mechanisms.

Next in this chapter, the two main controversies of metastatic research will be outlined. Firstly, whether metastasis occurs as an early or a late event in tumour progression and secondly the concept of cancer stem cells in metastatic dissemination. Following this the metastatic cascade will be described in detail, including the role of micro-environmental factors and host elements. The involvement of the endothelial cell layer and endothelial adhesion proteins in the inflammatory processes will be outlined and finally, experimental evidence of tumour cell interactions with endothelial cells at the metastatic site will be presented.

1. Early or late?

Controversy exists in metastatic research as to whether cancer cell dissemination occurs early or late during the progression of the primary tumour. This question carries further implications, since if cancer cell dissemination occurs early in tumour development, a large percentage of the primary tumour cell population would acquire metastatic capacity. In contrast, if dissemination is initiated at the later stages of tumour progression, only a small percentage of the population will carry the metastatic potential. [6,27,34,35]

The classical, widely accepted view was established in 1977 by Fidler *et al*.[27] It states that during tumour progression, a small subpopulation of cancer cells acquire the genetic alterations required for them to be able to metastasize to specific organs, postulating the"3S hypothesis", where metastasis is sequential and selective with stochastic elements.[1,36-38] As these cells would arise as a result of a progressive and cumulative process, they would be rare within the heterogeneous population and would arise late in tumour progression. In a way this models Darwinian evolution on a micro-scale, where sequentially acquired characteristics would mediate progression in tumourigenesis. [1,3,7,27,39] This was best illustrated by genome sequencing studies of pancreatic ductal adenocarcinomas, providing genetic evidence for clonal evolution to metastasis.[8,40-43] This hypothesis would also imply that the metastatic lesion and the primary tumour giving rise to it should be genetically quite similar. This is supported by work of Kuukasjarveu *et al*[44], who analysed the genetic composition of primary breast carcinomas and corresponding metastases and found that 69% had a high degree of clonality.[1,44] Another implication of clonal progression was proposed by Peter Nowell.[1,3,45] He postulated that the accumulation of genetic and epigenetic alterations required for the metastatic phenotype, suggests that metastatic cells should be genetically more unstable.[2,45,46] Using acquisition of mutations that confer drug resistance as the read-out, Fidler *et al* demonstrated that indeed metastatic cells were

three to seven fold more genetically unstable and the observation was further extended to include human models.[3,40,47] This model is called the late metastatic model or the model of metastatic evolution. [2,6,25,34,48] It is consistent with experiments utilising serial *in vivo* passaging of metastatic colonies in the lung, producing cell variants with different levels of metastatic efficiency.[27] However, it fails to explain the emergence of metastatic disease in patients long after excision of an early-detected small primary tumour.[2,25]

The second hypothesis was based on several gene-expression profiling studies showing that clinical prognosis can be established based on gene expression profiles of the primary tumour.[5,6,49-55] Some studies use retrospective analysis, where gene expression profiles of several primary tumours were grouped based on the clinical aggressiveness of the disease. This facilitated the characterisation of a gene expression profile predictive of metastatic relapse.[49,50,54- 56] Others compared the gene expression of several metastatic colonies to the gene expression profile of matched primary tumour mass. They found that the expression profile of a subset of primary tumours resembled that of the metastases and these were found to be more likely to be associated with metastatic disease and poor prognosis.[51,57] Both types of analysis allowed researchers to develop expression signatures associated with higher likelihood of metastatic dissemination and poorer prognosis.[25,51,53-57] These studies imply that the characterised genes of the "metastatic signature" are uniformly expressed across the population of the primary tumour. In other words, most of the cells in the primary tumour would possess metastatic potential and dissemination would occur early in tumour progression. Moreover, this also implied that metastasis was a genetic trait, dependent exclusively on the intrinsic properties of cancer cells.

The late metastasis model was also challenged by Bernards and Weinberg.[58] They argued that if the model of tumour progression was true, then the genes that govern metastasis would need to confer a survival benefit at the primary site for them to have developed as an evolutionary trait.[58] That this

may not be the case was demonstrated by Minn *et al*.[5,59] They knocked down the expression of genes that were shown to be specific to the cells responsible for lung colonization and observed that frequently this had no effect on the growth of the primary tumour. As “metastasis specific genes" would not confer an advantage to the cells in the primary tumour, these would remain extremely rare and metastasis would not proceed. Therefore, they proposed instead that the genetic changes required for tumourigenesis would also inadvertently confer the ability to metastasize. In this way, metastasis would be largely pre-determined by the combined actions of mutations that give rise to the primary tumour.[5,59,60]

This is also supported by a gene-expression study that identified the signalling pathways involved in breast cancer dissemination to the bone marrow. It showed the interlinking of genes responsible for proliferation and dissemination, namely Ras and Hif1α.[61,62] This results in three significant implications: it infers that the tendency to metastasize is acquired early in tumour progression, that metastasis specific genetic changes do not exist and as this is an early event, even a very small primary tumour mass can result in cell dissemination.[58,63] Consistent with this theory are the *in vivo* data demonstrating the presence of disseminated cancer cells in the bone marrow and lungs of early breast cancer patients and patients that never presented with overt disease. [34,64-66] Another argument in favour of early dissemination is the work by Husemann *et al* who showed dissemination of pre-malignant cells from the mammary tissue of transgenic animals to lungs and bone marrow when only atypical ductal hyperplasia presented in the mammary tissue.[34,48,63,64] Further, powerful arguments are provided by Podsypanina *et al*, [67] who showed that normal mouse epithelial cells, injected i.v. can arrest and survive in the lung[l]. When oncogene expression was induced, these cells initiated the process of colonization.[34,67]

[l] When these cells were re-implanted into the mammary fat pad tissue, they could form mammary glands.

However, the model based solely on pre-determined traits, outlined above, cannot explain the somatic evolution and general heterogeneity of primary tumours. [1,25,68] This heterogeneity, shown both in murine and human tumours, was confirmed by studies of clonal cell lines that were derived from late-stage carcinomas, showing that they differ in their metastatic potential.[27,69-73] The early dissemination model is also incompatible with the microarray data that identified specific tissue targeting genes and over 20 candidate metastasis suppressor genes that inhibited tumour cell spreading but had no effect on the growth of the primary tumour. [2,25,48,74-76]

A new hypothesis was proposed by Massague *et al* to bridge this gap, where the poor prognosis gene expression profile facilitates invasiveness of the tumour cells in the primary tumour mass and their entry into the circulation. Subsequently, these tumour cell acquire additional alterations in order to successfully metastasize in a tissue specific manner.[6] They used TGF-β to stimulate bone metastasis after i.v. tumour cell inoculation. This was followed by a series of *in vivo* passages and selections, resulting in sub-populations of cells with enhanced bone colonizing abilities. Following this, the transcriptome of these populations was compared to the parental cell line, leading to the identification of several genes with enhanced expression. They have been able to identify a poor-prognosis gene expression signature in the primary cell line, but the bone-seeking variant acquired additional changes.[6] Also along similar principles, Minn *et al* [5,59] established a lung seeking variant cell line (LM2). According to this model, the cells of the primary tumour possess the poor-prognosis gene expression signature and this allows invasion and escape of the cells from the primary tumour. In this new environment certain cells acquire additional functions in order to achieve the tissue specific phenotype and develop into aggressive metastases. Therefore the poor prognosis signature enables the emergence of metastatic cells, but the specific gene expression programmes would execute this potential[6,59]. As the microenvironments of different organs differ, so would the required sets of markers.[6]

2. Cancer stem cells in metastasis

In trying to answer the question of which cells can accomplish metastasis, in past years a large mass of research focused on cancer stem cells (CSCs).[2,77] The controversial "stem cell hypothesis" argues that, as the normal tissue consists of a hierarchy of cell lineages ranging from stem cells to terminally differentiated cells[4], the same architecture should be observed within tumour tissue[7,78-80]. These cancer stem cells are defined as being able to self-renew[II], survive in undesirable micro-environmental conditions (resistance to apoptosis) and be able to undergo multi-lineage differentiation. [1,7,79-89] This suggests that these would be the cells responsible for driving tumourigenesis and cancer development and for recreating the heterogeneous population analogous to the primary tumour at secondary sites.[1,8,77,78,88,90,91] The hypothesis also infers that there should be similarities in the mechanisms of stem cell renewal and cancer cell proliferation. Indeed dys-regulation of the stem self-renewal pathways would give rise to cancer.[78,92-94]

The CSC hypothesis is supported by the heterogeneity observed by Yachida *et al*,[43] when sequencing genomes of cancer cells micro-dissected from different sections of the same tumour[8] and by multiple studies finding that only a small proportion of cells in a tumour mass can support tumour growth.[3,4,95,96,101,103] This is not a new hypothesis, in 1855 Rudolph Virchow proposed a connection between tissue development processes and those required for tumour progression.[97,98] In 1867 Cohnheim postulated a role for immature embryonic cells in cancer development, thereby formulating the "embryonic theory" which then developed into the CSC hypothesis.[1,99]

However, CSCs are not proposed to be equivalent to normal tissue stem cells. Instead, they are defined as a small subpopulation of tumour cells possessing properties of both cancer cells and stem cells,[1,80,81,86,100] which grants them tumour-initiating potential and thereby metastatic potential.[7,100] If this is correct, the prevalence of CSCs in a given tumour would correlate with its metastatic potential

[II] The ability to maintain the original phenotype and endless proliferative capacity [1]

and tumour aggressiveness.[83,100-102] To date, the *in vivo* reports on the correlation between CSCs and clinical outcome have been contradictory. This is partly because the exact definition of CSCs is not clear.[1,95,101] This also leads to contradictory reports on the proportion of CSCs present in a tumour mass, which have sometimes been estimated to be as high as 90%.[88,95,101,103]

2.1. Origin of CSCs

There are two main hypotheses concerning the origin of CSCs.[81,98] The "top down" hypothesis argues that stem cells would be the only cells present in the tissues long enough to accumulate sufficient genetic abnormalities required to drive tumourigenesis[77,78,81,83,98]. Additionally, as the cells were already more resistant to apoptosis and able to self-renew, fewer genetic alterations would be required. This is in line with the similarity of surface markers observed between normal stem cells[1,78,79,102,104] and the CSCs as well as the amplification of mammary stem cells in breast cancer that occurs prior to cancer development.[81,105,106] In addition, experimental induction of the expression of Met and Myc oncogenes in progenitor cells was sufficient to drive mammary carcinoma development in mice.[81,107] The "bottom-up hypothesis" on the other hand, argues that CSCs may develop by mutations of progenitor cells derived from stem cells, thereby conferring the ability to self-renew.[81,83,108-110] This theory is supported by stronger experimental evidence, where ectopic expression of different oncogenes can reverse a committed progenitor cell differentiation. For example, Bcl-2 and BCR/ABL fusion protein expression in myeloid progenitor cells was sufficient to direct leukaemia development. [81,110,111] These two models were based on the development of colorectal adenomas[98], but can be expanded to the general principle. A controversial third hypothesis argues that CSCs arise from a fusion event between stem cells and other cells,[81,112,113] but no *in vivo* evidence exists to support this hypothesis.

These theories are not mutually exclusive and evidence suggests that different mutations may have different effects depending on tumour type.[8,80,81,114]

2.2. CSCs identification

Although CSCs had already been described in the 1970s[115,116] the concept of CSCs was more precisely defined in human acute myeloid leukaemia studies by John Dick *et al* in 1997.[79] Since they have also been defined in some solid tumours; in particular mammary carcinomas and tumours of neuroectodermal tissues.[8,80,81,104,117] As CSCs are postulated to be able to self-renew and reproduce the independent growth and heterogeneity of the primary tumour, they have been functionally defined by their ability to re-generate the original tumour[III] in mouse xenograft studies and serial transplantation assays.[1,8,81,90,91,98] The problems with this approach were best demonstrated by Quintana *et al*.[101,103] Using melanoma cells and serial *in vivo* passaging, they were able to increase the proportion of tumour-initiating cells, although CSCs should represent only a small population in a tumour mass.[101] On a molecular level CSCs have been labelled using techniques of normal stem cell identification. One technique relies on the use of DNA synthesis labels. As stem cells are proposed to divide more infrequently than the progenitor cells they generate in the asymmetric division, they would retain the label for longer, allowing identification of a label-retaining population. This has several downsides as not only is it not clear just how fast CSCs divide, some argue that to maintain genome integrity, the original strand (termed immortal strand) would always be retained in the parental stem cell. In this way any error generated during DNA synthesis (and the entire label incorporated) would always be passed onto the daughter progenitor cell and eventually be eliminated from the lineage.[98,118-121] All of these considerations make the use of DNA labelling in CSCs much more complicated.

Substantial work has been done to identifying cell surface markers of CSCs.[8,83,98,100,122,123] From the leukaemia studies by Dick *et al*, the tumour initiating cells were $CD34^{+}/CD38^{-}$.[1,79] Since then, populations of cells exhibiting distinct cell-surface expression profiles of different markers have been isolated and these show greatly increased tumour initiating ability as compared to the general

[III] Already in 1977 Hamburger and Klein shoed that in many human tumours only 1/1000 – 1/5000 of cells were able to form a macroscopic colony in soft agar[98]

population.[7,8,83] Some examples of these markers isolated in different types of cancer are: $CD44^+/CD24^-$ in breast cancer[88], $CD133^+$, $CD44^+$, $CD26^+$ in colorectal cancer[83,88] and aldehyde dehydrogenase in breast cancer.[88,100] This non-specific definition leads to extremely varied reports of the fraction of CSCs (as described above) and led Hanahan and Weinberg to propose that there could be a plasticity between CSCs and non-CSCs in a tumour.[8]

2.3. CSCs and metastasis

If the CSCs are the only cells in a tumour population that can initiate tumourigenesis and growth, they could also be the cells responsible for metastatic disease.[1] As proposed above, a higher prevalence of CSCs in a tumour mass would then correlate with the invasive and malignant phenotype and this has not been directly proven *in vivo*.[4,7] A link between CSCs and metastasis has only been circumstantial, based on the correlation between the frequency of cells with the ability to form metastasis in the primary tumour and the prevalence of CSCs, the similarity in cellular phenotypes (resistance to apoptosis, motility) and similarity in transcriptomes to that of normal stem cells.[1,8,124]

An example of such a link can be seen in a microarray analysis study by Glinsky *et al*[82], where stem cell genes were identified in a metastatic gene signature.[88] In addition, Hermann *et al* isolated a subpopulation of CXCR4-expressing CSCs (as defined by CD133 expression),[102] which proved to be responsible for the metastatic phenotype of the cell line.[83,102] This approach may be problematic as the main assay used to define CSCs remains the ability to initiate tumour growth in mouse xenograft studies. Kelly *et al* suggest that the assay neglects to take into account the importance of the microenvironment, leading to an underestimation.[95,125] Chaffer and Weinberg argue that this process is theoretically analogous to that of metastatic colony establishment.[7] Thereby the very traits that are used to define CSCs may in fact be pre-requirements for metastatic dissemination and colonization.[7,100-102]

THE METASTATIC CASCADE

The process tumour cell translocation and metastasis is complex, involving and depending on both intrinsic properties of tumour cells and their interactions with a variety of stromal cells.[1,8,9,126-131] In its simplest version the multi-step process of metastasis can be represented as a sequence of discrete steps: local invasion at the site of the primary tumour, intravasation of the disseminated cell into the haematogenous or lymphatic system, survival and transit of cells through that system, arrest in the distant capillary bed, transfer from the lumen into the parenchyma at the secondary site (extravasation), followed by proliferation and colonization.[1,3,8] This section focuses on outlining the events in the metastatic cascade and highlights any current controversies. It is presently believed that access to the majority of organs[IV] in the body is via the haematogenous route[7,14,125] and therefore this will be the main focus of this section.

1. Cell motility and local invasion

The first step in the process of dissemination requires tumour cells to break away from the primary tumour. In order to achieve this, these cells must acquire the ability to migrate and invade the surrounding tissue.[7] In normal tissue homeostasis, individual cells possess positional identity to precisely map their location and limit their numbers.[7,9] For example epithelial cells are tightly bound together via adherens junctions, tight junctions, gap junctions and desmosomes.[7,9,21,132,133] Metastatic cancer cells lose these constraints [4,7,21,132,134,135] and motile cells within a tumour were observed more than 100 years ago.[11,97,136]

[IV] Exceptions include lymph nodes and a separate type of invasion through the peritoneum.

1.1. Role of host components

A tumour is not only a mass of proliferating cells, but rather is a complex organ containing many different types of recruited stromal cells (endothelial cells, pericytes, neutrophils, mast cells, macrophages, fibroblasts, bone-derived cells) and other tissue components.[1,2,7,9,129-131,137-139] It is outside the scope of this review to outline each type of recruited cell and their effects and only a general overview will be offered.

The importance of the local microenvironment is demonstrated by Mintz *et al.*[140] After injection of cancer cells, the microenvironment of a mouse blastocyst not only suppressed further progression of teratocarcinoma cells, but also completely reprogrammed the cells, giving rise to normal chimeric mice.[9,140] In addition, other experiments, using a co-culture of fibroblasts and transformed prostate cells, demonstrated the suppressive role of stromal fibroblasts on epithelial cancer progression.[9,141-146] Historically the recruitment of inflammatory mediators into the tumour mass was seen as a failed attempt at tumour clearance.[9] It is now apparent that tumour cells not only develop mechanisms to avoid immune system detection, they are also able to modify the inflammatory response to tumour-promoting rather than tumour-suppressive.[4,9,129,131,147,148] The precise definition of different immune cells in tumour progression is more complicated however, as many different cell types (macrophages, T-cells, myeloid-derived suppressor cells, mesenchymal stem cells) can exhibit both tumour – promoting and suppressive phenotypes and the factors involved in controlling this balance are not well characterised.[1,9,129,149,150] For example following activation, macrophages can kill tumour cells *in vitro* as can *in vivo* injection of macrophage-activating agent.[1,151,152] Most studies demonstrating pro-tumour effects of macrophages disrupted wide populations of macrophages either via pharmacological (chlodronate-liposome) depletion or through genetic manipulation.[9,150,153-155] These caused a decrease in macrophage infiltration and subsequent inhibition of angiogenesis, tumour growth and metastatic spread.[1,9,150,153-156] However these experiments rely on elimination of all

macrophage populations and subsequently more elegant studies involving ablations of specific subpopulations have been performed. These identified different mechanisms promoting tumour cell invasion e.g. paracrine loop in breast cancer, where macrophage-secreted EGF stimulates CSF1 production by tumour cells, which in turn acts as a potent attractant for macrophages.[9,150,157] Interestingly, clinical studies have demonstrated an association between macrophage infiltration and poorer prognosis.[1,9,158,159]

Inflammatory mediators are thought to be recruited as a result of tissue disruption and general inflammation, rather than by a specific cancer antigen. In this way tumour cells exploit interactions with the microenvironment to orchestrate the progression, invasion, intravasation and acquisition of a motile phenotype.[2,9,160-162] This is supported by experimental data showing increased inflammatory recruitment into the tumour and a more aggressive phenotype in patients with concurrent chronic inflammatory conditions.[9]

Other environmental influences are also involved in promoting the metastatic phenotype and tone of the most important of these is hypoxia. Through stabilization of HIF-1, hypoxia promotes the outgrowth of malignant cells, promoting cell survival and invasion.[4,163] This is in agreement with clinical data, where Semenza *et al* found a correlation between HIF-1α stabilization, metastatic relapse and shorter survival.[4,163]

The tumour cell population is a heterogeneous mass of cells (as discussed above).[1,44,164] Urquidi *et al* provided direct evidence that within a tumour there are different subpopulations of cancer cells with variable metastatic potential.[1,71] Immunohistochemical techniques and *in vivo* imaging have proven the existence of islets of cells with increased motility, variable expression profiles and increased association with inflammatory mediators in human breast cancer.[9,165] It has been suggested that these so called “invasive niches” facilitate paracrine loops and lead to enhanced intravasation and metastatic spread.[9,162,166] Whether they are responsible for metastatic spread will need to be

validated, however their presence at the primary site correlates with metastasis development and is associated with a poorer prognosis.[167] In addition, Wyckoff *et al* demonstrated that the association of macrophages with cancer cells directly leads to an increased motility.[168,169]

1.2. Types of cell movement

Research into the mechanisms of cell movement has been facilitated by advances in *in vivo* imaging techniques. The three main mechanisms recognised today include collective motility, amoeboid motility and mesenchymal motility.[4,8,11,132,136,170-172] There is plasticity in switching between the different types.[11,132,172-174]

Collective invasion was first described in embryogenesis during epithelial sheet formation.[11,175,176] During this process clumps of cells invade the surrounding tissue as a group. It is reported to be characteristic of sqamous cell carcinomas[8] and has been observed in some clinical histological samples of solid tumours, but has not been directly observed in intravital microscopy studies. [11,175,176] This may be because collective invasion is proposed to be a slow process that occurs in the absence of highly motile individual cancer cells,[177,178] while experimental imaging usually occurs over short periods of time.[11]

Using intravital microscopy cancer cells have been shown to move at a higher speed (up to 15μm per minute) *in vivo* than is usually observed in 2D *in vitro* cultures. Additionally, they are able to rapidly change shape and direction in a manner not unsimilar to leukocyte movement.[4,11,132,162,171] This type of movement has been termed **amoeboid motility**[8,11,21,174,179,180] and is proposed to be mediated by cycles of acto-myosin contraction, actin polymerisation and cell adhesion to the extracellular matrix (ECM).[11,181] Experimental evidence supporting this model showed that inhibition of actin polymerisation resulted in a reduced metastatic burden in mouse models of breast cancer and melanoma.[11,181,182] In addition, the acquisition of this motile phenotype correlates with

metastasis.[4,59,183] In contrast to the other two types of migration, remodelling of the ECM is not required; instead, the plasticity in shape would allow the tumour cells to squeeze through existing spaces.[8,179,180]

Mesenchymal movement is mediated by epithelial-mesenchymal transition (EMT) that is subject to much controversy and is outlined below.

Microarray studies have led to the identification of several genes with differential expression between metastatic colonies and their primary tumour counterparts. In addition, molecular technologies have led to the identification of several molecules involved in the metastatic process.[1,185,186] These include a wide variety of mediators, including growth factors, ECM-remodelling factors and also transcription factors.[1] The role of transcription factors was proposed to involve orchestration of cellular changes during the invasive process.[1] Interestingly some of the more highly expressed transcription factors in metastatic cells (Snail, Slug, δEF1, Twist , FOXC2, Goosecoid and Zeb1[1,4,20,21,59,187-190]) were also implicated in epithelial-mesenchymal transition (EMT) - a highly conserved cellular programme of early embryogenesis.[7,8,20,21,191-194]

Epithelial cells are organised in layers, tightly connected to each other and to the basement membrane.[7] In pilot experiments Greenburg and Hay have shown that by the process of EMT, mesenchymal cells can develop from the epithelial cells. In contrast to the structured epithelial sheets, mesenchymal cells rarely establish contacts with neighbouring cells and can invade through the basement membrane. It is now recognised that EMT is defined by three changes in cellular phenotype, including morphological change into a spindle-shaped cell with protrusions and functional change allowing a motile phenotype with the ability to invade ECM. The third characteristic involves changes in the expression of markers on the cell surface, including cell-cell junction proteins, vimentin and fibronectin.[7,8,21,195-197] As the majority of human solid tumours are of epithelial cell origin[21], the

implication of EMT in the process of dissemination is not surprising. Here it is included as a cellular programme of motility, but it also mediates resistance to apoptosis, aids dissemination and facilitates disruption of basement membrane via proteolytic activities.[8,191,195,196,198-201]

Substantial experimental evidence supports the involvement of EMT in metastatic dissemination.[202-205] Increased expression of the transcriptional factors described above has been detected in certain invasive human and mouse tumour cell lines[8,20,188,190,192,193] and some have been implicated in poor prognosis signatures in gene expression studies of both neuroblastomas and melanomas.[21,202-204] In addition, Yang *et al* have demonstrated that Twist 1 is essential for the lung metastatic phenotype of a mouse mammary carcinoma.[20,21,205]

At post-transcriptional level, the role for EMT in metastasis has been implicated through observations of E-cadherin expression.[1,21,187,206-208] E-cadherin usually functions in maintaining cell-cell contact and the loss of its expression in EMT was defined in development. Loss of its expression has been associated with poor prognosis in both human and mouse tumours, thereby this supports the hypothesis of EMT occuring.[4,20,21,208] Experimentally, ectopic expression of E-cadherin leads to suppression of certain carcinomas, while blocking its function activates their metastatic potential.[8,21,209-213] Interestingly, E-cadherin expression in human carcinomas has been shown to be heterogeneous[21,214,215], which is in line with the heterogeneity observed in metastatic potential.[12,69]

The controversy of EMT involvement in metastasis arises from the difficulty in demonstrating the presence of cells in EMT in clinical samples.[7,21] There is some circumstantial evidence, from measuring either epithelial or mesenchymal markers in the primary tumours, but morphological changes characteristic of EMT are rarely detected.[1,21,207,216] Also arguing against it, are clinical observations showing that the majority of human breast metastases express E-cadherin.[207,216] Additionally several studies on mouse tumours demonstrate mechanisms of dissemination without obvious passage through EMT.[21,217] Weinberg proposes that this controversy is in part because EMT is depicted as a

permanent irreversible process. The reverse process (MET) has been described in embryogenesis[7,21,218] and has been implicated in metastasis by Thiery *et al*.[135,206] Additionally, tumour cells may pass through the process of EMT only partially and so maintain expression of both epithelial and mesenchymal markers.[7,21] It is also suggested that the process would be initiated and mediated via heterotypic interactions with the stromal cells.[7,8,126,195,196]

2. Intravasation

Intravasation is defined as the invasion of the blood vessels by the tumour cells. The molecular mechanisms governing this process are not well defined.[4] Limited experimental evidence suggests that metastatic cancer cells are able to orientate themselves towards blood vessels and when non-metastatic cells begin to intravasate, they are fragmented by the shear-stress of the blood stream.[11,169,219-222] This process is closely related to the controversy of the EMT transition and the types of tumour cell movement.[4]

As reviewed above the role of stromal cell interaction in inducing tumour cell motility and intravasation is now well established. In addition to the evidence presented above, macrophages have been found to directly promote the process of intravasation in intravital imaging of breast cancer models.[9,162] Moreover, Wyckoff *et al* utilizing the (CSF-1R)-GFP mouse model, demonstrated that cancer cells would intravasate only where perivascular macrophages were located.[9,160]

3. Survival in the systemic circulation and extravasation

3.1. Haematogenous circulation is a hostile environment

The environment in the haematogenous circulation is hostile for the tumour cells. This is not only due to immune surveillance by NK cells.[235] The vast majority of cancer cell clearance is induced by the mechanical stress of the shear forces of blood flow causing cells to rupture.[4,9,223] This in part accounts

for the inefficiency in the dissemination process, as it has been shown that millions of cells are shed by a tumour into the circulation in a 24h period[11,16,223] and only a very small proportion develop into macroscopic metastases[4,8,223,224]. Indeed, the blood of many carcinoma patients contains circulating cancer cells and this correlates with poorer prognosis.[7,14,225-227] The majority of studies of these cells has been performed *ex vivo*, on cells isolated from blood.[11] Interestingly, these cells have been shown to have a diameter of 20-30μm, making them far too large to be able to pass through capillaries[V] and yet they are able to persist in the circulation for 1-24h.[7,14,228] This phenomenon suggests that cancer cell physical plasticity may play an important role.

In order for tumour cells to survive they must also evade cell death that may be induced by loss of adhesive contact (anoikis).[4,229] Some *in vitro* data exist to support a role for different factors, but the relevance of anoikis in metastasis is unclear. One reason for this might be that in general the time for which tumour cells lack cell contact may not be sufficiently long for anoikis to be induced. This also implies that the time tumour cells can circulate before adhesion interactions are established is limited.[4,223,230] Furthermore if the tumour cell passed through the EMT as described previously, the need for adhesive contact would be eliminated.

Circulating cancer cells may also actively promote their own survival and one of the most important mechanisms is through co-option with platelets.[4,7,9,224,231-235] Tumour cells act to initiate the coagulation cascade and facilitate the formation of micro-thrombi.[9] This has been shown to mediate tumour cell survival through several mechanisms and is also involved in facilitating arrest and adhesion at the secondary site and subsequently extravasation.[9,224,233,235-238] Association with platelets is an extensively studied event that may involve several branches of the immune system. As it is also implicated in mediating tumour cell interaction with the endothelium, current research is outlined in further detail below.

[V] Capillary lumen is about 8um

3.2. Role of platelets, coagulation and immune system components in promoting metastasis

Correlation between cancer and clotting abnormalities was first noted by Armand Trousseau in the 19th century,[236,239,240] who described excessive blood clotting (venous thrombosis) in patients with occult carcinoma and proposed that such changes led to the inflammation of blood vessels, thereby supporting cancer progression.[236,240,241] The link between cancer development and platelet abnormalities has since been recognized and is supported both by clinical documentation and experimental data.[4,231,236,242-244]

Cancer patients exhibit signs of aberrant platelet activity and show a higher risk of blood clotting and developing thrombosis.[4,236,242,243,245-249] The symptoms can be incredibly diverse and are worse at the metastatic stage of the disease, when the most severe types of thrombosis include disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism.[236,243,248] In fact thromboembolic disease is one of the diagnostic factors and often the cause of death.[236,239,250-252] Even if overt thrombotic disease cannot be detected, cancer patients often present with enhanced platelet turnover and activation. A high platelet count is indicative of a poorer prognosis[VI] in many cancer types[9,236,239,251-256] and it is believed that up to 90% of patients with metastatic disease present with some type of coagulopathies.[245,246,257,258] However it has not been established whether a high platelet count actually constitutes a predisposition to metastatic disease.[239]

The functional association between platelets and tumour cells was first recognised in 1968 by Gasic *et al*[239,241,259], who demonstrated that tumour cells are able to aggregate platelets *in vitro*[VII] and that this is required for tumour cell survival in experimental pulmonary metastasis.[259] The significance of platelets in the metastatic process has since been demonstrated in different *in vivo* models of

[VI] This includes the following cancers: colon, breast, lung, gastric, renal, cervical, pancreatic, endometrial, oesophageal, ovarian and brain.[235,239]
[VII] Designated tumour cell-induced platelet aggregation (TCIPA)[239]

experimental metastasis and spontaneous metastasis.[4,231,232,236,239,241,260-263] Platelet depletion (genetic or antibody-induced) or inhibition of their aggregation resulted in a reproducible reduction in metastatic dissemination in numerous experimental models[232,235,236,239,259,260,264-266] Furthermore, reconstitution of platelets reversed this effect.[236,266] In addition, Jones *et al* showed arrested tumour cells surrounded by platelet emboli *in vivo*.[241] Hence a correlation was established between the ability of certain tumour cells to aggregate platelets *in vitro* and their metastatic dissemination.[239,241,266] The mechanism the tumour cells utilize to induce platelet aggregation and clot formation are outlined next.

A) Mechanisms by which tumour cells activate platelets and the coagulation cascade

Tumour cell can activate the coagulation cascade in three different ways.[246,267-269] They can express haemostatic factors which include pro-coagulant proteins and microparticles. They can also express adhesion proteins to facilitate direct interaction with platelets, leading to platelet activation and aggregation and they may also secrete inflammatory cytokines to induce haemostatic factor expression on endothelial cells and monocytes.

i) Production of haemostatic factors

The most important mechanism by which tumour cells initiate coagulation is by expression of the principal pro-coagulant protein tissue factor (TF).[245,265,268,270,271] Most tumour cell lines are reported to express a constitutively active form of TF on their surface.[241] Some reports even suggest that TF levels may be upregulated 1000-fold in the metastatic cells as compared to their non-metastatic counterparts.[245] Expression of TF correlates with advanced disease and poor outcome for multiple cancer types, including bladder, brain, colon, gastric cancer, lung, melanoma, renal cancer, ovarian and prostate cancer, pancreatic cancer, liver cancer and leukaemia.[245,268,270,272] The levels of expression vary, but it is generally higher in higher grade malignancies.[268,270,273]

TF is a 47kDa transmembrane glycoprotein.[245,265,270,271,274,275] It is the primary initiator of the physiologic coagulation cascade in blood (Diagram 1). [245,268,270,271,276] Under resting conditions, TF exposure to the blood vessel lumen is extremely low and its expression is tightly regulated. It is constitutively expressed in adventitial fibroblasts of the vessel wall [268,270,274,277-279] and would only be exposed to the haematogenous circulation under extreme conditions. These include: vascular injury, rupture of the endothelial lining, induction of TF on endothelial cells in angiogenesis and induction of TF expression on leukocytes.[245,271]

Coagulation is initiated by exposure of membrane bound TF to circulating Factor VII (FVII).[245,268,271,274,280] This allows rapid activation of the serine protease cascade, where TF binding facilitates activation of its protease activity,[245,265,270,271] while in absence of TF, FVII exhibits poor catalytic activity. The high affinity interaction leads to a bimolecular complex (TF/VIIa) that cleaves circulating factors FIX and FX to active forms FIXa and FXa, respectively.[265,268,271,274,276,281] FXa then initiates the conversion of pro-thrombin to thrombin.[245,271,274] Thrombin is also a serine protease that cleaves fibrinogen to fibrin.[236,274,282]

It is now recognised that only a fraction of FX and FIX are activated by TF/FVII complex. In addition in absence of activated FVa, FXa only generates trace amounts of thrombin. These are insufficient to initiate significant fibrin generation.[274,280,283] This stage of the coagulation cascade is called the initiation phase, where a 1:1 stochastic interaction between the TF and FVII is insufficient to support clot formation in itself. Instead, it serves as a trigger for further amplification events of the propagation phase.[274,280,281,283]

The propagation phase occurs independently of the TF-FVIIa complex. This complex is rapidly inactivated by the binding of TF pathway inhibitor (TFPI). TFPI is a potent inhibitor of FXa and FVIIa, consisting of three Kunitz-type domains and a basic carboxy-terminal region.[284] Site-directed mutagenesis showed that both the first and the second Kunitz-type domains are required to inhibit

the TF-FVIIa complex.[285] TF-FVIIa inhibition by TFPI occurs only when TFPI is bound by FXa. This allows for an efficient regulation of the reaction, where TFPI-mediated inhibition will only occur after FXa has been generated. [274,280,281,283] The thrombin that is generated interacts with non-proteolytic co-factors FV and FVIII, facilitating activation to FVa and FVIIIa, respectively.[274] This second arm of the cascade involves complex formation between FVIIIa and FIXa. This complex acts to amplify the formation of FXa, which further amplifies activation of thrombin.[245,271,274] FVa acts to orientate pro-thrombin for efficient catalysis and is required to facilitate full activation of FXa and thrombin generation. [274,280,281,283]

In addition to cleaving fibrinogen,[245,271] thrombin also activates platelets through a poly(ADP-ribose)-dependent mechanism.[270,286] It induces change of platelet shape and expression of different ligands on the platelet surface (e.g. P-selectin, integrin αIIbβ3, fibronectin).[239,287-289] These changes increase their adhesion to tumour cells, activate receptors on their surface and induce platelet cross-linking through αIIbβ3. This receptor is also responsible for binding fibrin and supporting platelet incorporation into clots. Together with fibrin cross-linking, activated platelets form platelet-fibrin thrombi around the tumour cell.[232,236,237,239,270,290] Platelet activation also leads to the release of additional fibrinogen and fibronectin to propagate thrombosis.[236,291] They also release platelet granules, containing proteases, growth factors, matrix proteins and chemokines. Release of these granules results in a further inflammatory response.[236,292]

The coagulation cascade is a complex network that is also regulated by negative feedback loops. For example thrombin interacts with the membrane bound receptor thrombomodulin (TM) and this alters its substrate specificity. In combination with TM it instead catalyses Protein C to its active form APC. This active form of the protein inactivates factors of the coagulation cascade (FVa and FVIIIa).[274,280,293,294]

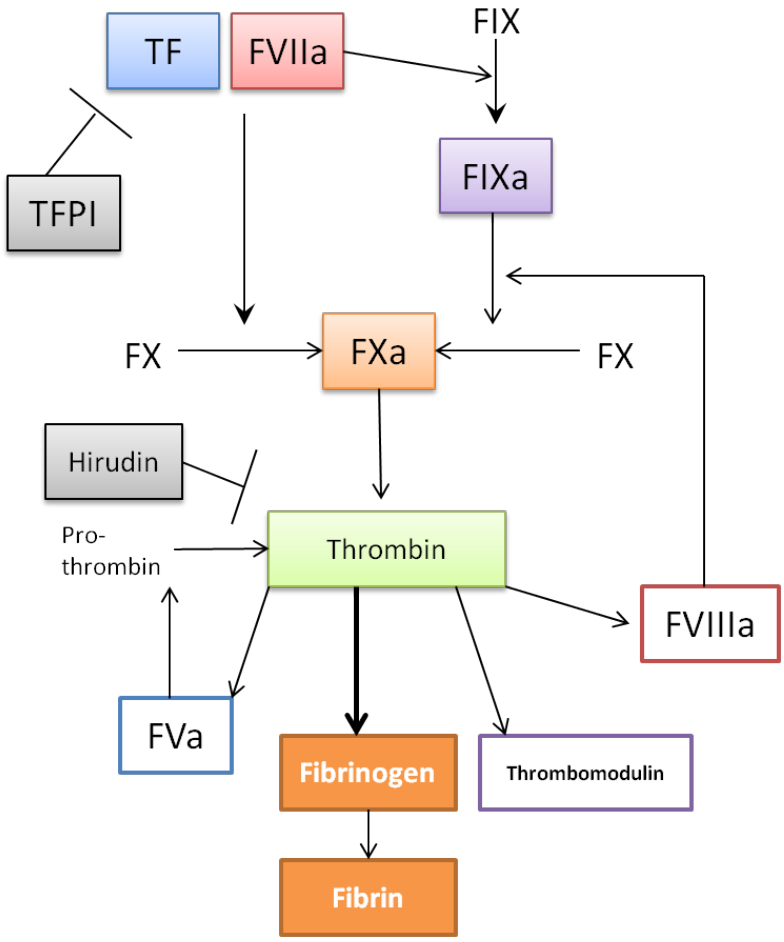


Diagram 1: Coagulation cascade

Interaction between TF and activated FVIIa facilitates rapid activation of the proteolytic cascade. The TF-FVIIa complex cleaves factors FIX and FX to active isoforms FIXa and FXa, respectively and FXa proceeds to initiate the conversion of pro-thrombin to thrombin. The TF-FVIIa complex is responsible for the formation of only a small amount of FXa, insufficient to generate a significant amount of thrombin. Instead these act as a trigger/ initiation for further events of the propagation phase of coagulation.
Thrombin generated during the initiation phase interacts with non-proteolytic co-factors, facilitating their activation to FVIIIa and FVa. FVIIIa forms a complex with FIXa and together they amplify the formation of FXa and thereby generation of thrombin. FVa co-factor acts to orientate the pro-thrombin molecules for efficient catalysis and is thereby required for efficient FXa reaction.
The binding of TFPI protein inactivates the TF-FVIIa complex, while hirudin inhibits the activity of thrombin.
Adapted from Mackman et al.[271]

Another pro-coagulant protein that may be produced by tumour cells is CP, a cysteine protease that activates FX directly (in the absence of FVIIA). Its expression has been found to be restricted almost exclusively to malignant tumour cells, however its role as a tumour marker and its clinical significance have yet to be established.[268,295,296]

Tumour cells have also been proposed to contribute to increased TF levels in circulation by release of microparticles (MPs).[247,268,297-299] These are membrane vesicles that were first demonstrated by Dvorak *et al* [297]and since confirmed in a variety of tumour cells.[270] They are released from cells traditionally following apoptosis or cell activation.[299] Clinically, MPs have been detected in the plasma of patients with different tumours.[VIII] They are proposed to contribute to thrombin generation through facilitating exposure to TF and release of MPs by tumour cells has been proposed to correlate with the pro-thrombotic status of cancer patients.[247,268,270,298] However, it remains to be demonstrated whether MPs from tumour cells contain active TF.[268,270,299]

ii) Release of inflammatory cytokines

Tumour cells can also promote coagulation indirectly by inducing TF expression on monocytes and endothelial cells. The cytokines synthesized and secreted by tumour cells can be pro-inflammatory (TNF-α, IL-1β) and pro-angiogenic (VEGF, bFGF). In support of this theory, macrophages harvested from experimental metastases, were shown to express elevated levels of TF. Clinically, circulating monocytes from the blood of patients with different types of cancer exhibited elevated TF activity. Endothelial cells, on the other hand were shown to increase the expression of TF, fibrinolysis inhibitors[IX] and to downregulate thrombomodulin expression.[268,300-302]

[VIII] These include colorectal cancer, leukaemia, lung carcinoma, pancreatic and breast carcinomas.[268]
[IX] This will allow the clot to persist for longer periods of time.

iii) Expression of adhesion proteins

Expression of adhesion proteins on the tumour surface is thought to facilitate direct contact with platelets, endothelial cells and/or leukocytes.[268,303-305] More specifically Laubli *et al* implicated the selectin family.[306] As described above, platelet activation and aggregation with the tumour cell has been shown to be mediated by thrombin [239,241,287,289] Thrombin can, in addition to platelets, also activate tumour cells.[239,241,307] Nierodzik *et al* demonstrated a 3-fold increase in adhesion when either tumour cells or platelets were treated with thrombin, but no additive effect was observed if both were treated.[241] This suggests an analogous mechanism and indeed, TF and PAR-receptor expression has been observed in some melanomas.[232,239,307-309]

Several platelet receptors have been described to be involved in mediating platelet functions in metastasis: integrin αIIbβ3,[310-312] P-selectin,[290,313,314] GPIbα,[315,316] ADP-receptors,[317,318] and thrombin receptors.[11,239,268,319,320] These interactions are proposed to facilitate further platelet activation, amplify thrombin production and clot formation.[231,236,268,320]

B) Mechanisms by which platelets and coagulation promote metastasis

Abundant experimental evidence established the crucial role for platelets and the coagulation cascade in promoting tumour cell metastasis. The proposed model of the mechanism states that upon intravasation of tumour cells into the blood circulation, they bind to activated platelets, a mechanism termed cohesion. This facilitates their interaction with leukocytes and assists their arrest at the vessel wall and survival in the circulation.[236,314,321,322] The supporting role of platelets is proposed to further extend to promoting extravasation and colonization events,[224,233,236,238,239,306,323] however the most significant role is likely to be performed during the early stages of metastasis.[239,259,266]

As outlined previously, TF plays a central role in these mechanisms. In addition to directly initiating thrombin generation, the TF/FVIIa-Xa complex also directly activates PAR-receptor signaling pathways and has been proposed to cooperate with TFPI that is immobilized in the tumour vessels in inducing

cell adhesion and migration.[241,264,268,324] In mice that were genetically deficient in platelet production and megakaryocyte maturation (NFE2 KO) experimental metastasis was almost completely abolished.[232,236] In addition, genetic deficiency in fibrinogen production or PAR –receptor KO, shows a significant reduction in metastasis formation of TF-expressing cells.[232,236,268,325-328] This suggests that the effects of TF in metastasis are mainly mediated via thrombin-dependent mechanisms.

In addition to the direct effect of thrombin on tumour cells, platelets and thrombus formation is also proposed to facilitate metastasis in several different ways. The formation of a fibrin and platelet aggregate around the tumour cell shields the cells physically from the haemodynamic forces of the circulation and thereby reduces the shear force that may be responsible for much cancer cell death.[4,9,231,253,264] The thrombus may also prolong tumour cell survival in the circulation by protecting it from innate immune system recognition.[4,9,261,264] Following experimental tumour cell injection, NK cell number in the lung was shown to increase by 80% within 60min[223,329] while already studies from the 1970s demonstrated their ability to lyze tumour cells *in vitro* and lead to a reduction in metastasis *in vivo*.[223,330] This was also demonstrated by studies where NK cells were depleted *in vivo*. This lead to a substantial increase of metastatic lesions.[264]

Most significantly, platelets were shown to impede the recognition of tumour cells by natural killer (NK) cells.[233-236,264] This is supported by experimental data using mice deficient in platelet shield formation (analogous to experiments described previously) either through inefficient platelet activation or fibrinogen deficiency.[231] When NK cells are present in these models, platelet activation was shown to be required for tumour cell survival. However concurrent NK cell depletion resulted in a reversal of metastatic ability.[236] The same group also recognized an NK cell – independent mechanism, but thrombin production was still required.[235,236,264] Additionally, platelets were shown to inhibit NK cell ability to lyze tumour cells both *in vitro* and *in vivo*.[9,264] Kopp *et al* demonstrated that upon tumour cell induced platelet activation, platelets secreted TGF-β. This led to a down-regulation of NKG2D

immunoreceptor expression on the NK cell surface and inhibition of granule mobilization, cytotoxicity and IFN-γ secretion.[233,239,331] The current hypothesis therefore proposes that platelets provide a physical barrier to NK cell contact and also exert paracrine suppression on NK cytolytic activity.[235,264] Platelet aggregate formation also increases the effective diameter of the tumour cell and provides a large active interfacing surface expressing numerous adhesion molecules. Hence, it has been proposed to facilitate interactions with the vasculature, arrest and adhesion of cancer cells.[9,232,234,239,308,309,332]

Additionally activated platelets are known to secrete platelet granules, containing proteases, growth factors, matrix proteins and chemokines. These may act directly on tumour cells[236] or may facilitate adhesion and extravasation past the endothelial cell layer. (e.g. heparanase[268] to dissolve EC junctions and growth factors[241] to promote endothelial cell activation.)

4. Tumour cell arrest and extravasation

The process of tumour cell arrest and extravasation in the metastatic microenvironment has proved to be difficult to study, as these interactions are difficult to observe *in vivo*. This is also due to anatomical inaccessibility of certain metastatic organs. For example the organ movement due to respiration, impedes accurate imaging of pulmonary metastasis. Furthermore the precise location of the site of metastatic dissemination is not known; thereby the determination field of view investigated is difficult. [11,236] By comparison the attachment and transmigration of leukocytes in inflammatory settings has been extensively studied.[236,333-337] Initially the process of tumour cell arrest and extravasation was therefore modelled on the behaviour of leukocytes.[179,338-343] Recently, research using genetic modifications and drug treatments *in vivo* has implicated specific adhesion molecules in the process and provided evidence of various interactions involved.[235,236,306,314]

4.1. Organ homing

The debate as to whether metastatic dissemination is an active or a passive process has been argued at least since James Ewing challenged Paget's "Seed and Soil hypothesis".[1,8,223] The most frequently described example of passive behaviour is that of colorectal carcinomas, that show a high prevalence of liver metastasis. This is believed to be due to the drainage of the portal circulation that delivers millions of tumour cells directly into the liver microvasculature.[7,223] This was confirmed by Weiss *et al*[344,345] who found an association between organ blood flow and the incidence of metastasis.[223251,252] Alternatively, an active process may occur where specific interactions between the tumour cells and the microvasculature play a role,[7,319,333,346-350] as is exemplified by Paget's "Seed and Soil" hypothesis.[4,24,223] This phenomenon is supported by clinical observations of metastatic patterns that cannot be explained purely by the anatomy of the circulation for example breast cancer metastasizes to the lung, liver, bone and brain and melanoma to the skin, liver and brain.[9,25,351,352] The precise molecular mechanisms that underlie the preferences of different cancers to metastasize to distinct organs are poorly understood.[9] Recent work involving genomic studies of properties inherent to tumour cells is described above, however it is now generally accepted that there is a micro-environmental component to this process as well.[9,11,160,168]

4.2. Mechanisms of tumour cell arrest

The two theories of the mechanism of metastasis carry with them another implication, namely the mechanisms that govern tumour cell arrest. Upon injection of tumour cells into the blood stream in experimental models of metastasis, they lodge in the capillary beds of distant organs within a few minutes.[11,14,17,223,333,353-359] Such tumour arrest can occur passively, simply by mechanical occlusion due to the size of the tumour cell or tumour cell cluster relative to the blood vessel diameter. This is called the mechanical trapping theory.[7,11,48,223,333,353-355,360-362] Alternatively tumour cells may actively participate in adhesion to the endothelial cells in the vessel wall. This is proposed to involve

heterotypic interactions between tumour cells and the components of the metastatic microenvironment. [7,11,14,230,333,348-350,352,362-365] The "adhesion theory" emphasises the role of organ microenvironment and hence organ specificity.[9,223,339] Nevertheless the two hypotheses are not mutually exclusive.[3,14,223,333] A powerful argument for this was the observation that non-metastatic cells are able to lodge in the vasculature at a secondary site, but are rapidly cleared and do not develop into metastatic lesions.[11,67,360] Hence, dissemination would be influenced by circulatory patterns, but the initial attachment and survival of the tumour cells would depend both on the intrinsic properties of the tumour cell and the permissive factors of the microenvironment.[4,9,333]

4.3. Extravasation

Extravasation is the process by which tumour cells translocate from the lumen of the vasculature, across the endothelial cell layer into the surrounding parenchyma of the colonised tissue.[333,349,366-369] The exact timing of this process can differ widely, but it lasts significantly longer compared to leukocyte extravasation.[4,11,339,360,368,370-373]

In some cases, tumour cells have been shown to proliferate within the vasculature prior to extravasation. This led to a rupture of the vessel wall, allowing tumour cell access to the parenchyma in the absence of an active transmigration process.[4,9,11,362,365,374] More often extravasation precedes clonal expansion. There are two main theories regarding tumour cell extravasation. The prevailing assumption is that tumour cells actively participate in the transmigration process. It has been hypothesized that this process would be analogous to that of leukocyte extravasation.[335,339,342,362] Additionally, it is proposed that many of the same molecules that have been shown to be involved in the process of intravasation of tumour cells would also play a role in extravasation.[11,335] However, not much is known of the precise mechanism and it is likely to differ between different tumour cell types.

An important concept to consider at this point is modulation of vascular permeability of the endothelial cell layer by the tumour cells , causing either alterations in endothelial cell junctions and/or modulation of endothelial cell function. Weis and Cheresh propose that tumour cells could actively induce changes in vascular permeability in the target organs via the VEGF and Src family kinase signalling.[4,375] Alternatively, cancer cells are proposed to extravasate passively according to the anatomical theory. This is based on the relative size of tumour cells, as they are almost twice the size of leukocytes.[339,361,376]

It is now accepted that both types of tumour cell arrest may occur depending on tumour type and the site of metastasis.[4,9,339]

4.4. Role of platelets and the inflammatory system in promoting tumour arrest and extravasation

As described previously, many tumour cells initiate platelet aggregation and activation of the coagulation cascade. Certain studies have suggested that platelets may also be able to support initial interactions with the endothelium in a similar way to that observed in leukocyte recruitment in inflammation.[236,377,378] Importantly inhibition of platelet activity reduces the number of metastatic lesions but does not affect organ distribution.[223,232,260,379]

As described above, adhesion receptors on platelets (most importantly αIIbβ3) can cross-link with ligands on cancer cells (e.g. integrin αvβ3).[236] They can also bind leukocytes and a model has been described by Gay *et al*, where a hetero-aggregate would form between tumour cells, platelets and leukocytes to help establish firm adhesion and arrest.[236,306] Laubli and Borsig propose that this interaction is mediated through P- and L-selectins. [306,321] Additionally, platelet activation and the secretion of different factors may influence expression of different adhesion molecules on the tumour cell surface.

Activated platelets can also modulate the integrity of the endothelial cell layer through the release of

platelet granules.[236,292] This has been shown to be involved in immune cells infiltration in inflammation.[377,378] Gay *et al* describe a similar mechanisms exploited by tumour cells.[236] Platelets are proposed to achieve this through the secretion of different molecules. E.g. S1P, LPA, serotonin, histamine. S1P and LPA are signalling molecules that affect cell-cell communication and endothelial permeability and may influence tumour cell extravasation.[236,380-382] Serotonin is taken up by platelets from the plasma and stored in granules. These molecules are released upon platelet activation and result in either vasoconstriction or vasodilatation. Serotonin receptor inhibition resulted in a reduced metastatic burden in the liver.[236,383,384] Finally, histamine has been shown to increase vascular leakage and to increase leukocyte extravasation.[385,749,750]

The generally accepted hypothesis states that tumour cells undergo similar dynamic interactions with the endothelium as leukocytes do and that the cell adhesion receptors implicated in tumour cell arrest and extravasation are also those facilitating parallel leukocyte processes.[179,333,334,340-343] Therefore, the experimental evidence for the involvement of specific molecules will be presented below, following an outline of the structure of the endothelium, its adhesion proteins and the process of leukocyte extravasation.

5. Colonization

Regardless of whether tumour cells have extravasated at the secondary site, the vast majority undergo apoptosis within 24h of arrest.[7,11,12,14,17,358,386] This was established by Kim *et al*, by visualising BAD-GFP fusion protein and its subcellular localisation. [11,386] This phenomenon has also been visualised via nuclear fragmentation and is proposed to delineate the difference between non-metastatic and metastatic cells.[11,386,387]

The experimental evidence showing the presence of multiple micrometastases that have successfully disseminated but never progressed to macroscopic metastatic tumours[1,3,8,388-390] prompted Hanahan

and Weinberg to propose that physical dissemination is distinct to the programme of colonization.[8] Thereby metastasis can be simply broken down into two phases, the physical translocation and the adaptation of the cells to the novel microenvironment, leading to successful colonization.[8] Indeed the environment at the secondary site is likely to significantly differ from that of the primary tumour and will likely demand substantial adaptation.[7] The controversies over whether this occurs at the secondary site or within the primary tumour have been outlined above.

Moreover, the adaptation of the metastatic tumour cells is likely to be affected by the microenvironment. Several models of metastasis have shown that the microenvironment of the secondary site may be affected by the primary tumour. Based on this, the concept of a pre-metastatic niche was developed, where the primary tumour mass influences the microenvironment at a distant site in a way that makes it more favourable to metastatic outgrowth.[7] This intriguing concept is further explained below, in the context of interactions between tumour cells, platelets and endothelial cells.

Experimentally several mechanisms have been described by which tumour cells are cleared. Fluorescent probe imaging showed NO production by endothelial cells, which through paracrine action is toxic to the tumour cells.[11,172,181] Tumour cells have also been shown to be cleared by the immune system at the colonization stage.[11,387,391] It has been speculated that the factors that would promote proliferation and local invasion in the primary tumour would also promote metastatic colony outgrowth. Indeed overexpression of Bcl2 and HRas increased metastatic cell survival and proliferation. [11,17,392]

ENDOTHELIAL CELL ADHESION MOLECULES AND THEIR IMPLICATION IN METASTASIS

Insight into the mechanisms of tumour cell arrest and extravasation has been provided by studies of parallel leukocyte processes.[339] Although the two may be similar, as endothelial cell-tumour cell interactions are difficult to visualize *in vivo*, this process has not been extensively analysed and it is unclear whether it is mediated by the same adhesive interactions and involves the same cell adhesion molecules as leukocyte migration.[339,361,393]

This section will first cover the process of leukocyte recruitment and extravasation, with particular focus on the cell adhesion molecules that may be involved. Following this, the evidence that these same interactions may be involved in mediating metastatic dissemination will be summarized.

1. Cell adhesion molecules in endothelial activation and leukocyte adhesion and extravasation

The process of leukocyte migration and extravasation was observed in the 19th century by Cohnheim, but was first identified as a multi-step process by Butcher *et al* and extended by Springer *et al* in the early 1990s.[334,336,394-396] Extravasating cells include T cells, NK cells, granulocytes, monocytes and macrophages.[342] This process facilitates access to tissue parenchyma in tissue surveillance, homeostasis inflammation, infection and injury.[335,339,397] In addition to the migrating cells, the endothelial cells lining the lumen are also active participants in this process.[335,339,397-400,746,748] In inflammation we observe a phenomenon called endothelial activation, where receptor/adhesion molecule expression on the endothelial surface is induced by pro-inflammatory cytokines and the cell-cell contacts in the endothelial cell layer are altered.[335,339,342,397,400,401,746,748]

Endothelial adhesion molecules have been broadly classified into 4 different families: selectins, integrins, the immunoglobulin superfamily and chemokines receptors.[334,335,337,396,397,402-407] In addition,

recent evidence suggests that certain enzymes expressed on the luminal surface of endothelial cells may also contribute to the leukocyte extravasation cascade.[408-410] The original model of leukocyte extravasation described by Butcher et al proposes a three-step paradigm of inflammatory cell recruitment consisting of rolling, activation and adhesion. These would then be followed by diapedesis – the movement of leukocytes across the endothelial cell layer.[334-336,342,396] It was proposed that each of these steps would be mediated by a distinct set of molecules in a sequential manner.[335,336,396] Namely, selectins would mediate the rolling movement on the surface of endothelial cells, chemokines trigger activation of leukocytes and integrin receptors mediate firm adhesion and arrest.[335,336]

Research over the past two decades has led to an expansion of the model postulated initially. The revised cascade also includes margination, slow rolling, adhesion strengthening and intra-luminal crawling. Additionally, the molecular mechanisms that mediate these processes have largely been identified, demonstrating that several adhesion molecules may play overlapping roles at several points in the process.[335,397,411-414] Furthermore, different mechanisms outlining the final migration movement across the endothelium have been investigated.[335,337,397,415]

1.1. Organ specificity

The structure of the endothelial layer can vary significantly between different tissues and this may have significant impact on leukocyte trafficking and vascular permeability.[342,416,417] Generally, blood vessels are composed of either a continuous monolayer of endothelial cells (lung, skin, brain) where the endothelial cells are closely connected to each other, a fenestrated endothelial layer (intestines), or a discontinuous/sinusoidal endothelial layer where large gaps between endothelial cells are common (in the liver).[342,416,417] In principle, leukocytes extravasate in the post-capillary venules. However the process differs in the lung, liver and lymph node vasculature,[342] which incidentally are

also the most common sites of metastasis.[8] In lung and liver the cells extravasate from the circulation in the microvasculature and in the lymph nodes from the high endothelial venules (HEV).[342,416-419]

Leukocyte concentration in the pulmonary circulation is 35-100 times higher than in the systemic circulation.[342,420,421] The lumen of pulmonary capillaries is too small to allow for the rolling movement of leukocytes and the role of selectins that would normally support this process is not entirely clear. Both selectin-dependent and selectin-independent mechanisms have been described. Similarly the liver also lacks the rolling phase and the endothelial cells in the liver lack selectin expression.[342,417,418,420]

1.2. Margination

Before initiating contact with the endothelium, leukocytes in the circulation move to the periphery of the blood stream in a process called margination. At the edges of vessels the flow rate is much lower than in the centre and this provides a higher probability for interactions with the endothelium. This process does not appear to be a rate-limiting for metastatic dissemination and there is no known inflammatory deficiency related to it. [330,422,423,336]

1.3. Rolling

Initially the cells loosely attach to the endothelium and are therefore still pulled along the endothelial surface by the shear force of the blood stream[335,342,424-426] and this results in a rolling motion along the endothelial cells. It is mediated by the selectin family of endothelial adhesion molecules: L-selectin, E-selectin and P-selectin. [334,335,342,411,424,426-430] Selectins are type I membrane proteins composed of a C-type lectin domain, EGFP-like motif, a series of short consensus repeats, a transmembrane motif and a short cytoplasmic tail.[224,362,430-435] L-selectin is mainly expressed on leukocytes, while E- and P-selectin are expressed on activated endothelial cells.[335,430,436] As mentioned previously P-selectin is also expressed on the surface of activated platelets. [224,303,305,362,435-437]

The main receptor responsible for the rolling motion of leukocytes on the HEV of lymph nodes is L-selectin. This is expressed on the leukocyte surface and facilitates the constitutive process of leukocyte recruitment into the tissues.[342,438,439] In other types of endothelium E and P-selectin are also required to regulate the process.[342,440,441]

As mentioned previously, the cells of the endothelial layer also actively participate in the process. They were shown to express both E-selectin and P-selectin.[335,362,429] E-selectin expression is upregulated within 2-6 hours of inflammatory stimulus,[303,306,362,430,442,443] whereas P-selectin is preformed and stored in Weibel-Palade bodies of the endothelium and α- granules of platelets. Activation of the cells leads to rapid mobilisation of the structures and upregulation of P-selectin on the membrane.[303,342,362,440,443,444] As these receptors bind their ligands constitutively, their regulation mainly occurs at the level of expression. Limited evidence exists for enhancing binding efficiency via posttranslational glycosylation, receptor clustering, conformational changes and receptor re-localisation.[411,445] These receptors are calcium-dependent transmembrane glycoproteins[303,342,433,434] that recognise and bind carbohydrate ligands that are heavily glycosylated via O-linked or N-linked side chains.[334,342,402,405,406] Their N-terminal extracellular domain consists of a lectin domain and 2-9 complement binding consensus repeats.[333,440,446,447]

Various ligands have been described, L-selectin interacts with CD34, GlyCAM-1 and MadCAM-1 and E-selectin interacts with the carbohydrates sialyl Lewis X (sLeX) and sialyl Lewis A (sLeA).[333,334,342,403,405,440] In addition, E-selectin recognizes proteins bearing these carbohydrate moieties, e.g. PSGL-1, CD44 and E-selectin ligand (ESL1).[335,448,449] The main ligands for P-selectin are P-selectin glycoprotein ligand 1 (PSGL1) and CD24. PSGL1 is only active if glycosylated correctly, but its expression spans almost all leukocytes.[333,440] Binding of PSGL1 to L-selectin also mediates leukocyte-leukocyte interactions and thereby facilitates recruitment of leukocytes that do not express E-selectin ligands to the site of inflammation. This is termed secondary tethering.[335,440,450,451]

Interestingly, interactions of L- and P-selectins with their carbohydrate ligands were shown to require the shear stress of blood flow, as the rolling leukocytes were shown to detach when blood flow was stopped.[335,424-426] The high speed of forming (and breaking) interactions therefore enables leukocytes to interact with the endothelial layer in the presence of flowing blood, where the force of it actually strengthens the interaction. [331,419-421] Interestingly, engagement of both selectins and their corresponding ligands leads to activation of signalling pathways via G-protein dependent signalling.[335,452] The slower the rolling velocity, the longer the transit times along the surface and higher the chance that other interactions may occur. Each of the selectin molecules was shown to support rolling at different velocities in the following order: L-selectin>P-selectin>E-selectin.[411,453,454] In addition to selectins, integrins were also shown to facilitate the rolling.[334,335,339,411,455-459] Integrins are heterodimers composed of α and β chains.[334,396,407] Eighteen different α chains and eight different β chains have been identified, giving rise to a potential 24 $\alpha\beta$ integrins with distinct binding potentials.[333,342,455,460] While the extracellular domain acts as a receptor and adhesion molecule, the intracellular domain is associated with focal adhesion proteins. In this way integrins participate in both outside-in (association of integrins with the focal adhesion complex) and inside out signalling, as most integrins require a conformational change in order to gain full adhesive potential.[333,334,407,413,461-463] Thus, the function of integrins is regulated both through expression regulation and at the post-translational level.

In vitro, α4β7 integrin expression enabled cell rolling on immobilised MadCam-1 and α4β1 expression enabled cells to roll on immobilised VCAM-1. In addition, β2 integrins were shown to be able to support rolling on ICAM-1.[335,339,458,464,465] Interestingly, this behaviour was shown to be enhanced by co-expression of E-selectin. Engagement of E-selectin and concomitant signalling activation is proposed to induce an activating conformational change of integrins from the intermediate affinity state.[335,465,466] In this way, a synergistic mode of action of both selectins and integrins results in a slower velocity of rolling.[335,411,466]

1.4. Activation and arrest

In an inflammatory setting the inflammatory cytokines will induce activation of endothelial cells and stimulate the production and expression of an array of chemokines and Ig-CAMs on the endothelial surface. This response is also potentiated by delivery of other chemoattractants from activated mast cells and platelets via circulating microparticles and exocytosed granules.[335,401,467-470]

Chemokines are soluble peptides of 8-14kDa. They are immobilized on the endothelial surface by glycosaminoglycans and this facilitates their role in leukocyte recruitment. In addition it may also protect them from proteolytic cleavage.[335,342,394,471-474] Their cognate receptors are G-protein coupled receptors (GPCRs) which are expressed on specific subsets of leukocytes. More than 50 different chemokines and 20 chemokine receptors have been characterised to date.[342,394,472,475,476] Structurally they have been divided into four groups based on the arrangement of cysteine residues on their N-terminus. These are either adjacent (CC chemokines) or separated by one or three amino acids (CXC and CX_3C chemokines).[394,475] However, functionally they can be divided into either constitutive chemokines or inflammatory chemokines.[342]

The rolling movement of leukocytes at low velocities prolongs contact with the endothelial surface.[334,335] This brings the leukocyte chemokine receptors into contact with chemokines immobilized on the endothelial cell surface. This high affinity interaction results in the activation of GPCRs and a complex intracellular signalling network, leading to modification of integrin intracellular domains. The molecular mechanism is not entirely clear, but is believed to include phosphorylation of the cytoplasmic domains of integrin chains and association of adaptor proteins e.g. talin-1. However, the process is likely to involve a complex network and over 47 proteins have been implicated in the regulation of this process.[335,342,413,463,477-480]

As mentioned previously, the inside-out signalling of integrin molecules causes a conformational change, shifting from a bent low-affinity to an extended high-affinity state, in which the ligand-

binding pockets become exposed.[335,342,413,460,463,481] Chemokines are the strongest physiological activators of integrins and the process occurs on a millisecond scale.[335,342,413,477,479] Integrins then interact with the immunoglobulin family of adhesion proteins, resulting in firm arrest of leukocytes on the endothelial surface.[335,342,467,482] Specificity of leukocyte recruitment and arrest is thereby regulated both by differential expression profiles of chemokine/chemokine receptors and integrin/integrin receptors and via chemokine-triggered intracellular signalling, which may have different effects in different cellular subsets.[335]

Integrins are the main adhesion proteins mediating leukocyte arrest.[342] The family is subdivided into 8 groups defined by the β chain and the most being β1 and β2.[335,339,342,413,477]

These bind to the members of the immunoglobulin superfamily on the endothelial cell surface.[334,335,396,397] The best characterised interactions are those of β2 integrins Mac-1 (αMβ2) and LFA-1 (αLβ2) with ICAM-1/2 and β1 integrin VLA-4 (α4β1) to VCAM-1.[334,342] Interestingly, in certain *in vitro* models E-selectin and P-selectin have also been shown to be able to mediate firm arrest.[334,483,484]

Integrins also mediate outside-in signalling, which is initiated by interaction with the immunoglobulin family. This is proposed to influence several cellular functions e.g. motility and is thought to be mediated through actin polymerization.[335,394,485-489]

Additionally, adhesion induced clustering of VCAM-1 and ICAM-1 also induces intracellular signalling in the endothelial cells. This event has been shown to be $Ca2^{+}$ dependent and involves activation of Rho-GTPases. It is believed that these signalling events may facilitate the formation of docking structures, representing a starting point for transcellular migration.[394,427,490]

Another type of molecule implicated in leukocyte arrest are the galectins. There are 15 galectins in mammals and they interact with β-galactose via a conserved carbohydrate recognition domain (CRD). Initially recognised for their role in apoptosis regulation, the galectins expressed on the endothelium

have also been shown to participate in leukocyte adhesion. Rather than functioning as classical adhesion receptors, they are believed to be involved in regulating integrin function.[342,491,492]

1.5. Transendothelial cell migration (TEM)

This is the final step in the cascade and occurs without disrupting the structural integrity of the vessel wall. Endothelial cells are connected by multi-protein complexes; the two main types being adherens and tight junctions.[339,427,493] The vast majority of TEM occurs at these endothelial borders and this is called the paracellular or junctional migration. Alternatively, migrating cells may traverse the endothelial cell layer through the endothelial cell, termed the transcellular route.[335,337,339,342,415,427,490,494] Immediately prior to crossing the endothelial layer, neutrophils and monocytes have been shown to display crawling behaviour in a Mac-1 ($\alpha M\beta 2$) and ICAM-1-dependent manner. When this is inhibited migration is delayed and preferentially occurred via the transcellular route.[335,495,496]

In order to traverse the endothelial layer, the cells need to pass the endothelial cells, the basement membrane and the pericytes covering the blood vessel. Indeed, the process is influenced by the ratio of endothelial cells to pericytes and therefore the composition of the basement membrane generated by the two cell types.[335,497,498] It is currently unclear what stimulates the process, but ICAM-1 and VCAM-1 binding, shear flow and luminal chemoattractants have been implicated.[335,499-501]
As mentioned previously, leukocytes can induce the formation of docking structures – endothelial projections rich in VCAM-1 and ICAM-1. Engagement of these is believed to be $Ca2^{+}$ dependent, involves Rac-1 GTPase and influences adherens junctions via ROS production.[335,337,339,499,500]

A) Paracellular route

In between the endothelial cells, leukocytes need to pass through tight junctions and adherens junctions. [342] This occurs when the connections between endothelial cells are substituted temporarily

by corresponding interactions forming between leukocytes and endothelial cells.[335,342,502-504]

PECAM-1 (CD31) is one of the most important molecules believed to be involved in this process. It belongs to the immunoglobulin superfamily and participates in homophillic binding. It is expressed both on endothelial cells and leukocytes and its blockade inhibits transmigration of a wide range of leukocytes including monocytes, NK cells and granulocytes.[334,335,337,342,371,394,503,505-508]

Another group of IgG superfamily members involved in this process are junctional adhesion molecules (JAM-A, JAM-B and JAM-C). These can participate in homophilic and heterophilic interactions inside the group and can also bind to integrins: JAM-A binds LFA-1, JAM-B binds VLA-4 and JAM-C binds Mac-1.[337,342,415,494,495,502,509-514]

A third molecule that is crucial in diapedesis of leukocytes is the non-IgG CD99. It is not related to any other molecule in the human genome, apart from a paralog CD99-like 2. It binds in homophilic fashion at the endothelial cell border and similarly to PECAM-1 is indispensible for the process. However, it appears to function at a later stage in transmigration as confirmed by sequential blocking studies of both CD99 and PECAM-1.[337,342,371,415,502,515-519]

B) Transcellular route

The transcellular mode of diapedesis occurs less frequently (5-20% of cells *in vitro*).[335,342,415,501] It has been demonstrated to occur in the central nervous system.[335,494,520]

The molecules that have been implicated in this process are the same as those in the paracellular movement, namely ICAM-1, PECAM-1, JAM-A and CD99. During this process ICAM-1 is proposed to redistribute to the site of migration and to the channel that surrounds the migrating leukocyte.[334,337,499,521,522]

The process may also involve the formation of vesiculo-vacuolar organelles. These are membrane-associated gateways that provide a direct link between the lumen of the blood vessel and the extracellular space. So far these have been described in mediating endothelial permeability, but may

be similar to lateral border recycling compartments (LBRCs) shown to be involved in TEM. [335,523,748] Furthermore these VVO structures are rich in PECAM-1, ICAM-1 and other adhesion molecules.[337,342,499,522,524]

It is presently unclear why would the cell choose the transcellular route as opposed to the paracellular route especially since the same adhesion molecules appear to be involved in both. Several factors have been proposed to influence the relative tightness of the endothelial junctions, the difficulty the migrating leukocyte encounters in reaching the junctions and the level of leukocyte activation that influences actin polymerisation events.[337]

After crossing the endothelial cell layer, the migrating cell encounters the basement membrane composed of vascular laminins (laminin-8, laminin-10) and collagen IV. Analysis of mouse cremaster blood vessels, led to the identification of areas with low expression of these proteins and concomitant lower pericyte coverage. These conditions are also permissive of transmigration and indeed neutrophils appear to preferentially migrate in these locations *in vivo*.[335,497,525-527]

1.6. A distinct class of endothelial adhesion molecules

A number of additional molecules have been proposed to be involved in mediating leukocyte extravasation.[408-410,528] Included are the group of proteins, expressed on leukocytes and endothelial cells, which possess an enzymatically active domain on the outer side of the cell: ecto-enzymes.[408,409,529,530] Some act as classical adhesion molecules, but most participate in adhesion via their catalytic activity. This is a large and diverse set of molecules, but the most essential in the leukocyte cascade are the nucleotidases (CD39, CD73), ADP-ribosyl cyclises (CD38, CD157), peptidases and proteases (CD10, CD13, CD26) and oxidases (VAP-1, NADPH).[408-410]

During the 1980s a unique endothelial adhesion molecule was discovered using a monoclonal antibody (generated in response to inflamed synovial endothelium) that inhibited leukocyte

extravasation by 50%. This was termed vascular adhesion protein 1 (VAP-1).[408,529,531-533] VAP-1 in endothelial cells is stored in intracellular granules and upon inflammatory stimulation is transported to the cell membrane. In addition, VAP-1 is also present in soluble form in plasma.[408,529,534-538]

The mechanism and regulation of its expression are not entirely clear and differ between organs with maximal expression is observed 6-8h after inflammatory stimulation.

VAP-1 is a transmembrane molecule composed of two 90kDa monomers of four domains each. It has a short cytoplasmic domain, a single transmembrane domain and a large extracellular domain with multiple linked O- and N-linked oligosaccharides. These may function in adhesion.[408,529,539-544] Cloning assays revealed that VAP-1 belongs to a family of semicarbazide-sensitive amine oxidases (SSAO).[408,532] Using copper and topaquinone as cofactors, these oxidatively deaminate amines to produce an aldehyde, ammonia and hydrogen peroxide. The reaction proceeds in two steps, where first the amine reacts with the topaquinone in a covalent interaction to produce a Schiff base. This transient binding is proposed to be responsible for mediating the adhesive roles of VAP-1 in the cascade. In the second step, the enzyme is reoxidised producing aldehyde, hydrogen peroxide and ammonia ($R\text{-}CH_2\text{-}NH_2+H_2O+O_2 \rightarrow R\text{-}CHO+H_2O_2+NH_3$).[408,543,545,546]

Antibodies against VAP-1 inhibited leukocyte binding to blood vessels *in vitro*. A small molecule inhibitor of SSAO activity and catalytically inactive mutants also inhibited rolling, firm adhesion and transmigration of leukocytes. [408,547-552]This is significant as binding of the antibodies against VAP-1 did not inhibit its enzymatic activity[408,548,549] suggesting that VAP-1 can serve a dual function. In this way, it could act as an adhesion molecule itself through antibody defined epitopes and also via the enzymatic reaction. In addition, the biologically active substances generated in the reaction (hydrogen peroxide and ammonia) may exert further effects in the microenvironment. Indeed recent evidence suggests they may elicit local inflammation and induce expression of selectins, the IgG superfamily and chemokines.[553,554] These effects were confirmed *in vivo*, where inhibition of VAP-1 results in increased

rolling velocities and decreasing the number of recruited leukocytes in the inflamed areas, thereby firmly establishing a role for VAP-1 in the cascade.[408,528,529,531,550,555,556]

2. Tumour cell interactions with the endothelium – evidence for cell adhesion molecules and interactions with the immune system and platelets

The role of endothelial cell surface molecules in mediating tumour cell attachment and extravasation has long been debated. This is not only due to the lack of definitive evidence that tumour cells follow the same path as transmigrating leukocytes, but also because this question carries the implication of whether tumour cells are actively recruited to the metastatic site through this action of CAMs and chemokines.[223,333,339,349,362] This is best demonstrated by numerous experiments that have utilised prior inflammatory stimulation to enhance metastasis.[349,557-563]

This section will summarize experimental and clinical evidence that implicates the involvement of selectins and their ligands, integrins and CAMs, chemokines and host responses in mediating specific steps in cancer cell interactions with the endothelial cell layer.

2.1. Rolling and selectins

Clinically, it has been recognised that tumours express high levels of selectin ligands and exhibit aberrant glycosylation patterns. Indeed, it has been established that selectin interactions are associated with a highly malignant phenotype and poorer prognosis.[303,333,349,362,564-578]

Despite the fact that a preferential adhesion of cancer cells has been demonstrated both *in vitro* and *in vivo*, the role of selectin-mediated rolling and its requirement for tumour cell arrest remains controversial.[230,339,579-584]

The rolling motion is rarely observed in cancer[339,349,585] and has only been reported *in vitro*, e .g. a bone-metastatic prostate cancer cell line was observed to roll on IL-1β stimulated endothelium; breast and colon cancer cells were able to roll on TNF-α –activated endothelium.[339,342,448,581,582,586] In contrast,

using intravital microscopy, Thorlacius *et al* failed to observe rolling behaviour in any of 6 cell lines expressing selectin ligands.[349,585] However a role for selectins in mediating tumour cell interaction with the endothelium is provided by expression of selectin ligands on a number of cancer cell lines (e.g. prostate cancer lines[342,586], colon carcinoma cell lines[342,587-589]). Additionally, a large body of experimental evidence focused on disrupting selectin interactions using selectin-ligand mimetics, modification or inhibition of the carbohydrate moieties that are needed for selectin interactions and direct inhibition of the function or expression of selectins.[362] Most of these manipulations resulted in reducing tumour cell adhesion and metastasis *in vivo*, suggesting that selectins mediate a crucial event in the interactions of tumour cells and the endothelium.[303,362,578,590-594] However, interpretation of these result in compounded by the fact that selectins are also expressed by platelets and leukocytes. As previously described, the interactions between tumour cells, platelets and leukocytes are well established. These may increase the effective diameter of the tumour cells, thereby promoting the arrest of the emboli via the mechanical theory. In addition, they may also bridge the interactions between tumour cells and the endothelium and eliminate the need for the rolling motion.[306,339,342,362,595-599]

2.2. Arrest and integrins

The contribution of integrins to the process of metastasis is not well established.[224,339,600] Similarly to selectins, some evidence exists from functional inhibition and depletion studies and several cell lines appear to express integrins. For example integrin $\alpha 4\beta 1$ which interacts with VCAM1, was shown to mediate adhesion of several cancer cell lines to the endothelium *in vitro*. These included melanoma, sarcoma, kidney and renal carcinoma cell lines.[339,601-603] Moreover, experimental inhibition of integrin $\alpha 6\beta 1$ diminished extravasation of melanoma cell lines.[339,604,605] Inhibition of $\beta 3$ in PC-3 prostate cancer cells resulted in 50% inhibition of transmigration *in vitro*.[339,606] Additionally, expression of $\alpha 4\beta 1$ enhanced the metastatic potential in inflammation.[342,561,562]

Clinically, integrin expression is often changed during malignant transformation.[333,607-609] These changes are best characterised for colorectal cancer,[610-613] melanoma[601,614-621] and breast carcinomas.[333,346,622-630] Blocking the function of integrins *in vivo* has been shown to produce anti-metastatic effects. Dissecting the role of integrins is further complicated as blocking also had anti-proliferative and anti-angiogenic effects, suggesting that integrins are involved at several point in tumour progression.[333,631-639] Furthermore interpretation of function blocking experiments is furhter compounded by the fact that both leukocytes and platelets also express integrins. For example, Bakewell *et al* used β3 null mice and demonstrated a reduction in melanoma bone metastasis formation.[224,600] Integrin αIIbβ3 is the most prominent integrin expressed on platelets and this experiment points to the essential role these play in metastatic dissemination.[224,236,239,307,640]

2.3. Diapedesis

The passage of the tumour cell across the endothelial cell layer has been characterised by a change in the shape of the tumour cell – flattening, lamellipodial and pseudopodial protrusion of tumour cells and retraction and redistribution of endothelial cells around these.[339,372] In contrast to leukocytes, tumour cells are proposed to be able to induce retraction of the endothelial cell and in some cases apoptosis or necrosis.[223,339,641] The precise mechanism of this is not entirely clear, but different mediators proposed include a lipid 12(S)HETE secreted by tumour cells[339,642-644], VEGF secreted by tumour associated components[339,645,646] and NO produced by endothelial cells themselves.[223,576,647]

2.4. Role of chemokines

Physiologically, chemokines mediate leukocyte homing, activation and arrest. In addition, they mediate tight adhesive interactions in endothelial cells, cytoskeletal rearrangements and cell migration.[333,339,648-653] In the process of metastasis they may be produced by cancer cells themselves or by the platelets and leukocytes recruited to them.[129,236,339,583]

During metastasis chemokines are proposed to cause activation of the endothelial cell layer (and perhaps the cancer cell) and induce the secretion of chemokines and thereby mediate adhesion and promote inflammation.[223,333,339,343,647] An example of these is IL-1β, which is secreted by B16 melanoma cells and promotes VCAM-1 mediated adhesion to the endothelium.[349,654] In addition, due to their solubility and chemotactic properties, chemokines have been implicated in facilitating recruitment of metastatic cells to specific sites in the body.[333,352,363,655-660] This is supported by evidence that TGF-β1 displays chemoattractant properties in bone-metastasizing tumours.[339,661,662]

Based on such evidence, a concept of a pre-metastatic niche was developed. This proposes that a primary tumour mass would influence the secondary site in such a way as to make it more permissive for metastatic dissemination.[9] This is proposed to be mediated by substances secreted by the primary tumour.[9] Based on the experimental evidence presented above, it is conceivable that such a permissive site might include recruited platelets and leukocytes that would result in further chemokine secretion. This would facilitate the recruitment of tumour cells and also endothelial activation to facilitate a permissive environment for metastatic cell survival and extravasation.

While the arrest of tumour cells may be a consequence of size restriction, cell adhesion molecules have been shown to have a significant impact on the success of metastatic dissemination.[349] However, in light of the conflicting evidence we decided to examine the effect disseminated tumour cell have on the pulmonary endothelium *in vivo*.

Aims of the study

In inflammation, endothelial adhesion molecules expressed on the luminal endothelial surface facilitate the recruitment, attachment and extravasation of activated leukocytes. In order to gain access to the secondary site during the process of metastasis, the disseminated tumour cells must interact with the endothelial cells lining the blood vessels. Therefore in the first step of the project we wanted to test whether endothelial activation also occurs during the process of metastasis. Furthermore the aim was to establish which of the endothelial activation antigens were induced and what the temporal dynamics of their expression were.

Upon entry into the circulation the tumour cells interact with several components of the host's system including platelets and immune system cells. As previously mentioned a platelet clot was shown to form around the tumour cells and different types of leukocytes have been shown to promote tumour cell dissemination. Firstly these two phenomena will be examined, to determine whether this is also the case in the models of metastasis used in this project. Next, provided that endothelial activation is induced, both the formation of a platelet clot and the recruitment of leukocyte population will be disrupted to evaluate their involvement in mediating the induction of endothelial activation.

Finally, the role of endothelial activation in mediating the process of tumour cell dissemination will be examined. More specifically whether disruption of endothelial adhesion protein function would inhibit metastatic dissemination and what the mechanism of these effects might be.

MATERIAL AND METHODS

1. Cell lines and tissue culture

4T1-GFP (murine breast cancer), 1205LU -GFP (human melanoma cancer, isolated from a lung colony), murine Lewis lung carcinoma, MC-38-GFP (murine colon carcinoma) and MDA-MB-231-GFP (human breast cancer) cells were grown in Dulbecco's modified medium -DMEM (Invitrogen) supplemented with 10% heat inactivated foetal bovine serum -FBS (Hyclone). The medium was also supplemented with penicillin/streptomycin at 100 units of penicillin and 100 µg of streptomycin /ml (Invitrogen). These cell lines had previously been transfected with a plasmid carrying EGFP and the selection medium was Geneticin (Invitrogen) at a concentration 0.4 mg/ml for most cell lines except for the 1205LU-GFP where the concentration required was 0.8mg/ml.

Met-1 (murine breast cancer) cells were grown in DMEM (Invitrogen) supplemented with 10% heat inactivated FBS (Hyclone) and with penicillin/streptomycin at 100 units of penicillin and 100 µg of streptomycin /ml (Invitrogen).

B16F10 (murine melanoma) cells were grown in RPMI without glutamine (Invitrogen) supplemented with 10% heat inactivated FBS (Hyclone), penicillin/streptomycin at 100 units of penicillin and 100 µg of streptomycin /ml (Invitrogen), 2mM L- Glutamine (Invitrogen) and 1M HEPES (Invitrogen). Additionally B16F10 transfected with either the murine TFPI (pcDNA3) or an empty Vector constructs (pcDNA3.2/Zeo) (kind gift of Dr. Amirkhosravi[265]; 2501 N. Orange Avenue, Suite 786, Orlando, FL 32804) were grown in selection medium with either Geneticin (Invitrogen; 0.3mg/ml) or Zeocin (Invitrogen; 0.4mg/ml) respectively.

All cells were grown in tissue culture flasks of 175cm^2 (Corning Life Sciences) in at least 20ml of complete medium. These were grown in an incubator with a humidified atmosphere at 37^{o}C at 5%

CO_2. The cells were grown to up to 90% confluency and were periodically tested for mycoplasma infection.

For storage, 10e6 cell were resuspended in 1ml of 90% FBS and 10% DMSO (Sigma Aldrich) and aseptically sealed in cryo-vials (Sigma Aldrich). These were placed in a freezing container (Sigma Aldrich) and stored at -80^{o}C. Thawing of the cells included first rapid dilution of the cell suspension in pre-warmed complete medium. This was followed by centrifuging the cell pellet (5min at 1200rpm) and re-suspension in complete medium.

2. Cell staining and preparation

B16F10 and Met-1 cell staining was performed using 12,5 µM CMFDA or CMRA solution (Invitrogen) as per manufacturer's instructions. In brief, the cells were seeded to give an approximately 30% confluence at the time of staining. The dye was reconstituted using DMSO (Sigma Aldrich) 10min prior to the staining procedure and a working dilution of 12,5 µM was obtained by diluting in pre-warmed serum-free medium. The cells were incubated with the dye for 30min and then washed with serum-free medium for another 30min.

Cell suspension preparation for injections: Cell culture flasks were first washed with PBS buffer and then incubated for up to 5 min either with 4mL of Trypsin 0.05% with EDTA (GIBCO) – for 4T1-GFP, 1205LU-GFP, MDA-MB-231-GFP, MC-38-GFP and LLC-GFP cells or with 4ml of Versene 0.2 g/L (Invitrogen) – for B16F10 and c-MET cells. The resulting cell suspension was then diluted in pre-warmed medium and centrifuged (5min at 1200rpm). After re-suspension in pre-warmed serum-free medium, the cell concentration was measured using the Nucleocounter NC-100 (Chemometec) as per manufacturer's instructions. The appropriate dilution was calculated for the live cell fraction and cell suspensions for injection were prepared in serum-free medium and transported and stored on ice.

3. Analysis of proteins

3.1. Preparation of protein extract

Cells were detached from the tissue culture flask and centrifuged for 5min at 1200rpm at 4^{o}C. The cell pellet was resuspended in 1x Pierce RIPA buffer (Thermo Scientific; 200μL per 10e6 cells). The RIPA buffer was supplemented with a protease inhibitor tablet (Roche; 1 tablet per 14ml of buffer). This was then incubated on ice for 30min with intermittent vortexing. After centrifuging at 13 000 rpm for 10min, the supernatant was collected to give the cell lysate.

3.2. Measuring the protein concentration

Protein concentration was measured using the BCA assay (Thermo Scientific) as per manufacturer's instructions. In brief, the protein standards (of bovine serum albumin) were prepared in the detection range 25-2000μg/ml (8 standard concentrations in total). The experimental protein samples were diluted appropriately in water and in triplicate. The assay is based on the colorimetric change of the bicinchoninic acid reaction. The acid reacts with $Cu1^{+}$ ions produced by reduction of $Cu2^{+}$ ions by peptide bonds. The chelation of bicinchoninic acid with $Cu1^{+}$ ion (2:1 ratio) results in a purple product that is analysed by absorbance at 562nm and is thereby proportional to the protein content in the solution.

Based on this principle, the solution added to the protein sample contains: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide. Just before adding to the protein sample we add 4% cupric sulphate (source of $Cu2^{+}$). The reaction was left to develop for 30 minutes at 37^{o}C and absorbance measured with a plate reader (TECAN) and analysed with Magellan software.

3.3. Western blotting

Cell lysates (30μg of protein) was diluted with LDS Sample buffer NuPAGE (Invitrogen), 0.1mM DTT and sterile water to a total volume of 20μL. These samples were then denatured at 90^{o}C for 5min and centrifuged. Using 12-well pre-cast gels (RunBlue, Expedeon Protein Solutions) of 4-12% gradient and accompanying SDS running buffer (RunBlue, Expedeon Protein Solutions), the protein samples were run for 2h at 200V (X Cell Sure Lock Mini Cell system, Invitrogen) with protein standards from BioRad (Precision Plus Protein Dual Color standards; 10μl per well).

Following the separation of protein by electroporation in polyacrylamide gels, these were transferred onto A PVDF membrane (Thermo Scientific) using a wet transfer system. The transfer buffer was used to soak the Whatmann paper and fill the chamber and the PVDF membrane (Thermo Scientific) was activated with methanol (Fisher Scientific). The transfer was run at 30Volts for 1h and 15minutes.

The membrane was then blocked in 5% milk powder (dried skimmed milk, Marvel Original) in TBS-Tween (Fisher Scientific) for 1h at room temperature. The membrane was incubated with the primary antibody, diluted in the 5% milk powder solution, overnight at 4^{o}C and the membrane washed in TBS-Tween three times for 10 minutes at room temperature. Secondary antibody conjugated to HRP was also diluted in 5% milk powder solution and incubated for 1h at room temperature, again followed by three washes in TBS-Tween. The signal was visualized using ECL kit (GE healthcare) on Medical x-ray Super Rx film (Fuji Film), which was developed in Compact X4 automatic processor (Xograph Healthcare)

3.4. Antibodies and reagents

Anti- human TF antibody was purchased from EMD chemicals and used 1/10000. Mouse anti-TF antibody (1/1000) was purchased from R&D Systems and mouse anti-β actin was purchased from Sigma Aldrich. Secondary antibodies used were conjugated to horseradish peroxidase: anti-Rat IgG (Zymed), anti-mouse IgG and anti-Goat IgG (GE Helathcare).

4. Animal work

4.1. Mouse strains

All mouse strains used were housed in individually ventilated cages with food and water provided *ad libitum.* All experiments were performed in accordance with the U.K. legal requirements "under the Animals (Scientific Procedures) Act 1986" and ethical guidelines defined by the wider research community for University of Oxford.

Female SCID (NOD.CB17-Prkdcscid/NCrHsd) mice were purchased from Harlan. C57BL/6 (C57BL/6N), FVB (FVB/NCrl and FVB/NHanTMHsd) and BALB/c (BALB/cOlaHsd and BALB/cAnNCr) mice were purchased from Harlan and Charles River Laboratories. For each experiment, female mice between the ages of 6-10weeks were included.

$CX_3CR1^{GFP/+}$ mice (B6.129P(Cg)-Ptprc^{a} Cx3cr1^{tm1Litt}/LittJ)[663] were purchased from the Jackson Laboratory. FVB-CSF-1R-GFP mice (FVB.*(tg(Csd1r-EGFP)1Jwp)*) were obtained from Professor J.W.Pollard[664] (Albert Einstein College of Medicine; Jack and Pearl Resnick Campus; 1300 Morris Park Avenue; Chanin Building, Room 607;Bronx, NY 10461) .Mac1-KO mice were a kind gift from Professor Siamon Gordon (University of Oxford; Sir William Dunn School of Pathology;South Parks Road, Oxford OX1 3RE).

4.2. Metastasis induction

For induction of experimental pulmonary metastasis, tumour cells (5x10e5 cells) were injected intravenously into the lateral tail vain. In these models used, upon i.v. injection the tumour cells metastasize efficiently to the lung. This type of metastatic model allows monitoring of a precise time-course after metastasis, as the time of dissemination is determined and equal for all tumour cells. Intravenous injections were performed by Dr. Sally Hill and Ms. Karla Watson. At specific times after tumour cell injection (from 15min to 2 weeks) mice were killed and lungs harvested as described below.

For induction of liver metastasis, MC38 cells (2x10e5) were injected into the spleen of C57BL/6 mice as this facilitated metastatic dissemination to the liver. Intrasplenic injections were performed by Dr. Sally Hill. Two weeks later the mice were killed and the liver harvested. The tissue was fixed by immersion in 4% PFA for 24h and cryo-protected in 25% sucrose for 48h.

For induction of spontaneous metastasis, 4T1-GFP cells (10e5 cells) were injected subcutaneously into BALB/c mice. These injections were performed by Dr. Sally Hill. 6 weeks later mice were killed and the lungs harvested as described below.

In the pre-metastatic niche model, B16F10 cells (5x10e3 cells) were injected subcutaneously in C57BL/6 mice. 3 weeks later mice were killed and the lungs harvested as described below.

4.3. Lung harvest for assessment of endothelial activation antigen expression

At the specified time-points animals were sacrificed by Schedule 1. Next, a cannula was inserted into the trachea and the left auricle of the heart was pierced. Using a 5ml syringe, PBS was infused into the right ventricle of the heart in order to clear the lung vasculature of blood. This was followed by perfusion of 5 ml of 4% PFA. Next, the lungs were removed and inflated with 4%PFA through the previously inserted cannula. The lungs were left in 4% PFA overnight and then put into 25% sucrose (Fisher Scientific) in PBS at 4^{o}C, which was changed daily over the next 2 days.

4.4. Lung harvest for ex vivo whole lung metastatic assay

Anaesthetic used was Fentanyl/fuanisone – Hypnorm (0.4 ml/kg) in combination with midazolam – Hypnovel (5 mg/ml). These were mixed in a ratio 2:1:1 of sterile water:hypnorm:hypnovel and administered at 0.1ml/10g of body weight.

Under terminal anaesthesia a tracheotomy was performed. Via this cannula mice were artificially ventilated with 5% CO_2/air (Pressure Controlled Ventilator Kent Scientific). In certain experiments, a labeling antibody was then injected into the vena cava [anti-CD31-PE, anti-VCAM-1-Alexa Fluor 647 (e-

Bioscience)] and then the vein was clamped for 5min to allow efficient labeling. This was followed by exsanguination through the vena cava. Next, the left auricle was excised and the lungs were perfused through an incision in the right ventricle via major blood vessels using gravitational force only, with Krebs Ringer Bicarbonate (K.R.B.) buffer. Following this, the lungs were removed and placed into a previously designed chamber. Here the cannula in the trachea was attached to an air valve that maintains air pressure in the lungs and subsequently the lungs were inflated with a syringe using 0.3ml of air. The bottom of the chamber consists of a cover-slip that allows optimal imaging.

Assessing metastatic dissemination

The survival of tumour cells in the lung 24h after injection was assayed with two different techniques (Figure 26). In the case of the B16F10-CMFDA metastatic model, the cells were injected i.v., the lung harvested and inflated and the cells counted on the lung surface *ex vivo.* This technique has been used extensively in our laboratory. Figure 26C shows the data for this experiment (n=3).

Injection of the same number of 4T1-GFP cells (5x10e5) results in a smaller number of metastatic cells at 24h (Fig.30). Therefore the error that could occur due to the experimental setup in the 4T1-GFP model e.g. lung folding, could affect the variability in the results more profoundly than in the B16F10-CMFDA model. For this reason we decided to harvest and process the 4T1-GFP metastatic tissue as for immunohistochemistry. Then in order to assess the metastatic efficiency, at least 10 tissue sections were counted for each animal (Fig.26& Fig.30)

As this methodology, in opposition to the intact *ex vivo* lung imaging technique, allowed us to inject and harvest several lungs at the same time, the experiments can include several types of treatment in addition to the control animals. In order to avoid any error that could occur due to any bias of the particular tumour cell suspension (as all animals are injected with the same batch of cells in the same

stage of growth), the experiment was repeated.[X]

This technique thereby allows a better and more reliable analysis of the effect on tumour cell survival. It eliminates variability in tumour cell counting between each injection. As both control and experimental animals are injected with the same cell suspension, the comparison of the two groups is more accurate. Additionally, as we obtain several tissue sections, the tumour cell number can be assessed in different planes of the lung tissue and not only on the lung surface. Another positive aspect of this technique is that other phenomena can be assessed, including apoptotic index, inflammation. Additionally, as several animals can be injected at the same time, different types of treatment can be compared against a single control group; complying with the 3Rs of animal experimental practice. The negative aspect of using this readout as opposed to the *ex vivo* analysis is that the sectioning must be performed in a consistent and systematic manner, where the same planes of the lung are analysed for each experimental group. Additionally, as mentioned previously, the experiments should be repeated to avoid any bias due to the single cell suspension used.

No significant difference was observed between the two approaches to analysing tumour survival in the B16F10-CMFDA model (Supplementary figure 1).

4.5. Lung harvest for assessment of leukocyte antigen expression

When tissue harvested by the previously described method was stained for leukocyte markers or platelet markers, staining proved to be quite sparse. This was noticed in comparison with freshly frozen tissue that was post-fixed with acetone (Data not shown).

There were several problems with using snap-frozen tissue and acetone post-fixation. The main problem was that as the GFP protein used to label the tumour cells is soluble, it was washed out during the procedure and the tumour cells could no longer be visualized. In addition non-fixed frozen

[X] In each experiment at least 3 animals were used per experimental groups and for each of these animals at least 10 sections were counted.

lung tissue was extremely brittle and difficult to section.

Following this, we attempted antigen retrieval techniques on the PFA fixed tissue. For enzymatic antigen retrieval a 0.4%- 5% range of solutions of pepsin (Sigma Aldrich) in 10mM HCL was tested as well as different incubation times (30min – 90 min at 37C). For heat antigen retrieval two different buffers were tested: Sodium Citrate buffer (10mM Sodium Citrate, 0.05% Tween 20; pH 6.0) and Tris-EDTA buffer(10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0). The samples were boiled in these buffers for 15-20 min using a microwave. In each case, these techniques led to the dissociation of the tissue section from the slide, either immediately or during the staining procedure. Therefore an alternative fixation protocol was optimized based on the lung isolation procedure used in the ex vivo metastatic assay. Here mice were artificially ventilated with 5% CO2/air under terminal anaesthesia (Hypnorm/Hypnovel), via a tracheotomy. This was followed by exsanguination. Lungs were then perfused via the heart and major blood vessels using gravitational force only, firstly with K.R.B. solution and then with 50mL of ice cold 4% PFA was infused using a syringe[XI]. Following lung harvest, they were inflated with 0.3 ml of 4%PFA and then cryoprotected in 25% sucrose solution in PBS for 48h at 4C (Supplementary Figure 2) .

5. Compounds and treatment

LPS: LPS (Sigma Aldrich) was reconstituted in sterile water and injected intraperitoneally – i.p. (0.5mg/ml in 200µl per 20g mouse).

Hirudin (refludan™): Mice injected i.p. with hirudin (reconstituted in sterile saline solution to 1mg/ml) at a dose of 20 mg/kg of body weight. This was administered 5 minutes before and 4h after tumour cell injection.

VCAM-1 blocking antibody: VCAM-1 antibody (Millipore) clone M/K-2 (0.5 mg/ml) and corresponding

[XI] When less than 50ml of PFA was used, this resulted in insufficient fixation; difficulty sectioning, weak GFP signal and tissue dissociation from the slide.

IgG1kappa control (GeneTex Inc.; 1mg/ml) were injected i.v. at 30µg per animal 4h prior to the experiment.

VAP-1 inhibitor: VAP-1 inhibitor PRX177636C- BA3115001 (Proximagen) was reconstituted in sterile saline solution and filtered to give a stock solution of 1.20 mg/ml (pH=6.4). It was injected in.p. at a dosage of 5ml/kg of body weight (6mg/kg of body weight).

6. Tissue processing and immunohistochemistry

The tissue was snap frozen using cryospray (Agar Scientific) and sectioned using a cryostat microtome at an optimal thickness of 18µm[XII]. The organs were sectioned in a sequential manner as follows: three sections from different locations of the organ were placed on one positively charged slide (Fisher scientific). These were then left to air-dry at least overnight at 37°C and further stored at -20°C.

6.1. Antibodies for immunohistochemistry

Rat anti-E-selectin (1/100) was purchased from ABR Affinity BioReagents, mouse anti-P-selectin (1/100) was purchased from Chemicon International and rat anti-VCAM-1 (1/100) and goat anti-MMP9 (1/100) from R&D Systems. Hamster anti- ICAM-1 (1/250) was purchased from Millipore. Rat anti-Cd11b (1/100), rat anti-F4/80 (1/50), rabbit anti-vWF, rat anti-CD68, chicken anti-GFP (1/500), rat anti-VAP-1 (1/50); hamster anti-CD11c (1/100) and rat anti-Ly6G (1/50) were purchased from Abcam. Rat anti-CD45 (1/100) and hamster anti-CD3ε (1/10) were purchased from BD Biosciences and rat anti-αIIb from Santa Cruz.

Permeabilization of the tissue was required for αIIb and vWF staining. This was achieved by incubating the tissue in 0.2% Triton-X (Sigma Aldrich) in TBS for 5minutes.

Primary antibody against GPF was detected using a goat anti-chicken antibody conjugated to Alexa

[XII] If the sections were cut thicker the tissue tended to dissociate, however at this thickness we did not experience problems with antibody penetration.

Fluor-488 (Invitrogen). Primary antibody against vWF was detected with a goat anti-rabbit antibody conjugated to Alexa Fluor-514 (Invitrogen).

For the horseradish peroxidase based amplification we used biotinylated secondary antibodies: goat anti-rat IgG and goat anti-mouse (Invitrogen) goat anti-hamster (Abcam) and horse anti-goat (Vector laboratories).

For the alkaline phosphatase based amplification we used Polink-2 Plus AP Detection System (Golden Bridge International)

In order to confirm the specificity of the secondary antibodies (staining negative control), a slide was stained using the same procedure where the primary antibody had been omitted. Instead the tissue was incubated with TNB alone.

For in vivo labeling we used anti-CD31-PE and anti-VCAM-1-Alexa Fluor-647 (e-Bioscience).

6.2. Horseradish peroxidase (HRP) based amplification

This form of amplification was used for staining of endothelial activation antigens (VCAM-1, E-selectin, P-selectin, ICAM-1 and VAP-1), αIIb, leukocyte markers (CD11b, CD11c, F4/80, CD68, CD45, Gr-1, and CD3ε) and MMP9.

The tissue was rehydrated in PBS and blocked for endogenous peroxidase activity as follows: for endothelial activation markers (VCAM-1, E-selectin and P-selectin) the slides were incubated in 99ml of methanol with 1ml of 30%w/v hydrogen peroxide (Fisher Scientific) and for other staining in 97ml of PBS with 3ml of 30%w/v hydrogen peroxide (Fisher Scientific). Next, the tissue was blocked for endogenous biotin and streptavidin with a blocking kit (Vector laboratories) for 15 minutes each at room temperature, in a dark humidified chamber. Next It-Fx image enhancer (Invitrogen) was used, which blocked background staining resulting from non-specific interactions of fluorescent dyes with tissue components. The incubation lasted for 30 minutes at room temperature, in a dark humidified

chamber. Each step was followed by a 5 minute wash with PBS. This was followed by a general block for at least 30 minutes at room temperature, in a dark humidified chamber, using 0.5% Roehinger milk buffer – TNB (PerkinElmer).The tissue sections were then incubated with primary antibodies diluted in TNB overnight at 4^{o}C in a humidified chamber. Excess of antibody was removed by washes in PBS. Primary antibodies were detected using biotinylated secondary antibodies, diluted in 0.5% TNB. Tissue was incubated for 30 minutes and then with Streptavidin-HRP complex (PerkinElmer) TNB (0.5%) dilution (1/200) for 30 minutes, both at room temperature, in a dark humidified chamber. Then the tissues were incubated with Tyramide Signal Amplification-biotin complex (PerkinElmer) diluted in the provided amplification buffer (1/100) for 7 minutes at room temperature, in a dark humidified chamber. The staining was finished by 30 minute incubation at room temperature, in a dark humidified chamber with streptavidin-fluorophore conjugates [streptavidin –Alexa Fluor-488 and streptavidin –Alexa Fluor-633 (Invitrogen); streptavidin-allophycocyanin (eBioscience); streptavidin-Cy3 (Biolegend)] diluted (1/100) in TNB buffer (0.5%). Each step was followed by washes in PBS to remove any excess of complexes. Next a coverslip was placed onto the hydrate tissue and sealed using clear nail polish.

6.3. Alkaline phosphatase (AP) based amplification

AP-based amplification was used when co-staining of two proteins was performed on the same tissue, both of which required substantial signal amplification (Figure 35). The tissue was rehydrated in PBS for 5 minutes and blocked using 0.5% Roehinger milk buffer – TNB (PerkinElmer) for 30 minutes at room temperature in a dark humidified chamber. Primary antibodies were diluted in TNB and tissues incubated overnight at 4^{o}C and non-bound primary antibody removed by PBS washes. Primary antibodies were detected using the Polink-2 Plus AP Detection system (Golden Bridge International) as per manufacturer's instructions. In brief, the tissue was incubated for 20min in the enhancer solution,

followed by a 30min-incubation with the polymer – alkaline phosphatase conjugated secondary antibody, both at room temperature in a dark humidified chamber. These steps were separated by 5 minute washes in PBS.

To optimise the phosphatase reaction the slides were first washed in pre-reaction buffer (30mM Tris-HCl, 100mM NaCl, 10mM $MgCl_2$, 0.1mM $ZnCl_2$; pH 7.5).

The fluorescent alkaline phosphatase substrate Elf97 (Invitrogen) was diluted (1/20) in reaction buffer (30mM Tris-HCl, 100mM NaCl, 10mM $MgCl_2$, 0.1mM $ZnCl_2$; pH 7.8).

The reaction was stopped after 10-20 min by incubation in a lower pH buffer containing TritonX-100 (Sigma Aldrich) and Levamisole (Vector Laboratories). This stop buffer consisted of 50mM Tris-HCl, 100mM NaCl, 100mM EDTA, 1mM Levamisole, 0.05% Triton X-100 (pH 7.2). Next the slides were washed in 30mM Tris-HCl an 100mM NaCl (pH7.2). Next a coverslip was placed onto the hydrate tissue and sealed using clear nail polish.

6.4. Two-step immunohistochemistry

Tissue sections were rehydrated in PBS and blocked in 0.5% Roehinger milk buffer – TNB (PerkinElmer) for at least 30min at room temperature. This was followed by incubation with the primary antibodies overnight at 4C in a dark humidified chamber. Any excess antibody was removed by corresponding 5 minute PBS washes. Secondary antibody, conjugated to the appropriate fluorophore, was diluted in TNB and incubated for 30min at room temperature in a dark humidified chamber.

6.5. M.O.M block

In the case of P-selectin antibody, where anti-mouse IgG secondary was used on mouse tissue, we used a mouse on mouse (M.O.M.) blocking solution (Vector Laboratories) prior to other staining. This is used to block endogenous IgG molecules , however the chemical composition of this solution is Proprietary information. A drop of the solution was diluted in 1.25ml of PBS as per manufacturer's

instructions and the tissues incubated for 60min at room temperature. After PBS washes, the tissues were stained using HRP-amplification as described previously.

6.6. Multiple staining

When several types of staining were performed on the same tissue, the blocking steps were performed first. This was followed by HRP amplification, while AP amplification reaction and staining were performed last.

Next a coverslip was placed onto the hydrate tissue and Vectashield mounting medium was used (Vector Laboratories), these were then sealed with clear nail polish.

7. Microscopy

7.1. Epifluorescent microscopy

A Leica DM IRB/E inverted epifluorescent microscope with a halogen lamp was used for imaging endothelial activation induction.

Objective: HC PL fluotar 20 x 0.50 M25; *Eyepiece:* HC PLAN 10x/20; *Software:* Simple PCI Digital Pixel (Hamamatsu; Version 6.5.2.0); *Camera:* Hamamatsu high resolution camera C4742-95 (Hamamatsu) *Halogne light source:* LEP ARC LAMP HBO100 DC IGN (Carl Zeiss) *Filter cubes:* L5 (excitation filter BP 480/40) for imaging of GFP and Alexa Fluor 488 and Y3 (excitation filter BP 545/40) for imaging the Cyanine 3 and Alexa Fluor 633.

Lung tissue is highly auto-fluorescent, especially in the range of emission of Cyanine 3 (peak emission is 570nm) therefore Alexa Fluor 633 was used instead. Although the Y3 filter cube is not optimal for imaging this fluorophore, control experiments performed in the confocal microscope with optimal settings showed no significant differences to images acquired with the Y3 filter (Supplementary figure 3). *The filter cube properties and the fluorophores used are summarised below.*

Fluorophore used	Peak excitation wavelength (nm)	Peak emission wavelength (nm)	Filter cube used/ Excitation filter	Dichromatic filter
GFP	488	509	L5 /BP 480/40	505
Alexa Fluor 488	499	519	L5 /BP 480/40	505
Alexa Fluor 633	632	648	Y3/BP545/40	565
Cyanine 3	550	570	Y3/BP545/40	565

7.2. Leica confocal microscope

Microscopy was performed using the Laser scanning confocal Leica TCS SP2 system with Leica DM LFSA microscope.

Objective lenses: Plan-Apochromat 20x/0.8 M27; Plan-Apochromat 40x/1.3 Oil DIC M27; *Optical sections (Z-stack):* Acquired at the recommended thickness depending on the beam splitters used (3 or 6). *Software:* LCSLite; *Image acquisition:* The images were acquired in a sequential fashion and the images were averaged by frame (frame average 10). *Image processing:* Average projections of Z-series displayed. *The laser lines and detection range for the fluorophores used are summarised below.*

Fluorophore used	Peak excitation wavelength (nm)	Peak emission wavelength (nm)	Laser line used	Detection range (nm)
GFP	488	509	488	499-532
Alexa Fluor 514	517	542	514	526-585
Alexa Fluor 633	632	648	633	648-751

7.3. Zeiss confocal microscope

LSM710 NLO laser scanning confocal system (Carl Zeiss) with AXIO observer Z1 microscope.

Objective lenses: Plan-Apochromat 20x/0.8 M27; Plan-Apochromat 40x/1.3 Oil DIC M27; Plan-Apochromat 63x/1.40 Oil DIC M27; *Size of optical sections (Z-stack):* Acquired at the recommended thickness depending on the dichroic filter used.; *Software:* ZEN 2009; *Image processing:* Maximum projections of Z-series are displayed in the figures.; *Image acquisition:* The images were acquired in a sequential fashion, where each fluorophore was excited individually. The images were averaged by

line (line average 4 or 8). *Tiles:* The motorized stage of the Zeiss 710 confocal scanning microscope facilitates tiled imaging of a larger surface area. The size of the tile scan is defined by the number of fields of view as determined from the central position. In this project, the tiles were acquired with the Plan-Apochromat 20x/0.8 M27 objective. As the ZEN software does not support extraction of individual images or positional information, individual images were extracted manually for quantification and are represented in Figure 37.

The laser lines and detection range for the fluorophores used are summarised below.

Fluorophore used	Peak excitation wavelength (nm)	Peak emission wavelength (nm)	Laser line used	Dichroic filter	Detection range (nm)
GFP	488	509	488	MBS 488	493-550
CMFDA	490	517	488	MBS 488	493-550
Elf 97	342	536	405	MBS 405	500-620
Alexa Fluor 488	499	519	488	MBS 488	493-550
CMRA	547	575	561	MBS 458/561	570-620
Cyanine 3	550	570	561	MBS 458/561	570-620
Alexa Fluor 633	632	648	633	MBS 488/561/633	637-747
Allophycocyanine (APC)	650	660	594	MBS 458/514/594	637-747
R-Phycoerythrin (PE)	488/561	570	561	MBS 488/561	493 - 620

8. Image analysis

Measurement of percentage of positive area: The percentage of positive area was measured by ImageJ (http://rsbweb.nih.gov/ij/). First the contrast was adjusted to normalize the histogram of the signal the image contains. The threshold was adjusted and the percentage positive area was measured from that image. (Supplementary Figure 4)

Quantification of endothelial activation: Images were acquired either on the Leica epifluorescent microscope or the Zeiss confocal microscope and the distance of an individual tumour cell to the

positively stained signal was measured with the simple PCI or ZEN2009 software, respectively. When this distance was less than 80μm[XIII], the tumour cell was considered to localise to the endothelial activation. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections (average number of cells analysed per animal was 493). Next, these values were averaged of all the mice in the experimental group (the number of mice per group depended on the specific experiment) to give the overall mean presented.

This type of image analysis yields binary values (where the tumour cell is considered either positive or negative) and it is important to note the caveat of this analysis when analysing the expression of VCAM-1 and VAP-1. When analysing the extent of endothelial activation, the level of expression of specific molecules is likely to be of importance and this type of "binary analysis" does not take this into account. This analysis was chosen due to the nature of the staining used to visualized endothelial marker expression in the tissues. For all endothelial expression molecules, enzymatic amplification was used (HRP). Therefore the strength of the signal will depend not only on the amount of the primary antibody bound (the amount of the antigen) but also on the activity of the enzyme, substrate availability and on the length of time the reaction is allowed to run. All these could vary between different experimental sets. Thereby the strength of the IHC signal could not be directly correlated with the level of expression of specific antigens.

Inflammatory cell characterisation: Maximum projections of images were analysed for the expression of different macrophage markers. CD11b is a transmembrane protein expressed by myeloid cells (monocytes, granulocytes, macrophages and NK cells) and has also been reported to be expressed by subsets of activated T-cells. CD11c is also a transmembrane protein that is expressed at the highest level on dendritic cells, but whose expression has also been reported on monocytes, macrophages, neutrophils and some B-cells. F4/80 is also a transmembrane protein expresses on cells macrophage

[XIII] This arbitrary distance was chosen as it is proposed to be the critical diffusion distance of oxygen.[665]

lineage. CD68 is a glycoprotein stored in intra-cellular granules of macrophages. CD3ε is a protein expressed on T- and NKT-cells and forms part of the T-cell receptor – CD3 complex involved in antigen recognition. Gr-1 (Ly6G) expression is localised to neutrophils and CD45 is a tyrosine kinase expressed by most cells of haematopoietic lineage (except for erythrocytes and platelets) and is therefore used as a pan-leukocyte marker.[666]

In the transgenic animal models, the co-localization with the GFP signal of the cells recruited to the tumour cell was counted. In the *wt* animal models (SCID, BALB/c and C57BL/6) the recruitment was assessed as described below.

The analysis criteria differ between the different markers as different numbers of recruited leukocytes are visualized by the different antigens. By adjusting the criteria to the specific data set, the variation between the experimental groups can be clearly visualized.

For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next, these values were averaged of all the mice in the experimental group (the number of mice per group depended on the specific experiment) to give the overall mean presented.

Platelet clot formation (αIIβ): Maximum projections of images were assessed for the tumour cell association with platelets. Initially, the volume of platelets around the tumour cell was measured, but this was proven to be dependent on the general size of the tumour cell. Therefore in order to avoid false positive results, we decided to assess platelet association based on the clot number. According to this the tumour cells were placed into one of the following categories: no clot associated with the tumour cell, 1-5 individual clots, over 5 individual clots or a continuous sheet of platelet clot formed around the tumour cell (Supplementary Figure 5). Comparing the number of tumour cells in each category allows us to compare clot sizes between different treatment groups. A tumour cell was considered to be associated with an extensive platelet clot, when more than 5 individual clots were

found. At least 15 cells were analysed for each animal.

For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next, these values were averaged of all the mice in the experimental group (the number of mice per group depended on the specific experiment) to give the overall mean presented.

CD11b recruitment: Maximum projections of images acquired on the Zeiss Scanning Confocal system were assessed for the number of CD11b cells recruited to the tumour cell. Accordingly the tumour cells were allocated to one of the following groups: no $CD11b^+$ cells recruited, 1-5, 6-10, 11-20 and over 21 $CD11b^+$ cells associated with the tumour cell (Supplementary figure 6). A tumour cell was considered to be associated with a CD11b cell cluster when more than 6 $CD11b^+$ cells were associated. At least 15 cells were analysed for each animal. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next, these values were averaged of all the mice in the experimental group (the number of mice per group depended on the specific experiment) to give the overall mean presented.

CD45 recruitment: Maximum projections of images acquired on the Zeiss Scanning Confocal system were assessed for the recruitment of $CD45^+$ cells. As the number of $CD45^+$ cells per field of view is much higher than that of $CD11b^+$ cells the same analysis criteria proved inefficient. Additionally, due to the high number of cells, the counting was more difficult and subjective. Therefore the cells were considered to be associated with a $CD45^+$ cell cluster when a distinct grouping of cells was visible around the tumour cell as opposed to the more or less homogenous distribution in the wt lung. (Supplementary figure 7) At least 15 cells were analysed for each animal. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next, these values were averaged of all the mice in the experimental group (the number of mice per group depended on the specific experiment) to give the overall mean presented.

Recruitment of leukocytes expressing other markers: Maximum projections of images acquired on the Zeiss Scanning Confocal system were also assessed for the recruitment of Gr-1$^+$ cells, F4/80$^+$ cells, CD68$^+$ cells and CD3e$^+$ cells. For Gr-1$^+$ cells, this was observed to be a very biphasic response, where either there was a continuous sheet of Gr-1 staining formed around the tumour cell or there were only a few individual Gr-1 cells per field of view (Supplementary figure 8). Accordingly, the cells were characterised as either with Gr-1 recruitment or without.

Similar analysis was also performed for F4/80 and CD68 expressing cells, where the cells were either classified to recruit these cells or not. CD3ε^+ cells were rarely shown to be recruited, and even then only individual cells were shown to interact with the tumour cell. In view of that, tumour cells were considered to recruit CD3$^+$ cells when more than 3 cells were associated.

9. Statistical analysis

Statistical analysis was performed with GraphPadPrism 5.02. The data were first analysed as to whether they follow a Gaussian distribution using D'Agostino and Pearson omnibus normality tests. If this distribution was observed, the data set was subjected to a one-way ANOVA test. Provided this test was significant ($p<0.05$), individual data groups were compared using Tukey's Multiple Comparison post-test. Additionally, if only two group sets were compared, we used unpaired t-test analysis.

In the case where data did not follow a Gaussian distribution, the data were subjected to Kruskal Wallis test, where specific groups were compared using Dunn's post test. In the case of only two data groups that failed to follow Gaussian distribution, we used Mann Whitney analysis. Accepted nomenclature states: $p> 0.05$ – NS; $p= 0.01$-0.05 - *; $p= 0.001$-0.01 - **; $p<0.001$ - *** In the figures the data are represented as the calculated mean $\pm$ standard deviation (SD).

10. Analysis of samples by Flow Cytometry (FACS)

In order to collect blood samples for FACS analysis, the female C57BL/6 mice were sacrificed by Schedule 1. The superior vena cava was cut and the blood was aspirated (about 500μL of blood per mouse) using a 1ml syringe containing 100μL heparin (Sigma Aldrich). The samples were then put on a rotator for 5-10 min, speed 35 per minute to evenly mix the solution and prevent blood clotting. Next, the samples were suspended in 5ml of ice-cold PBS and centrifuged for 5min at 1200rpm at 4^{o}C. The supernatant was discarded and the pellet resuspended in 10mL of ice-cold PBS. This was again centrifuged, but this time resuspended in pre-warmed Gey's solution for 3min to lyze red blood cells. In order to stop this reaction, the samples were diluted with 7ml of ice-cold FACS buffer (PBS with 2% FBS). In order to remove the Gey's solution, the samples were centrifuged for 5min at 1200rpm at 4^{o}C and again resuspended in FACS buffer. This population was counted using NC-100 (Chemometec) as per manufacturer's instructions. According to the dilution factor calculated, the cell suspension was adjusted to 10e6 cells per 100μL per FACS tube. These samples were then incubated with CD16/CD32 antibody (BD Bioscience) (1/50) for 5min to block the Fc fragment. Following this the samples were incubated with either the anti-Gr-1- Alexa Fluor 647 antibody (1/100; eBioscience) or with the isotype control RatIgG2b- Alexa Fluor 647 (1/100; eBioscience) for 40min on ice.

These were then washed with 3 ml of FACS buffer and resuspended in 200μL of FACS buffer. Immediately afterwards, they were analysed with a FACScalibur cytometer equipped with BD Cell Quest software (BD Biosciences) where 150 000 cells were acquired per sample (approx. 50 000 per myeloid gate) and processed using FlowJo software.

11. Buffers and solutions

TNB buffer : 0.1 M TRIS-HCl (pH 7.5), 0.15 M NaCl , 0.5% Blocking Reagent (supplied in the kit)

Gey's solution : 4.15g NH_4Cl, 0.5g $KHCO_3$ and 500 ml ddH_2O.

RIPA buffer: 25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS

SDS buffer (20x): 0.8 M Tricine,1.2 M Tris, 2% SDS, 50 mM Sodium Bisulfite; SDS buffer (1x) is made by dilution in water.

Transfer buffer (10x): 30.3 g Tris base, 144.1 g glycine and water to 1 liter; Transfer buffer (1x) : 10% transfer buffer 10x, 20% methanol and 70% water

TBS-Tween: 50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% tween20

RESULTS

CHAPTER 1: Endothelial activation in the lung

1. The dynamics of endothelial activation in the lung

The aim of this project was to examine the effect metastatic tumour cells have on the endothelial cell layer, in particular, whether they are able to induce endothelial activation, as characterised by expression of adhesion molecules on their luminal surface. As a large proportion of metastatic spread occurs to the lung, for the most part this research focuses on pulmonary metastasis.

Several endothelial activation molecules have been described to be induced in endothelial cells in response to inflammatory stimuli. Based on previous research,[335,342,378,431] we decided to focus on four endothelial activation antigens: E-selectin, P-selectin, VCAM-1 and ICAM-1. The roles of these in mediating leukocyte recruitment and extravasation have been well established.[335,337,339,342,378,502,667] Moreover, some have been implicated in metastatic spread through clinical data.[564-571,574,609,610,668] Additionally, we decided to include VAP-1. Although discovered recently, it has already been implicated in cancer pathology and the enzymatic nature of the protein presents a unique opportunity for its use in therapeutics.

In order to ensure that endothelial activation antigens were indeed induced by inflammation in pulmonary endothelium and that our experimental model allows for their efficient detection, we used a well established inflammation model, where a lipopolisaccharide (LPS) is injected intraperitoneally (i.p). LPS is a wall constituent of gram-negative bacteria and induces systemic inflammation upon introduction into the system.[669] We used LPS from E.Coli (O111:B4), injected into BALB/c mice at 0.5 mg/ml in 0.2ml i.p.; the lungs were harvested 4h later. In parallel, tissue was harvested from non-

treated mice in order to assess the basal expression level of the selected proteins. The tissue was then processed and stained with the corresponding immunohistochemistry (IHC) for the five endothelial antigens (Figure 1).

Interestingly ICAM-1 proved to be expressed homogenously and at a high basal level (Fig. 1A). Here, it is represented together with a staining control (where the primary antibody is omitted) in order to demonstrate ICAM-1 expression as opposed to autofluorescence of the lung tissue. This pattern and the high level of expression in untreated animals cause ICAM-1 to be unsuitable for this study as any induction in its expression would be impossible to distinguish from the background.

In contrast E-selectin expression was not detected at resting state (Fig. 1C) while it is sharply induced in inflammation (Fig. 1B). VCAM-1 expression was detected at basal level (Fig. 1C). However it only appeared to occur on isolated blood vessels in a scattered, low intensity manner. This is most notable in comparison to its expression observed in response to LPS stimulation, where it was shown to be highly upregulated (Fig. 1B).

Interestingly, a basal level of VAP-1 expression can be detected in the naïve lung and this does not appear to increase in response to LPS (Fig. 1B&C). However as the function of VAP-1 depends on its enzymatic activity, this might be induced in response to LPS. Even though expression of both VAP-1 and ICAM-1 can be observed at basal level, VAP-1 is more appropriate for this study. This is because its expression is not observed homogenously across most pulmonary endothelium and also due to its kinetic activity, where its function can be efficiently inhibited.

As mentioned previously, P-selectin expression is shared by endothelial cells and platelets. This can be observed at basal level (Fig. 1C). However, in response to inflammatory stimuli, it is upregulated on the pulmonary endothelium in a pattern distinct to that of platelets (Fig. 1B).

The staining controls, where the primary antibody has been omitted, for these are clear and the secondary antibodies exhibit specific binding affinities (Supplementary Figure 9).

Figure 1

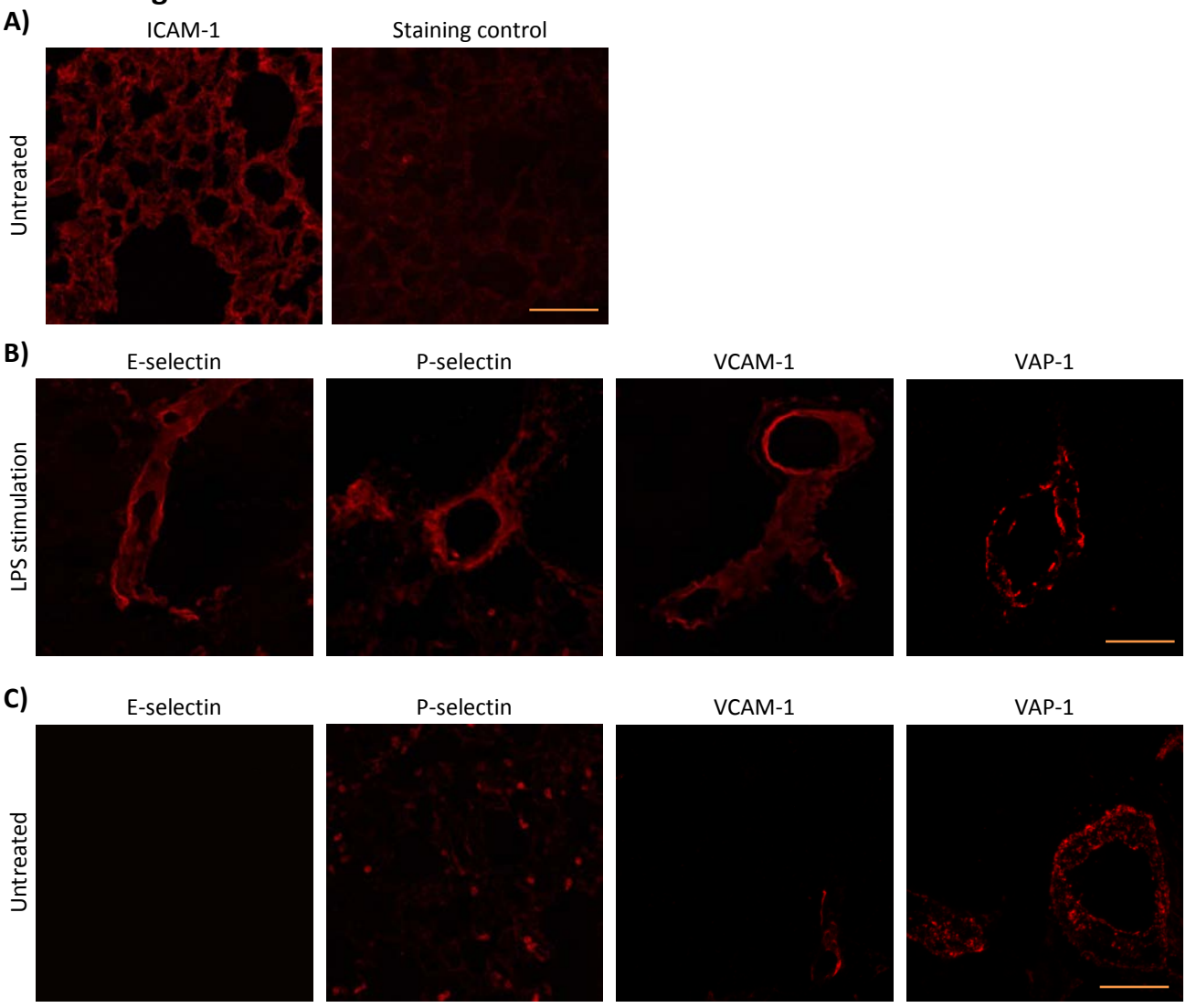


Figure 1: Expression of endothelial activation antigens in the lung

A) Immunohistochemical staining for ICAM-1 in a non-treated lung, with staining control, where the primary antibody was omitted, imaged under the same conditions.

B) Immunohistochemical staining of lung tissue for four different endothelial activation antigens: E- and P-selectins, VCAM-1 and VAP-1 (red – Alexa Fluor633). The lung tissue stained was harvested 4h post i.p. injection of 0.5mg/ml LPS. At this time, LPS had induced systemic inflammation and therefore widespread endothelial activation. Images were acquired with a Leica confocal microscope, apart from VAP-1 staining which was imaged with a Zeiss confocal microscope.

C) Immunohistochemical staining for the same endothelial activation antigens in lungs from a naïve, non-treated mouse.

Images were acquired by Leica confocal microscope. Scale =75µm

Next, we wanted to examine what the dynamics of the expression of the different endothelial activation markers are. We would expect them to be markedly different as some are produced and stored in intra-cellular granules (P-selectin and VAP-1), while others rely on transcriptional regulation (VCAM-1 and E-selectin). In order to investigate this, lung tissue was harvested at selected times after induction of inflammation (LPS injection), namely 15min, 2h, 4h, 6h and 8h. Later time-points were not possible due to the Home Office restrictions.

Based on the basal level of their expression and expression pattern we decided to focus on VCAM-1, E-selectin and VAP-1. Figure 2 shows representative images demonstrating endothelial expression of VCAM-1, E-selectin and VAP-1 at different time-points after LPS stimulation.

In order to quantify this, the percentage of positive signal per field of view of a Leica inverted epifluorescent microscope was measured, using the imaging software as described in Material and Methods (Figure 3). Immediately after LPS injection (15min) VAP-1 expression was as expected, where no difference was observed compared to that of non-stimulated tissue. Equally, E-selectin expression was minimal. In contrast, VCAM-1 was strongly upregulated at this time point.

VCAM-1 expression was sustained at this high level throughout the time-course. In contrast, E-selectin expression was upregulated at 2h, but appeared to be regulated in an almost parabolic fashion with the vertex at 4h. By 8h, the expression has been downregulated but not to that observed at basal level. Interestingly, VAP-1 expression induction was also increased, but this response is somewhat delayed as we did not observe any difference until at least hour 6.

This experiment illustrated the manner in which expression of these molecules is upregulated on the pulmonary endothelium by inflammation in the mouse. Next, we wanted to examine whether endothelial activation also occurred upon challenge with tumour cells and whether this involved the same endothelial activation antigen expression as in an inflammatory setting.

Figure 2

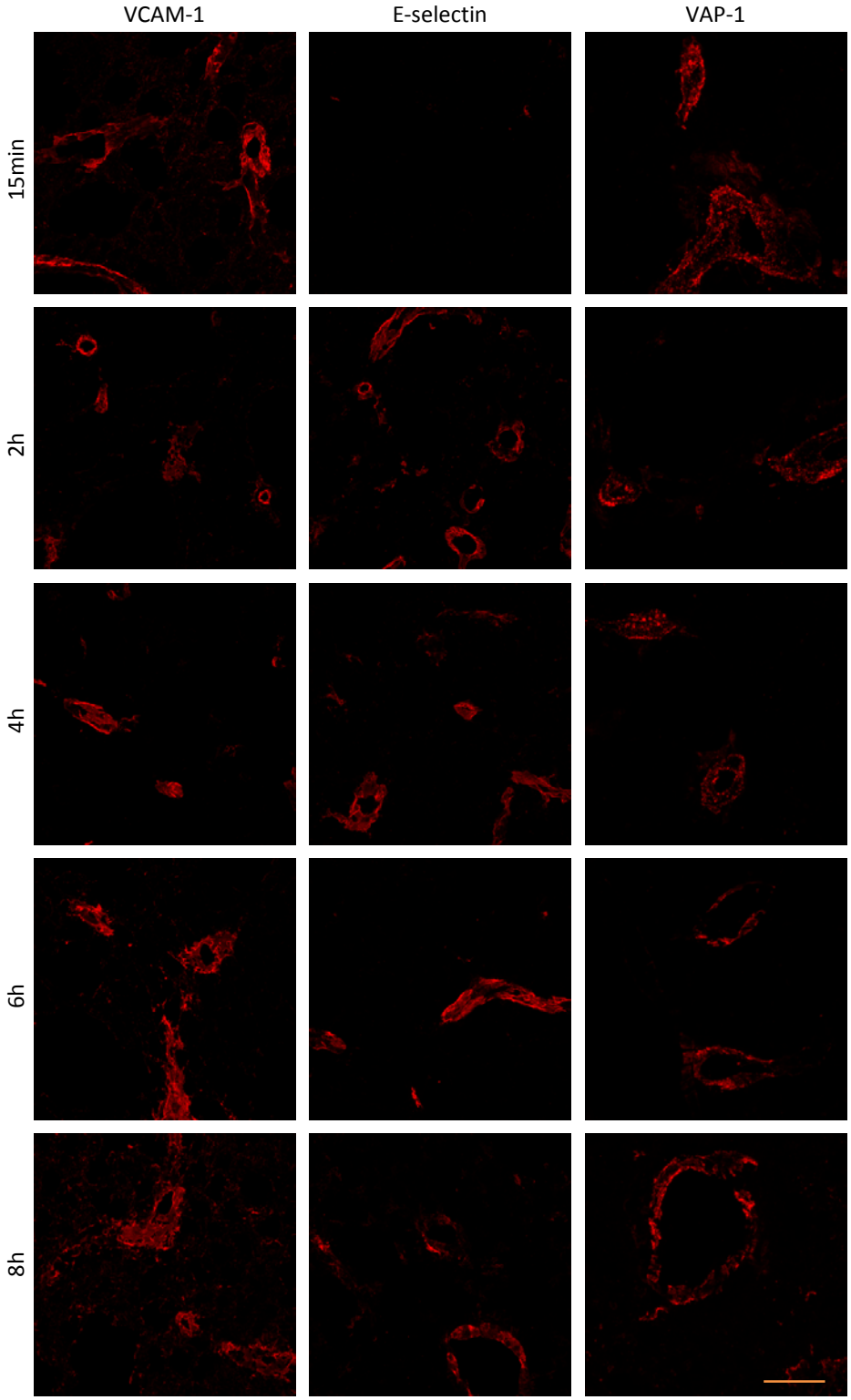


Figure 2: Dynamics of endothelial activation upon inflammatory stimuli
LPS was injected i.p. at 100µg in 200µL. At various time-points afterwards (15min, 2h, 4h, 6h and 8h) the lungs were harvested and assessed for the expression of VCAM-1, E-selectin and VAP-1 (red- Alexa Fluor 633) using fluorescent immunohistochemistry. The images were obtained with a Zeiss confocal microscope and the panel displays representative images for each of the time-points investigated. Scale =100µm

Figure 3

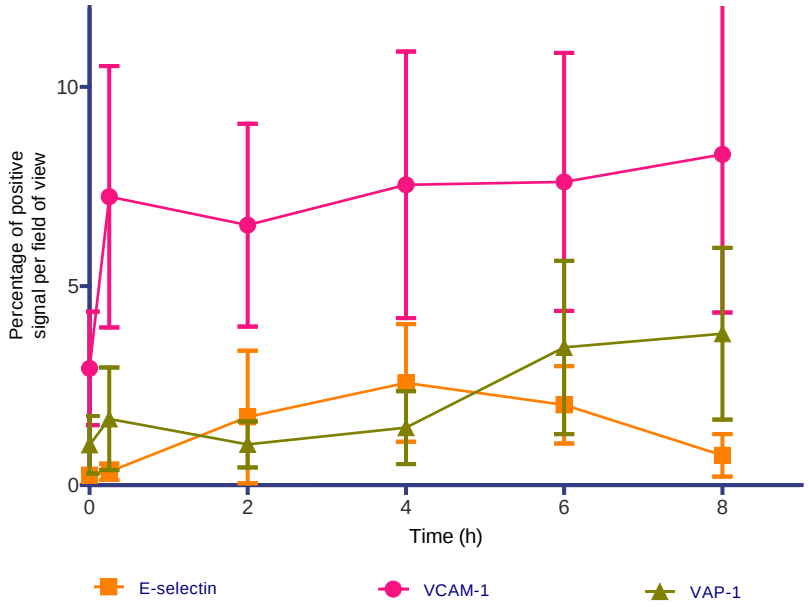


Figure 3: Quantification of endothelial activation in inflammation

Lung tissue was harvested at the different time-points (15 minutes - 8h) after injection of LPS. The 0h time-point on this graph represents a non-LPS injected control, showing the basal expression levels of the proteins. The endothelial activation proteins were visualised using fluorescent immunohistochemistry and imaged with a Leica epifluorescent inverted microscope (20x). The percentage of positive signal was evaluated by ImageJ tool analysis and thresholding in at least 10 fields of view and at least three sections for each time-point (n=1).). Statistical analysis was not possible as only one animal per time-point was analyzed.

2. Endothelial activation in metastasis

2.1 VCAM-1

The foremost endothelial activation antigen to be assessed was VCAM-1 (Fig. 4, 9&10), as its expression appeared to be most strongly induced in inflammation. VCAM-1 expression has also been described in haematopoietic cells[670], therefore in order to confirm that the VCAM-1 expression visualised was indeed upregulated on the endothelial cells, the tissue was co-stained for von Willebrand factor (vWF), a glycoprotein constitutively expressed in endothelial cells (Supplementary Fig 10).[671] Co-localization of the staining for VCAM-1 and vWF confirmed that VCAM-1 expression is induced in the endothelial cells

To facilitate the detection of VCAM-1 expression in response to tumour cell metastasis in the lung, three different experimental metastasis models were used. The first one was the mammary carcinoma cell line 4T1-GFP, that is syngeneic to BALB/c mice.

In addition, two human cell lines were used; the melanoma cell line 1205LU-GFP and the adenocarcinoma cell line MDA-MB-231-GFP. These were introduced into SCID (Severe Combined Immune Deficiency) mice in order to circumvent the adaptive immune system response to human antigens expressed on the tumour cells. All cells lines have been stably transfected with GFP, which allowed for efficient and specific imaging of the metastasized cells in the lung tissue.

The cells were resuspended in serum-free medium and injected i.v. At specific time-points after tumour cell injection (15min -2 weeks), the lungs were harvested and tissue processed as described in Material and Methods. Endothelial activation antigens were detected through fluorescent immunohistochemical staining with Alexa Fluor 633 fluorophore. As an experimental control, serum-free medium only was injected and this had no effect on endothelial activation in the lung (Supplementary Figure 11).

Induction of VCAM-1 expression was gauged by assessing each individual tumour cell or metastatic lesion for the presence of VCAM-1 positive blood vessels within 80μm.[XIV] The setting of this arbitrary boundary allowed for the assessment of the dynamics of endothelial activation in relation to metastatic spread. Because tumour cells appear to be involved in VCAM-1 induction, there is a correlation between the total VCAM-1 protein in the lung and the total number of tumour cells in the lung. The number of metastatic tumour cells in the lung diminishes rapidly over the first 24h (Supplementary figure 12) and this could produce a decrease of overall endothelial activation, where in relation to individual tumour cell its expression was maintained.

Thereby the output of this analysis is the percentage of total cells analysed that are localised to VCAM-1 expression (Figure 5). The caveat of this type of analysis is that if endothelial activation (e.g. VCAM-1 expression) is involved in promoting tumour cell survival in the lung tissue, the cells localised to the site of endothelial activation will preferentially survive through the time-course. Thereby, one should consider this type of analysis not as an objective measure of endothelial activation, but rather a measure of endothelial activation as it correlates to an individual metastatic cell/colony.

Surprisingly, the expression of VCAM-1 15 minutes after tumour cell injection was minimal (Figures 4&5). In the 4T1-GFP syngeneic model only 14.96% of cells were found to be associated with VCAM-1 staining. The percentage was even lower in the human cell lines, where 9.84% of 1205LU-GFP cells localised to VCAM-1 staining and only 0.79% of MDA-MB-231-GFP cells were found by VCAM-1 expression. (Fig. 4&5) This is interesting as VCAM-1 upregulation had occurred after 15 minutes in response to an inflammatory stimulus (Figure 2&3). Thereby the delay in VCAM-1 expression induction in response to metastasis was not likely to occur as a result of its expression regulation. This suggests that VCAM-1 may not function in a role analogous to that described for inflammatory

[XIV] This arbitrary distance was chosen because it is proposed to represent the critical oxygen diffusion distance in respiring tissue, thereby assuming that the tumour cells will have established within that parameter.[665] ibid.

cells, namely to recruit tumour cells and facilitate their adhesion. In contrast, in this setting it appears that the tumour cell themselves may be involved in mediating or initiating endothelial activation.

Figure 4

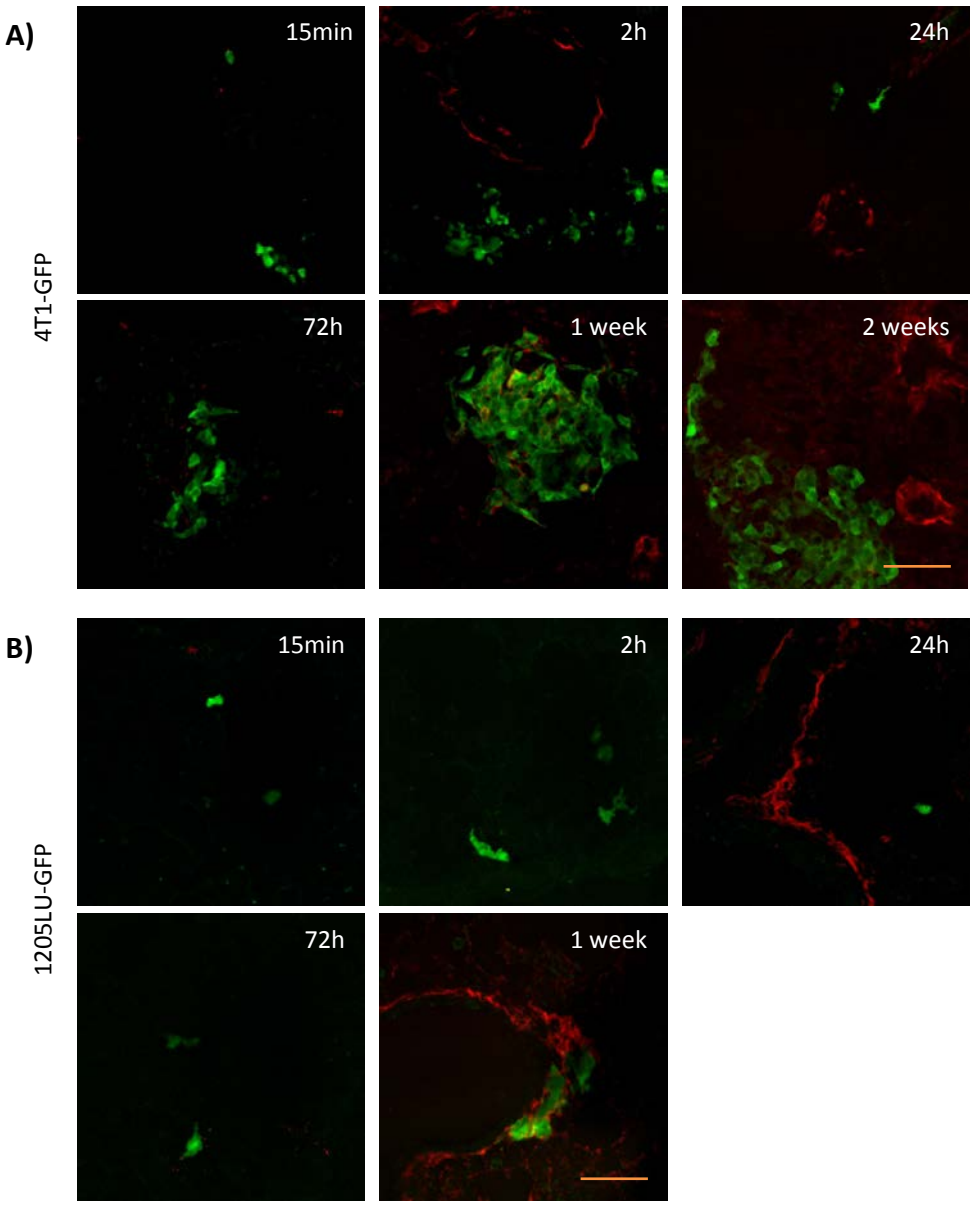
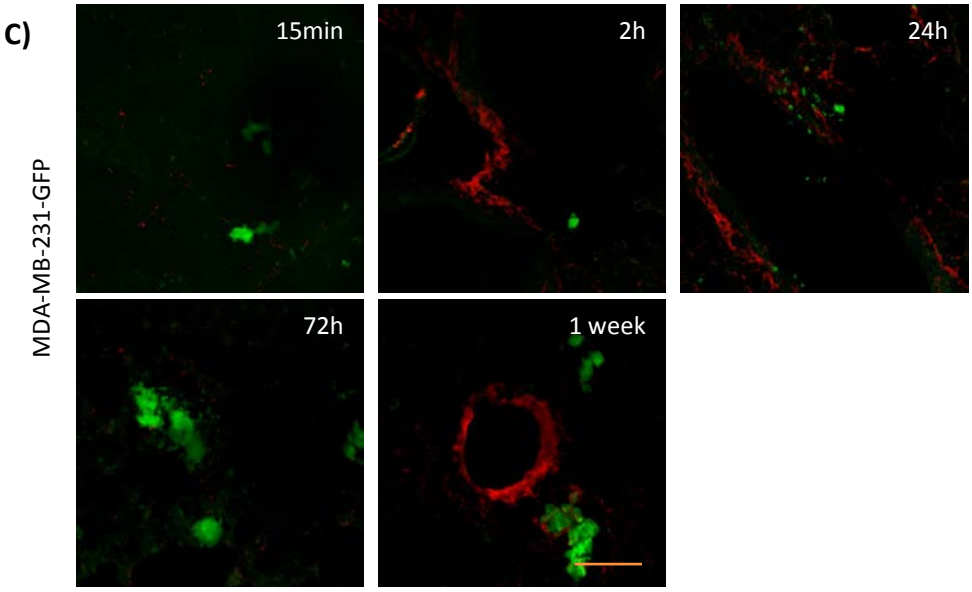


Figure 4: The expression of VCAM-1 is induced by pulmonary metastasis

Pulmonary metastasis models used were based on i.v. injection of tumour cells. At various time-point afterwards, from 15min to up to 2 weeks, the lungs were harvested and VCAM-1 expression investigated. A) Immunohistochemical staining of 4T1-GFP pulmonary metastasis (green) for VCAM-1 expression (red – Alexa Fluor 633). Shown are representative images of different time-points after i.v. cell injection. Imaging was performed with Leica Confocal microscope. Scale=75µm

B&C) VCAM-1 induction is also present in other metastatic models. Panels B and C illustrate two human tumour cell lines models: 1205LU-GFPs and MDA-MB-231- GFP (green) at various time post i.v. injection in SCID mice. VCAM-1 expression (Alexa Fluor 633-red). Imaging was performed with Leica Confocal microscope. Scale=75µm

Figure 4 continued



Analysis showed (Fig.5) that VCAM-1 expression was upregulated on the pulmonary vasculature in the vicinity of tumour cells after tumour cell challenge. This change in expression was found to be highly significant (One-way ANOVA with Tukey's Multiple comparison post-test: 4T1-GFP $p<0.0001$; 1205LU-GFP $p<0.0001$; MDA-MB-231-GFP $p=0.0180$).

When assessing VCAM-1 expression in response to tumour cell challenge, the lung tissue analysed is biased towards areas of the tissue containing tumour cell metastasis. Therefore a non-injected control was not included in the analysis. As no significant induction of VCAM-1 expression was observed 15 minutes after tumour cell injection, we conclude that the level of VCAM-1 expression localised to the metastatic cells at that time was due to its basal level of expression. Within the next hours the percentage of cells associated with VCAM-1 expression increased dramatically.

In the 4T1-GFP cell model VCAM-1 expression localized with 58.32% of tumour cells 2h after tumour cell injection and further to 74.48% of tumour cells localising to VCAM-1 staining at 24h. Interestingly the two human cell lines differed in the dynamics of VCAM-1 expression. Similarly to 4T1-GFP cells, 1205LU-GFP cells induced VCAM-1 expression through the next few hours. At 2h this had reached 52.62% of the tumour cells localised to VCAM-1 staining and at 24h this percentage increased to 62.68%. In contrast MDA-MB-231-GFP cells elicited a sharp increase already 2h post tumour cell injection (76.15%). Furthermore, at 24h this had decreased and only 36.57% of tumour cells co-localized with VCAM-1 expression.

By 72h, VCAM-1 expression was shown to decrease, with only 37.74% of 4T1-GFP tumour cells co-localised with VCAM-1 expression and in the 1205LU-GFP cell line model the association decreased to 32.00%. In the MDA-MB-231-GFP model the percentage of cells that localised to VCAM-1 protein (28.5%) was similar to that observed at 24h. Thereby, demonstrating that while lower VCAM-1 expression was already observed 24h after tumour cell injection in the MDA-MB-231-GFP cell model,

this likely represents a different temporal distribution of the same response observed at 72h in other cell models.

Later in metastatic colony progression and expansion (e.g. 1 week after injection), VCAM-1 expression was significantly upregulated again. In the 4T1-GFP cell model this increased to 83.38% association and in the human cell models, to 59.65% and 64.27% in 1205LU-GFP and MDA-MB-231-GFP cell models respectively.

In this way VCAM-1 induction appears to occur in a biphasic manner, where first an initial host inflammatory response within the first 24h of tumour cell arrival in the lung tissue induces its expression. During this time severe attrition of tumour cells in the lung occurs and only a small percentage are able to lodge and survive (Supplementary figure 12). VCAM-1 is then again upregulated during metastatic colony expansion. In the 4T1-GFP model this occurs at weeks 1 and 2 (Fig 4A & 5).

Other metastatic models

VCAM-1 induction in metastatic colony formation was also confirmed in other experimental models of metastasis (Fig.6). These include a spontaneous model of metastasis, where 4T1-GFP cells were implanted sub-cutaneously into BALB/c mice and grown for 6 weeks. The representative image shows a pulmonary metastatic colony that had developed from a spontaneously metastasized cell and VCAM-1 expression in its vicinity (Fig.6). As the tumour cells have been growing for over 6 weeks without selection medium for the GPF-expressing plasmid, the GFP signal is quite weak even after enhancing the GFP signal with IHC. However, the metastatic colony is morphologically extremely different from the remaining tissue and so easily identifiable. Another model used the LLC-GFP cells in C57BL/6 mouse. Upon injection it metastasized efficiently to the lung and induced expression of VCAM-1 on endothelial cells. In the third model MC-38 –GFP cells were injected into the spleen in order to facilitate efficient liver metastasis formation. The image shows that 10 days after injection

metastatic colonies were able to induce VCAM-1 expression in the liver. In contrast to the lung, upon harvest liver was not cleared of blood and was fixed only by incubating the organ in PFA overnight. Therefor the IHC was more diffcult and the signal detected weaker.

Figure 5

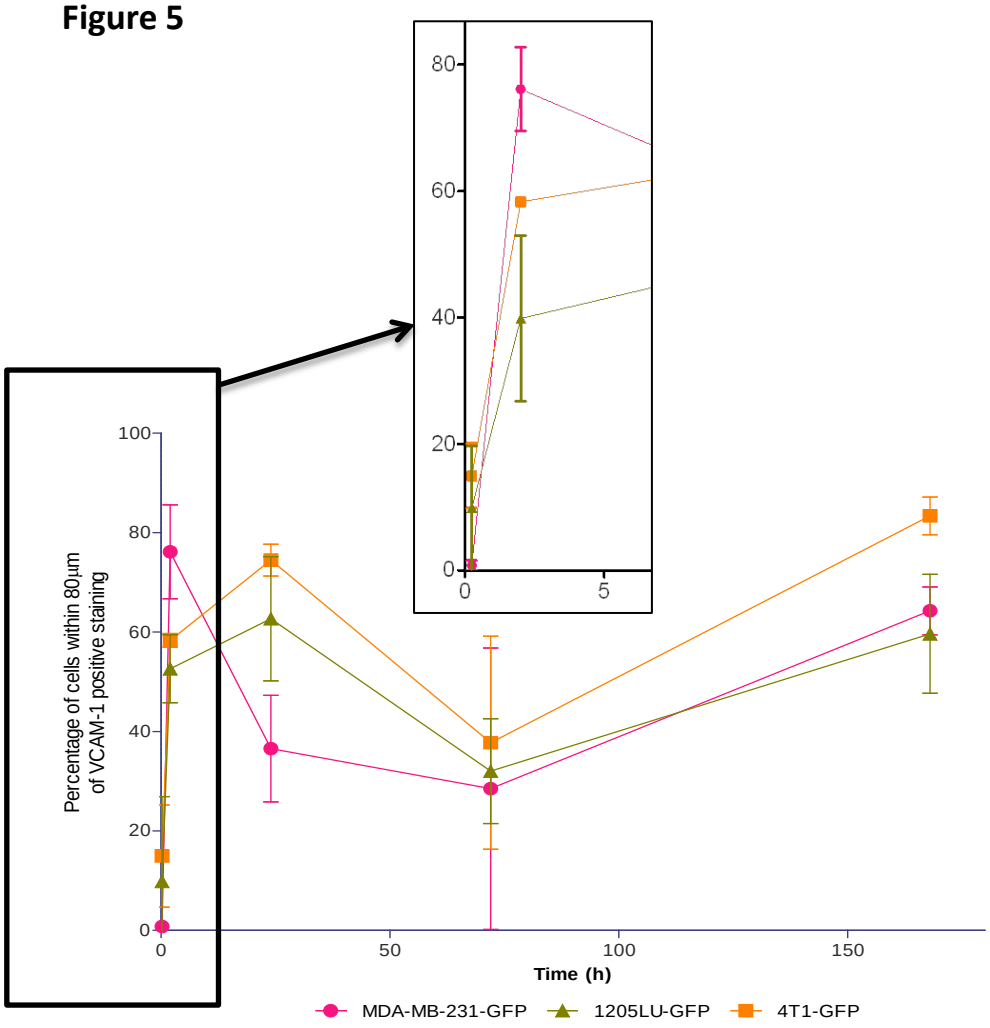


Figure 5: Quantification of VCAM-1 induction by pulmonary metastasis

VCAM-1 induction was quantified by assessing each individual tumour cell and whether it is located within 80µm of the VCAM-1 positive signal. This is expressed as percentage of all tumour cell assessed that were localised to VCAM-1 expression.
For each data point (15min, 2h, 24h, 72h and 1 week) first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next all these were averaged in either 4 animals (4T1-GFP and 1205LU-GFP) or 2 animals (MDA-MB-231-GFP) to give the overall mean presented. Statistical analysis – one-way ANOVA with Tukey's Multiple comparison post-test: 4T1-GFP $p<0.0001$; 1205LU-GFP $p<0.0001$.)

The insert represents a zoom into the first two time points (15min and 2h). Additionally in order to facilitate greater clarity, SEM is displayed rather than SD.

2.2 E-selectin

In order to evaluate the induction of E-selectin expression, we used the same three cell lines. E-selectin expression was analysed and quantified (Fig. 7C) in the same manner as VCAM-1 expression (Fig.5). In contrast to VCAM-1, E-selectin expression was not induced by the 4T1-GFP cells at any time after metastasis formation (Fig 7A). On the other, hand the human cell lines (1205LU-GFP and MDA-MB-231-GFP) appeared to induce a transient E-selectin expression (Fig. 7B&C).

This occurred faster in the MDA-MB-231-GFP cell line, where already at 2h 53.37% of cells were found to be associated with E-selectin expression. However at the same time only 4.45% of the 1205LU-GFP cells were localised to E-selectin expression. The situation had reversed by 24h, when only 1.61% of MDA-MB-231-GFP cells were found to be associated with E-selectin expression and 15.75% of 1205LU-GFP cells. This returned to the basal level by 24h in the 1205LU-GFP model as well.

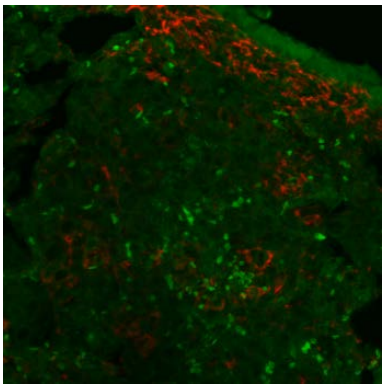
It appears that in contrast to VCAM-1, E-selectin is only up-regulated in an extremely transient fashion (Figure 7 and Supplementary Figure 13). This was similar to the endothelial activation dynamic measured upon inflammatory stimuli (Figures 2&3), where E-selectin expression was upregulated only briefly.

Another interesting aspect to note is that a similar difference occured in the timing of when a particular tumour cell line causes the endothelial activation. This may have to do with the metastatic efficiency of the particular cell line (Supplementary Figure 11). For example, MDA-MB-231-GFP cells are able to attach earlier and in both cases (VCAM-1 and E-selectin expression) have been shown to induce endothelial activation earlier.

In addition to E-selectin, some preliminary analysis of P-selectin expression was also performed (Supplementary Figure 14). P-selectin does not appear to be induced in response to 4T1-GFP tumour cells, however due to the platelet staining signal background it proved difficult to analyse. This could be solved by concurrent staining with either an endothelial cell marker or a platelet marker.

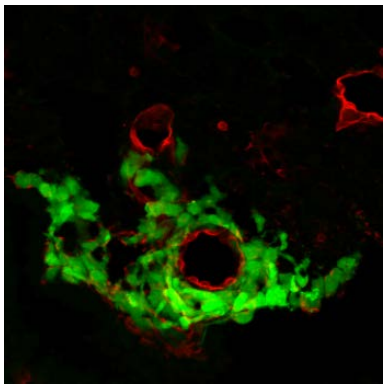
Figure 6

A)



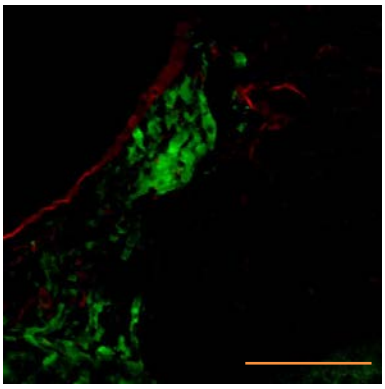
Spontaneous pulmonary metastasis of 4T1-GFP cells (green) injected sub-cutaneously at 6 weeks.

B)



Pulmonary metastasis of LLC cells (green) at day 10.

C)



Liver metastasis of MC-38 –GFP (green) cells injected intra-splenically at day 14.

Tumour cell VCAM-1

Figure 6: VCAM-1 expression is also induced by metastasis in other metastatic models

Upregulation of VCAM-1 expression (Alexa Fluor 633-red) was also observed in other models of metastatic spread. Tissues were imaged with a Leica confocal microscope. Scale = 100µm
A) 4T1-GFP cells were implanted subcutaneously and grown for 6 weeks. Shown is a representative image of a spontaneous pulmonary colony (green) and expression of VCAM-1 (red).
B) Pulmonary metastasis of Lewis Lung Carcinoma cells (green). These were injected into the C57BL/6 mice. VCAM-1 expression (red) was imaged with Alexa Fluor 633.
C) MC38 –GFP cells were injected into the spleen of C57BL/6 mice. Representative image shows a liver colony (green) and expression of VCAM-1 (red).

Figure 7

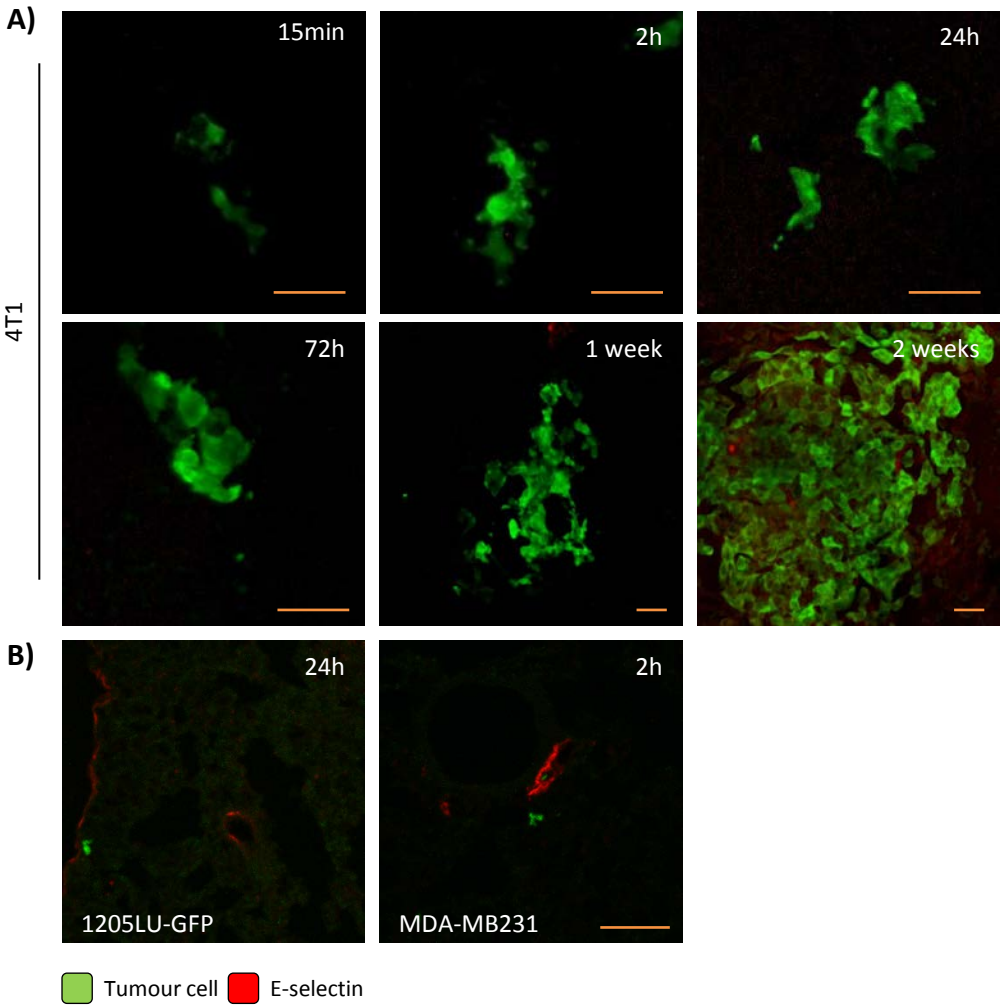


Figure 7: E-selectin induction by pulmonary metastasis

Pulmonary metastasis was induced by i.v. injection of tumour cells. At various time-point afterwards the lungs were harvested and E-selectin expression investigated.

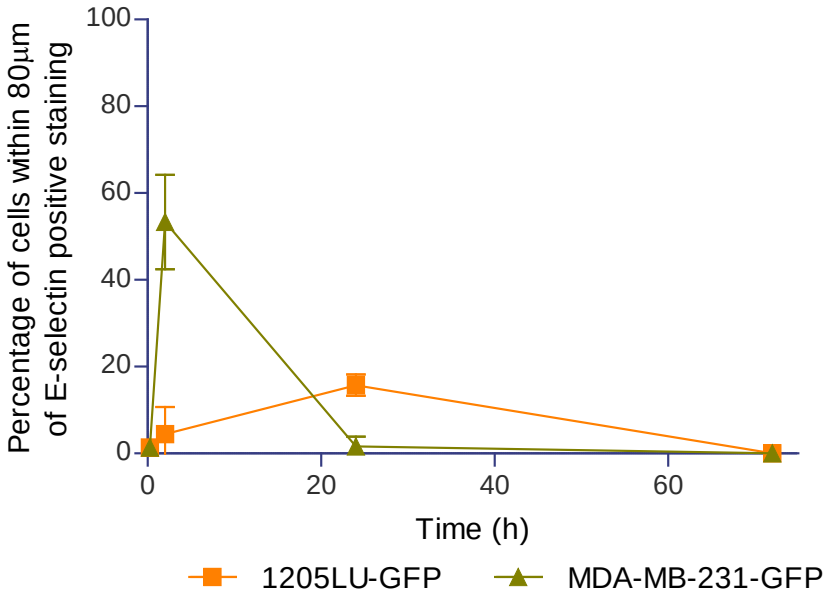
A) Immunohistochemical staining of 4T1-GFP pulmonary metastasis (green) for E-selectin expression (Alexa Fluor 633- red). Shown are representative images of different time-points after i.v. cell injection. The images were acquired with Leica Confocal microscope. Scale=30µm

B) In the two human tumour cell lines models: 1205LU-GFP and MDA-MB-231- GFP (green) E-selectin (red) is induced in a transient manner. Shown are two representative images at 2h and 24h time-point acquired with Zeiss Confocal microscope. Scale=100µm The other time points are shown in Supplementary figure 12.

C) E-selectin induction was quantified by assessing each individual tumour cell and whether it is located within 80µ of E-selectin staining. This is expressed as percentage of all tumour cell assessed that were localised to E-selectin expression. For each data point, first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next, all these were averaged in either 4 animals (1205LU-GFP) or 2 animals (MDA-MB-231-GFP) to give the overall mean presented. Statistical analysis – one-way ANOVA with Tukey's Multi-comparison post-test: 1205LU-GFP p=0.0166

Figure 7 continued

C)



2.3 VAP-1

The last endothelial activation molecule to be analysed was VAP-1 (Figure 8). We used the 4T1-GFP and the 1205LU-GFP cell lines and followed the same time course and methods of analysis as previously for VCAM-1 and E-selectin.

A low level of VAP-1 expression was observed in the lungs of non-treated animals (Fig.1). Upon inflammatory stimuli (LPS), this led to a slow induction of VAP-1 expression, however only 6-8h after stimulation. (Fig.2&3).

This is perhaps not surprising, as central to the mode of action of VAP-1 is its enzymatic activity. This may be regulated in many different ways, not relying solely on regulation of expression as do most other adhesion proteins.

Representative images of VAP-1 expression in response to 4T1-GFP and 1205LU-GFP pulmonary metastasis are shown in Figure 8. The analysis shows the percentage of metastatic tumour cells localising within 80μm of VAP-1 expression.

Interestingly VAP-1 did not appear to become significantly induced above the background level in response to metastatic spread of either cell line (Fig. 8C). Although a tendency was observed, no statistical differences were obtained for neither 4T1-GFP nor 1205LU-GFP cell lines. This highlights the necessity of exploring the enzymatic activity of VAP-1 rather than its endothelial expression.

Figure 8

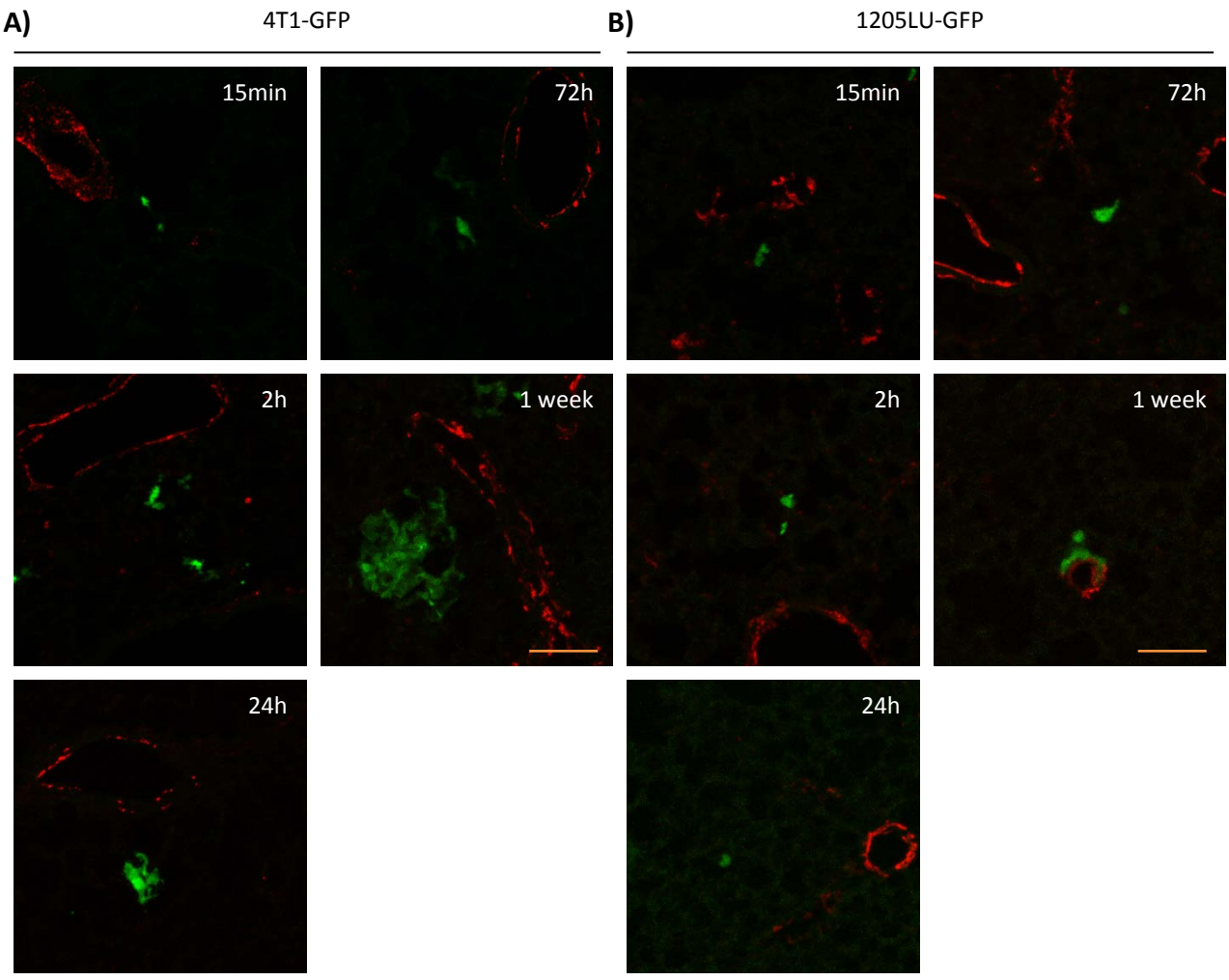


Figure 8: VAP-1 induction by pulmonary metastasis

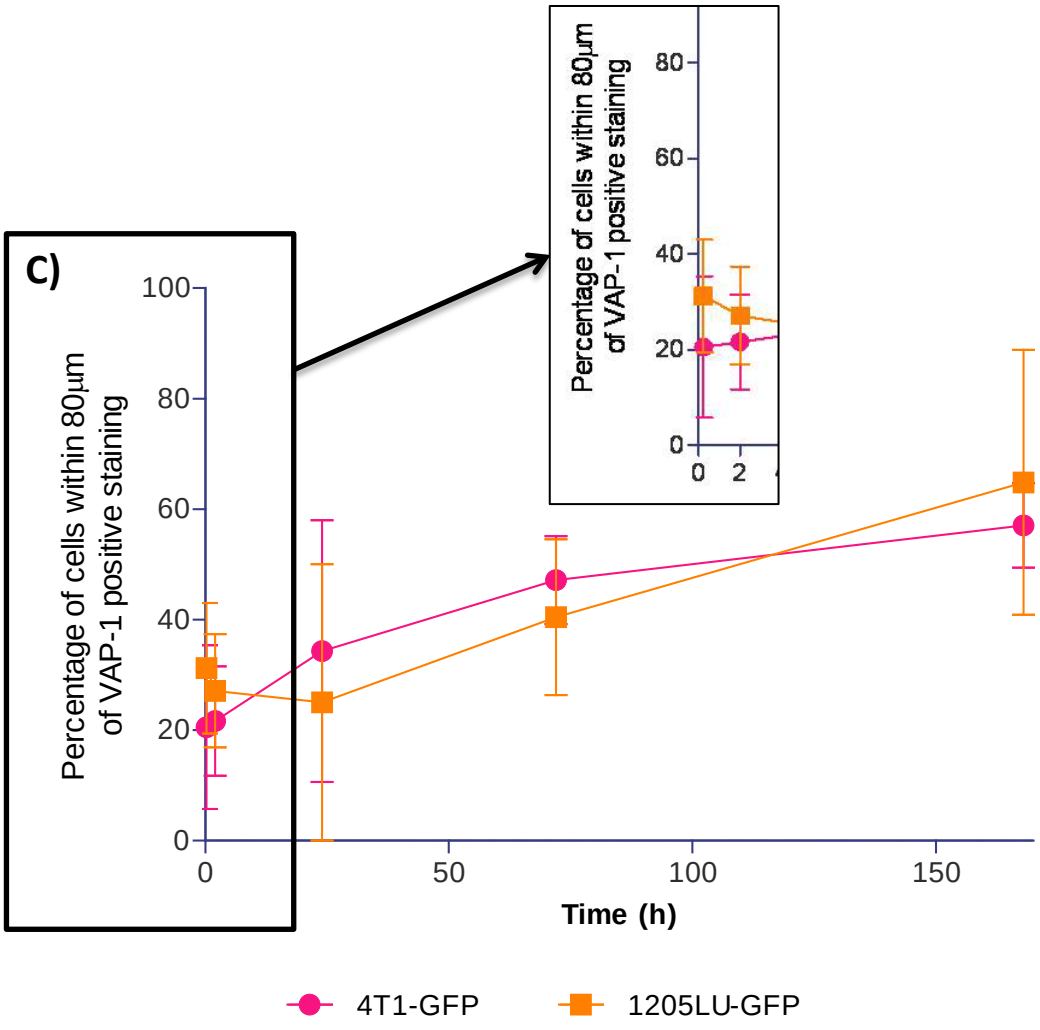
Pulmonary metastasis was induced by intra-venous injection of 4T1-GFP and 1205LU-GFP cells in BALB/c or SCID mice, respectively. At specific time-point afterwards (15min, 2h, 24h, 72h or 1 week) the lungs were harvested and VAP-1 expression assessed.

Immunohistochemical staining of 4T1-GFP (A) and 1205LU-GFP (B) pulmonary metastasis (green) for VAP-1 expression (Alexa Fluore633- red). Shown are representative images of different time-points after i.v. cell injection. Images were acquired with Zeiss Confocal microscope. Scale=100µm

C) (opposite page) VAP-1 induction was quantified by assessing each individual tumour cell and whether it is located within 80µm of VAP-1 staining. This is expressed as percentage of all tumour cell assessed that were localised to VAP-1 expression. For each cell line 3 animals were analyzed and at least 30 cells for each. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next the values for all three animals were averaged to give the overall mean presented.

Statistical analysis: one way ANOVA: 4T1-GFP: p=0.0593; 1205LU-GFP: p=0.0823.

Figure 8 continued



2.4 Endothelial activation within the initial 24h

The lack of E-selectin expression induction by 4T1-GFP cells (Fig.7) and the overall absence of VAP-1 expression induction within the first 24h (Fig.8) seemed irregular. If their expression was induced transiently in the time frame between 2h – 24h, as was observed for E-selectin in human metastatic models, it would remain undetected. Therefore, we decided to further investigate the initial inflammatory response within the first 24h in more detail (Figures 9, 10 &11).

Analysis of E-selectin expression at time-points of 1h, 4h, 8h and 16h, did not reveal induction of E-selectin expression by 4T1- GFP cells (Supplementary figure 15).

On the other hand, supplementing additional time-points allowed more detailed delineation of VCAM-1 expression induction. Acute upregulation of VCAM-1 expression was observed at these time-points (Fig. 9A & Fig. 10). Additionally, 4T1- GFP cells appear to induce a stronger response compared to that of 1205LU-GFP cells. Its expression was seen to increase steadily upon tumour cell injection, peaking at about 4-8. At 4h 69.49% and 45.94% of 4T1-GFP and 1205LU-GFP tumour cells were localised to VCAM-1 expression respectively. This had increased respectively from 66.37% and 40.27% observed at 4h. Such expression is maintained at this level all thorough the 24h, giving the expression profile a sigmoid appearance. At this time, 74.48% and 62.68% of cells were found to be associated with VCAM-1 expression in 4T1-GFP and 1205LU-GFP models respectively.

A small, transient upregulation of VAP-1 expression was observed in response to 4T1 –GFP cells (Fig.9B & Fig.11). This only occurred at 8h (53.08%) but was again decreased by 24h (34.08%) to essentially that observed at resting state. The induction of VAP-1 expression by 1205LU-GFP cells did not prove to be statistically significant ($p=0.0670$), but this was most likely due to the high level of variability observed at this time point.

Representative images (Figure 9B) illustrate the expression of VAP-1. In addition to the increase in the amount of VAP-1 positive blood vessels, it looks as if the expression itself is also intensified. Where previously the staining appeared scattered and discontinuous (e.g. at 1h), it is more uninterrupted at later time-points.

Based on these results we decided to focus on the time-frame of the initial 24h. Within this time, the tumour cells have been shown to interact with platelets and immune cells and here these have been demonstrated to induce endothelial activation. In the next stage the mechanism by which endothelial activation occurs are examined.

Figure 9

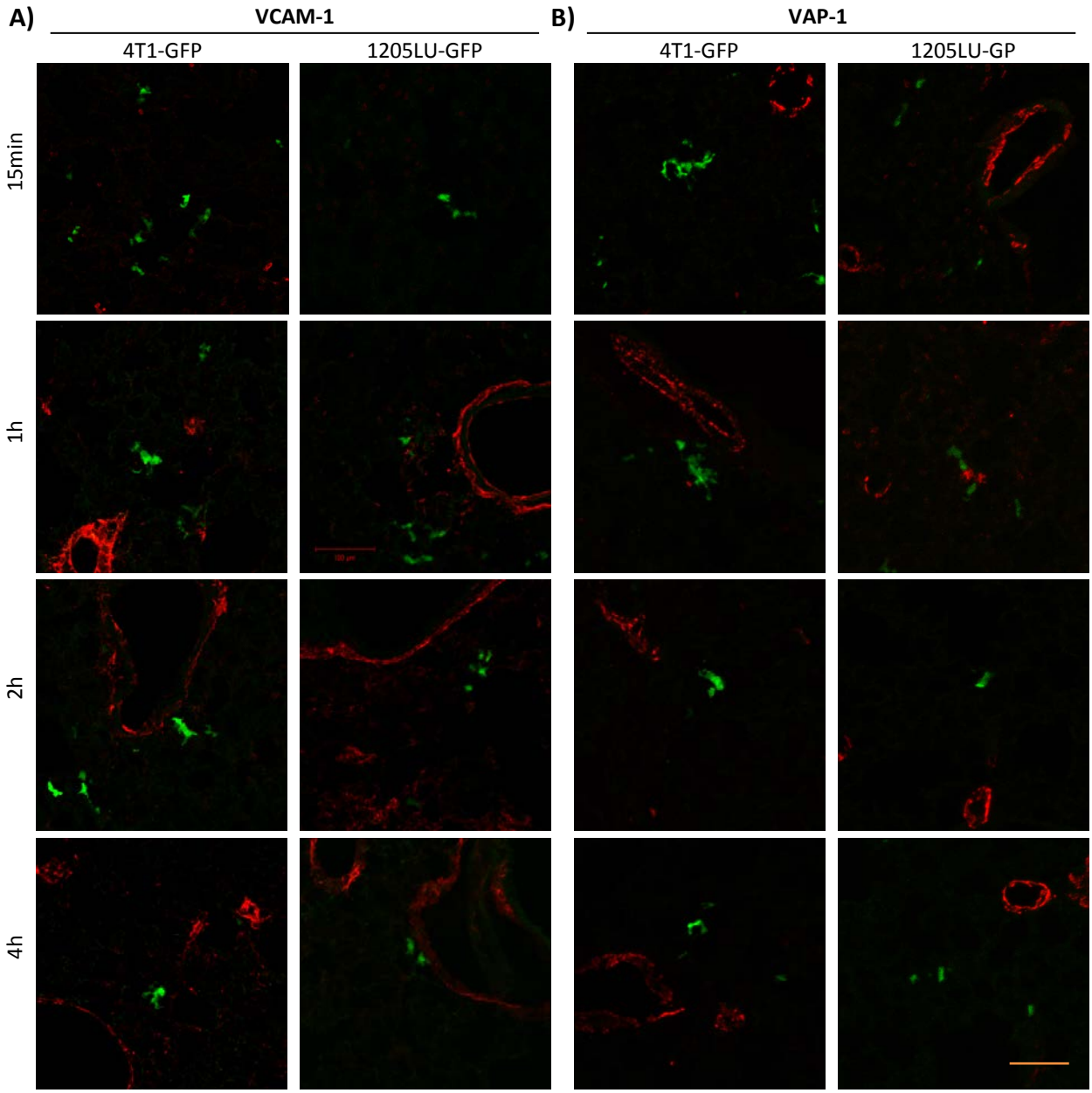


Figure 9: Induction of VCAM-1 and VAP-1 expression in 1205LU-GFP and 4T1-GFP metastatic models
Pulmonary metastasis was induced by intra-venous injection of 4T1-GFP and 1205LU-GFP cells in BALB/c or SCID mice, respectively. At specific time-point afterwards (15min, 1h, 2h, 4h, 8h and 16h) the lungs were harvested and the expression of both VCAM-1 and VAP-1 assessed. This figure contains representative images of immunohistochemical staining for VCAM-1 (A) and VAP-1 (B) expression (Alexa Fluor 633-red) on the lung endothelium in response to 4T1-GFP and 1205LU-GFP cell challenge (green). Images were acquired with a Zeiss Confocal microscope. Scale=100µm

Figure 9 continued

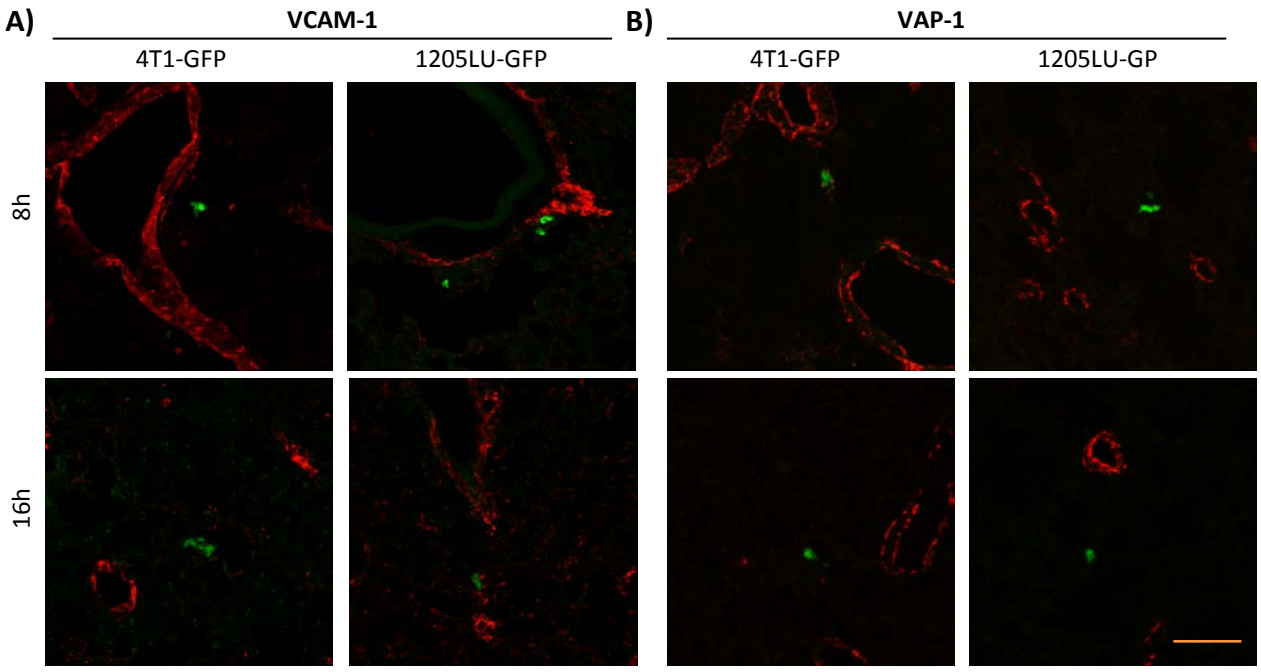
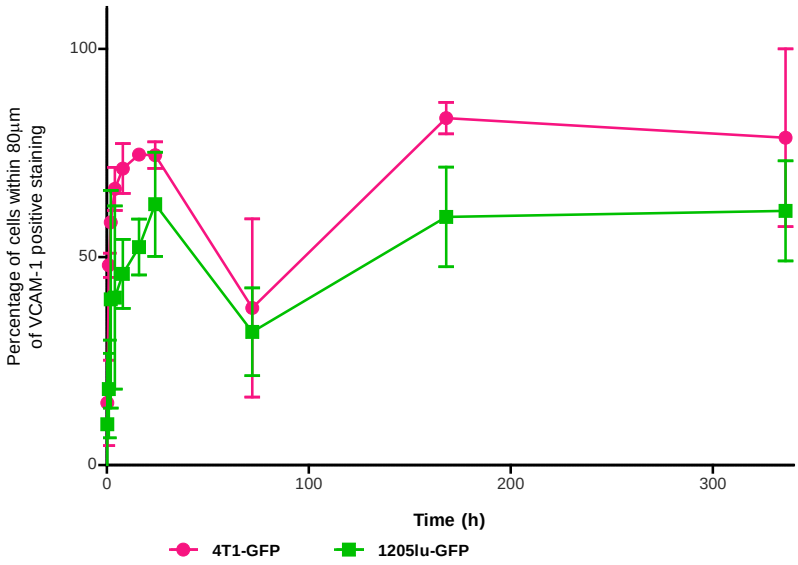


Figure 10

A)



B)

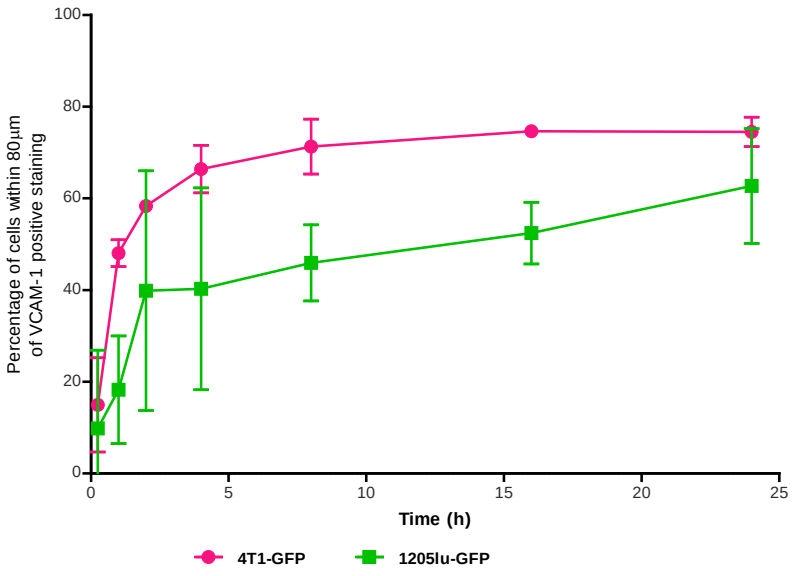


Figure 10: VCAM-1 expression is induced in response to pulmonary metastasis within 24h
A) A summary of the quantification data shown previously with additional time-points added in the 24h frame for VCAM-1 (n=4). The horizontal scale is interrupted between 30-70h. Statistical test – One way ANOVA with Tukey's Multi-comparison post-test (4T1-GFP: $p<0.0001$; 1205LU-GFP $p<0.0001$) B) Magnification of the initial 24h and illustrates the earliest endothelial activation in further detail. (n=4) VCAM-1 induction was quantified by assessing each individual tumour cell and whether it is located within 80µm of a VCAM-1 positive signal.

Figure 11

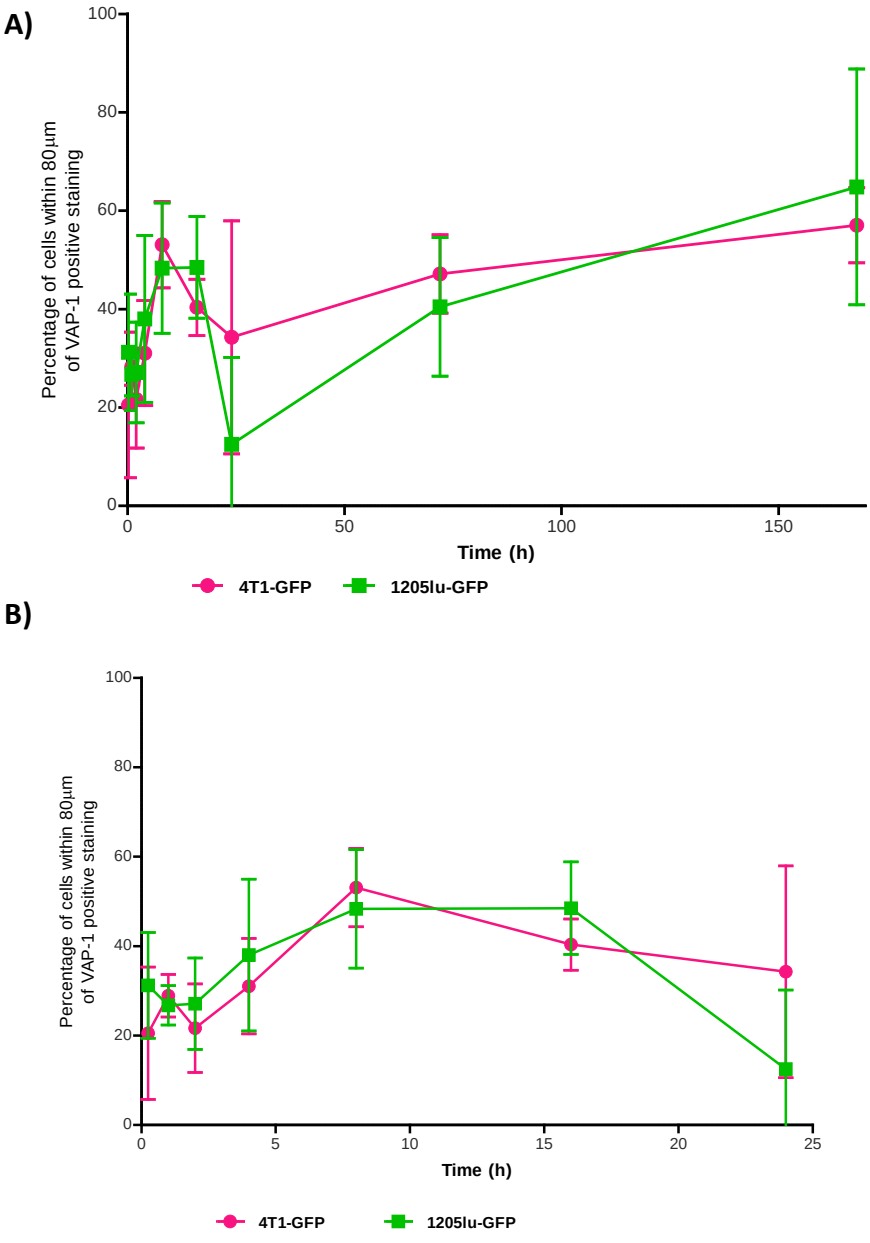


Figure 11: VAP-1 expression in response to pulmonary metastasis within 24h

A) A summary of the quantification data shown previously with additional time-points added in the 24h frame for VAP-1 (n=3). The horizontal scale is interrupted between 30-70h. Statistical test – One way ANOVA ANOVA with Tukey's Multi-comparison post-test(4T1-GFP: p=0.0017; 1205LU-GFP p= 0.0670) B) Magnification of the initial 24h and illustrates the earliest endothelial activation in further detail. (n=3) VAP-1 induction was quantified by assessing each individual tumour cell and whether it is located within 80µm of a VAP-1 positive signal.

CHAPTER 2: The mechanism of endothelial activation induction – role of platelets and the immune system

The importance of platelets and inflammatory mediators such as macrophages, monocytes or dendritic cells in metastasis has been recognised for some time.[8,125] Previous work in our laboratory (Gil-Bernabe *et al*, unpublished) identified a crucial role for the expression of the extracellular domain of tissue factor on the tumour cell in clot formation, recruitment of monocyte/macrophage population and tumour cell survival. This was shown to be essential in initiating activation of platelets and formation of a platelet clot surrounding the tumour cells. Accordingly, disruption of clot formation, by pharmacological or by genetic manipulation, dramatically reduced tumour cell association with monocytes/macrophages and subsequently, survival of tumour cells in pulmonary vasculature (Gil-Bernabe *et al*, unpublished).

In order to assay the role of the immune system on the formation of tumour cell-platelet-leukocyte complex and the survival of metastatic tumour cells, the use of two transgenic mouse models was reported (Gil-Bernabe *et al*, unpublished). In the first model, a C57BL/6 background, the fractalkine receptor (CX_3CR1) is interrupted by GFP. The fractalkine receptor is a G-protein coupled receptor (GPCR) expressed on monocytes/macrophages, brain microglia and has also been reported to be expressed on subsets of NK cells dendritic cells.[475] It has been shown to function both in adhesion as well as in chemoattraction of migrating leukocytes.[663] The insertion of GFP allowed not only the assessment of the role of the receptor in tumour cell survival and immune cell recruitment, but also facilitated imaging of the CX_3CR1 population *ex vivo.* These experiments relied on concomitant injections of labelled tumour cells (B16F10) and previously isolated and labelled platelets. Following lung isolation, this was imaged as a whole organ using confocal microscopy. Examining the three components (tumour cells, platelets and monocytes) over the 24h post injection demonstrated a recruitment of CX_3CR1-GFP cells to the tumour cell-platelet complex that peaked at 8h post tumour

cell injection. This was shown to be independent of the CX_3CR1 receptor by use of homozygous animals, which thereby lacked the functional receptor. On the other hand efficient platelet activation and clot formation was essential (Gil-Bernabe *et al*, unpublished).

This was confirmed using a second strain, which was on an FVB background and where the syngeneic Met-1 tumour cell line was used. The gene employed as reporter was the Colony stimulating factor 1 receptor (CSF1R), also known as CD115 or macrophage colony-stimulating factor receptor (M-CSFR), tagged with GFP and imaged *ex vivo* as described above (Gil-Bernabe *et al*, unpublished). CSF1R is a tyrosine kinase receptor that has shown to be essential for macrophage differentiation.[673] It is expressed on multipotent haematopoietic cells, phagocyte progenitor cells, monocytes, tissue macrophages, B-cells.[674] Moreover its deregulation has been observed in many tumours.[673] Subsequently the CD11b receptor was shown to be crucial in mediating the recruitment of leukocytes to the tumour cell-platelet complex (Gil-Bernabe *et al*, unpublished). Injection of tumour cells in mice deficient in CD11b resulted in a reduction in tumour cell survival in metastatic assays. Together, this demonstrated the importance of the tumour cell – platelet – $CD11b^+$ leukocyte complex formation in promoting tumour cell metastasis (Gil-Bernabe *et al*, unpublished).

Interestingly the time line for this phenomenon was similar to that observed in endothelial activation reported here (Fig. 10B). Additionally, platelets and inflammatory leukocytes have long been implicated in mediating metastasis.[8,236] Therefore the role of these components in the induction of endothelial activation was investigated.

1. Formation of tumour cell – platelet – leukocyte complex and its interaction with the endothelium

In previous work the formation of a platelet clot around the tumour cell was demonstrated. Firstly we wanted to confirm the recruitment of endogenous platelets to the tumour cells in the lung (Figure 12). Based on the work performed previously (Gil-Bernabe *et al*, unpublished), the time-point used was 8h after tumour cell injection. Not only was the recruitment of leukocytes shown to be most extensive at this time, endothelial activation studies demonstrated that both VCAM-1 and VAP-1 expression by the endothelial cell layer peaks at 8h (Fig.10&11).

In contrast to the tissue harvested when staining for endothelial activation antigens, immune cell markers were found to be much more sensitive to paraformaldehyde cross-linking and a prolonged fixation lead to a significant decrease in the staining efficiency (Supplementary figure 2). Therefore the tissue collected for this part of the project, was processed using perfusion fixation.

When lungs of CX_3CR1-GFP and CSF1R-GFP mice were harvested 8h after injection of tumour cells, extensive platelet clot formation was observed around the tumour cells. Furthermore this is accompanied by widespread recruitment of leukocytes (Fig.12A). (These experiments were executed in collaboration with Dr. Gil-Bernabe, where Dr. Gil-Bernabe performed cell preparation and lung harvest.)

Following this we wanted to confirm that platelet clot formation also occurs in a wt animal (Fig. 12B). In addition to the B16F10-CMFDA cell line, platelet clot formation was also assessed in the cell lines used previously: 4T1-GFP and 1205LU-GFP (Fig.12B). Interestingly, the 1205LU-GFP cell line did not appear to induce extensive platelet aggregation. This might be due to the lack of tissue factor expression (Supplementary figure 16). In order to confirm that the antibody used to recognise human tissue factor was efficient, a human cell line (A7) transfected with tissue factor was used (Gil-Bernabe

et al, unpublished).[675] The same protein extracts as used previously was utilised for the other cell lines. We can see that the human tissue factor antibody efficiently recognizes the protein and the lack of signal in the 1205LU-GFP cell line is in fact due to the absence of tissue factor protein expression. In contrast both B16F10-CMFDA and 4T1-GFP expressed tissue factor protein (Supplementary figure 16) and stimulated platelet clot formation (Fig. 12B and Supplementary figure 17).

Subsequently, we inspected the extent of endothelial activation in the CX_3CR1-GFP and CSF1R-GFP transgenic experimental models (Figure 13). In line with previous observations VCAM-1 expression was induced in response to both B16F10 and Met-1 metastasis to the lung tissue.

Figure 12

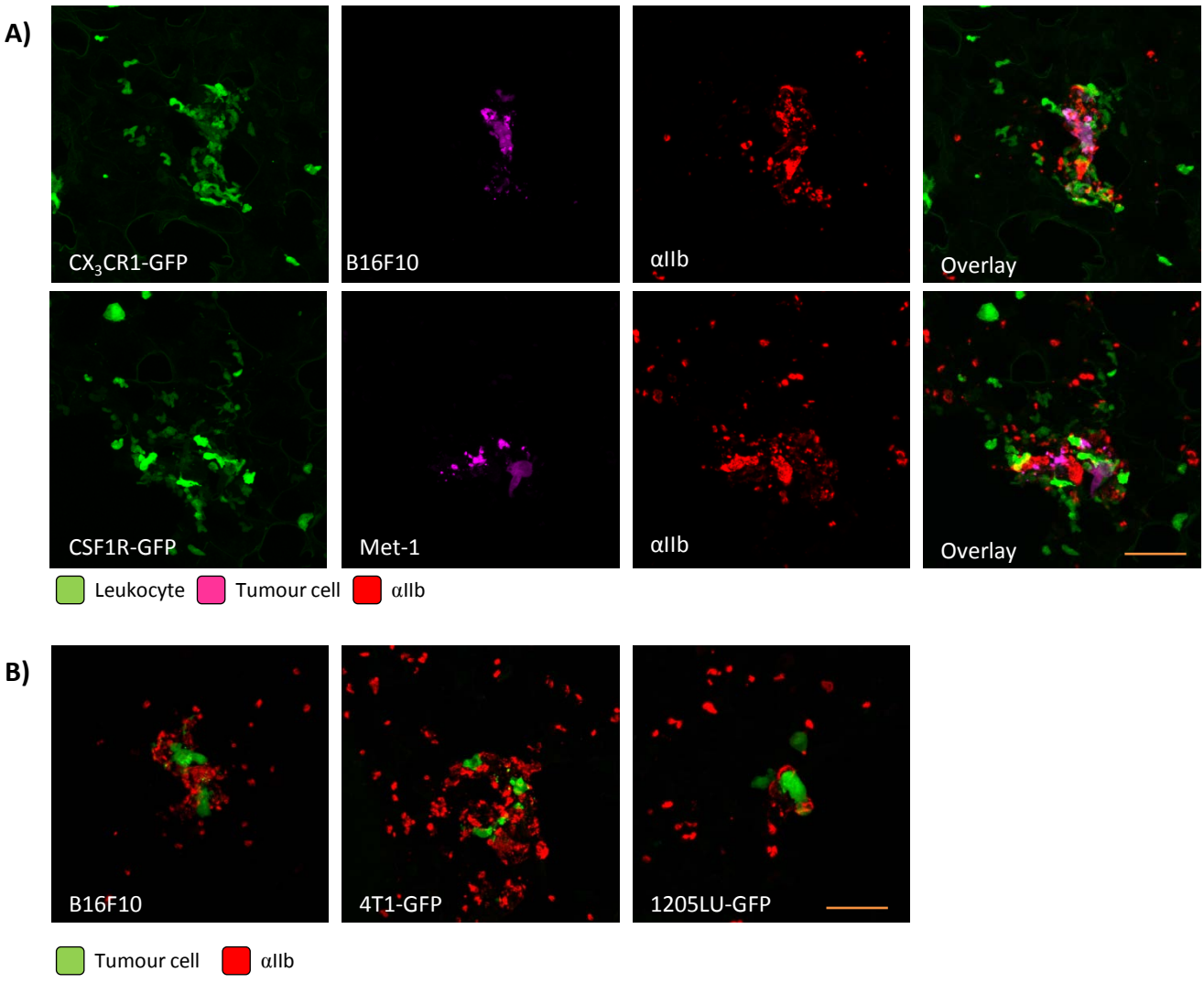


Figure 12: Tumour cell interaction with platelets and the immune system

A) CX_3CR1-GFP and CSF1R-GFP mice were injected i.v. with syngeneic B16F10 and Met-1 cells, respectively. These were labelled with CMRA *in vitro* prior to injection and the lungs were harvested at 8h. IHC staining for αIIb demonstrates platelet clot formation (Alexa Fluor 633-red) around the tumour cell (CMRA-magenta) in both models and concomitant recruitment of leukocytes (green). Scale=30µm B) Pulmonary metastasis was induced by i.v. injection of tumour cells (green) in non-transgenic animals. 4T1- GFP and 1205LU-GFP cells were injected into BALB/c and SCID mice respectively. B16F10 cells were labelled with CMFDA *in vitro* prior to injection. The lungs were harvested 8h later and this panel shows representative images of IHC staining for αIIb (Alexa Fluor 633 -red) of all three different tumour cell lines (green). Scale=30µm

Figure 13

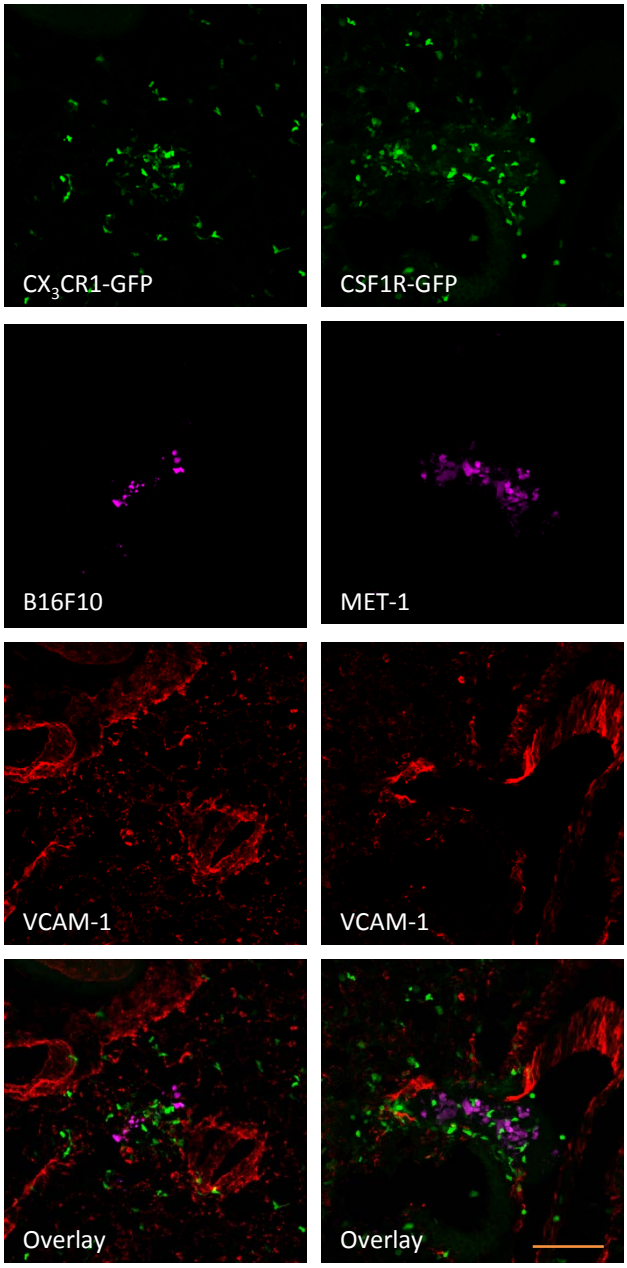


Figure 13: VCAM-1 expression is also induced in transgenic animal models
Two transgenic mouse models CX_3CR1-GFP and CSF1R-GFP were injected i.v. with B16F10 and Met-1 cells (5x10e5), respectively. These were labelled with CMRA *in vitro* prior to injection and the lungs were harvested at 8h. IHC staining for endothelial activation antigen VCAM-1 demonstrates its expression (Alexa Fluor 633-red) localizes to the tumour cell (CMRA -magenta) in both models and concomitant recruitment of inflammatory mediators (GFP-green). Scale=100µm

The CX_3CR1-GFP and CSFR1-GFP expressing cell recruited to the tumour cell could reflect a variety of myeloid cell types.[663,676] In order to characterise the cells that are being recruited to the tumour cell, the tissue sections of the two experimental models, 8h after tumour cell challenge, were stained for the different cell membrane markers: CD11b, CD11c, F4/80, CD68, CD45, Gr-1 and CD3ε (Figures 14, 15 &16). The leukocyte cell populations that express each of the proteins labelled are listed in the table below.

Marker	Description
CD11b	Also called Integrin αM and is expressed by monocytes, macrophages, granulocytes and natural killer cells
CD11c	CD11c is expressed at highest level by dendritic cells, but also mocytes/macropages and neutrophils
CD45	Is the pan-leukocyte marker, also called the common leukocyte antigen.
F4/80	F4/80 is specifically expressed by monocytes/acrophages
CD68	CD68 is specifically expressed by monocytes/macrophages
Gr-1	It is also called Ly6G and is expressed by neutrophils.
CD3ε	Forms part of the CD3 complex specific to T-cells.

Unsurprisingly, in both models the GFP-expressing cells stained positively for the pan-leukocyte marker CD45; 86.73% and 93.12% of CX_3CR1-GFP cells and CSF1R-GFP cells, respectively. Similarly, both stained highly positive for CD11b; 85.57% and 71.39% of CX_3CR1-GFP and CSF1R-GFP cells, respectively. This was expected in light of previous data where CD11b had been demonstrated to be crucial for the recruitment of the inflammatory mediators to the tumour cell. Interestingly, the cells recruited do not show high positivity for CD11c (21.28% and 24.66% by CX_3CR1-GFP and CSF1R-GFP respectively), suggesting that the majority of these were not dendritic cells.

Additionally, the expression of Gr-1 (9.24%) and CD3ε (3.25%), by the GFP-expressing cells recruited to the tumour cell, is minimal in the CX_3CR1-GFP model, suggesting that the recruited cells are neither neutrophils nor T-cells. As expected, the two experimental models differ slightly in the expression of investigated markers. CSF1R-GFP labels a wider population of leukocytes; therefore there are a few differences in the expression profile; a higher population of cells express Gr-1 (30.42%) and CD3ε

(11.65%).

On the other hand, most GFP^+ cells also expressed F4/80 and CD68 is in both models, reaching 84.40% and 78% in CX_3CR1-GFP experimental metastasis respectively and 66.94% and 70.23% in the CSF1R-GFP model respectively. Such expression profiles define the recruited leukocyte population as monocytes/macrophages.

Figure 14: Characterisation of recruited inflammatory mediators in CX_3CR1-GFP model
Transgenic mouse model CX_3CR1-GFP (green) was injected i.v. with B16F10 cells (magenta). These were labelled with CMRA *in vitro* prior to injection and the lungs were harvested at 8h. The tissue was then stained for different cell markers - CD11b, CD11c, F4/80, CD68, CD45, Gr-1 and CD3ε (red). The tissue was imaged with Zeiss 710 Confocal microscope and representative images are displayed. (n=1) Scale=100µm

Figure 15: Characterisation of recruited inflammatory mediators in CSF1R-GFP model
Transgenic mouse model CSFR1-GFP (green) was injected i.v. with Met-1 cells (magenta). These were labelled with CMRA *in vitro* prior to injection and the lungs were harvested at 8h. The tissue was then stained for different cell markers - CD11b, CD11c, F4/80, CD68, CD45, Gr-1 and CD3ε (red). The tissue was imaged with Zeiss 710 Confocal microscope and representative images are displayed. (n=1) Scale=100µm

Figure 14

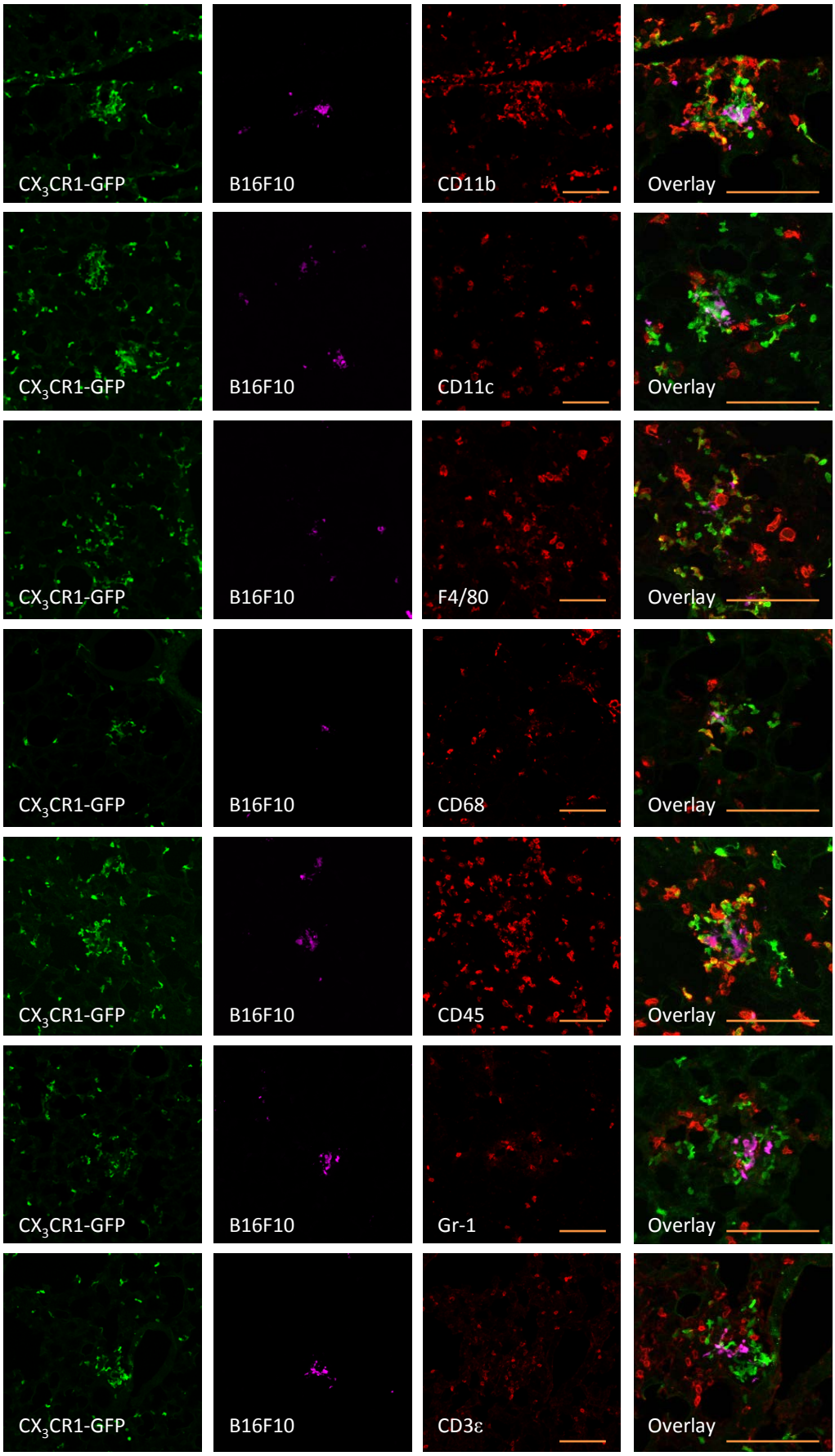

CX3CR1-GFP
B16F10
CD11b
Overlay
CX3CR1-GFP
B16F10
CD11c
Overlay
CX3CR1-GFP
B16F10
F4/80
Overlay
CX3CR1-GFP
B16F10
CD68
Overlay
CX3CR1-GFP
B16F10
CD45
Overlay
CX3CR1-GFP
B16F10
Gr-1
Overlay
CX3CR1-GFP
B16F10
CD3ε
Overlay

Figure 15

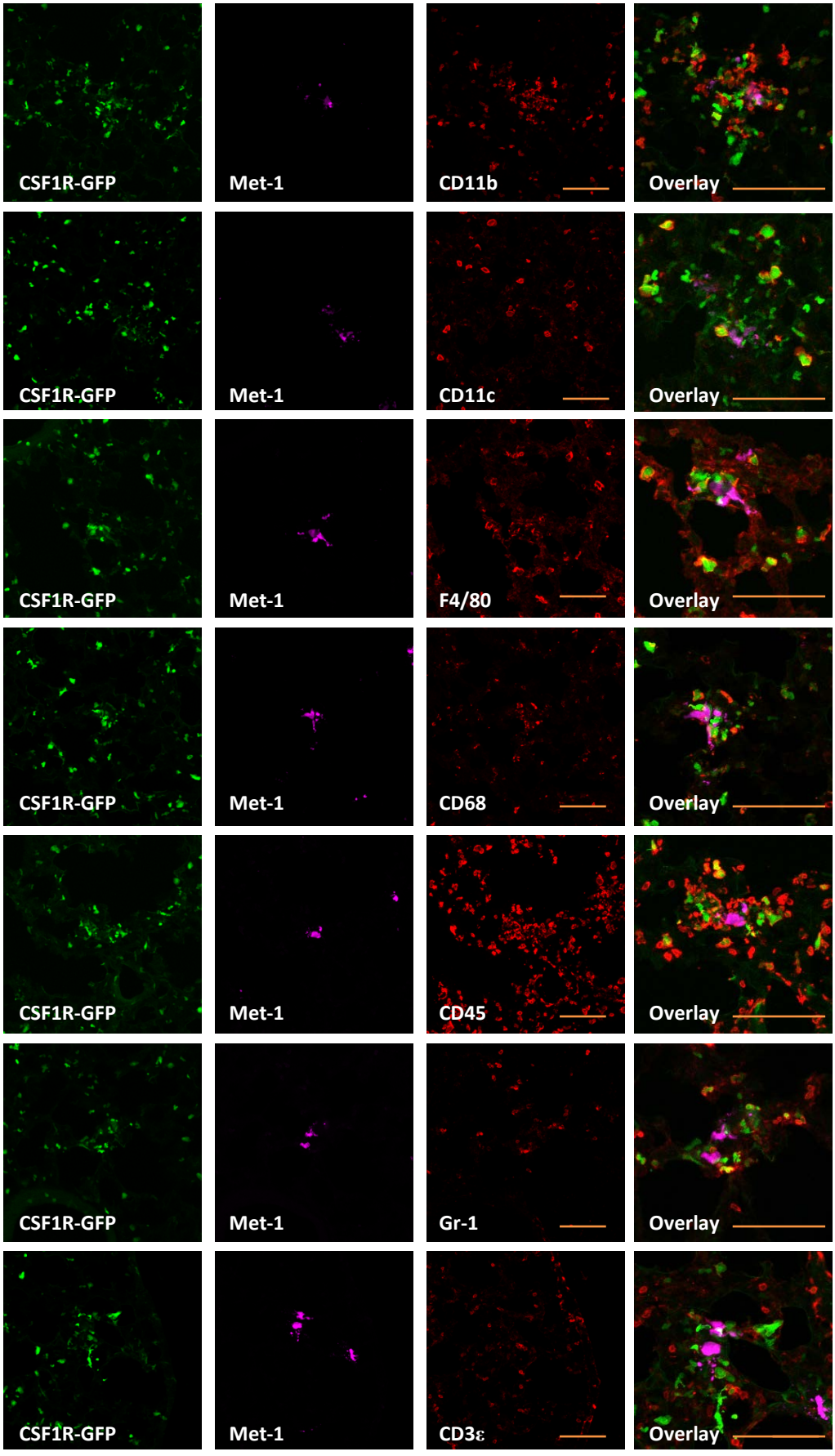
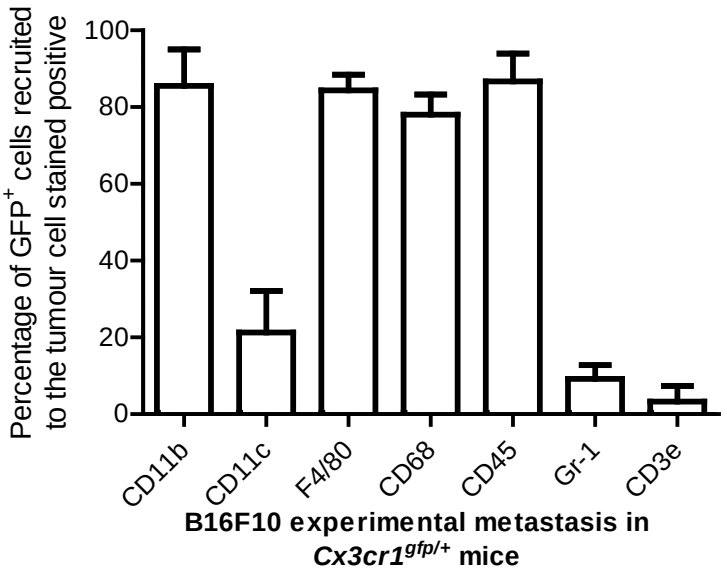

CSF1R-GFP
Met-1
CD11b
Overlay
CSF1R-GFP
Met-1
CD11c
Overlay
CSF1R-GFP
Met-1
F4/80
Overlay
CSF1R-GFP
Met-1
CD68
Overlay
CSF1R-GFP
Met-1
CD45
Overlay
CSF1R-GFP
Met-1
Gr-1
Overlay
CSF1R-GFP
Met-1
CD3ε
Overlay

Figure 16



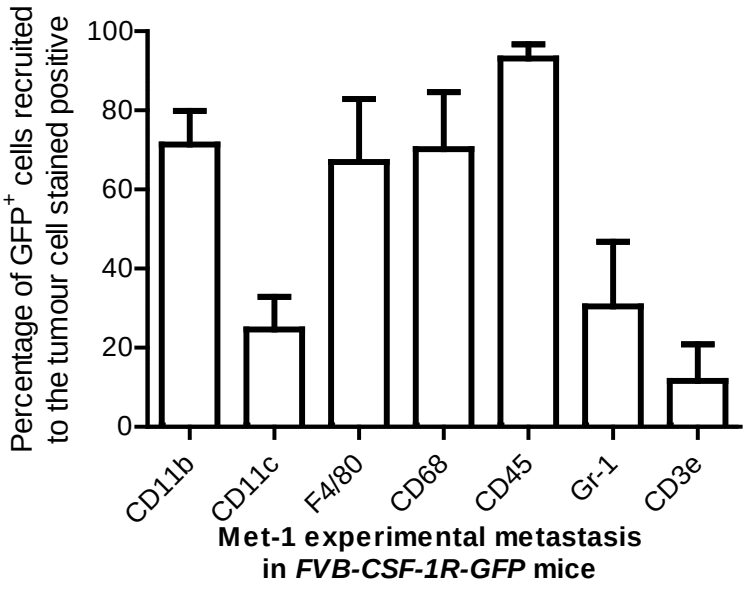


Figure 16: Characterisation of the recruited leukocytes in the transgenic models of metastasis
This figure demonstrates the quantification of the immunohistochemical staining presented.
In images presented previously, where genetic insertion of GFP (CX_3CR1 and CSF1R) facilitated visualisation of recruited myeloid population, the tissue was evaluated for different cell markers (CD11b, CD11c, F4/80, CD68, CD45, Gr-1 and CD3ε).
Quantification was achieved by assessing each individual GFP-expressing leukocyte cell recruited to the tumour cell for the expression of the particular protein. The results are expressed as the percentage of all GFP-expressing leukocytes recruited/analysed that were stained positively for the different markers.

In the next step, we investigated whether the same populations of leukocytes were recruited to the experimental metastasis models in wt mice; B16F10-CMFDA cells in C57BL/6 mice, 4T1-GFP cells in BALB/c mice and 1205LU-GFP cells in SCID mice. The tissues were harvested 8h after i.v. tumour cell injection and stained for the various cell surface markers (Figure 17). Quantification was performed by counting individual tumour cells and determining whether they display recruitment of leukocytes expressing the specific protein according to the criteria described in material and methods. The majority of 4T1-GFP and B16F10-CMFDA cells show recruitment of leukocytes (CD45 expression). In particular, 85.02% of 4T1-GFP cells and 75.00% of B16F10-CMFDA cells were found to have clustered with CD45-expressing cells. In contrast, only a small proportion (8.33%) of cells in the 1205LU-GFP model displayed any recruitment of $CD45^+$ cells. This again underlines previous observation that platelet recruitment by the tumour cell is essential to mediate recruitment of immune cells. In a similar way CD11b expressing cells were found to be recruited to 88.44% and 76.66% of 4T1-GFP and B16F10-CMFDA cells respectively. Again only a small percentage (8.67%) of 1205LU-GFP cells recruited $CD11b^+$ cells.

In line with the data from the transgenic models of metastasis, leukocytes recruited by both 4T1-GFP and B16F10-CMFDA were shown to express F4/80 (85.71% and 61.5% respectively) and CD68 (69.20% and 63.15% respectively). It is worth noting that the IHC staining for F4/80 and CD68 are far less efficient than CD11b and CD45. Therefore the percentages of cells that stain positive might be higher. Once more 1205LU-GFP cells did not appear to recruit F4/80 (16.67%) or CD68 (20.00%) expressing cells to the same degree as 4T1-GFP and B16F10-CMFDA cell lines.

None of the cell lines showed significant recruitment of CD11c expressing cells. Equally, neither B16F10-CMFDA nor 1205LU-GFP cells exhibited considerable recruitment of $Gr\text{-}1^+$ cells (10.50% and 0.00% respectively). In contrast, recruitment of Gr-1 positive cells was observed in the 4T1-GFP metastatic cell model (69.36%). This is supported by previous observations, where it has been

reported that 4T1-GFP cells elicit recruitment of Gr-1^{+}CD11b^{+} cells. These are termed myeloid derived suppressor cells (MDSCs)[677,678] The last cell membrane protein investigated is the T-cell marker CD3ε. As expected, the staining in 1205LU-GFP model is clear as SCID mice are deficient in T cells. Again, as observed previously, neither 4T1-GFP nor B16F10-CMFDA recruited a substantial number of CD3e expressing cells (19.79% and 0.00% respectively).

Taken together, these data confirmed that tumour cell initiation of platelet activation and clot formation is necessary to facilitate recruitment of leukocytes as described previously (Gil-Bernabe *et al*, unpublished). Additionally, these leukocytes appear to express CD45, CD11b, F4/80 and CD68. This suggests they are monocytes/macrophages but not T-cells, neutrophils, dendritic cells and in the 4T1-GFP cell model also MDSCs.

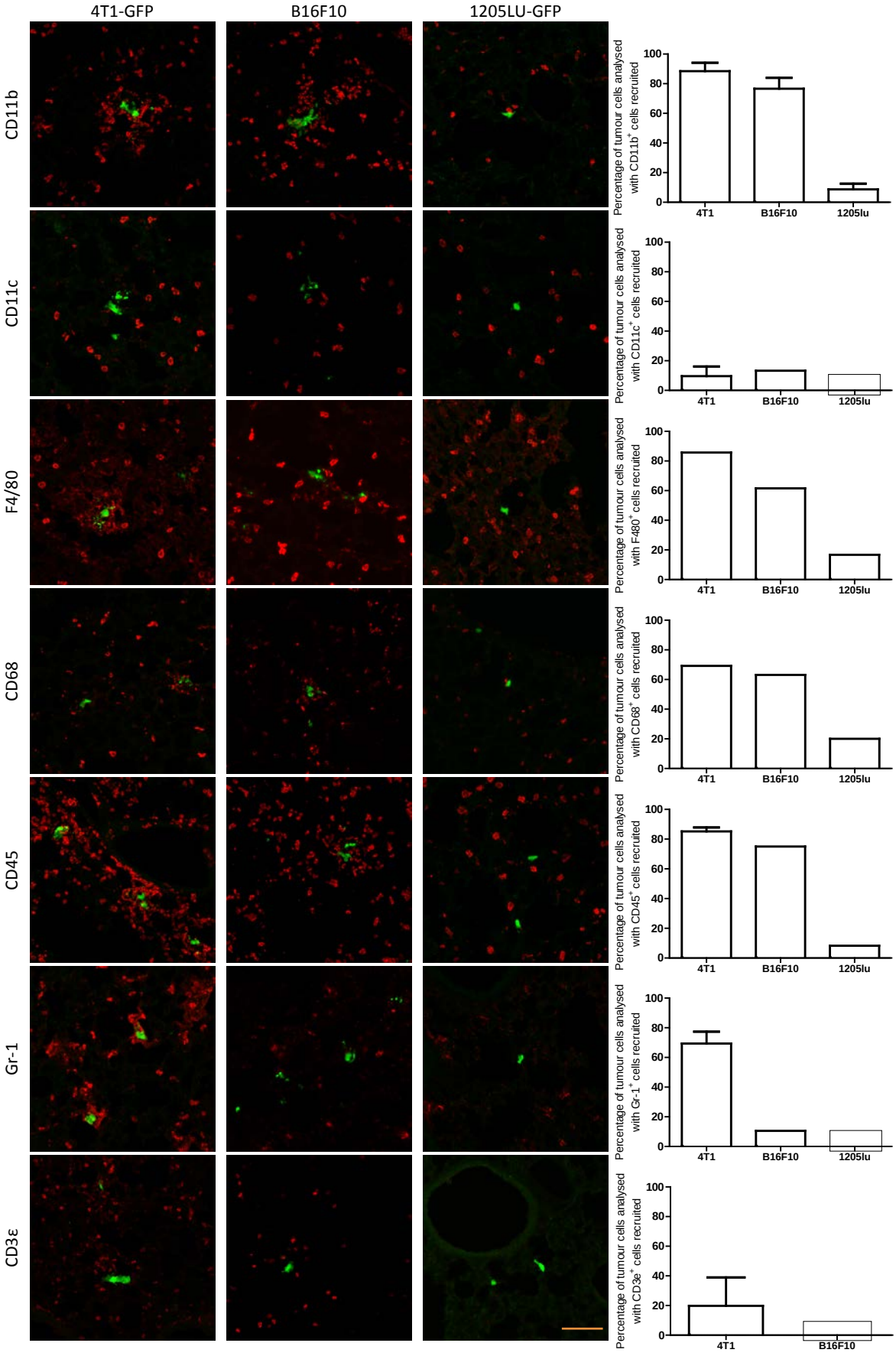
Next the role of the components of the tumour cell – platelet – monocyte complex in inducing endothelial activation was investigated.

Figure 17: Leukocyte recruitment in pulmonary metastasis

Recruitment of leukocyte population was characterised in non-transgenic animal models. Pulmonary metastasis was induced by i.v. injection of tumour cells (green). 4T1- GFP and 1205LU-GFP cells were injected into BALB/c and SCID mice respectively. B16F10 cells were labelled with CMFDA *in vitro* prior to injection and injected in C57BL/6 mice. The lungs were harvested 8h later and this panels shows representative images for different cell markers CD11b, CD11c, F4/80, CD68, CD45, Gr-1 and CD3ε (Alexa Fluor 633 -red).

The panels on the right-hand size display the percentage of tumour cells analysed that had recruited leukocytes expressing the particular cell marker, where at least 15 cells were analysed for each. (n=3 where SD is displayed, otherwise n=1) The tissue was imaged with a Zeiss Confocal microscope. Scale=100μm

Figure 17


4T1-GFP
B16F10
1205LU-GFP
CD11b
CD11c
F4/80
CD68
CD45
Gr-1
CD3ε
Percentage of tumour cells analysed with CD11b+ cells recruited
Percentage of tumour cells analysed with CD11c+ cells recruited
Percentage of tumour cells analysed with F480+ cells recruited
Percentage of tumour cells analysed with CD68+ cells recruited
Percentage of tumour cells analysed with CD45+ cells recruited
Percentage of tumour cells analysed with Gr-1+ cells recruited
Percentage of tumour cells analysed with CD3e+ cells recruited
0
20
40
60
80
100
4T1
B16F10
1205lu

2. Disruption of monocyte recruitment has no effect on endothelial activation induction

Cells of the monocyte/macrophage lineage become activated in response to inflammatory stimuli.[679,680] In fact different types of activation profiles have been characterised, including a classical (release of NO facilitate destruction of the pathogen in inflammatory loci) and an alternative phenotype (they induce proliferation and collagen production). Consequently these are able to produce an array of different cytokines and so regulate and orchestrate the progression of the inflammatory response of the host. Such cytokines would act to modulate other components of the immune system and also act on the recognised pathogen directly. Thereby it seems quite likely that tumour cell metastasis, consequent platelet activation and recruitment of a monocyte subset, would result in endothelial activation.

Previous work by our laboratory has demonstrated that Mac-1 KO leads to significant decrease in the number of B16F10 cells surviving in the lung in comparison to that seen in the wt mouse (Gil-Bernabe *et al*, unpublished). Subsequent analysis demonstrated that this was due to the essential role of CD11b in mediating monocyte recruitment and thereby aiding the survival of metastasizing tumour cells.

The hypothesis that the recruitment of monocyte/macrophage population and their production of a variety of cytokines also induced endothelial activation was investigated next. If so, interruption of monocyte/macrophage recruitment to the tumour cell should result in a decreased endothelial activation.

In order to disrupt monocyte recruitment to the tumour cell, we used a genetically modified strain of animals where Mac-1 (CD11b/CD18) expression has been knocked out. These were generated on a C57BL/6 background. In this experiment CMFDA labelled B16F10 cells were injected i.v. into either Mac-1 KO or wt animals and lungs were harvested after 8h. (These experiments were executed in

collaboration with Dr. Gil-Bernabe, where Dr. Gil-Bernabe performed cell preparation and lung harvest.)

Inhibition of macrophages through CD11b deletion did not affect platelet clot formation (Figure 18A). As the mouse model does not express CD11b (Supplementary figure 18), we used CD45 staining to evaluate leukocyte recruitment (Figure 18B). A decrease in the number of leukocytes recruited to the tumour cell was observed in the Mac-1 KO model compared to the wt. Following this, VCAM-1 expression was assessed as in previous experiments (Figure 18 C). Interestingly, there was no difference in VCAM-1 expression between the two strains of animals ($p=0.4869$).This suggests that endothelial activation does not occur as a consequence of monocyte recruitment. This disproves our hypothesis that monocyte interaction with the endothelial cells would result in their activation.

Figure 18

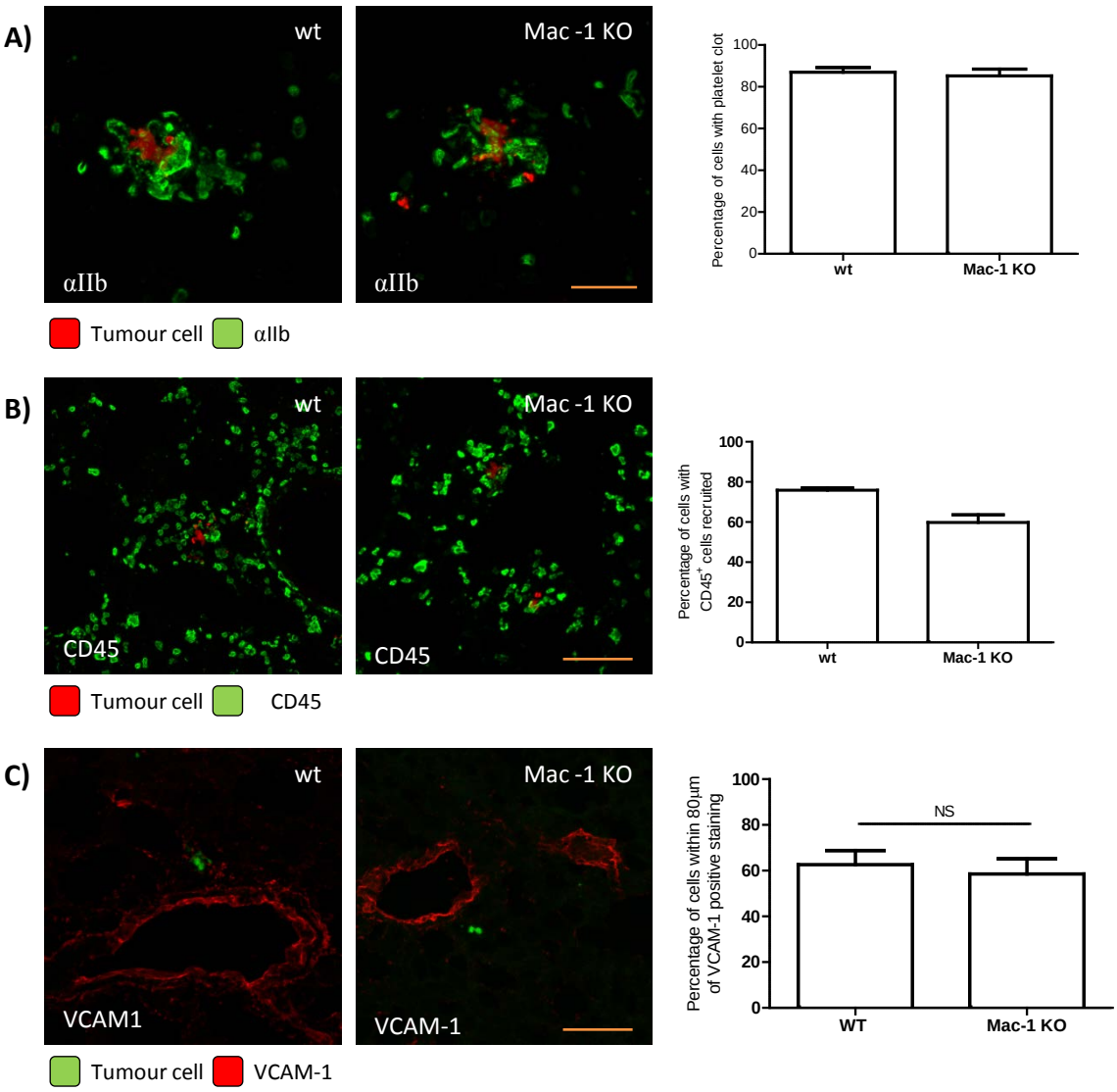


Figure 18: Mac-1 KO has no effect on endothelial activation by pulmonary metastasis at 8h

Pulmonary metastasis was induced by i.v. injection of *in vitro* labelled B16F10 cells into either wt C57BL/6 mice or Mac-1 KO mice. After 8h the lung was harvested and analysed for the different markers.

A) No effect is observed on platelet clot formation in Mac-1 KO animal. Representative images of IHC, where B16F10 (CMRA-red) and platelets: αIIb (Alexa Fluor 488-green) demonstrates platelet clot formation is not affected in Mac-1 KO animals. (n=2) Scale=30µm The quantification was performed as previously described in material and methods.

B) Representative images IHC , where B16F10 (CMRA-red) and CD45 (Alexa Fluor 488 -green) demonstrate a reduction in the amount of recruited leukocytes. The quantification was performed as previously described in material and methods. (n=2) Scale=100µm C) Representative images of IHC staining where B16F10 (CMFDA -green) and VCAM-1 (Alexa Fluor 633-red) demonstrate no difference in VCAM-1 expression. Scale=100µm VCAM-1 expression was scored with regards to the percentage of tumour cells localised to the VCAM-1 positive blood vessels. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next all these were averaged for 3 animals per group to give the overall mean presented. Statistical analysis: unpaired t-test; p=0.487.

3. Disruption of platelet clot formation inhibits endothelial activation

Activation of platelets does not only result in their aggregation and clot formation, but also induces the release of platelet granules that contain a wide variety of inflammatory cytokines.[236,681] We next postulated that it could be the activation of platelets, caused by the tumour cells, that is responsible for inducing activation of the endothelial cell layer.

3.1. Inhibition of thrombin

In the first experiment clot formation was disrupted by anticoagulant treatment. The animals were injected with a thrombin inhibitor, a recombinant version of hirudin: Lepirudin (Refludan). It differs from hirudin in one amino acid substitution (leucine for isoleucine) and one sulphate group on Tyrosine 63 and exhibits higher substrate specificity *in vivo*.[682,683] It acts by binding to thrombin in a 1:1 ratio and thereby inhibits its activity in a dose dependent manner. As its half-life is about 2.5h, the drug was injected 5min prior to tumour cell injection and then again 4h later via the i.p. route. (Both the dosage and the time of injection have previously been used by Dr. Gil-Bernabe.) As described previously, the lungs were harvested 8h after tumour cell injection, followed by tissue processing and imaging.

In the first step we confirmed the efficacy of the treatment by assessing clot formation in response to tumour cell metastasis (Figure 19 A, B &C). We observed that in the 4T1-GFP metastatic model the percentage of tumour cells associated with a platelet clot decreased significantly, from 90.22% to 20.00% ($p<0.0001$). Interestingly, even in the 1205LU-GFP metastatic model, where platelet clot formation is only induced by 15.5% of tumour cells, this was decreased by hirudin treatment to 1.58% of cells (Fig. 19B). In addition, this data is supported by assessment of the size of the clot associated with an individual tumour cell (Fig.19C).

Whether the disruption of platelet aggregation also leads to disruption of inflammatory cell recruitment was investigated next, as previously reported in a different model (B16F10) (Gil-Bernabe *et al*, unpublished). First the recruitment of CD11b-expressing cells was analysed. As expected, there was a marked decrease in the number of $CD11b^+$ cells recruited to the tumour cell (Fig.19D, E&F). This is best demonstrated when looking at the percentage of tumour cells that were associated with a cluster of $CD11b^+$cells. Therefore, in the 4T1-GFP metastatic model, the percentage of cells with leukocyte recruitment defined by $CD11b^+$ cell staining decreased from 88.44% in untreated animals to 23.49% when treated with hirudin ($p<0.0001$). This effect on 4T1-GFP cells was further confirmed by analysing the pan-leukocyte marker CD45 (Fig. 19 G&H). In this instance, treatment with hirudin reduced association from 85.20% to 36.50% of tumour cells.

In contrast, the recruitment of $CD11b^+$ leukocytes by 1205LU-GFP cells was minimal (8.67%) and the effect of hirudin treatment (to 3.50%) was insignificant. (Fig. 19E) Moreover when assessing the number of recruited $CD11b^+$ cells to each individual tumour cell, only a small decrease was observed (Fig. 19F). This was further confirmed by analysis of $CD45^+$ cell recruitment (Supplementary Figure 19).

In the initial characterisation of inflammatory mediators recruited to the tumour cells (Figure 17), 4T1-GFP cells were unique in their recruitment of Gr-1 expressing cells. Therefore we investigated whether this was also dependent on platelet activation and thereby influenced by hirudin treatment (Fig 19 G&H). We observed a significant decrease after treatment, where only 11.31% of cells were associated with $Gr\text{-}1^+$ after anti-coagulation in comparison to 69.36% observed in the non-treated control.

Figure 19

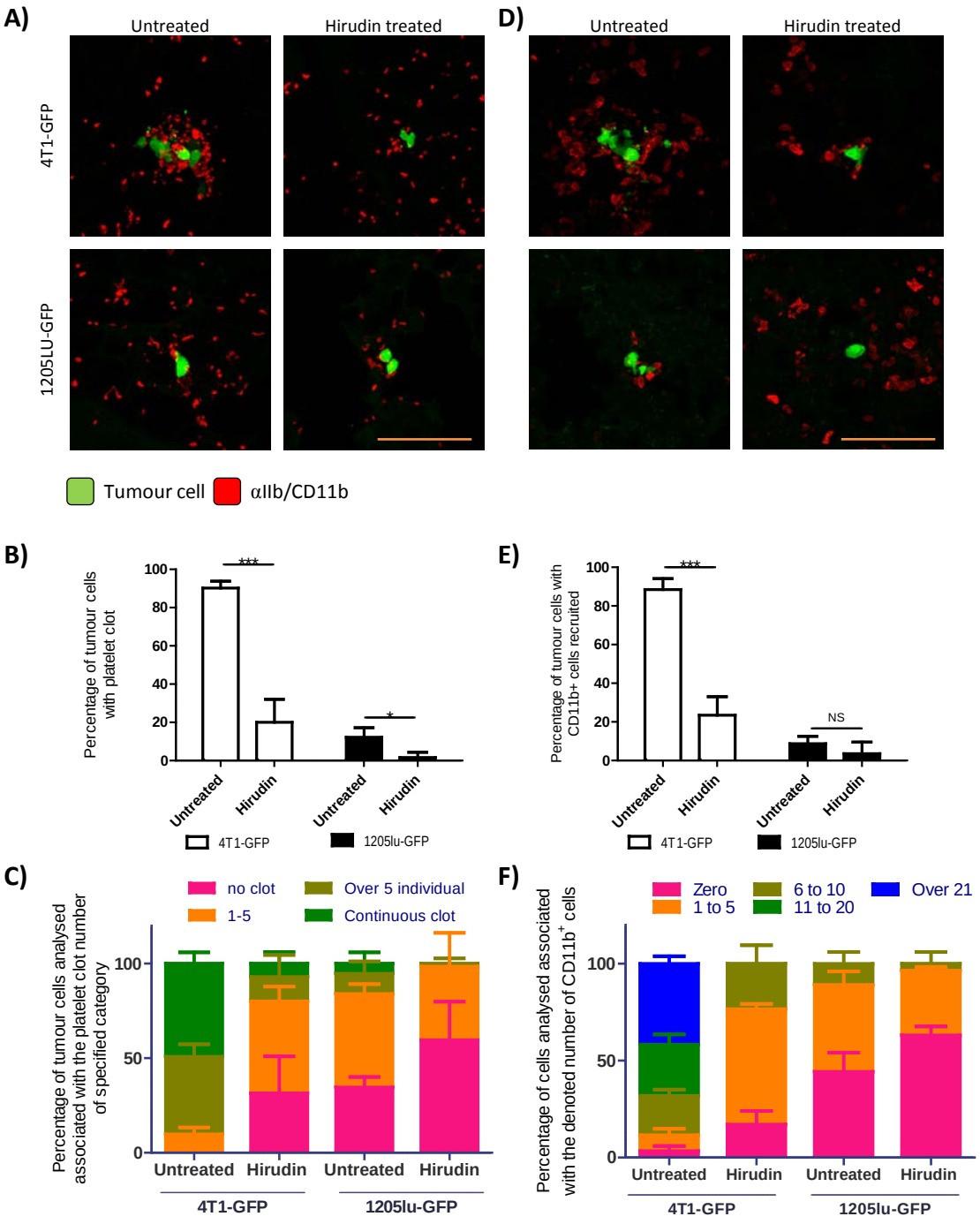


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Figure 19 continued

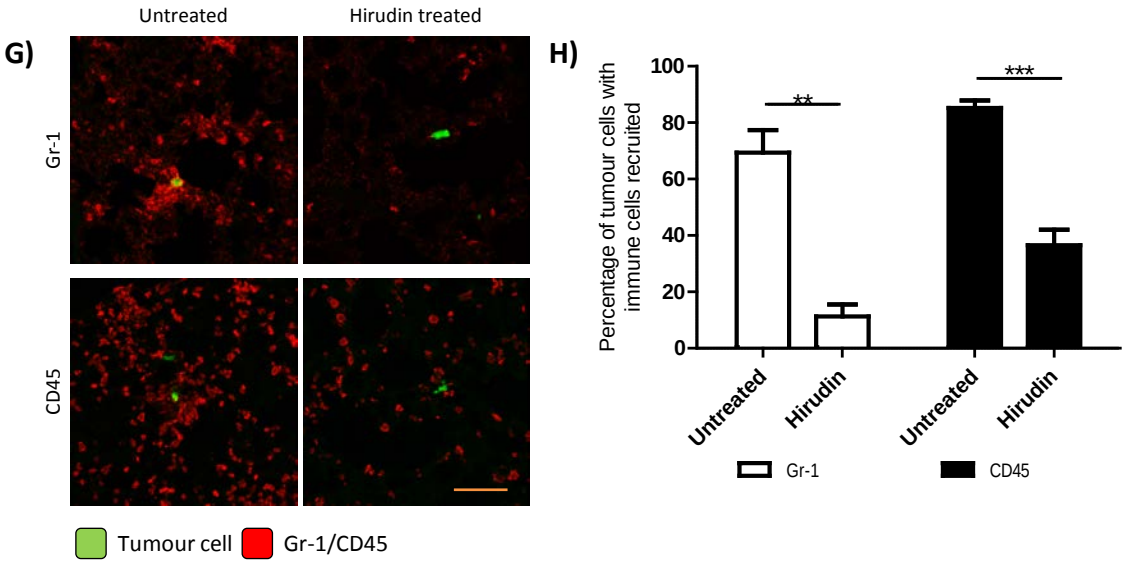


Figure 19: Disruption of platelet clot formation by hirudin reduced recruitment of monocytes

Treatment of mice with hirudin (at 20 mg per kg of body weight; 5min prior to tumour cell injection and 4h after) was effective in disruption platelet clot formation induced by the tissue factor expressed on the tumour cell surface. The imaging was performed with a Zeiss Confocal microscope.

A) Representative images of treated and non-treated lungs stained for αIIb (Alexa Fluor 633 - red) for both metastatic models: 4T1-GFP and 1205LU-GFP tumour cells (green). Scale=100µm

B) The quantification of the association of tumour cells with platelets, where the data represents the proportion of tumour cells analysed found to be associated with a clot. (n=3) Statistical analysis: unpaired t-test (4T1-GFP-p=0.0006; 1205LU-GFP p=0.0366)

C) Analysis of the clot size associated with the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with the size of clot corresponding to one of the 4 different categories. (n=3)

Platelet clot disruption inhibited immune cell recruitment.

D) Representative images of treated and non-treated lungs stained for CD11b (Alexa Fluor 633 - red) at 8h for both metastatic models: 4T1-GFP and 1205LU-GFP tumour cells (green). Scale=100µm

E) The quantification of the tumour cell induced recruitment of CD11b expressing cells. The data represents the proportion of tumour cells analysed found to be associated with more than 5 $Cd11b^+$ cells (n=3). Statistical analysis: unpaired t-test (4T1-GFP- $p<0.0001$; 1205LU-GFP – p=0.223)

F) Analysis of the number of $Cd11b^+$ cells recruited to the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with the number of CD11b+ cells corresponding to one of the 5 different categories. (n=3)

Hirudin treatment also inhibited recruitment of CD45+ cells and Gr-1+ cells by 4T1-GFP cells (G&H). (n=3)

G) Representative images of treated and non-treated lungs stained for CD45 and Gr-1 (Alexa Fluor-red) at 8h in the 4T1-GFP metastatic model. Scale=100µm

H) The quantification of the tumour cell induced recruitment of CD45- or Gr1-expressing cells. The data represents the proportion of tumour cells analysed found to be associated them. Statistical analysis: unpaired t-test (Gr-1-p=0.0028; CD45 $p<0.0001$)

As these data confirmed the efficacy of platelet clot formation disruption, next experiment assessed the effect of hirudin treatment on endothelial activation (Fig.20). The percentage of cells localising to VCAM-1 staining was decreased to 43.78% ($p<0.0001$) from 72.73% in the non-treated control. Both the representative images of immunohistochemical staining and the quantification of the endothelial expression of VCAM-1, demonstrated a significant decrease in the 4T1-GFP model. This confirms that clot formation is, at least in part, responsible for inducing endothelial activation and VCAM-1 protein expression. This was confirmed with the lack of effect of hirudin treatment on VCAM-1 expression in the 1205LU-GFP metastatic model. In this model, we do not observe a significant association of tumour cells with leukocytes and only minimal platelet clot formation.

Figure 20

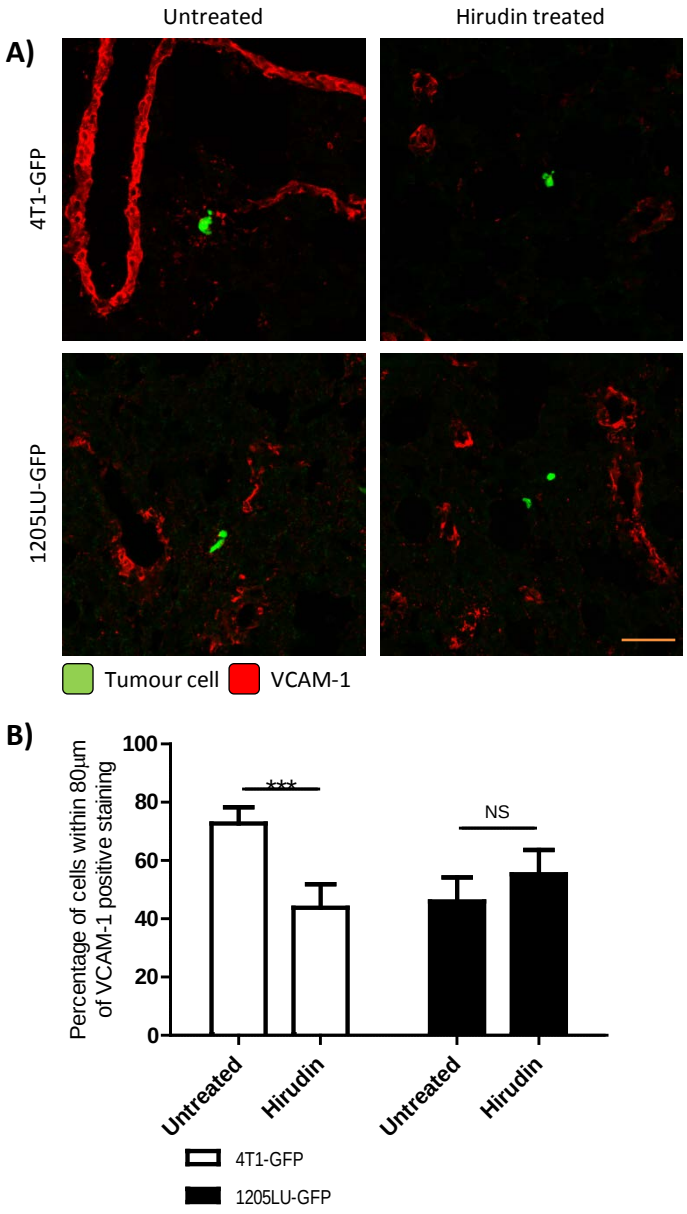


Figure 20: Disruption of platelet clot formation by hirudin reduced endothelial activation

Treatment of mice with hirudin (5min prior to tumour cell injection and 4h after) also resulted in reducing expression of VCAM-1

A) Representative images of treated and non-treated lungs stained for VCAM-1 expression (Alexa Fluor 633-red) for both metastatic models: 4T1-GFP and 1205LU-GFP tumour cells (green). Scale=100µm

B) Quantification of VCAM-1 induction was performed by assessing each individual tumour cell and whether it is located within 80µm of VCAM-1 staining. This is expressed as percentage of all tumour cell assessed that were localised to VCAM-1 expression. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next the values for all three animals were averaged to give the overall mean presented.

Statistical analysis: unpaired t-test (n=3); p values: $p<0.0001$ and 0.0831 respectively. Imaging was performed with Zeiss Confocal microscope.

3.2. Inhibition of the Tissue Factor Pathway

In a second set of experiments interrupting the coagulation cascade, platelet clot formation was inhibited through inhibition of the tissue factor pathway with TFPI (Tissue Factor Pathway Inhibitor). TFPI is a naturally occurring inhibitor, whose role is to modulate the coagulative cascade in a type of negative feedback loop.[275] This was exploited in this experiment by using B16F10-CMFDA cells stably transfected with murine TFPI. This cell line model has been established and characterised before.[265] In addition, a mock-transfected cell line (Vector) was used, where an empty plasmid is propagated by the cell (gift of Dr. Amirkhosravi).[265]

The ability of these cells to induce platelet clot formation when injected i.v. in C57BL/6 mice was confirmed (Fig. 21A, B&C). The data were acquired and analysed using the same techniques and analysis methods as in previous experiments. Previously reported decrease in clot size associated with the TFPI-expressing cells was observed here as well.[265] Additionally, the percentage of tumour cells associated with platelet clots decreased from 84.68% and 84.0% in wt and vector transfected cells respectively to 57.75% upon TFPI expression. As with hirudin treatment, the effect of TFPI expression on monocyte recruitment was investigated (Fig. 21 D, E&F). Even though the effect is more attenuated than that of hirudin, a significant inhibition of the number of $CD11b^+$ cell recruited was observed (Fig.21F). Inhibition by TFPI is less effective than that of hirudin, most likely because TFPI inhibits the activity of TF in the initiation phase of the coagulation cascade, while hirudin acts further downstream in the cascade, inhibiting the activity of thrombin (Diagram 1). In this way hirudin inhibits activation of the coagulation cascade in the propagation phase that can also be induced at low TF activity or in absence of TF activity as described previously. The percentage of tumour cells associated with a cluster of over 5 cells decreased from 76.66% to 39.13% upon TFPI expression (Fig. 21E). This was further confirmed using CD45 staining. (Supplementary figure 20)

Next, we focused on endothelial activation, i.e. VCAM-1 expression induction (Figure 22) and observed, that 75.00% of wt B16F10-CMFDA tumour cells localised to VCAM-1 positive staining, whereas only 50.48% of cells expressing TFPI did so. However this difference was not statistically significant (p=0.0521). This experimental data highlights the importance of the type of analysis used. As mentioned previously in the material and methods, the binary classification used to assess the induction of VCAM-1 expression could attenuate any difference occurring due to TFPI inhibition. As this is less efficient compared to hirudin treatment, the inhibitory effects on VCAM-1 expression could be more clearly seen through the measurement of the amount of expression. Regardless, the downward trend that was observed confirms our previous experiment, implicated platelet activation in inducing endothelial activation, as opposed to the possibility that VCAM-1 expression induction was an unrelated phenomenon.

Figure 21

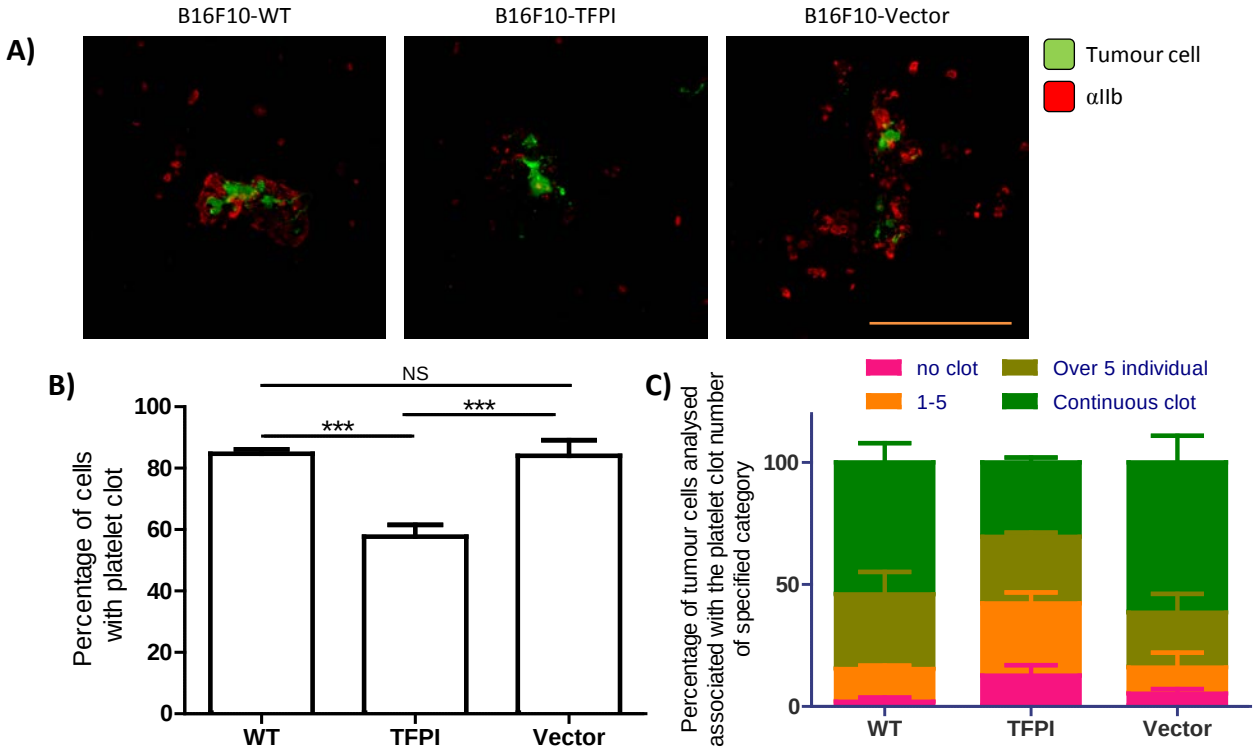


Figure 21: Disruption of platelet clot formation by TFPI

A second strategy to disrupting platelet clot formation was through expression of tissue factor pathway inhibitor protein, which acts to disrupt the Tissue Factor pathway inhibits platelet activation and aggregation. Thereby three B16F10 cells were used, a wt cell line (wt), TFPI- expressing cell line (TFPI) and a vector-transfected control (Vector). These were stained *in vitro* with CMFDA, injected i.v. in C57Bl/6 mice (n=3) and the lungs harvested 8h later. Imaging was performed with Zeiss Confocal microscope.

A) Representative images of tissue stained for αIIb (Alexa Fluor 633-red) for the three types of tumour cells (green) demonstrating a reduction in platelet clot formation. Scale=100µm

B) The quantification of the association of tumour cells with platelets, where the data represents the proportion of tumour cells analysed found to be associated with a clot. (n=3) (One-way ANOVA with Tukey's Multi-comparison test; p=0.0002)

C) Analysis of the clot size associated with the tumour cells. The data is represented as the percentage of the tumour cells analysed that are associated with the size of clot corresponding to one of the 4 different categories. (n=3)

Platelet clot disruption inhibits immune cell recruitment. (n=3)

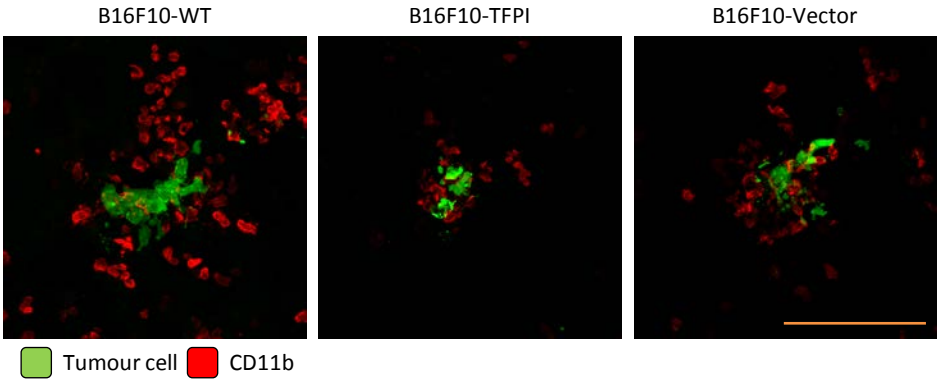
D) Representative images of tissue stained for CD11b (Alexa Fluor 633-red) at 8h in relation to the tumour cells (green) at 8h. Scale=100µm

E) The quantification of the tumour cell induced recruitment of CD11b expressing cells. The data represents the proportion of tumour cells analysed found to be associated with more than 5 $Cd11b^{+}$ cells (n=3). (One-way ANOVA with Tukey's Multi-comparison test; p=0.0181)

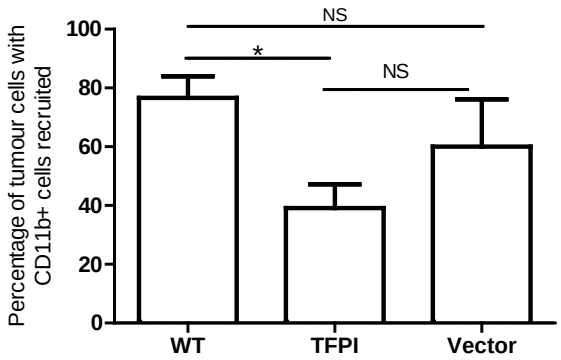
F) Analysis of the number of $Cd11b^{+}$ cells recruited to the tumour cells. The data is represented as the percentage of the tumour cells analysed that are associated with the number of CD11b+ cells corresponding to one of the 5 different categories.

Figure 21 continued

D)



E)



F)

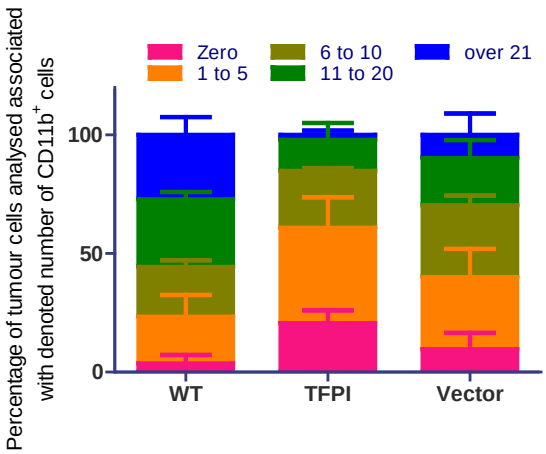


Figure 22

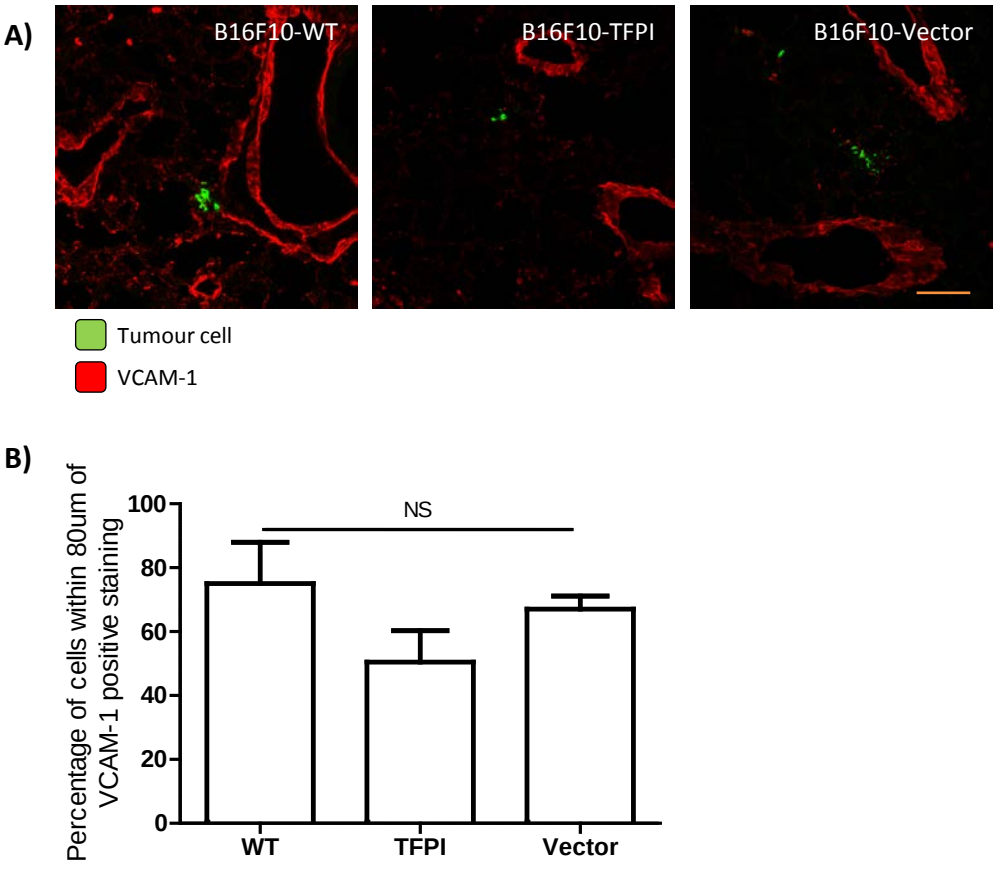


Figure 22: Effect of disruption of platelet clot formation by TFPI on endothelial activation
Expression of TFPI also reduced expression of VCAM-1(n=3) TFPI-expressing clone. The three clones of B16F10 cell line (wt, TFPI- and vector –transfected) were stained *in vitro* with CMFDA, injected i.v. in C57BL/6 mice (n=3) and the lungs harvested 8h later.
A) Representative images VCAM-1 expression (Alexa Fluor 633-red) for all three cell models(CMFDA-green). Scale=100µm
B) Quantification of VCAM-1 induction was performed by assessing each individual tumour cell and whether it is located within 80µm of VCAM-1 staining. This is expressed as percentage of all tumour cell assessed that were localised to VCAM-1 expression. For each data point first the average was calculated for each animal, where the cells were analysed in at least three non-sequential sections. Next the values for all three animals were averaged to give the overall mean presented. (Statistical test: One-way ANOVA; p= 0.0521)

CHAPTER 3: The function of endothelial activation in the process of metastasis

In the next part of our work, we focused on what role the endothelial activation antigens, which become expressed in response to tumour cells, perform during metastatic dissemination. Although platelets recruited by the tumour cells were shown to be at least in part responsible for inducing endothelial activation, this could just be due to the general inflammation of the host.

What seemed more likely however was that analogous to their role in inflammatory processes, endothelial adhesion proteins (VCAM-1 and VAP-1) would aid in the recruitment and attachment of activated leukocytes. Moreover, as previous work in our laboratory had shown, the monocyte/macrophage population that is recruited by the tumour cell promote its survival and aid the process of metastasis (Gil-Bernabe *et al*, unpublished). If that is the case inhibition of VCAM-1 and VAP-1 should result not only in inhibition of monocyte recruitment, but also inhibition of metastatic efficiency.

1. Inhibition of VCAM-1

VCAM-1 expression in the bone-marrow has been shown to play a role in mediating the attachment and retention of immature neutrophils in the bone-marrow. In these experiments blocking the function of VCAM-1, using a blocking antibody, resulted in the release of immature neutrophils into the peripheral blood.[684] This observation provided a useful readout for the efficacy of the blocking properties of commercially obtained antibody.

1.1. VCAM-1 blocking antibody

In this experiment, VCAM-1 blocking antibody (or the appropriate non-targeting isotype control) was injected i.v. at 1.5µg per g of body weight. The peripheral blood was harvested 4h later as previously reported.[684] The samples were stained for Gr-1 and analysed by FACS. A marked increase of the Gr-1^{+}

population in peripheral blood can be observed in response to VCAM-1 antibody injection (Figure 23). We analysed the cells of the myeloid cell population, as defined by forward and side scatter (Supplementary figure 21). Two populations of Gr-1^{+} myeloid cells were observed with medium and high levels, respectively (Gr-1^{high} and Gr-1^{medium}). In response to VCAM-1 antibody injection, the major increase occurred in the Gr-1^{medium} range (from 5.97% to 11.46% of the population analysed). Although a small increase was observed in the Gr-1^{high} expressing population as well (5.13% to 7.13%). When compared to the effectiveness of VCAM-1 blocking effects observed by Petty *et al,* this was shown to be less effective. While we observed an increase in neutrophil number of about 60%, they report an almost 130% increase. This may be due to a less effective VCAM-1 blocking antibody or it could be accounted for by a different analysis method. While we used FACS analysis and relied on Gr-1 marker staining, they use a hematology analyzer and thereby a larger population of cell could be analysed.

Regardless, this confirmed the effectiveness of the VCAM-1 blocking as well as the dosage and timing of the injection. Next, we proceeded with assessing the effect such inhibition of VCAM-1 had on metastatic spread.

Figure 23

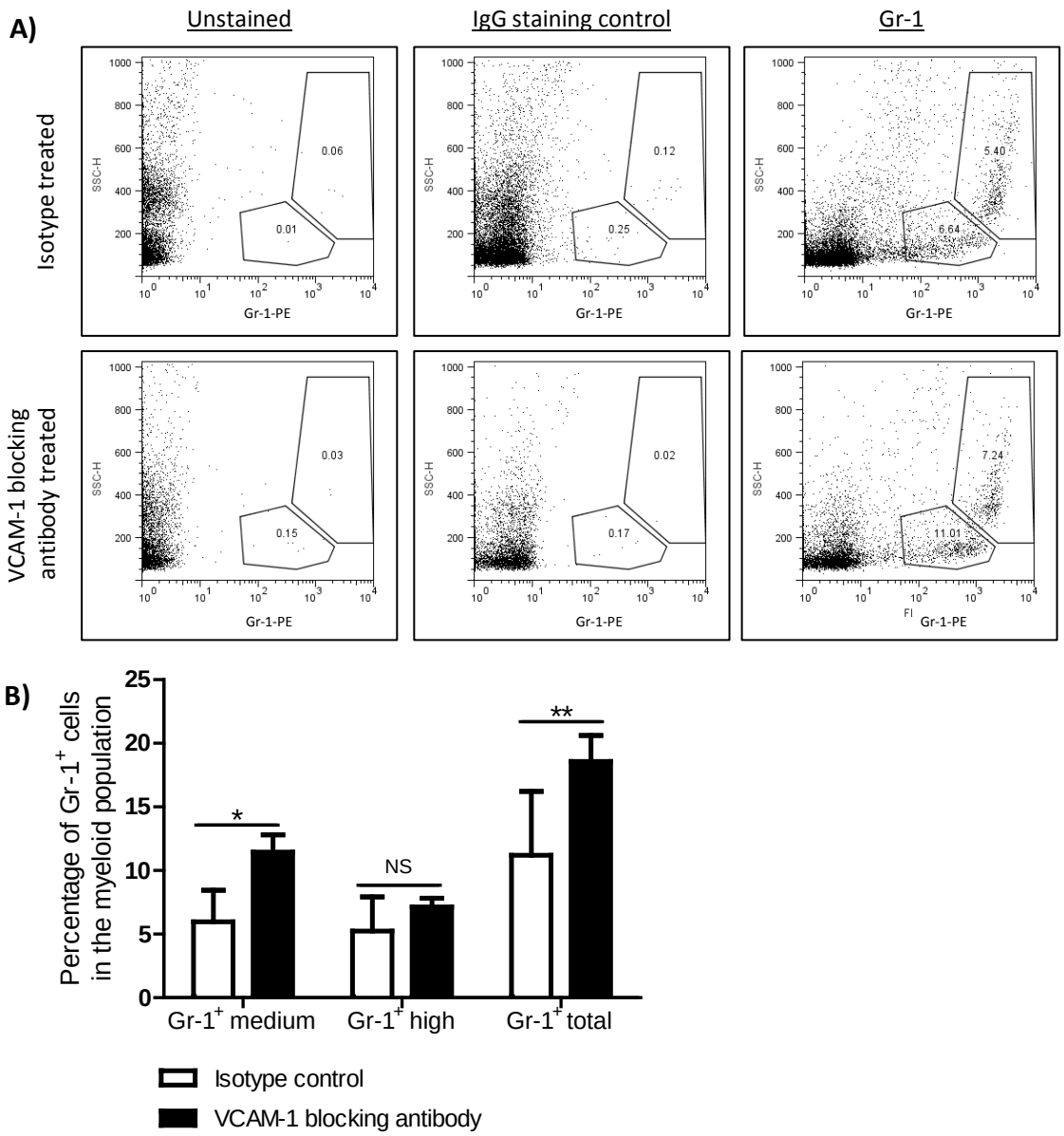


Figure 23: Validation of VCAM-1 blocking antibody in vivo

VCAM-1 blocking antibody or the corresponding non-targeting isotype control antibody (1.5µg per g of body weight) were injected i.v. in C57Bl/6 mice. After 4h peripheral blood was collected and the amount of circulating neutrophils was assessed by expression of Gr-1 in FACS analysis.

A) Representative FACS scatter plots of leukocytes within the myeloid gate for both the treated and control samples for three staining protocols: unstained sample, IgG isotype stained sample and Gr-1 stained sample.

B) Quantification graph of Gr-1 - expressing population in the peripheral blood sample of mice (n=5) treated either with Isotype control antibody or VCAM-1 blocking antibody. (One-way ANOVA with Tukey's Multiple Comparison post-test; $p<0.0001$)

1.2. Effect of VCAM-1 inhibition on monocyte recruitment and clot formation

In this experiment we focused on two experimental models of metastasis, 4T1-GFP breast mammary carcinoma cells and B16F10-CMFDA melanoma cells. First, we looked at the effect of VCAM-1 blocking by antibody on the host response 8h after tumour cell injection (Figure 24; n=4). No effect of the IgG isotype antibody, relative to an untreated control, was observed when the recruitment of $CD11b^+$ cells was analysed (Figure 24G). A significant decrease in the percentage of 4T1-GFP tumour cells that were associated with $CD11b^+$ cells (Fig.24A) was observed. This decreased from 88.44% in control lungs to 68.16% in treated lungs (p=0.0077). Concomitantly, the average number of recruited CD11b-expressing cells (Fig 24B) decreased when VCAM-1 blocking antibody was injected. In contrast, the decrease observed in the B16F10-CMFDA cell model (from 73.34% to 63.65%) was not significant (p= 0.177), although a trend in the decrease in the number of $CD11b^+$ cells recruited can be observed (Fig.24B). However if we compare the overall number of CD11b-expressing cells recruited by the B16F10-CMFDA cells, we can observed a large reduction in the number of tumour cells associated with more than 21 $CD11b^+$ cells. This suggests that it may be that VCAM-1 only affects the secondary wave of myeloid cell recruited. Hence VCAM-1 blocking antibody effectively reduces the overall number of CD11b-expressing cells recruited to the tumour cell. As B16F10-CMFDA tumour cell tend to recruit a lower number of myeloid cells overall, this is most apparent in this model. Representative images of $CD11b^+$ cell recruitment are shown in Figure 24 C and D.

The decrease in immune response exhibited by 4T1-GFP cells was further confirmed by CD45 staining and analysis (Fig. 24E&F). This was shown to decrease from 85.20% of tumour cells associated with $CD45^+$ cells in the control, to 53.84% in the treated animals (p<0.0001).

As shown previously, 4T1-GFP cells also exhibit a strong myeloid response. Surprisingly however, the decrease in Gr-1^+ cell association (from 69.36% in the control to 44.46%) was not significant (p=0.0588). This may also be due to the high level of variation that is observed in Gr-1^+ cell

recruitment in the treated tissue.

Next we assessed whether VCAM-1 inhibition treatment would have any effect on platelet clot formation by the tumour cells (Figure 25). Even though we observed a downward trend, where in the 4T1-GFP model VCAM-1 inhibition resulted in a decrease from 90.22% to 82.89% of cells associated with clots, the decrease was not significant in either model (Fig 25) and no significant difference was observed in the size of the clot (Supplementary Figure 22). Representative images of clot formation upon VCAM-1 blocking are shown in Figure 25B.

Figure 24

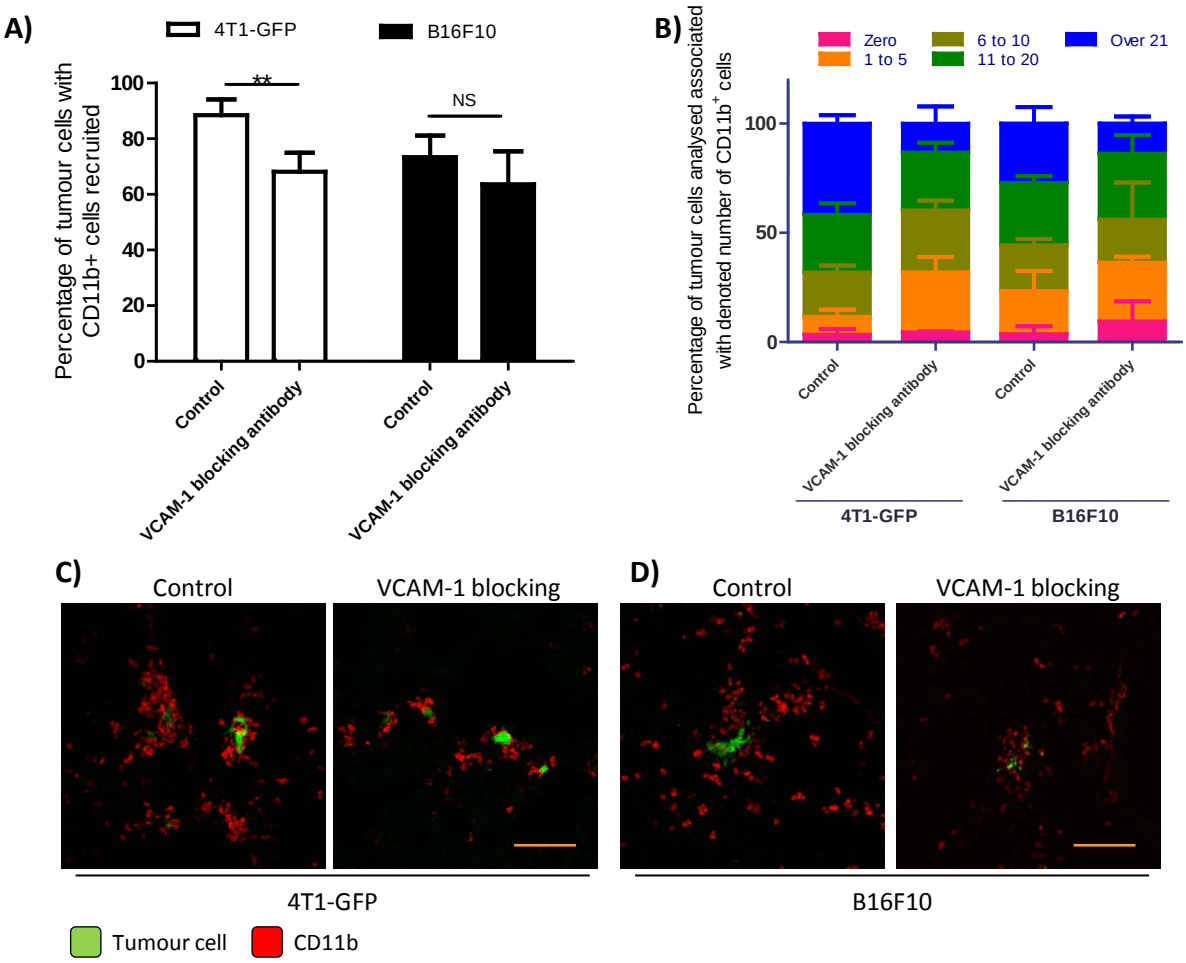


Figure 24: VCAM-1 blocking antibody inhibits inflammatory cell recruitment to the site of metastasis
VCAM-1 blocking antibody was injected i.v. 4h prior to the injection of tumour cells. Two experimental metastasis models were used, 4T1-GFP and CMFDA labelled B16F10 cells. After 8h, the lungs were harvested and analysed by fluorescent immunohistochemistry. Imaging was performed with Zeiss Confocal microscope. A) The quantification of the tumour cell induced recruitment of CD11b expressing cells. The data represents the proportion of tumour cells analysed found to be associated with more than 5 $Cd11b^{+}$ cells for each experimental group (n=4). Statistical analysis: unpaired t-test (4T1-GFP-p=0.0077; B16F10-p=0.1556) B) Analysis of the number of $Cd11b^{+}$ cells recruited to the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with the number of $CD11b^{+}$ cells corresponding to one of the 5 different categories. (n=4) C&D) Representative images of treated and control lungs stained for CD11b (Alexa Fluor 633-red) at 8h for both metastatic models: 4T1-GFP and CMFDA-labelled B16F10 tumour cells (green). Scale=100µm E&F) 4T1-GFP cells were also examined for recruitment of $CD45^{+}$ cells and $Gr\text{-}1^{+}$ cells (n=4) The quantification was performed as described previously (E), where the percentage of tumour cell analysed associated with the leukocyte cluster is presented. Representative images of treated and control lungs stained for CD45 and Gr-1(Alexa Fluor 633-red) are shown in F. Scale=100µm Statistical analysis: unpaired t-test (Gr-1-0.0588; CD45-p=0.0001) G) Treatment with IgG non-targeting antibody had no effect on recruitment of $CD11b^{+}$ cells as compared to non-treated lungs : Graph demonstrates the percentages of B16F10 tumour cell pulmonary metastases analysed found to be associated with more than 5 $Cd11b^{+}$ cells for each experimental group The statistical test performed was unpaired t-test (n=3; p=0.3949) Representative images show were obtained with Zeiss Confocal microscope. Scale=100µm

Figure 24 continued

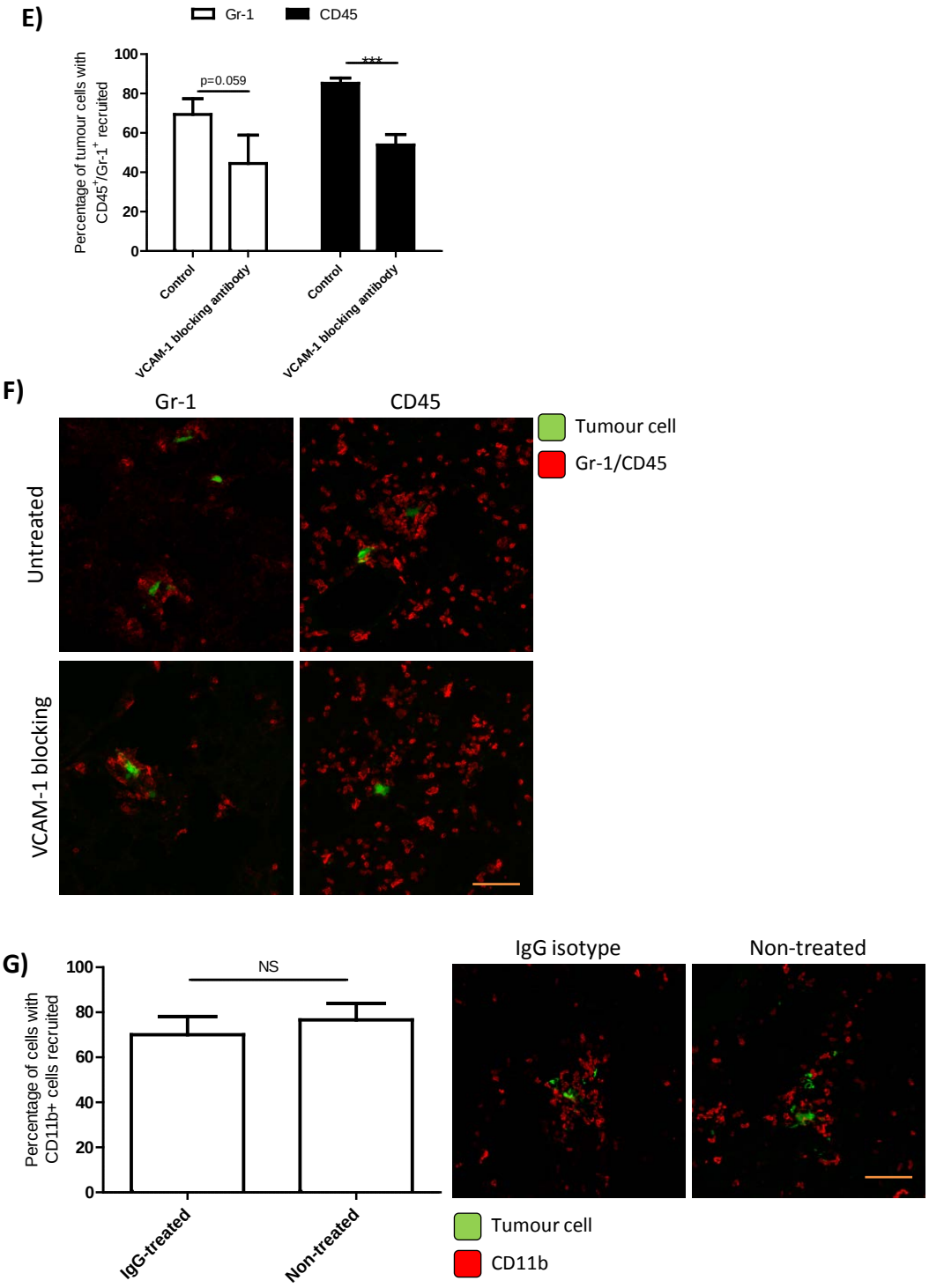
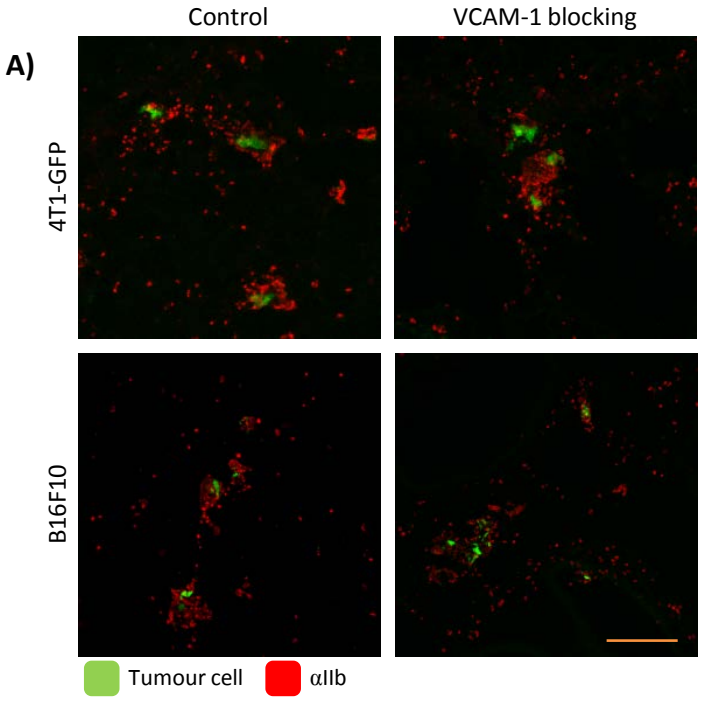


Figure 25



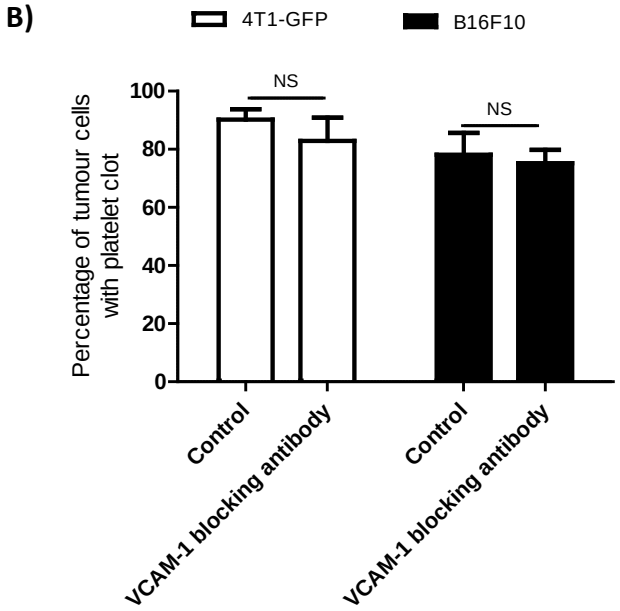


Figure 25: VCAM-1 blocking antibody does not affect platelet activation induction by the tumour cell

VCAM-1 blocking antibody was injected i.v. 4h prior to the injection of tumour cells. After 8h, the lungs were harvested and analysed by fluorescent immunohistochemistry. Two experimental metastasis models were used, 4T1-GFP and CMFDA-labelled B16F10 cells and imaging was performed with Zeiss Confocal microscope.

A) Representative images of treated and control lungs stained for αIIb(Alexa Fluor 633-red) in relation to the tumour cells (green) at 8h. Scale=100μm

B) The quantification of the tumour cell induction of platelet clot formation. The data represents the proportion of tumour cells analysed found to be associated with a platelet clot for treated and control mice (n=4). Statistical analysis: unpaired t-test (4T1-GFP-p=0.2191; B16F10-p=0.5679)

1.3. Effect of VCAM-1 blocking antibody on metastasis

In the next experiment, we assessed whether inhibition of VCAM-1 had any effect on the retention and survival of metastatic tumour cells in the lung 24h after i.v. injection. The experiments are presented in Figure 26A, where the effect of VCAM-1 blocking antibody is represented as the percentage of tumour cells retained in the treated lungs compared to the control lung (100%). The particular experiments for each cell line are shown in Fig 26 B and C, for 4T1-GFP and B16F10-CMFDA respectively. Injection of IgG control antibody has no effect on the metastatic cell survival *in vivo* (Supplementary Figure 23). A significant reduction in tumour cell survival in the lung was only observed in 4T1-GFP cells (the surviving fraction was reduced to 53.24%; n=7). This was also the experimental metastatic model that showed a significant reduction in monocyte recruitment after VCAM-1 inhibition (Figure 24). A representative experiment (Fig 26B) demonstrates that inhibition of VCAM-1 reduces the average number of surviving tumour cells from 136 to 74 per lung (n=5; p=0.0077). In contrast in the B16F10-CMFDA metastatic model VCAM-1 inhibition did not have a significant effect (n=3; p= 0.1767) on tumour cell survival (Fig. 26C).

Figure 26

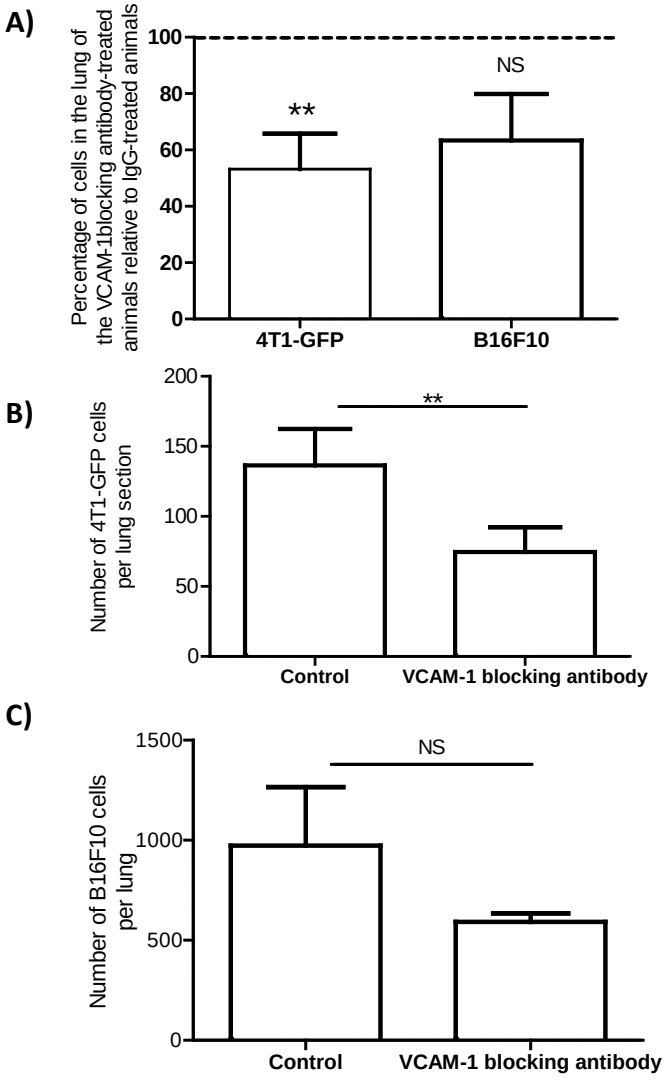


Figure 26: Effect of VCAM-1 blocking antibody on experimental tumour metastasis

Tumour cells (5x10e5) were injected i.v. 4h after the antibody was injected (1.5µg per g of body weight). Metastatic efficiency measure as tumour cell survival in the lung was measured 24h after injection. This was achieved either by whole lung imaging and counting the number of fluorescently labelled tumour cell on the surface of the lungs (B16F10 cells) or in the case of 4T1-GFP cells, the lungs were harvested as for IHC staining, sectioned and cells were counted in at least 10 sections to accurately gauge the surviving cell fraction.

A) The surviving cell fraction is represented as the percentage of cells in the treated lungs, where the 100% value is noted by the corresponding control lung (4T1-GFP n=7; B16F10 n=3). Each time the experiment was repeated twice.

B) Graph showing a representative experiment studying the effect of VCAM-1 blocking antibody on the survival of 4T1-GFP cells in the lung (n=4) (statistical analysis: unpaired t-test, p=0.0077)

C) Graph showing a representative experiment studying the effect of VCAM-1 blocking antibody on the survival of B16F10 cells in the lung (n=3) (statistical analysis: unpaired t-test, p=0.1767)

2. Inhibition of VAP-1

The VAP-1 is not a classical adhesion molecule, but rather a receptor with enzymatic activity. It has been shown to be involved in mediating leukocyte migration to the inflammatory sites and its enzymatic function was shown to be central to this process. Previous experiments have separated the inhibition of VAP-1 function into two components by either inhibiting its enzymatic function with small molecule inhibitors or sterically hindering the binding to surface epitopes (but not the enzymatic function) by use of blocking antibodies. However, blocking antibodies did not prove very effective, suggesting that the enzymatic activity is central to VAP-1 adhesion. Furthermore, enzymatic VAP-1 inhibition has been shown to reduce trafficking of leukocytes into the primary tumour and thereby inhibit its growth.[408,529]

Therefore, in our experiments we focused on inhibition of its enzymatic activity. Inhibition of VAP-1 protein was achieved by use of the following inhibitor: PRX177636C BA3166002 (Proximagen).

2.1. Effect of VAP-1 inhibition on monocyte recruitment and clot formation

As in previous experiments we first looked at the effect on leukocyte recruitment at 8h (Fig.27) and this was assessed in the three tumour cell line models used previously (4T1-GFP, B16F10-CMFDA and 1205LU-GFP). Previous data showed that the majority of leukocytes recruited to the tumour cells were $CD11b^+$ cells. We observed a very significant decrease in the percentage of tumour cells that were associated with a $CD11b^+$ cell cluster in both 4T1-GFP and B16F10-CMFDA cell lines (Fig. 27A). 4T1-GFP tumour cell association with $CD11b^+$ cells decreased from 88.4% of cells to 47.76% ($p<0.0001$; n=8) and B16F10-CMFDA cell association with $CD11b^+$ cells decreased from 76.66% to 25.79% after VAP-1 inhibition ($p=0.0029$; n=8). This inhibitory effect appeared much more pronounced than that of VCAM-1 blocking antibody. Consistent with the data showing a lack of platelet clot formation, lack of myeloid cell recruitment and lack of effect of VCAM-1 inhibition, inhibition of VAP-1 also had no effect on

recruitment of leukocytes by 1205LU-GFP cells (10.94% in wt animal and 17.93% after treatment; n=5; p=0.1164).

The role of VAP-1 can be further evaluated by looking at the number of $CD11b^+$ cells that were associated with individual tumour. This effect of VAP-1 inhibition on the number of $CD11b^+$ cells recruited to the tumour cells is shown in Fig.27B. Consistent with previous data, a reduction in the number of recruited $CD11b^+$ cells in both 4T1-GFP and B16F10-CMFDA metastatic models and no significant change in $CD11b^+$ cell association with 1205LU-GFP cells was observed. The representative images are shown in Fig. 27C.

As 4T1-GFP cells also induced the recruitment of Gr-1 expressing cells, we investigated the effect VAP-1 inhibition had in this case (Fig.28 D&E). Interestingly and in contrast to the effect of VCAM-1 blocking antibody (Fig.24 E&F), we observed a significant decrease in the percentage of tumour cells associated with a $Gr\text{-}1^+$ cell clusters. This was reduced from 69.37% in control animals to 38.55% in treated animals (n= 3; p=0.0166). Supplementary Figure 24 shows that in models that lack the recruitment of Gr-1+ cell (B16F10-CMFDA and 1205LU-GFP), VAP-1 inhibition had no effect.

This dramatic inhibition of leukocyte recruitment was further confirmed by analysis of the recruitment of CD45-expressing cells (Fig.28 D&E). Due to the high number of $CD45^+$ cells per field of view, it is much more difficult to determine which cells belong to the cluster associated with a tumour cell. This is especially apparent in comparison to the CD11b images. However, a significant reduction in the percentage of tumour cells with an extensive leukocyte association was observed from 85.20% in control to 46.64% in treated animals (n=3; p=0.0034).

Next, we looked at whether VAP-1 inhibition would affect platelet clot formation (Figure 29). Interestingly, we observed a small decrease in the percentage of tumour cells associated with clots in 4T1-GFP cells after VAP-1 inhibition (90.22% in control animals to 71.70% upon treatment; n= 3;

p=0.0152). In the B16F10-CMFDA model a small decrease was observed as well (from 84.69% to 75.88%; n=4), however this was not statistically significant (p=0.0887). In agreement with previous results, in the 1205LU-GFP cell model, there was no significant difference in clot association between non-treated and treated lung metastasis. This was 15.49% and 14.67% cells respectively (p=0.9257; n=5).

There have been no reports in the literature that platelets express VAP-1 or that VAP-1 has a function in the coagulation cascade. Therefore, the effect of VAP-1 inhibition on clot formation was quite surprising. It is possible that this is due to an overall inhibition in inflammation through inhibition of VAP-1. However, previous work (Gil-Bernabe *et al*, unpublished)[236] has postulated that the recruitment of leukocytes is also mediated through platelet clot formation. In this model, platelet clots on the surface of recruited leukocytes would interact with the clot formed around the tumour cell. In this way a reduction in the number of recruited leukocytes would also correlate with a reduction in platelet clot formation. This hypothesis is supported by the fact that the most profound effect on platelet clot formation was observed in cell lines with the most pronounced inflammatory response and extensive leukocyte recruitment, i.e. 4T1-GFP.

Figure 27

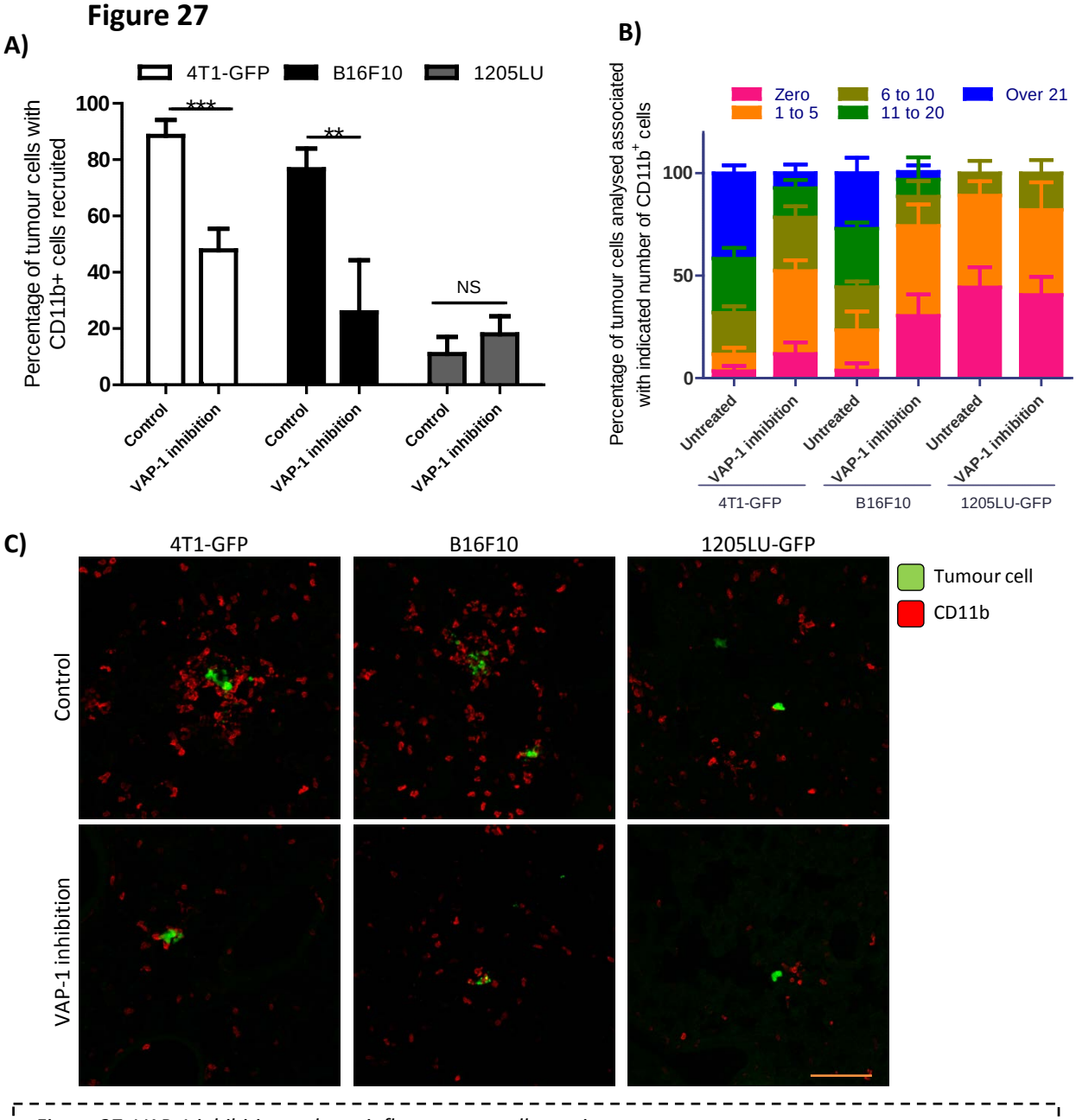
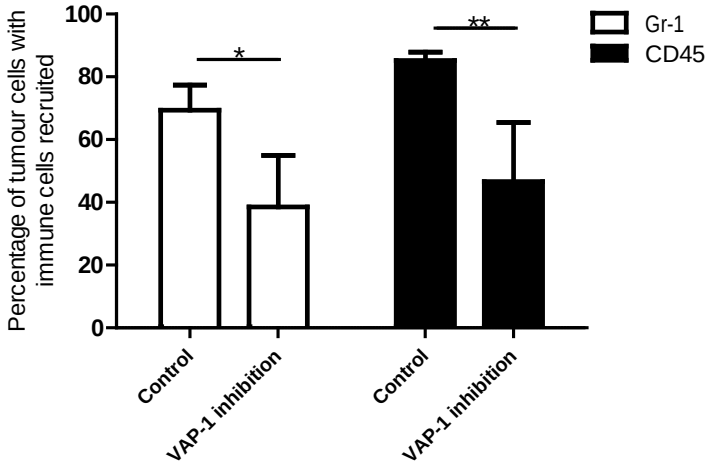


Figure 27: VAP-1 inhibition reduces inflammatory cell recruitment

VAP-1 inhibitor was injected i.p. 1h prior to the injection of tumour cells. Three experimental metastasis models were used, where 4T1-GFP, CMFDA-labelled B16F10 and 1205LU-GFP cells were injected i.v. in BALB/c, C57BL/6 or SCID mice respectively. After 8h, the lungs were harvested and analysed by fluorescent immunohistochemistry and imaging was performed with Zeiss Confocal microscope. (n=5 – 1205LU-GFP; n=8 – 4T1-GFP&B16F10) A) The quantification of the tumour cell induced recruitment of CD11b expressing cells. The data represents the proportion of tumour cells analysed found to be associated with more than 5 $Cd11b^+$ cells for each experimental group (statistical analysis: unpaired t-test; 4T1 –GFP-$p<0.0001$; B16F10-$p=0.0029$; 1205LU-GFP-$p=0.1164$) B) Analysis of the number of $Cd11b^+$ cells recruited to the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with the number of $Cd11b^+$ cells corresponding to one of the 5 different categories. C) Representative images of treated and control lungs stained for CD11b (Alexa Fluor 633-red) at 8h for all metastatic models: 4T1-GFP , B16F10 and 1205LU–GFP tumour cells (green). Scale=100µm

Figure 28

A)



B)

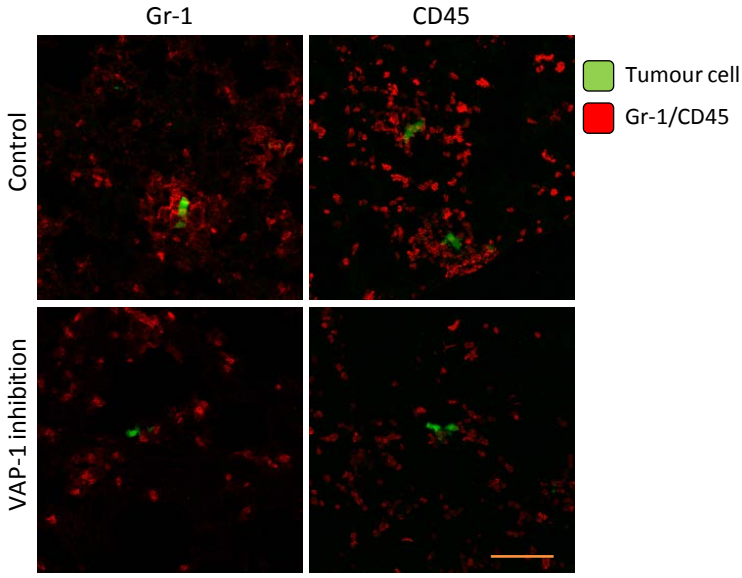


Figure 28: VAP-1 inhibition reduces inflammatory cell recruitment in the 4T1-GFP metastatic model

The effect of VAP-1 inhibition on the recruitment of Gr-1 and CD45 expressing cells was inspected in the 4T1-GFP metastatic model. VAP-1 inhibitor was injected i.p. 1h prior to the injection of tumour cells. After 8h, the lungs were harvested and analysed by fluorescent immunohistochemistry and imaging was performed with Zeiss Confocal microscope. (n=3)

A) The quantification of Gr-1 and CD45 expressing cells was performed, where the percentage of tumour cell analysed associated with the leukocyte cluster is presented. (Statistical analysis: unpaired t-test; Gr-1 – p=0.016; CD45-p=0.0034) Representative images of treated and control lungs stained for CD45 and Gr-1(Alexa Fluor 633-red) are shown in B. Scale=100µm

Figure 29

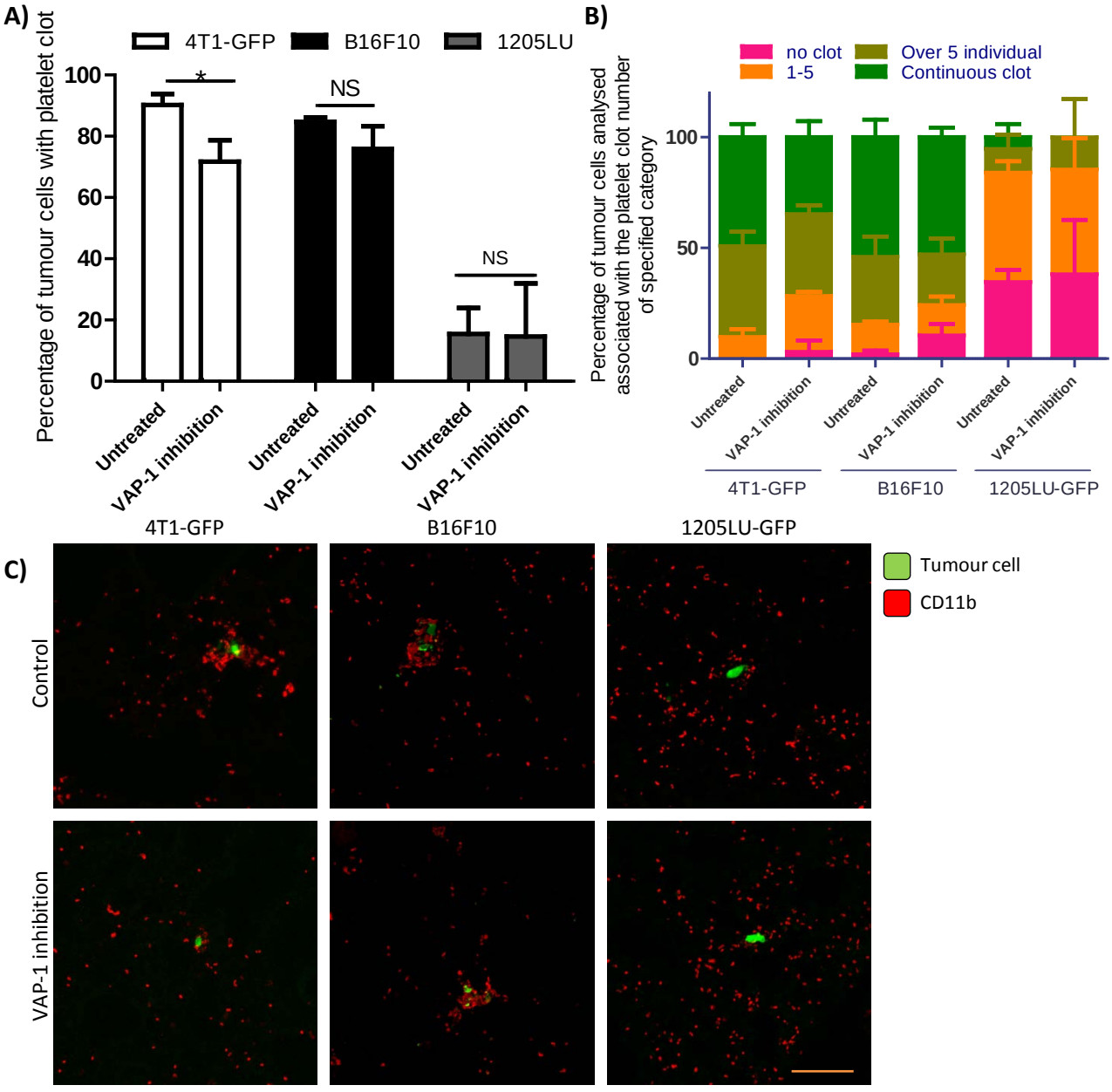


Figure 29: Effect of VAP-1 inhibition on platelet clot formation

The effect of VAP-1 inhibition on platelet clot formation was investigated in three experimental metastasis models: 4T1-GFP, CMFDA-labelled B16F10 and 1205LU-GFP cells were injected i.v. in BALB/c, C57BL/6 or SCID mice respectively. VAP-1 inhibitor was injected i.p. 1h prior to the injection of tumour cells. After 8h, the lungs were harvested and analysed by fluorescent immunohistochemistry and imaging was performed with Zeiss Confocal microscope. A) The quantification of the tumour cell induction of platelet clot formation. The data represents the proportion of tumour cells analysed found to be associated with a platelet clot for treated and control mice. (statistical analysis: unpaired t-test; 4T1-GFP- p=0.0152; B16F10-p=0.0887; 1205LU-GFP-p=0.9257) B) A graphic representation of the recruited clot size. The data is represented as the percentage of the tumour cells analysed that are associated with the size of clot corresponding to one of the 4 different categories. C) Representative images of IHC staining for αIIb- (Alexa Fluor 633-red) in relation to the tumour cells (green) at 8h Scale=100µm (n=3 - 4T1-GFP; n=5 -1205LU-GFP; n=4 – B16F10)

2.2. Effect of VAP-1 inhibition on metastasis

Next, we examined whether the dramatic reduction in leukocyte recruitment would lead to a reduced capacity for metastatic dissemination (Figure 30).

As in previous experiments the VAP-1 inhibitor was injected i.p. 1h prior to tumour cell injection. 24h after i.v. introduction of tumour cells, the lungs were harvested, sectioned and the number of tumour cells surviving in lung parenchyma was counted microscopically in at least 10 non-consecutive sections per lung.

The experiment was repeated at least twice for 4T1-GFP and B16F10-CMFDA cell lines and the overall number of tumour cells per lung section was found to differ between the repeats. For that reason, the overall survival of tumour cells after VAP-1 inhibition is expressed as the percentage of tumour cells in the treated lung, where the corresponding control represents 100%. The averages of all the experimental lungs across all the experiments are plotted in Figure 30A. Additionally, representative experiments, where the number of cells per lung is plotted, are shown in Fig. 30B, C&D.

VAP-1 inhibition inhibited the number of tumour cells in the lung at 24h for both B16F10-CMFDA and 4T1-GFP experimental models. In the B16F10-CMFDA survival was reduced to 67.65% of control and in 4T1-GFP cell model survival was inhibited to 58.06% of the control. In accordance with the effect on leukocyte recruitment, VAP-1 inhibition had a more profound effect than VCAM-1 blocking antibody. Not surprisingly, there was no effect of VAP-1 inhibition on the survival and metastasis establishment of the 1205LU-GFP cell line (Fig. 30A&D). This further confirms the direct relationship between leukocyte recruitment and tumour cell survival.

Figure 30

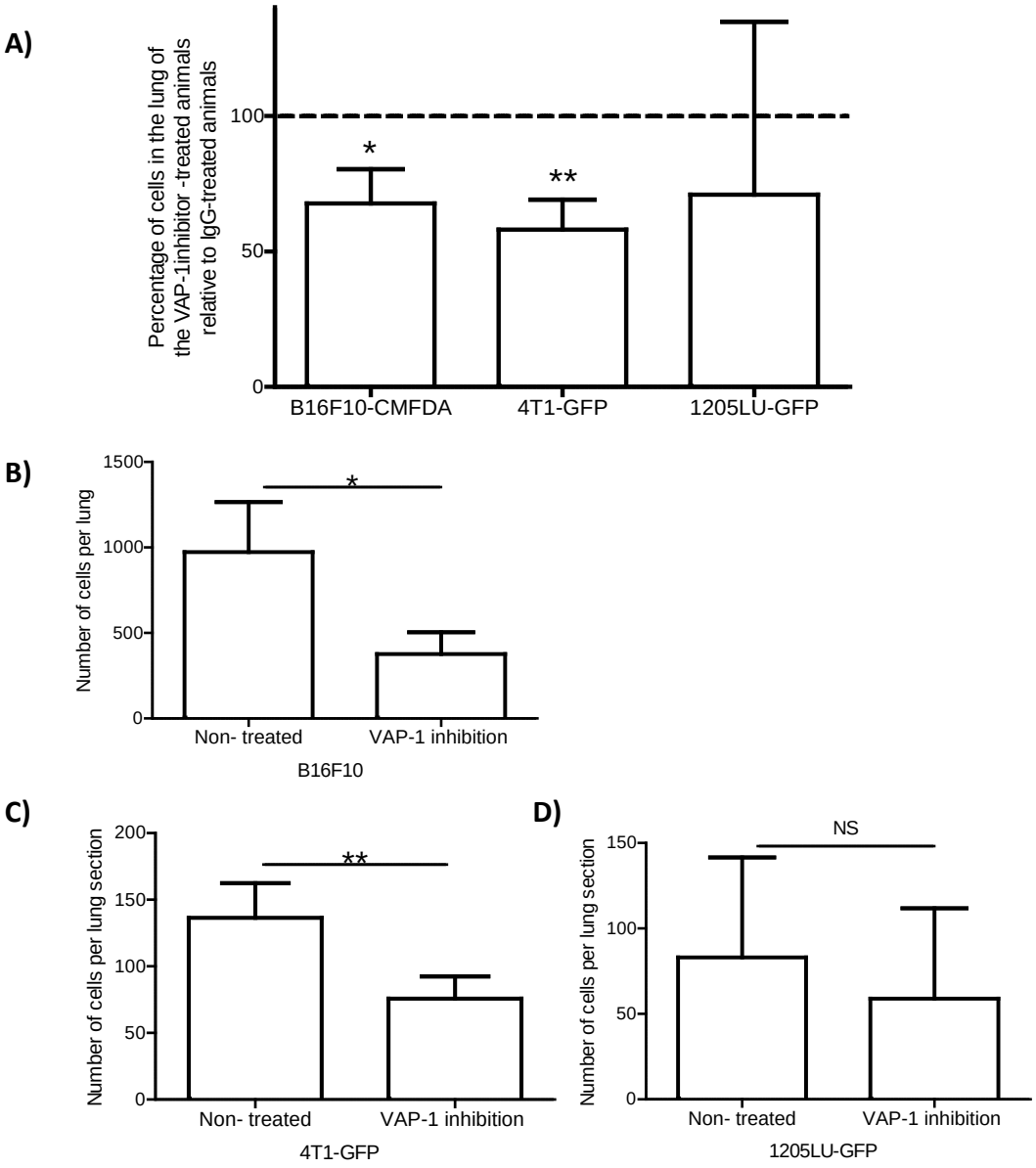


Figure 30: Effect of VAP-1 inhibition on experimental tumour metastasis

In order to assess the effect of VAP-1 inhibitor on the process of metastasis, again all three experimental metastatic models were used. Tumour cells (5x10e5)were injected i.v. 1h after inhibitor treatment. Metastatic efficiency/tumour cell survival in the lung was assessed 24h later. The lungs were harvested as for IHC staining, and cells were counted in at least 10 sections per animal to accurately gauge the surviving cell fraction.

A) The surviving cell fraction is represented as the percentage of cells in the treated lungs, where the 100% value is noted by the corresponding control lung (4T1-GFP n=10; B16F10 n=11; 1205LU-GFP n=5). For both 4T1-GFP and B16F10 models the experiment was repeated twice and the n number denotes the sum of all lungs analysed. For the 1205LU-GFP cell line, the experiment was performed once.

Graphs showing a representative experiment of the effects of VAP-1 inhibition are shown for each cell line: B16F10 (B), 4T1-GFP (C) and 1205LU-GFP (D). Statistical analysis: unpaired t-test (B16F10 p=0.0231; 4T1-GFP p=0.0064; 1205LU-GPF p=0.5148)

3. Concomitant inhibition of VCAM-1 and VAP-1

Previous experiments have demonstrated that VAP-1 plays a more significant role in mediating leukocyte recruitment and subsequent tumour cell survival than VCAM-1. In the next experiment, we wanted to investigate whether the two adhesion molecules were mediating these effects through the same mechanisms/pathway or whether double blocking would result in a synergistic effect.
Previously, the effects of VCAM-1 blocking antibody were only observed in the 4T1-GFP metastatic model. Furthermore, VAP-1 inhibition is much more effective in this model in inhibition of both recruitment of CD11b expressing cells and inhibition of metastatic dissemination. Therefore due to the strongest effects of blocking treatments observed in the 4T1-GPF metastatic model, this was mainly used to test any synergistic effects. As previously, VCAM-1 blocking antibody (1.5μg per g of body weight) was injected i.v. 4h prior to tumour cell injection and VAP-1 inhibitor was injected i.p. (6mh per kg of body weight) 1h prior to tumour cell injection.

3.1. Effect of VAP-1 and VCAM-1 inhibition on monocyte recruitment and clot formation

Firstly, we analysed the recruitment of inflammatory cells 8h after injection of 4T1-GFP tumour cell (Figure 31; n=3). As expected, we observed a significant reduction in the recruitment of $CD11b^+$ cells (Fig.31A). Association with $Cd11b^+$ cells was reduced from 88.45% to 63.33% (p=0.0021). Interaction with $CD45^+$ cells was inhibited from 85.20% to 56.14% (p=0.0011) and $Gr\text{-}1^+$ cell association decreased from 69.37% to 48.32% (p=0.0282). Representative images are shown in Figure 31C.
This effect was further confirmed when we analysed the overall number of $CD11b^+$ cells associated with individual tumour cells (Fig.31B).
Interestingly however, this inhibition was not significantly more pronounced than that observed with

VAP-1 inhibition alone (Figure 27). This would suggest that VAP-1 and VCAM-1 may function via the same mechanisms to facilitate tumour cell survival through leukocyte recruitment.

We also investigated the effect of this double inhibition on platelet clot formation (Figure 32) and have found no significant effect on either the percentage of tumour cells associated with platelets (Fig.32A) or with the size of the platelet clot (Fig. 32C). This is quite interesting as a decrease was observed when only VAP-1 was inhibited (Fig.29). It suggests that the effect of VAP-1 inhibition on platelet clot formation might not be of greater significance, but rather due to experimental error.

3.2. Effect of VAP-1 and VCAM-1 inhibition on metastasis

Thus, it appears that VCAM-1 and VAP-1 may act via the same mechanism to facilitate the recruitment of monocytes/macrophages. We then proceeded to examine whether the lack of synergistic effect was also observed in a tumour cell survival assay (Figure 33). Panel A demonstrates a reduction in survival for 4T1-GFP cells. This was reduced from 136.35 to 80.17 cells after treatment (n=3; p=0.0391) Figure 33B shows data for the metastatic survival assay upon double inhibition in the B16F10-CMFDA cell model. There is a downward trend (from 238.40 cells per lung to 161.27cells after treatment), but this did not appear significant (n=3; p=0.0735). The most likely reason for this is the technical difficulty of double i.v. injections in C57BL/6 mice.

Together these data confirm that VAP-1 and VCAM-1 adhesion molecules may work via shared pathways to promote leukocyte adhesion and subsequent tumour cell survival. Moreover, VAP-1 appears to be more effective, suggesting it may be placed further upstream in this cascade relative to VCAM-1.

Figure 31

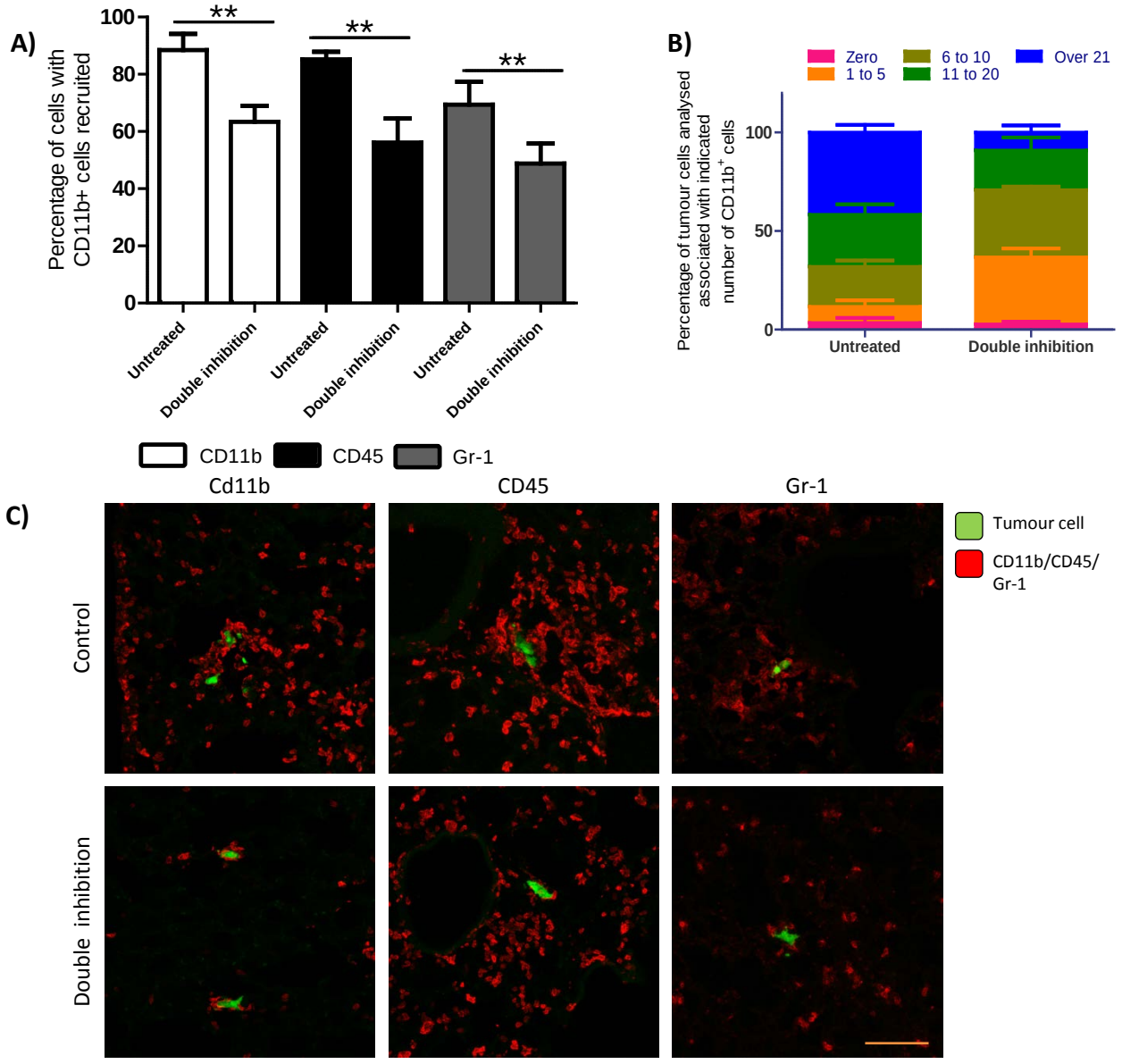


Figure 31: Effect of inhibition of both VAP-1 and VCAM-1 on inflammatory cell recruitment

The effects of concomitant inhibition of VCAM-1 and VAP-1 on the recruitment of leukocytes was investigated in the 4T1-GFP syngeneic model of metastasis. VCAM-1 blocking antibody was injected i.v. and this was followed by i.p. injection of VAP-1 inhibitor three hours later. One hour after treatment with VAP-1 inhibitor, tumour cell were injected i.v. and 8h later the lungs harvested. Imaging was performed with Zeiss Confocal microscope A) The quantification of the tumour cell induced recruitment of CD11b, CD45 and Gr-1 expressing cells. The data represents the proportion of tumour cells analysed found to be associated with more a leukocyte cluster characterised by the particular antigen for each experimental group (n=3). (statistical analysis: unpaired t-test; n=3) (Cd11b+ cells p=0.0021; CD45+ cell p=0.0011; Gr-1+cells p=0.0282) B) Analysis of the number of $Cd11b^+$ cells recruited to the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with the number of CD11b+ cells corresponding to one of the 5 different categories.(n=3) C) Representative images of IHC staining for CD11b/CD45/Gr-1- (Alexa Fluor 633 -red) in relation to the 4T1-GFP tumour cells (green) at 8h. Scale=100µm

Figure 32

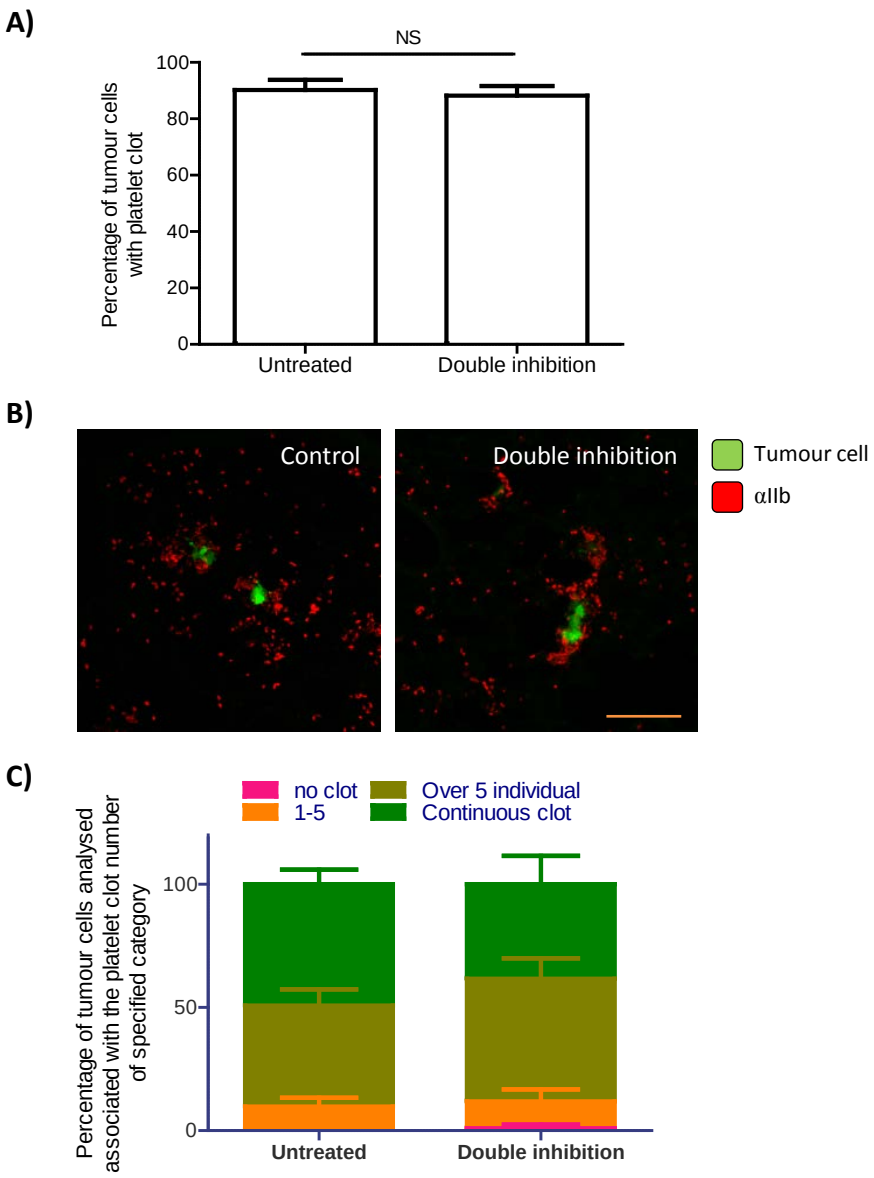


Figure 32: Effect of double inhibition of both VCAM-1 and VAP-1 on platelet clot formation

The effects of concomitant inhibition of VCAM-1 and VAP-1 on platelet clot formation was again investigated in the 4T1-GFP syngeneic model of metastasis. As described previously VCAM-1 blocking antibody was injected i.v. and this was followed by i.p. injection of VAP-1 inhibitor three hours later. One hour after treatment with VAP-1 inhibitor, tumour cell were injected i.v. and 8h later the lungs harvested. Imaging was performed with Zeiss Confocal microscope.

A) The quantification of the tumour cell induction of platelet clot formation. The data represents the proportion of tumour cells analysed found to be associated with a platelet clot for treated and control mice. (statistical analysis: unpaired t-test; n=3; p=0.5159) B) Representative images of IHC staining for αIIb (Alexa Fluor 633-red) in relation to the tumour cells (green) at 8h. Scale=100μm C) A graphic representation of the recruited clot size. The data is represented as the percentage of the tumour cells analysed that are associated with the size of clot corresponding to one of the 4 different categories. (n=3)

Figure 33

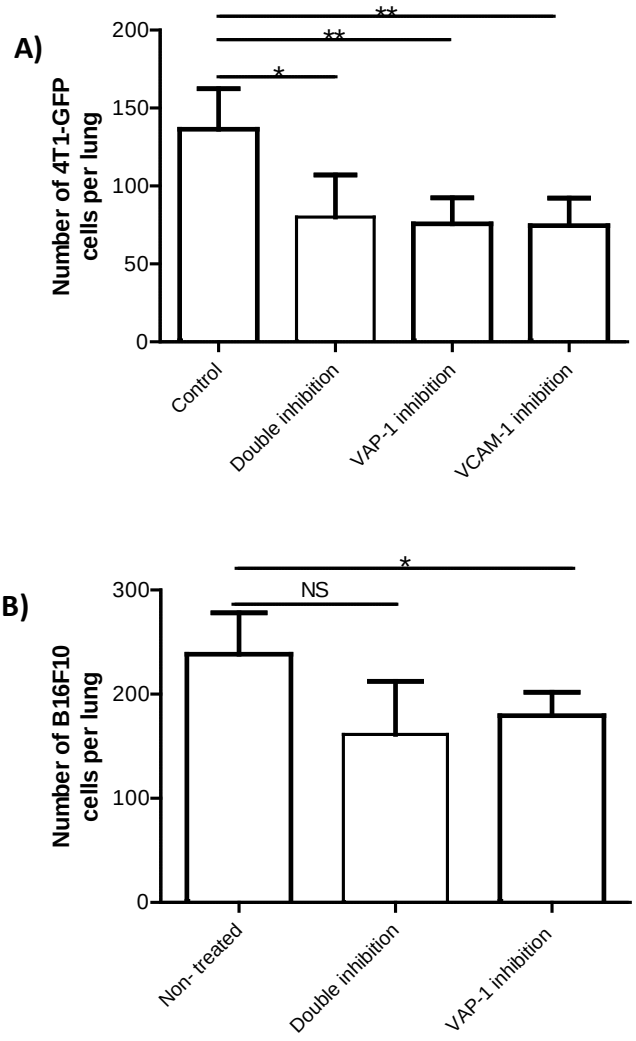


Figure 33: Effect of double inhibition of both VCAM-1 and VAP-1 on experimental tumour metastasis

The effects of concomitant inhibition of VCAM-1 and VAP-1 on metastatic dissemination were investigated in both 4T1-GFP and B16F10 cell models.

As described previously VCAM-1 blocking antibody was injected i.v. and this was followed by i.p. injection of VAP-1 inhibitor three hours later. One hour after treatment with VAP-1 inhibitor, tumour cell were injected i.v. Metastatic efficiency/tumour cell survival in the lung was assessed 24h later. The lungs were harvested as for IHC staining, and cells were counted in at least 10 sections to accurately gauge the surviving cell fraction.

A) Graph showing the effect of the treatments on the survival of 4T1-GFP cells in the lung (n=3) (statistical analysis: unpaired t-test, p=0.0391) For easier comparison effects of VCAM-1 blocking and VAP-1 inhibition alone are displayed (data from Fig.26 and Fig.30)

B) Graph showing a representative experiment studying the effect of the treatments on the survival of B16F10 cells in the lung (n=3) (statistical analysis: unpaired t-test, p=0.0735) For easier comparison effects of VAP-1 inhibition alone are displayed (data from Fig.30)

4. Long-term inhibition

The next experiment was designed to evaluate the effects of VAP-1 inhibition on long-term metastatic growth. We used both the B16F10 and 4T1-GFP experimental models and three treatment groups were applied. The control group received no VAP-1 inhibitor; only tumour cells were injected i.v. in the B16F10 cell model, 2x10e5 cells were injected into C57BL/6 mice and in the 4T1-GFP cell model, 5x10e5 cells were injected in the BALB/c mice. The first experimental group received VAP-1 inhibitor injection 1h before tumour cells were injected and then again 24h later. This group is in fact analogous to experiments described in Figure 30, where VAP-1 inhibitor was injected 1h before tumour cells and metastatic efficiency evaluated at 24h. In the second experimental group, VAP-1 inhibitor was not injected until after metastatic colonies were present in the lung (day5). After day 5, the VAP-1 inhibitor was injected every 48h, total of 4 times (days 5, 7, 9 and 11). The lungs were harvested on day 14 and the number of metastatic colonies on the surface of the lungs was counted.

We observed a significant decrease in both experimental metastatic models when VAP-1 was inhibited prior to induction of metastasis. In the 4T1-GFP cell model the average number of lung nodes per lung decreased from 164.4 in the control group to 103.0 after treatment (n=15). This is equivalent to 62.65% of cells surviving in the treated group in comparison to the control. This value is almost equal to the cell survival measured at 24h, confirming the validity of these results.

In the B16F10 tumour cell model, the inhibition of metastasis was equally efficient, decreasing from an average of 115.8 nodes per lung in the control group to an average of 44.10 in the treated group. This corresponds to a 62% reduction in tumour cell survival, where the treated group only retained 38.08% of lung nodes compared to the control. Surprisingly this was a much more significant inhibition in comparison to that observed at 24h.

Interestingly, in the second experimental group, where VAP-1 inhibitor was administered after day 5,

no significant difference in the number of metastatic nodes was observed. In the B16F10 tumour cell model, averages of 108.5 lung nodes were counted compared to the non-treated control which showed an average of 115.8. Similarly, the 4T1-GFP cells formed an average of 164.4 lung nodes when not treated and 150.5 upon treatment. These data suggest that VAP-1, and by extension endothelial activation, only have significant role for tumour cell metastasis in the initial stages of the process.

Figure 34

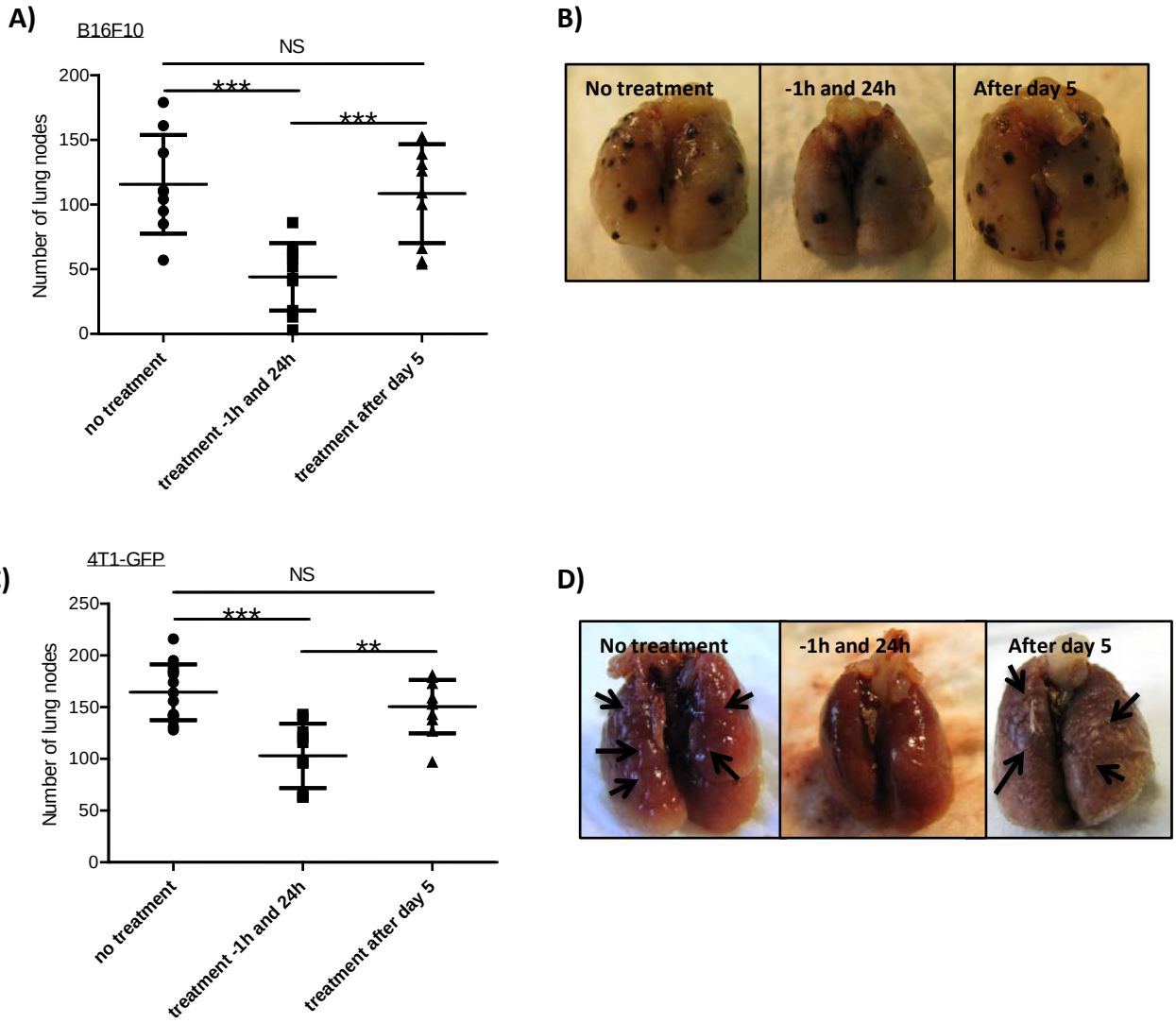


Figure 34: Effect of VAP-1 inhibition on metastasis

The effects of VAP-1 inhibition on the development of metastasis over 2 weeks was investigated using both 4T1-GFP and B16F10 metastatic models. Tumour cells were injected i.v. and metastatic colonies in the lung were counted 2 weeks later. Images were acquired using Canon PowerShot S5IS camera.

In the two experimental groups: 1.) VAP-1 inhibitor was injected 1h prior to tumour cell injection and then again 24h later and 2.) VAP-1 inhibitor injected after metastatic spread has established on days: 5, 7, 9 and 11.

A&B) Graph and representative images showing the effect of VAP-1 inhibition of the formation of lung nodules of B16F10 cells in the lung (n=10) (statistical analysis: One-way ANOVA $p=0.0009$; Tukey post-test)

C&D) Graph and representative images showing the effect of VAP-1 inhibition on the formation of lung nodules of 4T1-GFP cells in the lung (n=15) (statistical analysis: One-way ANOVA $p<0.0001$; Tukey post-test)

5. MMP9 and pulmonary metastasis

The importance of the interactions between the tumour cells, leukocytes and pulmonary endothelial cells has been shown to be crucial for the metastatic process. All the components of this axis have been shown to express and/or secrete different types of effector molecules. Alongside the previously outlined inflammatory cytokines, secreted proteases have also been implicated in mediating tumour invasion and metastasis.[236,685,686] Given the extent of matrix remodelling that occurs during metastasis, the production of proteases in this context is not at all surprising. In addition to protease secretion by the tumour cells, the stromal components have been shown to be crucial contributors to protease production.[152,687,688] Matrix metalloproteinases (MMPs) have been found to be frequently over-expressed in different types of cancer.[688] Of these, MMP9 has been implicated to be the rate-limiting protease of cell migration in *in vitro* experiments.[679] However it is not entirely clear whether this is its mechanism of action as migratory properties of cells are not affected in MMP9 KO animals.
It is however clear that MMP9 plays a significant role in the process of metastasis, as this is decreased at least two fold in MMP9 KO mice.[689] Moreover work by Hiratsuka *et al*[690] has shown that leukocytes recruited to the metastatic site are responsible for MMP9 induction and thereby metastasis. Inhibition of the recruitment of leukocytes and thereby induction of MMP9 expression, attenuated the metastatic efficiency.

As we have shown that leukocyte recruitment can be reduced by inhibition of endothelial activation antigens (VCAM-1 and VAP-1), we then proceeded to examine whether this also affected MMP9 expression.

MMP9 expression was visualized by fluorescence immunohistochemistry (Figure 35). The specificity of the staining was confirmed with the MMP9KO lung tissue (Supplementary figure 9). The expression of MMP9 in a non-treated naïve lung was very low (Fig.35C). Next, we looked at the MMP9

expression in response to tumour cell metastasis (Fig.35A&B). We observed little or no MMP9 expression 8h after 1205LU-GFP cell injection. In contrast, extensive MMP-9 deposition is visible around the 4T1-GFP tumour cells and upon quantification this was shown to occur in 60.71% of cases. Interestingly, B16F10-CMFDA metastasis did not evoke the same response; we did not observe the extensive continuous staining seen with 4T1-GFP cells. Instead, MMP9 expression appears to be induced by cells that do not localise to the metastatic tumour cell at 8h.

As 4T1-GFP cells appear to evoke the strongest MMP9 expression. we investigated the effects of different treatments on MMP9 induction in this metastatic model. Co-staining the 4T1-GFP metastatic cells for MMP9 and CD45 revealed that the two certainly coincide but do not appear to directly overlap. This suggests that MMP9 is not immobilised on the surface of the leukocytes.

Figure 35

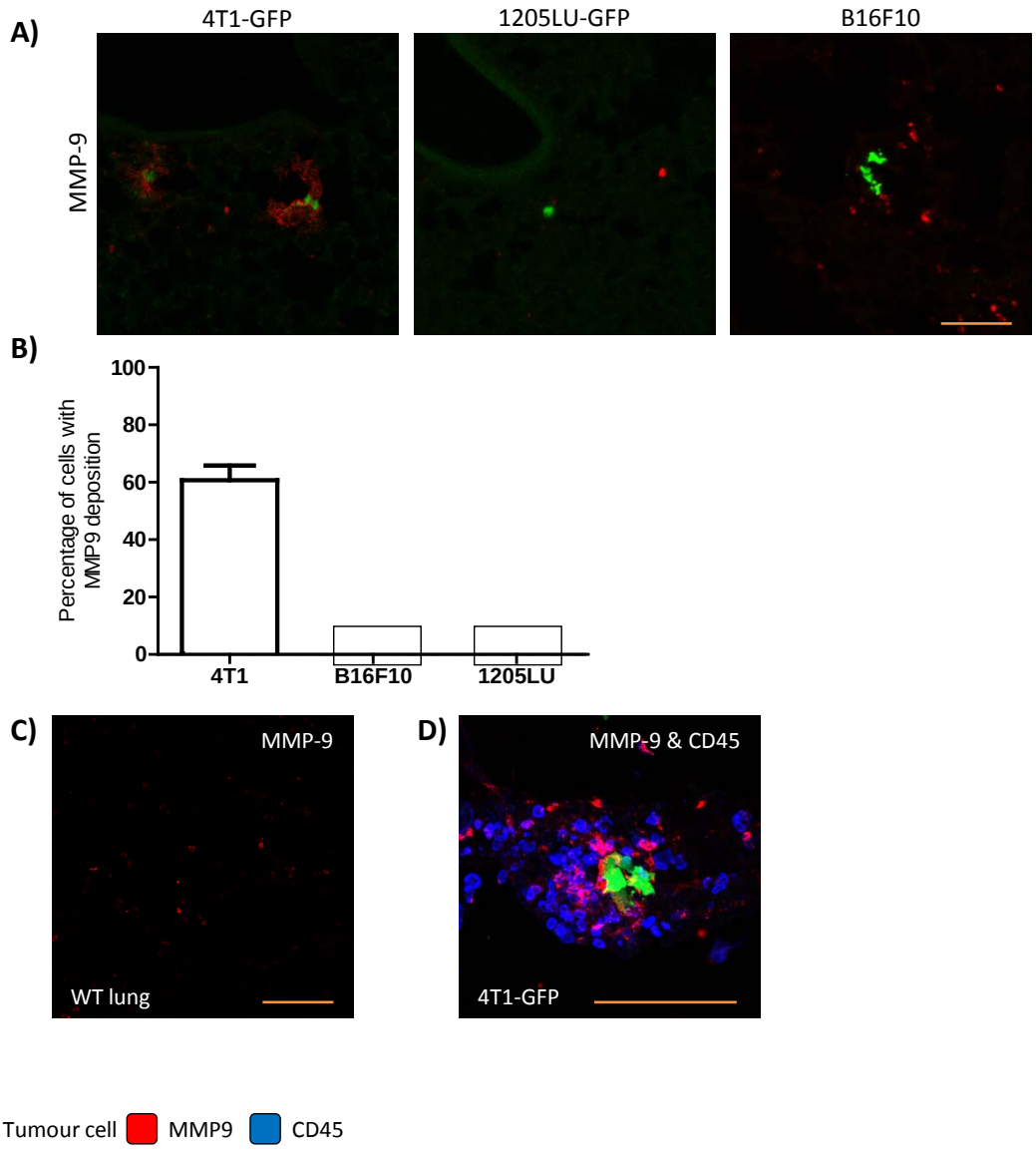


Figure 35: MMP9 expression in pulmonary metastasis

Expression of MMP9 (red) was investigated in response to tumour cell metastasis (green) in all three experimental models of metastasis. The lungs were harvested 8h after i.v. injection of tumour cells (4T1-GFP, 1205LU-GFP and B16F10). Imaging was performed with Zeiss Confocal microscope. Scale=100µm

A) Representative images lungs tissues harvested 8h after tumour cell challenged stained for MMP9 (Alexa Fluor 633-red) for all three metastatic models: 4T1-GFP, B16F10 and 1205LU-GFP tumour cells (green). Scale=100µm

B) Quantification of the MMP9 expression. The graph shows the percentage of tumour cell analysed that were found to have extensive MMP9 deposition (n=3).

C) Level of MMP9 expression in a naïve non-injected lung.

D)Double staining demonstrating the CD45-expressing cells (Elf97-blue) and MMP9 (Alexa Fluor-633-red) localisation around a 4T1-GFP (green) tumour cell.

Next we looked at the effect different treatments have on MMP9 expression in response to 4T1-GFP metastasis (Figure 36). Injection of hirudin, that led to a reduction in leukocyte recruitment (Fig. 19), resulted in a reduction in MMP9 expression where only 10.985% of tumour cells were surrounded by MMP9 upon treatment ($p=0.0094$). While MMP9 expression did not change in response to VCAM-1 inhibition (data not shown), VAP-1 inhibitor appears to lead to a small decrease, although this was not significant ($p=0.259$; $n=3$).

It appears that only 4T1-GFP cells are able to induce extensive deposition of MMP9. This would suggest that the recruited cells responsible are the Gr-1 – expressing cells, as we only observe the recruitment of these myeloid cells in the 4T1-GFP experimental model. Additionally, the recruitment of Gr-1^{+}myeloid cells was only affected by VAP-1 inhibition and not by blocking of VCAM- 1 function, in the same way as observed here for MMP9 expression.

The strongest inhibition of MMP9 expression is observed in response to anti-coagulation. This has been shown to reduce myeloid cell recruitment and through inhibition of platelet activation also reduce activation of endothelial cells. Therefore both endothelial cells and the recruited myeloid cells could be responsible for the MMP9 production. However, VAP-1 inhibition that only resulted in inhibition of leukocyte recruitment, did not significantly affect MMP9 production, suggesting that endothelial cells are the major producers of MMP9. This is also in line with the data presented by Hiratsuka *et al.*[690]

Figure 36

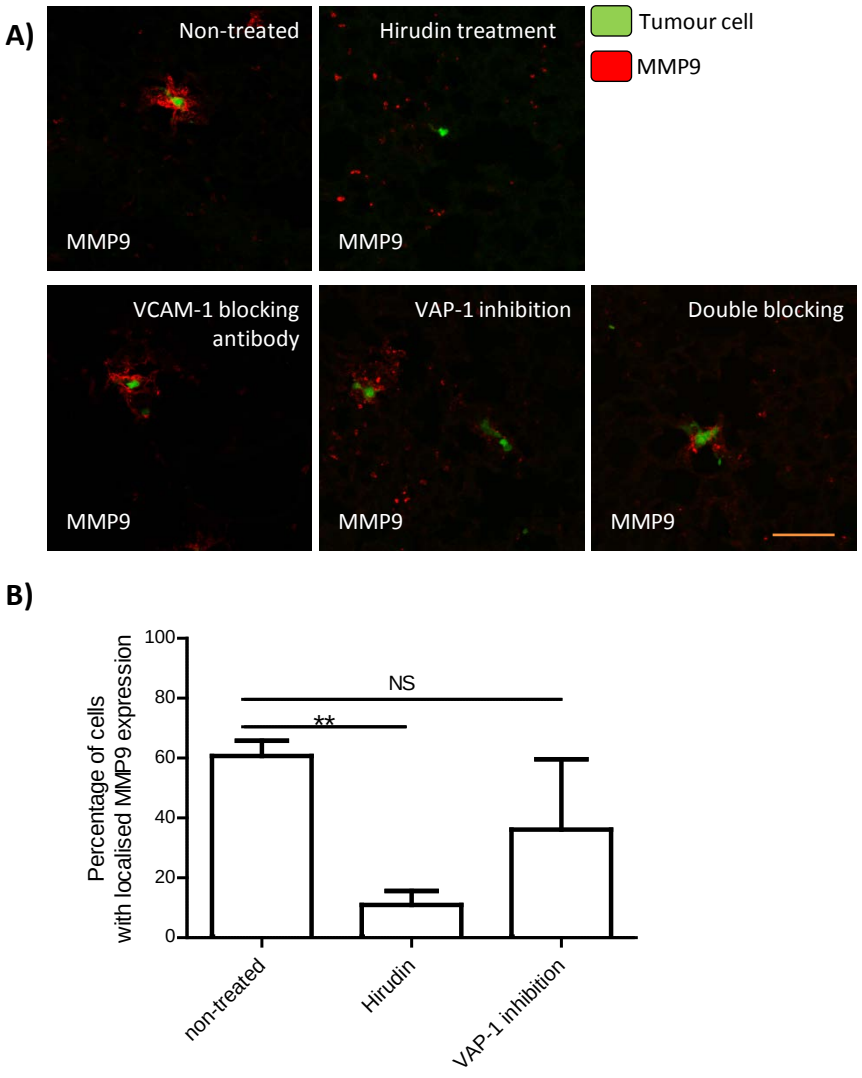


Figure 36: Effect of VAP-1 and VCAM-1 inhibition on MMP9 expression

The effects of hirudin treatment and of VCAM-1 and VAP-1 inhibition on MMP9 expression were investigated in the 4T1-GFP metastatic model. Hirudin was injected 5 min before and 4h after the tumour cells. VCAM-1 blocking antibody was injected 4h before and the VAP-1 inhibitor 1h before tumour cell injection.

A) Representative images of 4T1-GFP cell pulmonary metastases (green) stained for MMP9 (red). These images demonstrated MMP9 expression in response to 4T1-GFP metastasis and how this is affected by the different types of treatments. Imaging was performed with Zeiss Confocal microscope. Scale=100µm

B) Quantification of the MMP9 expression. The graph shows the percentage of tumour cell analysed that were found to have extensive MMP9 deposition (n=3). Statistical analysis: unpaired t-test; Hirudin treatment-p=0.0094; VAP-1 inhibition-p=0.259)

6. The premetastatic phase

In the past decade research has demonstrated that a primary tumour growth may act to pre-condition target organs for metastatic dissemination.[691-694] When comparing lung tissue from mice bearing a primary tumour to naive animals, Hiratsuka *et al*[690] noted a 1.5-2fold doubling in the number of Mac-$1^{+}MMP^{+}$ monocytes in the tissue. Moreover they have shown that these recruited monocytes act on the pulmonary endothelium via VEGFR-1 to facilitate induction of MMP9. Additionally Kaplan *et al*[693] demonstrated the formation of pre-metastatic niche by adoptive transfer, which facilitated labelling of bone marrow-derived cells (BMDCs) with GFP. Subsequent to tumour implantation, GFP-expressing BMDCs were shown to localise and cluster at discrete sites in lung tissue parenchyma. Using IHC and FACS analysis, they showed that these cells express CD11b, VEGFR1 and MMP9. In addition, they were shown to preferentially localise to the sites of increased fibronectin deposition. Subsequently, labelled and injected tumour cells were shown to disseminate, bind and establish at these sites. Other experiments identifying the components of this pre-metastatic niche included $CD11b^{+}Gr1^{+}$ cells.[695] These myeloid suppressor cells were proposed to inhibit inflammatory destruction of tumour cells by T-cell and NK-cell responses, in contrast to the $CD11b^{+}$ monocytes proposed to assist tumour cell survival by other means (e.g. chemokine secretion). However the presence of these MDSCs could in this case be due to the difference in tumour cell model as observed in our experiments. Additionally, Erler *et al* proposed lysyl oxidase (LOX) to be involved in mediating CD11b cell recruitment[696]. They have shown that LOX is secreted by hypoxic tumour cells, after which it accumulates at premetastatic sites to cross-link collagen IV and thereby mediate $CD11b^{+}$ cell recruitment.

The role of $CD11b^{+}$ cells, in mediating the initial stages of metastasis has been clearly established.[691] Therefore it is perhaps not surprising that these cells have been shown to be induced by the primary tumour to facilitate metastasis and to be crucial in establishment of the pre-metastatic niche.

Previous work in our laboratory demonstrated that recruitment of these $CD11b^+$ cells by the metastasizing tumour cell occurs via TF-induced coagulation (Gil-Bernabe *et al*, unpublished). *In vivo* experiments, where the pre-metastatic niche effect of the primary tumour implanted s.c. were interrupted by anti-coagulation treatment. When hirudin was injected through the course of primary tumour growth, care had to taken to avoid disrupting the growth of the primary tumour by the anti-coagulant. After 3 weeks tumour cells were injected i.v. and the efficiency of metastasis was evaluated. The number of metastases in naïve non-treated animals was lower than in animals with an implanted s.c. tumour. This confirmed the existence and effectiveness of a pre-metastatic niche. Furthermore anti-coagulation treatment induced a complete reversal of this enhancement. This effect was shown to be dependent on $CD11b^+$ cell recruitment.

Thereby, platelet activation was shown to be required to mediate recruitment of monocytes in the premetastatic phase. Data presented here proves that platelet clot formation is involved in mediating endothelial activation in the early stages of experimental metastasis (Fig.20). Moreover, inhibition of these endothelial adhesion antigens (VCAM-1 and VAP-1) resulted in inhibition of $CD11b^+$ cell recruitment (Fig. 24& Fig.27). This would suggest that endothelial activation may also be involved in mediating the establishment of the pre-metastatic niche. Therefore we wanted to investigate whether the components of the pre-metastatic niche included VCAM-1 and VAP-1.

In these experiments B16F10 cells were implanted s.c. and 3 weeks later the lungs were harvested. At this time there was no macroscopic evidence of spontaneous metastasis. (These experiments were performed in collaboration with Dr. Gil-Bernabe, where Dr. Gil-Bernabe developed the pre-metastatic animal model and performed cell preparation and lung harvest.)

First we concentrated on analysing the $CD11b^+$ cells in order to confirm the establishment of the premetastatic niche (Figure37). We observed an increase of about 1.3fold in the number of $CD11b^+$

cells per field of view in the tumour-bearing animals, which is consistent with the numbers reported by Hiratsuka *et al*.[691]

Next, the lungs were stained for VCAM-1 and VAP-1. Interestingly, a significant increase in the expression of VCAM-1 (~1.27 fold) was observed in the presence of a primary tumour. Perhaps not surprisingly no difference was observed in the expression of VAP-1. This is consistent with the data obtained previously, where VAP-1 expression was not significantly affected by tumour cell metastasis (Fig. 11). However, in comparison to VCAM-1, inhibition of VAP-1 has proven to be more effective in disrupting leukocyte recruitment in experimental metastasis. Moreover VAP-1 belongs to the same family of enzymes as LOX, an enzyme already shown to play a role in lung conditioning. Therefore the involvement of VAP-1 is to be expected. In contrast, VCAM-1 expression was induced in the early stages of metastasis and this also appears to be the case in the premetastatic phase.

In summary these data implicate endothelial activation in the establishment of the pre-metastatic niche, where it would facilitate the recruitment of inflammatory mediators. This could only be confirmed if inhibition of endothelial activation would result in disrupting the effect of the premetastatic niche in mediating metastasis and a reduction in $CD11b^+$ cell recruitment to the basal level.

Figure 37

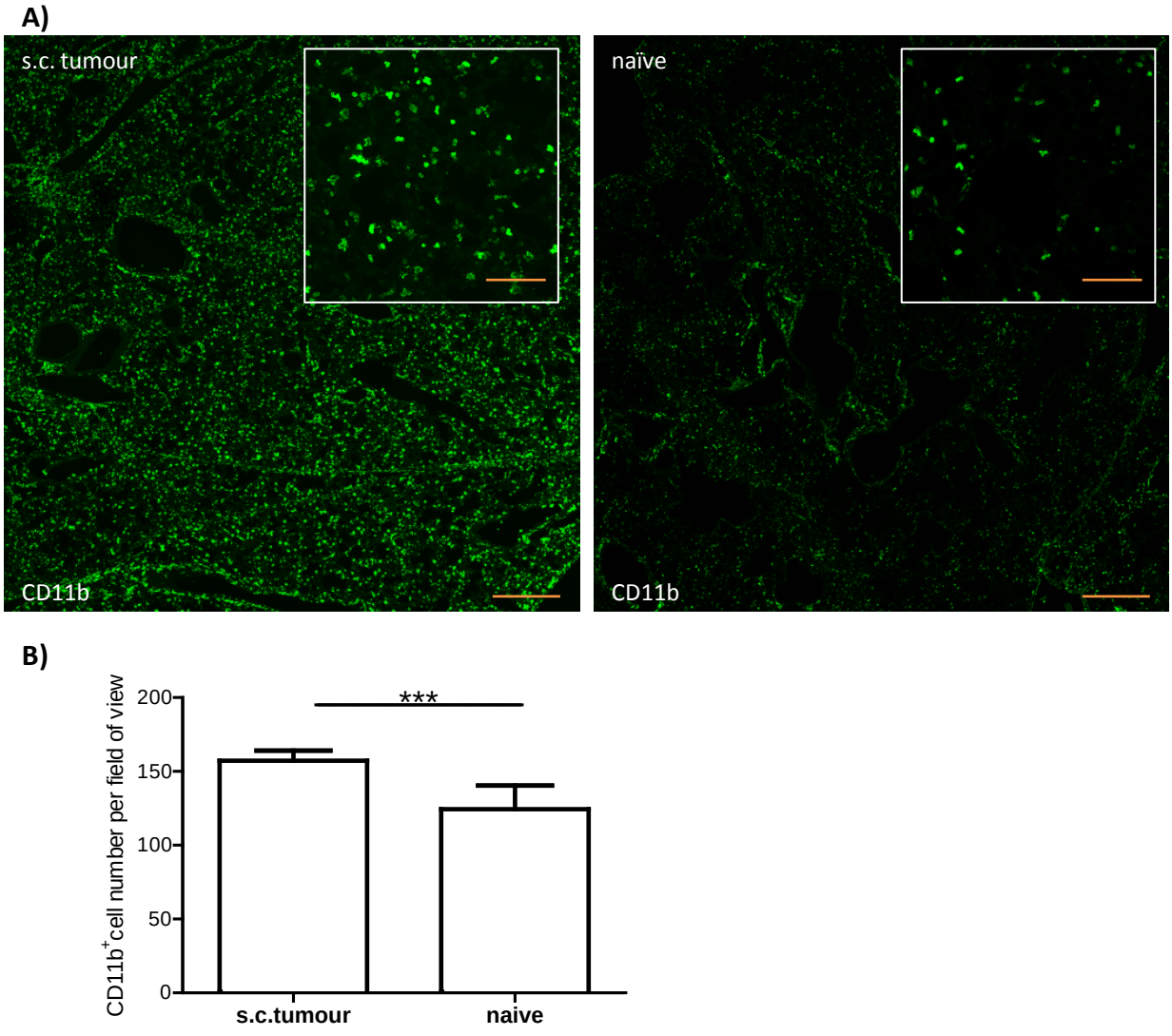


Figure 37: The number of CD11b-expressing cells is significantly increased in the pre-metastatic lung
The formation of a pre-metastatic niche was induced by the injection of B16F10 cells sub-cutaneously in C57BL/6 mice. Three weeks later the lungs were harvested and stained for different components.
A) Representative images of CD11b staining (green) from either a naïve animal or an animal bearing a s.c. primary tumour. The larger image is a mosaic scan acquired by 8x8 x fields of 20x objective using a Zeiss Confocal microscope. Scale = 400µm. The insert represent field of view of the 20x objective. (Scale = 100µm) B) Quantification of the number of CD11b-expressing cells per field of view (424.27µmx424.27µm) (Statistical analysis: unpaired t-test; p=0.0009)

Figure 38

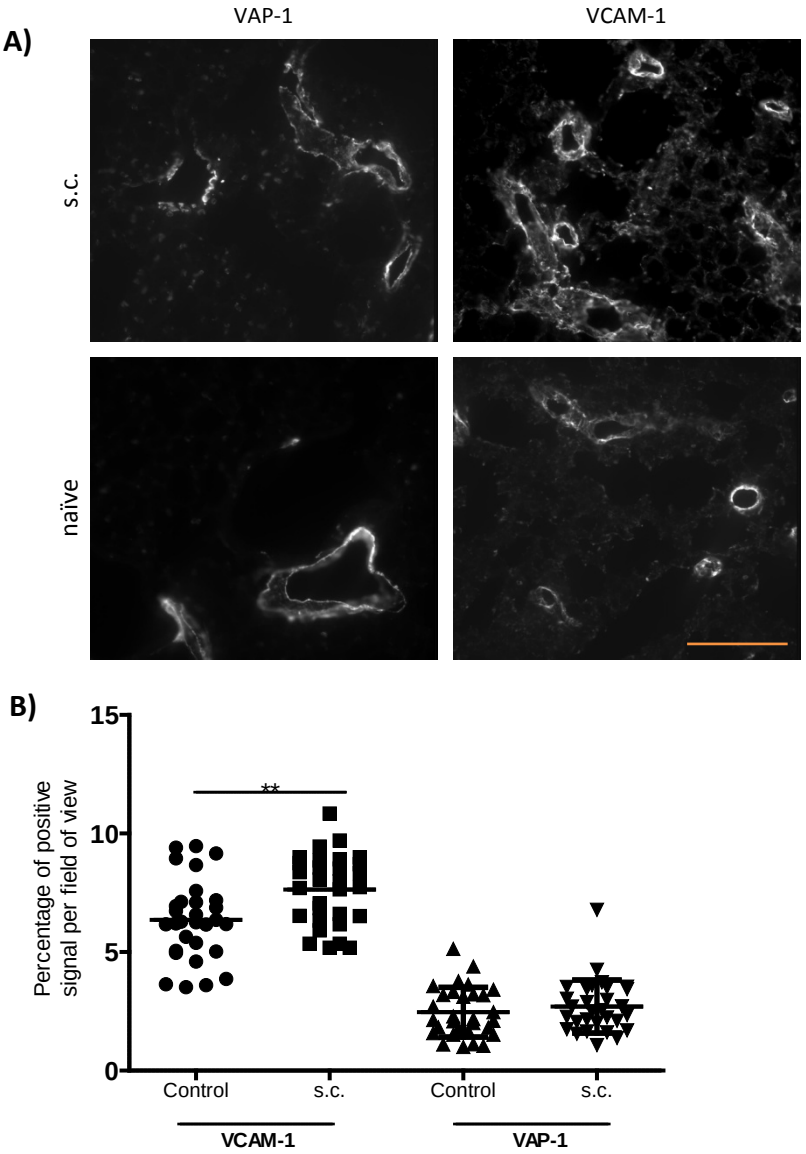


Figure 38: Expression of VCAM-1 and VAP-1 in the pre-metastatic lung
The formation of a pre-metastatic niche was induced by the injection of B16F10 cells sub-cutaneously in C57BL/6 mice. Three weeks later the lungs were harvested and stained for VCAM-1 and VAP-1. (n=3)
A) Representative images of VCAM-1 and VAP-1 staining in lung tissue from either naïve mice or mice with an implanted s.c. tumour. Images acquired by Leica Epifluorescent microscope.
B) Quantification of the percentage of positive signal per field of view. This was measured using ImageJ software, where at least 15 images were analysed per mouse. Statistical analysis for VCAM-1 expression: unpaired t-test; p= 0.0027 (Scale = 100µm)

DISCUSSION

Metastasis is the most devastating aspect of cancer, since more than 90% of cancer-related deaths are proposed to occur due to tumour cell dissemination.[15,43] During the process of cell detachment from the primary tumour, translocation to the secondary tissue and colonization of this site, tumour cells interact with numerous elements of the host system, including endothelial and immune cells. The ability to metastasize is central to the definition of a malignant cancer cell, as was defined by Hanahan and Weinberg in 2000.[125] However the contribution of micro-environmental components in this process has been shown to be critical[140,697] and this led to its recognition in the revised hallmarks of cancer in 2011.[8]

As outlined previously leukocytes have been shown to be involved in almost every step in metastatic dissemination. These include facilitating detachment from the primary site and intravasation into the vasculature, aiding survival in the circulation and facilitating attachment to the endothelium at the secondary site and are also proposed to pave the way for the tumour cells to extravasate into the tissue parenchyma. Moreover, it has been shown that tumour cells manipulate the inflammatory system in such a way as to aid their survival during tissue colonization and contribute to angiogenesis.

This project has focused on the effect metastatic dissemination has on the endothelium at the secondary site. The experimental metastasis models used relied on intravenous injection of different tumour cell lines. These were cultured *in vitro* and upon injection formed metastatic colonies in the lung. Initially the focus was on the induction of expression of different adhesion molecules on the endothelial surface, as occurs in inflammation. Due to the low basal level of adhesion molecule expression, the induction of expression in inflammation and their well established role in mediating leukocyte recruitment and extravasation, E-selectin, VCAM-1 and VAP-1 were investigated. Subsequently the mechanism of induction of endothelial activation was inspected, followed by assessment of the role of endothelial activation in promoting tumour cell metastasis.

1. The endothelial activation antigens do not appear to mediate direct interactions of tumour cells with the endothelium at the metastatic site

Endothelial activation has been implicated in promoting tumour cell adhesion and thereby metastasis in several *in vitro* models where pre-activation of the endothelial cells and thereby expression of various endothelial activation antigens enhanced tumour cell adhesion.[601,697-700] This led to translation of these conclusions into *in vivo* models, in which prior inflammation of the target organ was shown to increase the metastatic burden.[319,346,391,558-560,701,702] For example, injection of TNF-α or LPS has been shown to promote metastasis by Mannel *et al*.[391,559,560] Similarly, the induction of inflammation that acted through NF-κB transcription factor, was shown to promote lung metastasis.[701] Taranova *et al* have shown that allergic pulmonary inflammation also potentiates lung metastasis.[558]

Proof of the powerful effect inflammation has on promoting metastasis has also been seen in clinical situations where surgical trauma has long been recognized to increase metastasis to the sites.[703,704] An interesting observation supporting this also comes from Hirshberg *et al*, who noticed development of metastatic lesions at sites where a tooth had been extracted from previously. This was noted in 55 patients, most commonly with breast and lung cancer.[705]

At the heart of these observations lies the controversy of whether metastatic dissemination is mediated by specific adhesion antigens or whether it occurs passively as a result of mechanical occlusion. The metastasis promoting effects of prior inflammation were shown to be reversed, at least in part, by blocking the interactions between VLA-4 – VCAM-1.[559,618] Similarly blockade of endothelial activation by loss of G-coupled protein in the signaling cascade inhibits metastasis in a lung inflammation model. That this effect is dependent on interactions with VCAM-1 was confirmed in *ex vivo* adhesion assays.[558]

It is important to note however, that in contrast to *in vitro* situations, induction of inflammation *in vivo* has additional effects. In addition to inducing endothelial activation, inflammatory stimuli have other

systemic effects, most notably through modulation of the activity of the immune system. For example in these experiments injection of TNF-α reduces the activity of NK cells.[391,560]

In addition to these experiments, specific sets of adhesion molecules have been proposed to mediate the adhesion of tumour cells to the endothelium and thereby specific recruitment to the tissue.[223,230,319,333,346,367,601,611,615]

Tumour cell attachment and extravasation have proven very difficult to study *in vivo*, due to the lack of available models. Therefore, most of the current research had focused on *in vitro* models, *ex vivo* examinations and ultimately modelling the process on that of leukocyte migration. *In vitro* and *ex vivo* models do not take into account the contributions of the host components (leukocytes and platelets) or the effects of shear flow. Moreover, there is little or no direct evidence that tumour cells undergo the same types of interactions that occur in leukocyte attachment and extravasation. Together, the experiments outlined previously only demonstrate the significance of endothelial activation during metastasis and the role of the immune system. However the implication of endothelial adhesion molecules in directly facilitating tumour cell adhesion to endothelial cells and thereby metastasis is only circumstantial.

In the experimental setup used here, prior to tissue harvest the pulmonary circulation is cleared of blood and any non-adhered cells are removed, therefore the tumour cells assessed at any time are not free in circulation. In previous *in vitro* and *in vivo* experiments, VCAM-1 is the molecule most often implicated in mediating adhesion of tumour cells to the endothelium in the lung.[364,601,603,706] In our experiments however, the low level of VCAM-1 expression detected immediately (15 minutes) after tumour cell injection suggested that VCAM-1 does not function in the process of metastasis in the same role as in leukocyte extravasation, that is in mediating firm arrest. This was confirmed when VCAM-1 was blocked using a blocking antibody and the number of tumour cells present in the lung

tissue was again counted 15 min post injection. No difference was observed in the number of tumour cells adhering in the lung tissue (Supplementary Figure 25).

The observed low basal level of expression of this adhesion protein prior to tumour cell attachment suggested that instead of the VCAM-1 protein acting to facilitate direct adhesion of tumour cells to the endothelium, the tumour cells themselves might be involved in inducing the expression of these endothelial antigens.

In particular, in response to tumour cells, VCAM-1 was only upregulated 1h after tumour cell injection. In contrast, upon LPS stimulation, VCAM-1 expression was already upregulated after 15min. Therefore the delay observed in experimental metastasis assays could not be accounted for by the time required for the regulatory mechanism up-regulating the expression of VCAM-1. Hence the VCAM-1 upregulation observed must have occured as a result of tumour cell attachment.

VAP-1 belongs to a different class of adhesion molecules. Its expression is not regulated at the transcriptional and translational level. Instead, it is stored in intra-cellular granules distinct to Weibel-Palade bodies and upon stimulation is exported to the cell surface.[408,529]

It is important to note that even a short fixation with paraformaldehyde may permeabilize cells. Therefore, at least part of the VAP-1 expression detected was localised to the intra-cellular granules. This was confirmed by the discontinuous appearance of VAP-1 staining. The situation was further complicated by the mode of action of VAP-1 in facilitating adhesion, where its enzymatic activity is central to its adhesive function. Thereby it is likely to be further regulated through its enzymatic activity and based on the expression data alone, the role of VAP-1 in mediating initial adhesion could not be elucidated.

Previous research has implicated VAP-1 in mediating leukocyte recruitment.[335,707,708] However in order to establish its role in facilitating tumour cell interaction with the endothelium, functional analysis is

required; in particular, whether VAP-1 inhibition affects the number of tumour cells that adhere to the pulmonary vasculature shortly after i.v. injection.

In inflammation, selectins act to facilitate initial loose contacts between leukocytes and endothelium that result in a rolling motion along the endothelial surface. However the role of E-selectin in facilitating attachment and rolling of leukocytes on pulmonary endothelium has been questioned.[378,709] In contrast to other tissues in the body, the vessel diameter in the lung vasculature is considered to be too small to allow for margination and rolling movement.

While numerous reports implicate E-selectin in the process of metastasis, [228,300,336,446,551,563,573-583] its contribution to mediating initial contact of tumour cells is unclear.

Arguing against a role for selectins is the finding that rolling motion of tumour cells on the endothelial cell layer is rarely observed.[223,360,367] Additionally, Laubli and Borsig[303,306] showed that E-selectin expression in the lung prior to metastatic dissemination is not crucial in facilitating experimental metastasis. [300,320] In the experimental models used here, E-selectin expression was not detected at basal level. Additionally, its expression was only found to be induced after attachment of human tumour cells. Therefore it appeared unlikely that E-selectin would facilitate interactions and adhesion of tumour cells to the endothelial cell layer in the experimental models used here.

As described in the introductory chapter, tumour cells are also proposed to lodge in the vasculature passively, purely due to anatomical restrictions. While experiments presented here appeared to support this theory, this is also supported by experimental evidence demonstrating arrest by mechanical occlusion, using the same or similar metastatic models that have previously been used to show specific adhesion events.[48,223,355,356,360,710,711] Additionally, tumour cells are widely reported to be much larger than leukocytes and this was also evident in the experimental models used in this project. Together these provide circumstantial evidence that passive trapping of tumour cells in lung microvasculature may indeed occur. Indirect evidence for passive metastatic dissemination can also

be gathered from experiments where normal non-transformed epithelial cells are able to disseminate and embed in the lung vasculature.

According to this hypothesis, after tumour cell injection into the circulation, they would become trapped in the pulmonary vasculature by anatomical occlusion. This process would occur independently of specific interactions with the endothelial activation antigens. However Gassman *et al*[223] proposed that while evidence exists to support both models, the outcome is likely to be affected by the experimental model and technique used. In particular they highlight the significance of the site of injection, density of the cell suspension injected, the animal species investigated and the analysis techniques. Furthermore Martin *et al*[712] highlight the significance of the microenvironment of different organs. Using two-photon microscopy they compared early events in the metastatic process of one cell line to two most common sites of metastasis, the lung and the liver. They found that tumour cells extravasate at a much slower rate from the lung vasculature compared to the liver. They concluded that the difference occurs both due to the differences in the structure of the vasculature and also due to the difference in the interactions with the host cells in the specific microenvironment.

1.1. Role of the immune system in platelets in mediating tumour cell arrest

In physiological inflammation, endothelial adhesion molecules expressed on the luminal endothelial surface facilitate the recruitment, attachment and extravasation of activated leukocytes. As described previously, disseminating tumour cells have been shown to recruit and interact with different classes of leukocytes and are proposed to exploit this interaction to enhance metastatic efficiency. Indeed, the role of leukocytes in mediating metastatic dissemination has been extensively characterised and inhibition of leukocyte recruitment induces a reduction of metastasis (Gil-Bernabe *et al*, unpublished).[691]

In addition to providing a protective role from factors that would induce tumour cell death, leukocytes may also facilitate tumour cell adhesion to the endothelium analogous to the process that occurs in

inflammation. In response to interaction with the tumour cells, leukocyte activation will facilitate the adhesion of this complex to the activated endothelium. Indeed Liang *et al*[596] have shown that aggregation of tumour cells with leukocytes promots interaction with the endothelium. This is mediated via LFA-1 and Mac-1 and dependent on shear stress similar to leukocyte extravasation. Moreover Strell *et al*[342,713] proposed that leukocytes bridge the interactions between the tumour cells and the endothelium.

In this way tumour cells can arrest in the lung in the absence of specific adhesion interactions, as was observed by arrest of untransformed epithelial cells upon injection.[67] However, successful metastasis will then depend on leukocyte recruitment and interactions that will in turn mediate tumour cell attachment, survival and extravasation. Furthermore, such events may also explain that specific adhesion antigen interactions are necessary to mediate tumour metastasis, except that the interactions are bridged through recruited leukocytes. In this way, inhibition of specific endothelial adhesion proteins could result in inhibition of leukocyte recruitment and thus inhibition of metastasis without affecting tumour cell adhesion to the endothelium directly.

The interaction between tumour cells and leukocyte has also been shown to be dependent on platelet recruitment and clot formation, as disruption of platelet activation inhibits the recruitment of leukocytes. The implications of this will be further discussed below.

As mentioned previously, blocking of VCAM-1 function prior to tumour cell injection did not appear to have any effect on tumour cell adhesion to the endothelium (Supplementary figure 25). This could be due to the overlapping roles exhibited between different adhesion molecules in the leukocyte adhesion cascade, where other interactions would have compensated for the loss of the VCAM-1-VLA-4 interface. In order to confirm this hypothesis, several adhesion molecules would have to be simultaneously inhibited.

2. Endothelial activation is induced within hours of tumour cell metastasis

2.1. Induction of VCAM-1 expression

In the three different experimental metastatic models used, expression of endothelial activation proteins was induced on pulmonary endothelium following tumour cell injection. The most pronounced endothelial activation was observed for VCAM-1 expression (Fig 5&10). This is consistent with the level of expression induction observed in the inflammatory model (fig.3), where LPS injection caused induction of VCAM-1 expression immediately upon injection.

As outlined previously, in most tumour models there was a delay in VCAM-1 induction, where significant upregulation was only observed 1h post-injection and not immediately after tumour cell injection. As expected, there was some variation between the different cell lines. This appeared to correlate with the efficiency of tumour cell arrest and metastasis. For example the MDA-MB-231-GFP cells embeded in the lung parenchyma more efficiently (Supplementary figure 12) and in this model VCAM-1 expression was induced sooner. This further confirmed that the tumour cells themselves are involved in inducing endothelial activation.

Throughout the time-course analysed, VCAM-1 expression was assessed in relation to the tumour cell/ colony. As VCAM-1 expression was only analysed in the peritumoural region (within 80µm), no suitable internal control exists for this type of experimental data, where endothelial activation could be measured in the absence of metastasis. Therefore a more quantitative method would need to be developed, which would allow us to compare the level of endothelial expression upon tumour cell challenge to tissue expression in absence of metastatic colonies.

Furthermore if a suitable quantitative method could be established, this may also enhance the analysis techniques. Instead of the binary classification used here, using a quantitative technique

could provide information on the overall level of induction of expression of endothelial activation antigens.

However in contrast to systemic inflammation, analysis of overall VCAM-1 expression in response to tumour cell injection may be problematic. According to our data, VCAM-1 induction occured in response to tumour cell challenge; hence the total level of VCAM-1 protein in a lung would be correlated to the total number of tumour cells per lung. These declined rapidly over the investigated period (Supplementary figure 12) and such analysis could result in demonstrating VCAM-1 down-regulation even when VCAM-1 expression was maintained relative to the tumour cell. Therefore the measured level of expression of adhesion proteins would need to be normalized to the number of metastatic cells in the tissue.

In order to establish a more quantitative approach, *ex vivo* imaging was tested as well. Here under terminal anaesthesia anti-VCAM-1- Alexa Fluor 647 antibody was injected, into the vena cava 5min prior to lung harvest. However, most likely due to the low level of expression of VCAM-1, this technique proved not to be sensitive enough (Supplementary figure 26). If this could be optimised, stochastic binding of the antibody to the VCAM-1 protein could be correlated to the overall amount of VCAM-1 protein present. Furthermore as the whole lung tissue was imaged *ex vivo,* the overall level of the protein could be assessed.

These data are consistent with the work of Langley *et al*[618] who demonstrated that VCAM-1 expression is not required for pulmonary metastasis in a spontaneous metastatic melanoma model. Furthermore, they observe VCAM-1 upregulation after establishment of metastasis, as was demonstrated in the experimental models used here (Fig.10).

Induction of VCAM-1 expression occurred in a bi-phasic manner. The first phase encompassed the initial 24h. At this time VCAM-1 expression was induced within 1h of tumour cell entry into the circulation. This reached a maximum level at 8h and was maintained at this level throughout the 24h.

At the next time-point assessed (72h) the expression level of VCAM-1 had decreased and this was consistent across all experimental models of metastasis. However, the level of VCAM-1 expression was still found to be higher than at resting state. This suggests that while there is an attenuation of endothelial activation 72h after tumour cell attachment, it does not return to the resting state. Whereas the significance of this biphasic immune reaction is unclear, biphasic regulation is not an uncommon phenomenon in the systemic immune reaction to different pathogens. A suppression of the immune response has been observed 24h after infection and initial immune activation.[714] Therefore it is tempting to speculate that the two peaks could correspond to two phases in metastatic development (tumour cell arrest and extravasation; metastatic colonization) and hence two separate corresponding immune reactions.

2.2. E-selectin expression

In contrast to induction of VCAM-1 expression, E-selectin expression was only observed in response to challenge by human tumour cell lines. It is possible that this occured as a result of an immune reaction against human antigens expressed on the surface of circulating tumour cells. Despite using immunodeficient animals lacking the adaptive immune system, the antigens expressed on the surface of these tumour cells could elicit an immune reaction distinct to that induced by a disseminated tumour cell syngeneic to the strain. This in turn would result in induction of E-selectin on the pulmonary endothelium. Such a phenomenon would also explain why E-selectin expression induction was not observed in response to 4T1-GFP cells.

Another possibility is that as there is a wide variation in the different antigens expressed on the surface of different tumour cell lines, the interactions with the endothelium might differ as well. In particular, such distinctions have been made between different types of melanomas.[578,700,715,716] These have been shown to differ in relying on different adhesion proteins to facilitate their attachment to the endothelium, dependent on the expression of corresponding ligands on their surface (e.g. Sialyl Lewis x – negative melanoma cell line depends on VLA-4 for adhesion[700]). It stands to reason therefore that the effect these have on the endothelial cells might differ as well, which could account for the different endothelial activation profiles.

E-selectin expression was induced at a slower rate compared to VCAM-1 expression. This difference was accounted for by the dynamic of its expression regulation assayed in response to LPS. There VCAM-1 was upregulated 15min after stimulation, while E-selectin expression could only be detected 2h post injection. The induction of expression of both antigens could be induced by the same stimulus, however due to differential regulation of their expression the two adhesion molecules are induced at different times.

Moreover, E-selectin was expressed only transiently, whereas VCAM-1 expression was sustained

throughout the 24h. Such a difference was also observed in the inflammatory model when E-selectin upregulation was at a much lower level compared to VCAM-1 and occurred transiently, returning to resting state within 8h.

This transient nature of E-selectin upregulation supported the idea that it might indeed be a result of an inflammatory reaction in response to human tumour antigens, rather than a result of active induction by the tumour cells.

Another possibility has also been suggested through previous research. Khatib *et al,*[576] Mendoza *et al*[654] and Auguste *et al*[583] have shown that in response to tumour cell metastasis to the liver, local production of TNF-α results in induction of E-selectin. This in turn leds to the induction of VCAM-1 expression in the liver vasculature. If this was the case, E-selectin expression and receptor engagement represented the trigger that facilitates further endothelial activation and expression of other proteins. Such a model may account for leukocyte extravasation in most tissues, where the first contact between leukocytes and the endothelium forms via selectin-mediated rolling. Engagement of these receptors would then induce further activation of the endothelial cells and expression of other adhesion proteins to aid in tighter adhesion and extravasation.[576,583,654]

Similarly other studies have demonstrated endothelial activation in metastasis establishment in the liver.[306,577,583] Most implicate E-selectin upregulation to enhance tumour cell adhesion and arrest. This is especially interesting as E-selectin is not proposed to have a major role in mediating leukocyte recruitment and extravasation in this organ. However the role of selectins in pulmonary endothelium may be different. The size of lung microvasculature cannot support selectin – mediated rolling during the process of leukocyte extravasation. Due to the significant differences in the structure of the endothelium and the microenvironment between different organs,[712] the role of E-selectin in lung metastasis is likely to be different to that described in the liver.

In the metastatic models used in this project, E-selectin was only up-regulated by human tumour cell lines and is therefore less likely to represent a crucial adhesion molecule in this process. Moreover for the most part VCAM-1 upregulation preceded that of E-selectin. Therefore E-selectin did not appear to represent the initial trigger required for further endothelial activation.

2.3. VAP-1 expression in response to metastasis

The relationship between metastatic dissemination and regulation of VAP-1 protein expression was more difficult to elucidate compared to other adhesion molecules. In a naïve mouse a persistent basal level of VAP-1 expression was observed. This may be due to the detection of VAP-1 stored in intra-cellular vesicles, although it is also possible that VAP-1 is expressed at this level in the resting state. Salmi and Jalkanen report that VAP-1 is expressed on pericytes, smooth muscle cells and fat cells, while endothelial cells in the vasculature of most organs and leukocytes do not exhibit any VAP-1 activity.[529] This would suggest that the VAP-1 detected here is indeed intra-cellular and not available to the luminal surface.

While VAP-1 appeared to be upregulated in response to metastasis, in LPS induced inflammation, an increase was only observed 6-8h after stimulation. This is especially striking when compared to VCAM-1 and E-selectin, which were upregulated 15min and 2h post injection, respectively. Similar dynamics were observed in response to metastasis, where the increase in VAP-1 expression occurred slowly. In the same manner as observed for VCAM-1 it appeared to peak at 8h. However in contrast to VCAM-1, it decreased again to basal levels by 24h. Unfortunately the variation between the different samples at a given time-point was extraordinarily high. This was in stark contrast to the data for VCAM-1 expression. It is plausible that if intra-cellular VAP-1 represents part of the total VAP-1 expression assayed, this may be responsible for the variability observed. As the expression would not be related to tumour cell dissemination, its distribution would result in a high standard deviation.

Additionally, an increase in the expression of VAP-1 does not necessarily signify that the protein is active. As its role in mediating endothelial adhesion and facilitating interactions depends on its enzymatic activity, this should be assayed via zymographic techniques. In this way the availability of active VAP-1 could be assayed in response to tumour cell metastasis.

2.4. Endothelial activation during metastatic dissemination is (at least in part) induced by platelet clot formation

There are several possible candidates that could influence the endothelium during the metastatic process. While it was clear that the endothelial activation antigens were induced in response to tumour cell challenge, this could occur in response to soluble factors released by the tumour cells, either via direct interaction with the tumour cell or indirectly, where tumour cells act to activate other components (platelets, leukocytes) which would then proceed to trigger endothelial activation.
In the experiments presented, endothelial activation/ VCAM-1 expression was not exclusively localised to the site of metastasis, therefore it was clear that simple interaction between the tumour cell and endothelial cells could not account for the phenomenon observed.

Endothelial cells have been shown to become activated in response to different pathogens and a wide range of active substances including interleukins-1, -6, -8, -12, TNF-α, INF-γ. [339] An example of an active substance released by the tumour cells that could induce endothelial activation is nitric oxide,[717,718] and nitric oxide synthase (NOS) activity has been detected in tumour cells of different types of cancer. Moreover, tumour cells have been shown to secrete a wide range of cytokines, chemokines and other bioactive substances including IL-12, CCL5, VEGF, PDGF.[8,719,720] These have been proposed to mediate the “pro-metastatic” state by facilitating recruitment of lymphocytes that facilitate tumour cell survival, attachment and extravasation. In addition, tumour cell secreted cytokines are also proposed to facilitate immune evasion from tumour suppressive lymphocytes. Therefore it is likely that such

bioactive substances may also act on the endothelial cell layer to induce its activation.

While in the circulation disseminating tumour cells interact with and recruit different cells of the immune system. This leads to the activation of the recruited leukocytes and thereby secretion of different cytokines. In the experiments performed by Khatib *et al*[576] liver metastasis results in local secretion of cytokine (IL-1 and TNF-α) that facilitates endothelial E-selectin expression induction. Similarly Auguste *et al* [583] have shown that upon tumour cell metastasis in the liver, resident specialised macrophages – Kupffer cells release TNF-α which results in endothelial activation and upregulation of E-selectin and VCAM-1.

Upon entry into the circulation, several cell lines have also been shown to induce platelet activation and aggregation.[236,239] Platelet activation leads to the released of different bioactive substances and platelets have three different types of granules, released upon stimulation. These contain a wide range of proteins and small molecules (VEGF, CCL5, IL-1) that have been shown to modulate vascular inflammation.[681,721]

In addition to platelets, the leukocyte population recruited to the site of metastasis has previously been shown to promote metastasis (Gil-Bernabe *et al*, unpublished).[664] These metastasis promoting effects were shown to be dependent on the expression of CD11b on the leukocyte surface and the induction of clot formation by the tumour cells (Gil-Bernabe *et al*, unpublished). Using two transgenic models where two leukocyte receptors were tagged with the green fluorescent protein (fractalkine receptor and CSF-1 receptor), the leukocyte population recruited to the tumour cell was characterised. The interrupted receptors had previously been shown to have no effect on recruitment and clustering to the tumour cell (Gil-Bernabe *et al*, unpublished). The population recruited by the tumour cell was shown to be mainly $CD11b^+$, $F4/80^+$, $CD68^+$, $CD45^+$, $CD11c^-$, $Gr\text{-}1^-$ and $CD3e^-$. This showed that the cells recruited by the tumour cells are of myeloid lineage ($CD3^-$, $F4/80^+$, $CD68^+$). As they did not express Gr-1 or CD11c to any extent, these cells are most likely not neutrophils or

dendritic cells. Moreover, as the myeloid cells appeared to express F4/80, CD68 and CD11b, they were proposed to belong to the monocyte/macrophage population.

When these markers were translated to the WT animals and experimental metastasis of 4T1-GFP and B16F10-CMFDA cells was induced, the recruited population was found to be essentially the same. The only difference occurred in the 4T1-GFP cell model, which was shown to recruit an extensive Gr-1 positive population. This was not surprising as the 4T1-GFP metastatic model has been shown to elicit a strong myeloid reaction commonly with an associated hepatosplenomegaly.[722] Previous research reports recruitment and expansion of Gr-1^{+} and CD11b^{+} myeloid populations with granulocyte appearance in response to 4T1-GFP metastasis. These cells have been termed myeloid derived suppressor cells (MDSCs) due to their proposed role in suppressing T-cell responses. [677,723,724]

Interestingly, extensive MMP9 deposition was also only observed in the 4T1-GFP cell model. Therefore it appears likely that the MDSCs are responsible for the vast majority of MMP9 secretion.

The 1205LU-GFP cell line did not express tissue factor (Supplementary Figure 16) and therefore did not elicit significant platelet activation or clot formation. As expected the recruitment of the leukocyte population was therefore also disrupted.

Previous research has demonstrated that in the experimental metastasis models used here, maximum recruitment of leukocytes to the tumour cells occurs 8h after i.v. injection (Gil-Bernabe *et al*, unpublished). Likewise, induction of endothelial activation also peaked around 8h. Furthermore, VCAM-1 expression was shown to occur nearby the formation of these tumour cells – leukocyte complexes. Based on these observations the recruited leukocyte population represented a likely candidate for the trigger of endothelial activation. This would also be in line with the data obtained from liver metastasis models. Here tumour cell metastasis results in macrophage activation and subsequent endothelial activation.[576,583] As well as the formation of leukocyte clusters around the

tumour cells, platelet activation and clot formation has been shown to induce endothelial activation in inflammatory settings.[681,721,725]

Therefore the hypothesis postulated that the first trigger for endothelial activation would be provided by a combination of factors released by the tumour cells, the factors released by activated platelets recruited by the tumour cells and through the physical contact this tumour cell –platelet complex forms with the endothelial cell layer. Subsequently, factors released by the tumour cells and platelets would act to activate and recruit specific subsets of leukocytes. Here endothelial adhesion receptors expressed on the luminal side would facilitate the attachment of these leukocytes, which would in turn lead to further leukocyte activation and instigate their secretion of inflammatory mediators; thereby propagating the endothelial activation and further inflammation.

E-selectin expression was absent in the syngeneic experimental metastatic models used here and VAP-1 expression analysis was complicated by high variation and post-translation regulation. Therefore in the experiments determining the origin of the endothelial activation the research focused on the analysis of VCAM-1 expression. Previous research has demonstrated that CD11b/CD18 is crucial in facilitating the recruitment of leukocytes to the site of metastasis (Gil-Bernabe *et al*, unpublished).[268, 726] Using a Mac-1KO animal model, where leukocyte recruitment is significantly reduced and metastasis formation attenuated, no difference was observed in the expression of VCAM-1. This confirmed that, at least in this model, leukocyte activation and recruitment was not involved in the induction of endothelial activation.

When platelet activation and aggregation was disrupted, either by inhibition of thrombin or tissue factor, there was a significant decrease in the level of VCAM-1 expression. This proved that, at least in part, endothelial expression of VCAM-1 was induced through platelet activation and aggregation. Injection of hirudin (thrombin inhibitor) was extremely efficient in disrupting platelet aggregation. Previous research established that this also disrupts the recruitment of leukocytes (Gil-Bernabe *et al*,

unpublished). This was also the case in the experimental models used here, as visualised by CD11b and CD45 cell staining. Moreover, anti-coagulation also inhibited aggregation of Gr-1^{+} cells in the 4T1-GFP cell model. This suggested that platelet activation appears to be crucial in mediating the recruitment of this population as well. In the 4T1-GFP model, VCAM-1 expression was decreased upon hirudin treatment. However the same treatment had no effect on VCAM-1 expression in the 1205LU-GFP metastatic model. As platelet activation is absent in the 1205LU-GFP model, it is safe to conclude that the decrease in VCAM-1 expression observed in the 4T1-GFP model is in fact due to disruption of platelet activation and aggregation. Inhibition of tissue factor by tissue factor pathway inhibitor disrupts platelet aggregation much less efficiently than inhibition of thrombin. This is most likely why only a small decrease can be observed in expression of VCAM-1 in response to TFPI.

In summary the data suggested that upon tumour cell entry into the circulation, the tumour cells trigger platelet aggregation and activation that contributed to stimulation of endothelial activation. Platelet clot formation has also been proven crucial in mediating leukocyte recruitment and that population has been shown to promote tumour cell survival, extravasation and colonization (Gil-Bernabe *et al*, unpublished). The role of platelets in promoting metastatic potential has been well established.[236,266] The formation of a clot has been shown to promote survival of tumour cells either by shielding them from natural killer cell activity or independently of it. [264] In addition platelets have been shown to promote tumour cell adhesion and thereby metastasis[224,239,307,727] although Terraube *et al*[323] propose that their effects extend further to also mediating colonization.

In inflammation, the interaction between activated platelets and leukocytes has been shown to increase leukocyte adhesive properties and enhance their rolling and attachment to the endothelium.[342,441,721,728] It is possible that similar events may also take place during metastasis. Previous work has already demonstrated that disruption of platelet aggregation prevents leukocyte recruitment. In dissemination, activated platelets could thereby mediate leukocyte recruitment and

adhesion via several mechanisms. Firstly, bioactive proteins and small molecules secreted by platelets upon activation by the tumour cell could act on leukocytes and provide a chemotactic gradient. Secondly, direct interactions between the clot and leukocytes and subsequent enhancement of their migratory and adhesive properties, would promote firm adhesion of the metastatic cell complex.[306,379] Additionally, activated platelets have been shown to contribute to endothelial activation. Data presented here suggest that this does not affect tumour cell adhesion directly. Instead, it could act to facilitate recruitment and attachment of a specific leukocyte population. Moreover, platelet stimulation of the endothelial cell layer has also been shown to induce secretion of MCP-1 by the endothelial cells. Therefore the endothelial cell layer may also contribute to the active process of leukocyte recruitment.[721] This is in line with the findings of Laubli *et al*[729] who demonstrated that endothelial activation in metastasis results in upregulation and secretion of CCL5. This in turn facilitates recruitment of leukocytes to the site of metastasis and when this CCL5-dependent recruitment is inhibited, attenuation of metastatic dissemination occurs.

Even upon treatment with hirudin and thrombin inhibition, a significant level of VCAM-1 expression could be observed that is higher than at the basal level. One possibility is that because thrombin inhibition only inhibited platelet aggregation, cross-linking and clot formation, but not activation of the earlier enzymes in the coagulation cascade, a certain level of platelet activation by other factors in the cascade could still occur. In this way factors released from the platelet granules could facilitate VCAM-1 expression induction. This would seem unlikely however, because the coagulation cascade and clot formation are mediated through an amplification chain of reactions. The initial event of the cascade (exposure of tissue factor to the blood microenvironment) represents only the trigger for a series of subsequent reactions. This is then propagated through a pathway of reactions to facilitate efficient clot formation. Therefore, any platelet activation that occurs as a result of tumour cell interaction in the absence of coagulation cascade activation may be to be too small to account for the

induction of VCAM-1 observed. Consequently, it is likely that tumour cells themselves are able to induce endothelial activation directly, either via direct contact or by secretion of effector molecules.

The time required for the tumour cells to extravasate from the circulation can vary widely. Moreover, it will depend on the structure of the vasculature and therefore differ significantly between different tissues and sites of metastasis.[712] Additionally certain tumour cells have been reported to proliferate within the blood vessels(Supplementary figure 27).[365,374]

The leukocyte population recruited to the tumour cells has been shown to promote their survival through the initial 24h. Previous research has demonstrated that after this time immune cells recruited to the tumour cells start to disperse. Equally by 72h the expression of endothelial adhesion proteins has decreased.

Certain reports propose a role for leukocytes in also mediating tumour cell extravasation, where tumour cells follow extravasating leukocytes.[136,268,339,595-597,599,730] Taking all of this together, a hypothesis can be proposed, where the initial phase of endothelial activation (VCAM-1 expression) over the first 24h coincides with the first wave of myeloid cell recruitment through platelet activation. These recruited cells act to promote tumour cell survival and extravasation. Upon tumour cell extravasation the platelet clot is dissolved, resulting in the loss of endothelial activation stimuli and a decrease in VCAM-1 expression.

3. Endothelial activation antigens facilitate the recruitment of leukocytes

In order to investigate whether the role of endothelial activation antigens facilitated the process of metastasis or whether endothelial activation only occured as a by-product of platelet activation, their function was blocked. The adhesion properties of VCAM-1 were sterically hindered by binding of an antibody, while VAP-1 enzymatic activity was blocked by use of a small molecule enzymatic inhibitor (Proximagen). This allowed me to examine whether endothelial activation antigens promoted tumour cell survival in pulmonary metastasis.

Upon inhibition of these receptors, metastatic dissemination was less efficient and the number of tumour cells surviving in the lungs after 24h decreased significantly. Furthermore, when recruitment of the myeloid cell population was investigated, inhibition of the adhesion molecules resulted in a significant decrease in the number of leukocytes recruited. This signified that, while the CD11b antigen expressed on the leukocyte surface was crucial for the platelet clot – mediated recruitment of myeloid cells (Gil-Bernabe *et al*, unpublished), the recruitment and clustering was also facilitated by endothelial expression. Furthermore, the inhibitory effects on metastatic dissemination of blocking the endothelial activation antigens were mediated via the inhibition of recruitment of pro-metastatic leukocytes.

Based on these data a model can be established (Diagrams 2&3). Once tumour cells gain entry into the circulation, exposure of tissue factor expressed on their surface to the factors of the coagulation cascade triggers thrombin cross linking and platelet clot formation. The formation of this complex between the tumour cells and platelets together with inflammatory mediators released as a result, acts on the endothelium and induces endothelial activation. In the models of experimental metastasis used in this project, the endothelial activation molecules shown to be significant were VCAM-1 and

VAP-1. Furthermore, the tumour cell – platelet complex acts to recruit a subpopulation of myeloid cells and this recruitment was previously shown to depend on CD11b. Here, the recruitment and attachment of leukocytes to the site of metastasis proved to be dependent on the activated endothelial cell layer, both VCAM-1 and VAP-1.

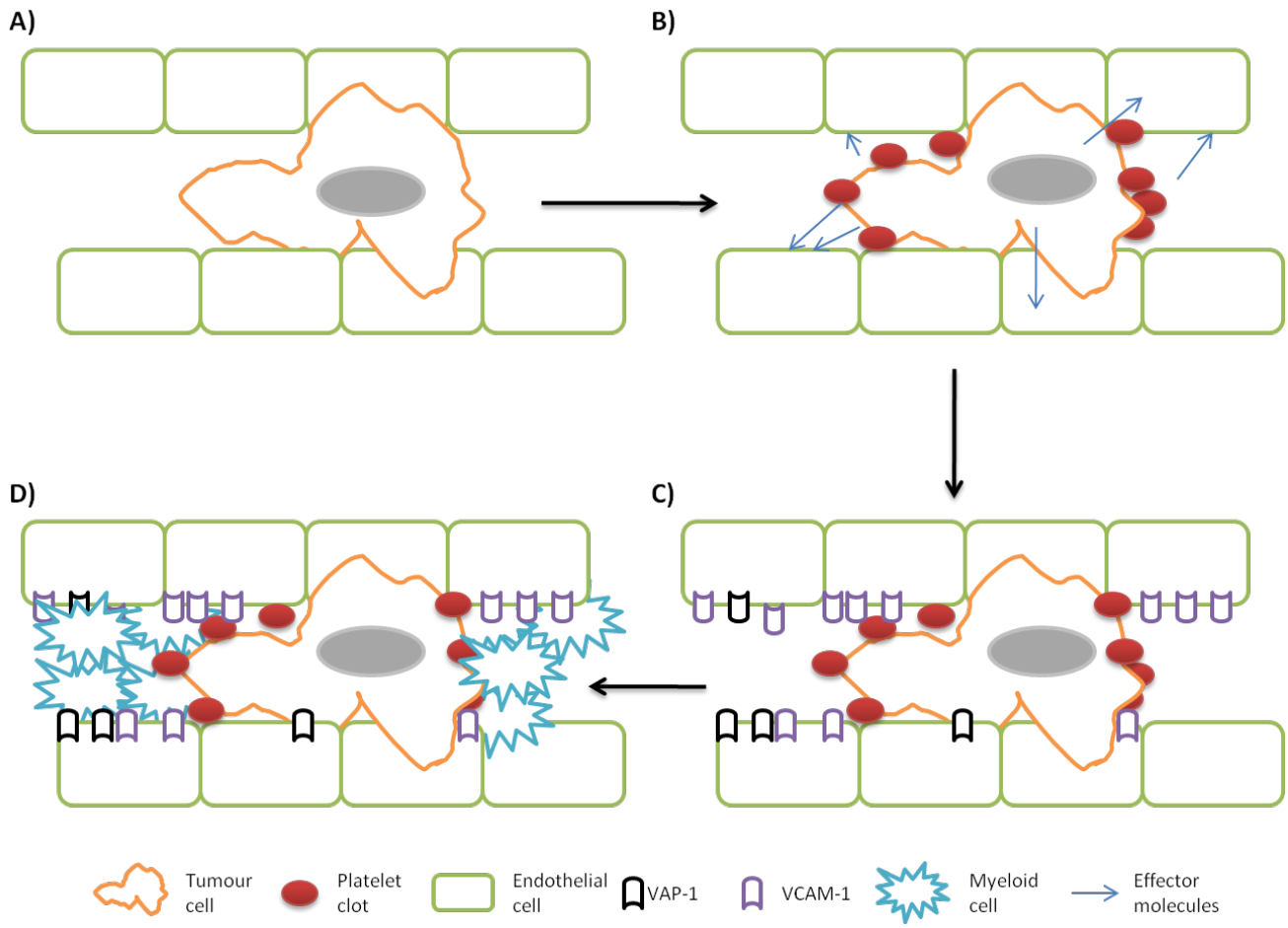


Diagram 2: Tumour cell interaction with platelets induces endothelial activation and mediates recruitment of leukocytes

Tumour cell intravasation/entry into the haematogenous circulation (A) and exposure of the tissue factor to the factors of the coagulation cascade present in the blood stream results in the activation of the coagulation cascade and the formation of platelet clot around the tumour cells (B). These shield the cell from NK cells, shear stress and promote tumour cell survival by providing adhesive surface and possibly through secretion of mitogenic molecules. The tumour cell – platelet complex act on the endothelial cell layer and induces endothelial expression of VCAM-1 and VAP-1. (C) The combination of secreted factors and endothelial activation facilitates the recruitment and adhesion of myeloid cells (D). These act together to promote tumour cell survival and metastasis.

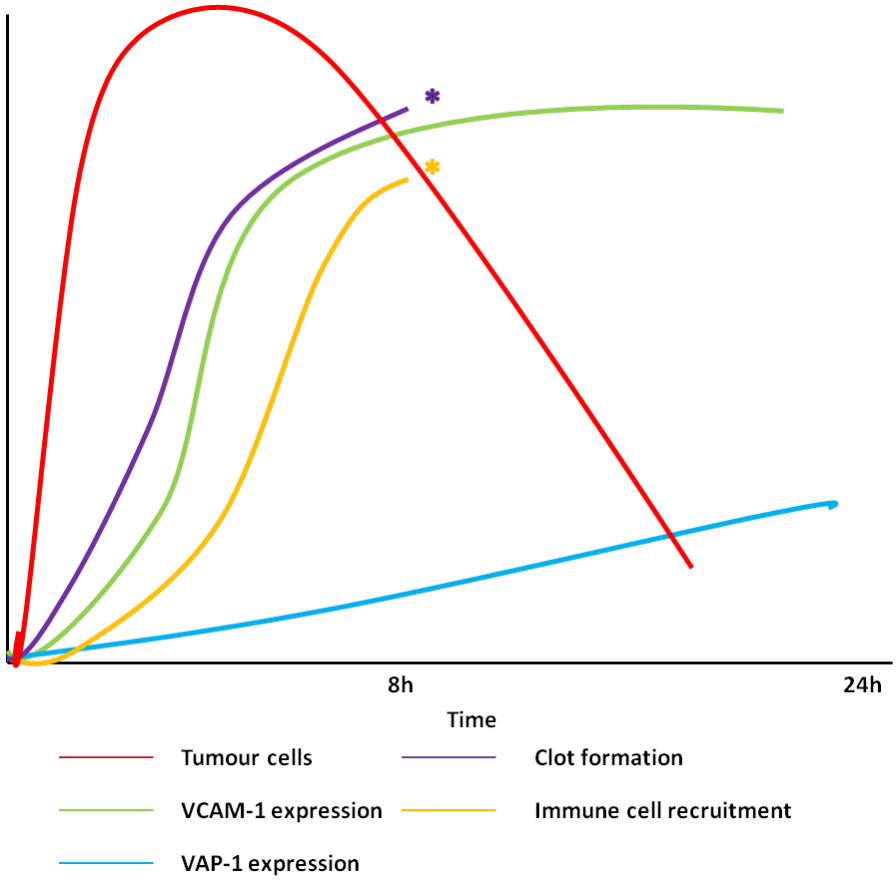


Diagram 3: The proposed time-course of tumour cell interactions with platelet, induction of endothelial activation and recruitment of leukocytes

This diagram represents the proposed time-course of the events described in Digram 2.Upon tumour cell injection into the blood stream these are retained in the lung within the first hours (red). Next within the first 24h (either through clearance by the immune system or through apoptosis) the overall number of metastases in the pulmonary circulation declines dramatically. Once in the circulation, a platelet clot forms around the tumour cells (purple). The complex of tumour cell and platelet clot then induces the expression of VCAM-1 on the endothelial cell surface (green). Together platelet activation and clot formation and endothelial activation facilitates the recruitment of the monocyte/myeloid population. The tumour cell-platelet clot complex may also induce expression of VAP-1 (blue), but this is a much slower response compared to VCAM-1 expression induction. The * denotes the interruption of the time-course, as clot formation and leukocyte recruitment were only monitored at 8h.

The idea that endothelial activation antigens support metastasis through the recruitment of myeloid cells is supported by several pieces of evidence. Previous work has demonstrated the crucial role of the CD11b-expressing myeloid lineage in promoting tumour metastasis where depletion of either CD11b molecules or CD11b-expressing cells reduce metastatic dissemination (Gil-Bernabe *et al*, unpublished). Inhibition of endothelial activation inhibited recruitment of $CD11b^+$ cells, therefore the inhibitory effects of VCAM-1 and VAP-1 blocking must be mediated through this pathway. Secondly, no effect was observed on the survival of the 1205LU-GFP cell line. As mentioned previously, these cells did not express the tissue factor protein and were therefore unable to trigger the activation of the coagulation cascade and the recruitment of leukocytes. As inhibition of VAP-1 had no effect on the survival of these cells, the positive effect on metastatic dissemination exerted by the adhesion protein must be through recruitment of the leukocyte population. Additionally the beneficial effects of inducing inflammation (and thereby endothelial activation) described previously, were shown to be due to the recruitment of macrophages by Stathopoulos *et al*.[701] They have demonstrated that concurrent induction of inflammation and depletion of the macrophage population results in an attenuation of metastasis enhanced by inflammation.[701]

However, in order for this model to be proven experimentally, inhibition of VAP-1/VCAM-1 and depletion of CD11b should be performed. Such double inhibition should have no further effect on the efficacy of metastasis, thereby confirming that the VAP-1 adhesion proteins facilitate metastasis through the recruitment of monocyte populations. Inhibition of both VCAM-1 and VAP-1 had no further effect on the formation of platelet clots. A decrease in platelet clot formation was only observed in the 4T1-GFP metastatic model upon VAP-1 inhibition. As VAP-1 expression is not reported on platelets, it was unlikely therefore that VAP-1 inhibition would work on platelets directly. Furthermore, the effect was only observed in one metastatic model. It was also possible that the smaller platelet clot formation occurred as a result of a feedback mechanism due to a lower number

of recruited leukocytes. As they have been reported to express tissue factor,[272,278] clot formation induction by the activated leukocytes may have contributed to clot formation around the tumour cells. Furthermore, reduction in leukocyte recruitment and subsequent damage to the tumour cell may have also resulted in it inducing a lower platelet clot formation. In line with this was the observation that this was only observed upon VAP-1 inhibition and not VCAM-1 blocking, since VAP-1 inhibition appeared more effective in the inhibition of leukocyte recruitment. Moreover, the effect was only observed in the 4T1-GFP cell line that has been shown to induce a particularly extensive immune response and recruitment.[677,678]

While VAP-1 inhibition also significantly reduced the recruitment of MDSCs by 4T1-GFP cells, the effect of VCAM-1 blocking antibody on this cell population was less pronounced. This could simply be due to the smaller role VCAM-1 appeared to play in mediating the recruitment of the general leukocyte population compared to VAP-1. On the other hand it was also possible that the recruitment of the MDSC cell population was less dependent on the endothelial antigens than other leukocytes.

As mentioned previously, VAP-1 inhibition was much more effective in inhibiting leukocyte recruitment and metastatic dissemination compared to VCAM-1 blocking antibody. It is possible that VAP-1 plays a bigger role in mediating leukocyte attachment and extravasation. However, it is more likely that the function of VAP-1 protein is placed higher in the inflammatory cascade.
The fact that endothelial adhesion proteins do not act in isolation has previously been described. For example monocyte engagement of one endothelial adhesion molecule may also increase interaction with other adhesion proteins by affecting a conformational change.[731] Furthermore, expression and interaction of E-selectin has been shown to induce VCAM-1 expression in liver metastasis.[583] Particularly interesting was the observation by Jalkanen *et al*, where they reported that activation/induction of VAP-1 can trigger expression of other endothelial activation proteins, where

engagement of VAP-1 results in up-regulation of both E- and P-selectin. Moreover this is dependent on the enzymatic activity of VAP-1 and is required to support leukocyte binding and recruitment.[553]

VAP-1 is an enzyme as well as an adhesion protein and its enzymatic activity is central to its function. Thereby its ability to induce endothelial expression of other proteins can occur through outside-in signalling upon VAP-1 binding or as a result of its enzymatic activity, since the product of the reaction is a biologically active substance. This may act on endothelial cells to induce the expression of other proteins like VCAM-1. If this was the case, following recruitment of the myeloid cells, their engagement and interaction with VAP-1 would provide the substrate for enzymatic reaction and so stimulate endothelial activation. As VCAM-1 upregulation was observed much sooner that extensive leukocyte recruitment, other substrates for VAP-1 interactions are likely to exist.

In order for the relationship between VAP-1 and VCAM-1 to be confirmed, the level of VCAM-1 expression should be assessed in response to VAP-1 inhibition. However the observation that double inhibition of both VCAM-1 and VAP-1 had no additional inhibitory suggested that the two functioned in the same pathway and facilitate the same mechanism exploited by the tumour cells.

In contrast to other metastatic tumour cell models used, the 1205LU-GFP cell line did not appear to exploit platelet clot formation and myeloid cell recruitment to promote its survival, however VCAM-1 induction was still observed. When compared to the 4T1-GFP cell line, the overall level of VCAM-1 expression was slightly lower. The level of VCAM-1 induction displayed by the 1205LU-GFP cells may therefore represent the proportion of VCAM-1 expression that occured as a result of direct effects of the tumour cells.

No difference was observed in the level of expression of VAP-1 in response to 1205LU-GFP cells (one-way ANOVA $p=0.0578$). This was not unexpected as the activity of VAP-1 was found most likely not to be regulated through its expression in other metastatic models as well. As mentioned previously,

there was a high level of variation between animals and any VAP-1 protein detected may not necessarily have been available at the membrane or be biologically active. Despite the difficulty in establishing whether tumour cell metastasis and concurrent platelet activation affect the level of VAP-1 expression, its role in promoting tumour cell metastasis was assessed through its inhibition. Inhibition of VAP-1 enzymatic activity had no effect on the metastatic dissemination of 1205LU-GFP cells. This again confirmed that the effects of VAP-1 in promoting metastasis were exerted within the model outlined above, where VAP-1 expression/bioavailability was exploited by the tumour cell – platelet clot complex to facilitate recruitment of pro-metastatic leukocyte populations. Therefore, even though the level of active VAP-1 was difficult to estimate through immunohistochemistry, as it could also be detected in intra-cellular compartments and the antibody also recognised enzymatically inactive protein, the fact that VAP-1 inhibition had no effect on 1205LU-GFP dissemination confirmed that VAP-1 activity in the process of metastasis acted through recruitment of myeloid cells to promote metastatic cell survival.

Interestingly, even though in the 1205LU-GFP cell model extensive platelet clot formation or myeloid cell recruitment was not induced, these cells were still able to metastasize successfully. Therefore a second mechanism must exist to mediate their survival. It is tempting to speculate that this mechanism may include E-selectin expression, as this was observed to be induced within a few hours of 1205LU-GFP cell attachment. However in the long term-metastatic assay, where lung colonization was assessed 6 weeks after injection, the 1205LU-GFP cell line was much less successful at the colonization compared to 4T1-GFP cells (Data not shown). This could pinpoint the crucial role of platelet clot formation and the formation of tumour cell – platelet clot – monocyte complexes in mediating successful metastatic dissemination.

3.1. Endothelial activation in the premetastatic lung

In the pre-metastatic phase, the primary tumour cell mass has been shown to condition secondary sites to enhance metastatic dissemination. Tumour cells at the primary site have been shown to secrete a wide range of proteins and these are proposed to act on both the endothelium and the myeloid cells at the secondary site.[690,691,693,694,696,732] This results in the recruitment of CD11b-expressing myeloid cells in the lung and a subsequent increase in metastasis formation.
The striking effects of the primary tumour secretions were also demonstrated by McAllister *et al* where a subcutaneously implanted carcinoma is able to instigate and support the growth of an otherwise indolent cell line when it is implanted subcutaneously in the opposing flank. The stimulatory effects are accompanied by myeloid cell recruitment and are dependent on osteopontin (OPN).[733] These events are parallel to the endocrine effects observed by others in the pre-metastatic niche establishment.

While the proteins secreted by the primary tumour to induce the formation of such a niche are still largely unknown, several candidates have been implicated. Erler *et al* have shown that secretion of lysyl oxidase (LOX) by the breast tumour cell is essential to mediate $CD11b^+$ cell recruitment. Inhibition of LOX results in inhibition of both metastatic dissemination and recruitment of myeloid cell populations.[696] Hiratsuka *et al* have shown that tumour cell release of VEGF-A, TGF-β and TNF-α are responsible for mediating effects on both the endothelial cell layer and the myeloid population at the secondary site.[691,734] Kaplan *et al* demonstrated that subcutaneous implantation of either melanoma or lung cancer cells results in recruitment of bone marrow-derived progenitor cells and the protein implicated was osteopontin.[694] Furthermore, the same effects are observed by injection of conditioned media, confirming the role of secreted factors.

The pre-metastatic niche model developed in our laboratory is based on B16F10 cell lines injected s.c. into C57BL/6 mice and was established by Dr. Gil-Bernabe. One of the most established components of the pre-metastatic niche is the recruitment of CD11b-expressing myeloid cells.[691,694] Indeed CD11b-expressing cells were found to be significantly increased in response to B16F10 primary tumour growth (Figure 37). Since endothelial activation antigens (VCAM-1 and VAP-1) were found to be crucial in mediating leukocyte recruitment in experimental metastatic models, it was proposed that they may exercise similar functions in the pre-metastatic phase (Figure 38).Indeed, VCAM-1 expression was shown to be up-regulated in the pre-conditioned lung tissue, suggesting that in the same way as observed in experimental metastasis, VCAM-1 facilitated myeloid cell recruitment and attachment. VAP-1 expression was shown not to differ and this is not surprising as it did not appear to be regulated through expression.

The role of these proteins in mediating the establishment of the pre-metastatic niche could reliably be established by testing whether their inhibition disrupts the recruitment of $CD11b^+$ cells and formation of the pre-metastatic niche and abolishes the beneficial effects on metastasis.

Conditioning effects on the endothelial cells have also been reported by others. In work presented by Hiratsuka *et al*[690,691,734] tumour secreted factors (VEGF-A, TGF-β and TNF-α) were shown to exert different effects on the endothelial cell layer. These factors were shown to induce secretion of S100A8 and S100A9 from lung endothelium. This facilitates the recruitment of CD11b –expressing cells. In addition they were also shown to enhance cancer cell motility.[691] Kaplan *et al* had previously demonstrated that recruitment of myeloid progenitor cells depended on their expression of VEGFR-1; therefore VEGF could also mediate myeloid cell recruitment.[694]

Secreted VEGF was shown to induce E-selectin expression on pulmonary endothelium.[734] This was shown to be mediated through activation of focal adhesion kinase (FAK) and exhibited focal areas of hyper-permeability in the lung vasculature. Moreover, tumour cells appeared to disseminate to these

sites. Therefore it is likely that in the same way as E-selectin, VCAM-1 and VAP-1 expression and activity are also part of the pre-metastatic niche. In fact Mendoza *et al* have shown that VEGF, secreted by the tumour cells, induces VCAM-1 expression in the liver.[735] In contrast, Laubli *et al* observed E-selectin upregulation only after metastasis colony formation and no effect was observed in E-selectin deficient mice.[306] Similarly, while finding that VCAM-1 expression correlates with the metastatic pattern, Langley *et* al did not observe any difference in VCAM-1 content in the lung tissue after conditioning. [618] This again points at the variation different tumour cell models exhibit in relying on different adhesion binding interactions.

In this way, secretion of different factors from the tumour cells of the primary mass facilitates activation and mobilisation of myeloid cells directly. In addition, these secreted factors also act on the endothelial cells to induce hyper-permeability of the vasculature and secretion of chemotactic factors by the endothelial cells to mediate myeloid cell recruitment and induce pulmonary endothelial activation. Endothelial activation and expression of various endothelial activation antigens may facilitate attachment and arrest of the recruited myeloid cell population. Furthermore, the interaction between the myeloid cells and the endothelium has been shown to induce secretion of MMP9 by both components to further mediate metastatic dissemination.[690] Coagulation and platelet activation have also been shown to be essential in mediating the recruitment of the CD11b-expressing myeloid population (Gil-Bernabe *et al*, unpublished). However, further research is required to elucidate the mechanism behind these events and to determine whether the process is analogous to that observed in experimental i.v. models of metastasis. While platelet activation is unlikely to occur within the primary tumour as conditioned medium has been shown to be able to recapitulate pre-metastatic niche formation, the precise function of coagulation remains to be determined. [694]

4. Endothelial activation during metastatic dissemination versus its role in colony expansion

In summary, these data demonstrated the role certain endothelial activation antigens exercise in mediating the initial stages of metastatic dissemination and extravasation. However, when VAP-1 inhibition was applied after tumour cell metastases had already established (after day 5), no effect on the number of metastatic colonies was observed. Equally, no effect was observed upon VCAM-1 blocking antibody injection (Supplementary figure 28). This showed that while endothelial activation antigens played a crucial role in the recruitment of myeloid cells initially, this may not be the case later on.

The infiltration of myeloid cells into the metastatic colonies has been extensively characterised. These cells have been shown to promote and contribute to tumour cell survival, metastatic colony expansion and neoangiogenesis.[736] Even though most of the research for this project focused on the time shortly after tumour cell intravasation into the circulation, it stands to reason that both VCAM-1 and VAP-1 may serve similar functions later in metastatic development i.e. facilitating the recruitment of leukocytes into the metastatic colony. Therefore, if the endothelial activation molecules investigated were also involved in mediating myeloid cell recruitment to the metastatic colony, their inhibition would be predicted to show an effect. Therefore it is more likely that, rather than leukocytes not playing a role in metastatic colonization, leukocytes are recruited via another mechanism separate to endothelial activation.

This phenomenon may also present a therapeutic challenge. If inhibition of endothelial activation is only effective prior to actual metastatic dissemination, its application in therapeutics could be fairly limited, especially since in most cases metastatic disease is present at the time of diagnosis and dissemination could also represent a very early step in cancer tumourigenesis.

However another conjecture should be considered. As outlined in the introduction, cancer cells are most likely to acquire further mutations and adapt to the new microenvironment at the site of metastasis. Such adaptations make these cells most suitable for further metastasis in the same organ and confer on them a survival advantage as compared to disseminating cells from the primary tumour.

In this way, inhibition of endothelial activation could prevent further metastatic dissemination, even when metastatic colonies were present at the time of diagnosis. Therefore inhibition of further metastasis may allow a different therapeutic approach, where if metastatic colonization could be confined to a localised area, surgical intervention may be possible.

If metastatic dissemination from metastatic colonies occurs, the reason why no reduction in metastasis was observed after VAP-1 and VCAM-1 inhibition could be the nature of the metastatic models and experimental setup. The models used relied on the i.v. injection of tumour cells, circumventing the need for a primary tumour. In order to accurately gauge the effect a treatment has on metastatic dissemination and lower the variability due to experimental error, the tumour cell number injected was high. Moreover, the level of dissemination was assessed 2 weeks post injection. As tumour cell dissemination from a metastatic colony is only likely to occur after the colony is established, later times should be considered.

5. Application in diagnostics and therapy

Further time-points that were analyzed for endothelial activation molecules were at 1 week and 2 weeks post tumour cell injection. At this time large tumour cell colonies had been formed. Interestingly, VCAM-1 expression was found to be upregulated again. Similarly, at this stage of metastatic colony growth, VCAM-1 upregulation was also detected in several other metastatic models. A fair amount of research has been dealing with the analysis of the neo-angiogenic blood vessels in the tumour colonies. Some reports suggest VCAM-1 expression is up-regulated on these newly formed blood vessels. [737-739]Due to the size of the tumour colonies at this point, one could be reasonably certain that angiogenesis had occurred, which could in part account for the increased expression of VCAM-1 at this time. However Langley *et al* reported that in contrast to most organs, VCAM-1 expression within metastatic tumour colonies decreases in the lung.[618] Therefore the increase we observed in VCAM-1 expression in response to the metastatic colony establishment must serve a different function to that in neo-angiogenesis.

As inhibition of endothelial activation antigens at these later times was shown to have no effect on the development of metastatic colonies, they must be a part of a different process to that characterized in the first phases of metastasis.

Additionally, it could be that endothelial activation occured as a byproduct of the general inflammatory response occurring as a result of tumour cell proliferation and colonization.[618]

Despite the relative lack of therapeutic value of inhibiting endothelial activation at these points, the endothelial activation profile could be used as a surrogate marker for early detection of metastatic colonies. Because the expression of endothelial activation markers is localized to the luminal side of endothelial cells, imaging agents targeted to these do not need to cross the endothelial cell barrier. Moreover, by attaching a suitable therapeutic agent to the targeting vehicle (e.g. antibody), the metastatic lesion could be specifically and systemically targeted.

This principle has already been tested in imaging of brain inflammation in imaging metastatic lesions.[740-742] In these experiments a VCAM-1 targeted antibody was conjugated to an MRI contrast agent (MPIO) which allowed efficient imaging of inflammation in the brain. This technique has been found to be unsuitable for imaging of the lung.[XV] Certain reports suggest that supplementation with air during anaesthesia with hyperpolarized gas may improve and enhance the contrast.[744,745] Nevertheless, conjugation to imaging tracers for SPECT or PET imaging might be advantageous.

As the VAP-1 inhibitor binds to all available VAP-1 (even enzymatically inactive) and the upregulation of VAP-1 protein expression has not been clearly established, VAP-1 was not considered a suitable marker for this study.

VCAM-1 expression occured upon tumour cell metastasis. In contrast to the VCAM-1 expression early in metastatic dissemination, where numerous tumour cells induced widespread inflammation, at the later time-points a smaller number of tumour colonies was found and VCAM-1 expression appeared closely localised to these metastatic lesion.

The antibodies injected into the circulation persist for a few days before being eliminated. In order to improve the time it takes to clear the contrast agent from the system, instead of the antibody, a peptide was targeted to VCAM-1. This was performed in collaboration with Dr. Yee Lim from the Chemistry Department of University of Oxford, who synthesised the peptide and conjugated it to either biotin of Fluorine-18.

The peptide was homologous to the VCAM-1 binding domain in VLA-4. The binding specificity of this peptide was confirmed by its binding to activated HUVECs *in vitro.* For this purpose the peptide was biotinylated and the biotin detected with fluorescently labelled streptavidin. Furthermore the specificity of the peptide binding was confirmed by competition with both unlabelled peptide and by soluble VCAM-1 protein (Data not shown).

[XV] Air in the lungs interferes with signal to noise ratio and yields very short relaxation times.[743]

Following this, the peptide was conjugated to a PET contrast agent Fluorine-18 and injected i.v. into metastasis bearing animals (Imaging performed by Dr. Veerle Kersemans.) Unfortunately the peptide had a clearance time of about 10min, which was not sufficient to support specific binding to VCAM-1. The correct combination of targeting and contrast agent is yet to be found for efficient imaging in the lung. These experiments nonetheless demonstrate a great potential for use of the endothelial activation profile for early detection of metastatic dissemination.

6. Summary

Dissemination of cancer cells from the primary site is the most devastating aspect of cancer progression. In the course of metastatic dissemination, tumour cells detach from the primary tumour and intravasate into the vasculature, of the haematogenous circulation or the lymphatic system. The relevance of the metastatic dissemination through the lymphatic circulation has been debated. While it is established that the presence of metastases in the lymph nodes are a prognostic factor for overall dissemination, it is not clear whether further dissemination occurs from these or whether they are just indicative of the malignant phenotype in general.[27,137] Some cancers also exhibit other pathways of migration, for example ovarian cancer spreads through the peritoneal cavity. However, as the vast majority of cancer cell dissemination occurs through the haematogenous circulation, this has been the focus of research here.

Upon intravasation, the tumour cells are faced with the hostile environment of the circulatory system, including the physical forces exerted by the shear stress of the blood flow and elimination by the immune system. Moreover, it has been proposed that cancer cell death can also be caused through anoikis induced by loss of adhesive interactions. Conversely, tumour cells support their survival through recruitment and interactions with the cells of the immune system and activation of the coagulation cascade. It has long been recognised that coagulation plays a large part in the pathogenesis of cancer. Indeed clinically different coagulopathies are associated with the vast majority of malignancies. One of the most important mechanisms tumour cells use to cause platelet activation is through the expression of tissue factor protein. Exposure of its extracellular domain to the blood stream triggers activation of a cascade that results in the cross-linking of thrombin, activation of platelets and the formation of a clot surrounding the tumour cell. Previously this has been shown to shield tumour cells from the physical forces of the blood stream and impede recognition by natural killer cells. Moreover, upon platelet activation, secretion of bioactive

substances has also been shown to inhibit natural killer cell cytotoxic activity directly and may also act in a paracrine fashion to promote tumour cell survival.

In addition to the coagulation pathway, tumour cells have also been shown to recruit a specific population of leukocytes. This recruitment was shown to be dependent on the formation of the clot around the tumour cell and the expression of CD11b on the leukocytes. While it is likely that this recruitment is facilitated via proteins secreted by the platelets and/or the tumour cell, the exact mechanism is yet to be established. The recruited leukocyte population was shown to promote tumour cell metastasis through an as yet unidentified mechanism.

At the secondary site the tumour cells arrest in the vasculature soon after gaining entry into the blood stream. It is debated whether this is a passive process or whether it depends on interactions of specific adhesion molecules. Evidence exists to support both models; however the results are likely to be dependent on the particular experimental models used and on subsequent analysis techniques. Adhesion molecule binding was implicated because the process of tumour cell translocation to the secondary site, attachment and arrest in the pulmonary vasculature and lastly extravasation into the underlying tissue has been modelled on that of immune cells in inflammation. In this situation an inflammatory stimulus will result in the activation of the endothelial cell layer and expression of various adhesion molecules on its luminal surface; these further mediate attachment and extravasation of immune cells into the underlying tissue. The investigation of whether similar adhesion molecules also mediate the process of tumour cell adhesion and extravasation has implicated specific molecules in this process. On the other hand tumour cells are much larger than leukocytes and arrest by anatomical occlusion has been observed *in vivo*. The endothelial activation antigens investigated here do not appear to be required to mediate tumour cell arrest in the experimental models used. Inhibition of VCAM-1 prior to injection of tumour cells into the blood

stream did not affect the number of tumour cells arrested in the pulmonary vasculature. Moreover, E-selectin expression was completely absent until a few hours after tumour cell arrest.

Interpretation of the experimental data is further complicated if we consider the adhesive interactions provided by platelets and leukocytes. Platelet activation leads to the mobilization of intra-cellular granules and release of bioactive proteins and small molecules. This has been shown to be one of the factors inducing endothelial activation upon tumour cell metastasis. That this is not the only component responsible for this induction is apparent both from the significant level remaining after inhibition of clot formation and the level of expression observed in an experimental metastasis model devoid of coagulation activity. Furthermore, disruption of leukocyte recruitment to the site did not affect the levels of VCAM-1 expression, eliminating leukocyte activation from the components involved.

An upregulation of endothelial activation antigens can be observed within a few hours of tumour cell metastasis challenge. While the specific endothelial activation profile appears to differ between different experimental models, the two most significant molecules investigated were VAP-1 and VCAM-1. VCAM-1 upregulation was observed within hours of metastatic cell arrest in the lung and the level of expression remained elevated throughout the first 24h.

On the other hand the role of VAP-1 in adhesion is dependent on its enzymatic function. Therefore looking only at its expression, no significant conclusion could be drawn regarding its regulation in response to metastasis. Data interpretation is further complicated by the fact that VAP-1 protein is stored in intra-cellular granules and then transported to the membrane upon stimulation.

Through inhibition, it was shown that endothelial activation is not only a side effect of the overall inflammatory process that occurs in response to metastasis. Inhibition of function resulted in an attenuation of metastatic dissemination, which has been shown to be mediated through a reduction in monocyte recruitment to the metastatic cell.

Double inhibition of both VCAM-1 and VAP-1 had no further effect on either metastatic dissemination or recruitment of the leukocyte population. This proves that the two proteins promote metastasis through the same mechanisms. Additionally VAP-1 inhibition was seen to be more effective than inhibition of VCAM-1. It is possible that this is purely due to the effectiveness of the inhibitors, where VAP-1 inhibitor could also inhibit enzymes with similar catalytic activity. This is commonly observed with small molecule inhibitors and interestingly there is an enzyme related to VAP-1: CD73.[408] However it could also be that VAP-1 functions further upstream than VCAM-1. According to this model, engagement of VAP-1 would result in inflammatory stimuli that would induce expression of further endothelial activation antigens. In order to confirm this, expression of VCAM-1 would need to be evaluated in response to VAP-1 inhibition.

Based on these data a model has been proposed, where platelet clots form around the tumour cells after these intravasate into the circulation. The combination of tumour cell and platelet secreted factors act to stimulate the endothelial cell layer at the secondary site and facilitate recruitment, activation and mobilisation of $CD11b^+$ cells, expression of VCAM-1 and VAP-1 on the pulmonary endothelium is then essential to mediate the attachment of the leukocyte population.

Another important group of adhesion antigens described in inflammation are the selectins. In the metastatic models used here, E-selectin upregulation was only observed in human metastatic models. Furthermore, the role of E-selectin in mediating leukocyte extravasation in the lung has been questioned. Similar to VAP-1, P-selectin is stored in intra-cellular granules and in response to stimuli these are transported to the membrane. P-selectin is also expressed on platelets. Thus endothelial P-selectin, possibly available on the luminal side of the endothelial cells, was difficult to identify. However it is important to note that both P- and L-selectins are proposed to mediate the interactions between platelets, tumour cells and leukocytes.[303]

As mentioned previously, platelets and leukocytes have been shown to be crucial in mediating tumour cell survival. Through these interactions the effective surface of the tumour cell complex is increased, thereby increasing the availability of adhesion proteins. In this way endothelial activation antigens could serve a similar roles in facilitating metastatic dissemination as that described in inflammation, namely facilitating the adhesion and arrest of patrolling leukocytes. Thus, in addition to facilitating immune evasion, protection from shear stress and mediating survival and the formation of the tumour cell – platelet – leukocyte complex may also facilitate interactions with the endothelium, adhesion and extravasation.

In the 1205LU-GFP cell model platelet activation, clot formation and subsequent leukocyte recruitment are minimal. Additionally VAP-1 inhibition has no effect on 1205LU-GFP cell metastatic efficiency. While we were still able to observe VCAM-1 upregulation, this was lower than that observed in other metastatic models. When the efficiency of metastasis was assessed long term, the 1205LU-GFP cell line was much less efficient than the 4T1-GFP cell line. This again points to the crucial role platelet exert in promoting metastatic dissemination.

The concept of the pre-metastatic niche states that the secondary organ is pre-conditioned for metastasis formation by the primary tumour. While the effector molecules secreted from the primary tumour have yet to be defined, conditioned lung tissue that supports increased metastasis formation has been reported to contain increased numbers of $CD11b^+$ cells, depositions of fibronectin and lox, localised areas of hyper-permeability and E-selectin expression. Moreover, platelet activation has been shown to be required for these effects.

As VAP-1 and VCAM-1 were shown to mediate CD11b-expressing cell recruitment in experimental metastasis models in response to platelet activation, it was plausible that the same mechanism may also be involved here. Indeed an increase in VCAM-1 expression was observed in conditioned lung tissue in comparison to the naïve tissue. However, for the role for endothelial activation antigens in

the pre-metastatic niche formation to be proven, the effect of inhibition of function of these on the enhancement of metastasis should be tested.

In the introductory chapter, the controversy, as to whether metastatic dissemination occurs early during tumour progression or as a progressive accumulation of traits at later stages, was outlined. If cancer cells are able to metastasize early, the metastatic traits would be acquired during tumour progression, should be inherent to the entire primary tumour population and could therefore be profiled in genetic profiling. In the case of cumulative accumulation on the other hand, only certain cells in the primary tumour would acquire the ability to metastasize.

The phenomenon of pre-metastatic niche establishment argues against the idea of metastatic dissemination occurring early during tumour progression. In the animal models, in order for the pre-metastatic niche to be established the primary tumour mass had to be present for at least two weeks (Gil-Bernabe *et al*, unpublished). Therefore, it appears that at least in these models metastasis is the result of a progressive process rather than an early event.

Equally one could argue that establishment of the pre-metastatic niche is the initial step in metastasis formation. Therefore, the characteristics predicting the metastatic phenotype characterised in numerous microarray genetic profiling studies could actually be identifying the traits required to mediate secretion of different factors and the establishment of the metastasis permissive environment.

The experimental setup of the pre-metastatic niche has also shed light on an important issue. Hiratsuka *et al* have shown that while VEGFR-1 KO animals displayed no difference in the growth of the primary tumour or of metastases when cells were injected i.v., significant differences occurred in spontaneous metastatic dissemination from the s.c. implanted primary tumour.[690] These findings highlight the significance of the experimental model chosen when studying the role of a specific antigen in metastatic dissemination.

Future directions

There are several interesting possible experiments that could be performed to extend the work presented in this project. These are summarized in the table below and some will be discussed in this section.

When analysing the distribution of endothelial activation in the pulmonary circulation upon metastasis, the tumour cells were characterised as localizing nearby positively stained signal or not. This resulted in a binary classification of data that failed to take into account the level of expression of the endothelial antigen. While the methodology of the staining used did not allow for this type of analysis in this instance, if an appropriate quantification method could be developed it could lead to a more accurate representation of the endothelial activation induced by the tumour cells. Additionally it may also highlight any more subtle differences in expression that would have been inaccessible to binary classification. Furthermore normalizing the data to the location of the metastasizing tumour cells did not allow for a control specimen, which would reveal the level of endothelial activation present in a naïve tissue. If a suitable quantification method could be established, the basal level of expression of the specific adhesion proteins could be taken into account.

Inhibition of VAP-1 activity had a more extensive inhibitory effect on leukocyte recruitment and metastasis as compared to VCAM-1 inhibition. As mentioned previously this may be due to off-target effects of the small molecule inhibitor. VAP-1 related molecules CD73 has been characterized[408] and it is plausible that the effects seen upon inhibitor injection may also be due to the inhibition of this receptor. Similar to VAP1, CD73 has also been reported to function in mediating leukocyte extravasation and may therefore support a similar role in metastatic dissemination. Therefore the enzymatic specificity of VAP-1 inhibitor should be tested. If this was proven to be specific, the efficacy of VAP-1 inhibition could be due to the interconnected regulation of the endothelial activation, where

engagement of the VAP-1 receptor would trigger further endothelial activation. In order to test this, expression of other endothelial activation antigens should be analysed following VAP-1 inhibition.

In the 1205LU-GFP metastatic model, no extensive platelet activation and leukocyte recruitment was observed. Despite this extensive endothelial activation was detected in response to metastasis. This pointed to the fact that endothelial activation may also play other roles in addition to mediating leukocyte recruitment. Therefore by using this model and elucidating the mechanism these cells use to successfully metastasize would lead to characterizing other mechanism by which endothelial activation promotes metastasis. This could involve modulation of endothelial cell function. As in the process of leukocyte extravasation, the endothelial cell layer is not a passive participant. Therefore the induction of endothelial activation may be an indication of alteration in endothelial cell function that ultimately aids in the process of metastatic dissemination.

Another interesting aspect discussed is the establishment of the pre-metastatic niche. Firstly in order to confirm the involvement of the endothelial activation proteins in the establishment of such pro-metastatic environment, we would need to test whether inhibition of endothelial function could disrupt its establishment and thereby eliminate the enhancement of metastatic dissemination observed in response to primary tumour growth.

Further it would be important to establish whether the niche components localize to specific locations in the lung or whether the effects observed are systemic. If the niche established at a precise location this could facilitate researchers to precisely map the locations where metastatic dissemination will occur. This would allow for precise imaging techniques to be established. Furthermore it may also highlight any other components of the niche and thereby aid in characterization of the hosts components involved in the process of metastasis.

Induction of endothelial activation by disseminated tumour cells	A better quantification method to be developed for the analysis of the expression of endothelial adhesion antigens e.g. western blotting or qRT-PCR
	Functional analysis of VAP-1 expression through assaying its enzymatic activity
	Analysis of the interconnectivity of the endothelial adhesion molecules. For example analysis of VCAM-1 expression after VAP-1 inhibition would show whether VCAM-1 expression is upregulated in response to VAP-1 enzymatic activity
The function and efficiency of the VAP-1 inhibitor	Test any off-target effects of VAP-1 inhibitors e.g.CD73
	Test the effect the inhibitor may have on the tumour cell directly
The role of adhesion molecules in initial adhesion of tumour cells	Assessing the effect VAP-1 inhibition has on the initial adhesion of tumour cells would evaluate any role this adhesion molecule has in tumour cell adhesion in the pulmonary vascuature
	Confirm the lack of effect of VCAM-1 blocking antibody on the initial adhesion in other metastatic models
	Blocking of function of multiple adhesion molecules could reveal any overlapping functions these adhesion molecules may exert during adhesion events
The role of adhesion molecules in leukocyte recruitment	Confirm the characterization of recruited monocyte population through a quantitative method (e.g. FACS)
	If there is a second wave of recruitment of a different myeloid population this could be analysed by investigating the recruitment at additional later time-points. Additionally analysing the effects of endothelial adhesion protein inhibition may separate the roles these proteins exert at different times.
	Investigate the mechanism of leukocyte recruitment through the endothelial adhesion antigens
Investigating the pre-metastatic niche	Investigate whether the pre-metastatic niche can be disrupted by VAP-1 inhibition
	Investigate whether the pre-metastatic niche establishes at specific foci in the vasculature rather than throughout the tissue
	Investigate the pre-metastatic niche establishment in other organs esp.liver
Role of adhesion molecules in metastatic dissemination in addition to leukocyte recruitment	Use the 1205LU-GFP metastatic model to investigate what if any role, endothelial activation antigens exert in the process of metastatic dissemination. This may also lead to the characterization of the inflammatory triggers responsible for inducing endothelial activation.

In summary endothelial activation has been proven to contribute to the efficiency of metastatic dissemination. While the adhesion proteins do not appear to mediate direct contact between the tumour cells and the pulmonary endothelium, they appear to function and/or become expressed within a few hours of tumour cell arrest. This is at least in part mediated through platelet activation. Inhibition of endothelial adhesion protein function attenuated metastasis and this appeared to be mediated through inhibition of leukocyte recruitment. The recruited leukocyte population relies on the activated endothelium for adhesion and has been shown to enhance tumour cell survival. Additionally they may also act to promote tumour cell extravasation. Together this work highlights how physiological processes of the host immune system are exploited by malignant cells. Additionally several opportunities for therapeutic intervention are presented, where the cascade of events in the models could be intercepted either via inhibition of platelet function or VAP-1 inhibition. Moreover the characterisation and targeting of the endothelial expression profile may facilitate the diagnosis of previously undetectable lesions.

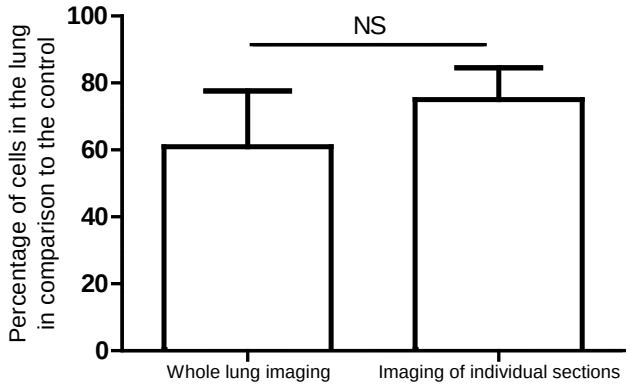
APPENDIX

SUPPLEMENTARY FIGURES

Supplementary Figure 1

There is no difference between the two experimental protocols for assessing pulmonary metastasis

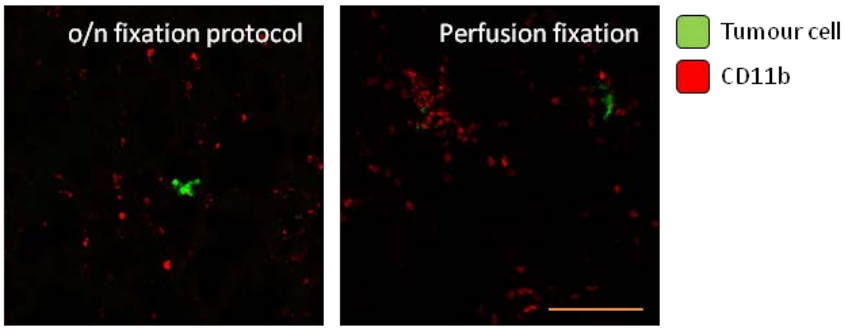
Tumour cells (B16F10-CMFDA) were injected i.v. 1h after inhibitor treatment. Metastatic efficiency of tumour cell survival in the lung was assessed 24h later. This was achieved either by whole lung imaging and counting the number of tumour cell on the surface of the lungs or in the lungs were harvested as for IHC staining, and cells were counted in at least 10 sections to accurately gauge the surviving cell fraction. This data is presented in Fig.26.The surviving cell fraction is represented as the percentage of cells in the treated lungs, where the 100% value is noted by the corresponding control lung. There was no significant difference between the two type of metastatic cell survival assay (unpaired t-test p=0.1389; n=5).



Supplementary Figure 2

Comparison of CD11b staining efficiency between over-night fixation and fixation perfusion

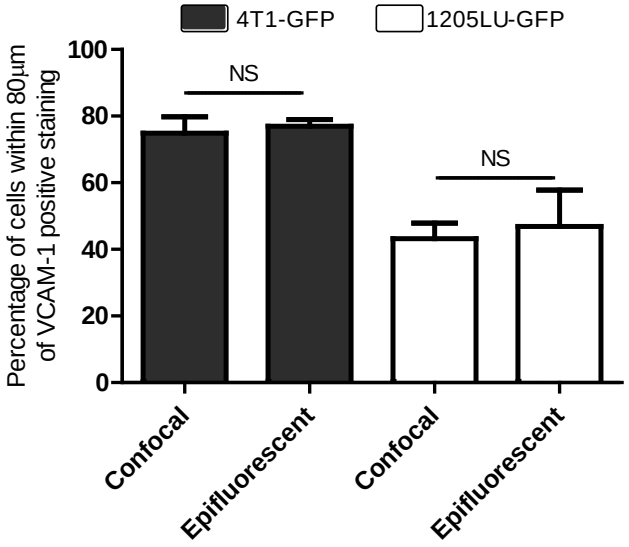
Both images are obtained from injection of B16F10-CMFDA cells i.v. into C57BL/6 wt mice. Left panel demonstrates CD11b staining, when the lungs were only perfused with 5ml of 4%PFA and then incubated in 4%PFA overnight. In the right panel the lungs were fixed solely by perfusion of 50mL of 4% PFA. In both cases the tissue was cryo-protected in 25% sucrose and processed as described. The tissues were imaged with Zeiss Confocal microscope. Scale =100µm



Supplementary Figure 3

There is no difference in Alexa Fluor 633 fluorophore imaging and analysis with either 633 laser/confocal microscope or Y3 filter/halogen lamp epifluorescence

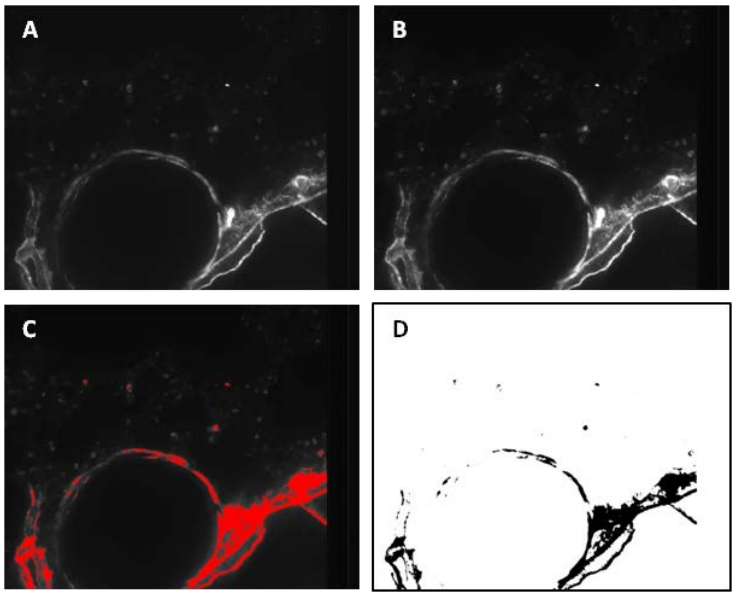
This graph shows the analysis of VCAM-1 expression 8h after tumour cell injection. Both 4T1-GFP and 1205LU-GFP challenged tissues were stained for VCAM-1 and visualised using Alexa Fluor 633 fluorophore.
In each case the tissue was analysed firstly using a Zeiss Confocal microscope (laser: 633, beam splitter: MBS: 488/561/33, objective: Plan-Apochromat 20x/0.8 M27, collection: 637 – 747) and then also by Leica epifluorescent microscope (halogen lamp excitation, filter Y3, objective: 20x HC PL fluotar 20x0.50).
Analysis shows there is no significant difference between the two types of imaging modalities. The data was statistically analysed using unpaired t-test (4T1-GFP: p=0.6431; 1205LU-GFP: p=0.6200).



Supplementary Figure 4

Analysis of the percentage of positive signal per field of view

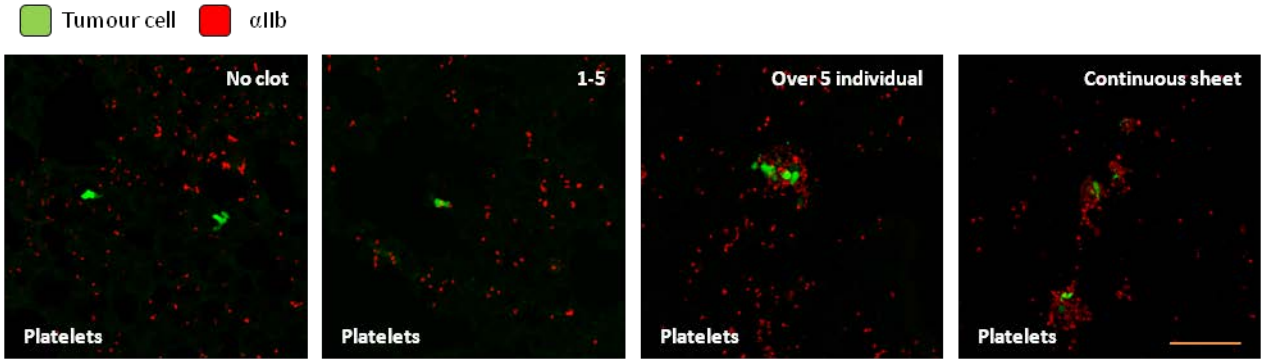
This panel shows the steps taken when analysing the percentage of positive signal per field of view using Image J. Panel A is the original raw image acquired by Leica Epifluorescent microscope using the 20x objective. Panel B is the same image after the histogram had been normalized. This step was taken because the distribution of staining intensities within a given image varies significantly, while the background signal is very low. This allowed for the low (underexposed) signal that may be underrepresented in an image with a high signal intensity to be taken into account. Next the threshold was set manually (C). And the signal considered positive measured (D).



Supplementary Figure 5

Representative images of different categories showing platelet clot formation at the tumour cells

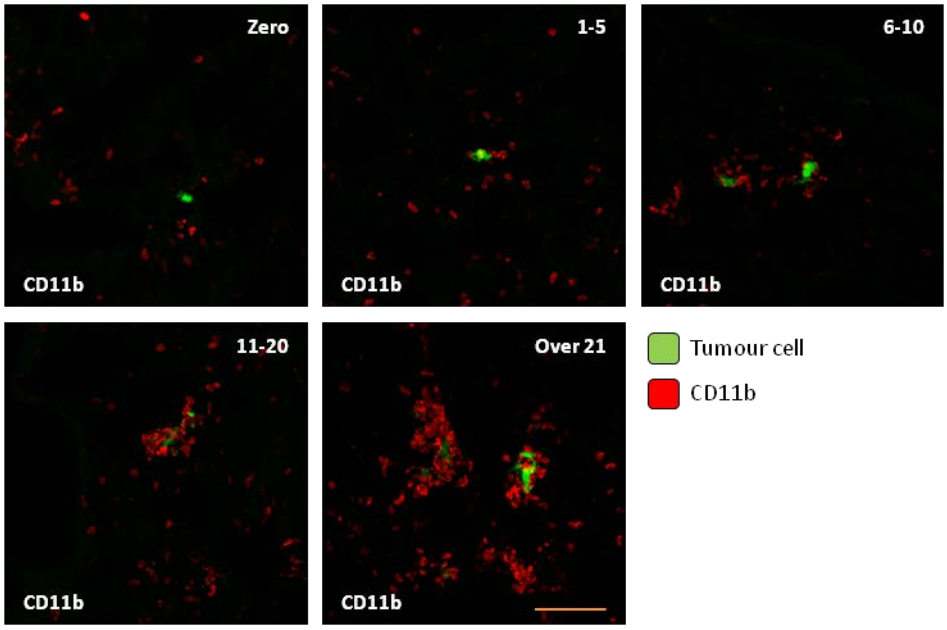
According to the number of associated platelet clots, the tumour cells were placed into one of the following categories: no clot associated with the tumour cell, 1-5 individual clots, over 5 individual clots or a continuous sheet of platelet clot formed around the tumour cell. This panel shows representative images of different cells corresponding to each category. The tissues were imaged with Zeiss Confocal microscope. Scale =100µm



Supplementary Figure 6

Representative images of different categories showing CD11b-expressing cell recruitment to the tumour cells

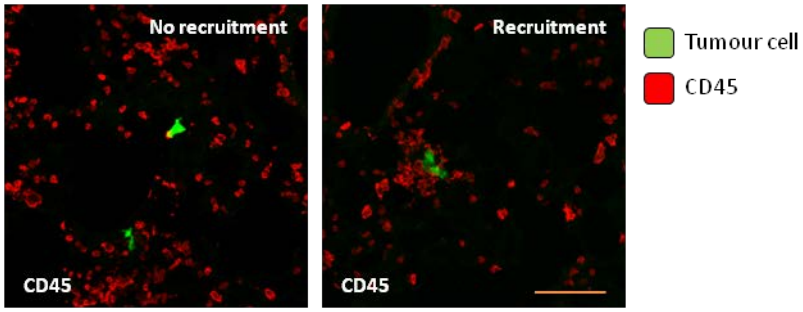
According to the number of associated CD11b$^+$ cells, the tumour cells were placed into one of the following categories: no cells associated with the tumour cell, 1-5 cells associated, 6-10 cells associated, 11-20 cells recruited or over 21 CD11b$^+$ cells clustered to the tumour cell. This panel shows representative images of different cells corresponding to each category.
The tissues were imaged with Zeiss confocal microscope.
Scale =100µm



Supplementary Figure 7

Representative images of different categories showing CD45-expressing cell recruitment to the tumour cells

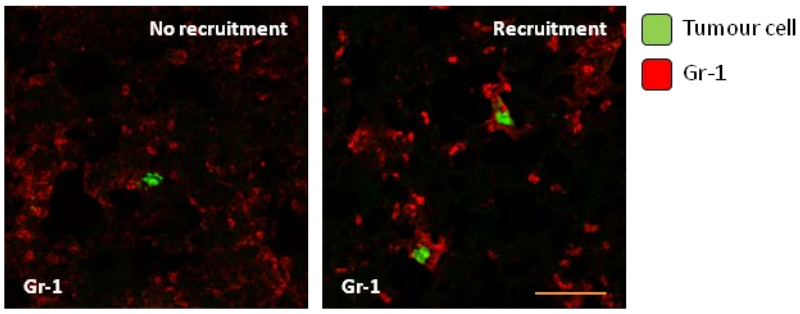
According to CD45$^+$ cell staining, the tumour cells were characterised as either inducing the clustering of CD45$^+$ cells or not. This panel shows representative images of different cells corresponding to each category.
The tissues were imaged with a Zeiss Confocal microscope. Scale =100µm



Supplementary Figure 8

Representative images of ting Gr-1-expressing cell recruitment to the 4T1-GFP tumour cells

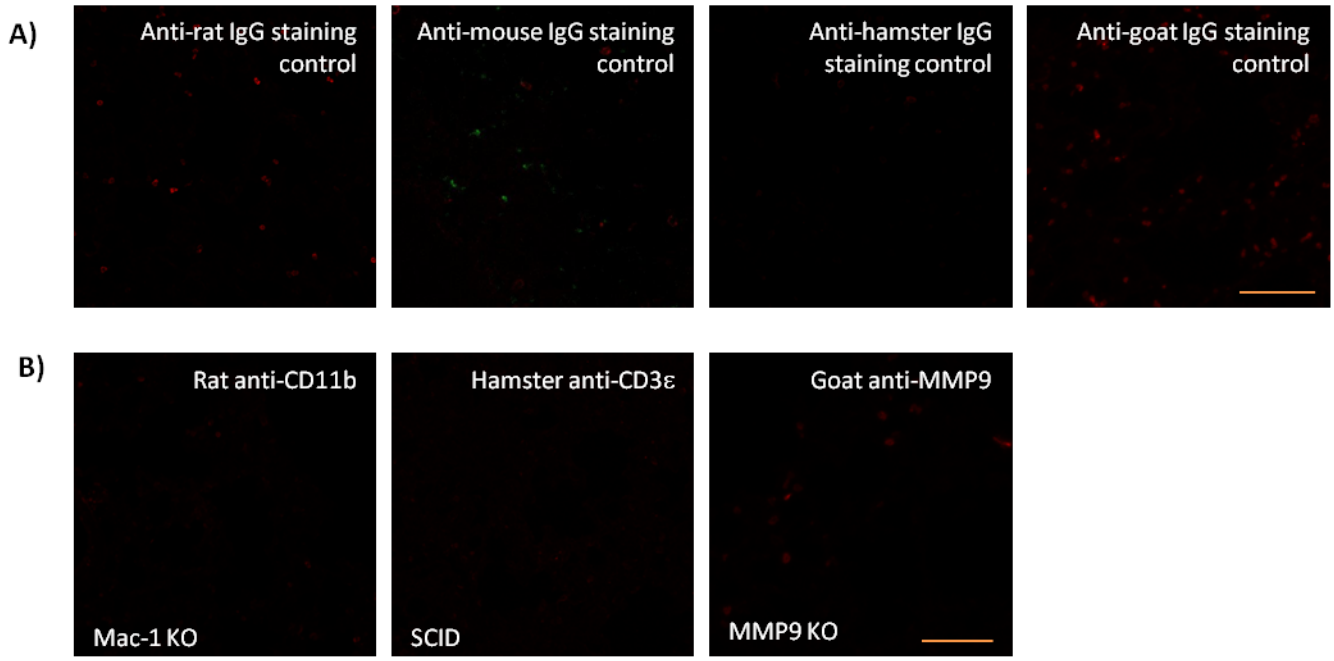
According to Gr-1 cell staining, the tumour cells were characterised as either having recruited Gr-1-expressing cells or not. This panel shows representative images of different cells corresponding to each category.
The tissues were imaged with Zeiss confocal microscope.
Scale =100µm



Supplementary Figure 9

Staining negative controls

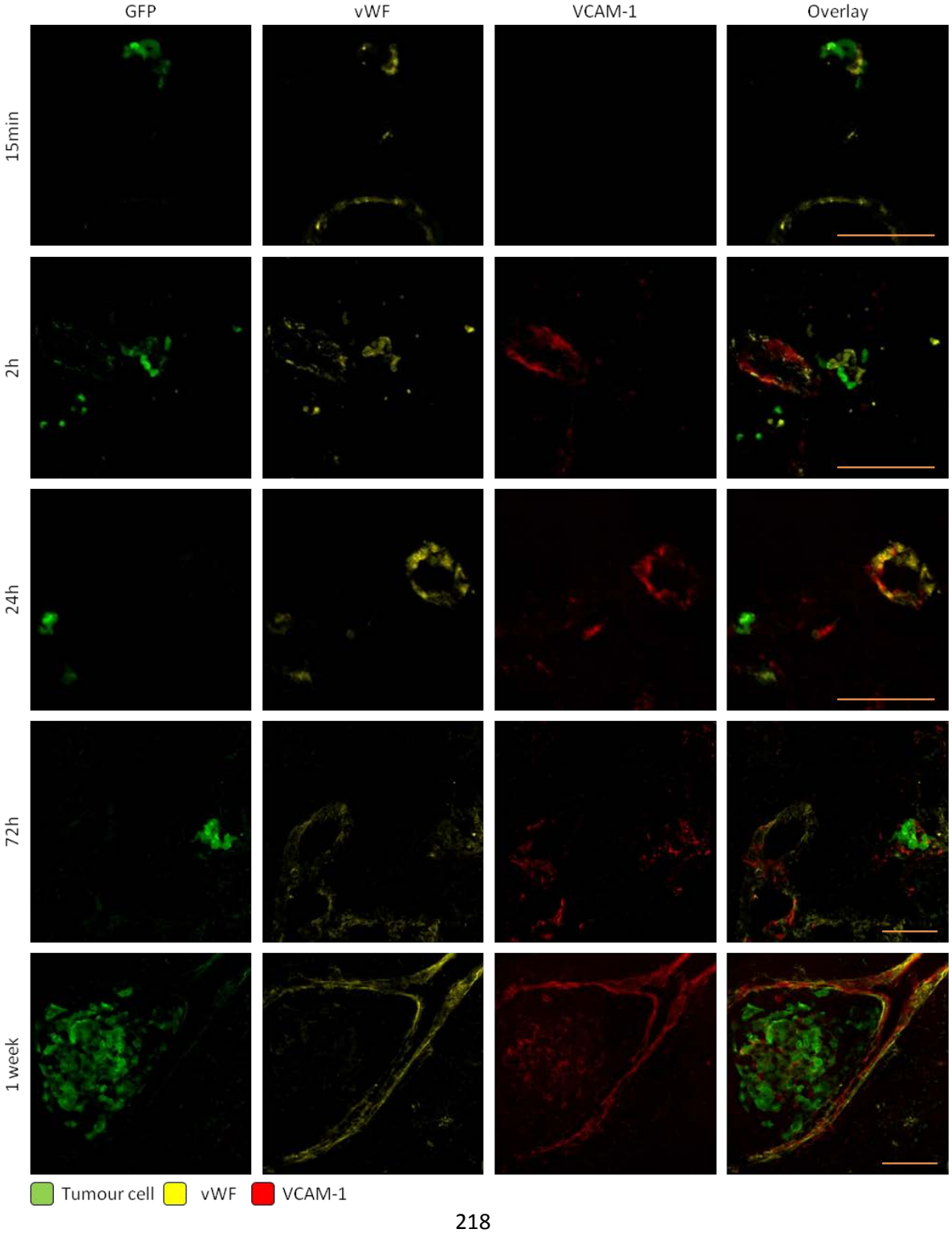
Staining negative controls (Alexa Fluor 633-red) demonstrate the unspecific binding of the secondary antibodies to the lung tissue.
A) Representative images of the signal produced due to unspecific binding of used biotynilated secondary antibodies and TSA amplification system. The lung tissue used for these staining was obtained by perfusion fixation with 50ml of 4% PFA. Images were obtained with either the Leica Confocal microscope (Anti-mouse IgG) or with a Zeiss Confocal microscope. Scale = 75µm
B) Additionally, we have also used (where possible) transgenic animals, where the protein targeted by the primary antibody is eliminated. Representative images show non-specific binding of the primary antibodies (CD11b, CD3ε and MMP9). Images were obtained by Zeiss Confocal microscope. Scale = 100µm



Supplementary Figure 10

Induction of VCAM-1 expression occurs on endothelial cells

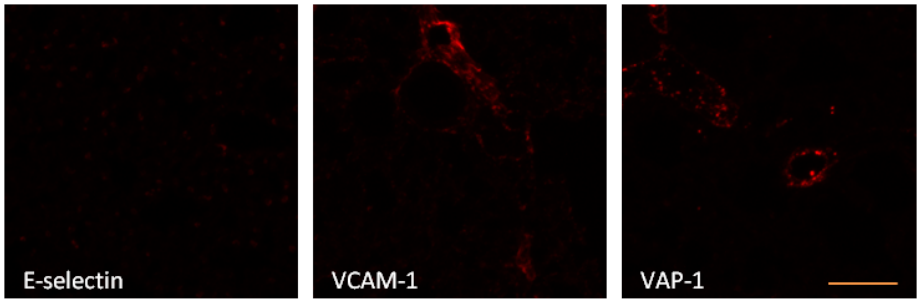
Female BALB/c mice were injected with 4T1-GFP i.v. (5x10e5) and lungs were harvested at the indicated time-points. This panel shows representative images, where the tissues were co-stained for both VCAM-1 (Alexa Fluor 633-red) and von Willebrand Factor (Alexa Fluor 514). We observe significant co-localisation, which demonstrates that VCAM-1 is indeed expressed on endothelial cells. The images were acquired using a Leica confocal microscope and average projections are displayed. Scale = 75µm



Supplementary Figure 11

The experimental procedure has no effect on endothelial activation

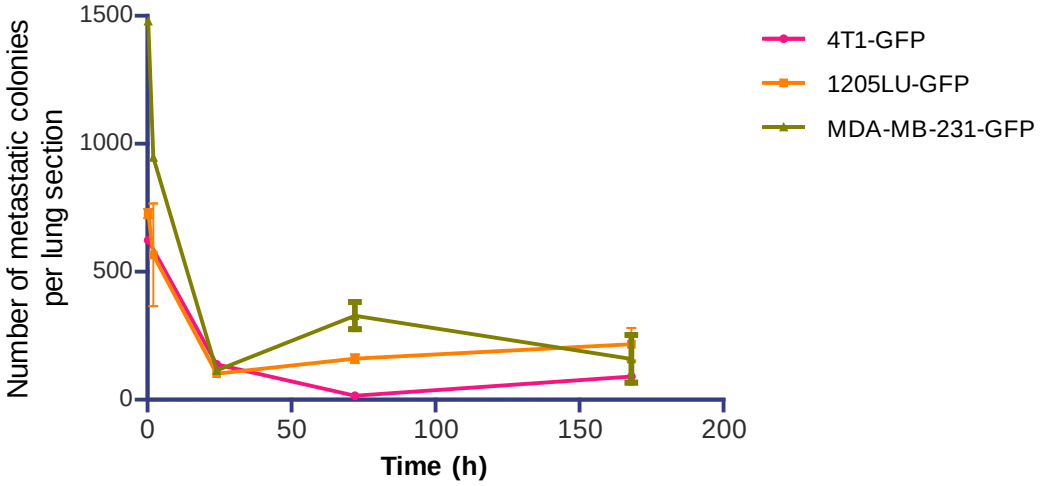
Female BALB/c mice were injected with serum-free medium (100µL) i.v. and the lungs were harvested 8h after. The endothelial activation antigens (E-selectin, VCAM-1 and VAP-1) were stained for as described and imaged (Alexa Fluor 633-red) with a Zeiss Confocal microscope. Scale = 100µm



Supplementary Figure 12

Attrition of metastasis in the lung over the first week after injection

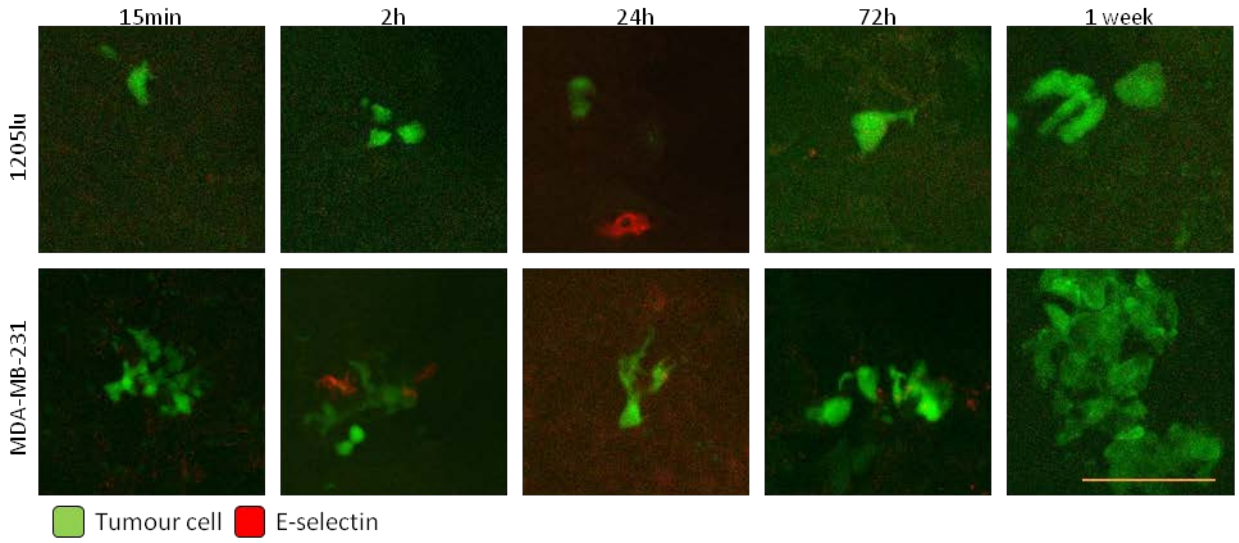
This graph portrays proportionally the numbers of metastatic tumour colonies across the time-course. These were assessed by counting at least three sections per each lung (n=2). As expected we were able to observe a decrease within the first 24h after which the number of metastasis remained essentially constant. The tumour cells (labelled with GFP) were counted using Leica epifluorescent microscope.



Supplementary Figure 13

E-selectin expression is induced by metastasis of 1205LU-GFP and MDA-MB-231-GFP cells

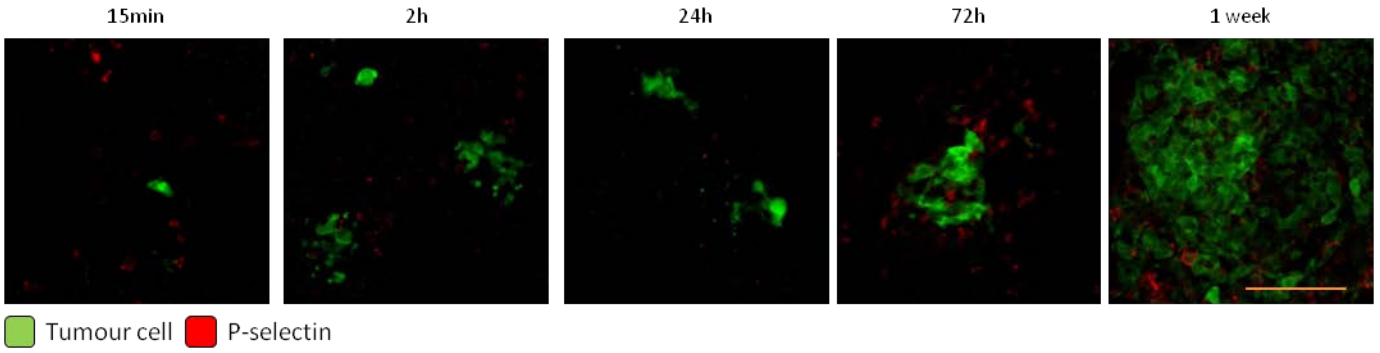
E-selectin expression (Alexa Fluor 633-red) in lung tissue challenged by human cancer cell lines labelled with GFP(green) demonstrate a transient nature of upregulation of E-selectin. Images acquired with a Leica Confocal microscope. Scale=80µm Representative images of the rest of the time course omitted from Figure 6.



Supplementary Figure 14

P-selectin expression in response to 4T1-GFP cell metastasis

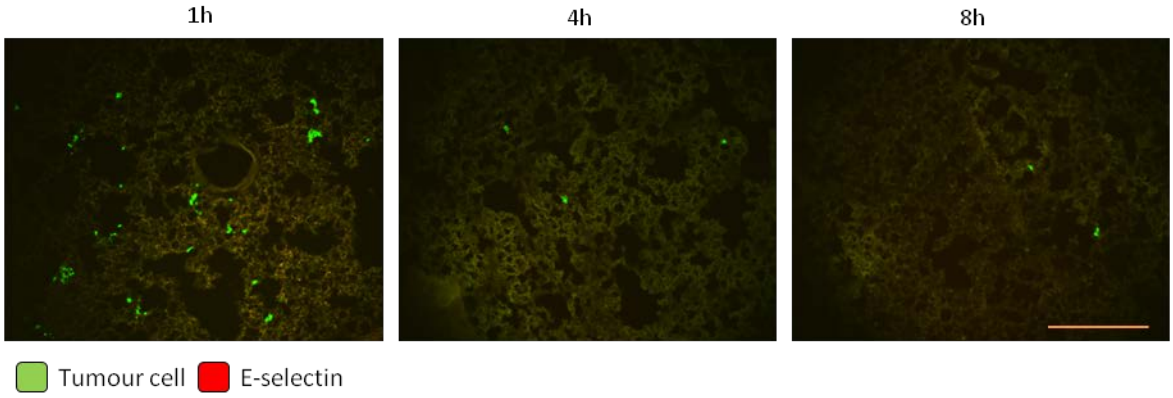
P-selectin expression (Alexa Fluor 633-red) is absent in lung tissue in response to 4T1-GFP metastasis (green). Images acquired with Leica Confocal Microscope. Scale=100µm Representative images demonstrate the lack of characteristically endothelial P-selectin expression.



Supplementary Figure 15

Metastasis of 4T1-GFP cells does not induce expression of E-selectin

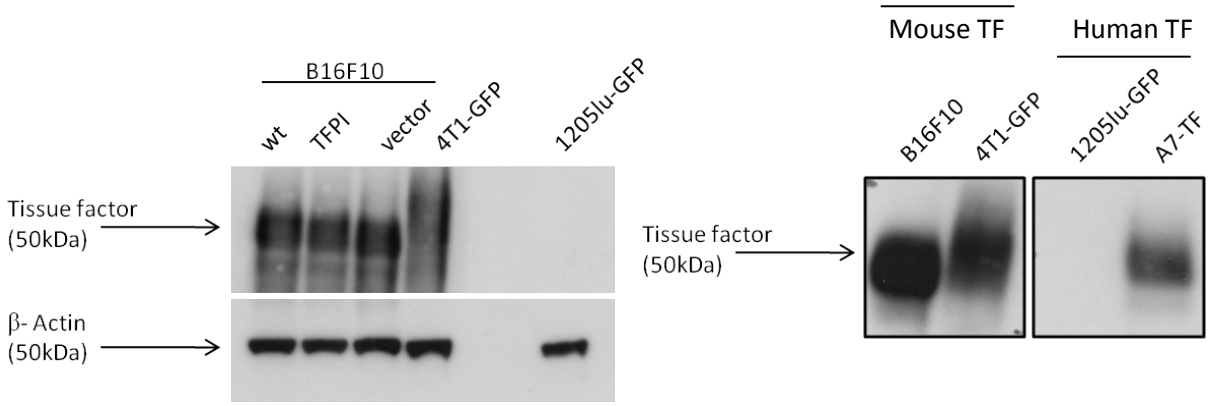
E-selectin expression (Alexa Fluor 633-red) is absent in lung tissue in response to 4T1-GFP metastasis (green). Images acquired with Leica epifluorescent microscope. Scale= 50µm Representative images demonstrate the lack of endothelial E-selectin expression 1h, 4h and 8h after i.v. injection (5x10e5) of 4T1-GFP cells.



Supplementary Figure 16

Tissue factor expression

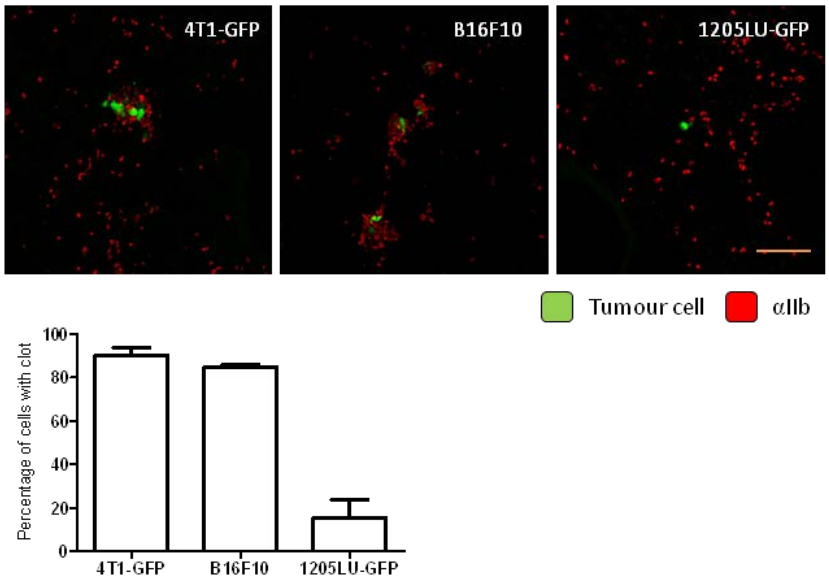
Western blot analysis was performed of the three cell lines for expression of tissue factor protein (50kDa). Both B16F10 and 4T1-GFP tissue factor was detected using an anti-mouse TF antibody. For 1205LU-GFP anti-human TF antibody was used. In order to establish whether the antibody used to recognise human tissue factor was efficient, a human cell line (A7) transfected with tissue factor was used.[675] The same protein extracts as used previously was utilised for the other cell lines. We can see that the human tissue factor antibody efficiently recognizes the protein and the lack of signal in the 1205LU-GFP cell line is in fact due to the absence of tissue factor protein expression. The protein lysate of the A7-TF cell line was a kind gift of Dr. Gil-Bernabe.
For B16F10 cell line three cell lines were used the parental wt and 2 transfectants that express murine TFPI (B16F10-TFPI) or an empty vector (B16F10-vector) Both B16F10 and 4T1-GFP tissue factor was detected using an anti-mouse TF antibody.



Supplementary Figure 17

Platelet clot formation induction in experimental metastasis models in wt animals

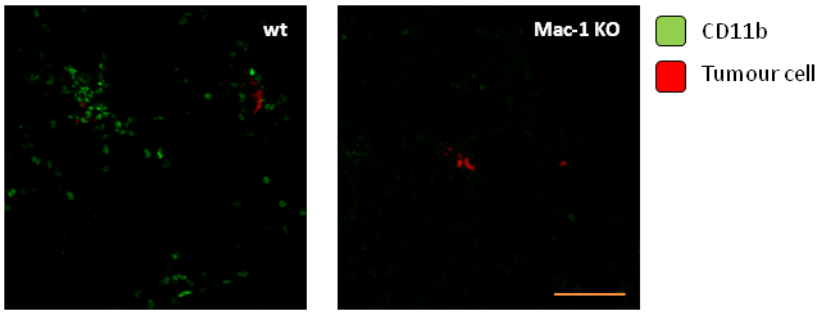
Platelet (αIIb) clot formation (Alexa Fluor 633-red) in lung tissue in response to tumour cell metastasis (green). Images acquired with a Zeiss Confocal microscope. Scale=100μm Representative images demonstrate the lack of clot formation in the 1205LU-GFP cell model in contrast to extensive platelet activation in the 4T1-GFP and B16F10-CMFDA metastatic models. Graph demonstrates the percentages of tumour cell pulmonary metastases that are associated with platelet clot. (n=3)



Supplementary Figure 18

Cd11b staining in lungs of Mac-1 KO animals

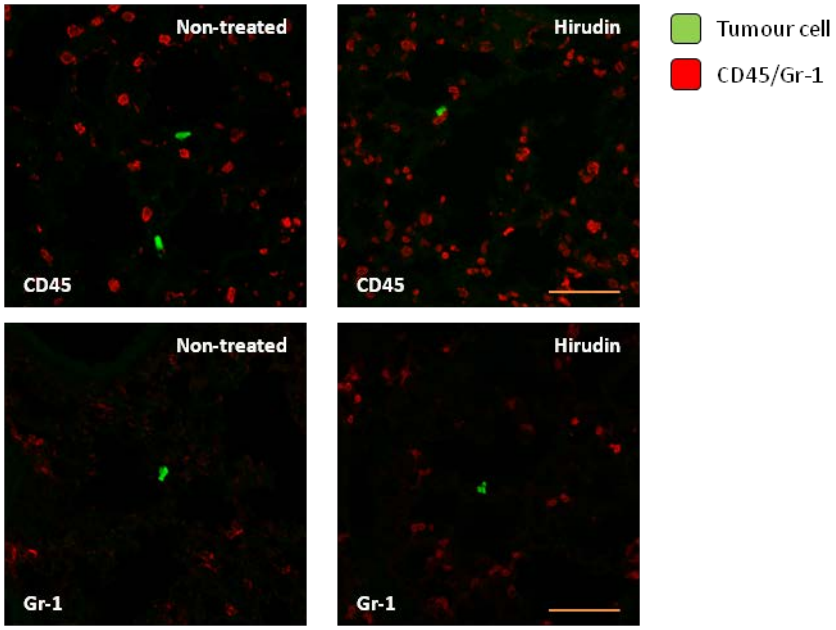
CD11b expression (Alexa Fluor 488) in lung tissue in response to CMRA-stained B16F10 metastasis (red). Images acquired with Zeiss Confocal microscope. Scale=100μm Representative images demonstrate the lack of CD11b expression in Mac-1 KO animals in comparison to its expression in wt animals.



Supplementary Figure 19

Recruitment of CD45$^+$ cells and Gr-1$^+$ cells by pulmonary metastasis of 1205LU-GFP cells at 8h CD45- expressing cells and Gr-1 – expressing cells (Alexa Fluor 633-red) in lung tissue in response to 1205LU-GFP metastasis (green). Images acquired with Zeiss Confocal Microscope. Scale=100µm

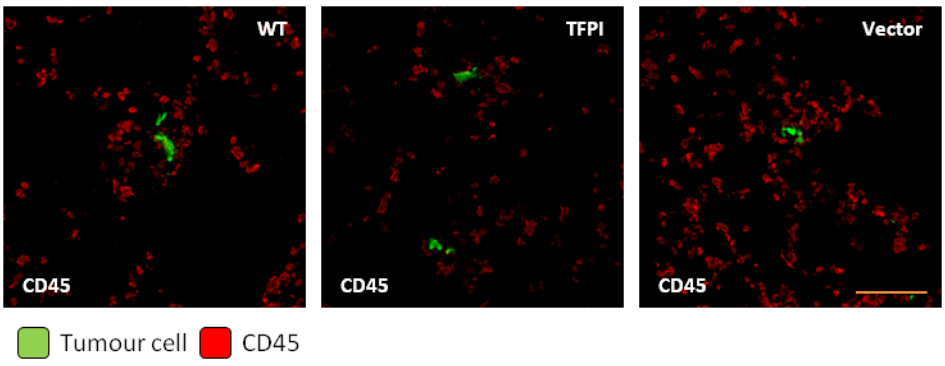
Representative images demonstrate the lack of leukocytes recruited to the 1205LU-GFP cells and hirudin treatment does not affect this.



Supplementary Figure 20

TFPI inhibits CD45$^+$ cell recruitment

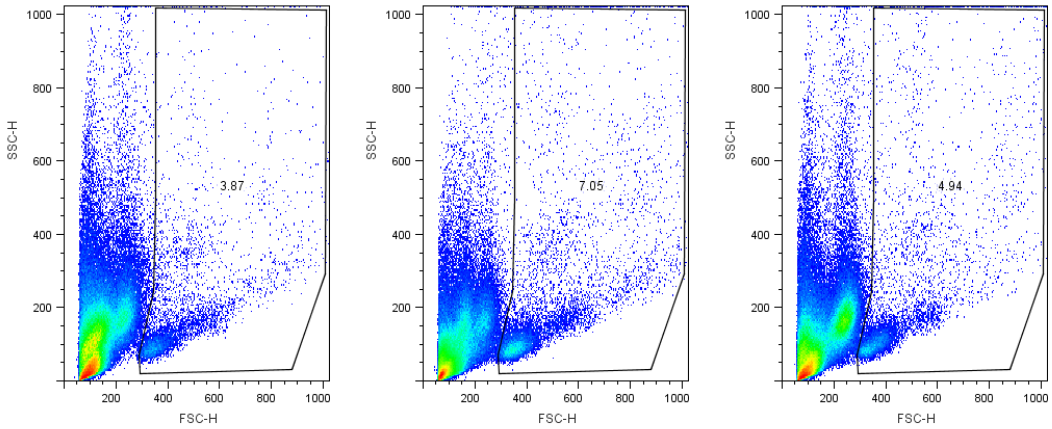
CD45- expressing leukocytes (Alexa Fluor 633-red) in lung tissue are recruited in response to CMFDA stained B16F10 cells (green). Images acquired with Zeiss Confocal microscope. Scale=100µm Representative images demonstrate inhibition by TFPI of the recruitment of CD45$^+$cells to the B16F10-CMFDA metastasis. In contrast, widespread recruitment is observed by wt and vector-transfected tumour cells.



Supplementary Figure 21

Myeloid cell gating in flow cytometry

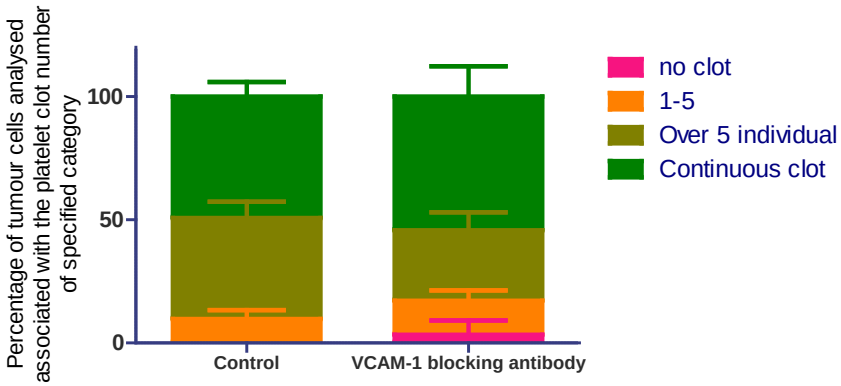
Flow cytometry was used to analyse the number of immature neutrophils present in the peripheral blood upon treatment with the VCAM-1 blocking antibody. 4h after treatment, the blood samples were collected and analysed. Shown are representative plots for the gating strategy of the for myeloid cell population based of forward and side scatter. Further analysis was then performed as described in material and methods and shown in Figure 23.



Supplementary Figure 22

Effect on the size of the recruited platelet clot by VCAM-1 blocking antibody

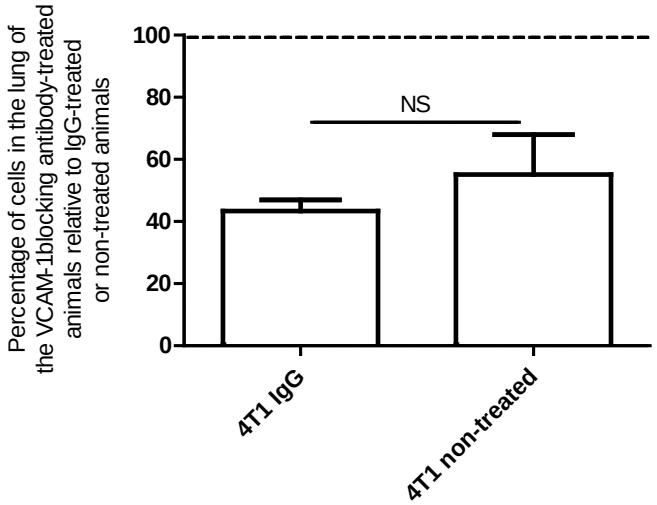
Analysis of the size of platelet clots formed around the tumour cells. In this graph the data is represented as the percentage of the tumour cells analysed that are associated with platelet clot size corresponding to one of the 4 different categories. (n=3)



Supplementary Figure 23

IgG non-targeting antibody has no effect on metastasis

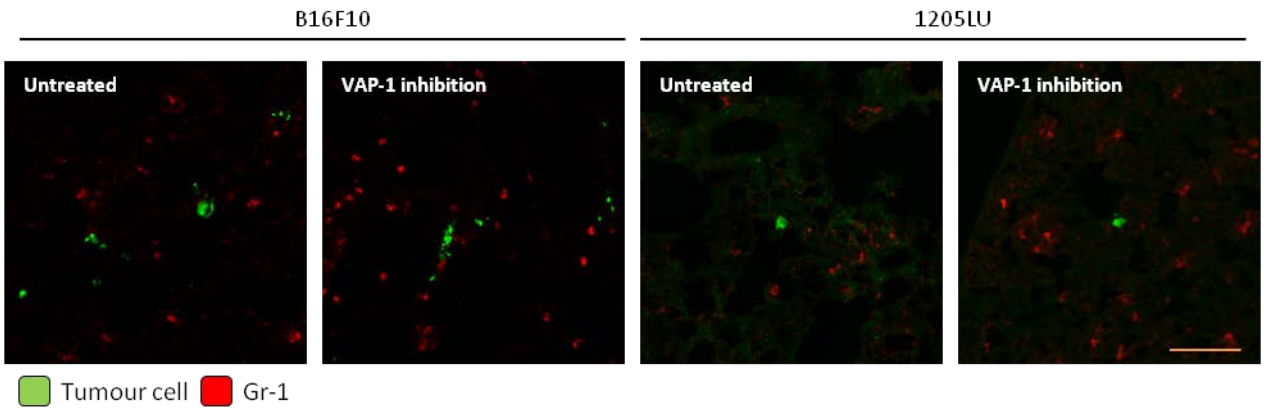
Tumour cells were injected i.v. 4h after VCAM-1 blocking antibody treatment. Metastatic efficiency measured as tumour cell survival in the lung was assessed. This data is presented in Fig.26A&B. The lungs were harvested as for IHC staining, and cells were counted in at least 10 sections per lung to accurately gauge the surviving cell fraction. The surviving cell fraction is represented as the percentage of cells in the treated lungs, where the 100% value is noted by the corresponding control lung. (n=3; Statistical test unpaired t-test; p= 0.2802)



Supplementary Figure 24

Lack of Gr-1^{+} cell recruitment is not affected by VAP-1 inhibition

Gr-1- expressing leukocytes (Alexa Fluor 633) in lung tissue are not recruited in response to either 1205LU-GFP or B16F10 metastasis (green). Therefore VAP-1 treatment has no effect. Images acquired with Zeiss Confocal microscope. Scale=100µm



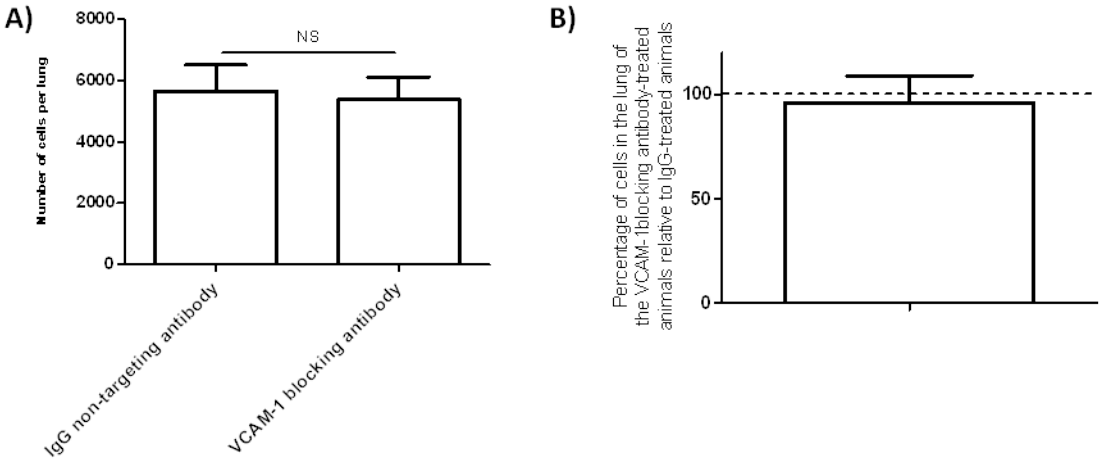
Supplementary Figure 25

There is no effect on tumour cell adhesion of B16F10-CMFDA cells by treatment with VCAM-1 blocking antibody

Tumour cells (B16F10-CMFDA) were injected i.v. 4h after either VCAM-1 blocking antibody or IgG antibody treatment. Metastatic efficiency/tumour cell survival in the lung was assessed 15min later. This was achieved by whole lung imaging and counting the number of tumour cell on the surface of the lungs (n=3).

A) There was no significant difference between the two experimental groups (unpaired t-test p=0.6795).

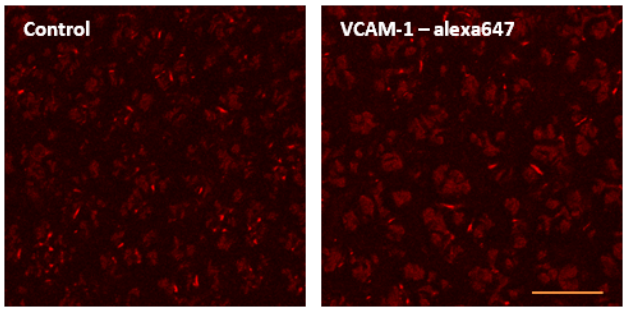
B) The surviving cell fraction is represented as the percentage of cells in the treated lungs, where the 100% value is noted by the corresponding control lung.



Supplementary Figure 26

Injection of a labelled VCAM-1- Alexa Fluor 647 antibody into the vena cava is not sufficient to allow *ex vivo* visualisation of VCAM-1 expression

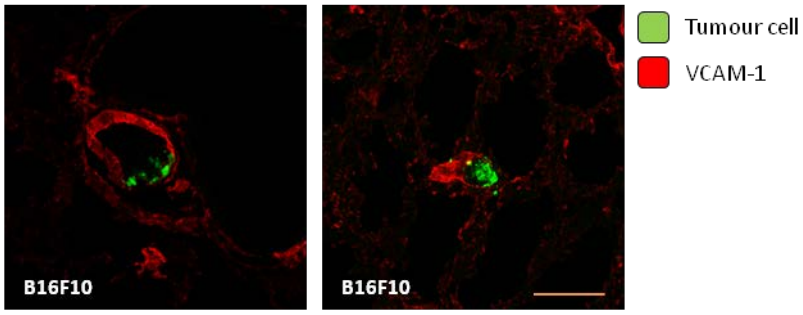
LPS was injected i.p. into female BALB/c mice and 4h later the lungs were harvested for *ex vivo* imaging. In one animal the anti-VCAM-1- Alexa Fluor 647 antibody was injected (100µl) into the vena cava under terminal anaesthesia (as optimised for the CD31-PE antibody). The lungs were imaged *ex vivo* using Zeiss Confocal microscope and representative images are shown. Scale=100µm



Supplementary Figure 27

Proliferation of tumour cells prior to extravasation may also occur in our models

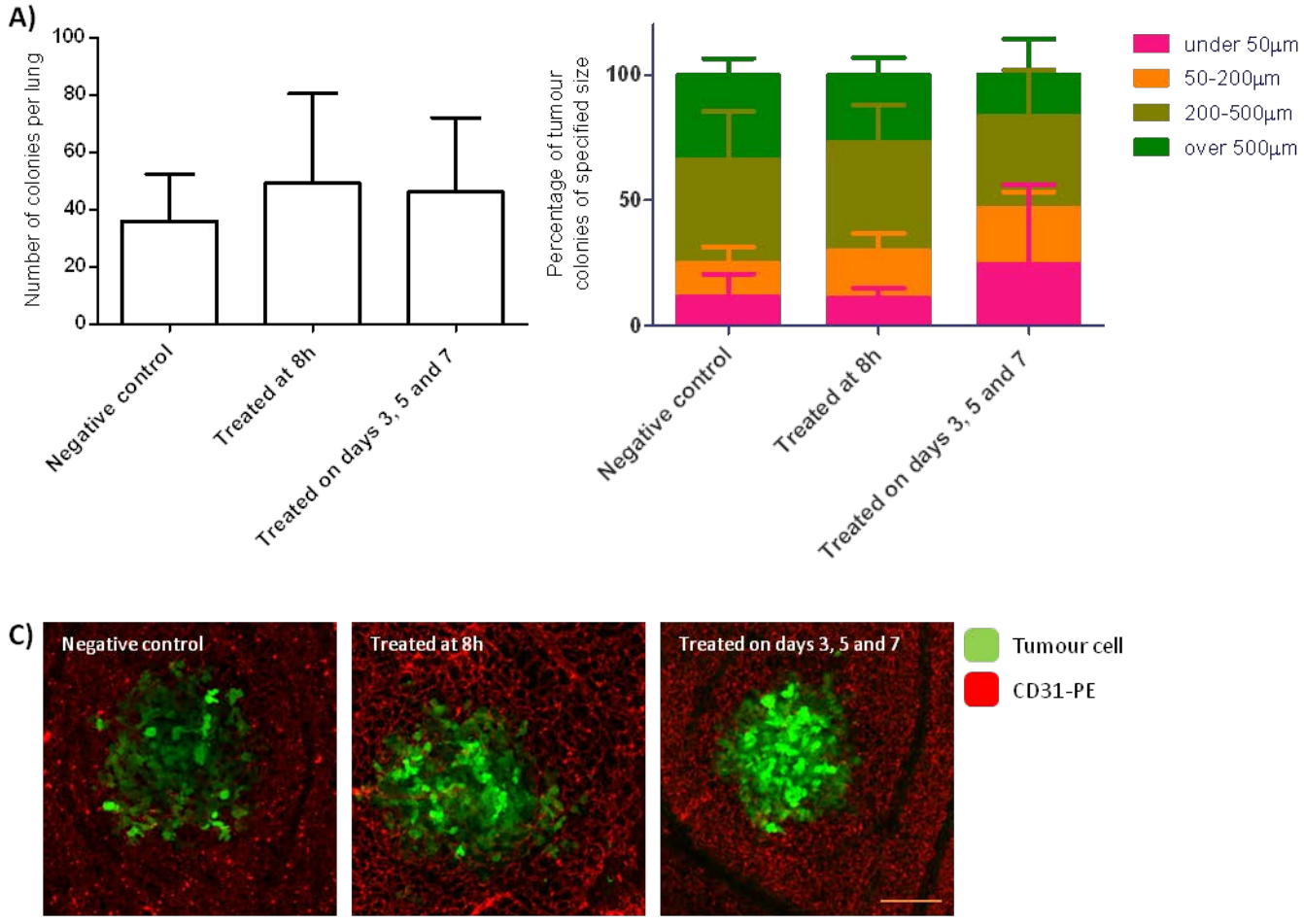
CMFDA-labelled B16F10 cells were injected i.v.(5x10e5) and lungs harvested 8h later.VCAM-1 (Alexa Fluor 633-red) expression in lung tissue was detected with fluorescent IHC and allowed for visualisation of pulmonary vasculature. Tumour cell clusters were observed in this tumour cell model and this may indicate proliferation of tumour cells prior to extravasation. Images acquired Zeiss Confocal microscope. Scale=100µm



Supplementary Figure 28

Inhibition of VCAM-1 by VCAM-1 blocking antibody post tumour cell injection has no effect on metastatic efficiency

Tumour cells (4T1-GFP) were injected at 5x10e5 i.v. into BALB/c mice.VCAM-1 blocking antibody was injected i.v. 8h after tumour cells injection in the first experimental group. In the second experimental group the antibody was injected on days 3, 5 and 7 after tumour cell injection while the negative control group received no VCAM-1 antibody. Lungs harvested 2 weeks after tumour cell injection. Under terminal anaesthesia and artificial lung ventilation, CD31-PE antibody (100µl) was injected into the vena cava. This was clamped for at least 5min, followed by lung harvest as described in material and methods. Imaging with a Leica epifluorescent microscope was used to count the number of metastatic nodes on the lung surface (A) and a Zeiss Confocal microscope was used to image and measure the size (B). Panel C contains representative images of 4T1-GFP lung colonies (green) and CD31-PE (red). The images were obtained using Zeiss Confocal microscope. Scale=100µm



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