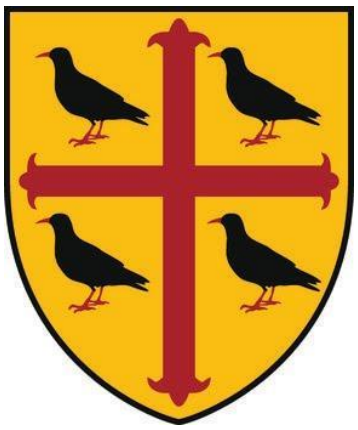
Deciphering the role of endolysosomal Ca^{2+} signalling via two-pore channel 2 (TPC2) in cancer: from bench to bedside



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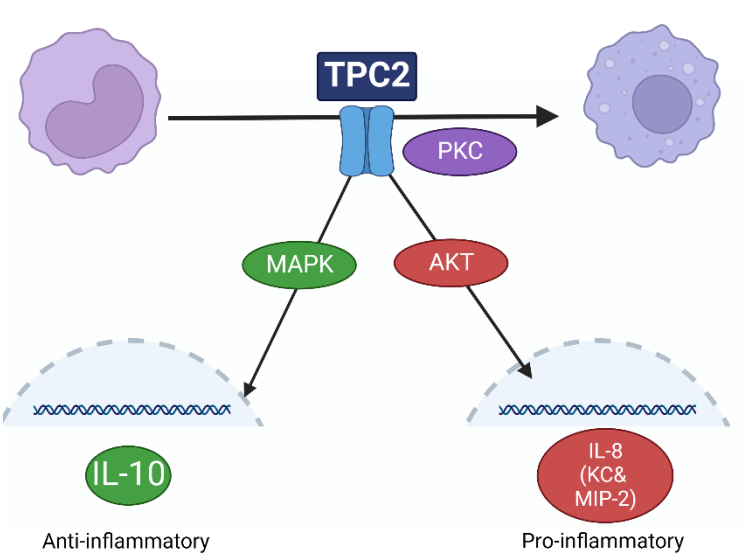
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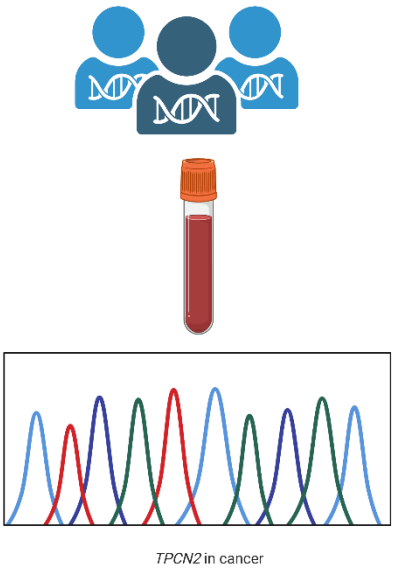
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Deciphering the role of endolysosomal Ca^{2+} signalling via two-pore channel 2 (TPC2) in cancer: from bench to bedside

Abstract: Cancer is a major public health problem and one of the leading causes of death worldwide, necessitating the development of innovative cancer biomarkers/targets to improve detection, diagnosis, prognosis, and therapy. Cancer biomarkers have evolved into clinical tools that can improve the efficiency of detection and guide the treatment of cancer patients by providing personalised therapy and information about expected cancer outcomes. There is increasing evidence that links two-pore channel 2 (TPC2, also known as TPCN2) to cancer, revealing the potential applications of TPC2 as a biomarker/ therapeutic target. My thesis studies have underscored the significant role of TPC2 in cancer immunology, biology, and genetics. Monocytes and macrophages are myeloid cells that play a vital role in inflammation, immunity, and cancer. The spleen is an important source of monocytes, which act as direct precursors of haematopoietic stem cell–derived macrophages. Proteins related to the physiological processes involved in the immune system and cancer were significantly altered in TPC1 knockout (KO), TPC2 KO, and TPC1/2 double KO mouse splenocytes, compared to WT controls, suggesting that TPCs may play significant roles in regulating innate immune system functions in various pathophysiological processes. My thesis studies have identified TPC2 as a major regulator of monocyte differentiation and macrophage development. In addition, my studies have revealed, for the first time, an important link between TPC2 and the release of two mediators that are opposed in their functions: IL-8 pro-inflammatory cytokine and IL-10 anti-inflammatory cytokine. Finally, I have discovered variants in the TPCN2 gene that are associated with cancer at the global level or 13 various cancer types at the local level, in the definition of tumour types, susceptibility, and prognosis in the UK-biobank. My thesis gives insights into the pathophysiological significance of TPC2 in cancer immunity and may contribute to the development of new genomics-based guidelines for cancer diagnosis and therapy.

Deciphering the role of endolysosomal Ca^{2+} signalling via two-pore channel 2 (TPC2) in cancer: from bench to bedside





TPCN2 in cancer

Abeer DPhil publications and contribution to science

- 2 review articles

Alharbi, A, Zhang, Y, Parrington, J. *Deciphering Ca^{2+}signalling in cancer metastasis: From the bench to the bedside*. Cancers (Invited to contribute to Special Issue Title: Targeting Cancer Metastasis) DOI: 10.3390/cancers13020179

Jin, X., Zhang, Y., Alharbi, A., Hanbashi, A., Alhoshani, A. and Parrington, J., 2020. *Targeting Two-Pore Channels: Current Progress and Future Challenges.* Trends in Pharmacological Sciences, 41(8), pp.582-594. DOI: 10.1016/j.tips.2020.06.002

- 2 Mini-review articles

Abeer F. Alharbi, John Parrington. *Endolysosomal Ca^{2+} signaling in cancer: The role of TPC2, from tumorigenesis to metastasis. Frontiers in Cell and Developmental Biology*, section Molecular and Cellular Oncology.DOI: 10.3389/fcell.2019.00302

Alharbi AF, Parrington J. *Deciphering the role of endolysosomal Ca2+ channels in immunity*. Frontiers in Immunology. 2021;12. DOI: 10.3389/fimmu.2021.656965

- 1 Original article

Abeer F. Alharbi, John Parrington. *The role of genetic polymorphisms in endolysosomal ion channels TPC2 and P2RX4 in cancer pathogenesis, prognosis, and diagnosis: a genetic association in the UK Biobank.* npj Genomic Medicine DOI: 10.1038/s41525-021-00221-9

- 1 Preview article

Abeer F. Alharbi, John Parrington. *TPC2 targeting evolution: Leveraging therapeutic opportunities for cancer*. Cell Chemical Biology DOI: 10.1016/j.chembiol.2021.07.020

- 1 Abstract

Abeer Alharbi, John Parrington. Deciphering the role of TPC2 in cancer immunology: from bench to bedside [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2021; 2021 Apr 10-15 and May 17-21. Philadelphia (PA): AACR; Cancer Res 2021;81(13_Suppl): Abstract nr 1764.

In preparation:

Abeer F. Alharbi, John Parrington. Endolysosomal *Ca^{2+} signalling in innate immune systems: the role of two-pore channels (TPCs), from monocyte differentiation to cytokine production*. Department of Pharmacology, University of Oxford, Oxford, UK.

Abeer career achievements during DPhil studies

2022	Simon, Arpi, Mari & John Simonian Prize for Excellence in Leadership, St Edmund Hall, University of Oxford, Oxford, UK
2021	2021 AACR-Woman in Cancer Research Scholar Award, American Association for Cancer Research, US
2021	Tony Doyle Graduate Science Prize, St Edmund Hall, University of Oxford, Oxford, UK
2019	ESMO Advanced Course on Biomarkers for Precision Medicine Travel Grant
2019	ESMO Fellowship-Translational Research in Oncology: From Bench to Bedside and Back
2018	Misk Fellowship, Riyadh, Saudi Arabia

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Abbreviations

Ca^{2+}	Calcium	**TLR**	Toll-like receptor
TPCs	Two-pore channels	**IL-10**	Interleukin-10
TRPs	Transient receptor potential cation channels	**TAMs**	Tumour-associated macrophages
TRPML	Transient receptor potential cation channel, mucolipin subfamily	**mTOR**	Mammalian target of rapamycin
P2X4R	P2X4 ATP-activated cation channel	**cADPR**	Cyclic ADP-Ribose
NAADP	Nicotinic acid adenine dinucleotide phosphate	**VEGF**	Vascular endothelial growth factor
$PI(3,5)P_2$	Phosphatidylinositol 3,5-bisphosphate	**VEGFR2**	Vascular endothelial growth factor receptor 2
CCL2	Chemokine (C-C motif) ligand 2	**CRISPR**	Clustered regularly interspaced short palindromic repeats
IL-8	Interleukin-8	**KO**	Knockout

CXCL1/KC	Keratinocyte-derived chemokine	**KD**	Knockdown
CXCL2/MIP-2	Macrophage inflammatory protein-2	**M1**	Macrophage type 1
M2	Macrophage type 2	**NF-κB**	Nuclear factor kappa-light-chain-enhancer of activated B cells
CD38	Cluster of differentiation 38	**MyD88**	Myeloid differentiation primary response 88
SLE	Systemic lupus erythematosus	**STAT3**	Signal transducer and activator of transcription 3
IL-1β	Interleukin 1 beta	**IL-10**	Interleukin 10
IL-6	Interleukin 6	**Mϕ**	Non-polarised adherent macrophages
IFN-γ	Interferon-gamma	**PMA**	Phorbol 12-myristate 13-acetate
TNF-α	Tumour necrosis factor	**CD11b**	Integrin Mac-1
PKC-alpha	Protein kinase C-alpha	**AML**	Acute myeloid leukaemia
MAPK	Mitogen-activated protein kinase	**SNP**	Single-nucleotide polymorphism
AKT	Protein kinase B	**SLE**	Systemic lupus erythematosus

Chapter 1: Introduction

I declare re-use of my published materials in this chapter:

- Alharbi AF, Parrington J. Endolysosomal Ca^{2+} signaling in cancer: the role of TPC2, from tumorigenesis to metastasis. Frontiers in Cell and Developmental Biology. 2019 Dec 4;7:302.
- Alharbi AF, Parrington J. Deciphering the Role of Endolysosomal Ca^{2+} Channels in Immunity. Frontiers in Immunology. 2021:1456.
- Alharbi AF, Parrington J. TPC2 targeting evolution: Leveraging therapeutic opportunities for cancer. Cell Chemical Biology. 2021 Aug 19;28(8):1103-5.

1. Chapter 1: Introduction

1.1. Introduction

1.1.1. General Introduction

Calcium (Ca^{2+}) signalling via endolysosomal ion channels that include two-pore channels (TPCs), transient receptor potential cation channels (TRPs), the mucolipin subfamily (TRPML), and the P2X4 ATP-activated cation channel (P2X4R), plays a significant role in various pathophysiological processes (1). These channels are localised in the endolysosomal system: lysosomes; early, late, and recycling endosomes; and autophagosomes (2,3,4,5,6). CD38 is a multifunctional ADP ribosyl cyclase that plays a role in signal transduction, cell adhesion, and intracellular Ca^{2+} signalling and is expressed in immune cells (7,8). It is known to generate both cADPR and NAADP (8). The exact mechanism of action of TPCs was debated by the scientific community (9) until the recent development of novel pharmacological agonists (TPC2-A1-N and TPC2-A1-P) by Gerndt et al. (2020) which provide an explanation for the ion permeability of these channels (10). They found that the release of Ca^{2+} or Na^{+} is ligand dependent (10). These ion channels can mediate Ca^{2+} signals upon binding to nicotinic acid adenine dinucleotide phosphate (NAADP) either directly or indirectly via other proteins, but they can also release Na^{+} in response to another intracellular modulator, phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) (4,11,12). The concept of a ligand-specific mechanism was suggested by Zhang et al. (2019) when they conducted high-throughput screening of FDA-approved drugs to discover activators of TPCs, followed by patch clamping (13). Alteration of endolysosomal Ca^{2+} signalling is emerging as a key player in cellular differentiation, migration, autophagy, phagocystosis, inflammation, immunity, and cancer development (2, 14,15,16). The immune system comprises two fundamental classes: the innate immune system and the adaptive immune system, both of which play significant roles in health and disease. Monocytes, phagocytes (macrophages and neutrophils), dendritic cells,

mast cells, basophils, eosinophils, and natural killer (NK) cells are components of the innate immune system. Accumulated data have shown that various physiological processes related to immune cell function, such as antigen presentation, phagocytosis, chemotaxis, release of proinflammatory mediators, and cellular trafficking, occur on endosomes or lysosomes (17,18,19,20,21). A recent study showed that TRPML2 mediated endolysosomal Ca^{2+} release upon ML2-SA1 (a selective agonist of TRPML2) binding plays a role in chemokine (C-C motif) ligand 2 (CCL2) trafficking and secretion from murine macrophages, which is part of the innate immune machinery (22). Recycling endosomes (RE), late endosomes and lysosomes, and the trans-Golgi-network are involved in the regulation of macrophage cytokine/chemokine release. Interleukin-8 (IL-8) is a proinflammatory chemokine mainly produced by macrophages and other immune cells that acts as a potent neutrophil chemoattractant (23). The keratinocyte-derived chemokine (CXCL1/KC) and macrophage inflammatory protein-2 (CXCL2/MIP-2) are murine IL-8 homologues produced by the toll-like receptor (TLR) signalling pathway in a distinctive manner, both by MYD88, whereas MIP-2 is produced through TRIF signalling (24). IL-8 levels were elevated in patients with scleroderma and other autoimmune diseases (25,26,27). It has also been shown that Ca^{2+} signalling induces IL-8 transcription and production in calcineurin-dependent pathways (28). Similarly, IL-8 signalling was shown to be involved in stimulating CD38 to raise the intracellular Ca^{2+} level, inducing the migration of lymphokine-activated killer cells (29). In 2015, the FDA approved daratumumab, which is the first human anti-CD38 monoclonal antibody to treat patients with multiple myeloma. Pharmacological modulation of IL-8 is a therapeutic strategy to treat patients with cancer that is still in the drug development stage. HuMax-IL-8 (BMS-986253), an anti-IL-8 monoclonal antibody, is in phase 2 clinical trials (NCT03400332, NCT04123379, NCT04848116, and NCT02451982) (30). IL-8 plays a role in inflammation and wound healing and acts as a bridge between inflammation and the tumour epithelial-mesenchymal transition (EMT) that facilitates tumour progression via autocrine and

paracrine pathways (31,32). In contrast, interleukin-10 (IL-10) is an anti-inflammatory and immunosuppressive cytokine produced by monocytes, stimulated macrophages, and other immune cells, with regulatory T-cells and helper T-cells being recognised as the major cellular sources of IL-10 (33). IL-10 plays a crucial role in hindering inflammatory and auto immune diseases (34). Inflammatory bowel disease developed in IL-10 knockout (KO) mice in an *in vivo* model following the colonisation of the gut with microorganisms (35). In the context of cancer, IL-10 exhibits anti-tumour effects and constrains tumour-promoting inflammation (36). Moreover, IL-10 KO mice have been shown to be susceptible to development of colon cancer, and human individuals with IL-10 deficiency developed lymphoma at an early age (37,38). The NAADP/TPC/Ca^{2+} signalling pathway is critical for cellular differentiation. Recent evidence has shown that TPC2 acts as a regulator of osteoclastogenesis (39). TPC2 is overexpressed in monocytes (40), which are recruited to target tumour tissues in response to tumour-derived factors, leading to the further differentiation of this cell type into macrophages (41). Tumour-associated macrophages (TAMs) are a part of the tumour microenvironment, where they play roles in tumour growth, angiogenesis, metastasis, immunosuppression and chemo- or radiotherapy resistance (42,43). Several clinical studies have shown a strong association between increased TAM density and poor survival in cancer patients (44). Circulating bone marrow monocytes are the precursors of macrophage-mediated pathological responses. One study has shown that circulating monocytes from prostate cancer patients enhance the invasion and motility of tumour cells (45). Activated STAT3 monocytes have been found to promote tumourigenesis in patients with liver cancer and in *in vivo* mouse models (46). Another study also demonstrated that adult neovascularisation relies on the recruitment of blood monocytes in a mouse model of vascular endothelial growth factor-induced liver angiogenesis (47). The specific elimination of a population of Tie2 (Angiopoietin-1 receptor) $^{+}$ monocytes reduces tumour angiogenesis and slows tumour progression in both mammary and glioma tumourigenesis (48).

These findings are consistent with the hypothesis that macrophages are the major source of angiogenic growth factors (49,50). The immunosuppression associated with TAMs might be attributed to the upregulation of programmed death-ligand 1 (PDL-1) and programmed death-ligand 2 (PDL-2) expression in blood monocytes and TAMs in cancer patients (41,51). Several processes, such as inhibition of the development of monocytes/macrophages or recruitment of monocytes to the tumor site, interfere with TAM development. A previous study showed that the half-life of circulating monocytes is short; thus, targeting the differentiation of monocytes into macrophages is a promising immunotherapeutic approach (43). Safety concerns regarding the depletion of non-tumour macrophages necessitate identifying a specific target or novel drug-based approach that specifically affects TAM function. It has also been shown that mammalian target of rapamycin (mTOR) pathway inhibition via rapamycin during peripheral monocyte differentiation modulates the pro-tumour function into anti-tumour effects (52). Trabectedin is a chemotherapeutic agent that was shown by several research groups to interfere with macrophages' development or functions. Recently, Belgiovine et al (2021) observed that pre-treating mice bearing tumours with trabectedin improved the response to PD-1checkpoint inhibitor via the activation of T-lymphocytes, which provides the first preclinical proof of concept for the candidacy of the combination therapy of trabectedin with PD-1/PD-L1 checkpoint inhibitors as therapeutic strategies to treat patients with cancer(53). The cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in monocyte differentiation and macrophage development and its links to inflammation, immunity, and cancer development have not yet been investigated. Indeed, research in this area is still in its infancy. In particular, the role of TPC-evoked endolysosomal Ca^{2+} signalling in intracellular trafficking and the release of chemokines and cytokines, particularly IL-8 and IL-10, have not been characterised or studied at the molecular level. Accordingly, my objectives in my DPhil studies were to examine NAADP/TPC2/Ca^{2+} signalling during monocyte differentiation and define the role of TPC2 as a potential downstream

regulator of monocyte differentiation or the functional microenvironment reprogramming of TAMs to restore anti-tumour capabilities and its role during immunosurveillance. This thesis reveals, for the first time, an important link between TPC2 and the release of IL-8 and IL-10. Furthermore, it shows that TPC2-mediated endolysosomal Ca^{2+} signalling plays a pivotal role in physiological processes related to the innate immune system and acts as a regulator of monocyte differentiation and it raises a question regarding the clinical utility of TPC2 as a novel biomarker or druggable target to block the development of macrophages and to modulate CD38/IL-8 levels as therapeutic strategies for future cancer therapy.

1.1.2. The ubiquity of Ca^{2+} and its role in health, disease, and therapy

The divalent cation calcium (Ca^{2+}) is a versatile second messenger, and its homeostasis is critical to maintaining health at both the intracellular and extracellular levels. The main source of Ca^{2+} is the diet and its release into the cell via Ca^{2+} channels and transporters is regulated by endogenous and exogenous modulators in the specific biological context. Intracellular Ca^{2+} signals are implicated in a wide range of physiological processes including proliferation, differentiation, metabolism, secretion, contraction, and fertilization (54). The dysregulation of Ca^{2+} homeostasis has been demonstrated in various pathophysiological processes, including apoptosis, autophagy, angiogenesis, inflammation, tumour invasion and migration. These, in turn, are linked to the development of various pathological condtions, such as cancer, cardiovascular diseases, diabetes, neurological defects, osteoporosis, and immunological disorders (55,56,57,58,59,60,61). Because of this, the regulation of spatiotemporal Ca^{2+} signals in the form of waves or oscillations has emerged as a topic of research relevant to human health and diseases. An established quantity of experimental evidence classified intracellular Ca^{2+} signalling pathways into two main categories, local and global, and their communication has drawn scientific attention for decades as a basis for understanding disease biology at the molecular level and for the development of therapies. Ca^{2+} signalling mediated by signalling proteins including inositol

1,4,5-trisphosphate (IP_3) receptors (IP_3Rs), transient receptor potential cation channels (TRPMLs, mucolipins), and two-pore channels (TPCs) or extracellular channels including calcium-sensing receptor (CaSR), and store-operated calcium entry (SOCE), are recognised as valuable therapeutic targets or biomarkers to optimise the diagnosis or the therapy of various diseases (62,63). In clinical settings across the globe, the pharmacological inhibition of Ca^{2+} signalling utilising Ca^{2+} channel blockers has been very valuable in controlling or treating many human disorders, including hypertension, supraventricular dysrhythmias, migraine headaches, subarachnoid haemorrhage, absence seizures, and chronic pain since they were discovered by Albrecht Fleckenste in 1964 (64). The indirect pharmacological approach that interferes with Ca^{2+} signalling via the inhibition of Ca^{2+}dependent proteins, such as cyclosporin-A (calcineurin inhibitor) has been widely utilised in clinical practice for a long time to modulate immune system functions in a dose-dependent manner (65) mainly to prevent rejection after solid organ transplantation (66) or to treat inflammatory disorders such as psoriasis (67) and rheumatoid arthritis (68). The potential therapeutic window of Ca^{2+} signalling modulations continues to attract researchers to decode the role of the different Ca^{2+} signals in the biology of various diseases and to discover novel therapeutic agents that precisely modulate the Ca^{2+} pathway that contributes to the development of certain diseases.

1.1.3. Intracellular Ca^{2+} channels in cellular development, cancer, and immunity

A growing body of literature is elucidating the biological significance of intracellular Ca^{2+} signalling in various physiological and pathological processes, and I am focusing mainly and briefly here on the intracellular Ca^{2+} channels that are involved in cell differentiation and development, immune cell biology and cancer pathology, which are related to my DPhil studies. Intracellular Ca^{2+} is mainly released from the endoplasmic reticulum (ER) and the sarcoplasmic reticulum (SR), which

are major intracellular Ca^{2+} repositories for ryanodine receptors (RyRs), inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the sarco-endoplasmic reticulum Ca^{2+} reuptake pump (SERCA). It is also released by the Ca^{2+} channels in the endolysosomal compartment, that is two-pore channels (TPCs) and transient receptor potential cation channels (TRPMLs, mucolipins). Previous studies have led to an understanding of the biological role of endolysosomal Ca^{2+} signalling in cell differentiation and development, which I will briefly review below (in section 1.1.2), but endoplasmic and sarcoplasmic reticular Ca^{2+} signalling at the molecular level in these processes is biologically less well characterised. Diverse immunological roles of endoplasmic and sarcoplasmic reticular Ca^{2+} signalling that are mainly mediated via inositol 1,4,5-trisphosphate receptors continue to attract researchers to understand immune cell biology, with recent evidence highlighting the immunological significance of RyR1 in T cell activation (69). Elucidating T lymphocyte regulation in the context of cancer was, and still is, a viable therapeutic target in the armamentarium against cancer. IP_3R (inositol 1,4,5-trisphosphate receptor) mediated Ca^{2+} release has been identified as a regulator of T lymphocyte apoptosis *in vitro* (69). In the same vein, Nagaleekar et al. (2008) deciphered the biological significance of IP_3R/Ca^{2+} signalling in other physiological processes within T cells, including differentiation and activation in a specific population of T lymphocytes – CD4 T cells (70). Recent research has found that triple knockout of IP_3R isoforms 1, 2 and 3 in mice *in vivo* caused the development of aggressive leukaemia in thymocytes that resembles human T cell acute lymphoblastic leukaemia, indicating that Ca^{2+} release via IP_3R is integral to prevention of malignant transformation and maintainance of normal thymocyte development (71). Similarly, Tang et al. (2017) showed that IP_3R-mediated Ca^{2+} signalling acts as a modulator of physiological processes (development, proliferation and survival) of B cells and their functions (IL-10 and antibody production) (72). Notably, the roles of IP_3R/Ca^{2+} signalling in physiological processes related to T and B cell functions are evolving, underscoring the importance of biologically and clinically fully characterising its functions in

lymphocytes, with a particular focus on T lymphocytes, for the development of immunotherapeutic approaches to treat cancer. Recently, Diercks et al. (2018) identified a pivotal role for NAADP-evoked Ca^{2+} release via RyR1 (intracellularly), with communication through Ca^{2+} signals triggered via ORAI1 and STIM (extracellularly) as a meditator of T cell activation (73). An evolving body of evidence continues to uncover the function of endoplasmic and sarcoplasmic reticular Ca^{2+} signalling in cancer biology. Importantly, IP_3R isoform 3 and SERCA3 are implicated in various types of cancer; however, the non-cancer specificity of these channels and the ubiquity of Ca^{2+} signals in our cells necessitates further investigation to discover the precise mechanisms underlying the action of these channels and to define a specific upstream or downstream target. While researching our mini-review articles, entitled 'Endolysosomal Ca^{2+} Signaling in Cancer: The Role of TPC2, From Tumorigenesis to Metastasis', and 'Deciphering the Role of Endolysosomal Ca^{2+} Channels in Immunity', I observed that evidence has started to grow for biologically characterising these endolysosomal Ca^{2+} signalling channels in the immune system and cancer, and I will discuss these data in the following section.

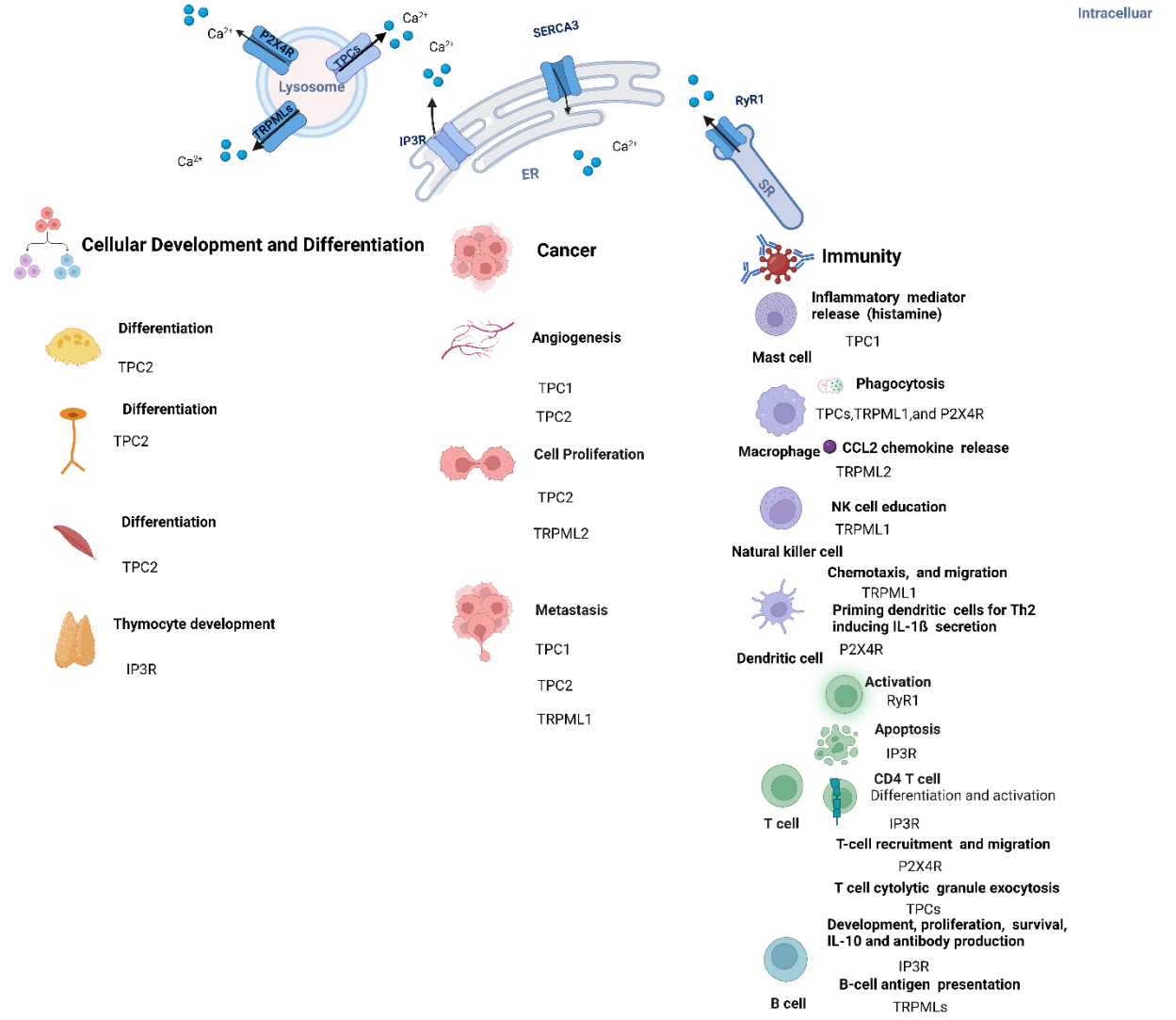


Figure 1. Schematic representation of intracellular calcium channels in cellular development, differentiation, cancer, and immunity. (Created with BioRender.com).

1.1.4. Endolysosomal Ca^{2+} Signalling and Lysosomes in Cellular Development, Cancer, and Immunity

The endolysosomal compartment is comprised of early, late, and recycling endosomes, and lysosomes. Two-pore channels (TPCs, also known as TPCNs); transient receptor potential cation channels; mucolipins (TRPMLs); and the P2X4 ATP-activated cation channel (P2X4R) are the main channels responsible for the endolysosomal Ca^{2+} signalling hub, which is recognized as a new biologically significant system of intracellular Ca^{2+} signalling. To my knowledge, the roles of endolysosomal Ca^{2+} signals in cellular development and differentiation have only been deeply investigated for TPCs. Although it has become clear that NAADP/TPC/Ca^{2+} signalling acts as a regulator of the differentiation process in various types of cells, including osteoclasts (74), neurons (75), and skeletal muscle cells (76), much remains to be discovered about the exact signalling mechanisms involved in the differentiation process of a specific type of cells. Our lab recently investigated the role of NAADP-Ca^{2+} signalling mediated by TPCs in embryonic development. Lysosomes were discovered by Christian de Duve, who won the Nobel Prize in Physiology or Medicine in 1974. Lysosomes are organelles with a plethora of functions, with particular significance in phagocytosis (77) and have been shown to exhibit different phenotypes in the context of cancer compared to normal lysosomes, in various aspects of their biology (78,79,80,81). Upregulation of protease activity was observed in cancer cell lysosomes and contributed to tumour progression biology (82,83). Ca^{2+} signalling mediated by endolysosomal channels, mainly via TRPML1 and TPC2, with recent interest in a role for P2X4R, is emerging as a player in processes related to cancer biology. Li et al. (2016) found that lysosomal Ca^{2+} via TRPML1 and calmodulin modulates m-TORC1 activity (84). m-TORC1 negatively regulates autophagy (85), which involves cancer biology, particularly in carcinogenesis and chemoresistance (86,87). PI3K/AKT/mTOR signalling is demonstrated in various physiological processes in cancer pathology and types of cancer (88). The transcription factor EB (TFEB) acts

as a regulator of various physiological processes. Its biological effects are well-studied in autophagy, which acts as the master modulator of this process, and in lysosomal biogenesis (89). In endothelial cells, TFEB was found to modulate post-ischemic angiogenesis in a process involving AMPKα signalling activation and autophagy. They also reported that TFEB mediates the upregulation in TRPML1 expression at the mRNA level and facilitates its binding to the MCOLN1 gene promoter (90), indicating that TEFB might play a role in angiogenesis through lysosomal Ca^{2+} signalling via TRPML1. Recently, TRPML1 was studied in triple-negative breast cancer cell biology by Xu et al. (2019) (91). TRPML1 modulation either by genetic deletion or by pharmacological inhibition by ML-SI11 attenuated cancer cell proliferation, migration and invasion *in vitro* (MDA-MB-231 cell line) and reduced tumour growth *in vivo* (91). The contradictory values of TRPML1 as a prognostic biomarker were demonstrated in HRAS mutant patients with bladder urothelial carcinoma or head and neck squamous cell carcinoma (92) and in glioblastoma patients (93). The loss of/reduced TRPML1 mRNA expression was correlated with favourable survival in HRAS mutant patients with bladder urothelial carcinoma or head and neck squamous cell carcinoma (92), whereas with poor survival in glioblastoma patients (93). TRPML2 and TRPML3 are less characterised in cancer biology. Upregulation in TRPML2 expression was found to facilitate glioma cell proliferation in *vitro* (T98 and U251 glioma cell lines) (94). TRPML3 acts as a player in endocytosis and cellular trafficking (95). Recently, the role of TRPML3 in gefitinib resistance in non-small cell lung cancer was revealed by Kim et al. (96). Non-small cell lung cancer developed resistance to gefitinib due to its positive effects on the lysosomal pH and trafficking (96). The loss of TRPML3 expression via genetic modulation enhanced the non-small cell lung cancer cells (*in vitro*) to gefitinib therapy. The authors did not illustrate the mechanism of how TRPML3 mediates gefitinib resistance in NSLC; they suggested that gefitinib enhances lysosomal pH, which leads to

lysosomal Ca^{2+} release via TRPML3 (acts as a sensor of lysosomal pH). This, in turn, drives lysosomal trafficking and exocytosis in NSCLC cells (96). Collectively, this evidence underscores the evolving roles of lysosomal Ca^{2+} via TRPML in cancer development, especially TRPML1, which warrants further investigation into its protumourigenic effects in several cancer hallmarks.

Purinergic receptor P2X4 is located in the late endosome, lysosome and plasma membranes (97). Lysosomal pH and luminal ATP mediate the Ca^{2+} release from this channel (97). Lysosomal Ca^{2+} signalling via P2X4R results in lysosomal membrane fusion in a calmodulin-dependent fashion (98). The role of P2X4R has been demonstrated mainly in cancer-associated pain (99,100); therefore, the pharmacological modulation of P2X4R represents a therapeutic approach to alleviate the pain associated with cancer. The evolving body of evidence underscores the biological significance of TPCs, particularly TPC2, in tumourigenesis, angiogenesis and metastasis (101,102,103,104,105). The role of TPC1 in cancer development has not yet been well studied (106). The TPC1 expression at the transcriptomic level was approximately three to eight times higher than TPC2 in the *in vitro* model of human breast cancer (SKBR3 cells) (101). Faris et al. (2019) reported that NAADP/TPC1/ Ca^{2+} mediates the activation of ERK and the PI3K/AKT signalling pathways, which facilitates metastatic colorectal cancer cell proliferation (107). This warrants further investigation of *in vivo* models to examine the therapeutic value of targeting TPC1 as a strategy to attenuate cancer growth in patients with metastatic colorectal cancer.

Recently Cui et al. (108) drew our attention to the role of lysosomes in macrophages as promoters of tumour progression. They developed a DNA nanodevice (E64-DNA) that precisely targets lysosomes inside the macrophages *in vivo* by inhibiting cysteine protease activity, thereby reducing tumour growth (108). It is therefore likely that such connections exist between lysosomes inside macrophages in the tumour microenvironment and during tumour growth. Further research

should be undertaken to investigate the possibility that cysteine protease present inside the lysosomes of macrophages acts as a promoter of tumour growth.

With the evolving knowledge regarding the roles of endolysosomal cation channels in health and disease, the functions of these channels in immunity have not yet been fully explored.

Therefore, I have reviewed extensively the critical roles of endolysosomal Ca^{2+} signalling in immunity and summarised their roles in various physiological processes in this system (in Figure 2 and Table 1). The roles of endolysosomal cation channels involving TPCs, TRPML1 and P2X4R are well-investigated in phagocytosis, which is a vital physiological process involved in immunity. (109, 110,111). Until now, TRPML2 is the only channel that has been shown to be directly involved in chemokine/cytokine release (22). TPC1 was reported to modulate the release of an inflammatory mediator (histamine), which contributes to the development of anaphylaxis (60). Zajac et al. (2013) recently discussed the approaches and challenges of imaging lysosomal Ca^{2+} signalling. They propose to use imaging with optogenetic probes to decipher Ca^{2+} signalling (112).

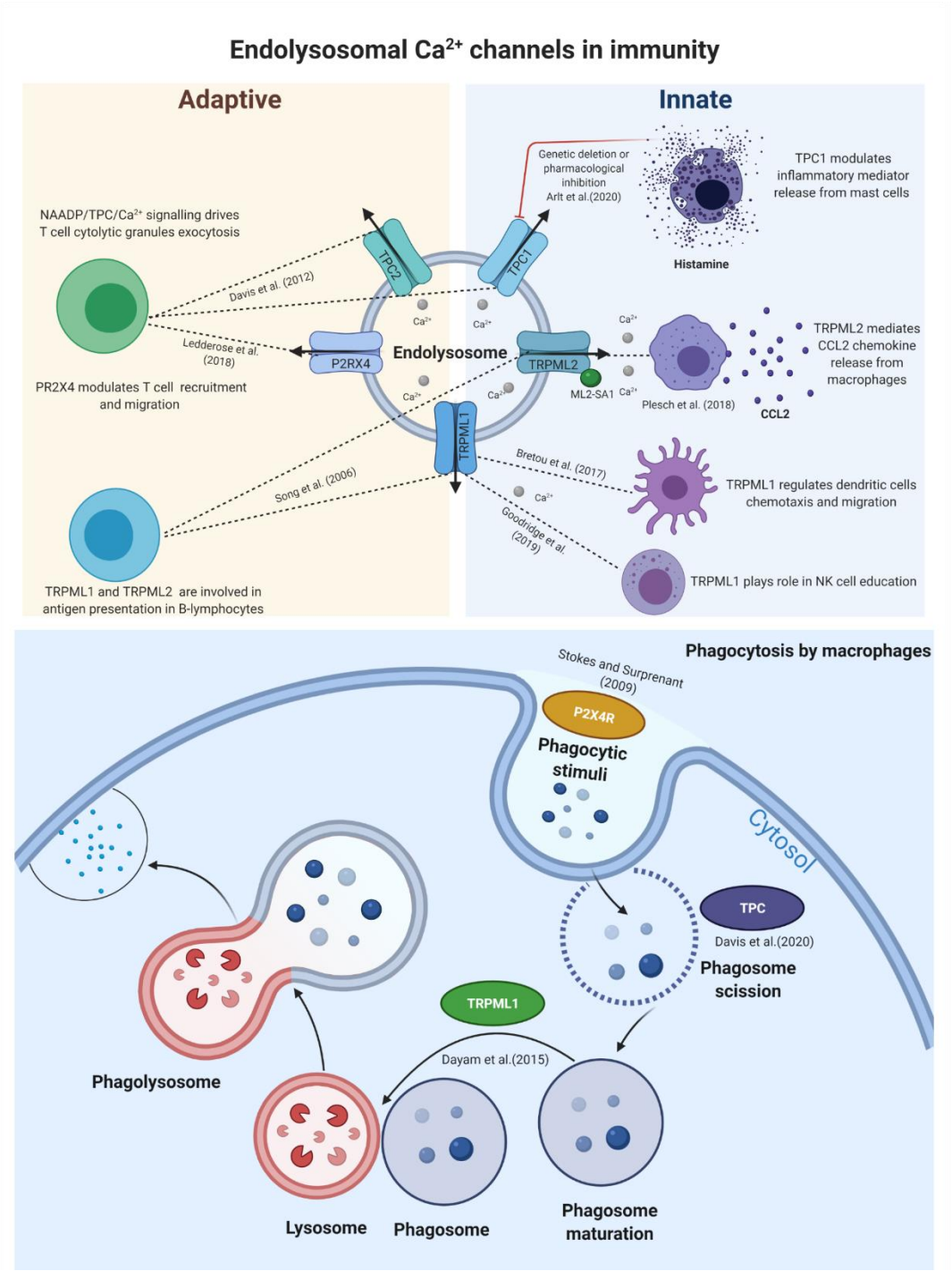


Figure 2. Endolysosomal channels in immunity (113). (The figure was reproduced from Alharbi, A. F., & Parrington, J. (2021), under copyright © 2021 Alharbi and Parrington).

Table 1. Endolysosomal Ca^{2+} signalling modulates physiological processes related to immunity. (The table was reproduced from Alharbi, A. F., & Parrington, J. (2021), under copyright © 2021 Alharbi and Parrington)				
Immune system	Endolysosomal Ca^{2+} channel	Process related to immunity	In vitro/in vivo	Ref.
Innate immunity				
Macrophages	TPCs	Phagocytosis	RAW 264.7, BMDM of WT, TPC1KO, TPC2KO, TPDKO and TRPML-1KO mice	109
	TRPML1			
	P2X4R		Human alveolar, THP-1, NR8383, J774, mouse peritoneal, and RAW264	110
	TRPML2	CCL2 chemokine release	BMDM of WT and TRPML-2KO mice	23
Dendritic cells	TRPML1	DC chemotaxis, and migration	BMDCs of WT and TRPML1 KO mice	114
	P2X4R	Priming dendritic cells for Th2 inducing *IL-1β* secretion	BMDCs of WT and P2X4R KO mice	115
Mast cells	TPC1	Inflammatory mediator release (histamine)	WT and TPC1 KO mice	111
NK cells	TRPML1	NK cell education	NK cells sorted from PBMC	116
Adaptive immunity				
B-cells	TRPMLs (TRPML1 and TRPML2)	B-cell antigen presentation	DT40 B-lymphocytes	117
T-cells	P2X4R	T-cell recruitment and migration	Primary T cells and Jurkat T cells	118
	TPCs (TPC1 and TPC2)	T cell cytolytic granule exocytosis	Jurkat 1G4 cells	119

1.1.5. Clinical investigations leveraging therapeutic opportunities of targeting endolysosomal Ca^{2+} channels in cancer and disorders related to the immune system (as biomarkers)

There is an increasing body of evidence that links two-pore channel 2 (TPC2) to cancer, indicating the potential applications of TPC2 as a biomarker. My DPhil studies have provided links between TPC2 and cancer biology and genetics. Recently, we have characterized for the first time the effects of genetic variants in TPCN2 and P2X4R on cancer at the global level and on various cancer types at the local level to reveal the potential applications of TPC2 and P2X4R as biomarkers in the definition of tumour types, susceptibility, and prognosis in the UK-biobank. Novel associations were reported between genetic variants of TPCN2 and P2X4R and cancer/cancer subtypes in the UK Biobank's population, which I will discuss in more detail in Chapter 5. An evolving body of literature underpins the significance of endolysosomal Ca^{2+} signalling in immunity at the expermintal level with limited data available at the clinical level. TPCN2 was recently reported as a one of four genetic biomarkers to identify subjects who are more likely to develop systemic lupus erythematosus (SLE) based on their genetic makeup (118). SLE is a chronic autoimmune disease, manifested by multiple organ disorders. Khalid et al.(2018) found that the protein expression of P2X4R was significantly upregulated in tissues of patients with hepatitis C virus-induced hepatocellular carcinoma compared to non-hepatitis C virus-induced hepatocellular carcinoma (120).This study provides prelminarly evidence regarding the possible role of P2X4R in hepatitis C virus-induced hepatocellular carcinoma biology; it raises a pharmacological question about the potential theraputic benefits of targeting this channel to prevent the development of hepatitis C virus-induced hepatocellular carcinoma,which warrants further investigations in cell or animal based biological models.

1.1.6. Structure and mechanisms of TPCs

TPC1 and TPC2 are expressed in mammals and located in the endolysosomal compartment. TPCN1 and TPCN2 genes are found on chromosome 11 region q13.3 and chromosome 12 region q24.13, respectively. The development of cryo-electron microscopy by Richard Henderson, Jacques Dubochet and Joachim Frank in 1990 has revolutionised the structural biology field by providing researchers with a valuable tool to identify the structures of unknown biological molecules (121). In 2018, the 3D structure of mouse TPC1 was confirmed by She et al. (2018) (122). Our lab is currently collaborating with Chengdu University of Traditional Chinese Medicine in China to identify the human structure of TPC1. Xiaoqi Pan and his colleagues have predicted the human TPC1 structure. She et al. (2019) revealed the structure of the human TPC2 protein (123); this has created opportunities to develop pharmacological modulators that modulate TPC2 function and to expand our knowledge of interactions that facilitate channel opening. In general, TPC1 and TPC2 structures are similar. TPC1 and TPC2 are homodimers composed of two identical subunits, and each subunit contains shaker-like 6 transmembrane domains (as shown in Figure 3 (124)). In contrast to TPC2, TPC1 uses voltage-dependent cation release, She et al. (2018) recognised arginine residues in the 6-TMII domain in the murine TPC1 structure as a sensor for voltage changes (122), whereas She et al. (2019) determined that the amino acid isoleucine at 551 (Ile551) of the human TPC2 structure provides the voltage-independent feature of this channel (123). She et al. (2018) and She et al. (2019) only provide insights into how phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) modulates mouse TPC1 or human TPC2 gating; little is known about the structural responsibility of these channels for ion release selectivity (122, 124). The first insight in terms of the gating mechanism of how NAADP activates TPC1 function came from Patel et al. (2017), when they identified arginine residues in the S4–S5 linker in the mouse TPC1 structure as a target responsible for NAADP-mediated cation release via TPC1 (125). TPC1 and TPC2 are cation

channels; the debate continues regarding whether these channels release Na^+ or Ca^{2+} upon binding by second messengers (nicotinic acid adenine dinucleotide phosphate (NAADP) or phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$). In 2009, TPC channels were initially recognised as Ca^{2+} meditated channels in mouse and human cells (126). In 2012, controversial data relating to the claim that NAADP is the main and only regulator of TPC function in the Ca^{2+} selectivity of these channels first came from Wang et al (2012) (4). They provided evidence to support their claim that TPCs are Na^+-selective, not Ca^{2+} channels, and modulated by $PI(3,5)P_2$, not by NAADP (4). These findings were critiqued by several research groups in the field to re-propose TPC as mainly Ca^{2+} mediated channels (127,128). Intriguingly, the chemical similarity of Ca^{2+} and Na^+ in terms of the ionic radius raises an additional question about the ion selectivity of these channels, which is challenged by the fact that the active form of calcium, Ca^{2+}, is smaller in size compared to Na^+ after losing its electron. From my perspective and based on previously published data, TPCs exhibit different actions in different biological contexts, and this could be attributed to the mediator (NAADP or $PI(3,5)P_2$) or cation release (Ca^{2+} or Na^+) in a specific biological context. Identification of TPC1 human structure and further investigations of TPC protein structure, molecular mechanisms of ligand gating and ion selectivity will contribute to advancing our biological knowledge of the role of these channels in health and disease.

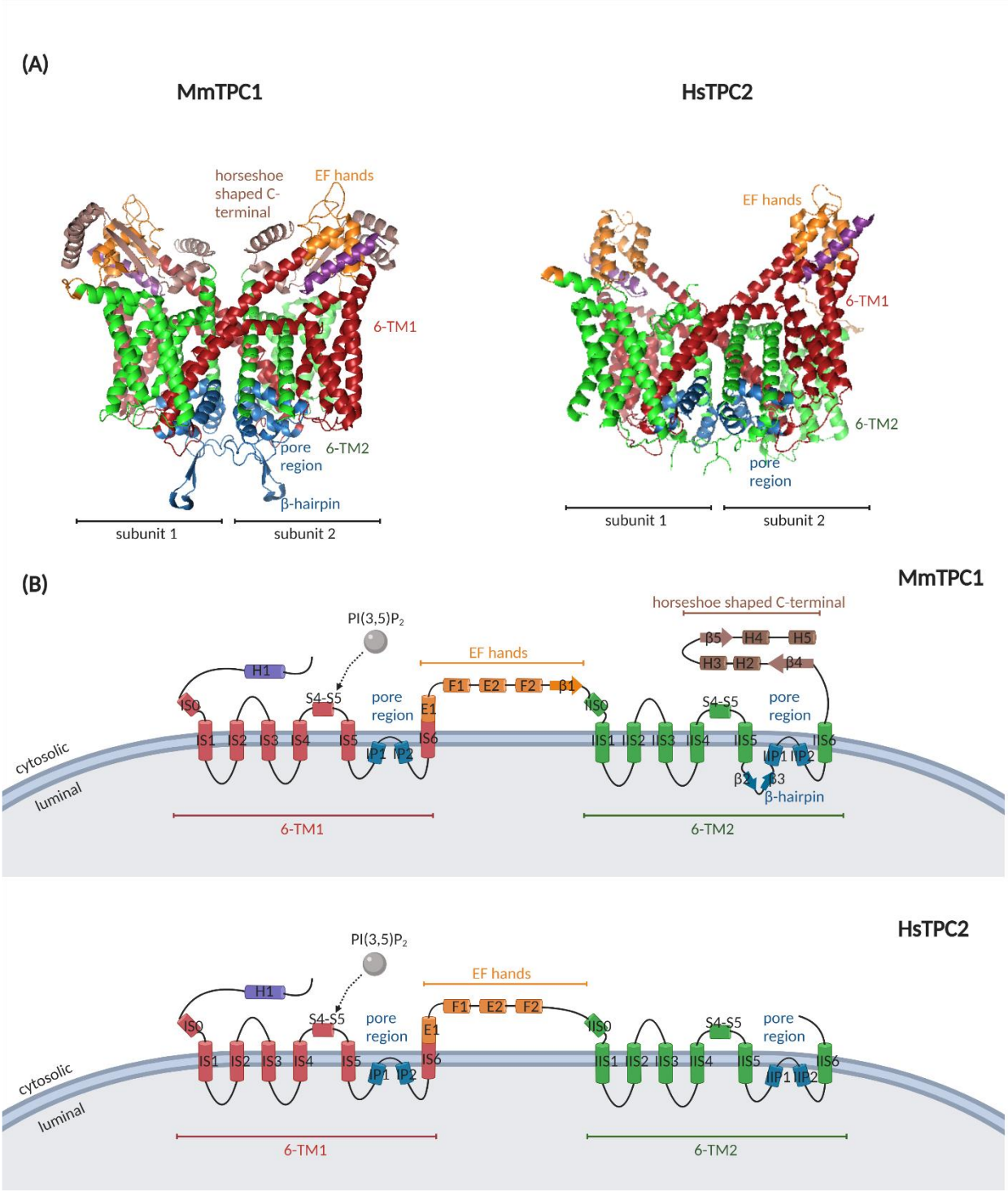


Figure 3. TPC1 murine and TPC2 human structures (124). (The figure was reproduced from Jin, Xuhui, et al, under Elsevier copyright)

1.1.7. Researching evolutionary aspects of TPC2 compared to TPC1 with therapeutic opportunities for various diseases

Over the last decade, TPC1 and TPC2 and their roles in human physiology have continued to attract scientists to unveil their distinctive and collective roles in human health and their potential therapeutic applications in the future. TPC1 and TPC2 sustain several pathological hallmarks; thus, TPC1 and TPC2 are viable, druggable targets for future therapy to treat various diseases developed in different physiological systems. Accumulated data have shown that Ca^{2+} signalling plays a significant role in the cardiovascular system. Recently, Trufanov et al. (2019) uncovered a role for endolysosomal Ca^{2+} signalling via TPC1 in norepinephrine (NE) induced vasoconstriction in rats (129). NAADP and cyclic ADP-ribose (cADPR) are key messengers involved in Ca^{2+} signalling, both being synthesized by the CD38 enzyme (130). CD38 deficient mice were shown to have decreased isoproterenol infusion-induced myocardial hypertrophy, compared to CD38 WT mice (131). In 2015, Capel et al. showed a significant role for TPC2 in cardiac hypertrophy development due to chronic β-adrenoreceptor stimulation (132). *In vitro* pharmacological inhibition of the TPC1/Ca^{2+} signalling pathway by Ned-K has protective effects, hindering ischemia and reoxygenation induced the death of cardiomyocytes (133). Interestingly, TPC1 inhibition either pharmacologically or through gene editing has been proven as a protective strategy against heart injury in *in vivo* mouse models (133). Despite the growing evidence that links endolysosomal Ca^{2+} signalling to pathophysiological processes related to the cardiovascular system, developing drugs to precisely modulate TPCs functions to treat cardiovascular disorders is still in its infancy. A recent study underpinned the immunological significance of TPC1 in anaphylaxis and allergic hypersensitivity, which raises translational questions regarding the potential therapeutic strategies to modulate TPC1 function to treat allergic reactions. Two pore channels (TPCs) are involved in the regulation of a wide spectrum of pathophysiological processes attributed to cancer biology. TPC2 roles in cancer biology have

been investigated much more extensively compared to TPC1. TPC1 was recently found to play a biological role in metastatic colorectal cancer and previously in breast cancer, whereas TPC2 has been implicated in various cancers including melanoma, bladder, breast and colorectal cancer. I will discuss in depth the oncological significance of TPCs in different biological systems. Future research should be conducted to reveal the underlying mechanisms and define TPCs' roles in various pathophysiological processes related to health and diseases with special focus on the role of TPC2 in cancer and TPC1 in cardiovascular, anaphylaxis, and allergic hypersensitivity.

1.2. Endolysosomal Ca^{2+} signalling via TPC2

1.2.1. TPC2 in Cellular Development

The role of TPC2-mediated endolysosomal Ca^{2+} signalling has been demonstrated in early developmental events linked to cell differentiation and development mainly in skeletal muscles, neurons and osteoclasts. Prior to the work conducted by Aley et al. (2010), the role of NAADP/TPC2/Ca^{+2} signalling in regulating muscle differentiation and development had not been revealed (134). They found that NAADP evoked Ca^{2+} signals are integral for skeletal muscle cell differentiation, and both TPC1 and TPC2 are involved in this process. When TPC2 function was impaired by genetic downregulation (siRNA), or by pharmacological inhibition (Ned-19), this reduced the differentiation process and fusion index (calculated as the percentage of nuclei in myosin heavy chain positive cells with at least three nuclei) in an *in vitro* mouse model of skeletal muscle differentiation (134). Several studies conducted by Kelu and his colleagues provided collective evidence that NAADP evoked TPC-endolysosomal Ca^{2+} release acts as a regulator and has a significant impact on the muscle cell differentiation process in zebrafish embryos. Recently, they identified ADP-ribosyl cyclase 1-like (ARC1-like; an enzyme known to generate NAADP) as the upstream target that modulate zebrafish myogenesis(135). ARC1-like modulation either by genetic downregulation or by pharmacological inhibition using nicotinamide was shown to disturb slow muscle cell development (135). They hypothesised that there is a direct link between ARC1

and NAADP mediated Ca^{2+} signalling via TPC2 in mediating slow muscle cell differentiation and development in zebrafish embryos. Interestingly, NAADP-mediated Ca^{2+} release via TPC2 has a distinctive role in neural differentiation of mouse embryonic stem cells (136). Thus, a study showed that TPC2 overexpression hampers murine embryonic stem cells' differentiation into neural progenitors, whereas TPC2 knockdown mediates the differentiation into neural progenitors (136). In the same vein, TPC2 acts as a mediator of osteoclastogenesis with a differential role in osteoclast development based on Mg^{2+} conditions (TPC2 acts as a promoter of osteoclastogenesis in normal Mg^{2+} conditions, whereas it acts as an inhibitor of this process under low-Mg^{2+} conditions) (137). Our lab recently identified a role for TPC2 in adipocyte differentiation *in vitro* via a mechanism involving cAMP signalling and calpain and is currently examining the role of NAADP/TP2C/Ca^{2+} signalling during embryogenesis in an *ex vivo* mouse model (138). Collectively, these findings highlight the biological significance of TPC2 as a regulator of cellular differentiation in various biological models, warranting further investigation of its role in different biological contexts that have not been explored yet and the relevance to human health and disease; in this thesis I study its role in monocyte differentiation into macrophages and conventional dendritic cells.

1.2.2. TPC2 in cancer

The number of publications investigating the cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in pathophysiological processes linked to cancer development, angiogenesis, migration, and autophagy started to increase since the middle of the last decade. While researching our mini-review article entitled 'Endolysosomal Ca^{2+} Signaling in Cancer: The Role of TPC2, From Tumorigenesis to Metastasis', I found evidence that TPC2 behaves differently in different pathophysiological processes of cancer or cancer types at different stages in different species. In this thesis study I have categorised the significant role of TPC2 in cancer biology into TPC2 as a therapeutic target or a biomarker to optimise cancer therapy.

1.2.2.1. TPC2 as a therapeutic target contributes to pathophysiological processes at the local level (including angiogenesis, cell proliferation, adhesion, motility, invasion, migration, autophagy and metabolism) and its effects on cancer development globally

Previous studies by our lab in collaboration with Prof. Antonio Filippini (University of Rome) utilising *in vitro* (human umbilical vein endothelial cells) and *in vivo* models (WT mice treated with Ned-19 or TPC2 KO mice), demonstrated that the VEGFR2/NAADP/TPC2/Ca^{2+} signalling pathway is integral for vascular endothelial growth factor (VEGF) mediated angiogenesis (80). In the same vein, pharmacological inhibition of TPC2 activity with naringenin was shown to hinder VEGF induced angiogenesis in human umbilical vein endothelial cells and in mice treated with naringenin (82). Recently Müller and his colleagues (2021) developed novel small molecules derived from tetrandrine (SG-005 and SG-094) that inhibit TPC2 function and exhibit downregulation of vascular endothelial growth factor receptor 2 (VEGFR2) downstream targets in human umbilical vein endothelial cells as assessed by western blot, suggesting the potential anti-angiogenic effects of these drugs and which warrant further investigation of their influence on VEGF-mediated angiogenesis *in vitro* and *in vivo* (139). These data provide pre-clinical evidence of the candidacy of TPC2 as a therapeutic target to develop novel anti-angiogenic pharmacological modulators.

In 2018, Wei Sun and his colleagues have shown that following TPC2 knockdown 4T1cells proliferated slowly compared to WT or TPC2 overexpressing 4T1 cells (an *in vitro* model of murine breast cancer) (140). This is consistent with the recent finding by Müller et al (2021), which found that TPC2 acts as a regulator of cell proliferation (*in vitro*) and tumour growth (*in vivo*) (139). TPC2-deficient RIL175 cells (an *in vitro* model of mouse carcinoma), generated by CRISPR/Cas9 gene knockout or pharmacological inhibition of TPC2 function by SG-005 and SG-094, showed a

significant decrease in cell proliferation compared to WT cells (139), suggesting that TPC2 represents a viable target to develop antiproliferative drugs.

Diminished TPC2 functions achieved by silencing using siRNAs reduces the migration and invasivity of T24 cells (an *in vitro* bladder cancer model) and the formation of lung metastases in an *in vivo* mouse mammary cancer model (141). Notably, silencing TPC2 using siRNAs or pharmacological inhibition (by Ned-19 or tetrandrine) in T24 cells impaired β1-integrin recycling (140), which plays a vital role in cell motility and invasion, and this provides an explanation for the observed reduction in tumour metastasis and how TPC2 modulate cancer cell invasion and migration. Controversially, our lab in collaboration with Prof. Antonio Filippini has found that knockout or knockdown of TPC2 promotes metastatic traits (adhesion and invasion) in an *in vitro* model of melanoma (142). They attributed the enhancement observed in adhesion and invasion of melanoma cells to the activation of YAP and TAZ, two key drivers of tumourigenesis and metastasis, although the mechanism underlying this activation remains to be fully understood (142). The previous studies investigated the role of TPC2 evoked Ca^{2+} signalling in cancer-related pathophysiological processes (including cancer cell adhesion and motility, tumour invasion, and migration) which interact with each other collectively at a local level to develop metastatic cancer at a global level, and indicated that TPC2 may act both to promote or enhance metastasis and it exhibits differential roles in different types or stages of cancer, including its role in the pathophysiological process which can be different in different biological contexts.

Autophagy is a physiological process that degrades and recycles proteins or organelles to maintain cell homeostasis (143). It exhibits a dual role in the context of cancer; it hampers normal tissue from reaching a tumorigenic state but it can enhance tumorigenic traits in cancer (144). Currently, the connection between autophagy and metabolism in cancer cells is a topic of great scientific interest. Showing a link between TPC2 and autophagy in a non-cancer context, TPC2-

deficient mouse skeletal muscle was reported to have a significant upregulation of autophagy flux characterised by an enhancement in autophagosome accumulation compared to WT muscle (145). In addition, overexpression of TPC2 in *in vitro* models (Hela human cervical cancer cells and 4T1 mouse breast cancer cells) inhibited the fusion between autophagosome and lysosome, which is manifested by a profound accumulation of microtubule-associated protein light chain 3 (LC3)-II and syntaxin 17 (STX17)-positive autophagosomes (140). Recent work conducted by Müller et al. (2021) investigated the metabolic significance of TPC2 in cancer biology (139). They studied the role of TPC2 in cancer metabolism by measuring ATP levels utilising an advanced live imaging approach based on forster resonance energy transfer (139). They found that ATP production was not affected after treating TPC2 KO RIL175 cells with a pharmacological modulator that hampers the glycolysis process compared to WT RIL175 cells (139). This is somehow consistent with our laboratory's recent findings and unpublished data; TPC2-deficient cells showed a significant increase in ATP production (*in vitro* cultured melanoma cells or mouse sperm cells) compared to WT cells. These data indicate that TPC2 may have an important role in cellular metabolism and warrants further investigation in *in vivo* models. Further research in this field would greatly expand our knowledge of TPC2's action at the molecular level and reveal the complex interplay between autophagy, metabolism and cancer.

1.2.2.2. TPC2 as a biomarker in cancer diagnosis and prognosis

Chromosome 11, region 13-region 14 amplifications are associated with cancer, and the TPCN2 gene location (chromosome 11, region 13.2) is within this reported region (146147). Similarly, TPC2 overexpression was observed in oral squamous cell carcinoma cell lines (148). These data raise a question regarding the candidacy of TPC2 as a biomarker in cancer diagnosis in general and in the definition of cancer type in oral squamous cell carcinoma specifically. Recently, TPC2 was reported as one of six genetic markers that serves as a biomarker of prognosis to predict postoperative biochemical recurrence in prostate cancer (149). In contrast, TPCN2 expression

was previously associated with increased survival in bladder cancer (150). Further investigation is needed to investigate its clinical utility as a potential prognostic marker for bladder cancer and there is a paucity of studies that categorise TPC2 clinically in cancer patients. Therefore, in this thesis, I have characterised the effects of genetic variants of TPCN2 on cancer to reveal the potential applications of TPC2 as a biomarker in the definition of susceptibility, prognosis and disease outcomes in the UK Biobank population.

A growing body of evidence has highlighted that endolysosomal Ca^{2+} dyshomeostasis via TPC2 contributes to cancer phenotypes (as shown in Figure 4), but there remains a lack of evidence regarding the distinctive and collective pathophysiological roles of TPC2 in cancer biology. Despite the substantial evidence that TPC2 sustains several cancer hallmarks (angiogenesis, autophagy, metabolism and metastasis) and TPC2 modulations, either by siRNA or pharmacologically, suggest that it is a promising therapeutic target for treating cancer; deeper understanding of TPC2's role in different fundamental processes of cancer and in different types of tumours could lead to the discovery of specific drugs that modulate TPC2 function and could be used to treat patients with various types of cancer. Further studies are required to reveal differential roles of TPC2 in different types or stages of cancer. I believe that TPC2 acts as a driver of tumorigenesis and the controversial data regarding its role in metastasis leads me to speculate that TPC2 exhibits both metastatic and anti-metastatic effects in a tissue- and genetic background-dependent manner. The collective evidence that underpins the potential application of TPC2 as a biomarker in cancer necessitates further investigation in large populations to provide the first in human evidence about the potential candidacy of TPC2 in clinical oncology settings. TPC2 pharmacological modulations provide *in vitro* evidence of the viability of TPC2 as a cancer therapeutic target (see Figure 4); however, TPC2 in terms of drug development is still in its infancy stage.

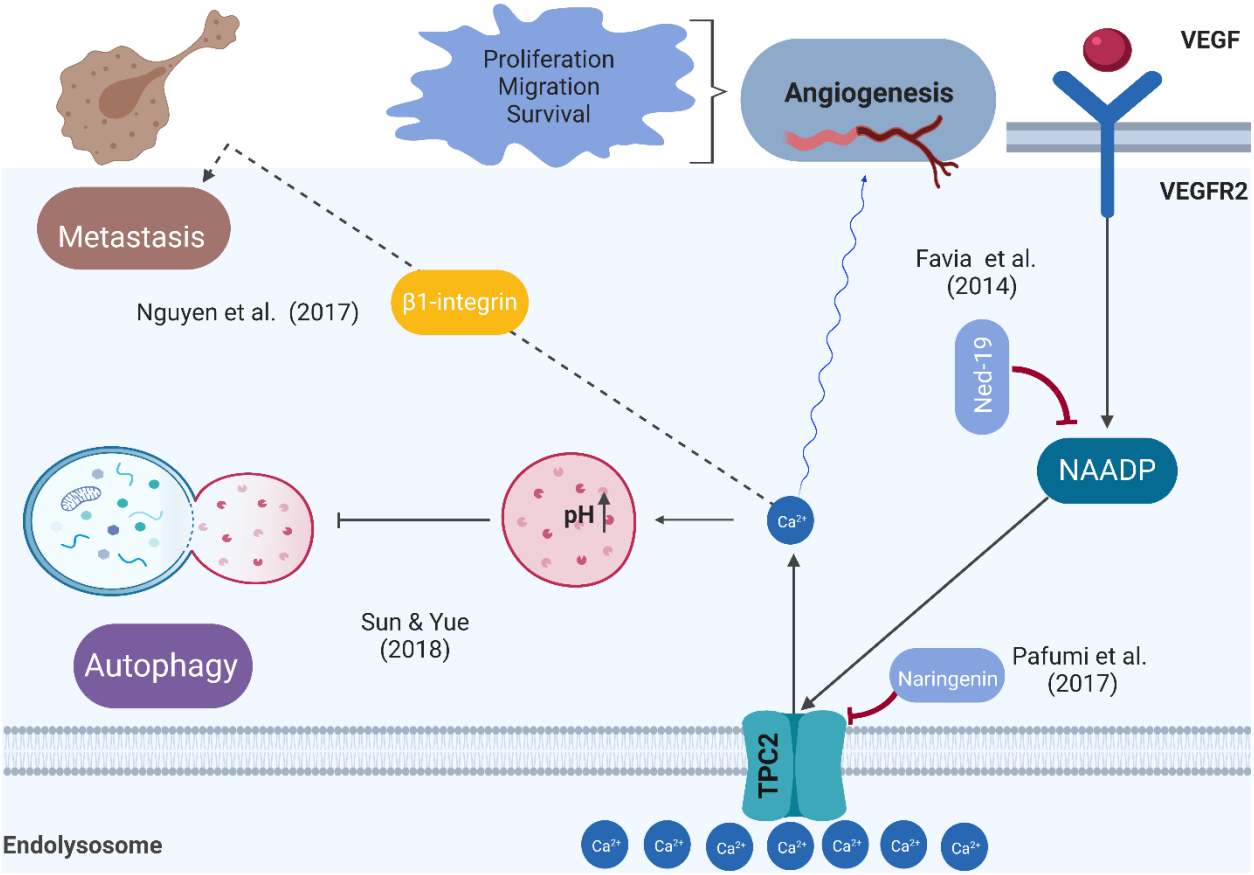


Figure 4. The biological significance of TPC2 as a modulator of cancer processes from tumour initiation to metastatic traits (2). (The figure was reproduced from Alharbi, A. F., & Parrington, J. (2021), under copyright © 2021 Alharbi and Parrington).

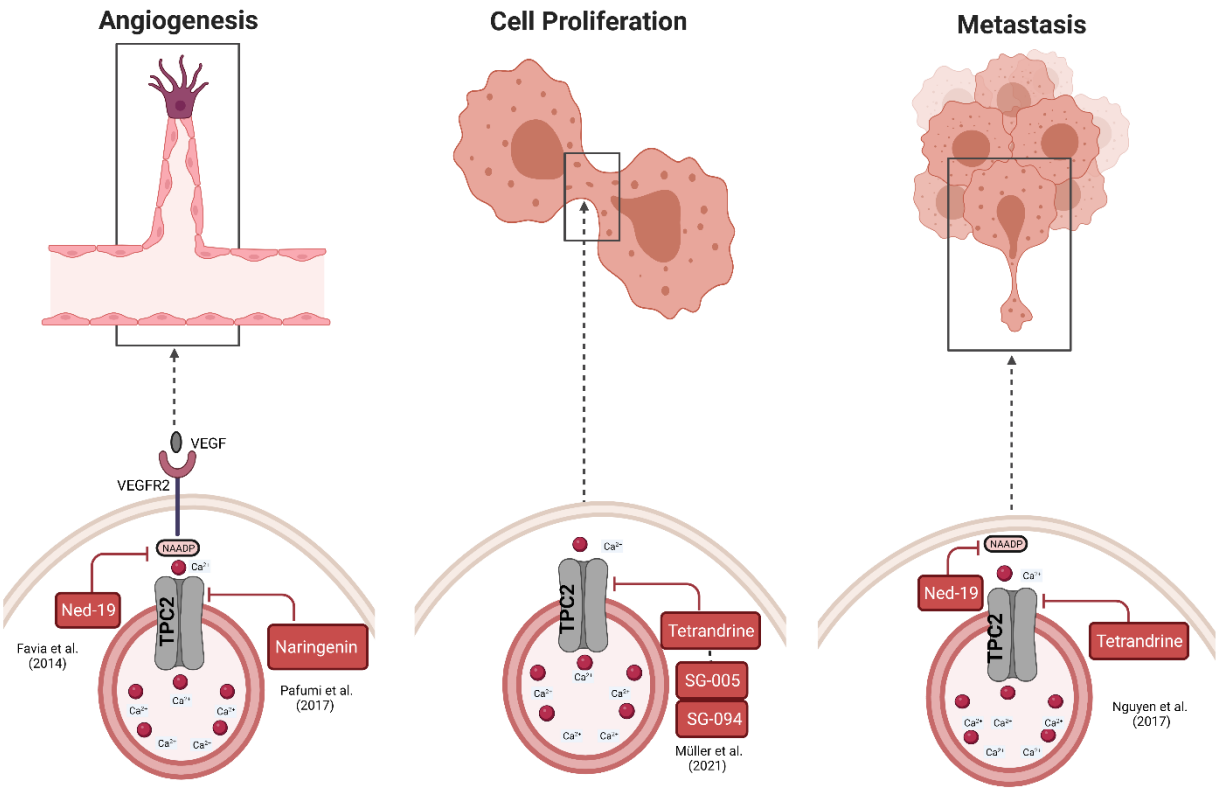


Figure 5. TPC2 in pathophysiological processes related to cancer biology and pharmacological modulations (151). (The figure was reproduced from Alharbi, A. F., & Parrington, J. (2021); as an author of the article, I retain the right to include it in a thesis based on Elsevier policies)).

1.2.3. TPC2 in immunity

There is an evolving knowledge relating to the contribution of two-pore channels to phagocytosis, viral infections, and T-lymphocyte biology (91). TPC2 is ubiquitous and expressed in various immune cell types including monocytes, macrophages, dendritic cells, mast cells, basophils, neutrophils, eosinophils, natural killer cells (innate immune system), and B cells and T cells (adaptive immune system) (152). The expression of TPC1 and TPC2 in immune cells and their functions was recently reviewed by Davis et al. (2022)(45). Figure 6 shows that TPCN2 is moderately expressed in classical monocytes and overexpressed in intermediate and non-classical monocytes compared to TPCN1(45). TPC2 protein expression was significantly elevated (G = 1.94) in M2 (anti-inflammatory and pro-tumoral effects) compared to M1 (inflammatory and anti-tumoral effects) murine macrophages derived from bone marrow by proteomics analysis (86). The proteomic analysis did not report the expression of other endolysosomal channels. However, LAMP1 (lysosome-associated membrane protein 1) (G = 0.27) and LAMP2 (lysosome-associated membrane protein 2) (G = 0.51) expression levels were reported to be significantly higher in M2 compared to M1 but not reaching the G-test (G-statistic) threshold of 1.5. LAMP 1 can be utilised as a lysosome marker. Although it is not entirely clear if the upregulation in TPC2 expression level is due to the increase in the number of lysosomes, I assume based on the data that it is unlikely because the LAMP-1 elevation did not reach the cut-off.

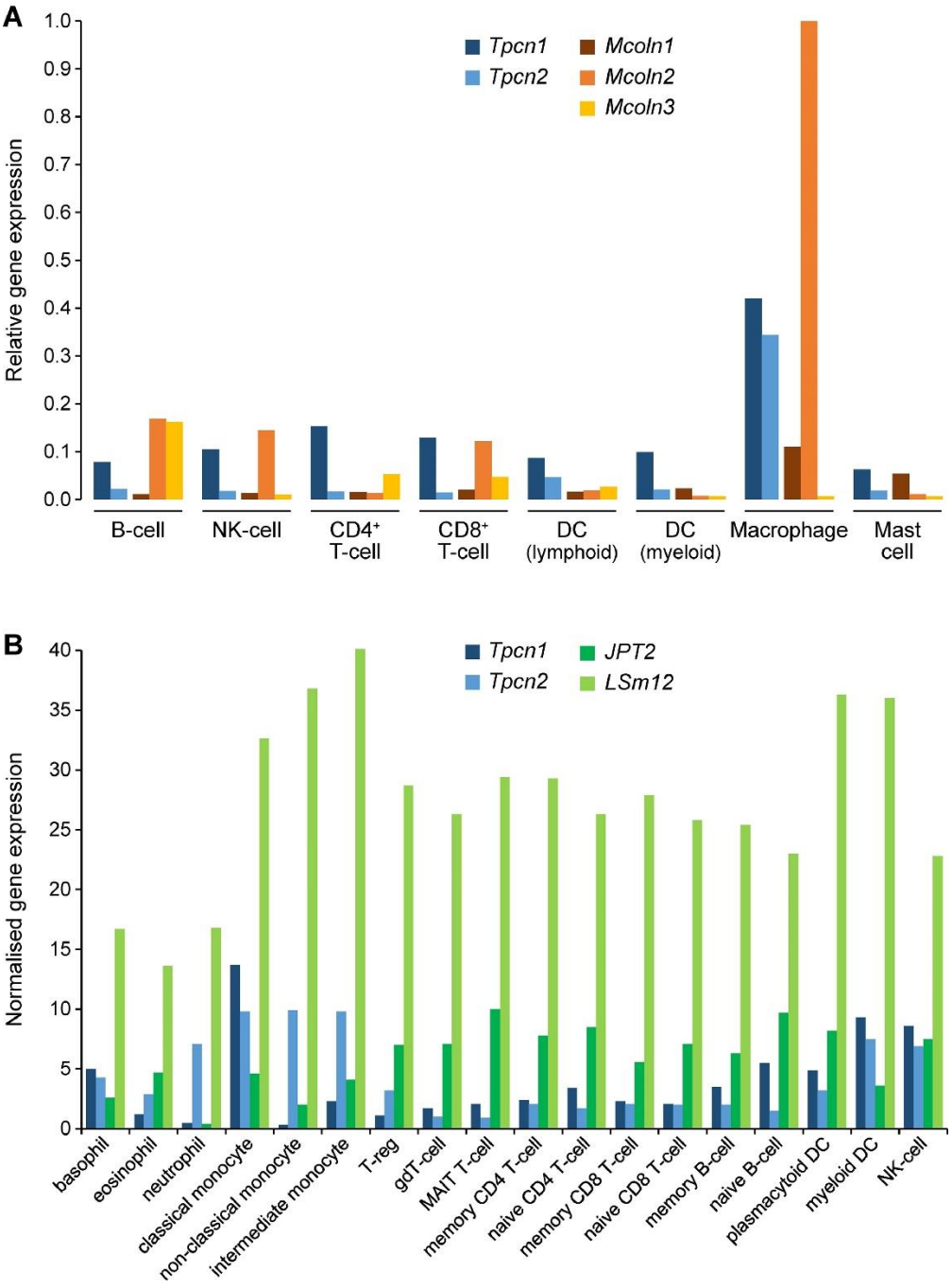


Figure 6. Gene expression of endolysosomal Ca^{2+} channels across immune cells with special focus on TPCs and their recently identified interactomes (58). The figure was reproduced from Davis et al. (2022), under the license number 5403210666014.

Phagocytosis by macrophages is a specialised form of endocytosis that internalises pathogens (bacteria, toxins, viruses) and tumour cells, which plays a vital role in various aspects of immunity. Recently, novel roles for NAADP/TPC/Ca^{2+} signalling from nanodomains were revealed in phagocytosis (109). The study demonstrated that TPCs act as a modulator of phagocytosis for small and large particles at the exit of phagosomes from the plasma membrane in macrophages in a process involving calcineurin and dynamine 2 (109).

Accumulated data has suggested a nonspecific role for TPC2 in virus trafficking that ultimately hindered infectivity of Middle East Respiratory Syndrome coronavirus (MERS-CoV) (153) and Ebola virus (154). Recently, Ou et al (2020) identified the virological significance of TPC2 in SARS-CoV2 infection biology (155). They found that TPC2 acts as a modulator of SARS-CoV-2 entry into human embryonic kidney 293- expressing recombinant human angiotensin converting enzyme 2 cells (HEK 293/hACE2) with phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), and cathepsin L (155). Consistent with Ou et al. (2020) findings, a recent study found that SARS-CoV2 infection was diminished by the inhibition of TPC2 function by siRNAs in human hepatoma-derived HuH-7 cells or naringenin in Vero E6 cells (156). Grimm and Tang (2020) reviewed the TPC functions in SARS-CoV-2 biology; suggesting that SARS-CoV2 has several therapeutic targets, such as targeting TPC1 and TPC2 specifically as therapeutic strategies to treat SARS-CoV2 infection (156). Further investigations utilising biomedical models (*in vitro* and *in vivo*) are required to leverage the candidacy of targeting TPC2 as an approach to develop anti-viral drugs to treat various viral infection, including the current SARS-CoV-2 outbreak. Recently, Li et al (2021) revealed the role of TPC2-evoked endolysosomal Ca^{2+} signalling in IL-1β (Interleukin 1 beta) (157). They demonstrated that pharmacological inhibition or genetic knockout of TPC2 reduced laser-induced choroidal neovascularization *in vivo* via attenuation of retinal VEGFA (vascular endothelial growth factor A) and IL-1β release (157), which ultimately interferes with angiogenesis and immune response activation. These data revealed the direct role of TPC2 in

inflammation and are in line with a previous study's findings that lysosomal Ca^{2+} release mediates lipotoxic inflammasome activation via the regulation of pro- IL-1β production and activation (158). Despite the evolving evidence demonstrating the biological effects of TPC2 in various processes attributed to immunity, the distinctive possible biological roles of lysosomal Ca^{2+} release via TPC2 in immune cell functions remains to be determined in addition to its underlying molecular mechanisms leading to pathological outcomes in the immune system. Notably, TPC2 reduced expression/over expression in various immune cells may have biological significance to mediate pathophysiological processes contributing to immunity. The research in this field is still in its infancy stage with expectations that the recent findings might attract scientists to explore TPC2 and its roles in human immunity.

1.2.4. Genomics aspects of TPCN2: involving polymorphisms identified in humans

The science underlying genomics has advanced rapidly since 1983, when James Gusella and his research team at Massachusetts General Hospital first suggested that genetics might be responsible for Huntington's disease (159). In medicine, genomics is defined as an emerging branch of science that uses genomic information to provide tailored health care based on an individual's genetic makeup. In 2003, the Human Genome Project (HGP) was completed, leading to an increase in our knowledge of human DNA and the role of genetic variations in the development of disease or response to therapy. More recently, the cost of DNA sequencing has decreased thanks to the development of advanced techniques for decoding DNA, such as next generation sequencing (NGS), whole exome sequencing (WES), and whole genome sequencing (WGS). These techniques have the potential to revolutionize modern medicine and help us achieve personalized healthcare, especially in diseases where genetics plays an important role in disease development, such as cancers and inherited diseases. The inclusion of genomic data in the electronic health record will therefore provide a clinical tool to stratify patients based on their genetic information and allow physicians to individualize care. It is well known that the

application of genomics has a positive impact on healthcare quality and costs. Although it has been observed that TPCN2 genetics is highly varied at the global level (160), the only TPCN2 polymorphisms reported to be associated with human conditions have been non-synonymous genetic variants (rs35264875 and rs3829241), which lead to gain of TPC2 activity at the functional level, and were associated with a shift from brown to blond hair (161,162). Further research in this field would be of great help in expanding our knowledge of TPCN2 genetics and could contribute to the identification of novel biomarkers to aid development of new diagnostic and therapeutic approaches, representing a further step towards personalised care and optimising human health. In this thesis study, I have investigated, for the first time, the impact of genetic variants in TPCN2 in cancer in the UK Biobank population to provide the first and initial insights into the possibility of utilising TPC2 as a biomarker in cancer.

1.3. Overall hypothesis, long-term goal, and objectives

My central hypothesis (Experimental Section 1A) is that TPC2 plays a pivotal role in the specific mechanisms of classical monocyte differentiation that lead to macrophage-mediated angiogenesis, tumorigenesis and metastasis, and that it is a candidate target for cancer immunotherapy and (Genetics Section 1B) knowing the genetic information about TPCN2 will enhance detection, diagnosis and prognosis, which will improve health outcomes and quality of life for cancer patients. This could be achieved by either retarding the development of tumour-associated macrophages (TAMs) via inhibition of TPC2 involvement in monocyte differentiation or TAM reprogramming to restore the anti-tumour effects of macrophages via mTORC-TPC2 signalling. This approach has the potential to be an adjuvant therapy to reduce chemo- or radioresistance, to synergise the effects of checkpoint inhibitors and to prevent cancer recurrence. The potential applications of TPC2 as a biomarker could be revealed by performing a study to identify the genetic variants in TPCN2 that are highly associated with cancer/cancer subtypes in the UK Biobank population.

My objectives are to examine NAADP/TPC2/ Ca^{2+} signalling during monocyte differentiation and define the role of TPC2 as a potential downstream regulator of monocyte differentiation or the functional microenvironment reprogramming of TAMs to restore anti-tumour capabilities and its role during immunosurveillance. In addition, my objectives are to characterise the effects of genetic variants in TPCN2 on cancer at the global level and on various cancer types at the local level to reveal the potential applications of TPC2 as a biomarker in the definition of tumour types, susceptibility, prognosis, response and cancer outcomes in the UK Biobank. The long-term goal is to identify innovative cancer biomarkers and therapeutic targets for cancer therapy and develop more effective, less toxic antineoplastic agents. This research provides novel insights into the cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in monocytes and provides a novel approach that enables clinicians to provide tailored therapy based on TPCN2 patient genetic information. Moreover, my thesis findings give us insight into the pathophysiological significance of TPCN2 in cancer, with the future possibility of targeting TPC2 as an innovative strategy in cancer treatment and may contribute to the development of genomics-based guidelines.

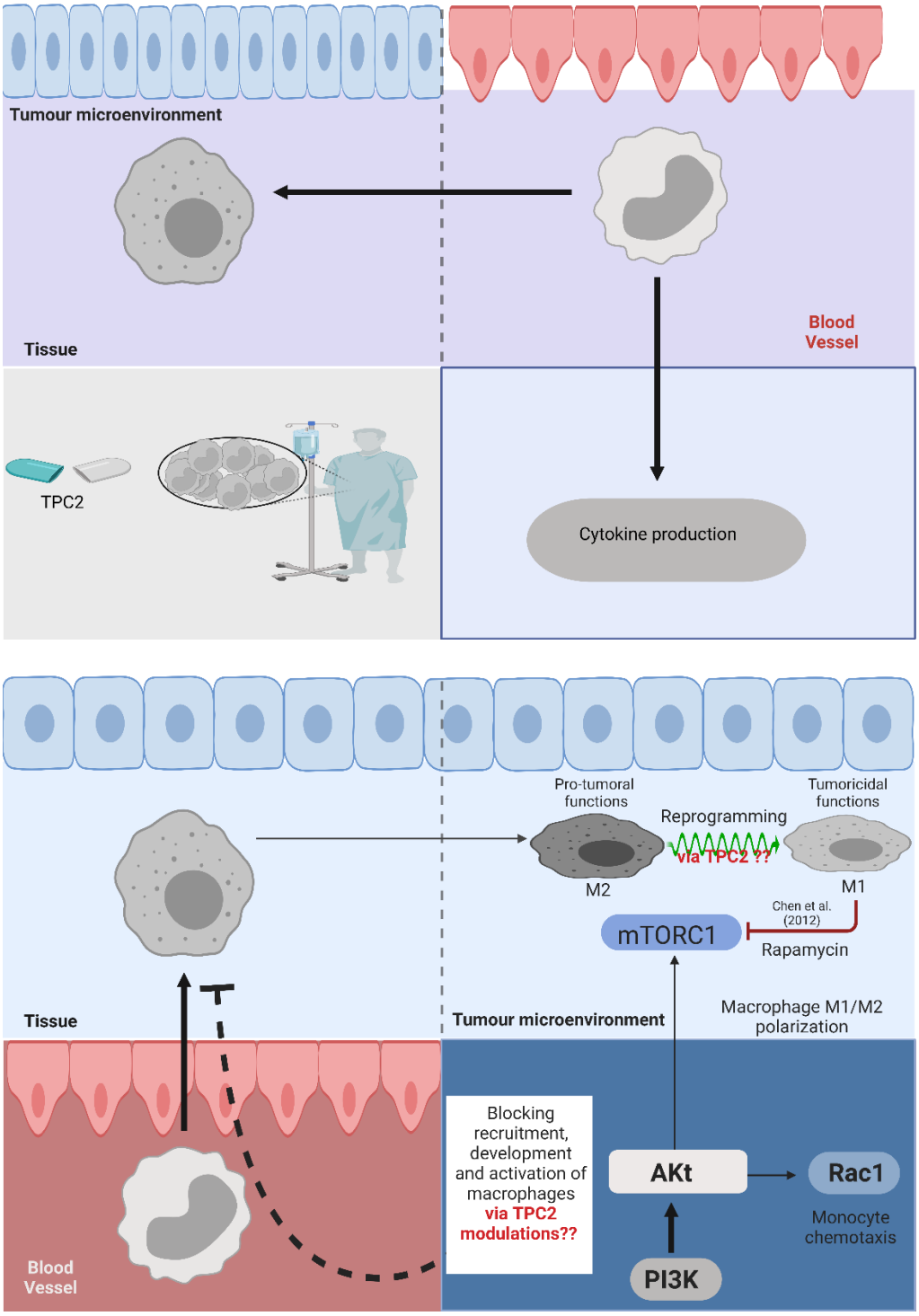


Figure 7. Overall hypothesis. (Created with BioRender.com).

1.4. Significance and innovation

Evolving knowledge relates to the contribution of two-pore channels (TPCs) to cancer biology, underscoring the potential health applications of targeting TPC2 function or the clinical utility of TPC2 as a diagnostic, prognostic and predictive biomarker in directing therapy in patients with cancer. The innate immune system is the first line of defence against both pathogens and tumour cells. While researching our mini-review articles entitled 'Endolysosomal Ca^{2+} Signalling in Cancer: The Role of TPC2, From Tumorigenesis to Metastasis' and 'Deciphering the Role of Endolysosomal Ca^{2+} Channels in Immunity', it became evident that endolysosomal Ca^{2+} signals mediated by TPCs play important roles in cellular differentiation, development, immunity and cancer; however, the cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in monocyte differentiation and macrophage development and its links to pathophysiological processes related to cancer development have not yet been investigated. The two main lacunae in our knowledge that this thesis addresses are the lack of molecular evidence that specifically characterises the role of TPC2 in monocyte differentiation and cytokine production and the paucity of studies that categorise TPC2 genetically in cancer patients. This thesis investigates a previously unrecognised role for TPC2 in monocyte differentiation and IL-8 release, revealing the candidacy of TPC2 as a viable target for cancer immunotherapy. Targeting TPC2 indirectly modulates immunological targets or enhances the response to targeted immunotherapies, especially checkpoint inhibitors.

It contains the first well-characterised, human genetic association study conducted to identify risk variants in the TPCN2 gene that contribute to cancer at the global level, as well as in 13 specific subsets of cancer in terms of cancer risk, metastasis, and disease recurrence in the UK Biobank's population.

1.5. Scientific Approach

In this section, I will discuss the approach used to address my working hypothesis: that TPC2 plays a pivotal role in monocyte differentiation and may serve as a novel cancer biomarker or immunotherapeutic approach to treat cancer patients. Monocytes and macrophages are myeloid cells that play a vital role in inflammation, immunity and cancer. The spleen is an important source of monocytes, which act as direct precursors of haematopoietic stem cell-derived macrophages. I investigated TPC2's role in monocyte differentiation, macrophage development, and IL-8 and IL-10 production, as well as its influence on monocyte chemotaxis, via the genetic/pharmacological modulation of TPC2 function. Because I identified significant changes in TPC2 expression at the RNA level in macrophages compared to monocytes, I established a TPC2-deficient U937 monocyte model and attempted to develop TPC2 deficient THP1 monocytes. This was done to utilise the model as a tool to expand our knowledge of the role of TPC2 in monocyte and macrophage biology since there is a lack of an available specific inhibitor for this channel, which I highlighted in our preview article entitled 'TPC2 targeting evolution: Leveraging therapeutic opportunities for cancer' that was published in *Cell Chemical Biology*. Proteomic analysis is used to study global changes in protein expression after knocking out TPC1 and/or TPC2 in murine solenocytes. In addition, I have also compared the TPC2 sequences from monocytes isolated from the blood of a healthy subject with U937 monocytes derived from a subject with acute lymphoblastic leukaemia. I have utilised UK Biobank data to characterise the effects of genetic variants in two endolysosomal channels (TPCN2 and P2RX4) on cancer at the global level and on various cancer types at the local level to reveal the potential applications of TPC2 and P2RX4 as biomarkers in the definition of tumour types, susceptibility, prognosis, response and cancer outcomes in the UK Biobank. Furthermore, I leveraged the UK Biobank data to investigate the effects of 71 genetic variants within, but not limited to, IL-8, IL-10 and TNF-α in cancer patients at a global level in terms of susceptibility, prognosis, disease recurrence and

overall survival.

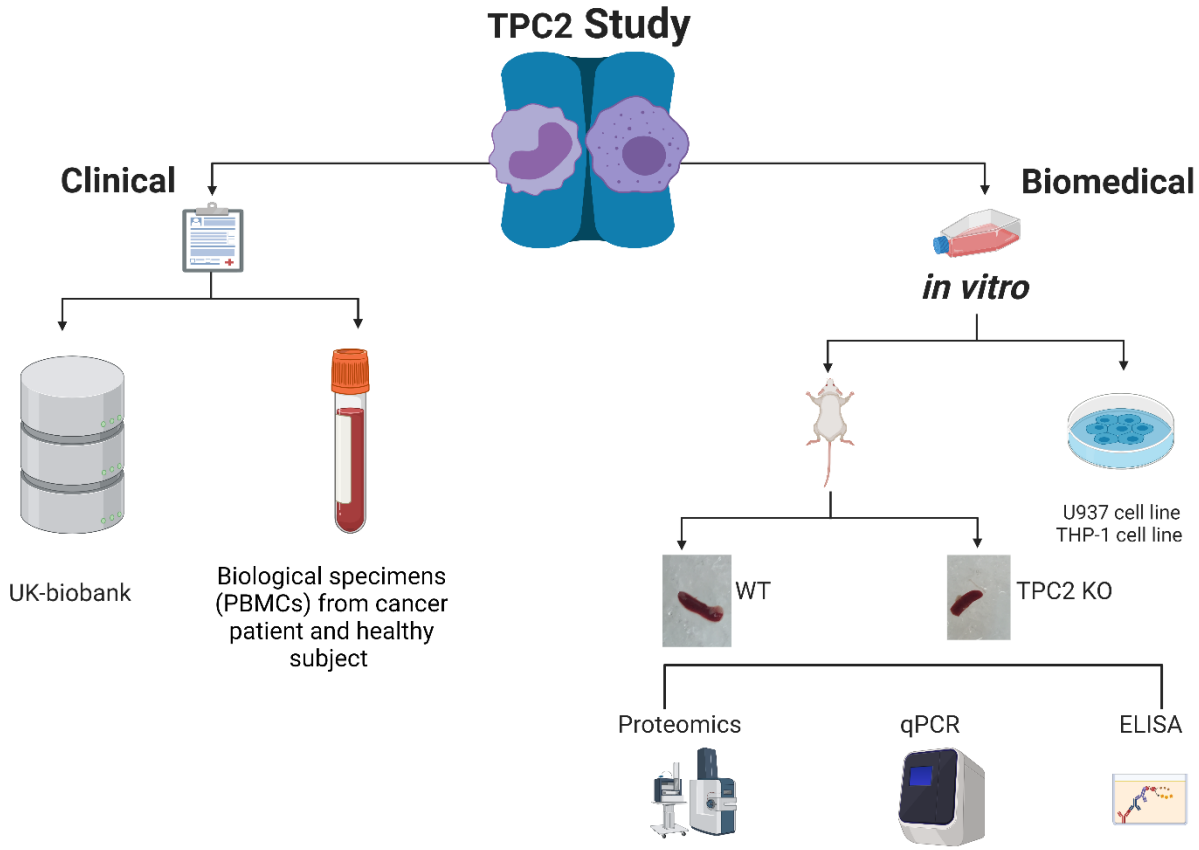


Figure 8. The overall study design.(Created with BioRender.com).

Chapter 2: TPC2 genome editing with CRISPR-Cas9 in U937 and THP-1 cells: challenges and applications

Chapter 2: TPC2 genome editing with CRISPR-Cas9 in U937 and THP-1 cells: challenges and applications

2.1.Introduction

2.1.1. Perspectives on genome editing using CRISPR-Cas9 as therapeutic approaches to treat cancer: are we there yet?

The term CRISPR stands for clustered regularly interspaced short palindromic repeats and was first introduced by Jansen and his colleagues in 2002 (163). The discovery of CRISPR-Cas9 is now considered to be breakthrough technology that revolutionises science and resultantly propels us toward a new era of medicine providing therapeutic approaches to treat diseases based on genetic modulations; it does however bring with it ethical and social dilemmas. Cas9 is DNA endonuclease Cas9, which in association with a guide RNA, enables scientists to precisely cut the target segment of DNA, and thereby introduce knockout or knockin mutations, or insert gene sequences, into a genome. The technology was developed in 2012 by Emmanuelle Charpentier and Jennifer Doudna who won the Nobel Prize in Chemistry in 2020 for their work. Cancer is a DNA based disease and the unique genetic makeup of the tumour, involving either germline or somatic mutations, correlates with the cancer in terms of diagnosis, prognosis and therapeutic outcomes. CRISPR-Cas9 is a powerful research tool that enables researchers to conduct functional assays to investigate the reported association between a genetic mutation and its phenotype in a cancer context to provide scientific evidence of a gene-phenotype causal relationship, which ultimately expands our cancer biology understanding. CRISPR-Cas9 technology can be utilised to generate a model that mimics the patient's tumour genetic information to screen drugs and provide tailored therapies. The number of Food and Drug Administration (FDA) approved drugs to treat cancer patients based on genetic information is evolving. Indeed, taking tumour genetic information into consideration continues to allow distinguished advances to be made in the treatment of cancer, allowing many patients justifiably

to hope for a cure. The therapeutic applications of genome editing are not yet prevalent within oncology settings, but may reach the clinic in the foreseeable future if such therapeutic strategies pass the initial clinical trial phases. To date, the number of clinical trials that utilise genome editing approaches to treat various types of cancer and are registered in the clinical trials database is approximately 20. The majority of these trials are using CRISPR/Cas9 mediated cancer immunotherapy to knock out PD-1 and generate genetically modified T cells derived from the cancer patients with the intention of enhancing the immune response. The first phase 1 trial based on CRISPR/Cas9 mediated cancer immunotherapy was approved by the FDA in 2018. The study was designed as a phase 1 single-centre, non-randomised, open-label, and parallel trial. Autologous T cells were genetically modified to express NY-ESO-1 (New York oesophageal squamous cell carcinoma 1) and knock out two TCR (T-cell receptor) chains, TCRα and TCRβ, as well as the programmed death protein-1 (PD-1) to enhance the immunotherapy response (164). Three patients were included in the study (two had advanced and refractory myelomas and one had a metastatic sarcoma). The study showed that using CRISPR-Cas 9 to genetically modify the autologous T cells is safe and tolerable, thus providing the first clinical evidence that translates experimental studies into potential clinical applications to continue the development of promising new treatments based on CRISPR-Cas9 technology (164). Gene editing is a promising field, but there are different views on it in the scientific and clinical community. Some oppose it because of implementation challenges, while other critics believe that the cost of using such technology in clinical practise would be too high and risks would increase due to off-target effects. In addition, there are many challenges that stand in the way of implementation in patient care, such as the paucity of clinical evidence, potential costs, healthcare infrastructure, and the need to educate and train healthcare providers in molecular genetics and genomics. Despite this, CRISPR-Cas9 based therapies have the potential to revolutionise the treatment of multiple diseases, especially cancers and inherited diseases, but the clinical research in this area is still in its infancy stage.

Thus, investigating the utilisation of gene therapy to treat various diseases will create a new era in treating patients and supply a clinical tool that will enable clinicians to individualise care, ultimately impacting positively upon health-care quality.

Table 2. The registered clinical trials based on CRISPR- Cas 9 technology cited on PubMed.

Disease	Clinical trial ID	Phase	CRISPR- Cas 9 based therapy
Oesophageal Cancer	NCT03081715	Phase 1	PD-1 Knockout T Cells
Human Papillomavirus-Related Malignant Neoplasm	NCT03057912	Phase 1	CRISPR/Cas9-HPV16 E6/E7T1 or CRISPR/Cas9-HPV18 E6/E7T2
Metastatic Non-Small Cell Lung Cancer	NCT02793856	Phase 1	PD-1 Knockout T Cells
Mesothelin Positive Multiple Solid Tumours	NCT03545815	Phase 1	CRISPR-Cas9 Mediated PD-1 and TCR Gene-knocked Out Chimeric Antigen Receptor (CAR) T Cells
Advanced Hepatocellular Carcinoma	NCT04417764	Phase 1	PD-1 knockout engineered T cells
Source: clinicaltrials.gov (Updated April 2022)			

2.1.2. TPCN2 genome editing status, applications, and limitations

TPC2 is a eukaryotic cation channel located mainly in the lysosome, and its gene is found on chromosome 11, region 13.2 (TPCN2, NCBI Gene ID: 219931). The TPCN2 gene is highly varied across different populations compared to other channel genes located in the endosomal–lysosomal compartment, including TPCN1, MCOLN1, MCOLN2 and MCOLN3. Its variations have been associated with different diseases (Table 43) (160, 165).The overexpression or knocking out of the TPCN2 gene in *in vitro*/*vivo* models reveals the association between TPC2 and key pathophysiological processes attributed to human health and disease, the potential of TPC2 as a novel hallmark, and whether it might represent a viable therapeutic target to treat patients with various diseases. In addition, the paucity of specific pharmacological modulators of TPC2 function necessitates the development of genetically modified models to be used as invaluable tools to underpin study of the role of NAADP-mediated Ca^{2+} signalling via TPC2 in health and disease. Our lab has successfully developed TPC2 genetically modified models in both cells and whole organisms in small and large animals (e.g. mice and pigs) using CRISPR-Cas9 technology. The majority of TPC2-deficient cells were generated recently to elucidate the molecular mechanisms underlying the roles of TPC2 in cancer development. D'amore et al. (2020) developed TPC2-deficient human CHL1 cells (an *in vitro* model of melanoma) using the CRISPR-Cas9 non-homologous end-joining approach to induce a frameshift mutation in exon 4 (142). Müller et al. generated TPC2 KO murine RIL175 cells (an *in vitro* model of hepatocellular carcinoma) based on the CRISPR-Cas9 gene editing assisted non-homologous end-joining to delete exon 2 (139). There are several approaches to generate TPC2 genetically modified mouse models (150). Our lab has developed a TPC2 KO germline mouse using genetically modified embryonic stem cells (carrying the 129P2 strain) developed using the mediated gene trap approach, that were injected into C57BL/6J mice blastocysts (126). In 2019, the first TPC2 KO pig was born in Spain,

developed using CRISPR-Cas9 based editing of embryos *in vitro* using oocytes that were microinjected before fertilization, by our lab in collaboration with Joaquin Gadea (166). Currently, the validation of TPC2 knockout can only be conducted at the DNA and RNA levels. The lack of an easily-useable specific commercially available antibody to detect the loss of TPC2 protein necessitates the development of a reliable tool to investigate TPC2 expression at the protein level.

Lysosomal patch clamping is used to measure the activity of the TPC2 channel, and Ca^{2+} imaging utilises it to measure intracellular calcium signalling. To my knowledge, we cannot investigate the Ca^{2+} release specifically from the TPC2 channel. However, we can utilise pharmacological modulators to control the release of Ca^{2+} from other channels located in the intracellular compartment. Although patch clamping is the gold standard and a solid technique for measuring the currents from any ion channel, some of the limitations of this technique are the potential risks of selection bias and altering/losing cytosolic factors that interfere with channel activity when dissecting lysosomes. The development of an innovative approach to measure calcium specifically from the ion channels that combine patch clamping and Ca^{2+} imaging in one solid, efficient and rapid technique that overcomes their limitations might advance the calcium channel field in the future.

Table 3. Mutations in TPCN2 reported in the Human Gene Mutation Database (HGMD®)							
Mutation ID	Mutation type	Codon change	Amino acid change	Protein	Functional analysis	Disease/phenotype	Ref
rs3750965	Missense Variant (c.1127A>G)	AAA-AGA	Lys376Arg	p.K376R	Mild gain of channel activity	Hair colour	160
rs35264875	Missense Variant (c.1450A>T)	ATG-TTG	Met484Leu	p.M484L	Gain of channel function	Hair colour	161, 162, 167
rs3829241	Missense Variant (c.2201G>A)	GGG-GAG	Gly734Glu	p.G734E	Gain of channel function	Hair colour	161, 162, 167
rs72928978	Missense Variant (c.655A>G)	ATC-GTC	Ile219Val	p.I219V	No reported significant effects on basal or PI(3,5)P2-stimulated channel TPC2 activity	Diabetes, type 2	160,168
rs61746574	Missense Variant (c.1160G>A)	GGC-GAC	Gly387Asp	p.G387D	Mild gain of channel activity	Increased activation levels	160

2.1.3. Genetically modified U937 and THP-1 models using CRISPR-Cas9 in drug discovery and therapeutic innovation

U937 and THP-1 are immortalised cell lines used as *in vitro* models of monocyte to macrophage differentiation and leukaemia development derived from the pleural effusion of a patient with histiocytic lymphoma or the blood of a patient with an acute monocytic leukaemia, respectively. U937 and THP-1 cells are cultured in suspension. From a technical perspective, U937 and THP-1 cells are difficult to transfect with a non-viral vector or a lipofection approach. Nucleofection is the most efficient approach to stably transfect U937 and THP-1 cell lines. Although there is a growing scientific interest in using U937 and THP1 CRISPR-Cas9 based genetically modified models, there is a paucity of publications in PubMed that have employed these powerful research tools. I navigated canSAR to determine TPCN2 expression in the U937 and THP-1 lines and

found that TPCN2 expression was upregulated in U937 by 4.13 and THP-1 by 3.84 (169). The majority of the studies that have developed CRISPR-Cas9 engineered U937 cells manipulated specific genes to decode the role of a particular gene's function in human myeloid leukaemia biology, which ultimately might lead to the development of therapeutic agents to treat or prevent myeloid leukaemia.

For example, Chen et al. (2020) generated transient receptor potential melastatin 2 (TRPM2)-deficient U937 cells to elucidate the role of TRPM2 in acute myeloid leukaemia (AML), focusing on cell proliferation and doxorubicin sensitivity (170). Knocking out TRPM2 via CRISPR-Cas9 served as a valuable tool that revealed the important roles of this channel in AML proliferation and chemotherapy sensitivity (170). Further studies using genetic/pharmacological modulators are required to decipher the precise mechanisms underlying the physiological processes of TRPM2 in AML.

The majority of published studies produced a gene knockout THP-1 model using CRISPR-Cas9 technology to underpin the function of a specific gene in innate immunity response, especially in human immunodeficiency virus type 1 or acute myeloid leukaemia. SAM domain- and HD domain-containing protein 1 (SAMHD1) deficient THP-1 cells generated by Bonifat et al. revealed that SAMHD1 regulates HIV-1 infection via reducing intracellular deoxyribonucleoside triphosphate (dNTP) pools (171). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) outbreak was revisited and the role of SAMHD1 in SARS-CoV2 infectivity investigated using CRISPR-Cas9 based genetically modified human embryonic kidney 293 cells but not the THP-1 cell line (172). The cellular mechanisms underlying the regulation of SAMHD1-modulated SARS-CoV2 infection warrants further investigation in THP-1 cells and animal models to help develop new pharmacological modulators for treating the coronavirus disease (COVID-19). High expression of CXC chemokine receptor 4 (CXCR4) is associated with AML relapse and poor

survival (173). Ren et al. showed that knockout of CXCR4 using CRISPR-Cas9 genome editing nanosystem hampered myeloid leukaemia cell migration and adhesion and induced apoptosis in THP-1 cells (174). Overall, these studies underline the significance of CRISPR-Cas9 technology as a tool to address scientific immunity questions regarding difficult-to-transfect suspension of U937 and THP-1 cells and warrant further investigations to develop simple and cost-effective protocols that facilitate efficient genome editing in these models. This may further the development of CRISPR-Cas9 based immunomodulators to treat inflammatory diseases and myeloid leukaemia.

2.1.4. TPC2 and imatinib in haematologic malignancies

Haematological malignancies are a heterogeneous group of cancers that affect the blood, bone marrow, and lymph nodes and are classified into three major categories: leukaemia, lymphoma, and multiple myeloma. The main types of leukaemia include acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL), and hairy cell leukaemia. Leukaemia is the twelfth most common type of cancer in cancer patients and ranked tenth among men in the UK in 2022 (175). The estimated mortality rate of leukaemia is 3.9%, and it is the sixth-leading cause of mortality among men in the US (176). AML is uncommon, and advances in survival outcomes for patients with AML have been slow (177). Recently, CRISPR-Cas9-based therapy has emerged as a novel effective strategy that offers remarkable advances in treatment for refractory AML by enhancing the tumour response rate and overall survival. Wang et al. (2019) identified an association between lysosomal membrane proteins (LAMP1, TPC1 and TPC2) and AML and discussed their potential as novel diagnostic, prognostic and predictive biomarkers in directing chemotherapy in AML patients (178). The authors found that high expression of TPCN2 in bone marrow correlated significantly with a favourable efficacy profile of chemotherapeutic agents in patients with AML, whereas high expression of LAMP1 was associated with a poor response to chemotherapy (178).

However, the data from this study should be interpreted with caution, since the subjects are not well characterised and the types of chemotherapeutic agents received are not clearly mentioned. Loss of TPC2 function was found to sensitise the vincristine-resistant CEM cells (an *in vitro* model of AML) to chemotherapy (including vincristine, doxorubicin and topotecan) (179), indicating the potential role of TPC2 in vincristine chemoresistance developed in acute lymphoblastic leukaemia (ALL). Hence, TPC2 profiling may represent a novel approach to enhancing patient prognosis and clinical response by identifying AML/ALL patients who are likelier to benefit from chemotherapy.

Imatinib mesylate is a multikinase inhibitor and is FDA approved for the treatment of several cancers, including Philadelphia chromosome-positive chronic myeloid leukaemia, and gastrointestinal stromal tumours (180). It works by targeting both BCR–ABL tyrosine kinase and the c-kit pathway in cancer cells and platelet-derived growth factor receptors in the tumour vasculature (180). The efficacy of imatinib as maintenance therapy was previously investigated in a phase 2 clinical trial (NCT00509093) in patients newly diagnosed with AML who were receiving chemotherapy (181). While the results of the trial have yet to be published, according to the results posted by the principal investigators, the median progression-free survival (calculated as the date of complete response to the date of relapse or death) in patients aged 60 years and older was 10.7 months (1.7 to 20.8) (181). In addition, imatinib efficacy and toxicity were examined in a phase 2 trial (NCT00376467) as a treatment for Ph+ acute lymphoblastic leukaemia with no data reported or published (182). Imatinib in combination with chemotherapy (NCT00358072) enhanced complete remission and stem-cell transplantation success rates in adult patients with Ph-positive ALL (183). In this chapter, I explore the cytotoxic effects of imatinib in U937 cells (an *in vitro* model of myeloid leukaemia and ALL) to investigate the efficacy of the

combination of TPC2 KD by CRISPR-Cas9 technology and a multikinase inhibitor as a treatment for AML.

2.1.5. Significance, innovation, hypothesis and objectives

CRISPRCas9-based gene editing has revolutionised medicine and opened the door to investigating gene function in disease development, which could, in future, lead to the application of gene-editing therapies in clinical practice.

Knocking out target genes is a powerful research approach to examining the function of a target gene in health and disease. In my thesis, for the first time, I generated a TPC2-deficient human monocyte model. A problem with knockout/knockdown of TPCN2 is that it is difficult to edit, particularly in U937 and THP-1 cells (cultured as suspension). Certain specific equipment is required to achieve efficient TPC2-based CRISPR-Cas9 gene editing. Recently, our preview article entitled 'TPC2 targeting evolution: Leveraging therapeutic opportunities for cancer', which was published in *Cell Chemical Biology*, discussed the paucity of chemicals that specifically modulate TPC2 function. Therefore, using pharmacological modulators alone is not a suitable model for investigating TPC2's role in monocyte differentiation, macrophage development and IL-8 and IL-10 production, as well as its influence on chemotaxis processes. TPCN2 knockdown in U937 monocytes was implemented to investigate whether NAADP/TPC2 signalling is specifically involved in monocyte differentiation. The development of TPCN2 knockout THP-1 monocytes was an attempt to confirm the findings in a similar biological context. The overall hypothesis is that the production of TPC2-deficient monocytes and study of them expands our knowledge about the molecular changes that occur during monocyte differentiation (a process linked to cancer) by studying the effect of knockdown or knockout of TPC2 on this process. The objective was to establish TPC2-deficient monocytes and use this model to examine the role of TPC2 in monocyte and macrophage biology because no inhibitor with a high degree of specificity for this channel is

currently available. I established a TPC2-deficient U937 monocyte model and sought to develop TPC2-deficient THP1 monocytes. Using this model greatly expanded our knowledge of the actions of TPC2 at the molecular level and revealed the complex interplay between monocytes and macrophages as key components of the innate immune system involved in inflammation and cancer. TPC2 thus has potential as a novel drug target for immunotherapy in patients with cancer or immune diseases. Nucleofection is the most efficient approach to stably transfect the U937 and THP-1 cell lines. For technical and financial reasons, including a lack of suitable equipment for conducting nucleofection-based CRISPR-Cas9 editing in our lab and the difficulty of TPC2 knockout, particularly in cells cultured in suspension, the efficiency of transfecting suspension cells with traditional lipofection methods is very low. I asked Synthego (a reliable supplier of gene editing services) to generate a pool of engineered U937 and THP-1 cells and perform a 200-step transfection/CRISPR condition process to allow for high efficiency of the transfection without the use of selection markers.

In this chapter, I discuss the challenges, limitations and applications of generating the TPC2 deficient monocyte model in collaboration with Synthego to address my thesis questions and answer other scientific questions related to immunity or cancer.

2.2.Methods

2.2.1. Generation of TPC2 Knockout U937 Cells

2.2.1.1. Nature of TPCN2 in the U937 cell line and CRISPR Editing by Synthego

TPCN2 is located on Chr11. U937 harbours a mix of diploid and triploid chromosome pairs. Chr11 appears to be diploid (as shown in Figure 10); hence the two copies of TPCN2 need to be inactivated. U937 TPCN2 knockdown (KD) pool cells were produced by Synthego (CA, USA). The nucleofection based transfection was conducted by CRISPR-Cas9 technology. sgRNA

(single guide RNA): CGUGCUGGUGAGUGAGGAUG was designed to target the fourth exon of the TPCN2 gene (Transcript ID: ENST00000294309).

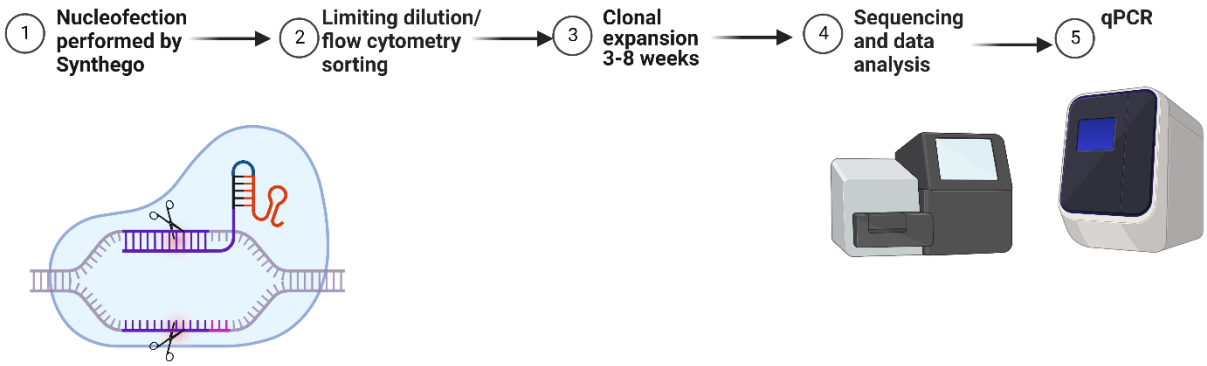


Figure 9. Schematic representation of the CRISPR/Cas9 work (created with BioRender.com).

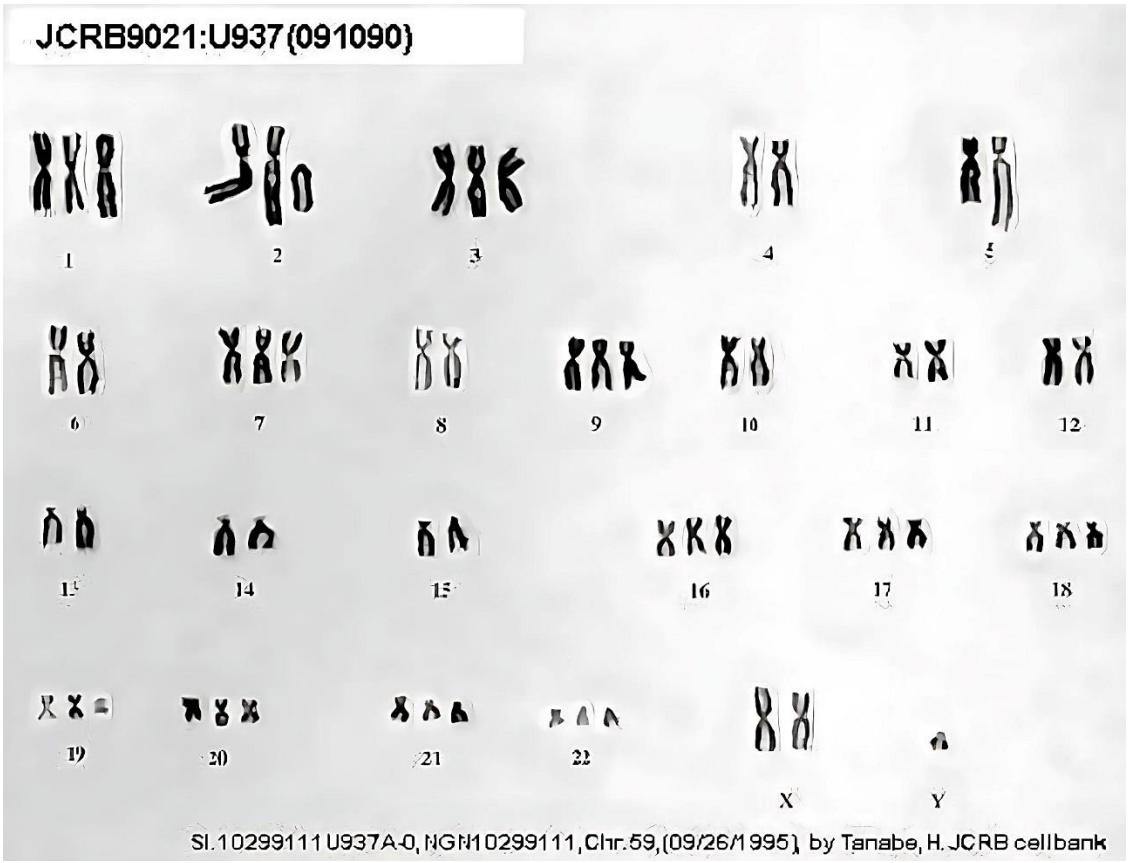


Figure 10. Schematic representation of the pairs of chromosomes in the U937 cell line (184)

2.2.1.2. Resuscitation of frozen cells and cell culture

The frozen pool of engineered cells (it contains a mixture of cells with heterogeneous genetic makeup; edited, non-edited and various undesired mutations) was thawed and cultured in Roswell Park Memorial Institute medium (RPMI 1640, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (12106C, H-I-FBS; Sigma-Aldrich) and 1% Penicillin-Streptomycin (P4333, Pen-Strep; Sigma-Aldrich). The pool of cell viability after thawing was above 90% and no sign of microbiological contamination was observed. The cells were incubated at 37°C in 95% humidified air and 5% carbon dioxide (CO_2) and were maintained in 1 well of a 24-well plate for 24 h before the limiting dilution step. The complete medium was utilised for other steps or culturing the cells.

2.2.1.3. Limiting dilution

U937 *TPCN2* knockdown (KD) pool cells were counted and diluted to 0.5-1 cells/100 µl of the complete medium (Roswell Park Memorial Institute medium (RPMI 1640, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (12106C, H-I-FBS; Sigma-Aldrich) and 1% Penicillin-Streptomycin (P4333, Pen-Strep; Sigma-Aldrich) by serial 10-fold dilutions performed according to Synthego's protocol. 1 cell/100 µl of the medium was plated in 10 96-well plates. After that, the cells were observed under the microscope to confirm that one cell was plated in each well and incubated at 37°C in 95% humidified air and 5% carbon dioxide (CO_2), and the medium changed if needed.

2.2.1.4. Clonal Expansion

The TPC2 genetically modified cells were observed under the microscope every 5-7 days. The single cells formed colonies after 3 weeks. When the established colonies reached 70% confluence, they were transferred into a 24-well plate, then into a 12 well plate, and after that into

a 6-well plate. A portion of cells from each colony was taken out for genotyping to determine which clone to maintain/discard.

2.2.1.5. DNA, and total RNA extractions, purifications, and quantifications

Genomic DNA and RNA were extracted using a DNeasy Blood & Tissue Kit and an RNeasy Mini Kit (QIAGEN, CA, USA) respectively, according to the manufacturer's protocols (which are summarised below).

2.2.1.5.1.DNA extraction

The appropriate number of cells were centrifuged for 5 min at 300 x g. The supernatant was discarded, and the pellet was resuspended in 200 µl PBS. Then, 20 µl of Proteinase K was added. 200 µl of Buffer AL was mixed with the sample to create a homogeneous solution. 200 µl ethanol (96–100%) was added to the homogenous solution and the contents mixed by vortexing. The homogenous solution was transferred into a DNeasy Mini spin column placed in a collection tube and centrifuged at ≥6000 x g (8000 rpm) for 1 min. After discarding the flow-through and collection tube, the spin column was placed in a new 2 ml collection tube, and 500 µl Buffer AW1 was added. The tube was centrifuged for 1 min at ≥6000 x g. After discarding the flow-through and collection tube, the spin column was placed in a new 2 ml collection tube, and 500 µl Buffer AW2 was added. The tube was centrifuged for 3 min at 20,000 x g. After discarding the flow-through and collection tube, the spin column was placed in a new 2 ml microcentrifuge tube, and 200 µl Buffer AE was added to elute DNA. The tube was incubated for 1 min at room temperature (15–25°C) and centrifuged for 1 min at ≥6000 x g.The DNA was stored at -20◦C.

2.2.1.5.2.RNA extraction

$1x10^6$ cells were centrifuged for 5 min at 300 x g. The supernatant was discarded, and the pellet was resuspended in 350 µl Buffer RLT.350 µl ethanol (70%) was added to the homogenous

solution and the contents mixed by pipetting. The homogenous solution was transferred into the RNeasy Mini spin column placed in a collection tube, and centrifuged at ≥8000 x g for 15 s. After discarding the flow-through and the collection tube, the spin column was placed in a new 2 ml collection tube, and 700 µl Buffer RW1 was added. The tube was centrifuged for 15 s at ≥8000 x g. After discarding the flow-through and the collection tube, the spin column was placed in a new 2 ml collection tube, and 500 µl Buffer RPE was added. The tube was centrifuged for 15 s at ≥8000 x g. After discarding the flow-through and collection tube, the spin column was placed in a new 2 ml collection tube, and 500 µl Buffer RPE was added. The tube was centrifuged for 2 min at ≥8000 x g. After discarding the flow-through and collection tube, the spin column was placed in a new 2 ml microcentrifuge tube, and 50 µl RNase-free water was added to eluate RNA. The tube was centrifuged for 1 min at ≥8000 x g. The RNA was stored at -80◦C.

2.2.1.5.3. Purifications and quantifications

A Nanodrop (Implen) was utilized to check the purity of the DNA and RNA (A260/280), with a ratio of 1.8 for DNA and 2 for RNA being acceptable limits, and to quantify the concentrations. 3.5 µl of DNA/ RNA was pipetted directly onto the middle of the NanoPhotometer Submicroliter Cell and the lid was placed on top. The NanoPhotometer quantifies the concentration based on the Beer-Lambert equation (A (absorbance) = E (wavelength-dependent molar absorptivity coefficient) * b (path length) * c(concentration)), which correlates absorbance with concentration.

2.2.1.6. Genotyping via PCR and Sanger sequencing

TPCN2 genomic DNA was amplified using a KAPA2G Robust HotStart ReadyMix PCR Kit (Kapa Biosystems, USA), according to the manufacturer's protocol, then the PCR products were sent to the Source Bioscience company for Sanger sequencing. PCR was conducted utilizing an oligonucleotide primer set for TPCN2 (FP; AAAGGAGACGGCCTGTGARTG, RP;

CACCTTCACAGAGAGGTCGG) (purchased from Life Technologies Ltd). A PCR reaction of 25 ul volume was calculated and prepared on ice as shown in the table below:

Material	Required volume	Finalconcentration
5X KAPA2G Robust HotStart ReadyMix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
DNA template	Variable (30-50 ng)	NA
PCR-grade water	Up to 25 µl	NA

The desired volumes of the prepared PCR mixture that contains the PCR master mix and the pair of primers were transferred into individual PCR tubes. DNA template was added (30-50 ng) to each tube, and the tube was sealed, mixed, and briefly centrifuged. The PCR reactions were performed using an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, USA), with the following thermal cycling programme.

Phase	Temperature	Duration	Cycles
Initial denaturation	95ºC	3 min	1
Denaturation	95ºC	15 sec	36
Annealing	62ºC	15 sec	
Extension	72ºC	15–60 sec/kb	
Final extension	72ºC	1 min/kb	1
Stored temporarily at 4°C and for the long-term stored at –20°C			

A 1% agarose gel stained with ethidium bromide was utilized to confirm the PCR product size. The 1% agarose gel was prepared as follows: 1x Tris-Borate electrophoresis buffer was prepared from 10 x Tris-Borate electrophoresis buffer after dilution with an appropriate volume of deionized water. For a 1% gel, 1 g agarose was added to 100 ml 1x TBE and heated in a microwave to dissolve the agarose. The appropriate amount of ethidium bromide (5 mg/ml) was added to the agarose solution after cooling it down with cold tap water. PCR products were electrophoresed at

90 V in TAE buffer and visualized with a UV transilluminator. An ExoSAP-IT Express PCR Cleanup Kit was utilized according to the manufacturer's protocol to purify PCR products for Sanger sequencing, which was conducted by the Source Bioscience company. The ExoSAP-IT™ Express reagent was thawed on ice. 5 µl of the PCR product was mixed thoroughly by vertexing with 2 µl of the ExoSAP-IT™ Express reagent and incubated at 37°C for 4 min, then 80°C for 1 min using an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, USA). The clean PCR product was stored temporarily at 4°C and for long-term use at –20°C.

2.2.1.7. Bioinformatics tools

The chromatogram quality was investigated using a CLC sequence viewer and chromas. The reads were assembled using the ICE analysis tool and CRISPR-ID, and TPC2 gene sequences of colonies were compared with the WT sequence (the amplicon size was ~350 bp).

2.2.1.8. Reverse transcription-quantitative PCR (RT-qPCR)

2.2.1.8.1. Reverse transcription (RT)

Complementary DNAs (cDNA) were synthesized by reverse transcription (RT) utilizing High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA), according to the manufacture's protocol. The kit reagents were thawed on ice. The reverse transcription reaction of 20 ul volume was calculated and prepared on ice as shown in the table below:

Material	Required volume	Final concentration
2X RT Buffer Mix	10.0 µl	1X
20X RT Enzyme Mix	1.0 µl	1X
RNA sample	Variable up to 9 µl (*up to 2 µg of total RNA per 20-µl reaction)	NA
Nuclease-free H_2O	Up to 20 µl	NA

The appropriate volumes of the prepared RT mixture were transferred into individual PCR tubes. The RNA template was added to each tube, and the tube was sealed, mixed, and briefly centrifuged. The RT reactions were performed using an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, USA), with the following thermal cycling programme:

Phase	Temperature	Duration	Cycles
1	37°C	60 min	1
2	95°C	5 min	1
Optional (hold)	4 °C	NA	NA
cDNA was stored at –20°C			

2.2.1.8.2. Quantitative PCR (qPCR)

qPCR was conducted utilizing PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. The kit components and the primers were thawed on ice. Our lab primers for detecting mRNAs corresponding to TPCN2 (FP; GTACCCCTCTTGTGTGGACG, RP; GGCCCTGACAGTGACAACTT), and β-actin(FP; CCAACCGCGAGAAGATGA, RP; CCAGAGGCGTACAGGGATAG), were purchased from Life Technologies Ltd. Values were normalized according to the relative amounts of the housekeeping gene β-actin , which was used as an endogenous control. The qPCR reaction of 20 ul volume was calculated and prepared on ice as shown in the table below:

Reagent	Required volume	Final concentration
PowerUp™ SYBR™ Green Master Mix (2X)	10.0 µl	1X
Forward and reverse primers (10 uM)	1.6 µl	800 nM
cDNA sample	Variable (20 ng)	NA
Nuclease-free H_2O	Up to 20 µl	NA

The desired volumes of the prepared qPCR mixture that contains PowerUp™ SYBR™ Green Master Mix and the pair of primers, was transferred into a 384 qPCR well plate. cDNA template was added (20 ng) to each well, and the plate was sealed, and briefly centrifuged. The qPCR

reactions were performed using LightCycler® 480 Roche machine (Roche, USA), with the following programme:

Stage	Temperature	Duration	Cycles
Stage1 (UDG activation)	50°C	2 min	Hold
Stage 2 (Dual-Lock™ DNA polymerase)	95ºC	2 or 10 min	Hold
Stage 3			
Denaturation	95ºC	15 sec	40
Annealing/ Extension	60ºC	1 min	
Stage 4(Cooling)	40ºC	30 sec	1

The relative qPCR quantitation was calculated as $2^{-\Delta\Delta Ct}$ (Ct= the value where the PCR curve crosses the threshold; Delta Ct = Ct TPCN2 – Ct β-actin; Delta Delta Ct = ΔCt KD – ΔCt WT).

2.2.2. Generation of TPC2 Knockout THP1 Cells

2.2.2.1. General method

Chr11 contains *TPCN2*, which is diploid in THP-1 cells (as shown in Figure 12). THP-1 *TPCN2* knockout pool cells were generated for us by Synthego using CRISPR-Cas9 technology nucleofection-based transfection (CA, USA). sgRNA (GCUCGAUGUGGCUUUACCGA) was designed to target exon 3 of the TPCN2 gene (Transcript ID: ENST00000294309). Resuscitation of frozen cells and cell culture, clonal expansion, genotyping via PCR and Sanger sequencing (FP; AGGTCTTGTGTGTTGGGAA, RP; CATCACAGGCCGTCTCCTTT), and reverse transcription-quantitative PCR (RT-qPCR), were conducted using the same methods that have been described above for the U937 cells, except for the TPCN2 engineered THP-1 cells single-

cell sorting was performed using flow cytometry, which I will describe in detail below. For the limiting dilution a different medium percentage of FBS was used (20% instead of 10%).

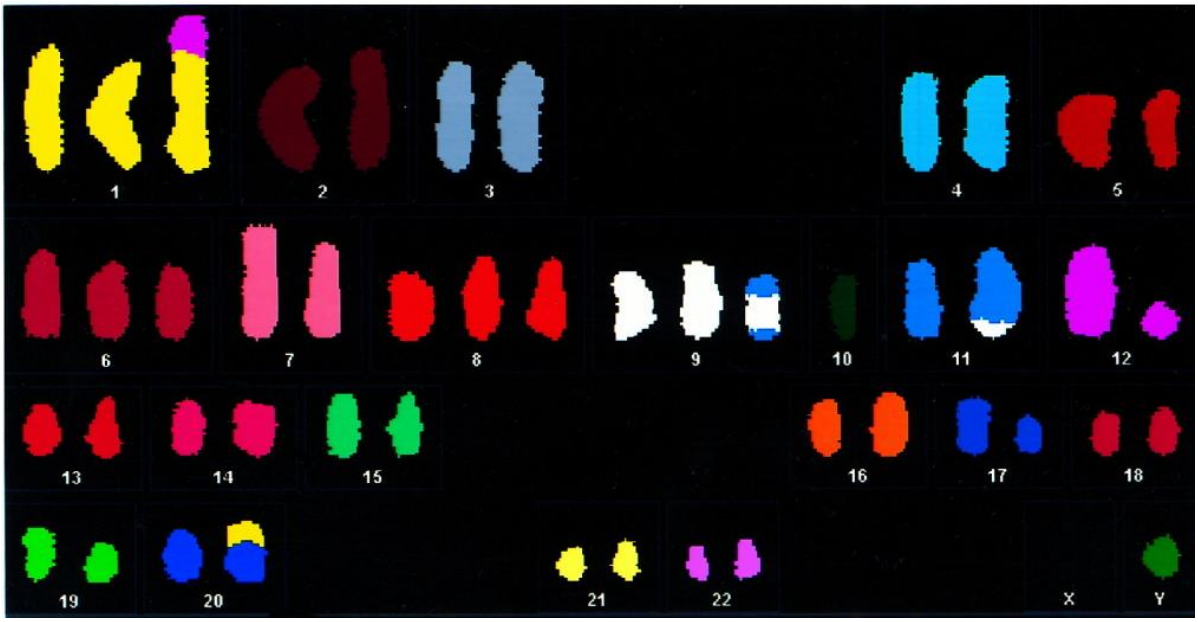


Figure 11. Schematic representation of the pairs of chromosomes in the THP-1 cell line (185). (The figure was reproduced from Odero et al. (2000), under the license number 5403840939993).

2.2.2.2. TPCN2-engineered THP-1 cells sorting by flow cytometry

1 million TPCN2-engineered THP-1 cells in 1 ml of the enriched medium (Roswell Park Memorial Institute medium (RPMI 1640), Thermo Fisher Scientific) supplemented with 20% heat-inactivated fetal bovine serum (12106C, H-I-FBS; Sigma-Aldrich) and 1% Penicillin-Streptomycin (P4333, Pen-Strep; Sigma-Aldrich) were sorted by flow cytometry, and plated at 1 cell per well in 96-well plates containing 100 µl/well of the enriched medium.

2.2.3. Cell proliferation by XTT assay

Proliferations of WT and TPC2 KD U937 cells were investigated using an XTT assay at different time points until day 3. $1x10^4$ U937 cells in 100 ul of complete medium were seeded into a 96-

well plate and incubated at 37°C in 95% humidified air and 5% carbon dioxide (CO_2). Prior to detection, XTT labelling reagent and electron-coupling reagent were thawed at 37°C and XTT labelling mixture was prepared according to the manufacturer's protocol. For detection, a 50 µl XTT labelling mixture was added into each well and incubated for 4 h at incubated at 37°C and 5% carbon dioxide (CO_2), subsequently, viable cells convert the XTT into a water-soluble orange formazan. Absorbance was measured using a microplate reader (Tecan) at a wavelength of 450 nm.

2.2.4. TPCN2 Characterization in Acute Lymphoblastic Leukaemia

2.2.4.1. TPCN2 via Sanger sequencing

TPC2 sequences from PBMCs isolated from the blood of healthy subjects and PBMCs derived from acute lymphoblastic leukaemia patients were examined for genetic variants in *TPCN2* using Sanger sequencing, as shown in Figure 12.

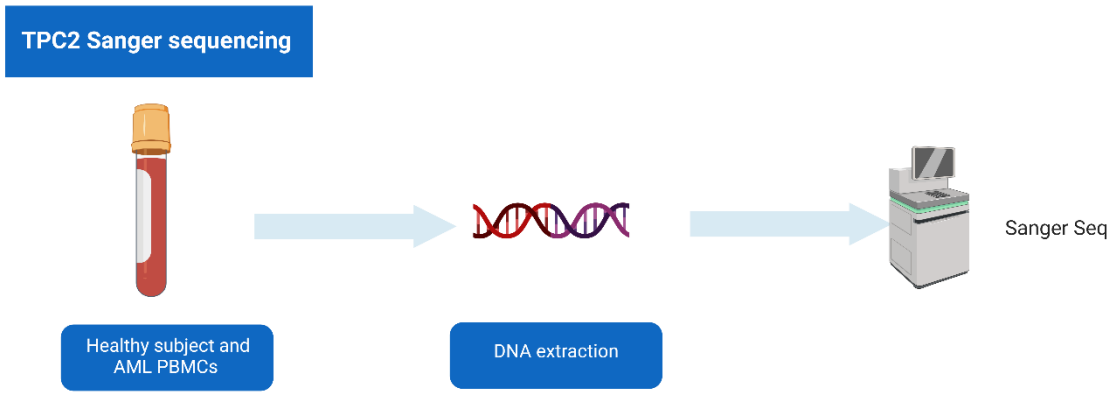


Figure 12. TPCN2 genotyping in PBMCs isolated from the blood of healthy subject and patients with acute lymphoblastic leukaemia (created with BioRender.com).

2.2.4.2. Cytotoxicity characterization of imatinib in TPCN2 deficient U937 cells by XTT assay

The TPC2 genetically modified human myeloid leukaemia cell line was used to investigate the role of TPC2 in imatinib cytotoxicity. The cytotoxicity responses of WT and TPC2 KD U937 cells were assessed, after treatment with different concentrations of imatinib. U937 cells were plated as $1x10^4$ in 100 ul of medium /well into a 96-well plate. The cells were treated with different concentrations of imatinib: 10, 50, and 100 uM. Then the cells were incubated at 37°C in 95% humidified air and 5% carbon dioxide (CO_2) with imatinib for 24h. For detection, as mentioned above, 50 ul of the XTT mixture was added into each well, incubated at 37°C for 4h, followed by observation using a microplate reader (Tecan) at a wavelength of 450 nm. Subsequently, the cell cytotoxicity of U937 cells was calculated, and cells that were not treated with imatinib were considered as a control.

2.2.5. Data presentation and statistical analysis

Results are presented as descriptive statistics. Analysis was performed utilizing R for Windows (version 3.4.1).

2.2.3. Results

2.2.3.1. TPCN2 CRISPR-Cas 9 engineered cell analysis

2.2.3.1.1. TPCN2 genetically modified U937 cell pool

Synthego failed to generate U937 *TPCN2* knock out (KO) pool cells with high editing efficiency (as shown in Table 4). By using the CRISPR-Cas9 technology targeting exon 4 of the TPCN2 gene, Synthego produced the first TPCN2-knockdown (KD) U937 pool cells with an initial editing efficiency of 52% however after cell expansion this dropped to 44% and a predicted deletion of nucleotides ranging from 1 to 6.

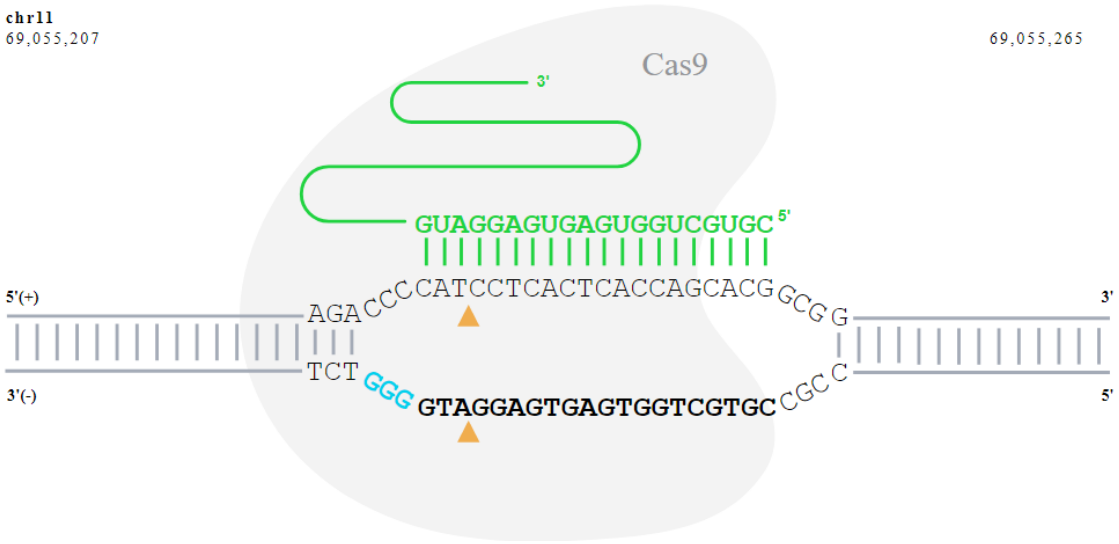


Figure 13. Guide RNA targting exon 4 of TPCN2 gene (figure provided by Synthego).

Table 4. TPCN2 engineered U937 pool cells analysis			
48 h Post-Transfection Editing Efficiency	Editing Efficiency after Expansion	Guide RNA	Exon Targeted
52.00%	44.00%	CGUGCUGGUGAGUGAGGAUG	Exon 4

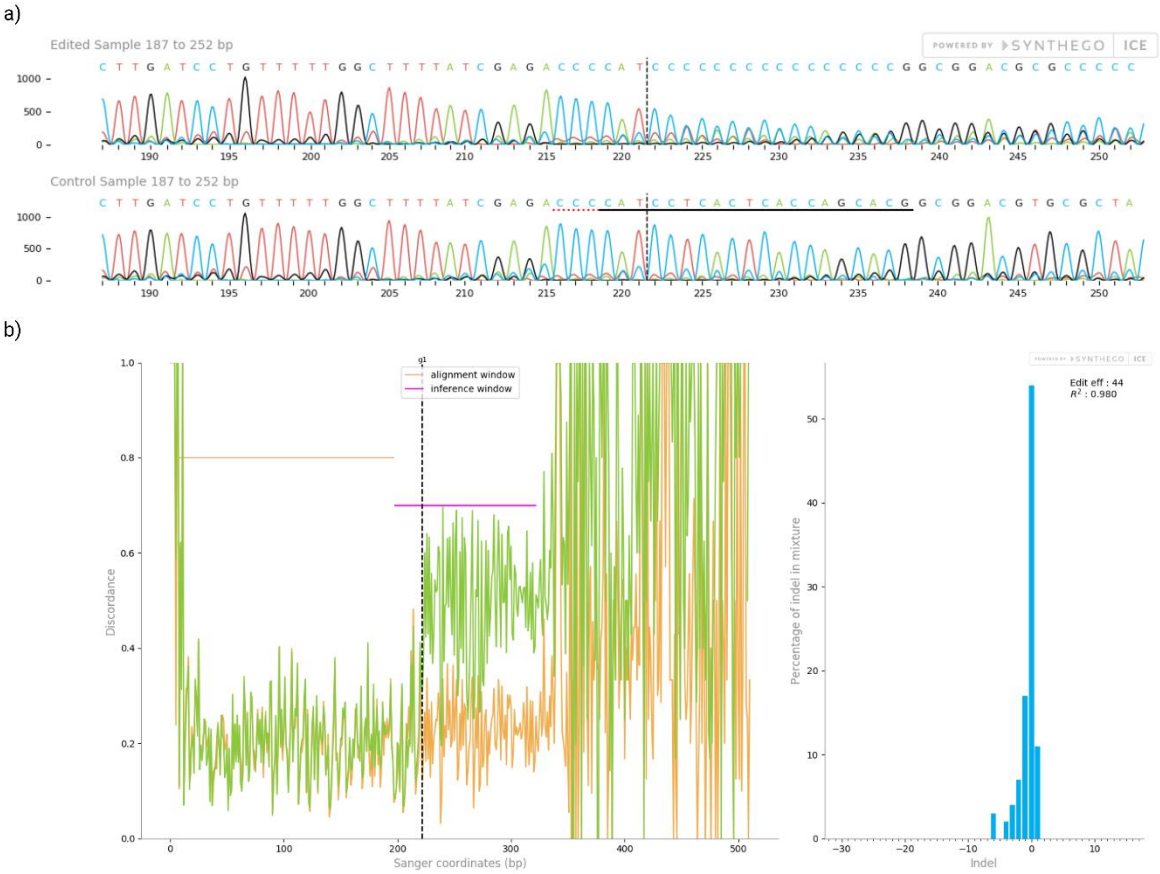


Figure 14. TPCN2-CRISPR-Cas 9 edits predicted by ICE (Figure provided by Synthego). A. TPCN2 sanger sequence.B. Discordance and Indel plots.

2.2.3.1.1.2. Clonal Isolation

Single cells were plated via limiting dilution, and 14 monoclonal populations of U937 cells were generated after 3 weeks and 5 days.

2.2.3.2. TPCN2 genetically modified THP-1 cell pool

2.2.3.2.1. TPCN2 knockout cell analysis

By using CRISPR-Cas9 technology to target exon 3 of the TPCN2 gene, Synthego succeeded in producing THP-1 *TPCN2* knock-out (KO) pool cells with high editing efficiency before and after cell expansion (as shown in Table 5). The predicted deletion of nucleotides ranges from 1 to 12.

2.2.3.2.2. TPCN2 genetically modified THP-1 cell sorting by Flowcytometry and Clonal Isolation

Single cells were plated via limiting dilution or sorted via flow cytometry (as shown in Figure 17) but this failed to produce monoclonal populations of THP-1 cells after 8 weeks.

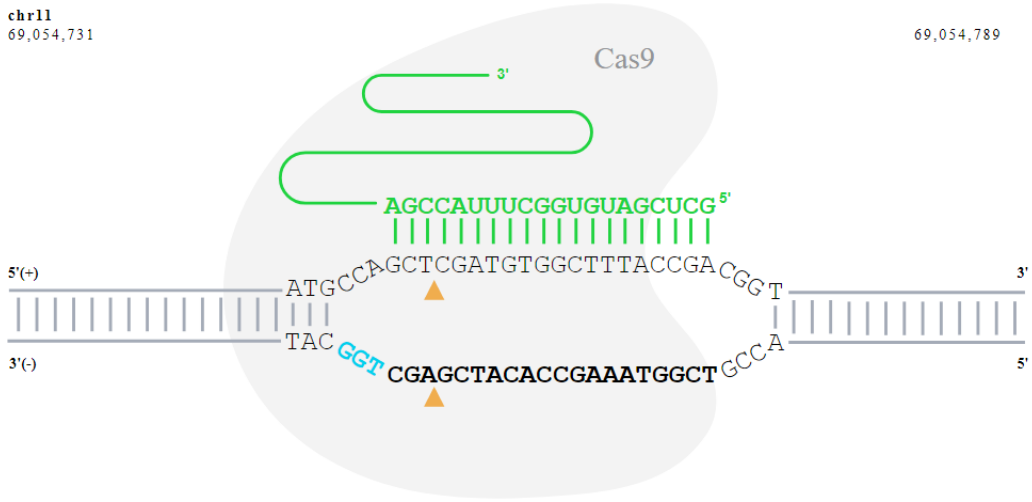
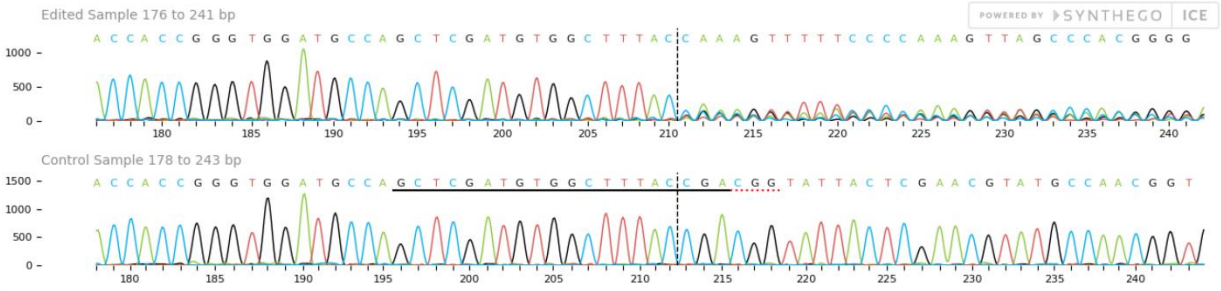


Figure 15. Guide RNA targting exon 3 of TPCN2 gene (figure provided by Synthego).

Table 5. TPCN2 based CRISPR-Cas 9 engineered THP-1 pool cells analysis.			
48 hrs Post-Transfection Editing Efficiency	Editing Efficiency after Expansion	(Guide RNA)	Exon Targeted
81.00%	70.00%	GCUCGAUGUGGCUUUACCGA	Exon 3

a)



b)

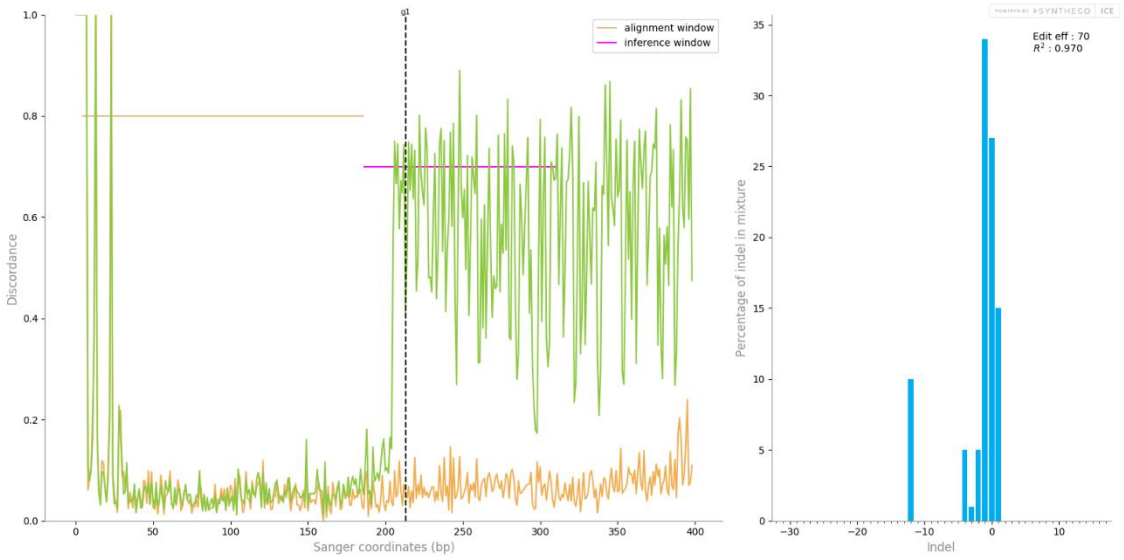


Figure 16. TPCN2-CRISPR-Cas 9 edits predicted by ICE (data provided by Synthego). a. TPCN2 sanger sequence.b. Discordance and Indel plots.

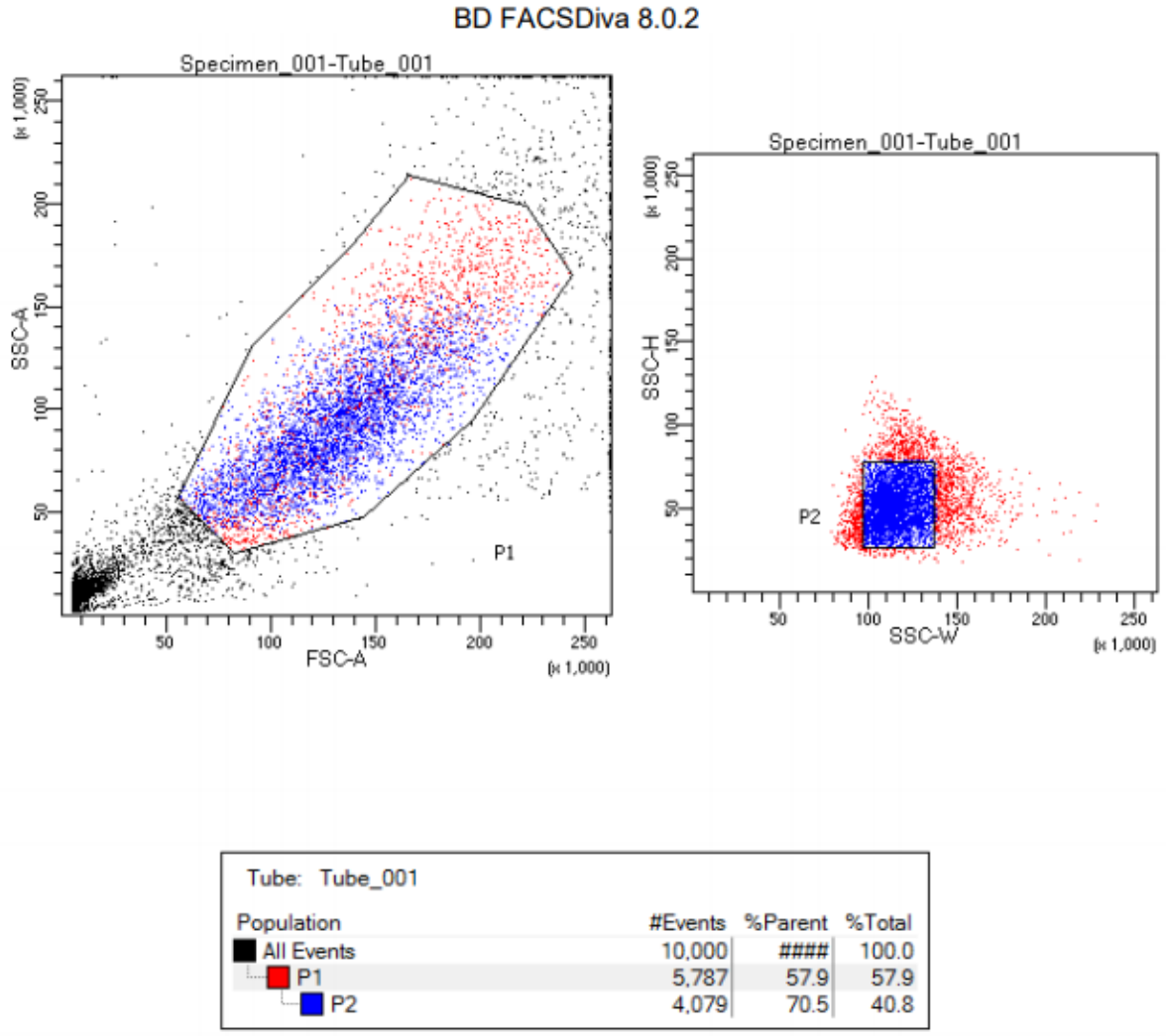


Figure 17. TPCN2-engineered THP-1 cells were sorted by flow cytometry.

2.2.3.3. Characterisation of the U937 clones at DNA and RNA levels

2.2.3.3. 1. Genotyping U937 clones

The TPCN2-CRISPR-Cas9 U937 clones' sequences were compared with wild-type sequences to detect the editing efficiency and KO score using the Inference of CRISPR Editing (ICE) bioinformatics tool. It can be seen from the data in Table S2.1 that 9 out of the 14 clones were within an indel percentage (Indel%) of 89% or above, which had been selected as the cut off to characterise the effect of these mutations on TPCN2 expression.

2.2.3.3.2. Validating TPCN2 expression depletion by qPCR

Figure 18a shows a reduction in TPCN2 expression at the mRNA level in U937 clones compared to WT U937. The significant decrease in TPCN2 expression ranging from 12% to 56%(ANOVA, p-value= 0.004) is confirmed by a company, indicating that mutation induced by CRISPR-Cas9 has affected the TPCN2 expression at RNA level, as shown in Figure 18b.

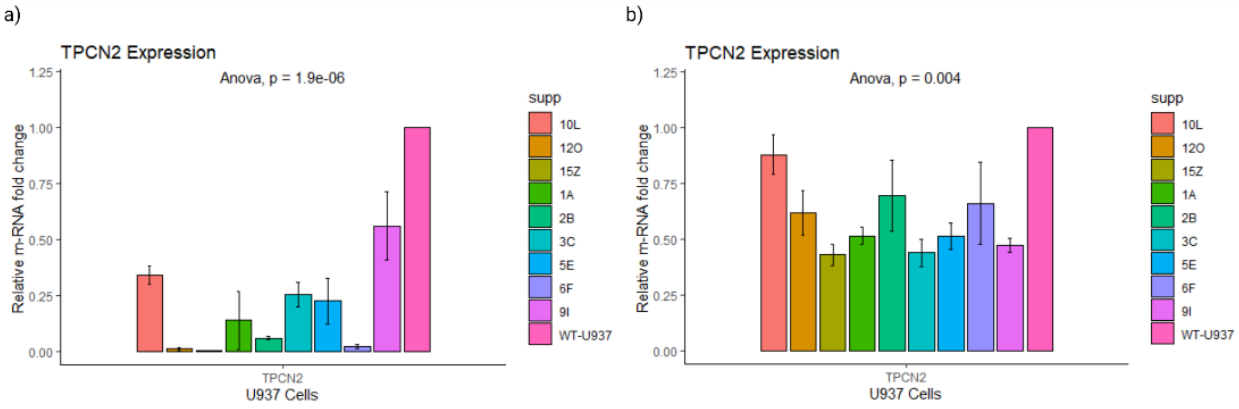


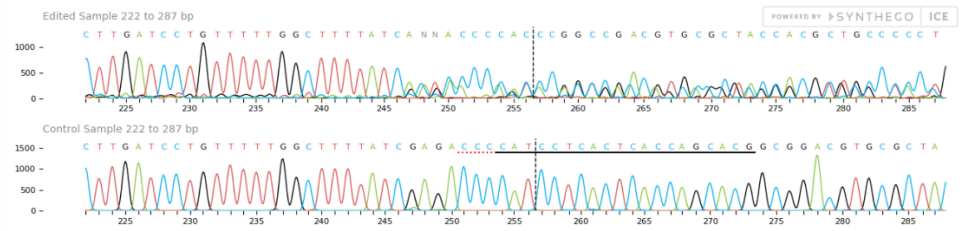
Figure 18. TPCN2 expression by qPCR. a. TPCN2 expression levels in TPCN2-engineered U937 cells (three replicates from three reactions, conducted in our lab). b. TPCN2 levels in TPCN2-engineered U937 (three replicates from three reactions, conducted by the company). The qPCR was conducted using Quantifast SYBR Green PCR mix (Qiagen) (The reactions were pipetted robotically by a CAS-1200; the cDNA concentrations were NA; cDNA was diluted 10-fold).

2.2.3.4. Selection of TPC2 KD clone

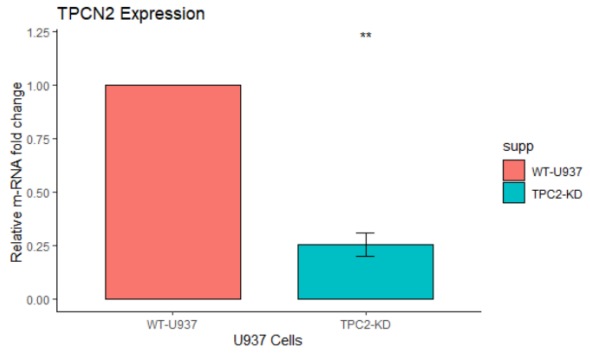
To select the right clone that is appropriate for downstream assays, subsequently referred to as the TPC2 KD U937 model, four parameters including knockout (KO) score, TPCN2 expression, similarity in the growth rate between WT and the clone, and homozygosity were assessed. To determine the KO score, I have compared the TPCN2 sequences from the 3C clone with WT U937 (as shown in Figure 19a). TPCN2 expression is significantly downregulated (two sample t-

test, p-value 0.005373) by 74.6% in the 3C clone compared to U937 WT (Figure 19b). As Figure 19c indicates, it is apparent that the 3C clone has a similar growth rate as the WT U937 cells (ANOVA, p-value= 0.776). The 3C clone shows a homogenous deletion at the predicted Cas9 cleavage site examined by CRISPR-ID (Figure 20).

a)



b)



c)

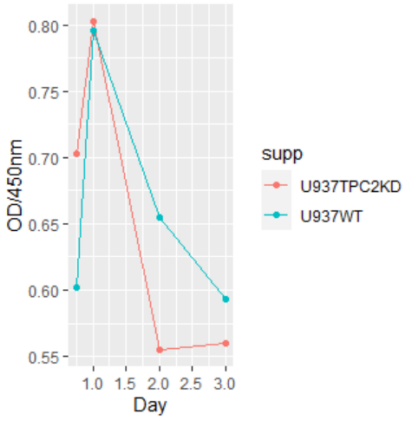


Figure 19. 3C clone defines as the TPC2 KD U937 model. a. TPCN2 sanger sequence of 3C vs WT b. TPCN2 expression in 3C vs WT (mean ± SEM). c. Proliferation of WT and TPC2 KD U937 cells using an XTT assay (mean).

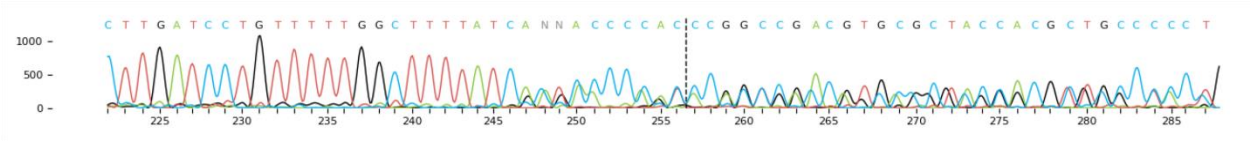


Figure 20. The 3C clone sequence at the predicted Cas9 cleavage site by CRISPR-ID.

2.2.3.5. Morphological and biological characterisation of the TPCN2 deficient U937cells

2.2.3.5.1. Morphological characterisation

Figure 21 illustrates the morphological characteristics of the WT and TPC2 KD U937 cells, which were monitored by Invitrogen EVOS FLoid cell imaging. Based on these data, we can see that there are no major differences in morphology between WT and TPC2 KD cells.

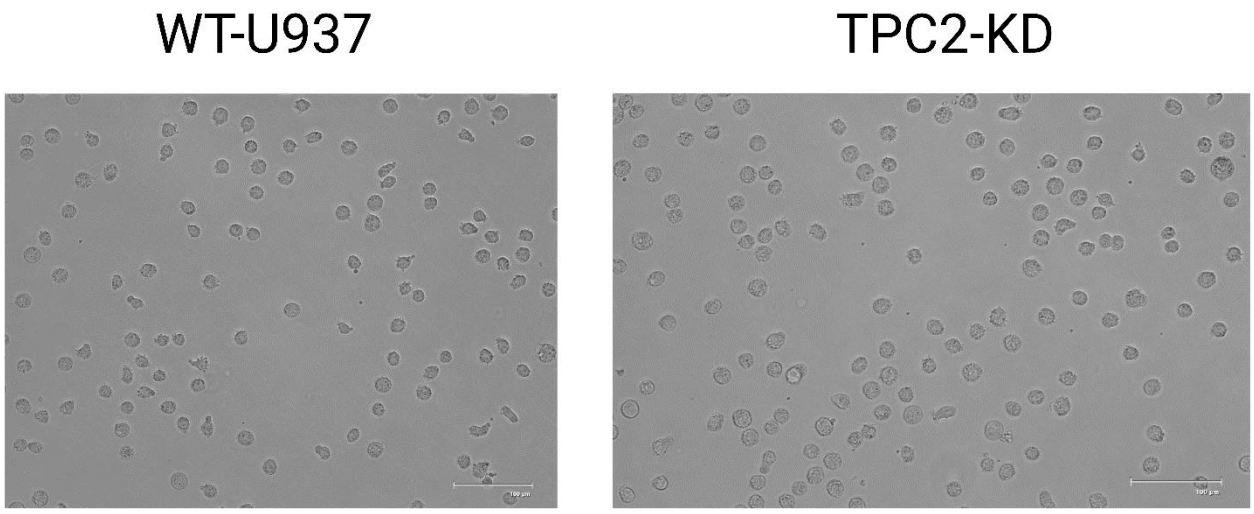


Figure 21. Morphology of U937 cells WT vs TPC2KD.

2.2.3.6. Biological characterisation

2.2.3.6.1. Cell proliferation

I investigated the effect of TPC2 in U937 cell proliferation using an XTT assay. No significant differences were found in cell proliferation between WT and TPC2 KD U937 cells (Day 1 [two sample t-testt, p-value 0.946], Day 2 [two sample t-test, p-value 0.289], and Day 3 [two sample t-test, p-value 0.642])

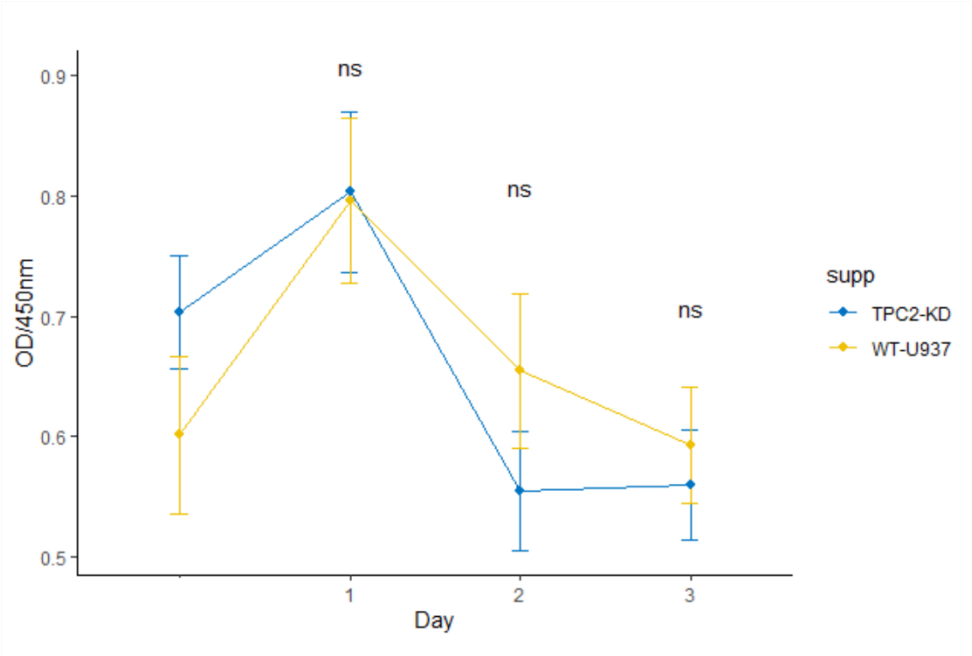


Figure 22. TPC2 knockdown role in U937 cell proliferation investigated by an XTT assay (mean ± SEM).

2.2.3.7. TPC2 in hematologic malignancies

A known SNP (rs3376) was found in the TPCN2 gene in PBMCs derived from acute myeloid leukaemia but not in PBMCs isolated from the blood of healthy subject, as shown in Figure 23. This result suggests that this SNP might be correlated with cancer. A known SNP (rs72917389) was found in the TPCN2 gene in monocytes prior to, during and after differentiation into

macrophages derived from AML, but not in monocytes isolated from the blood of healthy subjects (as shown in Figure supplementary S2.2. These results suggest that rs72917389 might be correlated with cancer.

Table 6. Subject characteristics						
Disease	Is the current disease primary or recurrent?	Diagnosis	Age	Gender	Patient race	Ethnicity
Acute myeloid leukaemia	Primary	Acute myeloid leukaemia	72	Male	Caucasian	Slavonic



Figure 23. Sanger sequences of PBMCs derived from acute myeloid leukaemia and healthy subject. SNP (rs3376) was identified in the TPCN2 gene of a subject with acute myeloid leukaemia.

2.2.3.8. Determination of the cytotoxicity of imatinib in TPC2 deficient myeloid leukaemia cells (U937 cell line) a combination therapy strategy

To explore the potential combination therapy of CRISPR Cas 9 TPCN2 knockdown and imatinib, U937 cells were treated with different concentrations of imatinib. The cell cytotoxicity was estimated with an XTT kit after 24 hours of treatment. As shown in Figure 24 b, no significant differences were found in the cytotoxicity of imatinib between WT and TPC2 KD U937 cells treated with different concentrations of imatinib (10 uM [two sample t-test, p-value 0.09696], 50 uM [two sample t-test, p-value 0.6871], and 100 uM [two sample t-test, p-value 1]).

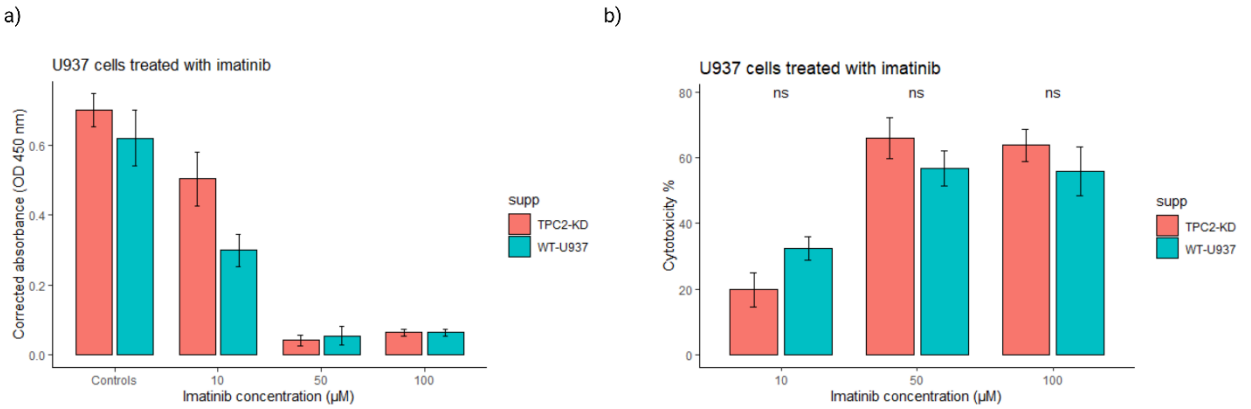


Figure 24. Cell cytotoxicities of WT and TPC2 KD U937 cells after receiving treatment with different concentrations of imatinib. The cell cytotoxicity was measured by an XTT assay.

2.4. Discussion

U937 and THP-1 cell lines are *in vitro* models of monocytes, macrophages and leukaemia. Monocytes and macrophages are myeloid cells that play a vital role in inflammation, immunity and cancer. Although TPC2 plays a role in cellular differentiation and cancer, the precise mechanisms underlying the action of TPC2 and its involvement in monocyte differentiation and cancer, particularly in leukaemia, remain unclear. Due to the paucity of available specific inhibitors of TPC2, I established a TPC2-deficient U937 monocyte model and attempted to develop TPC2-

deficient THP-1 monocytes twice, though these attempts were unsuccessful. Synthego generated a pool of TPCN2 genetically modified U937 and THP-1 cells. The appropriate approach to transfecting these cell lines is nucleofection; however, we lack the equipment to perform this in our lab. These cell lines are also very difficult to work with in relation to gene editing with high editing efficiency and low off-target effect, even with automation. When using U937, as the knockout of the target gene (TPCN2) is difficult to achieve, even after optimising 200 times and using multiple rounds of sgRNA design and transfection, Synthego failed to generate a pool of cells with high editing efficiency. The drop in editing efficiency observed after thawing the cells in both U937 or THP-1 was due to the cells' reduced ability to thrive after knockout.

A TPCN2 genetically modified pool of cells failed to form monoclonal populations of THP-1 cells after plating 1 cell/well in a 96-well plate using either the limiting dilution approach or flow cytometry and using different concentrations of FBS (10% or 20%) after monitoring the cells for up to eight weeks. Notably, this method failed to generate single clones in TPCN2-edited THP-1 cells, but there was no effect in TPCN2-edited U937 cells. These results demonstrate that the TPC2/Ca^{2+} signalling pathway may be critical for THP-1 proliferation, and further investigation to determine the effect of TPC2 in THP-1 (*in vitro* model of monocytes and leukaemia) proliferation is warranted. In addition, the inability of THP-1 to create monoclonal populations after TPC2 KO compared with U937 could be attributable to the nature of the cancer, origin and maturation stage of the cell line (U937 cells are derived from tissue of histiocytic lymphoma, metastasis state, whereas THP-1 cells are derived from the blood of patient with AML, primary state). qPCR was used to investigate the loss of TPCN2 expression at the transcriptomic level. Further, qPCR analysis was conducted by a company using the same primers to confirm the data. The qPCR data produced in our lab was consistent with the qPCR data conducted by the company in term of the conclusion but not the values. This discrepancy in the values could be due to the use of the different material Quantifast SYBR Green PCR mix (Qiagen). The reactions were pipetted

robotically by a CAS-1200, and cDNA was diluted 10-fold. A limitation of our developed model was that, unfortunately, performing further analysis to functionally validate the knockdown in TPC2 expression at protein level could not be achieved by western blot/flowcytometry analysis due to a lack of commercially available robust specific antibodies to confirm the loss of TPC2 protein. To my knowledge, I developed the first TPC2-deficent human U937 *in vitro* model to use as a tool for exploring the role of TPC2 in immunity and cancer biology, for which there is a paucity of specific pharmacological inhibitors. The possibility that the reduction in TPCN2 expression at the transcription level will not translate at the protein level is an important caveat to bear in mind when using this research tool to decode TPC2's role in pathophysiological processes attributed to immunity or cancer. Therefore, researchers should conduct other functional assays to investigate the activity of the TPC2 channel.

Imatinib is FDA approved for treating several types of leukaemia but is not yet approved for treating ALL. Many clinical trials registered on the clinical trials database have investigated imatinib safety and efficacy in ALL. However, few trials have reported the findings. One study found that the multi-kinase inhibitors sorafenib (IC50, 6.9 μmol/L) and sunitinib (IC50, 6.2 μmol/L) showed more potent antiproliferative activity in U937 cells compared with imatinib (IC50, 18 μmol/L) (186). Based on previous data on the antiproliferative activity of TPC2 knockout in different types of cancer, I speculated that treating U937 TPC2-deficient cells with imatinib could enhance the cytotoxic effect of imatinib. Therefore, I evaluated the *in vitro* cytotoxicity of imatinib in U937 WT and TPC2 KD cells using an XTT assay. I found that TPC2 knockout using CRISPR-Cas9 combined with imatinib did not enhance the cytotoxic activity of imatinib *in vitro*. Nevertheless, imatinib exhibited cytotoxic effects against U937 cells in the concentration range of 10 μM to 100 μM; therefore, it has the potential to exhibit antiproliferative activity in ALL. Further investigations to develop drugs to enhance its activity and tumour selectivity at clinically relevant

concentrations are warranted. Although I demonstrated the cytotoxic effect of imatinib in U937 cells, I did not investigate the underlying mechanisms.

rs72917389 (intron variant [minor allele frequency (MAF)], approximately 20% in Europeans) in TPCN2 was reported in U937 monocytes (in vitro model of monocytes) that were derived from but not in primary monocytes taken from a healthy subject(187).rs3376 in TPCN2 is a three prime untranslated region (3′-UTR) variant that may influence the expression levels of TPC2 at the post-transcriptional level. The effect of rs3376 on TPC2 protein structure or function and the correlation between this genetic variant with phenotype/disease are yet to be reported. I sought to predict the effect of this variant on TPC2 expression/protein using Genotype-Tissue Expression (GTEx) and Ensemble Variant Effect Predictor (VEP) but failed due to a lack of data. rs3376 is common among Europeans (minor allele frequency [MAF] >25%) and is likely to affect TPC2 at the functional level (188). Therefore, the presence of this genetic variant in the PBMCs of AML patients could be associated with cancer, which warrants functional characterisation of this genetic variant in cancer/cancer subtypes, particularly leukaemia. Our data regarding rs3376 in AML and how it influences the TPC2 expression/function point to a need for further research. Exciting research has revealed the potential role of TPC2 as a biomarker for estimating the response to chemotherapy, which could have significant implications for AML patient care. To develop a full picture of the role of TPCN2 expression to chemotherapy sensitivity in AML, studies on cohorts with diverse populations that are clinically and genetically well characterised should be conducted to explore the clinical utility of TPC2 expression in directing chemotherapy.

2.5. Conclusion

In this chapter, for the first time, I developed a powerful research tool for characterising the function of TPC2 in U937 cells (an *in vitro* model of human monocyte/macrophage cells or leukaemia). TPC2-deficient U937 cells with a 75% reduction in TPCN2 expression were

generated to explore the mechanisms that underlie the role of the endolysosomal TPC2 channel in different fundamental processes attributed to immunity or cancer. The lack of a specific antibody to determine the loss of TPC2 expression at the protein level is a major limitation that requires validation of the loss of TPC2 activity through different approaches. I reported finding a genetic variant in the TPCN2 gene in PBMCs derived from a patient with AML. While my finding of an association of rs3376 with AML development is intriguing, functional characterisation of this genetic variant's role in cancer is required at a global level and in patients with AML at a local level. Genetic modulation by CRISPR-Cas9 is a suitable approach to impair the function of TPC2 due to a paucity of specific pharmacological inhibitors; therefore, targeting TPC2 function could help develop new drugs and therapeutic agents with clinical relevance in treating cancer, particularly leukaemia. In addition, in the future, TPC2 genotyping could allow us to identify appropriate patients for therapy while avoiding the toxicities and costs for those patients who are unlikely to benefit, to subsequently be able to offer alternatives to them.

UNIVERSITY OF OXFORD

Chapter 3: Endolysosomal Ca^{2+} signalling in the innate immune system: the role of two-pore channels (TPCs), from monocyte differentiation to cytokine production

3.Chapter 3: Endolysosomal Ca^{2+} signalling in the innate immune system: the role of two-pore channels (TPCs), from monocyte differentiation to cytokine production

3.1. Introduction

3.1.1 Endolysosomal Ca^{2+} signalling via TPC2 in inflammation, immunity and cancer: facts and perspectives

Growing evidence links calcium (Ca^{2+}) signalling via TPC2 to inflammation, immunity and cancer; the role of TPC2 in different fundamental physiological processes attributed to inflammation, immunity and cancer and their therapeutic relevance were reviewed, published and discussed in Chapter 1. Here, I highlight the recent findings that have been published since mid-2021 in the context of inflammation, immunity and cancer and their potential therapeutic applications. For the first time (to my knowledge), the direct connection between TPC2/cation signalling has been underpinned in inflammation. NAADP-mediated cation signalling via TPC2 modulation either by genetic deletion or by pharmacological inhibition by trans-Ned-19, attenuated laser-induced choroidal neovascularization *in vivo* through diminishing the inflammatory response, which manifested in the significant reduction in IL-1β levels, and neoangiogenesis, which was exhibited by a decrease in VEGF-A levels (157). This study underscores the immunological significance of TPC2 function in immune response activation via the production of a potent pro-inflammatory cytokine (IL-1β) and is in line with the previously published data that identified TPC2 as a regulator of VEGF-induced angiogenesis (102,104). Although it has become clear that TPC2 activity plays pivotal roles in different cancer types, at different stages, and in different pathophysiological processes related to cancer (2), the precise mechanisms underlying the action of TPC2 in various aspects of cancer are still evolving(2). Recently, Müller et al. (2021) shed light on the role of TPC2 in hepatocellular carcinoma in terms of cellular proliferation and tumour growth at the local level and in cancer metabolism at the global level. At the local level, they found that knocking out TPC2 by CRISPR-Cas9 reduces both non-cancerous and cancerous hepatocyte proliferation (139).

Moreover, when C57BL/6-Tyr mice were injected with RIL-175 (mouse hepatocellular carcinoma cell line) WT and TPC2 KO cells, the tumour growth of the KO cells was reduced by 90% on day 10 *in vivo* compared to WT tumour growth (139). Together, these data indicate that impairing TPC2 activity exhibits antitumour effects, which is consistent with my perspective that TPC2 acts more as a critical driver than a passenger in tumourigenesis (involving cancer cell proliferation and growth). At a global level, they demonstrated that inhibition of the glycolysis by 2-deoxy-D-glucose (2-DG; glycolysis inhibitor) contributed to significant reduction in ATP levels in WT compared to TPC2 KO cells, which showed no changes in ATP production (139). These data might explain the antiproliferative effect of knocking out TPC2 *in vitro*/*vivo* through interfering with glycolysis and mitochondrial respiration (the Warburg Effect). The cellular mechanisms underlying the regulation of TPC2-mediated endolysosomal cation signals in cancer metabolism warrant further investigation to aid the development of new drugs that modulate TPC2 function, which indirectly interfere with aerobic glycolysis to overcome the toxicity associated with direct modulation of the glycolytic pathway. We highlighted the findings of Müller et al. (2020), focusing on the development of two small molecules that non-specifically diminish TPC2 function, in our preview article entitled 'TPC2 targeting evolution: Leveraging therapeutic opportunities for cancer', which was published in *Cell Chemical Biology* (151). The drug development of TPC2-specific pharmacological modulators is still in its infancy. The paucity of specific TPC2 pharmacological modulators that were tested in humans and approved by the FDA limits its potential clinical utility in the near future. The ubiquity of TPC2 and its roles in human physiology necessitate developing a drug that precisely modulates TPC2 activity in the pathological condition, while avoiding interfering with its physiological role before any potential therapeutic applications can be translated into a clinical setting.

3.1.2. Monocytes and macrophages, TPC2, and their therapeutic relevance in cancer

TPC2 is overexpressed in monocytes at both the RNA and protein levels; median expression was approximately 18 and 3.5, respectively (189). Monocytes are differentiated into macrophages or dendritic cells. Macrophages are generally classified into two major classes – macrophage type 1 (M1) and macrophage type 2 (M2) – that are opposed in their functions in immunity/cancer: M1 has a pro-inflammatory and anti-tumour function, and M2 has anti-inflammatory and pro-tumoural effects (190,191). TPCN1 and TPCN2 expression levels were found to be higher in bone marrow-derived macrophages than in mouse embryonic fibroblasts. This upregulation could be attributed to an increase in the number of lysosomes, but no data was reported regarding the number of lysosomes in this study (132). TPC2 protein expression was significantly increased in M2 macrophages compared with M1 mouse macrophages (129). Tumour-associated macrophages (TAMs) are part of the tumour microenvironment that exhibits M2-like macrophage phenotypes (192). TAMs have been correlated with poor prognosis in different types of cancer; several processes interfere with TAM development (as shown in Figure 25). TAMs play vital roles in physiological processes related to angiogenesis, tumour metastasis and progression, immunosuppression and chemo/radio therapy resistance. Recently, Cui et al. (2021) developed a DNA nanodevice (E-64 [protease inhibitor]-DNA) to precisely inhibit the activity of cysteine proteases that are found inside the lysosomes of TAM, which ultimately reduces tumour growth (129). The availability of drugs, some of which have been examined in humans, raises a translational question regarding the possibility of modulating TAM as an immune therapeutic approach to treat patients with cancer. This could be achieved by either depletion of TAM or TAM reprogramming (as summarised in Figure 26); for example, CD40 agonist antibodies developed to indirectly modulate TAM functions. Chi Lob 7/4 was tested in a phase I trial (NCT01561911) in twenty-eight patients with different types of cancer (193). Generally, it has acceptable safety and tolerability profiles, warranting further investigation in phase II trials (193). In contrast, magrolimab

(Hu5F9-G4) was developed to deplete TAM, and its safety and tolerability was assessed in cancer patients (NCT02216409) (194). It was relatively safe and tolerable at a priming dose of 1 mg/kg given on day 1, followed by up to 45 mg/kg weekly as a maintenance dose (194). A phase II study (NCT04788043) is currently being conducted to investigate the efficacy and safety of magrolimab and pembrolizumab in patients with relapsed or refractory classic Hodgkin lymphoma (195). The biological significance of TPC2 upregulation in monocytes and macrophages has not been investigated. Additionally, a full phenotypic characterization of macrophages and their subtypes are still an immunology puzzle; TPC2 might be considered a marker that categorises macrophage phenotypically, and its modulation might play a role in the reprogramming of TAMs to restore anti-tumour functions. In this chapter, I have examined the cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in monocyte differentiation and macrophage development and its links to inflammation, immunity and cancer development.

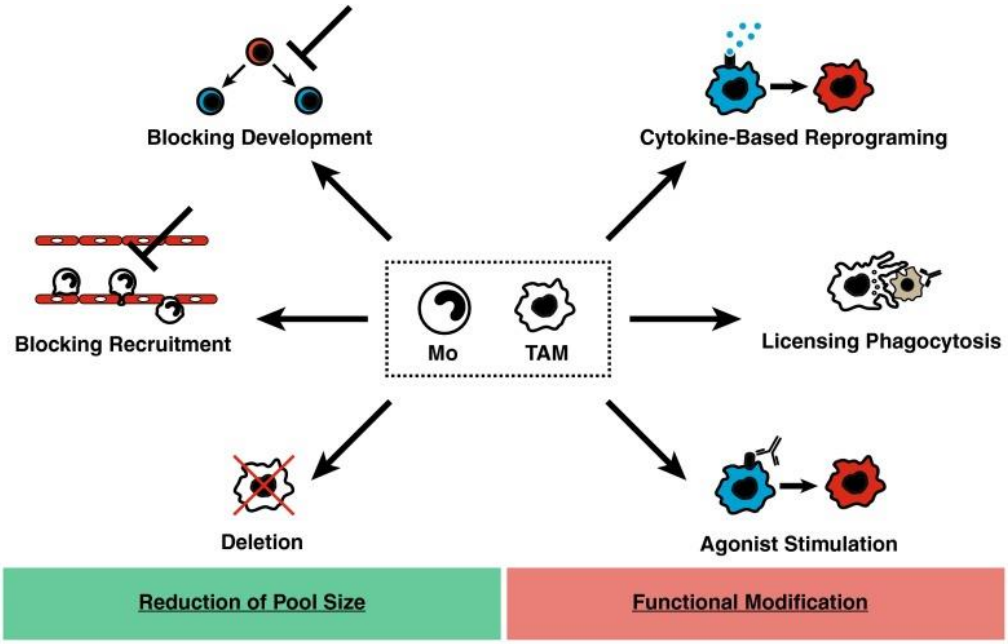


Figure 25. Schematic representation of processes that interfere with TAM (196). (The figure was reproduced from Richards et al. (2013) under Springer Science+Business Media Dordrecht 2012 copyright).

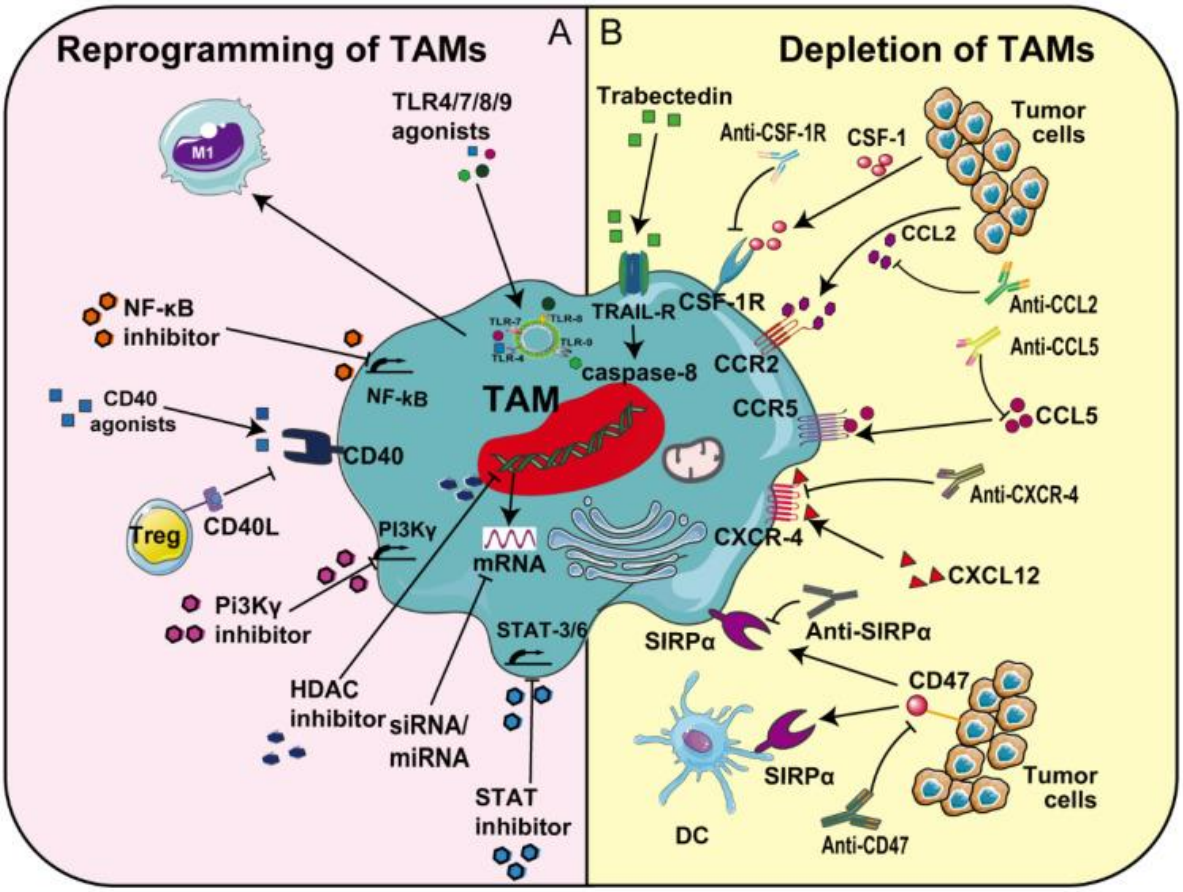


Figure 26. Schematic representation of therapeutic strategies to target TAM (197) (the figure was reproduced from He et al. under MDPI copyright).

3.1.3.CD38, PI3K/AKT, calpain, and PKC-alpha signalling pathways in inflammation, immunity and cancer

CD38 (cluster of differentiation 38) is a multifaceted ecto enzyme (a 45-kD glycoprotein) that is known to generate cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), two important messengers of intracellular Ca^{2+} signalling. CD38's biological significance evolved from a surface marker used to recognise T cells to an important

immunomodulator, especially in the context of cancer, recently to a valuable therapeutic target/biomarker (198). Figure 27 demonstrates that CD38 is highly expressed in various types of immune cells. It was shown that CD38 expression varies in cancer cells, and it was upregulated in multiple myeloma (malignant plasma cells) (199). CD38-mediated inflammation, development of autoimmune disorder, and cancer promotion have been a subject of increasing interest in recent years. Li et al. (2019) found that, after being induced by lipopolysaccharide (LPS), CD38-deficient mice developed a severe infection (200). The severity of the infection was explained by the increase in the proinflammatory mediators observed in CD38−/− mice, including IL-1β (interleukin 1 beta), IL-6 (interleukin 6), IFN-γ (interferon-gamma), and TNF-α (tumour necrosis factor) (200). The study also found that genetic deletion of CD38 in LPS-induced sepsis murine model contributed to severe kidney damage, which was elucidated due to upregulation in TLR4 via IFN-γ production (200). Many studies recognise CD38 as a vital immunomodulator in autoimmune diseases biology; it plays a dual role in autoimmunity, involving systemic lupus erythematosus (SLE) development (201). The beneficial line of evidence of CD38 in SLE demonstrated that CD38-deficient female mice exhibited pathological effects, manifested by upregulation in proteinuria levels, kidney damage, and other signs and symptoms of SLE; this effect was studied at the molecular level and linked to the lack of CD38-accelerated differentiation of T helper 2 cells and activation of autoreactive B cells (201), suggesting the protective role of CD38 against SLE development compared to WT mice through its possible regulatory effects on dendritic cells and thymic T cells' differentiation and activation. Most published data highlighting and confirming the pathological role of CD38 in SLE biology was reviewed by Piedra-Quintero et al. (2020) (202). It has recently been reported that CD38 expression in the non-classical monocytes was found to be significantly upregulated in patients with systemic lupus erythematosus (203), indicating the potential application of CD38 as a biomarker in SLE diagnosis, which warrants validation in a large prospective cohort of a diverse population. Despite

significant advances in using immune checkpoint inhibitors to treat patients with cancer in clinical settings, some patients show low successful response rates due to acquired resistance to immune checkpoint therapy; hence, underpinning the molecular mechanism underlying the development of immune checkpoint inhibitors' resistance biomarkers, by identifying the therapeutic target to optimise immunotherapy and overcome the resistance, is required. Recently, the novel role of CD38 was deciphered in mediating the resistance to PD-1/PD-L1 therapies. It was shown that inhibition of PD-1/PD-L1 via genetic/pharmacological modulations in murine models of lung cancer and melanoma resulted in increased expression of CD38 induced by all-trans retinoic acid and interferon β. Hence, CD38 acts as a regulator of resistance to anti-PD-1/anti-PD-L1 antibody treatment through a process involving attenuation of CD8+ T-cell infiltration via the activation of adenosine receptor signalling (204). Recently, an association has been reported between CD38 overexpression and poor survival, revealing the potential application of CD38 as a prognostic biomarker in patients with lung cancer (205). Inhibition of CD38/cADPR/TRPM2/intracellular Ca^{2+} signalling, either pharmacologically or by genetic knockout in LLC and A549 cells (*in vitro* and *in vivo* models), showed a significant reduction in lung cancer proliferation, tumour growth, progression, and metastasis by decreasing nuclear factor erythroid 2-related factor 2, which increases Kelch-like ECH-associated protein 1 (205). Figure 28 demonstrates that CD38 was implicated in several processes attributed to cancer pathology, particularly angiogenesis and metastasis, which are two hallmarks of cancer. The internalisation of CD38 II via endocytosis from the extracellular into the endolysosomal compartment is integral for exerting its action (generating NAADP-mediated intracellular Ca^{2+} release) (206). In my study, I observed a significant reduction in CD38 expression after knocking out TPC2 in murine and human cells. I speculate that CD38, TPC2, and cancer are closely linked. Therefore, investigating the biological significance of CD38/TPC2/Ca^{2+} signalling may serve as a viable therapeutic target for patients with cancer.

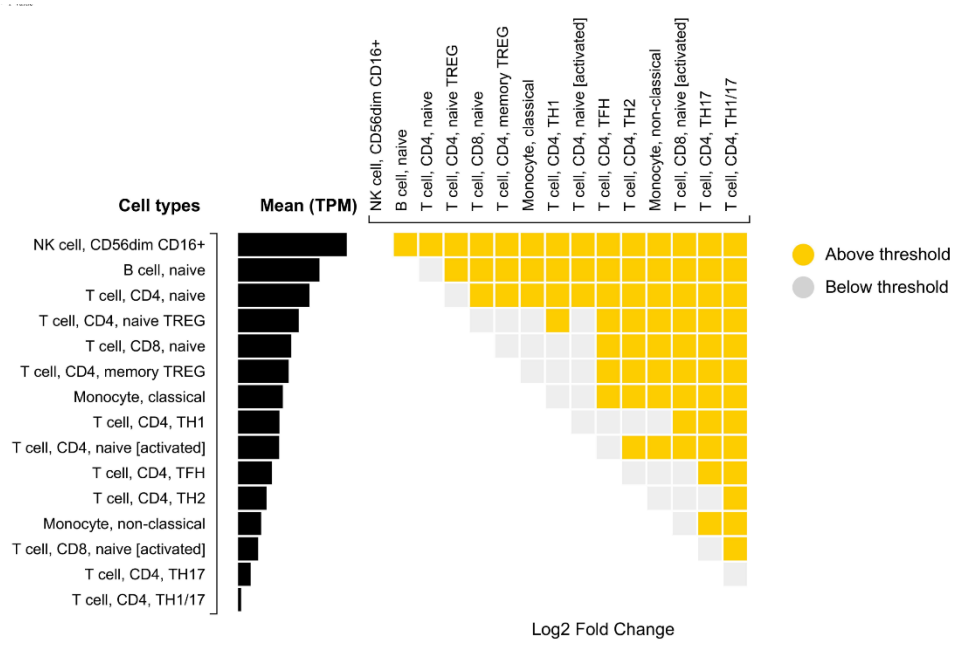


Figure 27. CD38 expression across immune cells (207).TPM; Transcripts Per Million.

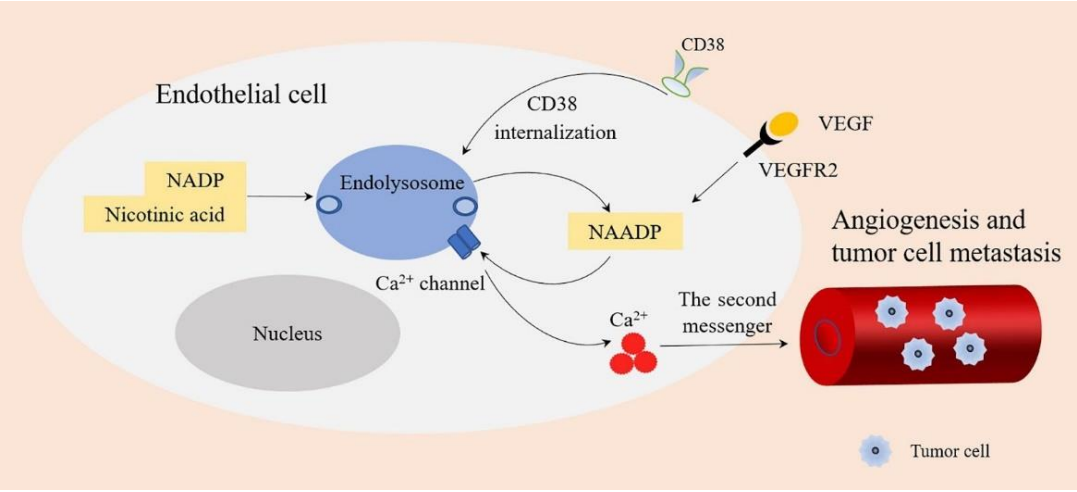


Figure 28. CD38 mediated intracellular Ca^{+2} signalling in cancer biology (206) (the figure was reproduced from Li et al. under Frontiers in Oncology copyright).

Overexpression of CD38 in cervical cancer induces PI3K/AKT pathway activation (208). PI3K/AKT signalling plays an integral role in inflammation, immunity and cancer pathogenesis (209,210,211,212,213). A recent review by Vergadi et al. (2017) discussed and summarised the biological significance of PI3K/AKT in macrophage biology (as shown in Figure 29) (214). Recent evidence has shown that S100A9 mediates the immune suppression effects in a process involving the activation of the PI3K/AKT signalling pathway in testicular macrophages (215). The calpain family consists of approximately 15 non-lysosomal intracellular Ca^{2+}-dependent cysteine proteases that play roles in health and disease (216). μ-calpain and m-calpain are the main isoforms of the calpain family (216) and have been extensively studied. The major difference between μ-calpain and m-calpain is the concentration of Ca^{2+} required for activation (μ-calpain at 20–60 μM Ca^{2+} and m-calpain at 0.3–1.3 μM) (216,217). An evolving body of evidence suggests targeting calpain as a therapeutic approach to treating inflammation and cancer (218,219). The pharmacological inhibition of calpain mediates monocyte migration in a process involving the activation of PI3K/AKT and MAPK signalling pathways (220). Recently, our lab has found that TPC2 modulates adipocyte differentiation via calpain by Zhang et al. (2021) (unpublished data) (221). Our lab showed gradual upregulation in calpain activity up to 8 hours after inducing the differentiation of WT and TPC2 KO murine preadipocytes, and observed a transient elevation of calpain activity at 24 hours only in WT, indicating that TPC2 modulates the differentiation in this system through a mechanism that requires calpain activation (221). The crosstalk between TPC2, calpain and monocyte differentiation has not yet been explored. In this thesis, I estimated the calpain activity in U937/THP-1 monocytes/macrophages and TPC2 KD U937 cells. The paradoxical roles of PKC-alpha signalling (as summarised in Figure 30) were recently reviewed by Black and Jennifer (2021), raising the intriguing question of whether PKC-alpha has a pathological or beneficial role in the context of cancer (222). This study shed light on the role of TPC2 in CD38, PI3K/AKT and PKC-alpha expression, drawing attention to the potential clinical

utility of TPC2 as an indirect regulator of these pathways to treat patients, especially with cancer, as an immunotherapeutic approach. Further research in this field could expand our knowledge of TPC2's action at the molecular level and reveal the complex interplay between TPC2 and these pathways in the context of cancer.

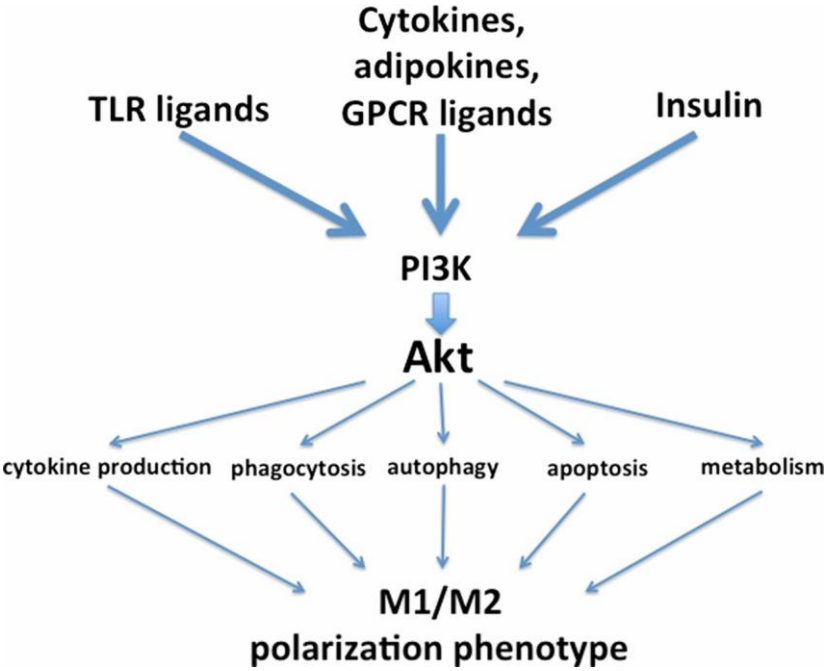


Figure 29. PI3K/AKT in macrophage biology (214) (the figure was reproduced from Vergadi et al. under the Journal of Immunology copyright).

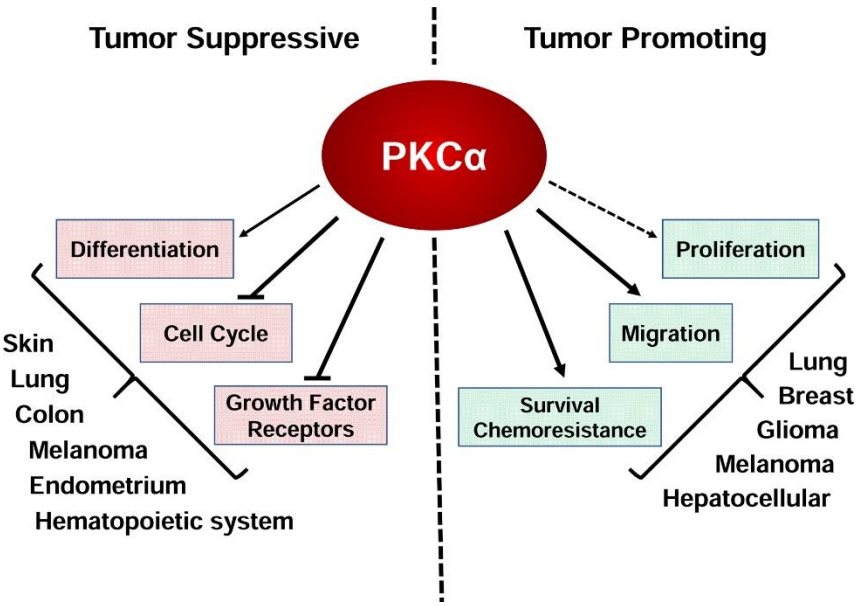


Figure 30. Controversy of PKC-alpha role in cancer (222) (the figure was reproduced from Black et al., under the license number 5405440187510).

3.1.4. Biological characterisation of human IL-8

Interleukin-8 (IL-8) is the first discovered proinflammatory chemokine that exhibits potent angiogenic properties and is produced by monocytes, macrophages, and other immune cells. It plays a vital role in neutrophil chemotaxis, activation, and recruitment to the site of inflammation (223). Murine IL-8 homologues include keratinocyte-derived chemokine (CXCL1/KC; a protein of 72 amino acids) and macrophage inflammatory protein-2 (CXCL2/MIP-2; a protein of 73 amino acids). In the history of chemokines/cytokines, IL-8 was first recognised as a monocyte-derived neutrophil chemotactic factor (MDNCF) by Teizo Yoshimura and Kouji Matsushima, who purified it from LPS-stimulated media of human PBMCs, consequently cloning the cDNA (224). As a result, they demonstrated that MDNCF protein is formed of 99 amino acid residues (224).

Serine proteases, including plasmin and thrombin, are responsible for the proteolytic cleavage of the IL-8 isoform, consisting of 99 peptides, into the IL-8 isoform of 72 amino acids (the major form is 10 times more active than the 77 amino acid isoform secreted by monocytes and macrophages)

and the IL-8 isoform of 77 amino acids (secreted by nonimmune cells) (225). A previous study found that thrombin was mainly responsible for the proteolytic conversion of the IL-8 77 amino acid isoform into the IL-8 72 isoform (225). Figure 30 illustrates IL-8 expression modulation via interleukin-1 (IL-1) or tumour necrosis factor alpha (TNF-α). Its expression is synergistically activated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in conjunction with activating protein-1 (AP-1) or CCAAT-enhancer-binding proteins (C/EBP) (224). When IL-8 binds to its two receptors, CXCR1 and CXCR2, it activates downstream targets that are involved in several physiological processes (as shown in Figure 32).

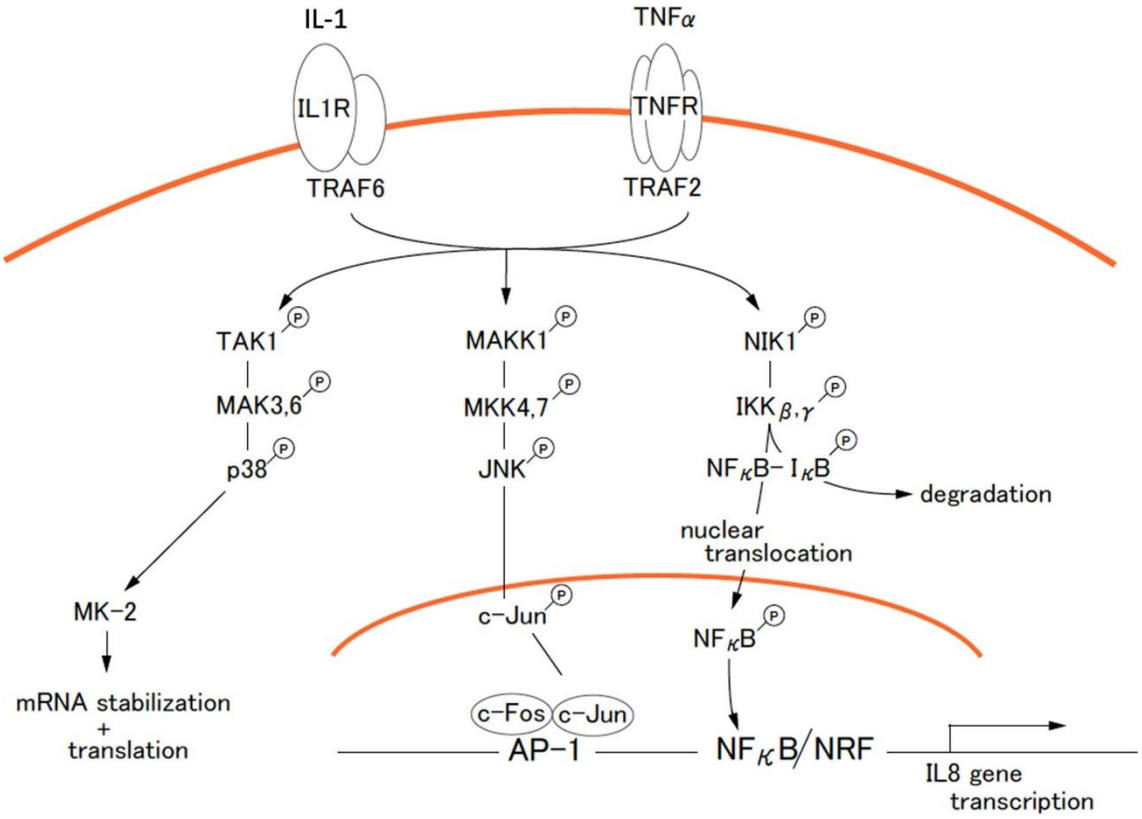


Figure 31. IL-8 expression modulated by IL-1 or TNF-α (224) (the figure was reproduced from Matsushima et al., under the license number 5405440480903).

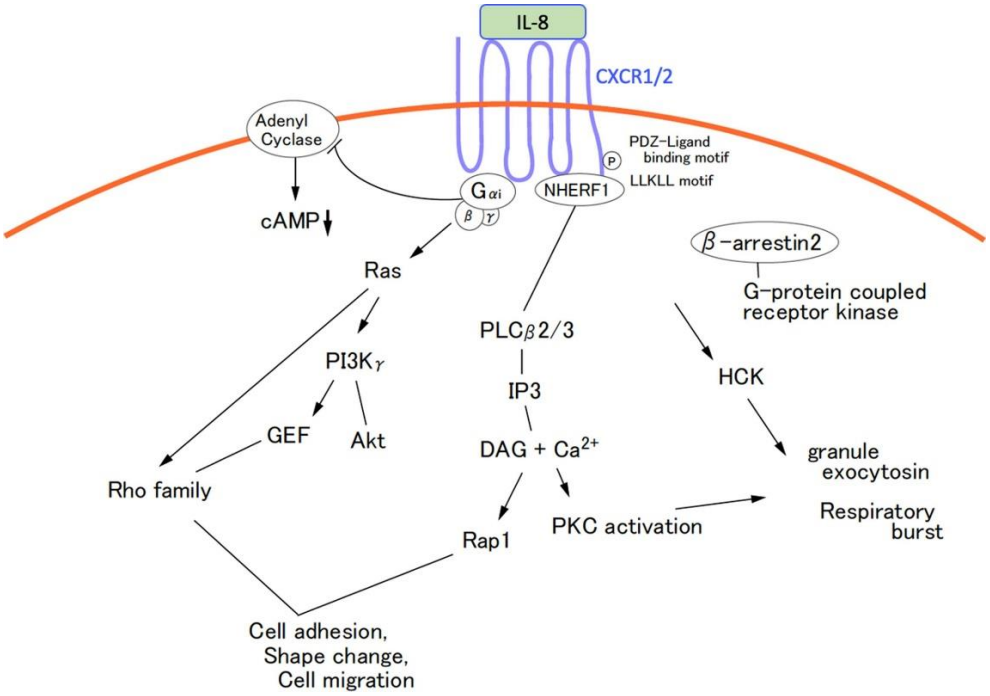


Figure 32. Schematic representation of IL-8 action in physiological processes (224) (the figure was reproduced from Matsushima et al., under the license number 5405440480903).

IL-8 acts a player of the inflammation and wound healing. It is implicated in several key pathophysiological processes attributed to cancer biology, involving angiogenesis, cancer cell survival, proliferation, progression, migration, and chemotherapeutic resistance (224). The measurement of circulating IL-8 in the serum of cancer patients is a useful biomarker to predict prognosis, tumour burden, and response to immunotherapy (226). This raises a translational question regarding the possibility of targeting IL-8 as an immunotherapeutic approach to cancer treatment. In 1998, it was reported that thapsigargin-mediated intracellular Ca^{2+} signalling–induced IL-8 expression at both mRNA and protein levels in a process that involved calcineurin activation in human peripheral blood neutrophils (227). Similarly, Mitsuyama et al. (2004) found that Ca^{2+} signalling via the calcineurin pathway mediated IL-8 gene expression through the activation of NF-κB inhuman osteoblast-like cells (228). In this chapter, the role of intracellular Ca^{2+} signalling via TPC2 in IL-8 expression and production, mainly in U937 monocytes, is

highlighted, and scientific speculations of the underlying molecular mechanisms of TPC2 modulates IL-8 and potential therapeutic applications are presented.

3.1.5. Biological sketch of interleukin-10

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that exhibits immunosuppressive effects. It is produced by monocytes, stimulated macrophages, regulatory T-cells, helper T-cells and other immune cells (229). IL-10 was discovered by Fiorentino et al. (1989); consisting of 160 amino acids, it is known as a secreted cytokine synthesis inhibitory factor (CSIF) from murine T-helper-1 cells (230). There are several molecular mechanisms underlying IL-10 production in different immune cells that are initiated via stimulation with various stimulators; however, these mechanisms have not yet been fully delineated. Figure 33a demonstrates LPS (lipopolysaccharide)-induced IL-10 production through the MyD88(Myeloid differentiation primary response 88)-mediated MEK/ERK/RSK and p38 signalling pathways (230). What is known about the IL-10 receptor (IL-10R) and signalling pathway was revised and summarised by Rutz and Ouyang in 2016 (231). As shown in Figure 33b, it is involved in the activation of the signal transducer and activator of transcription 3 (STAT3).

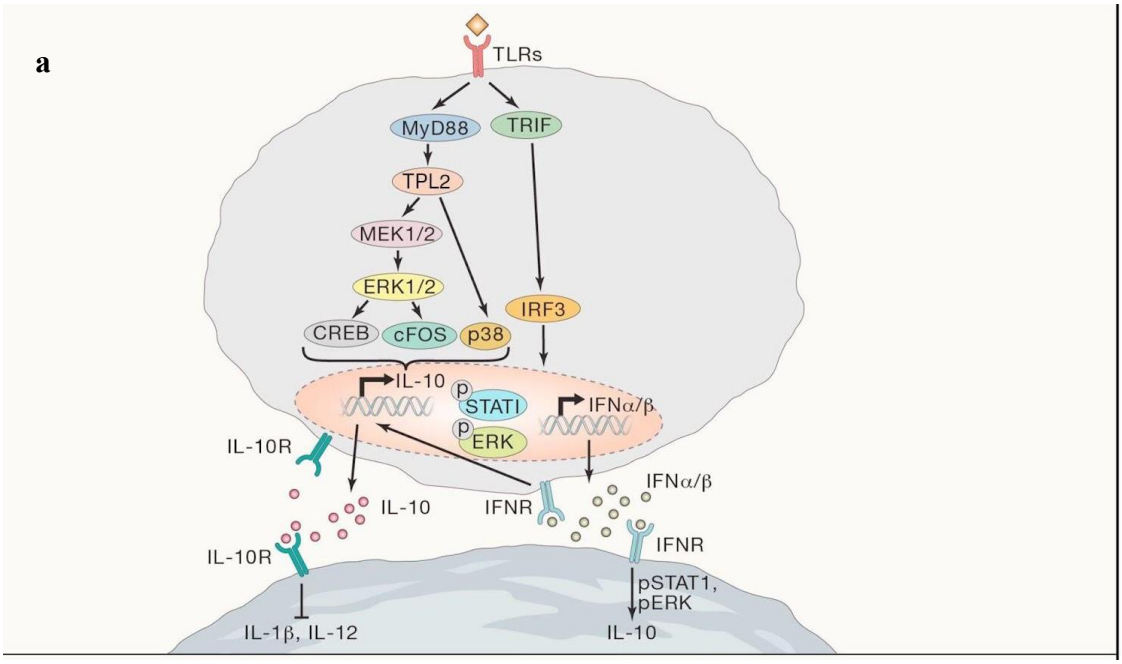


Figure 33a. IL-10 production via TLR signalling (232) (the figure was reproduced from Ouyang et al., under the license number 5405440788570).

The pleiotropic role of IL-10 in immunity and cancer has encouraged scientists to develop pegylated IL-10 to treat pathological conditions attributed to immunity and cancer. IL-10 hampers the development of infections and autoimmune diseases (233). Indeed, data from several sources have shown that IL-10 plays a dual role in cancer. Yet, despite the substantial evidence demonstrating the anti-tumour effects of IL-10, a much-debated question is whether IL-10 constrains or promotes tumours. IL-10 has been detected in the tumour microenvironment (tumour-associated macrophages) and tumour cells (234,235,236, 237,238), with elevated IL-10 expression levels found to be associated with poor prognosis in various types of cancer (232,238,239). Still, the underlying molecular mechanisms of IL-10 as a driver of tumorigenesis require further investigation.

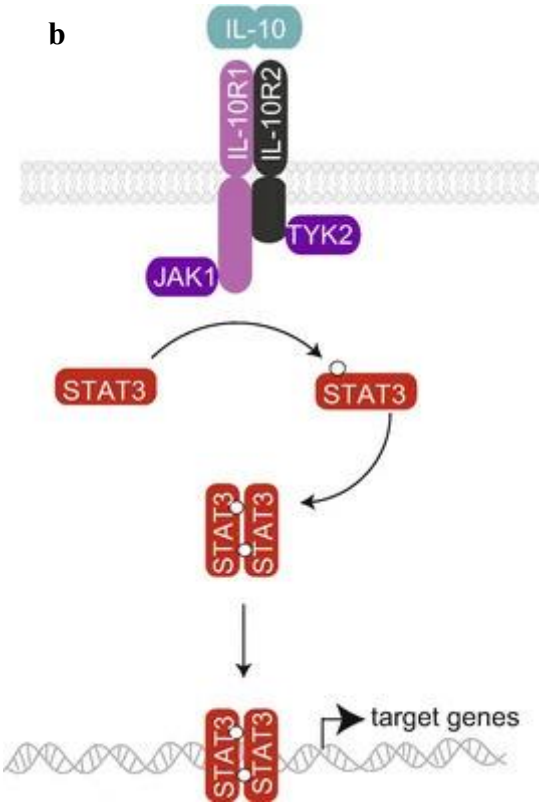


Figure 33b. IL-10 signalling via TYK2/JACK1/STAT3 activation (232) (the figure was reproduced from Rutz and Ouyang, under the license number 5407150554524).

Major histocompatibility complex (MHC) class I and II expression (232, 236, 237) mediates suppression of IFN-γ-production-induced cytokine release, particularly IL-12, as well as downregulation of the activation of CD4+ T-cells (antitumour activity). However, researchers have suggested that IL-10 contributes to tumour promotion through the attenuation of this mechanism, ultimately allowing tumours to evade immune responses. Nevertheless, in an *in vivo* model of skin cancer, IL-10-deficient mice showed a significant increase in tumour development and reduction in immune surveillance compared with WT mice (240). In the same vein, tumour cell overexpression of IL-10 has been shown to hinder tumourigenesis and metastasis (241). Notably, IL-10 exerts anti-tumour activity via the activation and proliferation of CD8+ T cells (cytotoxic function) or the inhibition of proinflammatory cytokine production, including the production of IL-6 and IL-23, which are known to facilitate tumour growth by inducing chronic inflammation, as

shown in Figure 34 (232,237).

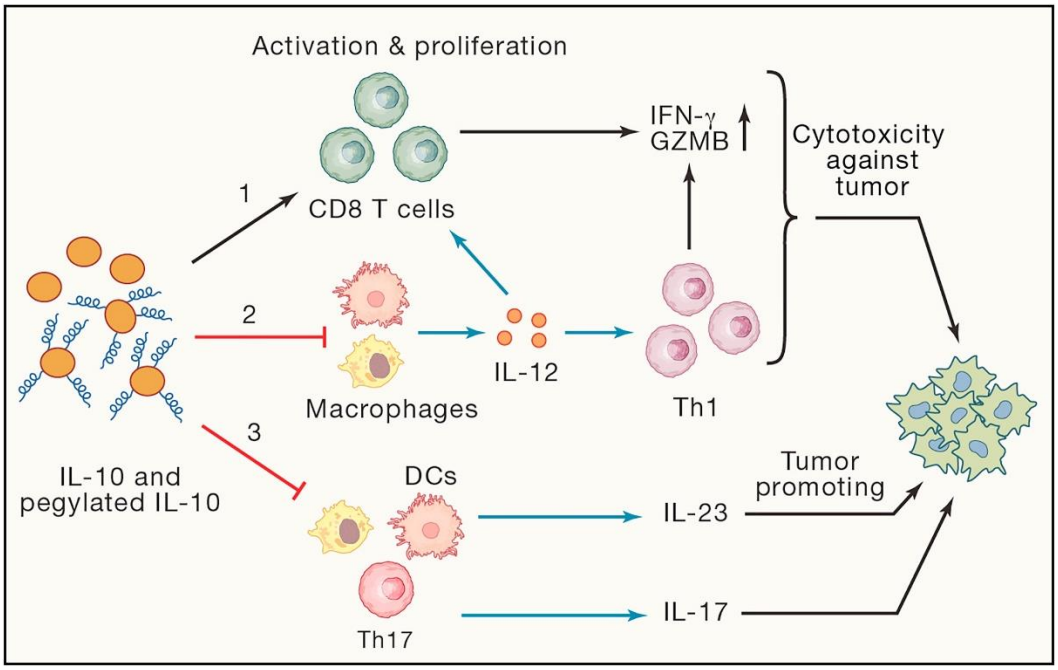


Figure 34. Multifaceted roles of IL-10 in cancer biology (233) (the figure was reproduced from Ouyang et al., under the license number 5405440788570).

Overall, the findings underline the multifaced and distinctive roles of IL-10 in cancer immunity and warrant further investigation to fully characterize the paradoxical functional role of IL-10 in cancer. Despite the growing evidence linking IL-10 signalling to tumourigenesis, recent clinical data on PEGylated IL-10 (Pegilodecakin) show that it functions as an immunomodulator to restore anti-tumour immunity and impair tumour-associated inflammation (242). This indicates its candidacy as a valuable therapeutic agent that warrants further clinical investigation in combination with immunotherapy to treat cancer patients.

Recently, the emerging roles of the Ca^{2+} signalling pathway as, a regulator of IL-10 production induced by zymosan in human primary macrophages (243) or by HIV-1 gp120 in human monocytes (244), have raised intriguing questions regarding the possibility of intracellular Ca^{2+} signalling involvement in IL-10 production. Here, for the first time in the literature, I characterise

the IL-10 expression/production in TPC2-deficient human and murine cells to underpin the potential modulation of IL-10 production via the TPC2/Ca^{2+} signalling pathway.

3.1.6. The immuno-oncological implications of utilising or antagonising CD38, PI3K/AKT, PKCα, IL-8 and IL-10

Cancer is one of the most common causes of death worldwide. In the UK, every four minutes, someone dies of cancer (Cancer Research UK) (245). Surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy and stem cell transplantation are the current approaches to treat cancer patients in clinical settings. However, despite the significant advances in cancer therapy, some patients still have low response rates to these treatments or experience moderate to severe side effects. A recent analysis found that, in non-smoking patients with conditions conducive to high first-line cure rates, peak attributable treatment failure ranges from 50 to 65% (246). This finding necessitates the identification of novel tumour hallmarks and the development of innovative cancer biomarkers to optimise detection, diagnosis, prognosis and therapy. CD38, PI3K/AKT, PKC-alpha and IL-8 pathways are emerging as key players in tumour initiation, proliferation, metastasis and processes related to immune cell function, contrasted with IL-10 signalling, which exerts mainly antitumor effects. There are several monoclonal antibodies that target CD38, including daratumumab, isatuximab (first generation), mezagitamab (TAK079), SAR442085 and felzartamab (second generation) (247, 248). Daratumumab was first approved by the FDA in November 2015 as a monotherapy to treat patients with relapsed/refractory multiple myeloma (RRMM). More recently, in March 2020, isatuximab (in combination with pomalidomide and dexamethasone) was approved by the FDA to treat RRMM. Other anti-CD38 drugs are currently under investigation in phase 1 clinical trials (including NCT04776018, NCT04000282 and NCT01421186), mainly in RRMM (249). The PI3K inhibitors approved by the FDA are copanlisib and duvelisib to treat lymphoma (250). There are several PI3K inhibitors, including AZD8186, taselisib and SAR26031, still under clinical investigation. The modulators of PI3K under

clinical investigation as well as the approved PI3K inhibitors are proposed to be given as a combination therapy, as the majority of these molecules did not show promising data as a mono-therapeutic agent. Midostaurin is a recent (in 2017) FDA-approved PKC-alpha inhibitor to treat patients with acute myeloid leukaemia with FLT3 mutation-positive (250). UCN-01 and enzastaurin are PKC-alpha inhibitors that were clinically investigated but did not pass the drug development pipeline or were not approved in the clinic as anticancer therapeutics due to no observed significant clinical benefits (251).

In a phase 1 clinical trial (NCT02536469), HuMax-IL-8 (BMS-986253) was found to be well-tolerated in patients with metastatic or unresectable solid tumours, with an encouraging clinical response (73% of patients with a stable disease) (252). Currently, it is under investigation in combination with Nivolumab (NCT04848116) in a phase 2 clinical trial in patients with head and neck squamous cell carcinoma (253).

The tolerability, safety, pharmacokinetics and efficacy of PEGylated IL-10 are being investigated in several clinical trials (including NCT02009449, NCT02923921 and NCT03381547). PEGylated IL-10, which works by activating and proliferating CD8+ T cells (254), is a promising novel therapeutic approach to optimise cancer therapy, especially when it is given as a combination therapy with PD-1/PDL-1 inhibitors. This strategy was examined in a phase 1b clinical trial (NCT02009449) on patients with renal cell carcinoma and non-small-cell carcinoma, in which it was administered in combination with pembrolizumab or nivolumab (250). It was found to be safe and well-tolerated, with promising antitumor effects, warranting further investigation in phase 2 trials (250).

The data from this chapter provides preliminary evidence that targeting TPC2 modulates several cancer hallmarks. This evidence necessitates the development of specific drug candidates that attenuate TPC2's function in cancer.

3.1.7. Significance, innovation, overall hypothesis and objectives

The presence and the role of TPC2 in the innate immune system remain unknown; there is a lack of molecular evidence that specifically characterises the role of TPC2 in monocyte differentiation, macrophage development, and IL-8 and IL-10 production, as well as its influence on monocyte chemotaxis via genetic/pharmacological modulation of the TPC2 function. My central hypothesis is that TPC2 plays a pivotal role in the specific mechanisms of classical monocyte differentiation that lead to macrophage-mediated angiogenesis, tumourigenesis and metastasis, and that it is a candidate target for cancer immunotherapy. My objectives are to determine whether TPC2 signalling is specifically involved in monocyte differentiation, macrophage development and cytokine production and underpin the therapeutic potential of modulating TPC2 function for immunotherapy. My research question is: Does TPC2 act as a novel regulator of monocyte differentiation and the release of cytokine, and how is this relevant to developing potential therapies to treat patients with cancer/immune diseases in the foreseeable future? To address this question, I utilised the TPC2-deficient U937 human monocyte model that I established (which was discussed in detail in Chapter 2) and TPC2-deficient murine splenocytes (the spleen is an important source of monocytes and macrophages). This chapter highlights a previously unrecognised role for TPC2 in monocyte differentiation and IL-8 release, suggesting the candidacy of TPC2 as a viable target for cancer immunotherapy. It provides the first preclinical proof of concept for the candidacy of TPC2 as a potential target to block the development of macrophages and to modulate CD38/IL-8 levels as therapeutic strategies for cancer patients.

3.2. Methods

3.2.1. U937 and THP-1 cell culture

Human Monocytic U937 cells (ATCC® CRL1593.2™), purchased from the American Type Culture Collection (Manassas, VA, USA), and U937 TPCN2 knockdown (KD) were generated in collaboration with Synthego (CA, USA). Cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (12106C, H-I-FBS; Sigma-Aldrich) and 1% Penicillin-Streptomycin (P4333, Pen-Strep; Sigma-Aldrich). The cells were incubated at 37°C in 95% humidified air and 5% CO_2 and were maintained at a cell density between 2 x 10^5 and 2 x 10^6 viable cells/ml, with the medium changed every 2–3 days. The development of TPC2 deficient human monocytes is described in detail in Chapter 2.

3.2.2 Mice and isolation of murine splenocytes

Wild-type and TPC2 KO mice (6–8 weeks old) were used. TPC2 KO mice derived from TPCN2Gt(YHD437) Byg, an embryonic stem cell line harbouring a gene trap mutation in the TPCN2 gene, were generated as described in Calcraft et al. (2009) (126). Mice were used as per the guidelines of the UK Animals (Scientific Procedures) 1986 Act, conducted under PPL no. P6058EA41 and performed by PIL no. I73812540 holder. Animals were kept on a 12-hour light and 12-hour dark cycle at 19–23°C and 45–65% relative humidity in the Biomedical Science Building at the University of Oxford. After termination of mice by cervical dislocation, whole spleens were dissected into single-cell suspensions of splenocytes and then washed with cold PBS. The subsequent procedure is delineated below and was performed on ice.

The spleen was placed into a standard 90 mm petri dish that contained at least 10 ml of the completed media, as it is essential that the spleen be covered with the media during isolation. The spleen was cut into small parts using forceps then gently mashed by the plunger end of a

syringe. It is important that this is done carefully to prevent damage to the cells that leads to DNA release and poor cell viability. Next, the spleen cells were transferred through a cell strainer into a 50 ml tube, and the petri dish was washed several times with 20–30 ml of completed media. The cell strainer was rinsed with completed media and discarded. The spleen cells were centrifuged at 800 x g for 3 min (preferably at 4ºC). The supernatant was discarded, and the pellet was washed three times with cold PBS and resuspended with the desired media. Splenocytes were counted using trypan blue by Countess 3 Automated Cell Counter. For subsequent experiments, cells were centrifuged at 500 or 600 x g for 5 min at approximately 4ºC.

3.2.3. Differentiation into non-polarized (Mϕ) U937/THP-1 macrophages

U937 and THP-1 human cells at 5 × 10^5 cells/ml were differentiated into macrophages after 48 h of incubation with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; P8139, Sigma-Aldrich). On day 3, cells were washed at least twice with complete medium, and fresh media was added. On day 4, macrophages were harvested using trypsinisation and washed with PBS.

3.2.4. DNA and RNA extractions, purifications and quantifications

Genomic DNA and RNA were extracted using a DNeasy Blood and Tissue Kit and an RNeasy Mini Kit (QIAGEN, CA, USA), respectively, according to the manufacturer's protocols. A Nanodrop was utilised to check the purity of the DNA and RNA (A260/280), with a ratio of 1.8 for DNA and 2 for RNA being acceptable limits, and to quantify the concentrations. The procedures are described in detail in Chapter 2.

3.2.5. Genotyping via PCR and sanger sequencing

PCR genotyping was conducted utilising an oligonucleotide primer set for TPCN2 (FP; AAAGGAGACGGCCTGTGARTG, RP; CACCTTCACAGAGAGGTCGG) purchased from Life

Technologies Ltd., to amplify TPCN2 genomic DNA using a KAPA2G Robust HotStart ReadyMix PCR Kit (Kapa Biosystems, USA) following the manufacturer's protocol. The procedures are delineated in Chapter 2.

3.2.6. RT-PCR

RT-PCR was performed utilising a OneStep RT-PCR kit (QIAGEN, CA, USA) as per the manufacturer's recommendations. A RT-PCR reaction of 50 µl volume was calculated and prepared on ice as shown in the table below.

Material	Required volume	Final concentration
5X QIAGEN OneStep RT-PCR Buffer	10 µl	1X
dNTP mix (10 mM each)	2 µl	
10 µM Forward Primer	3 µl	0.6 µM
10 µM Reverse Primer	3 µl	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	2 µl	
RNA template	Variable (300 ng)	NA
RNase-free water	Up to 50 µl	NA

The desired volumes of the prepared RT-PCR mixture containing the reagents and the pair of primers were transferred into individual PCR tubes. An RNA template was added (300 ng) to each tube, and the tube was sealed, mixed and briefly centrifuged. The RT-PCR reactions were performed using an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, USA) using the following thermal cycling programme.

Phase	Temperature	Duration	Cycles
Reverse transcription	50ºC	30 min	1
Initial PCR activation step	95ºC	15 min	1
Denaturation	94ºC	30-60 sec	36
Annealing	58–62ºC	30-60 sec	
Extension	72ºC	60 sec	
Final extension	72ºC	10 min	1
Stored overnight at 4°C and for the long term at -20°C			

3.2.7. qRT-PCR

Reverse transcription (RT) utilising SuperScript™ III Reverse Transcriptase (Invitrogen™) was used to synthesise cDNA, and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used for qPCR. The primers for detecting mRNAs corresponding to TPC2,m-TOR, PI3K, AKT, CD11b, IL-8, KC, TLR4, MAPK-14, CD38, CD11b, CD14, CD68, PKC-alpha, μ-Calpain, m-Calpain, Cdc42, Rac1, RhoA, and GAPDH, which are all listed in Table 7, were purchased from Life Technologies Ltd. Values were normalized according to the relative amounts of the housekeeping genes β-actin and GAPDH, which were used as endogenous controls. The procedures are described in detail in Chapter 2.

Table 7. Primers for RT-PCR and RT-qPCR

	FP	RP
Human		
TPCN2	GTACCCCTCTTGTGTGGACG	GGCCCTGACAGTGACAACTT
PKC-alpha	CAAGCAATGCGTCATCAATGT	ATCCGCCCCCTCTTCTCA
PI3K	CTCTCCTGTGCTGGCTACTGT	GCTCTCGGTTGATTCCAAACT
CD38	TGCTGATGACCTCACATGGT	CCATTGAGCATCACATGGAC
Akt	GGACAACCGCCATCCAGACT	GCCAGGGACACCTCCATCTC
TLR4	GCAGTTTCTGAGCAGTCGTG	CTGTCCTCCCACTCCAGGTA
IL-8	AAGAGAGCTCTGTCTGGACC	GATATTCTCTTGGCCCTTGG
m-TOR	AGGCCGCATTGTCTCTATCAA	CAGTAAATGCAGGTAGTCATCCA
CD11b	GGCATCCGCAAAGTGGTA	GGATCTTAAAGGCATTCTTTCG
CD14	ACGCCAGAACCTTGTGAGC	CATGGATCTCCACCTCTACTG
μ-Calpain	GAAGGCAACGAGTTCTGGAG	AAAGTGATGGCCTCCATGTC
m-Calpain	AGGCATACGCCAAGATCAAC	CACCAGCTTCTGAAACGTGA
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
β-actin	CCAGAGGCGTACAGGGATAG	CCAACCGCGAGAAGATGA
Cdc42	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Rac1	CTACCACATCCAAGGAAGCA	TTTTCGTCACTACCTCCCCG

RhoA	AGGAAGATTATGATCGCCTG	TTCTAAACTATCAGGGCTGTC
MAPK-14	CCCGAGCGTTACCAGAACC	TCGCATGAATGATGGACTGAAAT
Murine		
KC/CXCL1	GCTTGAAGGTGTTGCCCTCAG	AAGCCTCGCGACCATTCTTG
GAPDH	GAG CCA AAC GGG TCA TCA	CAT ATT TCT CGT GGT TCA CAC C
CD38	CTGCTGGAGAGGTTATTCCTCG	GGAAGAGTGAGCGGCGCATCAAGG
CD11b	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
MAPK-14	GGGACACCCCCTGCTTATCT	TCCCTGCTTTCAAAGGACTGG

3.2.8. ELISA assay and determination of IL-8 (KC and MIP-2 in murine) and IL-10 levels

3.2.8.1. Sample/standard preparation

The cytokines were assessed and determined utilizing Single Analyte ELISA Kits (QIAGEN, CA, USA) or Invitrogen™ ELISA kits (Thermo Fisher Scientific, USA). Cultured cell supernatant samples were prepared according to the manufacturers' protocols by centifugation (MEGAFUGE 1, Thermo Fisher Scientific) and stored at ≤-20°C until analysed. The WT U937 and TPC2 KD U937 cells, U937 macrophages at density 5 x10^5 were cultured for 24 h, then supernatants were obtained. U937 wild-type cells 5 x10^5 in 1 ml of medium treated with anti-TPC2 pharmacological modulator (Ned 19 100 um), or ML1-SA1 (a selective agonist of TRPML at 10um). U937 cells at 5 × 10^5 cells/ml were differentiated into macrophages after 48 hours of incubation with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (P8139, Sigma-Aldrich). On day 3, cells were washed twice with complete medium, and fresh media was added. On day 4, macrophages were treated with a combination of Ned 19 (100 um) and 10 ng/ml LPS and LPS alone for 24 h. Cells were centrifuged, then supernatants were taken. Murine splenocytes from the spleens of wild-type and TPC2 KO mice and wild-type murine splenocytes at 1 x 10^6 in 1 ml were cultured for 24 h. The standards were reconstituted with appropriate diluent; then serial dilutions of the standard were

made according to the manufacturers' protocols.

3.2.8.2. Sandwich ELISA procedure in general

All reagents were prepared based on the manufacturers' protocols, and equilibrated to room temperature. 50 µl of all standards, controls, and samples were loaded into pre-coated plates with capture antibodies in duplicates. Then, 50 µl of antigen Biotin Conjugate solution was added. The plate was gently mixed, covered and incubated for apprioximatly 2 h at room temperature on a plate shaker. The solution was aspirated and washed at least 4 times with 1X Wash buffer. 100 µl of 1X Streptavidin-HRP solution was added into each well and the plate was covered and incubated for 30-60 min at room temperature on a plate shaker. The solution was aspirated from wells and washed with 1X Wash buffer at least 4 times. 100 µl of the substrate solution was added to all wells, and the plate was incubated in the dark for 30 min at room temperature. 100 µl of Stop Solution was added to each well and the plate was gently tapped. The absorbance was read at 450 nm using a microplate reader within 30 min of stopping the reactions. The data were analysed by Curve Expert 1.4.

3.2.9. *In vitro* time dependent and IL-8 simulation release assays

For time dependent IL-8 release and cell culture supernatant samples were obtained from U937 cells and U937 macrophages at 0, 12, 24, and 48 h. For the stimulation assay, WT and TPC2 KD U937 cells were treated with 10 ng/ml of LPS, and Cell Culture Supernatants were obtained at 0, 12, 24, and 48 h. The IL-8 ELISA assay was conducted using Invitrogen™ Human IL-8- ELISA Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocols. The procedure was described above.

3.2.10. Beta-hexosaminidase activity

Beta-hexosaminidase is a hydrolytic enzyme found in lysosomes to distinguish between LE/LY and EE/RE as possible secretion pathways of IL-8, the activity of this enzyme was assessed using Beta-Hexosaminidase Activity Colorimetric kit (Tribioscience, USA). The TPC2 KD U937 and WT U937 cells were seeded at 1 x 10^6 cells/ml in 6 well plates and treated with 10 ng/ml of LPS for 24 h. Then, cells were centrifuged at 2000 xg for 5 min (preferably at 4ºC). The supernatant was discarded, and the pellet was washed three times with cold PBS.Treated and non-treated cells were resuspended with 1 ml of cold PBS and centrifuged at 14,000 xg for 10 min at 4°C. Supernatants were taken to be assayed. 20 µl of each sample/standard in duplicates was transferred into a clear flat bottom 96-well plate (100 µl of stop reagent was used as the blank). 80 µl of the substrate solution was added to all wells. The plate was incubated at 37°C for 30 min. 100 µl of Stop Reagent was added to each standard/sample. The absorbance was detected at 405 nm by microplate reader (Tecan).

3.2.11. Monocyte chemotaxis assay

U937 and U937 TPC2 KD chemotaxis was assessed using the Chemotaxis Assay Kit (96-well, 5 µm (Abcam, USA). U937 and U937 TPC2 KD cells were centrifuged and resuspended in a 0.1 % serum media at 1 x 10^6 cells/ml. In the bottom chamber, 150 µl of 0.1 % serum media was added into a 96-well plate. Then, the top chamber was placed back into the bottom chamber. In the top chamber, 50 µl of cells were added, the 10 ng/ml of LPS used as a stimulator. The volume was made up to 100 µl by 0.1% serum media. The plate was covered and incubated at 37°C in the CO_2 incubator for 2 h. After that, the media was aspirated from the top chamber without puncturing the membrane and the cells were removed carefully from the top chamber using a cotton swab. The U937 cells that migrated into the bottom chamber were counted by Invitrogen EVOS FLoid cell imaging.

3.2.12. Protein extractions, purifications and quantifications

Protein was extracted using NP40 Cell Lysis Buffer (Thermo Fisher Scientific) supplemented with 1 mM PMSF and protease inhibitor cocktail. A Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) was used to determine the concentration of protein.The BCA working reagent (WR) was prepared by mixing 50 ml of BCA Reagent A with 1 ml of BCA Reagent B.25 µl of each albumin standard or unknown sample was pipetted into a 96 well plate.200 µl of the WR was added to each well and the plate was mixed on a plate shaker for 30-60 sec.The plate was incubated at 37°C for 30 min.The absorbance was detected at 562 nm by microplate reader (Tecan).

3.2.13. Western blot analysis

25 µg of total protein per sample was analysed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (90%) and transferred to Immobilon PVDF membrane. AKT and PKC-alpha primary antibodies was added to the membrane for 1 hr (1:1000 dilution). After washing the membrane, a secondary antibody was added. (1:10000 dilution) for detection by Odyssey infrared imaging system. The Western blot analysis is described in detail below:

3.2.13.1. Sample preparation

First, 20–25 ug of protein was transferred into sterile 1.5 ml tubes. Then, an appropriate amount of 5 x loading buffer was added to each tube and the volume was equalized using double-distilled H_2O. The samples were heated for 5 min at 95ºC.

3.2.13.2. Electrophoresis

The gel was placed inside the electroporator and connected to a power supply. The running buffer was poured into the electroporator (the inner and outer chambers). The samples and load markers were loaded and the gel was run for approximately one hour.

3.2.13.3. Electrotransfer

The transfer buffer (IL) was prepared; 100 ml 10X transfer buffer; 200 ml Methanol; 700 ml ddH_2O and stored at 4ºC. Two filter sheets and one polyvinylidene fluoride (PDVF) membrane were cut to have similar dimensions. The sponge and filter paper were soaked with transfer buffer. The PDVF membrane was wetted with methanol for 1 min and then in H_2O for 1 min. The transfer sandwich was generated as follows: Sponge filter paper gel PVDF filter paper sponge and placed in the transfer apparatus. The transfer buffer was poured to cover the membrane, and the transfer process was conducted on ice for 90 or 120 min. Then, the transferred proteins were checked by Ponceau S stain. After that, the membrane was washed at least three times, first using PBS and then PBS/Tween.

3.2.13.4. Blocking and incubation with primary and secondary antibodies

The membrane was blocked with a 5 ml blocking buffer for one hour at room temperature. Then, the primary antibody in the blocking buffer was added to the membrane and incubated overnight at 4ºC on a shaker. After that, the membrane was washed three times with 5 ml of TBST for 5 min on a shaker at room temperature. The secondary antibody was added to the blocking buffer, and the membrane was incubated for one hour. The membrane was washed three times with 5 ml of TBST for 5 min on a shaker at room temperature. The membrane was visualised in the dark room and stored at 4ºC in PBS.

3.2.14. Calpain activity assay

Calpain activity was assessed using Calpain Activity Assay Kit (ab65308, Abcam). 50 ug of cell lysates adjusted to the volume of 85 µL per well with extraction buffer (extraction buffer was utilised as a negative control and active calpain was used as a positive control). 10 µL of 10X reaction buffer was pipetted into a 96 well plate. 5 µL of Calpain Substrate was added to each

well. The plate was incubated at 37°C and protected from light for 60 min. The fluorescent signal was quantified using a multimode plate reader (FLUOstar Omega) at excitation and emission wavelengths of 380 nm and 520 nm, respectively. Quantitative fluorescence data was presented as relative fluorescence units (RFU).

3.2.15. Cell viability by XTT assay

WT and TPC2 KD U937 cells were treated with either 10 ng/ml of LPS and 100 um of Ned-19 to investigate the effect of these chemicals on the cell viability using the XTT assay (Abcam). I utilised a similar procedure that has been illustrated in Chapter 2.

3.2.16. Cell viability by 0.4% trypan blue

WT and TPC2 KD U937 cells, U937 macrophages were treated with 100 ng/ml to investigate the cytotoxic effects of PMA on the cells after 48 h treatment using 0.4% trypan blue. 10 µL of the cell suspension was mixed with 10 µL of 0.4% trypan blue dye. 10 µL of the mixture was pipetted into the slide chamber. Cells viability was counted by Countess™ II automated cell counter (Invitrogen).

3.2.17. Data presentation and statistical analysis

Results are presented as descriptive statistics, including proportion, mean ± SEM. Data analysis was performed utilizing R for Windows (version 3.4.1).

3.3. Results

3.3.1. Changes in CD11b expression at the RNA level after TPC2 knockout in murine splenocytes

The spleen is an important source of both monocytes and macrophages. CD11b is widely utilised as a macrophage marker. To determine the possible role of TPC2 in macrophage development, I first assessed CD11b expression at the level of RNA; for this analysis, murine splenocytes from the spleens of wild-type TPC2 and TPC2 knockout (KO) mice were isolated. I observed that CD11b mRNA expression was significantly downregulated by 62% in TPC2 KO murine spleen cells. All the splenic data should be interpreted with caution due to the heterogeneity of splenic cell populations, and phenotyping of these populations was not carried out by flow cytometry. However, the consistency of the myeloid marker CD11b and the data from 3 biological replicates indicates that there is a link between TPC2 and CD11b expression at the mRNA level in the murine spleen.

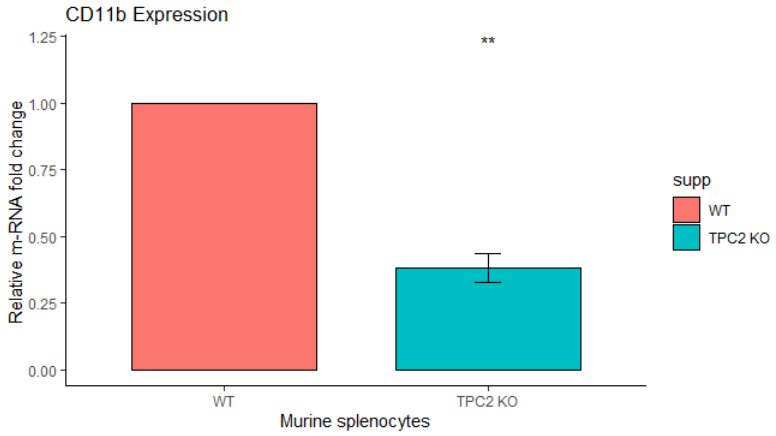


Figure 35. Real-time PCR analysis of CD11b mRNA expression, with glyceraldehyde 3-phosphate dehydrogenase used as an internal control for murine samples of three biological replicates in three independent experiments (mean ± SEM, two sample t-test, p-value = 0.007729).

3.3.2. Morphological characterisation and development of non-polarised (Mϕ) adherent U937/THP-1 macrophages

The differentiation of U937/THP-1 monocytes into non-polarised (Mϕ) adherent U937/THP-1 macrophages was induced with a phorbol 12-myristate 13-acetate (PMA) concentration of 100 ng/ml. After two days of incubation with PMA and one day of resting in complete RPMI 1640-media, the U937/THP-1 cells developed pseudopods and shifted from round-shaped U937/THP-1 suspension cells into U937 adhesion cells with a macrophage-like morphology. Figures 36 and 37 illustrate the morphological changes of the U937/THP-1 cells, which were monitored by Invitrogen EVOS FLoid cell imaging.

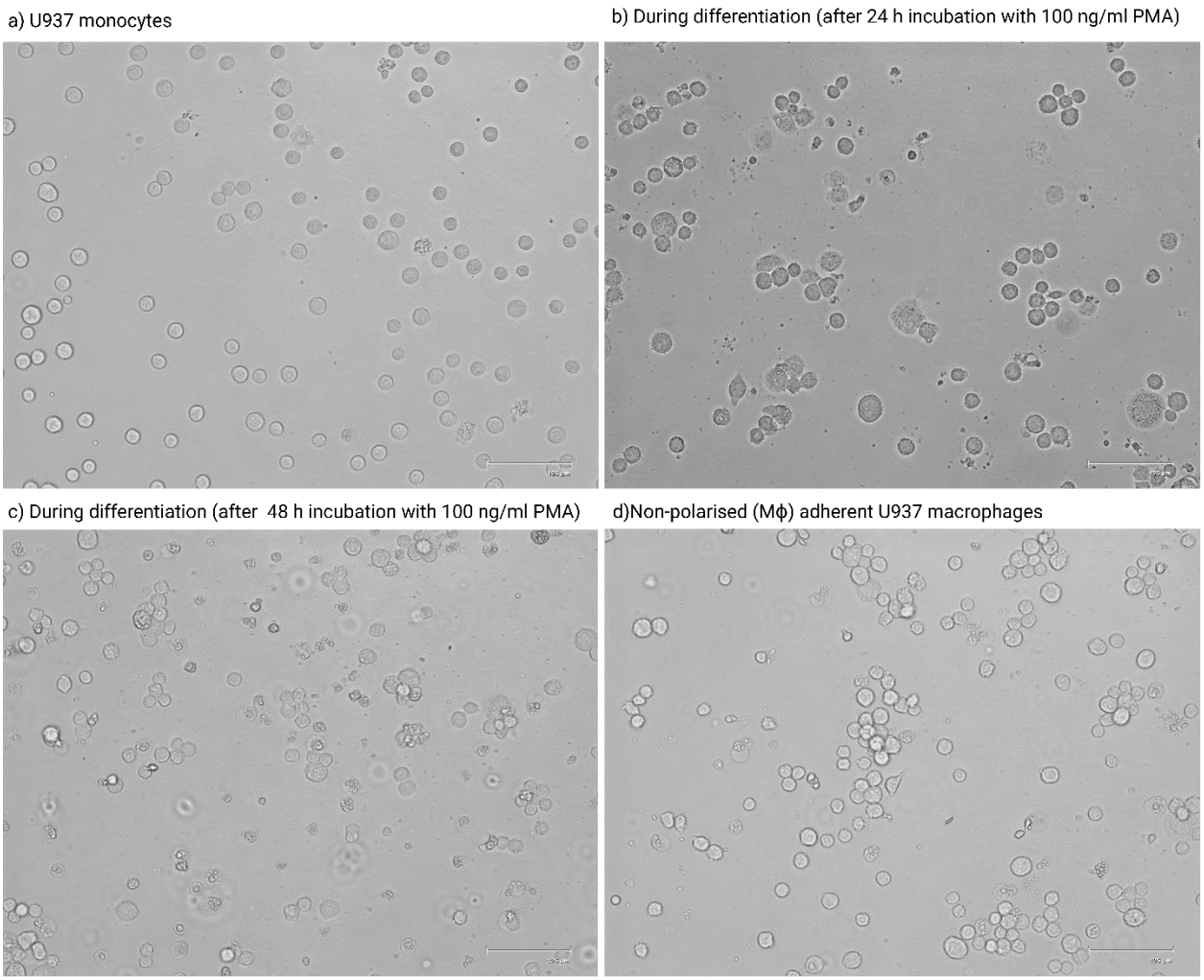


Figure 36. Morphology of U937 cells treated with 100ng/ml PMA for 48 h. Cells were then washed twice with PBS and cultured in complete RPMI 1640-media for 24 h. The U937 monocytes fully differentiated into non-polarised (Mϕ) adherent macrophages. (a) U937 cells (monocytes). (b) During differentiation (after 24 h incubation with 100 ng/ml PMA). (c) During differentiation (after 48 h incubation with 100 ng/ml PMA). (d) Macrophages (after 48 h incubation with 100 ng/ml PMA and 24 h rest).

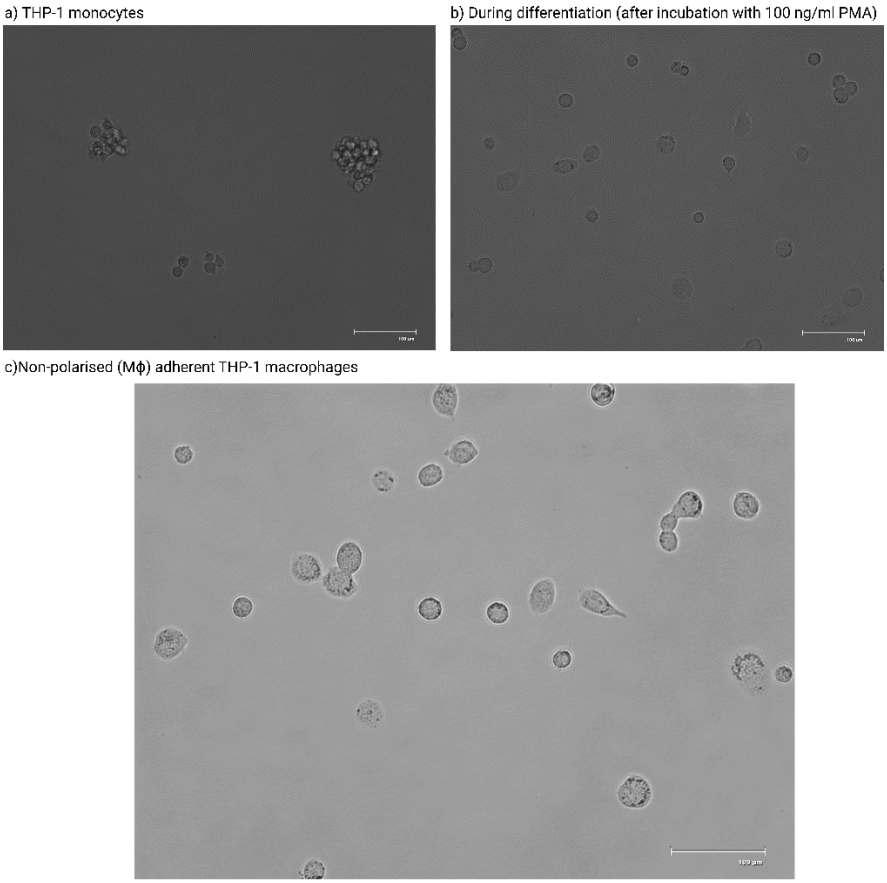


Figure 37. Morphology of THP-1 cells treated with 100ng/ml PMA for 48 h. Cells were then washed twice with PBS and cultured in complete RPMI 1640-media for 24 h. The THP-1 monocytes fully differentiated into non-polarised (Mϕ) adherent macrophages. (a) THP-1 cells (monocytes). (b) During differentiation (after incubation with 100 ng/ml PMA). (c) Macrophages (after 48 h incubation with 100 ng/ml PMA and 24 h rest).

3.3.3. RT-PCR and qPCR to confirm the development of U937 macrophages

Based on these data, we can see that TPC2 is expressed at the RNA level in U937 monocytes, U937 cells during differentiation and in U937 macrophages. In Figure 38, there is a clear trend of decreasing CD14 expression at the RNA level, confirming the differentiation of monocytes into macrophages. In addition, qPCR data shows a non significant increase in CD11b expression in macrophages compared to monocytes (*p*-value = 0.1194).

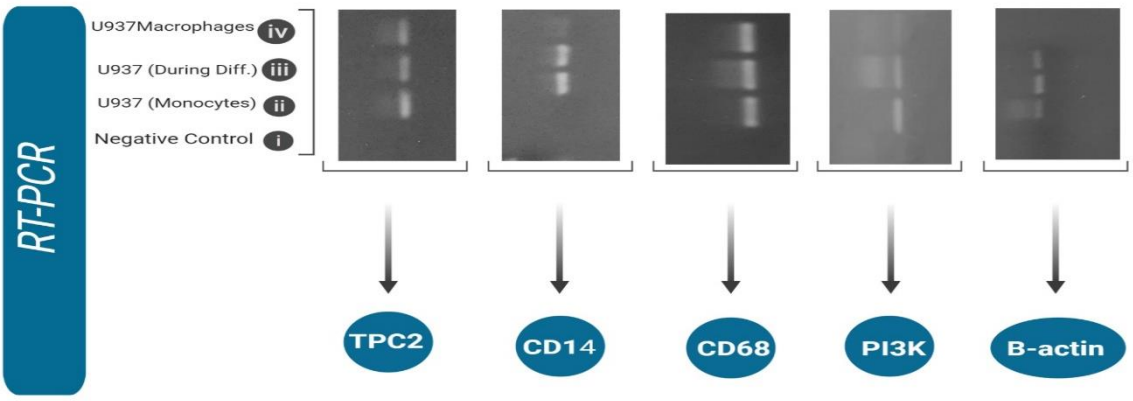


Figure 38. RT-PCR products of TPC2, CD14, CD68, PI3K andβ-actin.

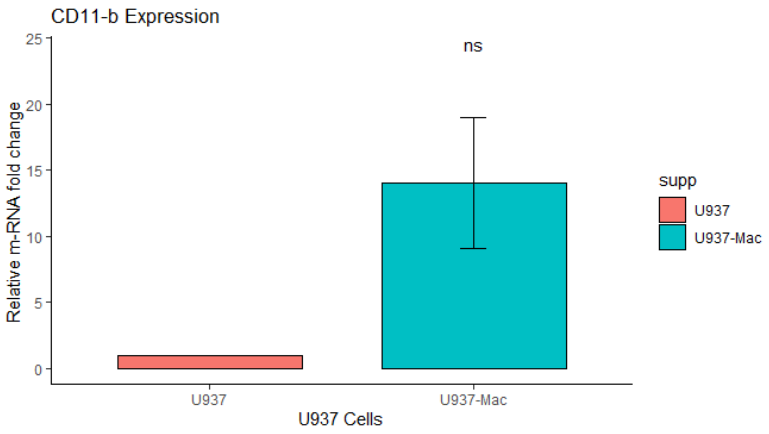


Figure 39. Real-time PCR analysis of CD11b mRNA expression, with β-actinused as an internal control for human samples of three biological replicates in three independent experiments (mean ± SEM, two sample t-test, *p*-value = 0.1194).

3.3.4. Expression of TPCN2 in macrophage development and mTORC1-TPC2 signalling regulate monocyte differentiation via PI3K pathway

To determine whether NAADP/TPC2 signalling is involved in monocyte differentiation and macrophage development, I investigated the expression pattern of TPCN2 during this process of cell differentiation and macrophage development. The expression of TPCN2 mRNA was determined by qRT-PCR. PMA induced differentiation of monocytes into macrophages via PI3K/AKT signalling. I investigated the mRNA expression of AKT, PI3K and m-TOR, Rictor, Raptor, and Deptor in U937 and U937 macrophages. I observed a significantly higher expression of TPCN2 in non-polarised (Mϕ) adherent U937/THP-1 macrophages, and this might be attributed to the increase in the number of lysosomes. The significant decrease of mTOR expression by approximately 91% might be linked to increasing TPC2 expression during the differentiation process and macrophage development because m-TORC1 negatively regulates lysosomal Ca^{2+} release via TPC2 (Figure 41). AKT, PI3K and Deptor expression levels were significantly downregulated in Mϕ U937. A non-significant difference was observed in total AKT protein levels in U937 macrophages (as shown in Figure 42). To determine the possibility of involvement of other channels that form part of the endolysosomal Ca^{2+} signalling machinery, I examined TPC1 and TRPML-1 mRNA expression, and these were also upregulated (as shown in Figure 43). Although the underlying mechanism by which endolysosomal Ca^{2+} channel expression increases during monocyte differentiation remains undetermined, my findings indicate that endolysosomal Ca^{2+} signalling, particularly via TPC2, could be a monocyte differentiation indicator.

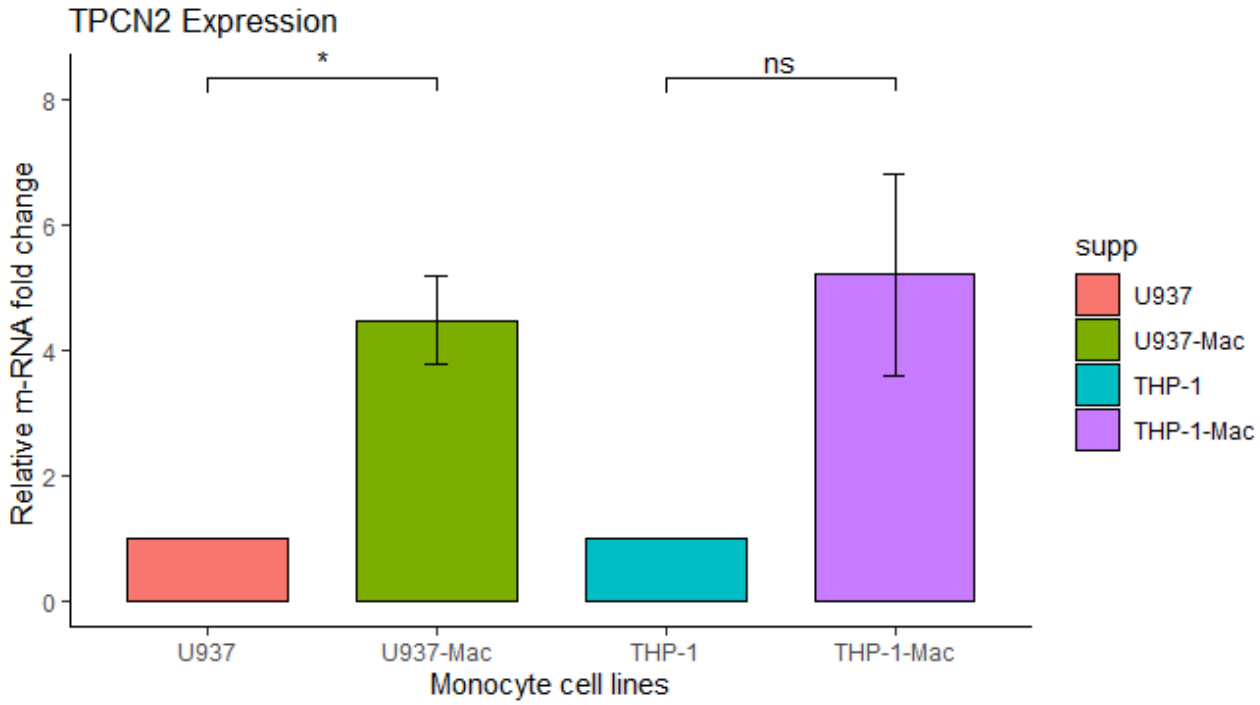


Figure 40. Real-time PCR analysis of the mRNA expression levels of TPCN2 in U937 and THP-1 cells. β-actinwas used as an internal control for human samples of three biological replicates in three independent experiments. mRNA expression levels of TPCN2 (mean ± SEM, two sample t-test, with *p*-values = 0.039 and 0.12 for U937 and THP-1, respectively).

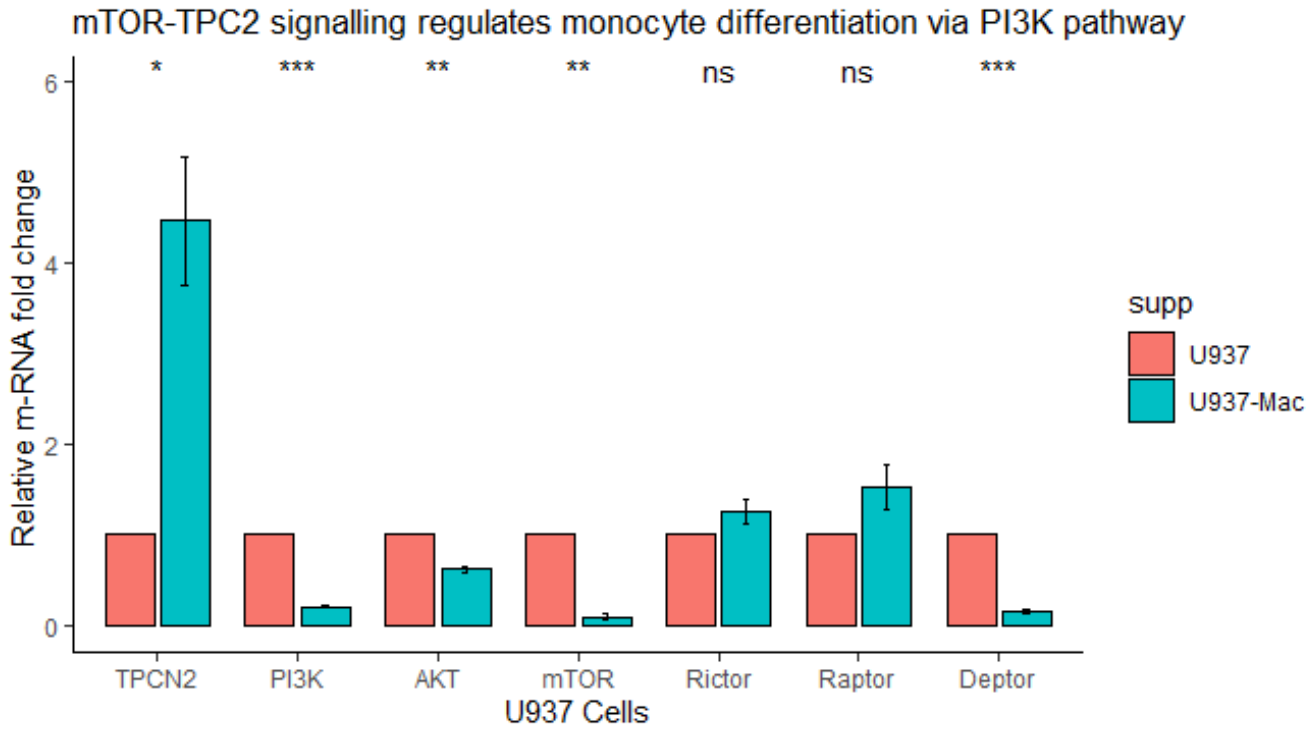


Figure 41. mTOR-TPC2 signalling regulates monocyte differentiation via the PI3K pathway. Real-time PCR of the mRNA expression levels of TPCN2, PI3K, AKT, mTOR, Rictor, Raptor, and Deptor (mean ± SEM of three biological replicates). β-actinwas used as an internal control. Two sample t-test, p-values of TPCN2 (0.03949), PI3K (0.0001612), AKT (0.00878), m-TRO (0.001031), Rictor (0.2213), Raptor (0.1681), and Deptor (0.0007914).

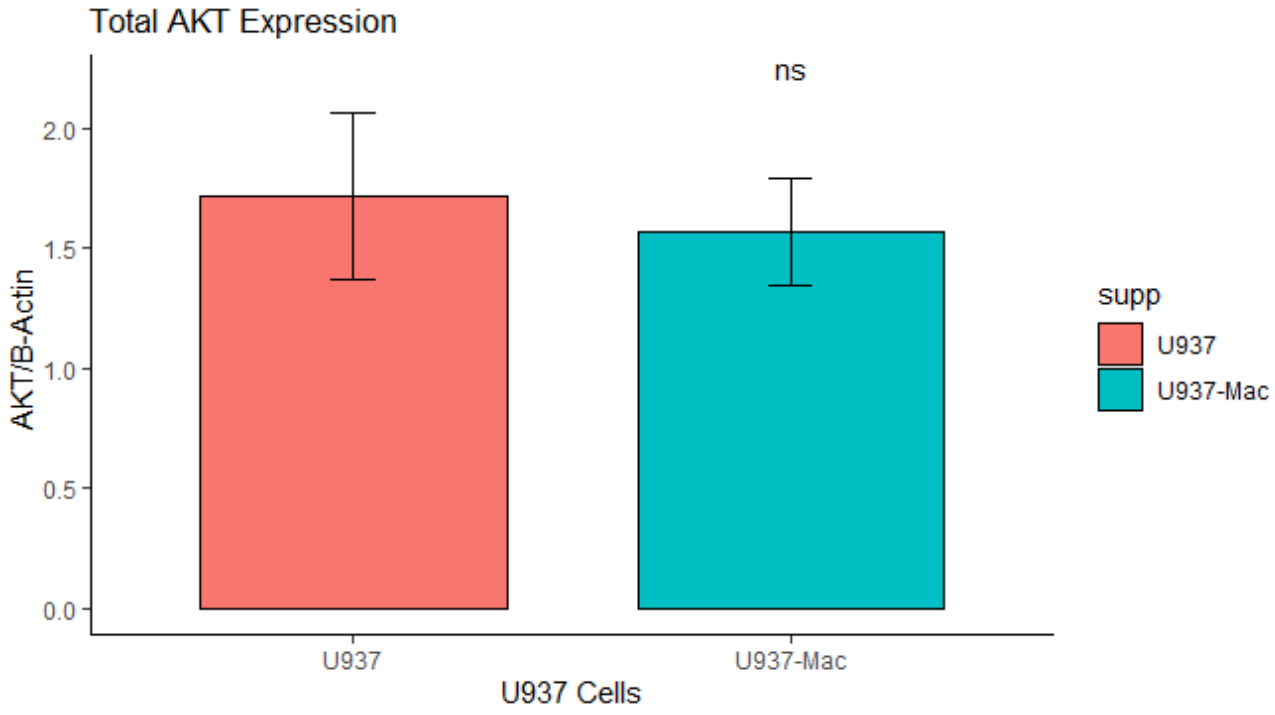


Figure 42. AKT expression was detected by Western blot analysis (WT U937 vs U937 m φ (mean ± SEM, two sample t-test, *p-value* = 0.7419).

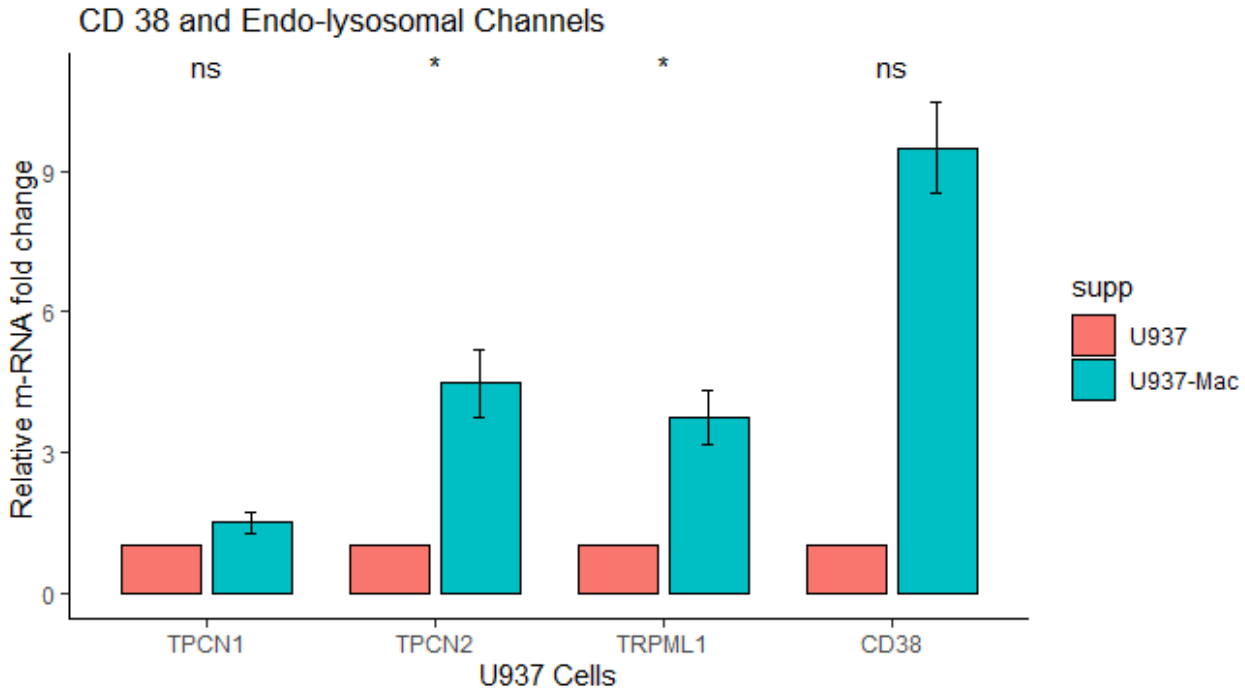


Figure 43. mRNA expression of CD 38 and endolysosomal Channels. Real-time PCR of the mRNA expression levels of CD38, TPCN1, TPCN2 and TRPML1 (mean ± SEM). β-actinwas used as an internal control. Two sample t-tests *p*-values of CD38 (0.07258), TPCN1 (0.2645), TPCN2 (0.03949) and TRPML1 (0.04352).

3.3.5. The development of TPC2-deficient human monocyte cell line

I generated a TPC2-deficient U937 monocyte model with a 75% significant reduction in TPCN2 expression (as shown in Figure 44) and attempted to develop TPC2-deficient THP1 monocytes. This was done to use the TPCN2 knockdown in U937 monocytes as an appropriate biological model to determine whether NAADP/TPC2 signalling is specifically involved in monocyte differentiation, due to the paucity of specific TPC2 inhibitors.

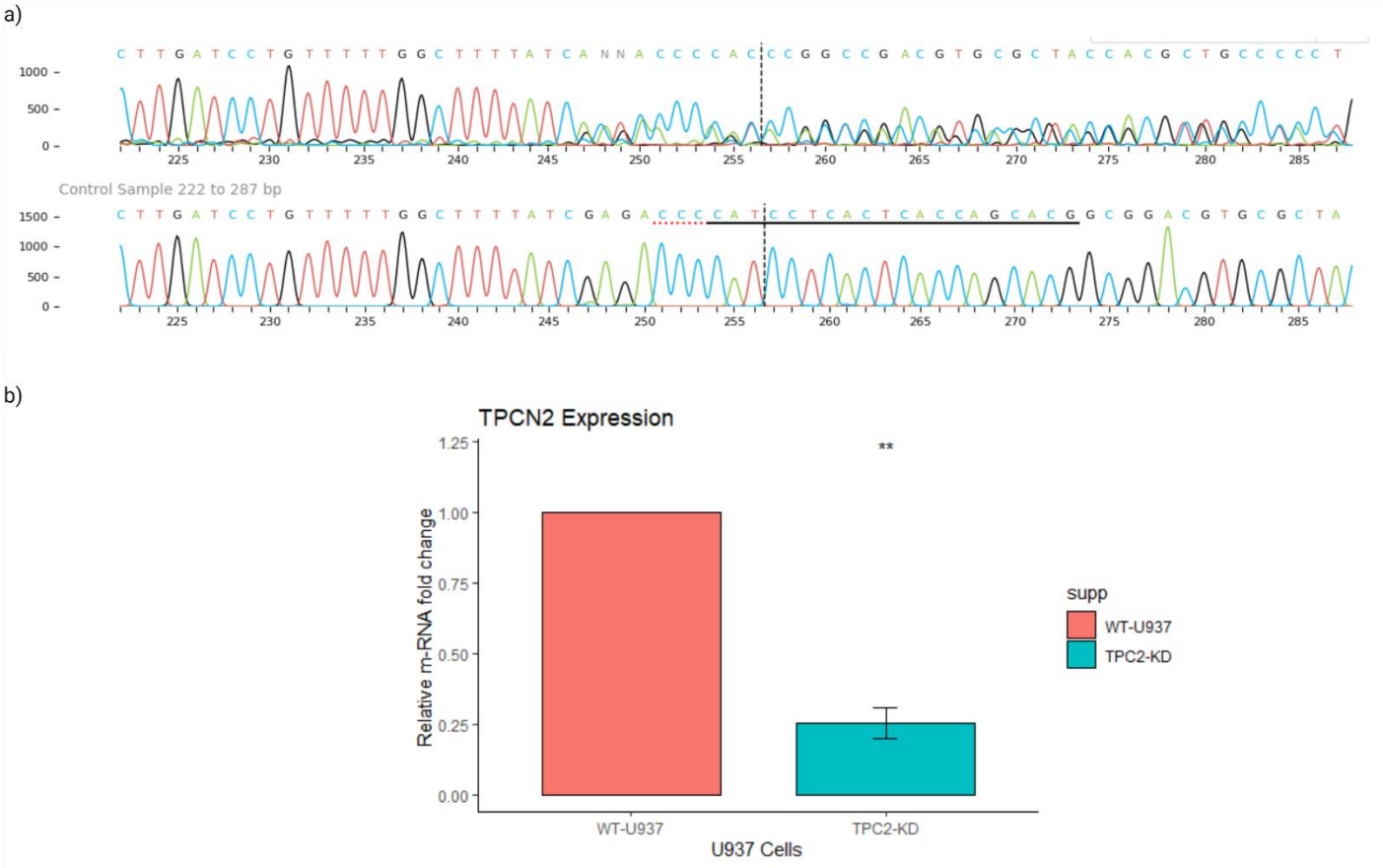
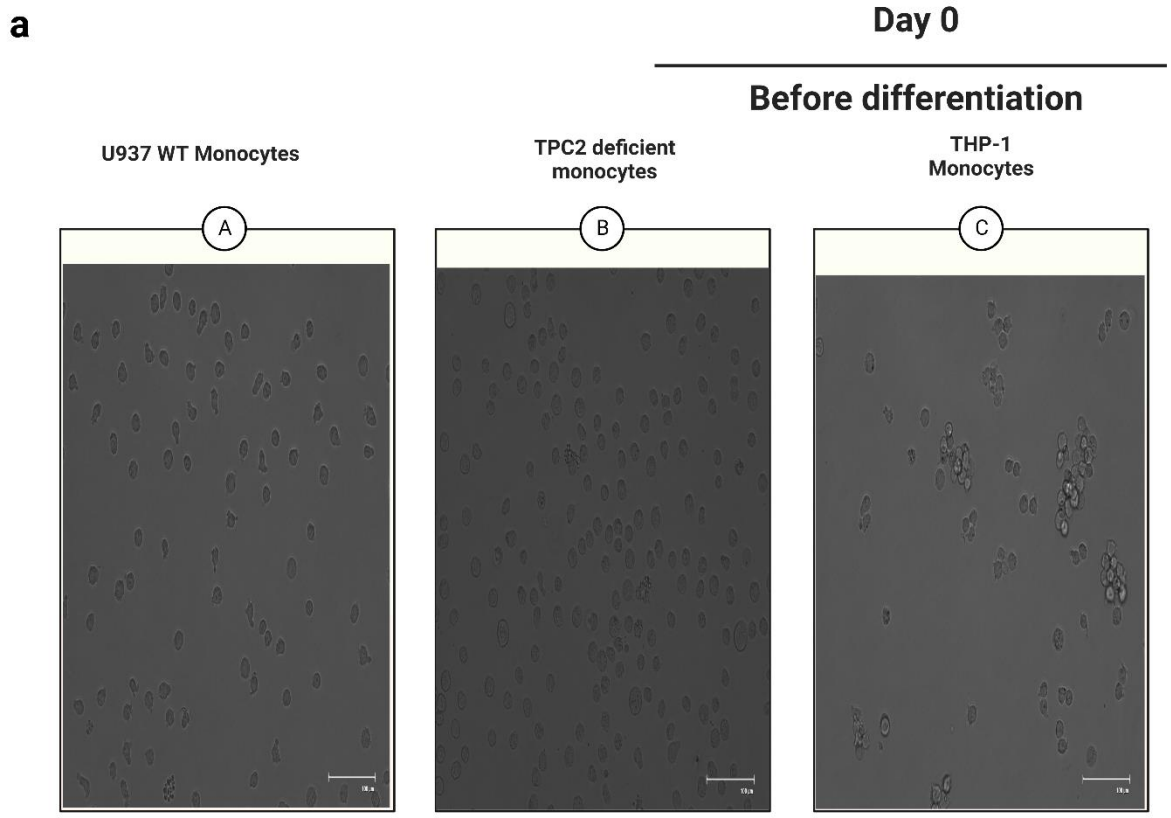


Figure 44. TPC2 KD U937 model. A. TPCN2 Sanger sequence of KD vs WT; B. TPCN2 expression in KD vs WT ((mean ± SEM), two sample -t-test, *p*-value = 0.005373).

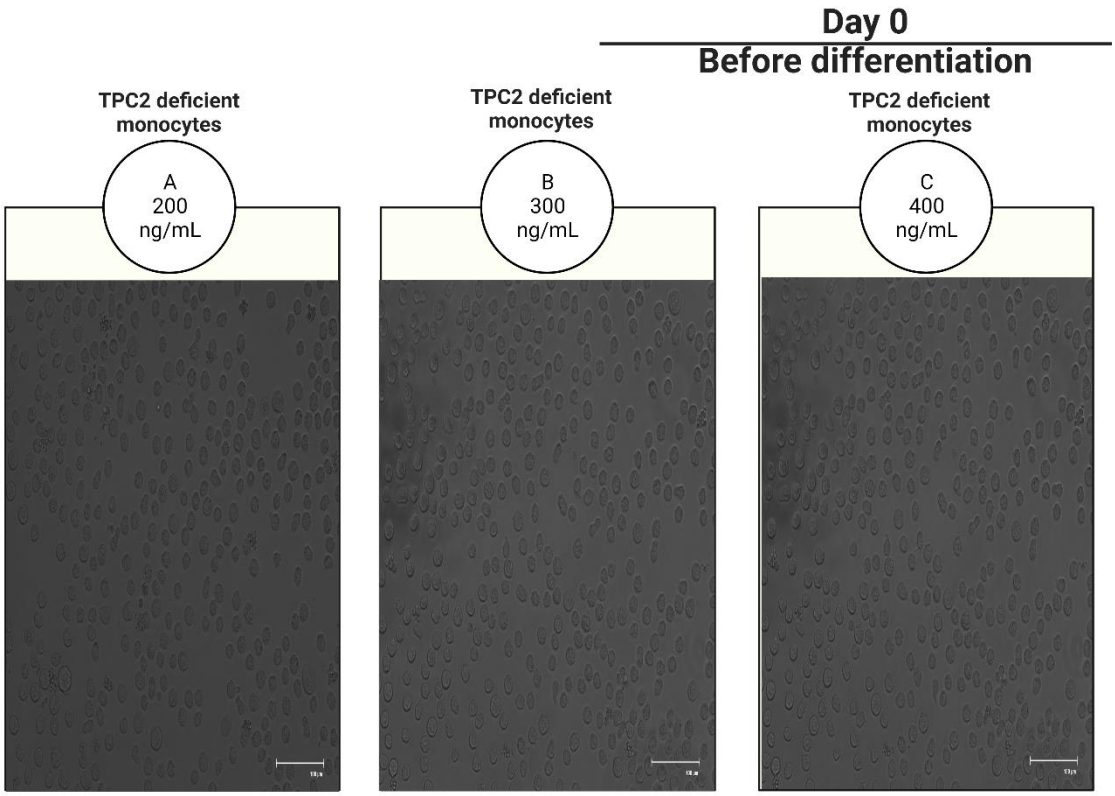
3.3.6. TPC2-deficient U937 monocytes are resistant to monocyte differentiation and macrophage development

To study the effect of knocking down TPC2 on the monocyte differentiation process into macrophages (Mϕ), I induced the monocyte differentiation by phorbol 12-myristate 13-acetate (PMA). Figure 45 shows that TPCN2 genetically modified U937 monocytes were resistant to monocyte differentiation after treatment with increasing concentrations of PMA ranging from 100 ng/mL to 400 ng/mL. TPC2-deficient U937 monocytes were not differentiated into Mϕ. The

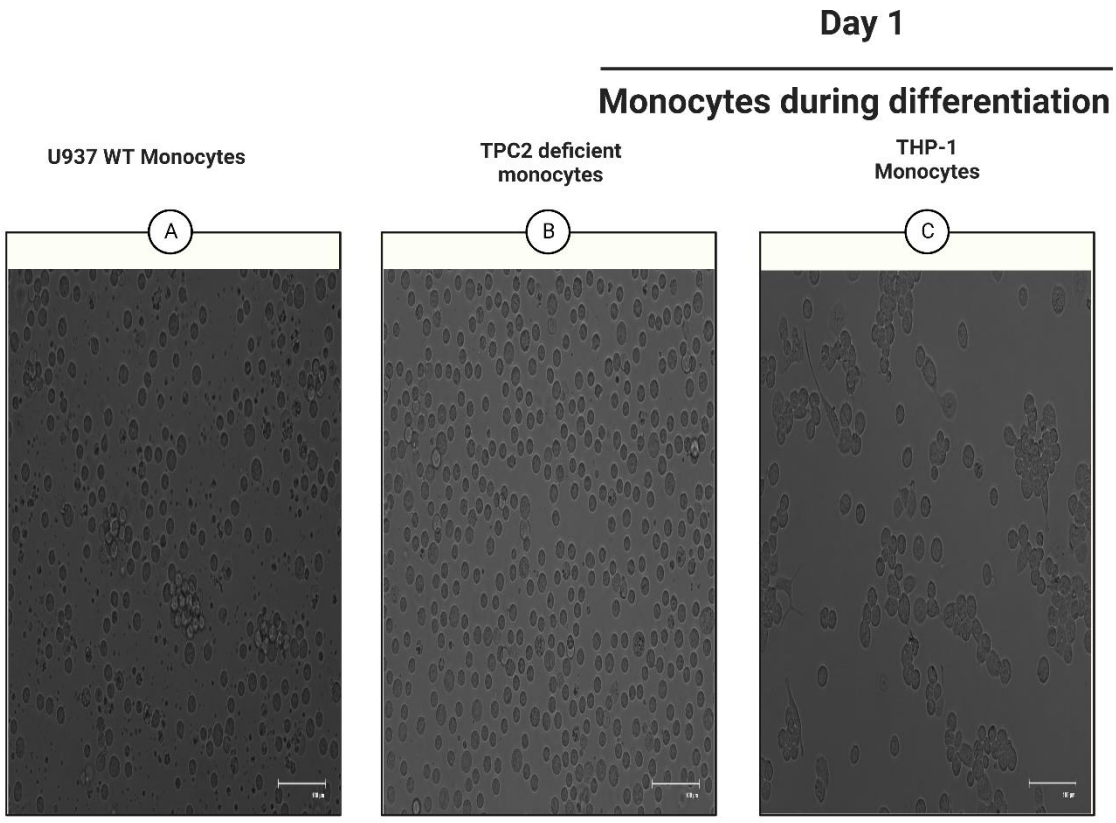
number of non-polarised (Mϕ) adherent macrophages in U937 WT compared to U937 TPC2 KD was significantly reduced (t-test, *p*-value = 0.009573), as presented in Figure 47.



b

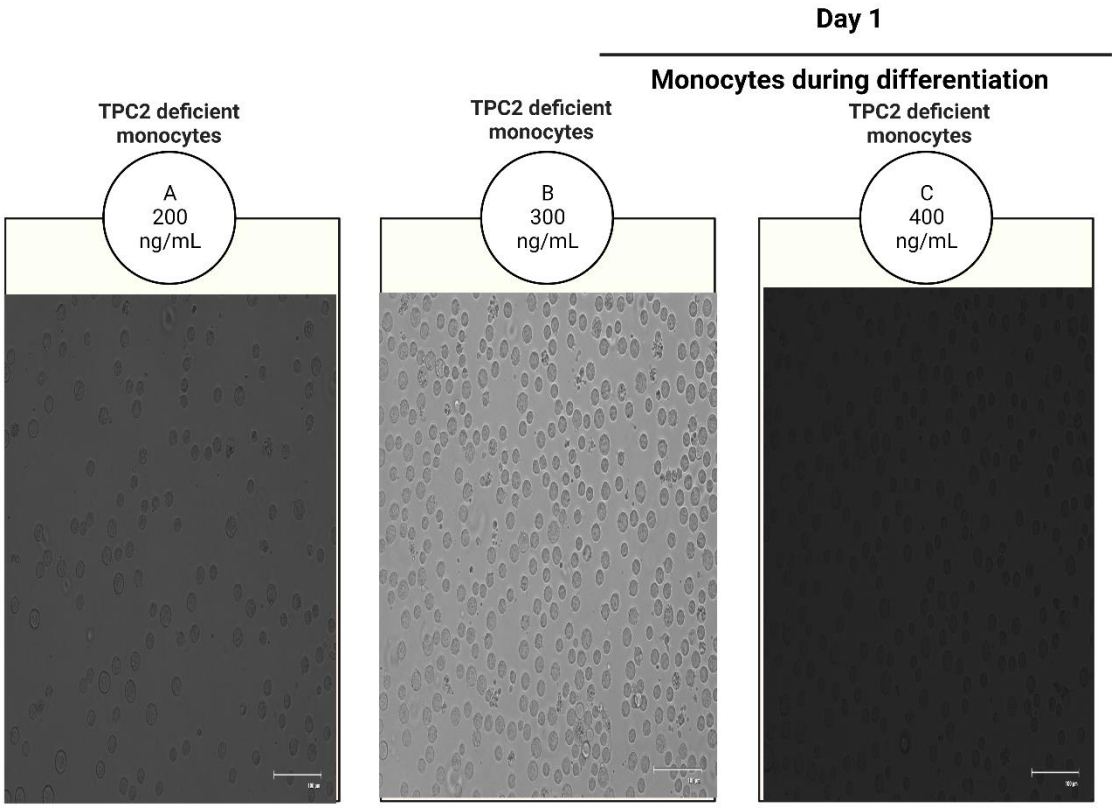

Day 0
Before differentiation
TPC2 deficient monocytes
A
200
ng/mL
TPC2 deficient monocytes
B
300
ng/mL
TPC2 deficient monocytes
C
400
ng/mL

c



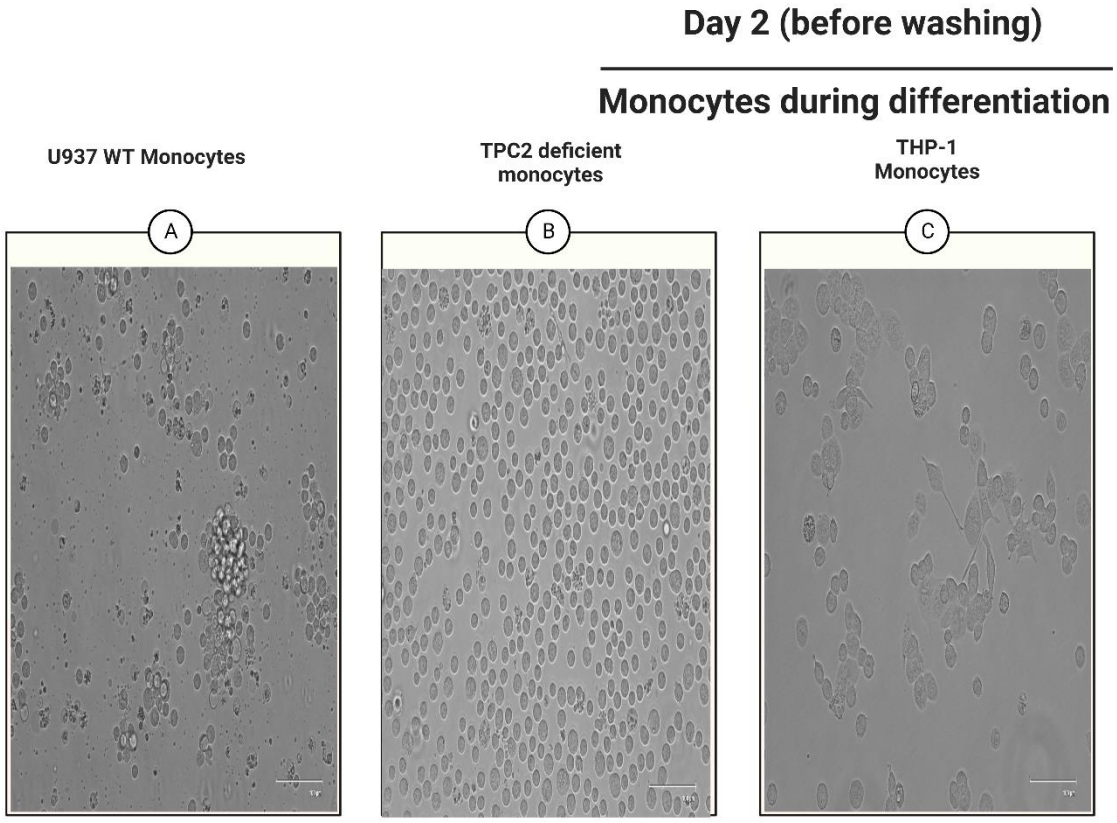
(after 24 hours of incubation with 100 ng/mL of PMA)

d



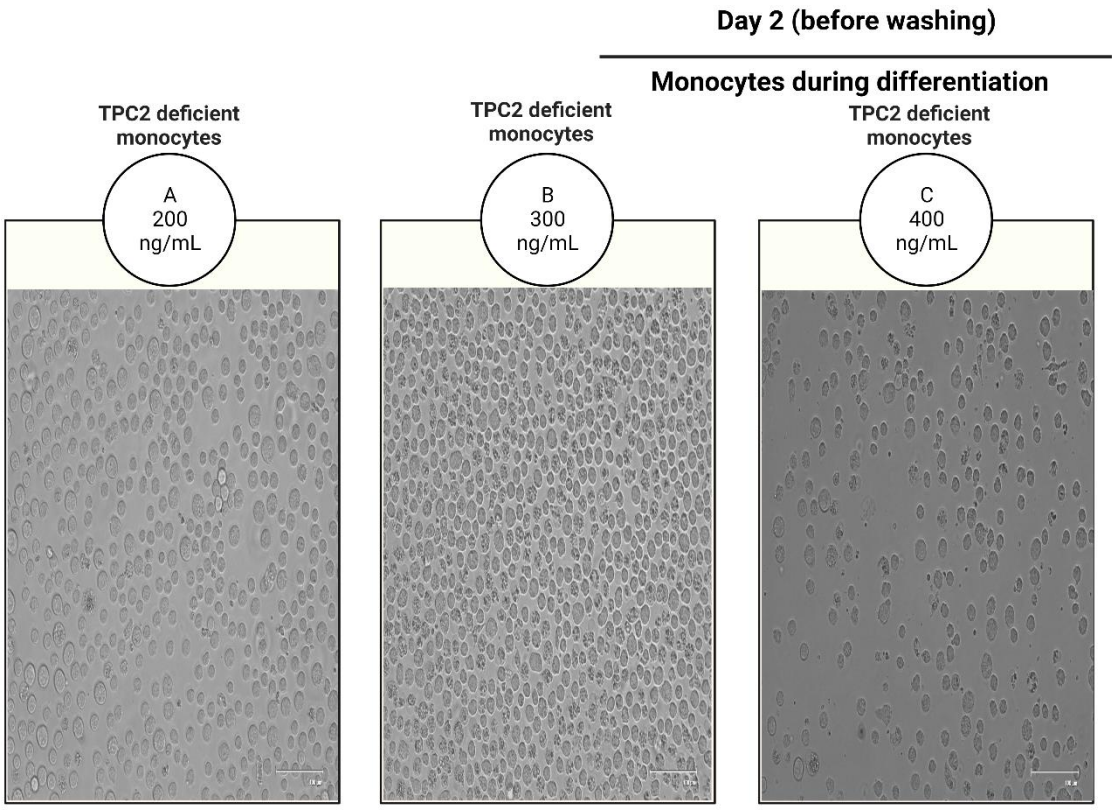
(after 24 hours of incubation with 200, 300 and 400 ng/mL of PMA)

e



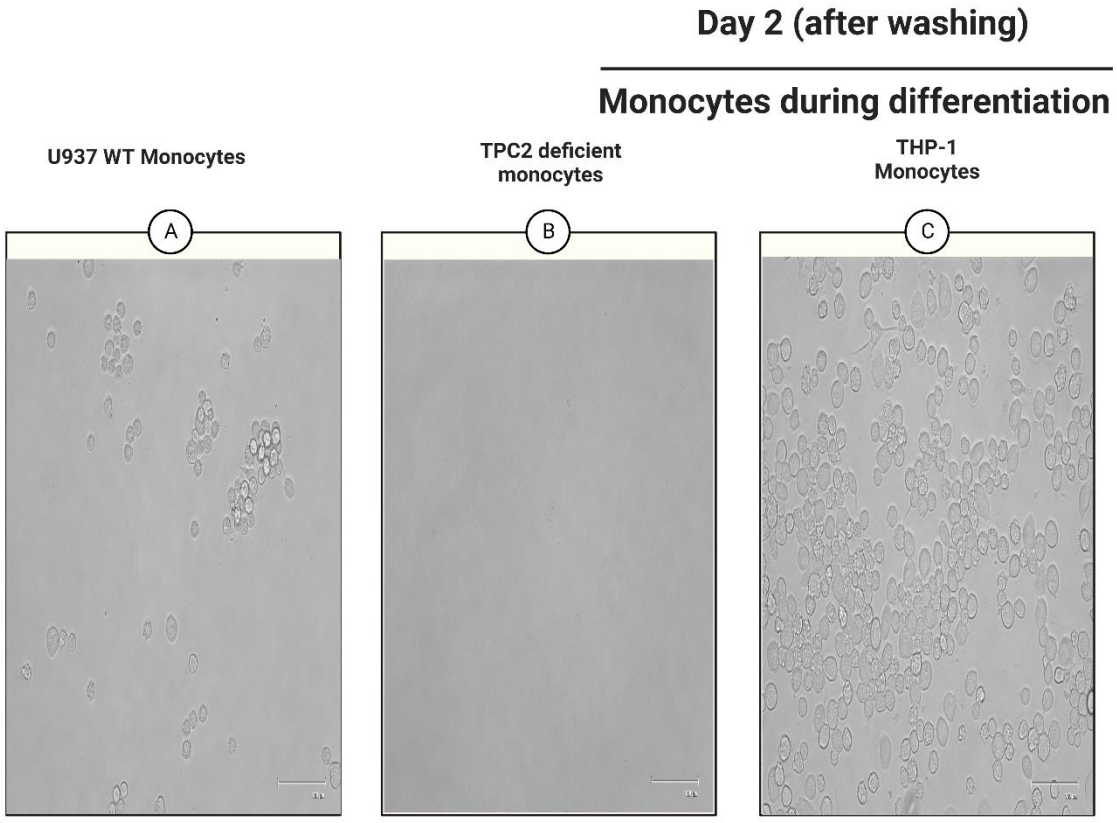
(after 48 hours of incubation with 100 ng/mL of PMA)

f



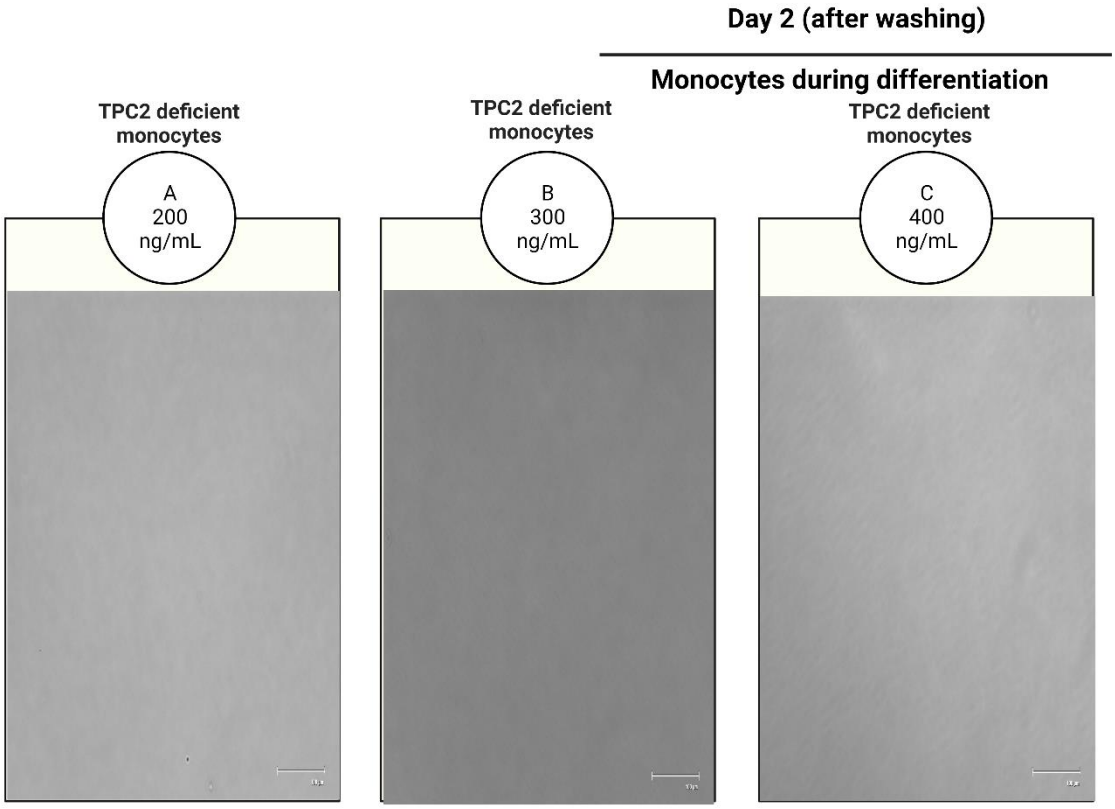
(after 48 hours of incubation with 200, 300 and 400 ng/mL of PMA)

g



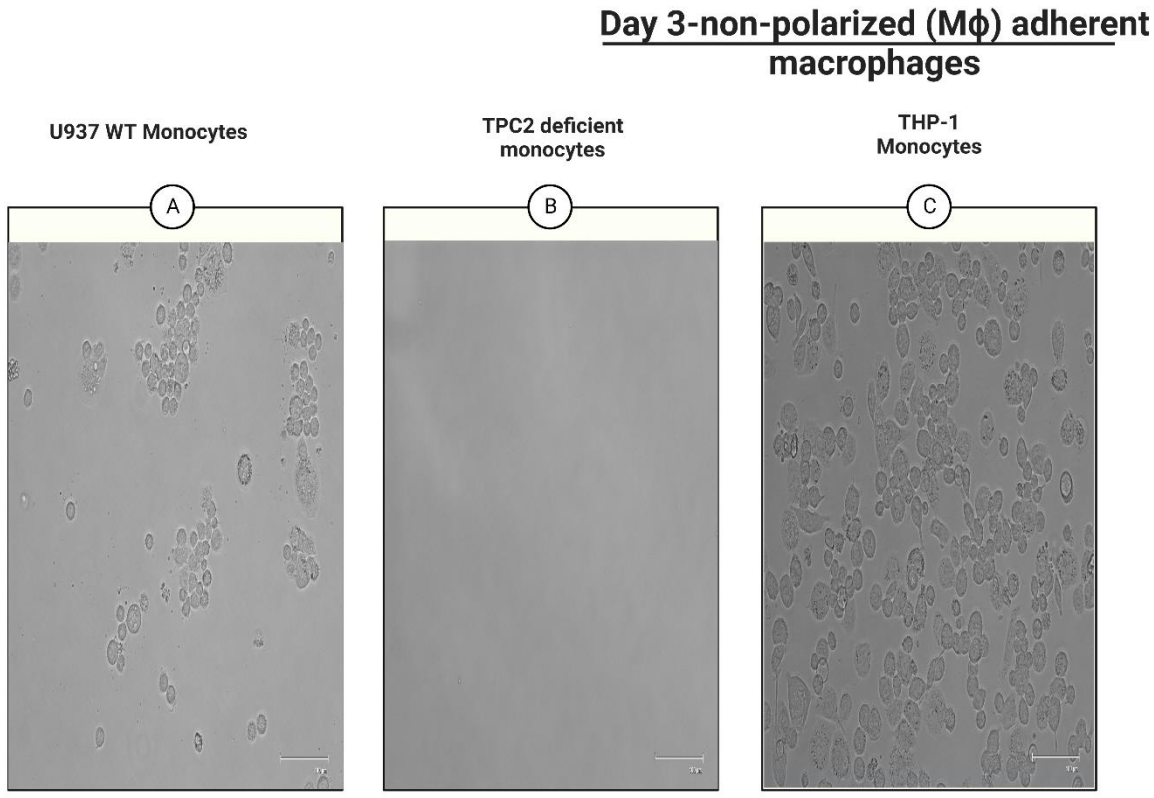
(after 48 hours of incubation with 100 ng/mL of PMA)

h



(after 48 hours of incubation with 200, 300 and 400 ng/mL of PMA)

i



(after 48 hours of incubation with 100 ng/mL of PMA and 24 hours resting in completed RPMI 1640-media)

j

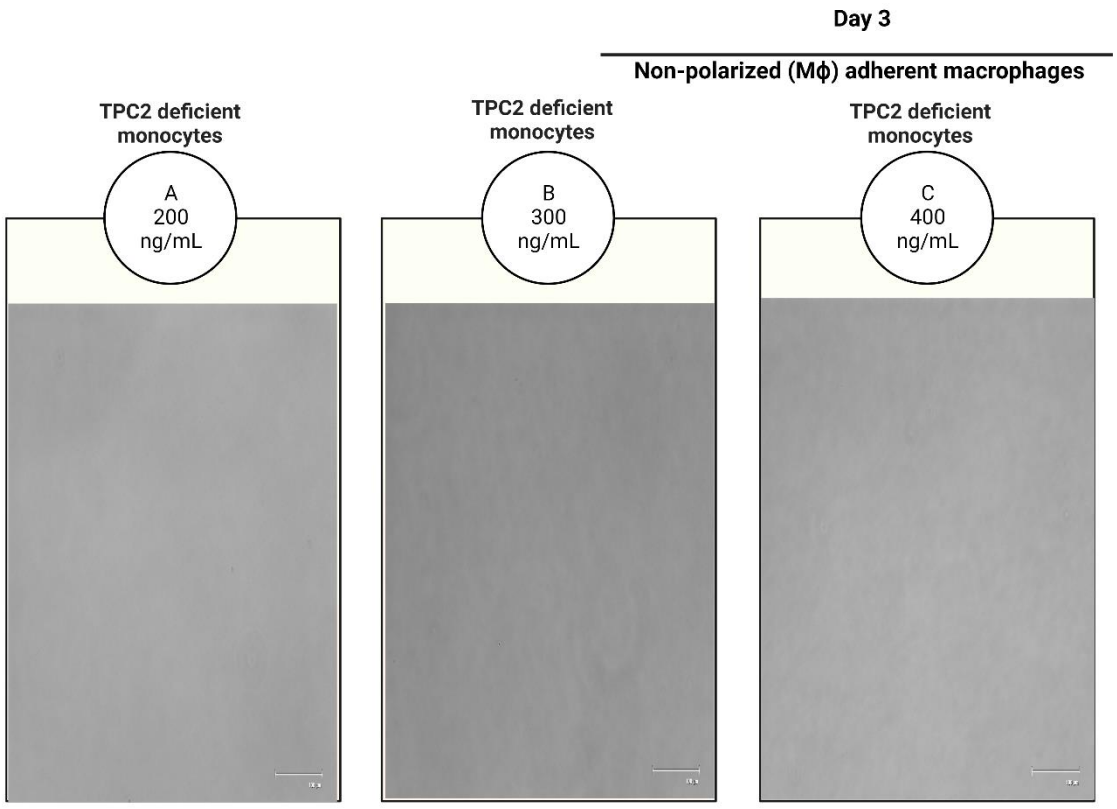


Figure 45. Morphology of U937, TPC2-deficient U937 and THP-1 human cells treated with PMA for 48 h. Cells were then washed twice with media and cultured in complete RPMI 1640-media for 24 h. The TPC2-deficient U937 monocytes were not differentiated into non-polarised (Mϕ) adherent macrophages.

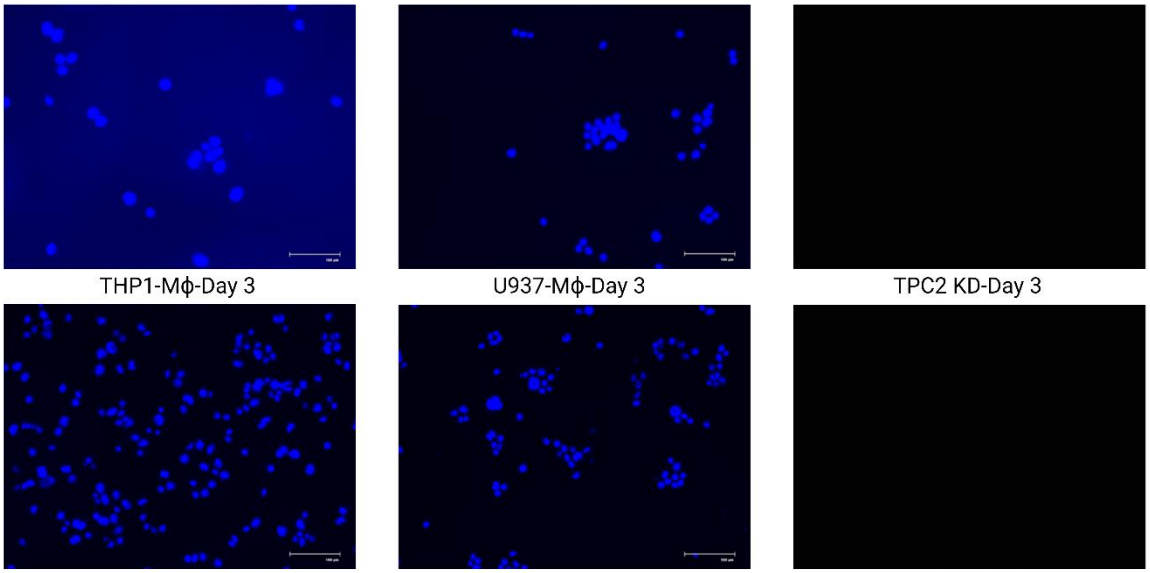


Figure 46. Morphology of U937, TPC2-deficient U937 and THP-1 human cells treated with PMA (100 ng/mL) for 48 h. Cells were then washed with media and cultured in complete RPMI 1640-media for 24 h. The non-polarised (Mϕ) adherent macrophages were dyed with Hoechst stains.

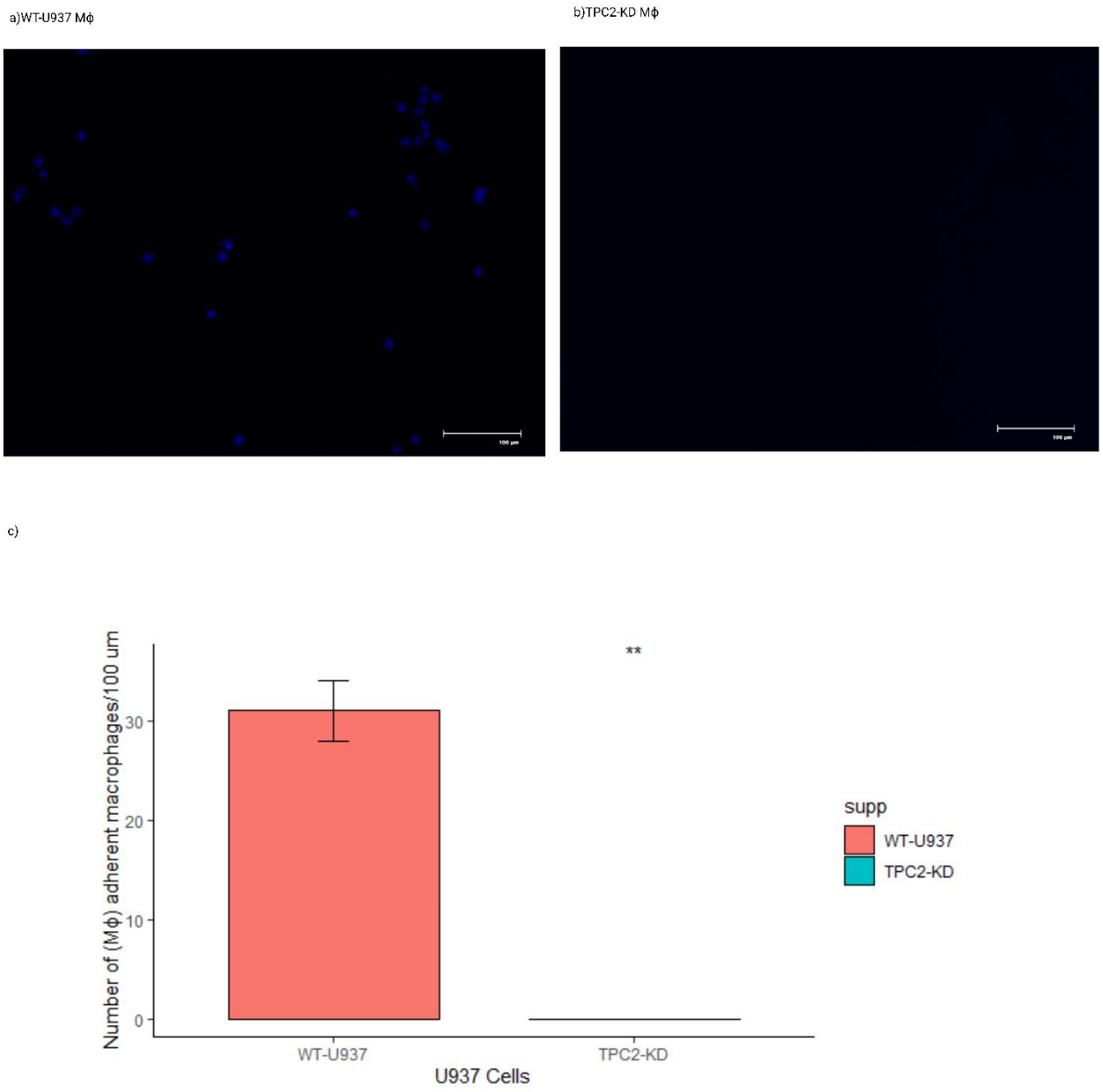


Figure 47. Morphology of U937 and TPC2-deficient U937 non-polarised (Mϕ) adherent macrophages. a, b. The non-polarised (Mϕ) adherent macrophages were dyed with Hoechst stains. c. Number of non-polarised (Mϕ) adherent macrophages in U937 WT compared to U937 TPC2 KD ((mean ± SEM), two sample -t-test, *p*-value = 0.009573).

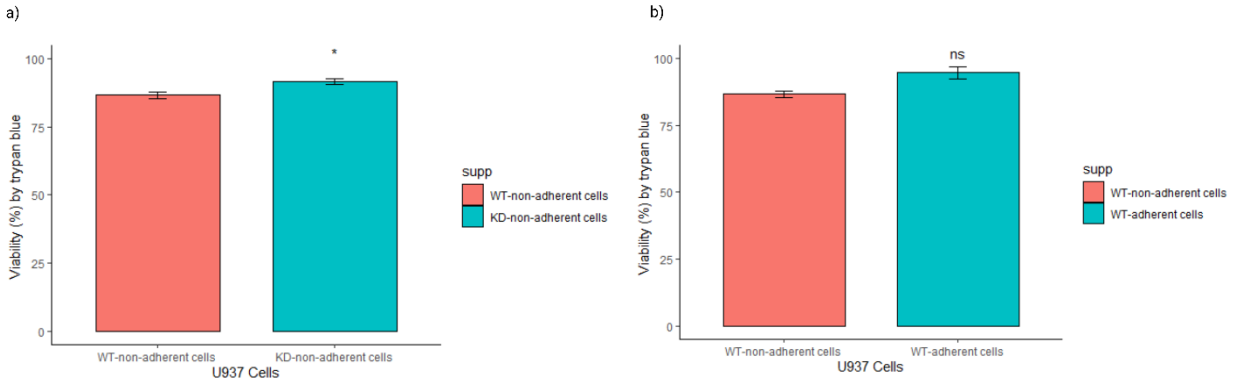


Figure 48. Viability assay by trypan blue of U937 and TPC2-deficient U937 on day 2 after 48 h treatment with PMA. (a) The non- adherent WT-U937 and TPC2 KD cells ((mean ± SEM), two sample t-test, *p*-value = 0.03526), (b). Non adherent WT-U937 and non-polarised (Mϕ) adherent macrophages on day 2 after 48 h treatment with PMA ((mean ± SEM), two sample -t-test, *p*-value = 0.05459).

3.3.7. TPC2 acts as a major regulator of monocyte differentiation by modulating PKC-alpha via calpain

PMA acts as a stimulator of PKC during monocyte differentiation, which then activates a wide range of downstream targets. Therefore, I investigated the KC-alpha expression level in KD compared to WT, and I found a significant (p-value = 0.003582) reduction in the PKC-alpha expression in TPC2-deficient monocytes (as shown in Figure 49), indicating that knocking down TPC2 downregulates the PKC-alpha expression, which plays an important role in monocyte differentiation and Mϕ development. A significant difference was observed in PKC-alpha expression at protein level in U937 macrophages (0.04319). To determine the regulator that links TPC2 to PKC-alpha, I examined calpain, which is known to negatively modulate PKC-alpha at RNA and protein levels. I observed a non-significant difference between KD and WT U937 cells in μ-calpain/m-calpain expression, whereas I observed a significant up-regulation in calpain

activity in TPC2-deficient monocytes compared to WT monocytes. This result indicates that calpain might be a potential downstream regulator of monocyte differentiation. There were no significant changes in calpain expression at RNA and protein levels in Mϕ compared to monocytes in both U937 and THP1 cells.

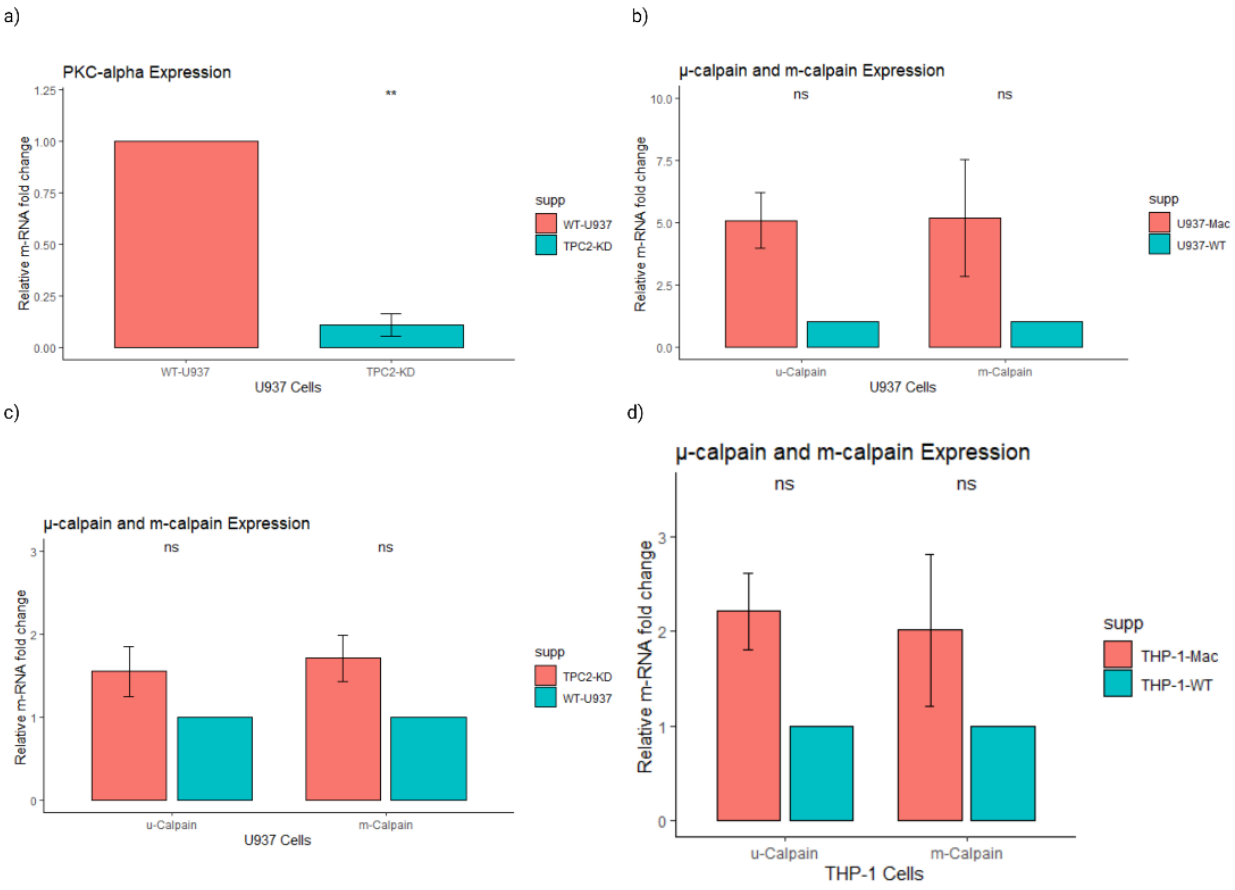


Figure 49. Real-time polymerase chain reaction (PCR) of the mRNA expression levels of TPCN2, m-calpain, μ-calpain and PKC-alpha ((mean ± SEM) of 3 biological replicates). β-actinwas used as an internal control. Two sample -t-test, p-values of PKC-alpha (0.003637), U937 macrophages μ-calpain (0.06577) and m-calpain (0.2184), U937 cells μ-calpain (0.2074) and m-calpain (0.1233),) and THP-1 macrophages μ-calpain (0.09503) and m-calpain (0.3352).

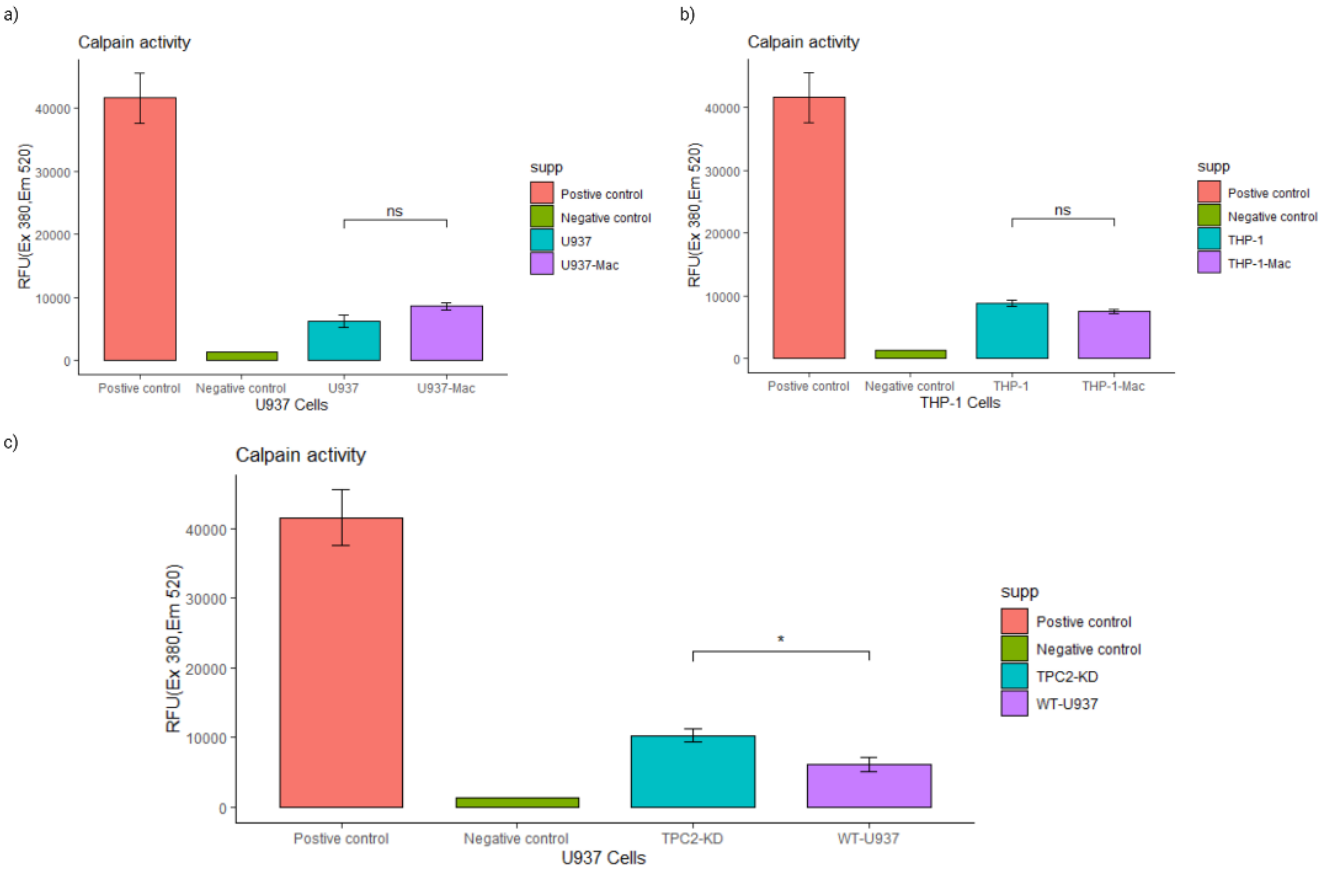


Figure 50. Calpain activity (mean ± SEM of 3 biological replicates) in U937 (two sample -t-test,*p*-value = 0.1182), THP-1 cells (two sample -t-test, *p*-value = 0.07903), and U937 TPC2 KD (two sample -t-test,*p*-value = 0.04122).

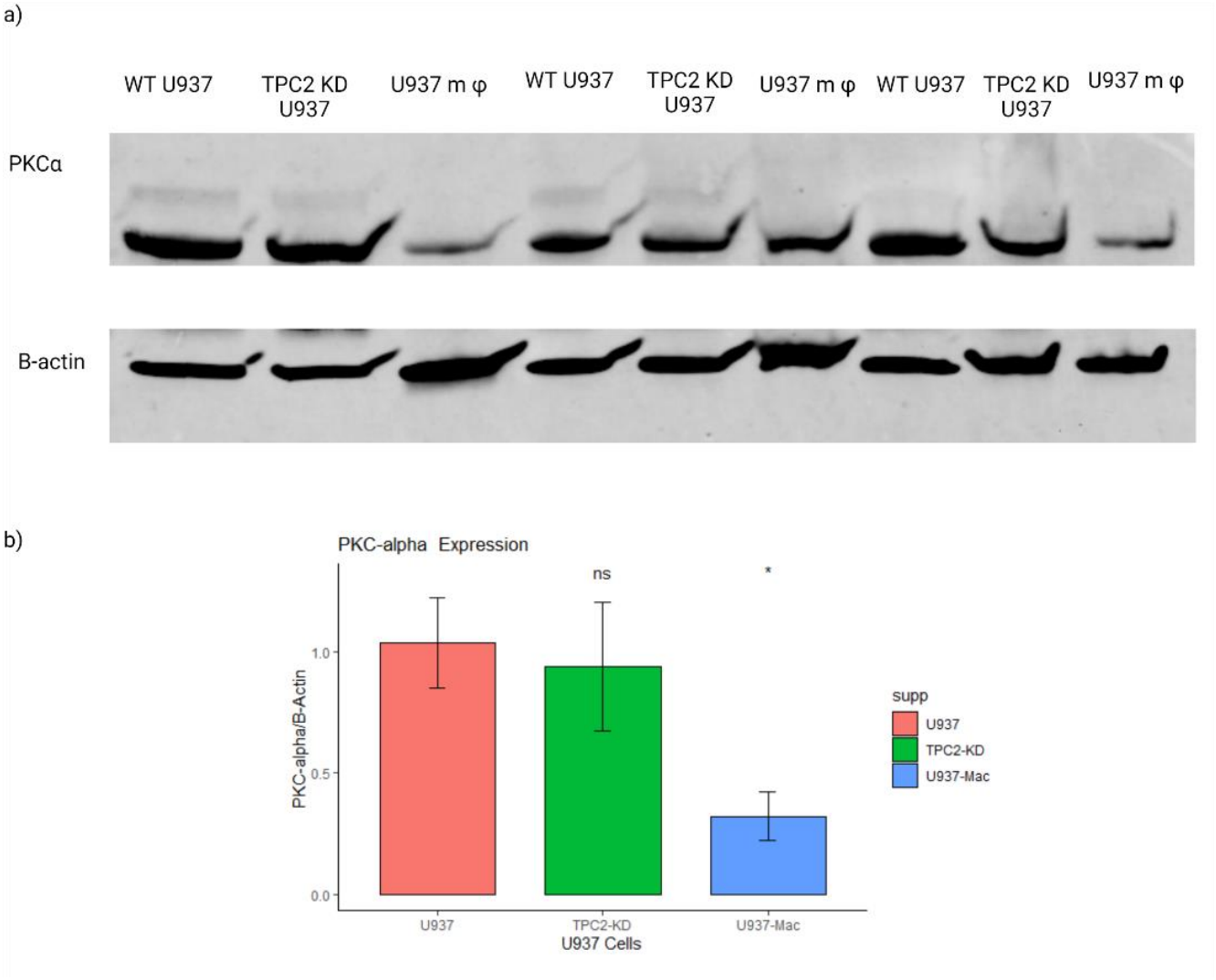


Figure 51. PKCα expression was detected by Western blot analysis ((mean ± SEM) WT U937 vs U937 m φ (two sample-t-test, *p*-value = 0.04319), WT U937 vs U937 TPC2 KD (two sample-t-test, p-value = 0.7827).

3.3.8. Inhibition/loss of TPC2 attenuates IL-8 production

To determine whether NAADP/TPC2/Ca^{2+} signalling is involved in IL-8 production, I first examined the levels of KC and MIP-2 (IL-8 homologues) in murine splenocytes after TPC2 KO. The MIP-2 level was significantly reduced by 3.72-fold in TPC2 KO splenocytes (as shown in Figure 52). To determine if differences in IL-8 murine homologue (KC) could be attributed to differences in their mRNA expression, I also compared KC mRNA levels in TPC2 KO splenocytes. The KC mRNA expression was observed to be downregulated in murine splenocytes after or TPC2 KO (as shown in Figure 52). I confirmed the data in an established different biological TPC2 KO model before selecting and establishing a suitable biological model to examine the role of TPC2 in IL-8 production in human monocytes and Mϕ.

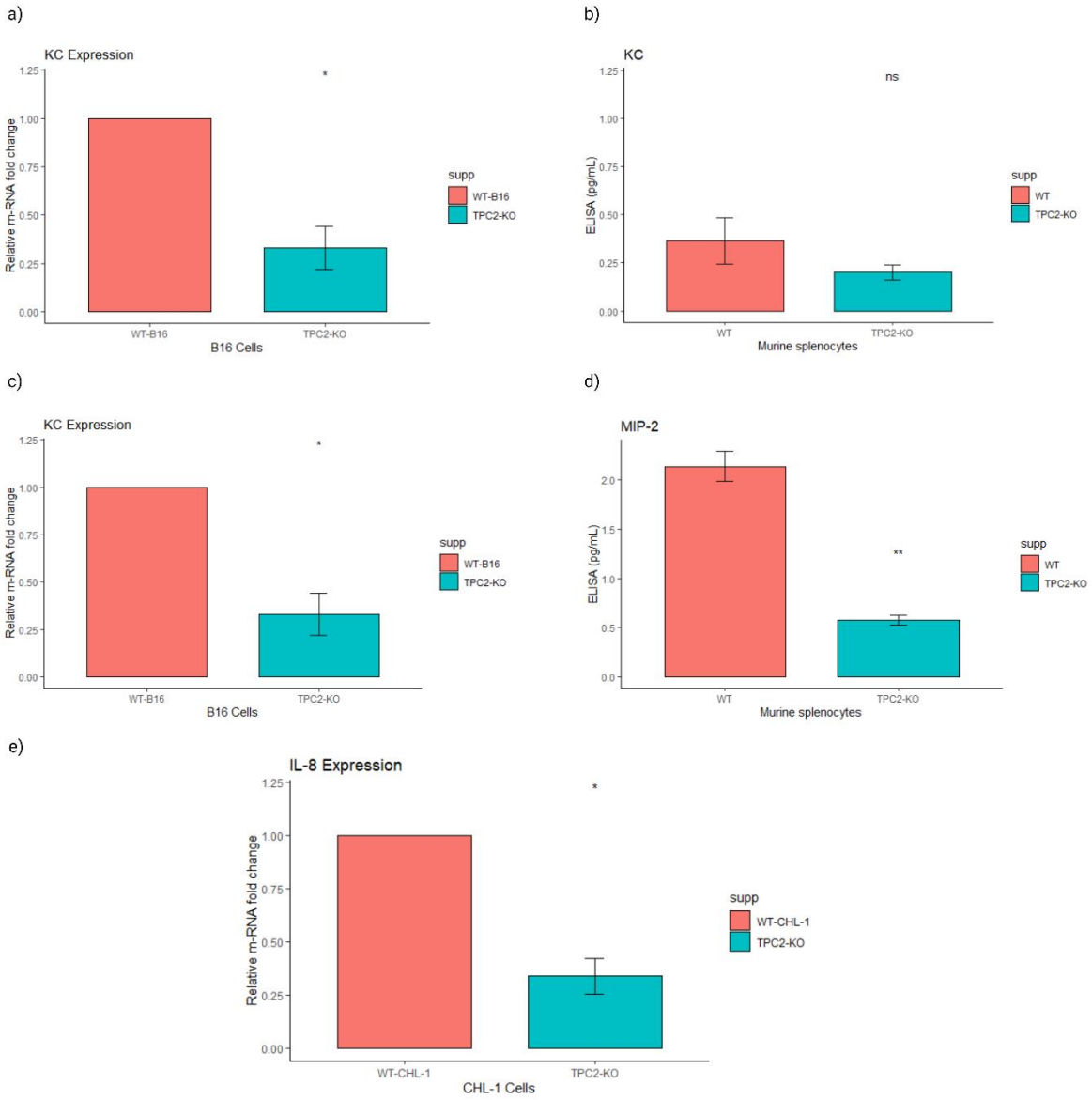


Figure 52. IL-8 murine homologues (KC and MIP-2) and IL-8 (mean ± SEM). (a) and (b) KC expression at both RNA (two sample -t-test,*p*-value = 0.004092) and protein (two sample -t-test, *p*-value = 0.3931) levels in TPC2 KO splenocytes; (c) KC mRNA expression in B16 cells (*p*-value = 0.02693); (d) MIP-2 protein (two sample -t-test,*p*-value = 0.005708) in TPC2 KO splenocytes; (e) in CHL-1 cells (two sample -t-test, *p*-value = 0.01511), with β-actinused as an internal control for human samples and glyceraldehyde 3-phosphate dehydrogenase used as an internal control for murine samples (*c,e: these are Ali Hanbashi's cDNA samples)*

Then, I measured the amount of IL-8 released by U937 monocytes and non-polarised (Mϕ) adherent U937 macrophages. I observed that IL-8 levels were increased in the medium of non-polarised macrophages compared to U937 monocytes (Figure 53 c, d). IL-8 mRNA was also highly expressed in non-polarised macrophages compared to U937 monocytes (as shown in Figure 53 a). Hence, the enhanced levels of secreted IL-8 protein in the medium correlate with an increase in IL-8 mRNA expression in cells. Then, I characterised the biological role of TPC2 in IL-8 release in U937 monocytes. I found that inhibition of TPC2 function via pharmacological means by Ned-19 at 100 µm or knockdown by CRISPR-Cas9 significantly attenuated IL-8 production *in vitro* (as shown in Figure 53 e,f). To determine that the involvement of endolysosomal Ca^{2+} via TPC2 is specifically attributed to the changes in IL-8 production, I treated U937 cells with ML1-SA1 (a selective agonist of TRPML). No significant difference was found in the level of IL-8 in the medium between U937 treated with ML1-SA1 at 10 µm or U937 (t-test,*p*-value 0.2367). Overall, these results indicate that the production of the proinflammatory chemokine IL-8 was drastically suppressed in splenocytes (source of monocytes and macrophages)/U937 monocytes by blocking lysosomal Ca^{2+} signalling either with the NAADP/TPC pharmacological modulator Ned-19 or by TPC2 KO.

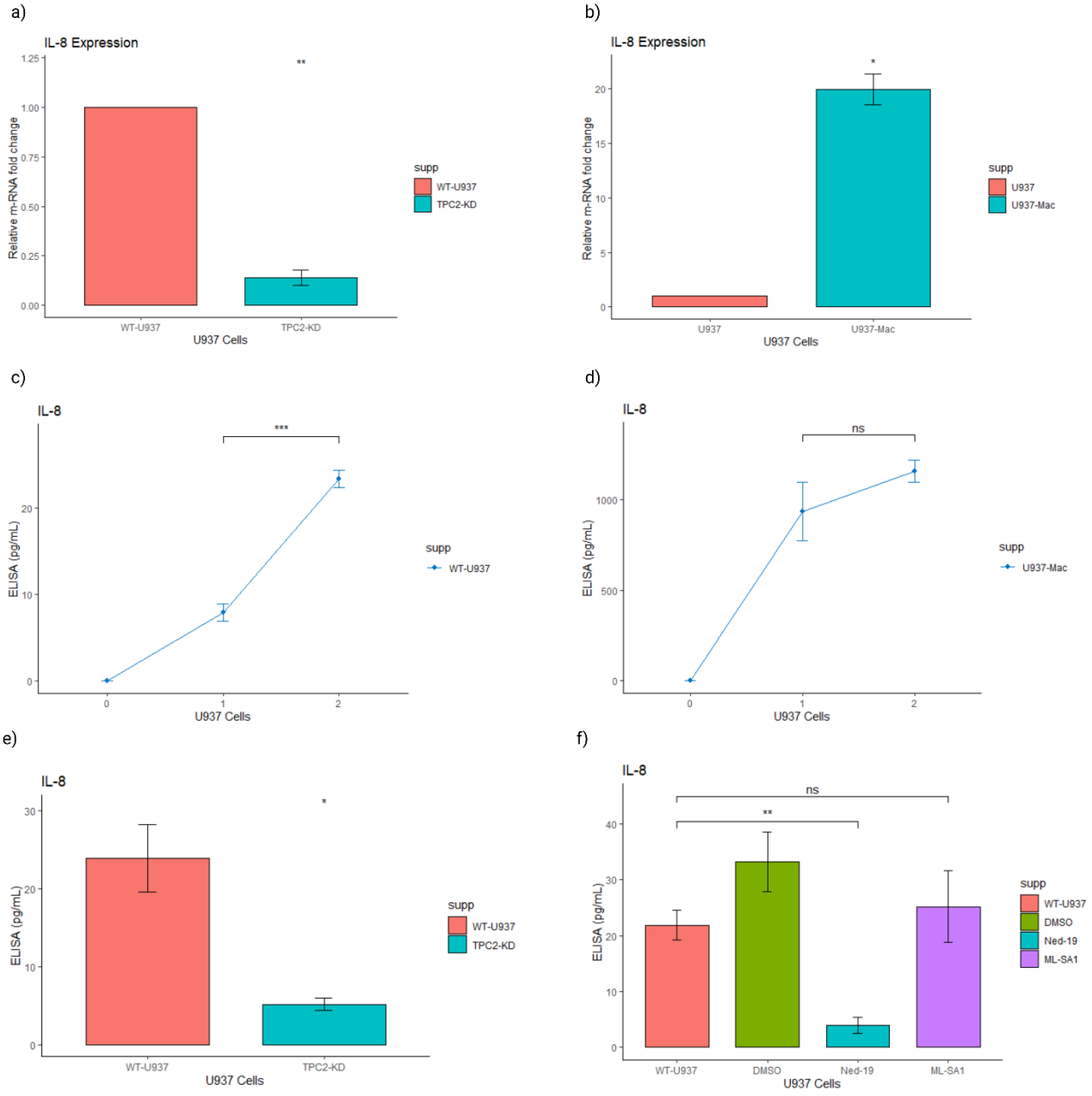


Figure 53. IL-8 protein and mRNA in U937 monocytes, TPC2 deficient U937 cells and U937 macrophages (mean ± SEM). (a). Comparison of IL-8 mRNA expression in U937 monocytes and U937 macrophages (two sample -t-test, *p*-value = 0.04707). (b). Comparison of IL-8 mRNA expression in U937 monocytes and TPC2 deficient U937 monocytes (two sample -t-test, *p*-value = 0.01731). (c, d). Time-dependent IL-8 protein production in U937 monocytes and U937

macrophages. (c). IL-8 protein levels in human U937 cells (Day 1 vs Day 2, two sample -t-test, *p*-value = 0.0003845). (d). IL-8 protein levels in human U937 macrophages (Day 1 vs Day 2, two sample -t-test, *p*-value = 0.3921). (e). IL-8 protein levels in human U937 cells (*p*-value = 0.04446). (f). Comparison of IL-8 levels in U937 cells treated with Ned-19 at 100 μm or ML-SA1 at 10 μm (Ned-19 vs WT, two sample -t-test, *p*-value = 0.009647, ML-SA1 vs WT, two sample -t-test, *p*-value= 0.2367).

3.3.9. LPS-mediated IL-8 production via AKT and MAPK signalling pathways in TPC2 deficient monocytes

To determine the possible pathway of intracellular Ca^{2+} signalling in regulating IL-8 production, I stimulated the IL-8 production by LPS; I found a significant difference in LPS-mediated IL-8 protein release in TPC2 deficient monocytes compared to WT U937 monocytes (as shown in Figure 54). No significant difference was found in LPS-mediated-IL-8 production in U937 macrophages treated or not treated with Ned-19 at 100 μm (p-value = 0.1031). CD38 has a profound influence on TLR4, which may contribute to the dysregulated expression of inflammatory IL-8. I have observed a significant reduction in CD38 expression level in TPC2 KD cells (Figure 54), and this could be attributed to the reduction in IL-8 levels in TPC2 deficient monocytes. In addition, a non-significant upregulation in CD38 expression was found in U937 macrophages. Therefore, I have recently investigated the expression of TLR-4 in TPC2 KD cells. I have found a non-significant difference in TLR-4 expression level in TPC2 deficient monocytes compared to WT U937 monocytes. AKT and MAPK play roles in IL-8 production in various types of cells. I examined PI3K/AKT and MAPK expression levels in TPC2 KD cells, and I observed significant reductions in AKT and MAPK expression levels in such cells. This study found that TPC2 modulation by genetic reduction reduced the LPS-mediated release of the pro-inflammatory mediator (IL-8) from monocytes. Although the molecular mechanism by which TPC2 regulates IL-8 production and release in monocytes remains to be clarified, a potential regulation pathway of

TPC2 signalling is via PI3K/AKT and MAPK (as shown in Figure 56). In addition, I have conducted a B-hexosaminidase assay to investigate the role of TPC2 in IL-8 release and trafficking and to distinguish between LE/LY and EE/RE as possible secretion pathways. Based on the preliminary data (as shown in Figure 57), I speculate that TPC2 in EE/RE is involved in LPS-mediated IL-8 production. Thrombin and plasminogen are responsible for cleaving the IL-8 isoform of 99 amino acid residues to yield biologically active IL-8 isoforms, with peptides of 72 (major form secreted by monocytes and macrophages), and 77 amino acids (secreted by non-immune cells). Therefore, I investigated the expression levels of prothrombin, u-PA and PAI-1 in U937 TPC2 KD cells. The mRNA expression level of prothrombin was undetectable by q-RT-PCR. Figure 57 shows that there are non-significant lower levels of u-PA and PAI-1 in U937 TPC2 deficient monocytes. Despite the exploratory nature of the evidence highlighting the role of endolysosomal Ca2+ signalling via TPC2 in IL-8 release, the findings provide the first insight into the role of the NAADP/TPC2/Ca2+ signalling as a regulator of the IL-8 release.

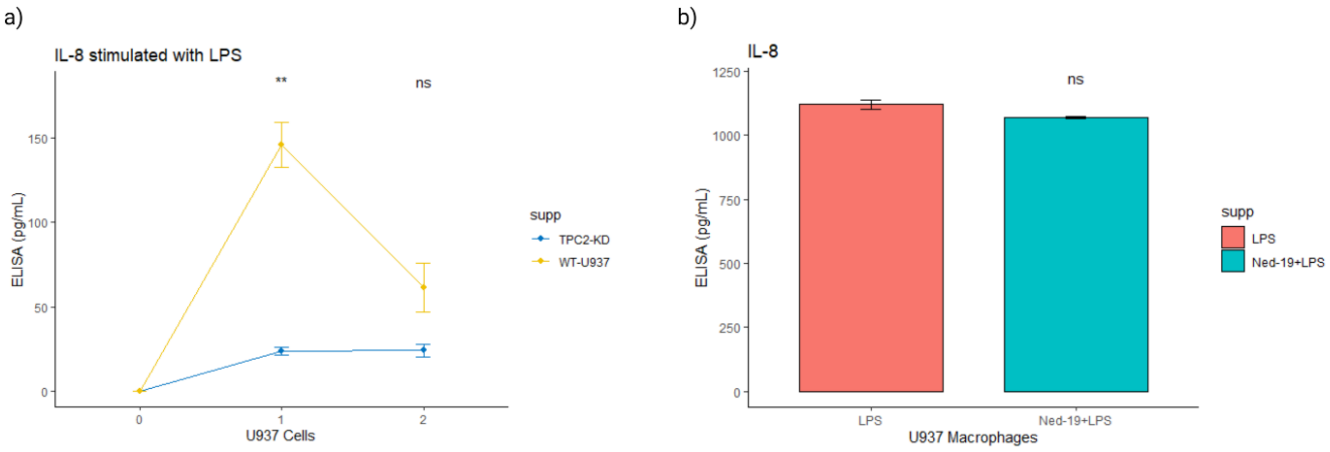


Figure 54. IL-8 protein stimulated with LPS (mean ± SEM). (a), IL-8 protein stimulated with LPS in U937 cells (Day 1 WT vs Day 1 TPC2KD, two sample -t-test, *p*-value = 0.00977), (Day 2 WT vs Day 2 TPC2KD, two sample -t-test, *p*-value = 0.115). (b), IL-8 protein levels in human U937 macrophages (two sample -t-test, *p*-value = 0.1031).

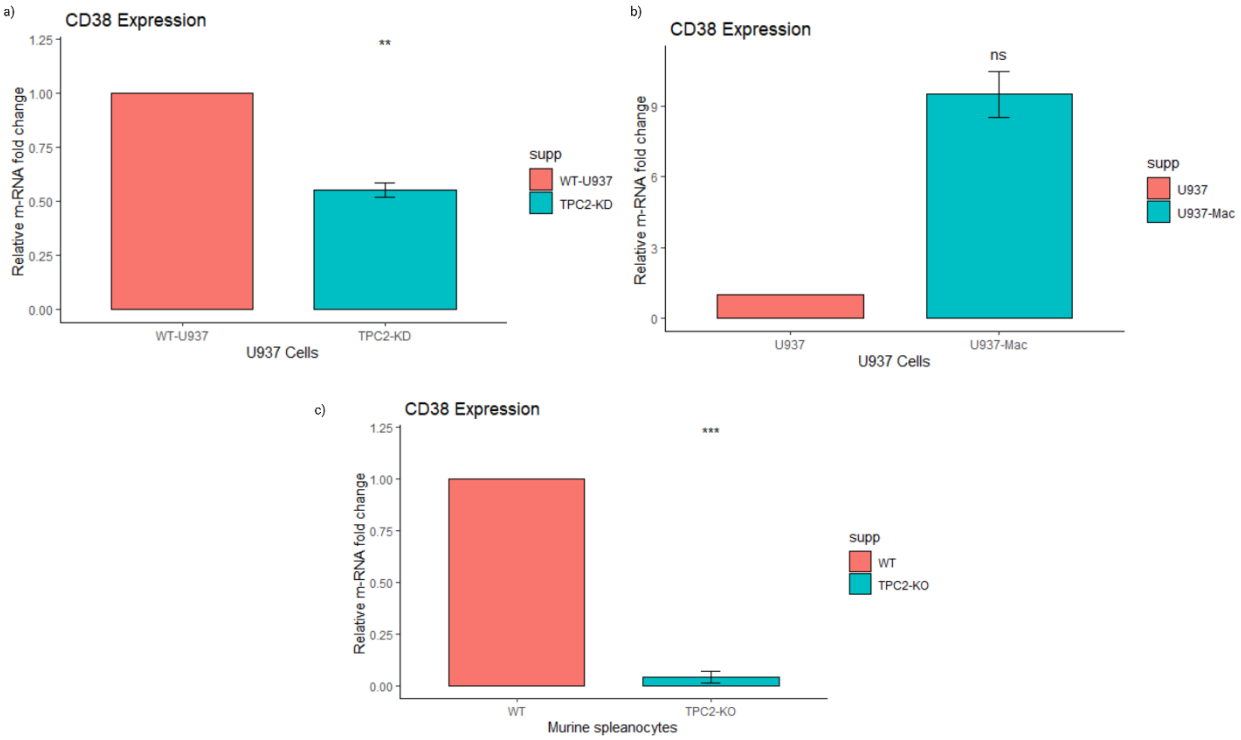


Figure 55. The mRNA expression level of CD38 (mean ± SEM). (a), in U937 cells (two sample -t-test, *p*-value = 0.00565), (b), in U937 macrophages (two sample -t-test, *p*-value = 0.07258), c, in murine spleen cells (two sample -t-test, *p*-value = 0.0008786).

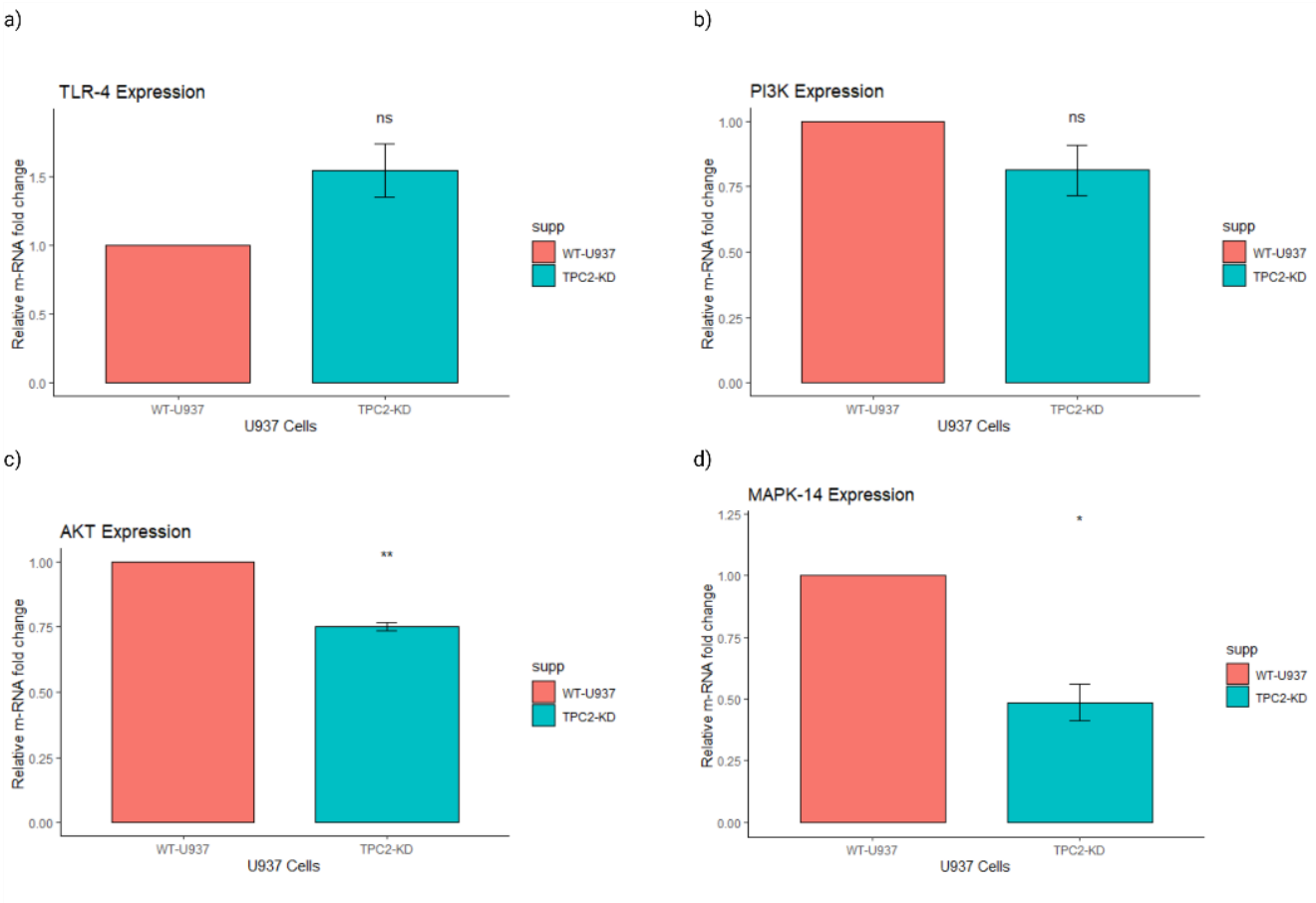


Figure 56. Real-time polymerase chain reaction (PCR) of the mRNA expression levels of TLR-4 (a), PI3K (b), AKT (c) and MAPK-14 (d) (Mean of three biological replicates (mean ± SEM). β-actin was used as an internal control. Two sample -t-test, *p*-values of TLR-4 (0.1054), PI3K (0.1952), AKT (0.004306) and MAPK-14 (0.01999).

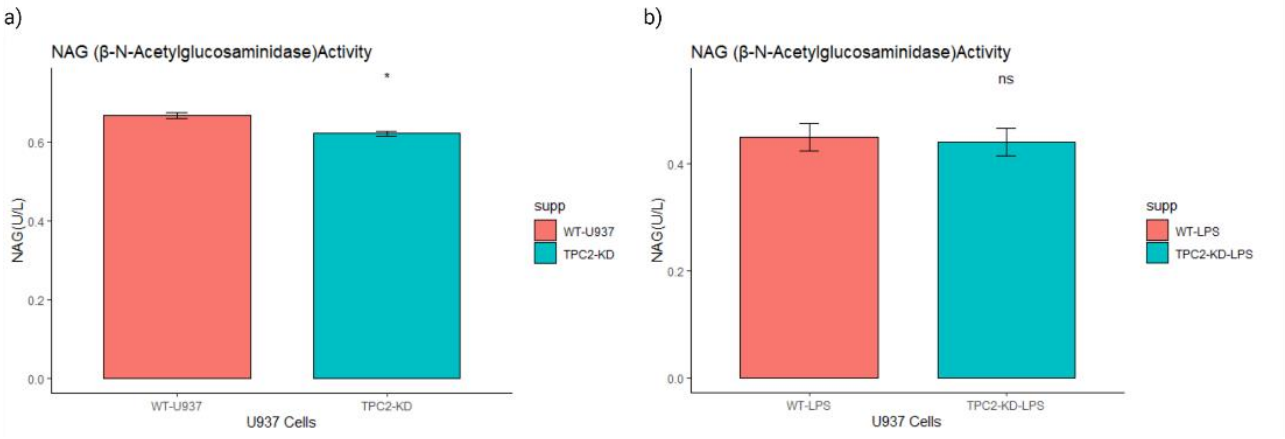


Figure 57. N-Acetylglucosaminidase Activity in U937 cells (mean ± SEM). (a) WT vs TPC2KD (Two sample -t-test, *p*-value = 0.0213), (b) WT-LPS vs TPC2KD-LPS (two sample -t-test, *p*-value = 0.8316).

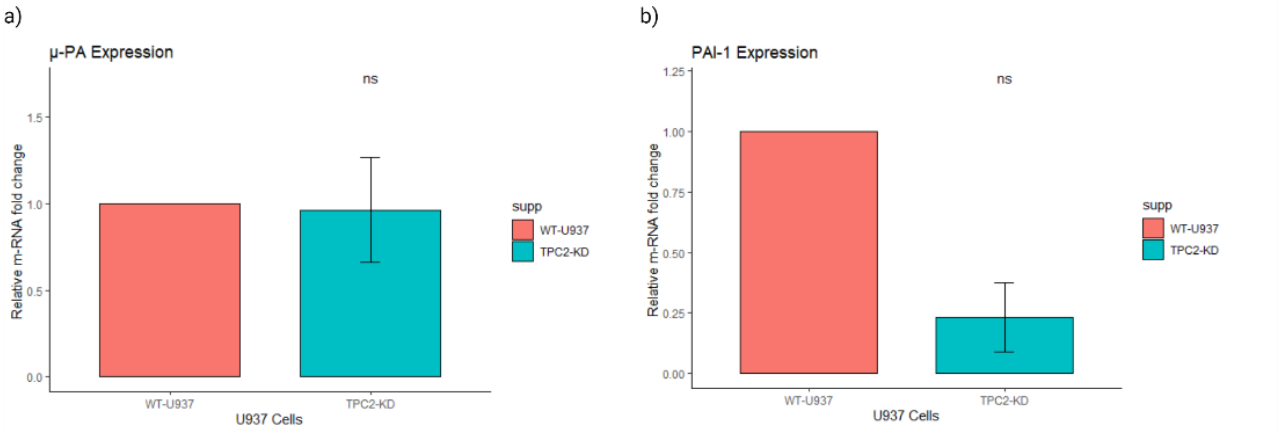


Figure 58. u-PA and PAI-1 expression levels (mean ± SEM) in TPC2 deficient monocytes. β-actinused as an internal control. P-values of (a)u-PA (two sample -t-test, *p*-value= 0.1158), and (b)PAI-1 (two sample -t-test, *p*-value =0.915).

3.3.10. Inhibition/loss of TPC2 enhances IL-10 production

To investigate whether murine spleen cells would also show changes in functionally different cytokines released by monocytes and macrophages, IL-10 levels were examined in the absence of TPC2. The production of the anti-inflammatory cytokine IL-10 was enhanced in TPC2 deficient splenocytes by 2.7 fold (as shown in Figure 59 a), and this enhancement was confirmed at the RNA level in the different biological models (as shown in Figure 59 b). To assess endolysosomal Ca^{2+} signals via TPC2 modulation of IL-10 production, I utilised the TPC2 deficient monocytes as my biological model since monocytes are one of the major sources of IL10 in the human body. Figure 59 c shows that there are non-significant higher levels of secreted IL-10 in U937 TPC2 KD by 1.4-fold when compared to U937 WT.

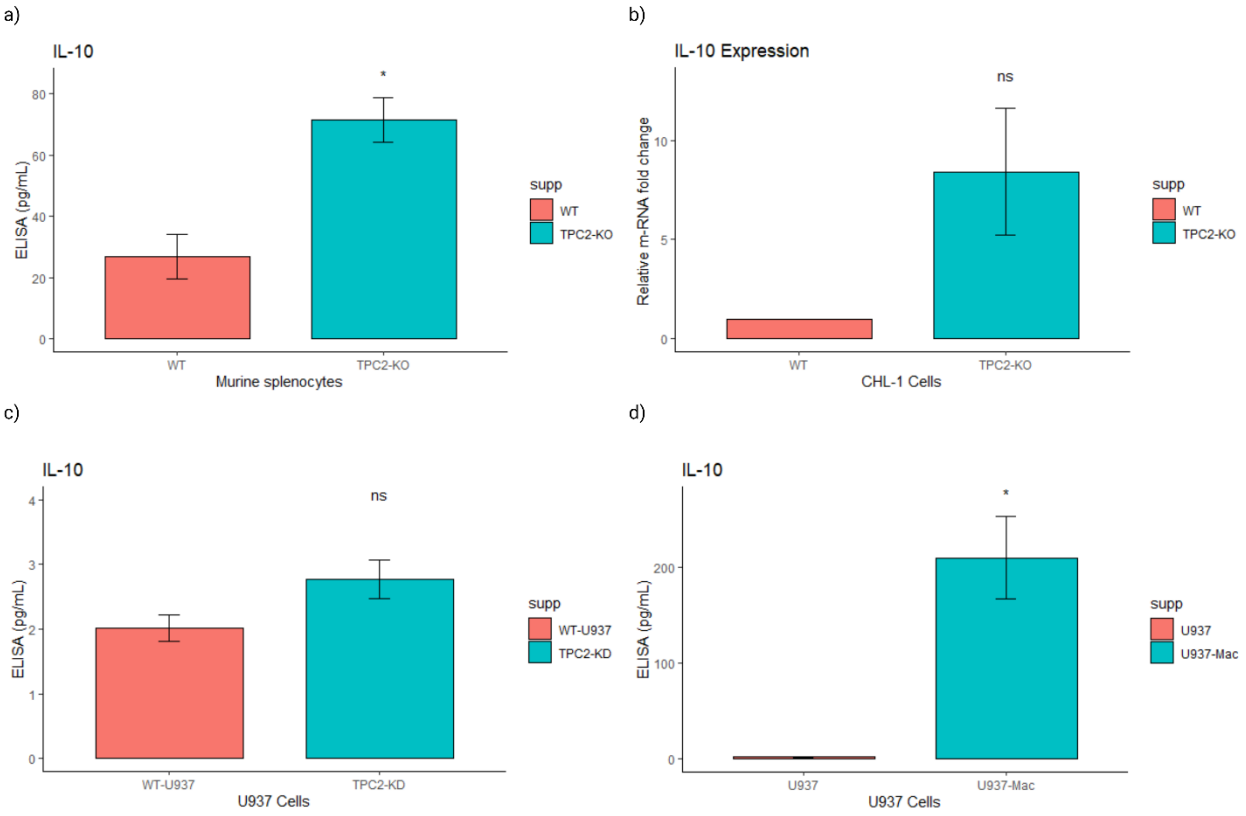


Figure 59. IL-10 expression and protein levels (mean ± SEM). (a), IL10 protein (two sample -t-test, *p*-value = 0.01211) in TPC2 KO splenocytes. (b), Real-time PCR analysis of IL10 expression with β-actinused as an internal control for human samples of three biological replicates in three independent experiments (two sample -t-test,*p*-value = 0.1463, these are Ali Hanbashi's cDNA samples). (c), IL-10 protein levels in human TPC2 deficient U937 cells (two sample -t-test,*p*-value = 0.07289). (d), IL-10 protein levels in human U937 macrophages (two sample -t-test,*p*-value = 0.04032).

To examine the possible pathway that could interact with NADDP/TPC2/ Ca^{2+} and IL10 production via MAPK14 signalling, I investigated the MAPK14 expression levels in TPC2 deficient monocytes/murine spleen cells. I observed significant differences in MAPK14 expression at the RNA level (as shown in Figure 60).

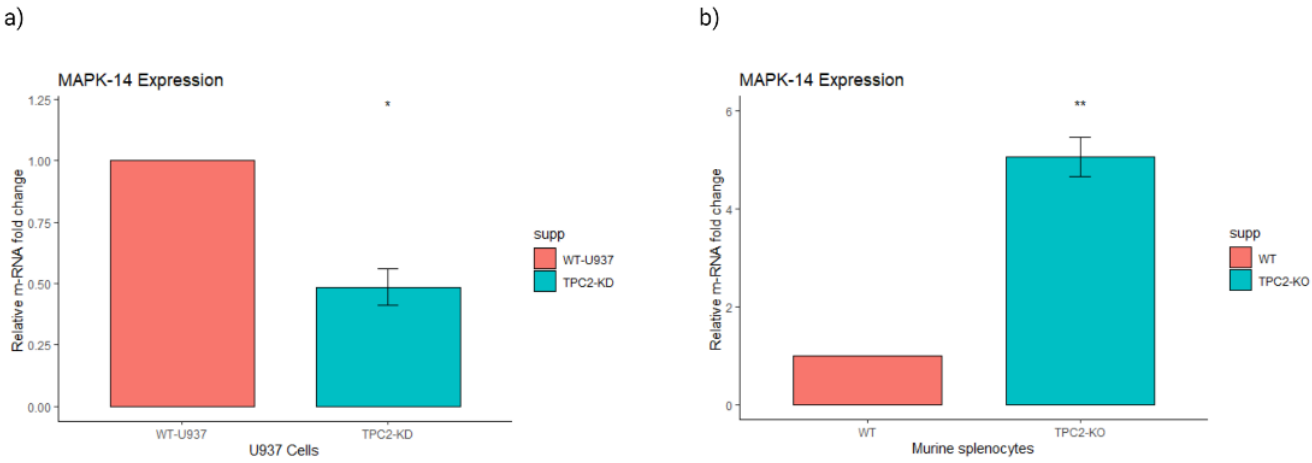


Figure 60. MAPK-14 expression levels in TPC2 deficient monocytes/murine solenocytes (mean ± SEM). (a), In U937 TPC2 KD (Two sample -t-test, *p*-value=0.01999). (b), In TPC2 KO spleen cells (two sample -t-test,*p*-value=0.009515).

3.3.11. The role of TPC2 in monocyte chemotaxis

To characterise TPC2 function in monocyte chemotaxis, U937 and U937 TPC2 KD were utilised to investigate the expression of Cdc42, Rac1 and RhoA, which are markers of chemotaxis. I observed that RhoA mRNA expression was significantly upregulated by 94 % in TPC2 deficient U937 cells. The U937 TPC2 deficient monocytes were non-significantly highly migratory toward the chemoattractant compared to WT U937 monocytes (as shown in Figure 62).

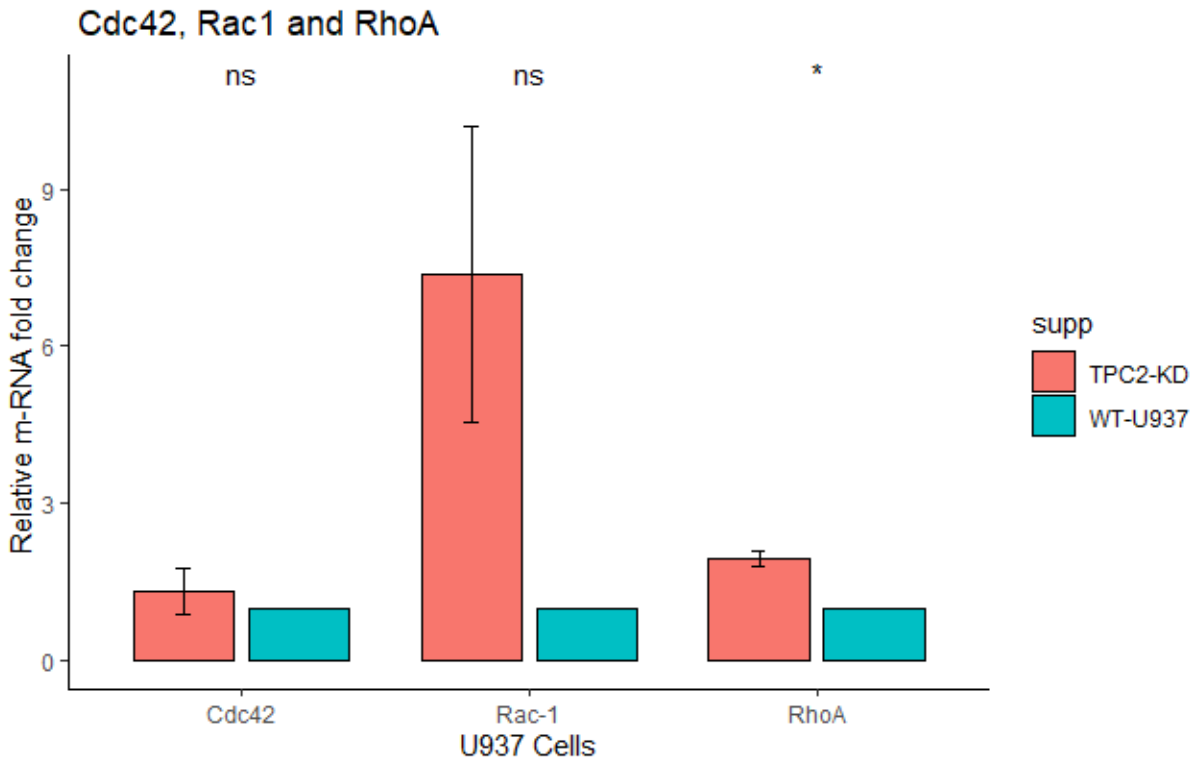


Figure 61. Cdc42, Rac1 and RhoA expression levels in TPC2 deficient monocytes. β-actinwas used as an internal control of three biological replicates in three independent experiments (mean ± SEM). Two sample t-test, *p*-values of Cdc42(0.5465), Rac1 (0.1521), and RhoA (0.02345).

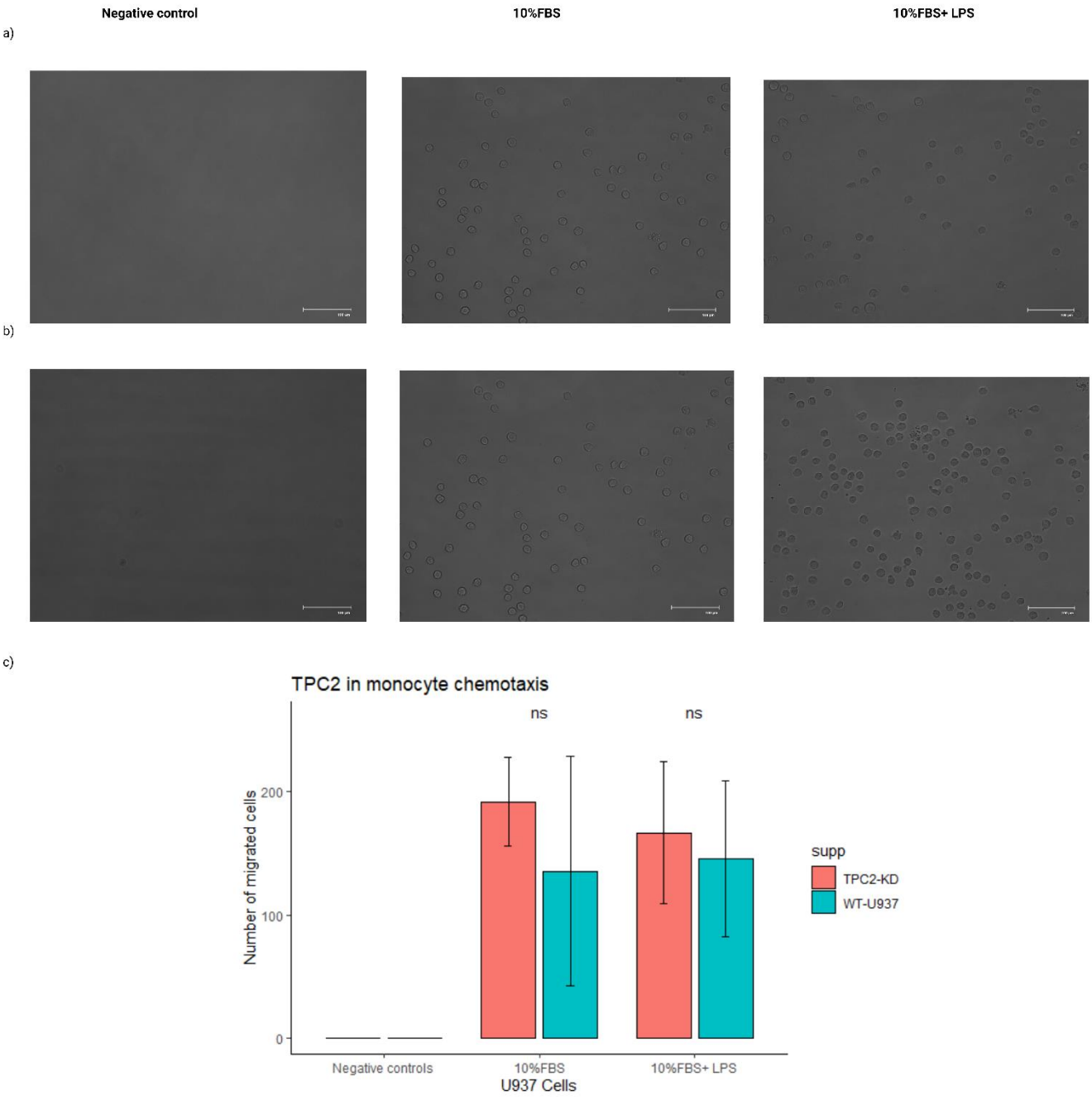


Figure 62. Microscopically characterised role of TPC2 in monocyte chemotaxis (mean ± SEM). FBS 10% WT vs TPC2 KD (Two sample t-test, *p*-value = 0.6195), FBS 10% +LPS WT vs TPC2 KD (two sample t-test,*p*-value = 0.8139).

3.4. Discussion

Recently, there has been an increasing interest in the role of endolysosomal ion channels in many aspects of immune cell function. While most previous studies have focused on TRPML channels (mucolipins), this is the first study to investigate and characterise the role of endolysosomal Ca^{2+} signals mediated through TPC2 in innate immune cells, with no previous direct links. Both members of the TPC subfamily are expressed in monocytes and macrophages, these cell types being key components of the innate immune machinery. TPC1 and TPC2 are localised in the endolysosomal system, which regulates various physiological processes related to inflammation and immunity, such as phagocytosis, intracellular trafficking and the release of inflammatory mediators (257,258). Cytokines/chemokines are crucial for intracellular communication between immune and non-immune cells, and are released from different immune cell types. Each type of immune cell secretes cytokines/chemokines through distinctive secretion pathways. Monocytes and macrophages are key components of the innate immune system machinery. It has been reported that macrophages are the major type of immune cells activated during inflammation and they lack secretory granules; hence, they mediate cytokine/chemokine release either directly through the trans Golgi network (TGN) or indirectly via recycling endosomes, late endosomes and lysosomes (259,260,261).

Until now, there has been no information about the role of TPC2 either in the regulation of monocyte differentiation and macrophage development or in the release of inflammatory mediators, especially IL-8 and IL-10, from monocytes and macrophages. Although the bone marrow is recognised as the major reservoir of monocytes, the blood and spleen are also significant sources of these cells and the biological model that contains both monocytes and macrophages. In general, the immune reactivity of splenocytes reflects the immune responses in the host (262), and as such the spleen is an appropriate model for deciphering the role of TPC2

in the release of cytokines/chemokines. To elucidate this further, we isolated the splenocytes of wild-type and TPC knockout mice. The integrin Mac-1 (CD11b) is expressed on the surfaces of a variety of immune cells, including monocytes, phagocytes (macrophages and neutrophils), granulocytes, and natural killer cells (263). We observed changes in the expression of CD11b, which is widely utilised as a macrophage marker in TPC2 KO murine splenocytes. The heterogeneity of splenic cell composition and its intervariability is a major scientific issue in studies that utilise the spleen as their biological model. To reduce bias, phenotypic characterisation should be taken into account. This study looked at the correlation between TPC2 and biological markers in the spleen at the global level without conducting characterisation by flow cytometry to study a specific population. Such findings suggest that TPC2 plays a role in the regulation of CD11b expression, which mediates cell adhesion and migration, and that these processes are associated with macrophage development. A previous study reported that ERK signalling is crucial for macrophage development but did not find any difference in CD11b expression between ERK1/ERK2 flox/flox Lyz2 Cre/Cre and WT murine macrophages(264). More recently, CD11b activation has been found to drive antitumour innate immunity by regulating tumour macrophage polarisation, vasculogenesis, and T cell recruitment (265). The possibility that there is crosstalk between such CD11b activation mechanisms and TPC2 in the context of antitumour innate immunity remains to be explored. Further studies, both *in vitro* and *in vivo*, aimed at uncovering the distinctive roles of TPC2 in murine monocyte differentiation, tumour macrophage polarisation, vasculogenesis, and T cell recruitment processes, are thus required.

Phorbol-12-myristate-13-acetate (PMA) is a potent inducer of protein kinase C, which leads to the activation of ERK signalling cascades (266). Using an *in vitro* model of human monocytes, we have shown that the expression of TPC2 in U937 nonpolarised (Mϕ) adherent macrophages increases after U937 monocytes differentiation stimulated with PMA (a chemical used to induce differentiation). PMA induced the differentiation of the U937 cells into macrophages. The

molecular mechanism of PMA in monocyte differentiation and macrophage development is still unknown. PMA acts as a stimulator of PKC, which then activates a wide range of downstream targets. RNA seq analysis revealed that PMA-induced differentiation of monocytes into macrophages via PI3K/AKT signalling (267). Similarly, we found significant differences in PI3K, AKT, and m-TRO expression levels in U937 macrophages compared to U937 monocytes. This finding demonstrates for the first time, the likely involvement of NAADP/TPC2/Ca^{2+} signalling in monocyte differentiation, indicating that TPC2 might act as a positive regulator of macrophage activation. As a previous study showed that the mammalian target of rapamycin (mTOR) pathway inhibition via rapamycin during peripheral monocyte differentiation modulates the protumour function into antitumour effects (268), I suggest a novel pathway involving M1/M2 polarisation via PI3K/AKT/m-TORC1 – whereby mTORC1-inhibition leads to intracellular Ca^{2+} signalling from endolysosomal TPC2, which in turn restores the anti-tumour effects of the macrophages.

I generated a TPC2-deficient U937 monocyte model to underpin the role of TPC2 in monocyte differentiation and macrophage development biology since there is a lack of an available specific pharmacological modulator that diminishes TPC2 function. I discovered that the TPC2-KD U937 cells were resistant to differentiation upon treatment of the cells with increasing concentrations of PMA, indicating that TPC2 acts as a major regulator of this process. Cell viability assay by trypan blue showed that the average viability was 92% after treating the TPC2-deficient cells with 100 ng/ml PMA for 48 hours. Therefore, the inability of TPC2-deficient monocytes to differentiate into macrophages is less likely to be due to the cytotoxic effects of PMA on TPC2 KD U937 cells. There are several theories regarding how TPC2 activity mediates monocyte differentiation into macrophages. It has been reported that mTORC1 negatively regulates TPC2 function. The distribution of TPC2 function in the TPC2-deficient monocytes could interfere with PMA-induced differentiation via the PI3K/AKT pathway that ultimately hinders the monocyte differentiation and macrophage development (illustrated in Figure 63 below). A previous study showed that calpain

negatively regulates the PI3K/AKT signalling pathway (269). I have observed a significant upregulation in calpain activity in TPC2-deficient monocytes that could disturb this pathway (as shown in Figure 64 below).

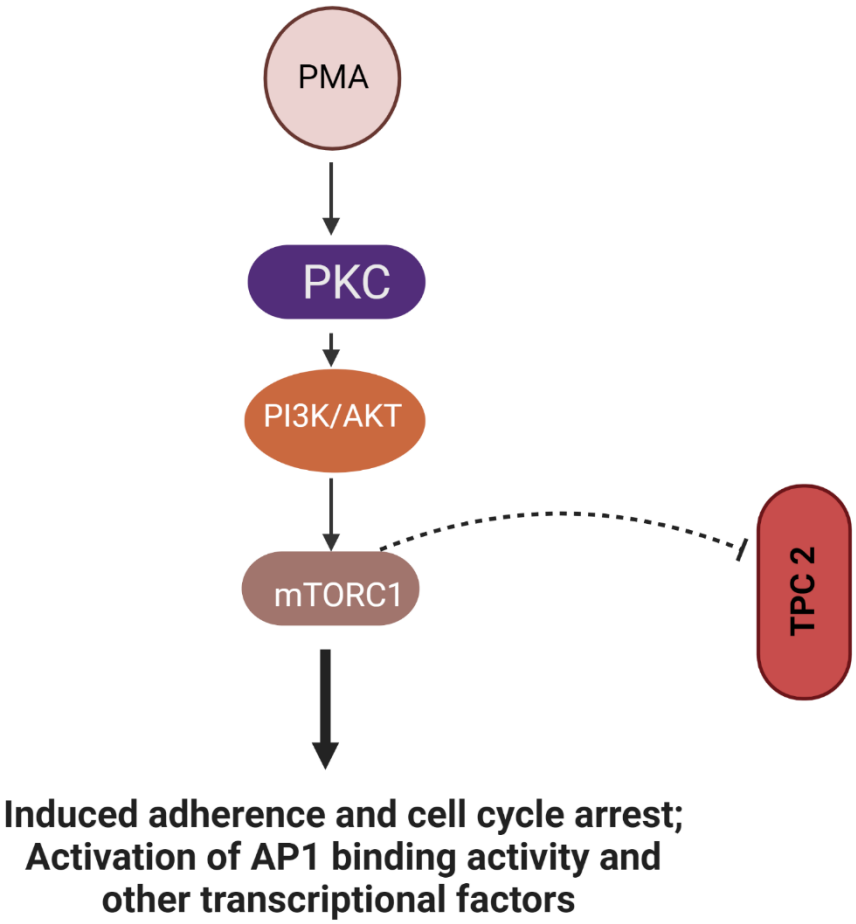


Figure 63. The PI3K/AKT pathway involving TPC2 in PMA-induced differentiation (created with BioRender.com).

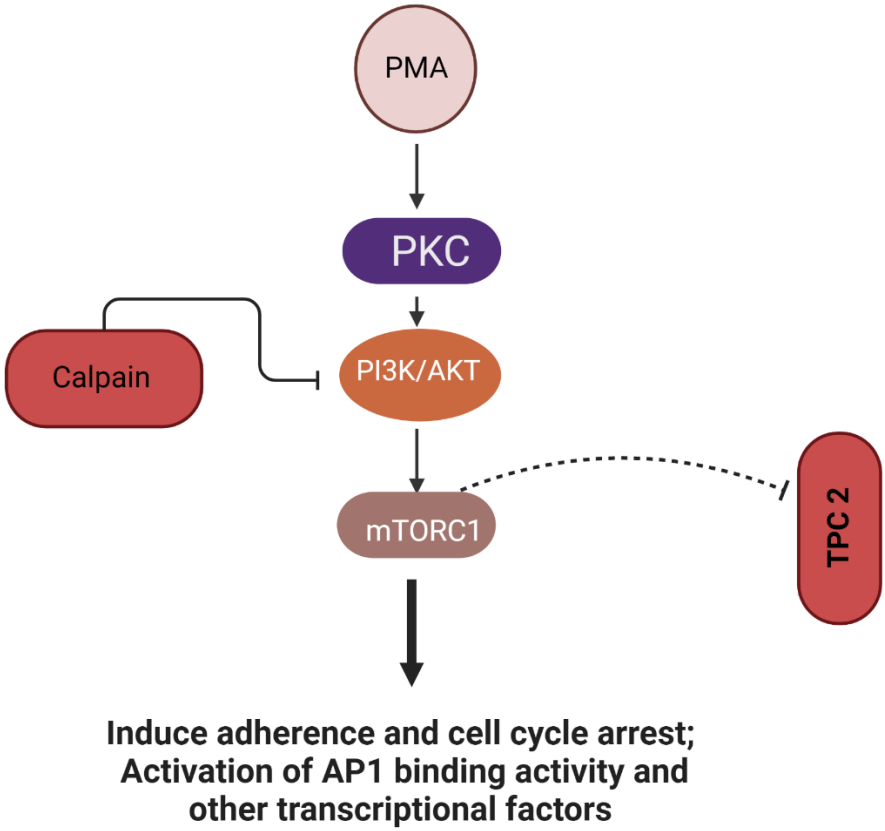


Figure 64. Calpain negatively regulates the PI3K/AKT pathway in a process involving TPC2 (created with BioRender.com).

PKC-alpha was identified as a promoter of the differentiation process (270). Along these lines, a previous study showed that inducing classical human monocyte differentiation into macrophages by stimulators (PMA or GM-CSF) requires the translocation of PKC-alpha from the cytosol to the plasma membrane (271). Therefore, PKC-alpha existence and translocation are integral to monocyte differentiation towards macrophages; upon the translocation of PKC-alpha, it becomes

activated and then phosphorylates downstream substrates. I found a significant reduction in the PKC-alpha expression at RNA level and non-significant decreased in protein level due to an outlier in TPC2-deficient monocytes, indicating that TPC2 modulates PKC-alpha expression, which plays a critical role in monocyte differentiation towards macrophages. A previous study reported that calpain negatively regulates PKC-alpha (272). I observed a significant upregulation in calpain activity in TPC2-KD U937 compared with WT U937. These data provide evidence that TPC2 acts as a major regulator of the differentiation by modulating PKC-alpha via calpain. Many questions remain to be answered, especially how calpain negatively modulates PKC-alpha. How might TPC2 affect PKC-alpha expression? My hypothesis is that knockdown of TPC2 in the U937 cells leads to an increase in some aspect of intracellular Ca^{2+} signalling, which then upregulates calpain activity (as described in Figure 65 below). Similarly, Scotto et al. (2022) have shown that the TPC2 pharmacological activator (TPC2-A1-P) compensate for the loss of function of TRPML1 in fibroblasts of Mucolipidosis type IV (MLIV) patients *in vitro* or murine models of MLIV *in vivo* (273), indicating a potential crosstalk between lysosomal cation channels. This might provide an explanation for the rise in calpain activity in TPC2 KD U937 cells.

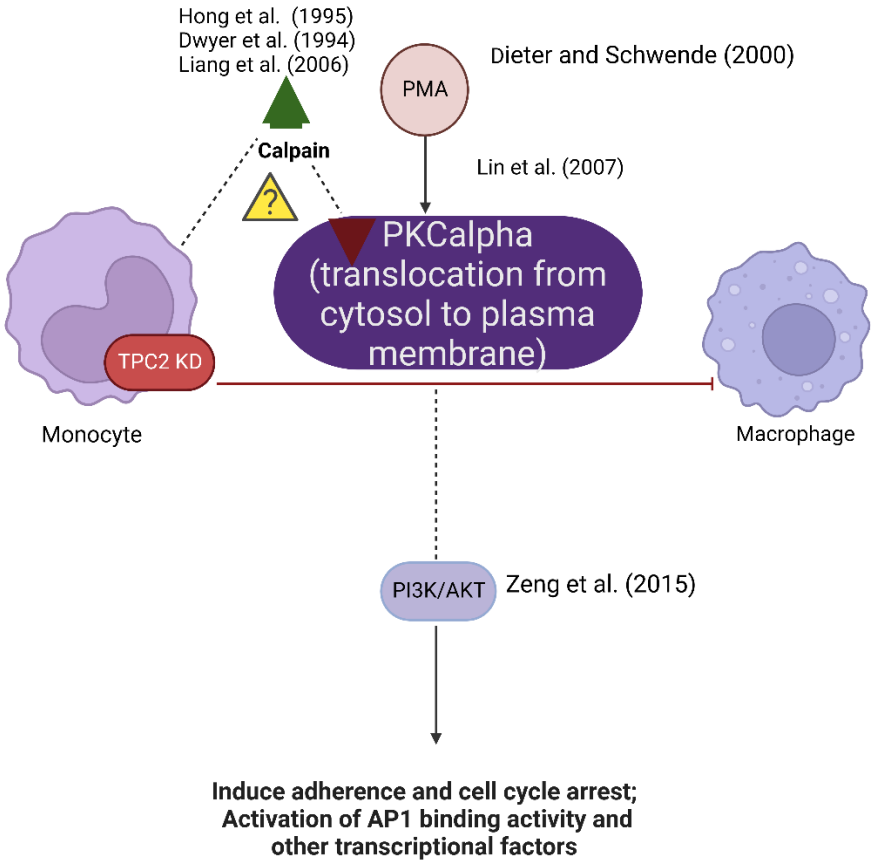


Figure 65. PKC-alpha, intracellular Ca^{2+} signalling and calpain in monocyte differentiation towards macrophages (created with BioRender.com).

However, U937 is derived from acute lymphoblastic leukaemia; hence, it still remains to be shown whether our findings also apply to macrophages developed from monocytes isolated from the blood of healthy subjects, as phenotypic and molecular characteristics of the macrophages might differ. The data highlights a previously unrecognised major role for TPC2 in monocyte differentiation and macrophage development, suggesting the candidacy of TPC2 as a viable target for cancer immunotherapy. This could be achieved by either retarding the development of TAMs via the modulation of TPC2 involvement in monocyte differentiation or TAM

reprogramming. Such a strategy is in line with a clinically approved chemotherapeutic agent that is active against different types of cancer – trabectedin, which targets macrophages by depleting circulatory monocytes, TAM and related angiogenesis (274). A recent investigation demonstrated that trabectedin elicits immunomodulatory effects in chronic lymphocytic leukaemia (275). This approach has the potential to be an adjuvant therapy to reduce chemo- or radioresistance, synergise the effects of checkpoint inhibitors and prevent cancer recurrence.

Furthermore, we sought to reveal the role of TPC2-mediated endolysosomal Ca^{2+} signalling in chemokine and cytokine production-mediated in the context of inflammatory and autoimmune diseases, and in cancer-associated inflammation. IL-8 is a proinflammatory chemokine, which has two major forms. Thrombin and plasminogen are responsible to cleave the IL-8 isoform of 99 amino acid residues. A previous study found that thrombin was mainly responsible for the proteolytic conversion of IL-8 77 isoform into IL-8 72 isoform (249). The form containing 72 amino acids is mainly found in monocytes and macrophages.

I found that IL-8 expression levels are elevated in U937 non-polarised (Mϕ) adherent macrophages compared to U937 monocytes. These results are consistent with previously published data (276). There is also prior evidence of the involvement of intracellular Ca^{2+} signalling in regulating IL-8 expression (277,278). The present study has demonstrated, for the first time, that NAADP/TPC2/Ca^{2+} signalling modulates IL-8 release from monocytes. The NAADP/TPC2/Ca^{2+} signalling mechanism regulating this IL-8 release has not yet been fully delineated. However, since pharmacological inhibition of intracellular Ca^{2+} signalling from endolysosomal TPC2 significantly reduces IL-8 levels in human cell lines, it is likely that this mechanism is endolysosomal Ca^{2+} release via TPC2 in IL-8 signalling. To further investigate these findings, I used TPC2 KO and WT mouse splenocytes to assess the levels of KC and MIP-2 (murine IL-8 homologues). Notably, the levels of MIP-2 but not KC were significantly reduced in TPC2 KO murine splenocytes compared to WT controls. Thus, TPC2 appears to be involved in

IL-8 secretion in both humans and mice. In contrast to KC at protein level, the KC expression level was significantly reduced in TPC2-deficient murine splenocytes. It is not clear why the changes in KC expression is not correlated with secreted protein levels. A previous study has shown that monocytes/macrophages are a source of both KC and MIP-2; they are synthesised through the MYD88 pathway (279). IL-8 and IL-8 homologues (KC and MIP-2) are responsible for the recruitment of neutrophils, which is the first fundamental step of innate immune defence against a pathogen (280). It has been reported that IL-8 acts as a messenger to cross-link inflammation and tumour epithelial-mesenchymal transition (EMT), involving MAPK signalling (281). IL-8 also plays a pivotal role in inflammation and cancer development, from tumour initiation to metastasis. Therefore, indirectly inhibiting the effects of IL-8 signalling through the modulation of TPC2 function may serve as a viable therapeutic target for patients with inflammation, immune diseases and cancer.

TPC2 modulation, either by genetic reduction or by pharmacological inhibition by trans-Ned-19, reduced the release of the pro-inflammatory mediator (IL-8) from monocytes. Accordingly, in this study, the release of IL-8 was stimulated by LPS to define the cellular mechanisms underlying the regulation of TPC2-mediated endolysosomal Ca^{2+} signals in monocytes. There is prior evidence of the involvement of intracellular Ca^{2+} signalling in regulating IL-8 expression. Intriguingly, CD38 levels were significantly decreased in TPC2 KO murine splenocytes or TPC2-deficient monocytes. CD38 has a plethora of functions, ranging from generating NAADP and involvement in the IL-1 response to a role in cancer, particularly in multiple myeloma (282). This finding suggests the modulation of TPC2 as a potential therapeutic approach for treating cancer, particularly in patients with haematological malignancies. In 2015, the FDA approved DARZALEX® (daratumumab), which is an anti-CD38 monoclonal antibody (mAb) used to treat multiple myeloma (283). Taken together, endolysosomal Ca^{2+} signalling partially mediated by TPC2 represents a novel candidate target for inflammatory and cancer immunotherapy.

A published abstract found that CD38 has a profound influence on TLR4, possibly contributing to the dysregulated expression of inflammatory factors (TNF-α, MCP-1 and IL-6) (see Figure 66) (284). This finding is contrary to that of Li et al. (2019) who found that TLR4 expression was significantly enhanced in $CD38^{-/-}$ mice (200). I observed a significant reduction in the CD38 expression level in TPC2-KD cells, and this could be attributed to the reduction in IL-8 levels in TPC2-deficient monocytes. Therefore, I investigated the expression of TLR4 in TPC2-KD cells and found a non-significant difference in the TLR4 expression level in TPC2-deficient monocytes compared with WT U937 monocytes.

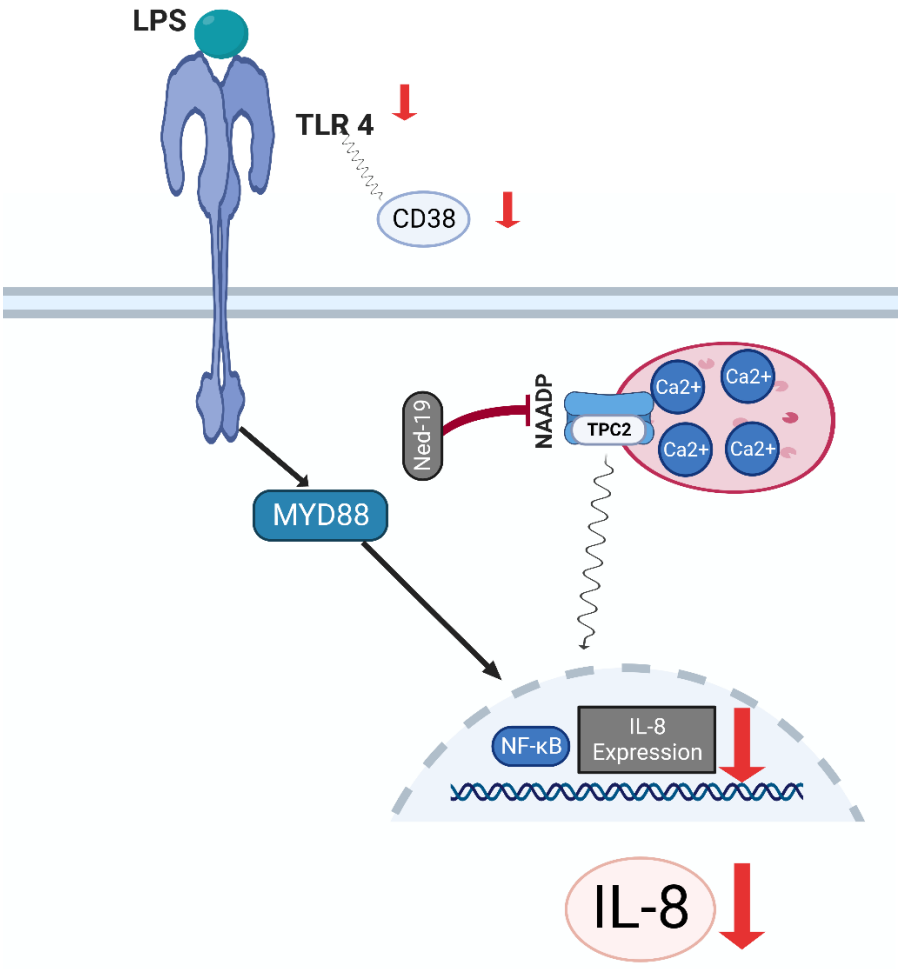


Figure 66. CD38 regulates IL-8 via TLR4 (created with BioRender.com).Although the molecular mechanism by which TPC2 regulates IL-8 production and release in monocytes remains to be clarified, a potential regulation pathway of TPC2 signalling in IL-8 production exists. Recently, I observed significant reductions in AKT and MAPK expression levels in TPC2-KD cells. Previous studies implied the vital roles of PI3K/AKT and MAPK in IL-8 secretion in various types of cells (as shown in Figure 67). In addition, I further investigated the role of TPC2 in IL-8 release and trafficking and attempted to distinguish between LE/LY and EE/RE as possible secretion pathways. Based on the data, TPC2 in EE/RE is involved in LPS-mediated IL-8 production.

IL-8 levels were elevated in cancer patients and correlated with poor prognoses (285,286). HuMax-IL-8 (BMS-986253), an anti-IL-8 monoclonal antibody that under clinical investigation in phase 2 trial (287). .Knocking out TPC2 was identified as having an influence on IL8 and CD38 expression levels in U937. However, the bioinformatic data utilising Correlation AnalyzeR illustrates significant positive weak correlations between TPCN2, IL-8 and CD38 in cancer at a global level (see Figure S3.3 Supplementary). In the correlation analysis, all types of cancer were included. Therefore, the observed impact of TPC2 on IL8 and CD38 expression levels could be specific to the biological context.Despite the exploratory nature of our evidence highlighting the role of endolysosomal Ca^{2+} signalling via TPC2 in IL-8 release, my findings provide the first insight into the role of NAADP/TPC2/Ca^{2+} signalling as a regulator of IL-8 release, raising questions regarding the clinical utility of TPC2 as a biomarker or immunotherapy target to modulate immune responses. Many questions remain to be answered. For example, how does TPC2 affect IL-8 release at the molecular level?

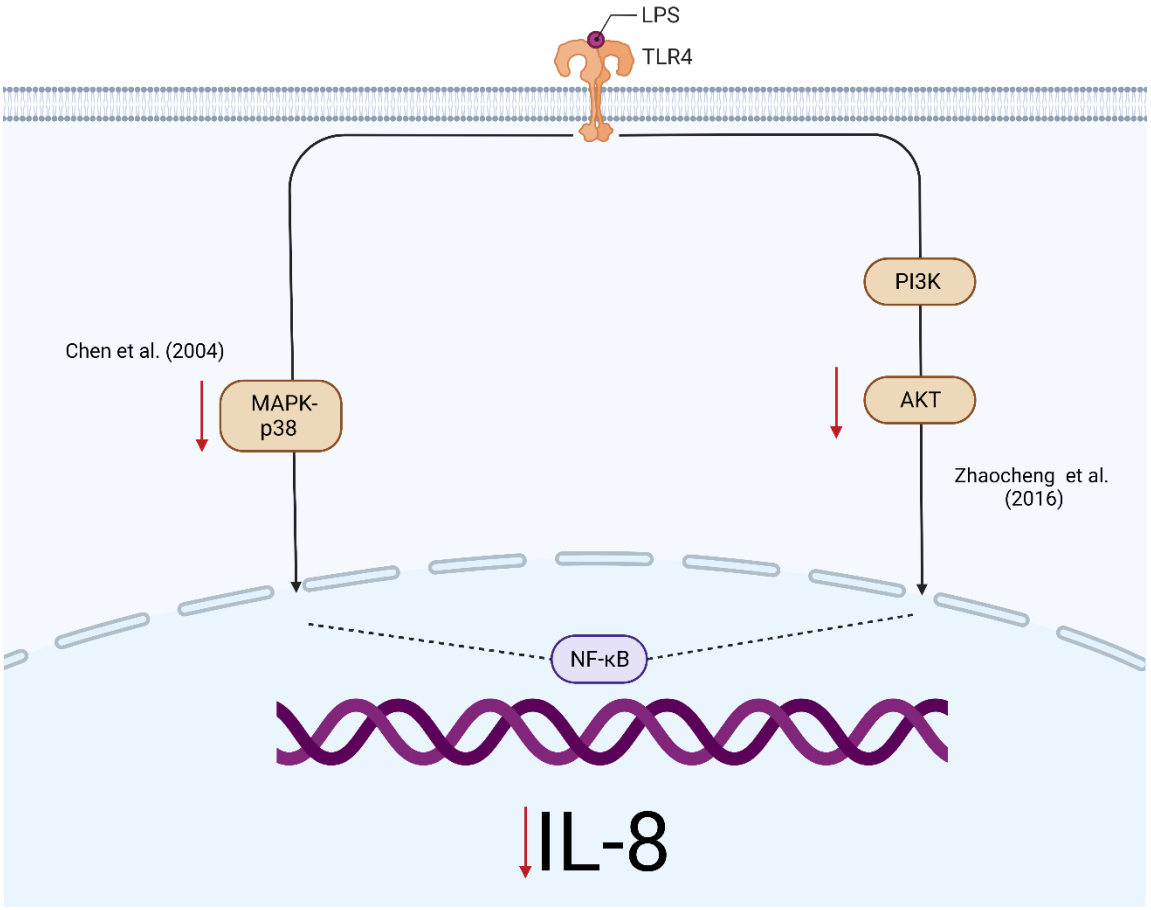


Figure 67. TPC2 mediates IL-8 release via PI3K/AKT and MAPK signalling pathways (created with BioRender.com).

In contrast to our finding that the inhibition of NAADP/TPC2/Ca^{2+} signalling reduced IL-8 (pro-inflammatory chemokine) levels in U937 monocytes, elevated non-significant levels of IL-10 (anti-inflammatory cytokine) were found in the same system. Enhanced IL-10 production was also observed in TPC2 KO murine splenocytes, but it reached the significance level. It has been reported that the MAPK cascade is involved in IL-10 production (288). My suggested signalling pathway involving a role for TPC2 for IL-10 production is described in Figure 68.

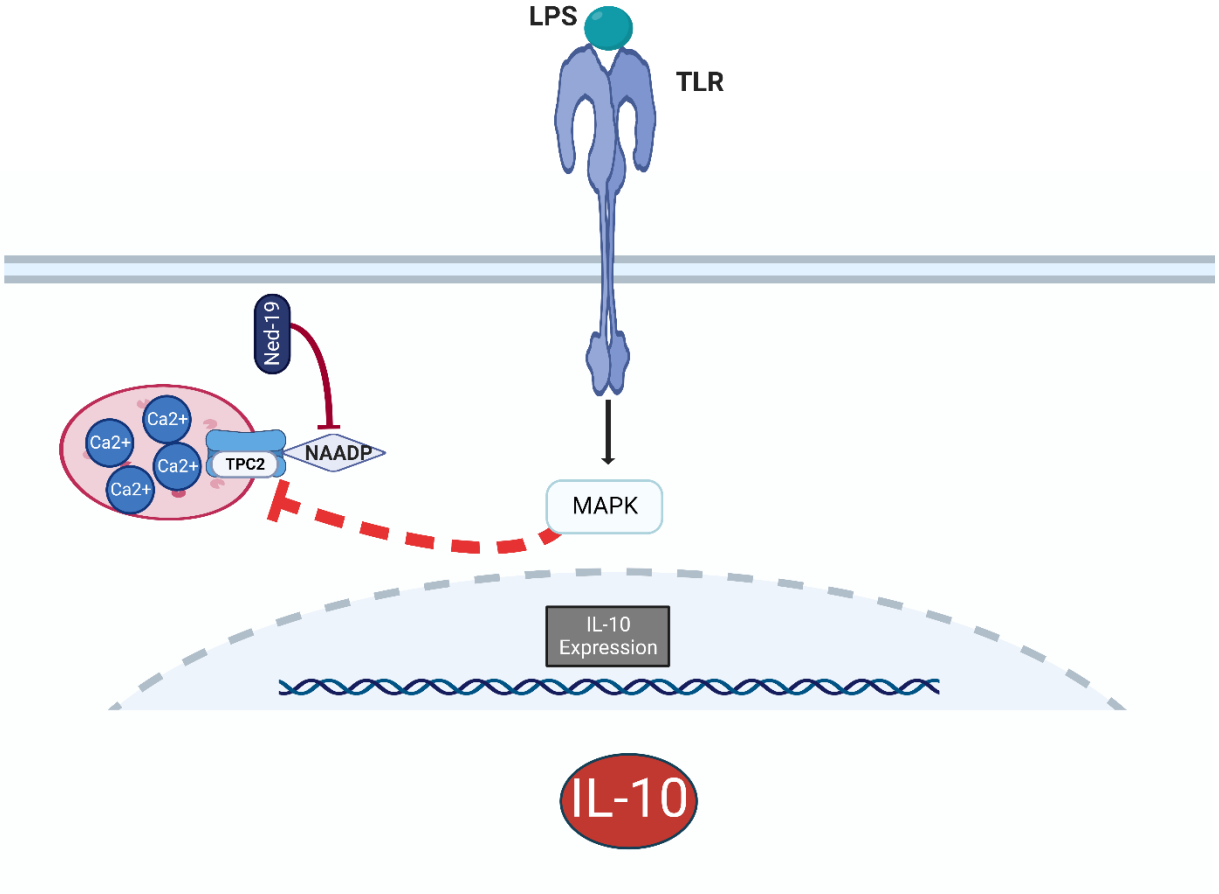


Figure 68. The role of TPC2 in IL-10 production via MAPK signalling (created with BioRender.com).

These data demonstrate that NAADP/TPC2/Ca^{2+} signalling is, biologically and significantly, involved in IL-10 production in both humans and mice and statistically significant only in mouse models. IL-10-deficient mice were found to be more susceptible to chemically induced skin cancers (37). In the same vein, it has been reported that mice and humans deficient in IL-10 have developed tumours spontaneously and at high rates (38). In addition, tumour rejection has been observed in an IL-10 overexpression model of B16-F1 melanoma injected into syngeneic mice or after treatment with recombinant human IL-10 in *in vivo* mouse models of colorectal carcinomas, sarcomas, or melanomas (289, 290). Genome-wide association studies have shown a correlation between IL-10 and various inflammatory diseases (291). Therefore, I believe that a further

understanding of how TPC2 inhibition or absence results in greater IL-10 levels will reveal the complex interplay between TPC2, IL-10 production, and key pathophysiological processes. Several IL-10 trials focusing on inflammatory diseases have been registered with the U.S. National Library of Medicine (clinicaltrials.gov) (292). However, the short half-life of IL-10 in patients, as well as the ubiquity of targeted therapy, may limit its therapeutic benefits. Consequently, a better understanding of the molecular mechanisms underlying IL-10 production, alongside the identification of related, novel, and specific downstream targets for drugs, requires further investigation.

I explored the role of TPC2 in monocyte chemotaxis, I observed that TPC2 knockdown non-significantly upregulated the monocytes chemotaxis and significantly enhanced RhoA expression at RNA level. These data must be interpreted with caution until the role of TPC2 in monocyte chemotaxis is quantitively characterised using appropriate approaches.

3.5. Conclusion

In summary, with growing evidence linking endolysosomal Ca^{2+} signalling to cellular development, inflammation, immunity, and cancer, this study reveals the possibility of the involvement of endolysosomal Ca^{2+} release via NAADP/TPC2/Ca^{2+} signalling in the innate immune system, from monocyte differentiation to cytokine production. I observed increased levels *in vitro* of TPC2 mRNA in human macrophages. TPC2-deficient human monocytes were resistant to the differentiation and to macrophage development. In addition, the levels of IL-8 were significantly reduced after treating U937 cells with Ned 19 (an NAADP antagonist) or TPC2 knockdown. MIP2 levels, but not KC, were significantly reduced in murine TPC2 KO splenocytes compared with WT controls. IL-10 levels were non-significantly elevated in TPC2-deficient human cells, but it was significantly increased in TPC2-deficient murine splenocytes compared with WT splenocytes. My data showed changes in CD11b after knocking out TPC2. These findings suggest that TPC2 may

act as a regulator of monocyte differentiation into macrophages and of chemokine and cytokine release. Furthermore, these findings raise important questions regarding the clinical utility of targeting TPC2 to eliminate tumour-associated macrophages via DNA nanodevice to deliver directly and precisely to the site of cancer without affecting the healthy cells or to indirectly modulate IL8 release, opening the possibility of innovative and novel strategies to treat patients with inflammatory and immune diseases or cancer.

Chapter 4: Proteomic analysis reveals the possible roles of TPC2 in pathophysiological processes attributed to cancer immunity

Chapter 4: Proteomic analysis reveals the possible roles of TPC2 in pathophysiological processes attributed to cancer immunity

4.1. Introduction

4.1.1. Proteomic analyses in haematology, immunity and oncology: Recent advances, and opportunities

The term proteome was derived from protein and genome, and introduced to the scientific field by Wilkins et al. in 1996 (293). It refers to the full profile of protein in cells/tissue/biological sample/condition. Recently proteomics has revolutionised biology and biomedical sciences by providing a powerful research tool to study the changes in the protein levels in two or more conditions. Mass spectrometry (MS)-based proteomics has led to important new scientific insights, revealed fundamental aspects in biology and derived valuable biomedical discoveries and medically applicable information. MS characteristics (low sample requirements, high sensitivity and ability to identify and measure the protein expression, the availability of the technology in various research institutions across the world), and its current status that is built on the maturity of the technology, makes it a suitable tool to perform proteomic analysis.

The comprehensive proteomics analysis of the haematological system components opens doors to tackle complex scientific questions regarding their function, communication at molecular level, contribution to develop pathological condition, and possible therapeutic application. A previous blood cell proteome analysis conducted by Bryk and Wiśniewski (2017) discovered and reported for the first time that drug transporter and choline transporters involving ABCA7, SLC44A1, and SLC44A2 are located on the red blood cell membrane (294). In 2016 Geyer and his colleagues developed rapid (~ 3 hours), robust, and high throughput plasma proteome profiling technique to serve as a biomedical tool in clinical settings by delivering the complete plasma profile of the

subject that contributes ultimately to formulate a clinical decision (295). The evolving knowledge acquired from utilising MS-based proteomics in transfusion medicine might optimise blood transfusion therapy by providing valuable data regarding the changes in the protein profile especially during the processing and storage phases and by delivering significant information with therapeutic relevance (296).

A recent investigation of the role of the platelet proteomic signature in adverse transfusion reactions (ATR) revealed that differentially expressed proteins in blood platelet attributed to ATR (297), this exploration of platelet proteome profiling shed the light on possible informative clinical tool that might shift the paradigm of blood transfusion therapy in the future.

Proteomics is a valuable resource that helps us characterise and elucidate the complexity of the immune cells through delivering informative proteomic signature of immune cell components in health and disease states. Rieckmann et al. (2017) deciphered the architecture of immune cell communication by proteomic analysis (298). They found that some proteins are highly expressed in a specific immune cell type and some immune cells formed a dense cluster of proteins (298). These findings highlight the significance of utilising proteomics to allow discovery of new markers to identify an immune cell/population and to provide insights into immune cell communication and which cells are biologically similar at functional level. In the era of COVID-19, the mass spectrometry proteomics greatly advances our understanding especially regarding the biological basis of multisystem inflammatory syndrome (MIS) pathology in children and has revealed new prognostic/diagnostic biomarkers, including ferritin light chain, tenascin C, QSOX1, FCGR3A, classical complement cascade(C1qA, C1qB, and C1qC), histidine-rich glycoprotein (HRG), sex hormone–binding globulin (SHBG), and complement component 7 to assess severity of the MIS in the paediatric population (299). Recently, Nie et al. (2021) identified the proteins that are differently expressed in 7 organs of 19 COVID-19 patients (as shown in Figure 69) to expand our scientific knowledge of COVID-19 biology (300). Other than the SARS-CoV2 virus,

the proteomics landscape of the hepatitis B virus offers possible therapeutic and diagnostic applications (301).

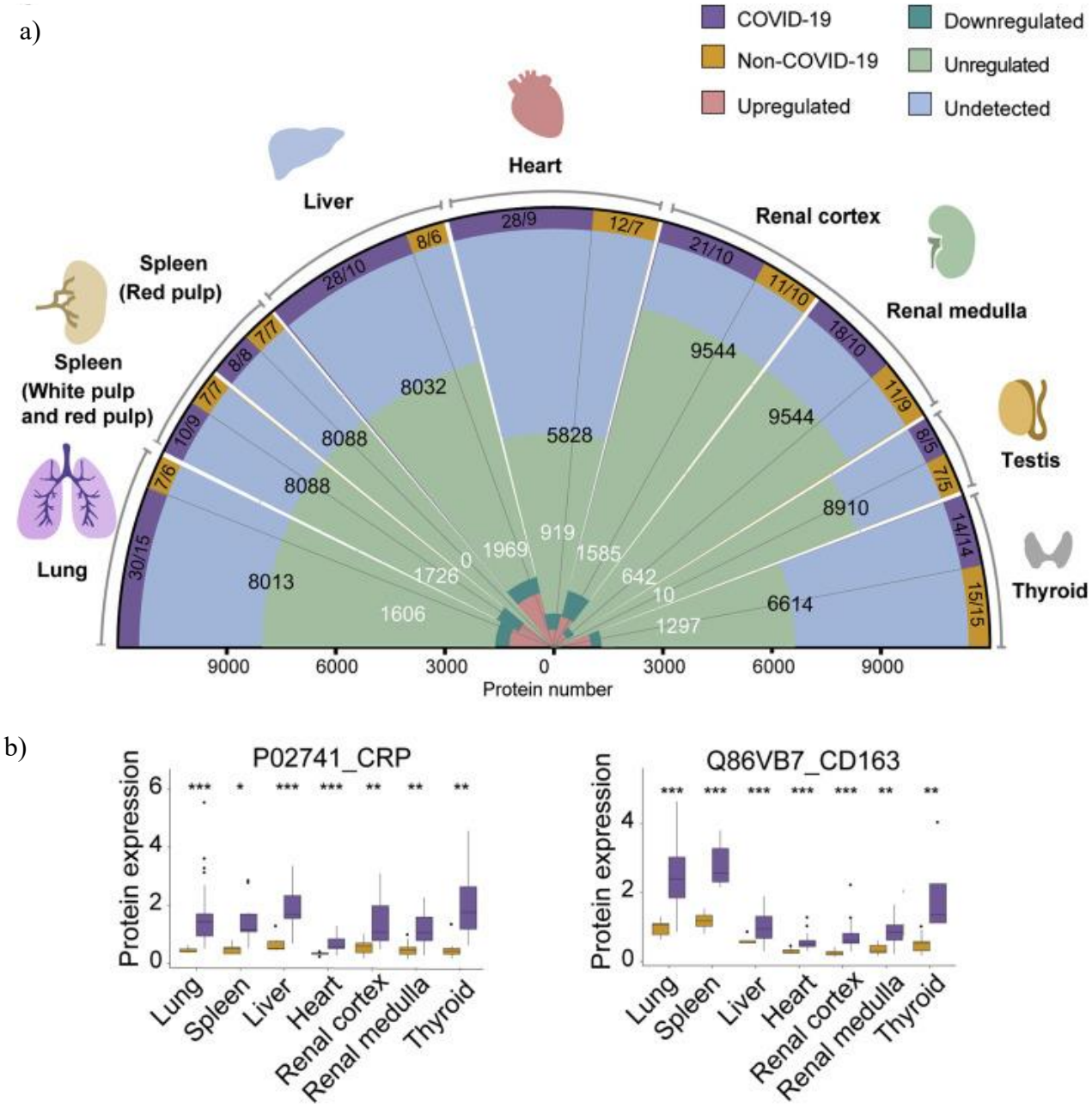


Figure 69. The multi-organ proteomic signature in COVID-19 biology (300) (the figure was reproduced from Nie et al., under Elsevier copyright)

The major drawback of this technique is its inability to discover low abundance proteins in the presence of highly expressed proteins, and difficulties in using it to measure the hydrophobic intensity, low or high molecular mass proteins, or proteins with an extreme isoelectric point or poor solubility (302,303,304). Oncology proteomics is a step towards precision medicine, as it reflects a promising approach for revealing the cellular mechanisms underlying cancer pathology and identifying new molecular targets/biomarkers to enhance cancer patients' therapy. It provides for the pre-candidacy of biological markers from correlation and enrichment analyses to diagnostic, prognostic and therapeutic biomarkers. Recently, MS-based proteomics identified the metabolic signatures of acute myeloid leukemia that might lead to the development of novel therapies to overcome the resistance to chemotherapy (305). Plasma proteome profiling of cancer patients before and after the treatment with Immune checkpoint blockers (ICB), underscored the biological and clinical significance of leukemia inhibitory factor (LIF) in directing immune checkpoint inhibitors (306). The upregulation of LIF at the protein levels were correlated with poor clinical outcomes in cancer patients treated with ICB (306). Despite the advancement in cancer therapy brought by the discovery of Immune checkpoint blockers, developing resistance limits its therapeutic application in some patients. Decoding the role of LIF in immune checkpoint blockers resistance might lead to the development of therapeutic agents to optimise ICB therapy and identify subjects who are likely to benefit from immunotherapies.

Proteome profiling delivers an informative tool to advance therapy and enhance diagnosis in various diseases, but it's still in research phase and not translated into the clinics. In the foreseeable future, un/ or targeted proteomics might be implemented in daily clinical practice to pave the way for personalized medicine. From my perspective, one of the potential pitfalls that hinder proteomics based clinical applications is that physicians are not prepared to comprehend proteome data during their medical training and the lack of availability of health care programs

that integrates proteomics data to deliver clinical-decision suggestions based on the subject proteome landscape.

4.1.2. The landscape of TPC2 genetically modified model proteomic profiling status and potential therapeutic significance

Proteomic analysis of genetically modified models at the cell or animal level has expanded our knowledge of gene function and its role in health and disease. Two pore channels make up a superfamily of cation channels, located on the endolysosomal system, that have two major subtypes in mammalians: TPC1 and TPC2. Despite the accumulated data that highlighted the vital roles of the NAADP/TPC2/Ca^{2+} signalling pathway in various pathophysiological processes in different biological systems, to my knowledge, TPC2 inhibition through gene editing impact on the proteome landscape has not yet been explored. The utilisation of spleen cells as a research tool to expand our scientific knowledge of human cancer biology is still considered a valuable source of research. However, the heterogeneity of spleen tissue is one of the drawbacks of this model. The phenotypic characterisation of the specific spleen-cell population by flow cytometry reduces inter-individual variability. In my thesis, I am presenting the first proteome data of murine splenocytes after TPC2 KO to reveal the impact of endolysosomal Ca^{2+} signalling via TPC2 in physiological processes attributed to haematology, immunity and oncology, which might lead to biomedical and therapeutic discoveries.

4.1.3. Unveiling the proteome landscape of splenic murine cells to understand complex and integrated biological systems in mice and provide insights into human biology

The spleen is a valuable biological model that contains heterogenous populations of cells, including stem cells with multi-lineage capacities, myeloid and dendritic cells. In addition, it is responsible for filtering blood-borne antigens and maintaining erythrocytes as a part of its function as a secondary lymphoid organ. Monocytes and macrophages are myeloid cells that play a vital

role in inflammation, haematology, immunity, and cancer. The spleen is an important source of monocytes, which act as direct precursors to haematopoietic stem cell-derived macrophages. Murine monocyte frequency in the spleen ranges from 3.5% to 5%, whereas in peripheral blood it is 2% to 3%. Circulating peripheral blood monocytes are known as circulating (non-resident) precursors of macrophage-mediated physiological/pathological responses. Paradoxically, splenic monocytes reside in the spleen and do not circulate. When triggered, they move to the site of the injury to modulate inflammation (307). Swirski et al. (2009) conclude that splenic monocytes and blood monocytes in mice are relatively similar morphologically, transcroipmically, proteomically, and functionally (307). In general, murine spleen biology resembles that found in humans. However, human monocyte markers are CD14 and CD16, whereas the murine monocyte identifier is Ly6C. Human monocytes are classified into three main subsets: classical (CD14+CD16-), non-classical (CD14dimCD16+), and intermediate (CD14+CD16+). Murine monocytes fall into two main populations: Ly6C+ and Ly6C-.

The utilisation of spleen cells as a research tool to expand our scientific knowledge of biology is still considered a valuable source of research. The use of a defined subpopulation of splenic cells as a research tool to expand our scientific knowledge of leukaemia was introduced by Dieguez-Acuña et al. (2010). They found that some proteins (illustrated in Figure 70) attributed to cancer pathology were expressed in Hox11+D45− (non-lymphoid) splenic stem cells and in Hox11+ T-cell lymphoblastic leukaemia (T-ALLL) (308). The proteome of splenic murine cells indicates the immunological significance of 10-hydroxydec-2-enoic acid in murine biology. Fan et al. (2020) observe that proteins involved in enhancing the immune system are highly upregulated after treating immunocompromised mice generated by cyclophosphamide with 10-hydroxydec-2-enoic acid, which highlights the potential immune protective effect of 10-hydroxydec-2-enoic acid (309). This warrants further investigation.

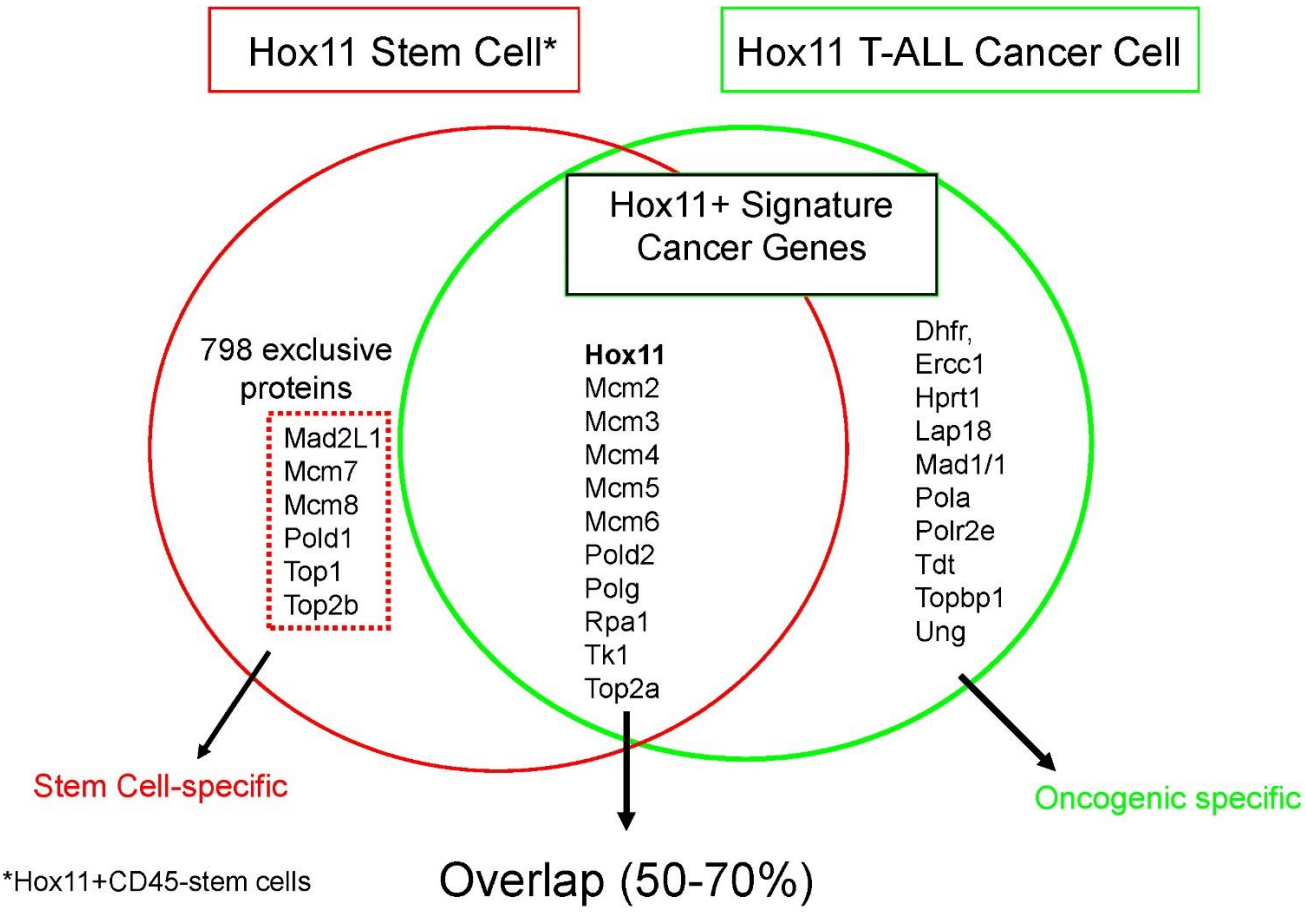


Figure 70. MS-based splenic cell proteomics reveal that cancer genetic signatures overlap with stem cell-specific proteins in the spleen and Hox11 T-ALL cancer cells (308) (the figure was reproduced from Dieguez-Acuña et al., under the license number 5405460642691).

The diversity of splenic cells and their involvement in various biological systems make them useful research tools in determining the role of two-pore channels (TPCs) in the fundamental processes of various diseases. Besides this, the analysis is intended to serve as a go-to resource to provide scientific insights into TPC2 functions in the pathophysiological processes related to haematology, immunity, and cancer.

4.1.4. Significance, innovation, overall hypothesis and objectives

TPCs are cation channels involved in the regulation of a wide range of pathophysiological processes. Recently, increasing evidence links endolysosomal Ca^{2+} signalling via TPCs with cancer and immunological and haematological disorders. Despite the growing proof that links endolysosomal Ca^{2+} signalling with the pathophysiological processes related to the development of various diseases, there is a paucity of studies that categorise TPC functions in health and disease at the proteome level. This study utilised label-free protein analysis of splenic murine cells to uncover the possible roles of endolysosomal Ca^{2+} in signalling via TPC in inflammation, immunity, haematology, and cancer. I hypothesise that TPC2-mediated endolysosomal Ca^{2+} signalling plays a pivotal role in physiological processes related to haematological and immune systems and contributes to the development of inflammation, cancer, and haematological and immunological diseases. My objective was to profile the splenic proteome and study global changes in proteins expressing or not expressing TPC1/TPC2 in murine splenocytes. This research was mainly conducted to provide novel insights into the cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in cancer immunity. This process could be linked with the pathophysiological processes related to cancer, with the future possibility of targeting TPC2 as an innovative strategy in immunotherapy cancer treatment.

4.2. Methods

4.2.1. Mice and Isolation of murine splenocytes

Wild-type, TPC2 KO, TPC1 KO, and TPC1/TPC2 double KO mice (6-8 weeks) were utilised. Mice were generated as illustrated in Calcraft et al. (2009) (126) and utilised according to the guidelines of the UK Animals (Scientific Procedures) 1986 Act. Mice were kept on a 12-hour light and 12-hour dark cycle at 19-23°C and 45-65% relative humidity. After termination of mice by cervical

dislocation, whole spleens were dissected into single-cell suspensions of splenocytes, and then washed twice with cold PBS. The procedures are described in detail in Chapter 3.

4.2.2. RNA extraction, quantification, purification and qRT-PCR

RNA was extracted by RNeasy Mini Kit (QIAGEN, CA, USA) according to the manufacturer's protocols. Nanodrop was utilized to quantify the concentrations and check the purity of the RNA (A260/280), with 2 for RNA as acceptable limits. Complementary DNAs (cDNA) were synthesized by reverse transcription (RT) utilizing SuperScript™ III Reverse Transcriptase (Invitrogen™). qPCR was conducted utilizing PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The primers for detecting Hcls1, CD38, and GAPDH which are listed in Table 8 were purchased from Life Technologies Ltd. Values were normalized according to the relative amounts of GAPDH, which was used as endogenous control. The procedures are described in detail in Chapter 2.

Table8. Primers for RT-qPCR.

	FP	RP
Murine		
GAPDH	GAG CCA AAC GGG TCA TCA	CAT ATT TCT CGT GGT TCA CAC C
Hcls1	CCTTGCTGCCCATTAGGCAGAC	CAGAGTCCTAGGGGGCAGAGC
CD38	CTGCTGGAGAGGTTATTCCTCG	GGAAGAGTGAGCGGCGCATCAAGG

4.2.3. Protein extraction, purification and quantification

Protein was extracted using NP40 Cell Lysis Buffer (Thermo Fisher Scientific), and the concentration of protein was assessed by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were sent to the Discovery Proteomics Facility of the Target Discovery Institute initiated by the Nuffield Department of Medicine and the University of Oxford for proteomics analysis using mass spectrometry. The procedures for protein extraction and

assessment are delineated in Chapter 3.

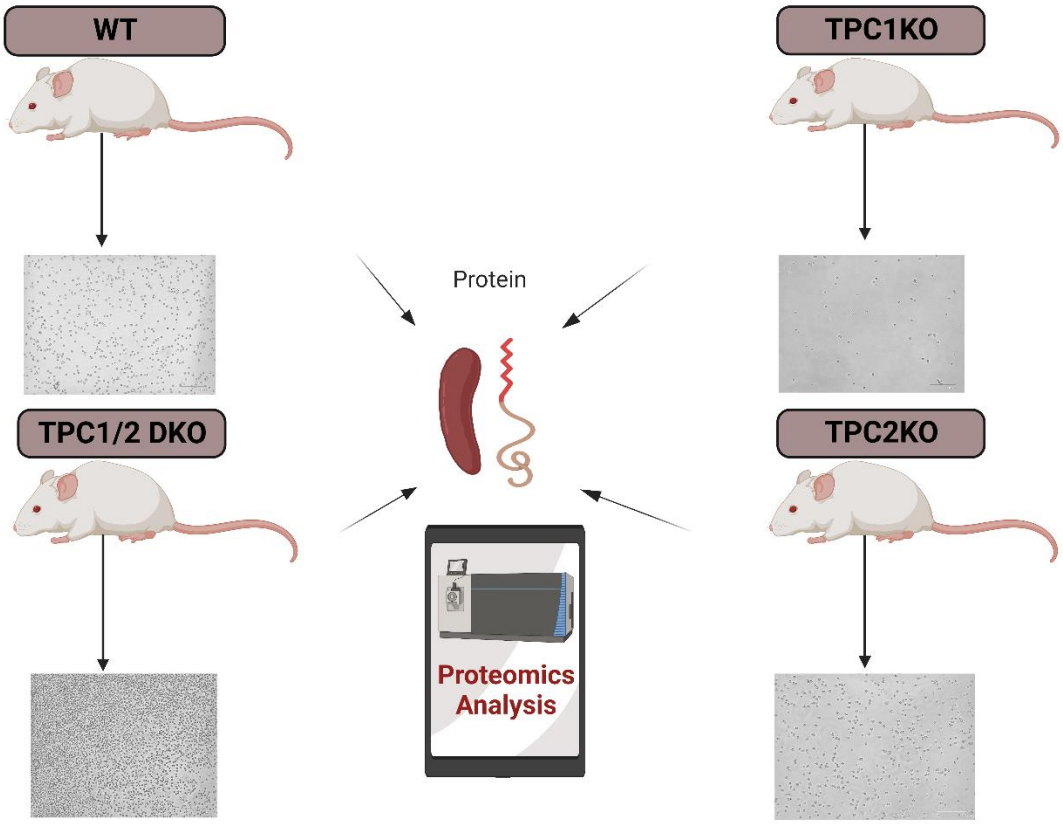


Figure 71. Murine splenocytes from the spleens of wild-type, TPC1KO, TPC2 KO, and TPC1/TPC2 double KO mice were isolated. The protein samples were sent to the Discovery Proteomics Facility for proteomic analysis.

4.2.4. Protein digestion and liquid chromatography-mass spectrometry analysis

Prior to injection into liquid chromatography-mass spectrometry, protein samples were digested into peptides. The approach is described in detail in Messina et al. (2022) (310).

4.2.5. Label-free quantification and proteomics analysis

Proteomic analysis was performed utilizing Perseus (version 1.6.5.0). The raw file was searched against the UniProt Mus Musculus Database. Data filtration, transformation, normalisation,

imputation, differential expression analysis, clustering, and functional analysis were conducted according to knowledge acquired from hands-on tutorials and theoretical lectures provided by the Perseus and Tyanova and Cox (2018) chapter "Perseus: a bioinformatics platform for integrative analysis of proteomics data in cancer research" in the book Cancer Systems Biology (311). The criteria to report the difference in protein expression levels was 1-fold change either above or below regardless of the significant level, or any fold change values that reached significance level. The significance of the difference was assessed utilising a two-tailed t-test. Significantly differentially expressed proteins were further analysed by STRING (STRING: functional protein association networks (string-db.org)).

4.2.6. Data presentation and statistical analysis

Results are presented as mean ± SD/SEM. R version 3.4.1 (ggplots, devtools and ggvenn packages) was utilised for data analysis and visualisation.

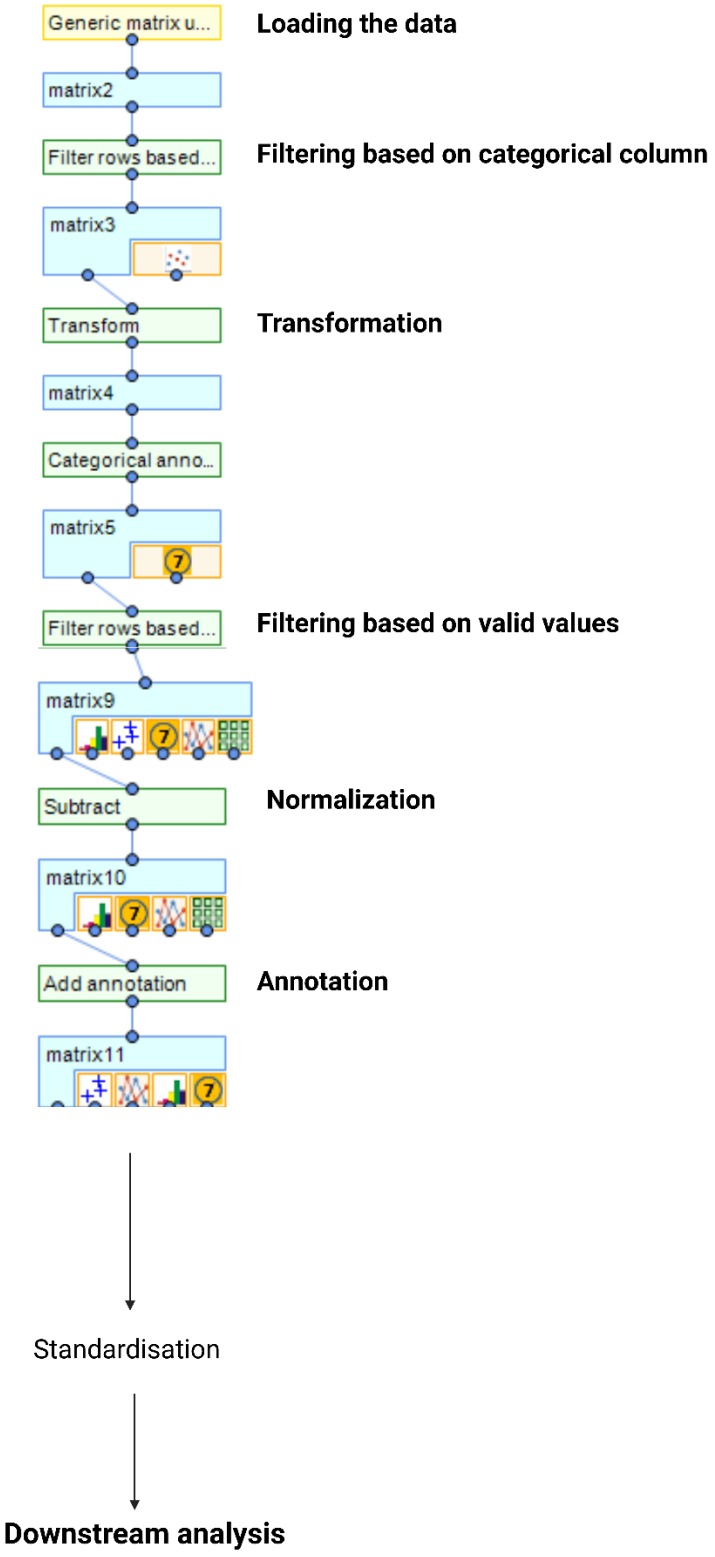


Figure 72. The workflow of splenic proteome analysis by Perseus.

4.3. Results

4.3.1. Altered protein levels of splenic cells by TPC1/TPC2 KO

To determine whether alterations in the levels of expression of proteins involved in the immune system cell development, cell differentiation, and physiological processes related to cancer (including cellular adhesion, cell migration, motility, angiogenesis, cellular proliferation, cell growth apoptosis, autophagy, phagocytosis, and inflammatory responses) could be attributed to endolysosomal Ca^{2+} signalling, we conducted label-free proteomics analysis in murine splenocytes after TPC1 KO, TPC2 KO, or TPC1/TPC2 double KO. I identified 22 (Table 9 and Supplementary Table 4.1), 7 (Table 10 and Supplementary Table 4.2), and 11 (Table 11 and Supplementary Table 4.3) upregulated proteins and 10, 85 and 16 downregulated proteins involved in immune system processes in TPC1 KO, TPC2 KO, and TPC1/TPC2 double KO murine splenocytes., respectively, compared to WT. Complement C3 was significantly downregulated by 5.3 fold, 0.89 fold, and 2.7 fold in TPC1 KO, TPC2 KO, and TPC1/TPC2 double KO murine splenocytes, respectively. Although chitinase-like protein 3 and neutrophilic granule protein were significantly upregulated in TPC1-deficient (by 1.35 and 1.49 fold, respectively), and TPC1/TPC2-deficient murine splenocytes (by 2.29 and 2.36 fold, respectively), we did not observe differences in fold change after TPC1 KO or TPC1/TPC2 double KO.

Chitinase-like protein 3 and neutrophilic granule protein levels appeared to be unaffected by TPC1/TPC2 double KO. Vitamin D–binding protein was significantly downregulated by 1.09 fold and 2.18 fold in TPC2KO and TPC1/TPC2 double KO murine splenocytes, respectively, compared to WT.

As shown in Table 10 and Supplementary Table 4.2, interestingly, we found that 66 different proteins that play vital roles in the immune system showed significant changes in expression in TPC2-deficient murine splenocytes (7 overexpressed proteins; 59 down-expressed proteins), compared to WT. This indicated that NAADP/TPC2/Ca^{2+} signalling could represent a viable target

to regulate processes related to the immune system. Three important proteins involved in monocyte differentiation and macrophage development were significantly altered ($p < 0.05$): haematopoietic lineage cell-specific protein and H-2 class II histocompatibility antigen gamma chain were decreased by 1.32 fold and 0.94 fold, respectively in TPC2 KO murine splenocytes compared to WT (as shown in Table 10). I observed significant changes in the protein expression levels involved in the regulation of the toll-like receptor 4 signalling pathway, chemokine and cytokine production, or the response to cytokine were after TPC1 KO, TPC2 KO, or TPC1/TPC2 double KO in murine splenocytes. These proteins included the following: lactotransferrin (overexpressed in all); bone marrow proteoglycan (overexpressed in TPC1KO or TPC1/TPC2 double KO); cathelin-related antimicrobial peptide (overexpressed in TPC2KO or TPC1/TPC2 double KO); stimulator of interferon genes (down-expressed in TPC2KO); pro-interleukin-16 genes (down-expressed in TPC2KO); H-2 class II histocompatibility antigen; K-B alpha chain, a beta chain, gamma chain and A-B alpha chain (down-expressed in TPC2 KO); SAM and SH3 domain-containing protein 3 (down-expressed in TPC2KO); pyrin and HIN domain-containing protein 1 (down-expressed in TPC2KO).

A closer inspection of Tables 10 and 11 reveals that proteins that showed significant differences in expression in TPC1 KO or TPC2 KO murine splenocytes compared to WT – which includes protein S100-A8, protein S100-A9, lymphocyte-specific protein 1, proto-oncogene Vav, and legumain – play a role in monocyte or neutrophil chemotaxis. Forkhead box protein P1, which acts as a regulator of monocyte differentiation, was only expressed in TPC1-deficient murine splenocytes, as shown in Table 4.4 supplementary. These findings suggest there is an association between endolysosomal Ca^{2+} signalling, particularly via TPC2, and the immune system. We further identified several proteins involved in cell development, cell differentiation, and physiological processes related to cancer that were also differentially expressed in murine

TPC1 KO, TPC2 KO, or TPC1/TPC2 double KO splenocytes (Tables 12–14 and Tables 4.5-4.8 supplementary).

To characterise the main TPC form that could play vital roles in cancer and immunity, further analysis was conducted to shed light on its potential as a regulator of cancer immunity. Figure 76 shows that 3 (upregulated), 28 (3 upregulated; 25 downregulated) and 2 (upregulated) significantly altered proteins involved in cancer immunity were found in TPC1 KO, TPC2 KO and TPC1/TPC2 double KO murine splenocytes, respectively. S100 calcium-binding protein A8, which is expressed in various cancer cells, was upregulated 2.74 fold and 2.02 fold in TPC1 KO and TPC2 KO murine splenocytes, respectively. This suggests that endolysosomal Ca^{2+} signalling via TPC1/TPC2 could be attributed to the regulation of inflammation-associated cancer. Similarly, I observed a significant increase in lactotransferrin levels, which modulates immune responses and exhibits anti-cancer and anti-inflammatory activities by 3.5 fold, 2 fold, and 3.3 fold after TPC1, TPC2 or TPC1/TPC2 double KO in murine splenocytes, respectively. The data shows that TPC2 is potentially the main modulator of TPCs in physiological processes relevant to cancer immunity. To understand the possible influence of TPC2 activity on proteins attributed to cancer immunity and to provide insights into pathways affected by TPC2 inhibition, I performed a further bioinformatics analysis using STRING. Figure 77 b illustrates that differently expressed proteins were significantly biologically related. In addition, an enrichment analysis revealed that genes that are part of biologically and clinically significant pathways are affected by TPC2 KO (Figure 77 c).

Proteins involved in pathophysiological processes related to haematological and cardiovascular diseases, including angiogenesis, blood coagulation, platelet activation, aggregation, degranulation, blood pressure and cholesterol homeostasis, were differentially expressed at the protein level after TPC1 KO, TPC2 KO or TPC1/TPC2 double KO in murine splenocytes. As shown in Supplementary Table 4.9, I found 24 (2 overexpressed; 22 down-expressed), 59 (5 overexpressed; 54 down-expressed) and 28 (2 overexpressed; 26 down-expressed) significantly

changed proteins involved in cardiovascular system processes in TPC1 KO, TPC2 KO and TPC1/TPC2 double KO murine splenocytes, respectively, compared with WT.

Table 9. Changes in expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC1.

Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value <0.05		
Upregulated		
Lactotransferrin	Ltf	3.507059
Protein S100-A8	S100a8	2.737471
Neutrophilic granule protein	Ngp	2.286671
Protein S100-A9	S100-A9	2.050961
Bone marrow proteoglycan	Prg2	1.788418
Chitinase-like protein 3	Chil3	1.348005
Igh protein	Igh	1.043617
Downregulated		
Complement C3	C3	-5.30447
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	-4.09356
Histone H4	Hist2h4	-3.49568
Histone H3	Hist1h3e	-3.01581
Histone H2B type 1-K	Hist1h2bk	-2.76598
Ferritin	Ftl1	-2.45398
Alpha-1-acid glycoprotein	ORM1	-1.79865
Ferritin heavy chain	Fth1	-1.59587

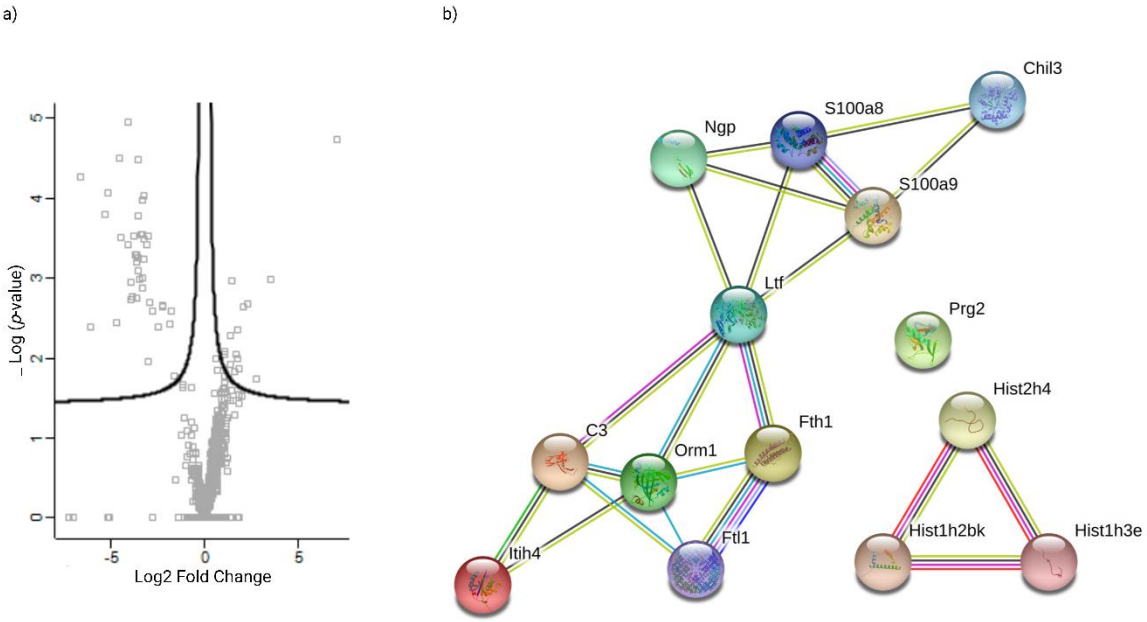


Figure 73. Altered proteins in splenic cells following TPC1 KO. (a). Volcano plot of proteins quantified using label free quantification technique in TPC1 deficient murine splenocytes. (b). Significantly altered gene ontology pathways involved in immune system in TPC1 KO spleen cells. It was created with string-db.org (protein–protein interaction enrichment P Value: < 1.0e-16).

Table 10. Changes in expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC2.

FDR-adjusted *p*-value <0.05		
Protein name	**Gene name**	**x-fold change**
Upregulated		
Protein S100-A8	S100a8	2.02
Lactotransferrin	Ltf	2
Cytoplasmic dynein 1 heavy chain 1	Dync1h1	1.28
C-1-tetrahydrofolate synthase	Mthfd1	1.17
Cathelin-related antimicrobial peptide	Camp	1.05
Costars family protein ABRACL	Abracl	1
Downregulated		
Histone H4	Hist2h4	-3.71
Histone H2B type 1-K	Hist1h2bk	-3.53
Histone H3.2	Hist2h3b	-3.2
Spectrin beta chain, non-erythrocytic 1	Sptbn1	-2.09
Pyrin and HIN domain-containing protein 1	Pyhin1	-1.81
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Slc9a3r1	-1.46
Hematopoietic lineage cell-specific protein	Hcls1	-1.32
Alpha-2-HS-glycoprotein	AHSG	-1.31
Apolipoprotein A-I	APOA1	-1.25
Galectin-1	Lgals1	-1.25
Myosin-9	Myh9	-1.2
Far upstream element-binding protein 1	Fubp1	-1.15
Wiskott-Aldrich syndrome protein family member 2	Wasf2	-1.14
Proteasome subunit beta type-8	Psmb8	-1.13
SAM and SH3 domain-containing protein 3	Sash3	-1.1
Vitamin D-binding protein	GC	-1.09
BTB/POZ domain-containing protein KCTD12	Kctd12	-1.07
Legumain	Lgmn	-1.07
Filamin-A	Flna	-1.04
Glucosidase 2 subunit beta	Prkcsh	-1.03
Inter-alpha-trypsin inhibitor HC2 component homolog	ITIH1	-1.01
Dipeptidyl peptidase 1	Ctsc	-0.99
Stimulator of interferon genes protein	Tmem173	-0.99
B-cell antigen receptor complex-associated protein alpha chain	Cd79a	-0.98
H-2 class II histocompatibility antigen, A-B alpha chain	H2-Aa	-0.98
Ena/VASP-like protein	Evl	-0.97
Lymphocyte-specific protein 1	Lsp1	-0.95
Pro-interleukin-16	Il16	-0.95
F-actin-capping protein subunit beta	Capzb	-0.94

H-2 class II histocompatibility antigen gamma chain	Cd74	-0.94
H-2 class II histocompatibility antigen, A beta chain	H2-Ab1	-0.94
Transcription intermediary factor 1-beta	Trim28	-0.94
Vitronectin	VTN	-0.94
40S ribosomal protein S19	Pld4	-0.93
5'-3'exonuclease PLD4	Rps19	-0.91
Proto-oncogene vav	Vav1	-0.9
Ig mu chain C region	Ighm	-0.9
Complement C3	C3	-0.89
Heterogeneous nuclear ribonucleoprotein U-like protein 1	Hnrnpul1	-0.89
Proteasome activator complex subunit 1	Psme1	-0.89
Palmitoyl-protein thioesterase 1	Ppt1	-0.88
F-actin-capping protein subunit alpha-1	Capza1	-0.87
Proteasome subunit beta type-1	Psmb1	-0.87
SUMO-activating enzyme subunit 2	Uba2	-0.86
Protein-tyrosine-phosphatase	Ptprc	-0.83
Transcription elongation factor B polypeptide 1	Tceb1	-0.83
F-actin-capping protein subunit alpha-2	Capza2	-0.81
Rho-related GTP-binding protein RhoG	Rhog	-0.8
H-2 class I histocompatibility antigen, K-B alpha chain	H2-K1	-0.79
Programmed cell death protein 4	Pdcd4	-0.79
Neutrophil cytosol factor 1	Ncf1	-0.77
Thioredoxin	Txn	-0.76
Ras-related protein Rap-1b	Rap1b	-0.75
Vesicle-fusing ATPase	Nsf	-0.75
Serine/threonine-protein kinase PAK 2	Pak2	-0.71
Proteasome subunit alpha type-5	Psma5	-0.69
Sorting nexin-3	Snx3	-0.68
Glutathione S-transferase P 1	Gstp1	-0.63
Protein CREG1	Creg1	0.54
DNA mismatch repair protein Msh2	Msh2	0.77

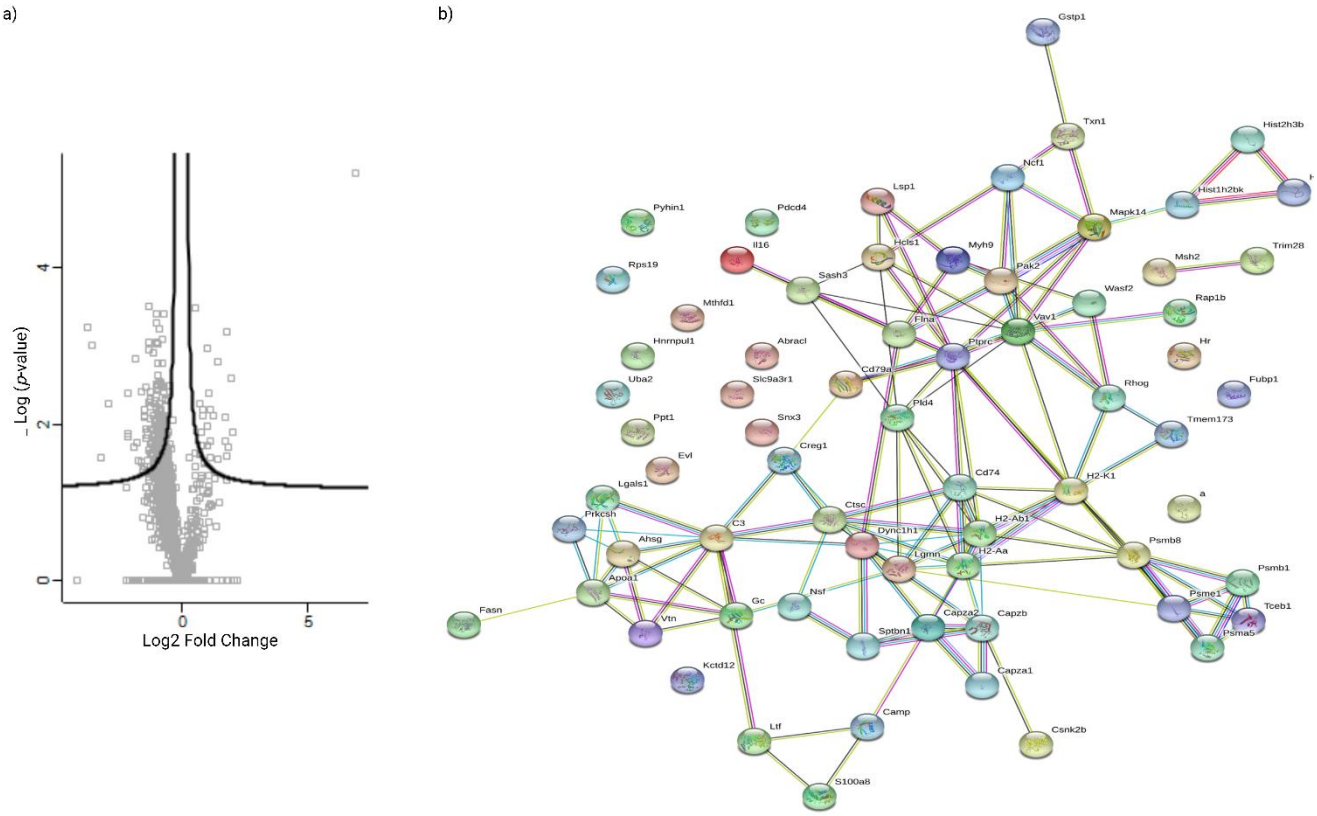


Figure 74. Altered proteins in splenic cells following TPC2 KO. (a). Volcano plot of proteins quantified using label free quantification technique in TPC2 deficient murine splenocytes. (b). Significantly altered gene ontology pathways involved in immune system in TPC2 KO spleen cells. It was created with string-db.org (protein–protein interaction enrichment P Value: < 1.0e-16).

Table 11. Changes in expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC1/TPC2.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value <0.05		
Upregulated		
Lactotransferrin	Ltf	3.343201
Neutrophilic granule protein	Ngp	2.363774
Cathelin-related antimicrobial peptide	Camp	2.12241
Myeloperoxidase	Mpo	1.804322
Chitinase-like protein 3	Chil3	1.48733
Downregulated		
Complement C3	C3	-2.68949

Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	-2.65088
Adiponectin	ADIPOQ	-2.54747
Vitamin D-binding protein	GC	-2.17768
Alpha-1-acid glycoprotein	ORM1	-2.0798
Alpha-2-HS-glycoprotein	AHSG	-2.02708

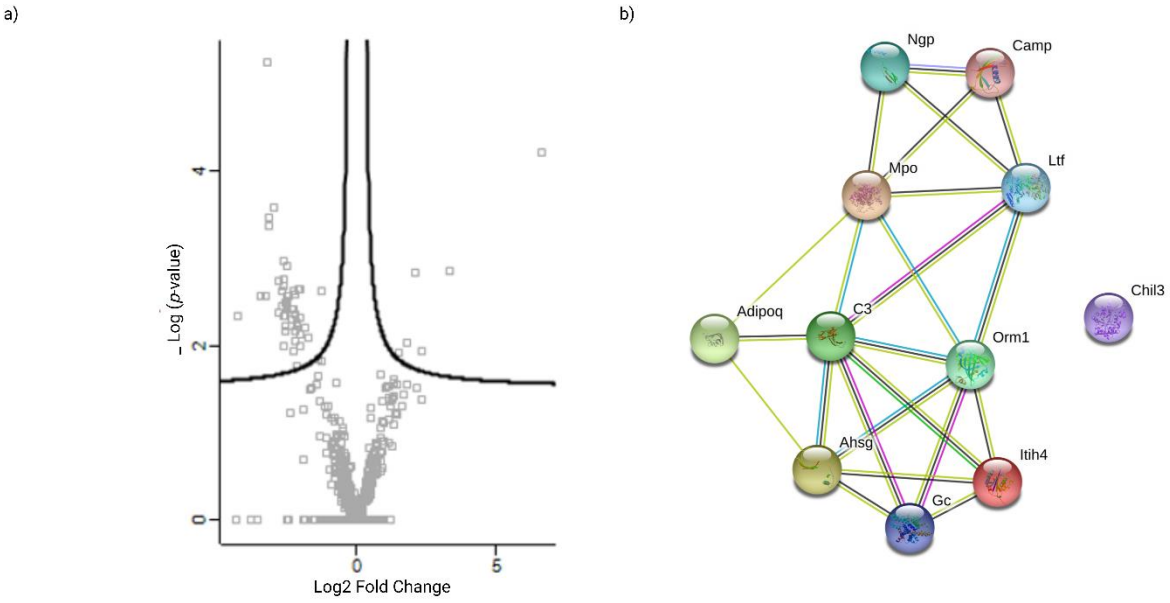


Figure 75. Altered proteins in splenic cells following TPC1/TPC2 double KO. (a). Volcano plot of proteins quantified using label free quantification technique in TPC deficient murine splenocytes. (b). Significantly altered gene ontology pathways involved in immune system in TPC1/TPC2 double KO spleen cells. It was created with string-db.org (protein–protein interaction enrichment P Value: < 1.0e-16).

Table 12. Changes in expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC1.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value <0.05		
Upregulated		
Lactotransferrin	Ltf	3.507059
Neutrophilic granule protein	Ngp	2.286671
Protein S100-A8	S100a8	2.737471
Protein S100-A9	S100a9	2.050961

Table 13. Changes in expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC2.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value <0.05		
Upregulated		
Protein S100-A8	S100a8	2.01847
Lactotransferrin	Ltf	1.995309
Phosphatidylinositol-binding clathrin assembly protein	Picalm	1.160064
Cathelin-related antimicrobial peptide	Camp	1.046663
DNA mismatch repair protein Msh2	Msh2	0.769385
Downregulated		
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Slc9a3r1	-1.45788
Hematopoietic lineage cell-specific protein	Hcls1	-1.31897
Myosin, light chain 12A, regulatory, non-sarcomeric	Myl12a	-1.22462
Myosin-9	Myh9	-1.20344
Wiskott-Aldrich syndrome protein family member 2	Wasf2	-1.14241
SAM and SH3 domain-containing protein 3	Sash3	-1.09967
Legumain	Lgmn	-1.06523
Filamin-A	Flna	-1.03754
Stimulator of interferon genes protein	Tmem173	-0.98954
Dipeptidyl peptidase 1	Ctsc	-0.98899
Chloride intracellular channel protein 1	Clic1	-0.98468
B-cell antigen receptor complex-associated protein alpha chain	Cd79a	-0.98093
Ena/VASP-like protein	Evl	-0.97024
Lymphocyte-specific protein 1	Lsp1	-0.95361
Pro-interleukin-16	Il16	-0.94569
H-2 class II histocompatibility antigen gamma chain	Cd74	-0.93984
Nucleophosmin	Npm1	-0.9387
Annexin	Anxa2	-0.93202
Phospholipase D4	Pld4	-0.92569
40S ribosomal protein S19	Rps19	-0.90845
Proto-oncogene vav	Vav1	-0.90341
Ig mu chain C region	Ighm	-0.90198
Palmitoyl-protein thioesterase 1	Ppt1	-0.87964

Beta-hexosaminidase	Hexb	-0.87093
Actin-related protein 2/3 complex subunit 5	Arpc5	-0.84746
40S ribosomal protein S3a	Rps3a	-0.84266
Receptor-type tyrosine-protein phosphatase C	Ptprc	-0.83347
Rho-related GTP-binding protein RhoG	Rhog	-0.80305
Protein RCC2	Rcc2	-0.79824
Programmed cell death protein 4	Pdcd4	-0.79308
Neutrophil cytosol factor 1	Ncf1	-0.7719
SH3 domain-binding glutamic acid-rich-like protein 3	Sh3bgrl3	-0.76409
Ras-related protein Rap-1b	Rap1b	-0.75338
Dihydropyrimidinase-related protein 2	Dpysl2	-0.74673
DNA-(apurinic or apyrimidinic site) lyase	Apex1	-0.73787
Serine/threonine-protein kinase PAK 2	Pak2	-0.70695
2-oxoglutarate dehydrogenase, mitochondrial	Ogdh	-0.64843
Glutathione S-transferase P 1	Gstp1	-0.62799
Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	Aimp2	-0.46604
Prostaglandin G/H synthase 1	Ptgs1	-0.43115
Alanine--tRNA ligase, cytoplasmic	Aars	0.848101

Table 14. Changes in expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC1/TPC2.

Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value <0.05		
Upregulated		
Lactotransferrin	Ltf	3.343201
Chitinase-like protein 3	Chil3	1.48733

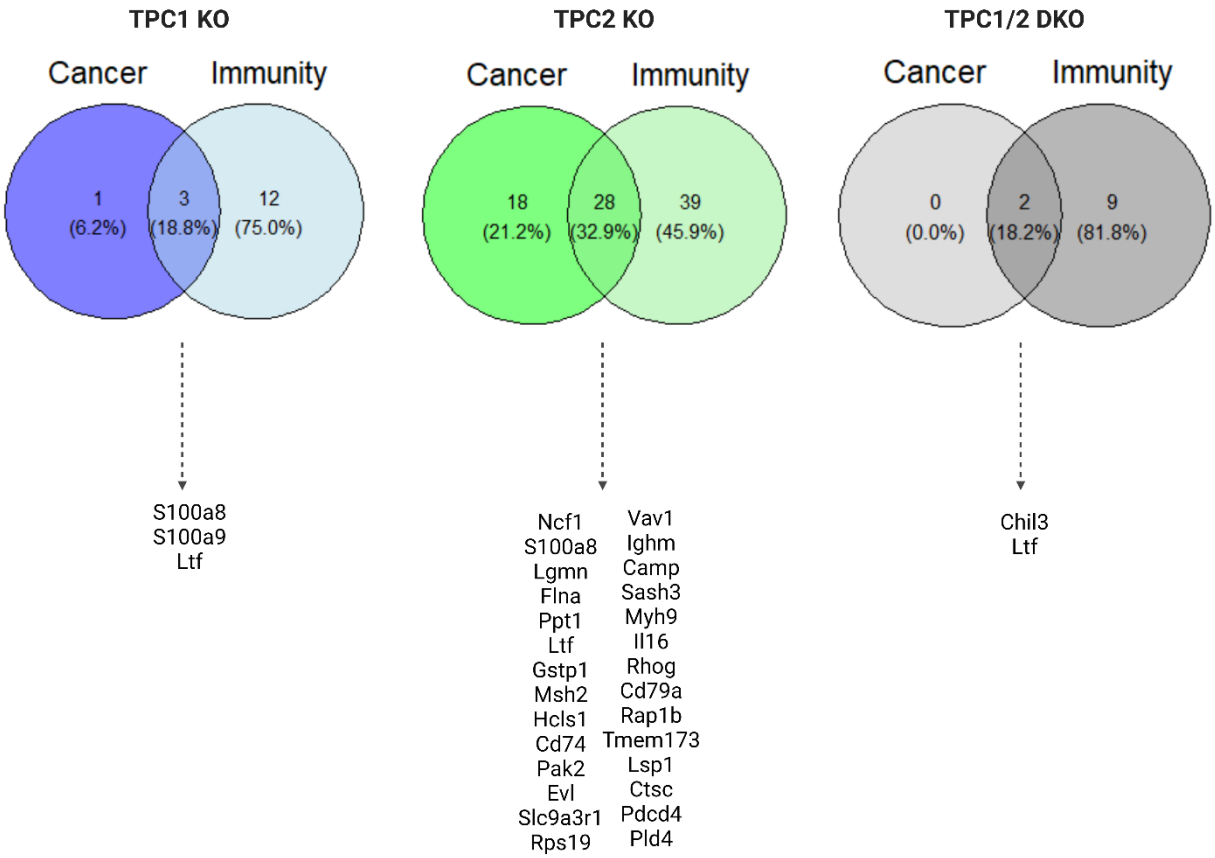
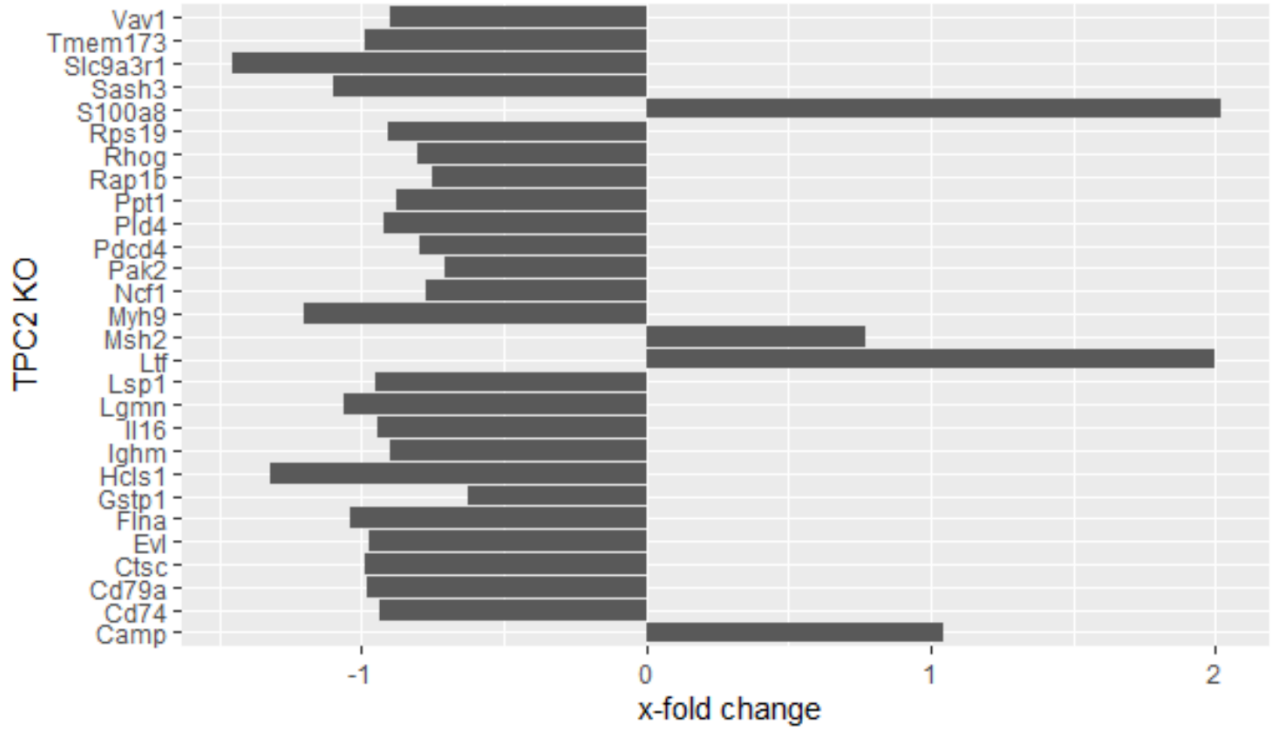
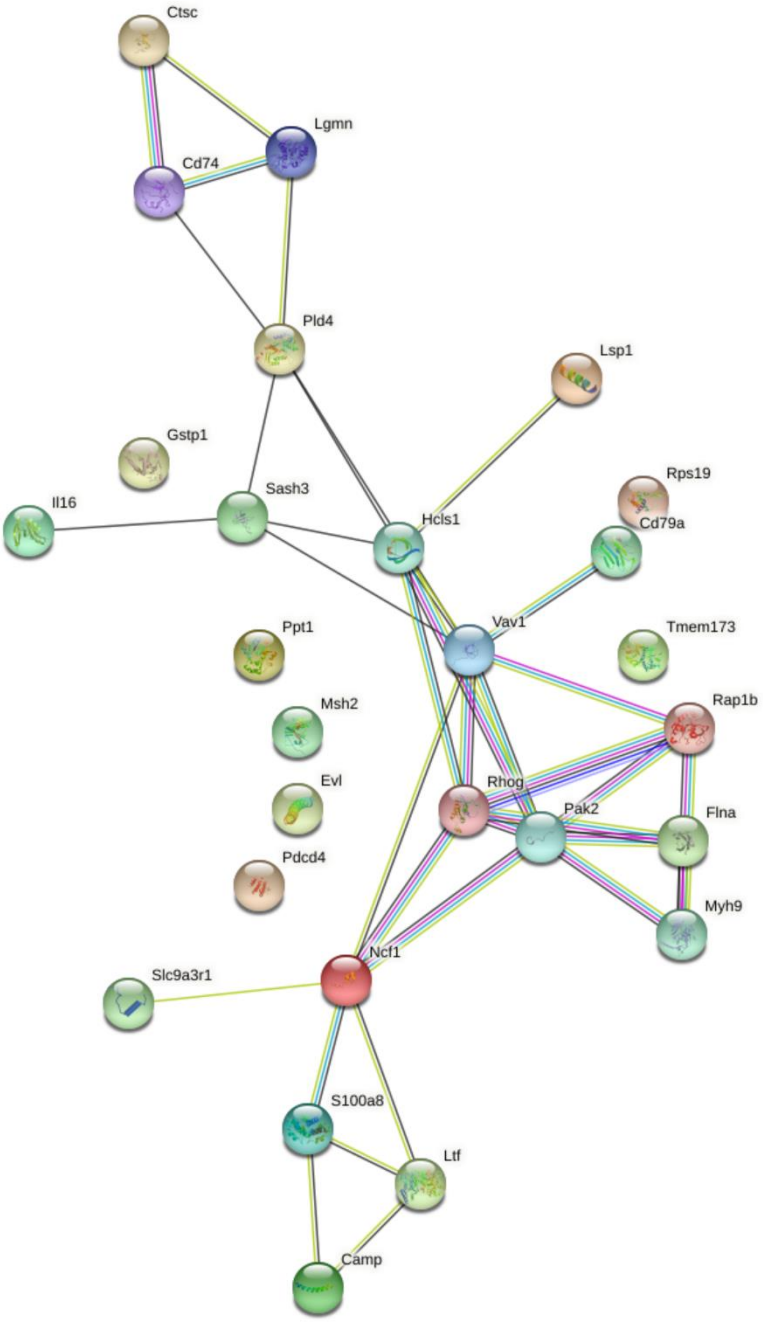


Figure 76. Differentially expressed proteins involved in physiological processes related to cancer or immunity after knocking out TPC1/ TPC2 KO in splenic cells.

a)


Vav1
Tmem173
Slc9a3r1
Sash3
S100a8
Rps19
Rhog
Rap1b
Ppt1
Pld4
Pdcd4
Pak2
Ncf1
Myh9
Msh2
Ltf
Lsp1
Lgmn
Il16
Ighm
Hcls1
Gstp1
Flna
Evl
Ctsc
Cd79a
Cd74
Camp
TPC2 KO
-1
0
1
2
x-fold change

b)


Ctsc
Lgmn
Cd74
Pld4
Lsp1
Gstp1
Rps19
Il16
Sash3
Hcls1
Cd79a
Vav1
Ppt1
Tmem173
Msh2
Rap1b
Evl
Rhog
Pak2
Flna
Pdcd4
Myh9
Ncf1
Slc9a3r1
S100a8
Ltf
Camp

c)

KEGG and Reactome Pathways				
Pathway	Gene	Observed gene count	Background gene count	Strength
KEGG Pathway				
Focal adhesion	Vav1,Pak2,Flna,Rap1b	4	196	1.22
Proteoglycans in cancer	Vav1,Hcls1,Flna,Pdcd4	4	199	1.22
Leukocyte transendothelial migration	Vav1,Ncf1,Rap1b	3	113	1.34
Lysosome	Lgmn,Ppt1,Ctsc	3	127	1.29
Reactome Pathway				
Immune System	Il16,Cd79a,Vav1,Ncf1,Myh9,Lgmn,Pak2,Ctsc,Ltf,S100a8, Rap1b,Pld4,Cd74,Rhog,Camp,Tmem173,Gstp1	17	1621	0.93
Innate Immune System	Vav1,Ncf1,Myh9,Lgmn,Pak2,Ctsc,Ltf,S100a8, Rap1b,Pld4,Rhog,Camp,Tmem173,Gstp1	14	949	1.08
Signaling by Rho GTPases	Vav1,Ncf1,Myh9,Evl,Pak2,Flna,S100a8,Rhog	8	381	1.23
RHO GTPase Effectors	Ncf1,Myh9,Evl,Pak2,Flna,S100a8,Rhog	7	246	1.37
Neutrophil degranulation	Ctsc,Ltf,S100a8,Rap1b,Rhog,Camp,Tmem173,Gstp1	8	519	1.1
Adaptive Immune System	Cd79a,Vav1,Ncf1,Lgmn,Pak2,Ctsc,Rap1b,Cd74	8	701	0.97
RHO GTPases activate PAKs	Myh9,Pak2,Flna	3	20	2.09
Metal sequestration by antimicrobial proteins	Ltf,S100a8	2	4	2.61
CD28 dependent Vav1 pathway	Vav1,Pak2	2	12	2.13
Fcgamma receptor (FCGR) dependent phagocytosis	Vav1,Myh9,Pld4	3	82	1.48
Antimicrobial peptides	Ltf,S100a8,Camp	3	88	1.44
VEGFA-VEGFR2 Pathway	Vav1,Ncf1,Pak2	3	92	1.43
Platelet activation, signaling and aggregation	Vav1,Flna,Rap1b,Rhog	4	253	1.11
Hemostasis	Vav1,Flna,Rap1b,Cd74,Rhog	5	524	0.89
RHO GTPases Activate NADPH Oxidases	Ncf1,S100a8	2	24	1.83
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	Cd79a,Vav1	2	24	1.83

Figure 77. Significantly altered proteins related to cancer immunity in TPC2 deficient murine splenocytes. (a). The fold-change of proteins that were significantly modulated by TPC2 knockout in cancer immunity. (b). Protein interaction map of protein that crossed in cancer and immunity and created with string-db.org (protein–protein interaction enrichment P Value: 2.22e-15). (c). Significantly enriched genes from the crossed differentially expressed proteins in cancer immunity involved in KEGG and Reactome pathways.

4.3.2. CD38, and Hcls-1transciptomics expression levels by q- RT-PCR

I observed that CD38 mRNA expression was significantly downregulated by 95% in TPC2 KO murine spleen cells, whereas Hcls-1 mRNA expression was significantly upregulated by approximately 300% (as shown in Figure 78).

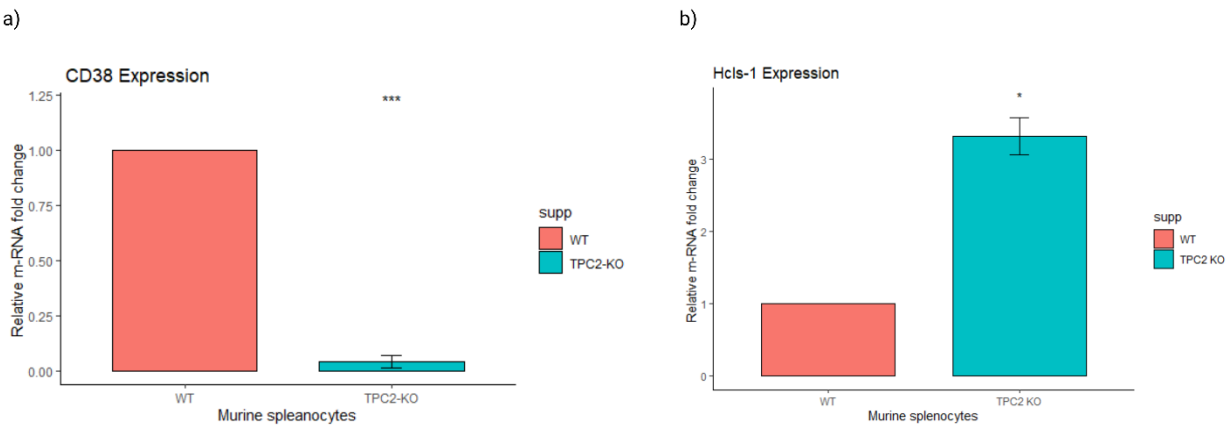


Figure 78. The mRNA expression levels of CD38, and Hcls-1 in murine splenocytes of three biological replicates in three independent experiments. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (mean ± SEM). Two sample t-test, *p*-values of CD38 (0.0008786), and Hcls (0.01213) in murine spleen cells.

4.4. Discussion

Despite the growing evidence that links endolysosomal Ca^{2+} signalling with pathophysiological processes related to immunity and cancer, research in this area is still in its infancy. Our study utilised a label-free protein analysis to reveal the impact of endolysosomal Ca^{2+} with signalling via TPC inhibition on protein expression on the whole splenic proteome architecture. The proteomics data show, for the first time, that changes in proteins playing vital roles in physiological processes related to the immune system and cancer occur after knocking out TPC2 or TPC1 in murine splenocytes. This indicates that the TPC channel subfamily might be involved in key immune cell functions and cancer cell biology. There were 14, 66 and 11 significantly differentially expressed proteins attributed to immune cell functions found in TPC1 KO, TPC2 KO and TPC1/TPC2 DKO murine splenic cells, respectively. This finding supports the hypothesis that TPC2 modulates immune cell biology and drives scientific questions regarding the immunological significance of TPC2 in murine/human biology.

Eight, five and three proteins were significantly decreased after TPC2 KO and were involved in mitogen-activated protein kinase cascades and RAF mediated MAP signalling and RHO GTPases activated P21-Associated Kinases (PAKs) pathways, respectively (as illustrated in Figure 79). This finding indicates the involvement of TPC2 in biologically and therapeutically important pathways that modulate health and disease states. The exact contribution of TPC2 in these signalling pathways that lead ultimately to immunological disease or cancer remains to be established.

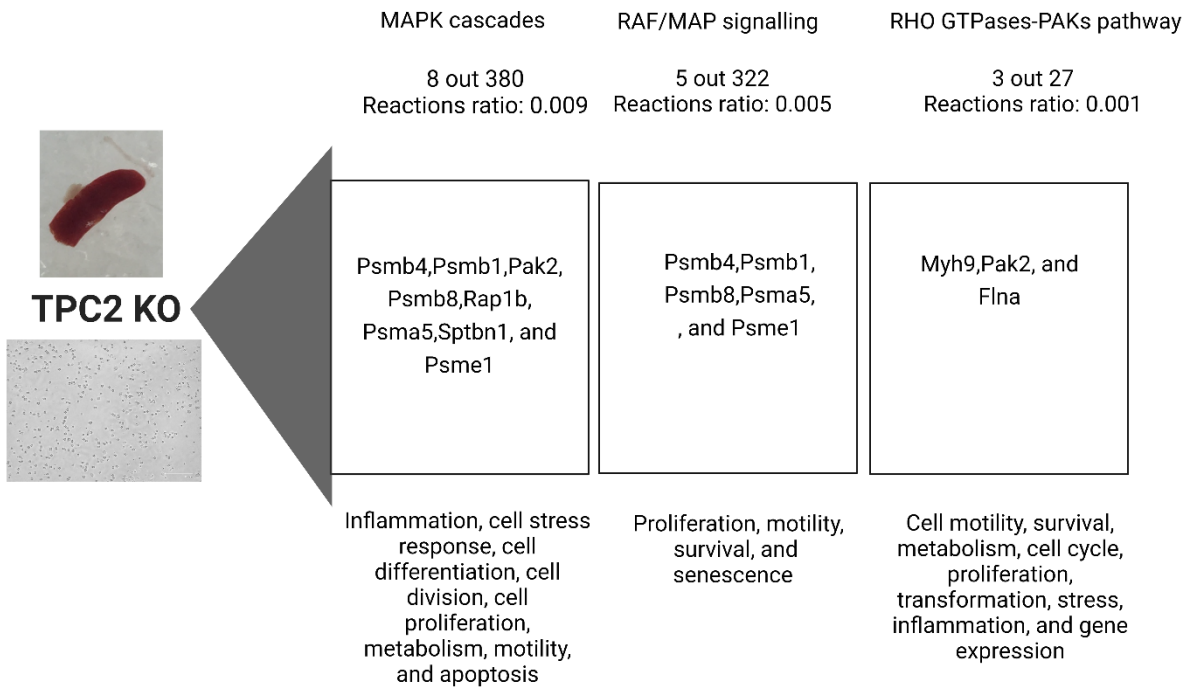


Figure 79. Significantly downregulated proteins involved in MAPK, RAF, and PAK signalling pathways in TPC2 deficient murine splenocytes. Reactions ratio: the proportion of reactions involving this pathway from the selected genes. Data was obtained from https://reactome.org.

In the same vein, TPC2 KO murine splenocytes exhibited a significant reduction in the Na/H exchanger regulatory factor (a scaffold protein). Although the Na/H exchanger regulatory factor has historically acted primarily as a connector in orchestrating interactions between receptors and cellular proteins, recent studies have also defined it as a dual player for either a cancer promoter or inhibitor based on its subcellular localisation (312, 313, 314) (Figure 80 a and c). In the plasma membrane, it behaves as an inhibitor, whereas in cytoplasm, it is a facilitator of tumorigenesis and cancer progression (315). Accumulated data shows that the Na/H exchanger regulatory factor also plays a role in cellular structure and trafficking, in inflammatory responses and in pathophysiological processes related to cancer, such as tumourigenesis and cancer cell progression (316, 317). In the current study, the Na/H exchanger regulatory factor was only significantly decreased by 1.46 in TPC2-deficient spleen cells, which raises a question regarding the dual role of the Na/H exchanger regulatory factor in cancer biology: does it constrain or promote cancer development, and how might endolysosomal Ca^{2+} signalling via TPC2 modulate its activity at a molecular level?

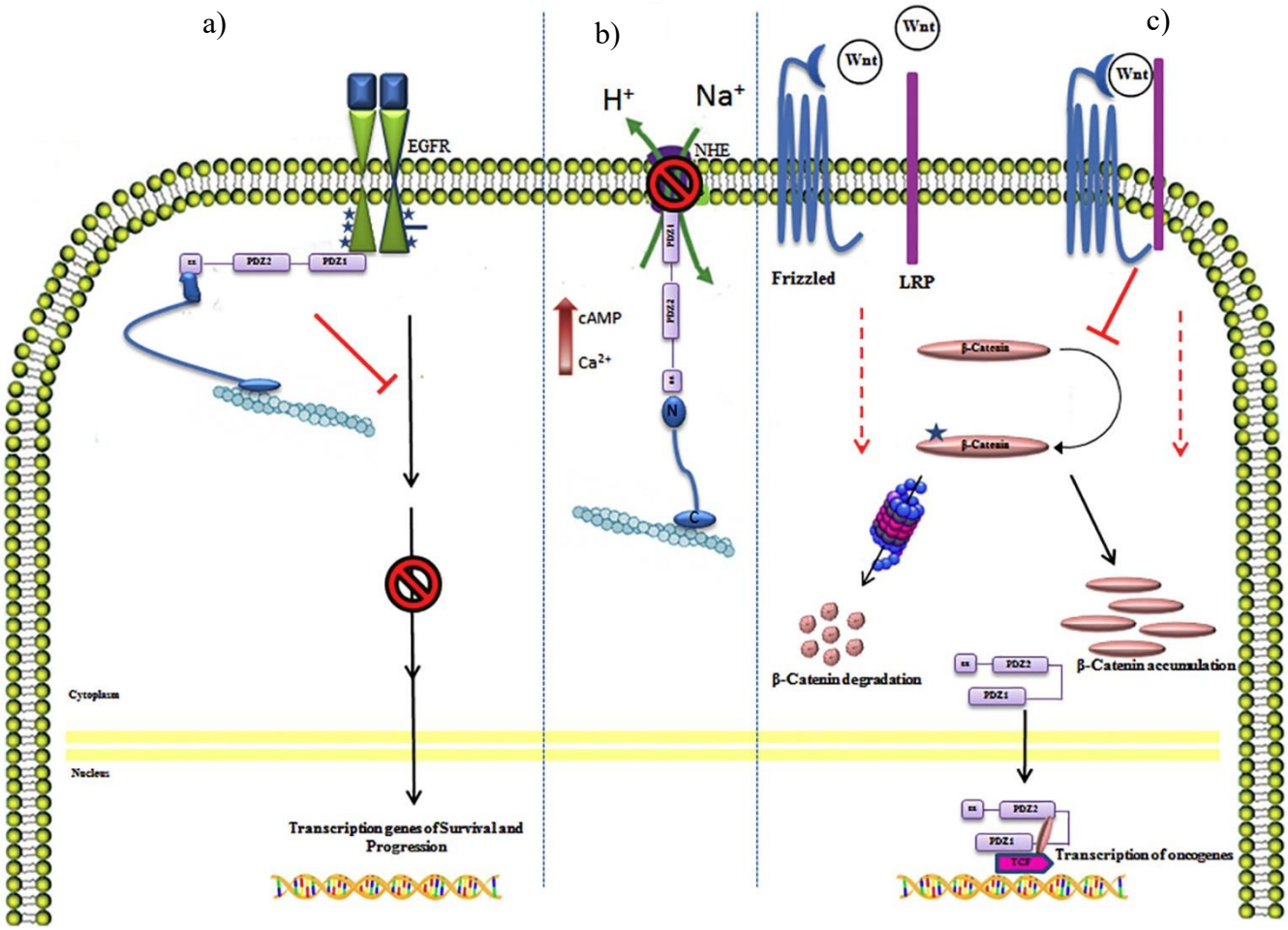


Figure 80. The dual roles of Na/H exchanger regulatory factor in cancer biology as promoter or suppressor (315) (the figure was reproduced from Centonze et al, under Elsevier copyright).

In contrast, Protein S100-A8, a Ca^{2+} binding protein, was significantly elevated in TPC1 KO or TPC2 KO spleen cells. Protein S100-A9 was only significantly upregulated in TPC1 KO cells. The overall data from Figure 81 imply that the biological role of S100-A8/9 is in inflammation and cancer (318, 319, 320). It has been shown that S100A8 expression levels were significantly downregulated at the RNA level in TPCN2 knockdown Jurkat and THP-1 cells (321). These findings highlight the potential involvement of lysosomal Ca^{2+} channels in mediating inflammation

and inflammation-induced cancer. However, further studies are required to uncover the molecular mechanisms underlying the TPC modulation of S100-A8/9 in *in vitro* and *in vivo* models.

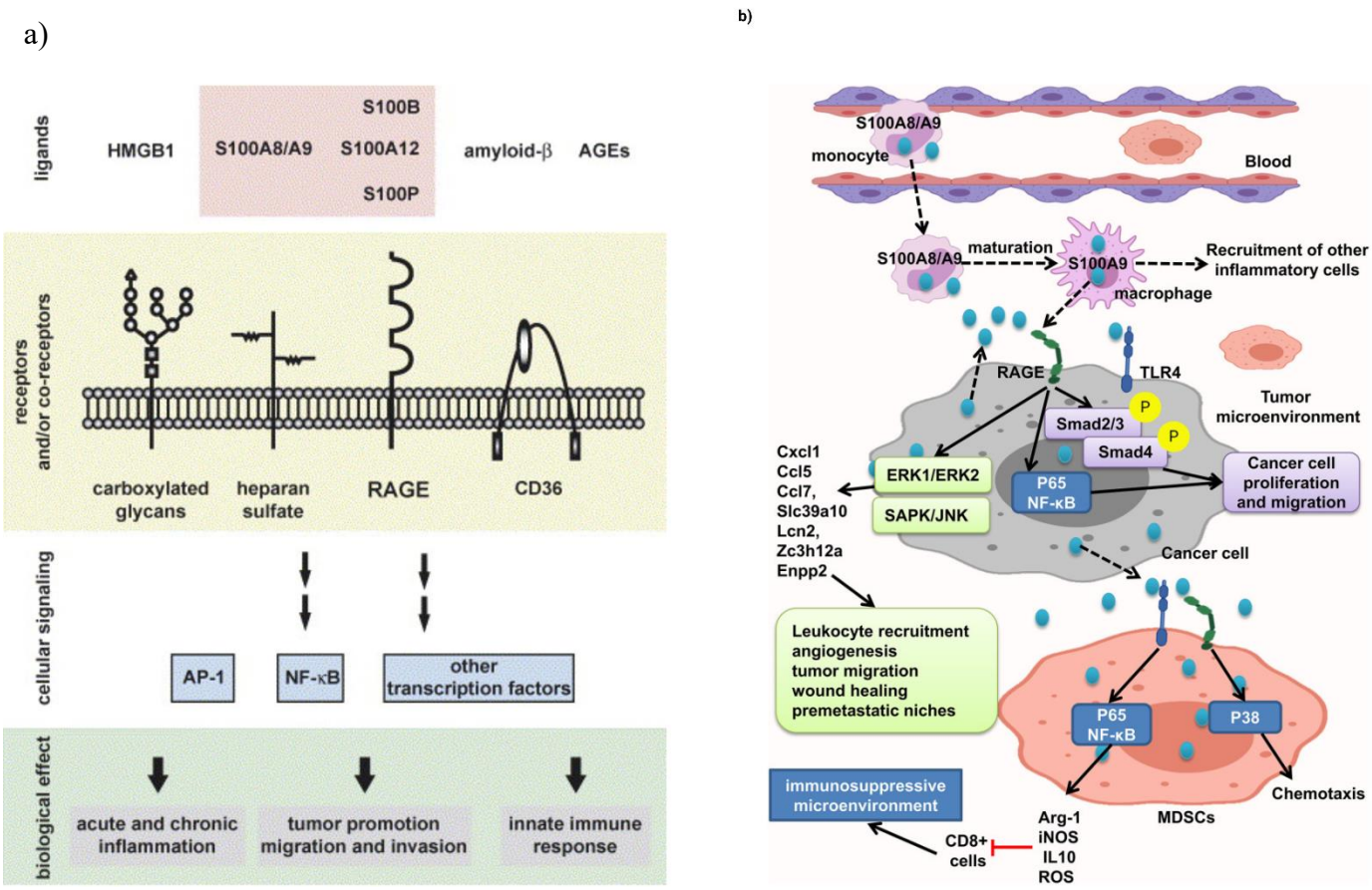


Figure 81. S100-A8/9 roles in some physiological processes attributed to cancer/immunity (319, 320) (the figures were reproduced from Gebhardtet al. and Azizian et al., under Biochemical Pharmacology and Frontiers copyrights)

Lactotransferrin, a multifunctional protein and iron-binding glycoprotein that exhibits anti-cancer and anti-inflammatory effects (322), was significantly increased after TPC1 KO, TPC2 KO or TPC1/TPC2 double KO in murine splenic cells. Although the anti-tumour activity of lactotransferrin and its derivatives is still not fully understood at the molecular level, it has been investigated in

several clinical trials (323). Talactoferrin is an orally administered recombinant human lactoferrin that reached phase-three trials (NCT00707304) in combination with the best standard of care in patients with non-small cell lung cancer who have failed at least two lines of therapy (323). No data has been reported from this trial. Previously, Moastafa (2014) et al. found that it has beneficial effects when administered at 250 mg/day in combination with 5-FU and calcium leucovorin for three months to patients with colorectal cancer (324). These findings highlight the potential involvement of lysosomal Ca^{2+} channels in controlling lactotransferrin protein expression. The complex interplay between TPC and lactotransferrin deserves further investigation.

In the context of cancer immunity, I found a significant elevation in chitinase-like protein 3, which plays a pathological role in the development of cancers and non-cancerous diseases (325). Zhaoet al. (2020) summarise the roles of chitinase-like protein 3 in cancer biology, ranging from tumorigenesis to metastasis (325) (as shown in Figure 82). This suggests that endolysosomal Ca^{2+} signalling, particularly via TPC1, may act as a mediator of pathological conditions related to cancer, including tumorigenesis, angiogenesis, progression, invasion and metastasis via chitinase-like protein 3.

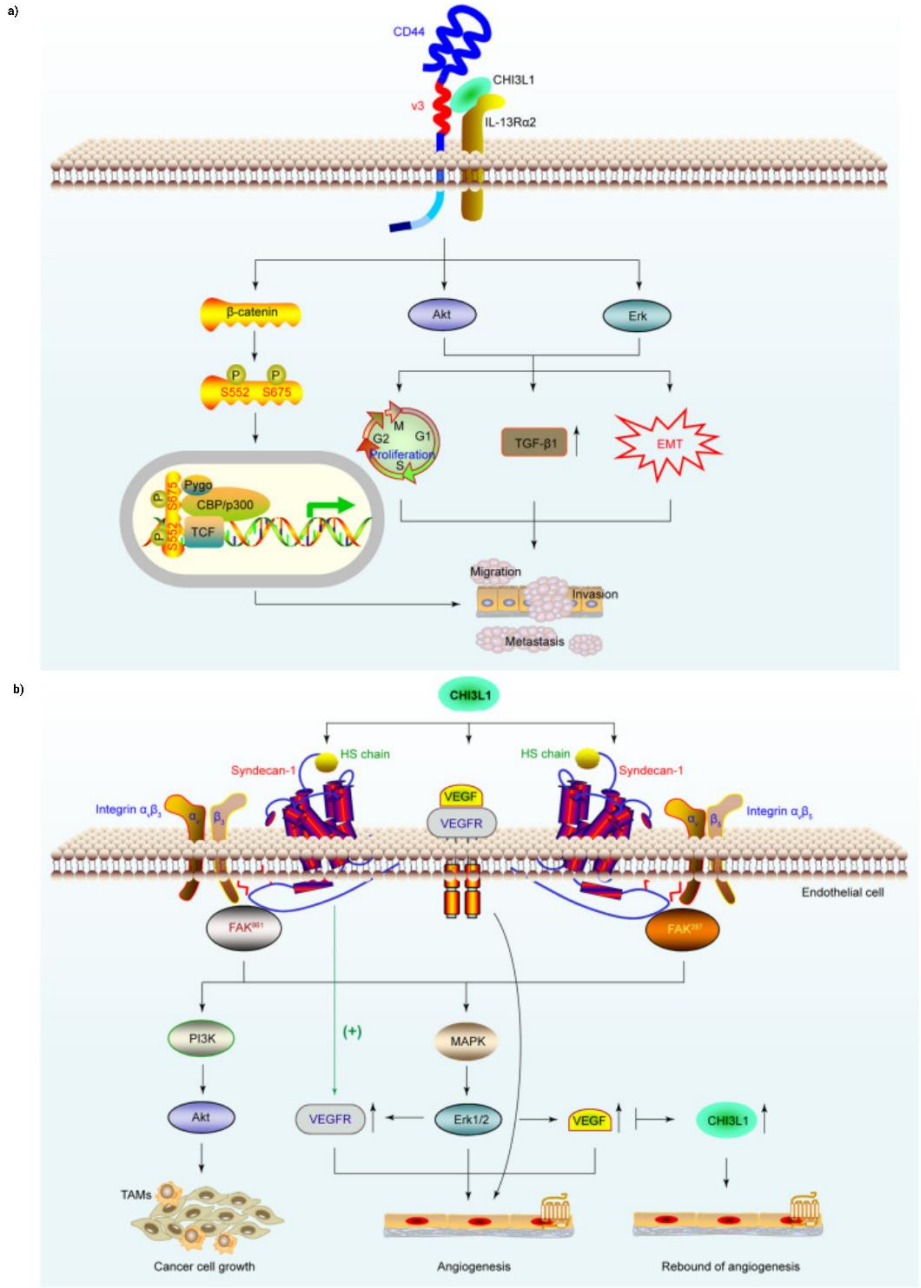


Figure 82. The vital role of Chitinase-like protein 3 in various pathophysiological processes related to cancer (325). (a) Invasion and metastasis; (b) Tumorigenesis, angiogenesis, and cancer cell proliferation (the figure was reproduced from Zhao et al., under signal transduction and targeted therapy copyright).

Adiponectin acts as a positive regulator of IL-8 production (331) and its levels were significantly decreased after TPC2 KO. In contrast, an overexpression of annexin A1, which acts as a negative regulator of IL-8 secretion (327), was found after TPC1 KO or TPC1/TPC2 double KO. The differences in the levels of adiponectin and annexin, which distinctively regulate IL-8, support the ELISA data, which indicates that connections exist between TPC2 and the release of IL-8. In accordance with the present results, Iwasa et al. (2012) found that annexins A1 and A4 repressed MIP-2 or IL-8 production in macrophages (328). Furthermore, it is interesting to note that pro-IL-16, haematopoietic lineage cell-specific protein 1 (HCLS1), NF-kappa B p105 subunit, SAM and SH3 domain-containing protein 3, and Wiskott–Aldrich syndrome (WAS) protein family member 2 were significantly downregulated by a one-fold change or above in TPC2-deficient murine splenocytes. Pro-IL-16 is a precursor of IL-16(a pro-inflammatory cytokine) (329) that can be cleaved by caspase-3 to exhibit its biological functions as a modulator of T-cell growth and a facilitator of the recruitment of CD4+ or CD9+ immune cells in the context of immunity (330). IL-16 is involved in cancer and several processes of the immune system, such as the regulation of T-cell activation and IL-6 and IL-12 secretion and the induction of chemotaxis (329,330,331). Pro-interleukin-16 was statistically downregulated (fold change, 0.95) after TPC2 KO. This suggests that the inhibition of the TPC2 function might interfere with inflammation through the modulation of the proteins involved in this process.

NFKB1 plays a dual role in cancer: it promotes carcinogenesis, as it is involved in apoptosis and immune responses (two important hallmarks of cancer) and constrains tumourigenesis by its tumour-suppressor effects (332). IL-10 is known to play a vital role in immune tolerance, as it inhibits interferon-gamma and tumour necrosis factor production (333). Our data showed a significant decrease in the levels of SAM and SH3 domain-containing protein 3, which acts as a positive regulator of interferon-gamma and tumour necrosis factor production. This finding can be linked to an increase in IL-10 production that we observed after blocking the NAADP/TPC2/Ca^{2+}

signalling pathway with knocking out TPC2 in spleen cells. The observed decrease in the level of HCLS1 protein, which acts as a positive modulator of macrophage differentiation, could be attributed to NAADP/TPC2/Ca^{2+} signalling being halted after TPC2KO, which provides further preliminary evidence to support the possibility of the involvement of NAADP/TPC2/Ca^{2+} signalling in macrophage development. Dehring et al (2011) revealed the corelationship between HCLS1 and WAS protein in promoting dendritic cells migration. It was found that HCLS1 significantly decreased at protein level in TPC2 KO murine spleen cells (334). In addition, WAS protein was significantly downregulated after TPC2 KO; it has been implicated in the regulation of actin cytoskeleton-dependent cellular processes, involving cell migration and cytokine release at the local level, and ultimately, has effects on innate and adaptive immunity globally (335). One possible question raised from these observations is whether TPC2 might be involved as an important regulator maintaining the homeostasis of the expression of these proteins.

Surprisingly, the decrease in the cluster of differentiation 38 (CD38) levels was only non-statistically significant in TPC2 KO murine splenocytes. CD38 is responsible for the generation of NAADP, which is a substrate for two pore channels. This finding points to the modulation of TPC2 as a potential therapeutic approach for treating cancer, particularly in patients with haematological malignancies. Taken together, endolysosomal Ca^{2+} signalling, partially mediated by TPC2, may represent a novel candidate for inflammatory and cancer immunotherapy.

Notably, proteins involved in blood coagulation, which involves haemoglobin, prothrombin, heparin cofactor 2, and plasminogen, were significantly downregulated after TPC1 KO, TPC2 KO, or TPC1/TPC2 double KO in murine splenic cells. A deficiency of antithrombin III is a serious inherited condition manifested by blood clots; hence, TPC gene therapy might provide beneficial effects for treating this disorder. Ankyrin-1 expressed in red blood cells was found to be downregulated by 1.2 in TPC1-deficient murine splenocytes compared with WT. I observed

differences in protein levels in processes that contribute to platelet biology at the local level and ultimately their effects on cardiovascular disease development globally from activation (i.e. prothrombin), including degranulation (i.e. protein disulphide-isomerase TMX3) to aggregation (i.e. vasodilator-stimulated phosphoprotein) after TPC or TPC2 KO in spleen cells. Our lab collaborator in Spain, Prof Joaquin Gadea, identified significant differences in haematological parameters (The Average Corpuscular Volume (VCM), Average Corpuscular Hemoglobin (HCM), Mean Cell Haemoglobin Concentration (MCHC) and Red Cell Distribution Width (RDW)) after knocking out TPC2 in pigs. These statistically significant findings are not relatively important at biological level. Further investigation of these biological parameters in murine blood after TPC1 KO, TPC2 KO, and TPC1/TPC2 double KO is required to inform us further about the role that Ca^{2+}/TPC signalling plays in the haematological system.

Intriguingly, none of the proteins that were differentially expressed after TPC2 KO in murine melanoma cells (B16) (an *in vitro* model) reported by my colleague Xuhui Jin in his MSc thesis were replicated in my proteomic analysis. However, there are similarities in the processes that these proteins are involved in, such as their response to viral infection, cell proliferation, cell migration and metastasis. These observations from the splenic proteome in differences in vital protein expressions that modulate immunity, cancer, and haematology raise a question regarding the possibility of targeting the $Ca^{2+/}$TPC pathway that regulates several vital proteins in cancer pathology as a novel approach for treating cancer and the dual/distinctive role of endolysosomal Ca^{2+} signalling in these systems: does it constrain or promote phenotypes related to cancer and immune and haematological disorders, and will the TPC subtypes behave differently and counteract with each other in the same biological context?

4.5. Conclusion

Cancer and immune diseases are serious health conditions, resulting in substantial morbidity and mortality on a global scale. TPCs are cation channels involved in the regulation of a wide spectrum of pathophysiological processes. Recently, increasing evidence links endolysosomal Ca^{2+} signalling via TPC with cancer and immunity. This study utilises a label-free protein analysis and murine blood profiles to underpin the role of TPCs in various fundamental processes involved in cancer and immune diseases. Proteins related to the physiological processes involved in the immune system or cancer biology were significantly differentially expressed at protein level in TPC1 KO, TPC2 KO, and TPC1/2 double KO splenocytes, compared with WT murine controls. Our study highlights the emerging roles of endolysosomal Ca^{2+} signalling via TPC in cancer immunity in mice. Despite our novel findings, mouse splenic biology might differ from human biology. However, our study highlights the role of TPCs in the immune system. Future research should be conducted to reveal the underlying mechanisms and define TPCs' roles in various pathophysiological processes related to immunological disorders at a local level and cancer immunity at a global level by utilising animal and human models. We suggest investigating TPCs' expressions in patients with immune diseases and cancer followed by in-depth demographic and clinical characterisations to expand our scientific knowledge about the potential for clinical translation as a biomarker for diagnosis and management of diseases or a target for cancer immunotherapy. In addition, our findings unexpectedly provide initial insights into the roles of TPCs in regulating the functions of the haematology system in various pathophysiological processes. This suggests the potential of TPC as a novel biomarker and a target for future cardiovascular/ haematology therapy.

Chapter 5: Exploring the Genetic Aspect of Endolysosomal Ion Channels, Monocytes and Macrophages in Cancer Pathogenesis, Prognosis and Diagnosis: A Genetic Association in the UK Biobank

I declare re-using of my published materials in this chapter:

Alharbi AF, Parrington J. The role of genetic polymorphisms in endolysosomal ion channels TPC2 and P2RX4 in cancer pathogenesis, prognosis, and diagnosis: a genetic association in the UK Biobank. NPJ genomic medicine. 2021 Jul 12;6(1):1-2.

5. Chapter 5: Exploring the Genetic Aspect of Endolysosomal Ion Channels, Monocytes and Macrophages in Cancer Pathogenesis, Prognosis and Diagnosis: A Genetic Association in the UK Biobank

5.1. General introduction:

5.1.1. Cancer genomics: status, promises, applications and challenges

Cancer is a major public health problem and one of the leading causes of death worldwide. The projected number of cancer cases that will be diagnosed in the United Kingdom will reach 243,690 in females and 270,261 in males, by 2035 (336). Moreover, approximately 1 in 2 people in the UK will be diagnosed with cancer at some point during their lifetime (337). Cancer genomics is an emerging discipline of science that applies human genetics to ensure that the best possible tailored diagnosis and therapy are provided to each patient in clinical oncology settings. This changes the process from a 'one-size-fits-all' approach based on a hypothetical average patient to an approach that evaluates each patient's information. The aim of this is to provide an accurate and individualised prevention and treatment plan, taking human genetic information, at a global level (germline) or tumour at a local level (somatic), into consideration. The genetics factor provides a clinical tool to stratify patients into three groups: responders, non-responders, and patients who are more likely to develop side effects of therapy, and serves as a prognostic or diagnostic biomarker. There is a growing body of literature that recognises the importance of genetic information and its contribution to cancer medicine. Recently, candidate gene- and genome-wide association studies have identified genetic variants associated with cancer at a global level and in cancer subtype at a local level. These studies have been conducted among paediatric and adult populations. Some of the findings of these studies were translated into clinics after receiving approval from the FDA. In November 2018, the FDA approved Larotrectinib, a tropomyosin receptor kinase (TRK) inhibitor. Larotrectinib is the first FDA-approved drug for

treating cancer patients with solid tumours based on the genetic makeup of the tumour (harbouring neurotrophic receptor tyrosine kinase gene fusion) and not based on the tumour origin (338). In oncology, 221 FDA-approved drugs contain genetic information that guide the provided therapy to cancer patients by clinicians. Zanubrutinib, an irreversible Bruton tyrosine kinase inhibitor, was approved by the FDA in 2019 to treat cancer patients with mantle cell lymphoma (MCL) who have previously received at least one line of therapy (339). In a phase III trial (NCT03053440), Zanubrutinib was effective in treating patients with Waldenström's macroglobulinemia carrying WT genetic status of Myeloid differentiation primary response gene (MYD88) (340). Genetic biomarkers are not only utilised in direct therapy, but have also been shown to optimise diagnosis and predict the risk and prognosis of cancer at research and clinical levels. The FDA-approved F1CDx and MSK-IMPACT tests are based on targeted next-generation sequencing to profile the genetic signature of the tumour and to detect genetic mutations in 324 and 505 genes, respectively (341,342,343). Despite the importance of preventing cancer development, a paucity of causal genetic risk has been discovered and moved into the clinical practice stage to predict or prevent cancer. Testing for BRCA1 and BRCA2 is the historical example of utilising genomics as an approach to identify subjects who are likely to develop breast and ovarian cancer in various populations at a global level (344). The advances in genetic technologies open the doors to reveal new genomic biomarkers to prevent or optimise cancer therapy as an approach to precision medicine. The field of cancer genomics is evolving and has made distinguished advances in the treatment and prevention of cancer. From my perspective, this field is shifting the paradigm in cancer medicine, allowing many patients hope for a cure and preventing many subjects from developing cancer. Genetic-based predictive testing will enable clinicians to evaluate the risks of developing cancer or undergoing direct therapy. This will lead to tailored therapy plans based on patient genetic information, reduce morbidity and mortality associated with irreversible side effects, lower the cost of complications, and eliminate negative

consequences on patient health – whether psychological, social or economic. One of the major limitations in the application of cancer genomics in daily clinical practice is that health professionals are not trained to understand this type of information and do not know how to interpret the results of genetic tests, nor how to resolve and address the ethical, legal, and social issues in the application of genomics in clinical practice. Therefore, I suggest establishing a cancer genomics unit as a part of the oncology clinic, built of a team from various disciplines, to help clinicians provide the best care to patients and incorporate cancer genomics in all health care practitioners' training in order to be competent in genomic-directive therapy.

Table 15. FDA- approved drugs labels contain genetic information in oncology.

Drug	Biomarker	Type of information	FDA label
Cisplatin	TPMT	Adverse Reactions (ototoxicity)	018057s083lbl.pdf
Trastuzumab	HER2	Indications	103792s5345lbl.pdf
Trametinib	RAS	Warnings and Precautions	204114s001lbl.pdf
https://www.accessdata.fda.gov			

5.1.2. UK Biobank and evaluation of cancer biomarkers

The UK Biobank is one of the world's largest and most comprehensive databases, containing in-depth genetic and health information from half a million participants in the UK. Leveraging the UK Biobank might lead to the discovery of new cancer hallmarks and biomarkers. The UK Biobank database allows cancer genomics to provide insights into the role of a specific gene in humans and will take researchers to the next level in conducting translational studies based on the evolving scientific knowledge of its genomic aspects in cancer. Recently, researchers identified new genetic risks in the UK Biobank population. Song et al. (2022) investigated the link between circulating cytokines and the likelihood of developing cancer at the genetic level (345). They suggested the involvement of 48 cytokines in cancer pathology in developing various cancer

subtypes, involving IL-17 in acute myeloid leukaemia and IL-18 in stomach cancer, which warrants further investigation (345). Similarly, Yarmolinsky et al. (2022) found that carrying 34536443:CC was associated with a higher risk of developing lung cancer and non-Hodgkin lymphoma (Lung cancer OR: 1.15, 95% CI: 1.09–1.23, *p*-value= 2.29×10^{-6}, non-Hodgkin lymphoma OR: 1.18, 95% CI: 1.05–1.33, P = 5.25×10^{-3} vs. G/G) (346). These recent data underscore the significance of utilising large databases to decode the gene's role in human health and disease states, which might ultimately lead to potential translational applications. In this thesis, I conducted two projects with the UK Biobank population to provide a novel approach that enables clinicians to provide tailored therapy based on patient genetic information, reduce morbidity and mortality, and eliminate negative consequences on patient well-being, whether psychological, social or economic. Moreover, these studies give us insight into the pathophysiological significance of candidate genes in cancer and may contribute to the development of genomics-based guidelines in the future.

5.2. General approach and methods

5.2.1. Study participants and data availability

Case-control genetic association studies were conducted with the UK Biobank population, which includes more than 500,000 clinically and genetically assessed participants. Participants with complete sex and genetic data, DNA concentration ≥ 10 ng/ul, and cluster CR ≥ 97 were eligible for the analysis. Metastasis and malignancy were defined by the behaviour of cancer tumour data (40012-0.0), and cancer recurrence was defined by reported occurrences of cancer (≥ 2 [40009-0.0]). The UK Biobank data can be retrieved by applying to the UK Biobank (https://www.ukbiobank.ac.uk/register-apply/).

5.2.2. DNA, genotyping and genetic analysis

Genomic DNA was extracted from the UK Biobank participants and stored according to the UK Biobank's protocols. Genotyping was performed according to the UK Biobank procedures utilising the GeneTitan® Multi-Channel Instrument at Affymetrix Research Services Laboratory in Santa Clara, California, USA. Genetic associations with the risk of cancer phenotypes were assessed using univariate and multivariate logistic regression analyses.

5.2.3. Bioinformatic analyses

Several public databases and tools involving the Genotype-Tissue Expression (GTEx) (347), Combined Annotation Dependent Depletion (CADD) GRCh38-v1.6 (348), FATHMM-XF (349), Mutation Assessor release (350), Human Protein Atlas (351), Ensembl Variant Effect Predictor (352), and UALCAN (353) were navigated to predict and assess the potential effect of the significant genetic variants in the corresponding gene expression/activity in similar biological contexts to their phenotypic association.

5.2.4. Statistical analysis

The findings have been presented using basic descriptive statistics such as proportion, mean ± SD, median, and range. R version 3.4.1 (ggplots package) was utilised for data analysis and visualisation statistical analysis was conducted utilising the appropriate test approach for the type of data, a two-sample t-test for numerical variables and a Chi-square test for categorical variables.

5.2.5. Ethical approval

The first UK Biobank study (ID-51249) and the second UK Biobank study (ID-51797) were approved by the UK Biobank committee, which included the approval from the North West Multi-Centre Research Ethics Committee and the Patient Information Advisory Group.

UNIVERSITY OF OXFORD

Chapter 5: Section 1

The role of genetic polymorphisms in endolysosomal ion channels TPC2 and P2RX4 in cancer pathogenesis, prognosis and diagnosis: a genetic association in the UK Biobank

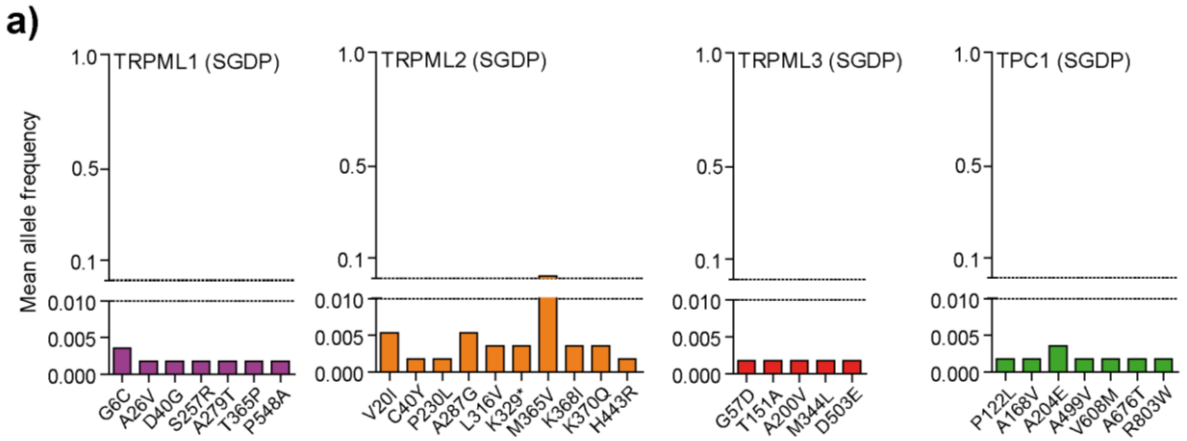
5.2. Section 1. The role of genetic polymorphisms in endolysosomal ion channels TPC2 and P2RX4 in cancer pathogenesis, prognosis and diagnosis: a genetic association in the UK Biobank

5.2.1. Introduction

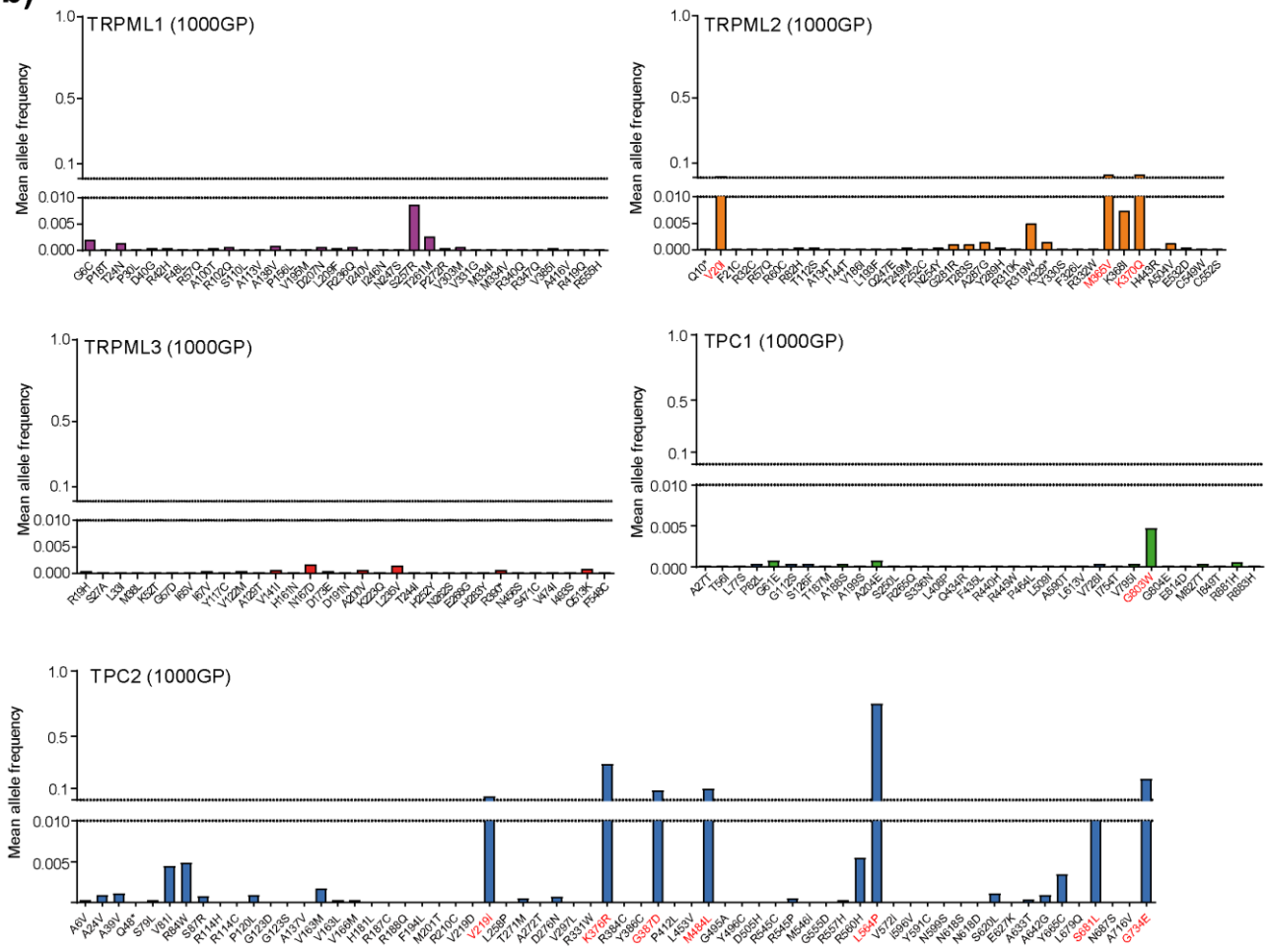
5.2.1.1. Endolysosomal cation channels at the genomic level in health and disease states

Endolysosomal Ca^{2+} signalling via TPCs, TRPML and ATP-regulated P2X4 channels plays a role in health and disease. Recently, the genetic variability in endolysosomal cation channels has been a topic of great interest that is drawing scientific attention to the importance of decoding endo lysosomal cation channel genes' role in health and disease. Böck et al. (2021) identified 7, 22, 7, 10, and 5 genetic variations in TPC1, TPC2, TRPML1, TRPML2, and TRPML3, respectively, in the Simons Genome Diversity Project dataset (160), indicating the higher frequency of TPC2 mutations compared to other endolysosomal cation channels. This finding was confirmed by Böck and his colleagues in two different datasets, the 1000GP and the gnomAD datasets, including the genetic variants (33, 381), (63, 412), (34, 272), (34, 278) and (31, 260) in TPC1, TPC2, TRPML1, TRPML2 and TRPML3, respectively (as shown in Figure 83) (160). There is a paucity of published data that links polymorphisms in TPC1, TPC2, TRPML1, TRPML2, TRPML3 and P2X4 genes to phenotypes. In the same study, Böck and his colleagues conducted GWAS analysis to underpin the genomic landscape of TPC2 in disease. Figure 84 shows genetic variants in the TPCN2 gene with phenotypes relevant to health and disease, including bone mineral density, type 2 diabetes, and hair colour (160). Similarly, two polymorphisms, rs35264875 and rs3829241, in the TPCN2 gene encoding amino acid alterations that change M484L and G734E, respectively, were found to lead to a gain in function of TPC2, manifested by substantial pore dilation (160,161,162). The polymorphisms rs35264875 and rs3829241 were associated with a shift from brown to blond hair colour (160,161,162). In 2015, Stokes et al. found that

rs28360472 (encodingTyr315>Cys) in the P2X4 receptor gene that leads to loss of function was associated with a higher pulse pressure in the white population (354). Overall, these data indicate that there is a paucity of studies that genetically characterise endolysosomal cation channels in human and disease states.



b)


TRPML1 (1000GP)
TRPML2 (1000GP)
TRPML3 (1000GP)
TPC1 (1000GP)
TPC2 (1000GP)
Mean allele frequency
1.0
0.5
0.1
0.010
0.005
0.000

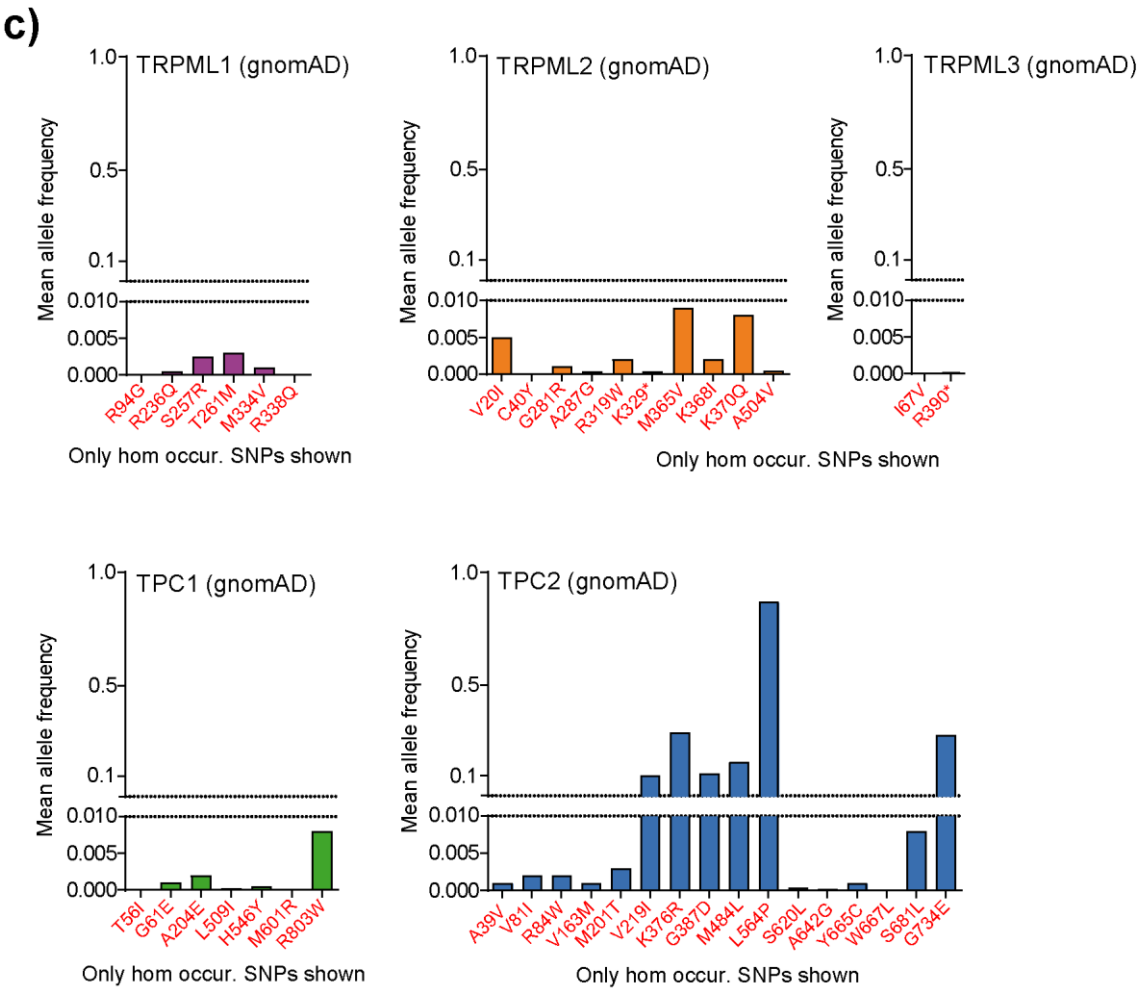


Figure 83. Endolysosomal cation channels' genomic landscape in various datasets: (a,) SGDP; (b) 1000GP; (c) gnomAD (160) (the figure was reproduced from Böck et al., under PLoS Genet coyright).

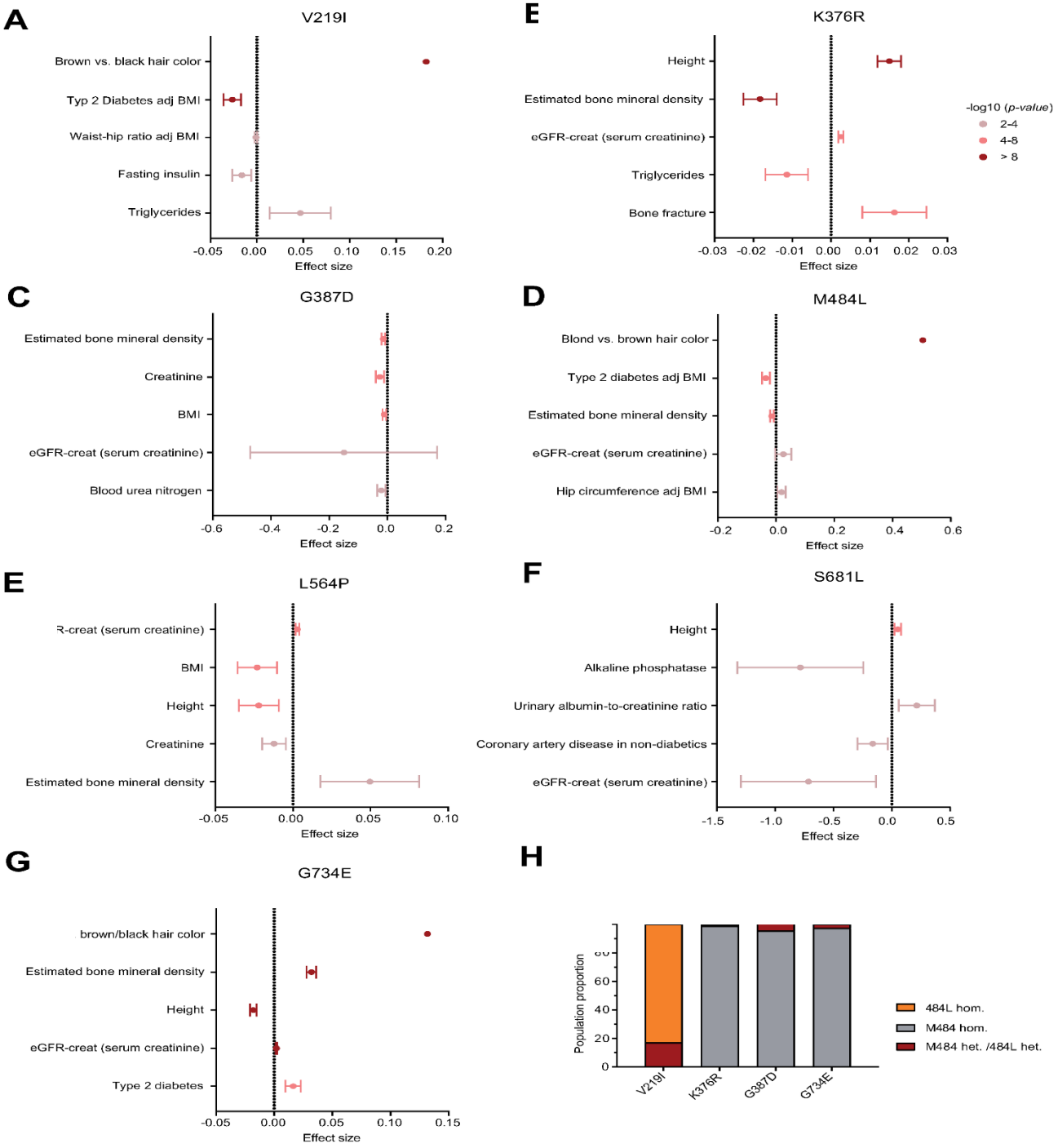


Figure 84. Genetic and phenotype association in TPC2 (160) (the figure was reproduced from Böck et al., under PLoS Genet coyright).

5.2.1.2. Endolysosomal cation channels focusing on two-pore channel type 2 as a biomarker in cancer/cancer subtype diagnosis and prognosis: an approach to precision medicine

The role of endolysosomal Ca^{2+} signalling has been demonstrated in various pathophysiological processes attributed to cancer, including cancer cell proliferation, apoptosis, angiogenesis, autophagy, invasion, migration, and metastasis. The evolving field of endolysosomal Ca^{2+} signalling in cancer, however, has a paucity of human data. Remarkably, TPCN2 genetic mutations were more common compared to other endolysosomal cation channels in genetic analyses conducted by Böck and his colleagues utilising different datasets (160). Recently, Li et al. (2019) identified TPCN2 as one of six gene architectures to predict postoperative biochemical recurrence, highlighting the prognostic role of TPCN2 in cancer (149). Rs72932540 is located on GRCh38: 11:69,154,575, and the distance from the variant to the transcription start site (TSS) of TPCN2 is 105,643 Kb. This genetic variation in close proximity to TPCN2 might influence tumour development. RNA-sequencing analysis conducted by Howarth et al. (2021) found that TPCN2 exon 5 was fused to RSF1 exon 4 in the MDA-MB-175 cell line (an *in vitro* model of breast cancer (355). In our recent publication in collaboration with Prof. Antonio Filippini at the Sapienza University of Rome in Italy, TPCN2 was found to be significantly downregulated in metastatic vs primary tumours in patients with skin cutaneous melanoma utilising The Cancer Genome Atlas (TCGA) dataset (142). In contrast, higher expression of TPCN2 was associated with poor survival outcomes in patients with bladder cancer using TCGA data (150). These data underscore the translational relevance of TPC2 as a potential novel biomarker in cancer.

5.2.1.3. Significance, innovation, overall hypothesis and objectives

There is increasing evidence that links two-pore channel 2 (TPC2) to cancer (2), revealing the potential applications of TPC2 as a biomarker in the definition of tumour types, susceptibility, prognosis, response, and cancer outcomes. Our current studies have begun to provide links between TPC2 and cancer biology. However, there is a lack of studies that have genetically and clinically characterised TPCN2 in cancer patients. I hypothesised that genetic polymorphisms in TPCN2 contribute to the development of cancer/cancer subtype, behaviour (malignant/metastatic), and recurrence. Therefore, I aimed to identify genetic associations in TPCN2 with the risks for developing cancer or cancer subtypes, for developing malignant/metastatic tumours and for cancer recurrence in the UK Biobank population. The goal of the project was to provide preliminary evidence about the clinical utility of TPC2 as a diagnostic, prognostic and predictive biomarker in directing therapy in patients with cancer, which ultimately might lead to the optimisation of cancer therapy. To my knowledge, this is the first study that has characterised the effect of genetic variants in endolysosomal cation channels (TPC2 and P2RX4) and cancer with a large-scale and comprehensive in-depth analysis of various types of cancer. I have also researched several databases to provide, in a preliminary fashion, the biological significance of these variants at the local level and TPC2 and P2RX4 expression/activity at the global level in cancer.

5.2.2. Methods

5.2.2.1. Study participants

Figure 86 shows that the study population consisted of two major cohorts – defined as cases (cancer) and controls (free of cancer). Then, the cancer cohort was classified into 13 sub-cohorts to perform the analysis of various subtypes of cancer, including brain, breast, bronchus, lung,

colon, lymphoid and hematopoietic systems, skin, ovary, prostate, rectum, thyroid gland, lip, oral cavity, pharynx, and urinary tract. The cancer type was defined by type of cancer ICD-10 (40006-0.0), which was originally retrieved from the cancer register.

5.2.2.2. Genetic analysis

UK Biobank's large-scale data was leveraged to investigate the associations between endolysosomal cation channels and cancer by looking at single nucleotide polymorphisms (SNPs) in TPCN2 (rs3750965, rs35264875, rs34510004, and rs3829241), a variant in close proximity to the TPCN2 gene (rs72932540), and P2RX4 (rs25644 and rs28360472), and cancer/cancer type (see figure 85). The minor allele frequencies were 33%, 16%, 0.13%, 39%, 9%, 12% and 2% for rs3750965, rs35264875, rs34510004, rs3829241, rs72932540, rs25644 and rs28360472, respectively. All genetic variants were in Hardy–Weinberg equilibrium (p-value > 0.05) – except for rs3829241 (p-value < 0.05), which was excluded from the analysis (see Supplementary Table 5.1). Genetic correlations with the risks of developing cancer at the global level or various types of cancer at the local level, of developing malignant or metastatic tumours, and of cancer recurrence were assessed using univariate and multivariate logistic regression analysis after adjusting for significant general cofounders (including sex, ethnicity and age). The genetic association risk among cases and controls was first investigated and then the significant associations were determined using logistic regression analysis. Three logistic regression models – Model A: univariate logistic regression, Model B: multivariate logistic regression after adjustment for gender and ethnicity, and Model C: multivariate logistic regression after adjustment after adjusting for age (as a categorical variable), sex and ethnicity – were performed to assess the significant genetic correlations in the UK Biobank population.

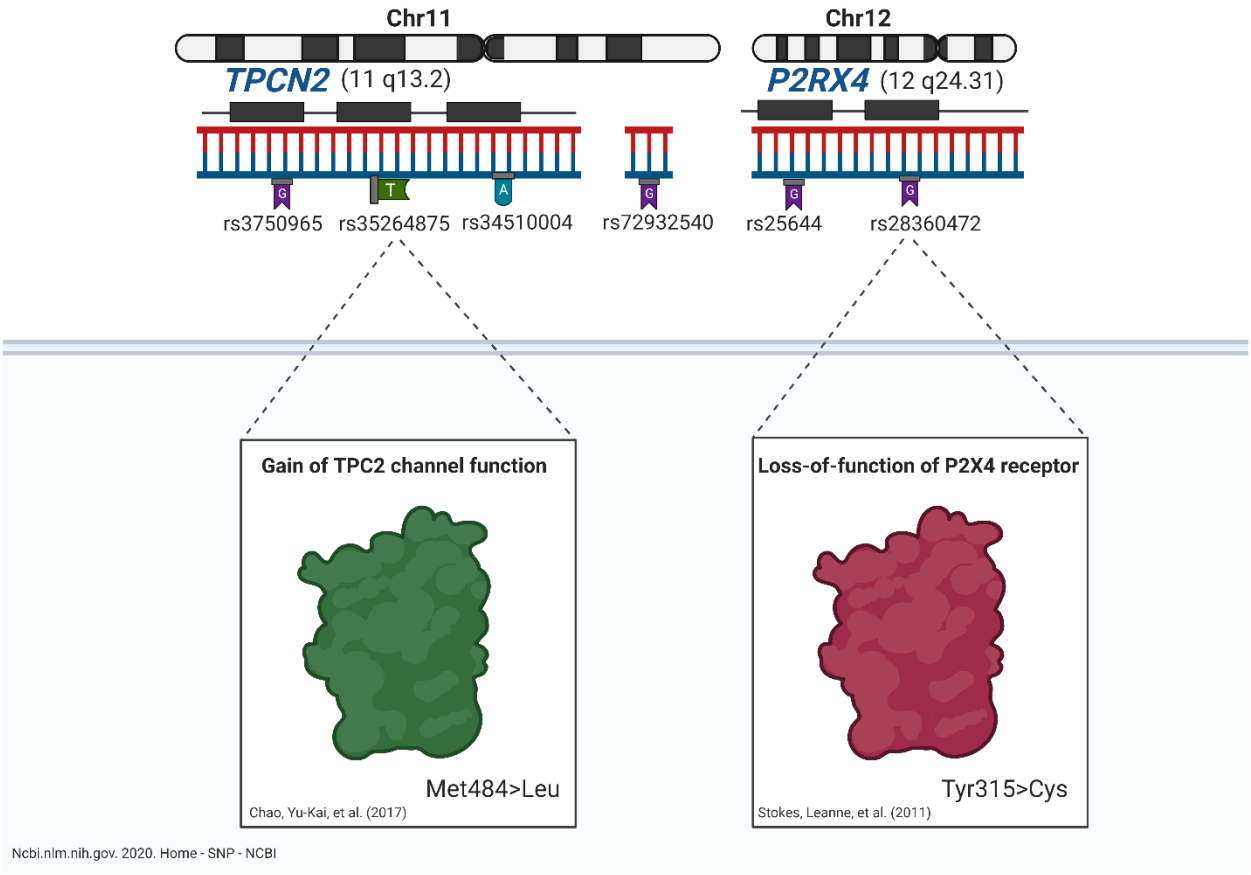


Figure 85. Single nucleotide polymorphisms (SNPs) in *TPCN2* and *P2RX4* that were included in our analysis (356) (the figure was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

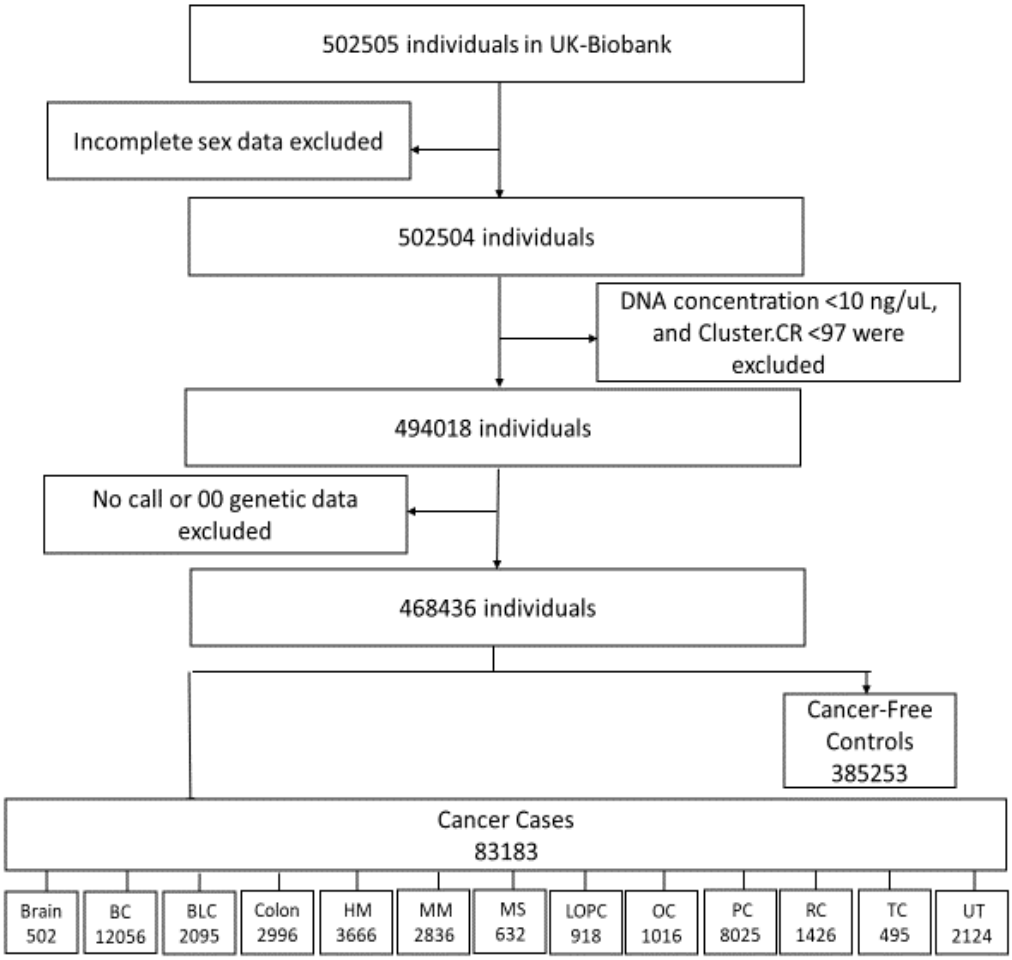


Figure 86. Schematic representation of the sample selection strategy (356) (the figure was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).The abbreviations in the figure correspond to the following types of cancer: BC is breast cancer; BLC bronchus and lung cancer; HM malignant neoplasms, stated or presumed to be primary of lymphoid, haematopoietic and related tissue; MM malignant melanoma; MS malignant neoplasms of mesothelial and soft tissue; LOPC malignant neoplasm of the lip, oral cavity and pharynx; OC malignant neoplasm of the ovary; PC malignant neoplasm of the prostate; RC malignant neoplasm of the rectum; TC malignant neoplasm of the thyroid gland; and UT malignant neoplasm of the urinary tract (the figure was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

5.2.3. Results

The data presented in this section were previously published in Alharbi et al. (2020)(356); therefore, I briefly highlight the major findings here. A total of 468,436 subjects were reviewed: 385,253 (82.24%) as cancer-free controls and 83,183 (17.76%) as cancer cases. The median age at diagnosis was 59.90 years (5.20–79.20), as shown in Table 16. Figure 87a and Table 17 show that carrying the TPCN2 rs3750965 allele GG was associated with a lower risk of developing cancer (OR: 0.97, 95% CI: 0.95–0.997, *p*-value = 0.029* vs A/A), whereas carrying rs35264875:TA and rs72932540:GA (TPCN2) was associated with an increased risk of cancer susceptibility (OR: 1.03, 95% CI: 1.01–1.05, *p*-value = 0.001** vs A/A and OR: 1.07, 95% CI: 1.05–1.09, *p*-value = 4.51e-10*** vs A/A, respectively) after adjusting for sex and ethnicity. There were no significant correlations between the genetic variants included in the analysis and cancer in terms of whether it was malignant or benign and whether there was cancer recurrence.

Table 19 and Figure 87 b illustrate that carrying the TPCN2 rs35264875 allele TA is associated with higher odds of developing breast cancer (OR: 1.06, 95% CI: 1.02–1.1, *p*-value = 0.006** vs A/A), colon cancer (OR: 1.03, 95% CI: 1.01–1.05, *p*-value = 0.00138** vs A/A), and malignant neoplasms stated or presumed to be primary, and of lymphoid, haematopoietic and related tissue (OR: 1.09, 95% CI: 1.02–1.18, *p*-value = 0.015* vs A/A). Subjects who were carriers of genotype TA or TT in rs35264875 (TPCN2) showed a higher risk of developing malignant neoplasms of the prostate (OR: 1.073, 95% CI: 1.02–1.13, *p*-value = 0.005** vs A/A and OR: 1.18, 95% CI: 1.03–1.35, *p*-value = 0.016* vs. A/A, respectively), whereas genotype TT in rs35264875 showed higher odds of developing malignant neoplasms of the rectum (OR: 1.37, 95% CI: 1.02–1.84, *p*-value = 0.034* vs A/A).

Genotype GA in rs3750965 in TPCN2 showed a lower risk of developing malignant melanoma (OR: 0.91, 95% CI: 0.84–0.99, *p*-value = 0.02334* vs A/A). Genotype GG in rs3750965 showed lower odds of developing malignant neoplasms of mesothelial and soft tissues (OR: 0.72, 95% CI: 0.53–0.97, *p*-value = 0.032 *, vs. A/A) but is correlated with higher odds of developing malignant neoplasms of the lip, oral cavity and pharynx (OR: 1.25, 95% CI: 1.02–1.53, p-value = 0.0311* vs A/A). Subjects who were carriers of genotype GA or GG in rs3750965 (TPCN2) were associated with a decreased risk of malignant neoplasms of the prostate (OR: 0.94, 95% CI: 0.90–0.99, *p*-value = 0.017* vs A/A and OR: 0.86, 95% CI: 0.80–0.93, p-value = 0.0002*** vs A/A, respectively). The TPCN2 rs3750965 allele GG was associated with high rates of cancer recurrence in patients with prostate cancer (OR: 1.07, 95% CI: 0.94–1.22, p-value = 0.018* vs A/A). In contrast, the TPCN2 rs3750965 allele GA was correlated with high rates of cancer recurrence in patients with rectal cancer (OR: 1.4, 95% CI: 1.05–1.85, *p*-value = 0.0199* vs A/A) (see Table 20 and Figure 87 d).

In various types of cancer at the local level, the carriage of rs72932540:GA was associated with an increased risk of breast cancer (OR: 1.1, 95% CI: 1.05–1.16, *p*-value = 7.2e-05*** vs A/A), malignant melanoma (OR: 1.15, 95% CI: 1.042–1.26, *p*-value = 0.00512** vs A/A) and malignant neoplasm of the rectum (OR: 1.15, 95% CI: 1.004–1.32, *p*-value = 0.044* vs A/A). Table 19 and Figure 87 b show that subjects carrying genotypes GA and GG in rs72932540 have a higher risk of developing malignant neoplasms of the prostate (OR: 1.11, 95% CI: 1.04–1.18, *p*-value = 0.0007*** vs A/A and OR: 1.31, 95% CI: 1.04–1.65, p-value = 0.024*, respectively). Genotype GA in rs28360472 (P2RX4) was associated with an increased risk of breast cancer susceptibility (OR: 1.1, 95% CI: 1–1.22, *p*-value = 0.0478* vs A/A) but was also associated with a decreased risk of colon cancer susceptibility (OR: 0.77, 95% CI: 0.61–0.97, p-value = 0.028* vs A/A). Genotype GG was associated with higher odds of malignant neoplasms of mesothelial and soft tissue susceptibility (OR: 10.8, 95% CI: 2.7–43.7, *p*-value = 0.0009*** vs A/A). GG in rs25644 in P2RX4

in cancer at the global level was correlated with lower rates of cancer recurrence (OR: 0.52, 95% CI: 0.28–0.96, *p*-value = 0.038* vs A/A), but at the local level, it was associated with an increased risk of malignant neoplasms of the prostate (OR: 1.23, 95% CI: 1.03–1.47, *p*-value = 0.02175* vs A/A). No significant association was observed between the genetic variants included in the analysis and cancer in terms of whether it was malignant or benign. The potential and predicted impact of these genetic variants on TPCN2/PR2X4 expression and activity levels showed significant correlations with cancer at the global level. Various types of cancer, in terms of susceptibility and outcomes, in the UK Biobank population were investigated in a similar biological/clinical context and are summarised in Table 21.

Table 16. Subject characteristics and univariate analysis. (The table was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

	UK Biobank ID	Total	Cancer Cases	Cancer-Free Controls	*p*-value
Number (n,%)		468436 (100%)	83183 (17.76%)	385253 (82.24%)	
Demographic factors					
Age at cancer diagnosis (Median (range))	40008-0.0		59.90 (5.20-79.20)		
Male (n,%)	31-0.0	214419 (45.77%)	35918 (43.18%)	178501 (46.33%)	< 2.2e-16
Female (n,%)		254017 (54.23%)	47265 (56.82%)	206752 (53.67%)	< 2.2e-16
White (n,%)	21000-0.0	424542 (90.73%)	77109 (92.70%)	347433 (90.18%)	< 2.2e-16
Clinical factors (n,%)					
Underlying (primary) cause of death					
Diseases of the circulatory system	40001-0.0	3903 (20.80%)	819 (7.12%)	3084 (73.65%)	
Reported occurrences of cancer		83183 (17.76%)	83183 (100%)		
Reported recurrence of cancer	40009-0.0	20998 (4.48%)	20998 (25.24%)		
Histology of cancer tumour					
Epithelial	40011-0.0		70912 (89.28%)		
Behaviour of					

cancer tumour					
Malignant	40012-0.0		66057 (83.12%)		
Genetic factors (median, range)					
DNA concentration	22024-0.0	34.42 (10-713.84)	34.35 (10-655.87)	34.43 (10-713.84)	0.13
Affymetrix quality control metric "Cluster.CR"	22025-0.0	99.52 (97-99.92)	99.52 (97.01-99.1)	99.52 (97-99.92)	0.23
Affymetrix quality control metric "dQC"	22026-0.0	0.978 (0.88-1)	0.977 (0.89-1)	0.9784 (0.88-1)	0.12
SNP (genotypes) (n,%)					
rs3750965	affy5779511				
A A		211180 (45.08%)	37638 (45.25%)	173542 (45.05%)	HWE=0.65
G A		206786 (44.14%)	36762 (44.19%)	170024 (44.13%)	
G G		50470 (10.77%)	8783 (10.56%)	41687 (10.82%)	
rs35264875	affy5779776				
A A		332599 (71.00%)	58604 (70.45%)	273995 (71.12%)	HWE=0.34
T A		124338 (26.54%)	22512 (27.06%)	101826 (26.43%)	
T T		11499 (2.45%)	2067 (2.48%)	9432 (2.45%)	
rs34510004	affy5779828				
A A		1 (0.0002%)	0 (0%)	1 (0.0003%)	HWE=0.77
A G		1179 (0.25%)	230 (0.28%)	949 (0.25%)	
G G		467256 (99.75%)	82953 (99.72%)	384303 (99.75%)	
rs3829241	affy5780031				
A A		72734 (15.53%)	13208 (15.88%)	59526 (15.45%)	HWE=0
A G		218297 (46.60%)	39316 (47.26%)	178981 (46.46%)	
G G		177405 (37.87%)	30659 (36.86%)	146746 (38.09%)	
rs72932540	affy5782262				
A A		390424 (83.35%)	68628 (82.50%)	321796 (83.53%)	HWE=0.41
G A		74511 (15.91%)	13929 (16.75%)	60582 (15.73%)	
G G		3501 (0.75%)	626 (0.75%)	2875 (0.75%)	
rs25644	affy7015041				
A A		365875 (78.11%)	65014 (78.16%)	300861 (78.09%)	HWE=0.47
G A		96176 (20.53%)	16990 (20.42%)	79186 (20.55%)	
G G		6385 (1.36%)	1179 (1.42%)	5206 (1.35%)	
rs28360472	affy7015101				
A A		453577 (96.83%)	80430 (96.69%)	373147 (96.86%)	HWE=0.1
G A		14721 (3.14%)	2724 (3.27%)	11997 (3.11%)	
G G		138 (0.03%)	29 (0.03%)	109 (0.03%)	

Table 17. Univariate and multi-variate logistic regression analyses of genetic variants in TPCN2 and P2RX4 that significantly associated with the risk of cancer in the UK Biobank. (The table was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

Gene	SNP	Genotype	Cancer-Free (Controls)	Cancer (Cases)	Probability of Cancer	Model A		Model B	
			n=385253	n=83183		(OR 95 %CI)	*p*-value	(OR95 %CI)	*p*-value
TPCN2	rs3750965	GA(n)	170024	36762		1 (0.98-1.01)	0.702	1(0.98-1.01)	0.591
		GG(n)	41687	8783	⬇	0.97 (0.95-0.997)	0.026 *	0.97(0.95-0.997)	0.029 *
	rs35264875	TA(n)	101826	22512	⬆	1.03 (1.02-1.05)	0.0001***	1.03(1.01-1.05)	0.001 **
		TT(n)	9432	2067		1.03 (0.98-1.08)	0.325	1.022(0.97-1.07)	0.374
Close to TPCN2	rs72932540	GA(n)	13929	60582	⬆	1.08 (1.06-1.10)	2.76e-13 ***	1.07(1.05-1.09)	4.51e-10 ***
		GG(n)	626	2875		1.02 (0.94-1.113)	0.639	1.012(0.93-1.103)	0.793
P2RX4	rs28360472	GA(n)	2724	11997	⬆	1.053 (1-1.099)	0.0159 *	1.04(0.99-1.08)	0.091
		GG(n)	29	109		1.23 (0.82-1.9)	0.314	1.23(0.81-1.85)	0.331

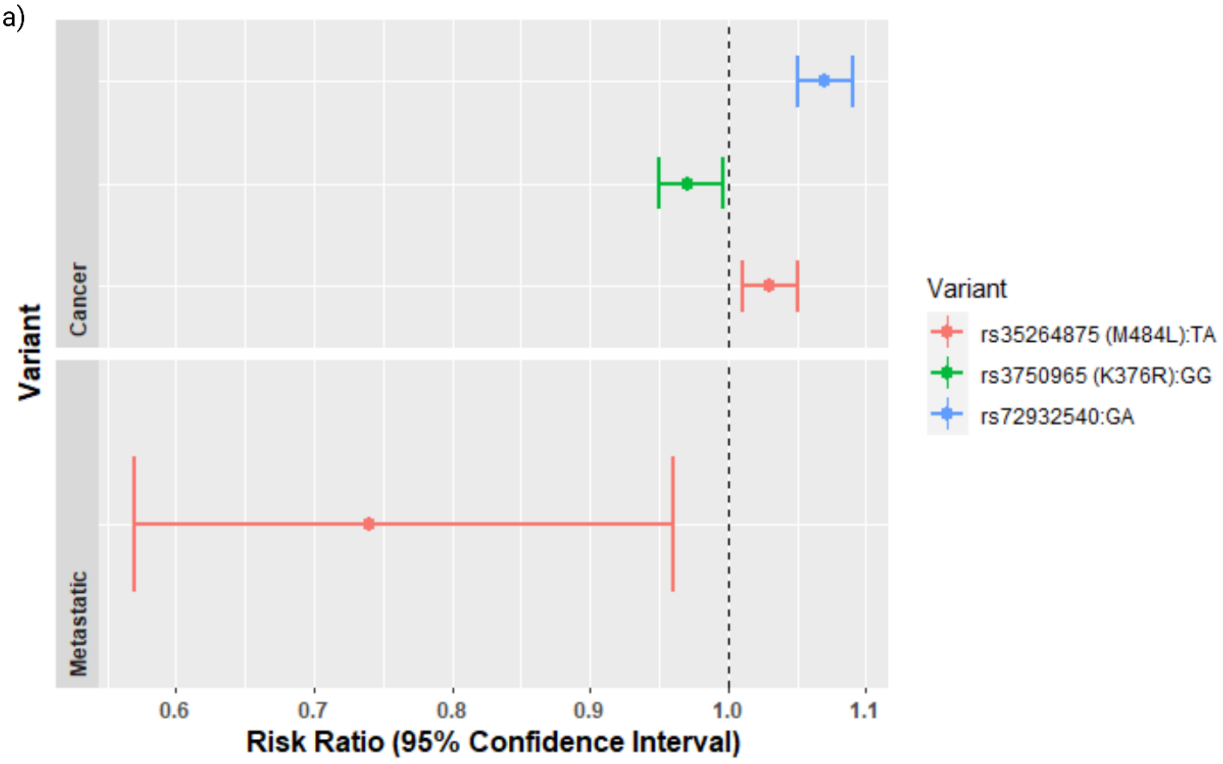
Abbreviations: OR, odds ratio; CI, confidence intervals; model A: univariate logistic regression; model B multivariate logistic regression, adjusted for sex, and ethnicity.

Table 18. Univariate and multi-variate logistic regression analyses of a genetic variant (rs35264875) that significantly associated with risk of metastatic cancer. (The table was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

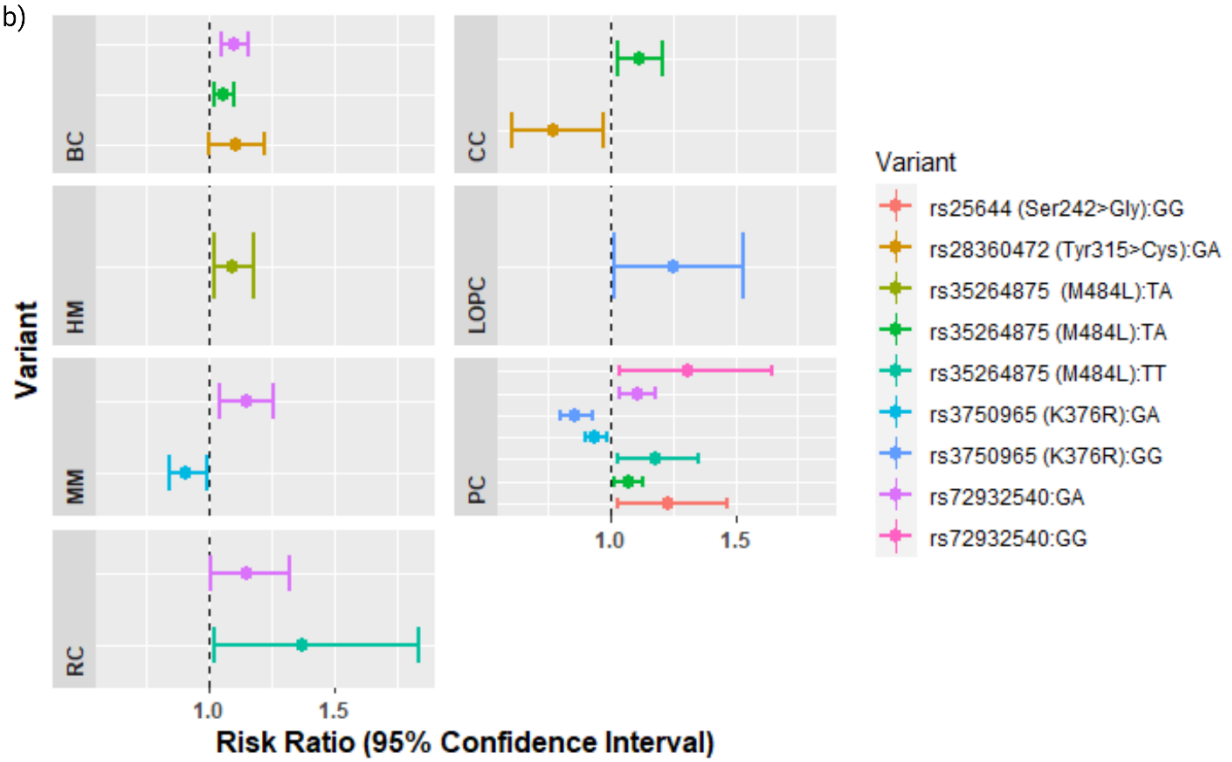
SNP		Primary cancer	Metastatic cancer		Model A		Model B		Model C	
		(Controls) n=65559	(Cases) n=334		(OR95 %CI)	*p*-value	(OR95 %CI)	*p*-value	(OR95 %CI)	*p*-value
rs35264875	TA	17864	73	⬇	0.74(0.57-0.96)	0.024 *	0.74(0.57-0.96)	0.0235 *	0.74(0.57-0.96)	0.025 *
	TT	1654	7		0.77(0.36-1.6)	0.49	0.77(0.36-1.63)	0.4911	0.77(0.36-1.63)	0.49

Abbreviations: OR, odds ratio; CI, confidence intervals; model A: univariate logistic regression; model B: multivariate logistic regression, adjusted for sex, and ethnicity; model C: multivariate logistic regression, adjusted for age, sex, and ethnicity.

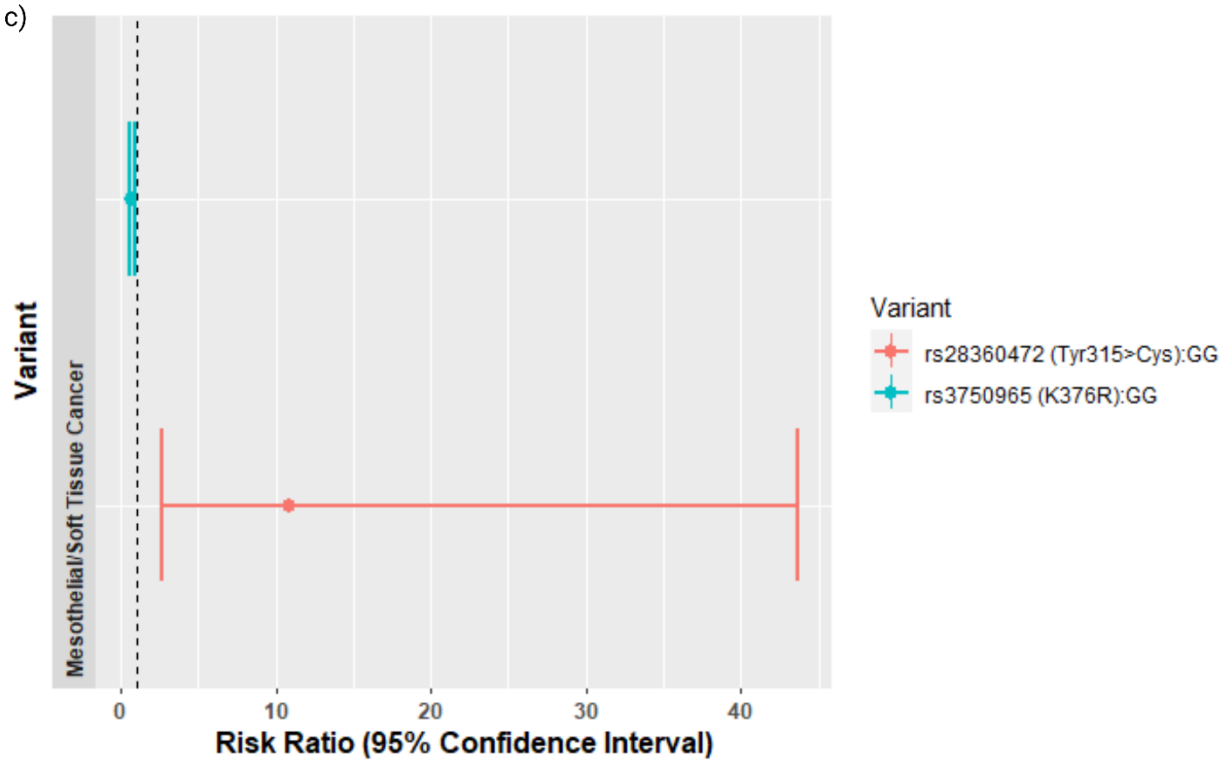
a)


Variant
Cancer
Metastatic
0.6
0.7
0.8
0.9
1.0
1.1
Risk Ratio (95% Confidence Interval)
Variant
rs35264875 (M484L):TA
rs3750965 (K376R):GG
rs72932540:GA

b)


Variant
BC
CC
HM
LOPC
MM
PC
RC
1.0
1.5
1.0
1.5
Risk Ratio (95% Confidence Interval)
Variant
rs25644 (Ser242>Gly):GG
rs28360472 (Tyr315>Cys):GA
rs35264875 (M484L):TA
rs35264875 (M484L):TA
rs35264875 (M484L):TT
rs3750965 (K376R):GA
rs3750965 (K376R):GG
rs72932540:GA
rs72932540:GG

c)


Variant
Mesothelial/Soft Tissue Cancer
0
10
20
30
40
Risk Ratio (95% Confidence Interval)
Variant
rs28360472 (Tyr315>Cys):GG
rs3750965 (K376R):GG

d)

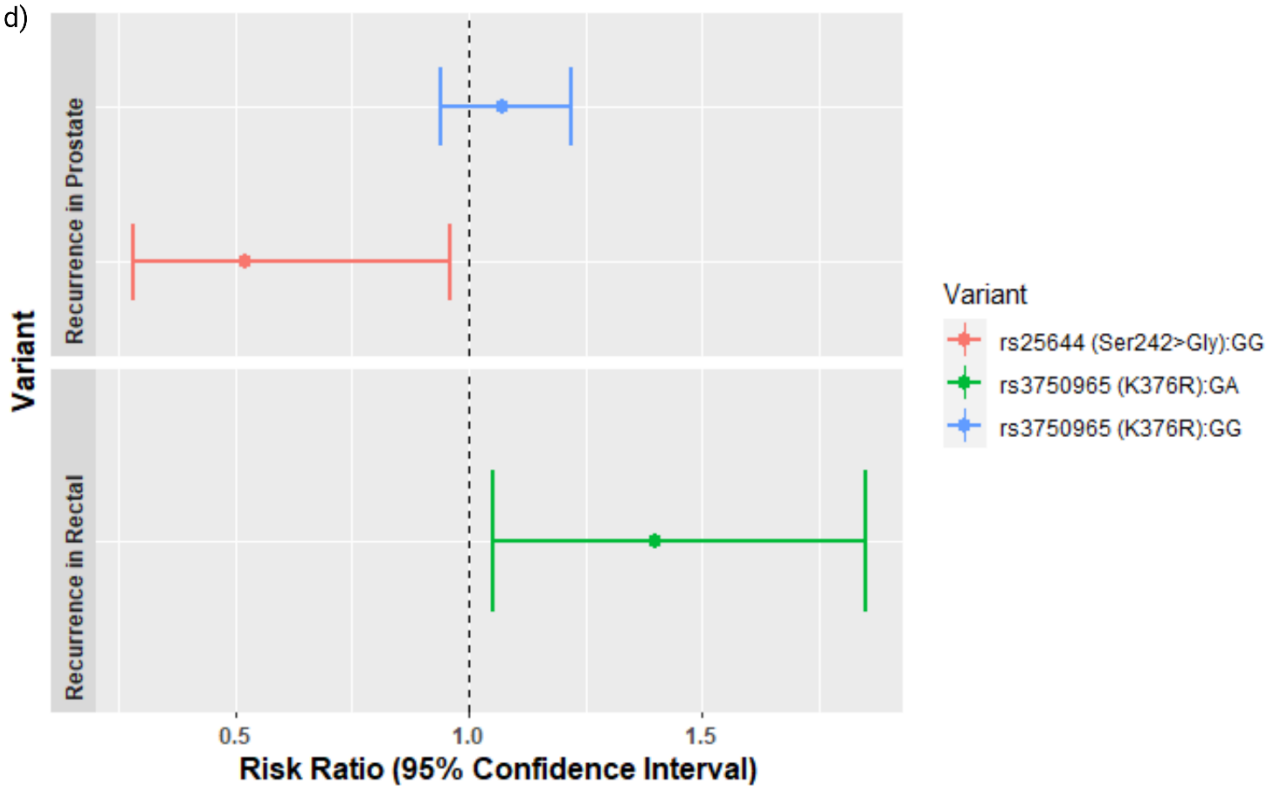

Variant
Recurrence in Prostate
Recurrence in Rectal
0.5
1.0
1.5
Risk Ratio (95% Confidence Interval)
Variant
rs25644 (Ser242>Gly):GG
rs3750965 (K376R):GA
rs3750965 (K376R):GG

Figure 87. Forrest plot showing genotypic ORs for significant SNPs in *TPCN2/ P2RX4*. (a), (b), (c): estimates were derived using logistic regression after adjusting for sex and ethnicity for cancer at a global level or various specific types of cancer at a local level. d: estimates were derived using logistic regression after adjusting for age, sex and ethnicity. Abbreviations: BC: Breast cancer, HM: Malignant neoplasms, stated or presumed to be primary, of lymphoid, haematopoietic and related tissue, MM: Malignant melanoma, PC: Malignant neoplasm of prostate, RC: Malignant neoplasm of rectum, LOPC: Malignant neoplasm of lip, oral cavity and pharynx (the figure data was reproduced from Alharbi et al., under NPJ Genom Medicine copyright).

Table 19. Univariate and multivariate logistic regression analyses of genetic variants in *TPCN2* and *P2RX4* significantly associated with the risk of various cancers at the local level (The table was reproduced from Alharbi et al., under Npj Genom Medicine copyright).

SNP	Genotype	Cancer-free (controls) N =385253	Cancer subtype (Cases)	Cancer subtype	Cancer subtype cohort sample size (n)	Model A		Model B	
						OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
rs3750965	GA(n)	170024	381	LOPC	918	0.94 (0.82-1.08)	0.3975	0.94 (0.82-1.08)	0.3839
			3494	PC	8025	0.95 (0.91-0.996)	0.035*	0.94 (0.90-0.99)	0.017*
			299	MS	632	1.07 (0.91-1.3)	0.386	1.07 (0.91-1.3)	0.385
			1204	MM	2836	0.915 (0.85-0.99)	0.027*	0.91 (0.84-0.99)	0.023*
	GG(n)	41687	124	LOPC	918	1.25 (1.02-1.53)	0.0296*	1.25 (1.02-1.53)	0.0311*
			290	MM	2836	0.9 (0.79-1)	0.103	0.9 (0.8-1.02)	0.106
			781	PC	8025	0.87 (0.80-0.94)	0.0003***	0.86 (0.80-0.93)	0.0002***
			49	MS	632	0.72 (0.53-0.97)	0.033*	0.72 (0.53-0.97)	0.032*
rs35264875	TA(n)	101826	385	RC	1426	1.04 (0.93-1.17)	0.492	1.03 (0.92-1.16)	0.575
			2240	PC	8025	1.085 (1.03-	0.001**	1.073	0.005**

						1.14)		(1.02-1.13)	
			1038	HM	3666	1.1 (1.02-1.18)	0.012*	1.09 (1.02-1.18)	0.015*
			859	CC	2996	1.12 (1.03-1.21)	0.005**	1.12 (1.03-1.21)	0.006**
			3322	BC	12056	1.06 (1.02-1.1)	0.00578**	1.06 (1.02-1.1)	0.006**
	TT(n)	9432	47	RC	1426	1.37 (1.02-1.84)	0.034*	1.37(1.02-1.84)	0.034*
			228	PC	8025	1.19 (1.04-1.36)	0.01*	1.18 (1.03-1.35)	0.016*
			83	HM	3666	0.95 (0.76-1.18)	0.63	0.95 (0.76-1.18)	0.623
			75	CC	2996	1.06 (0.84-1.33)	0.641	1.06 (0.84-1.34)	0.625
			294	BC	12056	1.012 (0.9-1.14)	0.84415	1.01 (0.9-1.14)	0.821
rs72932540	GA(n)	60582	255	RC	1426	1.12 (1.02-1.34)	0.025*	1.15 (1.004-1.32)	0.044*
			1390	PC	8025	1.13 (1.06-1.19)	7.34e-05***	1.11 (1.04-1.18)	0.0007***
			508	MM	2836	1.17 (1.06-1.3)	0.002**	1.15 (1.042-1.26)	0.005**
			2063	BC	12056	1.11 (1.05 - 1.16)	3.67e-05***	1.1 (1.05-1.16)	7.2e-05***
			12	RC	1426	1.16 (0.7 -2.05)	0.6121	1.16 (0.7-2.05)	0.61

	GG(n)	2875	76	PC	8025	1.3 (1.03-1.63)	0.0261*	1.31 (1.04-1.65)	0.024*
			93	BC	12056	1.05 (0.85-1.29)	0.635	1.03 (0.84-1.27)	0.7679
			19	MM	2836	0.92 (0.59-1.45)	0.722	0.91(0.58-1.42)	0.667
rs25644	GA(n)	79186	1648	PC	8025	1.002 (0.95-1.06)	0.9297	1.008 (0.95-1.07)	0.79076
			538	MM	2836	0.91 (0.83-1)	0.0423*	0.92 (0.83-1.01)	0.073
	GG(n)	5206	44	MM	2836	1.13 (0.84-1.52)	0.4303	1.16 (0.86-1.56)	0.33
			131	PC	8025	1.21 (1.02-1.44)	0.0314*	1.23 (1.03-1.47)	0.02175*
rs28360472	GA(n)	11997	19	MS	632	1.002 (0.95-1.06)	0.9297	1.008 (0.95-1.07)	0.79076
			73	CC	2996	0.78 (0.62-0.98)	0.034*	0.77 (0.61-0.97)	0.028*
			418	BC	12056	1.12 (1.01-1.23)	0.0283*	1.11 (1-1.22)	0.0478*
	GG(n)	109	2	MS	632	11.21 (2.8-45.48)	0.0007***	10.8 (2.7-43.7)	0.0009***
			1	CC	2996	1.17 (0.16-8.4)	0.875	1.14 (0.16-8.2)	0.897
			3	BC	12056	0.88 (0.280-2.78)	0.8312	1.02 (0.32-3.27)	0.9737

Abbreviations: OR, odds ratio; CI, confidence intervals; Model A, univariate logistic regression; Model B, multivariate logistic regression, adjusted for sex and ethnicity; BC, breast cancer, HM, malignant neoplasms, stated or presumed to be primary, of lymphoid, haematopoietic and related tissue; MM, malignant melanoma; MS, malignant neoplasms of mesothelial and soft tissue; LOPC, malignant neoplasm of lip, oral cavity and pharynx; PC, malignant neoplasm of the prostate; RC, malignant neoplasm of the rectum.

Table 20. Univariate and multivariate logistic regression analyses of genetic variants in *TPCN2* and *P2RX4* significantly associated with the risk of cancer recurrence in uk biobank (The table was reproduced from Alharbi et al., under Npj Genom Medicine copyright).

Gene SNP		Cancer occurrence once (controls), n	Cancer recurrence (cases), n	Cancer subtype	Cancer cohort (n)	Model A		Model B		Model C	
						OR, 95% CI	*p*-value	OR, 95% CI	*p*-value	OR, 95% CI	*p*-value
TPCN2 rs3750965	G A	525	2969	PC	8025	1.08 (0.94-1.23)	0.268	1.07 (0.941.22)	0.294	1.29 (1.04-1.58)	0.312
		141	483	RC	1426	1.36 (1.03 -1.8)	0.028*	1.37(1.04-1.81)	0.026*	1.4 (1.05-1.85)	0.0199*
	G G	135	646	PC	8025	1.27 (0.94-1.57)	0.023*	1.28 (1.04-1.57)	0.020*	1.07 (0.94-1.22)	0.018*
		27	134	RC	1426	0.94 (0.6-1.5)	0.798	0.97 (0.61-1.54)	0.887	1.01 (0.63-1.6)	0.981
P2RX4 rs25644	G A	240	1408	PC	8025	0.96 (0.83-1.12)	0.645	0.96 (0.82-1.12)	0.589	0.95 (0.82-1.11)	0.5482
	G G	11	120	PC	8025	0.52 (0.28-0.97)	0.038*	0.52 (0.28-0.96)	0.038*	0.52 (0.28-0.96)	0.038*

Abbreviations: OR, odds ratio; CI, confidence intervals; model A: univariate logistic regression; model B multivariate logistic regression, adjusted for sex and ethnicity; Model C, multivariate logistic regression, adjusted for age, sex, and ethnicity; PC, malignant neoplasm of prostate, RC: Malignant neoplasm of the rectum.

Table 21. Predictions of the association between significant snps and *TPCN2/* P2RX4expression/activity levels using bioinformatics tools (The Table was reproduced from alharbi et al., under Npj Genom Medicine copyright).

Gene; SNP		Example of a significant association in our study	Pathogenicity prediction			Predicted potential impact on protein (AA variant)	Protein expression levels in this type of cancer	Published evidence
						(Mutation Assessor release 3)	(Human Protein Atlas)	
			(CADD GRCh38-v1.6)		(FATHMM-XF)			
			PHRED	Raw score	Score			
TPCN2 rs3750965	GG	Decreased risk of MM	6.6	0.51	0.07	medium (K376R)	Medium/high expression of the protein was detected in 3 out of 11 patients with MM	NA
TPCN2 rs35264875	TA/TT	Increased risk of PC	9.81	0.84	0.18	Low (M484L)	Medium/high expression of the protein was detected in 10 out of 11 patients with PC	Gain of TPC2 function
P2RX4 rs25644	GG	Increased risk of PC	23.5	3.04	0.28	medium (S242G)	Medium/high expression of the protein was detected in 12 out of 12 patients' PC	NA
P2RX4 rs28360472	GA/GG	Increased risk of MS	26.1	3.86	0.69 (pathogenic)	medium (Y315C)	NA	Loss of function of P2RX4

Abbreviations: eQTL, expression quantitative trait loci; GTEx, genotype-tissue expression project database; HM, malignant neoplasms (stated or presumed to be primary, of lymphoid, haematopoietic and related tissue); MM, malignant melanoma; MS, malignant neoplasms of mesothelial and soft tissue; PC, malignant neoplasm of the prostate (main sources: https://www.gtexportal.org;https://cadd.gs.washington.edu/snv); FATHMM-XF, predict the functional consequences of single nucleotide variants (SNVs) with extra features (biocompute.org.uk); http://mutationassessor.org/r3/; The Human Protein Atlas.

5.2.4. Discussion

The genetic makeup of tumours provides valuable information for tailored therapies, paving the way towards personalised cancer care. The UK Biobank is one of the world's largest and most comprehensive databases, containing in-depth genetic and health information from half a million participants in the UK. The evolving field of endolysosomal Ca^{2+} signalling in cancer (15,357,358,359), however, has a paucity of human data. This is the first study that has characterised the effect of genetic variants in endolysosomal cation channels (TPC2 and P2RX4) and cancer with a large-scale and comprehensive analysis of 13 subtypes of cancer. Novel associations between genetic variants of TPCN2/P2RX4 and cancer in the definition of tumour types, susceptibility, prognosis and cancer outcomes in the UK Biobank population were revealed for the first time by Alharbi AF, Parrington J. 2020 (356), which briefly discusses the major findings in this section. rs35264875, a missense genetic variant (a substitution of methionine with leucine) (Europeans, MAF>15%) encoding M484L) (360) was correlated with a shift in hair colour from brown to blond (160,161,162).It was functionally examined by endolysosomal patch-clamp techniques, leading to a gain of TPC2 function through conformational changes within the pore (160,162). The genetic variant rs35264875:TA, in cancer at the global level, was associated with higher odds of cancer susceptibility ([OR: 1.03, 95% CI: 1.01–1.05, *p*-value = 0.00138**, vs A/A] and lower odds of cancer metastasis (OR: 0.74, 95% CI: 0.57–0.96, *p*-value = 0.025 * vs A/A). At a local level (subtype of cancer), the rs35264875 was correlated with an increased risk of developing five subtypes of cancer involving the breast, colon, malignant neoplasms, stated or presumed to be primarily lymphoid, haematopoietic and related tissues, prostate and rectum cancers. The effect of heterozygote rs35264875 on TPCN2 expression in the prostate was found to lead to upregulation in TPCN2 gene expression (See Table 21). TPCN2 overexpression was reported as a risk factor for developing skin cancer (361), whereas a significant reduction was observed in TPCN2 expression in patients with primary human skin cutaneous melanoma

compared to metastatic patients (142).Taken together, this data indicates that TPC2 overexpression might promote tumorigenesis, and its downexpression may hamper metastasis, which warrants further investigation in cell-based and animal models that harbour this genetic mutation.

The impact of the missense genetic variant rs3750965 (Europeans (MAF approximately 32%), encoding K376R, substitution of lysine with arginine) (362) on TPC2 channel activity has not yet been fully functionally established. In our analysis, GG in rs3750965 (TPCN2) was correlated with lower odds of cancer susceptibility (OR: 0.97, 95% CI: 0.95–0.997, *p*-value = 0.0292*, vs A/A), and genotypes GA/GG with significantly lower odds of developing three subtypes of cancer, including malignant melanoma (GA OR: 0.91, 95% CI: 0.84–0.99, *p*-value = 0.02334 *), malignant neoplasms of mesothelial and soft tissues (GA OR: 0.72, 95% CI: 0.53–0.97, P = 0.032 *), and malignant neoplasm of the prostate (GA OR: 0.94, 95% CI: 0.90–0.99, *p*-value= 0.017*) (GG OR: 0.86, 95% CI: 0.80–0.93, *p*-value = 0.0002 ***). In the GTEx database, GG genotype rs3750965 in TPCN2 expression leads to a downregulation of its expression levels in skin that is not exposed to the sun. These findings provide preliminary evidence that rs3750965 might be considered a protective mutation that reduces the risk of developing cancer at a global level, or melanoma, mesothelial, soft tissue and prostate cancer at the local level. Contradictorily, the homozygosity of this genetic variant was correlated with higher odds of developing malignant neoplasms of the lip, oral cavity and pharynx, and GG/GA increased rates of cancer recurrence in patients with prostate and rectal cancers, indicating that TPC2 genetic mutations or functions might exhibit distinct roles in various stages or types of cancers. No previous data has been reported regarding the association of this genetic variant and cancer, which require further validation for this association in the external cohort and functional characterisation of this genetic variant in cancer biology. The genetic variant rs72932540 is in close proximity (the distance from the variant to the TSS of TPCN2 is 105,643 Kb) to TPCN2 (it is located on GRCh38: 11:69,154,575 (363, 364).

Being located close to the TPCN2 gene might influence its expression level. The GA genotype in rs72932540 was found to increase the odds of developing cancer at the global level, and four subtypes of cancer involving breast cancer, malignant melanoma ,rectal and prostate cancer (OR: 1.1, 95% CI: 1.05–1.16, *p*-value = 7.2e-05 ***, OR: 1.15, 95% CI: 1.042–1.26, *p*-value = 0.00512 **, OR: 1.15, 95% CI: 1.004–1.32, *p*-value = 0.044*, OR: 1.11, 95% CI: 1.04-1, *p*-value =0.0007 *** 18, respectively, vs A/A). In European populations, rs72932540 was correlated with breast cancer (p-value 4 x 10^{-8}) (365). This data indicates the potential candidacy of the genetic variant rs72932540 as a genetic marker for breast cancer. A recent study identified TPCN2 as one among six gene markers to help predict postoperative biochemical recurrence in prostate cancer (149), highlighting the need to conduct research to reveal the roles of TPC2 at the genetic level and its potential use as a biomarker as well as to investigate the causal mechanism underlying these genetic variants in cancer pathology.

P2RX4 is mainly implicated in cancer pain (366). This study investigates the associations between rs25644 (Ser242>Gly) and rs28360472 (Tyr315>Cys) (367) in the P2RX4 gene and cancer in the UK Biobank population. Overexpression of P2RX4 was associated with prostate cancer (368). The pharmacological inhibition of P2RX4 by 5-(3-Bromophenyl)-1, 3-dihydro-2Hbenzofuro [3,2-e]-1, 4-Diazepin-2-one (5-BDBD) hampered the cancer growth in vitro and in vivo models of prostate cancers (368). In our analysis, the GG genotype in rs25644 (P2RX4) is correlated with higher odds of developing prostate cancer (OR: 1.23, 95% CI: 1.03–1.47, *p*-value = 0.02175 *vs A/A) and with lower rates of cancer recurrence in patients with prostate cancer (OR: 0.52, 95% CI: 0.28–0.96, *p*-value = 0.038 *, vs A/A). The impact of homozygote genotypes of rs25644 was predicted by utilising bioinformatics tools (GTEx and UALCAN). Upregulated expression of P2RX4 in the prostate was observed, and its expression was significantly (*p*-value, 2.23310259173104E-12) overexpressed in patients with prostate adenocarcinoma vs healthy subjects (353). This evidence highlights the possibly significant roles of P2RX4 in prostate

biology, representing potentially viable therapeutic strategies for the treatment of patients with prostate cancer and warrants further investigation in cell-based and animal models of the prostate.

The genetic variant rs28360472 was previously characterised functionally and reported as a found a loss-of-function of the P2RX4 through the distribution of the agonist binding site (369). Heterozygotes and homozygotes in rs28360472 were reported to be correlated with higher odds of breast cancer and of malignant neoplasms of mesothelial and soft tissue susceptibility and with lower odds of colon cancer. In the UALCAN database, significant P2RX4 downexpression was found in patients with mesothelioma stratified by the presence/absence of TP53 mutation (*p*-value, 3.569600E-01) (353). By utilising bioinformatics tools (CADD GRCh38-v1.6 and FATHMM-XF, pathogenicity scores of 26.1, 0.69), this variant was identified as a pathogenic variant which might explain its correlation with an increased risk of developing breast cancer and malignant neoplasms of mesothelial and soft tissue. Regardless, the preliminary nature of the evidence of the loss of function of P2RX4 might contribute to cancer development in mesothelioma, which warrants further investigation. P2RX4 overexpression/downexpression might have a distinct impact on cancer development in different biological contexts.

Several databases were navigated to provide, in a preliminary fashion, the biological significance of these variants at the local level and TPC2 and P2RX4 expression/activity in cancer at the global level. This study provides insights into the role of these channels in humans and will take the research to the next level in conducting translational studies based on the evolving scientific knowledge of these channels' genomic aspects in cancer.

In the above analysis, heterozygous genotypes of genetic variants reached significant levels, whereas the homozygous genetic variants failed to reach significant levels with our large sample size. These genotypes did not reach significant levels because the sample size may not be

sufficient for the detection of significant differences between the cases and controls, since similar case/control ratios were observed between homozygous genotypes and heterozygous genotypes in our analysis. It is speculated that the significant difference detected in our population is due to a higher frequency of these heterozygous genotypes than of other genotypes in cases versus controls. The similarity between heterozygous and homozygous case/control ratios provides evidence that the sample size is not the main driving reason for this association. The genomic evolution of endolysosomal cation channels (TPC2 and P2X4) might provide scientific insights into these interesting findings in the future. Is TPC2 another piece of the cancer biomarker puzzle? Regardless of the novelty of the presented associations in this study, external validations in large external cohorts of diverse populations and functional characterisations of these genetic variants in cancer biology are required before investigating these genetic variants in prospective clinical studies.

TPC2 profiling in cancer could enable the discovery of a novel cancer biomarker and hallmark and represent a further step towards personalised cancer treatment.

UNIVERSITY OF OXFORD

Chapter 5: Section 2

Novel associations between genetic variants in monocytes and macrophage genes and cancer in terms of susceptibility and cancer outcomes in the UK Biobank population

5.3. Section 2. Novel associations between genetic variants in monocytes and macrophage genes and cancer in terms of susceptibility and cancer outcomes in the UK Biobank population

5.3.1. Introduction

5.3.1.1. Decoding monocyte/macrophage and cytokine pathway genetic makeup in cancer: lessons from the human genome

The crosstalk between monocytes/macrophages and cancer has been implicated in various aspects of cancer immunity (370). Monocytes are recruited to target tumour tissues in response to tumour-derived factors, leading to their further differentiation into macrophages. Tumour-associated macrophages play a role in tumour growth and metastasis (44). A recent study has shown that circulating monocytes from prostate cancer patients enhanced the invasion and motility of tumour cells (45). In the same vein, the specific elimination of a population of Tie2+ monocytes reduced tumour angiogenesis and slowed tumour progression in mammary and glioma tumorigenesis investigations (48). Cancer is a genetic disease. Hence, the genetic variations involved in monocyte/macrophage genes and their association with cancer might help in elucidating the mechanisms that underpin the complex interplay between monocyte, macrophage and cancer. Tyrosine kinase 2 is highly expressed in monocytes (371), and it is an important pathway in cancer immunity (372,373). Recently, polymorphism rs34536443 in TYK2 tyrosine kinase 2, a gene that leads to partial loss of function, was significantly associated with a higher risk of developing lung cancer and non-Hodgkin lymphoma (346). CD86 is expressed in macrophages. A previous study showed that carrying the CD86 rs1129055 allele, GA or AA, was associated with a higher risk for developing pancreatic cancer (OR: 1.52, 95% CI: 1.09-2.38, *p*-value = 0.026, OR: 1.90, 95% CI: 1.21-3.01, *p*-value = 0.007, respectively vs G/G) (374). Recently, genotype AG/AA in the TNF-α gene (rs1800629) showed a significantly higher risk of

developing cervical cancer (375). Similarly, rs1800896: AG/AA (IL-10), was associated with an increased risk of cervical cancer susceptibility (376). Genotypes GG in IL-6 rs1800795 showed a higher incidence of metastatic cancer in patients with primary breast cancer (377). Overall, these data highlight the role of human genetics regarding monocytes/macrophages and cytokines in cancer development and prognosis. Further studies are required to expand our scientific knowledge and map the genomics, transcriptomics and proteomics in order to tackle complicated cancer questions.

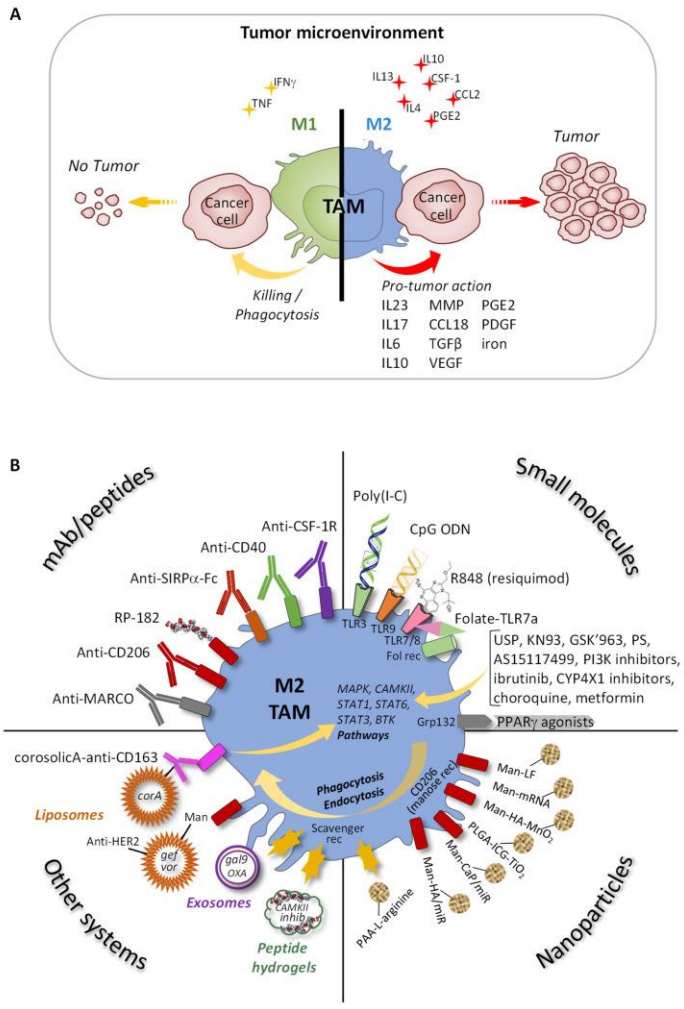


Figure 88. Tumour-associated macrophages in cancer biology and their therapeutic applications (370) (The figure was reproduced from Degboé et al., under MDPI copyright).

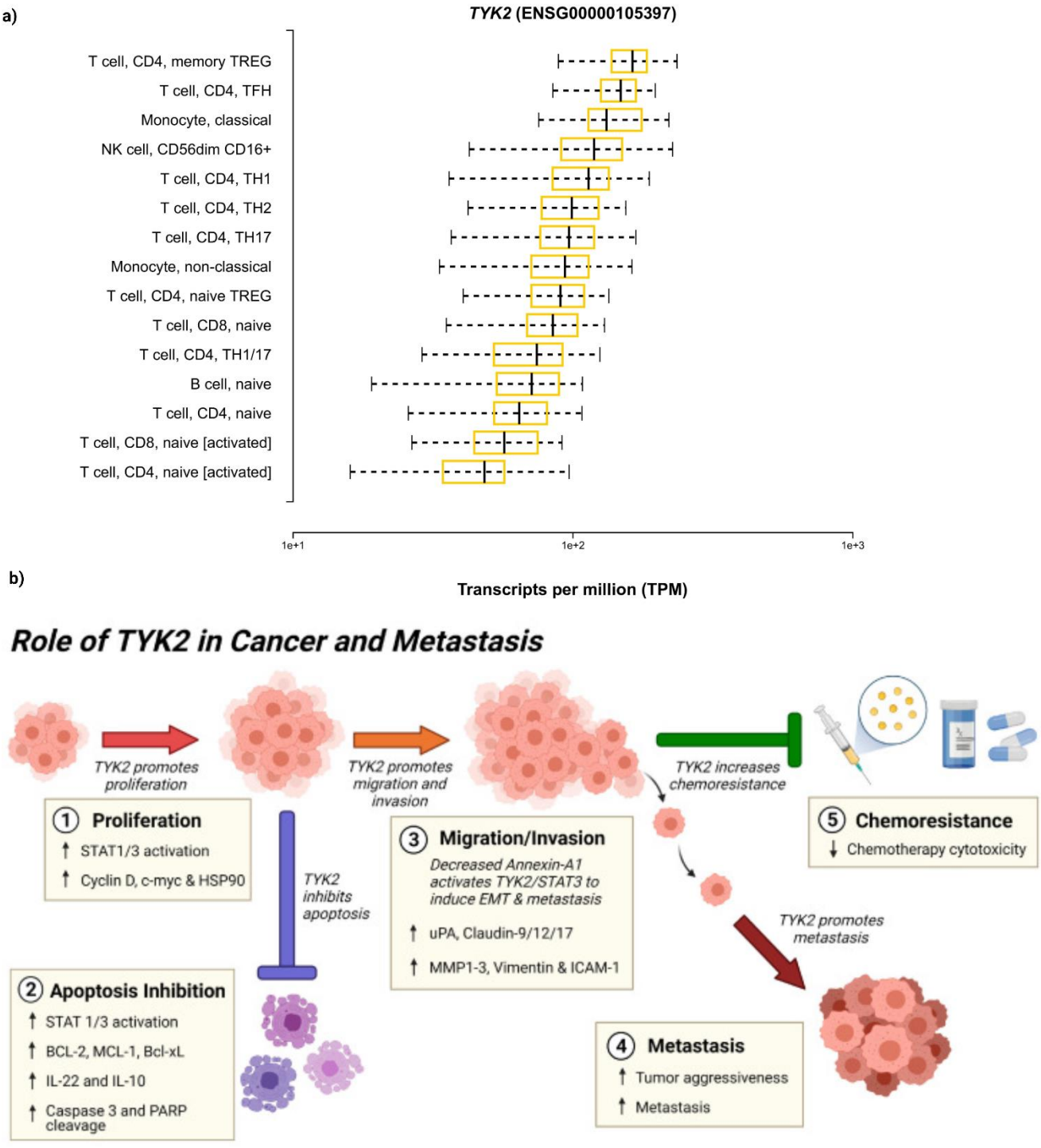


Figure 89. Tyrosine kinase 2 in immune cells and cancer. A, The expression TYK2 in various immune cells (372); B, The role of TYK2 in various pathophysiological processes attributed to cancer (372). (The figure was reproduced from Borcherding et al., under MDPI copyright)

5.3.1.2. Significance, innovation, overall hypothesis and objectives

My DPhil studies have begun to underpin new roles played by the two-pore channel subtype 2 (TPC2) in cancer genetics and its complex interplay between monocyte differentiation, the release of cytokines/chemokines and cancer biology. The genetic landscape underlying the regulation of monocyte/macrophage and cytokine pathways in cancer has not yet been deeply investigated in a large dataset. The long-term goal of this project is to establish genetic biomarkers to help clinicians to optimise cancer detection, diagnosis, prognosis and therapy. I aim to investigate the genetic variability underlying genes involved in monocyte/macrophage and cytokine signalling within, but not limited to, CD68, CD86, IL6, IL10, IL-8 and TNFα in cancer, thereby providing a tailored therapy based on patient information. The central hypothesis is that knowing the genetic information of monocyte/macrophage and cytokine will enhance the detection, diagnosis and prognosis of cancer, improving health outcomes and the quality of life for cancer patients. Thus, I performed a candidate gene approach study to investigate and identify associations between 79 genetic variants and cancer at a global level in the UK Biobank population.

5.3.2. Methods

5.3.2.1. Study participants and genetic analysis

UK Biobank subjects were reviewed and stratified into two cohorts, cancer-free controls and cancer cases. Single nucleotide polymorphisms (SNP) in TPCN2, IL10RA, IL-10, IL19, CD163, SACS, IL4R, IL27, IL23R, CD68, TYK2, IL12RB1, IFNL4, IL28B, IL1RN, IL-8, CXCR1, CD86, AFP, AFM, CXCL8, IL3, IL13, IL4, TMCO6/CD14, TNF, IL17F, IL6, CD36 and cancer came from the UK Biobank data. Out of 71 SNPs, 29 were in Hardy–Weinberg equilibrium (p-value > 0.05), shown in Supplementary Table S5.2. Genetic associations with the risks for developing cancer, for developing malignant tumours, for developing metastatic cancer and for cancer recurrence

were determined using univariate (Model A) and multivariate logistic regression after adjusting for sex and ethnicity (Model B) or sex, ethnicity and age (Model C).

5.3.2.2. Survival analysis

Data regarding overall survival (OS) were collected and OS was defined as the time from cancer diagnosis to death from neoplasm. OS was estimated using the Kaplan–Meier method utilising R version 3.4.1 (survival packages). Subjects were stratified according to their genotypes.

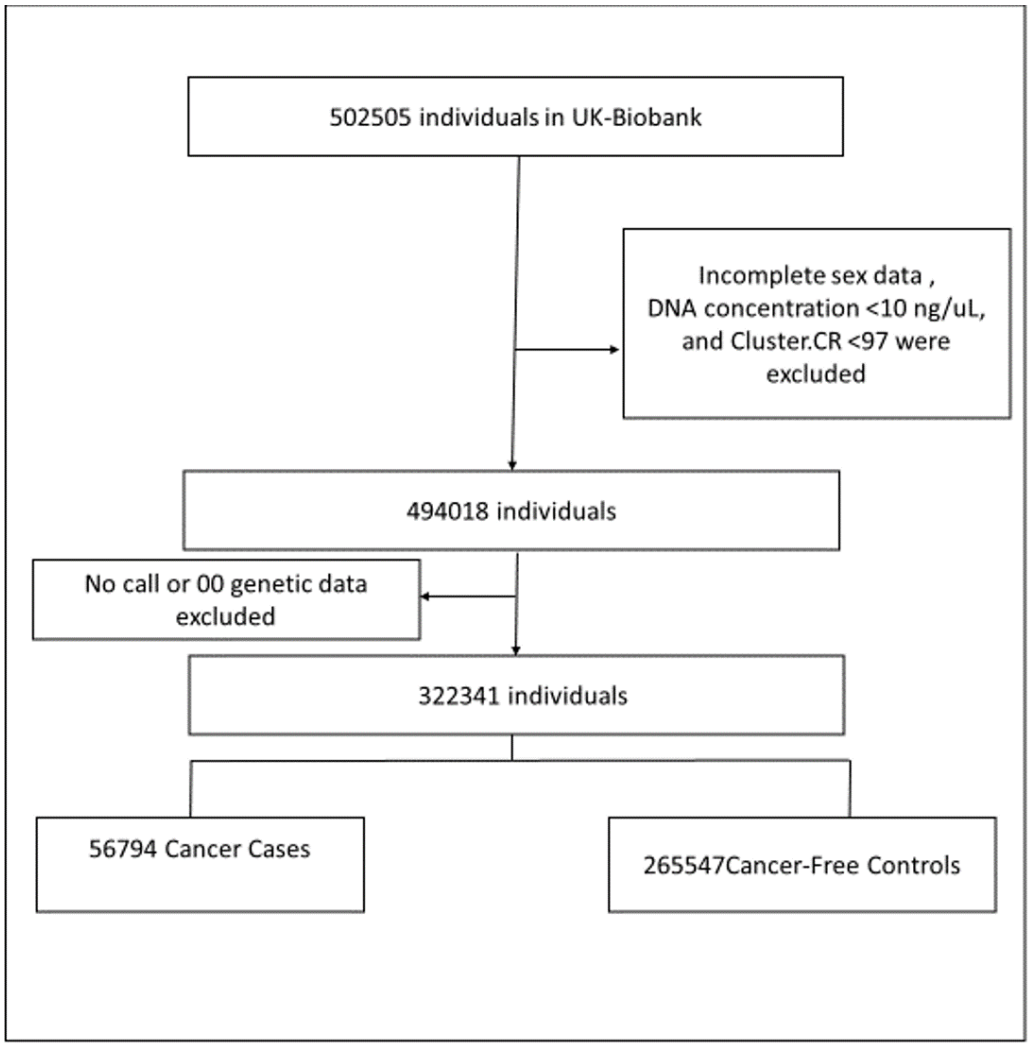


Figure 90. Sample selection strategy.

5.3.3. Results

5.3.3.1. Participants' characteristics

A total of 322,341 subjects were reviewed: 265,547 cancer-free controls and 56,794 with a median age at cancer diagnosis of 59.90 (5.20–79.20); all types of tumours were included in the analysis (see Table 22).

Table 22. Subject characteristics.

	UK Biobank	Total	Cancer cases	Cancer-free controls
	ID			
Number		322,341	56,794	265,547
Age at cancer diagnosis (years) (median, range)	40008-0.0	59.9 (5.20-79.2)	59.9 (5.20-79.20)	NA
Sex	31-0.0			
Male (n)		146211	24284	121927
Female (n)		176130	32510	143620
Ethnic background	21000-0.0			
White		291089	52540	238549
Age at death (years)	40007-0.0	68.8	68.6	69
Median (range)		(41.1-80.5)	(41.10-80.50)	(41.6 -80.10)
Clinical factors				
Type of cancer (first occurrence)	40006-0.0			
Malignant neoplasm of breast		8364	8364	
Malignant neoplasm of bronchus and lung		1225	1225	
Malignant neoplasm of colon		2071	2071	
Malignant neoplasms stated or presumed to be primary, of lymphoid, haematopoietic and related tissue		2470	2470	
Malignant melanoma		1952	1952	
Malignant neoplasms of mesothelial and soft tissue		421	421	
Malignant neoplasm of the prostate		5479	5479	
Histology of cancer tumour	40011-0.0			
Epithelial		48436	48436	
Connective tissue		1319	1319	
Hematologic (leukaemias,		2761	2761	

lymphomas and related disorders)				
Nervous system		837	837	
Behaviour of cancer tumour	40012-0.0			
Benign		678	678	
Uncertain whether benign or malignant		1570	1570	
Carcinoma in situ		6841	6841	
Malignant		45215	45215	
Reported occurrences of cancer (once)	40009-0.0	42539	42539	0
Reported recurrence of cancer		14255	14255	0
Genetic factors				
DNA concentration (median (range))	22024-0.0	(10-655.87)	(10.00-655.87)	(10-629.22)
Affymetrix quality control metric 'Cluster CR' (median (range))	22025-0.0	99.58(97 - 99.9)	99.58(97.01 - 99.91)	99.58(97.00-99.92)
Affymetrix quality control metric 'dQC' (median (range))	22026-0.0	0.98 (0.90- 1)	0.98 (0.90- 1)	0.98(0.90-1)

5.3.3.2. Genetic variants in in monocyte/macrophage and cytokine pathway and the risk of developing cancer at a global level, malignant, metastatic cancer and cancer recurrence

UK Biobank data was leveraged to characterise the effects of genetic variants in monocyte/macrophage and cytokine pathway genes in cancer patients at a global level in terms of susceptibility, prognosis, disease recurrence and overall survival (OS). Genotype TC in rs4645843 (TNF-α) had a significant lower risk for developing cancer (OR: 0.81, 95% CI: 0.67–0.98,*p*-value = 0.031* vs. C/C), whereas genotypes TG in rs2069827 (IL-6), TG in rs2255214 (ILDR1/CD86), GA in rs375947 (IL12RB1), GA in rs72932540 (close to TPCN2), and CA or CC in rs3024508: (IL-10), showed higher odds of cancer susceptibility ([OR: 1.03, 95% CI: 1–1.05, *p*-value = 0.042* vs. G/G], [OR: 1.025, 95% CI: 1–1.05, *p*-value = 0.029* vs. G/G], [OR: 1.02, 95% CI: 1–1.04, *p*-value = 0.035* vs. A/A], [OR: 1.06, 95% CI: 1.03–1.09, *p*-value = 2.76e-06 ***vs. A/A], [OR: 1.07, 95% CI: 1.02–1.12, *p*-value = 0.006** vs. A/A])) and [OR: 1.54, 95% CI: 1.03–2.32, *p*-value = 0.037* vs. A/A], respectively) after adjusting for sex and ethnicity (see Table 23). Table 24 illustrates that carrying the rs12720356:CA allele (TYK2) and the rs1799964: CT allele

(TNF) were associated with a lower risk of metastatic cancer after adjusting for age, sex and ethnicity [OR: 0.65, 95% CI:0.43–0.97, *p*-value = 0.036 * vs. A/A)] and [OR: 0.5, 95% CI: 0.28–0.9, *p*-value = 0.022* vs. C/C], respectively). Genotype CC in rs12720356 was associated with a lower incidence of cancer recurrence after adjusting for age, sex and ethnicity (OR: 0.78, 95% CI: 0.63–0.97, *p*-value = 0.026* vs. A/A), whereas genotype AG/GG in rs11209026 was found to have higher rates of cancer recurrence after adjusting for age, sex and ethnicity (AG OR: 1.45, 95% CI: 1.03–2.04, *p*-value = 0.034* and GG OR: 1.41, 95% CI: 1.01–1.98, *p*-value = 0.046* vs. A/A), shown in Table 25.

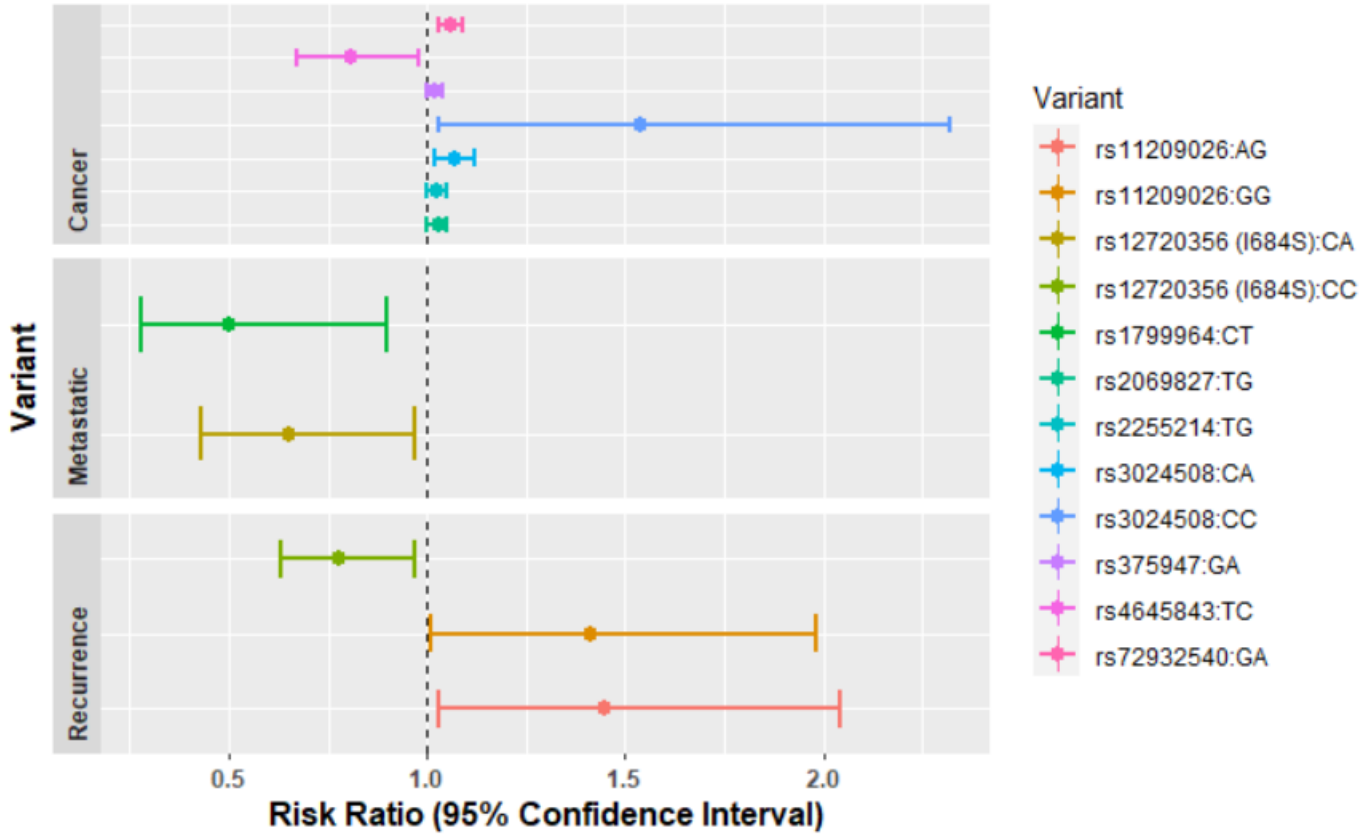


Figure 91. Forrest plot showing genotypic ORs for significant SNPs and cancer. Estimates were derived using logistic regression after adjusting for sex and ethnicity or age.

Table 23. Univariate and multivariate logistic regression analyses of genetic variants significantly associated with cancer risk in the uk biobank.								
			Cancer-cases	Cancer-free controls	Model A		Model B	
					OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Total (n)322341			56794	265547				
Female(n) 176130			32510	143620				
Male(n) 146211			24284	121927				
SNP	Gene	Genotypes (n)						
rs2069827 (affy30198890)	IL6	GG	46951	220846				
		T G	9394	42574	1.04 (1.01-1.06)	0.003**	1.03 (1-1.05)	0.042*
		T T	449	2127	0.99 (0.9-1.1)	0.892	0.98 (0.89-1.09)	0.706
rs4645843 (affy28459555)	TNF	CC	56672	264823				
		T C	122	724	0.79 (0.65-0.95)	0.0147*	0.81 (0.67-0.98)	0.03058*
rs2255214 (affy21053125)	ILDR1/CD86	GG	14703	70074				
		T G	28541	132390	1.03 (1-1.05)	0.015*	1.025 (1-1.05)	0.029*
		T T	13550	63083	1.02 (1-1.05)	0.074	1.02 (1-1.05)	0.068
rs375947 (affy15635575)	IL12RB1	A A						
			26341	124596				
		G A	24717	114371	1.02 (1-1.04)	0.0242*	1.02 (1-1.04)	0.035*
		G G	5736	26580	1.02 (0.99-1.05)	0.201	1.02 (0.99-1.05)	0.25
rs72932540 (affy5782262)	Close to TPCN2	A A	46928	221923				

		G A	9452	41653	1.07 (1.05-1.1)	1.54e-08***	1.06 (1.03-1.09)	2.76e-06***
		G G	414	1971	0.99 (0.89-1.05)	0.902	0.98 (0.88-1.09)	0.729
rs3024508 (affy6635044)	IL-10	A A	54476	255540				
		C A	2287	9914	1.08 (1.03-1.13)	0.000858***	1.07 (1.02-1.12)	0.00604**
		C C	31	93	1.56 (1.04 - 2.35)	0.031175*	1.54 (1.03-2.32)	0.03721*
Genetic variants were significantly associated with the risk of developing cancer in univariate-logistic regression but did not remain significant after adjusting for sex and ethnicity.								
rs16858811 (affy19110488)	CXCR1	AA	53472	249448				
		C A	3261	15825	0.96 (0.92-1)	0.0464*	0.97 (0.93-1.005)	0.08
		C C	61	274	1.04 (0.79-1.37)	0.7894	1.07 (0.81-1.4)	0.65
rs35264875 (affy5779776)	TPCN2	A A	46488	188562				
		T A	9762	70474	1.03 (1.01-1.05)	0.0117*	1.02 (1-1.04)	0.05169
		T T	544	6511	1.01 (0.95-1.07)	0.7442	1.01 (0.95-1.07)	0.86
rs12720356 (affy15521078)	TYK2	AA	46488	218900				
		C A	9762	44337	1.04 (1.01-1.06)	0.003**	1.02 (1-1.05)	0.06
		CC	544	2310	1.11 (1.01-1.22)	0.03103*	1.1 (0.99-1.2)	0.07
rs11575934 (affy15635686)	IL12RB1	CC	5615	25750				
		CT	24679	113752	0.99 (0.96-1.03)	0.76	1 (0.97-1.03)	0.83
		TT	26500	126045	0.96 (0.93-1)	0.024*	0.97 (0.94-1)	0.05218
Abbreviations: OR, odds ratio; CI, confidence intervals; Model A, univariate logistic regression; Model B, multivariate								

logistic regression, adjusted for sex and ethnicity.

Table 24. Univariate and multivariate logistic regression analyses of a genetic variant significantly associated with the risk of metastatic cancer.

			Metastatic cancer	Primary cancer	Model A		Model B		Model C	
			(Cases)	(Controls)	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Total (n)45098			228	44870						
Female (n) 23739			115	23624						
Male (n)21359			113	21246						
SNP	Gene									
rs12720356	TYK2	A A	197	36694						
		C A	27	7748	0.65 (0.43-0.97)	0.036*	0.65 (0.44-0.98)	0.037*	0.65 (0.43-0.97)	0.036*
		C C	4	428	1.74 (0.64-4.71)	0.275	1.74 (0.64-4.71)	0.275	1.74 (0.64-4.71)	0.275
rs1799964	TNF	CC	14	2018						
		C T	52	14827	0.51 (0.28-0.91)	0.024*	0.50 (0.28-0.91)	0.023*	0.5 (0.28-0.9)	0.022*
		T T	162	28025	0.83 (0.39-1.78)	0.513	0.83 (0.48-1.43)	0.494	0.82 (0.47-1.41)	0.471

Abbreviations: OR, odds ratio; CI, confidence intervals; Model A, univariate logistic regression; Model B, multivariate logistic regression, adjusted for sex and ethnicity; Model C, multivariate logistic regression, adjusted for age, sex, and ethnicity.

Table 25. Univariate and multivariate logistic regression analyses of a genetic variant significantly associated with the risk of cancer recurrence.

			Cancer-occurrence reported once (controls)	Cancer recurrence (cases)	Model A		Model B		Model C	
					OR (95% CI)	*p*-value	OR(95 %CI)	*p*-value	OR (95 %CI)	*p*-value
Total (n) 56794			42539	14255						
Female (n) 32510			23505	9005						
Male (n) 24284			19034	5250						
SNP	Gene	Genotypes (n)								
rs11209026	IL23R	A A(n)	188	44						
		A G (n)	5153	1758	1.46 (1.05-2.03)	0.026*	1.45 (1.04-2.02)	0.029*	1.45 (1.03-2.04)	0.034*
		G G (n)	37198	12453	1.43 (1.03-1.99)	0.033*	1.43 (1.03-1.98)	0.034*	1.41 (1.01-1.98)	0.046*
rs12720356	TYK2	A A(n)	34775	11713						
		C A(n)	7335	2427	0.98 (0.93-1.03)	0.4892	0.98 (0.93-1.03)	0.507	0.99 (0.94-1.04)	0.658
		C C(n)	429	115	0.8 (0.65-0.98)	0.0305*	0.80 (0.65-0.99)	0.036	0.78 (0.63-0.97)	0.026*

Abbreviations: OR, odds ratio; CI, confidence intervals; Model A, univariate logistic regression; Model B, multivariate logistic regression, adjusted for sex and ethnicity; Model C, multivariate logistic regression, adjusted for age, sex, and ethnicity.

5.3.3.3. The role genetic variants in in monocyte/macrophage and cytokine pathway in cancer overall Survival

5202 cancer patients were included in the analysis; significant differences were observed in OS. The results showed that carriers of genotype CA/CC in rs16858811 (CXCR1) had a lower rate of survival, whereas genotype TG /TT in rs2255214 (ILDR1/CD86) showed a favorable survival (p = 0.006 and p = 0.01, respectively), as shown in Figures 92 and 93.

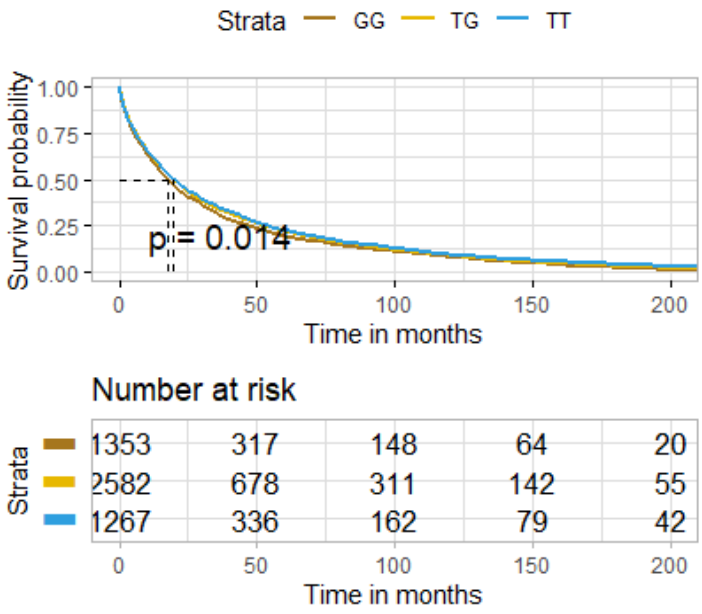


Figure 92. Kaplan-Meier Curve of OS in 5202 patients with cancer stratified rs2255214 (ILDR1/CD86) genotypes.

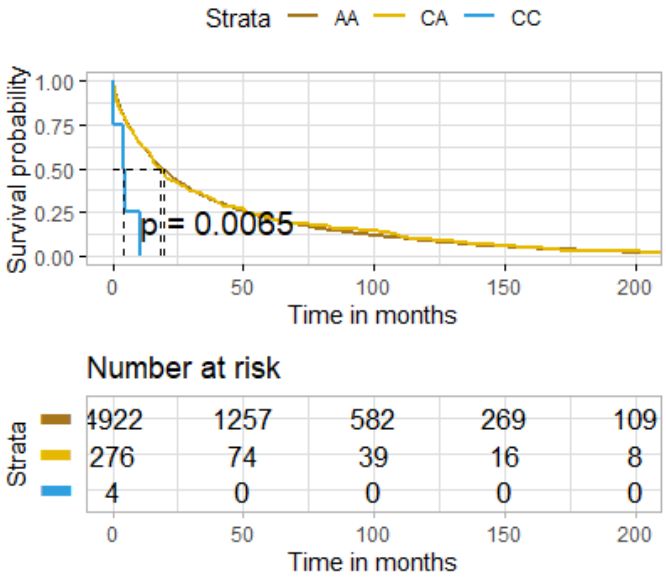


Figure 93. Kaplan-Meier Curve of OS in 5202 patients with cancer stratified by rs16858811 (CXCR1) genotypes.

Table 26. Genetic variants that significantly associated with overall survival.					
OS in 5202 patients with cancer					
Total (n)	5202				
Female (n)	2170				
Male(n)	3032				
SNP	**Gene**	**Genotypes**	**n**	**Median (95% CI) Months**	***p*-value**
rs16858811	CXCR1	A A	4922	19.25 (18.333 - 20.3)	p= 0.006
		CA	276	18.12(15.310 - 22.3)	
		CC	4	4.42 (0.526 - NA)	
rs2255214	ILDR1/CD86	G G	1353	17.5 (16.1 - 19.5)	p= 0.01
		T G	2582	19.5 (18.4 - 21.3)	
		T T	1267	19.8 (17.8 - 22.2)	

5.3.4. Discussion

Cancer biomarkers have emerged as clinical tools that can improve the efficiency of detection and guide the treatment of cancer patients by providing tailored therapy and information about expected cancer outcomes. Our study characterises the effects of 71 genetic variants within, but not limited to IL-8, IL-10 and TNF-α, in cancer patients regarding susceptibility, prognosis, disease recurrence and overall survival (OS). The present study found a novel association between genetic variants and cancer in terms of susceptibility and cancer outcomes in the UK Biobank population. To my knowledge, two out of 10 SNPs (Single-nucleotide polymorphisms) that showed significant cancer-related associations in the analysis were previously reported in cancer/cancer type. Therefore, this study reveals novel correlations between genetic variants in monocyte/macrophage and cytokine pathway genes. A significant association has been shown between a reduction of approximately 50% in metastatic risk and heterozygosity of rs1799964 (CT), revealing the potential application of rs1799964 as a prognostic biomarker in cancer patients. Li et al. (2018) found that rs1799964 was significantly correlated with an increased risk of cervical cancer in the Chinese population (378). Recently, this finding was supported by

Kosałka-Węgiel et al (2022). They reported that rs1799964 showed higher odds of developing cancer in patients with systemic sclerosis in the European population (379). Rs1799964 is an upstream_gene_variant in the TNF-α gene located in the protein-coding region. Generally, rs1799964 CT leads to loss/reduction in TNF-α expression at RNA and protein levels (379, 380). TNF-α biologically behaves in cancer as a sword with two edges. The fostering edge of tumorigenesis and metastasis is relevant to its vital roles in angiogenesis and cancer cell proliferation, invasion and migration (381, 382). The hindering edge of TNF-α in cancer biology is exhibited in mediating cancer cell death (381, 382). My explanation is that carriers of rs1799964 allele CT were less likely to develop metastasis due to a potential reduction in TNF-α alpha levels. This data warrants further investigations, including characterisations of this polymorphism in cancer patients at both RNA and protein levels, to reveal potential applications of rs1799964 as a prognostic biomarker in the European population. I believe that TNF-α is a complex player between cancer and immunity, and it plays a distinct role in various processes/tissues. Rs375947 in IL12RB1 is a missense variant with high MAF (minor allele frequency) in the European population, approximately at 32%(383); it is a coding sequence variant that substitutes T(ATG) in the amino acid codon with C (ACG), ultimately leading to change of amino acid methionine into threonine at protein level predicted by Ensembl Variant Effect Predictor (VEP). To my knowledge, this genetic variant's functional effect has not yet been deeply investigated. In the UK Biobank population, harbouring the rs375947 GA genotype increases the risk of developing cancer. This data contradicts the previous finding by Quan et al. (2014) regarding rs375947 AG/GG genotypes, which showed tyhem as associated with lower odds of developing oestrogen receptor-negative breast cancer in African Americans (384). Previous data should be interpreted with caution due to the sample size. The correlation was investigated and reported in homozygosity or heterozygosity analyses alone, and it was significant in the combined analysis of heterozygosity and homozygosity versus WT.

Significant and novel correlations were found between genetic variants (rs11209026, rs12720356, rs2069827, rs2255214, rs4645843, rs72932540, rs3024508 and rs16858811) and cancer in the definition of cancer susceptibility, metastasis, overall survival and cancer recurrence in the UK biobank. I could predict the effects of three (rs11209026, rs12720356 and rs4645843) out of eight SNPs that showed novel cancer-related associations on amino acid substitution, which ultimately predicate the function of protein in humans utilising PolyPhen-2 (Polymorphism Phenotyping v2). Figure 94 shows that two of these genetic variants (rs11209026 and rs12720356) were confidently predicated to be damaging.

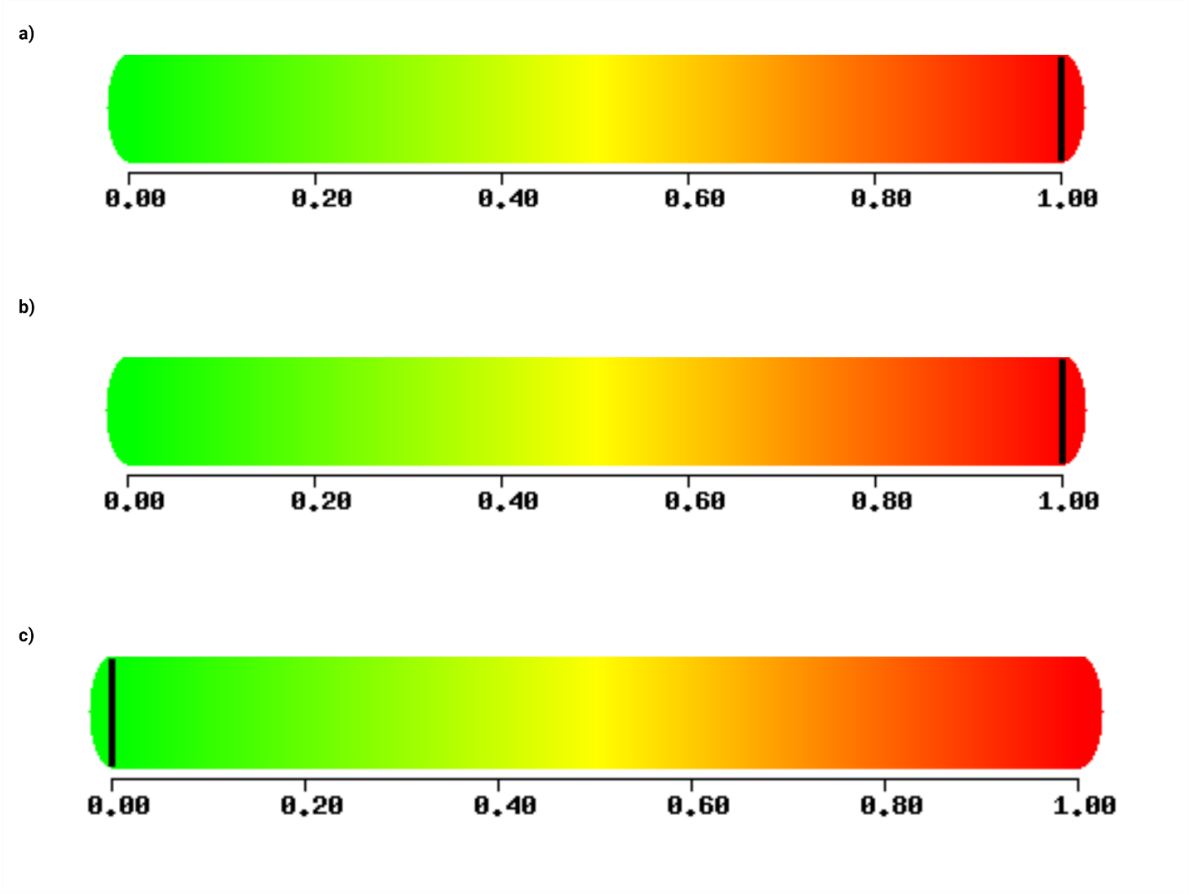


Figure 94. Predicts a genetic variant's potential impact on a human protein's structure and function. (a) rs11209026, (b) rs12720356 and (c) rs4645843. Score scale: 0.0 to 0.15 (benign) and 0.15 to 1.0 (possibly damaging). 0.85 to 1.0 (variants that are confidently damaging).

The influence of rs11209026 (IL-23R) genotypes on serum cytokine concentrations were previously characterised in patients with rheumatoid arthritis (a different disease model than cancer), involving IL-1β, IL-12, IL-17, IL-21, IL-22, IL-23 and TNF-α. The only significant difference (reduction) was observed in IL-17 serum levels in rs11209026 AG/GG (394.51±529.72) alleles compared to AA (176.11±277.32) (385). In the analysis, we found that rs11209026 AG/GG correlated with an increased risk of cancer recurrence. The controversial and variable roles of IL-17 in cancer pathology have been recently reviewed by Vitiello and Miller (2020) and summarised in Figure 93 (386). I speculate that the higher odds of cancer recurrence observed in the UK Biobank population carriers of AG/GG is due to the reduction of IL-17, which plays a significant role in anti-tumour immunity through recruiting neutrophils to the tumour site to mediate T cell response. This speculation is based on previous data that found patients with high IL-17 levels had a significantly favourable overall survival, indicating the prognostic and protective role of IL-17 in patients with colorectal cancer (387). However, several questions remain to be answered before investigating this genetic variant as a prognostic marker, about the impact of this polymorphism on IL-17A concentrations either in the serum of cancer patients or cancer tissues.

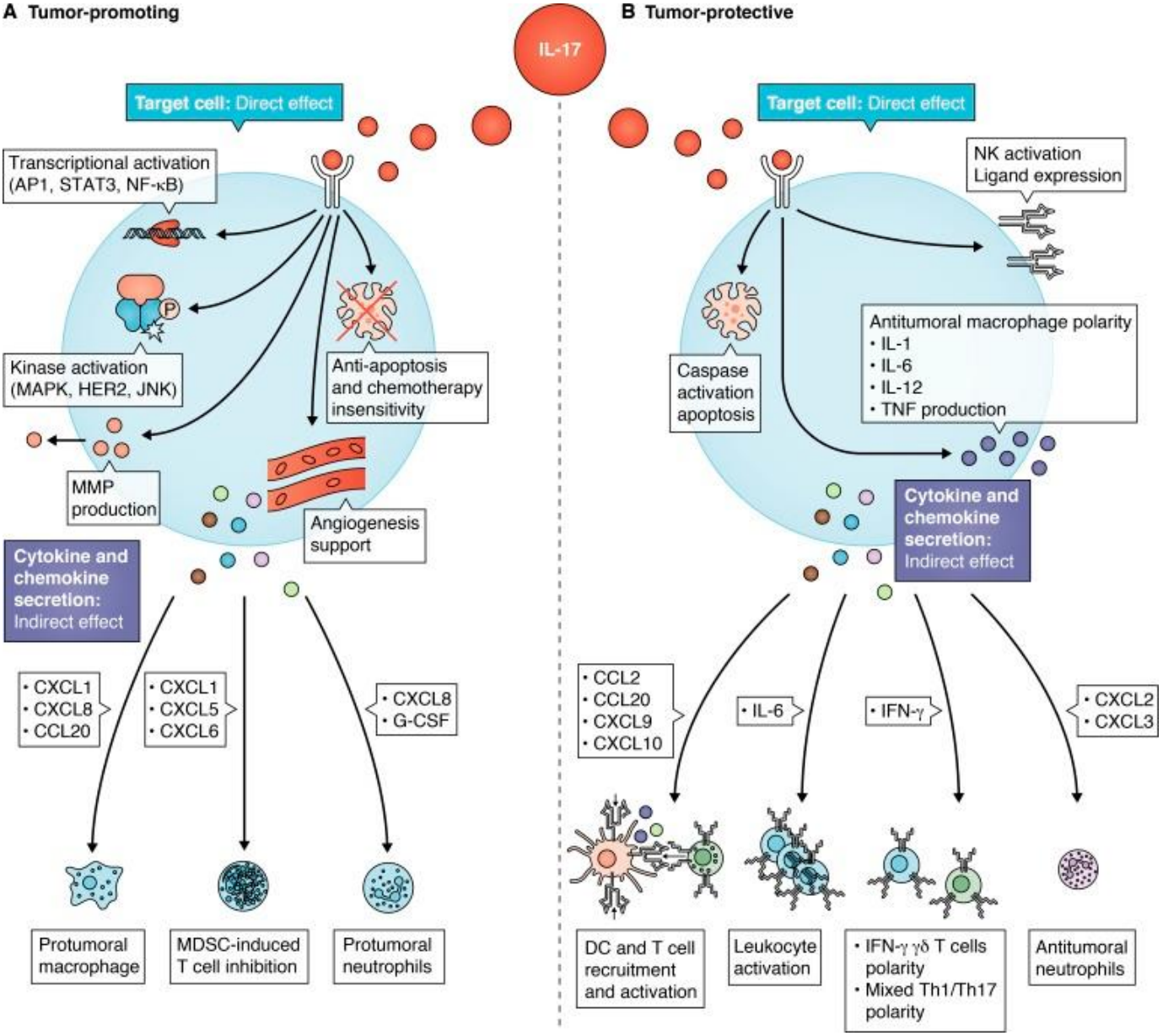


Figure 95. Tumour and antitumour activities of IL-17 (387) (the figure was reproduced from Vitiello et al., under Journal of Experimental Medicine copyright).

Rs12720356 is a missense variant with the MAF%, which is approximately 9% in Europeans, leading to a modification in amino acid position 684 from isoleucine to serine (388, 389). In the UK Biobank, heterozygosity of rs12720356 (TYK2) was associated with a decreased risk of metastasis, whereas homozygosity was correlated with a low incidence of cancer recurrence. A non-significant difference was detected in TYK2 mRNA expression levels in CA compared to AA in patients with rheumatoid arthritis (390). The polymorphism in rs12720356 implicated protection and risk effects in various diseases (391), necessitating the genetic/transcriptomic/proteomic characterisation of rs12720356 in cancer/cancer subtype to explain the observed protective role of the heterozygous in developing metastatic cancer and homozygous in cancer recurrence.

The OS was significantly associated with rs16858811 in CXCR1 and rs2255214 in ILDR1/CD86. Rs16858811 CA/CC showed lower survival rates than AA, whereas rs2255214TG/TT found favourable OS compared to GG in 5202 cancer patients. However, the potential translational utility of these findings is limited due to the lack of human data for investigation of the genotype effects of rs16858811 and rs2255214 on CXCR1 and CD86 at both transcriptomic and functional levels. Further studies are required to assess these polymorphisms in cancer at mRNA and protein levels. Hence, preliminary evidence and biological reasons should be provided before confirming these data in a prospective cohort as possible genetic biomarkers to predict the overall survival outcomes. The inability to estimate the upper confidence interval limit of homozygous is because of skewed data and the small sample size in this cohort.

In this analysis, I re-included the genetic variants in the TPCN2 gene to further investigate the interaction between genetic variants in TPCN2 and polymorphisms in monocyte/macrophage and cytokine genes in this cohort. In comparison with the first project in the UK Biobank, TPCN2 rs35264875: TA as an increased cancer risk was significant in the univariate logistic regression and near the significance level (0.05) in the multivariate logistic regression. The TPCN2

rs3750965:GG in cancer risk and TPCN2 rs35264875: TA in metastatic risk findings were not replicated in the second project because the sample size was smaller by 32% compared to the first project. Therefore, I conducted a further analysis of the association between genetic variants in TPCN2 that were not replicated due to the sample size in this cohort. I utilised the UK Biobank data after cleaning the missing or incomplete data for sex, DNA concentration, cluster.CR and the genotype of each variant separately (see Tables 28 and 29). I found that carriers homozygous for rs3750965 had decreased odds of developing cancer at a global level, and carriers heterozygous for rs35264875 showed a decreased risk of metastatic cancer, which is consistent with our previous findings. Therefore, the non-replication was due to an insufficient sample size and the poor power of these cohorts (<80%) to reach the significance level.

For the first time, I deciphered novel associations between genetic variants and cancer in the UK Biobank's population. This work might accelerate the evolution of novel biomarkers in terms of susceptibility and cancer outcomes. However, these findings should be validated in prospective cohorts with diverse populations. A functional analysis of significant genetic variants in similar biological contexts must be performed to reveal their potential clinical utility in the near future. Such genomic profiling in cancer may enable the discovery of novel cancer biomarkers and hallmarks, and it represents a further step toward personalised cancer care.

Table 27. Univariate and multi-variate logistic regression analysis of rs3750965 and the risk of cancer in the UK Biobank large cohort.				
	Cancer (Cases)	Cancer-Free (Controls)	Model A	
			OR (95 %CI)	*p*-value
Total (n)	84,558	391,377		
rs3750965				
GA			1(0.98-1.01)	0.5912
GG			0.93(0.88 - 0.99)	0.0218 *
Abbreviations: OR, odds ratio; CI, confidence intervals; Model A: univariate logistic regression				

Table 28. Univariate and multi-variate logistic regression analysis of a genetic variant that is significantly associated with a risk of metastatic cancer.				
	Metastatic cancer (Cases)	Primary cancer (Controls)	Model A	
			OR(95 %CI)	*p*-value
Total (n)	343	66,867		
rs35264875				
TA			0.77 (0.59-0.99)	0.0398 *
TT			0.85 (0.42-1.72)	0.648
Abbreviations: OR, odds ratio; CI, confidence intervals; Model A: univariate logistic regression				

5.5. General conclusion

Genomic profiling in cancer may enable the discovery of novel cancer biomarkers and hallmarks, representing a further step towards personalised cancer care. TPC2 is implicated in cancer biology, and my studies begin to provide evidence that links TPC2 to cancer immunity. There have been a lack of studies that have genetically characterised TPC2 in cancer at a global level and in 13 types of cancer at the local level. The UK Biobank studies unravelled novel associations between TPCN2 and monocytes/macrophages and cytokine genes in cancer in terms of susceptibility and cancer outcomes that were reported for the first time. Significant associations were discovered between 14 genetic variants and cancer. These findings lay the groundwork for future research to validate the findings in prospective cohorts with large and diverse population and to characterise these genetic variants functionally. The translational utility of these potential

genetic biomarkers have not yet reached the stage of clinical application. However, the findings at this stage might help identify a potential pathway that TPC2/monocytes/macrophages/cytokines might play a role in the pathophysiological processes that are related to cancer. In addition, the data provides evidence to further investigate the potential translational relevance of this data as diagnostic/prognostic biomarkers in clinical settings. This could enable providing tailored therapy based on individual genetic makeup as an application of cancer precision medicine.

UNIVERSITY OF OXFORD

Chapter 6: Discussion

6. Chapter 6: Discussion

6.1. General discussion

The overexpression of two-pore channel 2 (TPC2) in monocytes (40) and in *in vitro* models of human monocytes and myeloid malignancies (as shown in supplementary Figure 6.1) as well as the paucity of data that biologically, genetically, and clinically characterise TPC2 function in monocyte and macrophage development and its complex interplay in cancer immunology attracted my scientific curiosity to decipher the role of TPC2 in monocyte differentiation as a novel therapeutic target in cancer. In the first weeks of starting my DPhil, I found a known SNP (Single-nucleotide polymorphism) (rs72917389) in the TPCN2 gene in monocytes prior to, during and after differentiation of monocytes into macrophages derived from acute lymphoblastic leukaemia but not in monocytes isolated from the blood of healthy subjects. Then, I extracted DNA from the PBMCS of a healthy subject and an acute myeloid (AML) leukaemia patient to investigate genetic variants (in location Chr.11: 69088951-69089447) in the TPCN2 gene by Sanger sequencing.
rs3376 is a common genetic variant in the TPCN2 gene (MAF of approximately 24%) identified by Sanger sequencing in DNA samples derived from an AML patient. These data provide preliminary evidence of existing mutations in the TPCN2 gene (rs72917389 and rs3376) that might be correlated with cancer, indicating the possible role of TPC2 at the genetic level in haematological malignancies. During that time, an interesting article by Wang et al. (2019) found a positive correlation between TPCN2 expression in the bone marrow and the response to chemotherapy in Chinese patients who were newly diagnosed with AML (180). The identified frequency of TPCN2 mutation in protein and cancer illustrated in Figure 96, varies from one biological context to another. Therefore, I navigated the DepMap 22Q2 expression public database, and I investigated the expression of TPCN2 in non-cancerous lymphocytes and 67 various cell lines of leukaemia and lymphoma, and I observed a significant (*p*-value =0.003965)

upregulation in TPCN2 expression in *in vitro* models of leukaemia and lymphoma vs non-cancers (as shown in Figure 97). CanSar predicts the ligand ability score of TPCN2 structure is approximately 14% in cancer and 31% in other diseases. These data highlight the unrecognised function of TPC2 – pharmacologically, genetically, transciptomically, and proteomically in cancer.

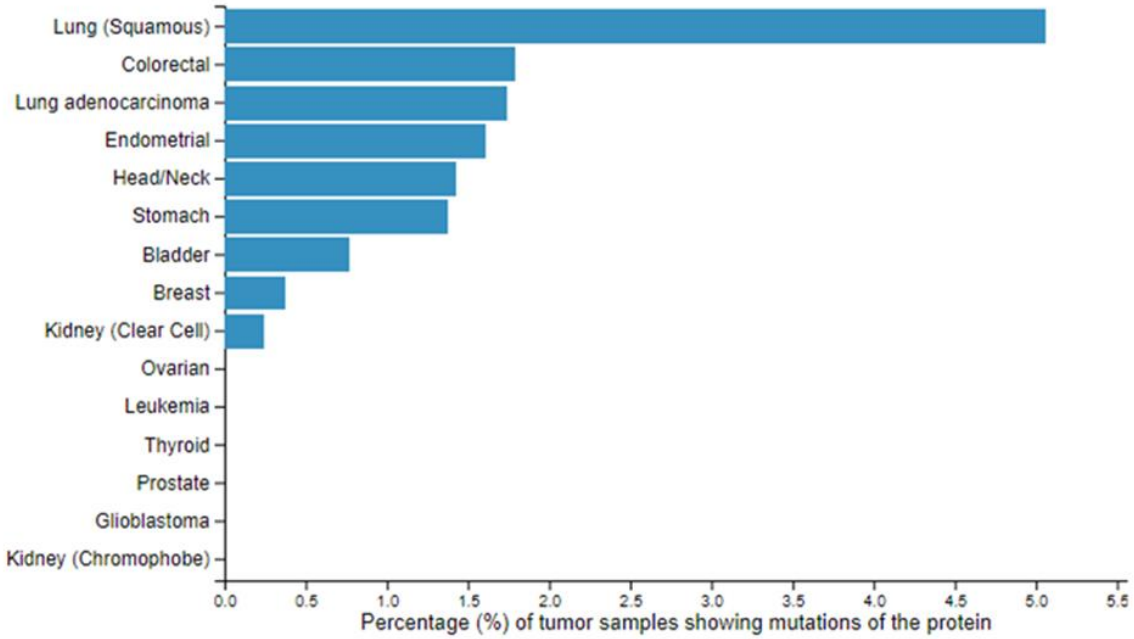


Figure 96. Protein Mutation Frequency in TPCN2 and Cancer (392).

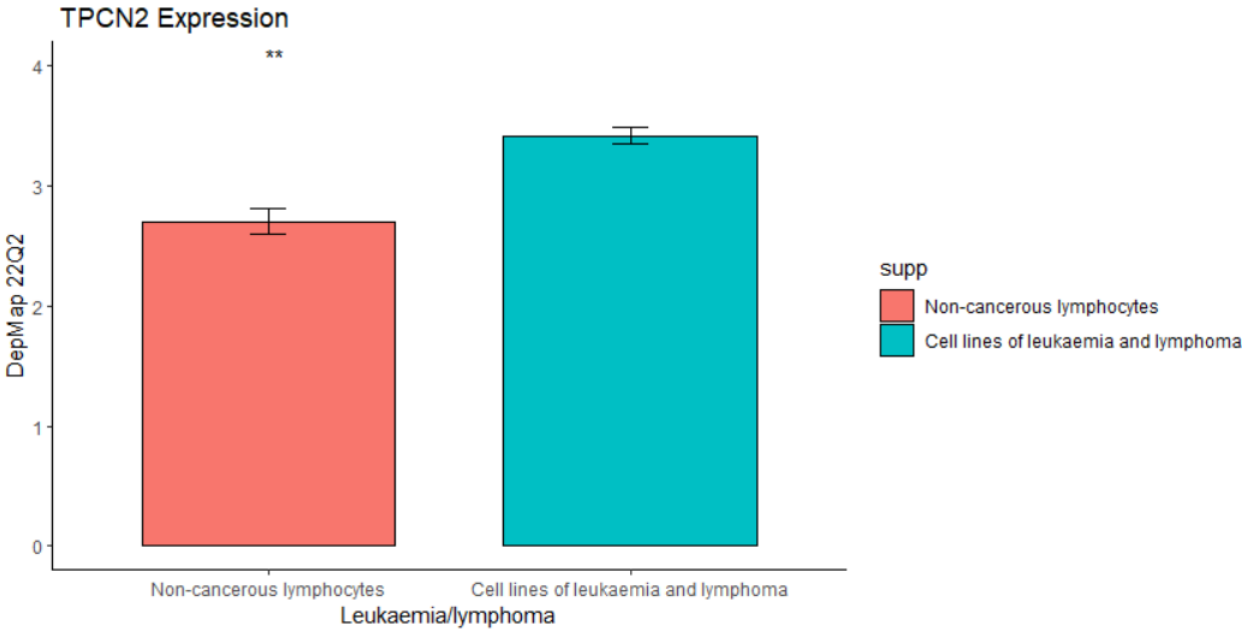


Figure 97. TPCN2 expression in non-cancerous lymphocytes and 67 various cell lines of leukaemia and lymphoma (mean ± SEM, two sample t-test, *p*-value = 0.003965) (393).

While treating the U937 cells with Ned-19 at 100 μM for 24 h and then inducing differentiation by PMA, I qualitatively observed that the yield of non-polarised macrophages was smaller compared to the untreated U937 cells (unpresented data). Hence, I hypothesise that Ca^{2+} signalling via TPC2 plays a pivotal role in monocyte differentiation and macrophage development that involves physiological processes related to immunity and cancer, and it is a candidate target for cancer immunotherapy. To provide insights and directions and get preliminary data to support the hypothesis, I conducted proteomic analysis utilising our lab resources (TPC knockout mice) to confirm that TPC2 is the main TPC channel that is involved in inflammation, immunity and cancer. I chose the spleen as a suitable model because it contains both monocytes and macrophages. I analysed the data focusing on the role of TPC2 in immunity or cancer, and I found that 87 proteins were significantly altered in TPC2-deficient murine solenocytes compared to the WT, indicating the potential roles of TPC2 in physiological processes attributed to cancer immunity.

Selecting a suitable model to investigate a hypothesis is a rate-limiting step of any scientific project. The evolving biological roles and potential therapeutic value of TPC2 have attracted the scientific community's attention to developing pharmacological modulators that interfere with TPC2 channel activity. In 2020, Gerndt et al. (10) discovered two highly selective lipophilic agonists to TPC2 vs TPC1, namely TPC2-A1-N (1) and TPC2-A1-P, which activate TPC2 function in a NAADP- or PI(3,5)P2-like fashion. Müller et al. (2020) recently developed TPC2 inhibitors (SG-005 and SG-094) that are not highly selective for TPC2. SG-005 has an inhibitory effect on TPC1 and TRPML1, whereas SG-094 has an inhibitory effect on TPC1 and minimally inhibits TRPML1 (139). Due to the lack of available chemicals that specifically inhibit TPC2 activity, utilising this pharmacological inhibitor alone is not the best suitable approach for investigating the role of TPC2 in monocyte and macrophage biology and its influence on physiological processes attributed to cancer. I selected two *in vitro* models of human monocytes that were derived from cancer patients and were different in their origin (blood vs tissue) as my biological context to address my DPhil experimental question: is TPC2 a regulator of monocyte differentiation and macrophage development and a possible candidate target of cancer immunotherapy? The appropriate approach to transfect U937 and THP-1 cells is nucleofection, and our lab lacks the required equipment to conduct it; therefore, I contacted Synthego to perform the transfection and send me the pools of TPCN2 genetically modified human monocytes (U937 and THP-1). I established a TPC2-deficient (approximately 70% reduction in TPCN2 expression) U937 monocyte model and attempted to develop TPC2-deficient THP-1 monocytes twice, but it failed. The failure to develop the TPC2 KO THP-1 model might be due to the potential major role of TPC2 in THP-1 cell survival and proliferation. However, this speculation should be interpreted with caution since Li et al. (2022) (394) were recently able to develop a TPC2 KO THP-1 model by transient transfection through a lentiviral vector; therefore, the suggested role of TPC2 in THP-1 growth could be attributed to the status of the transfection, transient or permanent, and the

approach utilised to knockout TPC2 in a THP-1 cell line. The successful development of TPC2 U937 KO cells is limited due to the lack of appropriate data presented in this thesis that defines the TPCN2 gene editing on a functional/protein level. In future work, I am aiming to utilise advanced and traditional approaches to assess the impact of TPC2 activity. Due to the lack of commercially available antibodies that specifically interact with TPC2 at the protein level, I tried to stain TPC2 intracellularly by flow cytometry. This requires permeabilisation of the cells and fluorescent conjugated secondary antibodies and I asked the Alomone lab to share a sample with me. I recall that it showed good binding, but it will require further experiments and funding to reach a solid result. Due to the impact of the COVID-19 pandemic on the capacity of each lab and the flow cytometry facility, I will postpone this experiment for future work, and I would like to continue what I have started to phenotypically characterise human monocytes and macrophages using the panel that I worked on optimising (presented in Supplementary Table 6.1) in early 2020. The phenotypic characterisation of monocytes and macrophages is a hot topic in the immunology field that might provide insights and answers to tackle complex and unanswered scientific questions related to oncoimmunology. In the near future, I am interested in polarised macrophages and characterising each type of TPC2 polymorphism, phenotypically and functionally. In addition, in future work, I aim to investigate the effects of TPC2 knockdown upon the global expression of RNA and proteins in U937 cells that can be utilised as *in vitro* models of both monocytes and myeloid leukaemia. I will relate the information that I obtain about such RNA and protein expression to the phenotypic findings that I have found to identify molecular mechanisms underlying the action of TPC2 in pathophysiological processes attributed to cancer immunity. The correlation analysis of transcriptomic and proteomic data will be useful when I establish a lab in my home country focused on translational cancer immunity, as I am planning to focus on haematological malignancies due to the high frequency of these tumours in Gulf state countries compared to other countries in the world.

Proteomic data revealed a significant change (downregulation) in haematopoietic cell-specific Lyn substrate 1 level, which was confirmed by qPCR but in an opposite direction (upregulation) in TPC2-deficient murine splenocytes. TPC2 might act as a mediator of haematopoietic stem cells in all blood lineage cells, especially monocytes, warranting investigation in a murine model. Interestingly, the HCLS1 change in protein level was not directly related to the change in mRNA. It could be attributed to post-transcriptomic regulations that happen after mRNA production and before the translation into a protein. HCLS1-deficient mice had differential effects on the numbers of peripheral blood immune cells, including monocytes, eosinophils and neutrophils; neutrophils were significantly lower, whereas monocytes and eosinophils were not significantly higher as compared to WT mice (395). The oncological role of HCLS1 has been investigated, showing it to be a player in physiological processes related to cancer biology involving tumorigenesis and metastasis. The crosstalk between TPC2, HCLS1 and myeloid cell differentiation in mice biology and its link to cancer pathology has not yet been investigated.

The ability to speculate about this data in the context of murine myeloid cell differentiation is limited because the number of monocytes and other myeloid cells was not counted in the peripheral blood of TPC2-/- mice or in the spleen compartment. There is a lack of knowledge regarding the biological role of TPC2 in murine myeloid cell differentiation based on insights from splenic proteome data. I made a preliminary hypothesis that TPC2 might have distinctive functions in myeloid cell differentiation in a process involving the HCLS1 pathway. Lee et al. 2017 (396) discovered that mTORC1-S6K1-Myc signalling is a major mediator of myeloid differentiation. The disruption of this pathway via raptor-inducible knockout in mice leads to monocyte and neutrophil depletion. mTORC1 negatively regulates TPC2 function (103). Further studies are required to define the role of TPC2 in murine myeloid cell differentiation at the global level and in monocytes at the local level.

In this thesis, I provide scientific evidence to address the following question: is TPC2 another piece of the cancer immunology puzzle? I investigated the role of TPC2 in monocyte differentiation, macrophage development, and IL-8 and IL-10 production, as well as its influence on monocyte chemotaxis via the genetic/pharmacological modulation of TPC2 function utilising molecular biology techniques such as ELISA, qPCR and western blot. I observed that TPC2-deficient U937 monocytes were resistant to monocyte differentiation and macrophage development upon treatment with PMA. To my knowledge, this is the first time that TPC2 has been demonstrated to be an important mediator of PMA-induced monocyte differentiation and macrophage development. In Chapter Three, I proposed several pathways based on the data acquired during my DPhil study that provides an initial explanation of how TPC2 impacts this process. The main proposed pathway is TPC2 signalling, which regulates monocyte differentiation via PKC-alpha and AKT-signalling pathways involving calpain activity, which is summarised in Figure 98. While conducting my DPhil project, I observed that TPC2 has a major role only in monocyte differentiation into macrophages and not into dendritic cells. PKC alpha and beta are conventional isoforms of PKC, stimulated by Ca^{2+} signals and diacylglycerol (DAG). A previous study showed that only PKC-alpha, not PKC-beta, is required to differentiate the monocytes into macrophages, and PKC-alpha has a distinct role in monocyte differentiation into macrophages, not into dendritic cells (271). PKC-alpha activity is essential for monocyte differentiation; however, another research group claimed that translocation is important but not integral for the activation. To my knowledge, PKC-alpha has to translocate to the plasma membrane to be activated and then phosphorylates other substrates (in our context, the AKT pathway). RNA seq analysis showed that PMA-induced differentiation of monocytes into macrophages is mediated via PI3K/AKT signalling (267). In my study, I observed a significant difference in PI3K, AKT, mTOR, and TPCN2 expression levels in macrophages compared to monocytes. In addition, calpain activity was upregulated in TPC2-deficient U937 monocytes. I

tried to define the dynamic changes in calpain activity during monocyte differentiation during my DPhil, but I did not achieve consistent data overall. I speculate that TPC2 KD leads to a compensatory mechanism through upregulation of one of the intracellular compartment channels, which ultimately elevates the calpain activity. Calpain is a negative regulator of PI3K/AKT and PKC-alpha.

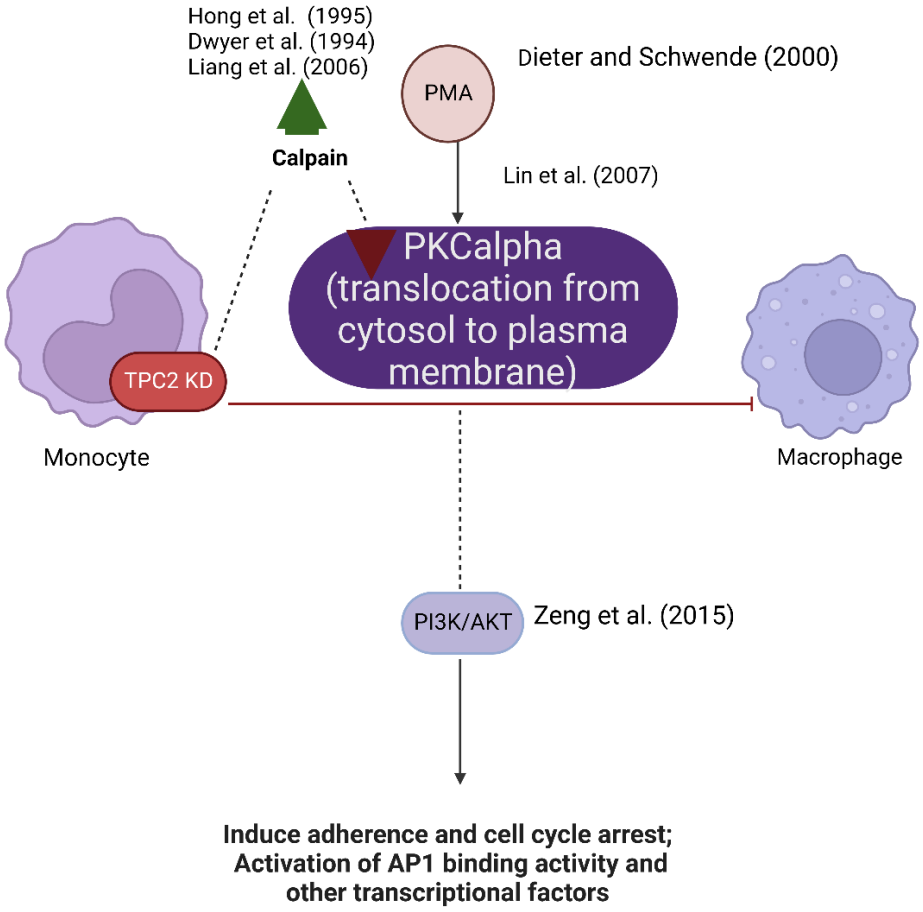


Figure 98. TPC2 in monocyte differentiation via PKC-alpha and AKT-signalling pathways.

The thesis findings identified TPC2 as a player in the release of two mediators that are opposed in their functions: the IL-8 pro-inflammatory cytokine and IL-10 anti-inflammatory cytokine. IL-8 expression was significantly reduced in two different human biological models harbouring loss of TPCN2 expression. Similarly, the murine homolog of IL-8 (KC) was drastically decreased in two different murine biological models harbouring loss of TPCN2 expression. The loss of IL-8 expression was significantly correlated with the reduction of IL-8 level in TPC2 KD U937, whereas the reduction in KC expression was not significantly associated with a decrease in KC level in TPC2 deficient spleen cells. The statically non-significant change in KC level might be explained due to the presence of an outlier that deviates from the conclusion. However, the non-statically significant results might have a biological or therapeutic significance. The MIP-2 expression in TPC2 deficient splenocytes was not reported due to the inconsistency of the data, whereas I observed a significant reduction in MIP-2 level. I further investigated the possible substrate that is responsible for the TPC2 role in MIP-2 utilising ELISA. Unexpectedly – which might be due to utilising a different plate reader – I obtained a reading from the blank that is higher than the unknown samples, which makes it unreliable to correct the absorbance by subtracting the samples from the blank readings. However, I observed that the absorbance readings were reduced by approximately 44-fold when WT splenocytes were treated with Ned-19 (unpresented data), indicating that NAADP might be the main activator of TPC2 channel to exhibit its function on MIP-2 release. The murine data regarding the role of TPC2 in KC/MIP-2 release are somehow limited because the time-dependent release was not conducted, and the level was only measured after 24 h utilising ELISA. I utilised ELISA as the only approach to detect the levels of KC (a 72 amino acid protein) and MIP-2 (a 73 amino acid protein) without confirmation of the data by other techniques such as WB and flow cytometry. The focus of my DPhil was to explore the role of TPC2 as a player in IL-8 production in humans, not in mice. Therefore, I conducted time-dependent release for up to 48 h in monocytes and macrophages and stimulated IL-8 release in

WT monocytes vs TPC2 KD monocytes. The significant reduction found in IL-8 levels in monocytes treated with Ned-19 (NAADP antagonist) indicates that TPC2 modulates IL-8 expression at RNA and protein levels through NAADP. In Chapter Three, I presented several theories based on the data that I acquired during my study in Oxford on how TPC2 impact the IL-8 expression and level. From my perspective, there are several processes associated with IL-8 production, and TPC2 might have a distinctive or collective impact on this. I measured the IL-8 utilising the ELISA kit that is suitable for measuring the IL-8 isoform of 72 amino acids. The IL-8 isoform of 99 amino acid residues is not biologically active and it undergoes proteolytic cleavage by thrombin and plasmin to yield active forms (peptides of 72 or 77); the 72 amino acids form is mainly produced by thrombin, and it is the major IL-8 form that is secreted by monocytes and macrophages. The splenic proteome analysis identified a significant reduction in thrombin and plasminogen levels in the TPC2 KO murine model, which led me to assume that TPC2 regulates IL-8 release in human monocytes through the modulation of two serine proteases that are responsible to yield the biologically active 72 peptide form. Therefore, I conducted qPCR to investigate plasminogen activator inhibitor-1 (PAI-1), urokinase-type plasminogen activator (uPA) and prothrombin mRNA levels. No significant changes were observed in PAI-1 and uPA expression levels between WT and TPC2 KD U937 cells. Prothrombin was not detected in either model. The proposed mechanism of how TPC2 mediates LPS- IL-8 production in monocytes via AKT signalling is shown in Figure 99. No significant difference was detected in IL-8 levels after treating the monocytes with ML-SA1 (TRPML agonist), indicating that TPC2 has a biologically distinctive role compared to this other endolysosomal channel in IL-8 release.

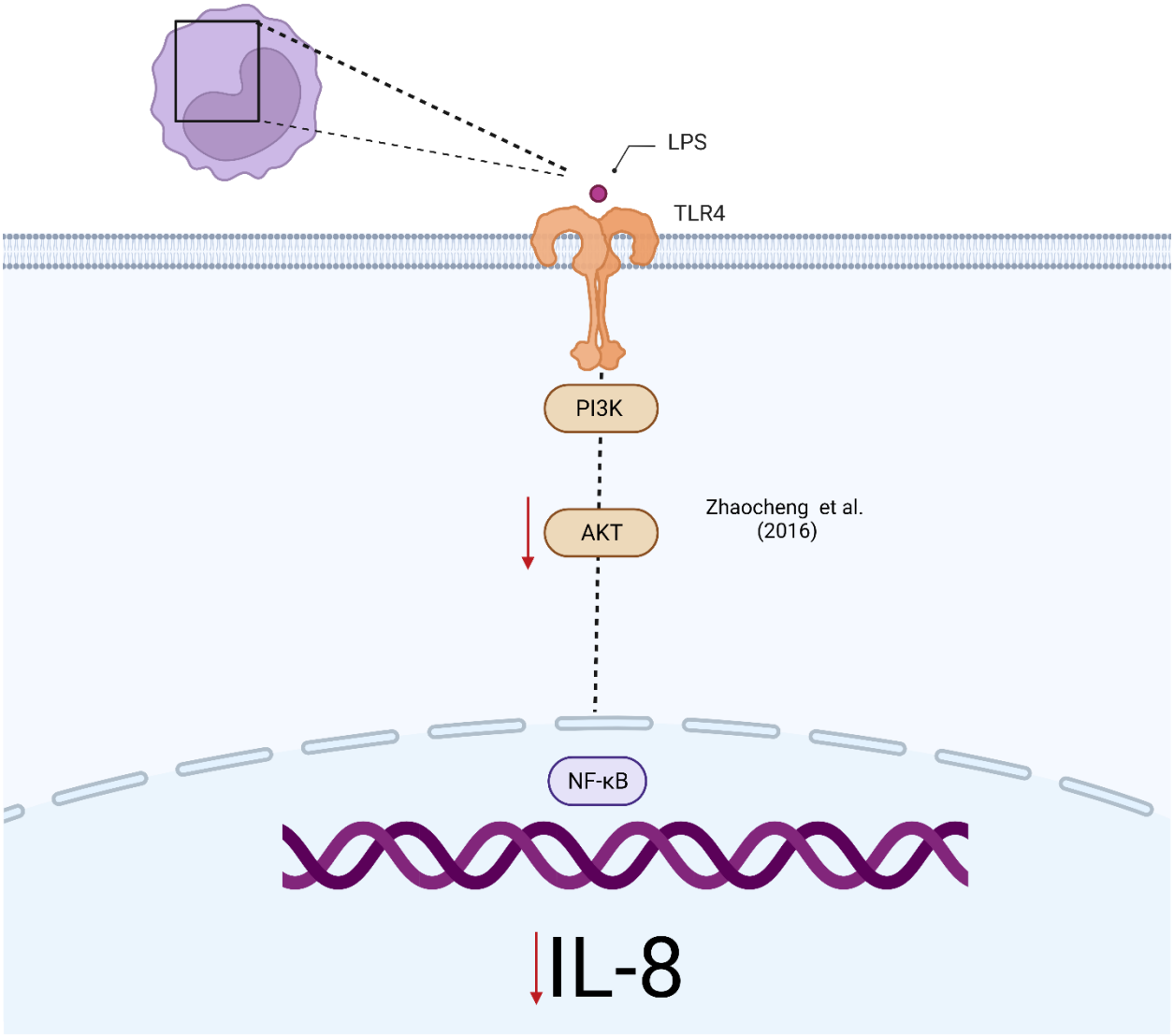


Figure 99. TPC2 role in IL-8 release via AKT pathway.

IL-10 release was statistically significantly enhanced in TPC2 KO murine model and not significantly increased in TPC2 deficient monocytes. The upregulation of IL-10 in murine cells with loss of TPC2 activity could be explained by the drastically significant increase in mitogen-activated protein kinase 14 (also called p38-α) expression in TPC2-deficient splenocytes. This finding supports the involvement of TPC2 and p38-α in IL-10 release. It has also been reported that MAPK is involved in the regulation of TPC2 function (103). MAPK/p38 negatively modulates the TPC2 function. This is supported by the observed enhancement in p38-α expression in TPC2 deficient spleen cells. The pharmacological inhibition of MAPK/p38 signalling via SB-203580 profoundly reduced IL-10 expression at RNA and protein levels in lymphocytes derived from septic animals (397). The exact contribution of TPC2 and mitogen-activated protein kinase 14 to IL-10 production remains to be established. The major caveats in generalising this data to the global impact of TPC2 in IL-10 production is that time-dependent release and stimulation release utilising various types of cells (involving macrophages) have not been investigated in this study. Utilising only one approach to measure the cytokines is one of the limitations of this study.

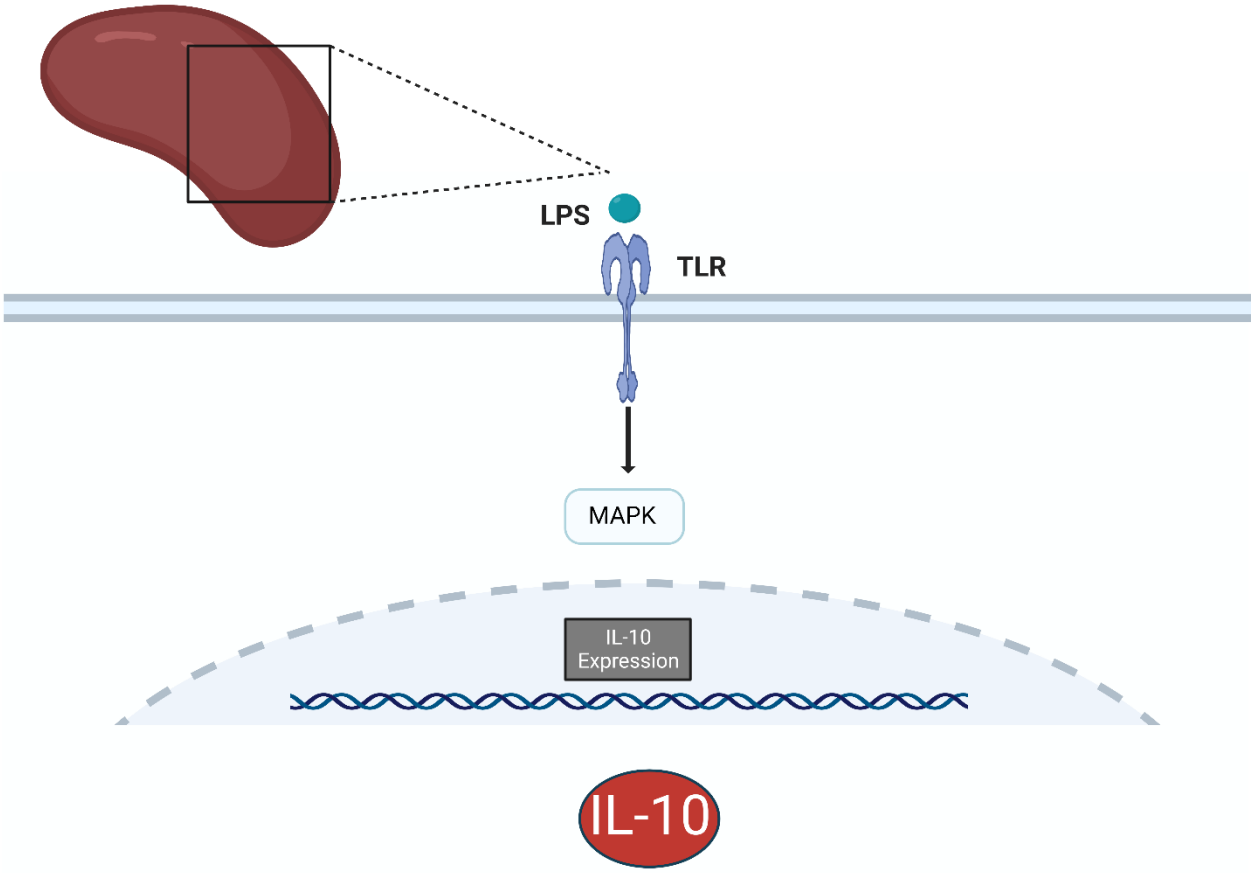


Figure 100. TPC2 role in IL-10 production via MAPK signalling.

In addition, the proteomic data underpinned the potential roles of TPC2 in cytokine production or regulation. Significant changes in protein levels of biologically important proteins attributed to this process involve pro-interleukin-16, SAM and SH3 domain-containing protein 3, and adiponectin. Novel genetic associations were reported for the first time in genes involved in cytokine signalling and cancer in terms of susceptibility, metastasis, recurrence and overall survival. The heterozygous alleles in rs2069827(IL-6), rs2255214(ILDR1/CD86), rs375947(IL12RB1), rs3024508(IL-10) or homozygous allele in rs3024508(IL-10) were correlated with a higher risk of cancer susceptibility, whereas the heterozygous allele in TNF-α rs4645843 was correlated with a

lower risk for developing cancer. In terms of metastatic cancer, heterozygous genotypes in rs12720356 (TYK2) and rs1799964 (TNF) are associated with a lower incidence of metastatic cancer. In cancer recurrence, homozygous genotypes in rs12720356(TYK2) are associated with lower odds, whereas homozygous /heterozygous genotypes in rs11209026(IL23R) are correlated with higher odds. Homozygous/heterozygous genotypes in rs16858811(IL-8RA/CXCR1) showed low favourability in terms of overall survival compared to WT, whereas homozygous/heterozygous genotypes in rs2255214(ILDR1/CD86) had a good prognosis. Overall, these data indicate the biological significance of cytokine signalling in cancer and provide insights into the possible translation relevance of these genetic associations as novel cancer biomarkers. IL-8 and IL-10 play vital roles in processes related to immunity and cancer. The discovered genetic correlations between genetic variants in IL-10 or IL-8RA and cancer warrant functional investigation of these mutations in IL-10 or IL-8RA due to limited data regarding their influences at the functional level.

In this thesis, I was not able to investigate the role of TPC2 in phagocytosis due to the resistance of TPC2 deficient U937 cells to differentiation. However, the splenic proteome analysis showed a significant downregulation in neutrophil cytosolic factor (NCF), which plays a role in phagocytosis, in TPC2 KO spleen cells, (398). Although I found that TPC2 deficient monocytes were non-significantly highly migrated towards chemoattractant, I identified a significant upregulation in RhoA expression. RhoA is mainly known to modulate focal adhesion, whereas RhoG regulates cell migration. Proteomic analysis revealed a lower expression in RhoG in TPC2 deficient spleen cells. These preliminary data warrant further investigation into the potential role of TPC2 in primary monocyte chemotaxis and neutrophil biology, especially in chemotaxis. I suggest that neutrophils could be cultured in the media from WT and TPC2 KO monocytes to assess the chemotaxis. In my DPhil studies, I found a significant reduction in IL-8 at both RNA and protein levels and significant downregulation of CD38 in TPC2 deficient monocytes. The observed reduction in CD38 could be attributed to the loss of the TPC2 channel, as CD38 is involved in

synthesis of NAADP, which is one of the main substrates of this channel. It was shown that IL-8-mediated neutrophil chemotaxis is a process involving CD38 activation (399). Recently, IL-8-CD38-NAADP-Ca^{2+} signalling was found to be involved in lymphokine-activated killer cells chemotaxis as shown in Figure 101 (400). This thesis provides insights into a possible role of TPC2 in neutrophil chemotaxis either directly or indirectly through IL-8 release, which warrants further investigations.

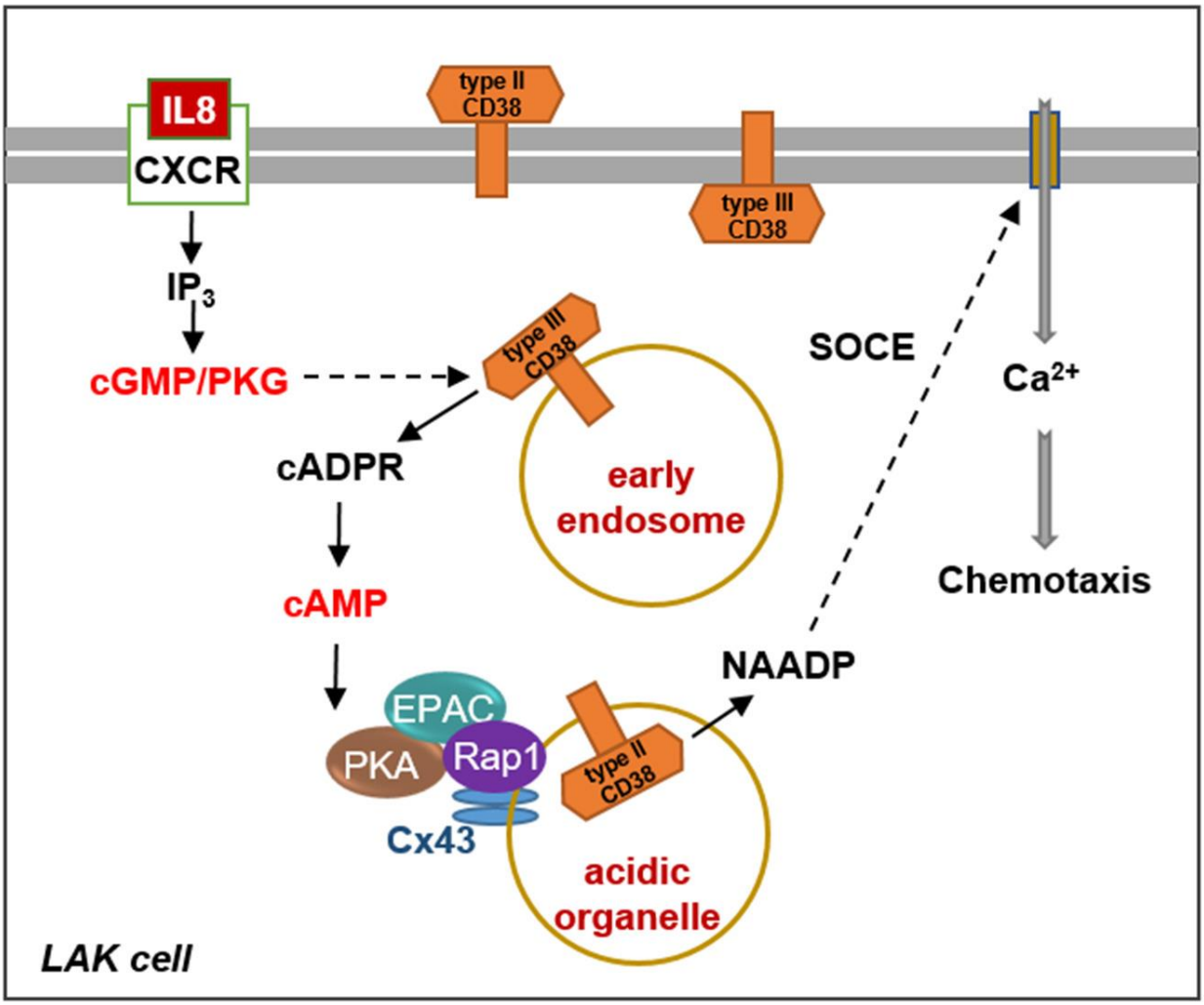


Figure 101. IL-8-CD38-NAADP-Ca^{2+} signalling in chemotaxis (400) (the figure was reproduced from Nam et al., under FASEB copyright).

The expression of TPCN2, other endolysosomal channels, and other key markers in various cell lines related to monocytes or haematological malignancies, that TPC2 was found to regulate their expression in this thesis, have been summarised in Table 29. In U937 cells, TPCN2, CD38, IL-8, AKT2 and mTOR expression are positively correlated, which is consistent with the transcriptomic data in the case of TPC2 expression loss. In addition, significant positive but weak correlations were observed between TPCN2 and IL-8 and between IL10 and CD38 in cancer when utilising Correlation AnalyzeR, as shown in Chapter Three supplementary (401). These data underscore the additional existence of biological links between TPC2 and other cancer markers, as demonstrated in this thesis for the first time, warranting further investigation.

Table 29. Endolysosomal ion channels and key markers of this thesis at the transcriptomic level.

Cell Line	TPCN2	TPCN1	CXCL8	IL-10	IL-10RA	IL-10RB	CD38	AKT2	RhoA	MTOR	MCOLN1	P2RX4
HL-60	2.98	3.12	6.25	0	0.99	4.28	0.61	4.61	8.11	4.07	3.53	2.64
THP-1	3.84	6.28	2.45	0.01	2.6	6.43	4.82	5.98	8.78	5.01	3.07	4.76
K-562	3	4.49	4.68	0	4.01	4.78	0.31	5.89	8.09	5.38	3.26	5.03
U937	4.13	4.81	1.05	0	2.74	5.84	2.07	6.05	9.05	4.94	2.74	4.72

Source: canSAR: update to the cancer translational research and drug discovery knowledge base, Coker EA et al., Nucl. Acids Res. 2019;47(D1):D917-D922. doi: 10.1093/nar/gky1129

The recent evolving scientific interests in the frequency of monocyte subsets in cancer and their protumoral or antitumor effects necessitate expanding our knowledge of how these cells differentiate and identifying major regulators of less well-defined biological processes. The possible therapeutic relevance of genetically modifying TPCN2 expression in human monocytes derived from cancer patients is because of its modulation of macrophage development, IL-8, IL-10, CD38, PKC-alpha, and AKT. Targeting circulating monocytes (trabectedin), IL-8 (BMS-986253 in clinical trials), IL-10(pegilodecakin), CD38 (daratumumab), PKC (sotrastaurin) and AKT (capivasertib) have been implicated as therapeutic strategies to treat cancer patients. Hence, targeting TPC2 or downstream/upstream regulators of this channel activity is a promising

therapeutic approach to treat cancer in the future. The lack of pharmacological modulators that specifically regulate TPC2 function with high sensitivity might be a considerable obstacle to investigating TPC2-based therapy in *in vivo* models of cancer. Further TPC2-based drug screening is required to be conducted in normal and cancer cells to identify chemicals that only interfere with TPC2 function. The ubiquity of TPC2 adds another challenge to its potential translational relevance, which suggests the need to develop novel approaches to target specifically TPC2 in cancer or physiological processes relevant to cancer pathology. The findings of this thesis about the potential therapeutic relevance of TPC2 are generalisable to other diseases related to inflammation and immunity. In the COVID-19 pandemic era, the possible therapeutic benefit of TPC2 modulations in COVID-19 and cancer as a treatment for both conditions simultaneously warrants further investigation. While conducting my DPhil project, I provided scientific insights into the potential major role of TPC2 in blood coagulation and haematological disease biology. During the COVID-19 pandemic, we had a fruitful discussion with Dr Susie Shapiro, a consultant haematologist. She suggested further investigating the data using an *in vivo* model. Future research should be conducted to shed light on the role of TPCs, especially TPC2, in blood coagulation and their clinical utility as a biomarker or target for haematological diseases.

Further research is required to delineate in detail the molecular mechanism of how TPC2 mediates the differentiation process by exploring the potential cross-talk between the intracellular Ca^{2+} channels and extracellular Ca^{2+} channels during the differentiation and identifying the possible intracellular Ca^{2+} channel that is upregulated due to loss of TPC2 function. The major finding that TPCN2 genetically modified U937 monocytes are resistant to monocyte differentiation after treatment with PMA, indicating TPC2 as a major regulator of monocyte differentiation, is limited to U937 cells and using PMA as a stimulator. We cannot extend this finding to primary human or murine monocytes and other cell lines. An important next step will be to investigate the

cellular mechanisms underlying the regulation of Ca^{2+} signalling via TPC2 in monocyte differentiation and macrophage development in primary monocytes. Human and murine monocytes will be sorted by flow cytometry and will be differentiated into macrophages. A further step would be to investigate this finding as it relates to cancer metastasis in an *in vivo* study. Such an *in vivo* model would be used to investigate the correlation between TPC2, metastasis, TAMs (tumour-associated macrophages), and cytokine levels (focusing on IL-8 and IL10). 4T1 cells (mouse models of metastasis) will be injected into wild-type, TPC1 KO, TPC2 KO and TPC1/TPC2 double KO mice, and wild-type mice will be treated with TPC2 pharmacological modulators, E64, DNA, E64-DNA, and cyclophosphamide. After that, imaging will be performed to detect the metastatic sites on Day 7 and the metastatic tumours will be dissected to measure the size and the volume. TAMs will be isolated, and imaging and transcriptional regulation assays will be conducted to examine how expression of TPC2 is affected by progression towards the metastatic state. Single cell seq will be utilized to identify cell populations in murine TAM isolates. Circulating monocytes and macrophages will be characterized using flowcytometry (Extracellular markers: CD14, CD16, CD86, CD206, Intracellular markers: CD68, TPC2), and KC, MIP-2 and IL10 levels will be measured utilizing ELISA.

The completion of the Human Genome Project (HGP) in 2003 revolutionised medicine through expanding our knowledge regarding the role of genetic variation in the development of disease and the response to therapy, improving both diagnosis and treatment, particularly of cancer. The cost of DNA sequencing has decreased, allowing for the application of genomic based clinical decisions in daily clinical practice. Currently, the importance of decoding human DNA in modern medicine has been illustrated by the award of the 2022 Nobel Prize in Medicine and Physiology to Prof Svante Pääbo. My great interest in cancer genomics started when I was an MSc student at the University of Minnesota and continued through my doctoral study, during which I participated in advanced courses offered by international institutions (ESMO advanced course on

Biomarkers for Precision Medicine). I applied to leverage the UK Biobank data to characterise TPC2 genetically in cancer. The number of SNPs that have been discovered in the TPCN2 gene are above 19,000 in the DbSNP database. TPCN2 germline mutations were approximately correlated with six diseases, including melanoma, skin neoplasms, diabetes mellitus type 2, melanosis, prostatic neoplasms, and lung neoplasms, according to the Public Health Genomics and Precision Health Knowledge Base (v7.9). The type of somatic mutations in the TPCN2 gene in 731 cancer samples are nonsense substitution: 11 (1.50%), missense substitution: 232 (31.74%), synonymous substitution: 134 (18.33%), frameshift insertion: 3 (0.41%), inframe deletion:1 (0.14%) and other: 22 (3.01%) (380). The pan-cancer analysis of TPC2 mutations was conducted in TCGA studies (as shown in Figure 102) (402). The unpublished data from our lab with collaborator Prof. Colin Goding, highlights the potential significance of TPC2 as a biomarker. Prof. Goding found that TPCN2 low expression was significantly correlated with metastasis and favourable prognosis in melanoma in the TCGA database. There is a paucity of genetic association studies that decipher the role of genetic variants in the TPC2 gene in cancer in terms of prognosis and diagnosis. In this thesis, I revealed the potential applications of TPC2 as a biomarker in the definition of tumour types, susceptibility, prognosis, response and cancer outcomes in the UK Biobank. Carriage of TPCN2 rs3750965:GG was associated with a lower risk for developing cancer (OR: 0.97, 95% CI: 0.95-0.997, *p*-value=0.0292*, vs. A/A), whereas carriage of TPCN2 rs35264875: TA was associated with an increased risk of cancer susceptibility (OR: 1.03, 95% CI: 1.01-1.05, *p*-value=0.00138 **, vs. A/A), after adjusting for sex and ethnicity. Carriage of rs35264875: TA was associated with a higher general risk of developing various cancer subtypes (breast cancer OR: 1.06, 95% CI: 1.02–1.1, *p*-value = 0.006**; colon cancer OR: 1.03, 95% CI: 1.01–1.05, *p*-value = 0.00138 **; malignant neoplasms, stated or presumed to be primary, of lymphoid, haematopoietic and related tissue OR: 1.09, 95% CI: 1.02–1.18, *p*-value = 0.015 *, vs. A/A). Genotype GA in rs3750965 in TPCN2 was associated with a decreased

risk of developing malignant melanoma (OR: 0.91, 95% CI: 0.84–0.99, p-value = 0.02334 *, vs. A/A). GA in rs3750965 in TPCN2 showed lower odds of malignant neoplasms of mesothelial and soft tissues (OR: 0.72, 95% CI: 0.53–0.97, *p*-value = 0.032 *, vs. A/A), whereas it was associated with higher odds of malignant neoplasms of the lip, oral cavity and pharynx (OR: 1.25, 95% CI: 1.02–1.53, P = 0.0311 * vs. A/A). Genotypes GA/GG in rs3750965 were associated with a decreased risk of malignant neoplasms of the prostate (OR: 0.94, 95% CI: 0.90–0.99, P = 0.017*; OR: 0.86, 95% CI: 0.80–0.93, *p*-value = 0.0002 ***, respectively, vs. A/A). Carriage of TPCN2 rs35264875: TA/TT was associated with an increased risk of malignant neoplasms of the prostate (OR: 1.073, 95% CI: 1.02–1.13, P = 0.005 **; OR: 1.18, 95% CI: 1.03–1.35, *p*-value = 0.016 *A/A). Carriage of TPCN2 rs35264875:TT or rs72932540:GA was associated with a higher risk of developing malignant neoplasm of the rectum (OR: 1.37, 95% CI: 1.02–1.84, P = 0.034 *; OR: 1.15, 95% CI: 1.004–1.32, *p*-value = 0.044*, respectively, vs. A/A). In addition, I trained and supervised an MSc student (Abdulla Alashkar), and with him showed that carriage of rs35264875: TA was significantly correlated with a higher risk of developing skin cancer at a general level and in those who had never used UV protection at a local level. Overall, the identified novel links between TPC2 polymorphisms and cancer in humans is limited because this study was conducted retrospectively in the UK population. In the future, I propose to conduct a multi-centre observational clinical study to investigate the utilization of two single-nucleotide polymorphisms (rs3750965 and rs35264875) in the TPCN2 gene as biomarkers for cancer/ cancer subtype detection, progression, and prognosis, as well as a transcriptomic analysis of TPCN2 gene expression. Adult healthy subjects and oncology patients (primary and metastatic, involving breast cancer; colon cancer; malignant neoplasms, stated or presumed to be primary, of lymphoid, haematopoietic, and related tissue; malignant melanoma; malignant neoplasms of mesothelial and soft tissues; malignant neoplasms of the lip, oral cavity and pharynx; malignant neoplasms of the prostate; and malignant neoplasm of the rectum) naive to chemotherapy and

radiation therapy in the European population will be recruited and divided into cases and controls groups. Genomic DNA and RNA will be extracted from the participants, and genotyping of the single nucleotide polymorphisms will be performed using the GeneTitan® Multi-Channel Instrument at Affymetrix Research Services Laboratory. Gene expression analysis will be performed in our lab using qPCR, followed by an in-depth clinical characterization of their 12-months progression-free survival (PFS) after receiving their cancer therapy in a cancer patients' cohort. The primary endpoint will be to detect genetic polymorphisms in TPCN2 in healthy subjects and cancer patients (involving primary and metastatic ones). A secondary endpoint will be to identify changes in TPCN2 expression in healthy subjects vs. cancer patients, and primary cancer patients vs. metastatic patients. A third endpoint will be to study progression free survival (PFS) and overall response rate after receiving the standard therapy.

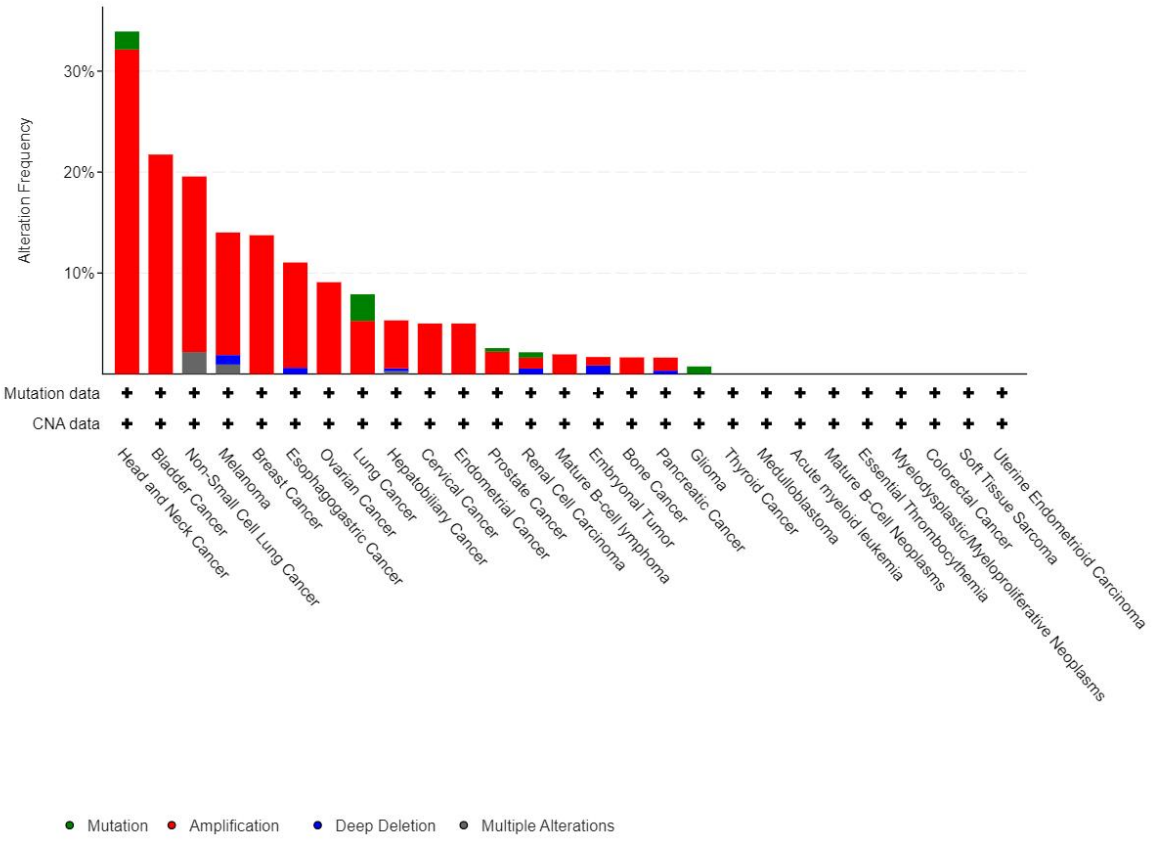


Figure 102. Pan-cancer analysis of TPC2 mutations in TCGA studies of 2565 patients (403).

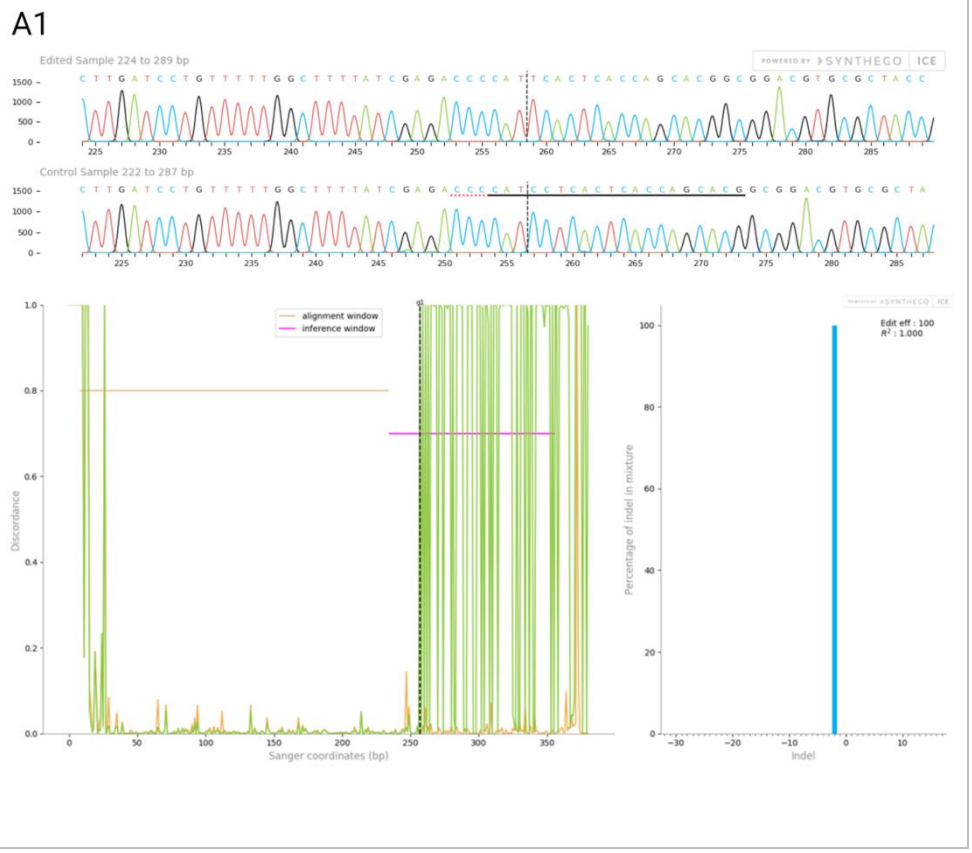
6.2. Conclusion

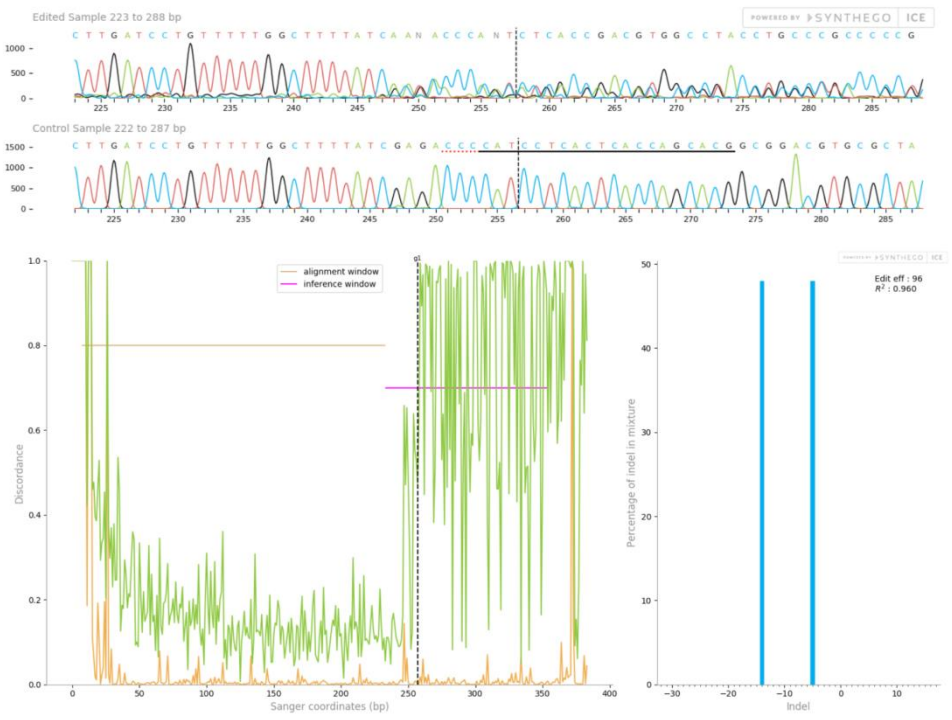
In summary, building on the growing evidence linking endolysosomal Ca^{2+} signalling to cellular development, inflammation, immunity, and cancer, this study reveals the involvement of endolysosomal Ca^{2+} release via NAADP/TPC2/Ca^{2+} signalling in the innate immune system, from monocyte differentiation to cytokine production. The thesis data underscore the unreported biological significance of TPC2 in monocyte differentiation and IL-8/IL10 release. Specifically, it sheds light on the role of TPC2 as a major mediator in monocyte differentiation into macrophages. In addition, the levels of IL-8 were significantly reduced after treating U937 cells with Ned-19 (an NAADP antagonist). KC and MIP2 levels were reduced in murine TPC2 KO splenocytes, compared with WT controls. IL10 levels were elevated in TPC2 KO murine splenocytes, compared to WT splenocytes. Proteins related to the physiological processes involved in the immune system or cancer were significantly altered in TPC1 KO, TPC2 KO, and TPC1/2 double KO splenocytes, compared to WT murine controls. Novel associations were discovered between genetic variants in TPC2 gene/cytokine signalling genes and cancer in the definition of tumour types, susceptibility, prognosis, and cancer outcomes. These findings suggest that TPC2 may act as a major regulator of monocyte differentiation into macrophages as well as of chemokine and cytokine release. These findings raise important questions regarding the clinical utility of targeting TPC2 to eliminate monocytes or to modulate chemokine or cytokine production, which opens up the possibility of innovative and novel strategies to treat patients with inflammatory and immune diseases or cancer. The thesis raises a question regarding the translational relevance of TPC2 as a novel cancer biomarker. Further research in this field would be of great help in expanding our knowledge of TPC2's action at the molecular level in various processes attributed to immunity/cancer. The discovered novel genetic association warrants further investigation in a prospective study.

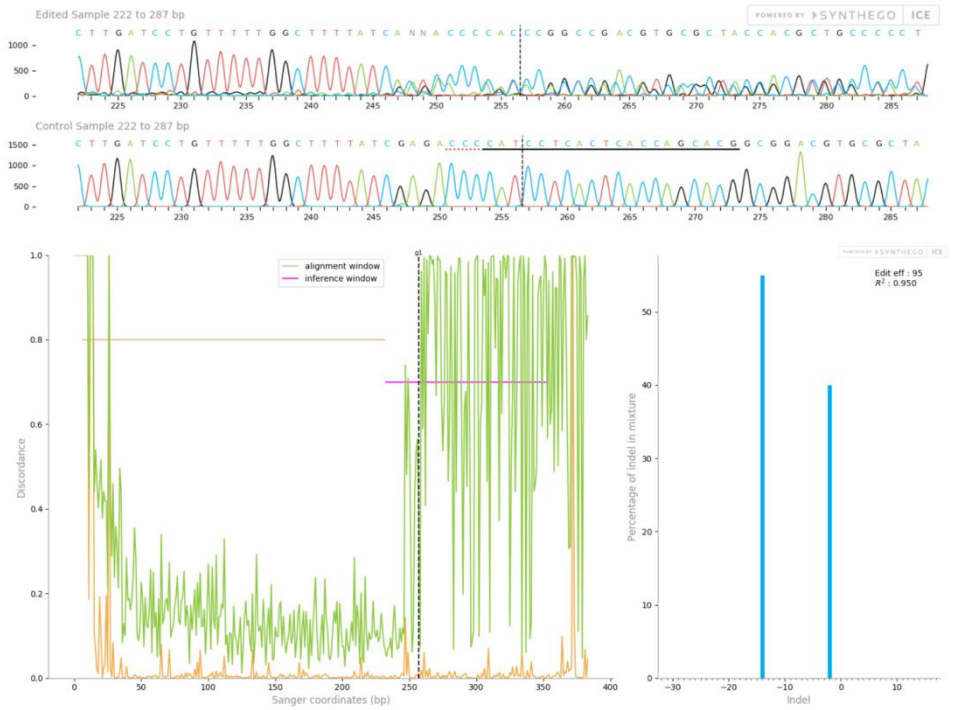
7. Appendixes

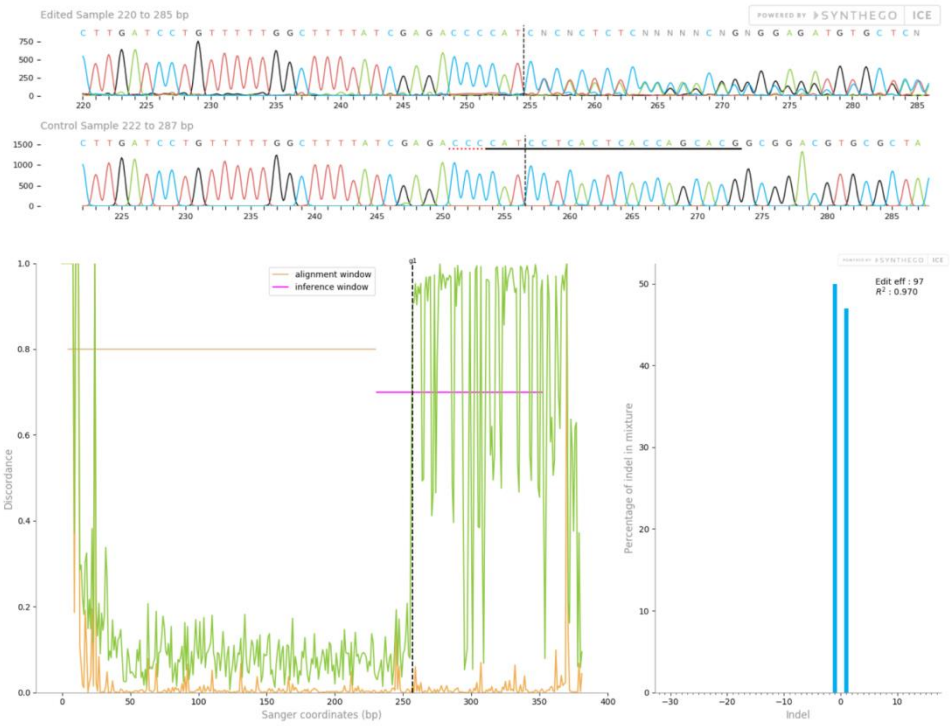
7.1. Appendix- Chapter 2

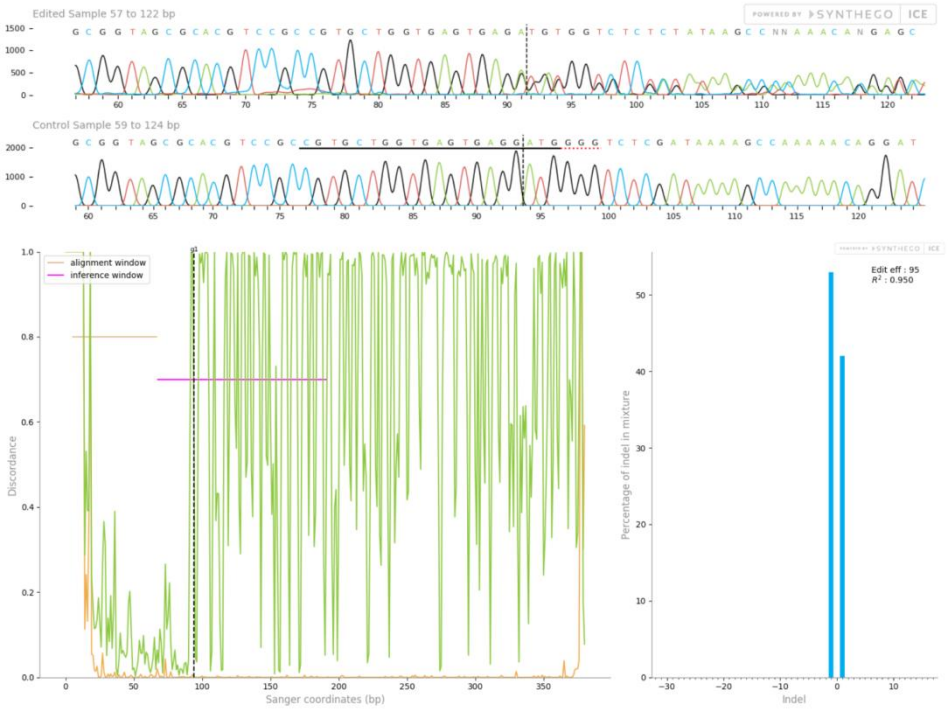
Appendix- Chapter 2

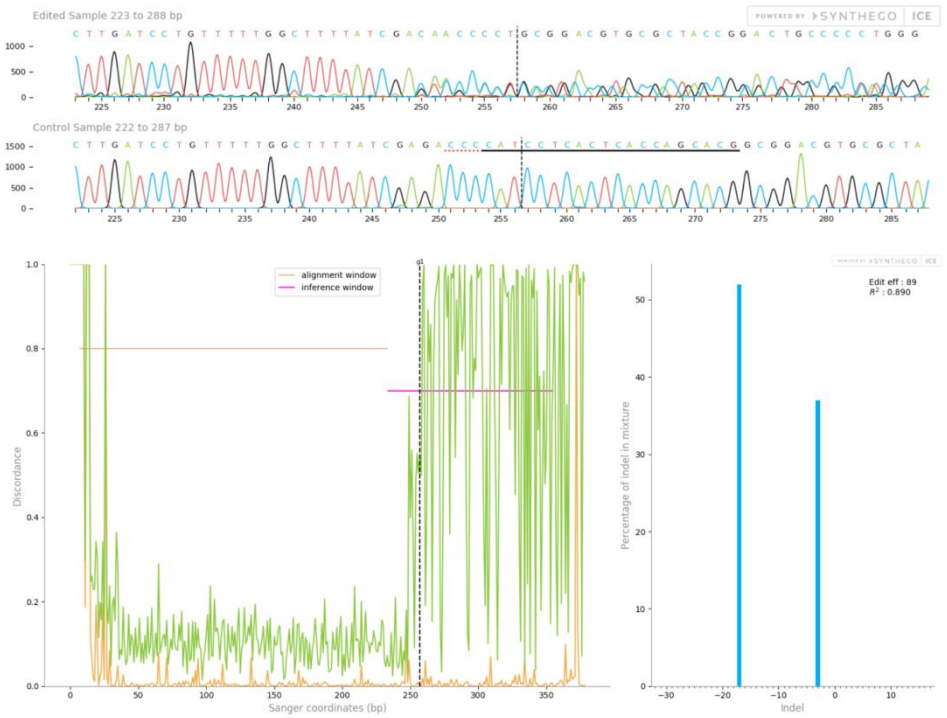
Table S2.1. Genetic characterisation of TPCN2-CRISPR-Cas9 U937 clones predicted by ICE (Interference of CRISPR Edits)(Guide RNA sequence: CGUGCUGGUGAGUGAGGAUG)			
Strain	Indel%	KO (score)	Sanger sequence and Discordance and Indel plots
A1	100	100	

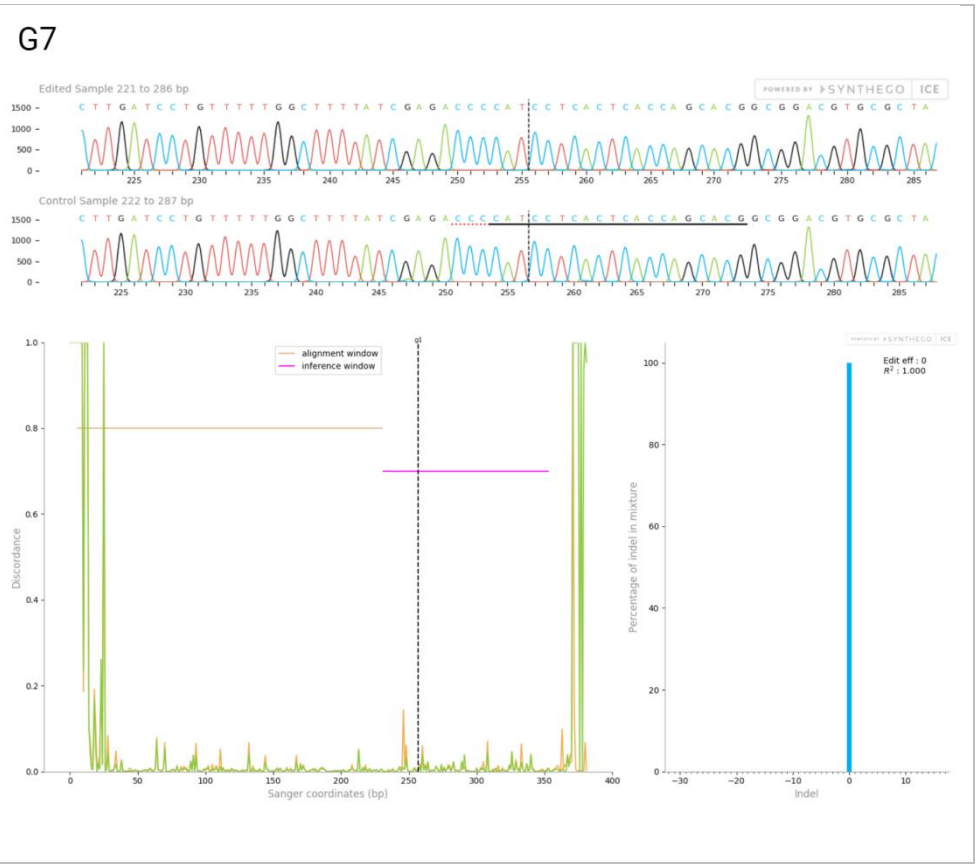
B2	96	96	B2  Edited Sample 223 to 288 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Percentage of indel in mixture Indel Edit eff : 96 R^2 : 0.960

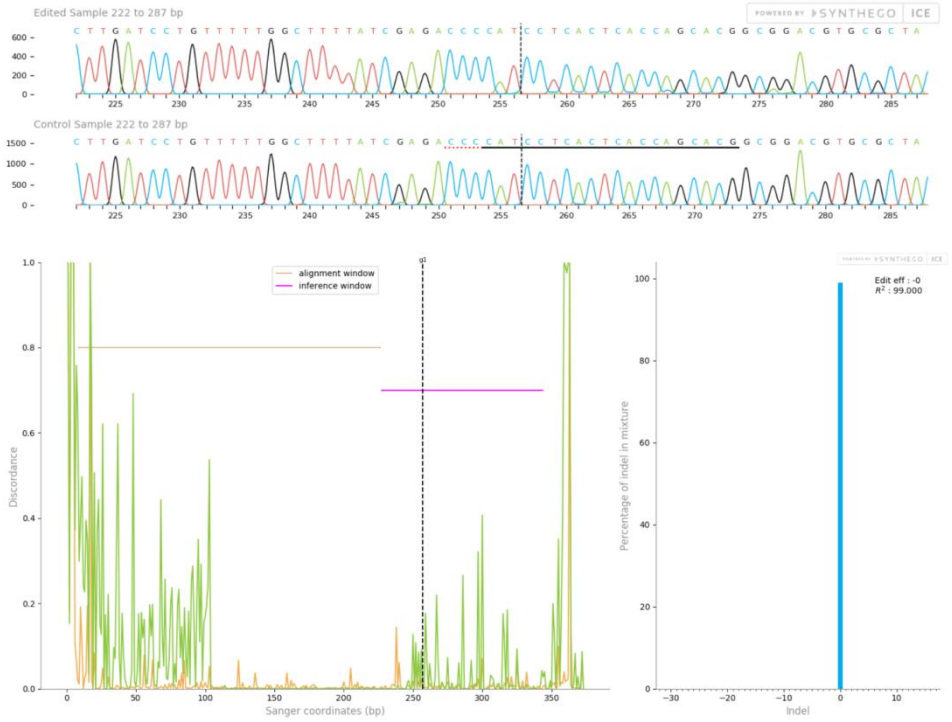
C3	95	95	C3 Edited Sample 222 to 287 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 95 R^2 : 0.950 Percentage of indel in mixture Indel

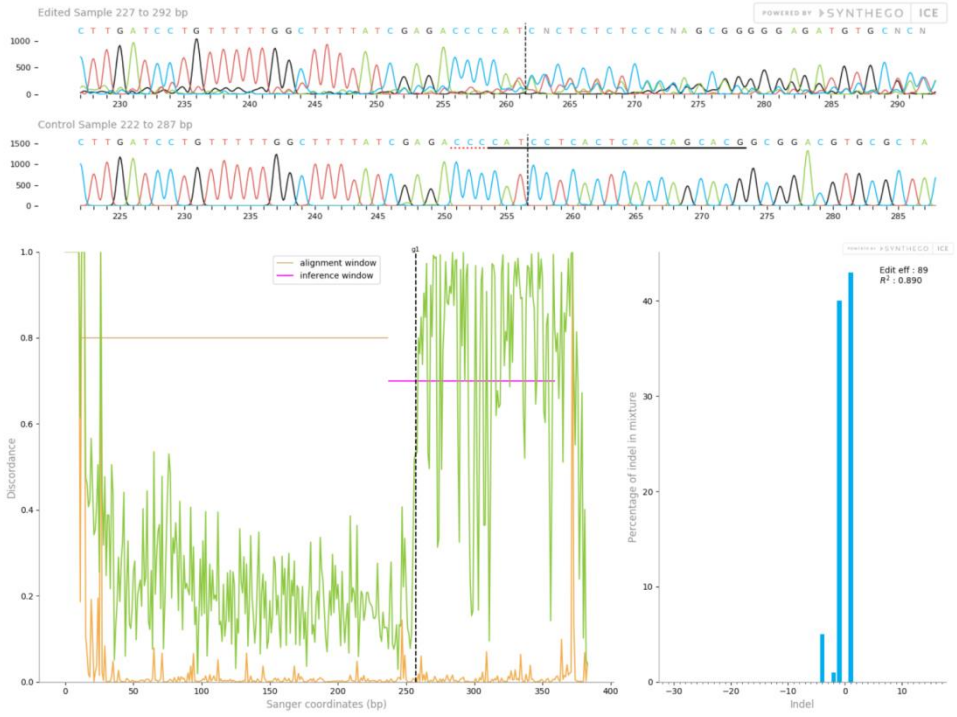
D4	97	97	D4 Edited Sample 220 to 285 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 97 R^2 : 0.970 Percentage of indel in mixture Indel

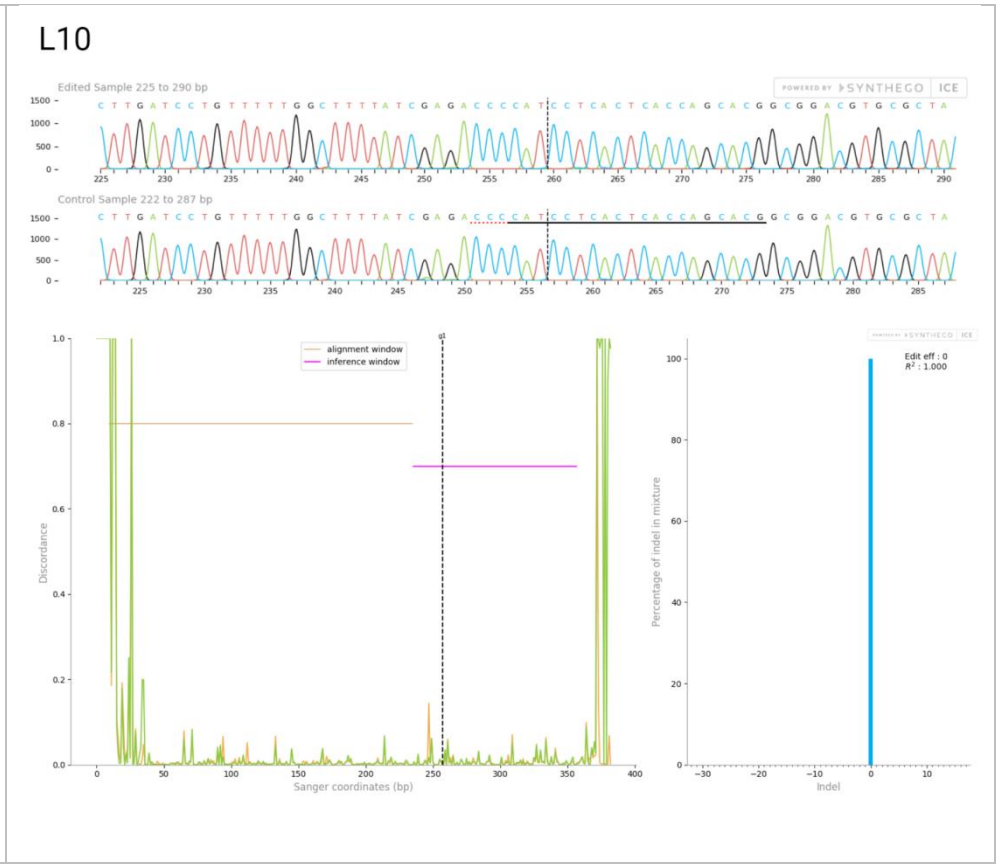
E5	95	95	E5 Edited Sample 57 to 122 bp Control Sample 59 to 124 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 95 R^2 : 0.950 Percentage of indel in mixture Indel

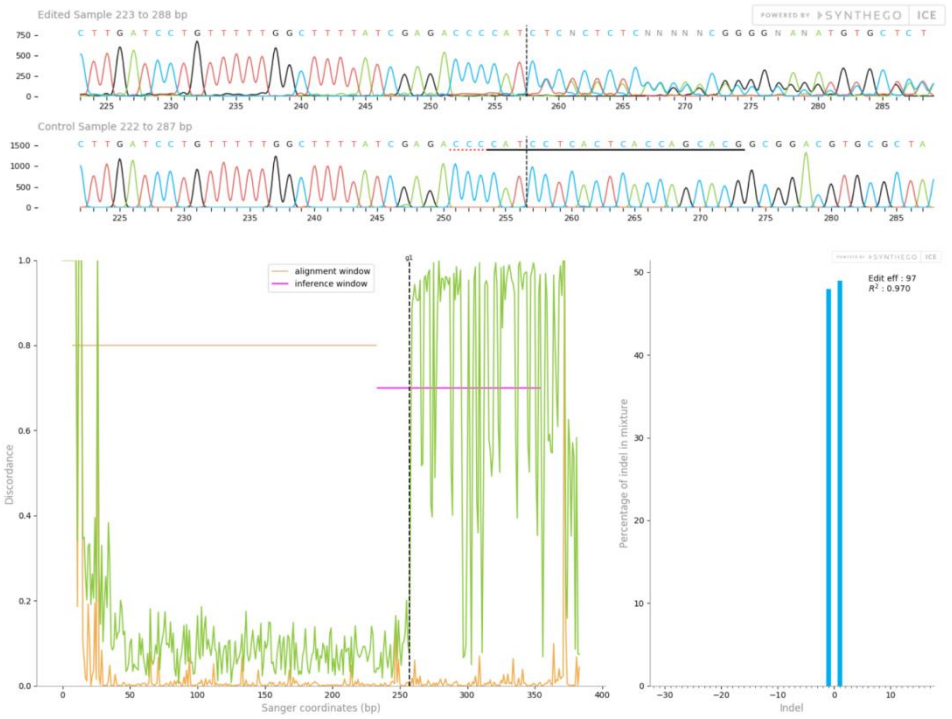
F6	89	52	F6  Edited Sample 223 to 288 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 89 R^2 : 0.890 Percentage of indel in mixture Indel

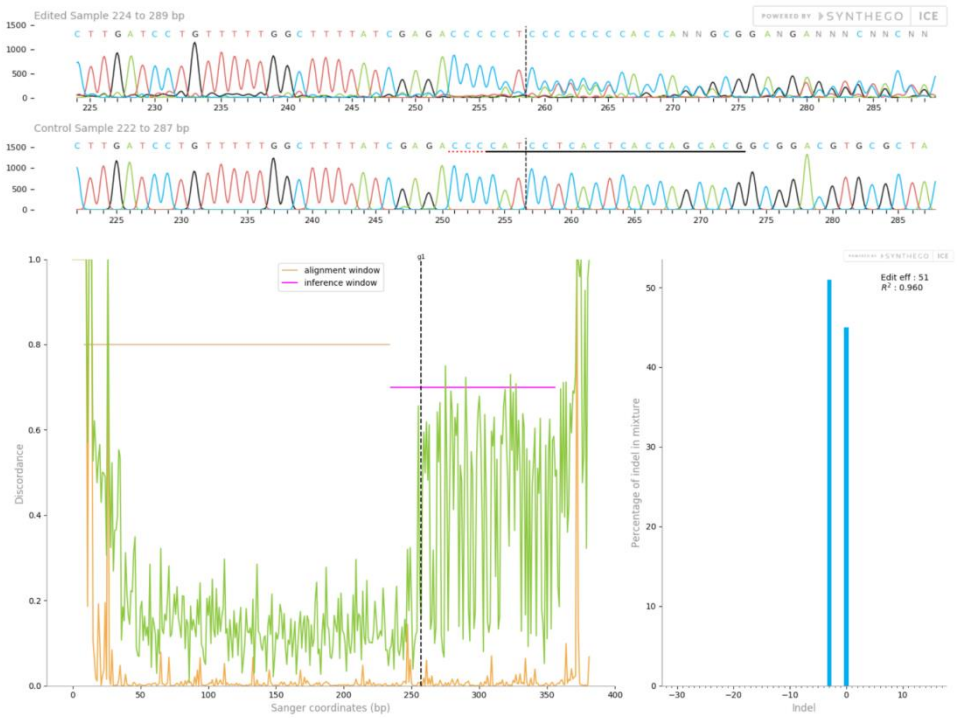
G7	0	0	G7 Edited Sample 221 to 286 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 0 R^2 : 1.000 Percentage of indel in mixture Indel

H8	0	0	H8 Edited Sample 222 to 287 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Percentage of indel in mixture Indel Edit eff : -0 R^2 : 99.000

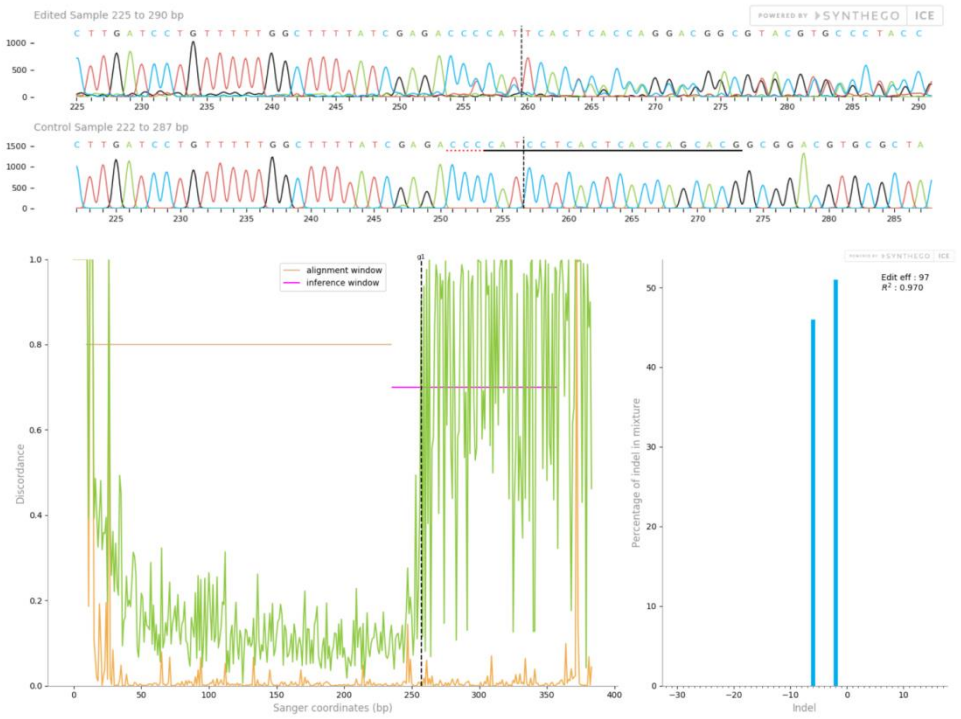
I9	89	89	I9 Edited Sample 227 to 292 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Percentage of indel in mixture Indel Edit eff : 89 R² : 0.890

L10	0	0	L10 Edited Sample 225 to 290 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 0 R^2 : 1.000 Percentage of indel in mixture Indel

O12	97	97	O12 

R13	51	0	R13  Edited Sample 224 to 289 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 51 R^2 : 0.960 Percentage of indel in mixture Indel

S14	31	27	S14 Edited Sample 222 to 287 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 31 Percentage of indel in mixture Indel

Z15	97	51	Z15  Edited Sample 225 to 290 bp Control Sample 222 to 287 bp alignment window inference window Discordance Sanger coordinates (bp) Edit eff : 97 R^2 : 0.970 Percentage of indel in mixture Indel

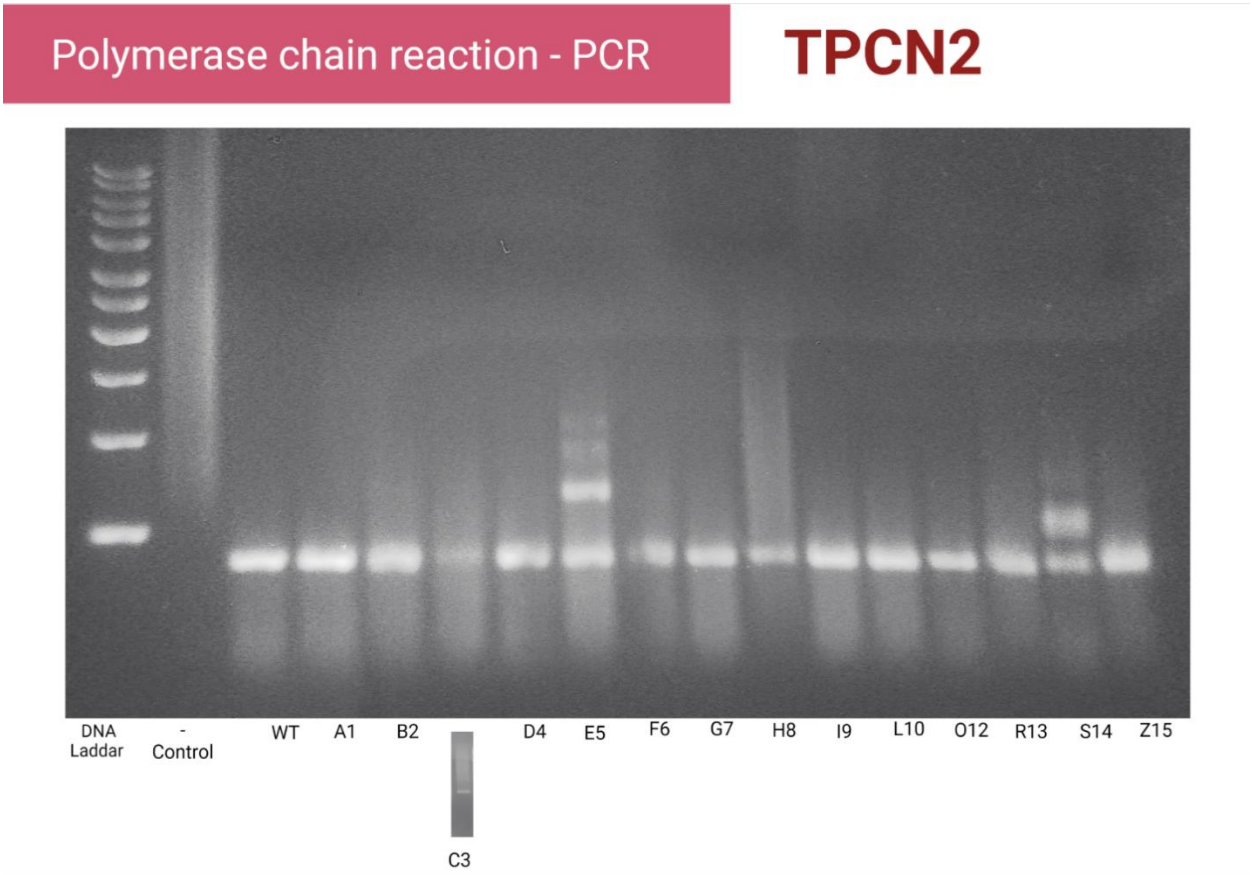


Figure S2.1. PCR and agarose gel analysis of U937 clones using primers spanning exon four sequence.

S2.2. TPCN2 in hematologic malignancies

Human monocytes were isolated from PBMCs from a healthy subject (ATCC® PCS-800-011™), obtained from the American Type Culture Collection (Manassas, VA, USA), utilising a Pan Monocyte Human Isolation Kit (130-096-537), (Miltenyi Biotec, CA, USA), as per the manufacturer's recommendations. TPCN2 gene sequences before, during and after differentiation into macrophages were then compared using Sanger sequencing (amplicon size was ~520 bp). In addition, TPCN2 sequences from monocytes isolated from the blood of a healthy subject were also compared with U937 monocytes derived from AML (amplicon size was ~520 bp).

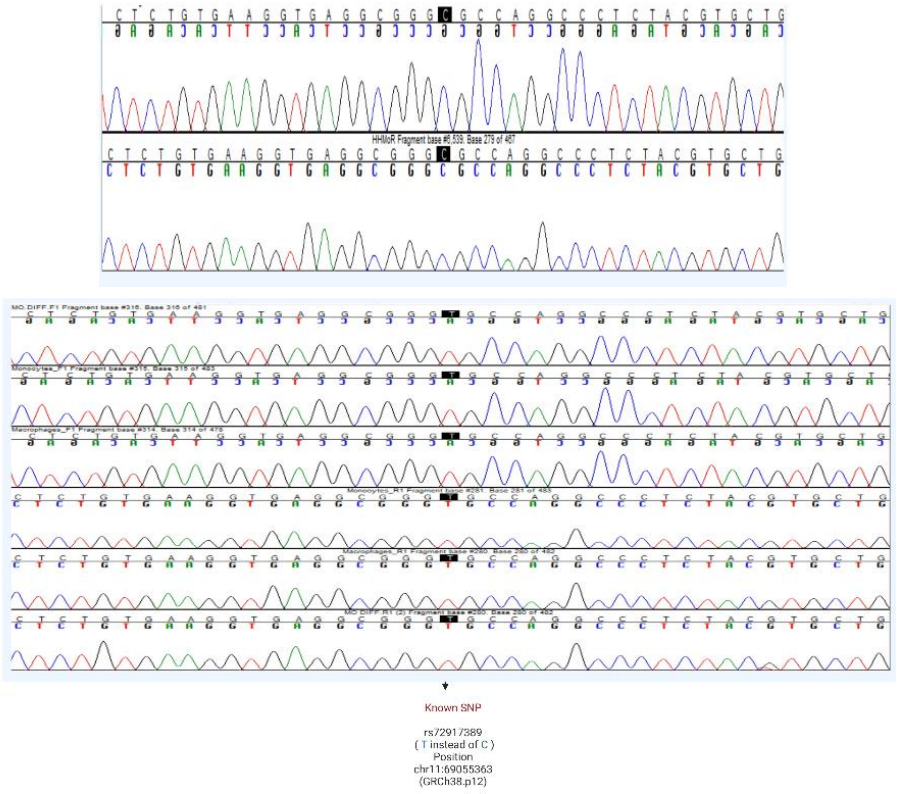


Figure S2.2. Sanger sequences of U937 monocytes prior, during and after differentiation into macrophages. SNP (rs72917389) was identified in the TPCN2 gene of U937 cell line.

7.2.Appendix- Chapter 3

Appendix- Chapter 3

S3.1.PCR of TPCN1 and TPCN2 genomic DNA

As Figure S3.1. indicates, it is apparent that TPCN1 and TPCN2 genes are found in U937 monocytes, U937 cells during differentiation, and U937 macrophages, at the DNA level.

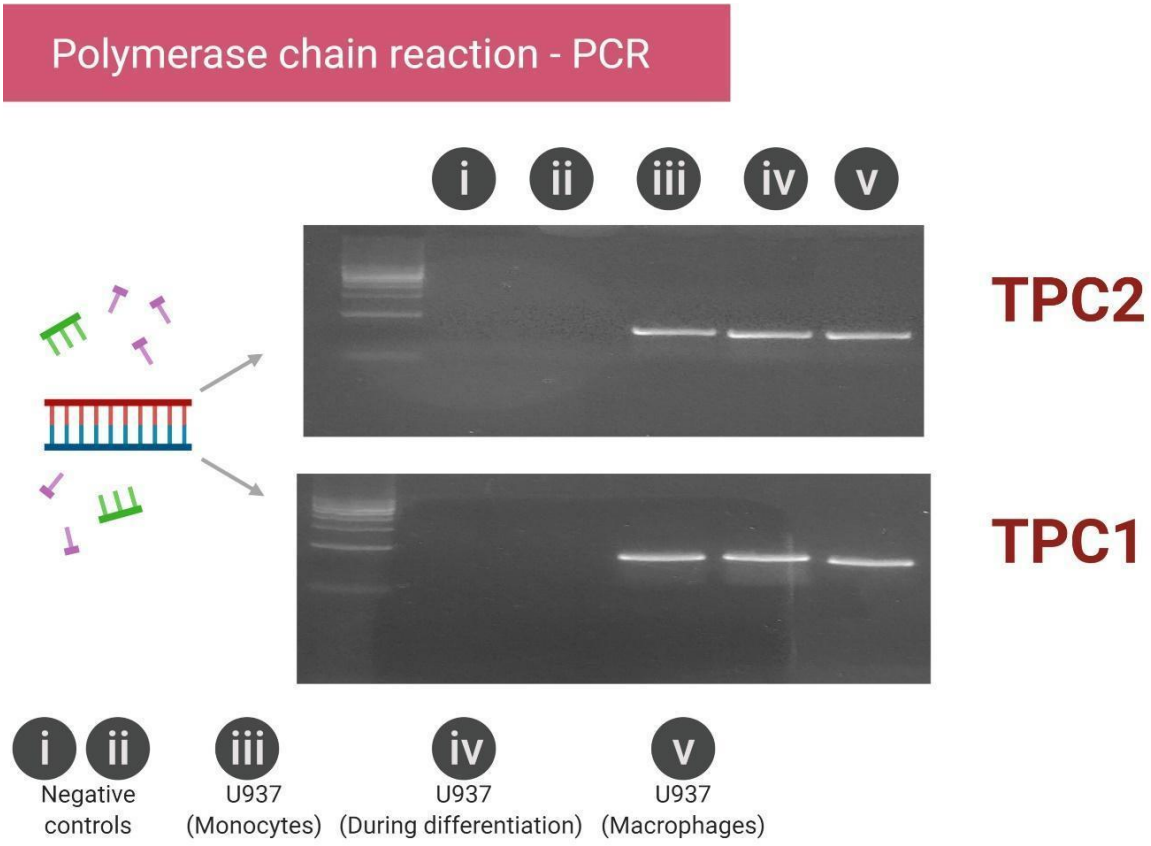


Figure S3.1. DNA of U937 monocytes, U937 during differentiation and non-polarized U937 macrophages were amplified by PCR. The existence of TPCN1 and TPCN2 genomic DNA was examined via agarose gel electrophoresis.

S3.2.Cell viability of U937 WT and TPC2 KD cells treated with LPS/Ned-19

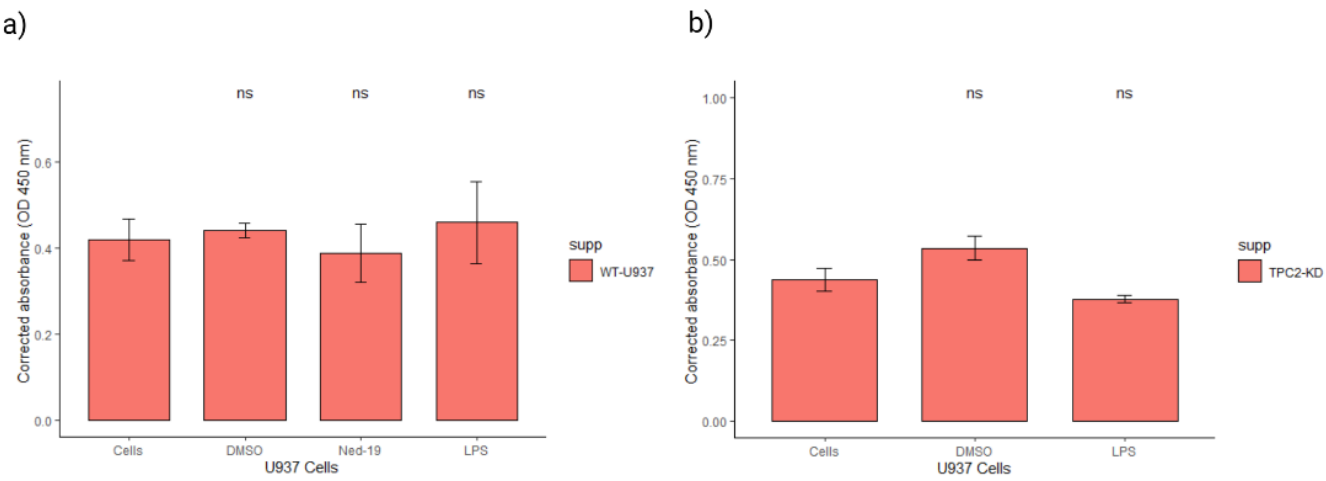


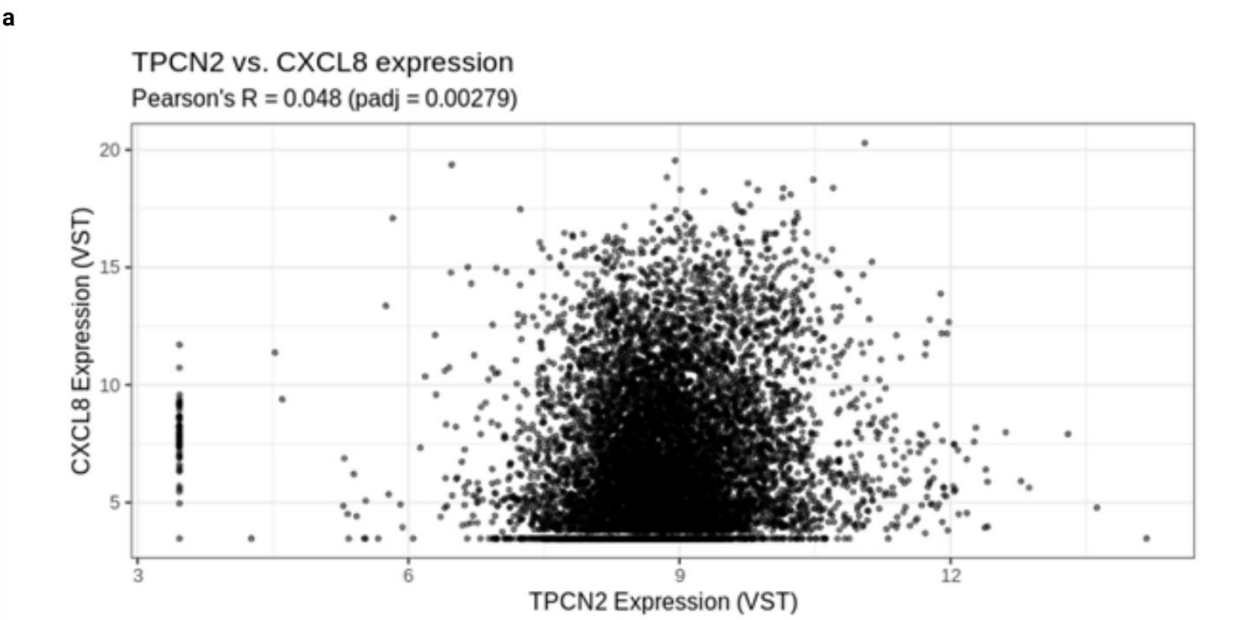
Figure S3. 2.Cell viability of (a) U937 WT and (b) TPC2 KD cells treated with LPS/Ned-19 examined by XTT assay ((mean ± SEM), two sample -t-test). Ned-19-WT (*p*-value= 0.7337), LPS-WT (*p*-value= 0.7274), and LPS-KD (*p*-value= 0.2356).

S3.3. The association analyses were performed using Correlation AnalyzeR to predict the co-expression correlations between TPCN2 and IL-8, and IL10 and CD38 in cancer

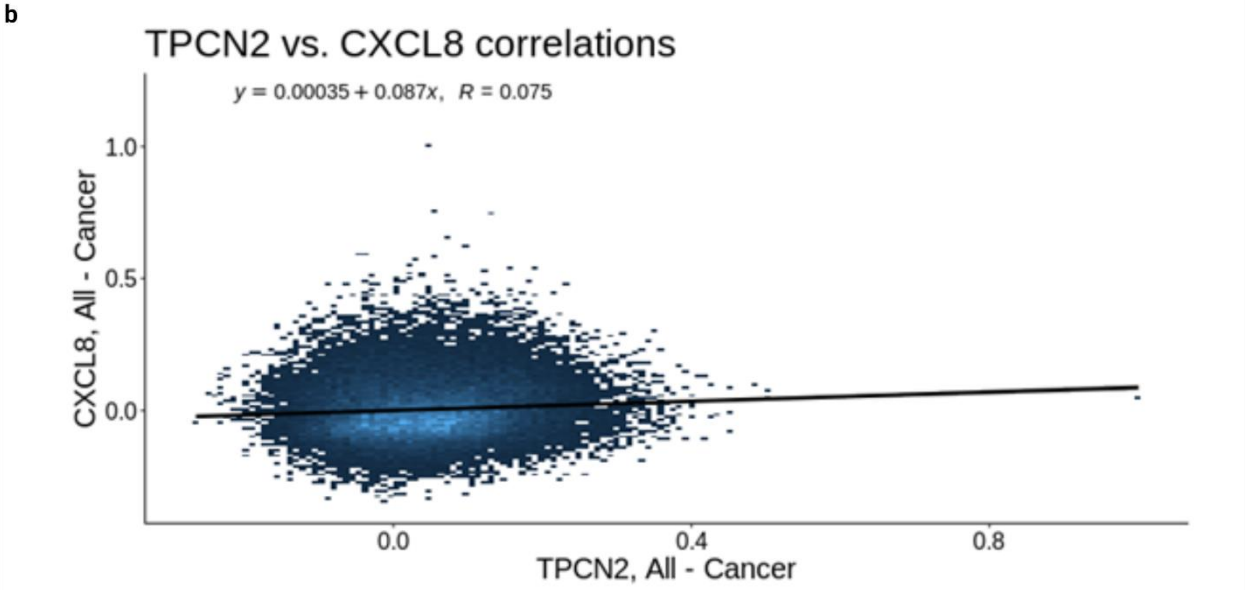
Figures S3.3.1- S3.3.3 show significant positive weak correlations between TPCN2 and IL-8, IL10 and CD38 in cancer at a global level.

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a



b



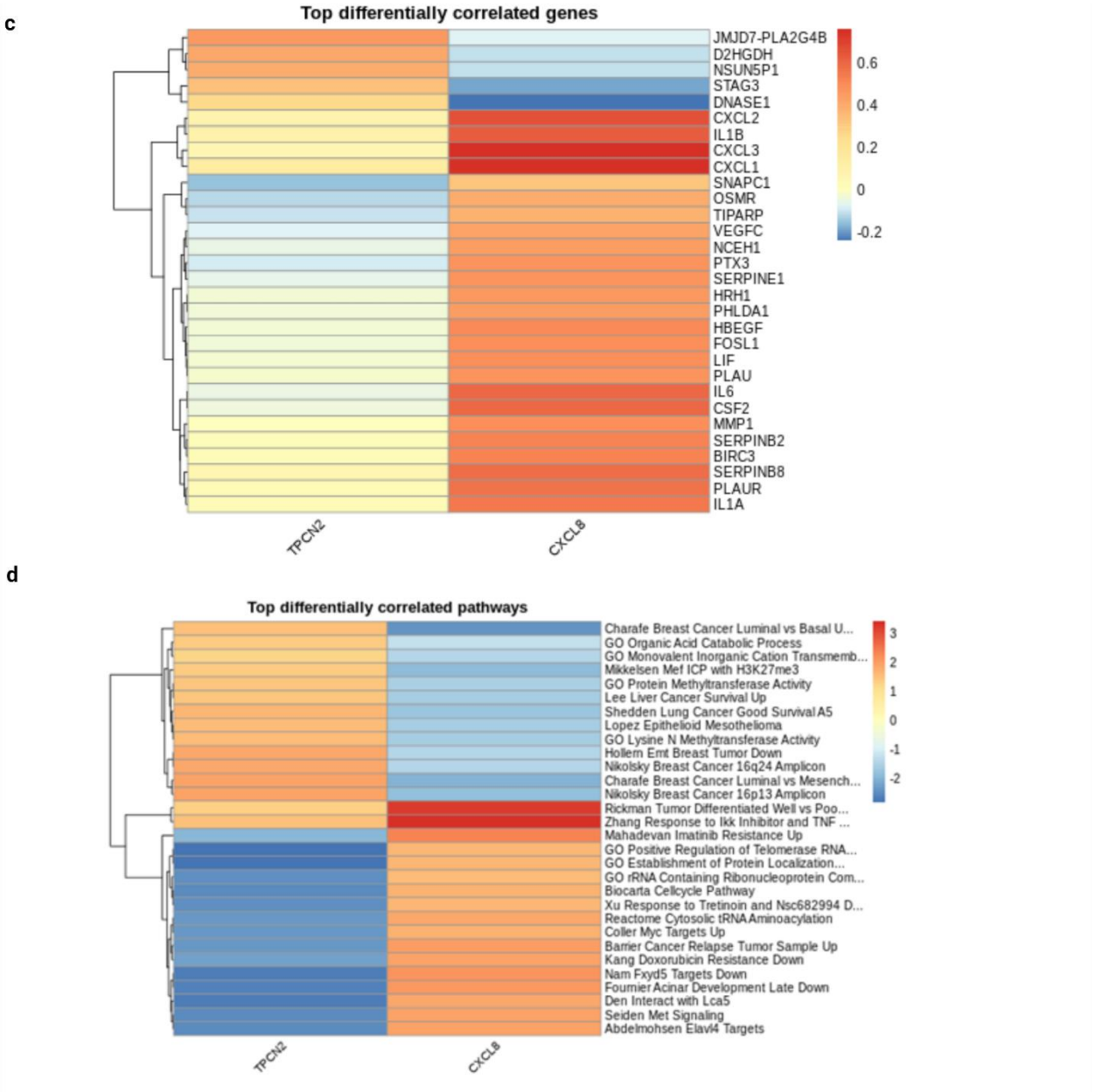
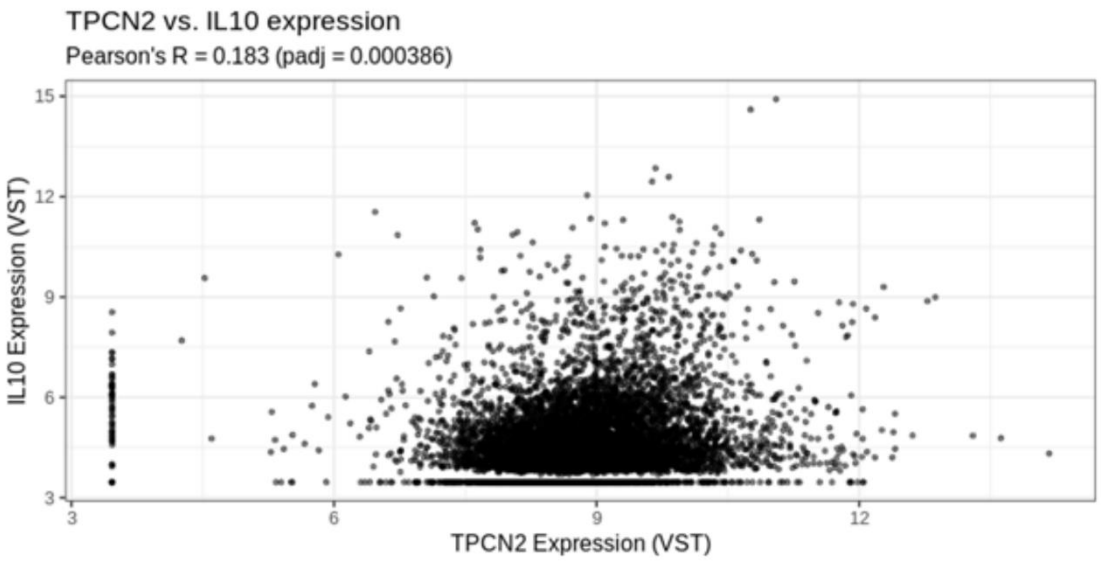
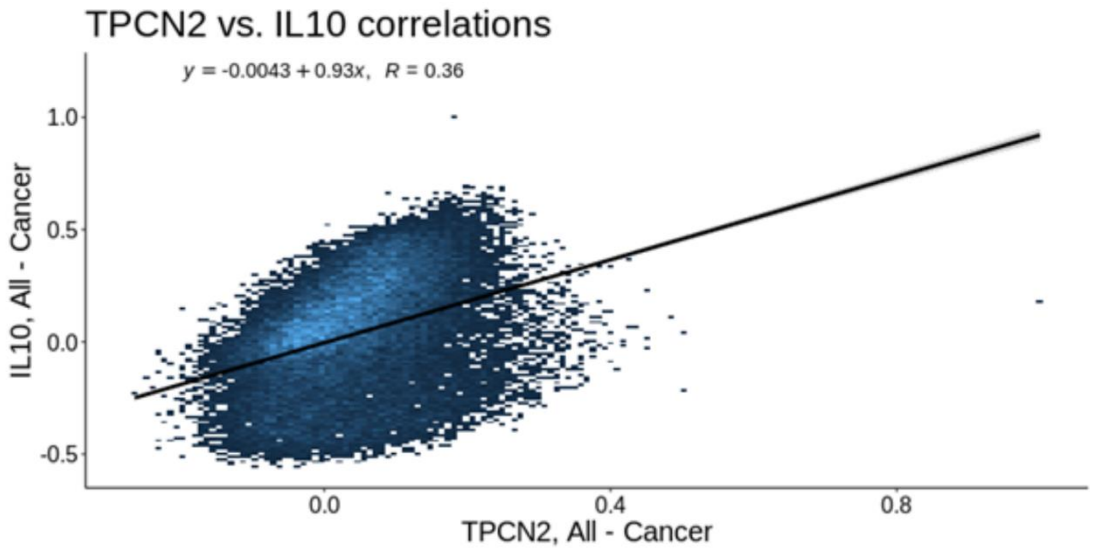


Figure S3.3.1. Correlation between TPCN2 and IL-8 in all cancer tissues (379). a, b: Scatter plots; c, d: Heatmaps.

a


TPCN2 vs. IL10 expression
Pearson's R = 0.183 (padj = 0.000386)
IL10 Expression (VST)
TPCN2 Expression (VST)
15
12
9
6
3
3
6
9
12

b


TPCN2 vs. IL10 correlations
y = -0.0043 + 0.93x, R = 0.36
IL10, All - Cancer
TPCN2, All - Cancer
1.0
0.5
0.0
-0.5
0.0
0.4
0.8

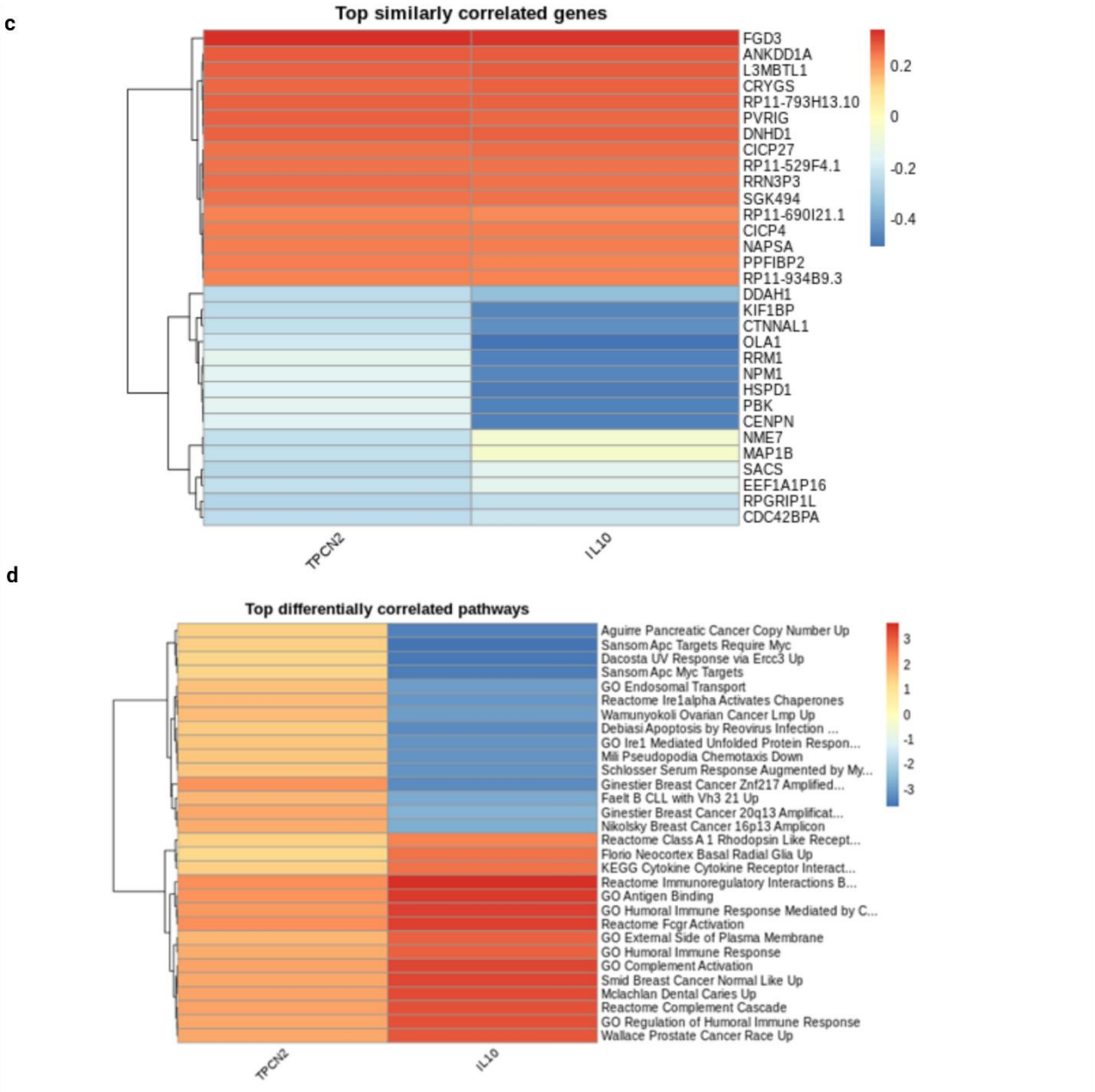
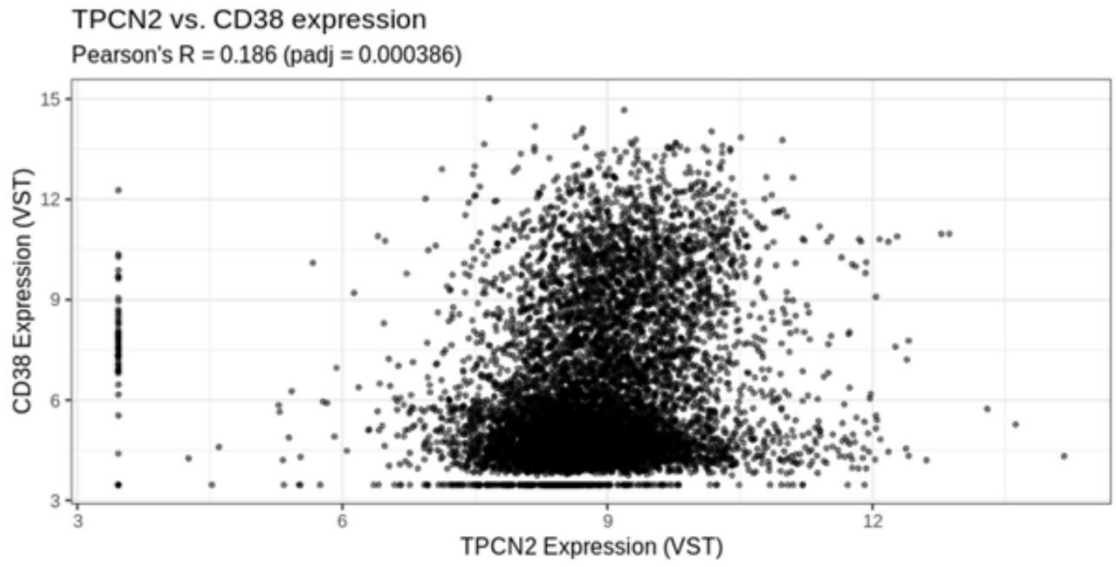
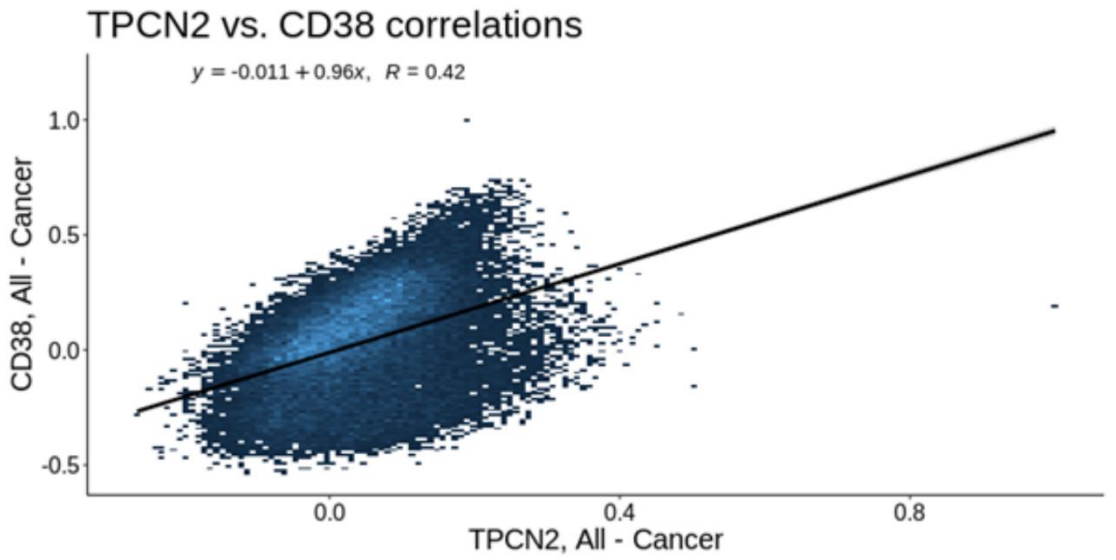


Figure S.3.3.2. Correlation between TPCN2 and IL10 in all cancer tissues (379). a, b: Scatter plots; c, d: Heatmaps.

a


TPCN2 vs. CD38 expression
Pearson's R = 0.186 (padj = 0.000386)
CD38 Expression (VST)
TPCN2 Expression (VST)
15
12
9
6
3
3
6
9
12

b


TPCN2 vs. CD38 correlations
y = -0.011 + 0.96x, R = 0.42
CD38, All - Cancer
TPCN2, All - Cancer
1.0
0.5
0.0
-0.5
0.0
0.4
0.8

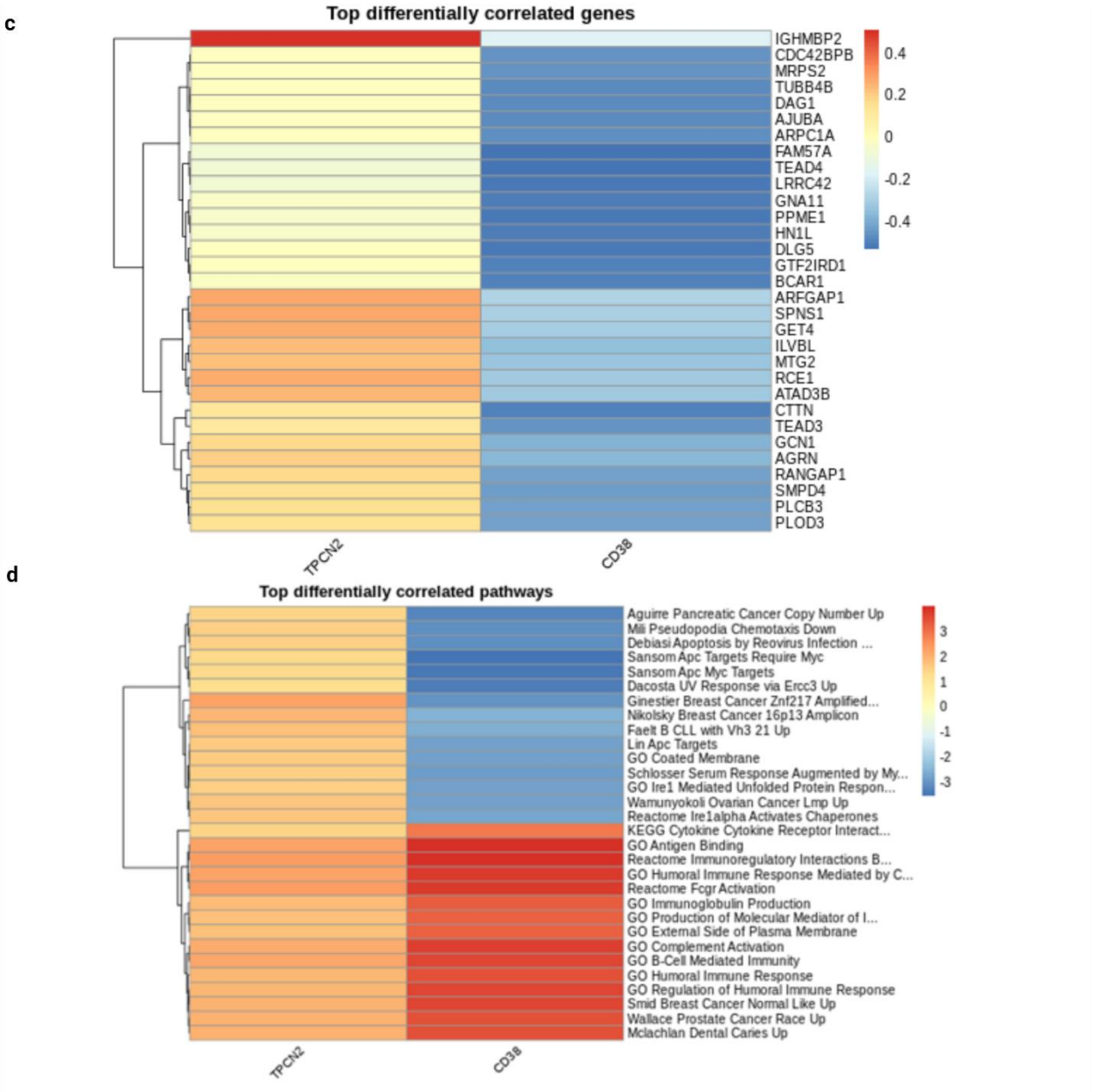
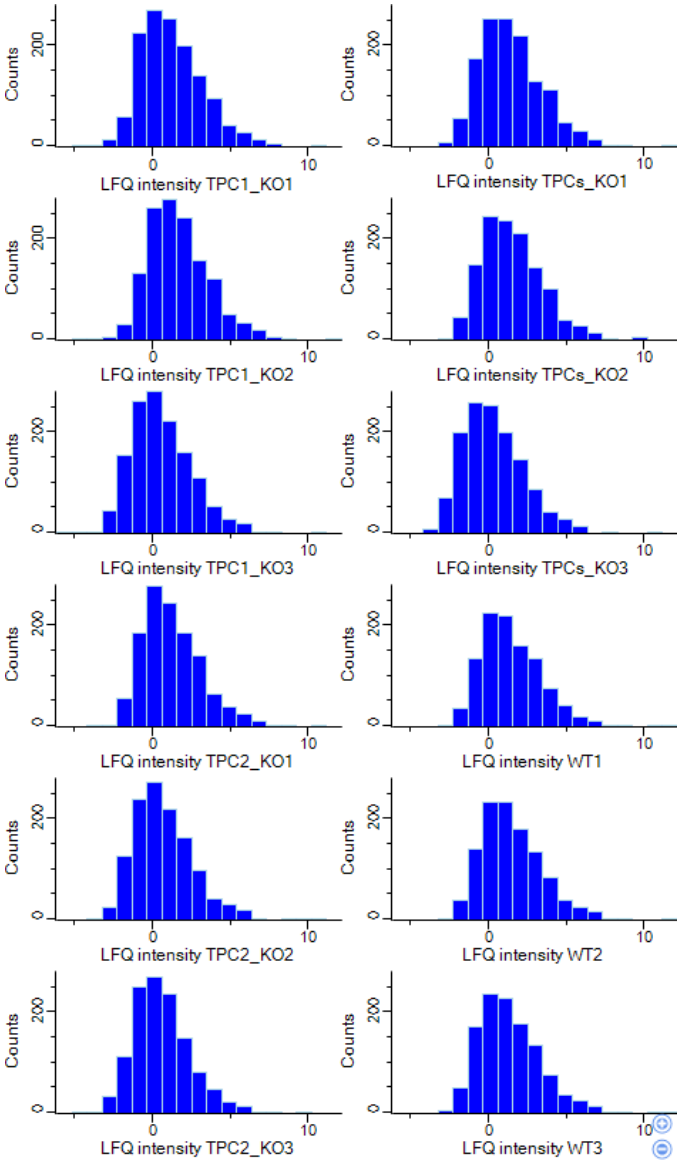


Figure S3.3.3. Correlation between TPCN2 and CD38 in all cancer tissues. a, b: Scatter plots; c, d: Heatmaps.

7.3.Appendix- Chapter 4

Appendix- Chapter 4

S4.1. The protein intensity distributions in TPC1 KO, TPC2 KO, and TPC1/TPC2 DKO splenic cells.



Figures S4.1. The protein intensity distributions in TPC1 KO, TPC2 KO, and TPC1/TPC2 DKO splenic cells.

Table S4.1. Changes in the expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC1.		
FDR-adjusted *p*-value >0.05		
Protein name	**Gene name**	**x-fold change**
Upregulated		
A-kinase anchor protein 12	Akap12	1.376307
Annexin	Anxa1	1.44911
Cathelin-related antimicrobial peptide	Camp	1.977494
Cytoplasmic dynein 1 heavy chain 1	Dync1h1	1.063599
Histone deacetylase	Hdac1	1.105787
Matrin-3	Matr3	1.223165
Myeloperoxidase	Mpo	1.357203
Non-specific protein-tyrosine kinase	Lyn	1.001497
26S protease regulatory subunit 6B	Psmc4	1.149707
RNA binding motif protein, X-linked-like-1	Rbmxl1	1.231012
Serine/threonine-protein kinase 10	Stk10	1.04833
Signal transducer and activator of transcription 3	Stat3	1.17863
Spectrin beta chain, non-erythrocytic 1	Sptbn1	1.07786
Thy-1 membrane glycoprotein	Thy1	1.055706
Ubiquitin-associated protein 2-like	Ubap2l	1.045672
Downregulated		
FAS-associated factor 2	Faf2	-1.11465
Interferon-induced guanylate-binding protein 2	Gbp2	-1.21416

Table S4.2. Changes in the expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC2.		
FDR-adjusted *p*-value >0.05		
Protein name	**Gene name**	**x-fold change**
Upregulated		
Signal recognition particle subunit SRP68	Srp68	2.19
Downregulated		
Adiponectin	ADIPOQ	-1.01
ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	Cd38	-1.17
B-cell antigen receptor complex-associated protein beta chain	Cd79b	-1.85
B-lymphocyte antigen CD20	Ms4a1	-1.15
Calmodulin-like protein 3	Calml3	-1.02
Catalase	Cat	-1.01
Clusterin	CLU	-1
Cytoplasmic dynein 1 intermediate chain 2	Dync1i2	-1.34
Fibrinogen alpha chain	FGA	-1.08
Gamma-interferon-inducible lysosomal thiol reductase	Ifi30	-1.1
High mobility group protein B2	Hmgb2	-1.29

Ig delta chain C region secreted form	Ighd	-1.07
Leucyl-cystinyl aminopeptidase	Lnpep	1.06
MB2q1 protein	H2-DMb2	1.34
Nuclear factor NF-kappa-B p105 subunit	Nfkb1	-1.15
Poly [ADP-ribose] polymerase 1	Parp1	-1.23
14-3-3 protein beta/alpha	Ywhab	-1.07
RNA binding motif protein, X-linked-like-1	Rbmxl1	-1.05
Transcription elongation factor SPT5	Supt5h	-1.24
Transcription factor BTF3 homolog 4	Btf3l4	1.85
UBA_e1_C domain-containing protein	Uba7	-1.01
Ubiquitin-40S ribosomal protein S27a	Rps27a	-1.08
Unconventional myosin-If	Myo1f	1.12
Vasodilator-stimulated phosphoprotein	Vasp	-1.11

Table S4.3. Changes in the expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC1/TPC2.

FDR-adjusted *p*-value >0.05		
Protein name	**Gene name**	**x-fold change**
Upregulated		
Annexin	Anxa1	1.247425
Bone marrow proteoglycan	Prg2	1.183201
Protein S100-A8	S100a8	2.32076
Protein S100-A9	S100a9	1.803398
RNA binding motif protein	Rbmxl1	1.305667
Transferrin receptor protein 1	Tfrc	1.14758
Downregulated		
B-cell scaffold protein with ankyrin repeats	Bank1	-1.33614
CD180 antigen	Cd180	-1.0947
Complement factor H	CFH	-4.32686
Ferritin heavy chain	Fth1	-1.10412
Heat shock protein 105 kDa	Hsph1	-1.01333
Histone H2B type 1-K	Hist1h2bk	-1.65046
Histone H4	Hist2h4	-1.67611
Interferon-induced guanylate-binding protein 2	Gbp2	-1.89219
Non-specific protein-tyrosine kinase	Hck	-1.00868
Ubiquitin-conjugating enzyme E2 D3	Ube2d3	-1.08033

Table S4.4. Changes in the expression of proteins that play vital roles in physiological processes related to the immune system, after knocking out TPC1/TPC2.					
Protein name	**Gene name**	**WT**	**TPC1KO**	**TPC2KO**	**TPC1/TPC2 KO**
Forkhead box protein P1	Foxp1	NA	+	NA	NA
FYN-binding protein	Fyb	NA	+	NA	NA
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Gnb1	NA	+	NA	+
Integrin alpha-M	Itgam	NA	+	NA	+
Matrix metalloproteinase-9	Mmp9	NA	+	NA	NA
Nucleolar RNA helicase 2	Ddx21	NA	+	NA	+
Protein PML	Pml	NA	+	NA	+
Ras-related protein Rab-8A	Rab8a	NA	+	+	NA
Serine protease inhibitor A3M	Serpina3m	NA	NA	+	+
T-cell surface glycoprotein CD5	Cd5	NA	+	NA	NA
Transcription activator BRG1	Smarca4	NA	+	NA	+
NA; Not applicable					

Table S4.5. Changes in the expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC1.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value >0.05		
Upregulated		
Annexin	Anxa1	1.44911
Cathelin-related antimicrobial peptide	Camp	1.977494
Histone deacetylase 1	Hdac1	1.105787
Pre-mRNA-processing factor 19	Prpf19	1.517163
Serine/threonine-protein kinase 10	Stk10	1.04833
Signal transducer and activator of transcription 3	Stat3	1.17863

Table S4.6. Changes in the expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC2.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value >0.05		
Upregulated		
Nuclear autoantigenic sperm protein	Nasp	1.796547
Peptidyl-tRNA hydrolase 2, mitochondrial	Ptrh2	2.064964
Ras suppressor protein 1	Rsu1	1.197671
Unconventional myosin-If	Myo1f	1.11625
Downregulated		
ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	Cd38	-1.16885
Cartilage oligomeric matrix protein	Comp	-1.1142
Catalase	Cat	-1.00666
Cellular nucleic acid-binding protein	Cnbp	-1.25746
Craniofacial development protein 1	Cfdp1	-1.07593
Emerin	Emd	-1.31602
Galectin-1	Lgals1	-1.2506
Gamma-interferon-inducible lysosomal thiol reductase	Ifi30	-1.09881
High mobility group protein B2	Hmgb2	-1.29436
Ig delta chain C region secreted form	Ighd	-1.07091
Moesin	Msn	-0.85984
Nuclear factor NF-kappa-B p105 subunit	Nfkb1	-1.14866
Platelet-activating factor acetylhydrolase IB subunit alpha	Pafah1b1	-1.06252
Prostaglandin E synthase 3	Ptges3	-1.27648

Table S4.7. Changes in the expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC1/TPC2.		
Protein name	**Gene name**	**x-fold change**
FDR-adjusted *p*-value >0.05		
Upregulated		
Annexin	Anxa1	1.247425
Pre-mRNA-processing factor 19	Prpf19	1.701955
Protein S100-A8	S100a8	2.32076
Protein S100-A9	S100a9	1.803398
D**ownregulated**		
CD180 antigen	Cd180	-1.0947
Cytoplasmic FMR1-interacting protein 2	Cyfip2	-1.31435
Heat shock protein 105 kDa	Hsph1	-1.01333
Non-specific protein-tyrosine kinase	Hck	-1.00868
Ubiquitin-conjugating enzyme E2 D3	Ube2d3	-1.08033

Table S4.8. Changes in the expression of proteins that play vital roles in physiological processes related to cancer, after knocking out TPC1/TPC2.					
Protein name	**Gene name**	**WT**	**TPC1KO**	**TPC2KO**	**TPC1/TPC2 KO**
Arachidonate 15-lipoxygenase	Alox15	NA	+	+	+
Calcium-activated chloride channel regulator 3A-1	Clca3a1	NA	NA	NA	+
Complement C3 beta chain	C3	NA	+	+	+
COP9 signalosome complex subunit 8	Cops8	NA	+	+	+
DNA topoisomerase 2-beta	Top2b	NA	NA	NA	+
Double-strand-break repair protein rad21 homolog	Rad21	NA	+	NA	+
Eosinophil peroxidase;Eosinophil peroxidase light chain;Eosinophil peroxidase heavy chain	Epx	NA	+	+	+
Forkhead box protein P1	Foxp1	NA	+	NA	NA
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Gnb1	NA	+	NA	+
Integrin alpha-M	Itgam	NA	+	NA	+
Matrix metalloproteinase-9	Mmp9	NA	+	NA	NA
Paxillin	Pxn	NA	+	NA	NA
Proliferation marker protein Ki-67	Mki67	NA	NA	NA	+
Protein PML	Pml	NA	+	NA	+
Spectrin alpha chain, erythrocytic 1	Spta1	+	NA	NA	NA
Transcription activator BRG1	Smarca4	NA	+	NA	+
UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	Ogt	NA	+	NA	NA
NA; Not applicable					

Table S4.9. Changes in the expression of proteins that play vital roles in physiological processes related to the cardiovascular system, after knocking out TPC1/TPC2.

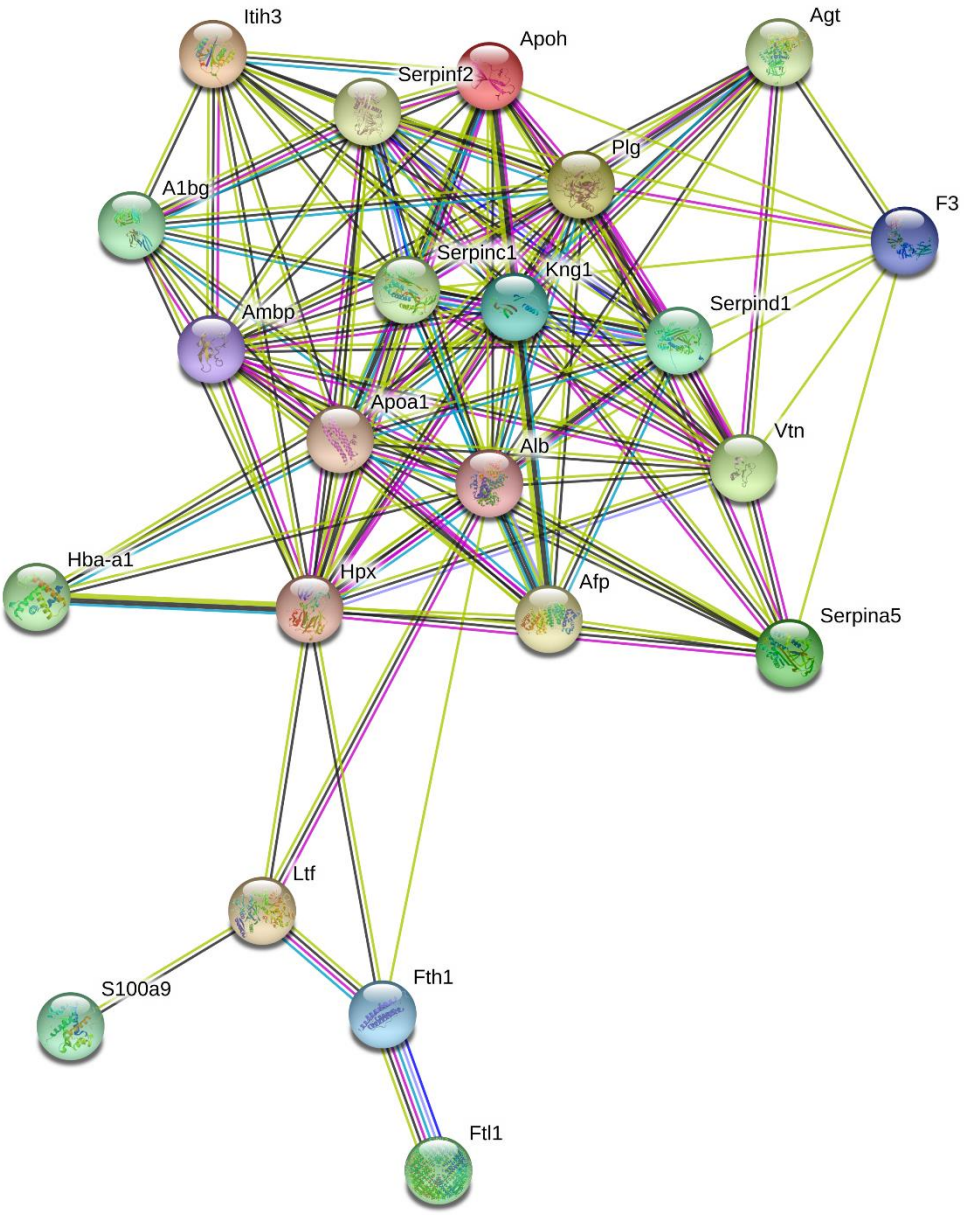
Protein name	Gene name	X-fold change
TPC1 KO (FDR-adjusted *p*-value <0.05, T-test)		
Upregulated		
Lactotransferrin	Ltf	3.507059
Protein S100-A9	S100a9	2.050961
Downregulated		
Alpha-1-antiproteinase	SERPINA1	-3.34652
Alpha-1B-glycoprotein	A1BG	-3.24449
Alpha-2-antiplasmin	SERPINF2	-3.69901
Alpha-fetoprotein	AFP	-2.22376
Angiotensinogen	AGT	-3.52789
Antithrombin-III	SERPINC1	-3.90771
Apolipoprotein A-I	Apoa1	-3.61276
Beta-2-glycoprotein 1	Apoh	-2.96063
Factor XIIa inhibitor	N/A	-4.71779
Ferritin	Ftl1	-2.45398
Ferritin heavy chain	Fth1	-1.59587
Hemoglobin subunit alpha	Hba	-3.4204
Hemopexin	HPX	-6.07382
Heparin cofactor 2	Serpind1	-3.54773
Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	-5.15015
Kininogen-1	KNG1	-4.555
Plasma serine protease inhibitor	SERPINA5	-3.93108
Plasminogen	Plg	-4.48095
Protein AMBP	AMBP	-4.11101
Serotransferrin	TF	-3.03367
Serum albumin	Alb	-3.50974
Vitronectin	VTN	-3.32397
TPC1 KO (FDR-adjusted *p*-value >0.05, T-test)		
Upregulated		
Dihydrofolate reductase	Dhf	1.046508
Non-specific protein-tyrosine kinase	Lyn	1.001497
Ubiquitin-associated protein 2-like	Ubap2l	1.045672
Downregulated		
Ankyrin-1	Ank1	-1.19517
Clusterin	CLU	-2.7708
Prothrombin	F2	-5.10339
TPC2 KO (FDR-adjusted *p*-value <0.05, T-test)		
Upregulated		
Hydroxymethylglutaryl-CoA lyase, mitochondrial	Hmgcl	1.013297
Lactotransferrin	Ltf	1.995309

Methylenetetrahydrofolate dehydrogenase	Mthfd1	1.166922
NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	Ndufs8	1.452835
Phosphatidylinositol-binding clathrin assembly protein	Picalm	1.160064
Downregulated		
Acyl-CoA synthetase family member 2, mitochondrial	Acsf2	-0.78404
Acyl-CoA synthetase family member 2, mitochondrial	Acsf2	-0.78404
Adiponectin	ADIPOQ	-1.00684
Alpha-1B-glycoprotein	A1BG	-1.03196
Alpha-fetoprotein	AFP	-1.0137
Angiotensinogen	AGT	-0.89466
Annexin A2	Anxa2	-0.93202
Apolipoprotein E	APOE	-0.98853
Beta-hexosaminidase	Hexb	-0.87093
Chloride intracellular channel protein 1	Clic1	-0.98468
Clusterin	CLU	-1.00101
Complement factor H	CFH	-1.50708
Cytochrome b5 type B	Cyb5b	-0.9298
Dolichol-phosphate mannosyltransferase subunit 1	Dpm1	-0.64225
Dynamin-1-like protein	Dnm1l	1.85882
Ena/VASP-like protein	Evl	-0.97024
5'-3'exonuclease PLD4	Pld4	-0.92569
Factor XIIa inhibitor	N/A	-1.45285
Fatty acid synthase	Fasn	0.835366
Fibrinogen alpha chain	FGA	-1.0772
Filamin-A	Flna	-1.03754
H-2 class II histocompatibility antigen gamma chain	Cd74	-0.93984
Hematopoietic lineage cell-specific protein	Hcls1	-1.31897
Heme oxygenase 2	Hmox2	-1.00493
Heme oxygenase 2	Hmox2	-1.00493
Hemoglobin fetal subunit beta	N/A	-1.36648
Hemoglobin subunit alpha	HBA	-1.01314
Hemoglobin subunit beta	HBB	-1.6853
Hemoglobin subunit beta-1	Hbbt1	-0.7693
Heparin cofactor 2	SERPIND1	-1.09375
Inositol monophosphatase 1	Impa1	-0.94823
Kininogen-1	KNG1	-1.22824
Kininogen-2	KNG2	-1.35683
Membrane-associated progesterone receptor component 2	Pgrmc2	-0.94345
Myosin, light chain 12A, regulatory, non-sarcomeric	Myl12a	-1.22462
Myosin-9	Myh9	-1.20344
Na(+)/H(+) exchange regulatory cofactor NHE-RF	Slc9a3r1	-1.45788
Neutrophil cytosol factor 1	Ncf1	-0.7719
NSFL1 cofactor p47	Nsfl1c	-1.02261

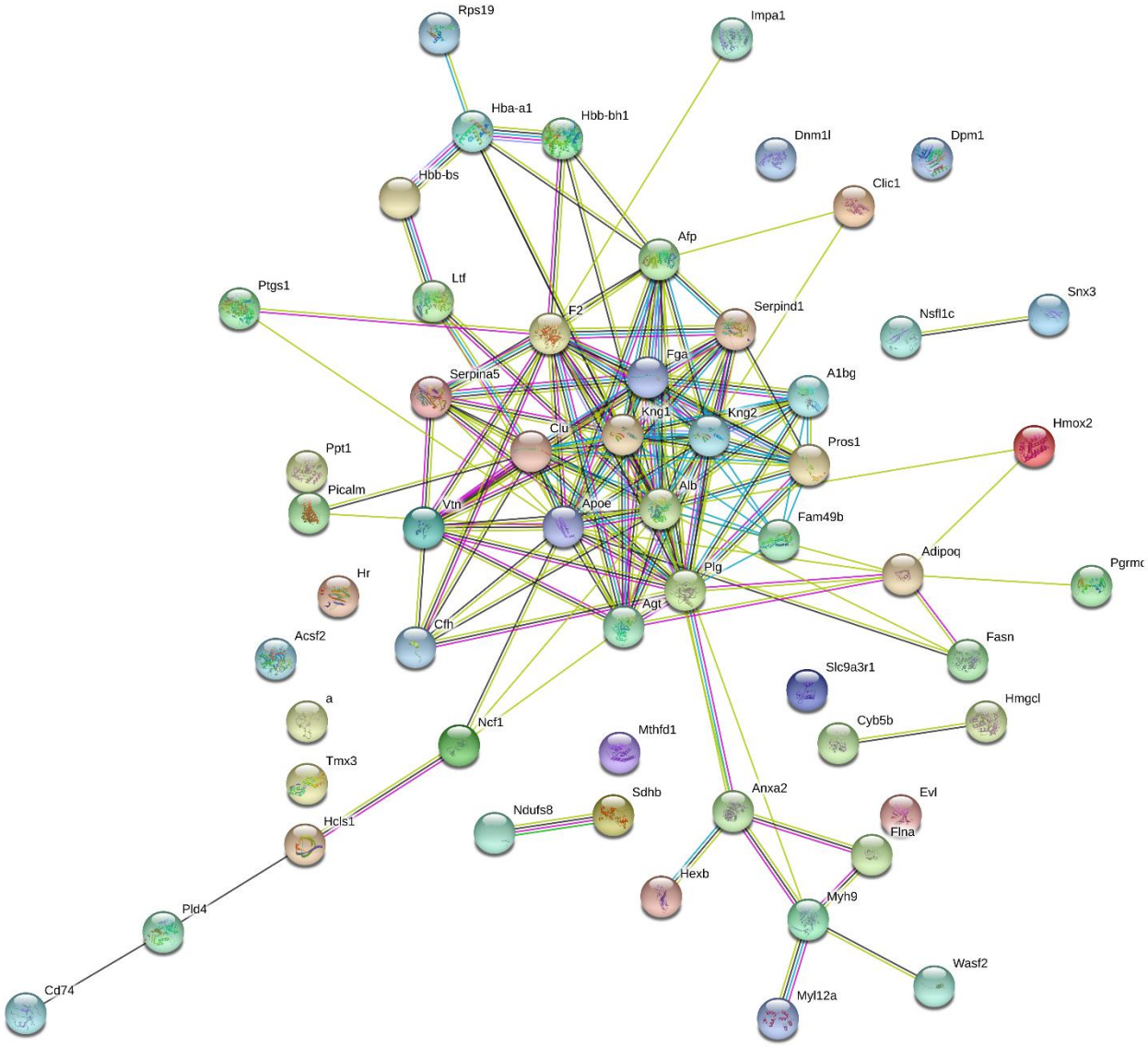
Palmitoyl-protein thioesterase 1	Ppt1	-0.87964
Plasma serine protease inhibitor	SERPINA5	-0.7392
Plasminogen	PLG	-1.24197
Prostaglandin G/H synthase 1	Ptgs1	-0.43115
Prostaglandin G/H synthase 1	Ptgs1	-0.43115
Protein disulfide-isomerase TMX3	Tmx3	-0.95245
Protein FAM49B	Fam49b	-0.73477
Prothrombin	F2	-1.03961
40S ribosomal protein S19	Rps19	-0.90845
Serum albumin	ALB	-1.1229
Sorting nexin-3	Snx3	-0.67939
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	Sdhb	-0.93732
Vitamin K-dependent protein S	PROS1	-1.17795
Vitronectin	VTN	-0.93887
Wiskott-Aldrich syndrome protein family member 2	Wasf2	-1.14241
TPC2 KO (FDR-adjusted *p*-value >0.05, T-test)		
Downregulated		
Apolipoprotein A-I	Apoa1	-1.25385
Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 1	Acap1	-1.55911
Beta-2-glycoprotein 1	APOH	-1.38415
Cartilage oligomeric matrix protein	Comp	-1.1142
Catalase	Cat	-1.00666
Galectin-1	Lgals1	-1.2506
High mobility group protein B2	Hmgb2	-1.29436
Platelet-activating factor acetylhydrolase IB subunit alpha	Pafah1b1	-1.06252
Prostaglandin E synthase 3	Ptges3	-1.27648
Protein disulfide-isomerase A6	Pdia6	-0.34389
Vasodilator-stimulated phosphoprotein	Vasp	-1.10458
TPC1/2 double KO (FDR-adjusted *p*-value <0.05, T-test)		
Upregulated		
Lactotransferrin	Ltf	3.343201
Myeloperoxidase	Mpo	1.804322
Downregulated		
Alpha-1-antiproteinase	SERPINA1	-2.34796
Alpha-1B-glycoprotein	A1BG	-2.30791
Alpha-2-antiplasmin	SERPINF2	-2.49023
Alpha-2-HS-glycoprotein	AHSG	-2.02708
Alpha-fetoprotein	AFP	-2.14733
Angiotensinogen	AGT	-2.2213
Antithrombin-III	SERPINC1	-2.59868
Apolipoprotein A-I	Apoa1	-2.23143

Beta-2-glycoprotein 1	APOH	-2.15698
Clusterin	CLU	-2.31188
Factor XIIa inhibitor	N/A	-4.27496
Ferritin	Ftl1	-2.01025
Hemoglobin fetal subunit beta	N/A	-1.87975
Hemoglobin subunit alpha	HBA	-2.01084
Hemopexin	HPX	-2.81132
Heparin cofactor 2	SERPIND1	-2.6517
Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	-3.22491
Kininogen-1	KNG1	-2.28595
Plasma serine protease inhibitor	SERPINA5	-2.95686
Plasminogen	PLG	-2.65974
Protein AMBP	AMBP	-2.60141
Prothrombin	F2	-3.26152
Serotransferrin	TF	-2.25877
Serum albumin	ALB	-1.76959
Thyroxine-binding globulin	SERPINA7	-3.45345
Vitronectin	VTN	-2.52621
TPC1/2 double KO (FDR-adjusted *p*-value >0.05, T-test)		
Upregulated		
Annexin A1	Anxa1	1.247425
ATP-dependent RNA helicase A	Dhx9	1.345394
CAD protein	Cad	1.131849
Protein S100-A9	S100a9	1.803398
Serine/arginine-rich splicing factor 1	Srsf1	1.436403
Transferrin receptor protein 1	Tfrc	1.14758
Downregulated		
CAAX prenyl protease 1 homolog	Zmpste24	-2.42426
Ferritin heavy chain	Fth1	-1.10412

a)


Itih3
Apoh
Agt
Serpinf2
Plg
A1bg
F3
Serpinc1
Kng1
Ambp
Serpind1
Apoa1
Vtn
Alb
Hba-a1
Hpx
Afp
Serpina5
Ltf
S100a9
Fth1
Ftl1

b)


Rps19
Impa1
Hba-a1
Hbb-bh1
Dnm1l
Dpm1
Hbb-bs
Clic1
Afp
Ltf
Ptgs1
F2
Serpind1
Snx3
Nsfl1c
Fga
Serpina5
A1bg
Kng1
Kng2
Hmox2
Clu
Pros1
Ppt1
Picalm
Alb
Vtn
Apoe
Fam49b
Adipoq
Pgrmc
Plg
Hr
Agt
Cfh
Acsf2
Fasn
Slc9a3r1
a
Hmgcl
Ncf1
Cyb5b
Mthfd1
Tmx3
Sdhb
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Wasf2
Cd74
Myl12a

c)

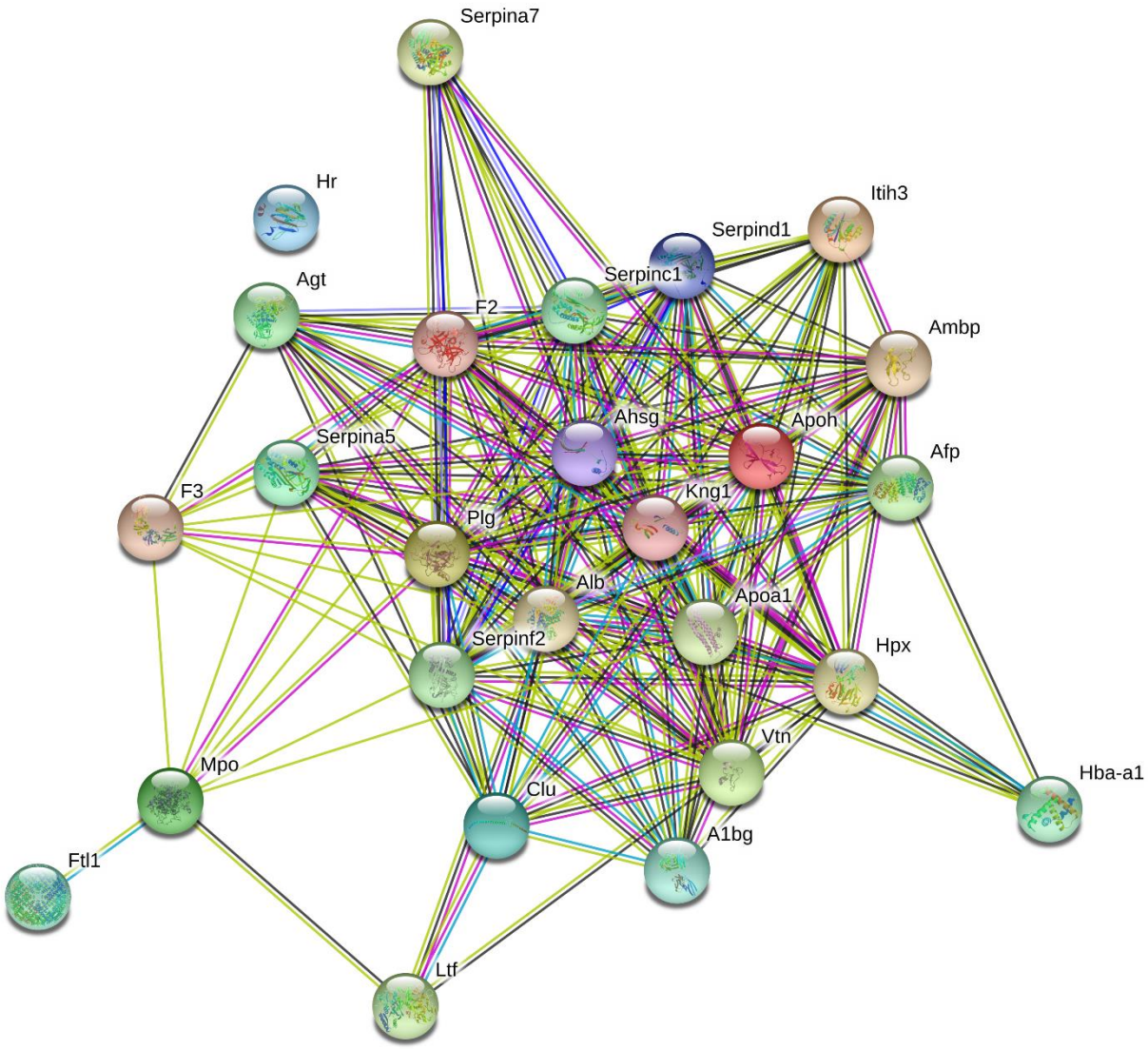


Figure S4.2. Significantly altered gene ontology pathways involved in the cardiovascular system in spleen cells. (a) KO. (b). TPC2 KO. (c). TPC1/TPC2 double KO.

7.4.Appendix- Chapter 5

Appendix- Chapter 5

Supplementary Table 5.1. Allele Frequencies and Hardy Weinberg equilibrium of endolysosomal ion channel polymorphisms							
SNP	Gene	Type	Location (GRCh38)	Alleles	Global MAF	MAF in our population	HWE
							(*p*-value)
rs3750965	TPCN2	SNV	11:69072692	A>C,G	G=0.31	G=0.33	0.65
rs35264875	TPCN2	SNV	11:69078931	A>C,T	T=0.17	T=0.16	0.34
rs34510004	TPCN2	SNV	11:69081448	G>A	A=0.00102	A=0.0013	0.77
rs3829241	TPCN2	SNV	11:69087895	G>A	A=0.359398	A=0.39	0
rs72932540	In close proximity to the TPCN2 gene	SNV	11:69154575	A>G	G=0.0951	G=0.09	0.41
rs25644	P2RX4	SNV	12:121228843	A>G	G=0.121560	G=0.12	0.47
rs28360472	P2RX4	SNV	12:121232473	A>G	G=0.01337	G=0.02	0.095
Ncbi.nlm.nih.gov. 2020. Home - SNP - NCBI							

Supplementary Table 5.2. Hardy Weinberg equilibrium of 71 genetic polymorphisms					
UDI	rs-ID	Gene	Type	Location	HWE
					(*p*-value)
affy4300170	rs9610	IL10RA	SNV	11:118001371	0.084
affy5779511	rs3750965	TPCN2	SNV	11:69072692	0.645
affy5779776	rs35264875	TPCN2	SNV	11:69078931	0.156
affy5779828	rs34510004	TPCN2	SNV	11:69081448	0.525
affy5780031	rs3829241	TPCN2	SNV	11:69087895	0
affy5782262	rs72932540	TPCN2	SNV	11:69154575	0.418
affy6634881	rs55705316	IL-10	SNV	1:206760172	0
affy6634986	rs3024505	IL-10	SNV	1:206766559	0
affy6635044	rs3024508	IL-10	SNV	1:206770065	0.724
affy6635056	rs3024493	IL-10	SNV	1:206770623	0
affy6635064	rs1518111	IL10	SNV	1:206771300	0
affy6635095	rs1800871	IL10	SNV	1:206773289	0
affy6635272	rs17351243	IL19	SNV	1:206786182	0.000017
affy8486628	rs6488336	CD163	SNV	12:7479287	0
affy8488682	rs10743940	CD163	SNV	12:7498542	0
affy9098753	rs4770433	SACS	SNV	13:23329652	0.0006
affy12604008	rs1801275	IL4R	SNV	16:27363079	0
affy12618596	rs17855750	IL27	SNV	16:28503907	0.485
affy13181524	rs1884444	IL23R	SNV	1:67168129	0.971
affy13187550	rs11805303	IL23R	SNV	1:67209833	0.004

affy13192389	rs11209026	IL23R	SNV	1:67240275	0.437
affy13194489	rs1343151	IL23R	SNV	1:67253446	0
affy14368477	rs9901673	CD68	SNV	17:7580783	0.000001
affy14368561	rs17607	CD68	SNV	17:7581464	0
affy14368564	rs9901675	CD68	SNV	17:7581494	0.447
affy15521078	rs12720356	TYK2	SNV	19:10359299	0.095
affy15635575	rs375947	IL12RB1	SNV	19:18069641	0.296
affy15635686	rs11575934	IL12RB1	SNV	19:18075808	0.87
affy15940455	rs12979860	IFNL4	SNV	19:39248147	0
affy15940502	rs8099917	IL28B	SNV	19:39252525	0.0056
affy17292335	rs315952	IL1RN	SNV	2:113132727	2.70E-06
affy18205507	rs10191411	Interleukin 8	SNV	2:148630399	0
affy19110488	rs16858811	CXCR1	SNV	2:218165120	0.06
affy21053125	rs2255214	ILDR1/CD86	SNV	3:122051692	0.63
affy21053385	rs4308217	CD86	SNV	3:122074340	0
affy21053435	rs9282641	CD86	SNV	3:122077921	0.43
affy21053741	rs2681417	CD86	SNV	3:122106350	0
affy21053868	rs1129055	CD86	SNV	3:122119472	0.000001
affy21053887	rs17281995	CD86	SNV	3:122120794	0.35
affy24914929	rs2298839	AFP	SNV	4:73445127	0.00001
affy24915421	rs1894292	AFM	SNV	4:73483441	0.033
affy24918888	rs4073	CXCL8	SNV	4:73740307	0.023
affy25632757	rs40401	IL3	SNV	5:132060785	0
affy25639631	rs1881457	IL13	SNV	5:132656717	0.47

affy25639637	rs1800925	IL13	SNV	5:132657117	0.0002
affy25639686	rs1295686	IL13	SNV	5:132660151	0
affy25639687	rs20541	IL13	SNV	5:132660272	0.007
affy25639862	rs2243250	IL4	SNV	5:132673462	0
affy25726722	rs17208187	TMCO6/CD14	SNV	5:140643653	0.53
affy28459490	rs1799964	TNF	SNV	6:31574531	0.3
affy28459505	rs4248160	TNF	SNV	6:31574916	0.704
affy28459517	rs1800750	TNF	SNV	6:31575186	0.63
affy28459519	rs1800629	TNF	SNV	6:31575254	0.038
affy28459532	rs2228088	TNF	SNV	6:31575828	0
affy28459538	rs1800610	TNF	SNV	6:31576050	0
affy28459548	rs3093662	TNF	SNV	6:31576412	0.213
affy28459555	rs4645843	TNF	SNV	6:31576785	0.46
affy28459557	rs3093664	TNF	SNV	6:31576865	0.182
affy28459564	rs3093665	TNF	SNV	6:31577614	0.249
affy28459580	rs3093668	TNF	SNV	6:31578718	0.4
affy28762720	rs763780	IL17F	SNV	6:52236941	0.0013
affy30198890	rs2069827	IL6	SNV	7:22725837	0.32
affy30198920	rs1800795	IL6	SNV	7:22727026	0
affy30198956	rs1474347	IL6	SNV	7:22728505	0
affy30199019	rs13306435	IL6	SNV	7:22731420	0
affy30947505	rs13236689	CD36	SNV	7:80606698	0.000039
affy30947798	rs10499859	CD36	SNV	7:80629494	0
affy30948290	rs5956	CD36	SNV	7:80663133	0.33

affy30948321	rs3211908	CD36	SNV	7:80664600	0.000169
affy30948409	rs3211931	CD36	SNV	7:80668857	0.0314
affy30948462	rs1527483	CD36	SNV	7:80672184	0.000064
Ncbi.nlm.nih.gov. 2020. Home - SNP - NCBI					

7.5.Appendix- Chapter 6

Appendix- Chapter 6

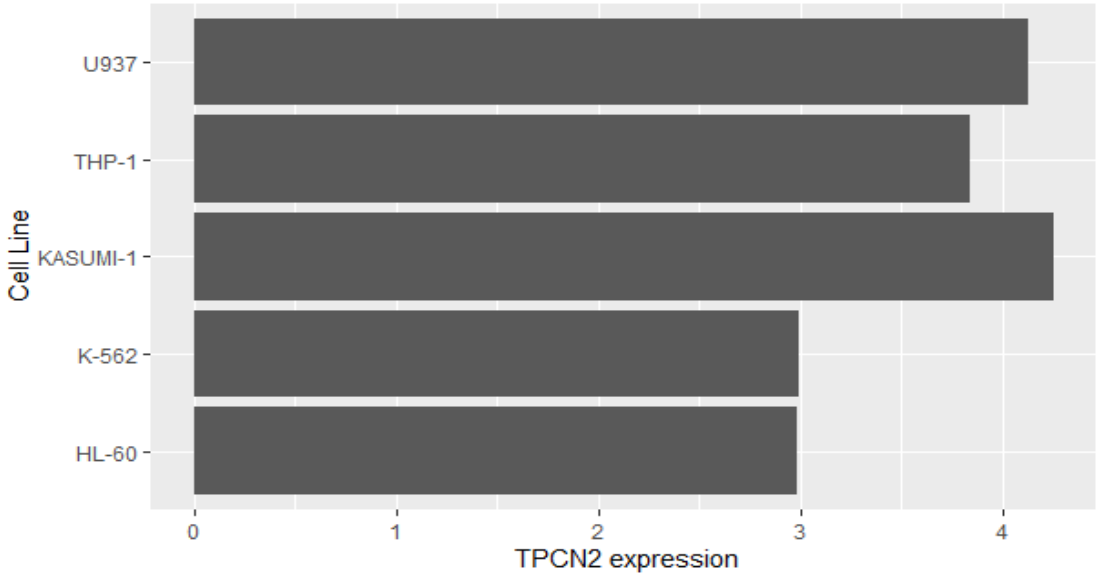


Figure S6. 1.TPCN2 expression across various types of cell lines.

Table S6.1. Flow cytometry panel			
Marker/ Antigen	location	Proposed fluorochrome	Antibody
CD14	Extracellular	BV711	BV711 Mouse Anti-Human CD14
CD16	Extracellular	BV605	BV605 Mouse Anti-Human CD16
CD86	Extracellular	PE	PE Mouse Anti-Human CD86
CD68	Intracellular	BV421	BV421 Mouse Anti-Human CD68
CD206	Extracellular	BB515	BB515 Mouse Anti-Human CD206
TPC2	Intracellular	AF647	Anti-TPCN2 Antibody Source: Rabbit Type: Polyclonal BV421 Goat Anti-Rabbit IgG Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647)
Live/dead cells marker Fixable Viability Stain 780			

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