

The effect of prostaglandin E₂ on natural killer cell activity

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Abstract

Tumour cells and other cell types within the tumour micro-environment (TME) are immunosuppressive and enable cancer cells to avoid immune-mediated destruction. One mechanism of immune escape is through secretion of immunosuppressive molecules. Several cancers are known to secrete prostaglandin E₂ (PGE₂) in the TME, which inhibits various functions of immune cells, via engagement with the PGE₂ receptors EP1-4. This study aims to explore the inhibitory effect of PGE₂ on NK cell cytotoxicity against cancer cells.

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. Whole PBMCs or isolated NK cells were pre-treated with synthetic PGE₂ and activated with cytokines (IL-2, IL-15, IL-12 and IL-18) and reovirus. Flow cytometry was used to assess NK cell activation and degranulation. RT-PCR was used to detect the expression of EP1-4. Enzyme linked immunosorbent assays were used to detect IFN- γ in cell supernatants. Western Blot were used to detect the inhibitory effect of PGE₂ on pSTAT pathways in IL-15 mediated-NK cells.

NK cells expressed the EP2 and EP4 PGE₂ receptors. PGE₂ inhibited cytokine-mediated increases in NK cell CD69 expression, IFN- γ secretion and degranulation against tumour cell targets. Moreover, the use of EP2 and EP4 receptor inhibitors restored NK cytotoxicity to some extent, revealing that PGE₂ exerts its inhibitory effects on NK cells at least in part through the EP2 and EP4 receptors. Importantly, PGE₂ suppresses IL-15-mediated activation of the pSTAT5 pathway in NK cells, revealing that PGE₂ blocks a fundamental pathway in NK cell activation.

Tumour cells can change and maintain the conditions for their own survival and development through autocrine and paracrine secretion, thereby promoting the growth and development of tumours. PGE₁ alcohol can

induce cancer cells to produce PGE₂ and inhibit NK cell cytotoxicity through EP3 and EP4. When tumour cells were co-cultured with TAMs and MSCs in 3D model, spheroids produced large amounts of PGE₂.

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Abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ADCC	antibody-dependent cellular cytotoxicity
ANOVA	analysis of variance
APC	antigen presenting cell
APM	antigen processing and presenting machinery
Arg	arginase
BCA	bicinchoninic acid
cAMP	cyclic adenosine monophosphate
CAR-NK	engineered NK cell
CCR	C-C chemokine receptor
CD	cluster of differentiation
CLR	C-type lectin receptor
COX	Cyclooxygenase
CREB	cAMP response element binding
CSC	cancer stem cell
Ct	cycle threshold
CTL	cytotoxic T cell
DC	dendritic cell
DD	death domain
DED	death effector domain
DHA	docosahexaenoic acid
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNAM-1	DNAX accessory molecule-1
DRG	dorsal root ganglion
EB	ethidium bromide

EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EPA	eicosapentaenoic acid
Erk	extracellular signal-regulated kinase
ES	ewing sarcoma
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	foetal bovine serum
FDA	Food and Drug Administration
FLUC	firefly luciferase
FSC-A	forward scatter-area
GBM	glioblastoma multiforme
GC	gastric cancer
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
Gzm	granzyme
HA	hemagglutinin
HCC	hepatocellular carcinoma
HCMV	human cytomegalovirus
HIF	hypoxia-inducible factor
HLA-G	MHC-I ligands
HN	hemagglutinin-neuraminidase
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
IFN	Interferon
IKK	I κ B kinase
IL	Interleukin
ILC	innate lymphoid cell

iNOS	inducible nitric oxide synthase
intILC	intermediate type 1 innate lymphoid cell
IP3	inositol 1,4,5-trisphosphate
ISVP	Infectious subviral particles
ITAM	Immunoreceptor tyrosine activation motifs
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITT	immunoreceptor tyrosine tail
Jak	janus tyrosine kinase
KIR	killer-cell immunoglobulin-like receptor
LAMP-1	lysosomal associated membrane protein-1
LAP	latency-associated peptide
LIR	leukocyte immunoglobulin-like receptor
LOX	lipoxygenase
LPS	lipopolysaccharide
LTB	leukotriene B
LTBP	latent TGF- β binding protein
MAPEG	membrane-associated protein involved in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
MC	mast cell
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility class
MICA/MICB	MHC class I polypeptide-related sequence A/B
MMP	matrix metalloproteinase
mNK	Memory NK
mPGES	membrane-bound prostaglandin E synthase
MPS	Mononuclear phagocyte system
MR	mannose receptor
MRP	multidrug resistance-associated protein

MS	multiple sclerosis
MTOC	microtubule organizing centre
NCR	natural cytotoxicity receptors
NF-κB	nuclear factor κB
NHSBT	National Health Service Blood and Transplant
NK	natural killer
NLR	nod-like receptor
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
NSB	non-specific binding
OV	oncolytic virus
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD-L1	programmed cell death ligand-1
PFU	Plaque forming units
PG	prostaglandin
PGG	prostaglandin G
PGH	prostaglandin H
PGT	prostaglandin transporter
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
p-NK	peripheral blood NK
pNPP	p-Nitrophenyl phosphate
PSGL	P-selectin glycoprotein ligand
PTG	prostaglandin transporter
PUFA	polyunsaturated fatty acid

PVR	poliovirus receptor
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor κ B ligand
RLR	RIG-I-like receptor
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park memorial institute
RT	reverse transcription
RUNX	runt-related transcription factor
SEM	standard error of mean
SH	src homology
SLC	small latent TGF- β complex
SSC-A	side scatter-area
STAT	signal activator of transcription
STR	short tandem repeat
tBid	truncated Bid
TCGF	T cell growth factor
TCGF	T cell growth factor
TCR	T cell receptor
TGF- β	transforming growth factor β
Th	T helper
Ti-NK	tumour-infiltrating NK
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
T-VEC	talimogene laherparepvec
TXA	thromboxane A

ULBP	UL16-binding proteins
UMAP	uniform manifold approximation and projection
VEGF	vascular endothelial growth factor
3D	three- dimensional

Chapter 1

Introduction

1.1 The immune system

The human immune system is a complex and sophisticated defence network designed to protect the body from pathogens (such as bacteria, viruses, fungi and parasites) and other harmful substances. The immune system is composed of multiple immune organs, cells, molecules and immune pathways that work together to detect, identify and eliminate foreign invaders and abnormal cells, such as cancer cells, in the body. The immune system includes a series of specialized organs and tissues, such as the bone marrow, thymus, lymph nodes and spleen, which provide sites for the generation, maturation, activation and interaction of immune cells. In addition, the immune system relies on cytokines (such as interleukins, interferons, etc.) and chemical mediators to coordinate immune responses and promote communication and cooperation between immune cells. The balance and normal function of the immune system are essential for health. An overactive immune system may lead to allergies and autoimmune diseases, while an underactive or dysregulated immune system may lead to increased susceptibility to infection or immunodeficiency diseases.

1.1.1 Innate and adaptive immunity

Innate immunity and adaptive immunity are the two major parts of the human immune system. These two types of immunity each play a unique role and work together to form a comprehensive defence system. Adaptive immunity is specific and has memory, enabling a more precise response to specific antigens and a more rapid and effective response when exposed

to the same antigen again (Netea et al., 2019). The adaptive immune system consists of two types of lymphocytes: B cells and T cells. T cells are initially produced in the bone marrow, and their maturation process mainly occurs in the thymus. Similar to T cells, B cells also originate in the bone marrow, but their maturation process occurs entirely in the bone marrow. The process of adaptive immunity can be divided into several major stages, including antigen recognition, activation, response and memory. T cells and B cells each have unique antigen recognition capabilities in adaptive immunity, which is achieved through antigen receptors on their surface. The antigen receptor of T cells is the T cell receptor (TCR), while the antigen receptor of B cells is the B cell receptor (BCR), which is similar in structure to antibodies (Prisco and De Berardinis, 2012). The antigen receptors of both types of cells are generated through a process of gene rearrangement, allowing them to recognize an almost unlimited number of different antigens. Once the antigen is recognized, T cells begin to proliferate and form a large number of specific T cell clones. Cytotoxic T cells (Tc cells/ CTL), are a group of CD8⁺ T cells, directly recognize and kill virus-infected or cancerous cells, and induce apoptosis of target cells by releasing cytotoxins. Activated helper T cells (CD4⁺ T cells) active other immune cells such as B cells by secreting cytokines (Luckheeram et al., 2012; Crotty, 2015). B cells begin to proliferate after activation, forming a large number of specific B cell clones. Activated B cells differentiate into plasma cells and produce a large number of specific antibodies and cytokines (Sharonov et al., 2020). During the immune response, some T cells and B cells differentiate into memory cells. These memory cells have a long lifespan and can respond quickly when encountering the same antigen in the future. When exposed to the same antigen again, memory cells will quickly recognize and initiate an immune response, thereby providing a faster and stronger immune response. This memory response is also the basis of vaccination (Prisco and De Berardinis, 2012).

Innate immunity is the body's first line of defence against invasion by external pathogens. It is non-specific; it can respond quickly to a variety of foreign pathogens, but it is not targeted at specific pathogens. Unlike adaptive immunity, innate immunity does not require exposure to pathogens to form and has no memory ability. The first layer of innate immunity is the physical and chemical barriers, which mainly include barriers of organ systems such as the skin, mucous membranes, digestive tract and respiratory tract (Goodarzi et al., 2007). For example, the skin can effectively prevent external pathogens from entering the body. The mucus secreted by mucosal epithelial cells can capture pathogens and expel them from the body through coughing, sneezing, etc. Secretions such as tears, saliva, and gastric acid contain antibacterial substances, such as lysozyme, which can directly destroy the cell walls of pathogens. When pathogens break through physical barriers, a variety of immune cells in the innate immune system immediately participate in defence. These cells recognize foreign pathogens through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (Hoffmann and Akira, 2013). These receptors can recognize molecular patterns unique to pathogens, called pathogen-associated molecular patterns (PAMPs). For example, TLRs, one of the most important pattern recognition receptors in innate immunity, are located on the cell membrane or endosomal membrane and can recognize a wide range of PAMPs (Medzhitov et al., 1997).

Macrophages are important cells in the innate immune system, responsible for identifying, engulfing and clearing foreign pathogens and damaged tissues and cell debris in the body. There are a variety of pattern recognition receptors on the cell surface, such as TLRs and mannose receptors (MRs), which can recognize PAMPs, such as bacterial lipopolysaccharide (LPS) (TLR4 recognition) or viral double-stranded RNA (TLR3 recognition) (Bode

et al., 2012; Suresh et al., 2019). When macrophages recognize pathogens, they will encapsulate the pathogens in phagosomes formed by cell membranes through phagocytosis, and then fuse with lysosomes in the cells to form phagolysosomes. Digestive enzymes and toxic free radicals in lysosomes can destroy and digest pathogens (Murray and Wynn, 2011). Although macrophages mainly function as phagocytes, they are also important antigen presenting cells (APCs). After engulfing and degrading pathogens, macrophages present the pathogen's antigenic peptides to helper T cells through major histocompatibility complex (MHC) class II molecules to help activate adaptive immune responses.

DCs are also activated by PRRs and are the most important APCs of innate immunity (Man and Jenkins, 2022; Tong et al., 2023). DCs help activate and regulate the function of T cells by presenting antigens and providing co-stimulatory signals. This connection is crucial for regulating the nature and intensity of immune responses. They engulf and process pathogens or foreign antigens in peripheral tissues, breaking down antigens into short peptides. The processed antigens are presented to T cells through two pathways: (1) MHC class I molecules are presented to CD8⁺ T cells: mainly used to present antigens from infected host cells (such as viral antigens). This pathway activates CTLs to directly kill infected cells or tumour cells (Fu and Jiang, 2018). (2) MHC class II molecules are presented to CD4⁺ T cells: mainly used to present exogenous antigens (e.g. such as those derived from bacteria) (Nakayama, 2014). This pathway activates helper T cells. The co-stimulatory molecules also expressed on the surface of dendritic cells bind to receptors on the surface of T cells to form co-stimulatory signals. This signal works together with the antigen-MHC signal to help T cells complete activation, proliferation and differentiation.

NK cells work closely with macrophages and DCs, cytokines produced by these cells (e.g., IL-12) can activate NK cells, while IFN- γ secreted by NK

cells can enhance the effects of other immune cells.

1.1.2 Immune cell classification

Immune cells are derived from hematopoietic stem cells (HSCs), which are found in the bone marrow and have the ability to self-renew and multi-directionally differentiate. HSCs differentiate into various cell types in the immune system, including innate immune cells and adaptive immune cells (Zhang et al., 2019). The process of HSC differentiation can be divided into two main pathways: lymphoid and myeloid. The main cell types of the immune system that lymphoid progenitor cells differentiate into are T cells, B cells, and NK cells. Myeloid progenitor cells differentiate into a variety of innate immune cells, including monocytes (macrophages and dendritic cells), neutrophils, eosinophils, basophils, and mast cells (Figure 1.1).

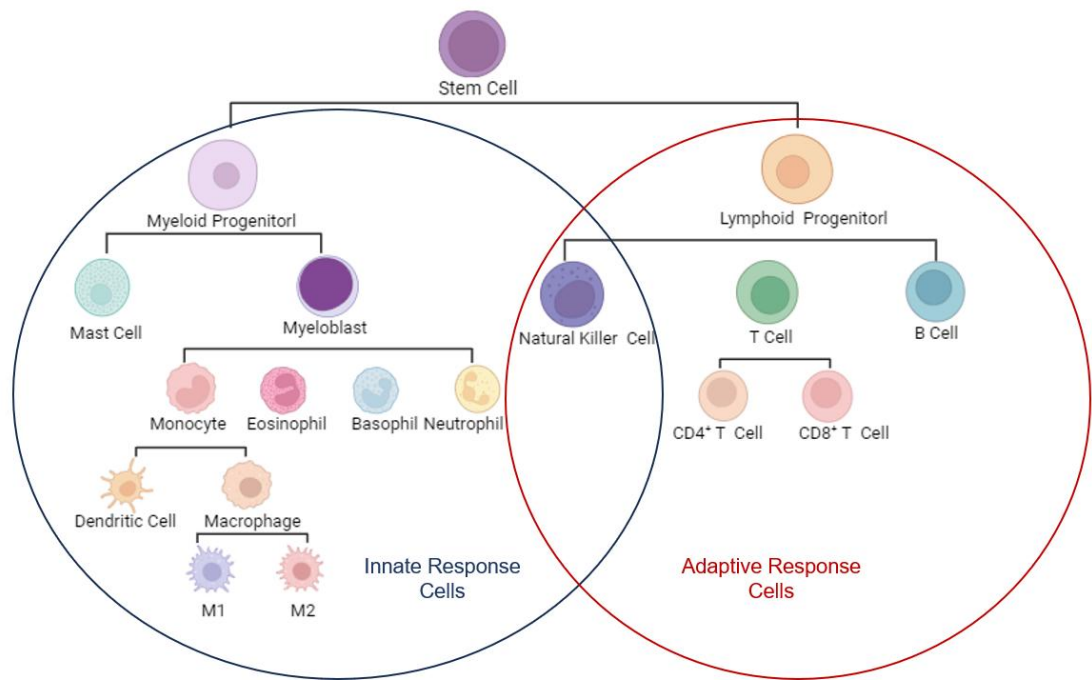


Figure 1.1: Immune cell classification. Hematopoietic stem cells differentiate into lymphoid and myeloid progenitor cells, which further differentiate into cell types of immune cells associated with adaptive and innate immunity. NK cells lack antigen receptors and are designated as part of innate immunity. However, they do show some features of adaptive immunity, such as a limited memory response. Figure generated using Biorender.

1.2 Natural Killer Cells

1.2.1 Identification

Natural killer cells (NK cells) are a very important type of lymphocytes in the immune system. They can kill target cells without prior sensitization, so they play a vital role in immune responses such as antiviral and anti-tumour. The discovery of NK cells can be traced back to the 1970s. At that time, scientists' understanding of the immune system was still in its infancy, and the functions and classification of lymphocytes were not yet clear. In 1975, Professor Kiessling of Sweden discovered this type of special lymphocyte in the spleen of mice, which has the function of "lysing cells" and is specific to mouse Moloney leukaemia cells. And these cells kill tumour cells without immune pre-activation, and they do not require antigen presentation or the initiation of adaptive immunity. Because these cells have "natural" killing functions, they are named "Natural Killer Cells" (Kiessling et al., 1975). This discovery made it clear for the first time that there is an immune cell in the innate immune system that can respond quickly and does not require specific antigen recognition. Then in 1978, human natural killer cells were discovered. Before that, reports on natural killer cells were all from mice (Daniela Santoli et al., 1978).

Human NK cells account for 5% to 15% of peripheral blood and 25% to 50% of liver (Sun et al., 2015; Fasbender et al., 2016). The proportion of NK cells in mice peripheral blood is lower, only 2% to 5%. The surface markers of mouse and human NK cells are different. The main marker of human NK cells is CD56, while mouse NK cells do not express CD56. CD49b (DX5) and NK1.1 are usually used to mark mouse NK cells (Pellicci et al., 2005). The main activation receptors of human NK cells include NKG2D, DNAM-1 and the natural cytotoxicity receptors (NCRs), NKp30, NKp44 and NKp46. The activation receptors of mouse NK cells also include NKG2D, DNAM-1

and NKp46, but NKp30 and NKp44 do not exist in mice (Hollyoake, 2005).

1.2.2 The working principle of NK cells

The "missing-self hypothesis" is an important theory for understanding the mechanism of NK cell recognition, because NK cells can directly kill target cells without antigen-specific recognition. This theory was first proposed in 1986 by Swedish scientist Klas Kärre et al., who proposed the mechanism of the "missing-self" theory and its importance in NK cell recognition in 1990 (Kärre et al., 1986; Ljunggren and Kärre, 1990). They explain how NK cells recognize and attack target cells that lack major histocompatibility complex class I (MHC I) molecules. Healthy cells usually express MHC I molecules on their surfaces, which can transmit "self" signals to NK cells, which inhibits NK cell cytotoxicity. This is because NK cells sense the presence of this "self" signal by binding to MHC I molecules through their inhibitory receptors. However, when cells are infected by viruses or become cancerous, the expression of MHC I may decrease. For example, many viruses reduce expression of MHC class I to evade T cells. When the "self" signal is insufficient, the inhibitory signal of NK cells is lost, NK cells are activated, and target cells are recognized and eliminated by NK cells.

This suggests that NK cell activity depends on a dynamic balance between inhibitory and activating receptors. The inhibitory receptors mainly expressed by NK cells are killer-cell immunoglobulin-like receptors family (KIRs), NKG2A/CD94 and leukocyte immunoglobulin-like receptor 1 (LIR-1). KIR mainly binds to classical MHC I (HLA-A, HLA-B, HLA-C) molecules, recognizes self, and inhibits NK cell activity (Anfossi et al., 2006; Rehermann, 2016). NKG2A can form heterodimers with CD94, and after binding to its ligand MHC I-related non-classical molecule HLA-E, it activates the inhibitory signalling pathway in the cell and inhibits NK cell

activity (Fisher et al., 2022). LIR-1 mainly binds to non-classical MHC-I ligands (HLA-G), and it is also considered a target of immune evasion because it binds to HLA I homolog UL18 encoded by human cytomegalovirus (HCMV) and is expressed in the tumour microenvironment (Li et al., 2011; Heidenreich et al., 2012).

NK cell activating receptors play a key role in controlling various viral infections and cancers by promoting NK cell cytotoxicity and cytokine production. NK cell activating receptors mainly include NKG2D, NCR, DNAX accessory molecule-1 (DNAM-1) and CD16 (Pazina et al., 2021). NKG2D can bind to non-classical MHC I molecules, MHC class I polypeptide-related sequence A/B (MICA/MICB) and UL16-binding proteins (ULBPs), these ligands are expressed more on stressed cells (Sutherland et al., 2001; Xing and Ferrari de Andrade, 2020). When NKG2D binds to ligands, it activates downstream activation signals to activate NK cells. NKp30, NKp44, and NKp46 are NCRs, which are the first discovered NK cell activation receptors. The first discovered NCR ligands came from viruses, influenza virus hemagglutinin (HA) and Sendai virus hemagglutinin-neuraminidase (HN) can bind to NKp46 then activate lysis by NK cells (Mandelboim et al., 2001). The ligand of NKp30 is B7-H6, which is expressed on the surface of tumour cells. After NKp30 binds to the ligand, NK cells produce interferon- γ (IFN- γ) and kill target cells (Fiegler et al., 2013). The ligands of DNAM-1 (CD226) are CD112 (Nectin-2) and CD155 (PVR, Poliovirus receptor), of which CD155 has a higher affinity with DNAM-1 and is highly expressed on infected cells and some tumour cells (Cifaldi et al., 2023). After binding to the ligand, DNAM-1 can promote the adhesion and polarization of NK cells to target cells (Fiegler et al., 2013). CD16 (Fc γ RIII) is a low-affinity IgG receptor that can bind to the Fc region of IgG molecules and promote antibody-dependent cellular cytotoxicity (ADCC) of NK cells, then activate and proliferate NK cells (Romee et al.,

2013a; Kim et al., 2023). However, CD16 only has low affinity for IgG and NK cells are only triggered by clustered IgG, e.g cells expressing a viral glycoprotein bound by a corresponding antibody.

The core mechanism of NK cell activation involves specific tyrosine-based motifs and downstream signalling pathways (Figure 1.2). Immunoreceptor tyrosine activation motifs (ITAM) are present in the signal transduction subunits of some activating receptors, such as CD3 ζ , DAP12, and FcR- γ . Fc γ RIII, NKp30 and NKp46 bind to FcR γ and/or CD3 ζ chains, and NKp44 binds to the adaptor protein DAP12 (Karimi et al., 2005; Long et al., 2013; Barrow et al., 2019). NKG2D does not have ITAMs but instead binds to the adaptor molecule DAP10, which has activating Tyr-based motif YxxM. DNAM-1 enhance NK cell activation by binding to an immunoreceptor tyrosine tail (ITT)-like motif and phosphorylation at a conserved tyrosine (Y) in its cytoplasmic domain (Zhang et al., 2015). When an activating receptor binds to its adaptor ITAM-associated protein, the tyrosines in the ITAM are phosphorylated by Src family tyrosine kinases. Phosphorylated ITAMs recruit and activate the Tyr kinases ZAP-70 and Syk through src homology 2 (SH2) domain-based interactions, thereby activating downstream signalling pathways including the phosphoinositide 3 - kinase (PI3K), phospholipase C (PLC)- γ pathway and guanine nucleotide exchange factors Vav (Kumar, 2018; Medjouel Khlifi et al., 2022). NK cells interact with ligands on target cells through activating receptors on their surface, recognize target cells and activate signalling pathways, causing intracellular granules (perforin and granzymes) to polarize toward the synapse. The microtubule organizing centre (MTOC) also polarizes toward the synapse. Afterwards, LFA-1 on the surface of NK cells recognizes ICAM-1 on target cells and forms a stable immune synapse structure. When the immune synapse is formed, NK cells release cytolytic granules to target cell and induce cell apoptosis (Topham and Hewitt, 2009; Huse, 2012).

The inhibitory receptors of NK cells all signal through immunoreceptor tyrosine-based inhibitory motif (ITIM) present in the intracellular portion of the inhibitory receptors. When it is phosphorylated, it can recruit inhibitory phosphatases (SHP-1, SHP-2) to dephosphorylate signalling molecules and inhibit activation of signalling pathways (Figure 1.2) (Kumar, 2018). Similar to the immune synapse, after NK cell inhibitory receptors bind to the MHC-I ligands of target cells, inhibitory synapses are formed between them. Inhibitory synapses can effectively prevent LFA-1-induced degranulation and thus protect target cells from being killed (Kumar, 2018).

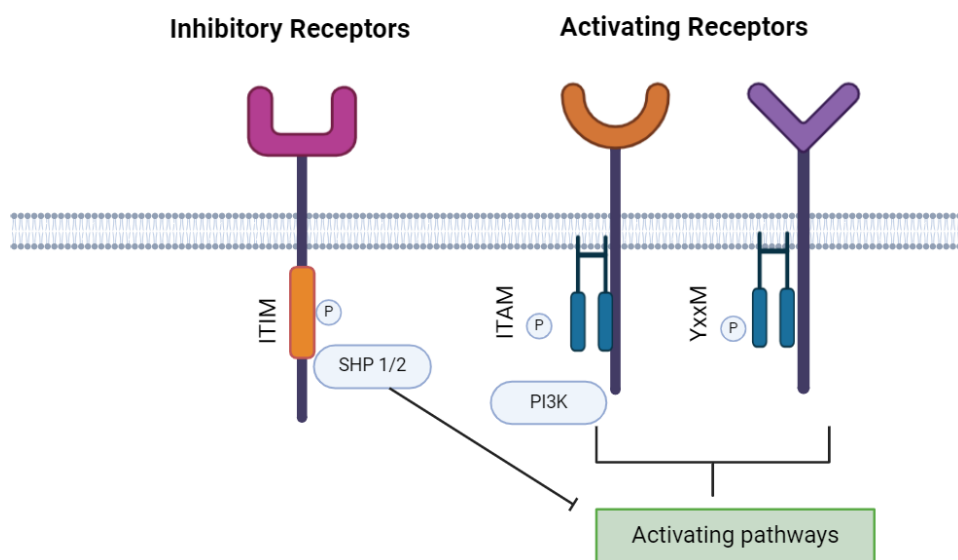


Figure 1.2: NK cell activating and inhibitory receptor mechanisms. NK cell inhibitory receptors recognize HLA class I molecules, leading to phosphorylation of the ITIMs, then activate SHP-1/2 and inhibit downstream signalling pathways. Activation of NK cell activating receptors leads to phosphorylation of ITAM or YxxM, and then PI3K is activated to activate the downstream signalling pathway. Figure generated using Biorender.

1.2.3 The mechanisms of NK cell killing

The killing mechanism of NK cells includes granule-dependent cytotoxicity (degranulation), ADCC, death receptor-mediated apoptosis and cytokine secretion (Figure 1.3).

Granule-dependent cytotoxicity is the main and most direct pathway for NK cells to kill target cells. As mentioned in 1.22 above, after NK cells recognize target cells, NK cells contact target cells to form an immune synapse. After recognizing target cells, the cytoplasmic granules in the NK cells are rapidly moved along microtubules by motor proteins, causing them to converge at the MTOC (microtubule-organizing centre), then the MTOC and the granules are polarized to the immune synapse. After polarization, the granules pass through the synapse and connect to the NK cell membrane to form temporary pores, releasing the cytotoxic granules into the external environment of the synaptic area (Ogbomo and Mody, 2017). These granules mainly contain perforin and granzymes. In 1988, human perforin was discovered, perforin is a glycoprotein with a similar structure to complement component C9 (Cullen and Martin, 2008). The main function of perforin is to form pores on the target cell membrane. This process requires the participation of Ca^{2+} ions. The pore size is 5-20 nanometres, providing a channel for the entry of granzymes. Perforin binds to target cells through membrane phospholipids, and the pores will destroy the cell membrane of the target cell, causing the cell contents to flow in and out freely, indirectly inducing the death of the target cell (Osińska et al., 2014). Granzymes are a class of serine proteases. There are five main types of granzymes in humans, granzymes A, B, H, K, and M, which induce apoptosis by activating different death pathways. Granzymes A and B are the most abundant and widely studied, and the cytotoxicity of GzmA is much lower than GzmB. When GzmA enters target cells, it does not depend on the activity of caspases and cleaves other substrates. GzmA disrupts

mitochondrial function and fragments DNA in target cells (Lieberman, 2010). As for GzmB, after it enters the target cell, GzmB will initiate the apoptosis cascade by cleaving and activating pro-caspase-8 and pro-caspase-10. After caspase-8 and caspase-10 are activated, proteases 3 and 7 are activated, directly inducing cell apoptosis. In addition, caspase-8 and caspase-10 can also activate Bid (one of the Bcl-2 family proteins) to form truncated Bid (tBid), causing mitochondria to release cytochrome c, activate caspase-9, and ultimately lead to DNA fragmentation and cell death (Thompson and Cao, 2024).

After NK cells are activated, they can also cause target cell death through death receptor-mediated apoptosis. This process depends on the interaction between death ligands on the surface of NK cells and death receptors on the surface of target cells. Death receptors are a class of transmembrane proteins belonging to the tumour necrosis factor receptor (TNF receptor) superfamily. Death receptors contain a special death domain (DD) that can bind to specific death ligands and induce cell apoptosis. The major death receptors include Fas receptor (CD95 or APO-1), tumour necrosis factor receptor 1 (TNFR1) and TRAIL receptor 1/2 (DR4/DR5). The main death ligands of NK cells are tumour necrosis factor α (TNF α), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), they belong to the TNF family. When death ligands bind to their receptors, DD induces receptor trimerization and recruits the Fas-associated death domain (FADD). FADD further binds to caspase-8 or caspase-10 containing the death effector domain (DED) to form the death-inducing signalling complex (DISC) and activate downstream apoptosis signalling pathways (Ramírez-Labrada et al., 2022). Because the composition of the signalling complex formed after ligand-receptor binding is different, different cell death pathways are activated.

ADCC has been mentioned in Chapter 1.22. NK cells activated in this way

kill cells through the release of cytotoxic granules, TNF family death receptor-mediated signalling and secretion of cytokines and chemokines. The main cytokines secreted by NK cells are IFN- γ , TNF- α and granulocyte-macrophage colony-stimulating factor (GM-CSF). These cytokines recruit and activate other immune cell types and contribute to the activation of the adaptive immune response (Abel et al., 2018).

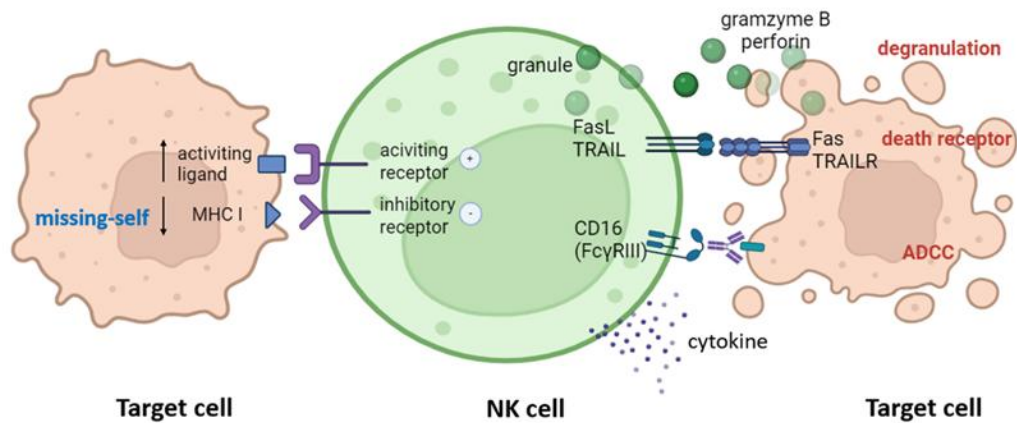


Figure 1.3: The mechanisms of NK cell killing target cell. NK cells are activated through a balance of activating receptors (induction of activating signals) and inhibitory receptors (absence of inhibitory signals). NK cells kill target cells in several ways, including granule-dependent cytotoxicity (degranulation), ADCC, death receptor-mediated apoptosis, and cytokine secretion. Figure generated using Biorender.

1.2.4 NK cell subset classification

NK cell subsets are classified according to surface markers. Based on the expression levels of CD16 and CD56, NK cells are divided into two major subsets, CD56^{bright} CD16⁻ and CD56^{dim} CD16⁺. CD56^{dim} NK cells account for most NK cells in peripheral blood, accounting for about 90% of NK cells and CD56^{bright} NK cells account for about 10% of NK cells in peripheral blood (Poli et al., 2009). CD56^{dim} NK cells are the main cytotoxic subset, which directly kills tumour cells and virus-infected cells, mainly through degranulation. In addition, CD56^{dim} NK cells highly express CD16, which enables these cells to active ADCC by recognizing target cells marked by antibodies, further enhancing their killing effect (Romee et al., 2013b). CD56^{bright} NK cells have low levels of CD16 at the most and only show very weak ADCC activity. CD56^{bright} NK cells regulate immune responses mainly by secreting cytokines, including IFN- γ , TNF- α , GM-CSF, IL-10 and IL-13 (Poli et al., 2009). These cytokines can activate macrophages, DCs, and T cells, promoting antigen presentation and activation of adaptive immunity. In the uterus, CD56^{bright} NK cells (known as decidual NK cells) are mainly involved in promoting placental angiogenesis and playing a key role in pregnancy (Poli et al., 2009).

KIR receptors are highly expressed in CD56^{dim} NK cells, and CD56^{bright} NK cells highly express NKG2A/CD94. CD56^{bright} and CD56^{dim} NK cells both express NKG2D. The differential expression of these receptors may be related to the killing effect of NK cells. Functional L-selectin (CD62L), an adhesion molecule that mediates interaction with vascular endothelial cells, and C-C chemokine receptor type 7 (CCR7), a chemokine receptor involved in regulating the migration and homing of immune cells, are highly expressed in CD56^{bright} NK cells (Pesce et al., 2016; Lima et al., 2017). CD56^{dim} NK cells express PEN5, a sulphated lactosamine epitope that binds to P-selectin glycoprotein ligand 1 (PSGL-1) (André et al., 1999).

Therefore, selectins can bind to both subsets of NK cells, suggesting that the molecular mechanisms of their migration may differ.

The classification of mouse NK cell subtypes is similar to that of human NK cells, but there are also some unique differences. Mouse NK cells do not express CD56. Mouse mature NK cells highly express CD11b while immature NK cell markers are CD27⁺CD11b⁻ and CD27⁺CD11b⁺ (Chiossone et al., 2009). Mouse mature NK cells have high levels of cytotoxicity, and immature NK cells mainly secrete pro-inflammatory cytokines.

1.2.5 NK cells and Transforming Growth Factor- β

Transforming growth factor- β (TGF- β) is a cytokine produced by many healthy cell types (Schuster and Krieglstein, 2002). Amongst its many roles, TGF- β has anti-proliferative action on many immune cell types (including NK cells) and can inhibit immune activation. TGF- β is highly produced in the tumour microenvironment where it contributes to immunosuppressive activity. (Trotta et al., 2008; Wilson et al., 2011; Slattery et al., 2019). TGF- β is not only produced by cancer cells, but also by stromal cells in the TME (Figure 1.4) (Xue et al., 2020; Chan et al., 2023).

In mammals, the three isoforms of TGF- β (TGF- β -1, -2 and -3) are highly homologous and encoded by separate genes. They are all synthesized as inactive precursors known as pro-TGF- β (Kubiczkova et al., 2012). The pro-TGF- β is cleaved, but the N-terminal portion (known as latency associated peptide or LAP) remains associated with the C-terminal TGF- β cytokine, rendering TGF- β inactive. This inactive TGF- β is further associated with another protein, latent TGF- β binding protein (LTBP) and secreted from the cell (Taylor, 2009; Kubiczkova et al., 2012). TGF- β activation requires

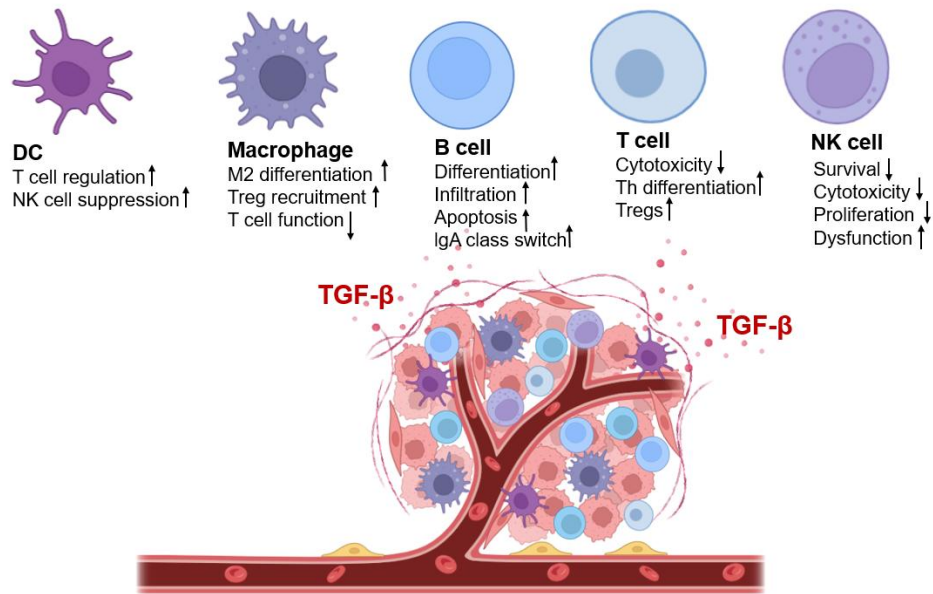


Figure 1.4: TGF- β functions in cancer immune surveillance. TGF- β plays a key immunomodulatory role in different immune cells in TME. TGF- β inhibits T cell proliferation and effector functions, and promotes Treg differentiation. TGF- β reduces the toxicity, proliferation, and survival of NK cells. TGF- β promotes the polarization of M2 type macrophages and inhibits the function of M1 type. Figure generated using Biorender.

release from LAP and LTBP and this process requires LAP to undergo conformational changes or be proteolytically hydrolysed, releasing active TGF- β (Taylor, 2009). Activated TGF- β can bind to TGF- β receptors and initiate downstream signalling.

The TGF- β receptor is a tetrameric structure composed of two type I TGF- β receptors (TGF- β RI) and two type II TGF- β receptors (TGF- β RII). Both receptors are serine/threonine kinase receptors. TGF- β first binds to TGF β R II, which then recruits type I receptors, and type II receptors phosphorylate type I receptors. Type I receptors then initiate the canonical SMAD-dependent signalling pathways by inducing phosphorylation of SMAD2 and SMAD3. Phosphorylated SMAD2/3 binds to SMAD4 to form a complex, which then enters the nucleus to repress or activate the expression of target genes and direct the cellular response to TGF- β (Figure 1.5). TGF- β canonical signalling ultimately affects multiple functional pathways such as cell cycle, apoptosis, differentiation, immunity, and extracellular matrix (ECM) remodelling (Zhang et al., 2017; M. Ferrão et al., 2018; Deng et al., 2024). For example, TGF- β inhibits tumour proliferation by upregulating tumour suppressor genes (such as p15 and p21) and reducing the expression of the tumour-promoting gene c-Myc (Zhang et al., 2017). TGF- β regulates epithelial-mesenchymal transition (EMT) by modulating the EMT-related transcription factors Snail and Slug (Motizuki et al., 2024). TGF- β activates the ECM gene transcription through the SMAD2/3-SMAD4 pathway by directly activating ECM genes, upregulating crosslinking enzymes, inhibiting degradation factors, and driving the activation of fibroblasts, ultimately leading to the excessive production and deposition of ECM proteins (collagen, fibronectin, and proteoglycans) (Biernacka et al., 2011; Giarratana et al., 2024). TGF- β receptors can also signal via non-SMAD (“non-canonical”) signalling pathways. These pathways include the mitogen-activated protein kinase

(MAPK) pathway (ERK, p38, and JNK), the PI3K/AKT pathway, and the Rho-like GTPase pathway and can be regulated by phosphatases such as PP2A (Griswold-Prenner et al., 1998; Sebestyén et al., 2007; Zhang, 2009). Non-canonical pathways often work synergistically with the SMAD pathway to regulate cell migration, metabolism, and immune regulation.

TGF- β inhibits NK cell production of IFN- γ and T-BET (a positive regulator of IFN- γ) in proinflammatory cytokine responses through SMAD2, SMAD3, and SMAD4 (Yu et al., 2006). In addition, TGF- β signalling inhibits the activation of the kinase mTOR, thereby downregulating NK cell metabolism and cytotoxicity (Viel et al., 2016). In gastric cancer (GC), TGF- β downregulates the expression of NK cell activating receptors NKG2D, NKp30, NKp46 and DNAM-1, weakening their ability to recognize and kill tumour cells (Han et al., 2018). It has also been found in glioblastoma multiforme (GBM) that TGF- β produced in the TME also inhibits the activating receptor NKG2D on NK cells and CD8⁺ T cells (Crane et al., 2010). In breast cancer, TGF- β was found to inhibit the function of human peripheral blood NK (p-NK) cells and malignant breast tumour-infiltrating NK (Ti-NK), such as inhibiting the secretion of IFN- γ and TNF- α , CD69 expression, and CD107 degranulation (Mamessier et al., 2011). NK cells can inhibit tumour metastasis, but TGF- β in the TME transforms NK cells into intermediate type 1 innate lymphoid cells (intILC) and innate lymphoid cells (ILC1), which are unable to control local tumour growth and metastasis, helping tumour escape the surveillance of the innate immune system (Gao et al., 2017). In mouse lung carcinoma and melanoma, SMAD3 inhibits NK cell development and effector function by repressing E4BP4/NFIL3, one of the main transcription factors for NK cell differentiation (Tang et al., 2017).

In order to restore NK cell function, several groups have proposed a variety of anti-TGF- β strategies, including the use of TGF- β inhibitors to improve the anti-tumour ability of NK cells by blocking the TGF- β signalling pathway,

these include antibody-based molecules as well as small molecule inhibitors of TGF- β receptor signalling (Otegbeye et al., 2018; Zaiatz-Bittencourt et al., 2018; Liu et al., 2021). However, although these approaches often show value in preclinical models, they have had disappointing results in clinical trials. This is often due to toxicity related to the widespread expression of TGF- β and its many physiological roles. Other approaches are being developed including engineered NK cells (CAR-NK) to block TGF- β 1 signalling by releasing specially designed peptide (known as P6), which can be used to overcome the NK-mediated immunotherapy barriers caused by TME (Shin et al., 2025). Importantly, TGF- β has similar inhibitory effects on CD8⁺ T cells and NK cells and, in addition, can promote the differentiation of regulatory T cells (Treg) which themselves inhibit NK cells and CD8⁺ T cells. This and other effects of TGF- β are summarised in Figure 1.4.

TGF- β is the most extensively studied immunosuppressive factor in the tumour microenvironment, where it profoundly inhibits NK and T cell activity and promotes tumour progression. For this reason, we describe in detail what is known about TGF- β . Other immunosuppressive molecules present in the TME include cytokines such as IL-6 and IL-10, the enzyme IDO, immune checkpoint ligands such as PD-L1, and prostaglandin E₂ (PGE₂). While TGF- β has served as a paradigm of immune suppression, the mechanisms by which PGE₂ regulates NK cell function are comparatively less well defined. This thesis therefore focuses on delineating the role of PGE₂ in shaping NK cell responses in cancer.

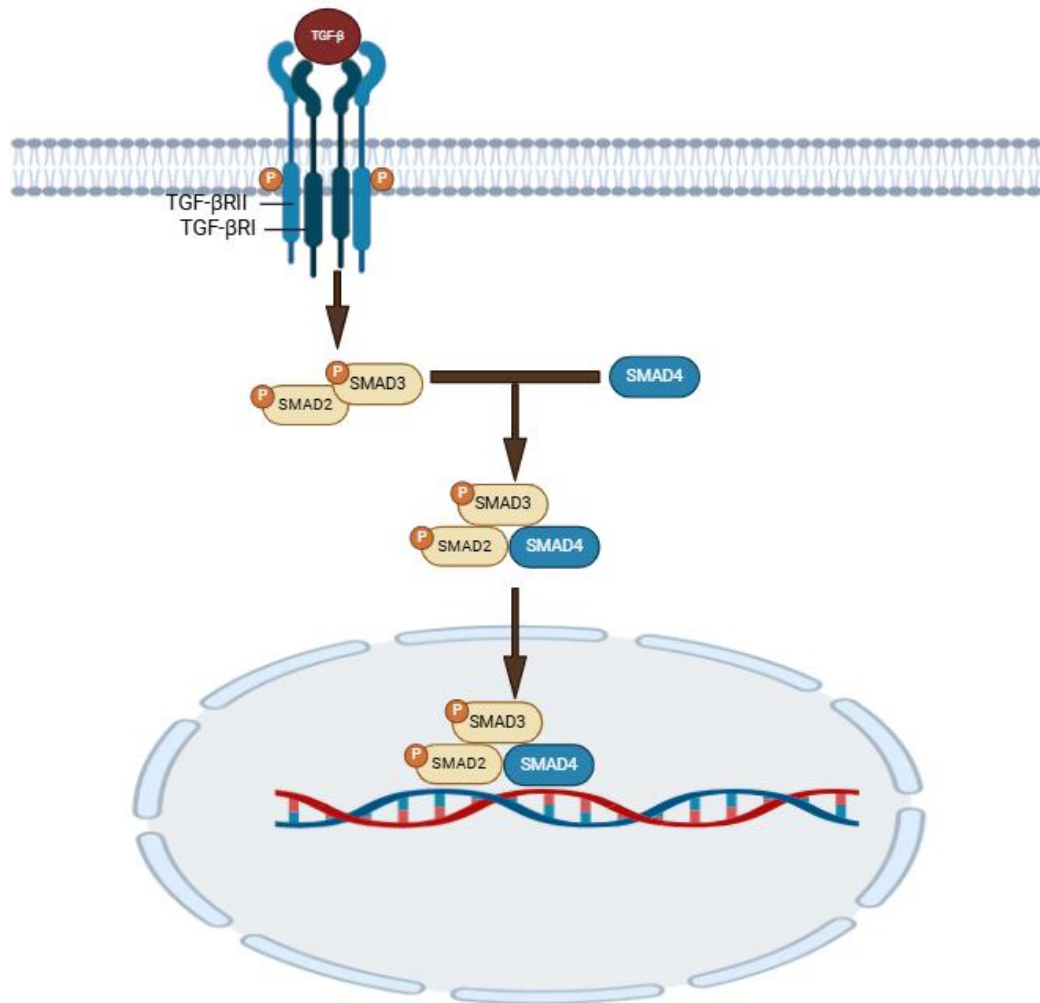


Figure 1.5: Canonical pathway of TGF- β signalling. Activation of the TGF- β RI and TGF- β RII heterotetrametric complex results in phosphorylation and activation of SMAD proteins. The SMAD2/3 complex then binds to SMAD4 and translocate to the nucleus, inducing gene transcription. Figure generated using Biorender.

1.3 Prostaglandin E₂ (PGE₂)

1.3 1 Discovery of PGE₂

The history of prostaglandin (PG) research can be traced back to 1930, when two American gynaecologists, Kurzrok and Lieb, observed that dropping fresh human semen onto the human uterine muscle layer would produce a contraction or relaxation response (Kurzrok and Lieb, 1930). The substance was later named PG after its purification (Goldblatt, 1933; Euler, 1935). Purification confirmed that the substance was a fat-soluble acid and was believed to be produced by the prostate, so it was named "prostaglandin" (Euler, 1935). It was later discovered that prostaglandins in human semen do not come from the prostate but from the seminal vesicles (Eliasson R, 1959). Later, people discovered that PG is widely present in other tissues, and PG has multiple functions, but the term PG is still used today. The basic structure of PG is a 20-carbon unsaturated fatty acid consisting of a five-membered aliphatic ring and two side chains. According to the different substituents (mainly hydroxyl and hydrogen) on the five-membered aliphatic ring, PG is divided into A, B, C, D, E, F and other types, which are represented by PGA, PGB, PGC, PGD, PGE, PGF, etc., and the number in the lower right corner represents the number of double bonds in the side chain.

PGE₂ is an important member of the prostaglandin family and the most abundant prostaglandin in the human body (Figure 1.6a). It was shown that PG is a general term for several closely related compounds and that arachidonic acid is a precursor of PGE₂. The numbering of the PGE series (1, 2, 3) is determined by the degree of unsaturation of their fatty acid precursors, which is the number of double bonds. PGE₁ contains only one double bond. PGE₂ retains two cis double bonds in the side chains of its ring structure. PGE₃ contains three double bonds. (Bergström et al., 1964).

1.3 2 The synthetic pathway and signalling pathway of

PGE₂

PGE₂ levels are usually elevated in inflamed tissues. The precursor of PGE₂ is arachidonic acid (AA), which is hydrolysed from the cell membrane by phospholipase A2 (PLA2). Next, AA is converted into prostaglandin G2 (PGG₂) and prostaglandin H2 (PGH₂) by cyclooxygenase (COX, including COX-1 and COX-2). Finally, PGG₂ and PGH₂ are converted into PGE₂ catalysed by PGE synthase (mPGES-1, mPGES-2 and cPGES) (Figure 1.6b) (Ricciotti and FitzGerald, 2011). mPGES-1, mPGES-2 and cPGES mediate the biosynthesis of PGE₂ through different COX coupling modes.

Membrane-bound prostaglandin E synthase-1 (mPGES-1) is a perinuclear-enriched protein belonging to the MAPEG (membrane-associated protein involved in eicosanoid and glutathione metabolism) family and is induced by cytokines, growth factors or lipopolysaccharide and downregulated by anti-inflammatory glucocorticoids (Murakami et al., 2003; Sampey et al., 2005). mPGES-1 requires glutathione (GSH) as a cofactor, is coupled to COX-2 and is highly expressed during inflammatory responses (Chang and Meuillet, 2011). This is the major pathway for PGE₂ synthesis under inflammatory conditions. mPGES-2 is also a membrane-bound PGE synthase. Unlike the former, it is constitutively expressed and can bind to both COX-1 and COX-2 and its function is not yet fully understood (Murakami et al., 2003). The cytosolic enzyme (cPGES), which is constitutively expressed in many organs and tissues, couples to COX-1 and requires the molecular chaperone HSP. cPGES generates PGE₂ under normal physiological conditions (Tanioka et al., 2000; Tanioka et al., 2003).

COX-1 is constitutive, and COX-2 is inducible, which are isoforms of COX and are targets of nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs competitively bind to COX-1 or COX-2 to inhibit prostaglandin

production. COX-2 is mainly expressed in inflamed tissues. It is involved in the production of inflammatory mediators and increases significantly when tissues are damaged or stimulated by inflammation (Sahu et al., 2023). Therefore, inhibiting COX-2 plays an anti-inflammatory role by reducing the production of inflammation-related prostaglandins. COX-1 is normally expressed in various tissues in the body, especially in the stomach, kidneys, and platelets (Zidar et al., 2009). The prostaglandins it produces are important for maintaining the integrity of the gastric mucosa, renal blood flow, and platelet aggregation (Adelizzi, 1999). Therefore, NSAIDs inhibition of COX-1 can lead to gastrointestinal adverse reactions. COX-2 is the main source of prostaglandin formation in inflammation, but COX-1 is also expressed in inflamed tissues. NSAIDs reduce prostaglandin synthesis by inhibiting the activity of COX enzymes, but the degree of inhibition of COX-1 and COX-2 varies (Brooks et al., 1999). Traditional NSAIDs (such as aspirin, ibuprofen, naproxen) inhibit both COX-1 and COX-2, which can effectively relieve inflammation, but may also cause side effects such as gastrointestinal damage and bleeding (Tai and McAlindon, 2021). Selective COX-2 inhibitors such as celecoxib target COX-2, reducing gastrointestinal effects but may still increase the risk of cardiovascular events (Gong et al., 2012).

PGE₂ signals by binding to four different G protein-coupled receptors (GPCRs), EP1-EP4 (Sugimoto and Narumiya, 2007). EP1 receptor activation (coupling to Gq) can increase intracellular Ca²⁺ concentration through phospholipase C (PLC). Activated PLC hydrolyses phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 activates calcium signalling pathways, and DAG activates protein kinase C (PKC), which subsequently activates extracellular signal-regulated kinases (ERK) through RAF (Rundhaug et al., 2011). EP3 has multiple different isoforms, and the isoforms of EP3

receptors are mainly formed by different C-terminus splicing variants. These variants allow EP3 receptors to couple with different G proteins and activate different cell signalling pathways. EP3 α and EP3 β isoforms mainly couple to G_i proteins and reduce intracellular cyclic adenosine monophosphate (cAMP) levels by inhibiting adenylate cyclase (AC) (IRIE et al., 1993; Schaid et al., 2023). EP3 γ subtypes can not only couple to G_i proteins, some EP3 γ isoforms can also couple to G_s proteins, activating the PLC signalling pathway, thereby increasing intracellular Ca²⁺ concentration (Negishi et al., 1996). Both EP2 and EP4 receptors are coupled to G_s and increase intracellular cAMP levels through AC stimulation (Wang et al., 2022), while activation of EP4 can also increase protein kinase B (AKT/PKB) by stimulating PI3K (George et al., 2007). PGE₂ mediates different physiological and pathological processes through EP1-EP4 receptors, and each receptor exerts unique functions through different signalling pathways. This diversity explains the complex role of PGE₂ in various biological phenomena, including pain perception, inflammation regulation, fever response and blood pressure regulation.

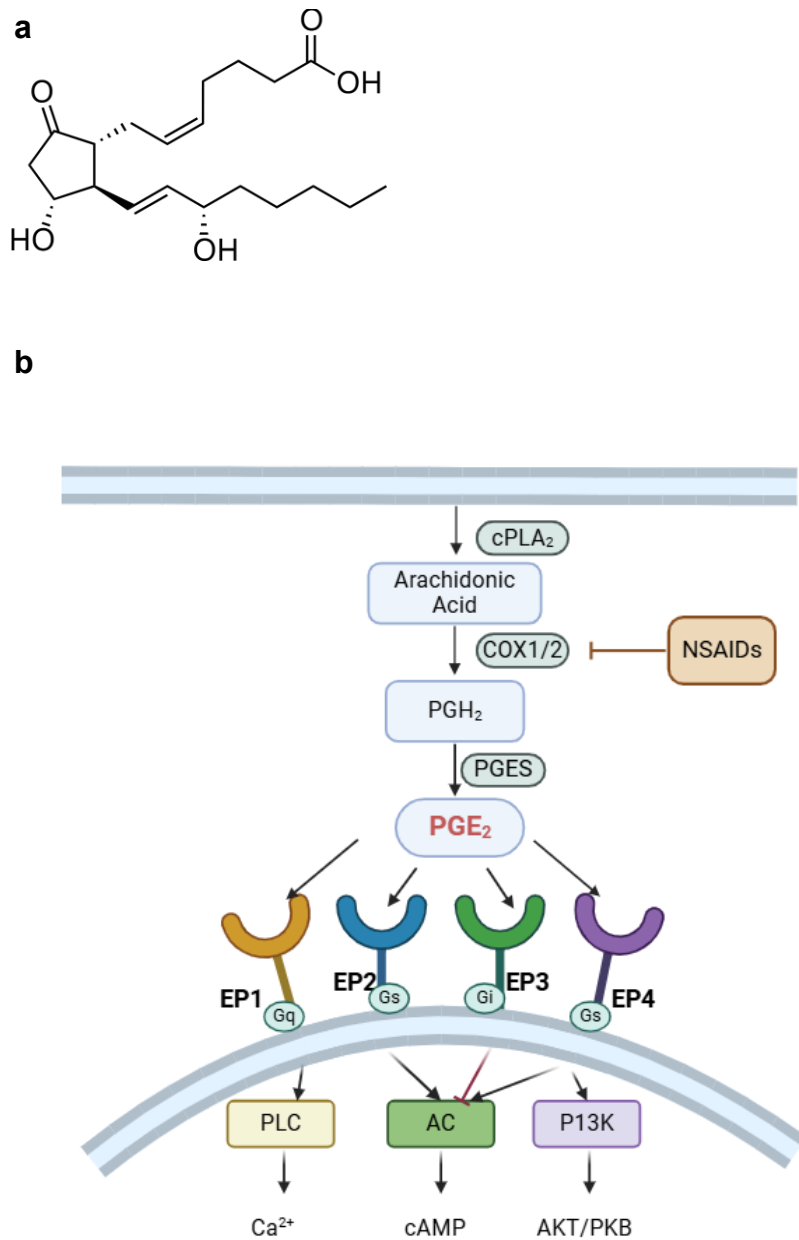


Figure 1.6: The synthetic pathway and signalling pathway of PGE₂. (a) Chemical structure of PGE₂, C₂₀H₃₂O₅. (b) PGE₂ biosynthesis and signalling. Phospholipase A₂ releases arachidonic acid from the cell membrane, which is converted to PGH₂ by COX. NSAIDs inhibit COX-1 and COX-2. PGE₂ is produced by PGE synthase, binds to different EPs, and activates downstream signalling pathways. Figure generated using Biorender.

1.3 3 Pathological and physiological functions of PGE₂

PGE₂ exerts a variety of pathological and physiological functions by binding to specific receptors (EP1, EP2, EP3, and EP4). In physiological processes, PGE₂ regulates body temperature, reproduction, renal function, pain, gastrointestinal, cardiovascular and inflammation. PGE₂ is a pyrogenic mediator, when the body temperature is normal, COX-1 is induced, but when fever occurs, COX-2 is induced to produce a large amount of PGE₂ (Esh et al., 2021). PGE₂ in the central nervous system acts on the hypothalamus to regulate the rise in body temperature, thereby causing a fever response, and this process works through the EP3 receptor (Conti, 2016). PGE₂ plays a key role in female reproduction. It participates in different stages of reproduction and has positive effects, including ovulation, fertilization, embryonic development and early implantation. PGE₂ promotes cumulus cell expansion by upregulating egg genes (including Areg, Ereg, Has2 and Tnfaip6) through EP2 receptor and increasing intracellular cAMP. PGE₂ can also optimize sperm ability to enhance its ability to bind to oocytes. PGE₂ maintains corpus luteum function through EP2 receptor, increases progesterone and promotes embryonic development (Niringiyumukiza et al., 2018). In the kidney, PGE₂ regulates vascular tone by acting mainly in sodium excretion and diuresis through EP1 and increasing renin expression in cells (Wang et al., 2018). PGE₂ maintains normal renal function by affecting tubular water reabsorption and glomerular hemodynamic (Li et al., 2017; Nasrallah et al., 2020). PGE₂ has a protective effect on the gastric mucosa, it is produced and stored in the gastric and duodenal mucosa (Park et al., 1992). PGE₂ can promote the production of gastric epithelial mucus and the secretion of bicarbonate, and inhibit the secretion of gastric acid and pepsin, helping to maintain the acid-base balance in the stomach (Carter et al., 1973; Johansson and Kollberg, 1979; Feldman, 1983; Hoshino et al., 2003). PGE₂ can increase

intracellular cAMP by binding to EP2 and EP4 receptors, leading to vasodilation and lowering blood pressure, however EP1 and EP3 receptors have vasoconstrictive and hypertensive effects (Yang and Du, 2012). PGE₂ stimulates the proliferation of vascular smooth muscle cells through EP2 receptor and regulates the blood supply to local tissues (Yau and Zahradka, 2003).

PGE₂ is widely involved in the pathological processes of pain, inflammation, immunosuppression, tumour formation, and some diseases (such as vascular disease and kidney disease). PGE₂ is a pain mediator that mainly increases intracellular cAMP levels through the EP4 receptor, mediating inflammatory responses and neuropathic pain (St-Jacques and Ma, 2014). In the transmission of pain in inflamed tissues, the PGE₂/EP4 pathway initiates and increases the sensitivity of pain-sensing dorsal root ganglion (DRG) neurons in inflamed tissues to amplify pain signals, so inhibition of EP4 has been shown to alleviate osteoarthritis and rheumatoid arthritis (Caselli et al., 2018; Kimourtzis et al., 2024). In addition to EP4 receptor, EP1 receptor also plays a major role in inflammatory pain, and inhibition of EP1 can reduce PGE₂-induced inflammatory pain sensitivity (Johansson et al., 2011; Wei et al., 2024). Therefore, NSAIDs relieve pain and reduce fever by inhibiting the synthesis of PGE₂. PGE₂ plays a bidirectional regulatory role in inflammation and is involved in both acute and chronic inflammatory diseases (Ricciotti and FitzGerald, 2011). In the acute inflammatory stage, PGE₂ usually promotes inflammatory response and helps the body fight pathogens. Erythema, fever, swelling and pain are the four major symptoms of acute inflammation. Erythema and fever reactions are caused by increased local blood flow due to vasodilation, while swelling reactions are caused by increased vascular permeability and leukocyte recruitment. Erythema and fever reactions are mainly caused by PGE₂ relaxing vascular smooth muscle through EP2 and EP4 receptors to

increase blood flow (Kawahara et al., 2015; Tsuge et al., 2019). Swelling reactions are caused by PGE₂ activating mast cells (MCs) through EP3 receptors, allowing extracellular Ca²⁺ to enter MCs and activate the PI3K-AKT pathway, ultimately leading to excessive vascular permeability and neutrophil recruitment (Kawahara et al., 2015). In acute inflammation, once pathogens and damaged tissues are cleared, usually within hours or days, acute inflammation is relieved, tissues are repaired, granulocyte recruitment stops, proinflammatory cytokines are downregulated and cleared, and then recruited granulocytes are cleared by exocytosis. However, acute inflammation that becomes chronic is often the root cause of various chronic diseases, such as autoimmune diseases, neurodegenerative diseases, vascular and metabolic diseases, and cancer (Yao and Narumiya, 2019). Antigens produced at the site of infection are presented by APCs, such as DCs and macrophages, which present antigens to T cells and activate the adaptive immune system. Activated APCs also produce various cytokines that prompt activated T cells to differentiate into T helper (Th) cells, which are Th1, Th2, and Th17. Th1 and Th17 cells are mainly involved in inflammatory diseases and autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and contact dermatitis (Bouma and Strober, 2003; Steinman, 2007; Lowes et al., 2007; Steinman, 2008; Kawahara et al., 2015). Th1 differentiation is induced by PGE₂-EP2/EP4 signals. This process requires cells to be stimulated by TCR and CD28 at the same time, followed by activation of PI3K, leading to the production of IL-12 and IFN- γ , ultimately inducing Th1 differentiation (Yao et al., 2009; Yao et al., 2013). It has been shown that PGE₂ stimulates Th17 cell expansion in the presence of IL-23. Activated dendritic cells produce IL-23, and PGE₂ can also induce dendritic cells to produce IL-23. Therefore, PGE₂-EP2/EP4 signals induce Th17 cell expansion by inducing DCs to secrete IL-23 through the cAMP-PKA pathway (Yao et al., 2009; Ma et al., 2016). PGE₂ not only promotes

the differentiation of Th17 cells, but also increases the secretion of cytokines such as IL-17, IL-21 and IL-22. They can recruit neutrophils and other inflammatory cells, enhance local pro-inflammatory responses, and thus aggravate tissue damage and inflammation. Therefore, these cytokines play an important role in a variety of chronic inflammatory diseases (Qu et al., 2013). Different from Th1 and Th17 cell, PGE₂ enhances the secretion of cytokines such as IL-4, IL-5, and IL-13 in Th2 cells, which are essential for the production of plasma cells, the formation of IgE antibodies, and the recruitment of eosinophils. These mechanisms play an important role in allergic reactions such as allergic rhinitis, asthma, and atopic dermatitis (Fort et al., 2001; Yao and Narumiya, 2019; Vlaykov et al., 2020). However, PGE₂ also has a certain anti-inflammatory effect. Since PGE₂ can regulate the differentiation direction of Th cells. It inhibits Th1 type response and enhances Th2 type response. Th2 response is more of an anti-inflammatory effect, while Th1 response is a pro-inflammatory effect. Excessive pro-inflammatory response can cause tissue damage (Berger, 2000).

PGE₂ also plays a key role in the occurrence, development and metastasis of cancer. The relationship between inflammation and cancer is very complex and close. Studies have shown that chronic inflammation is likely to increase the risk of cancer (Piotrowski et al., 2020). One of the reasons is that during the inflammation process, immune cells produce a large amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can damage DNA and increase the risk of gene mutation, thus providing a basis for the occurrence of cancer (Piotrowski et al., 2020; Iqbal et al., 2024). In addition, PGE₂ participates in cancer development by promoting angiogenesis, tumour cell proliferation, and immunosuppression. It is reported that PGE₂ is expressed in large quantities in human solid tumours, such as colon cancer, gastric cancer, lung cancer, prostate cancer,

hepatocellular carcinoma, breast cancer, and melanoma (Koga et al., 1999; Soslow et al., 2000; Jang, 2004; Khor et al., 2007; Panza et al., 2016). In the tumour microenvironment, PGE₂ is not only produced by tumour cells, but also secreted by immune cells. These PGE₂ promote tumour growth through autocrine and paracrine mechanisms, activating EP receptors present in cancer cells, which activate a series of intracellular signalling pathways and enhance tumour invasiveness, including growth promotion, evasion of apoptosis, transcriptional activation of tyrosine kinase growth factor receptors and induction of angiogenesis (Finetti et al., 2020). In squamous cell carcinoma of the skin and vulvar cancer, the EP1 expression level in well-differentiated tumour cells was significantly higher than that in poorly differentiated tumour cells (Lee et al., 2005; Buchholz et al., 2023). Studies have shown that PGE₂ phosphorylates ERK through the EP1 receptor, and ERK phosphorylates the transcription factor cyclic adenosine monophosphate response element binding protein (CREB) at ser133 through the Ca²⁺ pathway, enhancing the expression of matrix metalloproteinase (MMP2) (Sun et al., 2013). MMP2 is a key enzyme in tumour invasion. Therefore, the EP1/Ca²⁺/Erk pathway enhances tumour invasion (Sun et al., 2013). In NSCLC cells, PGE₂ activates the epidermal growth factor receptor (EGFR)-independent MAPK/Erk pathway through the EP1 receptor, promoting cancer cell proliferation (Krysan et al., 2005). EP1 receptor also promotes hepatoma cell adhesion and migration by regulating FAK phosphorylation through activation of PKC/c-Src and EGFR signalling pathways (Bai et al., 2013). Both EP2 and EP4 receptors are associated with G_{as} proteins and act through the cAMP-PKA pathway, and they can promote the production of vascular endothelial growth factor (VEGF) and induce cancer cell invasiveness (Spinella et al., 2004). EP2 and EP4 also activated the PI3K/AKT-GSK3 β - β -catenin pathway through PGE₂, enhancing the stability of β -catenin, β -catenin promotes the expression of angiogenic factors such as VEGF and leading to epithelial-

mesenchymal transition (EMT) to promote tumour cell proliferation and survival (Chun et al., 2010; Hsu et al., 2017; Mangelsen et al., 2020; Guo et al., 2024). In mouse skin cancer, tumour formation was delayed after EP3 receptor knockout, and it was found that EP3 receptor promoted mast cell activation and IL-6 production, leading to cancer occurrence (Shoji et al., 2005). EP3 can also promote tumour metastasis and angiogenesis by upregulating MMP-9 and VEGF in endothelial cells (Amano et al., 2009). PGE₂ promotes angiogenesis by stimulating the EP3 subtype of receptors and phosphorylating fibroblast growth factor receptor 1 (FGFR-1), thereby providing blood supply to proliferating tumours (Finetti et al., 2008). PGE₂ activates PI3K and MAPK signals through the EP4 receptor, activates the NF-κB pathway, and induces the formation, maintenance, and expansion of cancer stem cells (CSCs), which are considered to be the main cause of tumour recurrence and treatment resistance (Wang et al., 2015).

1.3.4 Docosahexaenoic acid and eicosapentaenoic acid inhibit PGE₂

DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) are omega-3 polyunsaturated (PUFA) fatty acids that play an important role in human immune regulation (Yang et al., 2014). DHA and EPA affect the production of PGE₂ through competitive metabolism and regulation of inflammatory signalling pathways and play an important role in inflammation and cancer. The synthesis of PGE₂ mainly depends on AA, while DHA and EPA can compete with AA as substrates of COX enzymes, inhibiting the enzymatic activity of the two COX isozymes (COX-1 and COX-2) and downregulating PGE₂ (Ringbom et al., 2001; Yang et al., 2014). EPA can also generate anti-inflammatory mediators, prostaglandin E₃ (PGE₃) and leukotriene B₅ (LTB₅) through the COX and 5-lipoxygenase (5-LOX) pathways. Compared with

PGE₂ and leukotriene B₄ (LTB₄), PGE₃ and LTB₅ tend to have anti-proliferative and anti-inflammatory activities (Calder, 2010). LTB₄ is generated by 5-LOX catalysing AA, and its main function is to strongly promote inflammation and activation of neutrophil functions (Tatsuno et al., 1990). Furthermore, DHA and EPA can reduce the activation of NF-κB (nuclear factor κB), which is an important transcription factor for COX-2 expression, thereby inhibiting COX-2 production and PGE₂ synthesis (Mishra et al., 2004; Starkweather et al., 2020). DHA and EPA also reduce the release of proinflammatory cytokines (such as IL-1β, TNF-α and IL-6), thereby indirectly reducing the expression of COX-2, ultimately leading to a decrease in PGE₂ (Calder, 2010; Mullen et al., 2010). Therefore, EPA and DHA can reduce PGE₂ levels, inhibit tumour growth, and improve chemotherapy effects.

1.4 Suppression of the immune system by PGE₂

In the TME, PGE₂ can inhibit anti-tumour immune responses and help tumours escape the surveillance of the immune system. PGE₂ inhibits the immune response by reducing the activity of effector T cells, inhibiting the function of NK cells, and increasing the activity of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells (Figure 1.7) (Finetti et al., 2020).

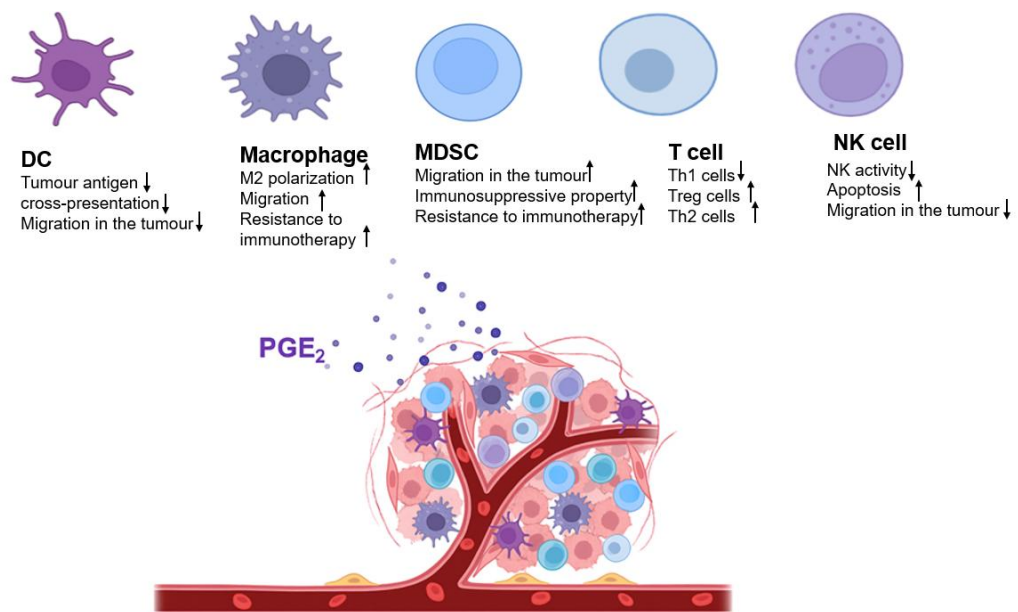


Figure 1.7: The roles of PGE₂ in the regulation of tumour microenvironment. PGE₂ is secreted by cancer cells and immune cells in the TME. The main functions of PGE₂ on MDSCs, tumour-associated macrophages, DCs, NK cells, and T cells are depicted. Figure generated using Biorender.

1.4.1 PGE₂ and myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) were discovered in tumours from patients and in tumour mouse models in the 1970s and 1980s (Young et al., 1987; Seung et al., 1995). MDSC is a heterogeneous cell population, which is an immature myeloid cell that plays an anti-tumour immunity role in TME and promotes tumour growth and metastasis. MDSCs inhibit the anti-tumour response of immune cells by secreting immunosuppressive factors, ROS, nitric oxide (NO), arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS) (Condamine and Gabrilovich, 2011). PGE₂ secreted by tumours can promote the differentiation, recruitment and immunosuppressive function of MDSCs through multiple mechanisms. The research has shown that in mouse breast cancer, MDSCs express all EP receptors and PGE₂ mediates MDSCs differentiation through EP1, EP2 and/or EP4 receptors (Sinha et al., 2007). Moreover, EP2 KO mice have slowed tumour growth and reduced MDSCs accumulation. It was found that the use of COX-2 inhibitors can reduce PGE₂ synthesis and thus delay tumour progression and MDSC differentiation (Sinha et al., 2007). Monocytes can be induced by PGE₂ to produce MDSC-related immunosuppressive factors, as mentioned above. These immunosuppressive factors and PGE₂ induce COX-2 production, leading to the autocrine production of endogenous PGE₂ in MDSCs (Obermajer et al., 2011). PGE₂ can also recruit MDSCs into the TME through chemotaxis, further enhancing the immunosuppressive microenvironment. where the chemokine CCL2 plays an important role in the migration process, while CTLs induce tumour cells to secrete PGE₂, attracting MDSC aggregation and promoting tumour progression (Fujita et al., 2011; Yang et al., 2015). Due to the important role of PGE₂ in MDSCs regulation, targeting PGE₂ or its synthesis pathway to inhibit the function of MDSCs has become a potential anti-tumour strategy.

1.4.2 PGE₂ and Dendritic Cells

PGE₂ has a paradoxical effect on DCs. PGE₂ can regulate the maturation of DCs. It does not induce DC maturation on its own but when it works synergistically with IL-1 β and TNF- α , it can exert its effects at very low concentrations. PGE₂ induces the production of IL-12-deficient DCs and biases naive Th cells toward Th2 development (Kaliński et al., 1998). In addition, PGE₂ upregulates IL-10 and inhibits IL-12 production via EP2 and EP4 to affect the antigen-presenting capacity of DCs (Harizi et al., 2003a). PGE₂ can cause DCs to secrete CCL22, thereby recruiting Tregs to further suppress the immune response (Muthuswamy et al., 2008). Studies have shown that high concentrations of PGE₂ inhibit DC migration, while low concentrations promote cell migration (Diao et al., 2020). A large amount of PGE₂ is produced in the TME, so the migration ability of DCs in the TME is restricted.

1.4.3 PGE₂ and T cells

PGE₂ mainly inhibits IL-2-mediated gene expression and IL-2 receptor expression by producing cAMP, ultimately inhibiting T cell proliferation and activation (Sreeramkumar et al., 2012). PGE₂ inhibits the production of IL-2 and IFN- γ by Th1 cells, but does not inhibit the production of IL-4 and IL-5 by Th2 cells (Betz and Fox, 1991). PGE₂ inhibits T cell function by inducing prolactin expression in T cells through the Ca²⁺ and cAMP signalling pathways of EP3 and EP4 receptors (Gerlo et al., 2004). PGE₂ also enhances the differentiation and function of Th17 cells through EP2 and EP4 (Kalinski, 2012).

1.4.4 PGE₂ and NK cells

PGE₂ has multiple inhibitory effects on NK cells. PGE₂ not only inhibits NK cell proliferation but also increases cell apoptosis (Li et al., 2016). PGE₂ inhibits NK cell cytotoxicity by inhibiting NK receptors through the cAMP/PKA pathway mediated by EP2 and EP4 receptors (Martinet et al., 2010). PGE₂ inhibits the secretion of IFN- γ by NK cells mainly through EP4 receptor (Ma et al., 2013), it also attenuates IL-12- and IL-18-induced NK cell IFN- γ expression via the EP2 receptor (Walker and Rotondo, 2004).

1.5 Aims of this project

Tumour cells and other cell types within the tumour micro-environment are immunosuppressive and enable cancer cells to avoid immune-mediated destruction. One mechanism of immune escape is through secretion of immunosuppressive molecules. Several cancers are known to secrete PGE₂ in the TME, which inhibits various functions of immune cells, via engagement with the PGE₂ receptors EP1-4.

This research aims to determine the mechanisms of tumour-derived PGE₂ inhibition of NK cells and ultimately find ways to inhibit PGE₂ for cancer therapy. Because the mechanism of PGE₂'s inhibitory effect on NK cells is not clear in the literature, this work focuses on the inhibitory pathways of PGE₂ on NK cells.

The main aims were:

1. To determine how PGE₂ inhibits NK cell activation.
 - Evaluate the effect of PGE₂ on NK cell activation by cytokines and oncolytic viruses.
2. To elucidate the mechanisms underlying PGE₂-mediated inhibition of NK cells.
 - Identify the signalling pathways affected by PGE₂ in NK cells.
3. To investigate the mechanisms of PGE₂ production in tumours and its impact on NK cells.
 - Examine how tumour-derived PGE₂ inhibits NK cell functions within the tumour microenvironment.

Chapter 2

Materials and methods

2.1 Cell culture

2.1.1 Cell culture

All cells were cultured in Corning® flasks or tissue culture plates, and maintained in a humidified atmosphere (5% CO₂ and 37°C). Cells were routinely passaged when near confluence. Firstly, cells were washed with phosphate buffered saline (PBS, Sigma), and then 1-2 mL of pre-warmed 1x trypsin-EDTA solution (Sigma) added to cells for 3-5 minutes at 37°C. Cell culture was performed under aseptic conditions using Nuair Class II biological safety cabinets.

2.1.2 Cell lines

Cell lines and their complete culture medium are summarised in Table 2-1. Two Ewing sarcoma cell lines (SK-N-MC and TC-32) harbouring characteristic EWSR1:FLI1 gene fusion and two osteosarcoma cell lines (MG63 and HOS) were from the American Type Culture Collection (ATCC). The HCA-7 colorectal adenocarcinoma cell line was kindly provided by Nametso Khumo (University of Leeds). The identity of cell lines had been previously confirmed using short tandem repeat (STR) profiling.

Table 2.1: Cell lines

Cell line	Origin	Complete growth medium
SK-N-MC	Ewing Sarcoma	DMEM +10% FBS
MG63	Osteosarcoma	DMEM +10% FBS
HOS	Osteosarcoma	DMEM +10% FBS
TC32	Ewing Sarcoma	RPMI1640 +10% FBS
HCA7	Colon adenocarcinoma	RPMI1640 +10% FBS

2.1.3 Primary mesenchymal stem cells

Primary mesenchymal stem cells (MSCs) were isolated from bone marrow of patients (recovered during trauma surgery), and provided by Dr Heather Owston (University of Leeds). Cells were cultured in Dulbecco's modified eagles medium (DMEM) (Sigma-Aldrich, #D6429) or Roswell Park Memorial Institute (RPMI1640) (Sigma-Aldrich, #8758) medium supplemented with 10% foetal bovine serum (FBS) (Gibco), 100 units of penicillin and 0.1 mg/mL streptomycin.

2.1.4 Cryopreservation

For long-term storage, cell lines were stored in complete growth medium with 10% dimethyl sulfoxide (DMSO). Cells were then frozen at -80°C in a Mr Frosty™ freezing container for 24 hours, and finally transferred to gas phase liquid nitrogen.

2.1.5 Recovering frozen cells

Cryovials containing cell lines were thawed in a 37°C water bath for 2 mins. Then the cells were diluted in 10 mL complete growth medium in a 15 mL falcon tube, and cells were spun down at 400 xg for 5 minutes, the supernatant was discarded. Cell pellets were resuspended in 10 mL complete growth medium. The cell suspension was put into a T75 flask and adding another 5 mL medium to ensure that the media is covering the whole area of the flask. The cells were maintained in a humidified atmosphere (5 % CO₂ and 37°C).

2.1.6 Peripheral blood mononuclear cells (PBMCs) isolation and culture

Since PBMCs and red blood cells have different densities, the density gradient centrifugation process can separate the cells in lymphoprep (STEMCELL Technologies, #07851), PBMCs were isolated from apheresis cones. Apheresis cones were purchased from National Health Service Blood and Transplant (NHSBT) as a waste product from healthy donors. Hanks Balanced Salt Solution (Sigma-Aldrich, #9394) was used to dilute the blood in a 50 mL falcon tube, to a final volume of 40 mL. Next, 15 mL

Lymphoprep™ was added to two 50 mL falcon tubes, and 20 mL of diluted blood was carefully layered onto the solution. Tubes were centrifuged at 800 *xg* for 20 minutes (Brake 3 and acceleration 0). Post-centrifugation the PBMC fraction (white layer as shown in Figure 2.1) was removed using a 2 mL Pasteur pipette and transferred into a 50 mL Falcon tube. Then 40 mL of Hanks Balanced Salt Solution was added to the PBMCs before centrifugation at 200 *xg* for 20 minutes (Brake 9 and acceleration 3). The supernatant was discarded and the PBMCs were washed again in 40 mL Hank buffer before centrifugation at 400 *xg* for 10 minutes. The supernatant was discarded and PBMCs were cultured in a plate or T75 flasks in RPMI with 10% FCS at a density of 2×10^6 cells/mL and kept in a humidified atmosphere (5% CO₂, at 37°C). Figure 2.1a shows the detection of PBMCs under flow cytometry.

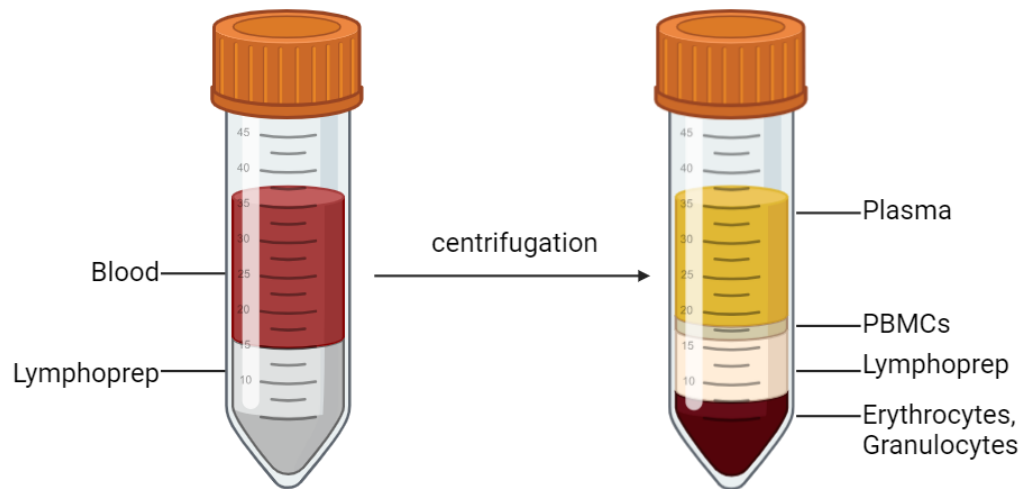


Figure 2.1: Density gradient centrifugation to isolate PBMCs. The volume, shape and specific gravity of mononuclear cells are different from those of other cells in the peripheral blood. Therefore, the difference between the specific gravity of various blood cells and mononuclear cells can be used to separate various blood cells from mononuclear cells. Before centrifugation (left) upper layer: blood, lower layer: Lymphoprep. After centrifugation (right), it is divided into four layers (from top to bottom): Plasma, PBMCs, Lymphoprep and Erythrocytes/ Granulocytes. Figure generated using Biorender.

2.2 Cell selection using magnetic beads

2.2.1 CD56 positive selection of NK cells

Magnetic beads coated with anti-CD56 antibody, termed CD56 MicroBeads (Miltenyi Biotec, #130050401) were developed for the positive selection of NK cells from human PBMCs. Most NK cells express CD56 antigen, and a minor T cell subset (CD3⁺CD56⁺ NKT cells) also express CD56 antigen on their surface (as Figure 2.2a shows).

NK cells were isolated from PBMCs by positive selection using CD56 beads (Miltenyi Biotec). Firstly, PBMCs were centrifuged at 400 *xg* for 5 minutes then resuspended in cold MACS buffer (PBS with 0.1 % FCS and 0.5M EDTA) and mixed with CD56 beads. After incubating for 15 minutes at 4°C, cells were washed using MACS buffer and centrifuged at 400 *xg* for 5 minutes. Finally, cells were resuspended in MACS buffer. LS columns (Miltenyi Biotec) were placed in a magnetic stand and cells applied onto the LS column and washed with 3 x 3 mL MACS buffer. The unlabelled cells passed through after washing and purified NK cells were collected by releasing the column from the magnet and flushing the column with 5 mL MACS buffer. NK cells were cultured in RPMI 1640, supplemented with 10 % FCS at 37°C, 5 % CO₂.

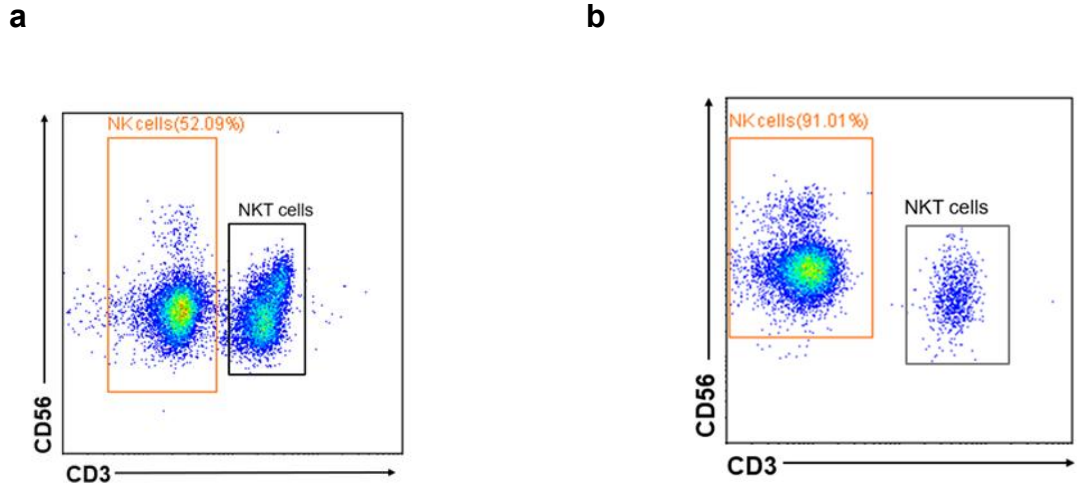


Figure 2.2: NK cell selection using magnetic beads. (a) Gating on lymphocyte population using a FSC-A (forward scatter-area) – SSC-A (side scatter-area) dot plot. (b) Gating on the CD56⁺CD3⁻ NK cell population after using CD56 positive selection. (c) Gating on the CD56⁺CD3⁻ NK cell population after using negative selection.

2.2.2 Negative selection of NK cells

The NK Cell Isolation Kit (Miltenyi Biotec, #130092657) was developed for the untouched isolation of NK cells from human PBMCs. The cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail in this kit can magnetically label the non-NK cells, such as T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells; this procedure selects out all other cells without touching the NK cells and, by depleting non-NK cell types, NK cells can be isolated at high purity.

NK cells were isolated from PBMCs by negative selection using an untouched kit (Miltenyi Biotec). PBMCs were resuspended in cold MACS buffer and Biotin-Antibody after spun down at 400 *xg* for 5 minutes. Biotin-Antibody labelled other cells in PBMCs except NK cells. After incubating for 5 minutes at 4°C, cells were mixed with MACS buffer and anti-biotin magnetic beads, and then had a further incubation of 10 minutes at 4 °C. Then LS column was placed in a strong magnetic stand, and cells were applied onto the column and washed by 3 mL MACS buffer. NK cells were collected after washing, and finally cultured in RPMI 1640, supplemented with 10 % FCS at 37°C, 5 % CO₂.

This procedure selects the non-NK cells (using antibodies against monocytes, T cells and B cells), leaving NK cells untouched; figure 2.2c shows that after the purification, the purity of NK cells reaches ~90% (Figure 2.2b). This is higher than the purity of NK cells isolated by direct selection (anti-CD56 beads) shown in Figure 2.2a (about 50%). Both purification methods result in the isolation of NKT cells, although the negative selection resulted in fewer residual NKT cells than positive selection (Figure 2.2).

2.3 Cell treatments

2.3.1 Reovirus treatment

Reovirus was provided by Dr Tyler Barr. Plaque assay was used to determine the viral titers of reovirus stocks, and Dr Tyler Barr performed this assay. Briefly, L929 cells were seeded into 6 well plates at a density of 5×10^5 per well, and left to adhere overnight. Reovirus stocks were diluted in 10-fold serial dilutions in serum free DMEM. Dilutions ranging from 10^{-3} to 10^{-8} were added to wells in 100 μ L of serum free DMEM, with an additional 500 μ L media, and incubated for 2.5 hours. Samples were then removed and replaced with 2 mL DMEM + 10% FBS with 1.6% carboxymethylcellulose (w/v; CMC) at a ratio of 2:1. Plaque formation was monitored for 2-4 days, once plaques were visible, medium was removed and cells washed with PBS, fixed in 1% PFA and 0.5 mL of 1% (w/v) methylene blue in ethanol and distilled H₂O (1:1) was added to wells for 2 minutes. Plates were washed in H₂O and viral titre quantified as plaque forming units (pfu) per mL of virus stock.

PBMCs were seeded into cell culture plates (Corning) at 2×10^6 cells per mL, and NK cells were seeded at 1×10^6 cells per mL. Then, reovirus was added to the cells at 0.1 pfu/mL or 1 pfu/mL (plaque forming units per mL). Plates were returned to standard cell culture conditions (5 % CO₂, at 37°C) and cultured for 48 hours. After the treatment, the cells were spun down at 400 $\times g$ for 5 minutes and washed twice with PBS for flow cytometry. The supernatants were carefully moved into Eppendorf tubes and frozen at -20°C for IFN- γ ELISA assays.

2.3.2 Cytokine treatment

PBMCs were seeded into cell culture plates (Corning) at 2×10^6 cells per mL, and NK cells were seeded at 1×10^6 cells per mL. IL-15 or IL-2 was added to cells at 5 ng/mL or 10 ng/mL (Table 2.2), and plates were returned to standard cell culture conditions (5 % CO₂, at 37°C) and cultured for 48 hours. After the treatment, the cells were spun down at 400 xg for 5 minutes and washed twice with PBS for flow cytometry. At the same time, the supernatant has been frozen at -20°C for IFN- γ ELISA assays.

Table 2.2: Cytokines for cell activation

Cytokine	Manufacturer	Stock concentration
Human Interleukin 2 (IL-2)	Miltenyi Biotec	20 μ g/mL
Human Interleukin 15 (IL-15)	PeptoTech	5 μ g/mL
Human Interleukin 12 (IL-12)	PeptoTech	10 μ g/mL
Human Interleukin 18 (IL-18)	PeptoTech	10 μ g/mL

2.3.3 PGE₂ treatment

PGE₂ (Selleckchem) was added to the cells at 1 μM or 10 μM and cultured for 1 hour at 37 °C, 5 % CO₂. The cells were treated with reovirus or cytokines for stimulation following the PGE₂ treatment.

2.3.4 PGE₁ alcohol treatment

PGE₁ alcohol is a mimic of PGE₂. Firstly, cells were seeded into cell culture plate at 1x10⁵ cells per mL for 24 hours. PGE₁ alcohol was added to the cells at 1 μM for 48 hours, then the plates were returned to standard cell culture conditions (5 % CO₂, at 37°C). After the treatment, cells were removed by centrifugation (at 400 xg, 5 minutes), cell culture supernatants were collected and stored at -20°C.

2.3.5 Use of PGE₂ receptor inhibitors

NK cells were pre-treated with 10 μM EP2 inhibitor (AH 6809, Cayman) and EP4 inhibitor (GW 627368X, Cayman) for 1 hour. 10 μM PGE₂ was added to the cells for another hour. After those two treatments, cells were treated with 10 ng/mL IL-15 for 48 hours at 37 °C, 5 % CO₂. Then the supernatant was collected for IFN-γ ELISA.

2.4 Flow cytometry

Flow Cytometry was performed using a Cytoflex LX flow cytometer (Beckman Coulter) and data was analysed using the analysis software CytExpert 2.1.

For all flow cytometry experiments, PBS was used for washing and 1% PFA/PBS (v/v) solution was used to fix cells. Cells were centrifuged at 400 *xg* for 5 minutes, unless otherwise stated. The antibodies against CD56 and CD3 were added to the cells, in order to identify the CD3⁻ CD56⁺ NK cells within PBMCs. In addition, cells were stained with isotype-matched control antibodies to assess background staining levels for markers of interest. A gate was set at 2 % for positive staining based on the isotype control. Table 2.3 shows the list of antibodies were used in this thesis.

2.4.1 Live/Dead™ cell staining

Cell viability was determined using the LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (ThermoFisher). The working principle of this assay is to use the difference in fluorescence intensity to distinguish live and dead cells (Figure 2.3). In live cells, the dye only acts on the amines on the cell surface, resulting in low fluorescence intensity. In dead cells, because the cell membrane of dead cells is compromised, the dye will react with free amines inside the cells and on the cell surface, producing strong fluorescent staining; the difference in fluorescence intensity between live cells and dead cells is usually greater than 50 times, so it is very easy to distinguish. The excess reactive dye can be subsequently washed away. LIVE/DEAD™ yellow stain was reconstituted in 50 μ L DMSO and stored at -20°C for up to two weeks.

After cell treatment, cells were harvested and transferred into 5 mL round bottom FACS tubes (BD Falcon™), then centrifuged at 400 xg for 5 minutes. The supernatant was discarded carefully and then cells were washed in PBS for three times. After washing, cells were then resuspended in the LIVE/DEAD™ yellow stain solution (LIVE/DEAD™ stain: PBS; 1:1000) at 1×10^6 cells per mL. Cells were incubated at 4 °C for 30 minutes protected from light. After incubation, cells were washed twice with PBS and then fixed in 1% PFA/PBS (v/v) solution. Flow cytometry was used to detect the fluorescence intensity of the samples (Figure 2.4).

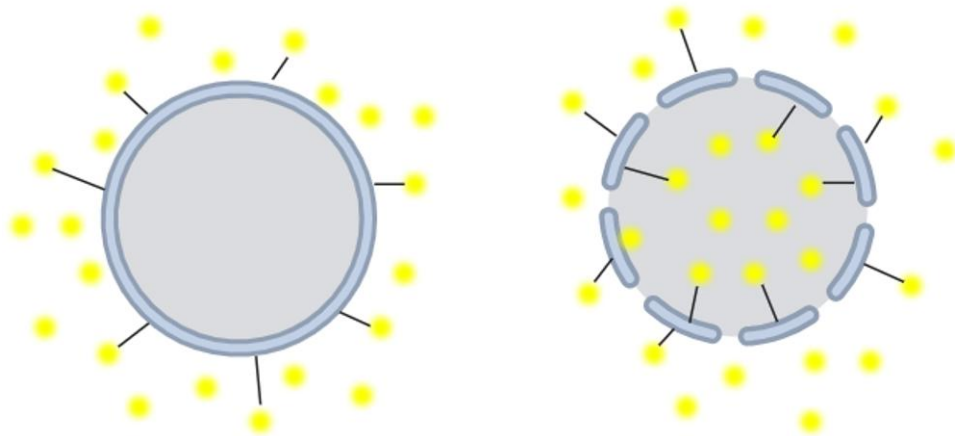


Figure 2.3: The working principle of LIVE/DEAD™ cell staining. Live cells (left) react with the LIVE/DEAD™ dye only on their surface. Dead cells with compromised membranes (right) react with the dye, both in the cell interior and on the cell surface. Figure generated using Biorender.

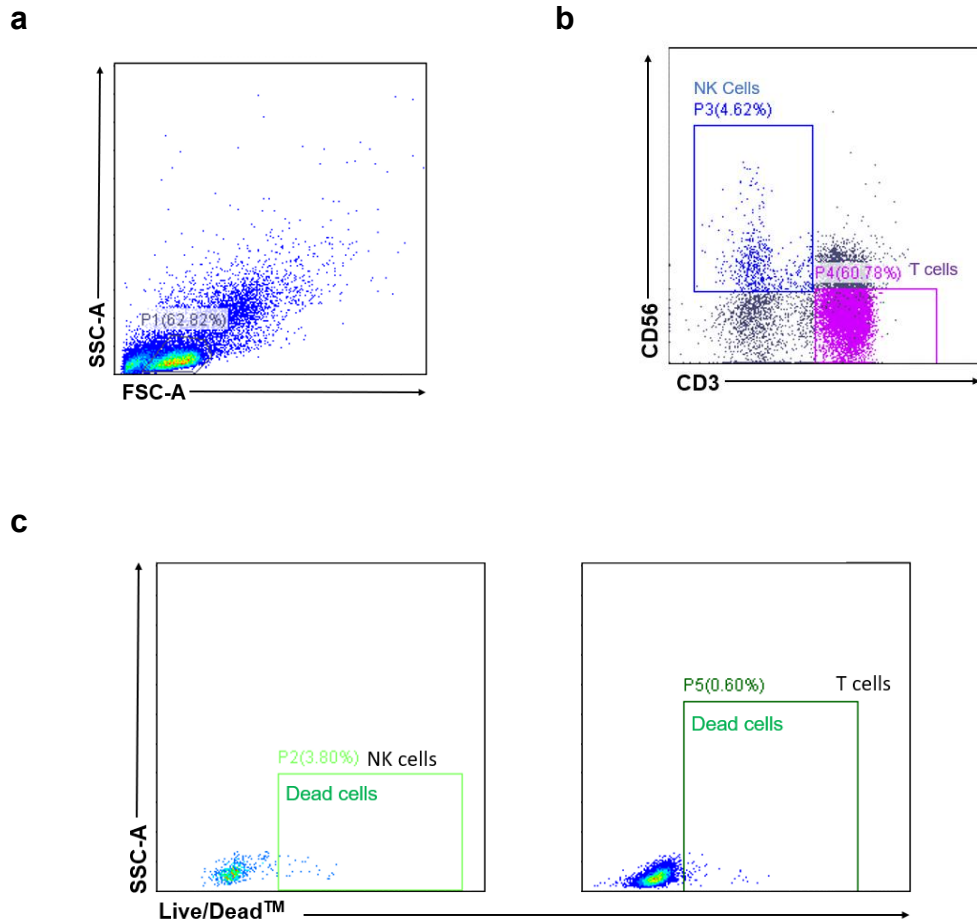


Figure 2.4: Gating strategy for LIVE/DEAD™ cell staining. (a) Gating on lymphocyte populations using a FSC-A- SSC-A dot plot. (b) Gating on the CD56⁺CD3⁻ NK cell population and CD56⁺CD3⁺ T cell population. (c) Gating on dead cells to determine the percentage of dead cells.

2.4.2 Lymphocyte activation assays

After the treatments, PBMCs were spun down at 400 *xg* for 5 minutes, cells were harvested into 5 mL round bottom polystyrene tubes (FACS tubes) and washed twice in PBS. Then the cells were resuspended at approximately 1×10^6 cells/100 μ L in PBS buffer with Anti-CD3 anti-CD56, anti-CD69 or IgG1 isotype antibodies (Table 2.3). After the incubation for 30 minutes at 4°C in the dark, cells were then washed twice by PBS (at 400 *xg* for 5 minutes) and then fixed in 200 μ L 1% PFA/PBS (v/v) solution. Cells were kept in 4°C and protected from light before flow cytometric analysis. Flow cytometry was used to detect the expression of CD69 (a marker of lymphocyte activation) on NK cells.

2.4.3 NK cell degranulation assays

CD56 purified NK cells (Figure 2.2b) or PBMCs were co-cultured with tumour target cells in a 12-well cell culture plate (Corning), at a 10:1 (effector: target) ratio for an hour at 37°C. After the incubation, anti-CD3, anti-CD56, anti-CD107a (LAMP-1) antibodies (Table 2.3) and Brefeldin A (BioLegend) were added to the cells for a further 4 hours at 37°C. After the treatment, cells were harvested into 5 mL FACS tubes and washed twice in PBS (at 400 *xg* for 5 minutes). Cells were resuspended in 200 μ L 1% PFA/PBS (v/v) solution, and kept in 4°C in the dark before flow cytometric analysis. Staining was analysed on a flow cytometer and NK cell degranulation was quantified by assessing expression of cell surface CD107a (Figure 2.5). Figure 2.5c showed that only adding the target cells also increased NK cell degranulation.

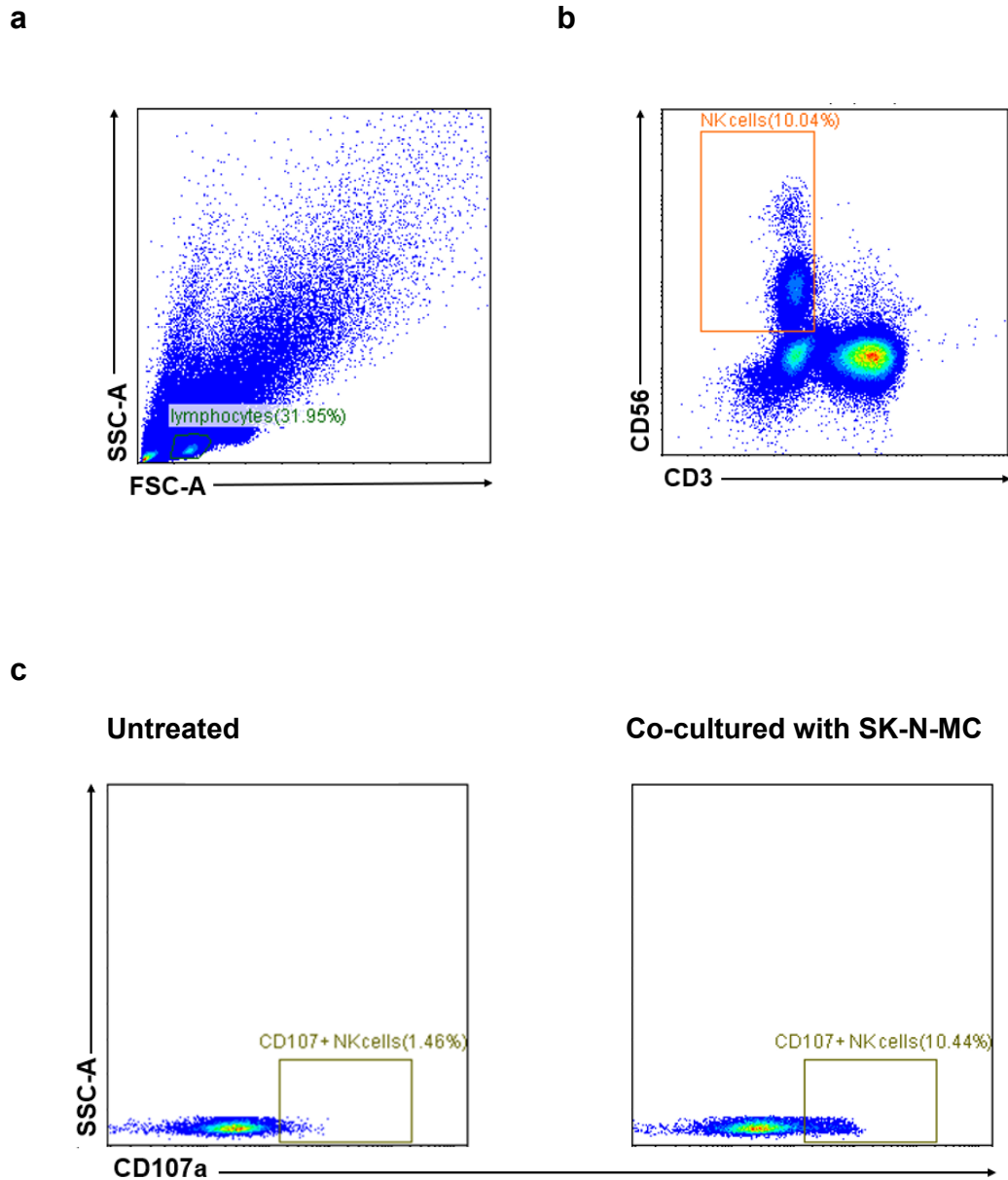


Figure 2.5: Gating strategy for NK cell degranulation assays. (a) Gating on lymphocyte population using a FSC-A- SSC-A dot plot. (b) Gating on CD3⁺CD56⁺ NK cells. (c) Gating to establish the percentage of CD107⁺ degranulating NK cells. This example uses the Ewing sarcoma cell line SK-N-MC as a tumour target cell.

2.4.4 Intracellular staining

Intracellular staining was used to determine levels of phosphorylated STAT proteins. After the treatment, cells were collected into the 5 mL FACs tubes and washed with staining buffer (BD Biosciences) (at 400 *xg* for 5 minutes). Cells were resuspended at 1×10^6 cells/100 μ L staining buffer, anti-CD3 and anti-CD56 antibodies were added to the cells for 30 minutes incubation at 4°C, in the dark. Then cells were washed once in staining buffer and resuspended in staining buffer.

Cytofix fixation buffer was pre-warmed to 37°C (BD Biosciences), then added in equal volume to the cell suspension and incubated at 37°C for 10 minutes. After centrifugation (at 400 *xg* for 10 minutes), the pellet was resuspended in 1 mL of per-cold Perm Buffer III (-20°C) (BD Biosciences) and incubated on ice for 30 minutes. Samples were resuspended in staining buffer (approximately $1 \times 10^6/100 \mu$ L) containing the appropriate anti-phospho-STAT antibodies or isotype controls (Table 2.3) for 30 minutes at room temperature, protected from light, following three washes with 3 mL staining buffer. Samples were washed twice in staining buffer (at 400 *xg* for 5 minutes) and resuspended in 200 μ L staining buffer (at 4°C in the dark) before flow cytometric analysis.

Table 2.3: Flow cytometry antibodies

Antibody	Conjugate	Volume per 10 ⁶ cells (μ L)	Manufacturer (Product number)
NK cell activation			
Anti-CD3	PerCP	1	Miltenyi Biotec #130113131
Anti-CD56	eFluor450	4	Invitrogen™ #48056642
Anti-CD69	PE	5	BioLegend #310906
IgG1 isotype control	PE	5	BioLegend #400112
NK cell degranulation			
Anti-CD107a	PE	2	Miltenyi Biotec #130111621
Anti-CD3	PerCP	1	Miltenyi Biotec #130113131
Anti-CD56	eFluor450	4	Invitrogen™ #48056642
IgG1 isotype control	PE	5	BioLegend #400112
NK cell intracellular staining			
pY694 STAT5	PerCP Cy5.5	10	BD Biosciences #560118
Human IgG1 isotype control	PerCP Cy5.5	10	BD Biosciences #552834
pY701 STAT1	APC	10	Miltenyi #130105092

Human IgG1 isotype control	APC	10	Miltenyi #130113450
pY693 STAT4	PE	6	BD Biosciences #562073
Human IgG1 isotype control	PE	6	BD Biosciences #559529

2.5 Enzyme-linked immunosorbent (ELISA) assays

After cell treatments, cell free supernatants were collected by centrifugation of samples at 400 xg for 5 minutes and carefully pipetting the supernatants and transfer into Eppendorf tubes. Samples were stored at -20°C until needed.

2.5.1 ELISA for interferon γ

96 well MAXIsorp plates (Thermo Fisher Scientific) were used for the sandwich ELISA assay. Blocking buffer (PBS + 10% FCS) was used for block step and PBST washing buffer (PBS + 0.05 % Tween) was used for wash steps.

At first, the IFN- γ capture antibody (Table 2.4) was 1:250 (capture antibody: coating buffer) diluted in the ELISA coating buffer (0.1M NaHCO₃ pH8.2), to a final concentration of 10,000 pg/mL, and added to the 96 well plate at 100 μ L per well. The plate was coated overnight at 4°C. The next day, the solution was discarded, and the plate was washed with PBST buffer for three times before adding the blocking buffer at 200 μ L per well for 2 hours at room temperature. The standards and cell supernatant samples were diluted in RPMI 1640 (Sigma) + 10 % FCS by following the plate three washes by PBST buffer and added to the plate. Recombinant IFN- γ (Miltenyi) of known concentration (from 0 to 10,000 pg/mL) were added to the plate to make a standard curve; IFN- γ was diluted from a stock of 2.5 μ g/mL. Firstly, the IFN- γ stock was diluted as 1:10 and did 1:250 dilutions, then with 6 sequential 1 in 2 dilutions and the blank standard was only the medium. All samples and standards were added in duplicate at 100 μ L per well and the plate was incubated overnight at 4°C. Next, the standards and samples were discarded, and the plate was washed six times in PBST

buffer. The IFN- γ detection antibody (Table 2.4) was 1:500 (detection antibody: blocking buffer) and added to the plate at 100 μ L per well. The plate was incubated for 2 hours at room temperature, then washed another 6 times by PBST buffer. Avidin conjugated alkaline phosphatase (Sigma, ExtrAvidin) was diluted in PBST buffer (1 in 5000) and added to the plate at 100 μ L per well for 1 hour incubation at room temperature. After incubation, the solution was discarded, the plate was washed three times with distilled water after washing with three times with PBST buffer. Finally, p-Nitrophenyl phosphate (pNPP) (Sigma, SigmaFast) substrate was added to the plate at 100 μ L per well. One pNPP tablet (1.0 mg/mL) and one 0.2 M Tris buffer tablet are dissolved in deionized water. The plate was incubated at room temperature for about 30 minutes, protected from light. Then absorbance of the plate was read at 405 nm using a Cytation 5 Imaging Plate Reader (Bio Tek).

Table 2.4: IFN- γ ELISA antibodies

Target molecule	Manufacturer	Role	Dilution
IFN- γ	BD Biosciences #554550	Detection (biotinylated)	1:500
IFN- γ	BD Biosciences #551221	Capture	1:250

2.6 PGE₂ ELISA

The concentration of PGE₂ was detected by PGE₂ ELISA (R&D System, # KGE004B). At the beginning, the working standards and samples were diluted with Calibrator Diluent RD5-56. PGE₂ standards of known increasing concentration (0 to 2500 pg) were added to the plate to make a standard curve. PGE₂ standard was diluted from 25,000 pg/mL, the standard stock was diluted 1 in 10 before with 6 sequential 1 in 2 dilutions and the blank standard was only Calibrator Diluent RD5-56. Calibrator Diluent RD5-56 was also added to the non-specific binding (NSB) wells at 200 µL per well. PGE₂ standards, and supernatants were added to the remaining wells at 150 µL per well on the goat anti-mouse IgG 96 well microplate (R&D Systems). After adding 50 µL of the PGE₂ Primary Antibody Solution to each well (excluding the NSB wells), all wells except the NSB wells would be blue in colour. The plate was incubated for 1 hour at room temperature on a shaker at 500 rpm following covered with a plate sealer. After incubation, PGE₂ Conjugate was added to each well at 50 µL except the NSB wells and the plate incubated for 2 hours at room temperature on the shaker. Following four washes with PGE₂ wash buffer (diluted 1:25 in deionized water), substrate solution (colour reagent A and B were mixed together in equal volumes within 15 minutes) was added to each well at 200 µL and incubated the plate for 30 minutes at room temperature, protected from the light. Using stop solution at 100 µL per well to stop the reaction, and the colour in the wells changed from blue to yellow. A Cytation 5 Imaging Plate Reader (Bio Tek) was used to read the plate at 450 nm and determine the optical density of each well.

2.7 Cyclic adenosine monophosphate (cAMP) ELISA

2.7.1 Cell lysis

After treatment, cells were centrifugated at 400 *xg* 5 minutes and supernatant was discarded. Cells were washed three times in cold PBS and resuspended in cell lysis buffer (diluted 1:5 in deionized water) to a concentration of 1×10^7 cells per mL. Cells needed to be lysed by repeating the freeze (-20°C) /thaw (with gentle mixing) cycles. Cell lysis was confirmed using trypan blue staining and microscopy (intact cells exclude trypan blue, lysed cells are intensely stained). After cell lysis, cellular debris was removed by centrifugation at 600 *xg* for 10 minutes at 4°C. Lysates were stored at -20°C until assayed.

2.7.2 cAMP ELISA

The concentration of cAMP was detected by cAMP ELISA (R&D System, # KGE002B). cAMP primary antibody solution was added at 50 μ L to all wells except the NSB wells on a goat anti-mouse IgG 96 well plate (R&D Systems). The plate was covered with an adhesive strip and incubate for an hour at room temperature on a microplate shaker at 500 rpm. The plate was washed by cAMP wash buffer (diluted 1:25 in deionized water) filling 400 μ L each well for a total of four washes. Remaining wash buffer needed to remove at the last wash, since complete removal wash buffer could show better performance. Then, cAMP conjugate was added to all wells at 50 μ L. The working standards, samples were diluted in Calibrator Diluent RD5-55. cAMP standards of known increasing concentration (240 pmol/mL-0 pmol/mL) as the standards were added to the plate, to make a standard curve. cAMP standard was diluted from 2400 pmol/mL, the standard stock was diluted 1 in 10 before with 6 sequential 1 in 2 dilutions and the blank

standard was only Calibrator Diluent RD5-55. Working standards and samples were added to the plate at 100 μ L per well, the plate was covered with a new adhesive strip and incubated for 2 hours at room temperature on a microplate shaker at 500 rpm. cAMP substrate solution (colour reagent A and B were mixed together in equal volumes within 15 minutes) was added to each well at 200 μ L and incubated the plate for 30 minutes at room temperature, protected from the light, after four washes with cAMP wash buffer. Stop solution was added to all wells at 100 μ L following the incubation, the colour in the wells changed from blue to yellow. To determine the optical density of each well, A Cytation 5 Imaging Plate Reader (Bio Tek) was used to read the plate at 450nm.

2.8 Western Blotting

2.8.1 Preparation of cell lysates

Cells were harvested into Eppendorf tubes, centrifuged at 400 xg for 5 minutes, then washed twice with 1 mL PBS. After that, cells were lysed with lysis buffer. Lysis buffer was made using the M-PER™ Mammalian Protein Extraction Reagent (thermos scientific) with adding 1% 0.5M EDTA and 1% Halt™ Protease & Phosphatase Inhibitor Cocktail (thermos scientific). Lysates were incubated on ice for 60 minutes on a shaker, then stored the samples at -20°C until used.

2.8.2 Protein quantitation using bicinchoninic acid (BCA)

The samples of total protein were quantified by the BCA protein Assay kit which is a detergent-compatible formulation based on bicinchoninic acid (BCA). Firstly, samples were spun down at 10,000 rpm for 5 minutes and

collected the supernatant into new eppendorf tubes. Samples were diluted 1:10 in deionized water. Then, the albumin standards (BSA) were prepared, standards of known increasing concentration (2000 µg/mL-0 µg/mL). Standards and diluted samples were added to the 96-well plate at 20 µL each well. BCA working reagent was mixed with BCA reagent A and reagent B (50:1) and added to the plate at 180 µL per well and mixed the wells. The plate was incubated for 30 minutes at 37°C. After the incubation, cool plate to room temperature and measure the absorbance at 540 nm on a Cytation 5 Imaging Plate Reader (Bio Tek).

2.8.3 SDS-PAGE

After determining the protein concentration of the samples, the amount to be loaded was calculated. On the day of use, samples were thawed and 4x Laemmli sample buffer (Bio rad) which was added with 2-mercaptoethanol (1:10, sample buffer: 2-Mercaptoethanol) was added to samples. Samples were kept on ice after samples were heated at 95°C for 5 minutes, to denature and reduce protein.

A 10% Mini-PROTEAN TGX Precast Protein Gel (Bio rad) was used for running. The gel was placed into a miniPROTEAN Tetra Cell electrophoresis tank (Bio rad) and submerged in 1x running buffer (Bio rad). Samples were loaded into the gel, and a Precision Plus Protein™ Dual Color Standards (Bio rad) was used to confirm the size of the target protein with a potential difference of 120V for 1.5 hours.

2.8.4 Western transfer

A Hybond-P polyvinylidene fluoride (PVDF) membrane (Bio rad) was activated in methanol for 30 seconds and washed twice with deionized water before immersing in 1x transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol). The gel was then placed on top of the membrane, between the two filters and sponges, so that the proteins were transferred from the gel to the membrane, they were placed into a miniPROTEAN Tetra Cell electrophoresis tank (Bio rad) and submerged in 1x transfer buffer (4°C), a potential voltage difference of 100V was applied for 1 hour with an ice box.

2.8.5 Antibody Staining

After transfer, the membrane with transferred protein was blocked in 1x blocking buffer (25 mM Tris base, 134 mM sodium chloride, with 0.1% Tween-20 and 5% skimmed milk) for an hour, with continuous agitation. The blocking buffer was discarded, the membrane was immersed in primary antibody (Table 2.5) (diluted in blocking buffer) at the specified dilution and incubated overnight at 4°C with continuous agitation following washed once with PBS. The primary antibody solution was removed, and the membrane was washed in TBST (1X Tris buffered saline with 0.1% Tween) for 10 minutes for a total three washes. The secondary antibody (Table 2.5) (diluted in TBST) was added at the specified dilution and the blot was incubated for 1 hour at room temperature, with continuous agitation. The secondary antibody solution was removed, and the membrane washed three times in TBST for 5 minutes. An enhanced chemiluminescence (ECL) solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, thermos scientific) was pipetted over the membrane for one minute, then the membrane was read using a ChemiDoc Imagers (Bio rad).

Table 2.5 Western Blotting antibodies

Target	Host species	Manufacturer (Product number)	Dilution
pSTAT1 (Tyr701)	rabbit	Invitrogen #610115	1:500
STAT1	mouse	BD Bioscience #612233	1:500
pSTAT5	rabbit	Cell signalling technologies #9359	1:1000
STAT5	rabbit	Cell signalling technologies #4459	1:1000
β -Actin	rabbit	Proteintech #811151RR	1:20000
COX-2	mouse	Santa Cruz Biotechnology # sc-19999	1:100
GAPDH	rabbit	Proteintech #805701RR	1:20000
Anti-Rabbit HRP	mouse	Cell signalling Technology # 93702S	1:5000
Anti-Mouse HRP	goat	BD Bioscience # 554002	1:1000

2.9 Quantitative reverse transcription (RT)-PCR

2.9.1 RNA extraction

A RNeasy plus kit (Qiagen) was used to extract RNA from cells after the incubation, according to the manufacturer's instructions. Cells were harvested into Eppendorf tubes, centrifuged at 400 *xg* for 5 minutes, then washed twice with PBS. Then PBS was removed, and cells were lysed and homogenized in the supplied lysis buffer (RLT buffer) (Qiagen). The lysates were passed through gDNA Eliminator spin columns, 70% ethanol was added to the flow-through and mixed well. Immediately, the samples were transferred to RNeasy spin columns. RNA banded to the membrane and contaminants were washed away following some wash steps (as in the protocol). RNA was eluted in 30 μ L of RNase-free water from the spin column membrane by centrifugation. The concentration and purity of RNA was determined by a Nanodrop Spectrophotometer (Thermo Fisher Scientific), and RNA was stored at -80°C until used.

2.9.2 cDNA synthesis

500 ng total RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. 2 μ L gDNA Wipeout Buffer (Qiagen) was added to the RNA sample, and added RNase-free water to a final volume of 14 μ L per reaction in a PCR tube. Each reaction was incubated at 42°C for 2 minutes in a thermal cycler (Bio rad) before placing on ice for >1 minute. 4 μ L Quantiscript RT Buffer (Qiagen), 1 μ L RT Primer Mix (Qiagen) and 1 μ L Quantiscript Reverse Transcriptase (Qiagen) was added to the template RNA to a final volume at 20 μ L. Reaction was then incubated at 42°C for 30 minutes then 95°C for 5 minutes in a thermal cycler (Bio rad). The concentration and purity of cDNA

was determined by a Nanodrop Spectrophotometer (Thermo Fisher Scientific), and cDNA was stored at -20°C until used.

2.9.3 TaqMan™ method

Each reaction was in a MicroAmp fast optical 96 well reaction plate (Applied Biosystems), 10 µL 1x Taqman gene expression master mix (Applied Biosystems) was combined with 2 µL cDNA, 1 µL 1x TaqMan™ gene expression assay probe (Table 2.6) and 7 µL nuclease free water to a final volume at 20 µL.

A QuantStudio® 5 (Applied Biosystems) was used to perform the reactions, using the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were automatically generated, the expression of target gene was analysed using the Δ Ct method (below) and calculated using Applied Biosystems software.

$$\Delta Ct = \text{Target Ct mean} - \text{Housekeeping Ct mean}$$

$$\Delta\Delta Ct = \Delta Ct \text{ control} - \Delta Ct \text{ treatment}$$

$$\text{Fold change expression over resting} = 2^{-\Delta\Delta Ct}$$

Table 2.6: TaqMan™ probes and primers

Gene	Assay ID	Manufacturer	Amplicon length	Dye
PTGER1	HS00909194_g1	Thermo Scientific	85	FAM-MGB
PTGER2	HS00168754_m1	Thermo Scientific	79	FAM-MGB
PTGER3	HS00168755_m1	Thermo Scientific	121	FAM-MGB
PTGER4	HS00168761_m1	Thermo Scientific	68	FAM-MGB
PRF1	HS00169473_m1	Thermo Scientific	106	FAM-MGB
IFNG	HS00989291_m1	Thermo Scientific	73	FAM-MGB
GAPDH	HS02786624_g1	Thermo Scientific	157	FAM-MGB

2.10 Firefly luciferase (FLUC) killing assay

MG63-FLUC and HOS-FLUC cells were used to measure the relative number of dead cells in a cell population. Firefly luciferase is a light-emitting enzyme that catalyses a biochemical reaction that produces light when it is exposed to oxygen and luciferin (substrate). Firefly luciferase-expressing osteosarcoma cell lines (MG63-FLUC and HOS-FLUC) were generated by lentiviral transduction (provided by Dr Tyler Barr, University of Leeds). When the target cells were killed, the released luciferase protein banded to luciferin and emits luminescence, and is detected using Cytation 5 plate reader (BioTek).

FLUC-expressing target cells were seeded in a 96 well plate at 1×10^4 cells per well in 50 μ L media. Then 1 μ L PGE₁ alcohol in 50 μ L media was added to the FLUC target cells for 48 hours. At the same day, PBMCs were treated with 0, 0.1 or 1 pfu/cell reovirus for 48 hours. After the treatments, the treated PBMCs and the treated FLUC target cells were co-cultured together for another 24 hours. At the end of treatments, luciferin was added to the samples at 0.5 μ L per well, and cultured samples at 37°C for 5 minutes. The luminescence was measured using a plate reader (Figure 2.6).

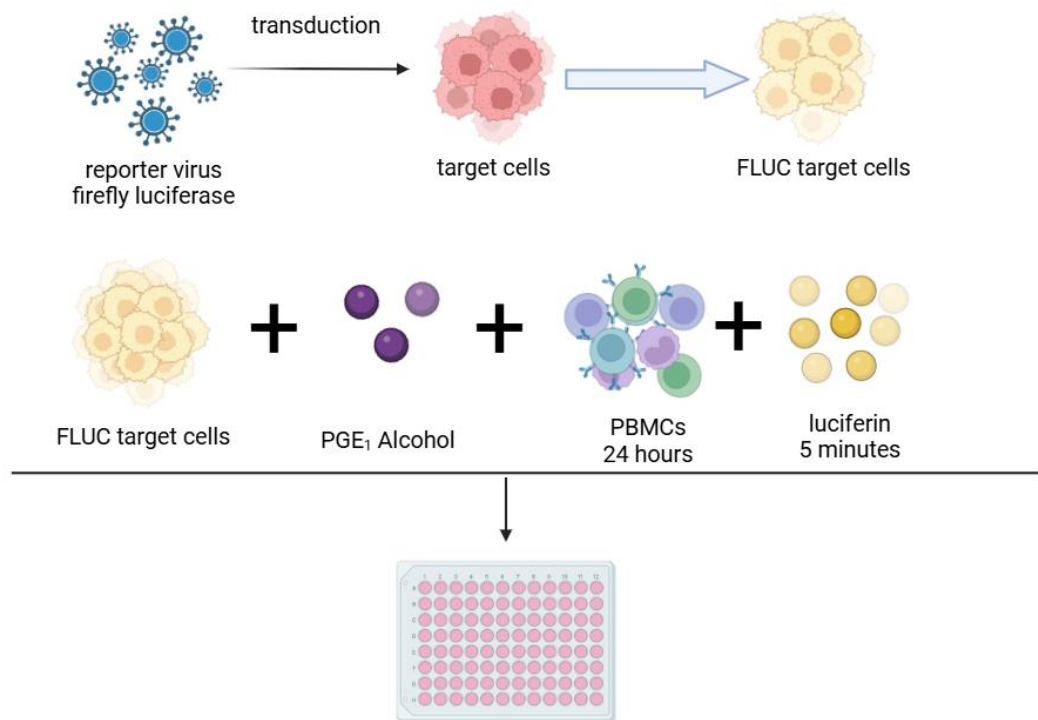


Figure 2.6: Firefly luciferase killing assay. Target cells were transfected by reporter virus with firefly luciferase to make FLUC target cells. FLUC target cells were treated with PGE₁ alcohol for 48 hours and co-cultured with PBMCs for another 24 hours. Luciferin was added to the samples and measured the luminescence. Figure generated using Biorender.

2.11 Three- dimensional (3D) culture-spheroid assay

MG63 cells were used to establish the 3D cell model. Firstly, MG63 cells were seeded in a 96 well low adhesion plate (Costar) at 1×10^4 cells per well in 50 μ L DMEM (+ 10% FBS). Then 5×10^3 CD14⁺ monocytes in 50 μ L RPMI (+ 10% FBS) and 2×10^3 MSC066 in 50 μ L MSC media (DMEM+10% FBS+ pen/step) were added into the MG63 cells (Figure 2.7a). The outer wells of the plate were filled with PBS. For 1x plate of MG63 cells alone, 1×10^4 MG63 cells were seeded in a 96 well low adhesion plate in 100 μ L DMEM per well and extra 100 μ L DMEM was added to make to 200 μ L per well. The plates were cultured for 7 days at 37 °C, 5 % CO₂. After 7 days, figure 2.7b shows that CD14⁺ monocytes were transferred to TAMs. After the treatment, the media were carefully removed from each spheroid well, the media from the same spheroid type and treatment was saved in the same 15 mL tube and kept in – 20 °C for the following PGE₂ ELISA.

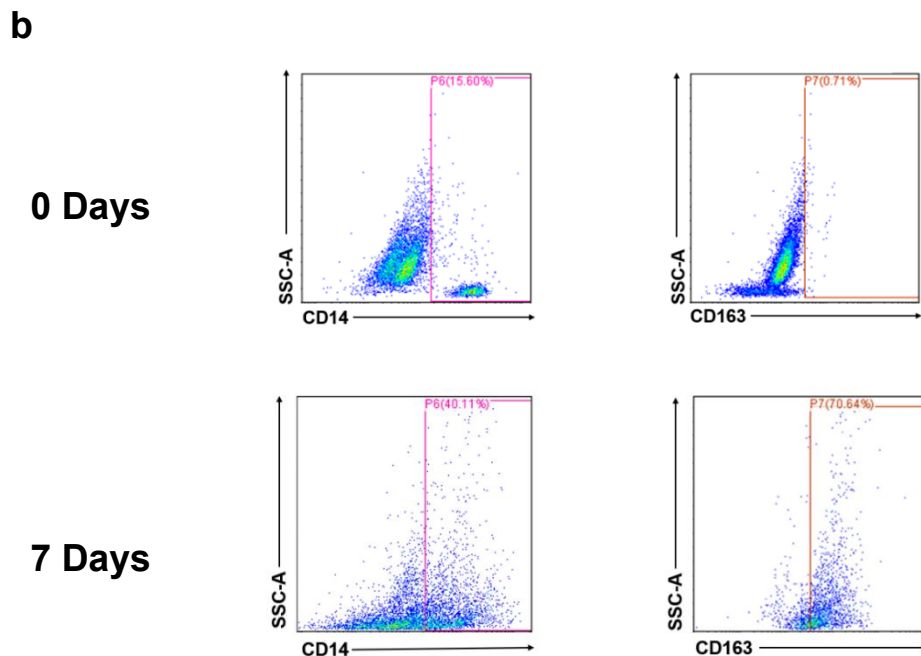
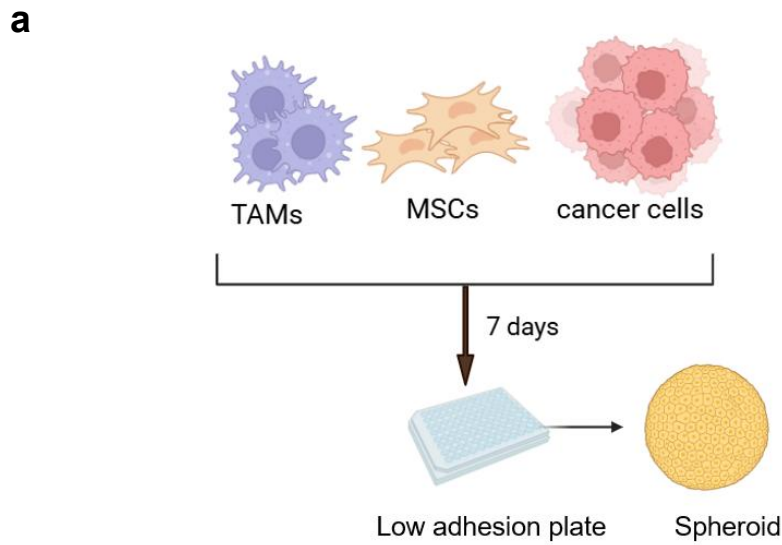


Figure 2.7: Establishment of 3D cell model. (a) Cancer cells, MSCs and monocytes were co-cultured in low adhesion plate for 7 days to make spheroid. Figure generated using Biorender. (b) CD14⁺ monocyte differentiation to TAM in spheroids was validated using flow cytometry. Cells from spheroids on day 0 and day 7 were stained with CD14 and CD163 antibodies and assessed using flow cytometry (Data generated by Tyler Barr).

2.12 Statistical analysis

Statistical analysis of results was performed using Graph Pad Prism 10 software. P-values were calculated using either paired t-tests (comparing two groups), one-way analysis of variance (ANOVA) or two-way ANOVA (comparing three or more groups). Levels of statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, absence of * or ns indicates non-significant result.

Chapter 3

Prostaglandin E₂ inhibits natural killer cell activation

3.1 Introduction:

PGE₂ is the most biologically active prostaglandin. It is produced by many cell types and sustains physiological and homeostatic functions, but also induces pathologic responses. PGE₂ is abundantly expressed in the tumour microenvironment, and increasing evidence demonstrates that PGE₂ is related to tumour cell proliferation, invasion, and metastasis, and inhibits the activation of the immune system (Santiso et al., 2024).

NK cells can directly recognise and attack tumour cells. In the TME, NK cells eliminate tumours through degranulation, ADCC, or FASL/TRAIL-induced apoptosis. In addition, NK cells can also secrete cytokines or chemokines to recruit other immune cells and upregulate their anti-tumour responses. Because of these characteristics of NK cells, NK cells play a pivotal role in anti-tumour immunity. However, in the TME, the anti-tumour function of NK cells is challenged, especially since most advanced tumours can escape NK cell killing and evade immune detection more broadly.

Previous studies have shown that PGE₂ can inhibit the function of NK cells (Hall et al., 1983; Agard et al., 2013). However, we do not know the mechanism underlying the inhibitory effect of PGE₂ on NK cells. Experiments in this chapter were designed to demonstrate the range of inhibitory effects of PGE₂ on activated NK cells under different conditions. NK cells were first activated using different cytokines or reovirus and the effect of PGE₂ on NK cell cytotoxicity and cytokine production was analysed.

3.2 Results

3.2.1 Reovirus treatment activates NK cells

Oncolytic viruses (OVs) therapy is an emerging cancer treatment method. OV can selectively replicate in tumour cells, lyse the tumour cells and release more virus particles, leading to extensive tumour lysis (Davola and Mossman, 2019). Furthermore, OV can induce anti-tumour immunity (Jafari et al., 2022). An advantage of OV therapy is that it has low adverse reactions and, to a large extent, does not cause cross-resistance with other cancer treatment drugs (Lin et al., 2023). Reovirus is a type of OV that can directly activate NK cells in vitro and in vivo and enhance the cytotoxicity of NK cells (El-Sherbiny et al., 2015; Wantoch et al., 2022).

Peripheral blood mononuclear cells were isolated from healthy donor blood by density gradient separation (section 2.2). Human NK cells are defined as CD56⁺ and CD3⁻ lymphocytes, enabling them to be identified within PBMCs by flow cytometry (Figure 3.1a). NK cells account for approximately 27% of all lymphocytes (Figure 3.1b). CD69 is expressed on the cell surface (where it can be detected by flow cytometry), and is upregulated after lymphocyte activation (Fig 3.1c) and is often used as an activation marker for NK cells.

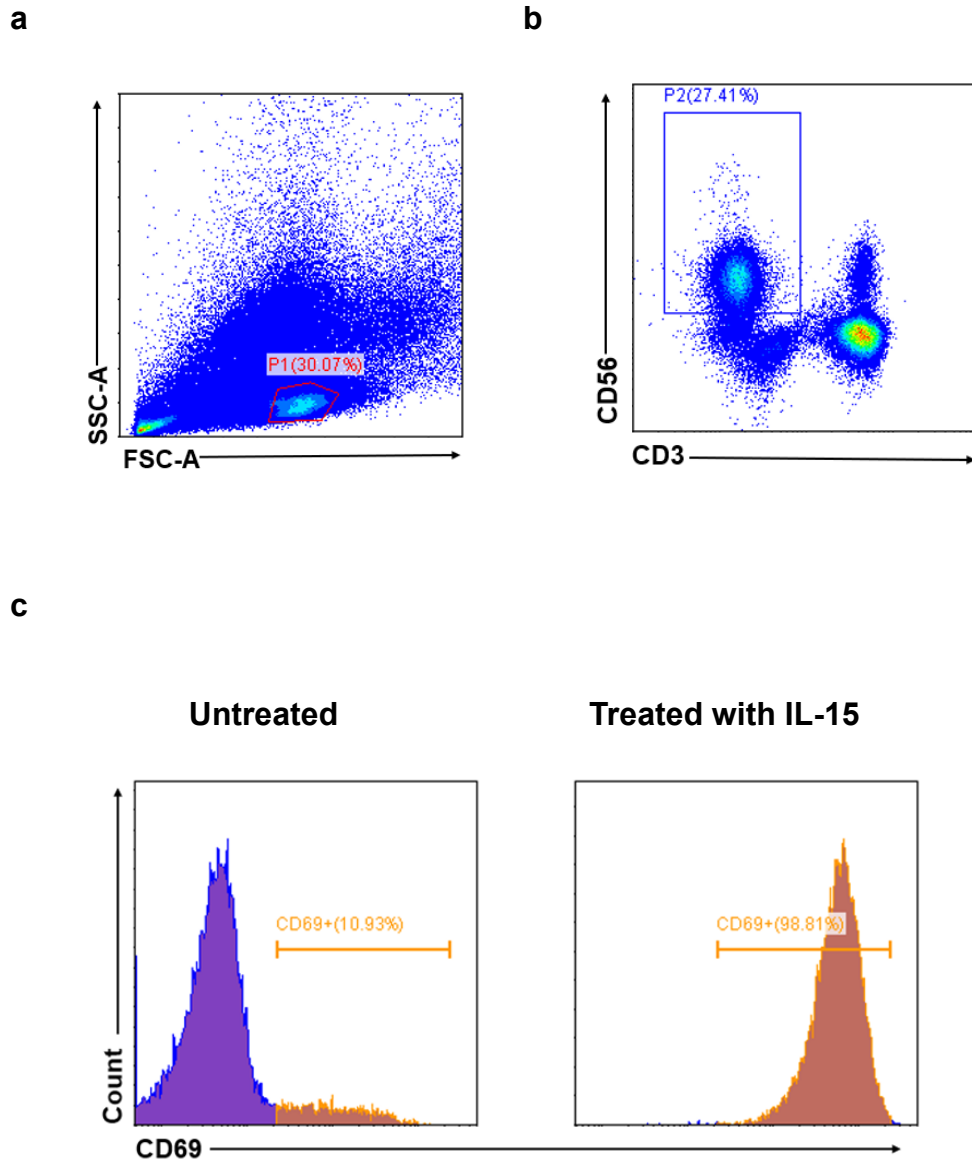


Figure 3.1: Gating strategy for NK cell activation. The figure shows (a) gating only lymphocyte population using a FSC-A- SSC-A dot plot. (b) Gating on CD3⁻CD56⁺ NK cells. (c) Gating to establish the expression of CD69 NK cells.

PBMCs were treated with increasing amounts of reovirus for 48 hours. Then the activation of NK cells within PBMCs was investigated using flow cytometry. The results showed that both 0.1 pfu/cell and 1 pfu/cell reovirus can activate NK cells significantly (Figure 3.2a).

IFN- γ is a cytokine produced by NK cells. IFN- γ promotes the cytotoxicity of T cells and NK cells and regulates immunity by promoting DC maturation and the expression of MHC class I and antigen presentation. and is therefore important in anti-tumour immunity (Cui et al., 2019; Lin et al., 2021). PBMCs were stimulated with reovirus for 48 hours and supernatants analysed by ELISA for IFN- γ . The results showed that treatment with reovirus enhanced the production of IFN- γ , but not significantly (Figure 3.2b). Because the IFN- γ was measured from PBMCs by ELISA, it might be coming from other cells (e.g. CD4⁺ T cells). Reovirus is known to activate type I IFN (IFN-I) production and this in turn can activate NK cells (El-Sherbiny et al., 2015; Wantoch et al., 2022) and it is likely that the IFN- γ is mainly derived from activated NK cells under these conditions.

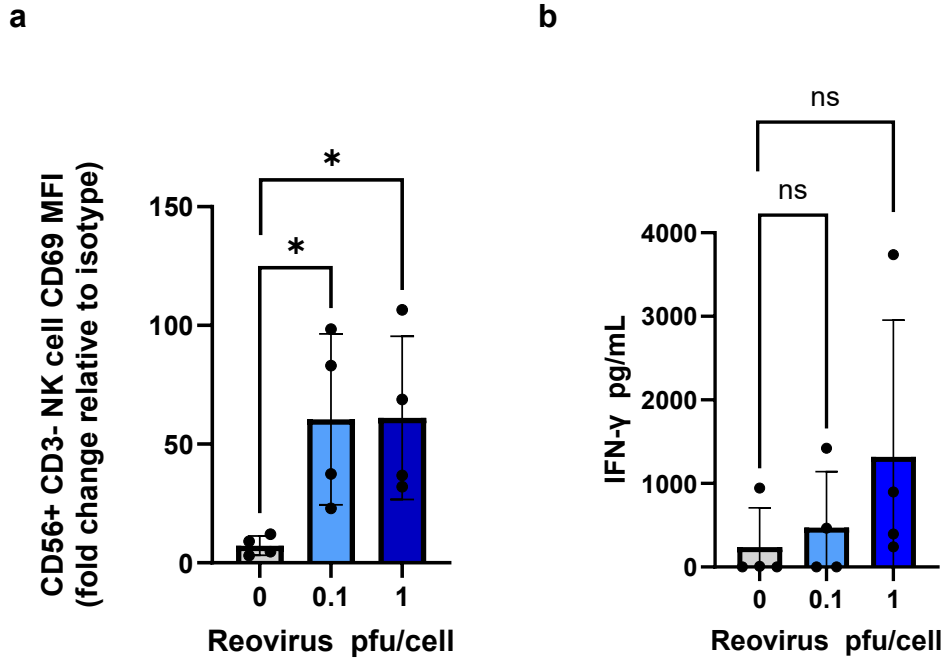


Figure 3.2: Activation of NK cells by reovirus. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with reovirus at 0, 0.1, 1 pfu/cell for 48 hours. CD3-CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm standard error of the mean (SEM), for n=4 independent experiments. (b) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with reovirus at 0, 0.1, 1 pfu/cell for 48 hours. The supernatants were collected and IFN- γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = $p < 0.05$, and ns, not significant.

3.2.2 IL-2 treatment activates NK cells

Interleukin-2 (IL-2) is an immunostimulatory cytokine, also known as T cell growth factor (TCGF), because it induces proliferation of activated T cells (Ross and Cantrell, 2018). IL-2 can also induce proliferation and cytotoxicity of NK cells (Wang et al., 1999).

PBMCs were treated with 0, 5 or 10 ng/mL IL-2 for 48 hours and NK cell activation investigated using flow cytometry. Figure 3.3a shows that the expression of CD69 was increased significantly on NK cells after IL-2 treatment. An IFN- γ ELISA showed that both concentrations of IL-2 increased the IFN- γ release by NK cells, but not significantly (Figure 3.3b). However, there was donor variability, and these results did not reach statistical significance.

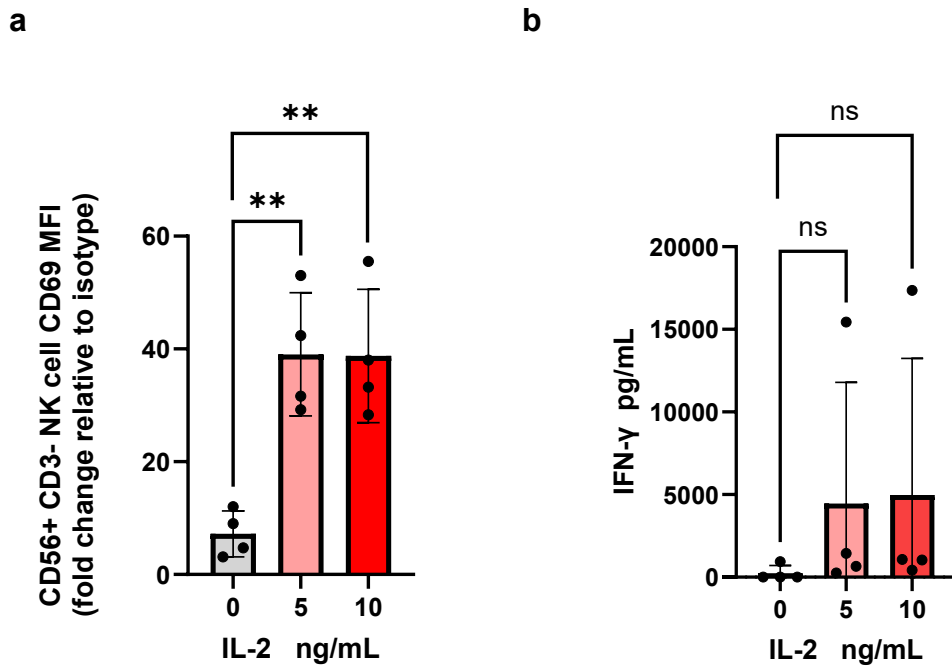


Figure 3.3: Activation of NK cells by IL-2. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-2 for 48 hours. CD3⁻CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for n=4 independent experiments. (b) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-2 for 48 hours. The supernatants were collected and IFN- γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = $p < 0.05$, ** = $p < 0.01$, and ns, not significant.

3.2.3 IL-15 treatment activates NK cells

The lymphokine interleukin-15 (IL-15), like IL-2, can also activate NK cells. IL-15 binds to the receptor IL-15R α and signals through the β chain and γ chain signalling complexes to activate JAK1 and JAK3, then phosphorylate the signal activator of transcription (STAT, mainly STAT5 in the case of IL-15). Because IL-15 and IL-2 share the β chain of the signalling complex, IL-15 has some similar functions to IL-2, but it has a wider range of applications than IL-2 and targets more cells and tissues (Perera et al., 2012).

PBMCs were treated with 0, 5, 10 ng/mL IL-15 for 48 hours. Then the activation within PBMCs of NK cells were investigated using flow cytometry. After IL-15 treatment, the expression of CD69 was increased (Figure 3.1c). Figure 3.4a shows with the IL-15 treatment, NK cells were significantly activated by both concentration (5 and 10 ng/mL), since CD69 expression was significantly increased. The IFN- γ release by PBMCs was not significantly increased after adding 5 and 10 ng/mL IL-15 for 48 hours (Figure 3.4b). Compared with reovirus and IL-2 treatments, the degree of NK cell activation and the amount of IFN- γ produced were the highest after IL-15 stimulation.

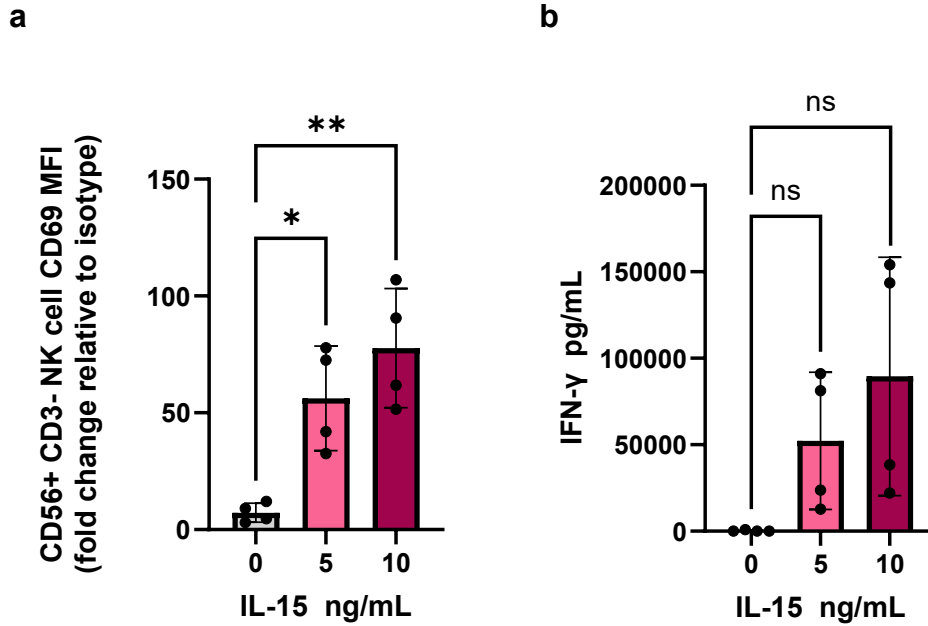


Figure 3.4: Activation of NK cells by IL-15. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-15 for 48 hours. CD3⁺CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for n=4 independent experiments. (b) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-15 for 48 hours. The supernatants were collected and IFN- γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = p < 0.05, ** = p < 0.01, and ns, not significant.

3.2.4 PGE₂ does not kill NK cells

I then used the assays described above to test the effects of PGE₂ on NK cell activation. It was important to demonstrate that PGE₂ did not kill NK cells in culture. PBMCs were treated with 0, 0.1, 1, 10 μM PGE₂ for 48 hours. Then the cells were stained with LIVE/DEAD™ as the gating shows on Figure 2.3. According to the flow cytometry results, these three concentrations of PGE₂ had no effect on the viability of NK cells (or T cells, seen in the Live/Dead™ data) (Figure 3.5).

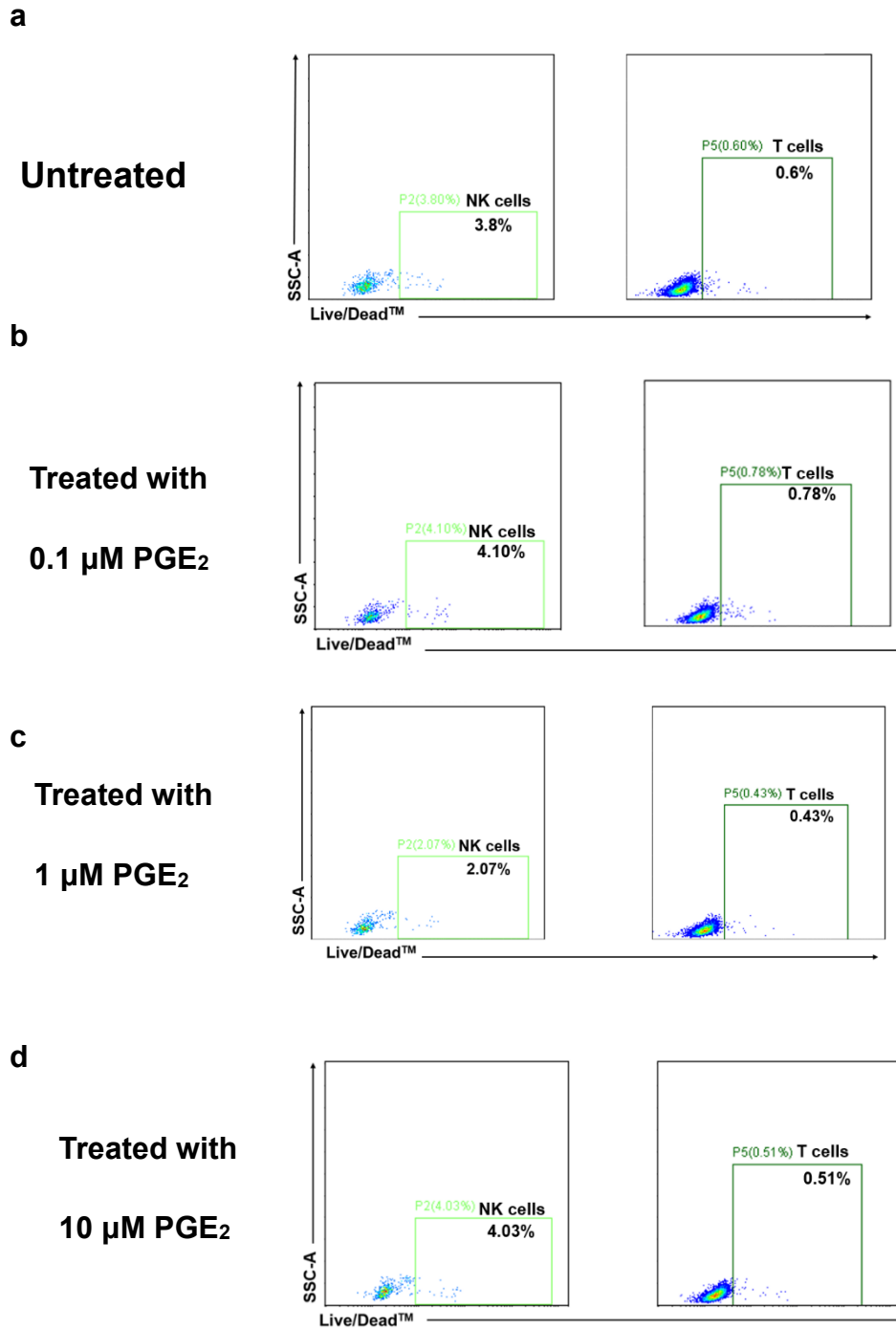


Figure 3.5: PGE₂ does not kill NK cells and T cells. (a) NK cells and T cells were untreated for 48 hours. (b) NK cells and T cells were treated with 0.1 μ M PGE₂ for 48 hours. (c) NK cells and T cells were treated with 1 μ M PGE₂ for 48 hours. (d) NK cells and T cells were treated with 10 μ M PGE₂ for 48 hours.

Furthermore, treatment of PBMCs with 0, 1 or 10 μM PGE₂ for 48 hours showed that PGE₂ treatment had no significant effect on the expression of CD69 on NK cells (Figure 3.6a) or on IFN- γ release by PBMCs (Figure 3.6b). Although IFN- γ appeared to be induced by PGE₂, the levels released were hundreds of pg/mL compared to tens of thousands of pg/mL when IL-15 was added to PBMC (as shown above in Figure 3.4).

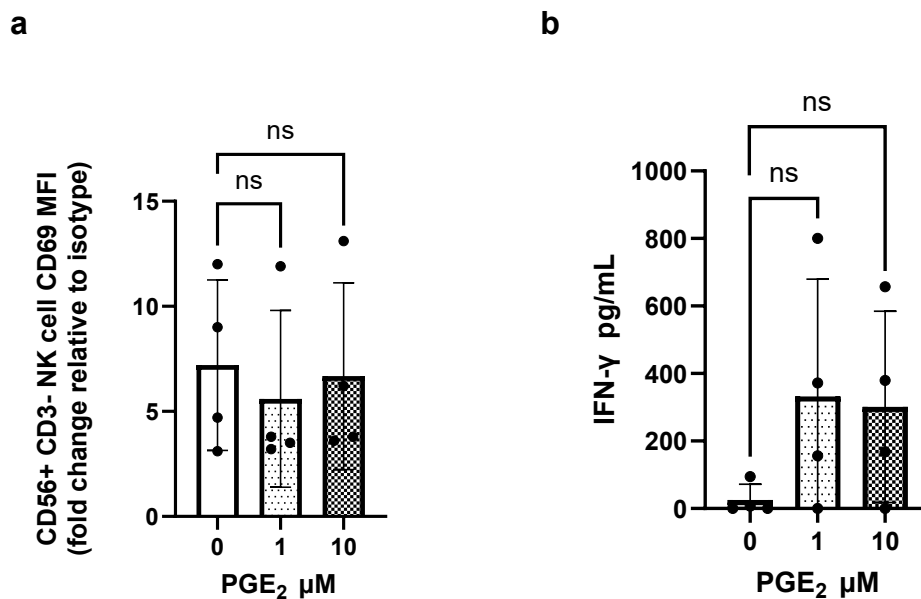


Figure 3.6: The effects of PGE₂ on NK cells only. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 1, 10 μM PGE₂ for 48 hours. CD3⁻CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for $n=4$ independent experiments. (b) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 1, 10 μM PGE₂ for 48 hours. The supernatants were collected and IFN- γ production was determined by ELISA ($n=4$). Statistical analysis was performed by paired t-test. ns, not significant.

3.2.5 Effect of PGE₂ on reovirus-mediated NK cell activation

I then tested the effect of PGE₂ on reovirus activation of NK cells; 1 μM and 10 μM PGE₂ were used to treat the PBMCs for one hour before reovirus treatment for another 48 hours. Figure 3.7 showed that PGE₂ reduced reovirus-mediated NK cell activation based on the NK cell expression of CD69 and IFN-γ production by the PBMCs culture (Figure 3.7b). However, there was variability between donors and these results did not reach statistical significance.

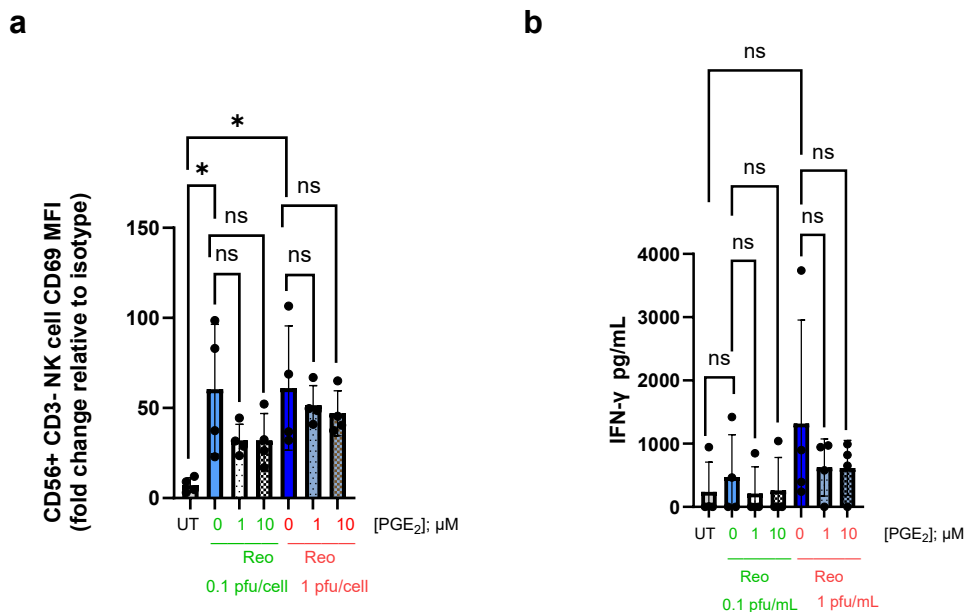


Figure 3.7: Effect of PGE₂ on reovirus mediated NK cell activation. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 0.1, 1 pfu/mL reovirus for 48 hours following treated with 1 μM, 10 μM or without PGE₂. CD3⁺CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for n=4 independent experiments. (b) The supernatants were collected and IFN-γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 and ns, not significant.

3.2.6 PGE₂ inhibits IL-2- mediated NK cell activation

IL-2 activates NK cells (Figure 3.3), so PGE₂ treatment was used to detect whether PGE₂ has an inhibitory effect. PBMCs were treated with 0, 1 μM, 10 μM PGE₂ for an hour, then 0, 5, 10 ng/mL IL-2 were added to the samples for another 48 hours. Flow cytometry results showed that both concentrations of PGE₂ significantly inhibited IL-2-mediated NK cell activation based on the expression of cell surface CD69 (Figure 3.8a). Although the Figure 3.8b showed that PGE₂ inhibited IFN-γ production by PBMCs, the results are not significant.

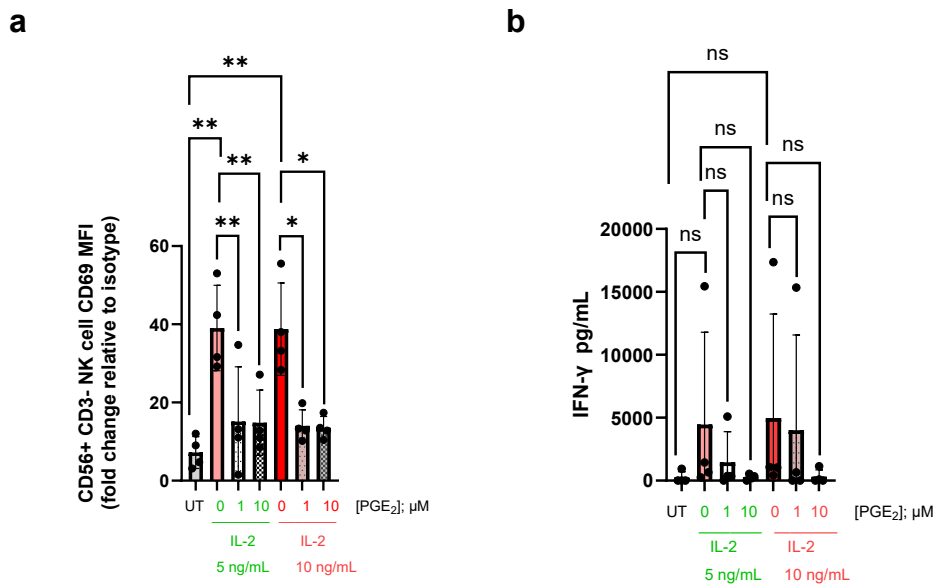


Figure 3.8: PGE₂ inhibits IL-2- mediated NK cell activation. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-2 for 48 hours following treated with 1 μM, 10 μM or without PGE₂. CD3⁺CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for n=4 independent experiments. (b) The supernatants were collected and IFN-γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 and ns, not significant.

3.2.7 PGE₂ inhibits IL-15- mediated NK cell activation

In the tumour microenvironment, IL-15 is an activation and survival factor for NK cells. Monocytes and dendritic cells in the tumour microenvironment are the main source of IL-15 (Sindaco et al., 2023) and IL-15 and PGE₂ in the TME might provide contrasting signals to NK cells. I therefore analysed the effect of PGE₂ on IL-15 activation of NK cells.

PBMCs were treated with 0, 1 μM ,10 μM PGE₂ for an hour, then 0, 5, 10 ng/mL IL-15 were added to the samples for another 48 hours. PGE₂ significantly inhibited the IL-15-mediated CD69 expression of NK cells (Figure 3.9a). Both concentrations of PGE₂ inhibited the production of IFN-γ by PBMCs but results were not significant due to donor variability (Figure 3.9b).

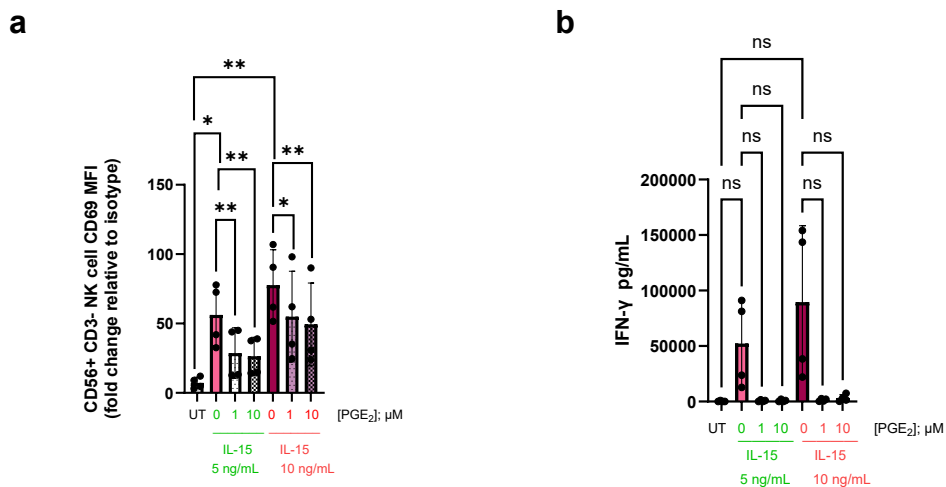


Figure 3.9: PGE₂ inhibits IL-15- mediated NK cell activation. (a) PBMCs were cultured in 6 well plates at 2×10^6 cells/well and were treated with 0, 5, 10 ng/mL IL-15 for 48 hours following treated with 1 μM ,10 μM or without PGE₂. CD3⁻CD56⁺ NK-cell expression of CD69 was determined using flow cytometry. All values represent means \pm SEM, for n=4 independent experiments. (b) The supernatants were collected and IFN-γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = $p < 0.05$, ** = $p < 0.01$, and ns, not significant.

I used the indirect isolation method to purify NK cells and used these cells to visualise the effects of IL-15 and PGE₂ on NK cell morphology. NK cells were cultured at 0.5x10⁶ cells/mL and left untreated, or treated with IL-15, PGE₂ or a combination. Figure 3.10a shows that IL-15 caused the NK cells to increase in size and to aggregate. This did not occur when NK cells were treated with PGE₂ alone. In addition, pre-treatment with PGE₂ inhibited the growth of NK cells following IL-15 treatment compared to the IL-15 only treatment and the NK cells were also prevented from aggregating. These images show that PGE₂ can inhibit NK cell growth and prevent NK cell activation after IL-15 induction. In addition, I repeated the IFN- γ ELISA on purified NK cells. Figure 3.10b shows the production of IFN- γ from NK cells was significantly increased by IL-15 and PGE₂ inhibited IFN- γ secretion.

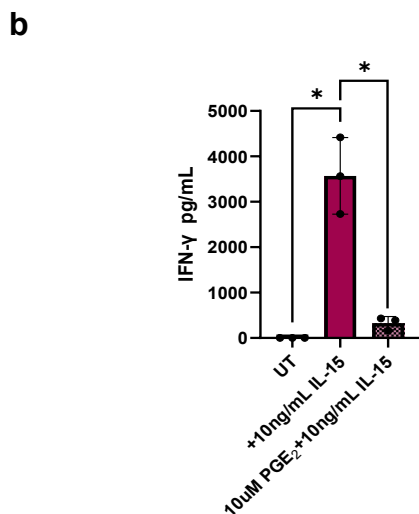
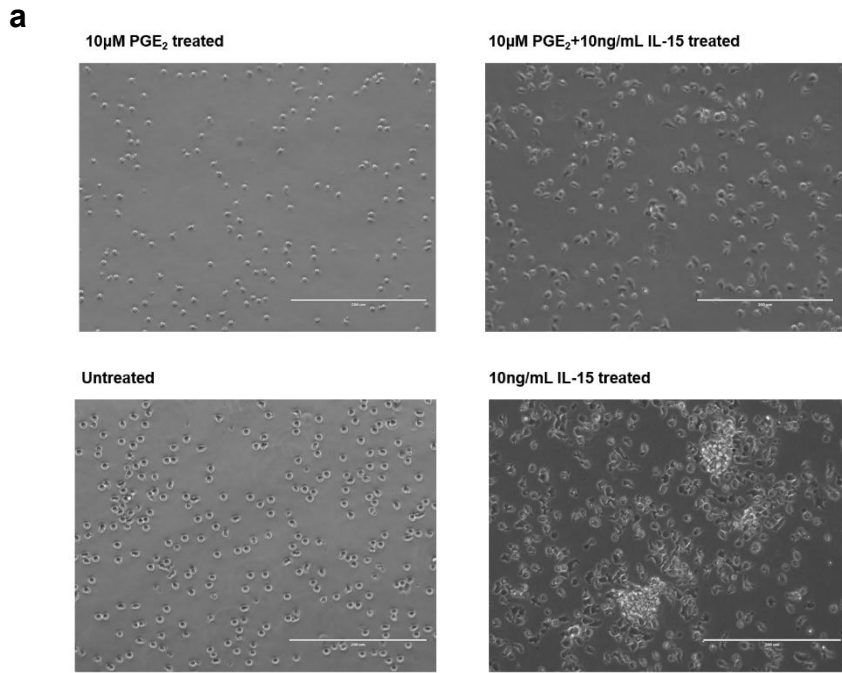


Figure 3.10: PGE₂ inhibits IL-15- mediated NK cells. (a) Purified NK cells were cultured in 6 well plates at 0.5×10^6 treated with 0 or 10 ng/mL IL-15 for 48 hours following treated with 10 µM or without PGE₂. Images were taken by EVOS. 200 µm scale bar (b) NK cells were cultured in 6 well plates at 0.5×10^6 cells/well and were treated with 10 ng/mL IL-15 for 48 hours following treated with 10 µM or without PGE₂. The supernatants were collected and IFN-γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = $p < 0.05$

3.2.8 PGE₂ inhibits IL-12 and IL-18- mediated NK cell activation

IL-12 and IL-18 are produced by monocytes during an immune response and play an important role in activating NK cells. I treated purified NK cells with IL-12, IL-18 or a combination in the presence or absence of PGE₂. The combination of IL-12 and IL-18 resulted in strong induction of IFN- γ , much greater than either cytokine alone. More importantly, IFN- γ induction by IL-12 + IL-18 was strongly and significantly inhibited by PGE₂ (Figure 3.11).

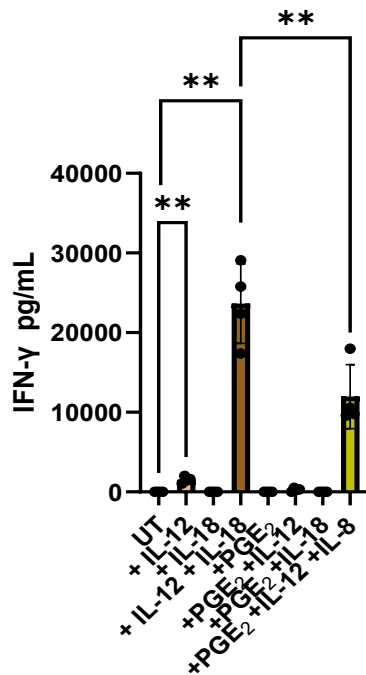


Figure 3.11: PGE₂ inhibits IL-12 and IL-18- mediated NK cell activation.

4 x10⁵ NK cells were treated with or without IL-12, IL-18 and the combination at 10 ng/mL for 48 hours after 10 μ M PGE₂ treatment. The supernatants were collected and IFN- γ production was determined by ELISA (n=4). Statistical analysis was performed by paired t-test. * = p < 0.05, ** = p < 0.01, and ns, not significant.

3.2.9 PGE₂ inhibits NK cell degranulation

NK cell degranulation is one of the important mechanisms of NK cell cytotoxicity. After activation, the secretory lytic granules (containing perforin and granzymes) move to the vicinity of the cell membrane and are exocytosed into the extracellular space. This process is called degranulation, and both NK cells and T cells release perforin and granzymes in this way to kill target cells.

Lysosomal associated membrane protein-1 (CD107a or LAMP-1) is a highly glycosylated transmembrane protein present in lysosomes and the secretory lysosomes of NK cells and T cells. The expression of CD107a on the membrane is a marker of degranulated CD8⁺ T cells and NK cells. In NK cells, CD107a is one of the most abundant proteins in cytolytic granules, located in the inner layer of the vesicle membrane, with its highly glycosylated part hidden in the lumen and the short tail exposed to the cytoplasm. At the end of degranulation, the outer membrane of the granules merges with the NK cell membrane, resulting in the exposure of CD107a molecules to the surface (Figure 3.12). Therefore, NK cell activation can be measured by detecting CD107a on the surface of NK cells using flow cytometry.

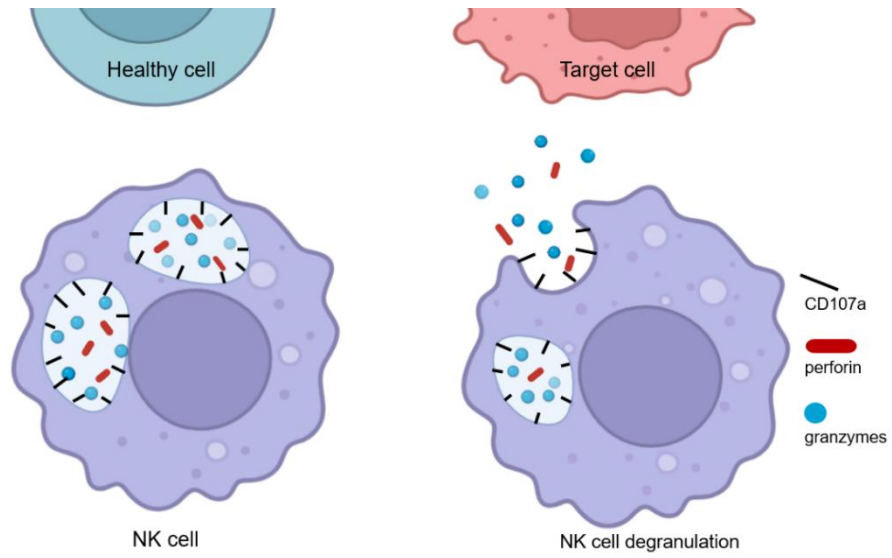


Figure 3.12: NK cell degranulation. NK cells release the contents of their lytic granules to kill target cells, and this process exposes the membrane protein CD107a to the cell surface. Figure generated using Biorender.

Addition of reovirus to PBMCs caused NK cell activation as determined by an increase in CD69 expression (Figure 3.2). I determined whether PGE₂ would inhibit this; PBMCs were pre-treated with PGE₂ for one hour and then reovirus was added for 48 hours. These treated PBMC were then co-cultured with a tumour target cell line (SK-N-MC cell line, an ovarian cancer cell line) to induce NK cell degranulation. Degranulation was determined by staining with NK cell markers (CD56⁺CD3⁻) and for cell surface CD107a. The results in Figure 3.13a show that reovirus significantly induced degranulation in response to SK-N-MC cells and that this degranulation was significantly inhibited by pre-treatment with PGE₂. I repeated this experiment using IL-15 based activation of NK cells instead of reovirus (Figure 3.13b). Figure 3.13c shows IL-15 significantly increased the degranulation of NK cells in response to target cells and 10 µM PGE₂ significantly inhibited this IL-15-mediated NK cell degranulation. PGE₂ also inhibited NK-cell degranulation in a dose responsive manner.

These results demonstrate that PGE₂ can inhibit NK cell activation and effector function. More precisely, PGE₂ inhibits both reovirus-mediated and IL-15 mediated NK cell activation (based on CD69 expression and IFN-γ release) and the degranulation of NK cells in response to tumour targets.

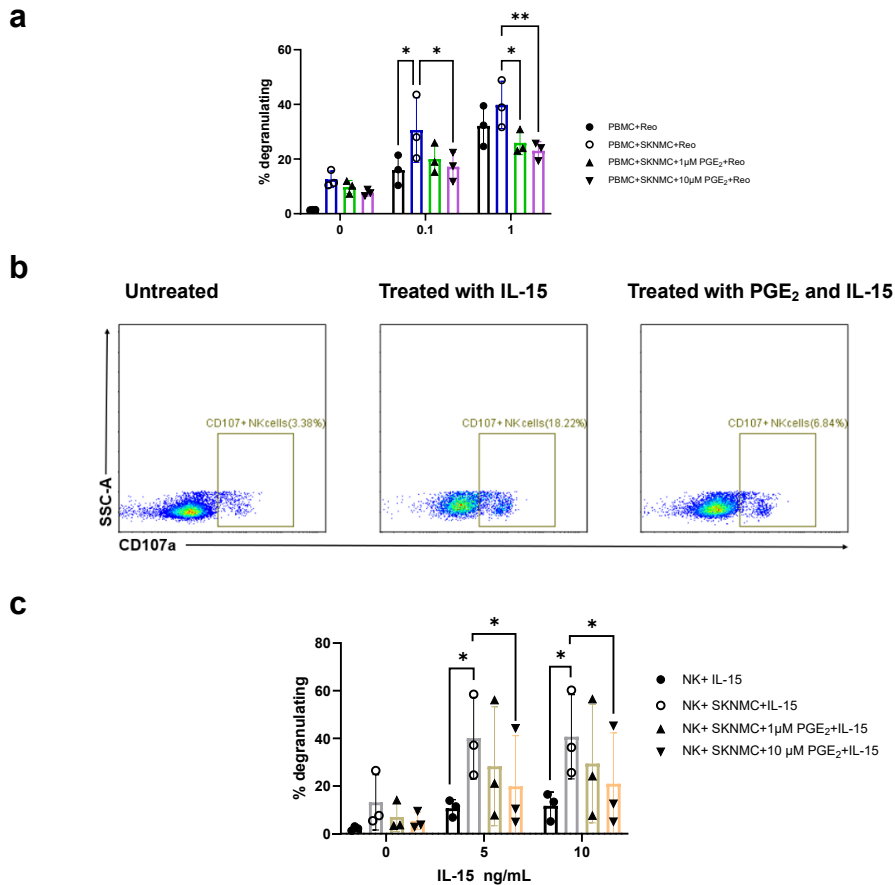


Figure 3.13: PGE₂ inhibits degranulation of isolated NK cells. (a) shows 4×10^5 PBMCs were treated with Reovirus (0, 0.1, 1 pfu/cell) for 48 hours following the treatment of PGE₂ (0, 1, 10 μ M) for 1 hour and cultured in 12 well plates. PBMCs were then co-cultured with 2×10^5 SK-N-MC cells for 4 hours, CD107a expression was then determined using flow cytometry. All values represent means \pm SEM, for n=3 independent experiments. Statistical analysis was performed using two-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$. (b) Gating to establish the percentage of CD107a degranulating NK cells. (c) shows 4×10^5 NK cells were treated with IL-15 (0, 5, 10 ng/mL) for 48 hours following the treatment of PGE₂ (0, 1, 10 μ M) for 1 hour and cultured in 12 well plates. NK cells were then co-cultured with 2×10^5 SK-N-MC cells for 4 hours, CD107a expression was then determined using flow cytometry. All values represent means \pm SEM, for n=3 independent experiments. Statistical analysis was performed using two-way ANOVA. * $p < 0.05$

3.3 Discussion

In summary, this chapter has demonstrated that PGE₂ inhibits NK cell activation. Reovirus, IL-2 and IL-15 treatments make NK cells produce IFN- γ and degranulate. Of these stimuli, IL-15 has the most pronounced effects. However, after PGE₂ treatment, the production of IFN- γ and NK cell degranulation were significantly inhibited.

Cytokines are essential for the maturation, activation and survival of NK cells (Figure 3.14). Cytokines can positively regulate NK cell function independently or synergistically. Activated NK cells synthesize perforin and granzymes and release them (via degranulation) to destroy target cells. In addition, they secrete a variety of cytokines and chemokines such as IFN- γ and TNF- α , which directly act on DCs, T cells and other cell types to regulate immune responses (Jiang and Jiang, 2023). The release of IFN- γ by NK cells bridges the innate and adaptive immune response as this cytokine acts upon DC to mediate maturation, aiding the priming of T cell responses. IFN- γ stimulates antigen processing and presentation and this enables DC to present antigen efficiently to T cells and IFN- γ acts on surrounding cells (e.g. tumour cells and stromal cells) to enhance antigen presentation (Castro et al., 2018). IFN- γ also favours the development of cytotoxic T cell responses from naïve T cells. The ability of PGE₂ to inhibit this activity as well as NK cell degranulation highlights the importance of PGE₂ in the tumour microenvironment in regulating anti-tumour immunity.

Several cytokines activate NK cells, including IFN- γ , IL-2, IL-15, IL-12, IL-18 and IL-21. When the NK cell function in the tumour microenvironment is weakened, these cytokines can stimulate NK cells and restore their function (Konjević et al., 2019).

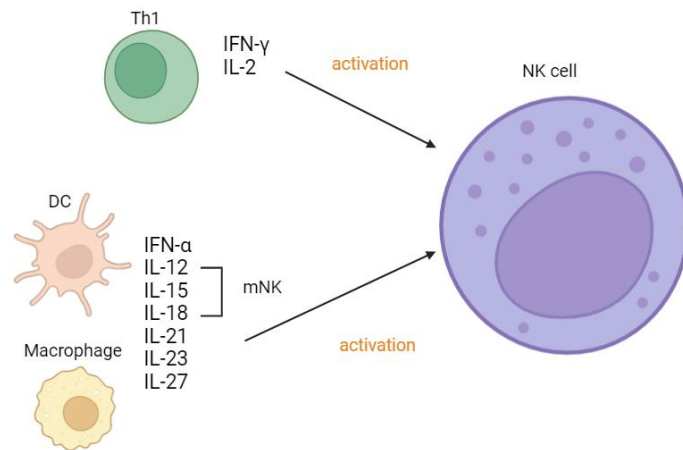


Figure 3.14: Cytokine activation of NK cells. Th1, DCs and macrophages secrete cytokines to active NK cells. Figure generated using Biorender.

Previous studies have found that some cytokines can stimulate NK cells to produce cytokine-induced memory NK (mNK) cells, and the frequency of IFN- γ produced after further stimulation is higher. These cytokines include IL-15, IL-12 and IL-18 (Min-Oo et al., 2013).

IL-2 and IL-15 belong to the γc cytokine family and can bind heterotrimeric receptor complexes with high affinity. The γc chain is an important component of these cytokine receptors, which binds to the Janus tyrosine kinase (Jak) required for signal transduction. Jak binds to the receptor and phosphorylates different downstream signals. IL-2 is mainly produced by Th1, which can induce the expansion of CD4⁺ and CD8⁺ T cells, activate B cells and differentiate them into plasma cells (Hipp et al., 2017), and activate and enhance the cytotoxic activity of NK cells. However, in the tumour microenvironment, Tregs and Th2 cells are dominant. When Tregs are stimulated by IL-2, their TCR is activated, then temporarily upregulating CD25 and expressing high-affinity IL-2R. Because Tregs express high levels of CD25, they can better compete for binding to IL-2 and prevent the

activation of NK cells by IL-2 in the TME. In addition, IL-2 causes Tregs to produce immunosuppressive cytokines TGF- β and IL-10, which also inhibit the immune effects of NK cells (Papillion and Ballesteros-Tato, 2021).

IL-15, as the most promising alternative to IL-2, is produced by activated monocytes, macrophages and DCs. Importantly, IL-15 can induce NK and CD8⁺ T cell activity but does not stimulate Tregs (Berger et al., 2009). IL-15, like IL-2, can activate expression of NKG2D, NCRs, and KIR receptors and modulate NK cell-mediated cytotoxicity by upregulating perforin and granzyme B. My experimental results also show that the CD69 expression and IFN- γ of NK cells induced by IL-15 are several times higher than those of IL-2.

IFN- γ is produced by NK cells and T cells. IFN- γ can activate CD8⁺ CTL and stimulate the upregulation of MHC class I, MHC class II and co-stimulatory molecules on APCs (Massa et al., 2023). It plays an important role in antitumor immunity by enhancing the cytotoxic response of NK and CD8⁺ T cells through inducing perforin, granzyme, and FasL expression, this initiates apoptosis in tumour cells.

My results show that PGE₂ inhibits NK cells from producing IFN- γ and undergoing NK cell degranulation. In the TME, tumour cells can secrete PGE₂ to suppress NK cells and other immune cells. According to previous studies, PGE₂ promotes tumorigenesis and helps tumours escape immune system by regulating multiple cancer signalling pathways, affecting cell apoptosis, survival, proliferation, angiogenesis, invasiveness and metastasis (Sun and Li, 2018). In the following chapters, I will focus on the signalling downstream of PGE₂ which inhibits NK cell function.

Chapter 4

The mechanism of PGE₂ mediated inhibition of NK cells

4.1 Introduction

PGE₂ acts through four G protein-coupled receptors known as EP1, EP2, EP3 and EP4. Each of these receptors has different signal transduction mechanisms and physiological functions (G. O'Callaghan and Houston, 2015). Furthermore, PGE₂ has different affinities for these different receptors; EP3 and EP4 are high-affinity receptors, EP1 and EP2 are low-affinity receptors, among which EP1 receptor has the lowest affinity (An et al., 2021). According to previous studies, EP1-4 receptors are involved in the invasion, migration and growth of many solid tumours (Von Der Emde et al., 2014). Therefore, EP receptors can be used as potential targets for cancer treatment.

The results described in chapter 3 suggest that reovirus, IL-2 and IL-15 induces NK cell activation and IFN- γ production. Since I focus on IL-15, in this chapter, I am trying to find how PGE₂ inhibits IL-15-mediated NK cell activation signalling pathways. Previous studies have shown that IL-15 is presented to NK cells in the form of IL-2/IL-15R $\beta\gamma$ heterodimers, following binding to IL-15R α on antigen-presenting cells, and directly activate the JAK/STAT signalling pathways, phosphorylated STAT5 and STAT1 proteins translocate to the nucleus and activates downstream genes (Gotthardt et al., 2019). The signal transduction mechanism of the JAK/STAT signalling pathway was proposed by Fu in 1992 (Fu, 1992; Hu et al., 2021). JAK/STAT signalling pathway is responsible for transmitting extracellular signals (such as cytokines and growth factors) to the cell nucleus and ultimately regulating gene expression, it is an important intracellular signal transduction mechanism (Hu et al., 2021). As Figure 4.1 shows cytokines

bind to transmembrane receptors, causing conformational changes in the receptors. JAK molecules are activated and catalyse the phosphorylation of tyrosine residues of the receptors, which provides sites for recruiting and activating STATs. JAKs then phosphorylate specific sites of STATs, and phosphorylated STAT proteins bind through the SH2 domain to form homo- or hetero-dimers. The dimers are transferred to the nucleus and bind to specific DNA sequences to ultimately activate or inhibit the transcription of target genes (Gotthardt et al., 2019; Hu et al., 2021).

PGE₂ is mostly inhibitory in regulating immune responses, and EP1-4 receptors are involved. EP1-4 receptors play a complex regulatory role in immune cells. These receptors are expressed in different types of immune cells and have a multifaceted impact on immune responses. In addition, the downstream pathways and genes regulated via EP receptors are also important to the NK cell activation. Therefore, in this chapter I aim to investigate the signalling pathways of NK cells activated by IL-15 and the inhibitory effect of PGE₂ on them.

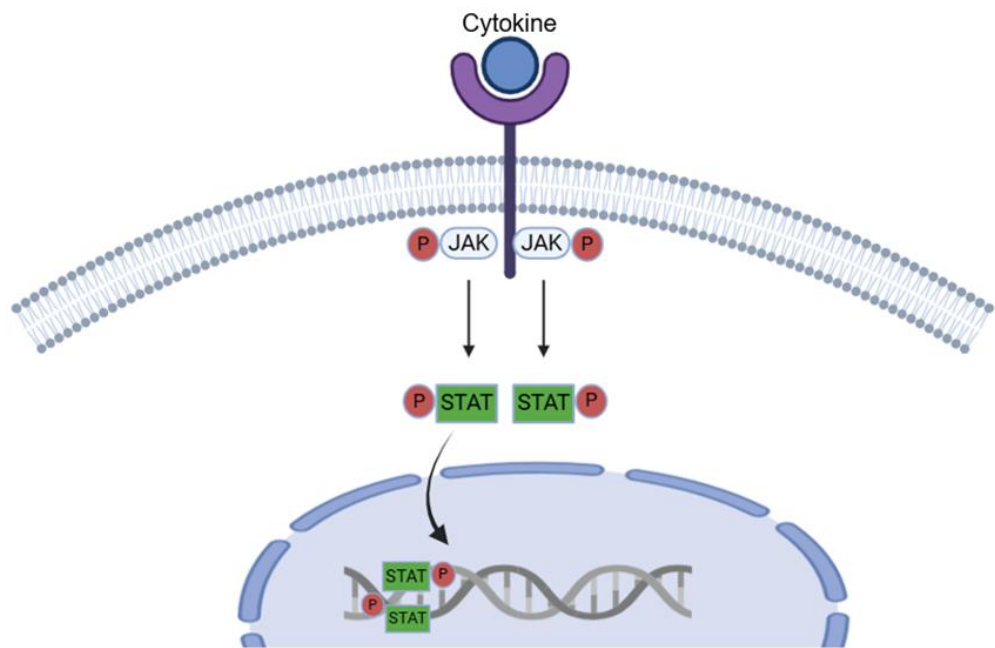


Figure 4.1: The JAK/STAT signalling pathway. After cytokines bind to their transmembrane receptors, JAK is activated and phosphorylates STAT proteins. pSTAT proteins are transferred into the cell nucleus in the form of dimers, binds to DNA and regulates the transcription of target genes. Figure generated using Biorender.

4.2 Results

4.2.1 NK cells express EP2 and EP4 receptors

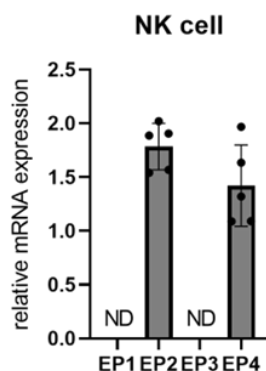
Because PGE₂ works through EP1-4 receptors, the expression of EP receptors in NK cells was analysed. NK cells were purified by negative selection, then total RNA was extracted from the purified NK cells and cDNA prepared. TaqMan RT-qPCR was used to detect the expression of EP receptors in NK cells. Figure 4.2a shows that NK cells only express EP2 and EP4 receptors, they do not express EP1 or EP3 receptors.

To confirm these results, I analysed single cell scRNAseq data using single cell portal (https://singlecell.broadinstitute.org/single_cell). C. Domínguez Conde et al, developed CellTypist, a comprehensive immune cell type reference database (Domínguez Conde et al., 2022). They used a classifier to divide immune cell populations in human tissue into 43 specific subtypes, and NK cells were identified (Figure 4.2b). Expression of genes encoding EP1-EP4 (*PTGER1-PTGER4*) were analysed. The results showed that *PTGER2* (EP2) and *PTGER4* (EP4) genes are expressed in most immune cells including NK cells, and *PTGER4* gene is more highly expressed than the *PTGER2* gene (Figure 4.2c). In addition, the gene expression of *PTGER1-4* in different types of immune cells was compared. Figure 4.2d shows that CD16⁺ NK cells expressed more *PTGER2* genes than CD56^{bright} NK cells, and more CD16⁺ NK cells expressed *PTGER2* genes compared to CD56^{bright} NK cells. As for the *PTGER4* gene, the expression levels in these two NK cell subsets are similar. The genes *NCR1*, *FCGR3A* and *GZMB* are used to identify NK cells. *NCR1* encodes the NK cell activation receptor NKp46, which is expressed by all NK cells. Because the CD56^{dim} NK cell population mainly induces apoptosis of target cells through exocytosis of pre-assembled cytolytic granules containing granzyme B and perforin, CD56^{dim}CD16⁺ NK cells express the *GZMB* gene at much higher

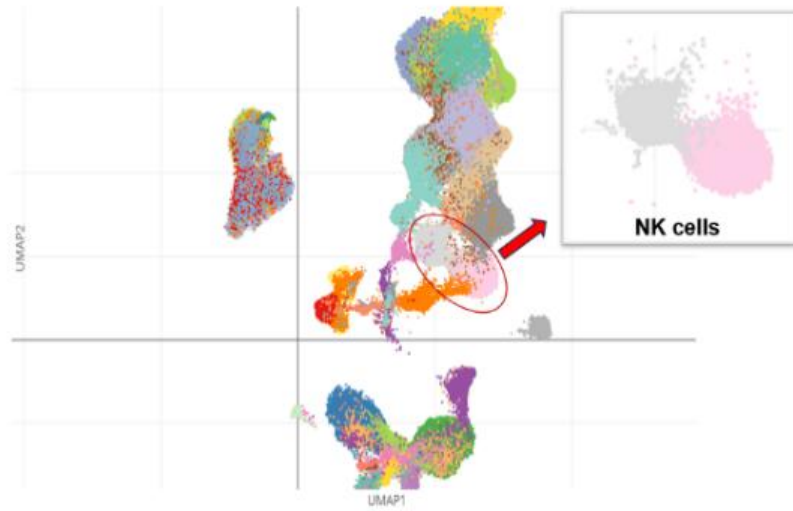
levels than CD56^{bright} NK cells. The *FCGR3A* gene encodes CD16, which plays an important role in the ADCC of NK cells. The results in Figure 4.2c also shows that some T cells and macrophages express the *PTGER2* gene, and more cells express *PTGER4* gene. These results demonstrate that the two main NK cell subsets express EP2 and EP4 receptors.

PGE₂ activates adenylate cyclase through its receptors EP2 and EP4, thereby increasing intracellular cAMP levels (Chapter 1.3.2 and Figure 1.4). In the tumour microenvironment, the cAMP signal from PGE₂ may promote tumour growth and immune escape. I looked at whether cAMP was produced by NK cells in response to PGE₂ using a cAMP ELISA. Firstly, NK cells were purified using the negative selection kit. After the ± IL-15 ± PGE₂ treatments, the cells were lysed, and the lysates were collected for cAMP ELISA. Figure 4.3 shows that IL-15 treatment did not alter cAMP levels of NK cells. However, after the addition of PGE₂, the cAMP level of NK cells increased significantly. Furthermore, treatment with IL-15 after PGE₂ treatment did not alter the cAMP level. These results indicate that PGE₂ signalling generates cAMP in NK cells, consistent with EP2 and EP4 activity shown in other cell types (Obermajer et al., 2011; Lee et al., 2019). Furthermore, my results show that IL-15 is not able to reverse the effects of PGE₂, suggesting that PGE₂ plays a dominant role in NK cell inhibition.

a

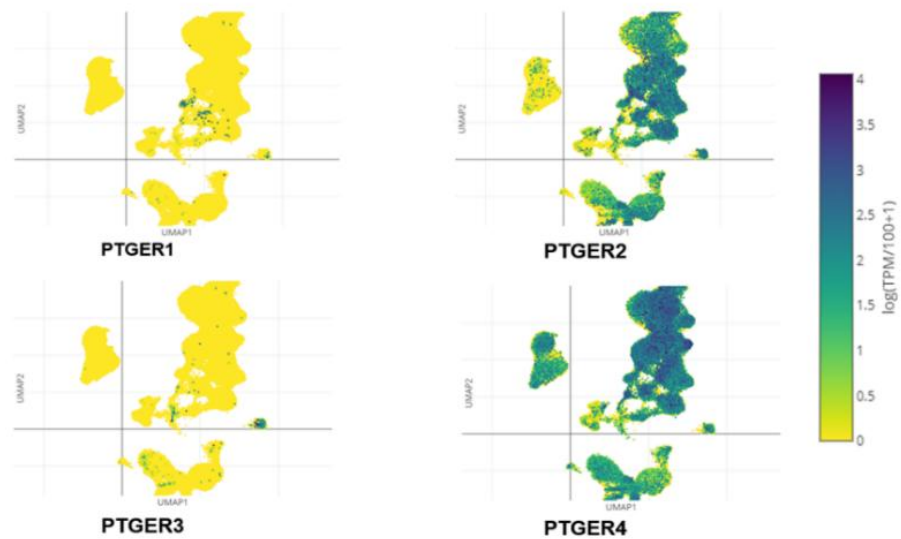


b



ABCs	1209	MAIT	3413	Progenitor	1518
Alveolar macrophages	3413	Mast cells	3291	T/B doublets	1458
Classical monocytes	3414	Megakaryocytes	317	T_CD4/CD8	3413
Cycling	1161	Memory B cells	3414	Teffector/EM_CD4	3414
Cycling T&NK	2126	migDC	262	Tem/emra_CD8	3413
DC1	356	MNP/B doublets	744	Tfh	3413
DC2	1147	MNP/T doublets	2508	Tgd_CRTAM+	3413
Erythroid	445	Naive B cells	3413	Tnaive/CM_CD4	3414
Erythrophagocytic m...	2103	NK_CD16+	3414	Tnaive/CM_CD4_act...	3413
GC_B (I)	369	NK_CD56bright_CD...	3413	Tnaive/CM_CD8	3413
GC_B (II)	203	Nonclassical monoc...	2420	Tregs	3413
ILC3	1312	pDC	713	Trm/em_CD8	3413
Intermediate macrop...	2236	Plasmablasts	1710	Trm_gut_CD8	3414
Intestinal macrophages	599	Plasma cells	3413	Trm_Tgd	3413
		Pre-B	75	Trm_Th1/Th17	3413
		Pro-B	39		

c



d

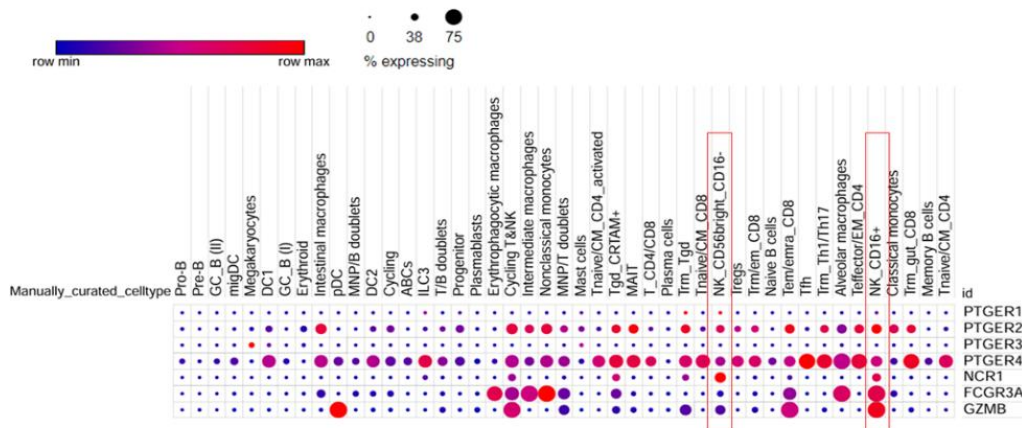


Figure 4.2: EP receptors expression in NK cells. (a) NK cells were purified from PBMCs by CD56 negative selection kit, and then extracted total RNA from purified NK cells. The expression of EP receptors was detected by TaqMan™ RT-qPCR. Expression of GAPDH was used as a control. Results presented are the mean \pm standard error of the mean (SEM) for n=5 independent experiments. (b) Analysis of single cell (sc) RNAseq data of blood cells from the study of (Domínguez Conde et al., 2022). Uniform manifold approximation and projection (UMAP) visualization of the immune cell compartment coloured by cell types. (c) UMAP visualization of the *PTGER1*, *PTGER2*, *PTGER3* and *PTGER4* genes expression in immune cells. Colour represents maximum-normalized mean expression of cells expressing marker genes (d) Dot plot for expression of marker genes of the identified immune cells populations. Colour represents maximum-normalized mean expression of cells expressing marker genes, and size represents the percentage of cells expressing these genes.

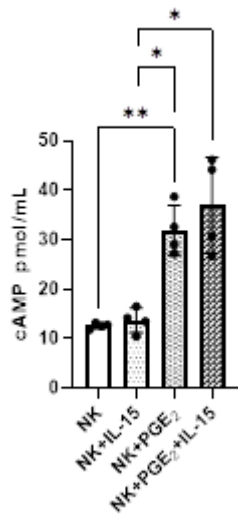


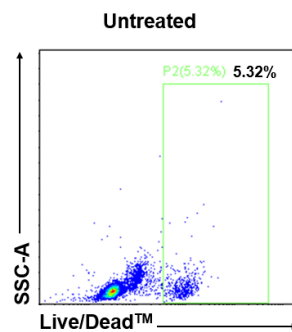
Figure 4.3: PGE₂ increased cAMP level in NK cells.

NK cells were purified from PBMCs by CD56 negative selection kit, and the cells was treated with PGE₂ for one hour, then IL-15 was added for the following one hour. After the treatments, the cells were lysed and then the lysates were collected for cAMP ELISA.

4.2.2 Pharmacological inhibition of EP2 and EP4 inhibitors

To test the importance of the EP2 and EP4 receptors I used small molecule inhibitors. However, it was important to establish that these inhibitors were not toxic to NK cells, as this would affect NK cell function. NK cells were purified from PBMCs using negative selection and then treated with EP2 or EP4 inhibitor alone or EP2 and EP4 inhibitors together. Figure 4.4 shows that EP2 and EP4 inhibitors have very little effect on NK cell viability, allowing them to be used in functional.

a



b

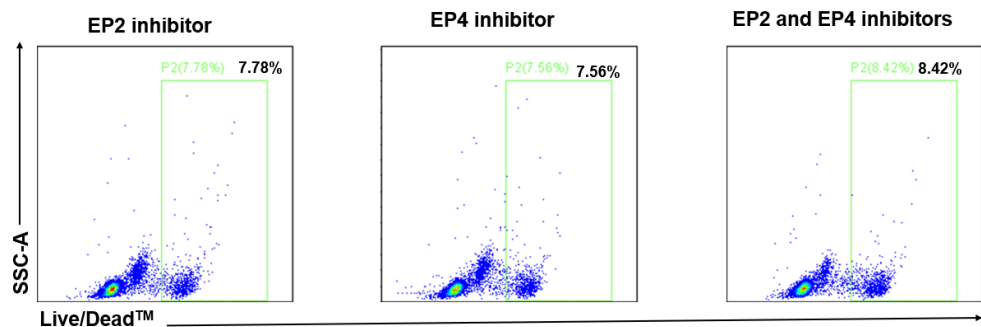


Figure 4.4: EP2 and EP4 inhibitors do not kill NK cells. (a) NK cells alone for 48 hours. (b) NK cells were treated with 10 μ M EP2, EP4 inhibitors alone or both for 48 hours. Experiments have done twice.

4.2.3 Inhibition of EP2 and EP4 receptors restores NK cell function in the presence of PGE₂

I then used the EP2 and EP4 inhibitors in functional tests of NK cell activity. NK cells were purified using negative selection. Then the NK cells were treated with inhibitors for one hour before the samples were treated with PGE₂ for one hour and then with IL-15 for another 48 hours, and IFN- γ production were measured using ELISA. Figure 4.5 shows that both EP2, EP4 inhibitor single and combination treatment can block the receptors and partly restored the IFN- γ production. The combination of EP2 and EP4 inhibitors had the greatest effect. However, it cannot completely restore the ability of NK cells to produce IFN- γ . These results suggest that NK cells are inhibited by PGE₂ using both EP2 and EP4 receptors.

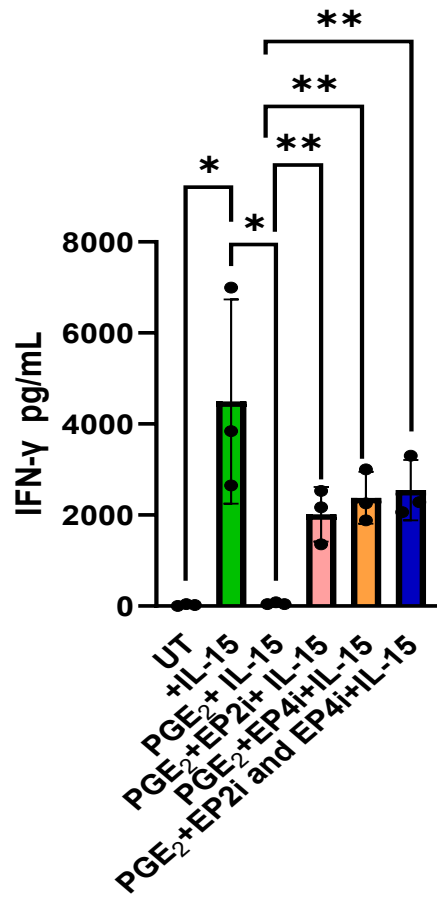


Figure 4.5: EP2 and EP4 inhibitors block the effect of PGE₂. NK cells were purified from PBMCs by CD56 negative selection kit. Purified NK cells were treated with 10 μM EP2 and EP4 inhibitors for 1 hour, then with PGE₂ for one hour before treating with IL-15 for a further 48 hours. Supernatants were screened for IFN-γ using ELISA. Results presented are the mean ± SEM for n=3 independent experiments.

4.2.4 IL-15 mediated STAT5 activation

I am also interested in and studying the pathways downstream of PGE₂- and EP2/EP4 on NK cells. Results in the previous chapter show that PGE₂ inhibits IL-15 mediated NK cell activation (Figure 3.8). IL-15 signals via the IL-15 receptor and JAK/STAT signalling and I hypothesised that PGE₂ might inhibit this activity, preventing IL-15 stimulation. NK cells were purified from PBMCs using CD56 negative selection kit, then NK cells were treated with or without 10 ng/mL IL-15 for 1h, 24h or 48h. After the treatments, the cells were harvested and STAT5 phosphorylation analysed by western blotting. Figure 4.6 shows that phosphorylated pSTAT5 protein (90 kDa) was induced at the 1h timepoint and that the levels then decreased with increasing time of IL-15; the optimal time to induce pSTAT5 is 1h compared to 24h and 48h. β -Actin (42 kDa) is a highly conserved cytoskeletal protein that is widely present in eukaryotic cells. β -Actin is often used as a suitable loading control because it is stably expressed and present in most cells and tissues.

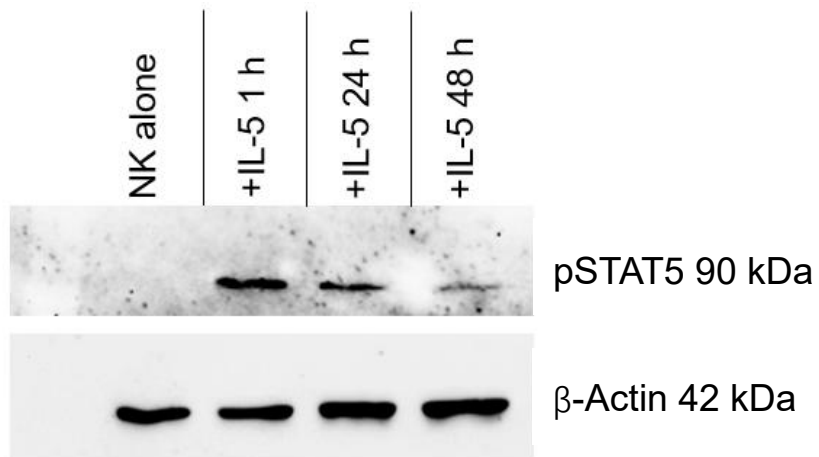


Figure 4.6: The timepoint of the expression of pSTAT5 protein in NK cells after IL-15 treatment. Each 1×10^6 NK cells were treated with or without 10 ng/mL IL-15 for 1h, 24h or 48h and cell lysates analysed for the presence of pSTAT5 or β-Actin.

4.2.5 PGE₂ inhibits NK cell activity through pSTAT1 and pSTAT5 pathways

Based on the above experiment, I chose 1 hour as a timepoint to determine the effect of PGE₂ on IL-15 signalling. NK cells were treated with PGE₂ for 1 hour and then with IL-15 for 1 hour following. Western blot was then used to detect the phosphorylation level of pSTAT1 (91 kDa) and pSTAT5 (90 kDa). pSTAT1 and pSTAT5 are the activated form of STAT1 and STAT5 proteins and are the same size as unphosphorylated STAT1 and STAT5 protein because phosphorylation only adds a small phosphate group to the protein and does not significantly change its molecular weight. Detection of total STAT1 and STAT5 protein can reflect the basal STAT levels in cells to measure whether changes in phosphorylation levels are caused by activation signals rather than changes in the total STAT proteins amount. β -Actin was used as a loading control protein. Figure 4.7 shows that both STAT1 and STAT5 proteins were phosphorylated after IL-15 treatment, indicating that the activation of NK cells would go through the pSTAT1 and pSTAT5 signalling pathways. Since the blots showed that the expression level of the pSTAT1 band was significantly lower than that of pSTAT5, IL-15 activated NK cells mainly by phosphorylating STAT5 protein. The addition of PGE₂ reduced pSTAT5 (and pSTAT1 proteins) in NK cells. This suggests that PGE₂ inhibits the phosphorylation of the pSTAT5 and pSTAT1 pathways in NK cells to prevent NK cell activation by IL-15.

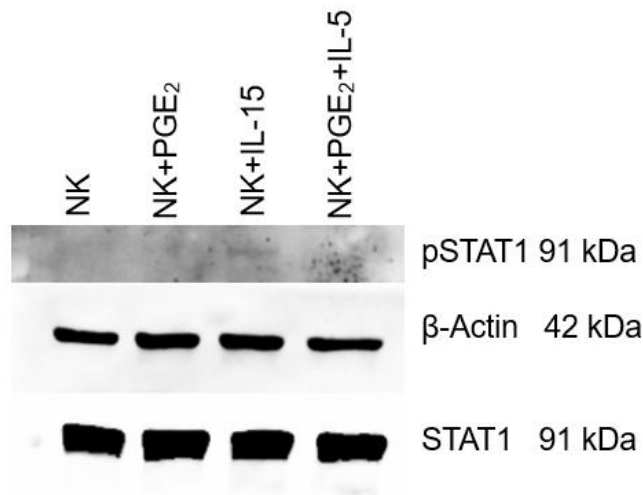
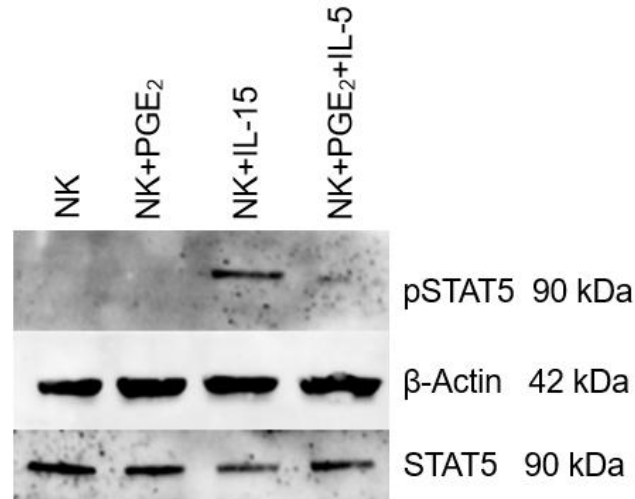


Figure 4.7: Expression of pSTAT5 and pSTAT1 proteins in NK cells after \pm IL-15, \pm PGE₂ treatment. NK cells were purified from PBMCs by untouched kit. Purified NK cells were treated with 10 μ M PGE₂ for 1 hour, and then treated with 20 ng/mL IL-15 for 1 hour. After lysing the cells, immunoblotting was used to detect the expression of pSTAT1 and pSTAT5 proteins in NK cells.

4.2.6 PGE₂ does not inhibit *IFNG* and *PRF1* gene expression downstream of JAK/STAT signalling in NK cells

Because cytokines regulate target genes through the JAK/STAT pathway, I analysed the downstream genes of the IL-15-mediated pSTAT5 and pSTAT1 pathways (Figure 4.8a). I chose *IFNG* and *PRF1* genes to test whether the addition of PGE₂ would affect their expression levels. The *IFNG* gene encodes IFN- γ and is located on the long arm of chromosome 12 (Chr 12q15). The *PRF1* gene encodes perforin and is located on the long arm of chromosome 10 (Chr 10q22). In figure 4.8b and c, the expression levels of *PRF1* gene and *IFNG* gene were detected by TaqMan qPCR. Surprisingly, the expression levels of *PRF1* and *IFNG* genes in IL-15-activated NK cells did not change after PGE₂ treatment.

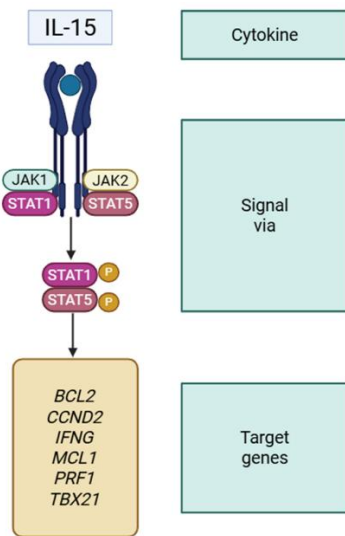
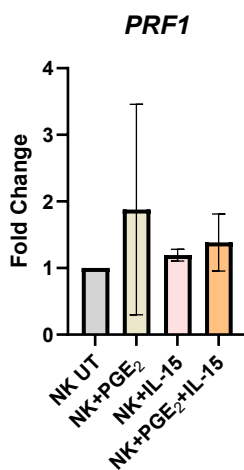
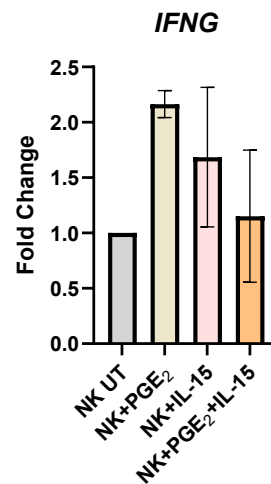
a**b****c**

Figure 4.8: Downstream genes of IL-15 activated NK cells. (a) IL-15 activates NK cells by activating downstream target genes through the JAK/STAT signalling pathway. Figure generated using Biorender. (b,c) NK cells were purified from PBMCs by CD56 negative selection kit, and then extracted total RNA from purified NK cells. The expression of *PRF1* and *IFNG* genes were detected by TaqMan™ RT-qPCR. *GAPDH* was used as a control. Results presented are the mean \pm SEM for n=2 independent experiments.

4.3 Discussion

My results show that PGE₂ inhibits NK cell function via EP2 and EP4 receptors. When the EP2 and EP4 receptors on NK cells are inhibited, it reduces the effect of PGE₂ and restores the IFN- γ production by NK cells.

PGE₂ plays a key role in tumour survival, and it is a major metabolite of COX-1 and COX-2. It is known that high levels of PGE₂ and high expression of COX-2 are observed in many solid tumours (Kawamori et al., 1998; Giles et al., 2002; Hasegawa et al., 2005; Cetin et al., 2005; Ohsawa et al., 2006). Currently, COX-2 inhibitors, mostly nonsteroidal anti-inflammatory drugs (NSAIDs), are used in clinical practice and clinical research, but these drugs can cause gastrointestinal reactions, liver, kidney and blood system damage and cannot completely inhibit the pathological activity of PGE₂. The differential expression of EP1-4 receptors means that PGE₂ plays has biological functions in many different cells, and studies have shown that EP receptors play an important physiological role in malignant tumours (Gray et al., 2009; Reader et al., 2011; Rundhaug et al., 2011; G O'Callaghan and Houston, 2015; Masato et al., 2021). Therefore, inhibitors of the EP1-4 receptors might be potential therapeutic agents for malignant tumours.

Activation of EP1 receptors is associated with the occurrence and progression of cancer, and it promotes cancer through multiple signalling pathways. PGE₂ must be at high levels to stimulate EP1 receptor activation. When the EP1 receptor is activated, it can activate phospholipase C (PLC), leading to the production of phosphatidylinositol 4,5-bisphosphate (PIP₂), which is then broken down into 1,2-diacylglycerol (DAG) and IP₃. DAG activates protein kinase C (PKC), which activates a variety of downstream signalling molecules, while IP₃ promotes the release of Ca²⁺ from the endoplasmic reticulum and increases intracellular calcium concentration, they promote cell proliferation and survival (Rundhaug et al., 2011a). The

PLC/PKC pathway has been detected in many types of cancer, such as colorectal cancer, breast cancer, and prostate cancer. In hepatocellular carcinoma (HCC), it has found that EP1 receptor may activate I κ B kinase (IKK) through PKC, I κ B protein is phosphorylated, NF- κ B is released into the cell nucleus, and cancer cell migration and invasion are promoted (Bai et al., 2014). Studies have reported that blocking EP1 receptors can reduce FasL expression in tumour cells in vivo and in vitro. Blocking EP1 receptors can also reduce Tregs and TAMs, and increase CD8⁺ T cells (O'Callaghan et al., 2013). In summary, EP1 receptors regulate the TME and immune cell function through multiple pathways, thereby helping tumour cells evade host immune surveillance.

The EP2 receptor is highly expressed in many cancers and plays an important role. The EP2 receptor is coupled to G proteins and activates AC mainly through the G α s protein, increasing the level of intracellular cAMP. My data shows that NK cells express EP2 and produce cAMP in response to PGE₂. The generated cAMP activates PKA, which in turn phosphorylates a variety of downstream target proteins, including transcription factors and enzymes, regulating gene expression and cell behaviour, and promoting tumour cell growth and survival. In addition, EP2 activation stimulates the secretion of IL-6 (Merz et al., 2016), a pro-inflammatory factor that directly acts on cancer cells to trigger STAT3 activation and the expression of STAT3 target genes; these proteins drive cancer cell proliferation and survival, promote angiogenesis, invasiveness, metastasis and immunosuppression- key hallmarks of cancer.

In addition to expression by NK cells, the EP2 receptor is expressed by a variety of immune cells, such as macrophages, dendritic cells, T cells and B cells (Punyawatthanakool et al., 2024). For example, activation of the EP2 receptor can promote the proliferation and function of Tregs and inhibit the function of Th1 cells (Fabricius et al., 2010; An et al., 2021; Goepp et

al., 2021). Thus, acting via EP2, PGE₂ will inhibit NK cells and favour immunosuppression. Due to the importance of EP2 in immune regulation, it has become a potential target for tumour immunotherapy. Blocking the EP2 signalling pathway may become a therapeutic strategy to enhance anti-tumour immune response.

The EP3 receptor, like EP2, belongs to the GPCR family. EP3 receptor activates different cell signalling pathways through different G protein coupling modes, playing a variety of complex roles in cancer, such as Gai/cAMP/PKA pathway (Zhang et al., 2024), Gαq/PLC pathway (Kim et al., 2013) and Gα12/Rho pathway (Macias-Perez et al., 2008). Studies have shown that EP3 enhances cancer cell proliferation and migration (Ye et al., 2020), and induces the expression of VEGF in cancer (Reader et al., 2011), favouring angiogenesis.

The role of EP4 receptor in tumours has been extensively studied and found to play an important role in promoting tumour growth, invasion and immune escape in various cancer types. For example, the EP4 receptor promotes prostate cancer cell growth and invasion by activating signalling pathways such as PI3K/AKT and ERK/MAPK, and EP4 promotes prostate cancer cell growth and invasion by inducing genes related to cell migration and invasion, such as MMPs, receptor activator of nuclear factor κB ligand (RANKL), and runt-related transcription factor 2 (RUNX2) (Xu et al., 2018). In breast cancer, EP4 also promotes tumour progression by regulating angiogenesis and lymphangiogenesis and inducing the expression of proinflammatory cytokines such as IL-8, IL-6, TNF-α, VEGF, and granulocyte colony-stimulating factor (De Paz Linares et al., 2021).

Since both EP2 and EP4 are highly expressed in most immune cells, EP4 is often studied together with EP2, and studies have shown that EP2 and EP4 are co-expressed on a variety of immune cells. EP2 and EP4 receptors inhibit T cell activation by increasing intracellular cAMP levels through

activation of the G α s protein-coupled signalling pathway. High levels of cAMP inhibit TCR signalling, reducing T cell proliferation and cytokine production, such as IFN- γ and IL-2. PGE $_2$ induces the production of IL-10 and inhibits the production of IL-12 through the EP2 and EP4 signalling pathways. EP2 and EP4 work together to inhibit the expression of MHC class II proteins, weakening antigen presentation and reducing T cell activation (Harizi et al., 2003). In addition, studies have shown that the activation of EP2 and EP4 also has an inhibitory effect on DC cells. They will activate NF- κ B signalling to promote active inflammation. In addition, EP2 and EP4 will enhance the immunosuppression of TME through the mregDC (mature DC enriched in immunoregulatory molecules)-Treg axis. This study also found that the use of EP2 and EP4 antagonists can effectively deplete Treg and upregulate T cell activity (Thumkeo et al., 2022). Therefore, as my research has shown, EP2 and EP4 receptors can be used as potential immune targets and effective inhibitors can be found.

Chapter 5

The mechanism of PGE₂ production in tumours and its effects on NK cells

5.1 Introduction

In cancer development, autocrine and paracrine signalling mechanisms play a key role in the occurrence and progression of cancer by promoting the growth, invasion and metastasis of tumour cells and regulating the tumour microenvironment. Tumour cells produce large amounts of PGE₂ in the TME. However, most tumour cells stop secreting PGE₂ when cultured *in vitro*. The reason for this is not clear but the lack of a physiological environment including other cell types is one possibility.

Prostaglandin E₁ (PGE₁) alcohol is a mimic of PGE₂ (Figure 5.1a), which can also bind to EP receptors, EP3 and EP4 receptors (Figure 5.1b). PGE₁ alcohol is an alcohol formulation of PGE₁. PGE₁ itself is a biologically active lipid. During the formulation process, its alcohol properties may be utilized for certain chemical modifications to facilitate better preservation, transportation, and application. I hypothesise that PGE₂ might stimulate its own synthesis (Figure 5.1b). To test this, I used PGE₁ alcohol to stimulate EP3 and EP4 receptors and then measured resultant PGE₂ production.

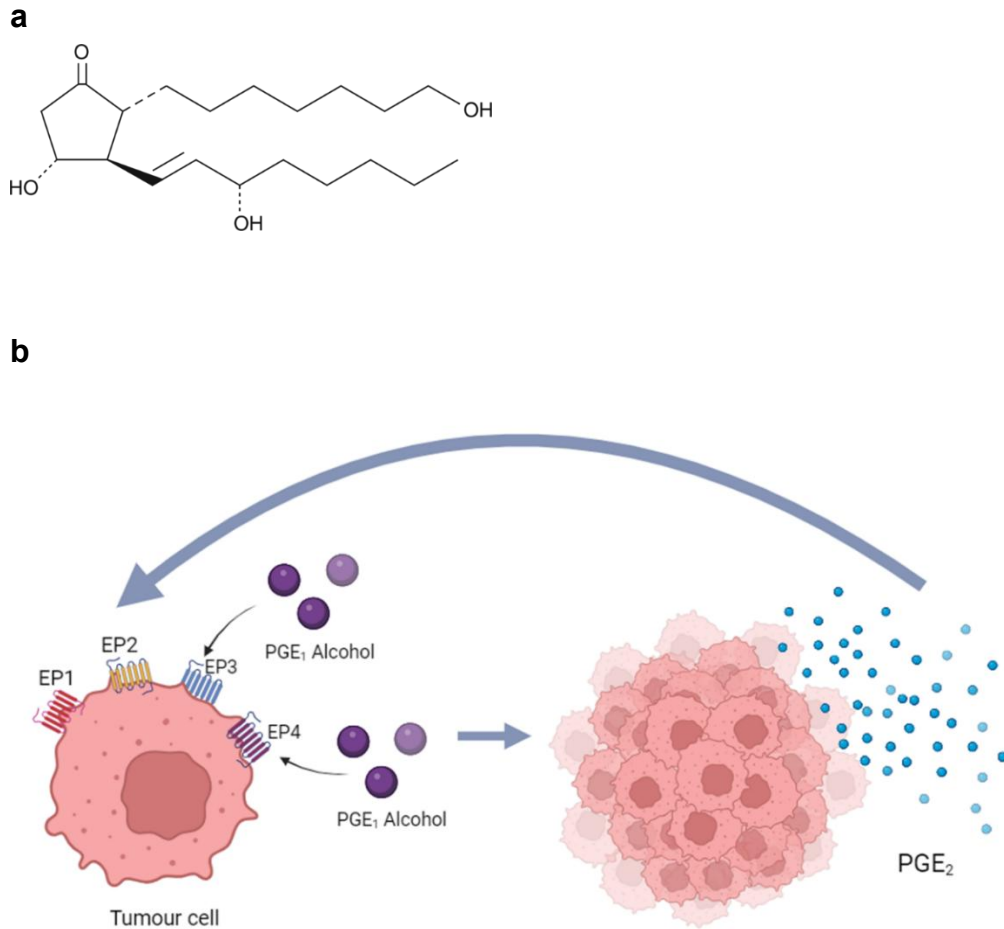


Figure 5.1: Structure and function of PGE₁ alcohol. (a) The structure of PGE₁ alcohol. PGE₁ alcohol and PGE₂ are similar in structure, mainly because they both belong to the PGE class of prostaglandins, have the same cyclic skeleton and functional groups, but the unsaturation (number of double bonds) of the side chains is different, which leads to their different activities *in vivo*. PGE₁ alcohol has only one double bond located on the α chain, while PGE₂ has one double bond each on the α chain and the β chain. (b) PGE₁ alcohol binds to EP3 and EP4 on the surface of cancer cells, causing the cancer cells to re-secrete PGE₂. The secreted PGE₂ can then bind to EP1-4 on the surface of cancer cells to produce more PGE₂. Figure generated using Biorender.

5.2 Results

5.2.1 Specificity of the PGE₂ ELISA

Detecting newly synthesised PGE₂ as a result of PGE₁ alcohol stimulation requires the ability to distinguish between the two compounds. The two molecules are highly related (Figure 5.1a) and I was concerned that the ELISA assay I used to quantify PGE₂ would also detect PGE₁ alcohol. I therefore set up a control ELISA, using the kit to detect a range of PGE₂ standards and a similar concentration range of PGE₁ alcohol. The PGE₂ ELISA kits use a competitive enzyme-linked immunosorbent assay format. PGE₂ in the sample competes with horseradish peroxidase (HRP)-conjugated PGE₂ tracer for binding sites on a mouse monoclonal anti-PGE₂ antibody. So higher PGE₂ in sample is fewer binding sites available and more HRP tracer remains unbound, then lower OD is showed. The results shown in Figure 5.2 show that the PGE₂ ELISA does not detect PGE₁ alcohol.

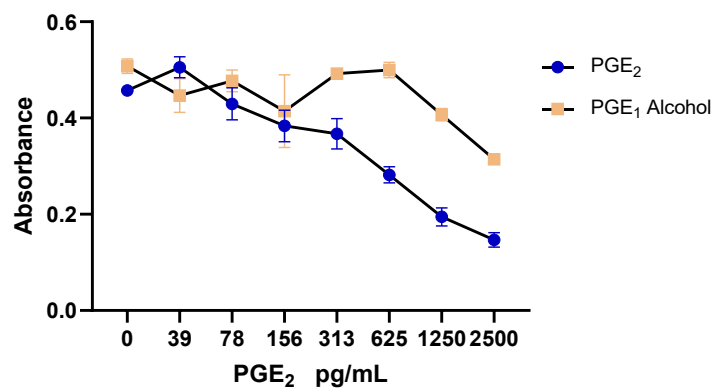
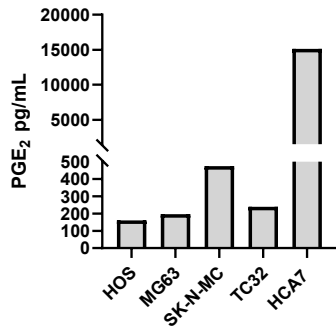
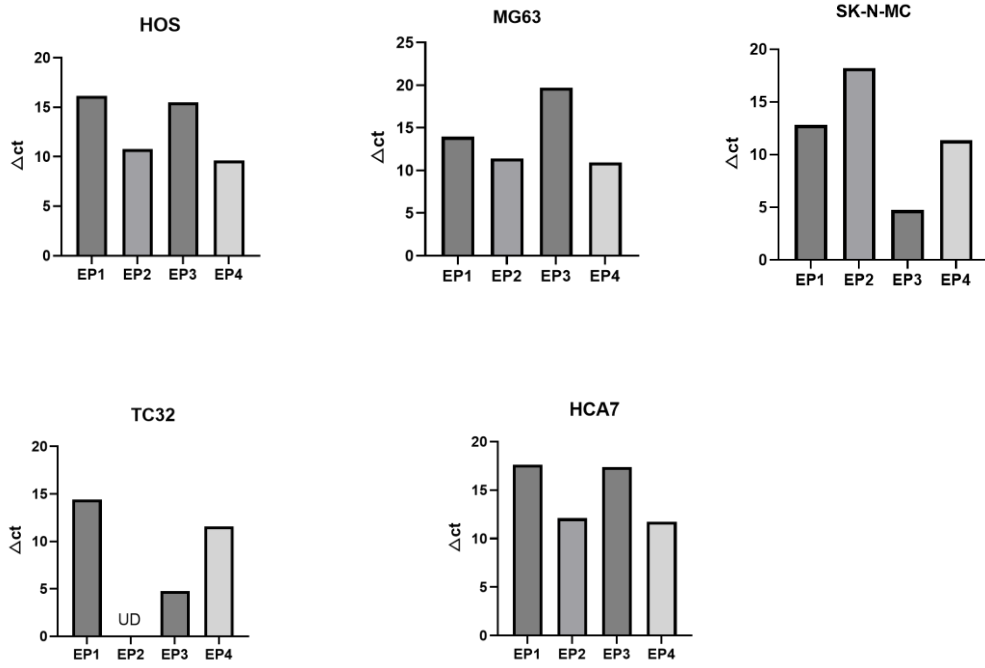
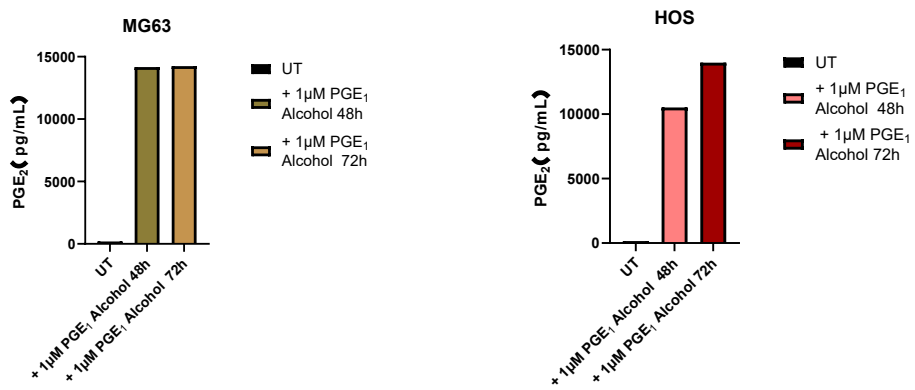


Figure 5.2: PGE₂ control ELISA for PGE₁ alcohol. PGE₁ alcohol was diluted to 2500 pg/mL and continue diluting to the PGE₂ standard concentration range, then detected using PGE₂ ELISA.

5.2.2 PGE₁ alcohol induces cancer cell lines to secrete PGE₂

Most tumour cell lines produce almost no PGE₂ under normal cell culture conditions *in vitro*. Here I selected several bone cancer cell lines (representing Ewing sarcoma and osteosarcoma) to test the amount of PGE₂ produced. It is known that the colorectal cancer cell line HCA7 produces a large amount of PGE₂ when cultured *in vitro*, so it was used as a positive control for this assay. Figure 5.3a shows that, without PGE₁ alcohol treatment, only HCA7 cells secreted high level of PGE₂, all the bone cancer cell lines secreted very little amount of PGE₂. Then Taqman™ qPCR was used to test the expression of EP receptors in each cancer cell line. The result shows that HOS, MG63 and SK-N-MC cells have all four EP receptors, TC32 does not have EP2 receptor, and HCA7 cells have EP1-4 receptors (Figure 5.3b). PGE₁ alcohol can bind to EP3 and EP4 receptors and all cell lines used in the experiments expressed these two receptors suggesting that PGE₁ alcohol can act upon them. The bone cancer cell lines were treated with 1 μM PGE₁ alcohol for 48 hours or 72 hours. After the treatment, all the supernatant from these four cell lines were collected and PGE₂ levels determined by ELISA. Figure 5.3c shows that after the PGE₁ alcohol treatment, the secretion of PGE₂ increased in all the bone cancer cell lines after 72 hours. The HOS and MG63 cell lines are osteosarcoma cell lines and SK-N-MC and TC32 cell lines are Ewing sarcoma cell lines. PGE₁ alcohol induces the osteosarcoma cell lines to produce large amounts of PGE₂, around 10-20 times greater than the amounts made by Ewing sarcoma cell lines.

a**b****c**

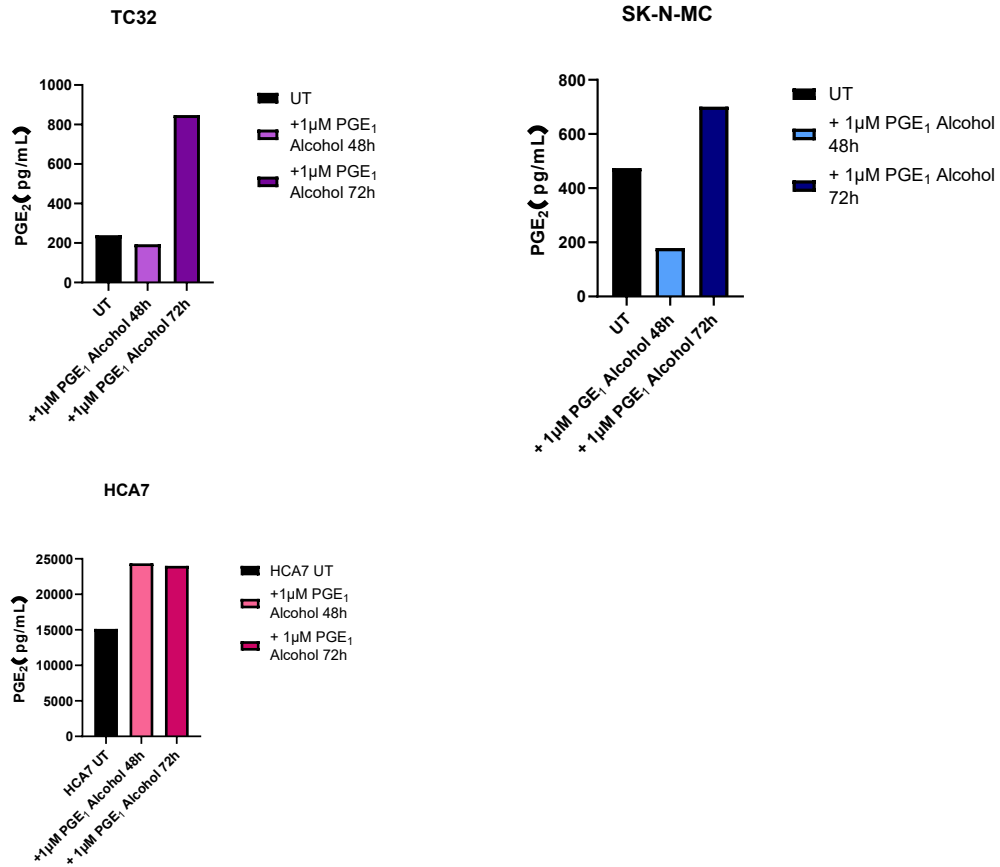


Figure 5.3: PGE₁ alcohol induces cancer cell lines to secrete PGE₂. (a) 2×10^6 HOS, MG63, SK-N-MC, TC32 and HCA7 cells were cultured in a 6 well plate for 48 hours. After culture, the supernatants were collected for PGE₂ ELISA. (b) 2×10^6 HOS, MG63, SKNMC, TC32 and HCA7 cells were cultured in 6 well plates for 48 hours and then the cells were harvested for EP1-4 expression using Taqman™ qPCR. (c) 2×10^6 HOS, MG63, SK-N-MC, TC32 and HCA7 cells were cultured in 6 well plates for 48 hours or 72 hours. After the treatment, the supernatants were collected for PGE₂ ELISA.

5.2.3 PGE₁ alcohol upregulates the expression of COX-2 in cancer cell lines

Results in Figure 5.3 show that PGE₁ alcohol stimulates the cell lines to produce more PGE₂. I used western blotting to detect the effect of PGE₁ alcohol treatment on the expression of COX-2 in these cancer cell lines. Surprisingly, although it has been shown that HOS, MG63, TC32 and SK-N-MC cells produce almost no PGE₂ when cultured *in vitro* (Figure 5.3a), they all express COX-2 under untreated conditions, and SK-N-MC cell expresses COX-2 at a higher level than HCA7 which produces the largest amount of PGE₂ (Figure 5.4a). After PGE₁ alcohol treatment, the expression of COX-2 in HOS, TC32 and SK-N-MC cells was upregulated (Figure 5.4a) suggesting that PGE₁ alcohol acting through EP receptors, stimulates COX-2 and causes them to secrete more PGE₂. As cAMP is known to be a downstream product of the PGE₂ signalling pathway, the production of cAMP was analysed following PGE₁ alcohol stimulation. However, Figure 5.4c shows that there is almost no difference in their cAMP production in HOS, MG63, TC32 and SK-N-MC cells after PGE₁ alcohol treatment. This experiment needs repeating and further investigation of the mechanism of PGE₂ production following PGE₁ alcohol stimulation is required.

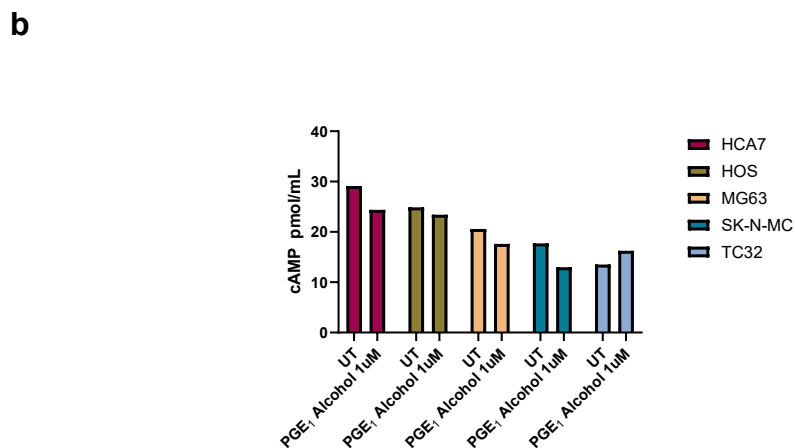
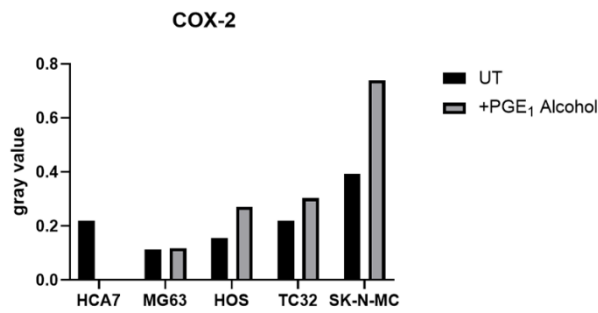
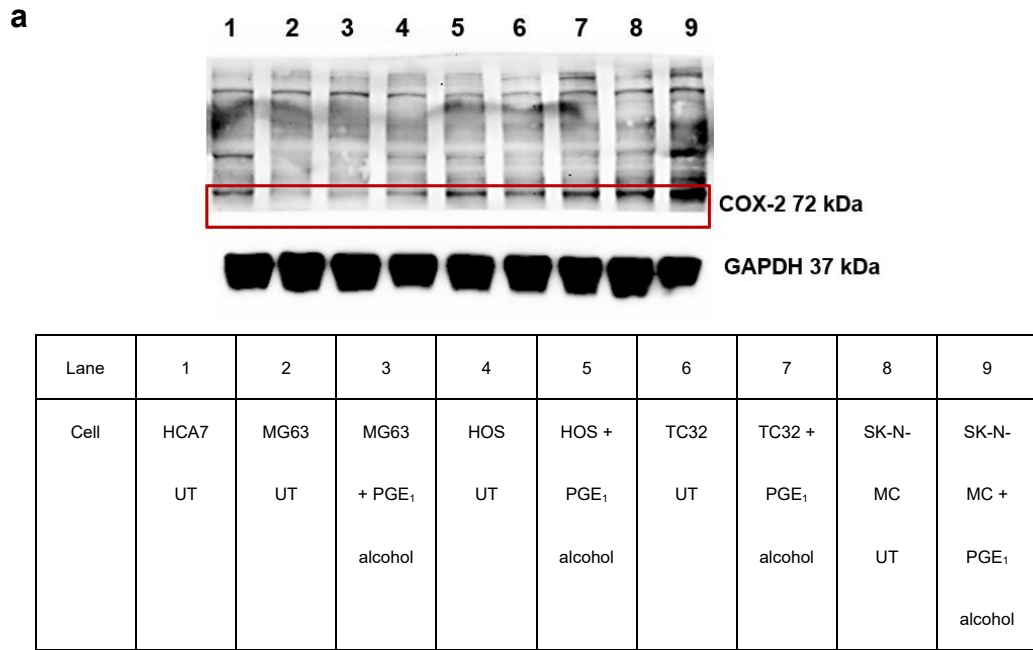


Figure 5.4: The expression of COX-2 and cAMP in cancer cell lines after PGE₁ alcohol treatment. (a) With or without PGE₁ alcohol treatment, 1×10^6 HCA7, HOS, MG63, TC32 and SK-N-MC cells were cultured in a 6

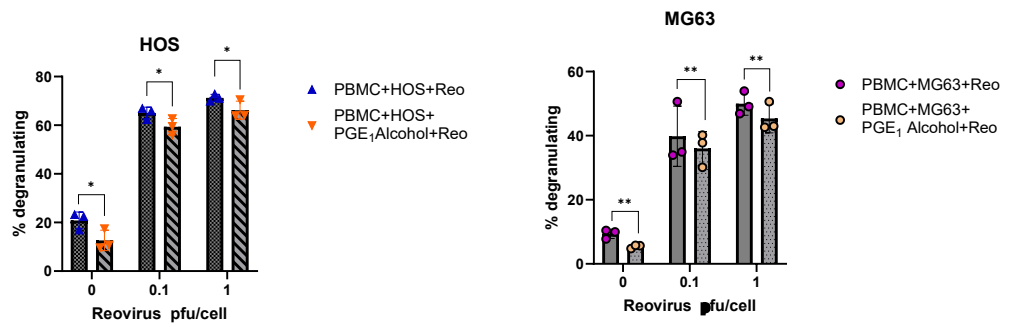
well plate. After 24 hours treatment, the cells were harvested. After lysing the cells, immunoblotting was used to detect the expression of COX-2 in these cells. GAPDH was used as the housekeeping standard. The gray value of protein expression was quantified by ImageJ. (b) With or without PGE₁ alcohol treatment, 1x10⁶ HCA7, HOS, MG63, TC32 and SK-N-MC cells were cultured in a 6 well plate. After 24 hours treatment, the cells were harvested for cAMP ELISA.

5.2.4 The impact of cancer cell derived PGE₂ on NK cell cytotoxicity

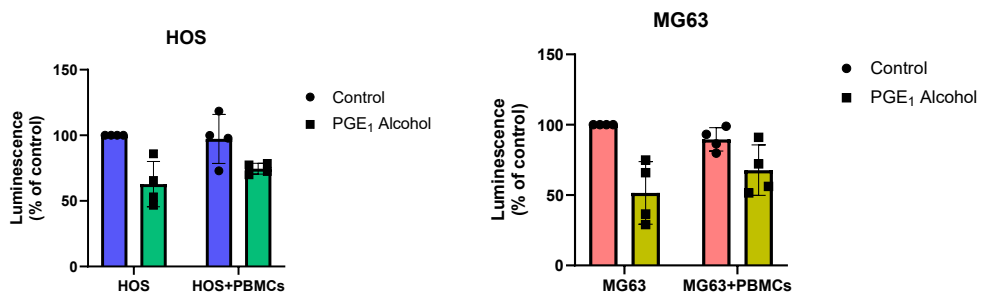
Because osteosarcoma cell lines HOS and MG63 produced more PGE₂ after the addition of PGE₁ alcohol, HOS and MG63 cell lines were selected for the following experiments. I examined whether inducing PGE₂ secretion by PGE₁ alcohol treatment inhibited NK cell cytotoxicity against osteosarcoma cell cultures. The degranulation capacity of NK cells against HOS and MG63 was analysed after co-culture of HOS and MG63 cells (with or without PGE₁ alcohol treatment) using PBMCs stimulated with/without reovirus for 4 h. Figure 5.5a shows that reovirus stimulates NK cell activity against these osteosarcoma cell lines and that NK cell degranulation is inhibited following PGE₁ alcohol treatment of the osteosarcoma cell lines. This is consistent with PGE₁ alcohol inducing PGE₂ production in the cell lines and the inhibition of NK cell activity by this PGE₂.

Then I made target cells expressing FLUC. The FLUC HOS and MG63 cells (with or without PGE₁ alcohol treatment) were co-cultured with PBMCs and I measured luminescence (as a marker of cell viability) to detect the killing by NK cells. Adding PGE₁ alcohol causes the HOS and MG63 cells to secrete PGE₂ (Figure 5.3c). PGE₁ alcohol treatment reduced the killing of the FLUC target cells but these results did not reach statistical significance (Figure 5.5b). In addition, the production of IFN- γ were analysed by ELISA. HOS and MG63 cells were treated with PGE₁ alcohol for 48 hours, then the supernatants were collected and were added to PBMCs for another 48 hours. Compared to the IL-15 treatment, supernatants from PGE₁ alcohol treated cells caused a reduction in IFN- γ secretion, consistent with the production of PGE₂ under these conditions.

a



b



c

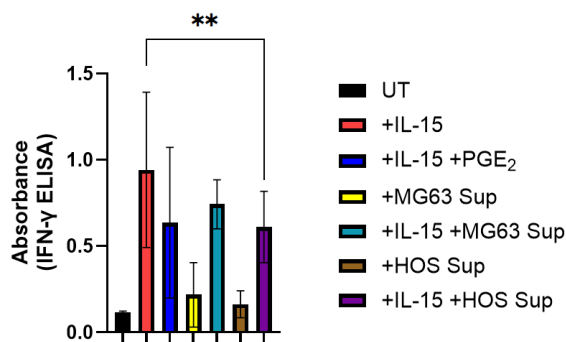


Figure 5.5: Cancer cell lines derived PGE₂ inhibits NK cell cytotoxicity.

(a) 1×10^5 HOS and MG63 cells were treated with or without 1 μ M PGE₁ alcohol for 48 hours. PBMCs were treated with 0, 0.1 and 1 pfu/cell reovirus for 48 hours. Then co-culture the target cells and PBMCs (1×10^6 PBMCs in 100 μ L RPMI each well) for 4 hours and assess NK cell degranulation using

CD3, CD56, CD107 using flow cytometry. Results presented are the mean \pm SEM for n=4 independent experiments (b) 1×10^4 HOS-FLUC and MG63-FLUC cells were treated with or without $1 \mu\text{M}$ PGE₁ alcohol for 48 hours. Then 2.5×10^5 PBMCs in $100 \mu\text{L}$ RPMI were added into the target cells and co-cultured for 24 hours. After the treatment, luciferin was added to the cells for 5 mins and read the luminescence. Results presented are the mean \pm SEM for n=4 independent experiments. (c) 2×10^6 HOS and MG63 cells were treated with or without $1 \mu\text{M}$ PGE₁ alcohol for 48 hours. The supernatants from the cells were collected and added to the PBMCs. PBMCs were treated with or without $1 \mu\text{M}$ PGE₂ for 1 hour and then treated with IL-15 for 48 hours. Supernatants were screened for IFN- γ using ELISA. Results presented are the mean \pm SEM for n=4 independent experiments. This experiment shows absorbance on the y axis rather than the concentration of IFN- γ because the standard curve did not work in this experiment.

5.2.5 Cancer cell 3D co-culture model secretes high levels of PGE₂

The results above suggest that PGE₂ induces its own synthesis in tumour cells. I speculated that, in the tumour microenvironment, different cell types might co-operate to regulate PGE₂ production. I therefore analysed PGE₂ production by spheroids containing MG63 alone or MG63 co-cultured with CD14⁺ monocytes and MSCs. These 3D cell models were cultured for 7 days in a low adhesion plate to become spheroids (Figure 5.6a). After the treatment, the supernatants of the spheroids were collected for PGE₂ ELISA, and these values were compared to the PGE₂ from the individual cell types. The co-culture model secreted a high level of PGE₂ (Figure 5.6b) suggesting that these cells do interact in the tumour to promote the production of PGE₂, leading to a highly immunosuppressive microenvironment.

a



b

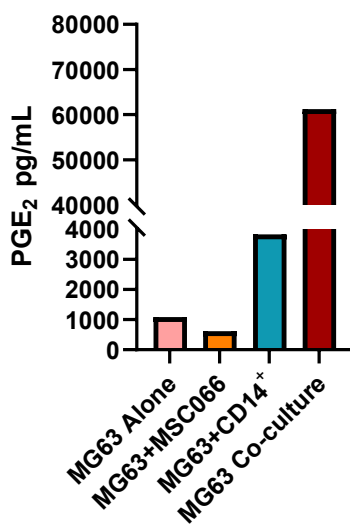


Figure 5.6: MG63 co-culture spheroid secretes high levels of PGE₂. (a) 1×10^4 MG63 alone or MG63 co-culture (1×10^4 MG63, 5×10^3 CD14⁺ monocytes and 2×10^3 MSCs) were cultured in 96 well low adhesion plate for 7 days. Images were taken by EVOS. 200 μ m scale bar (b) The media from MG63 alone MG63 + MSC066, MG63 + CD14⁺ monocytes and MG63 co-culture spheroid was collected and detected the PGE₂ secretion by PGE₂ ELISA kit. (MG63 + MSC066, MG63 + CD14⁺ monocytes data generated by Tyler Barr)

5.3 Discussion

In this chapter, I used two methods to induce PGE₂ secretion from cancer cell lines cultured *in vitro*. PGE₁ alcohol can cause the cells to secrete PGE₂ by acting through EP receptors on the surface of cancer cells. In addition, it was found that the production of COX-2 increased. However, cAMP, which is the downstream of the PGE₂ signalling pathway, did not change. PGE₂ produced by cancer cells after PGE₁ alcohol treatment has been shown to inhibit the cytotoxicity of NK cells.

PGE₁ alcohol acts on EP3 and EP4 receptors of the cell, while cAMP is produced through the downstream pathways of EP2 and EP4 receptors. Although EP2 and EP4 can increase intracellular cAMP levels by activating AC, EP4 can also couple with inhibitory G α protein (G α i) to inhibit cAMP (Fujino and Regan, 2006). Furthermore, EP4 is rapidly internalized after ligand binding, which may reduce further signalling of PGE₂ (Vleeshouwers et al., 2021). In addition, the newly produced PGE₂ in cells may activate EP3 and EP1 receptors more. Thus, the EP3 receptor may lead to the suppression of cAMP levels through Gi protein (Vio et al., 2012). Activation of EP1 receptor may trigger an increase in Ca²⁺-related signals rather than cAMP (Chi et al., 2014). In addition, COX-2 products are not only PGE₂, but also include PGI₂, PGD₂, PGF₂ α and TXA₂ (thromboxane A₂) (Brock et al., 1999). Some of these products may inhibit cAMP through the Gi pathway. After activation, PKA (cAMP-dependent protein kinase) may inhibit AC through a negative feedback mechanism, limiting further increase in cAMP. PGE₂ is mainly released into the extracellular space through PGE multidrug resistance-associated protein 4 (MRP4) and prostaglandin transporter (PGT), and then binds to EP receptors (Kochel and Fulton, 2015). If PGT expression is limited, even if intracellular PGE₂ increases, exogenous signals may still be insufficient to activate the cAMP pathway. This may explain why increased cAMP was not detected in the tumour cells after

PGE₁ alcohol treatment. However, this needs further testing.

When cancer cells, TAMs and MSCs were co-cultured in a 3D model, they produced high levels of PGE₂. This is the result of their combined effects, as single cancer cells, single TAMs, and MSCs can only produce very small amounts of PGE₂. This may be because TAMs and MSCs secrete a small amount of PGE₂, which then acts on cancer cells, stimulating cancer cells to produce PGE₂ through the EP1-4 receptors on cancer cells. Moreover, both TAMs and MSCs secrete related cancer-promoting molecules such as TGF- β , IL-10 and IL-6, which may also prompt cancer cells to produce more immunosuppressive factors, including PGE₂ (Figure 5.7). In addition, 3D cell culture is a cell culture method that simulates the *in vivo* microenvironment. Its main purpose is to be closer to physiological conditions and improve the biological relevance of the research. Compared with traditional 2D cell culture, 3D cell culture can better reflect cell-cell interaction, cell-matrix interaction and tissue structure. This can increase the interaction between cells, better simulate the TME, and make the autocrine and paracrine effects of cancer cells produce better effects (Kapałczyńska et al., 2016). Therefore, 3D cultures (spheroids) change cellular interactions and the microenvironment, and this may promote PGE₂ synthesis. Because the cells are densely packed in the spheroids, a hypoxic zone is easily formed inside the spheres, activating hypoxia-inducible factor 1 α (HIF-1 α) which promotes the upregulation of COX-2 expression and induces PGE₂ synthesis (Liu et al., 2002). In 3D culture, cancer cells, TAMs, and MSCs are in closer contact, and cell adhesion molecules (such as E-cadherin and N-cadherin) and integrin signals may be upregulated, thereby activating signalling pathways such as NF- κ B and P13K, which may increase COX-2 and PGE₂ levels (Dohadwala et al., 2006; Nam et al., 2013; Kariya et al., 2018; Loh et al., 2019). 3D culture may also change the morphology and stress distribution of cells, affecting mechanosensory

pathways such as YAP/TAZ, which may further promote inflammatory and immunosuppressive signals and upregulate PGE₂ (Pancieria et al., 2017; Jafarina et al., 2024). In spheroids, as the diameter of the spheroid increases, the supply of oxygen and nutrients to the inner regions gradually decreases, leading to the formation of pronounced hypoxic or severely hypoxic zones, particularly in the core. This physiological condition closely mimics the hypoxic microenvironment found within solid tumours and triggers a series of cellular adaptive responses, with the activation of the hypoxia-inducible factor (HIF) signalling pathway being the most central. Firstly, hypoxia induces DNA strand breaks, including double-strand breaks (DSBs) and single-strand breaks (SSBs), and it also weakens DNA repair pathways. Then under hypoxia, multiple signalling pathways (PI3K-mTOR, JAK-STAT3, NF-κB, MAPK, Wnt/β-catenin, Notch) enhance HIF expression. Loss of tumour suppressors (p53, PTEN) and increased ROS production further stabilize HIF. HIF translocates into the nucleus, and binds to hypoxia response elements (HREs), and activates a wide range of downstream genes, such as vascular endothelial growth factor (VEGF), glycolytic enzymes, and pro-survival factors. These molecules promote angiogenesis, metabolic reprogramming, and cellular adaptation to hypoxia, thereby enhancing tumour proliferation, migration, invasion, EMT, and angiogenesis (Chen, et al., 2023).

In summary, 3D culture may lead to increase PGE₂ synthesis through cell aggregation, hypoxia, enhanced cell interactions and changes in mechanical forces. For the future research, this 3D co-culture model can help us gain a deeper understanding of the upstream regulatory factors of PGE₂ in TME (such as COX-2, NF-κB, HIF) and their role in immunosuppression and inflammation regulation, providing new ideas for anti-cancer treatment targeting the PGE₂ pathway.

This model is not only for studying cancer cells themselves, but also for

studying how TAMs are polarized by tumour cells (M1 and M2), how MSCs promote immunosuppression or enhance tumour invasiveness, and how hypoxia regulates inflammation and immune escape. And whether other inflammatory factors (such as IL-6, TGF- β) can synergize with PGE₂. This is of great significance for understanding how the TME shapes cancer progression and finding ways to reverse immunosuppression. Therefore, it can provide theoretical support for the development of new immunotherapy strategies, such as PGE₂ inhibitors, and provide new evidence for combined immunotherapy.

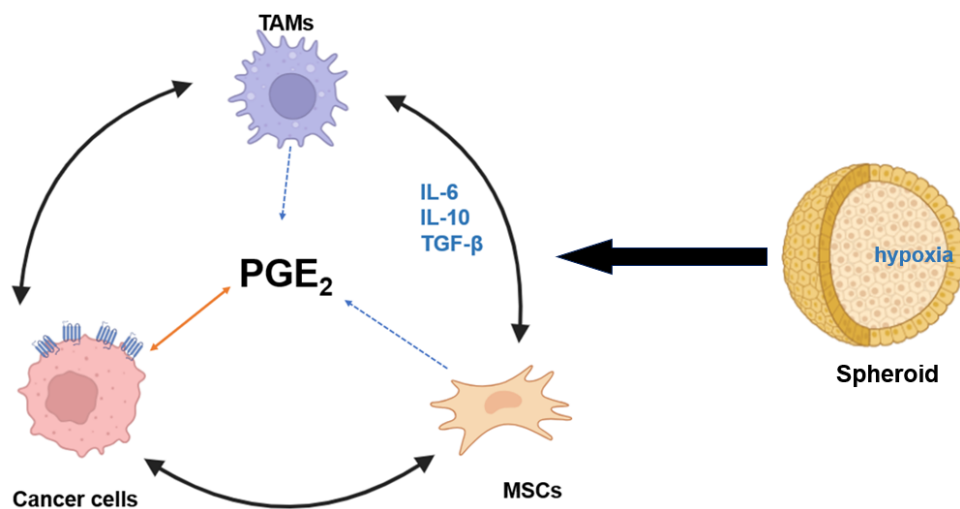


Figure 5.7: 3D co-culture cell model enables the production of large amounts of PGE₂. 3D co-culture enhances cell interactions, hypoxia, ECM signals, inflammatory cytokines, and mechanical changes, ultimately leading to the production of PGE₂. Figure generated using Biorender.

Chapter 6

Conclusions and future work

6.1 Principal findings and summary

In this study, I demonstrated that PGE₂ inhibits human NK cell activity *in vitro*. In Chapter 3, I showed that reovirus, IL-2 and IL-15 increased NK cell activity. However, PGE₂ pre-treatment of NK cells prior to reovirus or cytokine treatments inhibited NK cell activation, NK degranulation and IFN- γ secretion.

In Chapter 4, I found that NK cells express the PGE₂ receptors EP2 and EP4 but do not express EP1 and EP3 receptors. Importantly, PGE₂ upregulates the cAMP level in NK cells through EP2 and EP4 receptors, thereby inhibiting the pSTAT5 signalling pathway, which leads to a decrease in IL-15-mediated NK cell activity, reduced degranulation, and suppressed IFN- γ production. However, the mechanism by which STAT5 phosphorylation is inhibited is not yet clear. IL-15 binding to the IL-15 receptor causes the phosphorylation of JAK1 and 3 which then phosphorylate STAT5 (Lin and Leonard, 2018). As for Chapter 5, I used the PGE₂ mimic, PGE₁ alcohol, which signals via EP3 and EP4 and induces cancer cell lines to secrete PGE₂. PGE₁ alcohol pre-treatment of osteosarcoma cell lines MG63 and HOS inhibited NK cell degranulation. Moreover, this demonstrates that this PGE₂ mimic induces PGE₂ synthesis in tumour cells, indicating that while PGE₂ secretion is often lost in long established tumour cell lines, cells remain sensitive to autocrine and/or paracrine stimulation to secrete PGE₂.

I established that when cancer cell lines, TAMs and MSCs were co-cultured in 3D spheroids, a large amount of PGE₂ was produced, which suggests that in the tumour microenvironment, different cell types will synergistically

regulate the production of PGE₂. Overall, this will build up the production of PGE₂ and help to establish an immunosuppressive environment in the tumour. I have summarised my key findings in Figure 6.1.

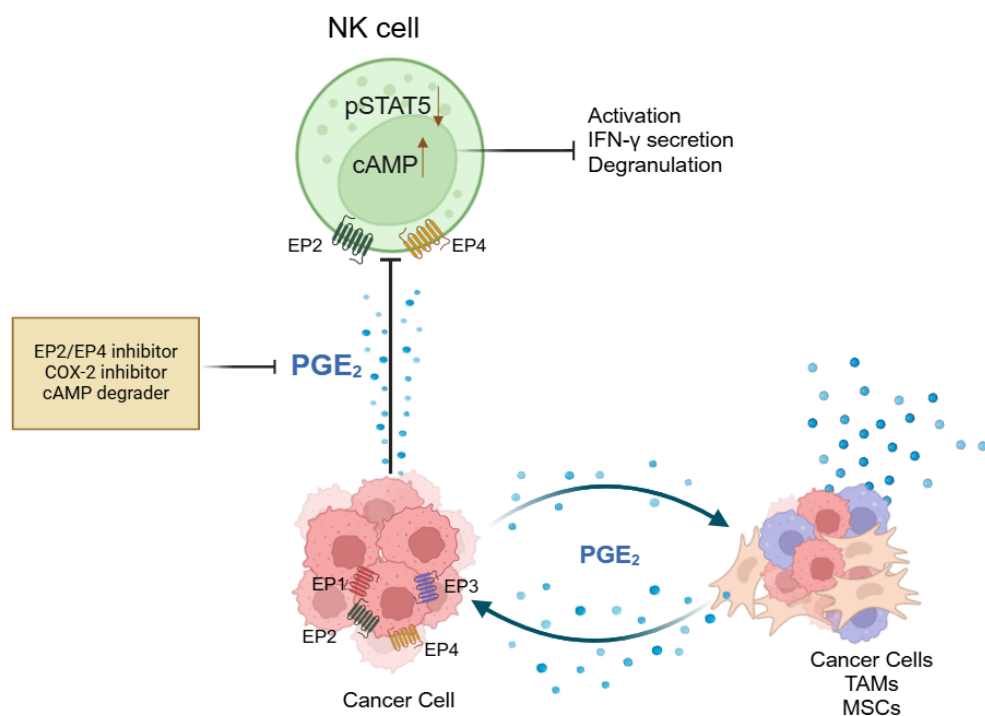


Figure 6.1: PGE₂ inhibits NK cell activity. Cancer cells produce PGE₂, which inhibits the activity of NK cells through the EP2/EP4 and pSTAT5/cAMP pathway on the surface of NK cells. When cancer cells, TAMs and MSCs are 3D co-cultured, a large amount of PGE₂ is produced. EP2/EP4 inhibitors, COX-2 inhibitors and cAMP degraders may block PGE₂ production. Figure generated using Biorender.

Several experiments using NK cells, especially those in chapters 3 and 4, show a high degree of variability of responses between donors. For example, Figure 3.9a shows that IL-15 induces strong IFN- γ production by NK cells and that this is almost completely inhibited by PGE₂ treatment. However, there is considerable variation between donors in the amount of IFN- γ produced when NK cells are stimulated with IL-15 and this variation meant that the inhibition of IFN- γ production by PGE₂ did not reach statistical significance. Variation in the activity of human NK cells between donors is well documented. There are at least two important sources of this variation, technical and biological. Technical variation between donors can result from differences in the age of the donated apheresis cone (whether cells have been sitting in storage or not for a period of time)- we do try and minimise this by requesting cells that are less than 24 hours old. Biological variation arises because humans (unlike laboratory mice) are genetically variable and there will always be variation in responses between human donors. For many years, it was puzzling that some human donors had very active NK cells, but others had only weakly active NK cells. Originally this was thought to arise from technical issues in experiments. However, we now know that NK cells undergo “education” or “licensing” which means that only those NK cells that can be inhibited by their own MHC class I molecules are functionally competent for example (Kim et al., 2005; Holmes et al., 2014; Tu et al., 2016). This is determined by two most polymorphic loci in the human genome, the KIR locus on chromosome 19 encoding the inhibitory receptors for MHC class I and the MHC class I locus on chromosome 6 (encoding KIR ligands). These two unlinked polymorphic loci determine NK cell activity in an individual and hence unrelated individuals have different proportions of active (licensed or educated) NK cells. Despite these issues in variability, my results show that PGE₂ is a powerful inhibitor of NK cell function.

Overall, these findings deepen our understanding of the mechanism of action of PGE₂ in NK cell immunosuppression and provide new ideas for how we can better model tumours *in vitro* to reflect PGE₂ output and its immunosuppressive activity against NK cells. This would allow testing of immunotherapies in more relevant systems, which incorporate immunosuppressive molecules, including PGE₂, which are upregulated by reciprocal interactions between tumour, immune and stromal cells. Many immunosuppressive soluble factors have been identified in the tumour microenvironment, including the cytokines TGF- β , IL-6, VEGF and IL-10, the enzyme ARG1 and the small molecules kynurenine, ATP and PGE₂. NK cells play a vital role in anti-tumour and anti-viral infection. However, PGE₂ is widely present in the tumour microenvironment and is one of the important factors involved in immunosuppression (Kim et al., 2019; Punyawatthanakool et al., 2024). This study systematically studied the molecular mechanism of PGE₂ affecting NK cells, providing a new theoretical basis for targeting the PGE₂ pathway to improve NK cell function. In particular, it revealed that PGE₂ affects the function of NK cells through the EP2/EP4 receptor and pSTAT5/cAMP pathway, and studied the mechanism of PGE₂ production in tumours and its inhibitory effect on NK cells, which aids our understanding of how the tumour microenvironment weakens the ability of immune system to fight cancer.

Previous studies have shown that PGE₂ can widely regulate the functions of various immune cells and exert immunosuppressive effects in the tumour microenvironment, thereby promoting tumour progression. For example, PGE₂ inhibits the differentiation and maturation of DCs through EP2 and EP4 receptors, weakening their ability to activate T cells. In addition, PGE₂ promotes the development of suppressive MDSCs and induces them to express immunosuppressive factors, IDO, arginase 1, IL-4R α , NOS2, and IL-10 (Obermajer et al., 2011). PGE₂ inhibits the cytotoxic effect of CD8⁺ T

cells by upregulating the expression of inhibitory receptor CD94/NKG2A through the cAMP pathway (Zeddou et al., 2005). PGE₂ activates the cAMP-PKA pathway through EP2/EP4 receptors, reducing the production of IFN- γ and TNF- α by NK cells, thereby weakening their anti-tumour ability and promoting tumour migration (Holt et al., 2011). In addition, PGE₂ has been found to affect the metabolic state of NK cells. PGE₂ can inhibit the mTOR signalling pathway, thereby reducing the energy metabolism and functional activity of NK cells. The activation of mTOR is downstream of IL-15 and pSTAT5 and my results showing that PGE₂ blocks STAT5 activation might help to explain why PGE₂ blocks mTOR activity. These studies and the work presented here show that PGE₂ not only affects the cytotoxicity of NK cells through conventional signalling pathways in the immune microenvironment, but may also further weaken the effector function of NK cells through metabolic regulation (Viel et al., 2017; Chen et al., 2021). The ability of PGE₂ to inhibit NK cell activity (and other immune cell types) suggests that agents targeting PGE₂ production (such as COX-2 inhibitors) or inhibition of EP2/EP4 receptors might be useful to enhance immune responses in cancer. COX-2 inhibitors are widely used to block inflammation associated with arthritis and are being evaluated for their anti-cancer activity (Mahboubi-Rabbani et al., 2024). Inhibitors of EP2 and EP4 might be more difficult to develop as drugs since these receptors are widely expressed and are involved in many physiological processes so that blocking them might be difficult without extensive side effects. In many ways this resembles the situation with TGF- β , where inhibitors (small molecules) and antibodies have failed clinical trials due to side effects resulting from its many roles.

6.2 Future work

Future work should analyse the molecular mechanism of PGE₂ inhibiting NK cell function, such as whether PGE₂ also affects NK cell function through other signalling pathways (such as PI3K/AKT, NF-κB). Although my results indicate that PGE₂ treatment of NK cells does not impact expression of *PRF1* and *IFNG* genes, RNA sequencing to analyse the changes in gene expression of NK cells after PGE₂ treatment could be a way to explore this more broadly. Secondly, use of EP2/EP4 receptor antagonists or cAMP degraders (such as PDE4 inhibitors) to detect whether NK cell function can be restored could be an interesting approach for future work (Houslay and Adams, 2003). In addition, using CRISPR/Cas9 silencing or knockout of EP2/EP4 receptors or downstream signalling molecules in NK cells could also be carried out to further confirm the key role of PGE₂ signalling. Studies in mouse models (e.g. using conditional knockouts of the EP2 and/or EP4) would be a useful way to study the effects of PGE₂ on NK cells in vivo. For therapy, knocking out the EP2 and EP4 receptors in NK cells that are then infused into patients (e.g., CAR-NK cells) would be a powerful way to target the activity of these receptors.

In addition, future work should explore the effect of PGE₂ on NK cells, such as NK cell activation, degranulation, and IFN-γ production in the 3D co-culture model. Future studies should further explore the mechanism of action of PGE₂-related signalling pathways and evaluate the potential of PGE₂/ COX-2 inhibitors as combination strategies to improve immune-based therapies for cancer.

References

- Abel, A.M., Yang, C., Thakar, M.S. and Malarkannan, S. 2018. Natural killer cells: Development, maturation, and clinical utilization. *Frontiers in Immunology*. **9**(1869), pp.1-23.
- Adelizzi, R.A. 1999. COX-1 and COX-2 in health and disease. *The Journal of the American Osteopathic Association*. **99**(11 Suppl), pp.S7-12.
- Agard, M., Asakrah, S. and Morici, L.A. 2013. PGE2 suppression of innate immunity during mucosal bacterial infection. *Frontiers in Cellular and Infection Microbiology*. **3**(45), pp.1-11.
- Amano, H., Ito, Y., Suzuki, T., Kato, S., Matsui, Y., Ogawa, F., Murata, T., Sugimoto, Y., Senior, R., Kitasato, H., Hayashi, I., Satoh, Y., Narumiya, S. and Majima, M. 2009. Roles of a prostaglandin E-type receptor, EP3, in upregulation of matrix metalloproteinase-9 and vascular endothelial growth factor during enhancement of tumor metastasis. *Cancer Science*. **100**(12), pp.2318–2324.
- An, Y., Yao, J. and Niu, X. 2021. The Signaling Pathway of PGE2 and Its Regulatory Role in T Cell Differentiation. *Mediators of Inflammation*. **2021**, pp.1–7.
- André, P., Spertini, O., Guia, S., Rihet, P., Dignat-George, F., Brailly, H., Sampol, J., Anderson, P.J. and Vivier, E. 2000. Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin. *PNAS*. **97**(7), pp.3400–3405.
- Anfossi, N., André, P., Guia, S., Falk, C.S., Roetynck, S., Stewart, C.A., Bresó, V., Frassati, C., Reviron, D., Middleton, D., Romagné, F., Ugolini, S. and Vivier, E. 2006. Human NK Cell Education by Inhibitory Receptors for MHC Class I. *Immunity*. **25**(2), pp.331–342.
- Bai, X., Wang, J., Guo, Y., Pan, J., Yang, Q., Zhang, M., Li, H., Zhang, L., Ma, J., Shi, F., Shu, W., Wang, Y. and Leng, J. 2014. Prostaglandin E2 stimulates β 1-integrin

expression in hepatocellular carcinoma through the EP1 receptor/PKC/NF- κ B pathway. *Scientific Reports*. **4**(6538), pp.1-9.

Bai, X., Wang, J., Zhang, L., Ma, J., Zhang, H., Xia, S., Zhang, M., Ma, X., Guo, Y., Rong, R., Cheng, S., Shu, W., Wang, Y. and Leng, J. 2013. Prostaglandin E2 receptor EP1-mediated phosphorylation of focal adhesion kinase enhances cell adhesion and migration in hepatocellular carcinoma cells. *International Journal of Oncology*. **42**(5), pp.1833–1841.

Barrow, A.D., Martin, C.J. and Colonna, M. 2019. The Natural Cytotoxicity Receptors in Health and Disease. *Frontiers in Immunology*. **10**(909), pp.1-20.

Berger, A. 2000. Science commentary: Th1 and Th2 responses: what are they? *BMJ*. **321**(7258), pp.424–424.

Berger, S.C., Berger, M., Hackman, R.C., Gough, M., Elliott, C., Jensen, M.C. and Riddell, S.R. 2009. Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood*. **114**(12), pp.2417–2426.

Bergström, S., Danielsson, H. and Samuelsson, B. 1964. The enzymatic formation of prostaglandin E2 from arachidonic acid prostaglandins and related factors 32. *Biochimica et Biophysica Acta (BBA) - General Subjects*. **90**(1), pp.207–210.

Betz, M. and Fox, B.S. 1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *Journal of immunology (Baltimore, Md. : 1950)*. **146**(1), pp.108–113.

Biernacka, A., Dobaczewski, M. and Frangogiannis, N.G. 2011. TGF- β signaling in fibrosis. *Growth factors (Chur, Switzerland)*. **29**(5), pp.196–202.

Bode, J.G., Ehltling, C. and Häussinger, D. 2012. The macrophage response towards LPS and its control through the p38 MAPK-STAT3 axis. *Cellular Signalling*. **24**(6), pp.1185–1194.

Bouma, G. and Strober, W. 2003. The immunological and genetic basis of

- inflammatory bowel disease. *Nature Reviews Immunology*. **3**(7), pp.521–533.
- Brock, T.G., McNish, R.W. and Peters-Golden, M. 1999. Arachidonic Acid Is Preferentially Metabolized by Cyclooxygenase-2 to Prostacyclin and Prostaglandin E2. *Journal of Biological Chemistry*. **274**(17), pp.11660–11666.
- Brooks, P., Emery, P., Evans, J.F., Fenner, H., Hawkey, C.J., Patrono, C., Smolen, J., Breedveld, F., Day, R., Dougados, M., Ehrich, E.W., Gijon-Baños, J., Kvien, T.K., Van Rijswijk, M.H., Warner, T. and Zeidler, H. 1999. Interpreting the clinical significance of the differential inhibition of cyclooxygenase-1 and cyclooxygenase-2. *Rheumatology*. **38**(8), pp.779–788.
- Buchholz, A., Vattai, A., Fürst, S., Vilsmaier, T., Zati Zehni, A., Steger, A., Kuhn, C., Schmoeckel, E., Dannecker, C., Mahner, S., Jeschke, U. and Heidegger, H.H. 2023. Prostaglandin E2 receptor EP1 expression in vulvar cancer. *Journal of Cancer Research and Clinical Oncology*. **149**(8), pp.5369–5376.
- Calder, P.C. 2010. Omega-3 Fatty Acids and Inflammatory Processes. *Nutrients*. **2**(3), pp.355–374.
- Carter, D.C., Karim, S.M.M., Bhana, D. and Ganesan, P.A. 1973. Inhibition of human gastric secretion by prostaglandin. *Journal of British Surgery*. **60**(10), pp.828–831.
- Caselli, G., Bonazzi, A., Lanza, M., Ferrari, F., Maggioni, D., Ferioli, C., Giambelli, R., Comi, E., Zerbi, S., Perrella, M., Letari, O., Di Luccio, E., Colovic, M., Persiani, S., Zanelli, T., Mennuni, L., Piepoli, T. and Rovati, L.C. 2018. Pharmacological characterisation of CR6086, a potent prostaglandin E2 receptor 4 antagonist, as a new potential disease-modifying anti-rheumatic drug. *Arthritis Research & Therapy*. **20**(1), p.39.
- Castro, F., Cardoso, A.P., Gonçalves, R.M., Serre, K. and Oliveira, M.J. 2018. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Frontiers in immunology*. **9**, article no: 847, pp.1–19.

- Cetin, M., Buyukberber, S., Demir, M., Sari, Ismail, Sari, Ibrahim, Deniz, K., Eser, B., Altuntas, F., Camci, C., Öztürk, A., Turgut, B., Vural, Ö. and Unal, A. 2005. Overexpression of cyclooxygenase-2 in multiple myeloma: Association with reduced survival. *American Journal of Hematology*. **80**(3), pp.169–173.
- Chan, M.K.-K., Chan, E.L.-Y., Ji, Z.Z., Chan, A.S.-W., Li, C., Leung, K.-T., To, K.-F. and Tang, P.M.-K. 2023. Transforming growth factor- β signaling: from tumor microenvironment to anticancer therapy. *Exploration of Targeted Anti-tumor Therapy*. **4**(2), pp.316–343.
- Chang, H.-H. and Meuillet, E.J. 2011. Identification and development of mPGES-1 inhibitors: where we are at? *Future medicinal chemistry*. **3**(15), pp.1909–34.
- Chen, Z., Han, F., Du, Y. et al. 2023. Hypoxic microenvironment in cancer: molecular mechanisms and therapeutic interventions. *Signal Transduction and Targeted Therapy*. **8**(70), pp.1-23
- Chen, Z., Yang, Y., Neo, S.Y., Shi, H., Chen, Y., Wagner, A.K., Larsson, K., Tong, L., Jakobsson, P., Alici, E., Wu, J., Cao, Y., Wang, K., Liu, L.L., Mao, Y., Sarhan, D. and Lundqvist, A. 2021. Phosphodiesterase 4A confers resistance to PGE₂-mediated suppression in CD25⁺/CD54⁺ NK cells. *EMBO reports*. **22**(e51329), pp1-17.
- Chi, Y., Suadicani, S.O. and Schuster, V.L. 2014. Regulation of prostaglandin EP₁ and EP₄ receptor signaling by carrier-mediated ligand reuptake. *Pharmacology Research & Perspectives*. **2**(4) e0051, pp.1-11.
- Chiossone, L., Chaix, J., Fuseri, N., Roth, C., Vivier, E. and Walzer, T. 2009. Maturation of mouse NK cells is a 4-stage developmental program. *Blood*. **113**(22), pp.5488–5496.
- Chun, K.-S., Lao, H.-C. and Langenbach, R. 2010. The Prostaglandin E₂ Receptor, EP₂, Stimulates Keratinocyte Proliferation in Mouse Skin by G Protein-dependent and β -Arrestin1-dependent Signaling Pathways. *Journal of*

Biological Chemistry. **285**(51), pp.39672–39681.

Cifaldi, L., Melaiu, O., Giovannoni, R., Benvenuto, M., Focaccetti, C., Nardozi, D., Barillari, G. and Bei, R. 2023. DNAM-1 chimeric receptor-engineered NK cells: a new frontier for CAR-NK cell-based immunotherapy. *Frontiers in Immunology*. **14**(1197053), pp.1-8.

Condamine, T. and Gabrilovich, D.I. 2011. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends in Immunology*. **32**(1), pp.19–25.

Conti, B. 2016. Prostaglandin E2 that triggers fever is synthesized through an endocannabinoid- dependent pathway. *Temperature*. **3**(1), pp.25–27.

Crane, C.A., Han, S.J., Barry, J.J., Ahn, B.J., Lanier, L.L. and Parsa, A.T. 2010. TGF- downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. *Neuro-Oncology*. **12**(1), pp.7–13.

Crotty, S. 2015. A brief history of T cell help to B cells. *Nature Reviews Immunology*. **15**(3), pp.185–189.

Cui, F., Qu, D., Sun, R., Zhang, M. and Nan, K. 2019. NK cell-produced IFN- γ regulates cell growth and apoptosis of colorectal cancer by regulating IL-15. *Experimental and Therapeutic Medicine*. **19**(2), pp.1400-1406.

Cullen, S.P. and Martin, S.J. 2008. Mechanisms of granule-dependent killing. *Cell Death and Differentiation*. **15**(2), pp.251–262.

Daniela Santoli, Giorgio Trinchieri and Hilary Koprowski 1978. Cell-Mediated Cytotoxicity Against Virus-Infected Target Cells in Humans: II. Interferon Induction and Activation of Natural Killer Cells. *J Immunol* . **121**(2), pp.532–538.

Davola, M.E. and Mossman, K.L. 2019. Oncolytic viruses: how “lytic” must they be for therapeutic efficacy? *OncolImmunology*. **8**(6) e1596006, pp.1-7.

- Deng, Z., Fan, T., Xiao, C., Tian, H., Zheng, Y., Li, C. and He, J. 2024. TGF- β signaling in health, disease and therapeutics. *Signal Transduction and Targeted Therapy*. 9(1), p.61.
- Diao, G., Huang, J., Zheng, X., Sun, X., Tian, M., Han, J. and Guo, J. 2020. Prostaglandin E2 serves a dual role in regulating the migration of dendritic cells. *International Journal of Molecular Medicine*. 47(1), pp.207–218.
- Dohadwala, M., Yang, S.-C., Luo, J., Sharma, S., Batra, R.K., Huang, M., Lin, Y., Goodglick, L., Krysan, K., Fishbein, M.C., Hong, L., Lai, C., Cameron, R.B., Gemmill, R.M., Drabkin, H.A. and Dubinett, S.M. 2006. Cyclooxygenase-2–Dependent Regulation of E-Cadherin: Prostaglandin E2 Induces Transcriptional Repressors ZEB1 and Snail in Non–Small Cell Lung Cancer. *Cancer Research*. 66(10), pp.5338–5345.
- Domínguez Conde, C., Xu, C., Jarvis, L.B., Rainbow, D.B., Wells, S.B., Gomes, T., Howlett, S.K., Suchanek, O., Polanski, K., King, H.W., Mamanova, L., Huang, N., Szabo, P.A., Richardson, L., Bolt, L., Fasouli, E.S., Mahbubani, K.T., Prete, M., Tuck, L., Richoz, N., Tuong, Z.K., Campos, L., Mousa, H.S., Needham, E.J., Pritchard, S., Li, T., Elmentaite, R., Park, J., Rahmani, E., Chen, D., Menon, D.K., Bayraktar, O.A., James, L.K., Meyer, K.B., Yosef, N., Clatworthy, M.R., Sims, P.A., Farber, D.L., Saeb-Parsy, K., Jones, J.L. and Teichmann, S.A. 2022. Cross-tissue immune cell analysis reveals tissue-specific features in humans. *Science*. 376(6594), pp.1-26.
- Eliasson R 1959. Studies on prostaglandin; occurrence, formation and biological actions. *Acta Physiologica Scandinavica. Supplementum*. 46(158), pp.1–73.
- El-Sherbiny, Y.M., Holmes, T.D., Wetherill, L.F., Black, E.V.I., Wilson, E.B., Phillips, S.L., Scott, G.B., Adair, R.A., Dave, R., Scott, K.J., Morgan, R.S.M., Coffey, M., Toogood, G.J., Melcher, A.A. and Cook, G.P. 2015. Controlled infection with a therapeutic virus defines the activation kinetics of human natural killer cells in vivo. *Clinical and Experimental Immunology*. 180(1), pp.98–107.

- Von Der Emde, L., Goltz, D., Latz, S., Müller, S.C., Kristiansen, G., Ellinger, J. and Syring, I. 2014. Prostaglandin receptors EP1-4 as a potential marker for clinical outcome in urothelial bladder cancer. *Am J Cancer Res.* **4**(6), pp.952-962.
- Esh, C.J., Christmas, B.C.R., Mauger, A.R., Cherif, A., Molphy, J. and Taylor, L. 2021. The influence of environmental and core temperature on cyclooxygenase and PGE2 in healthy humans. *Scientific Reports.* **11**(1), pp.6531.
- Euler, U.S. von 1935. The specific hypotensive substance from the secretions of the human prostate and seminal vesicles. *Klin. Wochschr.* **14**, pp.1182-1183.
- Fabricius, D., Neubauer, M., Mandel, B., Schütz, C., Viardot, A., Vollmer, A., Jahrsdörfer, B. and Debatin, K.-M. 2010. Prostaglandin E2 Inhibits IFN- α Secretion and Th1 Costimulation by Human Plasmacytoid Dendritic Cells via E-Prostanoid 2 and E-Prostanoid 4 Receptor Engagement. *The Journal of Immunology.* **184**(2), pp.677-684.
- Fasbender, F., Widera, A., Hengstler, J.G. and Watzl, C. 2016. Natural Killer Cells and Liver Fibrosis. *Frontiers in Immunology.* **7**(19), pp1-7.
- Feldman, M. 1983. Gastric bicarbonate secretion in humans. Effect of pentagastrin, bethanechol, and 11,16,16-trimethyl prostaglandin E2. *Journal of Clinical Investigation.* **72**(1), pp.295-303.
- Fiegler, N., Textor, S., Arnold, A., Olle, A.R., Oehme, I., Breuhahn, K., Moldenhauer, G., Witzens-Harig, M. and Cerwenka, A. 2013. Downregulation of the activating NKp30 ligand B7-H6 by HDAC inhibitors impairs tumor cell recognition by NK cells. *Blood.* **122**(5), pp.684-693.
- Finetti, F., Solito, R., Morbidelli, L., Giachetti, A., Ziche, M. and Donnini, S. 2008. Prostaglandin E2 Regulates Angiogenesis via Activation of Fibroblast Growth Factor Receptor-1. *Journal of Biological Chemistry.* **283**(4), pp.2139-2146.
- Finetti, F., Travelli, C., Ercoli, J., Colombo, G., Buoso, E. and Trabalzini, L. 2020. Prostaglandin E2 and Cancer: Insight into Tumor Progression and Immunity.

Biology. **9**(434), pp.1-26.

- Fisher, J.G., Doyle, A.D.P., Graham, L. V., Khakoo, S.I. and Blunt, M.D. 2022. Disruption of the NKG2A:HLA-E Immune Checkpoint Axis to Enhance NK Cell Activation against Cancer. *Vaccines*. **10**(12), pp1-17.
- Fort, M.M., Cheung, J., Yen, D., Li, J., Zurawski, S.M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S.D., Zurawski, G., Leach, M.W., Gorman, D.M. and Rennick, D.M. 2001. IL-25 Induces IL-4, IL-5, and IL-13 and Th2-Associated Pathologies In Vivo. *Immunity*. **15**(6), pp.985-995.
- Fu, C. and Jiang, A. 2018. Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment. *Frontiers in Immunology*. **9**(3059), pp1-11.
- Fu, X.-Y. 1992. A transcription factor with SH2 and SH3 domains is directly activated by an interferon α -induced cytoplasmic protein tyrosine kinase(s). *Cell*. **70**(2), pp.323-335.
- Fujino, H. and Regan, J.W. 2006. EP4 Prostanoid Receptor Coupling to a Pertussis Toxin-Sensitive Inhibitory G Protein. *Molecular Pharmacology*. **69**(1), pp.5-10.
- Fujita, M., Kohanbash, G., Fellows-Mayle, W., Hamilton, R.L., Komohara, Y., Decker, S.A., Ohlfest, J.R. and Okada, H. 2011. COX-2 Blockade Suppresses Gliomagenesis by Inhibiting Myeloid-Derived Suppressor Cells. *Cancer Research*. **71**(7), pp.2664-2674.
- Gao, Y., Souza-Fonseca-Guimaraes, F., Bald, T., Ng, S.S., Young, A., Ngiow, S.F., Rautela, J., Straube, J., Waddell, N., Blake, S.J., Yan, J., Bartholin, L., Lee, J.S., Vivier, E., Takeda, K., Messaoudene, M., Zitvogel, L., Teng, M.W.L., Belz, G.T., Engwerda, C.R., Huntington, N.D., Nakamura, K., Hölzel, M. and Smyth, M.J. 2017. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nature Immunology*. **18**(9), pp.1004-1015.
- George, R.J., Sturmoski, M.A., Anant, S. and Houchen, C.W. 2007. EP4 mediates PGE2 dependent cell survival through the PI3 kinase/AKT pathway.

Prostaglandins & Other Lipid Mediators. **83**(1–2), pp.112–120.

- Gerlo, S., Verdood, P., Gellersen, B., Hooghe-Peters, E.L. and Kooijman, R. 2004. Mechanism of Prostaglandin (PG)E₂-Induced Prolactin Expression in Human T Cells: Cooperation of Two PGE₂ Receptor Subtypes, E-Prostanoid (EP) 3 and EP4, Via Calcium- and Cyclic Adenosine 5'-Monophosphate-Mediated Signaling Pathways. *The Journal of Immunology*. **173**(10), pp.5952–5962.
- Giarratana, A.O., Prendergast, C.M., Salvatore, M.M. and Capaccione, K.M. 2024. TGF- β signaling: critical nexus of fibrogenesis and cancer. *Journal of Translational Medicine*. **22**(1), p.594.
- Giles, F.J., Kantarjian, H.M., Bekele, B.N., Cortes, J.E., Faderl, S., Thomas, D.A., Manshouri, T., Rogers, A., Keating, M.J., Talpaz, M., O'Brien, S. and Albitar, M. 2002. Bone marrow cyclooxygenase-2 levels are elevated in chronic-phase chronic myeloid leukaemia and are associated with reduced survival. *British Journal of Haematology*. **119**(1), pp.38–45.
- Goepp, M., Crittenden, S., Zhou, Y., Rossi, A.G., Narumiya, S. and Yao, C. 2021. Prostaglandin E₂ directly inhibits the conversion of inducible regulatory T cells through EP2 and EP4 receptors via antagonizing TGF- β signalling. *Immunology*. **164**(4), pp.777–791.
- Goldblatt, M.W. 1933. A depressor substance in seminal fluid. *J Soc Chem Ind*. **52**, pp.1056–1057.
- Gong, L., Thorn, C.F., Bertagnolli, M.M., Grosser, T., Altman, R.B. and Klein, T.E. 2012. Celecoxib pathways. *Pharmacogenetics and Genomics*. **22**(4), pp.310–318.
- Goodarzi, H., Trowbridge, J. and Gallo, R.L. 2007. Innate immunity: A cutaneous perspective. *Clinical Reviews in Allergy and Immunology*. **33**(1–2), pp.15–26.
- Gotthardt, D., Trifinopoulos, J., Sexl, V. and Putz, E.M. 2019. JAK/STAT Cytokine Signaling at the Crossroad of NK Cell Development and Maturation. *Frontiers in Immunology*. **10**(2590), pp1–16.

- Gray, S.G., Al-Sarraf, N., Baird, A.-M., Cathcart, M.-C., McGovern, E. and O'Byrne, K.J. 2009. Regulation of EP receptors in non-small cell lung cancer by epigenetic modifications. *European Journal of Cancer*. **45**(17), pp.3087–3097.
- Griswold-Prenner, I., Kamibayashi, C., Maruoka, E.M., Mumby, M.C. and Derynck, R. 1998. Physical and Functional Interactions between Type I Transforming Growth Factor β Receptors and $\beta\alpha$, a WD-40 Repeat Subunit of Phosphatase 2A. *Molecular and Cellular Biology*. **18**(11), pp.6595–6604.
- Guo, J., Jiang, X., Lian, J., Li, H., Zhang, F., Xie, J., Deng, J., Hou, X., Du, Z. and Hao, E. 2024. Evaluation of the effect of GSK-3 β on liver cancer based on the PI3K/AKT pathway. *Frontiers in Cell and Developmental Biology*. **10**(3398), pp.1-19.
- Hall, T.J., Chen, S.-H., Brostoff, J. and Lydyard, P.M. 1983. Modulation of human natural killer cell activity by pharmacological mediators. *Clin Exp Immunol*. **54**(2), pp.493-500.
- Han, B., Mao, F.-Y., Zhao, Y.-L., Lv, Y.-P., Teng, Y.-S., Duan, M., Chen, W., Cheng, P., Wang, T.-T., Liang, Z.-Y., Zhang, J.-Y., Liu, Y.-G., Guo, G., Zou, Q.-M., Zhuang, Y. and Peng, L.-S. 2018. Altered NKp30, NKp46, NKG2D, and DNAM-1 Expression on Circulating NK Cells Is Associated with Tumor Progression in Human Gastric Cancer. *Journal of immunology research*. **2012**, article no:652130, pp.1-9.
- Harizi, H., Grosset, C. and Gualde, N. 2003. Prostaglandin E2 modulates dendritic cell function via EP2 and EP4 receptor subtypes. *Journal of Leukocyte Biology*. **73**(6), pp.756–763.
- Hasegawa, K., Ohashi, Y., Ishikawa, K., Yasue, A., Kato, R., Achiwa, Y., Nishio, E. and Udagawa, Y. 2005. Expression of cyclooxygenase-2 in uterine endometrial cancer and anti-tumor effects of a selective COX-2 inhibitor. *International journal of oncology*. **26**(5), pp.1419–28.

- Heidenreich, S., Zu Eulenburg, C., Hildebrandt, Y., Stübiger, T., Sierich, H., Badbaran, A., Eiermann, T.H., Binder, T.M.C. and Kröger, N. 2012. Impact of the NK cell receptor LIR-1 (ILT-2/CD85j/LILRB1) on cytotoxicity against multiple myeloma. *Clinical and Developmental Immunology*. **2012**, article no: 652130, pp.1-13.
- Hipp, N., Symington, H., Pastoret, C., Caron, G., Monvoisin, C., Tarte, K., Fest, T. and Delaloy, C. 2017. IL-2 imprints human naive B cell fate towards plasma cell through ERK/ELK1-mediated BACH2 repression. *Nature Communications*. **8**(1443), pp.1-17.
- Hoffmann, J. and Akira, S. 2013. Innate immunity. *Current Opinion in Immunology*. **25**(1), pp.1–3.
- Hollyoake, M. 2005. NKp30 (NCR3) is a Pseudogene in 12 Inbred and Wild Mouse Strains, but an Expressed Gene in *Mus caroli*. *Molecular Biology and Evolution*. **22**(8), pp.1661–1672.
- Holmes, T.D., Wilson, E.B., Black, E.V.I., Benest, A. V, Vaz, C., Tan, B., Tanavde, V.M. and Cook, G.P. 2014. Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proceedings of the National Academy of Sciences of the United States of America*. **111**(52), pp.E5688-5696.
- Holt, D., Ma, X., Kundu, N. and Fulton, A. 2011. Prostaglandin E2 (PGE2) suppresses natural killer cell function primarily through the PGE2 receptor EP4. *Cancer Immunology, Immunotherapy*. **60**(11), pp.1577–1586.
- Hoshino, T., Tsutsumi, S., Tomisato, W., Hwang, H.-J., Tsuchiya, T. and Mizushima, T. 2003. Prostaglandin E2 Protects Gastric Mucosal Cells from Apoptosis via EP2 and EP4 Receptor Activation. *Journal of Biological Chemistry*. **278**(15), pp.12752–12758.
- Houslay, M.D. and Adams, D.R. 2003. PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochemical Journal*. **370**(1), pp.1–18.

- Hsu, H.-H., Lin, Y.-M., Shen, C.-Y., Shibu, M.A., Li, S.-Y., Chang, S.-H., Lin, C.-C., Chen, R.-J., Viswanadha, V.P., Shih, H.-N. and Huang, C.-Y. 2017. Prostaglandin E2-Induced COX-2 Expressions via EP2 and EP4 Signaling Pathways in Human LoVo Colon Cancer Cells. *International journal of molecular sciences*. **18**(6), pp.1-16.
- Hu, X., li, J., Fu, M., Zhao, X. and Wang, W. 2021. The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduction and Targeted Therapy*. **6**(1), p.402.
- Huse, M. 2012. Microtubule-organizing center polarity and the immunological synapse: protein kinase C and beyond. *Frontiers in Immunology*. **3**, article no:235, pp1-11.
- Iqbal, M.J., Kabeer, A., Abbas, Z., Siddiqui, H.A., Calina, D., Sharifi-Rad, J. and Cho, W.C. 2024. Interplay of oxidative stress, cellular communication and signaling pathways in cancer. *Cell Communication and Signaling*. **22**(1), p.7.
- Irie, A., Sugimoto, Y., Namba, T., Harazono, A., Honda, A., Watabe, A., Negishi, M., Narumiya, S. and Ichikawa, A. 1993. Third isoform of the prostaglandin-E-receptor EP₃ subtype with different C-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *European Journal of Biochemistry*. **217**(1), pp.313–318.
- Jafari, M., Kadkhodazadeh, M., Shapourabadi, M.B., Goradel, N.H., Shokrgozar, M.A., Arashkia, A., Abdoli, S. and Sharifzadeh, Z. 2022. Immunovirotherapy: The role of antibody based therapeutics combination with oncolytic viruses. *Frontiers in Immunology*. **13**(2022), pp.1-23.
- Jafarinia, H., Khalilimeybodi, A., Barrasa-Fano, J., Fraley, S.I., Rangamani, P. and Carlier, A. 2024. Insights gained from computational modeling of YAP/TAZ signaling for cellular mechanotransduction. *npj Systems Biology and Applications*. **10**(1), p.90.
- Jang, T.J. 2004. Expression of proteins related to prostaglandin E2 biosynthesis is

- increased in human gastric cancer and during gastric carcinogenesis. *Virchows Archiv.* **445**(6), pp.564–571.
- Jiang, H. and Jiang, J. 2023. Balancing act: the complex role of NK cells in immune regulation. *Frontiers in Immunology.* **14**, article no: 1275028, pp.1–13.
- Johansson, C. and Kollberg, B. 1979. Stimulation by intragastrically administered E₂ prostaglandins of human gastric mucus output. *European Journal of Clinical Investigation.* **9**(3), pp.229–232.
- Johansson, T., Narumiya, S. and Zeilhofer, H.U. 2011. Contribution of peripheral versus central EP1 prostaglandin receptors to inflammatory pain. *Neuroscience Letters.* **495**(2), pp.98–101.
- Kalinski, P. 2012. Regulation of immune responses by prostaglandin E₂. *Journal of immunology (Baltimore, Md. : 1950).* **188**(1), pp.21–8.
- Kaliński, P., Schuitemaker, J.H.N., Hilkens, C.M.U. and Kapsenberg, M.L. 1998. Prostaglandin E₂ Induces the Final Maturation of IL-12-Deficient CD1a+CD83+ Dendritic Cells: The Levels of IL-12 Are Determined During the Final Dendritic Cell Maturation and Are Resistant to Further Modulation. *The Journal of Immunology.* **161**(6), pp.2804–2809.
- Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, Ł. and Lamperska, K. 2018. 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. *Archives of Medical Science.* **14**(4), pp.910–919.
- Karimi, M., Cao, T.M., Baker, J.A., Verneris, M.R., Soares, L. and Negrin, R.S. 2005. Silencing Human NKG2D, DAP10, and DAP12 Reduces Cytotoxicity of Activated CD8+ T Cells and NK Cells. *The Journal of Immunology.* **175**(12), pp.7819–7828.
- Kariya, Yukiko, Oyama, M., Hashimoto, Y., Gu, J. and Kariya, Yoshinobu 2018. β 4-Integrin/PI3K Signaling Promotes Tumor Progression through the Galectin-3–

- N*-Glycan Complex. *Molecular Cancer Research*. **16**(6), pp.1024–1034.
- Kärre, K., Ljunggren, H.G., Piontek, G. and Kiessling, R. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. **319**(6055), pp.675–678.
- Kawahara, K., Hohjoh, H., Inazumi, T., Tsuchiya, S. and Sugimoto, Y. 2015. Prostaglandin E2-induced inflammation: Relevance of prostaglandin E receptors. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. **1851**(4), pp.414–421.
- Kawamori, T., Rao, C. V, Seibert, K. and Reddy², B.S. 1998. Chemopreventive Activity of Celecoxib, a Specific Cyclooxygenase-2 Inhibitor, against Colon Carcinogenesis¹. **58**(3), pp.409-12.
- Khor, L.-Y., Bae, K., Pollack, A., Hammond, M.E.H., Grignon, D.J., Venkatesan, V.M., Rosenthal, S.A., Ritter, M.A., Sandler, H.M., Hanks, G.E., Shipley, W.U. and Dicker, A.P. 2007. COX-2 expression predicts prostate-cancer outcome: analysis of data from the RTOG 92-02 trial. *The Lancet Oncology*. **8**(10), pp.912–920.
- Kiessling, R., Klein, E. and Wigzell, H. 1975. „Natural” killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European Journal of Immunology*. **5**(2), pp.112–117.
- Kim, J., Phan, M. -T T., Hwang, I., Park, J. and Cho, D. 2023. Comparison of the different anti-CD16 antibody clones in the activation and expansion of peripheral blood NK cells. *Scientific Reports*. **13**(1), p.9493.
- Kim, S., Poursine-Laurent, J., Truscott, S.M., Lybarger, L., Song, Y.-J., Yang, L., French, A.R., Sunwoo, J.B., Lemieux, S., Hansen, T.H. and Yokoyama, W.M. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. **436**(7051), pp.709–13.

- Kim, S.-H., Roszik, J., Cho, S.-N., Ogata, D., Milton, D.R., Peng, W., Menter, D.G., Ekmekcioglu, S. and Grimm, E.A. 2019. The COX2 Effector Microsomal PGE2 Synthase 1 is a Regulator of Immunosuppression in Cutaneous Melanoma. *Clinical Cancer Research*. **25**(5), pp.1650–1663.
- Kim, S.O., Dozier, B.L., Kerry, J.A. and Duffy, D.M. 2013. EP3 receptor isoforms are differentially expressed in subpopulations of primate granulosa cells and couple to unique G-proteins. *Reproduction*. **146**(6), pp.625–635.
- Kimourtzis, G., Rangwani, N., Jenkins, B.J., Jani, S., McNaughton, P.A. and Raouf, R. 2024. Prostaglandin E2 depolarises sensory axons in vitro in an ANO1 and Nav1.8 dependent manner. *Scientific Reports*. **14**(1), p.17360.
- Kochel, T.J. and Fulton, A.M. 2015. Multiple drug resistance-associated protein 4 (MRP4), prostaglandin transporter (PGT), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) as determinants of PGE2 levels in cancer. *Prostaglandins & Other Lipid Mediators*. **116**(117), pp.99–103.
- Koga, H., Sakisaka, S., Ohishi, M., Kawaguchi, T., Taniguchi, E., Sasatomi, K., Harada, M., Kusaba, T., Tanaka, M., Kimura, R., Nakashima, Y., Nakashima, O., Kojiro, M., Kurohiji, T. and Sata, M. 1999. Expression of Cyclooxygenase-2 in Human Hepatocellular Carcinoma: Relevance to Tumor Dedifferentiation. *Hepatology*. **29**(3), pp.688–696.
- Konjević, G.M., Vuletić, A.M., Mirjačić Martinović, K.M., Larsen, A.K. and Jurišić, V.B. 2019. The role of cytokines in the regulation of NK cells in the tumor environment. *Cytokine*. **117**, pp.30–40.
- Krysan, K., Reckamp, K.L., Dalwadi, H., Sharma, S., Rozengurt, E., Dohadwala, M. and Dubinett, S.M. 2005. Prostaglandin E2 Activates Mitogen-Activated Protein Kinase/Erk Pathway Signaling and Cell Proliferation in Non-Small Cell Lung Cancer Cells in an Epidermal Growth Factor Receptor-Independent Manner. *Cancer Research*. **65**(14), pp.6275–6281.

- Kubiczkova, L., Sedlarikova, L., Hajek, R. and Sevcikova, S. 2012. TGF- β – an excellent servant but a bad master. *Journal of Translational Medicine*. **10**(1), p.183.
- Kumar, S. 2018. Natural killer cell cytotoxicity and its regulation by inhibitory receptors. *Immunology*. **154**(3), pp.383–393.
- Kurzrok, R. and Lieb, C.C. 1930. Biochemical Studies of Human Semen. II. The Action of Semen on the Human Uterus. *Experimental Biology and Medicine*. **28**(3), pp.268–272.
- Lee, J., Aoki, T., Thumkeo, D., Siriwach, R., Yao, C. and Narumiya, S. 2019. T cell–intrinsic prostaglandin E2–EP2/EP4 signaling is critical in pathogenic TH17 cell–driven inflammation. *Journal of Allergy and Clinical Immunology*. **143**(2), pp.631–643.
- Lee, J.L., Kim, A., Kopelovich, L., Bickers, D.R. and Athar, M. 2005. Differential Expression of E Prostanoid Receptors in Murine and Human Non-Melanoma Skin Cancer. *Journal of Investigative Dermatology*. **125**(4), pp.818–825.
- Li, N.L., Davidson, C.L., Humar, A. and Burshtyn, D.N. 2011. Modulation of the inhibitory receptor leukocyte Ig-like receptor 1 on human natural killer cells. *Frontiers in Immunology*. **2**, article no:46, pp.1-15.
- Li, T., Zhang, Q., Jiang, Y., Yu, J., Hu, Y., Mou, T., Chen, G. and Li, G. 2016. Gastric cancer cells inhibit natural killer cell proliferation and induce apoptosis via prostaglandin E2. *Oncolmmunology*. **5**(2), p.e1069936.
- Li, Y., Wei, Y., Zheng, F., Guan, Y. and Zhang, X. 2017. Prostaglandin E2 in the Regulation of Water Transport in Renal Collecting Ducts. *International journal of molecular sciences*. **18**(12), p.2539.
- Lieberman, J. 2010. Granzyme A activates another way to die. *Immunological Reviews*. **235**(1), pp.93–104.

- Lima, J.F., Oliveira, L.M.S., Pereira, N.Z., Duarte, A.J.S. and Sato, M.N. 2017. Polyfunctional natural killer cells with a low activation profile in response to Toll-like receptor 3 activation in HIV-1-exposed seronegative subjects. *Scientific Reports*. **7**, article no:524, pp.1-9.
- Lin, D., Shen, Y. and Liang, T. 2023. Oncolytic virotherapy: basic principles, recent advances and future directions. *Signal Transduction and Targeted Therapy*. **8**, article no:156, pp.1-29.
- Lin, J.-X. and Leonard, W.J. 2018. The Common Cytokine Receptor γ Chain Family of Cytokines. *Cold Spring Harbor perspectives in biology*. **10**(9), p.a028449.
- Lin, Q., Rong, L., Jia, X., Li, R., Yu, B., Hu, J., Luo, X., Badea, S.R., Xu, C., Fu, Guofeng, Lai, K., Lee, M. chun, Zhang, B., Gong, H., Zhou, N., Chen, X.L., Lin, S. hai, Fu, Guo and Huang, J.D. 2021. IFN- γ -dependent NK cell activation is essential to metastasis suppression by engineered Salmonella. *Nature Communications*. **12**(1),p.2537.
- Liu, B., Zhu, X., Kong, L., Wang, M., Spanoudis, C., Chaturvedi, P., George, V., Jiao, J., You, L., Egan, J.O., Echeverri, C., Gallo, V.L., Xing, J., Ravelo, K., Prendes, C., Antolinez, J., Denissova, J., Muniz, G.J., Jeng, E.K., Rhode, P.R. and Wong, H.C. 2021. Bifunctional TGF- β trap/IL-15 protein complex elicits potent NK cell and CD8+ T cell immunity against solid tumors. *Molecular Therapy*. **29**(10), pp.2949–2962.
- Liu, X.H., Kirschenbaum, A., Lu, M., Yao, S., Dosoretz, A., Holland, J.F. and Levine, A.C. 2002. Prostaglandin E2 Induces Hypoxia-inducible Factor-1 α Stabilization and Nuclear Localization in a Human Prostate Cancer Cell Line. *Journal of Biological Chemistry*. **277**(51), pp.50081–50086.
- Ljunggren, H.-G. and Kärre, K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology Today*. **11**, pp.237–244.
- Loh, C.-Y., Chai, J.Y., Tang, T.F., Wong, W.F., Sethi, G., Shanmugam, M.K., Chong,

- P.P. and Looi, C.Y. 2019. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. *Cells*. **8**(10), p.1118.
- Long, E.O., Sik Kim, H., Liu, D., Peterson, M.E. and Rajagopalan, S. 2013. Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annual Review of Immunology*. **31**(1), pp.227–258.
- Lowes, M.A., Bowcock, A.M. and Krueger, J.G. 2007. Pathogenesis and therapy of psoriasis. *Nature*. **445**(7130), pp.866–873.
- Luckheeram, R.V., Zhou, R., Verma, A.D. and Xia, B. 2012. CD4 +T cells: Differentiation and functions. *Clinical and Developmental Immunology*. **2012**, article no:925135, pp.1–12.
- Ma, X., Aoki, T. and Narumiya, S. 2016. Prostaglandin E2-EP4 signaling persistently amplifies CD40-mediated induction of IL-23 p19 expression through canonical and non-canonical NF- κ B pathways. *Cellular & Molecular Immunology*. **13**(2), pp.240–250.
- Ma, X., Holt, D., Kundu, N., Reader, J., Goloubeva, O., Take, Y. and Fulton, A.M. 2013. A prostaglandin E (PGE) receptor EP4 antagonist protects natural killer cells from PGE₂-mediated immunosuppression and inhibits breast cancer metastasis. *Oncolmmunology*. **2**(1), p.e22647.
- Macias-Perez, I.M., Zent, R., Carmosino, M., Breyer, M.D., Breyer, R.M. and Pozzi, A. 2008. Mouse EP3 α , β , and γ receptor variants reduce tumor cell proliferation and tumorigenesis in vivo. *Journal of Biological Chemistry*. **283**(18), pp.12538–12545.
- Mahboubi-Rabbani, M., Abdolghaffari, A.H., Ghesmati, M., Amini, A. and Zarghi, A. 2024. Selective COX-2 inhibitors as anticancer agents: a patent review (2018-2023). *Expert opinion on therapeutic patents*. **34**(9), pp.733–757.
- Mamessier, E., Sylvain, A., Thibult, M.-L., Houvenaeghel, G., Jacquemier, J.,

- Castellano, R., Gonçalves, A., André, P., Romagné, F., Thibault, G., Viens, P., Birnbaum, D., Bertucci, F., Moretta, A. and Olive, D. 2011. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *The Journal of clinical investigation*. **121**(9), pp.3609–22.
- Man, S.M. and Jenkins, B.J. 2022. Context-dependent functions of pattern recognition receptors in cancer. *Nature Reviews Cancer*. **22**(7), pp.397–413.
- Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T.I., Bushkin, Y., Davis, D.M., Strominger, J.L., Yewdell, J.W. and Porgador, A. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. **409**(6823), pp.1055–1060.
- Mangelsen, E., Rothe, M., Schulz, A., Kourpa, A., Panáková, D., Kreutz, R. and Bolbrinker, J. 2020. Concerted EP2 and EP4 Receptor Signaling Stimulates Autocrine Prostaglandin E2 Activation in Human Podocytes. *Cells*. **9**(5), p.1256.
- Martinet, L., Jean, C., Dietrich, G., Fournié, J.-J. and Poupot, R. 2010. PGE2 inhibits natural killer and $\gamma\delta$ T cell cytotoxicity triggered by NKR and TCR through a cAMP-mediated PKA type I-dependent signaling. *Biochemical Pharmacology*. **80**(6), pp.838–845.
- Masato, M., Miyata, Y., Kurata, H., Ito, H., Mitsunari, K., Asai, A., Nakamura, Y., Araki, K., Mukae, Y., Matsuda, T., Harada, J., Matsuo, T., Ohba, K. and Sakai, H. 2021. Oral administration of E-type prostanoid (EP) 1 receptor antagonist suppresses carcinogenesis and development of prostate cancer via upregulation of apoptosis in an animal model. *Scientific Reports*. **11**(1), p.20279.
- Massa, C., Wang, Y., Marr, N. and Seliger, B. 2023. Interferons and Resistance Mechanisms in Tumors and Pathogen-Driven Diseases—Focus on the Major Histocompatibility Complex (MHC) Antigen Processing Pathway. *International Journal of Molecular Sciences*. **24**(7), p.6736.

- Medjouel Khelifi, H., Guia, S., Vivier, E. and Narni-Mancinelli, E. 2022. Role of the ITAM-Bearing Receptors Expressed by Natural Killer Cells in Cancer. *Frontiers in Immunology*. **13**, article no:898745, pp.1-14.
- Medzhitov, R., Preston-Hurlburt, P. and Janeway, C.A. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*. **388**(6640), pp.397-397.
- Merz, C., Von Mässenhausen, A., Queisser, A., Vogel, W., Andrén, O., Kirfel, J., Duensing, S., Perner, S. and Nowak, M. 2016. IL-6 overexpression in ERG-positive prostate cancer is mediated by prostaglandin receptor EP2. *American Journal of Pathology*. **186**(4), pp.974–984.
- M. Ferrão, P., M. Nisimura, L., C. Moreira, O., G. Land, M., Pereira, M.C., de Mendonça-Lima, L., C. Araujo-Jorge, T., C. Waghbi, M. and R. Garzoni, L. 2018. Inhibition of TGF- β pathway reverts extracellular matrix remodeling in T. cruzi-infected cardiac spheroids. *Experimental Cell Research*. **362**(2), pp.260–267.
- Min-Oo, G., Kamimura, Y., Hendricks, D.W., Nabekura, T. and Lanier, L.L. 2013. Natural killer cells: Walking three paths down memory lane. *Trends in Immunology*. **34**(6), pp.251–258.
- Mishra, A., Chaudhary, A. and Sethi, S. 2004. Oxidized Omega-3 Fatty Acids Inhibit NF- κ B Activation Via a PPAR α -Dependent Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **24**(9), pp.1621–1627.
- Motizuki, M., Yokoyama, T., Saitoh, M. and Miyazawa, K. 2024. The Snail signaling branch downstream of the TGF- β /Smad3 pathway mediates Rho activation and subsequent stress fiber formation. *The Journal of biological chemistry*. **300**(1), p.105580.
- Mullen, A., Loscher, C.E. and Roche, H.M. 2010. Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated

- with LPS stimulation in THP-1-derived macrophages. *The Journal of Nutritional Biochemistry*. **21**(5), pp.444–450.
- Murakami, M., Nakashima, K., Kamei, D., Masuda, S., Ishikawa, Y., Ishii, T., Ohmiya, Y., Watanabe, K. and Kudo, I. 2003. Cellular Prostaglandin E2 Production by Membrane-bound Prostaglandin E Synthase-2 via Both Cyclooxygenases-1 and -2. *Journal of Biological Chemistry*. **278**(39), pp.37937–37947.
- Murray, P.J. and Wynn, T.A. 2011. Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology*. **11**(11), pp.723–737.
- Muthuswamy, R., Urban, J., Lee, J.-J., Reinhart, T.A., Bartlett, D. and Kalinski, P. 2008. Ability of Mature Dendritic Cells to Interact with Regulatory T Cells Is Imprinted during Maturation. *Cancer Research*. **68**(14), pp.5972–5978.
- Nakayama, M. 2014. Antigen presentation by MHC-dressed cells. *Frontiers in Immunology*. **5**, article no:672, pp1-8.
- Nam, J.-M., Ahmed, K.M., Costes, S., Zhang, H., Onodera, Y., Olshen, A.B., Hatanaka, K.C., Kinoshita, R., Ishikawa, M., Sabe, H., Shirato, H. and Park, C.C. 2013. β 1-integrin via NF- κ B signaling is essential for acquisition of invasiveness in a model of radiation treated in situ breast cancer. *Breast Cancer Research*. **15**(4), p.R60.
- Nasrallah, R., Zimpelmann, J., Robertson, S.J., Ghossein, J., Thibodeau, J.-F., Kennedy, C.R.J., Gutsol, A., Xiao, F., Burger, D., Burns, K.D. and Hébert, R.L. 2020. Prostaglandin E2 receptor EP1 (PGE2/EP1) deletion promotes glomerular podocyte and endothelial cell injury in hypertensive TTRhRen mice. *Laboratory Investigation*. **100**(3), pp.414–425.
- Negishi, M., Hasegawa, H. and Ichikawa, A. 1996. Prostaglandin E receptor EP3 γ isoform, with mostly full constitutive Gi activity and agonist-dependent Gs activity. *FEBS Letters*. **386**(2–3), pp.165–168.
- Netea, M.G., Schlitzer, A., Placek, K., Joosten, L.A.B. and Schultze, J.L. 2019. Innate

and Adaptive Immune Memory: an Evolutionary Continuum in the Host's Response to Pathogens. *Cell Host and Microbe*. **25**(1), pp.13–26.

Niringiyumukiza, J.D., Cai, H. and Xiang, W. 2018. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. *Reproductive Biology and Endocrinology*. **16**(1), p.43.

Obermajer, N., Muthuswamy, R., Lesnock, J., Edwards, R.P. and Kalinski, P. 2011. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood*. **118**(20), pp.5498–5505.

O'Callaghan, G. and Houston, A. 2015. Prostaglandin E2 and the EP receptors in malignancy: Possible therapeutic targets? *British Journal of Pharmacology*. **172**(22), pp.5239–5250.

O'Callaghan, G., Ryan, A., Neary, P., O'Mahony, C., Shanahan, F. and Houston, A. 2013. Targeting the EP1 receptor reduces Fas ligand expression and increases the antitumor immune response in an in vivo model of colon cancer. *International Journal of Cancer*. **133**(4), pp.825–834.

Ogbomo, H. and Mody, C.H. 2017. Granule-dependent natural killer cell cytotoxicity to fungal pathogens. *Frontiers in Immunology*. **7**, article no:692, pp.1-6.

Ohsawa, M., Fukushima, H., Ikura, Y., Inoue, T., Shirai, N., Sugama, Y., Suekane, T., Kitabayashi, C., Nakamae, H., Hino, M. and Ueda, M. 2006. Expression of cyclooxygenase-2 in Hodgkin's lymphoma: its role in cell proliferation and angiogenesis. *Leukemia & Lymphoma*. **47**(9), pp.1863–1871.

Osińska, I., Popko, K. and Demkow, U. 2014. Perforin: An important player in immune response. *Central European Journal of Immunology*. **39**(1), pp.109–115.

Otegbeye, F., Ojo, E., Moreton, S., Mackowski, N., Lee, D.A., de Lima, M. and Wald,

- D.N. 2018. Inhibiting TGF-beta signaling preserves the function of highly activated, in vitro expanded natural killer cells in AML and colon cancer models. *PLOS ONE*. **13**(1), p.e0191358.
- Pancierà, T., Azzolin, L., Cordenonsi, M. and Piccolo, S. 2017. Mechanobiology of YAP and TAZ in physiology and disease. *Nature Reviews Molecular Cell Biology*. **18**(12), pp.758–770.
- Panza, E., De Cicco, P., Ercolano, G., Armogida, C., Scognamiglio, G., Anniciello, A.M., Botti, G., Cirino, G. and Ianaro, A. 2016. Differential expression of cyclooxygenase-2 in metastatic melanoma affects progression free survival. *Oncotarget*. **7**(35), pp.57077–57085.
- Papillion, A. and Ballesteros-Tato, A. 2021. The Potential of Harnessing IL-2-Mediated Immunosuppression to Prevent Pathogenic B Cell Responses. *Frontiers in Immunology*. **12**, article no: 67342, pp.1–14.
- Park, S.M., Yoo, B.C., Lee, H.R., Chung, H. and Lee, Y.S. 1992. Distribution of prostaglandin E2 in gastric and duodenal mucosa: possible role in the pathogenesis of peptic ulcer. *The Korean journal of internal medicine*. **7**(1), pp.1–8.
- De Paz Linares, G.A., Opperman, R.M., Majumder, M. and Lala, P.K. 2021. Prostaglandin e2 receptor 4 (Ep4) as a therapeutic target to impede breast cancer-associated angiogenesis and lymphangiogenesis. *Cancers*. **13**(5), pp.1–27.
- Pazina, T., Macfarlane, A.W., Bernabei, L., Dulaimi, E., Kotcher, R., Yam, C., Bezman, N.A., Robbins, M.D., Ross, E.A., Campbell, K.S. and Cohen, A.D. 2021. Alterations of nk cell phenotype in the disease course of multiple myeloma. *Cancers*. **13**(2), pp.1–22.
- Pellicci, D.G., Hammond, K.J.L., Coquet, J., Kyparissoudis, K., Brooks, A.G., Kedzierska, K., Keating, R., Turner, S., Berzins, S., Smyth, M.J. and Godfrey, D.I.

2005. DX5/CD49b-Positive T Cells Are Not Synonymous with CD1d-Dependent NKT Cells. *The Journal of Immunology*. **175**(7), pp.4416–4425.
- Perera, P.Y., Lichy, J.H., Waldmann, T.A. and Perera, L.P. 2012. The role of interleukin-15 in inflammation and immune responses to infection: Implications for its therapeutic use. *Microbes and Infection*. **14**(3), pp.247–261.
- Pesce, S., Moretta, L., Moretta, A. and Marcenaro, E. 2016. Human NK cell subsets redistribution in pathological conditions: A role for CCR7 receptor. *Frontiers in Immunology*. **7**, article no:414, pp.1–10.
- Piotrowski, I., Kulcenty, K. and Suchorska, W. 2020. Interplay between inflammation and cancer. *Reports of Practical Oncology & Radiotherapy*. **25**(3), pp.422–427.
- Poli, A., Michel, T., Thérésine, M., Andrès, E., Hentges, F. and Zimmer, J. 2009. CD56^{bright} natural killer (NK) cells: an important NK cell subset. *Immunology*. **126**(4), pp.458–465.
- Prisco, A. and De Berardinis, P. 2012. Memory immune response: A major challenge in vaccination. *Biomolecular Concepts*. **3**(5), pp.479–486.
- Punyawatthanakool, S., Matsuura, R., Wongchang, T., Katsurada, N., Tsuruyama, T., Tajima, M., Enomoto, Y., Kitamura, T., Kawashima, M., Toi, M., Yamanoi, K., Hamanishi, J., Hisamori, S., Obama, K., Charoensawan, V., Thumkeo, D. and Narumiya, S. 2024. Prostaglandin E2-EP2/EP4 signaling induces immunosuppression in human cancer by impairing bioenergetics and ribosome biogenesis in immune cells. *Nature Communications*. **15**(1), p.9464.
- Qu, N., Xu, M., Mizoguchi, I., Furusawa, J., Kaneko, K., Watanabe, K., Mizuguchi, J., Itoh, M., Kawakami, Y. and Yoshimoto, T. 2013. Pivotal Roles of T-Helper 17-Related Cytokines, IL-17, IL-22, and IL-23, in Inflammatory Diseases. *Clinical and Developmental Immunology*. **2013**, article no:968549, pp.1–13.
- Ramírez-Labrada, A., Pesini, C., Santiago, L., Hidalgo, S., Calvo-Pérez, A., Oñate, C., Andrés-Tovar, A., Garzón-Tituaña, M., Uranga-Murillo, I., Arias, M.A., Galvez,

- E.M. and Pardo, J. 2022. All About (NK Cell-Mediated) Death in Two Acts and an Unexpected Encore: Initiation, Execution and Activation of Adaptive Immunity. *Frontiers in Immunology*. **13**, article no:896228, pp.1-14.
- Reader, J., Holt, D. and Fulton, A. 2011. Prostaglandin E 2 EP receptors as therapeutic targets in breast cancer. *Cancer and Metastasis Reviews*. **30**(3–4), pp.449–463.
- Rehermann, B. 2016. Peptide-dependent HLA-KIR-mediated regulation of NK cell function. *Journal of Hepatology*. **65**(2), pp.237–239.
- Ricciotti, E. and FitzGerald, G.A. 2011. Prostaglandins and inflammation. *Arteriosclerosis, thrombosis, and vascular biology*. **31**(5), pp.986–1000.
- Ringbom, T., Huss, U., Stenholm, Å., Flock, S., Skattebøl, L., Perera, P. and Bohlin, L. 2001. COX-2 Inhibitory Effects of Naturally Occurring and Modified Fatty Acids. *Journal of Natural Products*. **64**(6), pp.745–749.
- Romee, R., Foley, B., Lenvik, T., Wang, Y., Zhang, B., Ankarlo, D., Luo, X., Cooley, S., Verneris, M., Walcheck, B. and Miller, J. 2013. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood*. **121**(18), pp.3599–3608.
- Ross, S.H. and Cantrell, D.A. 2018. Signaling and Function of Interleukin-2 in T Lymphocytes. *Annual Review of Immunology*. **36**, pp.411–433.
- Rundhaug, J.E., Simper, M.S., Surh, I. and Fischer, S.M. 2011. The role of the EP receptors for prostaglandin E 2 in skin and skin cancer. *Cancer and Metastasis Reviews*. **30**(3–4), pp.465–480.
- Sahu, A., Raza, K., Pradhan, D., Jain, A.K. and Verma, S. 2023. Cyclooxygenase-2 as a therapeutic target against human breast cancer: A comprehensive review. *WIREs Mechanisms of Disease*. **15**(3), p.e1596.
- Sampey, A. V, Monrad, S. and Crofford, L.J. 2005. Microsomal prostaglandin E

synthase-1: the inducible synthase for prostaglandin E2. *Arthritis research & therapy*. **7**(3), pp.114–7.

Santiso, A., Heinemann, A. and Kargl, J. 2024. Prostaglandin E2 in the Tumor Microenvironment, a Convolved Affair Mediated by EP Receptors 2 and 4. *Pharmacological Reviews*. **76**(3), pp.388–413.

Schaid, M.D., Harrington, J.M., Kelly, G.M., Sdao, S.M., Merrins, M.J. and Kimple, M.E. 2023. EP3 signaling is decoupled from the regulation of glucose-stimulated insulin secretion in β -cells compensating for obesity and insulin resistance. *Islets*. **15**(1), p.2223327.

Schuster, N. and Krieglstein, K. 2002. Mechanisms of TGF- β -mediated apoptosis. *Cell and Tissue Research*. **307**(1), pp.1–14.

Sebestyén, A., Hajdu, M., Kis, L., Barna, G. and Kopper, L. 2007. Smad4-independent, PP2A-dependent apoptotic effect of exogenous transforming growth factor beta 1 in lymphoma cells. *Experimental Cell Research*. **313**(15), pp.3167–3174.

Seung, L.P., Rowley, D.A., Dubey, P. and Schreiber, H. 1995. Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection. *Proceedings of the National Academy of Sciences*. **92**(14), pp.6254–6258.

Sharonov, G. V., Serebrovskaya, E.O., Yuzhakova, D. V., Britanova, O. V. and Chudakov, D.M. 2020. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nature Reviews Immunology*. **20**(5), pp.294–307.

Shin, S. hun, Lee, Y.E., Yoon, H.-N., Yuk, C.M., An, J.Y., Seo, M., Yoon, S., Oh, M.-S., Shin, S.C., Kim, J.H., Kim, Y.J., Kim, J.-C., Kim, S.C. and Jang, M. 2025. An innovative strategy harnessing self-activating CAR-NK cells to mitigate TGF- β 1-driven immune suppression. *Biomaterials*. **314**, p.122888.

Shoji, Y., Takahashi, M., Takasuka, N., Niho, N., Kitamura, T., Sato, H., Maruyama, T., Sugimoto, Y., Narumiya, S., Sugimura, T. and Wakabayashi, K. 2005.

Prostaglandin E receptor EP 3 deficiency modifies tumor outcome in mouse two-stage skin carcinogenesis. *Carcinogenesis*. **26**(12), pp.2116–2122.

Sindaco, P., Pandey, H., Isabelle, C., Chakravarti, N., Brammer, J.E., Porcu, P. and Mishra, A. 2023. The role of interleukin-15 in the development and treatment of hematological malignancies. *Frontiers in Immunology*. **14**, article no: 1141208, pp.1-10.

Sinha, P., Clements, V.K., Fulton, A.M. and Ostrand-Rosenberg, S. 2007. Prostaglandin E2 Promotes Tumor Progression by Inducing Myeloid-Derived Suppressor Cells. *Cancer Research*. **67**(9), pp.4507–4513.

Slattery, K., Zaiatz-Bittencourt, V., Woods, E., Brennan, K., Marks, S., Chew, S., Conroy, M., Goggin, C., Kennedy, J., Finlay, D.K. and Gardiner, C.M. 2019. TGFβ drives mitochondrial dysfunction in peripheral blood NK cells during metastatic breast cancer. **9**(2), p.e002044.

Soslow, R.A., Dannenberg, A.J., Rush, D., Woerner, B.M., Khan, K.N., Masferrer, J. and Koki, A.T. 2000. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer*. **89**(12), pp.2637–2645.

Spinella, F., Rosanò, L., Di Castro, V., Natali, P.G. and Bagnato, A. 2004. Endothelin-1-induced Prostaglandin E2-EP2, EP4 Signaling Regulates Vascular Endothelial Growth Factor Production and Ovarian Carcinoma Cell Invasion. *Journal of Biological Chemistry*. **279**(45), pp.46700–46705.

Sreeramkumar, V., Fresno, M. and Cuesta, N. 2012. Prostaglandin E₂ and T cells: friends or foes? *Immunology & Cell Biology*. **90**(6), pp.579–586.

Starkweather, K., Hales, K. and Hales, D. 2020. Abstract B45: Anti-inflammatory actions of DHA via inhibition of the NF-κB pathway. *Clinical Cancer Research*. **26**(13_Supplement), pp. B45–B45.

Steinman, L. 2007. A brief history of TH17, the first major revision in the TH1/TH2 hypothesis of T cell-mediated tissue damage. *Nature Medicine*. **13**(2), pp.139–

145.

- Steinman, L. 2008. A rush to judgment on Th17. *The Journal of experimental medicine*. **205**(7), pp.1517–22.
- St-Jacques, B. and Ma, W. 2014. Peripheral prostaglandin E2 prolongs the sensitization of nociceptive dorsal root ganglion neurons possibly by facilitating the synthesis and anterograde axonal trafficking of EP4 receptors. *Experimental Neurology*. **261**, pp.354–366.
- Sugimoto, Y. and Narumiya, S. 2007. Prostaglandin E Receptors. *Journal of Biological Chemistry*. **282**(16), pp.11613–11617.
- Sun, B., Rong, R., Jiang, H., Zhang, H., Wang, Y., Bai, X., Zhang, M., Ma, J., Xia, S., Shu, W., Zhang, L. and Leng, J. 2013. Prostaglandin E2 receptor EP1 phosphorylate CREB and mediates MMP2 expression in human cholangiocarcinoma cells. *Molecular and Cellular Biochemistry*. **378**(1–2), pp.195–203.
- Sun, C., Sun, H., Xiao, W., Zhang, C. and Tian, Z. 2015. Natural killer cell dysfunction in hepatocellular carcinoma and NK cell-based immunotherapy. *Acta Pharmacologica Sinica*. **36**(10), pp.1191–1199.
- Sun, X. and Li, Q. 2018. Prostaglandin EP2 receptor: Novel therapeutic target for human cancers (Review). *International Journal of Molecular Medicine*. **42**(3), pp.1203–1214.
- Suresh, M. V., Dolgachev, V.A., Zhang, B., Balijepalli, S., Swamy, S., Mooliyil, J., Kralovich, G., Thomas, B., Machado-Aranda, D., Karmakar, M., Lalwani, S., Subramanian, A., Anantharam, A., Moore, B.B. and Raghavendran, K. 2019. TLR3 absence confers increased survival with improved macrophage activity against pneumonia. *JCI Insight*. **4**(23).
- Sutherland, C.L., Chalupny, N.J. and Cosman, D. 2001. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer

cell functions. *Immunological Reviews*. **181**(1), pp.185–192.

Tai, F.W.D. and McAlindon, M.E. 2021. Non-steroidal anti-inflammatory drugs and the gastrointestinal tract. *Clinical Medicine*. **21**(2), pp.131–134.

Tang, P.M.-K., Zhou, S., Meng, X.-M., Wang, Q.-M., Li, C.-J., Lian, G.-Y., Huang, X.-R., Tang, Y.-J., Guan, X.-Y., Yan, B.P.-Y., To, K.-F. and Lan, H.-Y. 2017. Smad3 promotes cancer progression by inhibiting E4BP4-mediated NK cell development. *Nature Communications*. **8**(1), p.14677.

Tanioka, T., Nakatani, Y., Kobayashi, T., Tsujimoto, M., Oh-ishi, S., Murakami, M. and Kudo, I. 2003. Regulation of cytosolic prostaglandin E2 synthase by 90-kDa heat shock protein. *Biochemical and Biophysical Research Communications*. **303**(4), pp.1018–1023.

Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M. and Kudo, I. 2000. Molecular Identification of Cytosolic Prostaglandin E2 Synthase That Is Functionally Coupled with Cyclooxygenase-1 in Immediate Prostaglandin E2 Biosynthesis. *Journal of Biological Chemistry*. **275**(42), pp.32775–32782.

Tatsuno, I., Saito, H., Chang, K.-J., Tamura, Y. and Yoshida, S. 1990. Comparison of the effect between leukotriene B4 and leukotriene B5 on the induction of interleukin 1-like activity and calcium mobilizing activity in human blood monocytes. *Agents and Actions*. **29**(3–4), pp.324–327.

Taylor, A.W. 2009. Review of the activation of TGF- β in immunity. *Journal of Leukocyte Biology*. **85**(1), pp.29–33.

Thompson, R. and Cao, X. 2024. Reassessing granzyme B: unveiling perforin-independent versatility in immune responses and therapeutic potentials. *Frontiers in Immunology*. **15**, article no:1392535, pp.1–7.

Thumkeo, D., Punyawatthanakool, S., Prasongtanakij, S., Matsuura, R., Arima, K., Nie, H., Yamamoto, R., Aoyama, N., Hamaguchi, H., Sugahara, S., Takeda, S., Charoensawan, V., Tanaka, A., Sakaguchi, S. and Narumiya, S. 2022. PGE2-

EP2/EP4 signaling elicits immunosuppression by driving the mregDC-Treg axis in inflammatory tumor microenvironment. *Cell Reports*. **39**(10), p.110914.

Tong, C., Liang, Y., Han, X., Zhang, Z., Zheng, X., Wang, S. and Song, B. 2023.

Research Progress of Dendritic Cell Surface Receptors and Targeting.

Biomedicines. **11**(6) p.1673.

Topham, N.J. and Hewitt, E.W. 2009. Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology*. **128**(1), pp.7–15.

Trotta, R., Col, J.D., Yu, J., Ciarlariello, D., Thomas, B., Zhang, X., Allard, J., Wei, M.,

Mao, H., Byrd, J.C., Perrotti, D. and Caligiuri, M.A. 2008. TGF- β Utilizes SMAD3 to Inhibit CD16-Mediated IFN- γ Production and Antibody-Dependent Cellular Cytotoxicity in Human NK Cells. *The Journal of Immunology*. **181**(6), pp.3784–3792.

Tsuge, K., Inazumi, T., Shimamoto, A. and Sugimoto, Y. 2019. Molecular

mechanisms underlying prostaglandin E2-exacerbated inflammation and immune diseases. *International Immunology*. **31**(9), pp.597–606.

Tu, M.M., Mahmoud, A.B. and Makrigiannis, A.P. 2016. Licensed and Unlicensed NK

Cells: Differential Roles in Cancer and Viral Control. *Frontiers in immunology*. **7**, p.166.

Viel, S., Besson, L., Marotel, M., Walzer, T. and Marçais, A. 2017. Regulation of mTOR, Metabolic Fitness, and Effector Functions by Cytokines in Natural Killer Cells. *Cancers*. **9**(10), p.132.

Viel, S., Marçais, A., Guimaraes, F.S.-F., Loftus, R., Rabilloud, J., Grau, M., Degouve, S., Djebali, S., Sanlaville, A., Charrier, E., Bienvenu, J., Marie, J.C., Caux, C., Marvel, J., Town, L., Huntington, N.D., Bartholin, L., Finlay, D., Smyth, M.J. and Walzer, T. 2016. TGF- β inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Science Signaling*. **9**(415), p.ra19.

Vio, C.P., Quiroz-Munoz, M., Cuevas, C.A., Cespedes, C. and Ferreri, N.R. 2012.

Prostaglandin E₂ EP3 receptor regulates cyclooxygenase-2 expression in the kidney. *American Journal of Physiology-Renal Physiology*. **303**(3), pp. F449–F457.

Vlaykov, A.N., Tacheva, T.T., Vlaykova, T.I. and Stoyanov, V.K. 2020. Serum and local IL-4, IL-5, IL-13 and immunoglobulin E in allergic rhinitis. *Advances in Dermatology and Allergology*. **37**(5), pp.719–724.

Vleeshouwers, W., van den Dries, K., de Keijzer, S., Joosten, B., Lidke, D.S. and Cambi, A. 2021. Characterization of the Signaling Modalities of Prostaglandin E₂ Receptors EP2 and EP4 Reveals Crosstalk and a Role for Microtubules. *Frontiers in Immunology*. **11**, article no:613286, pp.1-13.

Walker, W. and Rotondo, D. 2004. Prostaglandin E₂ is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon- γ synthesis. *Immunology*. **111**(3), pp.298–305.

Wang, D., Fu, L., Sun, H., Guo, L. and DuBois, R.N. 2015. Prostaglandin E₂ Promotes Colorectal Cancer Stem Cell Expansion and Metastasis in Mice. *Gastroenterology*. **149**(7), pp.1884-1895.

Wang, J., Liu, M., Zhang, X., Yang, G. and Chen, L. 2018. Physiological and pathophysiological implications of PGE₂ and the PGE₂ synthases in the kidney. *Prostaglandins & Other Lipid Mediators*. **134**, pp.1–6.

Wang, K.S., Ritz, J. and Frank, D.A. 1999. IL-2 Induces STAT4 Activation in Primary NK Cells and NK Cell Lines, But Not in T Cells. *The Journal of Immunology*. **162**(1), pp.299–304.

Wang, L., Wu, Y., Jia, Z., Yu, J. and Huang, S. 2022. Roles of EP Receptors in the Regulation of Fluid Balance and Blood Pressure. *Frontiers in Endocrinology*. **13**, article no:875245, pp.1-10.

Wantoch, M., Wilson, E.B., Droop, A.P., Phillips, S.L., Coffey, M., El-Sherbiny, Y.M., Holmes, T.D., Melcher, A.A., Wetherill, L.F. and Cook, G.P. 2022. Oncolytic virus

treatment differentially affects the CD56dim and CD56bright NK cell subsets in vivo and regulates a spectrum of human NK cell activity. *Immunology*. **166**(1), pp.104–120.

Wei, D., Birla, H., Dou, Y., Mei, Y., Huo, X., Whitehead, V., Osei-Owusu, P., Feske, S., Patafio, G., Tao, Y. and Hu, H. 2024. PGE2 Potentiates Orai1-Mediated Calcium Entry Contributing to Peripheral Sensitization. *The Journal of Neuroscience*. **44**(1), p.e0329232023.

Wilson, E.B., El-Jawhari, J.J., Neilson, A.L., Hall, G.D., Melcher, A.A., Meade, J.L. and Cook, G.P. 2011. Human Tumour Immune Evasion via TGF- β Blocks NK Cell Activation but Not Survival Allowing Therapeutic Restoration of Anti-Tumour Activity. *PLoS ONE*. **6**(9), p.e22842.

Xing, S. and Ferrari de Andrade, L. 2020. NKG2D and MICA/B shedding: a 'tag game' between NK cells and malignant cells. *Clinical & Translational Immunology*. **9**, article no: e1230, pp.1-10.

Xu, S., Zhou, W., Ge, J. and Zhang, Z. 2018. Prostaglandin E2 receptor EP4 is involved in the cell growth and invasion of Prostate cancer via the cAMP-PKA/PI3K-Akt signaling pathway. *Molecular Medicine Reports*. **17**(3), pp.4702–4712.

Xue, V.W., Chung, J.Y.-F., Córdoba, C.A.G., Cheung, A.H.-K., Kang, W., Lam, E.W.-F., Leung, K.-T., To, K.-F., Lan, H.-Y. and Tang, P.M.-K. 2020. Transforming Growth Factor- β : A Multifunctional Regulator of Cancer Immunity. *Cancers*. **12**(11), p.3099.

Yang, F., Wei, Y., Cai, Z., Yu, L., Jiang, L., Zhang, C., Yan, H., Wang, Q., Cao, X., Liang, T. and Wang, J. 2015. Activated cytotoxic lymphocytes promote tumor progression by increasing the ability of 3LL tumor cells to mediate MDSC chemoattraction via Fas signaling. *Cellular & Molecular Immunology*. **12**(1), pp.66–76.

- Yang, P., Jiang, Y. and Fischer, S.M. 2014. Prostaglandin E3 metabolism and cancer. *Cancer Letters*. **348**(1–2), pp.1–11.
- Yang, T. and Du, Y. 2012. Distinct Roles of Central and Peripheral Prostaglandin E2 and EP Subtypes in Blood Pressure Regulation. *American Journal of Hypertension*. **25**(10), pp.1042–1049.
- Yao, C., Hirata, T., Soontrapa, K., Ma, X., Takemori, H. and Narumiya, S. 2013. Prostaglandin E2 promotes Th1 differentiation via synergistic amplification of IL-12 signalling by cAMP and PI3-kinase. *Nature Communications*. **4**(1), p.1685.
- Yao, C. and Narumiya, S. 2019. Prostaglandin-cytokine crosstalk in chronic inflammation. *British Journal of Pharmacology*. **176**(3), pp.337–354.
- Yao, C., Sakata, D., Esaki, Y., Li, Y., Matsuoka, T., Kuroiwa, K., Sugimoto, Y. and Narumiya, S. 2009. Prostaglandin E2–EP4 signaling promotes immune inflammation through TH1 cell differentiation and TH17 cell expansion. *Nature Medicine*. **15**(6), pp.633–640.
- Yau, L. and Zahradka, P. 2003. PGE2 stimulates vascular smooth muscle cell proliferation via the EP2 receptor. *Molecular and Cellular Endocrinology*. **203**(1–2), pp.77–90.
- Ye, Y., Peng, L., Vattai, A., Deuster, E., Kuhn, C., Dannecker, C., Mahner, S., Jeschke, U., von Schönfeldt, V. and Heidegger, H.H. 2020. Prostaglandin E2 receptor 3 (EP3) signaling promotes migration of cervical cancer via urokinase-type plasminogen activator receptor (uPAR). *Journal of Cancer Research and Clinical Oncology*. **146**(9), pp.2189–2203.
- Young, M.R., Newby, M. and Wepsic, H.T. 1987. Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors. *Cancer research*. **47**(1), pp.100–5.
- Yu, J., Wei, M., Becknell, B., Trotta, R., Liu, S., Boyd, Z., Jaung, M.S., Blaser, B.W., Sun,

- J., Benson, D.M., Mao, H., Yokohama, A., Bhatt, D., Shen, L., Davuluri, R., Weinstein, M., Marcucci, G. and Caligiuri, M.A. 2006. Pro- and Antiinflammatory Cytokine Signaling: Reciprocal Antagonism Regulates Interferon- γ Production by Human Natural Killer Cells. *Immunity*. **24**(5), pp.575–590.
- Zaiatz-Bittencourt, V., Finlay, D.K. and Gardiner, C.M. 2018. Canonical TGF- β Signaling Pathway Represses Human NK Cell Metabolism. *The Journal of Immunology*. **200**(12), pp.3934–3941.
- Zeddou, M., Greimers, R., de Valensart, N., Nayjib, B., Tasken, K., Boniver, J., Moutschen, M. and Rahmouni, S. 2005. Prostaglandin E2 induces the expression of functional inhibitory CD94/NKG2A receptors in human CD8+ T lymphocytes by a cAMP-dependent protein kinase A type I pathway. *Biochemical Pharmacology*. **70**(5), pp.714–724.
- Zhang, H., Liu, Y., Liu, J., Chen, J., Wang, J., Hua, H. and Jiang, Y. 2024. cAMP-PKA/EPAC signaling and cancer: the interplay in tumor microenvironment. *Journal of Hematology and Oncology*. **17**(5), pp.1-31.
- Zhang, P., Zhang, C., Li, J., Han, J., Liu, X. and Yang, H. 2019. The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. *Stem Cell Research and Therapy*. **10**(1), p.327.
- Zhang, Y.E. 2009. Non-Smad pathways in TGF- β signaling. *Cell Research*. **19**(1), pp.128–139.
- Zhang, Y., Alexander, P.B. and Wang, X.-F. 2017. TGF- β Family Signaling in the Control of Cell Proliferation and Survival. *Cold Spring Harbor perspectives in biology*. 9(4), article no: a022145, pp.1-22.
- Zhang, Z., Wu, N., Lu, Y., Davidson, D., Colonna, M. and Veillette, A. 2015. DNAM-1 controls NK cell activation via an ITT-like motif. *Journal of Experimental Medicine*. **212**(12), pp.2165–2182.

Zidar, N., Odar, K., Glavac, D., Jerse, M., Zupanc, T. and Stajer, D. 2009.

Cyclooxygenase in normal human tissues – is COX-1 really a constitutive isoform, and COX-2 an inducible isoform? *Journal of Cellular and Molecular Medicine*. **13**(9b), pp.3753–3763.