ELUCIDATING NOVEL FUNCTION(S) OF PROLACTIN INDUCIBLE PROTEIN IN REGULATING NATURAL KILLER (NK) CELL BIOLOGY

BY

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ABSTRACT

Prolactin-inducible protein (PIP) is found in various bodily fluids, including tears, saliva, sweat, amniotic fluid, and seminal plasma. This protein is frequently associated with breast cancers (BCs) and serves as a biomarker for identifying the origin of BC. Its presence has been linked to favorable prognostic outcomes in human BC. In a 4T1 breast cancer mouse model, ectopic expression of PIP led to an increased frequency of natural killer (NK) cells and dendritic cells within the tumor microenvironment, resulting in delayed tumor onset and reduced growth. While previous research has associated PIP with immune response regulation, the specific mechanisms by which PIP modulates NK cell biology remain unclear.

I hypothesized that PIP acts as a novel host factor influencing NK cell functionality. To investigate this, I examined its role in regulating NK cell migration, cytotoxicity, and cytokine production.

To assess PIP's impact on NK cell migration, a transwell migration assay was conducted. NK cells showed significantly enhanced migration toward conditioned media from PIP-expressing 4T1 cells compared to empty vector (EV) controls. Additionally, recombinant mouse PIP (mPIP) at a concentration of 1µg/600ul significantly induced NK cell migration compared to BSA. Treatment of NK cells with PIP also resulted in significant phosphorylation of p38 MAPK and IκBα, suggesting a potential mechanism for PIP's regulation of NK cell migration.

However, the expression of PIP did not affect NK cell-mediated killing or interferon-gamma (IFN-γ) production in response to parental, breast-derived, and lung-metastatic 4T1 cells in vitro.

In summary, these findings demonstrate that PIP promotes NK cell migration but does not directly influence NK cell-mediated cytotoxicity or cytokine production in vitro. This study provides novel insights into PIP's role in enhancing NK cell migration, contributing to a better understanding of its involvement in immune regulation.

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DEDICATION

This thesis is dedicated to the cherished memory of my late father, Chief Innocent Okeke, whose passion for education and unwavering love laid the foundation for my academic achievements.

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293T	Human embryonic kidney (HEK) cell line
4T1 EV-BR	4T1 control cells derived from primary tumor
4T1 EV-ML	4T1 control cells derived from metastatic lungs
4T1 PIP-BR	4T1 PIP expressing cells derived from primary tumor
4T1 PIP-ML	4T1 PIP expressing cells derived from metastatic lungs
4T1	Transplantable mouse TNBC cell line
AR	Androgen receptor
BALB/C	Mouse strain
BC	Breast Cancer
BCA	Bicinchoninic acid
BFA	brefeldin A
CD107a	Cluster of differentiation 107a
CD11c	Cluster of differentiation 11c
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD40	Cluster of differentiation 40
CD45	Cluster of differentiation 45
CD49b	Cluster of differentiation 49b
CD8	Cluster of differentiation 8
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CM	Conditioned media
CMV	Cytomegalovirus
CO2	Carbon dioxide
CXCL10	C-X-C motif chemokine ligand 10
CXCL5	C-X-C chemokine ligand 5
CXCR2	C-X-C chemokine receptor 2

CXCR3	C-X-C chemokine receptor 3
CXCR7	C-X-C chemokine receptor 7
DC	Dendritic cell
DC-LPS	LPS-matured dendritic cells
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DX5	Detecting antibody for CD49b
ECL	Enhanced chemiluminescence
ECM	Extra cellular matrix
EDTA	Ethylenediamine tetra acetic acid
EF1α	Elongation factor 1 alpha
EGF-1	Epidermal growth factor 1
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK1/2	Extracellular signal-related kinase 1 and 2
EV	Empty vector
FACS	Flow activated cell sorter
FBS	Fetal bovine serum
FN	Fibronectin
GCDFP-15	Gross cystic disease fluid protein
gp-120	Envelope glycoprotein 120
H-2Dd	MHC Class I (mouse)
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma

IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-1a	Interleukin-1 alpha
IL-1b	Interleukin-1 beta
IL-2	Interleukin-2
IL-22	Interleukin-22
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
ILCs	Innate Lymphoid Cells
IP	Immunoprecipitation
IκBα	nuclear factor of kappa B-cells inhibitor alpha
JNK1	c-Jun N-terminal kinase
kDa	Kilodalton
KO	Knockout
LAPM-1	Lysosomal-associated membrane protein 1
LPS	lipopolysaccharide
MAPK	Mitogen activated protein kinase
MET	Mesenchymal to epithelial transition
mL	Millilitre
mM	Millimolar
MMP	Metalloproteinase
MMP-2	Metalloproteinase-2
MMP-9	Metalloproteinase-9
mPIP	Recombinant Mouse Prolactin Inducible Protein (mouse)

mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
N	Number
NaCl	Sodium chloride
N-cad	N-cadherin
NK cells	Natural Killer Cells
Ns	Not significant
P	Probability
p38 MAPK	p38 mitogen-activated protein kinase
p65 NF-kappa-B	nuclear factor NF-kappa-B p65 subunit
PAGE	Polyacrylamide gel electrophoresis
Parent 4T1	Wild-type transplantable mouse TNBC cell line
Parent 4T1-EV	Transduced wild-type 4T1 containing empty vector
Parent 4T1-PIP	Transduced wild-type 4T1 expressing PIP
PBS	Phosphate buffered saline
PD-L1	Programmed cell death ligand-1
PI3Kα	Phosphoinositide 3-kinase alpha
PIP	Prolactin Inducible Protein
PMA	Phorbol myristate acetate
PR	Progesterone receptor
RAE-1	Retinoic acid early inducible 1
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	Seconds
SD	Standard deviation
SDS	Sodium dodecyl sulfate
STAT-1	Signal transducer and activator of transcription 1

STAT-5	Signal transducer and activator of transcription 5
Sup	Supernatant
TAM	Tumor associated macrophage
T-bet	T-box expressed in T cells
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween 20
TGF-β	Transforming growth factor-beta
Th1	T helper, type 1
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
V	Volts
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
μg	Microgram
μL	Microlitre
μM	Micromolar

CHAPTER ONE

1.0. INTRODUCTION

1.1. THE IMMUNE SYSTEM

The immune system consists of a collection of cells, tissues, and molecules that protect an organism from the effects of foreign antigens, pathogens, toxins, and cancers. Many organs play different roles in the immune system. For instance, skin and mucosal membranes serve as physical barriers against the invasion of pathogens or toxins. These are supported by antimicrobial peptides, stomach acids, and normal microbial flora that fight against foreign organisms and substances. Another group of organs in the immune system is the lymphoid organs, which consist of the primary and secondary lymphoid organs. The primary lymphoid organs include the bone marrow and thymus, where lymphocytes are made. The secondary lymphoid organs include the spleen, lymph nodes, mucosal layers of body tissues, and tonsils, which are the sites where lymphocytes carry out their function of fighting and eliminating pathogens, toxins, and cancers (Kaufmann 2019). The immune system is divided into 2 main arms: the adaptive and innate immune system.

1.1.1. The Adaptive Immune System

The adaptive immune system is the arm of the immune system that is characterized by the development of high specificity and immunological memory (Bonilla and Oettgen 2010; Kaufmann 2019). The immune response mounted by cells and their components in this arm is not spontaneous as observed in the innate arm, but they require time for naïve B or T cells to differentiate into antibody producing B cells or effector T cells respectively when sensitized by a specific antigen (Bonilla and Oettgen 2010). The naive CD4+ T cells become effector cells when their T cell receptors bind to MHC class II on antigen presenting cells (APCs) like dendritic cells,

macrophages and B cells. While CD8+ T cells differentiate into effector T cells by the interaction between the T cell receptor (TCR) and MHC class I found on target cells (Koretzky 2010; Luckheeram et al. 2012). CD4+ T cells differentiate into several subsets like T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or regulatory T cells (Tregs), depending on the available cytokines and transcription factors present. The various CD4+ T cell subsets are involved in the production of cytokines that shape immune response. On the other hand, effector CD8+ T cells carry out cytotoxicity by releasing granzymes and perforins that kills the target cells (Koretzky 2010; Luckheeram et al. 2012). B cells on their part are involved in the humoral immune response and secrete antibodies that carry out various functions like opsonization, neutralization of antigens, activation of complement proteins and antibody-dependent cell cytotoxicity (ADCC) (Schroeder and Cavacini 2010).

1.1.2. The Innate Immune System

The innate immune system is the arm of the immune system that serves as the first line of defense against pathogens, toxins and cancers (Chaplin 2010). The innate immune response is nonspecific and fast (Aristizábal and González 2013). The cells in this arm of the immune system recognize invading cells by discriminating between "self" and "non self" using toll-like receptors (TLRs) that can detect specific pathogen associated patterns or danger associated molecular patterns (PAMPs or DAMPs) (Takeuchi and Akira 2010). The components of the innate immune system include physical barriers (the skin, epithelial and mucous membrane surfaces) that prevent entry of foreign bodies. There are also phagocytes like neutrophils, monocytes and macrophages that are involved in eliminating foreign pathogens by engulfing them and can also secrete factors (cytokines and chemokines) that can modulate immune response of other cells (Lim, Grinstein, and Roth 2017). Complement, C-reactive protein, lectins such as mannose-binding lectin, and

ficolins) are serum proteins that shape inflammation, trigger membrane attack and opsonization (Aristizábal and González 2013; Dunkelberger and Song 2010; Wills-Karp 2007). The antimicrobial peptides such as defensins, cathelicidin help protect against foreign microbes (Huan et al. 2020). Natural killer cells eliminate viruses, cancers and other microbes by direct killing through the release of cytotoxic substances on the target cells (Sun and Lanier 2009).

1.1.2.1. ***Cellular Components of the Innate Immune System***

1.1.2.1.1. *Phagocytic Cells*

Macrophages: These long-lived immune cells and are residents in almost every tissue (Ovchinnikov 2008). They can detect pathogens through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Macrophages can engulf and degrade microbes, cancer cells, cellular debris and other foreign materials and secrete pro-inflammatory cytokines to recruit other immune cells (Gordon and Plüddemann 2018; Mahla et al. 2021).

Neutrophils: They are the most abundant leukocytes and are rapidly recruited to sites of infection or injuries. They are short-lived immune cells and can phagocytose pathogens, release antimicrobial peptides and reactive oxygen species (Amulic et al. 2012). They can also secrete neutrophil extracellular traps (NETs) that trap and eliminate extracellular microbes (Clark et al. 2007).

1.1.2.1.2. *Natural Killer (NK) Cells*

NK cells mediate cytotoxicity against virus-infected and transformed cells by releasing perforin and granzymes. They also secrete cytokines such as IFN-γ to modulate immune responses (Sun and Lanier 2009; Vivier et al. 2008) . NK cells will be discussed more extensively in a subsequent section.

1.1.2.1.3. *Innate Lymphoid Cells (ILCs)*

ILCs, including ILC1, ILC2, and ILC3 subsets are part of the most recently identified innate immune cells. They perform non-cytotoxic roles in tissue homeostasis, mucosal immunity, and inflammation by producing cytokines such as IL-5, IL-13, IL-17, and IL-22 (Vivier et al. 2018). These cells are mostly tissue-resident and their effector functions are also tissue specific (Kim, Ryu, and Kim 2021; Vivier et al. 2018).

1.1.2.1.4. *Dendritic Cells (DCs)*

DCs are specialized antigen-presenting cells (APCs), bridging the gap between innate and adaptive immunity. They can recognize microbes using PRRs like TLRs and phagocytose microbes, degrade their proteins into small bits and present these fragments on their cell surface using MHC molecules (Liu 2016). Plasmacytoid DCs are specialized in antiviral responses through Type I interferon production, while conventional DCs process and present antigens to T cells (Summerfield and McCullough 2009).

1.1.2.1.5. *Mast Cells*

Mast cells are cells that originate from the myeloid stem cell and are part of the immune systems, they possess several histamine and heparin rich granules. They secrete histamine and other mediators during allergic reactions and pathogen defense, contributing to inflammation and vascular permeability (Galli, Gaudenzio, and Tsai 2020). They are also involved in wound healing, angiogenesis, and immune tolerance (da Silva, Jamur, and Oliver 2014).

1.1.2.1.6. *Basophils*

Basophils are the largest type of granulocyte and like mast cells have granules filled with histamine. They play a key role in most allergic responses and are involved in the clearance of parasitic infections (Voehringer 2009). They serve as a clinical indicator of allergic responses mediated by IgE (Hoffmann et al. 2015).

1.1.2.1.7. *Eosinophils*

Eosinophils make up about 1-3% of all white blood cells (Uhm, Kim, and Chung 2012). During inflammation, eosinophils facilitate effector functions and innate immunity and can also modulate adaptive immune responses (Uhm et al. 2012). They are primarily involved in allergic responses and parasitic infections, releasing cytotoxic granules and cytokines that damage pathogens and modulate immune responses (Huang and Appleton 2016; Klein Wolterink et al. 2012).

1.1.2.2. ***Non-Cellular Components of the Innate Immune System***

1.1.2.2.1. *Physical and Chemical Barriers*

Epithelial barriers like the skin and mucosal surfaces form a mechanical barrier that are reinforced by tight junctions. They possess pattern recognition receptors (PRRs) and can also produce cytokines and antimicrobial peptides which eliminates microbes and enhances inflammation (Afshar and Gallo 2013; Szolnoky et al. 2001). Mucous membranes or mucosa in the digestive, respiratory, and genitourinary tracts consist of epithelia cells that prevents microbes from entering the host. These epithelia cells produce mucus and antimicrobial peptides such as defensins (Bals 2000; Danahay and Jackson 2005). The mucus traps pathogens and facilitates their clearance, while the antimicrobial peptides can directly kill bacteria through the disruption of cell membrane (Benfield and Henriques 2020; Schroeder 2019; Zhang and Gallo 2016).

1.1.2.2.2. *Complement System*

This comprises of a cascade of plasma proteins that can opsonize pathogens (coating of the pathogens cell surface with complement opsonins like C3b) which leads to the phagocytosis of the pathogens by phagocytic immune cells like neutrophils and macrophage (Gorgani et al. 2008; Ross and Větvicka 1993). They promote inflammation through the actions of the complement anaphylatoxins "C3a and C5a", and carry out targeted killing by forming membrane attack complexes that facilitate the lysis of microbial cell membrane (Bubeck 2014; Elsner et al. 1994; Murakami, Imamichi, and Nagasawa 1993). There are three (3) pathways of complement activation; classical, alternate and lectin dependent pathways.

1.1.2.2.3. *Acute Phase Proteins*

These are a group of proteins produced by the hepatocytes (liver cells) in response to inflammation or infection and are important players in the innate immune system (Gabay and Kushner 1999). Some studies have shown that non hepatocytes also produce some acute-phase proteins (De Buck et al. 2016; Reichhardt and Meri 2018). Proteins like C-reactive protein (CRP) and mannose-binding lectin (MBL) recognize pathogen-associated molecular patterns (PAMPs) and facilitate phagocytosis (Garlanda et al. 2002; Ip et al. 2009).

1.1.2.2.4. *Antimicrobial peptides (AMPs)*

AMPs are important components of the innate immune system and serves as a defense against microbial infections (Huan et al. 2020; Wang et al. 2019). These small, naturally occurring peptides have a wide range of activity against bacteria, viruses, fungi, and some parasites (Lei et al. 2019; Mohammed, Said, and Dua 2017; Torrent et al. 2012). They are evolutionarily conserved and found in various organisms, including humans, animals, plants, and even some

microorganisms (Kang et al. 2019). AMPs can carry out membrane disruption by binding to negatively charged microbial membranes, leading to cell lysis (Baek et al. 2016; Malanovic and Lohner 2016). They can also penetrate cells and inhibit microbial DNA, RNA, or protein synthesis and can modulate immune response (Mookherjee et al. 2020; Zhang and Gallo 2016). The antimicrobial peptides such as defensins, cathelicidin help protect against foreign microbes (Huan et al. 2020).

1.2. NATURAL KILLER (NK) CELLS

Natural killer (NK) cells are a subset of lymphocytes within the innate immune system, known for their ability to eliminate malignant cells and pathogens. Unlike other immune cells, NK cells can initiate immune responses without prior activation. They exhibit cytotoxicity and secrete various cytokines and chemokines, such as IFN-γ, TNF-α, and GM-CSF, which enhance and modulate the adaptive immune response. (Basílio-Queirós et al. 2022).

Natural killer (NK) cells were discovered in the early 1970s when a novel subpopulation of lymphocytes that exhibited the ability to spontaneously kill mouse tumor cells without prior activation were discovered. They were called “Natural Killers” cells because of their rapid cytolytic activity without being primed (Kiessling, Klein, Pross, et al. 1975; Kiessling, Klein, and Wigzell 1975). Subsequent study revealed that NK cells can also respond to viral infections and cells that lack self MHC I molecules on their membrane “missing self” by releasing cytotoxic substances and producing cytokines (Ljunggren and Kärre 1990).

NK cells constitute about 5-20% of the lymphocyte population in circulation. They are present in different organs and tissues and mostly populate secondary lymphoid tissues organs like the spleen, lymph node and tonsil (Grégoire et al. 2007; Perera Molligoda Arachchige 2021). Although

NK cells are generally classified as innate immune cells, they have also been observed to possess and exhibit adaptive-like immune features and responses like specific antigen recognition, clonal expansion, and long-term memory (Malone et al. 2017; Rölle, Pollmann, and Cerwenka 2013; Sun, Beilke, and Lanier 2009) .

1.2.1. Comparison of Natural Killer (NK) Cells with Innate Lymphoid Cells (ILCs)

Natural Killer (NK) cells are a major part of the innate immune system and are now recognized as part of the wider family of innate lymphoid cells (ILCs). This family comprises of three major groups: ILC1s, ILC2s, and ILC3s, that have different functions and transcriptional regulations. Though they share developmental pathways and some functional similarities, NK cells and the other ILC subsets exhibit key differences in cytotoxicity, receptor expression, and immune roles.

1.2.1.1. *Similarities Between NK Cells and Other ILCs*

1. *Lineage and Development*

Both NK cells and ILCs originate from common lymphoid progenitors (CLPs) and develop without requiring antigen receptor gene rearrangements (Spits and Di Santo 2011; Vivier 2021). The transcription factor ID2 (DNA-binding protein inhibitor ID-2) is important in the development, maturation and function of both NK cells and ILCs (Vivier et al. 2018).

2. *Rapid Response to Stimuli and Tissue Localization*

NK cells and ILCs respond promptly to pathogens and tissue damage without prior sensitization (Piersma 2024; Wiedemann 2022). Both secrete cytokines to amplify innate and adaptive immune responses, contributing to immune defense and homeostasis (Vivier et al. 2018). NK cells and

ILCs are widely distributed in peripheral tissues, particularly at barrier sites like the skin, where they act as immune sentinels (Boudkova et al. 2024).

Table 1: Differences between NK cells and other ILCs

Feature	**NK Cells**	**Other ILCs (ILC1, ILC2, ILC3)**
Cytotoxicity	Highly cytotoxic; use perforin and granzymes to lyse virus-infected and tumor cells.	Non-cytotoxic; mediate immune regulation via cytokine secretion.
Receptor Expression	Express activating and inhibitory receptors, e.g., NKG2D and KIRs, enabling target recognition.	Lack NK-specific receptors; respond to cytokine signals.
Cytokine Profiles	Secrete proinflammatory cytokine IFN-γ and TNF-α that help modulate immune response.	Secrete cytokines specific to their subsets: ILC1 (IFN-γ), ILC2 (IL-5, IL-13), ILC3 (IL-17, IL-22).
Transcription Factors	Depend on T-bet and Eomes for development and function.	Require distinct transcription factors: T-bet (ILC1), GATA3 (ILC2), RORγt (ILC3).
Immune Role	Directly eliminate infected or malignant cells and modulate immune responses.	Orchestrate tissue repair, barrier integrity, and immune homeostasis.

1.2.1.2. Functional Roles of NK cells and ILCs

NK cells are potent cytotoxic lymphocytes specializing in detecting and eliminating virus-infected and transformed cells by recognizing stress-induced ligands or reduced MHC-I expression, killing through perforin/granzyme pathways and enhancing the amplifying innate and adaptive immune response through the secretion of pro-inflammatory cytokines (Vivier et al. 2008).

ILC1 subset share similarities with NK cells in cytokine profiles and produce both IFN-γ and TNF-α, but they lack cytotoxic granules (perforin and granzymes). ILC1s are critical in intracellular pathogen defense (Eberl et al. 2015). On the other hand, ILC2 regulate type 2 immunity by producing IL-5 and IL-13 that promotes responses against helminths and allergens, while ILC3 produce IL-17 and IL-22 that are important for maintaining gut barrier integrity and defending against extracellular bacteria and fungi (Eberl et al. 2015).

1.3. IMPORTANCE OF NK CELLS

1.3.1. NK cell in anti-tumor immunity

NK cells play a crucial role in the immune system's response to tumors and have been widely studied in mice and humans (Smyth et al. 2002). They are primed to eliminate tumor cells because they can identify cells with “missing self” i.e., cells that downregulate MHC class 1 expression. These tumor cells evade CD8+ T cell immune response and NK cells are poised to eliminate them. In clinical studies involving humans, the presence of cytotoxic NK cells negatively correlates with cancer occurrence in patients. Also, an increased survival rate was observed in patients with increased presence of tumor-infiltrating NK cells (Guillerey, Huntington, and Smyth 2016). In

studies involving mice that were treated with NK cell-depleting antibodies and those with NK cell deficiency, a high occurrence and susceptibility to tumors was observed (Guillerey et al. 2016).

NK cells like CD8+ T cells eliminate tumor cells by degranulation. This is the process that leads to the release of cytotoxic molecules such as perforin and granzymes by cytotoxic immune cells. Studies have shown that perforin-deficient NK cells were incapable of killing YAC-1 (mouse thymoma cell line) or RMA-S (a lymphoma mutant cell line with decreased MHC I expression) targets in vitro (van den Broek et al. 1995; Kägi et al. 1994). Also, chemical-induced fibrosarcomas that are susceptible to NK cell cytotoxicity showed resistance to killing in perforin-deficient mice in vivo (Smyth and Godfrey 2000). Similarly, NK cells also lyse tumor target cells by releasing granzyme A and granzyme B (Shresta et al. 1995). NK cells express Fas-L and TRAIL and they can facilitate NK cell tumor cytolytic activity when they bind to the corresponding Fas and TRAIL receptors on tumor cells as observed in in-vitro and in-vivo experiments (Guillerey et al. 2016; Oshimi et al. 1996; Takeda et al. 2001).

NK cells also enable tumor elimination by secreting factors like cytokine and chemokines that modulate the anti-tumor immune response. Activated NK cells can secrete IFN-γ and TNF-α that can directly affect tumor cells and the tumor environments. These cytokines can also activate other innate immune cells (macrophages, neutrophils and dendritic cells) and adaptive immune cells (T and B cells) and help to shape their anti-tumor response (Guillerey et al. 2016; Jorgovanovic et al. 2020). The critical role played by IFN-γ in anti-tumor response has been observed in mice defective in their IFN-γ production. These mice developed multiple malignancies including lymphomas and carcinomas (Street et al. 2002). Chemokines such as CCL2, CCL3, CCL4and CXCL8 are produced by NK cells and they serve as chemoattractant to other innate and

adaptive immune cells, thereby amplifying the anti-tumor immune response (Narni-Mancinelli, Vivier, and Kerdiles 2011).

1.3.2. NK cell and immune response to infections

NK cells play a key role in the host immune response against several pathogens. Genetic mutations associated with NK defects have led to combined immunodeficiency syndromes in patients (Buckley et al. 1997; Notarangelo et al. 2000; Shibuya et al. 1999). These patients were observed to be very susceptible to viral infections including herpes simplex virus (HSV), Cytomegalovirus (CMV), and human papilloma virus (Jost and Altfeld 2013; Orange 2006). Children with mutated forms of the Fc receptor for IgG type IIIA (CD16) on their NK cells had recurrent viral infections linked to HSV, Epstein-Barr virus (EBV) and varicella-zoster virus (VZV) (Jawahar et al. 1996; de Vries et al. 1996). NK cells in the uterus (decidua) have been shown to play a role in controlling human immunodeficiency virus (HIV)-1 infection in pregnancy (Quillay et al. 2016). NK cells have also been reported to inhibit hepatitis C and influenza infections (Goodier et al. 2016; Guidotti and Chisari 2006). NK cells not only directly kill viral infected cells but also release pro-inflammatory cytokines that recruit and regulate the immune response of other cells (Cook, Kline, and Whitmire 2015; Waggoner et al. 2011).

Viral infections are not the only pathogenic infections that can be combatted by NK cells. Being among the first responders to pathogen invasions, NK cells have been shown to mount direct immune response against other infectious agents. Studies have shown that NK cells can directly respond to several Gram-positive and Gram-negative bacteria like *Mycobacterium tuberculosis*, *Bacillus anthracis*, *Escherichia coli* or *Salmonella typhi* by releasing perforin and granulysin (Brill et al. 2001; Gonzales et al. 2012; Lu et al. 2014). They have also been observed to exhibit antibacterial response to intracellular bacterial pathogens using the Fas-FasL and TRAIL pathways

that lead to the apoptosis of the cell (Zucchini et al. 2008). A study to confirm the antibacterial activity of NK cells in-vivo using an animal model showed that mice lacking T and B cells but have NK cells had a higher survival rate and lower titers of the *Shigella flexneri* bacteria compared to mice that lacked T, B and NK cells (Le-Barillec et al. 2005).

In fungal infections, NK cells have been observed to mount an immune response against some disease-causing fungi (*A. fumigatus*, *C. albicans* and some mucormycetes) both in-vitro and in-vivo (Schmidt et al. 2011, 2016; Voigt et al. 2014). Perforins derived from NK cells play a key role in the control of fungal infections and cytokines released by NK cells as well as modulate the antifungal innate and adaptive immune responses (Schmidt et al. 2013). Mouse model studies of the importance of NK cells in the antifungal immune response showed that the proliferation of NK cells in mice infected with *Aspergillus niger* positively corelates to the reduction in fungal growth (Benedetto et al. 1988). Also, the depletion of NK cells in mice infected with *C. neoformans* led to an increase in disease burden in the lungs when compared to untreated controls (Lipscomb et al. 1987). Similar findings showing the importance of NK cells in host immune response against fungal infections like *A. fumigatus*, *C. albicans*, and *Histoplasma capsulatum* have been observed (Morrison et al. 2003; Park et al. 2009; Quintin et al. 2014).

1.4. NK CELL FUNCTIONS

1.4.1. Effector functions

Natural killer cells mount immune response and lyse tumor cells, virus and other infected cells without prior priming and respond to target cells lacking the major histocompatibility complex (MHC) class I molecules through natural cytotoxicity and antibody dependent cell cytotoxicity (ADCC).

1.4.1.1. *Natural Cytotoxicity*

NK cells bind to a target cell and form an interface called the immune synapse. After target recognition, NK cells undergo degranulation, which is the release of their cytotoxic granules that contains perforin (a membrane-disrupting protein) and granzymes (serine proteases) through a Ca2+-dependent exocytosis in the immunological synapse. Perforin from the NK cells form a transmembrane pore on the target cells and this facilitates the entry of granzymes into the cytosol of the target. The granzymes activates several caspases and lead to the apoptosis of the target cells (Trapani and Smyth 2002). The cytotoxic granule membrane on the NK cells is covered by the lysosomal-associated-membrane protein-1 (LAMP-1, or CD107a). It has been observed that the up-regulation of LAMP-1 on NK cell membrane is an indicator of NK cell activation and lysis of target cells (Alter, Malenfant, and Altfeld 2004).

NK cell natural cytotoxicity can be carried out through a different mechanism that involves the death receptors. The binding of NK cell molecules that are members of the Tumor Necrosis Factor (TNF) family, such as FasL and the soluble TNF-related apoptosis-inducing ligand (TRAIL) to the Fas, and TRAIL receptors expressed on target cells can lead to lysis of tumor or infected cells. This mechanism is more involved in the clearance of auto-reactive lymphoid cells and maintenance of homeostasis. Several cells express the Fas (CD95) receptor which contains an intracytoplasmic "death domain" that is conserved. Its interaction with the Fas-ligand activates the cleavage of caspases and causes apoptosis of the target cell.

1.4.1.2. *Antibody dependent cellular cytotoxicity (ADCC)*

ADCC is another crucial effector mechanism employed by the immune system to eliminate viral infected cells and tumors. In ADCC, IgG antibodies that bind to the Fcγ receptors on effector cells

like NK cells cover the antigens of tumor or infected cells (Hashimoto, Wright, and Karzon 1983). The IgG antibody covering the target cells facilitates contact between the target cells and the NK cells, therefore leading to the subsequent lysis of the target cells.

Natural Killer cells express the FcγRIIIA or CD16, especially the CD56dim NK cells (Lanier et al., 1988). The binding of the CD16 on the NK cells to the Fc region of the IgG (its ligand) causes the degranulation of the NK cell and promotes the release of perforin and granzymes that cause apoptosis of the target cell (Wang et al. 2015). This effector mechanism has been utilized in breast cancer immunotherapy to produce antibodies that target specific antigens overexpressed by tumor cells (Wang et al. 2015). Trastuzumab and Rituximab are examples of humanized monoclonal antibody agents employed in the treatment of breast cancer and non-Hodgkin's lymphoma respectively (Cartron et al. 2002; Junttila et al. 2010).

1.4.2. Cytokine secretion

NK cells are also important producers of several proinflammatory cytokines, chemokines and growth factors that help to amplify immune responses. The cytokines secreted by NK cells include interferon gamma (IFN-γ), TNF-α, IL5, IL10 and IL13. They secrete the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines such as C-C motif chemokine ligand (CCL) 3, 4, 5 and IL8 (Fauriat et al. 2010). NK cells store IFN-γ and TNF-α in granules and they are released when they are activated. The cytokines secreted by NK cells are not target specific and can elicit responses from several cells at the same time (Reefman et al. 2010).

NK cells produce an abundance of IFN-γ, and this cytokine plays diverse immunomodulatory roles. This cytokine helps shape both innate and adaptive responses by promoting the maturation, activation and recruitment of dendritic cells, macrophages and T cells (Goldszmid et al. 2012; Kos

and Engleman 1995) and is crucial against tumor, viral infections and intracellular pathogens. IFN-γ induces the upregulation of both MHC class I and class II expression. It enhances macrophage activation, thereby supporting phagocytosis and facilitating the production of proinflammatory cytokines. It also controls cellular proliferation and differentiation of naïve CD4 T cells into Th1 effector subset (Schoenborn and Wilson 2007).

TNF-α is another proinflammatory cytokine secreted by NK cells that can induce direct apoptosis of tumor cells, recruit other immune cells, regulate pro-tumorigenic regulatory T (Treg) cells and enhance effector T cell immune response (Josephs et al. 2018). NK cells produce chemokines such as IL8, macrophage inflammatory protein 1a (MIP-1a) or CCL5 that aid in the recruitment of other immune cell subsets to the site of inflammation (Roda et al. 2006).

1.5. NK CELL TARGET RECOGNITION

NK cells possess different repertoire of receptors and can recognize targets by discriminating between the expression of self and non-self-molecules (Lanier 2005; Moretta et al. 2002; Orr and Lanier 2010; Pegram et al. 2011). The intricate shift in the balance between activating and inhibitory signals determines the functional state of an activated NK cell (O'Connor, Hart, and Gardiner 2006; Pegram et al. 2011). NK cell activation is believed to be strongly regulated, and different models have been proposed to explain the mechanisms involved in target recognition and self-tolerance in NK cells. One of such models is the "missing self-hypothesis" that was proposed by Ljunggren and Karre in 1986 and its dependence on the major histocompatibility complex MHC-I model of education of NK cells (Kärre et al. 1986; Ljunggren and Kärre 1990).

1.5.1. Missing self-hypothesis

The missing self-hypothesis hinges on the dogma that the function of NK cells is to recognize and eliminate cells that lack the expression of self-major histocompatibility complex (MHC) class I molecules (Ljunggren and Kärre 1990). The cells lacking or presenting with an altered MHC-1 are sensitive to NK cell killing. This can be seen in viral infections and cancer. The MHC-1 molecule is a marker of "self" and is widely expressed on normal cells and help to facilitate signals that inhibit NK activation. This model was backed by the discovery of the Ly49A and Killer cell immunoglobulin-like receptors (KIR) inhibitory receptors in mice and human NK cells (Karlhofer, Ribaudo, and Yokoyama 1992; Long, Colonna, and Lanier 1996).

Previously, NK cell education was shown to only occur in bone marrow. However, subsequent findings showed that NK cell effector functions can be regulated by altering the expression of MHC-1. It was observed that anergy in NK cells correlates with the deficiency in MHC-1 while NK cells from mice deficient in β2m or mutated transporter associated with antigen processing 1 (Tap1) became functionally activated after adoptive transfer to mice expressing MHC-1, clearly indicating that NK cell target recognition, education and activation is regulated by MHC-I (Elliott, Wahle, and Yokoyama 2010; Liao et al. 1991). However, this model presented a question as to how target cells are killed by NK cells by their lack of MHC-1 molecule alone. Researchers were uncertain if this was enough to activate NK cells or whether additional stimulatory mechanisms were also involved.

Studies have now shown that NK cells express a variety of other stimulatory receptors. The Ly49H in mice and NKp46 in humans are an example of molecules that recognize molecules of viral pathogens (Arase et al. 2002; Mandelboim et al. 2001). NKG2D receptor recognizes self-proteins that are highly expressed in diseased or tumor cells but are poorly expressed on normal cells

(Raulet 2003). The high affinity Fc receptor is another stimulatory receptor that facilitates NK cells mediated antibody dependent cellular cytotoxicity (ADCC) (Perussia 1998). These studies have shown that NK cell target recognition and activation requires the crosstalk and engagement of one or more stimulatory receptors. Though the role of these stimulatory receptors has been identified and is still to be characterized, their signals can be countered by a very strong engagement of the MHC-1 inhibitory receptors. This suggests that the absence of self MHC molecules alone is not sufficient to make a target more sensitive to NK cell killing, rather it also requires signals from the stimulatory receptors binding to their ligands.

1.5.2. Activating NK cell receptors

NK cells were thought to be solely activated by the downregulation of MHC-1 molecule. Subsequent studies have revealed the role of other receptors in regulating NK cell activation and function. Several activating receptors are present on the surface of NK cells and the binding of these receptors to their ligands activates NK cells. The ligands for the activating NK cell receptors are overexpressed on infected cells and tumor cells. Some of the already characterized NK cell activating receptors are the natural cytotoxic receptors (NCRs) (NKp30, NKp44 and NKp46), CD226 (DNAX Accessory Molecule-1, DNAM-1), natural killer group 2 member C and D (NKG2C and NKG2D), CD16 (FcgRIIIA) and killer immunoglobulin like receptors (KIRs) (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1) (Barrow, Martin, and Colonna 2019; Blunt and Khakoo 2020; Lanier 2015; Martinet and Smyth 2015; Molfetta et al. 2017). The KIRs with a short intracellular tail are activating receptors while those with a long intracellular tail are inhibitory receptors (Dębska-Zielkowska et al. 2021).

NCRs can recognize host- and pathogen- associated ligands like the viral hemagglutinins. NKG2D and DNAM-1 can recognize ligands on cancer cells. MHC class I polypeptide-related sequence A and B MICA/B and UL16-binding proteins (ULBP) are ligands for NKG2D while CD112 (Nectin-2) and CD155 (PVR, poliovirus receptor) are ligands for DNAM-1 (Bottino et al. 2003; Gasser et al. 2005). CD16 (FcgRIIIA) binds to the Fc region of immunoglobulins and facilitates ADCC of targets that have been opsonized by antibodies (Lanier et al. 1991). Adhesion molecules also play an important role in the activation of NK cells. The formation of immune synapse between NK cells and their target is enabled by these molecules. Integrins like the LFA-1 (CD11a/CD18), CD2 and the SLAM receptor 2B4 (SLAMF4, CD244) play a key role in this regard (Binder et al. 2020; Claus et al. 2019; Urlaub et al. 2017).

1.5.3. Inhibitory NK cell receptors

These are the receptors that facilitate the inhibition of NK activation and response, especially to prevent self-recognition. Many inhibitory receptors bind to major histocompatibility complex class 1 (MHC-I) molecules and many of the inhibitory receptors on NK cells belong to the KIR family. (Sivori et al. 2020). The binding of self-MHC-I with an inhibitory KIR dampens the activation of NK cells (Ljunggren and Kärre 1990). NKG2A (CD94) is another receptor that inhibits NK cells by binding to human leukocyte antigen E (HLA-E). Killer cell lectin-like receptor subfamily B, member 1 (KLRB1) and killer cell lectin like receptor G1 (KLRG1) are also inhibitory receptors that bind lectinlike transcript 1 (LLT1) and cadherins respectively.

Table 2: NK cells activating and inhibitory receptors

Receptor Type	Receptor	Ligands	HLA/Non-HLA
Activating Receptors			
	NKG2D	MICA, MICB, ULBP1-6	Non-HLA
	NKp30	B7-H6, BAG6, HSP70	Non-HLA
	NKp44	Viral hemagglutinin, PCNA	Non-HLA
	NKp46	Viral hemagglutinin, fungal components	Non-HLA
	DNAM-1 (CD226)	CD112 (Nectin-2), CD155 (PVR)	Non-HLA
	2B4 (CD244)	CD48	Non-HLA
	CD16 (FcγRIII)	Fc portion of IgG	Non-HLA
	NKG2C	HLA-E	HLA
	NKG2E	HLA-E	HLA
	KIR2DS1	HLA-C2	HLA
	KIR2DL1	HLA-C	HLA
	KIR3DS1	HLA-Bw4, HLA-F	HLA
	KIR2DS4	HLA-A11:02 and HLA-C05:01	HLA
	KIR2DS5		HLA
	KIR2DL4*	HLA-G	HLA
Inhibitory Receptors			
	TIGIT	CD112 (nectin-2), CD155 (PVR)	Non-HLA
	LAIR-1	Collagens	Non-HLA
	Siglecs	Sialylated glycans	Non-HLA
	KLRG1	E-, N-, and R-cadherins	Non-HLA
	CD96 (Tactile)	CD155 (PVR)	Non-HLA
	PD-1	PD-L1, PD-L2	Non-HLA
	TIM-3	Galectin-9, HMGB1, Ceacam-1 and Phosphatidyl serine – PtdSer	Non-HLA
	IL-1R8	IL-37	Non-HLA
	IRp60	Phosphatidylserine (PS) and Phosphatidylethanolamine (PE)	Non-HLA
	KIR2DL1	HLA-C2	HLA
	KIR2DL2/3	HLA-C1	HLA
	KIR3DL1	HLA-Bw4	HLA
	KIR3DL2	HLA-A3/A11	HLA
	CD94-NKG2A	HLA-E	HLA
	LIR-1 (ILT2)	HLA-G, HLA-A, HLA-B, HLA-C	HLA
	LAG-3	MHC-II	HLA
	KIR2DL5		HLA
	KIR2DL4*	HLA-G	HLA
	LILRB	HLA- A-C, HLA- E-F, and HLA-G, Angiopoietin-like proteins	HLA

KIR2DL4* acts as an inhibitory and activating receptor

1.6. NATURAL KILLER CELLS- DENDRITIC CELLS CROSSTALK

NK cell and dendritic cell (DC) crosstalk is part of the key mechanisms that bridge the innate and adaptive immune response to facilitate a more robust and increased immune response. This is a two-way interaction that involves both cell to cell contact and cytokine signaling (Pende et al. 2006; Wu and Laufer 2007).

NK cells and DCs can interact in lymphoid and nonlymphoid tissues (Borg et al. 2004; Buentke et al. 2002; Harizi 2013). DCs undergo their development in the bone marrow and immature DCs home to tissues such as lung mucosa, skin epithelia, nasal and gastrointestinal linings, which are in constant contact with the external environment to act as part of the first responders against foreign pathogens/antigens. Their expression of costimulatory molecules, such as CD80, CD86, CD83, and MHC II is low and they secrete low levels of proinflammatory cytokines, such as IL-12, IL-10, and TNF-α. These immature DCs phagocytose and process antigens and these lead to the over-expression of the costimulatory molecules such as CD40, CD80, CD86 and MHC-class II which are markers for DC maturation. The mature DCs secrete cytokines and chemokines, change their shape and express chemokine receptors like CCR7 and then migrate to lymph nodes to present the antigen to effector cells including NK cells (Ferlazzo et al. 2004; Marcenaro et al. 2012).

Some important areas of the NK-DC interactions are the lymph nodes and gut mucosa. In humans, NK cells are resident in the lymph node and represent about 0.5% of the immune cell population at this site. The population of NK cells within the lymph node increases during infection, as they are recruited via the CXCR3 pathway. There is a high expression of CCR7, L-selectin and CXCR3 in human NK cells (Harizi 2013; Marcenaro et al. 2012; Piqueras et al. 2006). Studies have also

shown that a population of human and mouse NK-like cells that express NKp44/NKp46 and IL-22 are positioned near resident DCs in the gut mucosa (Kirchberger et al. 2013).

DCs release several cytokines, chemokines and other signals that influences NK cell effector immune response and regulate their proliferation, activation and migration (Ferlazzo and Morandi 2014; Vujanovic et al. 2010). Cytokines from DCs such as IL-12 facilitate the derivation of IFN-γ producing NK cells. IL-1 and IL-8 derived from DCs are important in NK cells gaining the IL-12 receptor (Andrews et al. 2003). Other cytokines like IL-15 and TNF-α secreted by DC enhance NK cell survival, proliferation and promote the secretion of pro-inflammatory cytokines and cytotoxic molecules (Ferlazzo and Morandi 2014). On the other hand, NK induces MHC molecule expression leading to the activation and maturation of DCs. NK-cell promotion of DC activation relies on both tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) secretion and a cell-to-cell interaction via the NKp30 (Walzer et al. 2005).

IFN-γ promotes DC maturation and enhances the expression of MHC-I and II molecules, upregulation of co-stimulatory molecules and the processing of antigen to T cells (Pan et al. 2004). Also, IFN-γ activation of DCs induces the expression of IL-12 and IL-15 in DCs and they help to promote anti-tumor Th1 CD4 T cell and CD8 cytotoxic T cell immune responses (Kelly et al. 2002; Morandi et al. 2009; Walzer et al. 2005). NK cells regulate DC homeostasis or DC editing by selectively killing autologous immature DCs that have reduced expression of MHC molecules using the NKp30 and TNF-related apoptosis inducing ligand (TRAIL). Matured DCs escape this killing by their high expression of MHC molecules, hence are primed to effectively present antigen to T cells (Bald et al. 2020; Chiossone et al. 2018; Hayakawa et al. 2004). Studies have shown a reduction in the activation of DCs in the lymph node when NK cells are depleted (Zitvogel et al. 2006).

1.7. NK CELL MIGRATION AND HOMING

NK cells originate from hematopoietic stem cells in the bone marrow where they differentiate and then both mature and immature NK cells migrate via the circulating blood and home to secondary lymphoid and other peripheral tissues. NK cells are present in both lymphatic and non-lymphoid organs, making them effective to carry out immunosurveillance against cancer and infections (Björkström, Ljunggren, and Michaëlsson 2016; Freud et al. 2017). Their homing into these tissues is very dependent on their development level and activation status, enabling them to perform their functions in these tissues (Peng and Tian 2014; Yao and Matosevic 2021).

1.7.1. Factors responsible for NK cell migration

There are several factors that regulate NK cell migration. These factors include integrins, selectins, chemokine receptors and their ligands, cytokines, sphingosine-1-phosphate (S1P) and the microenvironments (Mayol et al. 2011; Shannon and Mace 2021; Yao and Matosevic 2021).

1.7.1.1. *Integrins*

Integrins are a collection of transmembrane receptors that have 18 identified α subunits and 8 identified β subunits. An integrin heterodimer is formed when individual α subunit binds with several β subunits (Shannon and Mace 2021). The individual integrin heterodimer can bind to several components of the extracellular matrix such as the selectins and cell adhesion molecules (CAMs), enabling them to interact with the extracellular matrix (Shannon and Mace 2021). Integrins have adaptable structures and functions and this helps to enhance the migration speed of cells (Shannon and Mace 2021).

NK cells express different integrins and they are involved in controlling NK cell migration and tissue homing and helps to identify tissue and non-tissue resident NK cells (Shannon and Mace

2021). β2 integrins are part of the integrins expressed by NK cells. These integrins facilitate the migration of NK cells through tissues and blood circulation. They are involved in cellular adhesion of the NK cells to other cell or tissue surfaces and initiate the formation of the immune synapse during the killing of target cells (van Spriel et al. 2001). In the blood and lymph nodes, NK cells express integrins such as αL (LFA-1)/β2, αM (Mac-1)/β2, αX (CR4)/β2, and αDβ2 that bind to the CAMs like vascular cell adhesion molecule-1 (VCAM), ICAM and MadCAM on extracellular matrix. This allows NK cells to migrate from circulation into tissues (Shannon and Mace 2021). Tissue resident NK cells commonly express αL (LFA-1)/β2, αM (Mac-1)/β2, α1 (VLA-1)/β1, α2 (VLA-2)/β1, α4 (VLA-4)/β1, and α5 (VLA-5)/β1 β1 and these integrins can bind with collagen, laminin, E-cadherin, fibronectin, VCAM-1, MAdCAM-1, and vitronectin and facilitate the trafficking of NK cells into tissue microenvironment and initiate NK cytotoxicity on target cells (Mace et al. 2010; Shannon and Mace 2021). In various organs and tissues such as lungs, liver, uterus, bone and spleen, the binding of various integrins to their ligands such as α1/β1 to collagen IV/laminin, αE(CD103)/β7 to E-cadherin, and α4/β7 to MadCAM-1, VCAM-1 and fibronectin facilitates tissue residency of NK cells in those tissues (Shannon and Mace 2021).

1.7.1.2. *Chemokine Receptors*

Chemokine receptors also called G-protein-coupled seven-transmembrane receptors are important factors in migration and homing of cells. These receptors are present on the surface of some cells and they bind to a type of cytokine called chemokines (Murphy et al. 2000). The binding of the chemokine receptors to chemokines leads to a change in the affinity of integrins and induces actin restructuring, thereby facilitating cell migration (Shannon and Mace 2021). Chemokine receptors have their ligands (chemokines) and these receptors are grouped by their structures: C-X-C chemokine receptor (CXCR), C-C chemokine receptor (CCR), C-X3-C chemokine receptor

(CX3CR), and XCR. Each has its corresponding chemokine ligands (Shannon and Mace 2021). In NK cells, different chemokine receptors participate in engendering migration and other functions. These chemokine receptors include CXCR1, CXCR3, CXCR4, CXCR6, CCR7, C–C motif chemokine ligand (CCL) ¾, regulated activation, normal T-cell expressed, and secreted (RANTES or CCL5), and C-X-C motif chemokine ligand (CXCL) 1 (Shannon and Mace 2021).

1.7.1.3. *Sphingosine-1-phosphate and sphingosine-1-phosphate 5 receptor*

The sphingosine-1-phosphate 5 receptor is a subtype of the sphingosine-1-phophate receptors that bind to sphingosine-1-phosphate (S1P). The sphingosine-1 phosphate (S1P) is a lysophospholipid that plays a key role in many cells, including NK cells. It is highly present in blood and lymph and binds to five different SIPR receptors (S1PR1–5) (Baeyens and Schwab 2020; Kihara et al. 2014). The receptors regulate migration, cytoskeleton organization, immune cell trafficking etc (Baeyens and Schwab 2020). In NK cells, S1PR5 have been reported to be involved in the migration of NK cells from the bone marrow and lymph nodes to the peripheral tissues (Björkström et al. 2016; Mayol et al. 2011; Peng and Tian 2014; Walzer et al. 2007). S1PR5 was reported to be highly expressed in matured NK cells and there was reduction in circulating NK cells in the blood, spleen and lungs of S1PR5 deficient mice, with an increase in the number of NK cells in the bone marrow and lymph nodes (Drouillard et al. 2018; Walzer et al. 2007). In NK cells, S1PR5 functions as a chemotactic receptor for S1P, enabling homing in blood, spleen and lungs.

1.7.1.4. *L-Selectin (CD62L)*

L-selectin is a cell adhesion molecule that is present on the cell surface of leukocytes. L-selectin have a key function in both the innate and adaptive immune responses by promoting leukocyte-endothelial cell adhesion (Ivetic, Hoskins Green, and Hart 2019). The binding of L-selectin to its ligands facilitates immune cell trafficking into tissues and homing to secondary lymphoid organs. It is expressed on most circulating immune cells (Ivetic et al. 2019). The ligands of L-selectin include GlyCAM-1, CD34, MadCAM-1 and PSGL-1. The initial attachment and slow rolling of immune cells to the walls of the high endothelial venules (HEV) is facilitated by L-selectin. In NK cells, L-selectin cross-linking stimulates LFA-1 adhesion by NK cell subsets (Frey et al. 1998). It was also reported that $CD56^{bright}$ NK cells expressed higher level of L-selectin compared to T cells, monocytes and neutrophils and NK cells expressed increased level of L-selectin in response to cytokines (IL-12, IL-10 or IFN-α) (Frey et al. 1998). NK cells have also been shown to bind to and transmigrate across endothelial cells in vitro at a faster rate than T cells (Allavena et al. 1994; Bianchi et al. 1993), making this molecule and important factor facilitating NK cell migration into tissues.

1.7.2. Chemokine induced mechanism in NK cell migration

Chemokines have been shown to play a key role in migration of leukocytes, but the pathways involved have not been fully outlined. There is an interface among several molecules that control cytoskeleton components of cells that facilitates migratory signals in cells (Vicente-Manzanares and Sánchez-Madrid 2004).

Migration in NK cells relies on chemokines, chemokine receptors, integrins and their signaling networks that activate several pathways. Studies have shown that the binding of LFA-1 to its

ligands activates Src and Syk kinase, though this has been linked to cytotoxicity and not migration in NK cells (Perez et al. 2004; Riteau, Barber, and Long 2003). Protein tyrosine kinases (PTK) play a role in different biological processes including cell migration. Using PTK inhibitors such as herbimycin A (general tyrosine kinase inhibitor), damnacanthal (specific Lck inhibitor) and piceatannol (Syk inhibitor), it was observed that the Src kinase and Lck play a role in CXCL12-induced NK cell migration (Inngjerdingen, Torgersen, and Maghazachi 2002).

Focal adhesion kinases (Fak) have been suggested to also facilitate migration in different cells. There are two members, the p125 focal adhesion kinase (p125Fak) and the proline-rich tyrosine kinase 2 (Pyk-2). NK cells have been reported to express p125Fak and it is activated by the binding of β1 integrin to its ligand (Rabinowich et al. 1996). Human peripheral blood NK cells were reported to express Pyk-2 that is linked with the cytoskeletal protein paxillin. The attachment of β1 or β2 integrins on human NK cells leads to the tyrosine phosphorylation of both Pyk-2 and paxillin. It was also shown that Pyk-2 functions upstream of β1 and β2 integrin-induced MAPK cascades and regulates NK cell cytotoxic response (Gismondi et al. 1997, 2000). The binding of NK cells to the endothelium activates Pyk-2 and Rac (a small GTP-binding protein), which regulates actin cytoskeleton change. Pyk-2 functions as a link between integrin and chemokine receptor signaling and the Pyk-2/Rac pathway is important in regulating NK cell transendothelial migration (Gismondi et al. 2003). interestingly, Pyk-2 has also been shown to colocalize with microtubule-organizing center (MTOC) in NK cells (Sancho et al. 2000).

1.8. PROLACTIN-INDUCIBLE PROTEIN (PIP)

Prolactin-inducible protein (PIP) was first identified by Shiu and Iwasiow (1985). It is a protein present in T47D human breast cancer (BC) cell lines (Shiu and Iwasiow 1985). It was also found to be present in gross cystic disease of the breast and was tagged gross cystic disease fluid protein 15 (GCDFP-15) (Haagensen et al. 1979; Mazoujian et al. 1983). PIP in humans is a 118 amino acid secreted polypeptide that has a molecular mass of 14-20 kDa and its molecular mass is dependent on its site of origin (Haagensen et al. 1979; Rathman et al. 1989; Schaller et al. 1991; Toth et al. 1988). PIP has been identified in bodily fluids such as tears, saliva and sweat, amniotic fluid, seminal plasm, and blood of pregnant women (Autiero, Abrescia, and Guardiola 1991; Haagensen et al. 1980; Schenkels et al. 1991).

PIP structural features consist of seven parallel β-sheets and seven β-turns and lacks the α-helix-type structures. The β-sheet motifs form a hollow, sandwich-like structure due to the hydrophobic properties of amino acid residues (M. I. Hassan et al. 2008). It has an actin-binding motif and possess CD4-binding and fibronectin-binding domains (Akiyama and Kimura 1990; Basmaciogullari et al. 2000; Caputo et al. 2003). The expression of PIP is regulated by androgen, prolactin and glucocorticoids (Haagensen et al. 1990; Shiu and Iwasiow 1985). PIP's expression is also regulated by cytokines like interleukins 1α, 4 and 13 and downregulated by 17β-estradiol and IL-6 (Dauvois et al. 1990; Labrie et al. 1990; Shiu and Iwasiow 1985).

1.8.1. Function of PIP

Prolactin-Induced Protein (PIP) exhibits an aspartyl protease activity, attributed to the presence of an aspartate residue at position 22 (Asp22), which mirrors the conserved aspartate residue at position 32 found in other aspartyl proteases such as cathepsin D, pepsin, and renin (Caputo et al.

2000). The aspartyl protease activity of PIP is implicated in the degradation of fibronectin and components of the extracellular matrix, suggesting a potential role in the facilitation of breast cancer progression. Moreover, PIP has been reported to interact with various proteins, although the full physiological implications of these interactions remain to be elucidated (Urbaniak et al. 2018). The proteins identified as binding partners of PIP include actin, fibrinogen, β-tubulin, serum albumin, zinc α2-glycoprotein, and the Fc fragment of immunoglobulin G (Md. I. Hassan et al. 2008; Kumar et al. 2012; Naderi and Vanneste 2014; Rathman et al. 1989; Schenkels et al. 1994).

PIP has been shown to have antimicrobial properties. Studies have suggested that PIP binds to several bacteria in humans. PIP/EP-GP from human saliva was also shown to bind to bacteria from the genera Gemella, Streptococcus and Staphylococcus, leading to bacteria aggregation (Lee, Bowden, and Myal 2002; Schenkels et al. 1994, 1997). In mice, PIP from saliva performs a similar function and binds to bacteria from the genus Streptococcus (Nistor et al. 2009). These findings indicate that PIP plays a role in the defense against bacteria pathogens in the mouth and the bacterial aggregation inhibits bacteria proliferation in the oral cavity (Lee et al. 2002; Urbaniak et al. 2018). PIP may affect the development of the submaxillary gland during the early stages of embryonic development in mice (Lee et al. 2002).

1.8.2. PIP and the Immune System

The localization of Prolactin-Induced Protein (PIP) within mucus and submucosal layers of tissues at critical pathogen entry points, such as the skin, saliva, bronchial submucosal glands, and lacrimal fluid, suggests its involvement in host immune defense mechanisms (Urbaniak et al. 2018). PIP's ability to bind to bacteria present in the oral cavities of both humans and mice further supports its antimicrobial role (Lee et al. 2002; Nistor et al. 2009). These findings imply that PIP

plays a role in the innate immune response, particularly in defense against certain pathogenic bacteria.

Multiple studies provide compelling evidence for the involvement of Prolactin-Induced Protein (PIP) in the adaptive immune response. PIP contains a CD4 binding domain, specifically identified as two fragments encompassing the N-terminal amino acids 1-35 and residues 78-105 (Basmaciogullari et al. 2000). CD4 molecules on T cells function as coreceptors in the recognition of antigenic peptides presented by MHC class II molecules and are also crucial for signal transduction during T cell activation (König 2002; Veillette et al. 1989). PIP, isolated from human seminal fluid (referred to as gp17/SABP) and breast cyst fluid (GCDF-15), has been demonstrated to bind to CD4 receptors on the surface of T cells, macrophages, and spermatozoa (Autiero et al. 1995; Bergamo et al. 1997; Caputo et al. 2003). Notably, further research revealed that PIP derived from breast cyst fluid, a pathological source, exhibited a lower binding affinity to CD4 compared to PIP obtained from normal physiological conditions, such as in human seminal fluid (Caputo et al. 2003). This differential binding affinity suggests that PIP may have distinct functional roles, with its interactions being influenced by the tissue type and the physiological or pathological state.

CD4 serves as a receptor for the gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1), facilitating viral entry into CD4+ T cells (Autiero et al. 1997; Klatzmann et al. 1984; Maddon et al. 1986). The binding of Prolactin-Induced Protein (PIP) to the CD4 molecule induces a conformational change that disrupts the interaction between CD4 and gp120, thereby inhibiting the attachment and subsequent entry of HIV-1 into CD4+ T cells (Autiero et al. 1997). This interaction between PIP and CD4 effectively prevents the binding of the viral gp120 glycoprotein to CD4, suggesting that PIP plays a protective role in the pathogenesis of HIV-1 infection (Autiero et al. 1997). Additionally, further studies have demonstrated that PIP inhibits

apoptosis of CD4+ T cells triggered by gp120/CD4 and TCR/CD3 activation (Gaubin et al. 1999). Given the critical role of CD4+ T cells in orchestrating adaptive immune responses and modulating innate immunity, the loss of these cells due to HIV-1 infection leads to immunodeficiency.

Mice lacking Prolactin-Induced Protein (PIP) gene (PIP KO) exhibited several immune-related pathologies, including enlarged lymph nodes adjacent to the parotid glands, lymphocytic infiltrations within the prostate lobes, hypertrophy of the thymic medulla, and the presence of cardiac arteritis and periarteritis (Blanchard et al. 2009). Further investigations revealed a significant reduction in the percentage of CD4+ T cells in the spleens of PIP KO mice compared to wild-type controls, which was associated with an impaired Th1 immune response and reduced nitric oxide (NO) production (Ihedioha et al. 2018; Li et al. 2015).

In a Leishmania infection model, PIP knockout (PIP KO) mice demonstrated increased susceptibility to infection attributed to deficiencies in their immune response. CD4+ T cells isolated from ovalbumin (OVA)-immunized PIP KO mice exhibited markedly reduced proliferation and interferon-gamma (IFN-γ) production following in vitro restimulation, indicating a compromised adaptive immune response. Additionally, macrophages derived from the secondary lymphoid organs of PIP KO mice showed diminished nitric oxide (NO) production, contributing to the impaired immune defense against Leishmania (Li et al. 2015). Subsequent studies revealed a reduction in pro-inflammatory cytokine production in PIP KO macrophages, which was associated with decreased phosphorylation of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) proteins upon stimulation with IFN-γ and lipopolysaccharide (LPS) (Ihedioha et al. 2018). Moreover, expression levels of suppressors of cytokine signaling (SOCS) 1 and 3, known inhibitors of IFN-γ and LPS signaling, were elevated in PIP KO macrophages compared to their wild-type counterparts (Ihedioha et al. 2018). These

findings underscore the critical role of PIP in modulating both innate and adaptive immune responses against pathogens and infectious agents.

1.8.3. PIP and Breast cancer

PIP is currently utilized as a prognostic marker of breast cancer (Darb-Esfahani et al. 2014). It also serves predictive biomarker of breast cancer response to chemotherapy treatment outcomes (Urbaniak et al. 2023). PIP is expressed in normal breast tissue and is linked to prolactin induced differentiation of luminal epithelial cells in the breast (Li et al. 2021; Naderi 2015; Shiu and Iwasiow 1985). Genomic studies have revealed that luminal A and molecular apocrine breast cancer subtypes highly express PIP (Baniwal et al. 2012; Doane et al. 2006; Teschendorff et al. 2006). Molecular apocrine breast cancer is a subtype that is estrogen receptor (ER)-negative, overexpresses ErbB2, and exhibits steroid-response gene markers such as androgen receptor (AR), FOXA1, and TFF3 (Doane et al. 2006; Farmer et al. 2005; Teschendorff et al. 2006). About 40–50 % of ER-negative breast tumors express AR and most of these tumors also overexpress ErbB2 (Guedj et al. 2012; Naderi and Hughes-Davies 2008; Park et al. 2010). PIP has been shown to be the most regulated molecular apocrine gene in both ER-negative breast tumors and cell lines (Naderi and Meyer 2012). In the same vein, PIP was revealed to be one of the characteristic biomarkers for the identification of molecular apocrine breast tumors using immunohistochemistry (Lehmann-Che et al. 2013). Previous studies have also confirmed the expression of PIP in axillary node metastasis at both mRNA and protein levels (Clark et al. 1999; Murphy et al. 1987; Wick et al. 1989). In contrasts, PIP expression is decreased in late-stage apocrine carcinomas and invasive breast tissue compared to adjacent normal tissue (Honma et al. 2005; Parris et al. 2014). A study by Gangadharan et al. indicated that the expression of the PIP gene showed a gradual decrease in higher grade and later stage of breast cancer (Gangadharan et al. 2018). The study also showed a

significantly higher levels of PIP expression at the mRNA and protein level in normal breast tissue than in breast cancer tissues with PIP being significantly downregulated in early stages of BC progression (Gangadharan et al. 2018).

Several studies have shown that the expression of PIP is associated with low grade breast cancers, indicating that an overexpression of PIP is a marker of favorable prognosis (Clark et al. 1999; Darb-Esfahani et al. 2014). Breast cancer patients presenting with high expression of PIP showed longer relapse-free survival and overall survival (Hähnel and Hähnel 1996; Pagani et al. 1994). Increased response to standard adjuvant chemotherapy (doxorubicin + cyclophosphamide) in breast cancer patients was seen to positively correlate with an increased level of PIP expression (Jablonska et al. 2016). Low level or lack of expression of PIP was linked with unfavorable response of breast cancer to chemotherapy treatment (Jablonska et al. 2016). The expression of PIP was also revealed to be lowest in the triple negative breast cancer subtype that is characterized with poor prognosis (Jablonska et al. 2016).

PIP knockout in mice affected T-helper 1 immune function (Li et al. 2015). A robust T-helper 1 immune response is linked with positive outcomes in patients with breast cancer (DeNardo et al. 2011). A decrease in T-helper 1 cytokines like IL-12 and IFN-γ have been observed in breast cancer patients with larger tumors (Emens 2012), indicating that PIP might be involved in immune response modulation during breast cancer development. An in vivo study using the 4T1 syngeneic mouse model demonstrated that 4T1 cells expressing PIP exhibited delayed tumor onset and slower growth compared to control cells with an empty vector (Edechi et al. 2021). It was also observed that there was an increase in the percentage of NK cells and DCs, with a reduction of T-helper 2 cells in the PIP-expressing tumors compared to the EV controls (Edechi et al. 2021). This suggests a possible NK immune regulatory function of PIP. Interestingly, the expression of PIP

caused upregulation of genes linked to metastasis and promoted metastasis to the lungs, indicating that PIP influences antitumor immunity and is pro-metastatic (Edechi et al. 2021).

1.9. STUDY RATIONALE

Analyses of the immune cell subsets identified in the primary breast tumor collected from the transplanted immunocompetent BALB/c mice showed that there was a higher percentage of NK cells and dendritic cells in the 4T1-PIP group when compared to the control 4T1-EV group (Edechi et al. 2021). Interestingly, transplantation of the 4T1-PIP in the Rag2-/-xγc-/- immunodeficient (NK cell, T cell and B cell-deficient) animals abrogated the effects of PIP on primary tumor growth but maintained the promotion of lung metastasis. This suggests an interaction between PIP and immune cells, facilitating tumor reduction and delayed onset at the primary tumor site (Edechi et al. 2021). Therefore, it is imperative to delineate the role of PIP in the regulation of NK cell biology and immune response in the primary and metastatic breast tumor microenvironment.

1.10. GLOBAL HYPOTHESIS

PIP is a novel host factor that regulates NK-cell migration and effector functions (cytotoxicity and/or cytokine-production).

Sub Hypothesis

PIP-expressing 4T1 cancer cells re-derived from primary breast and metastatic lung microenvironments exert different effects on NK cells.

1.11. STUDY OBJECTIVES

To investigate the role of PIP in regulating NK cell functions, the following objectives were formulated:

1. Determine the effect of PIP and conditioned media from PIP-expressing 4T1breast cancer cell lines re-derived from primary breast and metastatic lung tissues on NK-cell migration in vitro.
2. Determine in vitro NK cell cytotoxic and cytokine effector functions towards the 4T1breast cancer cell lines re-derived from primary breast and metastatic lung tissues.

CHAPTER TWO

2.0. GENERAL MATERIALS AND METHODS

2.1. ETHICS STATEMENT

Six- to eight-week-old BALB/c and C57BL/6 mice, sourced from the in-house breeding colony at the University of Manitoba's Central Animal Care Services, were used in this study. The mice were housed in a specific pathogen-free environment, maintained under standard light-dark cycles, provided with ad libitum access to food, and housed in plastic cages with wood chip bedding. They were handled in accordance with the guidelines established by the Canadian Council on Animal Care. The study protocol was reviewed and approved by the Research Ethics Boards of the University of Manitoba, Winnipeg, Canada.

2.2. 4T1 CELL LINES

The parent 4T1 (wild type) cell line was purchased from the American Type Culture Collection (ATCC, VA, USA). The PIP-expressing and empty vector control 4T1 cell lines were kindly provided by Dr. Yvonne Myal from the Department of Pathology, University of Manitoba. The PIP-expressing (Parent 4T1 PIP) and empty vector control (Parent 4T1 EV) 4T1 cells were generated via lentiviral transduction and analyzed by flow cytometry to assess the percentage of cells expressing enhanced green fluorescent protein (GFP) (GFP sequence part of lentiviral vector). Cells were subsequently sorted for further experimentation (Edechi et al. 2021).

The transduced Parent 4T1 cells (PIP and EV) were orthotopically injected into the fourth mammary fat pad of female wild-type BALB/c mice to establish tumors. Cells were re-derived from both the primary breast tumors (4T1 PIP BR and 4T1 EV BR) and metastatic lung tissue (4T1 PIP ML and 4T1 EV ML) (Edechi et al. 2021).

All 4T1 cell lines, including parent and re-derived cells, were cultured in T25 tissue culture flasks at 37°C using high-glucose Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/L glucose, Hyclone Laboratories Inc., UT, USA) supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 50 μg/mL streptomycin, 50 U/mL penicillin (Hyclone Laboratories Inc.), and 10 μg/mL bovine insulin (Sigma-Aldrich, ON, Canada). Cells were passaged every 2-3 days once they reached 85-100% confluency. For passaging, the cell culture supernatant was collected and stored in 15 mL tubes. The T25 flasks were rinsed with 3 mL of 1x phosphate-buffered saline (PBS), and adherent 4T1 cells were detached by treating with 1 mL of 0.05% Trypsin-EDTA (Gibco, Grand Island, NY, USA) for 5 minutes. The trypsin-EDTA activity was neutralized by adding 4 mL of complete DMEM, and the cells were pipetted 3-5 times to ensure proper dispersion. The cell suspension was transferred to 15 mL tubes, centrifuged at 1200 rpm for 5 minutes, and the supernatant was discarded. Cells were resuspended in fresh medium and counted, and 1×10^4 cells were seeded into a new T25 flask containing 4-5 mL of fresh medium for continued culture.

2.2.1. Cell Counting by Trypan Blue Exclusion

Ten microliters (10ul) of resuspended cells in fresh media was mixed with trypan blue (1:1) and counted before each cell passage using a TC-10 counter (Bio-Rad, ON, Canada).

2.3. 4T1 CELL CONDITIONED MEDIA PREPARATION

The cell culture supernatant from all 4T1 cell lines, including both parent and re-derived cells, was harvested after 2–3 days of growth in T25 tissue culture flasks at 37°C. Supernatant collection occurred when cells reached 90–100% confluency. To remove suspended cells, the supernatant was centrifuged at 1500 rpm for 10 minutes. The cell-free conditioned medium was then carefully

transferred into a fresh 15 mL tube using a pipette, aliquoted, and stored at -20°C for subsequent use in various assays.

2.4. WESTERN BLOT ANALYSIS

2.4.1. Preparation of Cell Lysates

Cells were cultured to confluency in T-25 flasks, then rinsed using 1x phosphate-buffered saline (PBS). The cells were lysed with 100 µL of RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, NY, USA) containing protease inhibitors (Roche Diagnostics) and 1x Halt phosphatase inhibitor cocktail (ThermoFisher Scientific, NY, USA) and transferred to 1.5 mL tubes and heated for 5mins at 90ºC. The lysates were sonicated for 15 seconds and centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble material. The supernatants were collected into fresh tubes, and protein concentrations were measured using the bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Cat Number: 23225) according to the manufacturer's instructions. The cell lysates were stored at -20°C for subsequent use in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.2. SDS-PAGE

A total of 20 µg of cell lysate was mixed in a 1:1 ratio (v/v) with 2X Laemmli Sample Buffer (containing 65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% [w/v] glycerol, 0.01% bromophenol blue, and 0.05% [v/v] β-mercaptoethanol) and heated at 70°C for 10 minutes. Proteins from the sample and molecular weight markers (Bio-Rad, ON, Canada) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% acrylamide/bis-acrylamide stacking gel and a 10% resolving gel. Electrophoresis was performed at 150V for 60 minutes.

Following electrophoresis, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada) using the Pierce™ 1-Step Transfer Buffer and the Pierce Power Blot Cassette (Thermo Fisher Scientific, NY, USA) at 25V for 7 minutes. The membrane was then blocked using the EveryBlot Blocking Buffer (Cat. No.: 12010020, Bio-Rad Laboratories, Mississauga, ON, Canada) for 1 hour.

Primary antibody (Cat number: sc-377171, Santa Cruz, USA) (PIP: 1:2000) were diluted in the blocking solution and incubated with the membrane overnight at 4°C with gentle shaking. The following day, the membrane was washed three times with TBS-T (Tween concentration: 0.5%) for 10 minutes each and then incubated with secondary antibodies (m-IgGk BP-HRP: 1:500, Cat number: sc-516102, Santa Cruz, USA) for 1 hour at room temperature with shaking. After secondary antibody incubation, the membrane was washed again three times with TBS-T for 10 minutes each and developed using an enhanced chemiluminescence (ECL) kit (BioRad Clarity Max) (Cat number: 1705060S, BioRad USA). The chemiluminescent signal was detected using a C-Digit blot scanner (Licor, NE, USA).

2.5. IMMUNE CELL ISOLATION AND CULTURE

2.5.1. Natural Killer isolation

Primary Natural Killer (NK) cells (CD3- DX5+) were isolated from the spleens of mice. Splenocytes were obtained by homogenizing the spleen, followed by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Sweden) according to the manufacturer's protocol. NK cell enrichment was performed using the EasySep™ Mouse NK Cell Negative Selection Kit (StemCell Technologies, Vancouver, BC). The NK cells were cultured at 37°C with 5% CO_2 in mouse culture medium supplemented with 10% fetal bovine serum (FBS; HyClone),

1% penicillin-streptomycin-glutamine (PSG; Invitrogen), 1.6 mmol/L 2-mercaptoethanol (2-ME), and interleukin-2 (IL-2) at a concentration of 1000 U/mL. NK cell purity was assessed prior to experimentation.

2.5.2. Whole splenocyte isolation

To obtain whole splenocytes, the mouse spleen was homogenized, incubated in ACK buffer for 2mins to lyse red blood cells and then centrifuged at 300g for 10mins. The whole splenocyte cells were cultured and incubated for 4 days at 37°C with 5% CO_2 in mouse culture medium supplemented with 10% fetal bovine serum (FBS; HyClone), 1% penicillin-streptomycin-glutamine (PSG; Invitrogen), 1.6 mmol/L 2-mercaptoethanol (2-ME), and interleukin-2 (IL-2) at a concentration of 1000 U/mL.

2.5.3. Primary mouse bone marrow derived dendritic cells

Bone marrow precursor cells were stimulated to differentiate into mature dendritic cells (DCs) as described by Zhang et al. (2009). Precursor cells were harvested from the femur and tibia, followed by a 2-minute incubation with ACK buffer to lyse red blood cells. Bone marrow cells (0.5-1 × 10^6 cells per well) were seeded in 24-well plates containing RPMI 1640 medium (Hyclone) supplemented with 1% penicillin-streptomycin-glutamine (PSG), 10% fetal goat serum (FGS), 1.6 mmol/L 2-mercaptoethanol (2-ME), and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech). On day 3, one-third of the culture medium was removed to eliminate non-adherent cells, and fresh GM-CSF-containing medium was added. On day 5, the cultures were replenished with GM-CSF-containing medium, maintaining a total volume of 1 mL per well. On day 8, lipopolysaccharide (LPS) was added at a concentration of 1 μg/μL for 24 hours to induce maturation of the DCs. Mature DCs were identified by the expression of surface markers CD40,

CD80, and CD86. Both untreated and LPS DCs, along with their corresponding conditioned media, were utilized in various experimental settings. All mature and immature cells were phenotyped before use in the experiments.

2.6. TRANSWELL BASED NK CELL MIGRATION

NK cell chemotaxis against mouse medium and various conditioned media (Parent 4T1, Parent 4T1-PIP, Parent 4T1-EV, 4T1-PIP-BR, 4T1-EV-BR, 4T1-PIP-ML, 4T1-EV-ML, and LPS-matured dendritic cell supernatant) was conducted using a transwell system. A total of 0.2×10^6 IL-2-activated murine NK cells (in 100 μL) were loaded into the upper chamber of a 5 μm pore transwell insert, while 600 μL of plain medium or conditioned medium was placed in the lower chamber, and the setup incubated at 37°C. In certain experiments, mouse recombinant PIP (Cloud-Clone Corp, USA) at concentrations of (100 ng, 300 ng, 600 ng, or 1 μg)/600 μL of plain medium or bovine serum albumin (BSA; 1 μg/600 μL of plain medium) was placed in the lower chamber. After a 90-minute incubation, cells that had migrated to the lower chamber were collected into polypropylene tubes, centrifuged at 300g for 10 minutes, and then counted. The number of migrated cells was quantified as an absolute count of the input cells, following the method described by Gustafsson et al. (2008).

2.7. CHEMOKINE AND CYTOKINE PROFILE ANALYSIS PROCEDURE

Conditioned media from all the 4T1 cell lines (parent and re-derived) were aliquoted into 15ml tubes and properly labelled. The samples were sent to Eve Technologies (Calgary, AB, Canada) for a Mouse Cytokine/Chemokine 32-Plex Discovery Assay® Array (MD32).

2.8. NK CELL FUNCTIONAL ASSAYS (CYTOTOXICITY AND CYTOKINE PRODUCTION)

To evaluate NK cell cytotoxicity and cytokine production in response to target cells, PIP and EV-expressing 4T1 cell lines (both parental and re-derived), the cell surface expression of lysosomal-associated membrane protein-1 (CD107a) and intracellular production of cytokine (interferon gamma) was assessed. CD107a is significantly upregulated on the surface of NK cells primed to kill a target and they produce proinflammatory cytokines like interferon gamma upon stimulation. IL-2-stimulated NK cells, harvested on day 4, were collected and counted. Adherent 4T1 cells were detached and counted as per previously described methods. NK cells and 4T1 cells were then seeded in a 5 mL flow tube at a 1:1 ratio (1×10^4: 1×10^4), and 3 mL of fresh medium was added. NK cells were also treated with 1ug of PIP or BSA in some experimental groupings.

The tubes were centrifuged at 1200 rpm for 10 minutes. After centrifugation, the supernatant was removed, and the cell pellet was resuspended in 200 µL of fresh medium. A cell stimulation cocktail containing phorbol 12-myristate 13-acetate (PMA) and ionomycin, along with protein transport inhibitors (Thermo Fisher Scientific, NY, USA), was added to the positive control sample. PMA and ionomycin are known to activate transcription factors involved in intracellular signaling and cytokine production in various immune cell types, including NK cells. One microliter (1 µL) of CD107a antibody was added to all test samples, and the tubes were gently vortexed and incubated for 1 hour at 37°C. Following this incubation, 1 µL of monensin and brefeldin A (BFA) (Thermo Fisher Scientific, NY, USA), were added to each sample to inhibit protein transport, and the samples were incubated for an additional 5 hours at 37°C.

At the end of the incubation period, the cells were washed with flow buffer and blocked by adding 100 µL of Fc blocking reagent (2.4G2 Hybridoma supernatant), followed by incubation on ice for

10 minutes. The cells were then washed again and stained with antibodies for surface NK/T cell markers (1 μL each of DX5 and CD3), incubating on ice for 30 minutes. After staining, the cells were washed with 1 mL of flow buffer. For intracellular staining for interferon gamma, the cells were fixed with 0.5 ml 2% paraformaldehyde (Sigma). Cell membranes were permeabilized using saponin dissolved in FACS buffer and samples incubated on ice for 15 minutes. The Cells were later washed with 2ml of saponin buffer and stained intracellularly for IFN-γ using Fluorochrome-conjugated antibody (eBioscience; each at 0.5 μL per tube). The cells were resuspended in 500 μL of flow buffer to stop the action of saponin and for acquisition using flow cytometry. Flow cytometric analysis was performed to assess the expression of CD107a, IFN-γ and other surface markers.

Table 3: List of fluorochrome conjugated antibodies used for flow cytometry

S/N	Antibody	Clone	Fluorochrome
1	CD49b	DX5	APC
2	CD3	17A2	Pacific Blue, APC/Cy7
3	CD107a (Lamp-1)	1D4B	FITC
4	IFN-γ	XMG1.2	PercCp/cy5.5
5	NK 1.1	PK136	Pacific Blue
6	CXCR3	CXCR3-173	APC
7	Ly49 C/I/F/H	14B11	FITC
8	RAE-1γ	CX1	PE
9	H-2D^{d}	34-2-12	PE
10	H-2K^{b}	AF6-88.5	PE
11	CD45R	RA3-6B2	PE
12	CD80	16-10A1	APC
13	CD11c	N418	FITC
14	CD86	GLI	APC
15	CD40	3/23	APC
16	Phospho IκBα	RILYB3R	PE
17	Phospho p38 MAPK	4N1T4KK	PE
18	Phospho ERK1/2	6B8B69	FITC
19	T-bet	eB104B10	PE/Cy7
20	Phospho NF-kappa B-p65	93H1	PE
21	Phospho STAT5	A17016B.Rec	APC

2.9. PHOSPHO-FLOW FOR INTRACELLULAR AND INTRANUCLEAR MOLECULES

To assess the phosphorylation of signaling molecules in pathways involved in NK cell migration in response to PIP stimulation, IL-2-stimulated NK cells (harvested on day 4) were collected, placed in a flow cytometry tube, and centrifuged. The cells were then resuspended in 100 µL of fresh medium containing IL-2 at a concentration of 1000 U/mL. NK cells were subjected to various stimuli and incubated at 37°C, with the treatments terminated at specific time points (1 min, 2 min, 5 min, 15 min, and 30 min) by adding 1 mL of prewarmed lyse/fix buffer. The samples were vortexed and incubated at 37°C for 12 minutes.

Following incubation, the cells were centrifuged at 600 g for 8 minutes, and the supernatant was discarded, leaving approximately 50 µL of residual volume. The tubes were vortexed to disrupt the cell pellets. The cells were then washed with 1 mL of stain buffer, centrifuged again at 600 g for 8 minutes, and the supernatant removed as before and then vortexed. To permeabilize the cells, 1 mL of perm/wash buffer 1 was added, and the tubes were gently mixed and incubated for 30 minutes at room temperature.

After incubation, the cells were centrifuged and vortexed as previously described. The cells were resuspended in 100 µL of perm/wash buffer 1 and stained with the appropriate antibodies. The mixture was incubated at room temperature for 60 minutes and protected from light by covering the tubes with aluminum foil. Subsequently, the cells were washed with 3 mL of perm/wash buffer 1, centrifuged at 600 g for 8 minutes, and the supernatant was removed, leaving approximately 50 µL of residual volume. The pellets were vortexed, resuspended in 500 µL of perm/wash buffer 1, and analyzed using flow cytometry.

Table 4: List of reagents used for Phospho-flow

Full Name	Short Name	Manufacturer	Catalog Number
BD™ Phosflow Lyse/Fix Buffer I	Lyse/Fix Buffer I	BD Biosciences	558049
BD™ Phosflow Perm/Wash Buffer I, 10x	Perm/Wash Buffer I	BD Biosciences	557885
BD Pharmingen™ Stain Buffer	Stain Buffer	BD Biosciences	554656

2.10. LIVE-CELL IMAGING IN 3D COLLAGEN CHAMBERS AND ANALYSIS

Live-cell imaging in 3D collagen chambers was conducted following the protocol outlined by Koh et al. (2020). Bovine collagen (PureCol) was used to prepare each chamber with a final concentration of 1.7 mg/mL. To assess NK cell migration in conditioned media from PIP or EV treatments, 1–2 million NK cells were labeled with either CellTracker Blue (CMAC; 30 µM) or CellTracker Red (CMTPX; 7.5 µM), washed, and embedded within the collagen matrix alongside the conditioned media. The chambers were allowed to solidify for 45 minutes at 37°C with 5% CO_2, after which they were placed on a custom-made heated platform connected to a temperature control system (Werner Instruments). A thermocouple continuously monitored and maintained the chamber temperature at 37°C.

For imaging, a multiphoton microscope equipped with two Ti:sapphire lasers (Coherent) was set between 780 and 920 nm to optimize excitation of the fluorescent probes. Four-dimensional recordings of NK cell migration were generated by acquiring stacks of 13 or 26 optical sections (512 x 512 pixels) with 4 µm z-spacing, at intervals of 15 or 30 seconds, capturing imaging volumes of 48 µm or 96 µm in depth. Emitted light was collected through dichroic filters (460/50

nm, 525/70 nm, and 595/50 nm) using non-descanned detectors. All images were obtained using a 20X 1.0 N.A. objective lens (Olympus XLUMPLFLN; 2.0 mm working distance).

2.10.1. Image analysis

Image analysis was conducted according to the methods described by Koh et al. (2020). Time-lapse micrographs were processed using Imaris 8.3 (Bitplane) to create maximum intensity projections (MIPs), which were subsequently exported as QuickTime movies. Automated 3D tracking of dendritic cell (DC) centroids was performed to evaluate motility, with cell tracking parameters such as arrest coefficient and mean displacement analyzed using a custom Matlab (Mathworks) script.

To assess the spatial distribution of NK cells, line profile analyses were conducted to generate fluorescent intensity profiles along the z-axis, where 0 corresponds to the uropodia and 1 to the leading edge. Cell surface area and perimeter were quantified using ImageJ, applying the wand (tracing) tool after color thresholding. Circularity was also measured in ImageJ, with a value of 0 representing a straight line and 1 indicating a perfect circle, as outlined by Usmani et al. (2019). Additionally, contact duration and NK cell migration velocities during cell-cell contacts were analyzed using ImageJ.

2.11. STATISTICS

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.), a commercial software for scientific graphing and statistical analysis. Data are presented as mean ± standard error of the mean (SEM). For comparisons between two groups, a two-tailed Student's t-test was employed. One-way analysis of variance (ANOVA) was used for comparisons involving more than two groups in all experiments. A p-value of <0.05 was considered statistically significant.

CHAPTER THREE

3.0. RESULTS

3.1. SOLUBLE PIP IS EXPRESSED IN THE CELL LYSATE AND CONDITIONED MEDIA FROM PIP-EXPRESSING 4T1 CELL LINES

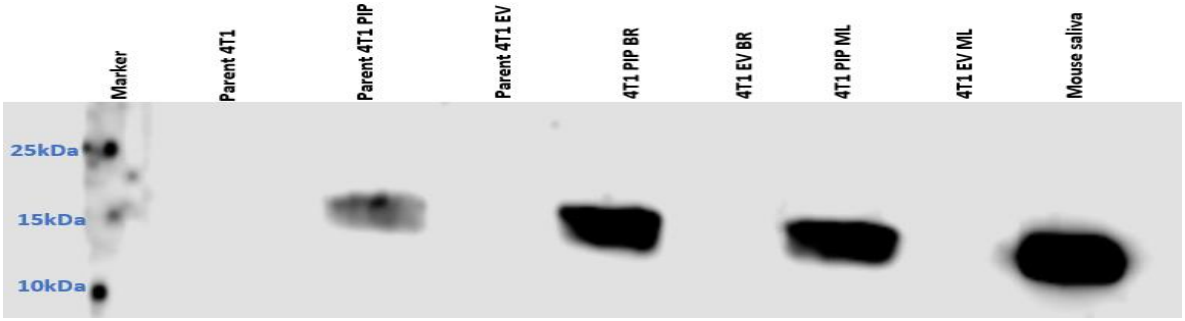
Murine triple-negative 4T1 breast cancer cells do not express endogenous mouse PIP. In our previous work, mouse PIP cDNA was introduced into 4T1 cells using a lentiviral vector to generate PIP-expressing cells (Parent 4T1-PIP). An empty vector (Parent 4T1-EV) served as a negative control. Western blot analysis was performed to confirm PIP protein expression in both parental and re-derived 4T1 cells from primary breast tumors (BR) and metastatic lungs (ML).

Cell lysates and conditioned media (CM) were prepared from 4T1, Parent 4T1-PIP, Parent 4T1-EV, 4T1-PIP BR, 4T1-EV BR, 4T1-PIP ML, and 4T1-EV ML cells as described in the "Materials and Methods." Protein concentrations were measured using the bicinchoninic acid (BCA) assay, and 50 µg of each lysate were loaded per lane for SDS-PAGE. I detected PIP protein expression using an anti-mouse PIP antibody, with mouse saliva serving as a positive control.

As expected, PIP protein was absent in the lysates of Parent 4T1, Parent 4T1-EV, 4T1-EV BR, and 4T1-EV ML cells (Fig. 1A). A 15-kDa band corresponding to the expected molecular weight of mouse PIP was detected in the lysates of PIP-expressing cells (Parent 4T1-PIP, 4T1-PIP BR, and 4T1-PIP ML) (Fig. 1A).

Since PIP is a secreted protein, its expression was also assessed in CM from these 4T1 cell lines. Consistent with the lysate data, mouse PIP proteins were detected in the CM from PIP-expressing 4T1 cells but not in CM from Parent 4T1 or EV-expressing cells (4T1-EV BR and 4T1-EV ML) (Fig. 1B).

A



B

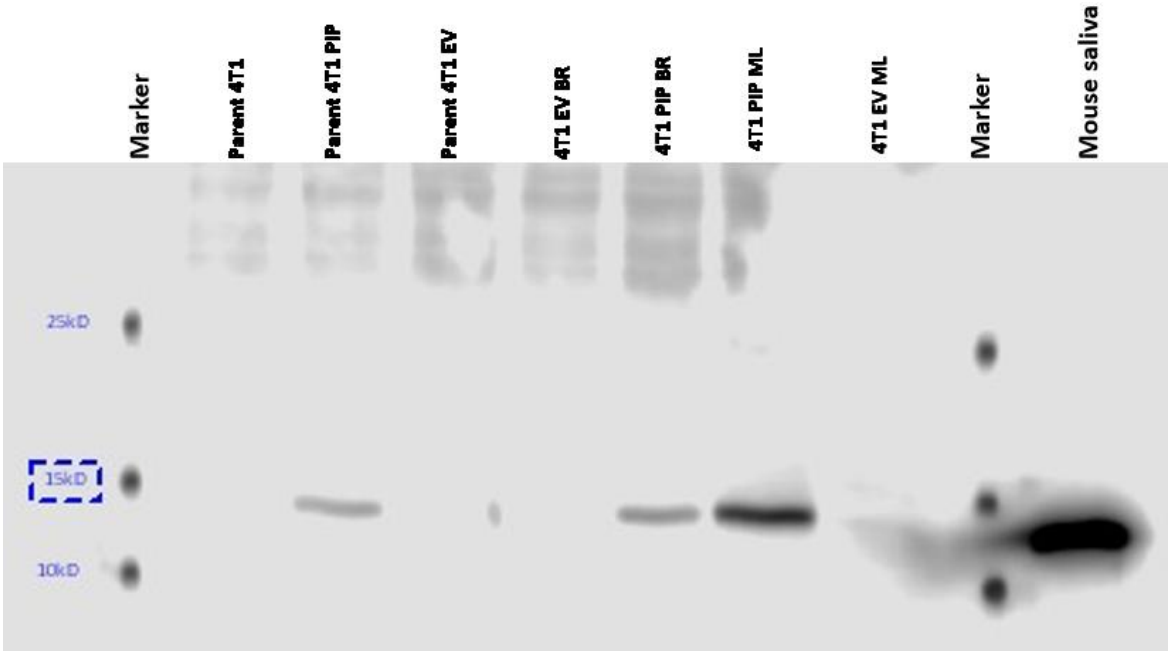


Fig. 1. Confirmation of PIP expression in the cell lysate and conditioned media from PIP-expressing 4T1 cell lines. (A) Showing blot for cell lysate. (B) Showing blot for conditioned media. The PIP band at 15kDa was identified using the mouse monoclonal PIP antibody in a Western blot analysis as described in "Materials and Methods". Mouse saliva was used as a positive control.

3.2. CONDITIONED MEDIA FROM PARENT 4T1-PIP CELLS PROMOTED NK-CELL MIGRATION IN VITRO

Previous in vivo studies in our laboratory demonstrated an increased percentage of NK cell infiltration in breast tumors of mice injected with PIP-expressing 4T1 cells compared to mice injected with empty vector controls. These mice also exhibited delayed tumor onset and reduced tumor growth. To determine whether conditioned media from PIP-expressing parental 4T1 cells could promote NK cell migration in vitro, I carried out transwell migration experiments using conditioned media from parental 4T1 cells.

Our lab has previously established that supernatants from LPS-matured dendritic cells (DC-LPS sup) induce NK cell migration, and this served as the positive control. DMEM media was used as the negative control. Conditioned media from wild-type 4T1 cells (Parent 4T1), PIP-expressing parental 4T1 cells (Parent 4T1-PIP), and empty vector controls (Parent 4T1 EV) were added to the basal chambers of the transwell plates along with the DC-LPS sup and DMEM media controls. IL-2-activated NK cells, cultured with 1000 U/mL IL-2 for 4 days, were seeded into the apical chambers and incubated for 90 minutes. Migrated cells in the basal chambers were then quantified.

The results indicated that DC-LPS sup significantly enhanced NK cell migration compared to DMEM media (Fig. 2). Furthermore, conditioned media from Parent 4T1-PIP cells significantly promoted NK cell migration compared to conditioned media from Parent 4T1 EV and wild-type Parent 4T1 cells (Fig. 2).

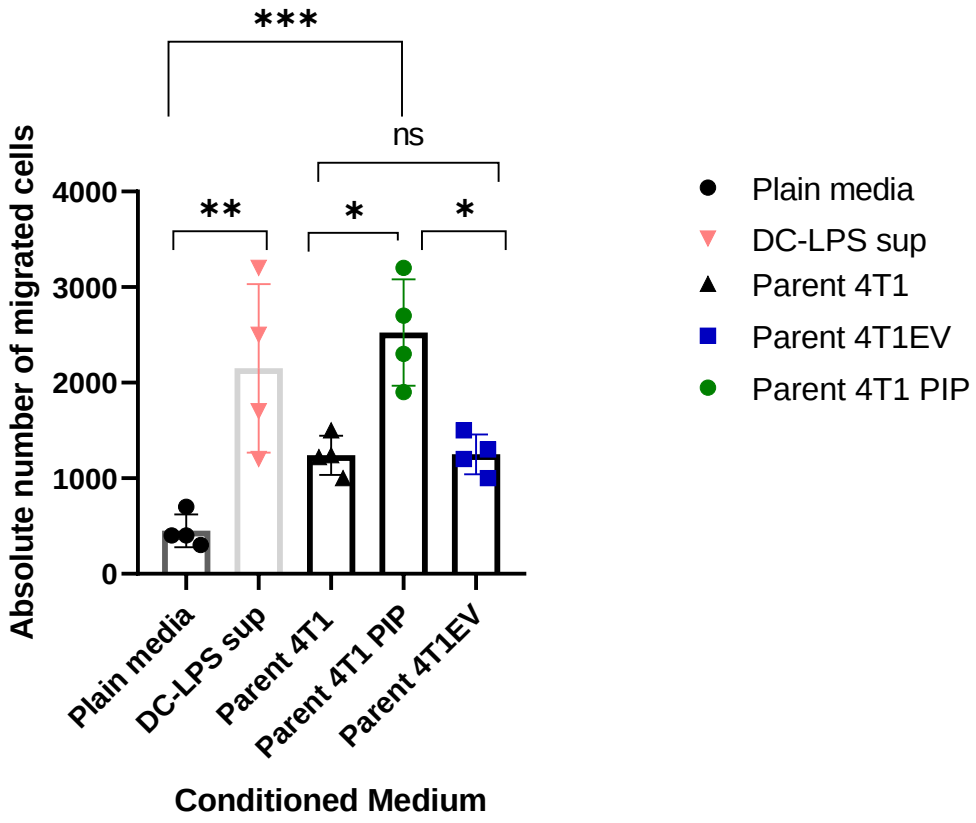


Fig. 2. Conditioned media from PIP expressing Parent 4T1 promoted NK cell migration. Cell culture supernatant from Parent 4T1, Parent 4T1-PIP and Parent 4T1-EV were placed in the basal wells of a transwell plate, while 5x104 NK cells well seeded in the apical wells and incubated, DC- LPS sup and media were used as positive and negative control respectively. After 1hour and 30 mins, the migrated NK cells in the basal cells were counted. *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, ns = not significant, ordinary one-way Anova.

3.3. CONDITIONED MEDIA FROM 4T1-PIP TUMOR CELLS RE-DERIVED FROM PRIMARY BREAST TISSUE OR METASTATIC LUNG TISSUE PROMOTED NK-CELL MIGRATION

As previously noted, increased NK cell infiltration was observed in PIP-expressing primary breast tumors compared to empty vector controls by Edechi et al. However, immune cell infiltration in metastatic lungs was not evaluated. To investigate whether conditioned media from 4T1-PIP tumor cells re-derived from primary breast tumors and metastatic lungs could drive NK cell migration in vitro, a transwell migration assay was performed as described previously.

Conditioned media from 4T1 cells re-derived from primary breast tumors (4T1 PIP-BR and 4T1 EV-BR) and metastatic lungs (4T1 PIP-ML and 4T1 EV-ML) were placed in the basal chambers of transwell plates. Purified IL-2-activated NK cells were seeded into the apical chambers and incubated for 90 minutes, after which migrated cells were counted.

Conditioned media from PIP-expressing 4T1 cells re-derived from primary breast tumors (4T1 PIP-BR) significantly enhanced NK cell migration compared to the empty vector control (4T1 EV-BR) (Fig. 3a). Similarly, conditioned media from PIP-expressing 4T1 cells re-derived from metastatic lungs (4T1 PIP-ML) significantly promoted NK cell migration compared to the empty vector control (4T1 EV-ML) (Fig. 3b).

A **B**

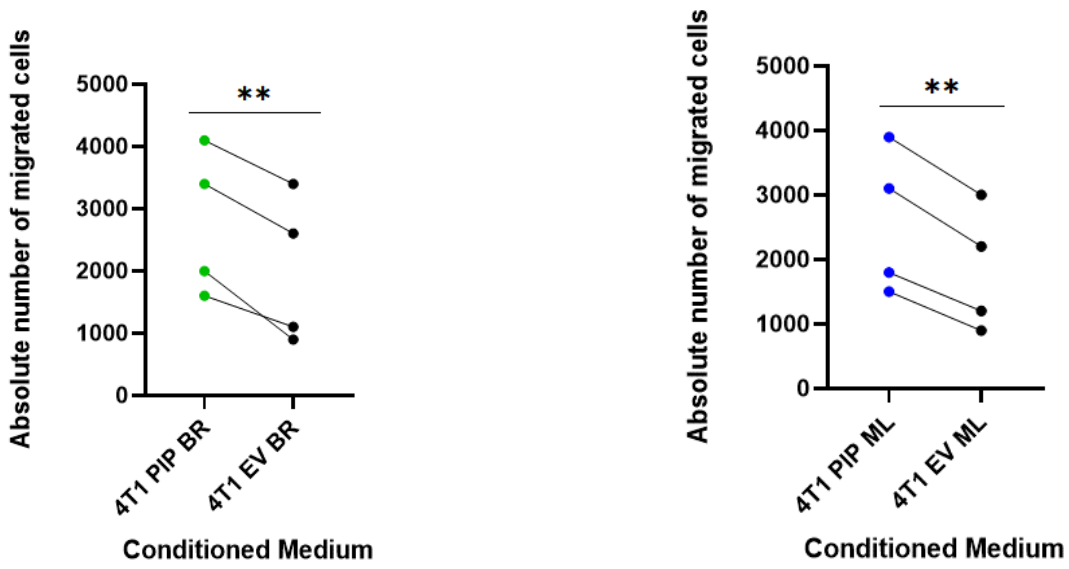


Fig. 3. Conditioned media from PIP expressing rederived 4T1 cells promoted more NK cell migration. Cell culture supernatant from rederived 4T1 cells were placed at the basal wells of a transwell plate, while $5x10^4$ NK cells well seeded at the apical wells and incubated. (A) Showing number of migrated NK cells to the conditioned media from 4T1 cells rederived from the breast (4T1 PIP-BR and 4T1 EV-BR). (B) Showing number of migrated NK cells to the conditioned media from 4T1 cells rederived from the metastatic lung (4T1 PIP-ML and 4T1 EV-ML). **$p \leq 0.01$, paired t-test.

3.4. NK CELL MIGRATION WAS NOT AFFECTED BY THE SITE OF REDERIVATION OF 4T1 CELLS (PRIMARY BREAST OR METASTATIC LUNG)

To investigate the impact of the tumor microenvironment (TME) on the migratory response of NK cells, the number of NK cells migrating to conditioned media from PIP-expressing 4T1 cells re-derived from primary breast tumors (4T1 PIP-BR) was compared to those from metastatic lungs (4T1 PIP-ML). Similarly, NK cell migration was assessed using conditioned media from empty vector (EV) 4T1 cells re-derived from primary breast tumors (4T1 EV-BR) and metastatic lungs (4T1 EV-ML). Significant difference in NK cell migration was observed between the conditioned media from 4T1 PIP-BR and 4T1 PIP-ML cells (Fig. 4a). Conversely, conditioned media from 4T1 EV-BR and 4T1 EV-ML cells showed no significant differences in promoting NK cell migration (Fig. 4b).

A **B**

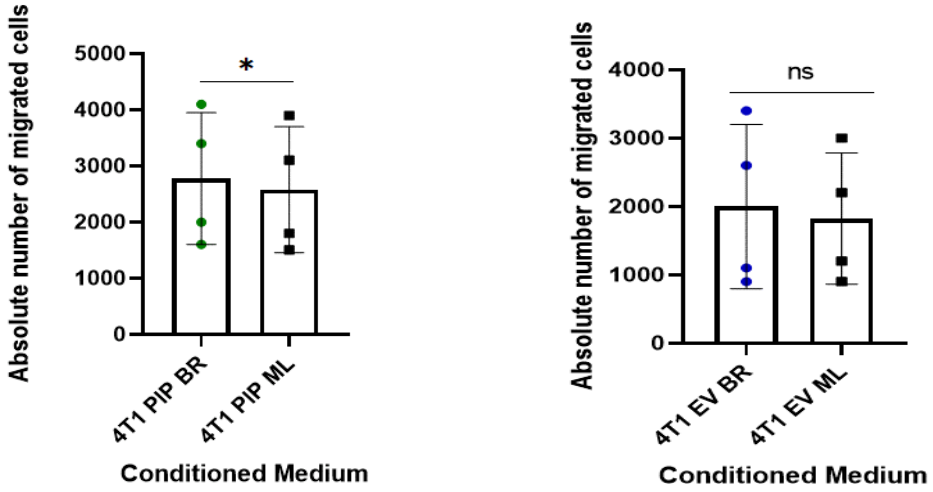


Fig. 4. TME/site of rederivation did not affect NK cell migration. (A) Showing number of migrated NK cells to the conditioned media from PIP expressing 4T1 cells rederived from the breast and lungs (4T1 PIP-BR and 4T1 PIP-ML). (B) Showing number of migrated NK cells to the conditioned media from empty vector control 4T1 cells rederived from the breast and lungs (4T1 EV-BR and 4T1 EV-ML). *$p \leq 0.05$; ns = not significant, paired t-test.

3.5. PIP IN CONDITIONED MEDIA FROM TRANSDUCED 293T PROMOTE NK CELL MIGRATION

Conditioned media from PIP-expressing parental 4T1 cells (Parent 4T1 PIP) and PIP-expressing 4T1 cells re-derived from breast tumors (4T1 PIP BR) and metastatic lung sites (4T1 PIP ML) were previously shown to promote NK cell migration (Fig. 2; Fig. 3a, b). To assess whether this migratory response is due to factors specific to 4T1 cells in the culture supernatant and to confirm the role of PIP in NK cell migration—including potential cross-reactivity between mouse NK cells and human PIP—293T epithelial-like cells derived from human embryonic kidney (HEK) were transduced to express human PIP (hPIP). Conditioned media from these transduced 293T cells and untransduced controls were used to assess mouse NK cell migration using a transwell migration assay.

Supernatants from LPS-stimulated mature dendritic cells (DC-LPS sup) and plain media were included as positive and negative controls, respectively. As expected, DC-LPS sup promoted NK cell migration. Similarly, conditioned media from hPIP-expressing 293T cells significantly enhanced NK cell migration compared to media from untransduced 293T cells (Fig 5).

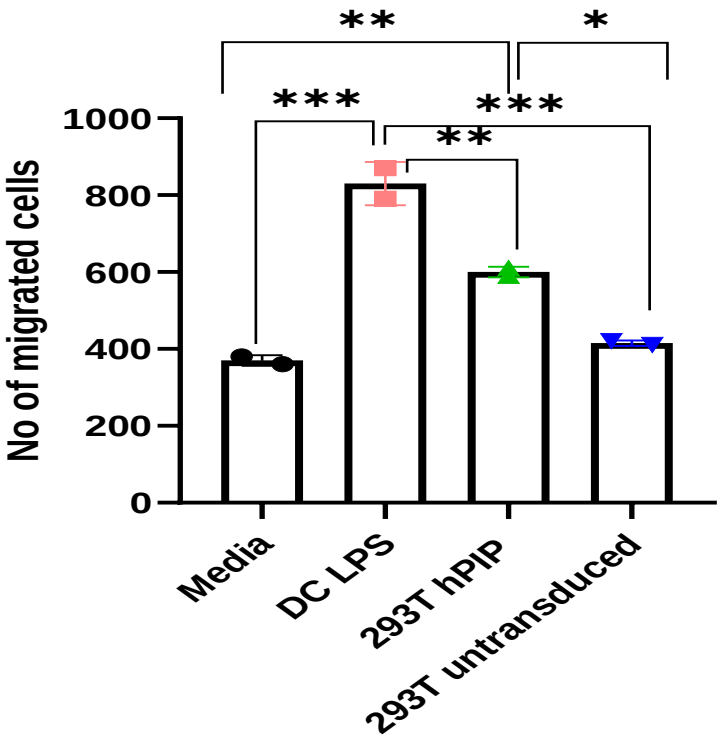


Fig. 5. Conditioned media from PIP expressing 293T transduced cells promoted NK migration. 293T cells well transduced to express human-PIP (hPIP) and cell culture supernatant from the cells was used in a transwell migration as described in the "Materials and Methods" using mouse NK cells. Cell culture supernatant from PIP expressing 293T cell promoted more mouse NK cell migration compared to untransduced 293T and plain media. n –value =2, *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001, ordinary one-way Anova

3.6. PIP-EXPRESSING 4T1 CELL CONDITIONED MEDIA INDUCED NK CELL MIGRATION NOT DRIVEN BY CHEMOKINES

The findings of this study demonstrated that conditioned media from PIP-expressing 4T1 cells (Parent 4T1 PIP, 4T1 PIP BR, and 4T1 PIP ML) significantly promoted NK cell migration compared to conditioned media from empty vector controls and wild-type 4T1 cells (Parent 4T1). Similarly, conditioned media from PIP-expressing 293T cells, a distinct cell line, also induced NK cell migration.

Given that conditioned media contains a complex mixture of factors, including chemokines and cytokines that could drive NK cell migration, it was necessary to identify and quantify these components. For this purpose, samples were subjected to multiplex cytokine analysis, and the complete panel of results is provided in the appendix.

The analysis revealed that certain chemokines and cytokines were highly expressed in the conditioned media of specific PIP-expressing 4T1 cells. However, no single chemokine or cytokine was consistently or significantly upregulated across all PIP-expressing 4T1 cell lines compared to their respective controls.

One notable finding was the significant upregulation of the chemokine IP10 (CXCL10) in the conditioned media from PIP-expressing 4T1 cells re-derived from primary breast tumors (4T1 PIP-BR) compared to empty vector controls (4T1 EV-BR) (Fig. 6b). In contrast, no significant differences in IP10 levels were observed among parental 4T1 cells (Fig. 6a) or cells re-derived from metastatic lung tissue (Fig. 6c).

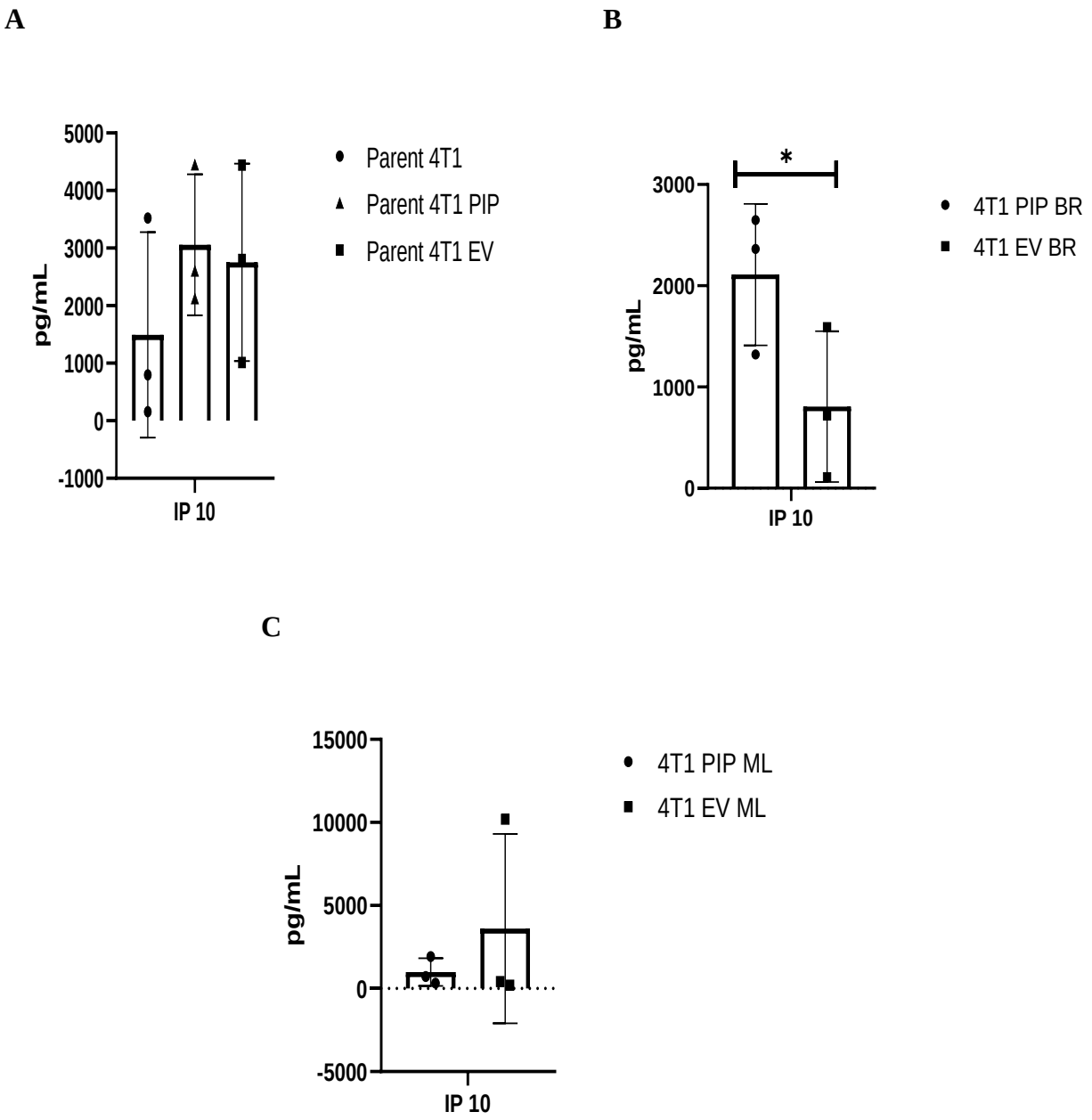


Fig. 6. Levels of IP10 chemokine present in the conditioned media of the 4T1 cell lines. (A) Analysis of the cell culture supernatant (conditioned media) from Parent 4T1, Parent 4T1-PIP and Parent 4T1-EV showed no significant increase in the abundance of IP10. (B) IP10 level was significantly higher in 4T1 PIP-BR than 4T1 EV-BR. (C) There is no significant difference in the level of IP10 between 4T1 PIP-ML and 4T1 EV-ML. n –value =3, *$p \leq 0.05$; unpaired t-test and ordinary one-way Anova.

The chemokine MCP-1 was highly expressed in the conditioned media of PIP-expressing 4T1 cells re-derived from primary breast tumors (4T1 PIP-BR) compared to empty vector controls (4T1 EV-BR); however, this difference was not statistically significant (Fig. 7b). In conditioned media from 4T1 cells re-derived from metastatic lung tissue, MCP-1 expression was higher in empty vector controls (4T1 EV-ML) than in PIP-expressing cells (4T1 PIP-ML) (Fig. 7c).

In parental 4T1 cells, MCP-1 expression was significantly higher in the conditioned media from empty vector controls (Parent 4T1 EV) compared to wild-type 4T1 cells (Parent 4T1 WT). However, no significant difference in MCP-1 expression was observed between PIP-expressing parental 4T1 cells (Parent 4T1 PIP) and their empty vector controls (Parent 4T1 EV) (Fig. 7a).

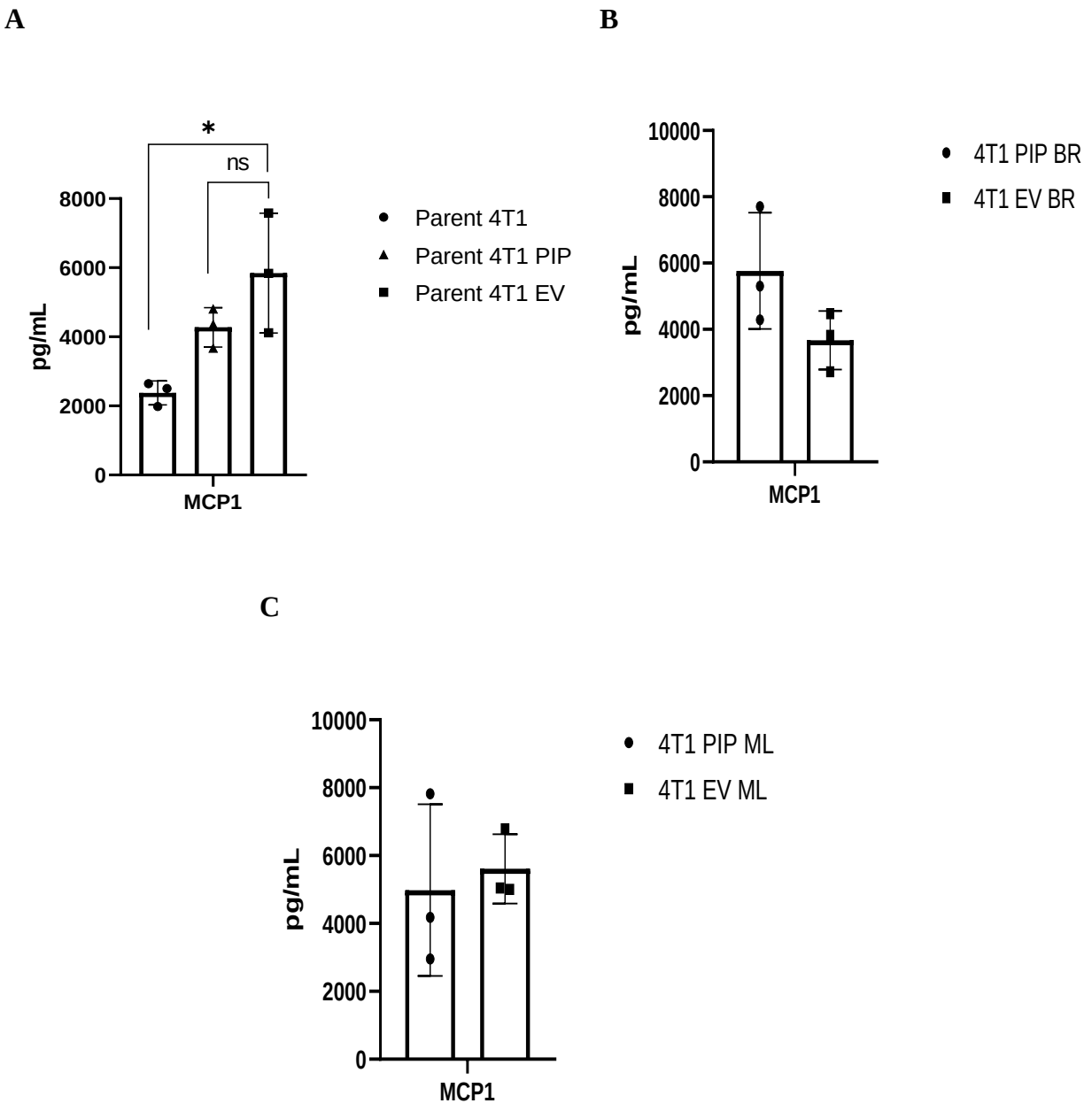


Fig. 7. Levels of MCP-1 chemokine present in the conditioned media of the 4T1 cell lines. (A) Analysis of the cell culture supernatant from Parent 4T1, Parent 4T1-PIP and Parent 4T1-EV showed that Parent 4T1-EV had a significant increase in MCP-1 levels compared to Parent 4T1 and Parent 4T1-PIP. (B) MCP-1 level was not statistically significantly higher in 4T1 PIP-BR than 4T1 EV-BR. (C) There is no significant difference in the level of MCP-1 between the conditioned media from 4T1 PIP-ML and 4T1 EV-ML. n –value =3, *$p \leq 0.05$; ns = not significant, ordinary one-way Anova and unpaired t-test.

3.7. PIP-INDUCED NK CELL MIGRATION OCCURS INDEPENDENTLY OF THE CONVENTIONAL CHEMOTACTIC GRADIENT MECHANISM

This study demonstrated that conditioned media from PIP-expressing 4T1 cells induces NK cell migration. However, the observed migration could not be attributed to the activity of specific chemokines or cytokines, as none of the detected chemokines were significantly upregulated across all PIP-expressing 4T1 cell lines. To investigate whether PIP induces NK cell migration through the conventional chemokine gradient mechanism, a transwell migration assay was performed using recombinant mouse PIP, BSA, and supernatants from LPS-matured dendritic cells (LPS-DC sup).

IL-2-activated NK cells were resuspended in media containing IL-2, media supplemented with recombinant PIP (1 µg/mL), media containing BSA (1 µg/mL), or LPS-DC sup. These cells were seeded in the apical inserts of the transwell plates. The basal chambers were filled with 600 µL of DMEM (negative control), LPS-DC sup (positive control), or media supplemented with PIP (1 µg/mL) or BSA (1 µg/mL). The plates were incubated for 90 minutes, after which migrated cells in the basal chambers were quantified.

The results showed that the chemotactic effect of LPS-DC sup was significantly reduced when used to seed NK cells in the apical well, indicating a disruption of the chemotactic gradient. LPS-DC sup in the basal well significantly promoted NK cell migration compared to its placement in the apical well (Fig. 8). Importantly, PIP promoted NK cell migration regardless of its placement in the apical or basal wells, demonstrating higher migration compared to BSA or LPS-DC sup-resuspended NK cells (Fig. 8).

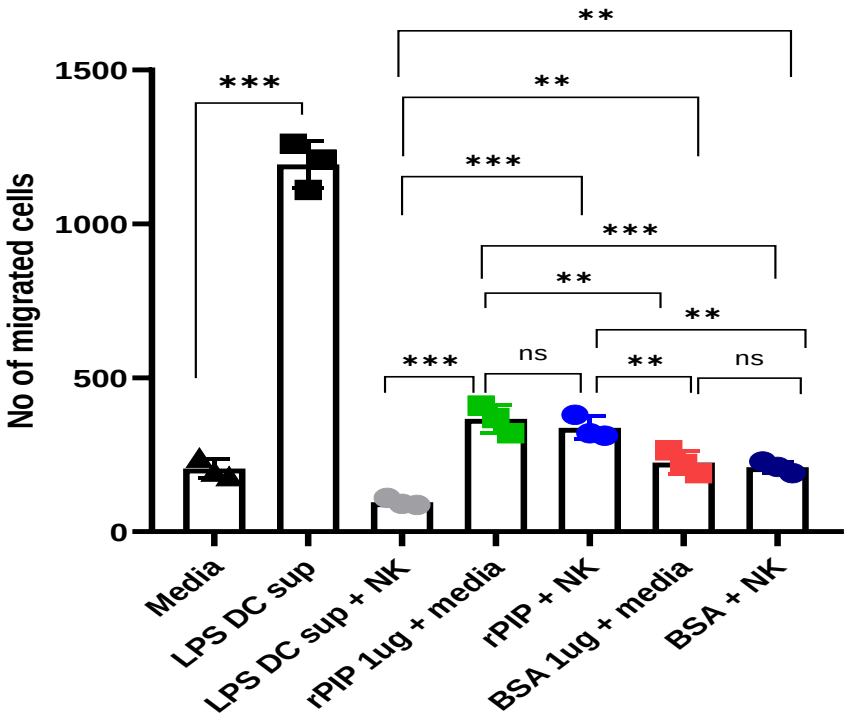


Fig. 8. PIP induced NK cell migration is independent of chemotactic gradient. PIP (1ug/mL), BSA (1ug/mL) and LPS DC sup were used to seed NK cells in a transwell assay to assess NK cell migration. PIP induced NK cell migration different from canonical chemokine pattern. **$p \leq 0.01$, ***$p \leq 0.001$, ns = not significant, ordinary one-way Anova.

3.8. MOUSE RECOMBINANT PIP PROMOTED NK-CELL MIGRATION

This study revealed that conditioned media from PIP-expressing 4T1 cells promotes NK cell migration; however, this migration is not mediated by chemokines or cytokines. This raises the question of whether PIP directly induces NK cell migration. To evaluate the direct effect of PIP on NK cell migration in vitro, commercially available recombinant mouse PIP (Cloud-Clone Corp, USA) was tested in a transwell migration assay at varying concentrations. Plain media and LPS-matured dendritic cell supernatant (DC-LPS sup) served as negative and positive controls, respectively.

Recombinant mouse PIP (mPIP) at concentrations of 300ng, 600ng, and 1µg was added to 600 µL of plain media and placed in the basal (lower) wells. Additionally, 1µg of BSA in 600 µL of plain media was included as a specificity control in a separate well. IL-2-activated NK cells cultured for four days were seeded into the apical inserts of the transwell plates, and the assay incubated for 90 minutes.

As anticipated, DC-LPS sup significantly induced NK cell migration compared to plain media (Fig. 9). Notably, PIP at a concentration of 1µg/600ul DMEM significantly promoted NK cell migration compared to BSA and DMEM (plain media) (Fig. 9). However, no significant difference in migration was observed across the different concentrations of mPIP (Fig. 9).

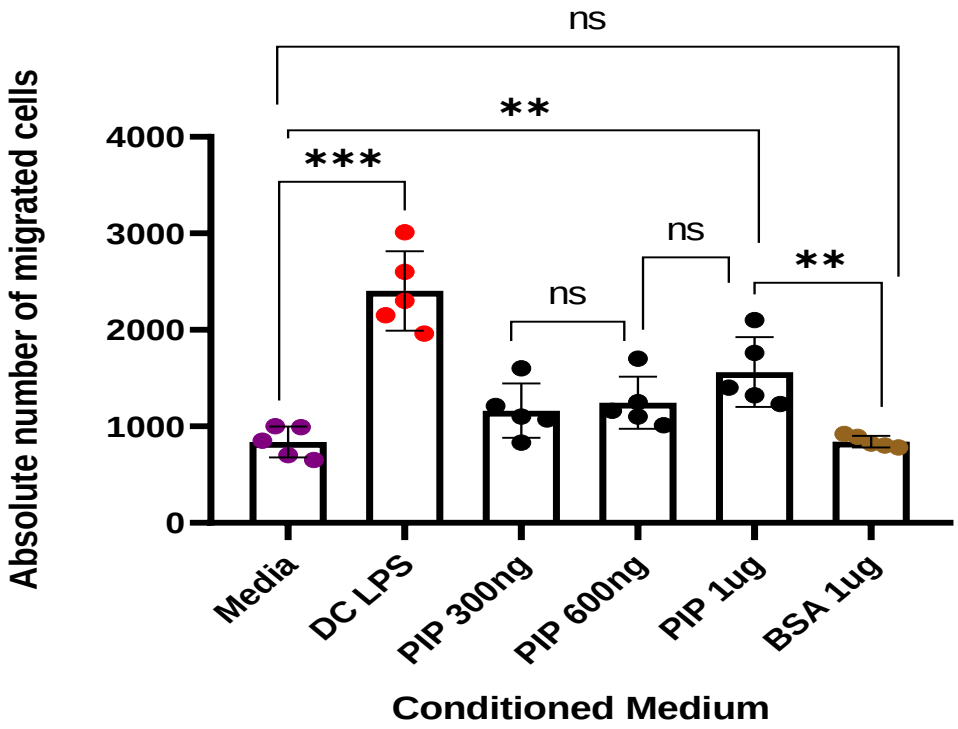


Fig. 9. Recombinant mouse PIP (mPIP) cells promoted NK migration. Different concentration of recombinant mouse PIP (300ng, 600ng and 1ug)/600ul DMEM and control (BSA 1ug/600ul DMEM) was used in a transwell assay to assess NK cell migration. 1ug of mPIP significantly promoted NK cell migration compared to BSA 1ug and media. **p ≤ 0.01, ***p ≤ 0.001, ns = not significant, ordinary one-way Anova.

3.9. TREATMENT OF NK CELLS WITH PIP INDUCED THE PHOSPHORYLATION OF P38 MITOGEN-ACTIVATED PROTEIN KINASE (P38 MAPK)

Conditioned media from PIP-expressing 4T1 cells was previously shown to promote natural killer (NK) cell migration. This effect was not attributable to the overexpression of specific chemokines in the conditioned media. Additionally, recombinant mouse PIP (1 μg) directly induced NK cell migration. These findings collectively suggest that PIP enhances NK cell migration; however, the underlying mechanism remains unclear. Given that NK cells employ diverse migratory mechanisms depending on their microenvironment, we investigated the potential impact of PIP on signaling pathways associated with NK cell migration. NK cell signaling pathways can be dependent or independent of the immunoreceptor tyrosine-based activation motifs (ITAMs) (Vély and Vivier 2005). The ITAM dependent and independent signaling pathways affect the polymerization and rearrangement of actin and involves mitogen-activated protein kinases (MAPK) signaling cascades (Watzl and Long 2010). P38 MAPK signaling pathway is activated in NK cells during ligation of β1 integrins (Mainiero et al. 2000) and PIP facilitates the binding of integrin-β1 (Naderi and Meyer 2012) and can directly bind to components of the cytoskeleton like actin and β-tubulin (Naderi and Vanneste 2014; Schenkels et al. 1994). Therefore, I went on to examine signaling pathways activated in NK cells in response to PIP.

IL-2 activated Day 4 NK cells were treated with 1μg of PIP or BSA for 2 and 5 minutes. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were used as positive control, while untreated NK cells served as a negative control. Following treatment, the cells were fixed, permeabilized, and stained for phosphorylated p38 MAPK, with data acquisition performed via flow cytometry.

As expected, PMA/ionomycin treatment induced robust phosphorylation of p38 MAPK in NK cells compared to the untreated control (Fig. 10A and 10B). Analysis of the mean fluorescence

intensity (MFI) for phosphorylated p38 MAPK revealed an increase in the PIP-treated group at 2 minutes compared to the BSA-treated group at the same time point. Although this difference was not statistically significant, it displayed a consistent trend (Fig. 10A). At 5 minutes, the levels of phosphorylated p38 MAPK were comparable between PIP-treated and BSA-treated groups (Fig. 10B).

A **B**

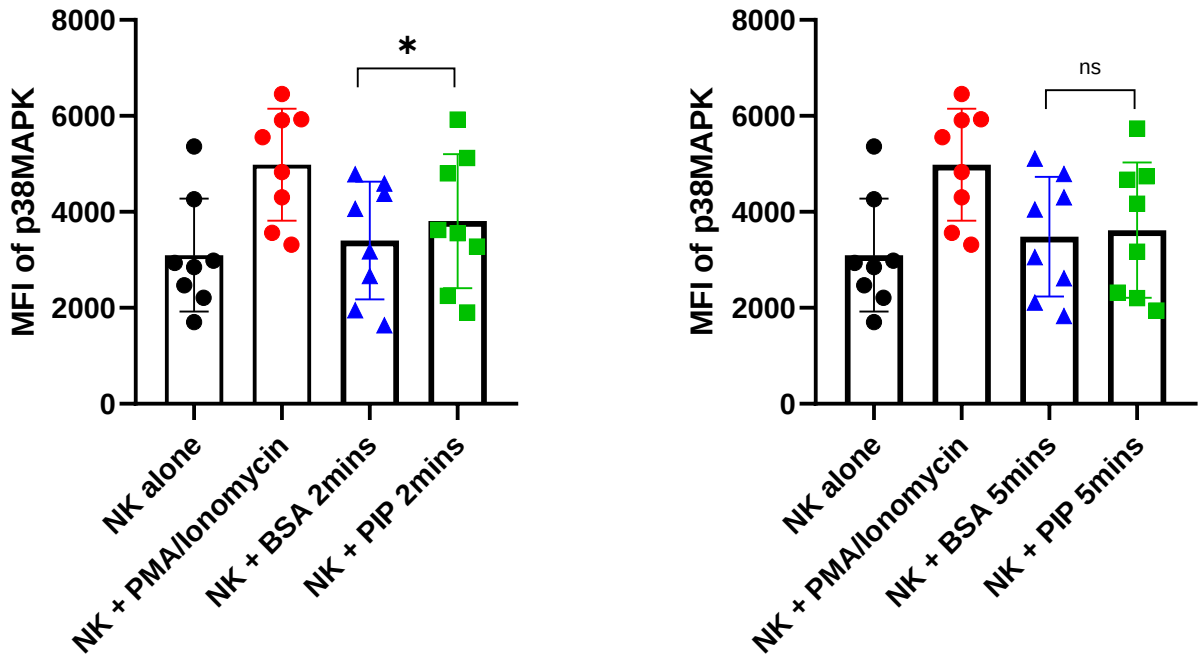


Fig. 10. NK cell treatment with PIP phosphorylates p38 MAPK. Day 4 purified NK cells activated with IL2 were treated with different stimulants at different timepoints. (A). Showing mean fluorescence intensity of p38 MAPK phosphorylation at 2mins (B). Showing mean fluorescence intensity of p38 MAPK phosphorylation at 5mins. *$p \leq 0.05$, ns = not significant, paired t-test.

3.10. PIP TREATMENT INDUCED THE PHOSPHORYLATION OF NF-KB INHIBITOR ALPHA (IKBα) IN NK CELLS

Previous research by Lee et al. (2021) demonstrated that PIP signals through toll-like receptor 2 (TLR-2) in invariant natural killer T cells (iNKT cells). However, no specific receptor for PIP has been identified in NK cells. Since extracellular TLRs universally signal via MyD88 (a known regulator of NK cell functions and an upstream activator of the NF-κB pathway), I investigated whether recombinant mouse PIP activates the NF-κB pathway in NK cells. Specifically, I assessed PIP-induced phosphorylation of IκBα, a key inhibitor of NF-κB nuclear translocation.

To test this, NK cells were treated with 1 µg of PIP or bovine serum albumin (BSA) at designated time points (2 and 5 minutes). PMA/ionomycin served as positive control, while untreated NK cells were included as negative control. After treatment, cells were fixed, permeabilized, and stained to detect phosphorylated IκBα, with flow cytometry used for data acquisition.

A significant increase in the mean fluorescence intensity (MFI) of phosphorylated IκBα was observed in NK cells treated with PIP for 2 minutes compared to BSA or untreated cells (Fig. 11A). No significant differences in IκBα phosphorylation were observed between groups at the 5-minute time point (Fig. 11B).

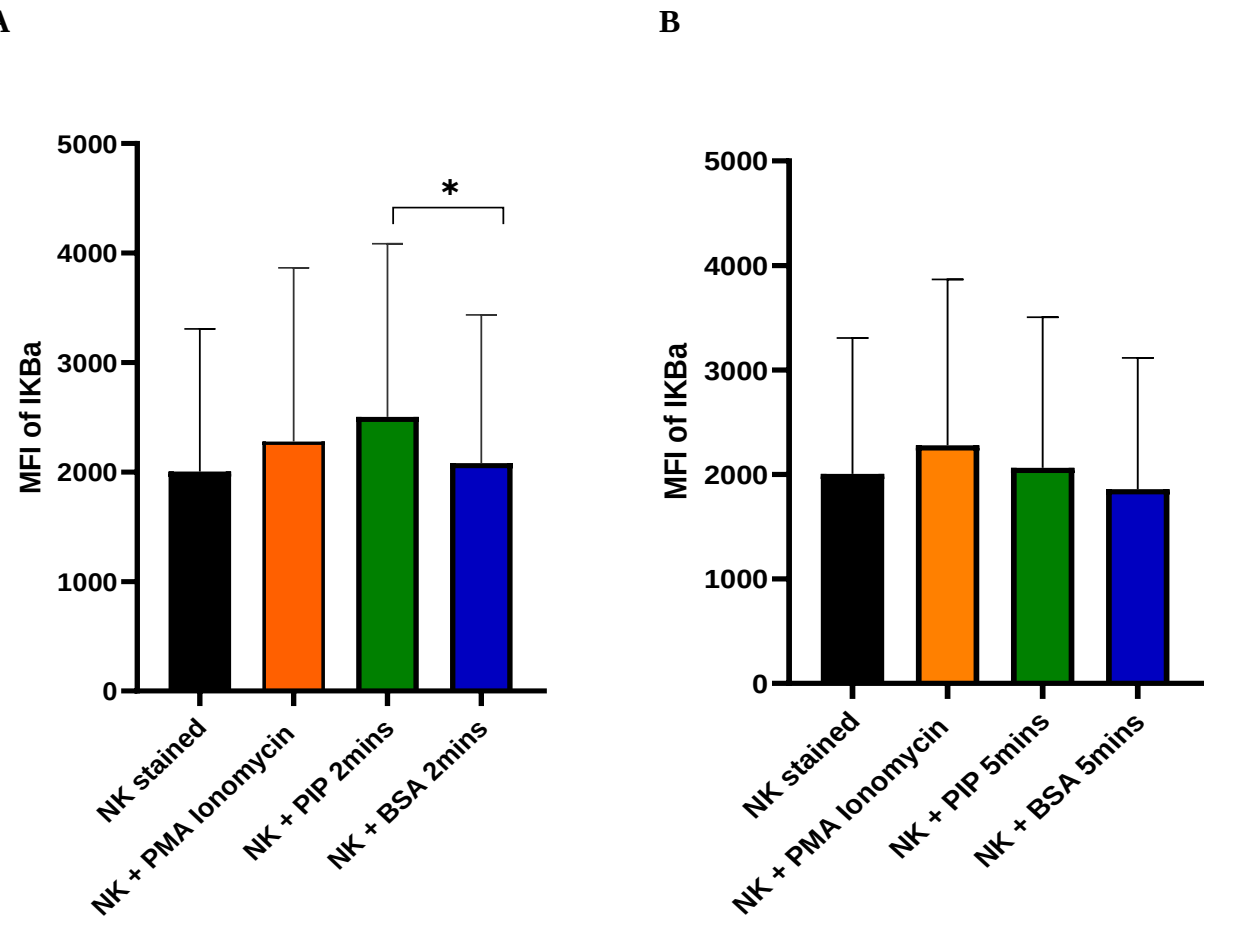


Fig. 11. Treatment of NK cells with PIP activated IκBα. Day 4 purified NK cells activated with IL2 were treated with different stimulants. (A). Showing mean fluorescence intensity of IκBα phosphorylation at 2mins (B). Showing mean fluorescence intensity of IκBα phosphorylation at 5mins. *$p \leq 0.05$, ns = not significant, paired t-test.

3.11. NK CELLS EXHIBIT CYTOTOXIC RESPONSE TOWARDS PARENT 4T1 CELLS

The present study demonstrates that PIP induces NK cell migration in vitro. NK cells play a critical role in immune surveillance by targeting and eliminating malignant cells. To ascertain the cytotoxic response of NK cells on Parent 4T1 cells IL-2-activated Day 4 NK cells were co-cultured in vitro with 4T1 cells. The expression of CD107a, a marker of NK cell degranulation, was assessed using flow cytometry as described in the "Materials and Methods" section. PMA/ionomycin-stimulated NK cells served as positive control, while unstimulated NK cells were used as a negative control.

As anticipated, NK cells treated with PMA/ionomycin exhibited significantly higher CD107a expression compared to untreated NK cells (Fig. 12). However, no significant difference in the percentage of CD107a-positive NK cells was observed between those co-cultured with 4T1 Wild-type (Parent 4T1), PIP-expressing Parent 4T1 cell (Parent 4T1-PIP) and Parent 4T1 empty vector control (Parent 4T1-EV) (Fig. 12).

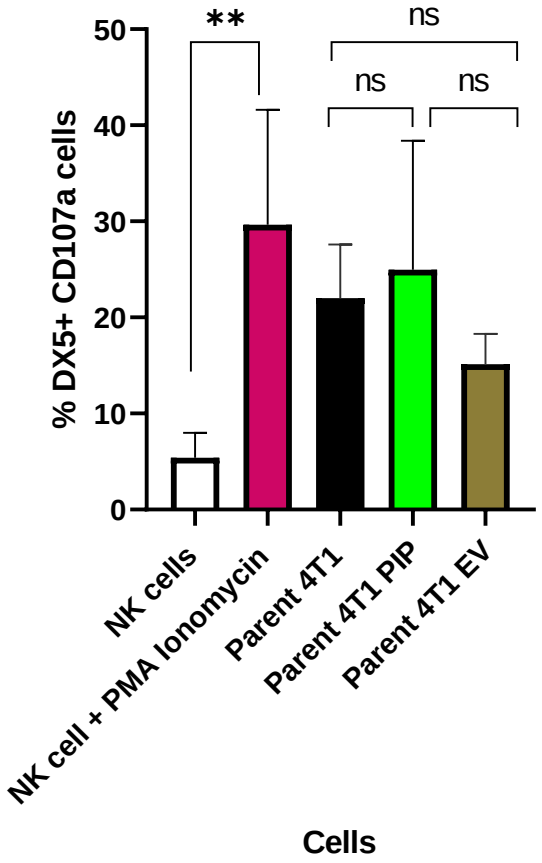


Fig. 12. CD107a expression in NK cells was not significantly different in response to Parent 4T1 cells (Parent 4T1, Parent 4T1 PIP and Parent 4T1 EV). NK cells and 4T1 cells were cocultured in an E:T ratio of 1:1 for 6 hours and assessed for CD107a expression by flow cytometry. **$p \leq 0.01$, ns = not significant, ordinary one-way Anova.

3.12. PIP EXPRESSION IN 4T1 CELLS REDERIVED FROM THE BREAST HAD NO EFFECT ON NK CELL DEGRANULATION IN VITRO

Previous work by Edechi et al. (2021) in our lab reported an increased percentage of NK cells within PIP-expressing primary breast tumors compared to tumors expressing an empty vector control. That study also observed delayed tumor onset and reduced tumor size in mice bearing PIP-expressing 4T1 cells. While these findings indicated a correlation between increased NK cell infiltration and reduced tumor burden in PIP-expressing tumors, the direct role of NK cells in this model remained unclear.

To evaluate the effect of PIP on the cytotoxic response of NK cells toward PIP-expressing 4T1 cells derived from the breast tumor microenvironment, IL-2-activated Day 4 NK cells were co-cultured in vitro with rederived 4T1 cells from primary breast tumors (4T1 PIP BR and 4T1 EV BR) and the expression of CD107a was assessed using flow cytometry as described in the "Materials and Methods" section. PMA/ionomycin-stimulated NK cells were used as positive control, while unstimulated NK cells served as negative control.

As anticipated, NK cells treated with PMA/ionomycin exhibited significantly higher CD107a expression compared to untreated NK cells (Fig. 13). However, no significant difference in the percentage of CD107a-positive NK cells was observed between those co-cultured with PIP-expressing 4T1 cells (4T1 PIP BR) and those with the empty vector control (4T1 EV BR) (Fig. 13).

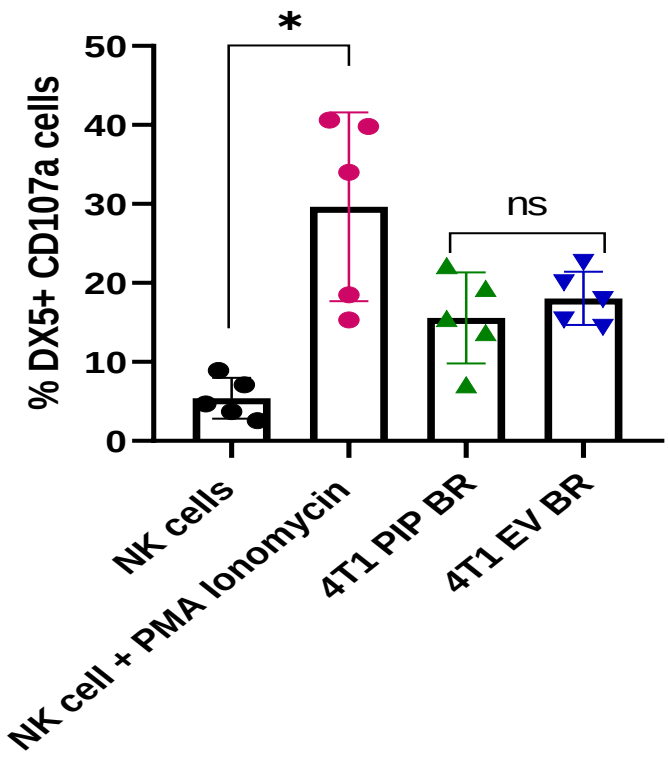


Fig. 13. CD107a expression in NK cells was not significantly different in response to 4T1 PIP BR and 4T1 EV BR. NK cells and 4T1 cells were cocultured in an E:T ratio of 1:1 for 6 hours and assessed for CD107a expression by flow cytometry. *$p \leq 0.05$, ns = not significant, paired t-test.

3.13. THE EXPRESSION OF PIP DID NOT AFFECT NK CELL DEGRANULATION IN RESPONSE TO 4T1 CELLS REDERIVED FROM METASTATIC LUNGS IN VITRO

Metastasis is the primary cause of cancer-related mortality and is associated with poor prognosis. A study by Edechi et al. (2021) in our lab demonstrated that PIP expression in primary breast tumors correlated with increased NK cell infiltration and reduced tumor size. However, the same study revealed that PIP expression significantly enhanced metastatic spread to the lungs compared to empty vector controls. This paradox raises the critical question of why immune cells, particularly NK cells, fail to control metastatic dissemination to the lungs.

To investigate the cytotoxic response of NK cells toward metastatic PIP-expressing 4T1 cells in the lungs, IL-2-activated Day 4 NK cells were co-cultured in vitro with rederived 4T1 cells from metastatic lung lesions (4T1 PIP ML and 4T1 EV ML). CD107a expression was measured using flow cytometry, as detailed in the "Materials and Methods" section. PMA/ionomycin-stimulated NK cells were included as positive control, and unstimulated NK cells served as negative control.

As expected, NK cells stimulated with PMA/ionomycin exhibited significantly higher CD107a expression compared to untreated NK cells (Fig. 14). However, no significant difference in the percentage of CD107a-positive NK cells was observed between those co-cultured with PIP expressing 4T1 cells from the metastatic lungs (4T1 PIP ML) and those with empty vector control cells from the metastatic lungs (4T1 EV ML) (Fig. 14).

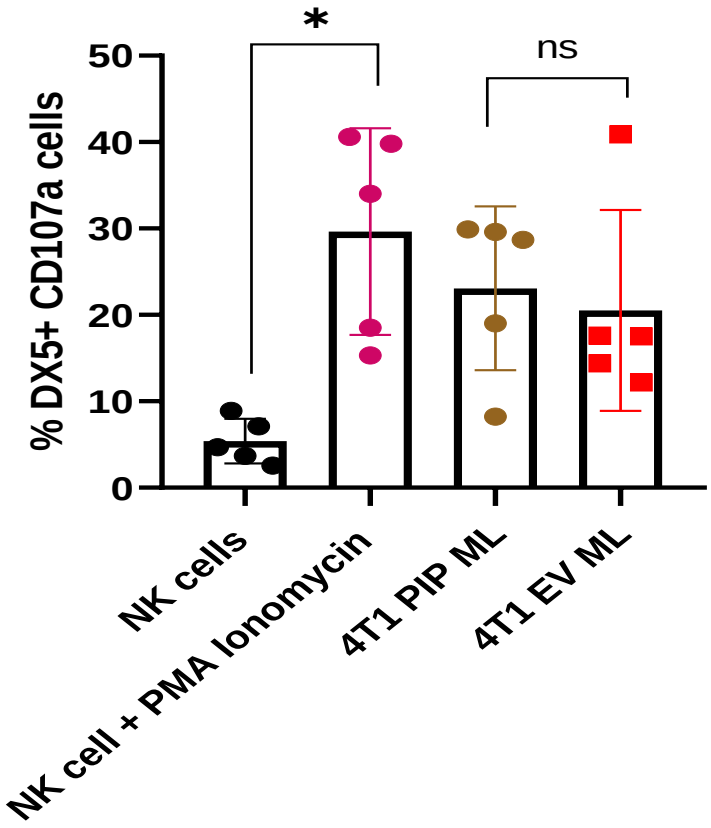


Fig. 14. CD107a expression in NK cells was not significantly different in response to 4T1 PIP ML and 4T1 EV ML. NK cells and 4T1 cells were cocultured in an E:T ratio of 1:1 for 6 hours and assessed for CD107a expression by flow cytometry. *$p \leq 0.05$, ns = not significant, paired t-test.

3.14. TUMOR MICROENVIRONMENT (SITE OF REDERIVATION) DID NOT AFFECT NK CELL DEGRANULATION

The tumor microenvironment (TME) is composed of various cellular components, including tumor cells, immune cells, and non-immune cells. Interactions among these components can profoundly influence tumor progression and the immune response (Giraldo et al. 2019). In this study, I have demonstrated that natural killer (NK) cell degranulation, as measured by CD107a expression, did not differ between PIP-expressing and empty vector (EV) control 4T1 cells re-derived from either primary breast tumors or metastatic lung sites.

To further investigate the impact of the TME (i.e., site of re-derivation site) on NK cell degranulation, I compared the NK cell response to PIP-expressing 4T1 cells re-derived from the primary breast tumor (4T1 PIP-BR) with those re-derived from metastatic lungs (4T1 PIP-ML). Additionally, NK cell responses to EV control 4T1 cells from the primary breast tumor (4T1 EV-BR) were compared with those to EV control cells from metastatic lungs (4T1 EV-ML).

The results revealed no significant difference in the percentage of NK cells expressing CD107a in response to PIP-expressing 4T1 cells re-derived from the primary breast tumor compared to those from metastatic lungs (4T1 PIP-BR vs. 4T1 PIP-ML) (Fig. 15a). Similarly, there was no significant difference in NK cell responses to EV control 4T1 cells re-derived from the primary breast tumor compared to those from metastatic lungs (4T1 EV-BR vs. 4T1 EV-ML) (Fig. 15b).

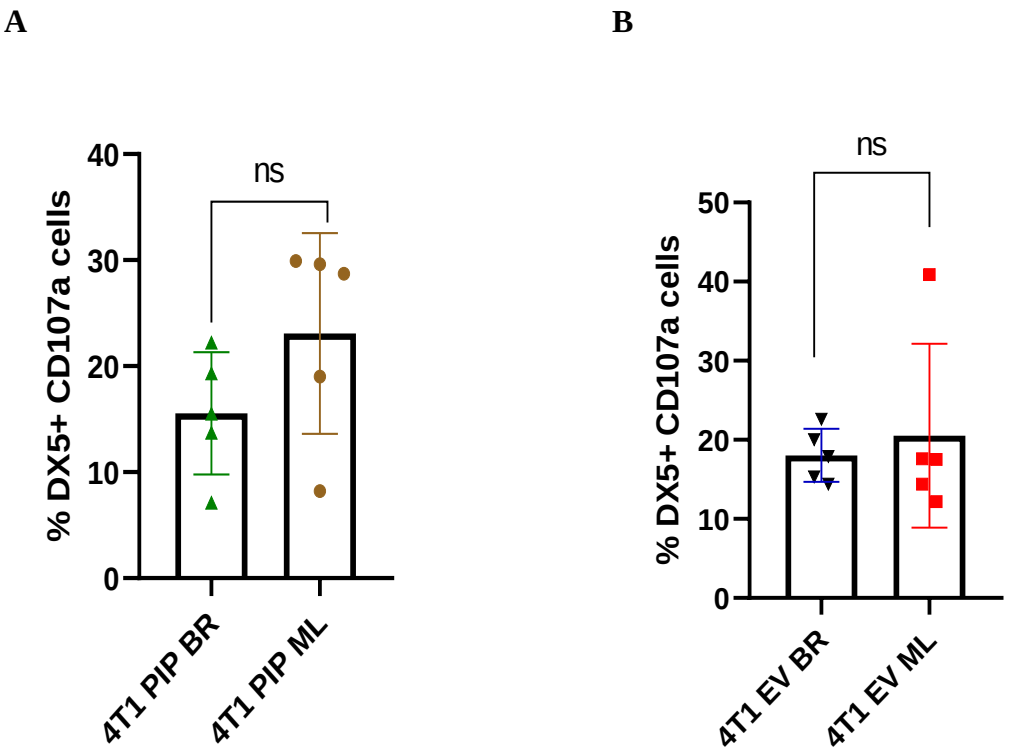


Fig. 15. TME/site of rederivation did not affect NK cell degranulation. (A) Showing percentage of degranulated NK cells in response to PIP expressing 4T1 cells rederived from the breast and lungs (4T1 PIP-BR and 4T1 PIP-ML). (B) Showing percentage of degranulated NK cells in response to empty vector control 4T1 cells rederived from the breast and lungs (4T1 EV-BR and 4T1 EV-ML). ns = not significant, paired t-test.

3.15. PARENT 4T1 CELLS INDUCED LOW INTERFERON GAMMA PRODUCTION IN NK CELLS

One of the ways NK cells facilitate the clearance of viruses and malignant cells is by the production of pro-inflammatory cytokines like IFN-γ and TNF-α. IFN-γ can directly or indirectly facilitates tumor clearance (Jorgovanovic et al. 2020). IFN-γ activates other immune cells (macrophages, dendritic cells and T cells) and help to shape their anti-tumor response (Guillerey et al. 2016; Jorgovanovic et al. 2020). I therefore investigated NK cells' cytokine production in response to the parental 4T1 cell lines (Parent 4T1, Parent 4T1-PIP and Parent 4T1-EV). To address this, IL-2-activated Day 4 NK cells were co-cultured in vitro with 4T1 cells. The percentage of NK cells producing IFN-γ (IFN-γ^+ NK cells) was assessed by flow cytometry. PMA/ionomycin-stimulated NK cells served as positive control, while unstimulated NK cells were used as negative control.

As anticipated, treatment of NK cells with PMA/ionomycin significantly elicited high IFN-γ^+ NK cells compared to untreated NK cells (Fig. 16). However, I also observed that there was no significant difference in the percentage of IFN-γ^+ NK cells in response to 4T1 Wild-type (Parent 4T1), PIP-expressing Parent 4T1 cell (Parent 4T1-PIP) and Parent 4T1 empty vector control (Parent 4T1-EV) (Fig. 16).

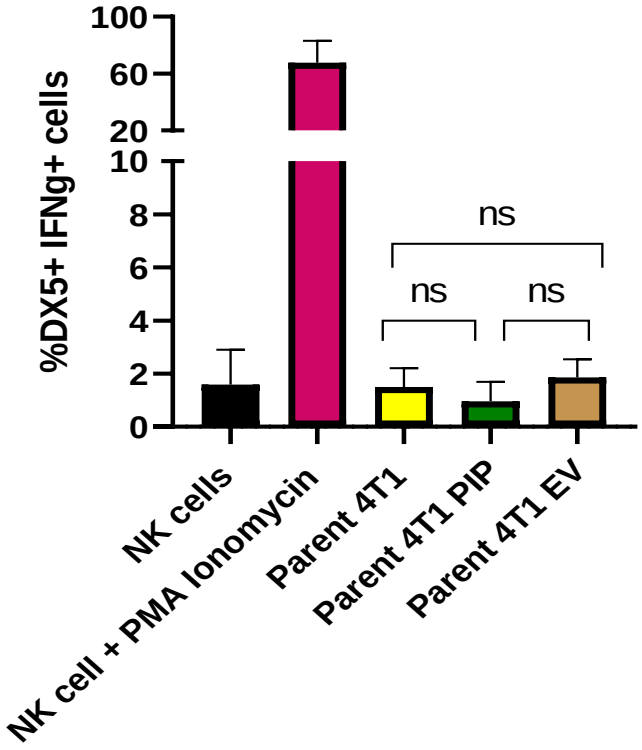


Fig. 16. NK cell secretion of IFN-γ was not significantly different in response to parent 4T1 cells (Parent 4T1, Parent 4T1 PIP and Parent 4T1 EV). NK cells and 4T1 cells were cocultured in an E:T ratio of 1:1 for 6 hours and assessed for IFN-γ production by flow cytometry. ns = not significant, ordinary one-way Anova.

3.16. PIP EXPRESSION IN 4T1 CELLS REDERIVED FROM THE PRIMARY BREAST AND METASTATIC LUNGS DID NOT AFFECT NK CELL INTERFERON GAMMA RESPONSE IN VITRO

I had previously shown in this study that PIP expression in 4T1 cells rederived from the primary breast and metastatic lungs microenvironment did not affect NK cell degranulation in vitro (Fig. 16 and 17). Studies have shown that NK cells help control tumor metastasis through the secretion of IFN-γ (Glasner et al. 2018; Putz et al. 2017; Street, Cretney, and Smyth 2001). But the study by Edechi et al. (2021) showed an increased metastasis to the lungs in PIP-expressing 4T1 cells compared to the EV control. Therefore, I decided to investigate NK cell interferon gamma production in response to the rederived PIP and EV-control 4T1 cells from the primary breast and metastatic lungs microenvironment.

4T1 cells rederived from primary breast (4T1 PIP BR and 4T1 EV BR) and metastatic lungs (4T1 PIP ML and 4T1 EV ML) were co-cultured with IL-2-activated NK cells and the percentage of IFN-γ+ NK cells were assessed using flow cytometry. PMA/ionomycin-treated NK cells were used as a positive control, and the negative control were unstimulated NK cells.

There was a significant increase in the percentage of IFN-γ^+ NK cells in the PMA/ionomycin treated NK cells compared to untreated NK cells (Fig. 17a and b). Interestingly, there was no significant difference in the percentage of IFN-γ^+ NK cells in response to PIP-expressing 4T1 cells from the primary breast (4T1 PIP BR) compared to empty vector control (4T1 EV BR) (Fig. 17a). In the same vein, the percentage of IFN-γ^+ NK cells in response to PIP-expressing 4T1 cells from the metastatic lungs (4T1 PIP ML) compared to empty vector control (4T1 EV ML) was not significantly different (Fig 17b).

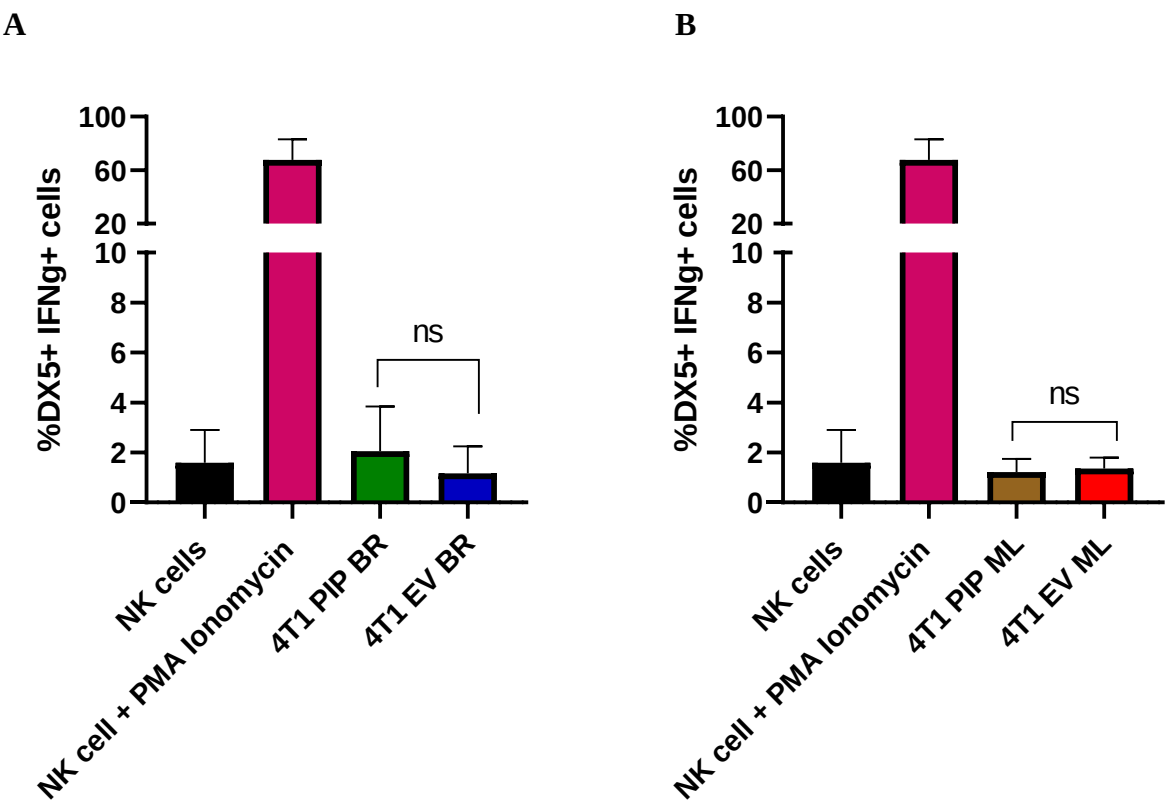


Fig. 17. IFN-γ production in NK cells in response to rederived 4T1 cells not affected by PIP expression. (A). Showing percentage of IFN-γ^+ NK cells in response to rederived cells from the primary breast (4T1 PIP BR) and empty vector control from the breast (4T1 EV BR). (B). Showing percent of IFN-γ^+ NK cells in response to rederived cells from the metastatic lungs (4T1 PIP ML) and empty vector control from the breast (4T1 EV ML). NK cells and 4T1 cells were cocultured in an E:T ratio of 1:1 for 6 hours and percent of IFN-γ^+ NK cells was assessed by flow cytometry. ns = not significant, paired t-test.

CHAPTER FOUR

4.0. DISCUSSION

The role and function of prolactin inducible protein (PIP) in the body is not fully understood, as the protein has not been widely studied. Most importantly, PIP's role in regulating and modulating immune response has not been adequately elucidated. Previous studies by our group reported potential immune regulatory role for PIP on NK cells in a 4T1 breast cancer model (Edechi et al. 2021). The study showed that PIP expressing 4T1 breast cancer cells from the primary site (breast) showed a significant increase in the percentage of NK cells and dendritic cells infiltrating the solid tumor compared to the empty vector control (Edechi et al. 2021). Other studies like those of Li et al. (2015) have reported impairment in Th1 immune response in PIP KO mice. The mice had a reduced percentage of CD4+ T cells in their spleen and these naïve CD4+ T cells had a defect in their ability to differentiate into IFN-γ producing CD4+ T cells (Li et al. 2015). Furthermore, PIP KO mice were reported to have morphologic defects in their spleens and lymph nodes (Blanchard et al. 2009). PIP has been reported to also play a role in immunity to HIV infection by binding to CD4 molecules on T cells (Basmaciogullari et al. 2000) and possess antimicrobial properties (Lee et al. 2002; Yousuf et al. 2022). Taken together, these findings suggest that PIP plays a crucial role in immunity. This current study presents evidence showing that prolactin inducible protein (PIP) enhances the migration of Natural Killer (NK) cells in vitro. The findings provide novel insight into the immunoregulatory role of PIP, highlighting its potential impact on immune cell trafficking and the broader immune response and adds to the body of knowledge by demonstrating a specific effect of PIP on NK cell biology, especially their migratory behavior.

4.1. RECOMBINANT PIP AND CONDITIONED MEDIA FROM PIP-EXPRESSING 4T1 CELLS PROMOTED NK CELL MIGRATION IN VITRO

To evaluate the effect of PIP on NK cell migration, conditioned media from PIP expressing 4T1 cells was used. I confirmed that PIP was present in both cell lysates and conditioned media from PIP expressing cells (Fig. 1). To study the effect of PIP on NK cell in vitro, a transwell migration assay was used. Transwell migration assay have been previously used in our lab to study the migratory response of NK cells to different conditions in vitro (Alamri et al. 2018). I observed an increase in the number of NK cells in the conditioned media of PIP expressing Parent 4T1 cells compared to Parent 4T1 and Parent 4T1-EV (Fig. 2). A similar trend was observed in NK cell migration between conditioned media from the rederived 4T1 cells from primary breast (4T1 PIP-BR and 4T1 EV-BR) and metastatic lungs (4T1 PIP-ML and 4T1 EV-ML). More NK cells migrated to the conditioned media from 4T1 PIP-BR and 4T1 PIP-ML than to their empty vector counterparts (Fig 3a and 3b).

Interestingly, NK cell migration was not affected by the site of rederivation of the 4T1 cells (Fig. 4a and 4b). These findings indicate that the presence of PIP can enhance NK cell migration and corroborates previous study by (Edechi et al. 2021) that showed an increase in the percentage of NK cells in PIP-expressing 4T1 primary tumors in murine breast. Also, the finding that conditioned media from PIP expressing 293T cells induced NK cell migration compared to the untransduced 293T cells further enhances the potential role of PIP in driving NK cell migration (Fig. 5). Though this experiment was done using purified mouse NK cells and conditioned media from a human cell line and presents a xenogeneic situation, the result still suggests a direct potential role of PIP in enhancing NK cell migration.

To understand the potential effect of PIP expression on the migration of other immune cell types like T cells, mouse splenocytes was used in a transwell migration. NK cells were observed to have increased migration to conditioned media from PIP-expressing 4T1 rederived from the primary breast compared to empty vector control. $DX5^+$ $CD3^-$ NK cells from inactivated (Day 0) and IL-2 activated (Day 4) splenocytes were observed to show increased migration to conditioned media from PIP-expressing 4T1 rederived from the primary breast (Fig. S3a and S4a). This finding is consistent with the previous observations when purified NK cells were used (Fig. 3a). Similarly, there was an increase in migration of $DX5^-$ $CD3^+$ T cells from IL-2 activated (Day 4) splenocytes in response to conditioned media from PIP-expressing 4T1 rederived from the primary breast compared to empty vector control (Fig. S4d), though $DX5^-$ $CD3^+$ T cells from inactivated (Day 0) splenocytes showed an opposite trend (Fig. S3d). These findings indicate that PIP may be driving the migration of T cells, and this phenomenon might be dependent on the activation state of the T cells. Though the effect of PIP on the migration of other immune cells like dendritic cells, B cells and macrophages were not assessed in vitro, previous in vivo study by Edechi et al. (2021) showed a significant increase in the percentage of $CD11c^+$ DC cells in the TME of PIP-expressing primary breast tumor compared to the EV control. An increase in $F4/80^+$ macrophages were also in the TME of PIP-expressing primary breast tumor, though not statistically significant Edechi et al. (2021).

Robust immune response involves crosstalk and interaction between immune cells. Increased NK cell migration induced by PIP can positively modulate inflammation and immune response to infections and tumor clearance. Increased presence of NK cells potentially increases the level of IFN-γ and TNF-α production which can promote DC maturation, improving antigen presentation to T cells (Pan et al. 2004). DC-NK interactions increases IL-12 production, further boosting Th1

immune responses and cytotoxic T lymphocyte activation (Kelly et al. 2002; Morandi et al. 2009). IFN-γ secretion by NK cells can also activate macrophages, promoting a pro-inflammatory (M1) phenotype that enhances pathogen clearance and tumor destruction (Vivier et al. 2011). The high infiltration of NK cells due to PIP can enhance the adaptive immune response by stimulating T cell proliferation and differentiation through the secretion of cytokines like IFN-γ (Vivier et al. 2008). Future studies will try to ascertain the direct immunomodulatory role of PIP on specific immune cells (in vitro and in vivo) and how it affects their interactions with other immune cells.

Using live-cell imaging in a 3D collagen chamber, I observed that NK cells placed in conditioned media from PIP-expressing 4T1 cells rederived from primary breast had a lesser arrest coefficient compared to those from empty vector control though statistically not significant (Fig. S5b). The arrest coefficient is a measure that quantifies the proportion of time a cell is stationary (arrested) during an observation period. This finding indicates that NK cells in the conditioned media from PIP-expressing 4T1 cells rederived from primary breast were more motile and less stationary than those in the conditioned media from EV control rederived from the primary breast. This implies that PIP might be influencing NK cell migration in these conditions by making them more motile.

Since I have established that there was increase in the number of NK cells that migrated to conditioned media from PIP expressing 4T1 and 293T cells compared to empty vector and untransduced controls respectively, I proceeded to confirm whether other factors like chemokines and cytokines in the conditioned media are responsible for the disparity in migratory responses observed in NK cells to the conditioned media. To check for a differential expression of cytokine or chemokines in the conditioned media, samples were shipped to Eve Technologies (Calgary, AB, Canada) for a Mouse Cytokine/Chemokine 32-Plex Discovery Assay® Array (MD32). The cytokine/chemokine analysis showed that IP10 was significantly increased in the conditioned

media from 4T1 PIP-BR compared to 4T1 EV-BR but there was no difference in IP10 level in Parent 4T1 group (Parent 4T1, Parent 4T1-PIP and Parent 4T1-EV) and the metastatic lung group (4T1 PIP-ML and 4T1 EV-ML) (Fig. 6). Also, the expression of PIP didn't affect the level of MCP1 in any of the 4T1 groups (Fig. 7) with similar result observed for other cytokines and chemokines (Fig. S1 and S2). These findings indicate that the 4T1 cells express different levels of chemokines and cytokines that could have been influenced by the expression of PIP (or not) and the interaction between the 4T1 cells and the TME (Parent, primary breast and metastatic lungs). More importantly, they indicate that chemokines or cytokines are not responsible for the enhanced NK cell migration observed in the conditioned media from PIP expressing 4T1 cells from the parent, primary breast or metastatic lungs, suggesting that PIP in the conditioned media of the PIP expressing 4T1 cells could be responsible for directly enhancing NK cell migration in vitro.

Having confirmed that the observed migration differences is not tied to any chemokine(s) or cytokine(s), I proceeded to determine the direct role of PIP in promoting NK cell migration. Recombinant mouse PIP was observed to enhance NK cell migration, albeit in a dose-dependent manner. PIP at 1ug/600ul DMEM promoted more NK cell migration than BSA 1ug/600ul DMEM (Fig. 9). The migration assay results indicate that PIP can directly act as a chemotactic factor, effectively influencing NK cell movement, which is crucial for immune surveillance and the prompt elimination of target cells, such as virus-infected or malignant cells.

My study also revealed that adding PIP to the top chamber during the transwell migration assay facilitated NK cell migration. The result showed that compared to BSA or adding LPS DC sup at the top, stimulating the NK cells with PIP at the apical (insert) well during the incubation period promoted migration, similar to what is observed when PIP is added to the basal well of the transwell (Fig. 7). The effect of concentration gradient in chemotaxis was clearly demonstrated as

NK cells migrated significantly to the basal well containing LPS DC sup but the migratory effect of the LPS DC sup was abrogated when NK cells were incubated with LPS DC sup in the apical well (Fig 7). Previous study in our group have demonstrated that conditioned media from LPS matured DCs can significantly drive NK cell migration (Alamri et al. 2018). This finding suggests that PIP might be influencing NK cell trafficking through a mechanism distinct from traditional chemokines, which typically function by establishing a concentration gradient. In the classical model, chemokines such as CXCL12 or CCL5 create a gradient that guides immune cells toward higher concentrations, facilitating chemotactic migration (Förster, Davalos-Misslitz, and Rot 2008; James E Moore, Brook, and Nibbs 2018; Schwarz et al. 2017; Weber et al. 2013). However, the ability of PIP to promote NK cell migration without an apparent gradient challenges this paradigm and points to a potential chemokinesis-like effect.

Chemokinesis refers to the random movement or enhanced motility of cells in response to a uniform stimulus, rather than directed movement toward a gradient (Chang et al. 2018). The observation that PIP in the top chamber increased NK cell migration suggests that PIP can also function as a motility-enhancing factor, stimulating NK cells to move more dynamically regardless of a gradient. This behavior is different with how chemokines operate and highlights the adaptability of mechanisms that can regulate immune cell trafficking.

A receptor for PIP has not been identified in NK cells, but the mechanism underlying PIP-induced NK cell migration may involve the interaction between PIP and receptors expressed on NK cells. Chemokines and other chemotactic factors are known to play pivotal roles in the homing and distribution of NK cells within tissues (Vicente-Manzanares and Sánchez-Madrid 2004). It is plausible that PIP influences NK cell migration by modulating signaling pathways akin to those triggered by classical chemokines, such as CCL5, CX3CL1 or CXCL10, which are well-

documented in mediating NK cell chemotaxis (Grégoire et al. 2008; Paust et al. 2010; Robinson et al. 2003; Trifilo et al. 2004). Researchers are unearthing different migratory phenomenon in immune cells. For example, recent studies have shown that DCs not only migrate along a concentration gradient of CCL19 using CCR7 but they were also able to modulate the local chemokine concentration by sinking CCL19 through the internalization of CCR7 (Alanko et al. 2023; Donnelly, Mandrou, and Insall 2023). The sinking of the CCL19 enhanced the migratory behavior of the DCs and also provided better migratory cues for other immune cells (Alanko et al. 2023). Though there is no evidence from my study that the migration of NK cells in response to PIP follow the mechanism described by Alanko et al., but the distinct behavior observed with PIP broadens our understanding of how immune cell migration can be regulated beyond the traditional chemokine model.

Another possible explanation for this observed migratory response is that PIP could activate intracellular signaling pathways that increase the general motility of NK cells. It is probable that PIP engages surface receptors on NK cells, leading to the activation of motility-related signaling cascades, such as the p38 MAPK pathway. The activation of p38 MAPK is well-known to influence cytoskeletal dynamics, which are crucial for cell movement (Bian et al. 2017; Heit et al. 2002). Therefore, PIP may act more as a general stimulator of cell motility rather than a directional chemotactic agent. However, further studies are needed to confirm the interaction between PIP and chemokine receptors expressed on NK cells and to delineate the precise interactions and downstream signaling events activated by the interaction of PIP and the chemokine receptor. Identifying PIP-specific receptors on NK cells will not only help foster a better understanding of how PIP regulate NK migration, but will further facilitate our understanding of its immunoregulatory functions.

4.2. PIP INDUCED PHOSPHORYLATION OF P38 MAPK IN NK CELLS

In addition to demonstrating that Prolactin Inducible Protein (PIP) promotes NK cell migration, I went further to elucidate the potential signaling mechanism underlying PIP-induced NK cell migration. My study showed that PIP also induces the phosphorylation of p38 Mitogen-Activated Protein Kinase (MAPK) in NK cells (Fig. 10). This phosphorylation event implies that the p38 MAPK signaling pathway could be a key mechanism driving PIP-mediated NK cell migration. These findings align with the well-known role of p38 MAPK in cellular migration and chemotactic responses, particularly within the immune system (Bian et al. 2017; Heit et al. 2002).

The p38 MAPK pathway is known to be a crucial regulator of cellular processes, including inflammation, apoptosis, and migration. Macrophages derived from PIP knockout (KO) mice exhibited reduced production of proinflammatory cytokines and nitric oxide (Ihedioha et al. 2018). This reduction was correlated with decreased phosphorylation levels of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 1 (STAT1) proteins. Previous studies have shown that the MAPK signaling pathway is involved in actin polymerization that control migration of vascular smooth muscle cells (Lee et al. 2016). In the context of immune cell trafficking, p38 MAPK activation has been associated with enhanced cytoskeletal rearrangements and increased chemotactic movement in neutrophils (Cara et al. 2001; Heit et al. 2002; Heuertz et al. 1999) and activation of dendritic cells (Zaru et al. 2007). NK cells, which are vital for innate immune surveillance, rely on rapid and efficient migration to eliminate virally infected or malignant cells. My observation that PIP triggers the phosphorylation p38 MAPK in NK cells is consistent with this well-characterized pathway's involvement in orchestrating immune cell migration.

Several studies have highlighted the importance of p38 MAPK in mediating immune response in NK cells. For instance, Mainiero et al. (2000) showed that the activation of RAC1/p38 MAPK led to the production of proinflammatory cytokine IL-8 through the stimulation of β1 integrins (fibronectin receptors). Xia et al. (2021) demonstrated that the activation of p38 MAPK after treatment with metformin promoted anticancer activity of NK cells. Pisegna et al. (2004) revealed that there was a TLR3-mediated increase in the cytotoxicity and cytokine production of human NK cells regulated by the activation of p38 MAPK. My findings extend these observations by identifying PIP as a novel activator of p38 MAPK in NK cells, suggesting that PIP might function similarly to classical chemotactic stimuli.

The mechanism by which PIP induces p38 MAPK phosphorylation in NK cells needs further exploration. It is possible that PIP interacts with surface receptors on NK cells, triggering intracellular signaling cascades that lead to p38 MAPK activation. Previous research on related proteins has shown that such activation can be mediated through receptor tyrosine kinases or G-protein-coupled receptors (GPCR), both of which are known to activate MAPK pathways (Yamauchi et al. 1997). In T cell, signaling via the β-adrenergic receptors (βAR) is involved in the activation of p38 MAPK (LaJevic et al. 2011). Grimsey et al. (2019) reported p38 activation stimulated endothelial inflammatory responses when GPCR were engaged by their agonists using an unorthodox TAB1-TAB2 and TAB1-TAB3-dependent pathway. Identifying the receptor(s) through which PIP exerts its effect could provide valuable insights into how NK cell migration is regulated.

Interestingly, the link between PIP and p38 MAPK activation introduces new potential therapeutic opportunities. If PIP's ability to enhance NK cell migration through p38 MAPK activation can be harnessed, it may pave the way for novel strategies to boost NK cell activity in immune-based

therapies. However, therapeutic modulation of this pathway would require a delicate balance, given that excessive p38 MAPK activation can also promote inflammatory responses and tissue damage.

My findings raise important questions about the broader role of PIP in immune regulation and signal transduction. For example, can PIP activation of the p38 MAPK pathway in NK cell be recapitulated in other immune cell subsets? Additionally, the dynamics of p38 MAPK activation and its downstream effects on NK cell function, such as cytotoxicity and cytokine production, need to be explored. Understanding these dynamics will help clarify whether PIP's role is restricted to migration or extends to other aspects of NK cell biology.

4.3. PIP INDUCED PHOSPHORYLATION OF IκBα IN NK CELLS

My study also observed that Prolactin Inducible Protein (PIP) induced the phosphorylation of IκBα (Fig. 11) and p65 NF-κB (Fig. S7), which are key components of the NF-κB signaling pathway. This finding suggests that PIP activates NF-κB signaling, and could be another mechanism by which PIP drives NK cell migration. NF-κB is a transcription factor that controls various cellular processes, including immune cell activation, survival, and motility (Hoffmann and Baltimore 2006). The observed phosphorylation of IκBα, which leads to its degradation and allows the nuclear translocation and DNA binding activities of NF-κB proteins like p65 and p50, can lead to the activation of inflammatory genes.

The activation of NF-κB in NK cells by PIP might be enhancing the expression of genes involved in cytoskeletal reorganization and cell motility, like those encoding chemokine receptors (e.g., CXCR4, CCR7) or adhesion molecules (e.g., integrins). This also can be a possible mechanism that can explain the increased migratory response observed in the presence of PIP. Furthermore,

NF-κB has been observed to play a role in regulating immune cell migration by previous studies. For instance, Baratin et al. (2015) reported a new aggregate of genes regulated by NF-κB that is specific to migratory DCs. Also, a study by Park et al. (2012) showed that deficiency of IKKβ (a key component of the NF-κB canonical pathway) reduced adhesion, migration, and uptake of lipid in macrophages. My findings agree with this framework, indicating that PIP might be acting upstream of NF-κB in NK cells, possibly through a yet to be identified or confirmed receptor or signaling complex.

Previous research has shown that NF-κB activation can also facilitate cell migration by upregulating motility-associated genes. Helbig et al. (2003) showed that NF-κB increases the expression of CXCR4 in breast cancer cells, facilitating their migration and metastasis. CXCR4 is a chemokine receptor that has been shown to be critical for directional migration in cancer cells and leukocytes (Goedhart et al. 2019; Helbig et al. 2003; Levy et al. 2019; Noda et al. 2011). PIP's activation of NF-κB may be similarly enhancing NK cell motility by influencing such receptor expression. Furthermore, since NF-κB has been observed to be involved in breast cancer cell migration, the reported increased metastasis of PIP expressing 4T1 cells from the primary breast site to the lungs by Edechi et al. (2021) could be due to the increased regulation of NF-κB in those cells, but this remains to be confirmed.

NF-κB is often activated in tumor microenvironments, where it modulates immune cell behavior. Taniguchi et al. (2018) opined that tumor-secreted factors activate NF-κB in surrounding immune cells, promoting their recruitment and sometimes altering their functional states. The study by Edechi et al. (2021) showed a reduction in tumor size of PIP expressing 4T1 cells at the primary site in breast and an increased presence on NK cells in the tumor. A study by Zhou et al. (2002) observed that NF-κB activation in NK cells controls the expression of perforin (a cytotoxic

molecule needed for target killing). In the same vein, Huang et al. (2006) reported an NF-κB binding location, that is involved in regulating granzyme B gene transcription in humans. PIP in the 4T1 cell model of Edechi et al., might play a similar role by acting as a tumor-derived factor that activates NF-κB in NK cells, promoting their infiltration into the tumor microenvironment and leading to the reduction in tumor size.

The activation of NF-κB signaling through the phosphorylation of IκBα and p65 also provides a probable mechanism by which PIP promotes NK cell migration. This mechanism not only aligns with previous studies on NF-κB's role in immune cell motility but also provides unique features of PIP-mediated signaling. Future research should aim to identify the specific upstream receptors or pathways involved in PIP-induced NF-κB activation in NK cells and investigate whether these effects are unique to PIP. This information could inform treatment strategies to enhance NK cell migration and function in cancer immunotherapy.

4.4. PIP EXPRESSION IN 4T1 CELLS FROM DIFFERENT TME DID NOT AFFECT NK CYTOTOXIC RESPONSE IN VITRO

I showed that PIP promoted NK cell migration in vitro. I further examined whether PIP modulated other NK cell functions such as cytotoxic response and cytokine production. As previously mentioned, a recent in vivo study in our lab by Edechi et al. showed increased NK cell infiltration into PIP-expressing breast tumors and there was a significant reduction in tumor growth at the primary site (Edechi et al. 2021). The current findings demonstrated that NK cells exhibit comparable degranulation responses to PIP-expressing and empty vector (EV) control 4T1 cells, irrespective of whether the cells were derived from the parent (Parent 4T1, Parent 4T1-PIP or Parent 4T1-EV) (Fig 12), primary breast tumor (4T1 PIP-BR vs. 4T1 EV-BR) (Fig. 13) or from metastatic lung sites (4T1 PIP-ML vs. 4T1 EV-ML) (Fig. 14). These observations suggest that the

expression of PIP did not significantly modulate NK cell degranulation against 4T1 tumor cells in vitro.

I further examined whether NK cells responded differentially to the tumors based on their microenvironment (site of rederivation). Natural killer (NK) cells play a crucial role in the immune system's ability to target and eliminate tumor cells, particularly in the context of metastasis, where tumor cells disseminate from the primary site to distant organs. The findings presented here highlight that NK cells exhibited comparable degranulation responses to tumor cells derived from primary breast tissue and metastatic lung environments. In this study, no significant differences in CD107a expression were observed in NK cells exposed to PIP-expressing 4T1 tumor cells from the primary breast (4T1 PIP-BR) compared to those from metastatic lung tissue (4T1 PIP-ML) (Fig 15a). This suggests that the metastatic microenvironment does not significantly alter the ability of NK cells to degranulate in response to PIP-expressing tumor cells. Similar results were observed with empty vector (EV) control tumor cells derived from both environments (4T1 EV-BR vs. 4T1 EV-ML) (Fig 15b), indicating that the degranulation response is independent of PIP expression and consistent across primary and metastatic tumor contexts. These findings align with previous research demonstrating that NK cell functionality is influenced by multiple factors, including tumor-derived signals, cytokines, and the immune microenvironment (Pegram et al. 2011; Vivier et al. 2011).

The characterization of the 4T1 cell groups revealed a similar pattern, a high expression of $H2D^d$ (MHC class 1) (Fig. S11) and low expression of RAE-1 (a stress-induced ligand of NKG2D) (Fig. S12). Similar observations of the properties of 4T1 cells have been reported by (Tallerico et al. 2017). The high expression of $H2D^d$ (MHC class 1) and low expression of RAE-1 (a ligand of NKG2D) makes 4T1 cells highly resistant to NK cell killing, compared to cancer cell line like

RMA-S. However, the lack of variation in degranulation responses also suggests that the intrinsic ability of NK cells to recognize and respond to 4T1 tumor cells is preserved across different microenvironments, at least in the case of PIP-expressing and control 4T1 cells in vitro.

These findings help to unravel some of the complexities underlying the observations of Edechi et al. (2021) that saw a reduced tumor size and delayed tumor onset in the breast of mice in the PIP group compared to the EV control group. Paradoxically, they also observed higher lung metastasis in the PIP-expressing group compared to the control.

The enhanced NK cell infiltration and reduced tumor growth at the primary breast site in mice bearing PIP-expressing tumors suggest that PIP may create a more immunologically active microenvironment that facilitates NK cell recruitment and perhaps temporarily restrains tumor growth. This aligns with the known roles of NK cells in tumor surveillance, where their presence is typically associated with improved control of primary tumors (Cerwenka, Baron, and Lanier 2001; Diefenbach et al. 2001). PIP could be functioning as a chemotactic or motility-enhancing factor, as demonstrated by my in vitro migration assays, thereby increasing NK cell presence in the tumor microenvironment. This increased infiltration could lead to improved immune surveillance and suppression of tumor expansion at the breast site.

However, my findings of equal NK cell killing sensitivity between PIP and EV-expressing 4T1 cells in vitro imply that PIP expression does not directly alter the susceptibility of tumor cells to NK cell-mediated lysis. This suggests that while PIP may recruit more NK cells to the primary tumor, its role in regulating NK cell direct killing of PIP-expressing 4T1 tumor in the breast as observed by Edechi et al., might be indirect. This could be a crucial factor in understanding why PIP's impact does not extend to controlling metastatic spread, particularly in the lungs in their study.

The higher incidence of lung metastasis in the PIP-expressing group, as reported by Edechi et al. (2021), indicates that PIP may have additional roles that promote metastasis, which antagonizes the benefits of increased NK cell recruitment at the primary tumor site. One possible explanation is that PIP could facilitate metastatic dissemination through mechanisms that are independent of immune cell killing. For example, PIP's pro-metastatic effects could include the remodeling of the extracellular matrix, enhancement of angiogenesis, or alteration of the tumor microenvironment to support cancer cell escape and seeding in distant organs.

Moreover, the lung microenvironment itself presents unique challenges for immune surveillance. Metastatic sites often exhibit an immunosuppressive milieu that can dampen NK cell activity, making it difficult for these immune cells to effectively control metastatic tumor cells (Liu et al. 2023). Factors such as immune checkpoint molecules, immunosuppressive cytokines, and stromal interactions in the lungs could contribute to the reduced efficacy of NK cells in metastatic settings, even when they are present. Thus, while PIP may enhance NK cell recruitment at the primary tumor, this effect may not translate to effective immune control at metastatic sites due to the distinct immune landscape of the lungs, even though my in vitro study showed that NK cells degranulated in response to the 4T1 cells (PIP-expressing and EV control) rederived from the metastatic lungs.

4.5. INTERFERON-GAMMA PRODUCTION NOT AFFECTED BY PIP-EXPRESSION AND SITE OF REDERIVATION OF 4T1 IN VITRO

Natural killer (NK) cells are pivotal in the innate immune defense against tumors, employing various mechanisms such as degranulation, cytokine secretion, and the induction of apoptosis to eliminate cancer cells. Among these mechanisms, the gamma response, primarily characterized by the secretion of interferon-gamma (IFN-γ), plays a critical role in tumor surveillance by

modulating the tumor microenvironment and enhancing the recruitment and activation of other immune cells (Vivier et al. 2011). The results of this study indicate that there was no significant difference in the gamma response of NK cells to the parent 4T1 cells (wild-type, PIP-expressing, and EV control) (Fig 16) or to the rederived 4T1 cells from the breast (4T1 PIP-BR and 4T1 EV-BR) and metastatic lungs (4T1 PIP-ML and 4T1 EV-ML) in vitro (Fig 17a and 17b).

The observed low NK cell gamma response to both parent and rederived 4T1 cells (PIP-expressing and EV control), suggests that the tumor cells, regardless of their PIP expression status, did not differentially modulate the NK cell's ability to produce IFN-γ in vitro. This finding collaborates with studies showing that tumor cells often alter immune responses by downregulating activating ligands or secreting immunosuppressive factors, such as transforming growth factor-beta (TGF-β) or interleukin-10 (IL-10), which can suppress NK cell IFN-γ production (Gao et al. 2017; Lanier 2008).

Additionally, the consistent interferon gamma response to rederived tumor cells from both the primary breast and metastatic lungs indicates that the process of metastasis and the metastatic microenvironment had no effect on the NK cell's ability to produce IFN-γ in response to these cells. Metastatic tumor cells often undergo significant phenotypic and functional changes that can impact immune recognition, including alterations in major histocompatibility complex (MHC) expression, stress-induced ligand expression, or secretion of immune-modulating factors (Hanahan and Weinberg 2011). My study showed that there was a high expression of $H2D^d$ (MHC class 1) and low expression of RAE-1 (a ligand of NKG2D) on the 4T1 cells rederived from the metastatic lungs and other groups from the primary breast and parental groups (Fig. S11 and S12). The uniform IFN-γ response could be due to the consistent expression of NK cell-activating and inhibitory ligands on the tumor cells across the different groups tested. Key ligands such as

NKG2D ligands (RAE-1) and MHC class I molecules (MHC class 1), remained unaffected by PIP expression or the metastatic process.

Interestingly, treatment of natural killer (NK) cells with recombinant prolactin-inducible protein (PIP) increased interferon-gamma (IFN-γ) production compared to bovine serum albumin (BSA)-treated and untreated NK cells (though not statistically significant) provides additional insights into the immunomodulatory role of PIP (Fig S10). The lack of a significant IFN-γ response in BSA-treated and untreated NK cells highlights the specificity of the PIP-induced effect. BSA served as a control to account for non-specific protein effects, suggesting that the observed increase in IFN-γ is not merely due to the presence of a protein substrate but is specific to PIP's functional properties. Untreated cells provide a baseline, further underscoring the functional enhancement induced by PIP treatment. PIP has been implicated in modulating immune responses, potentially influencing the activity of various immune cells, including NK cells. For instance, PIP-deficient mice exhibited compromised CD4+ T cell proliferation and IFN-γ production, indicating a potential impact on immune responses (Ihedioha et al. 2018). PIP may interact with specific receptors on NK cells, triggering intracellular signaling cascades that culminate in the transcription and translation of IFN-γ. This is similar to findings of Matalka (2003) that showed prolactin hormone can activate immune cells by binding to the prolactin receptor, leading to increased IFN-γ production. By increasing IFN-γ production, PIP may enhance the cytotoxic activity of NK cells against viruses and tumor cells.

These findings highlight the need for further mechanistic studies to elucidate the interplay between tumor-derived factors, immune microenvironments, and NK cell functionality. Understanding these interactions could inform the development of NK cell-based therapies for metastatic breast cancer.

4.6. SUMMARY AND CONCLUSION

This study explored the effects of Prolactin Inducible Protein (PIP) on natural killer (NK) cell behavior, focusing on its impact on NK cell migration and cytotoxicity in the context of breast cancer. The in vitro experiments demonstrated that PIP significantly enhances NK cell migration, suggesting that PIP could serve as a chemotactic or motility-enhancing factor for NK cells. The study also implicates p38 MAPK, IκBα and p65 phosphorylation as potential mechanisms driving this migratory effect.

There was no difference in NK cell-mediated killing and interferon gamma production observed between PIP-expressing 4T1 breast cancer cells and empty vector (EV) controls, for parental, breast-derived and lung metastatic 4T1 cells in vitro. These findings indicate that while PIP promotes NK cell recruitment, it does not directly affect the susceptibility of 4T1 tumor cells to NK cell-mediated cytotoxicity in vitro. Conversely, an increased percentage of IFN-γ^+ NK cells was observed in response to recombinant PIP treatment, highlighting a potential role of PIP in inducing interferon gamma response in NK cells.

When comparing my findings to previous work by Edechi et al. (2021), which reported increased NK cell infiltration and reduced tumor growth at the breast site but higher lung metastasis in PIP-expressing tumors, my study provides new insights into the complex role of PIP. I suggest that the enhanced NK cell presence at the primary tumor may help control local tumor growth but does not extend to effective suppression of metastatic spread. This may be due to differences in the metastatic microenvironment, potential immune evasion mechanisms, or the broader pro-metastatic effects of PIP.

My findings highlight a novel role for PIP in modulating NK cell migratory behavior and potentially enhancing IFN-γ secretion. The study underscores the potential of PIP's influence on NK cell biology, with potential implications for tumor and infection control. Future research is necessary to further dissect the mechanisms by which PIP regulates NK cell function and to evaluate its impact in more physiologically relevant in vivo models. This research could ultimately inform strategies to harness or modulate PIP's effects for therapeutic benefit in cancer treatment.

4.7. SIGNIFICANCE OF STUDY

My findings show in vitro evidence of PIP's ability to promote NK cell migration. These findings have potential implications for cancer immunotherapy and the fight against infections. Given the role of NK cells in tumor surveillance and viral infection clearance, the ability of PIP to promote NK cell migration and interferon gamma production may be leveraged to enhance the effectiveness of NK cell-based therapies. Primary breast tumor overexpressing PIP in mice have been shown to have a significant increase in NK cell infiltration compared to empty vector control (Edechi et al. 2021). PIP or its derivatives could be employed to improve the homing and cytokine production efficiency of NK cells to tumors and infection sites, thereby augmenting the immune response against cancers and other infections.

4.8. LIMITATIONS OF THE STUDY

In Vitro Model Limitations: The study primarily relied on in vitro assays to assess NK cell migration and cytotoxicity. While these models provided controlled environments to analyze NK cell behavior, they do not replicate the complexity of the in vivo microenvironment, including factors like blood flow, tissue architecture, and interactions with other immune and stromal cells.

Lack of In Vivo Validation: Although my findings suggest mechanisms of NK cell behavior in response to PIP, the absence of in vivo experiments limits the understanding of how PIP influences NK cell activity and tumor progression in a living organism. In vivo models could help validate whether the observed effects of PIP on NK cell migration translate to changes in NK immunosurveillance.

Limited Assessment of NK Cell Function: My study focused on NK cell migration, cytotoxicity and cytokine production in response to PIP expression in 4T1 cells but did not thoroughly investigate other critical functions of NK cells, such as immune synapse formation, or potential exhaustion markers. A more comprehensive analysis of NK cell functionality could provide a better understanding of how PIP influences NK cell behavior.

Simplified Tumor-NK Cell Interactions: The co-culture assays used in my study to assess NK cell killing and cytokine production in response to PIP-expressing 4T1 cells did not account for the influence of other immune cells or the broader tumor microenvironment. Tumor-immune interactions are highly complex, and the presence of other cell types, such as macrophages, dendritic cells, T cells, or myeloid-derived suppressor cells, could influence the results.

Limited study on PIP’s effect on other Immune Cells: My study primarily investigated the effect of PIP on NK cell migration; however, the impact of PIP on dendritic cells (DCs) and macrophages was not assessed. Additionally, a comprehensive analysis of PIP's role in modulating T cell function was not conducted. The lack of data on PIP’s influence on these immune cell populations limits our understanding of its potential regulatory effects on individual immune cell subsets and its broader role in shaping immune cell interactions within the tumor microenvironment.

4.9. FUTURE DIRECTIONS

Use of other in vitro models: This current study mainly employed transwell migration assay to study NK cell migration in response to PIP. Intravital imaging was not extensively used and other models like microfluidic systems could be used to further study PIP's effect on NK cell migration. With these additional models, an in-depth study of different aspects of NK cell migratory behavior will provide a better understanding of PIP's role in promoting NK cell migration in vitro.

Elucidating PIP Receptors on NK Cells: The finding of this study has shown that PIP can promote NK cell migration. No PIP receptor has been characterized on NK cells and identifying the receptors through which PIP interacts with NK cells is critical. This could involve proteomic or genetic screening to discover potential surface receptors on NK cells that mediate PIP's effects. Understanding these interactions would provide insights into the specific signaling pathways involved in NK cell migration in response to PIP. It is possible that PIP could be influencing NK cell migration by signaling through PAMPs like Toll-like receptors. Examining the upstream signaling that integrates p38 MAPK, IκBα and p65 could potentially lead to the identification of the receptor(s) involved in PIP signaling in NK cells.

Investigating Intracellular Signaling Pathways Activated by PIP: Further studies should explore how PIP affects the intracellular signaling cascades within NK cells. For example, given the observed phosphorylation of p38 MAPK, IκBα and p65, studies could be conducted to confirm the phosphorylation of these molecules in response to PIP using western blots. It is also important to confirm whether PIP also influences other pathways which are involved in NK cell activation, survival, and cytotoxicity. This can also be done by western blotting, flow cytometry, or advanced imaging techniques to monitor pathway activation.

Assessing NK Cell Functional Changes in Response to PIP: Beyond migration, it is essential to evaluate whether recombinant PIP modulates other NK cell cytotoxic functions, cytokine production, or degranulation capabilities. This could involve the treatment of NK cells with PIP to measure NK cell degranulation and production of cytokines using ELISA or flow cytometry

Examining PIP's Effect on NK Cell Exhaustion or Immune Evasion: There was no difference in NK cell degranulation in response to rederived 4T1 cells from the breast and metastatic lungs (PIP and EV control) in vitro and an in vivo study showed reduced tumor in primary breast site but increased lung metastasis due to the presence of PIP. Thus, investigating whether PIP contributes to NK cell dysfunction or exhaustion, especially in metastatic environments like the lungs, is crucial. This would include studying the expression of exhaustion markers such as TIM-3, PD-1, or LAG-3 on NK cells exposed to PIP-expressing tumor cells using flow cytometry. These studies will clarify whether PIP impacts NK cell longevity and effectiveness over time.

Investigating PIP's Effect on NK Cell Trafficking In Vivo: Using in vivo models, such as NK cell-tracking studies with fluorescence-labeled NK cells, their infiltration into PIP-expressing tumors compared to controls can be visualized and quantified. This would help confirm whether PIP indeed enhances NK cell recruitment in a live organism and whether this leads to a sustained anti-tumor effect or temporary immune surveillance.

Investigating the Immunomodulatory Effects of PIP on Various Immune Cell Populations: To comprehensively assess the impact of PIP on immune cell function, studies will be conducted to evaluate its effects on dendritic cells (DCs), macrophages, B cells, T cells, and other immune cell subsets. Transwell migration assays and additional in vitro approaches will be employed to examine PIP's influence on the migratory capacity of DCs, macrophages, B cells, and T cells. Furthermore, these immune cells will be treated with PIP, and their functional responses; including

cytotoxic activity, cytokine production, and MHC-II expression will be analyzed. Using in vivo studies I will characterize immune cell infiltration within PIP-expressing tumors in both the primary site and metastatic lung lesions, utilizing flow cytometry and immunohistochemistry. Collectively, these investigations will elucidate the role of PIP in modulating immune cell dynamics and function.

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APPENDIX

Supplementary Data 1

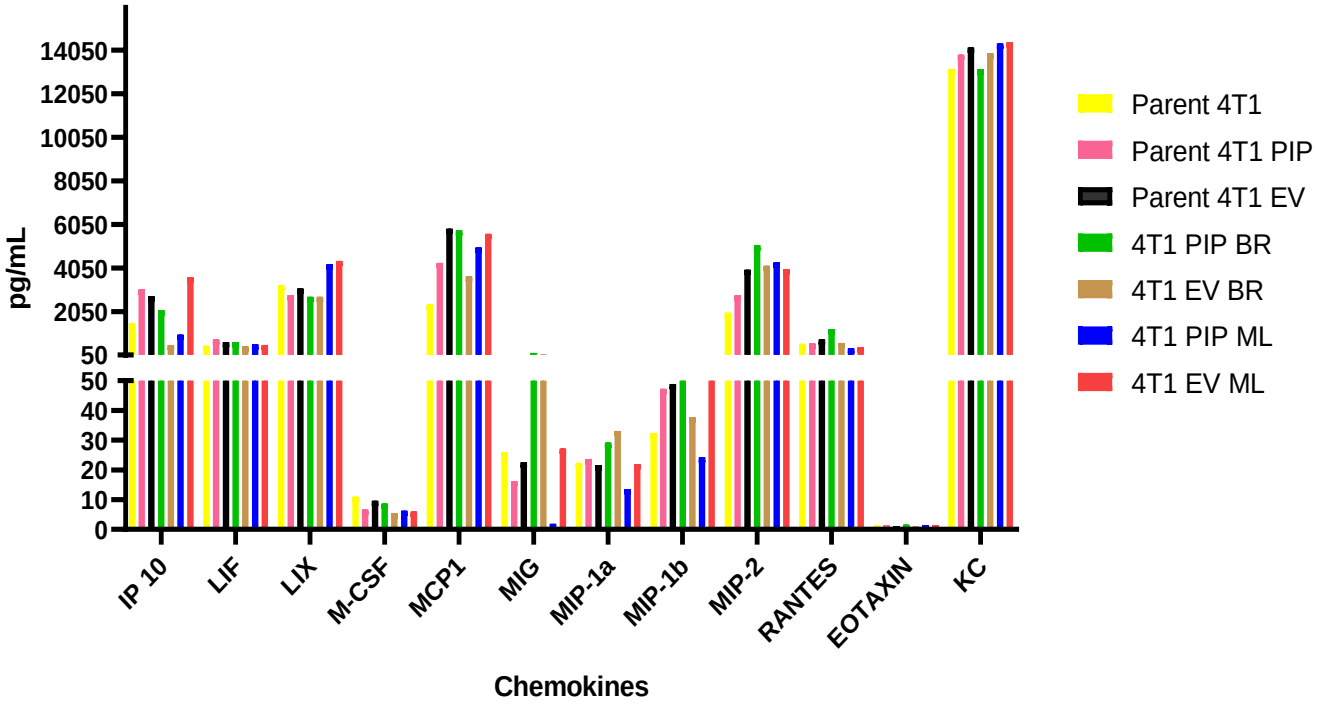


Fig S1: Analysis of chemokine profile of conditioned media from the 4T1 cell lines. Samples were analyzed) for Mouse Cytokine/Chemokine 32-Plex Discovery Assay® Array (MD32).

Supplementary Data 2

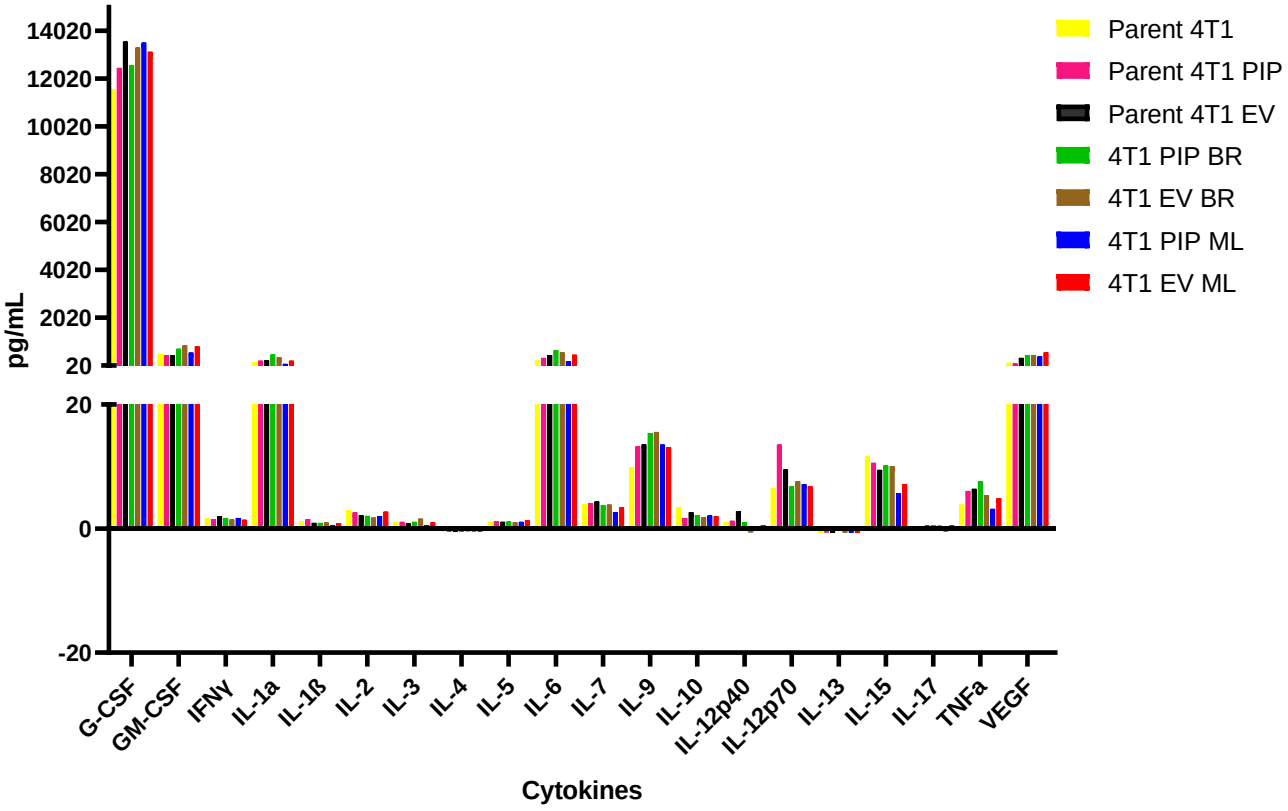
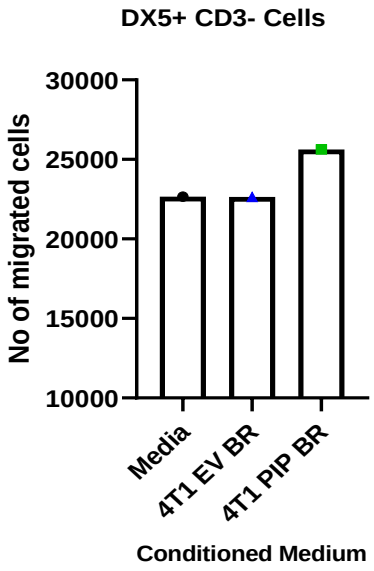


Fig S2: Analysis of cytokine profile of conditioned media from the 4T1 cell lines. Samples were analyzed) for Mouse Cytokine/Chemokine 32-Plex Discovery Assay® Array (MD32).

Supplementary Data 3

A



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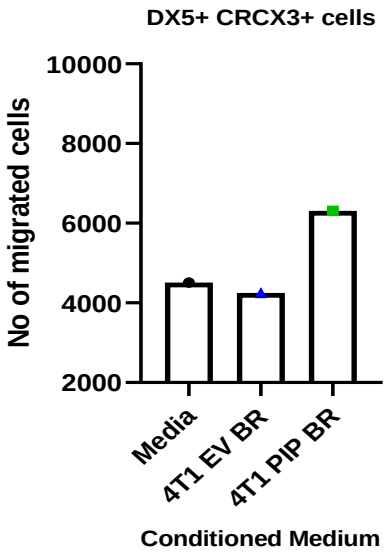

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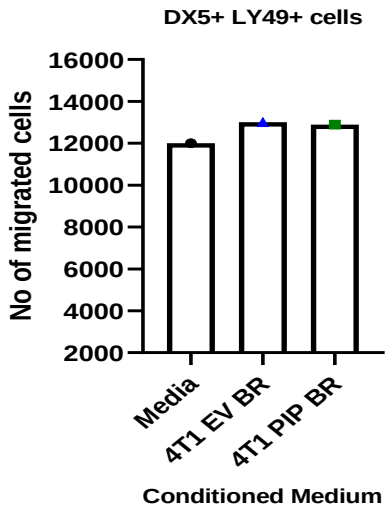

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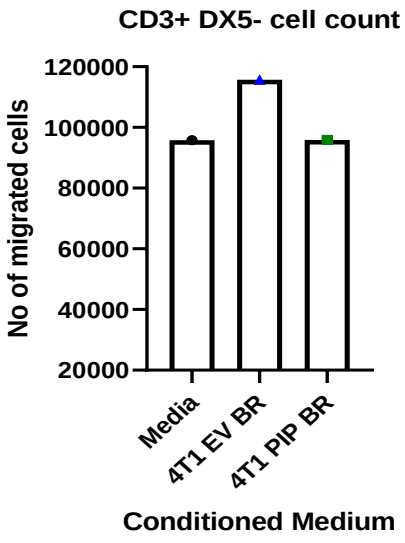


Fig S3. Condition media from PIP expressing 4T1 cells promoted NK cell migration in Day 0 splenocyte. Day 0 whole splenocytes were used in a transwell migration assay to assess migration. (A). Showing number of migrated DX5+ cells. (B). Represents the number of migrated DX5+ CXCR3+ cells. (C). Represents the number of migrated DX5+ LY49+ cells. (D) Showing number of migrated CD3+T cells.

Supplementary Data 4

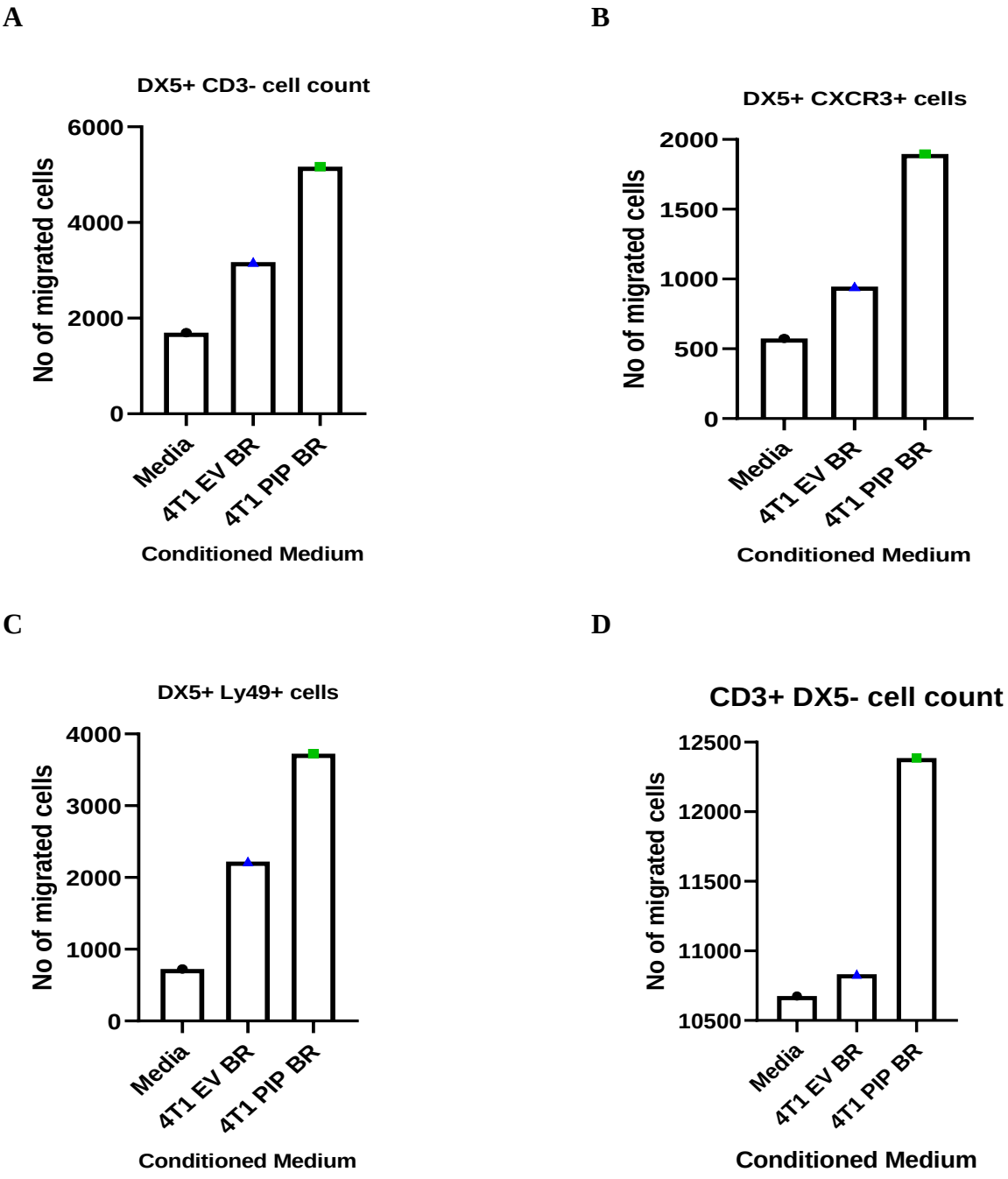


Fig. S4. Condition media from PIP expressing 4T1 cells promoted more Day 4 splenocyte migration. Day 4 whole splenocytes was used in a transwell migration assay to assess migration. (A). Showing number of migrated NK cells DX5+ CD3-). (B). Represents the number of migrated DX5+ CXCR3+ cells. (C). Represents the number of migrated DX5+ LY49+ cells. (D) Showing number of migrated T cells (CD3+ DX5-).

Supplementary Data 5

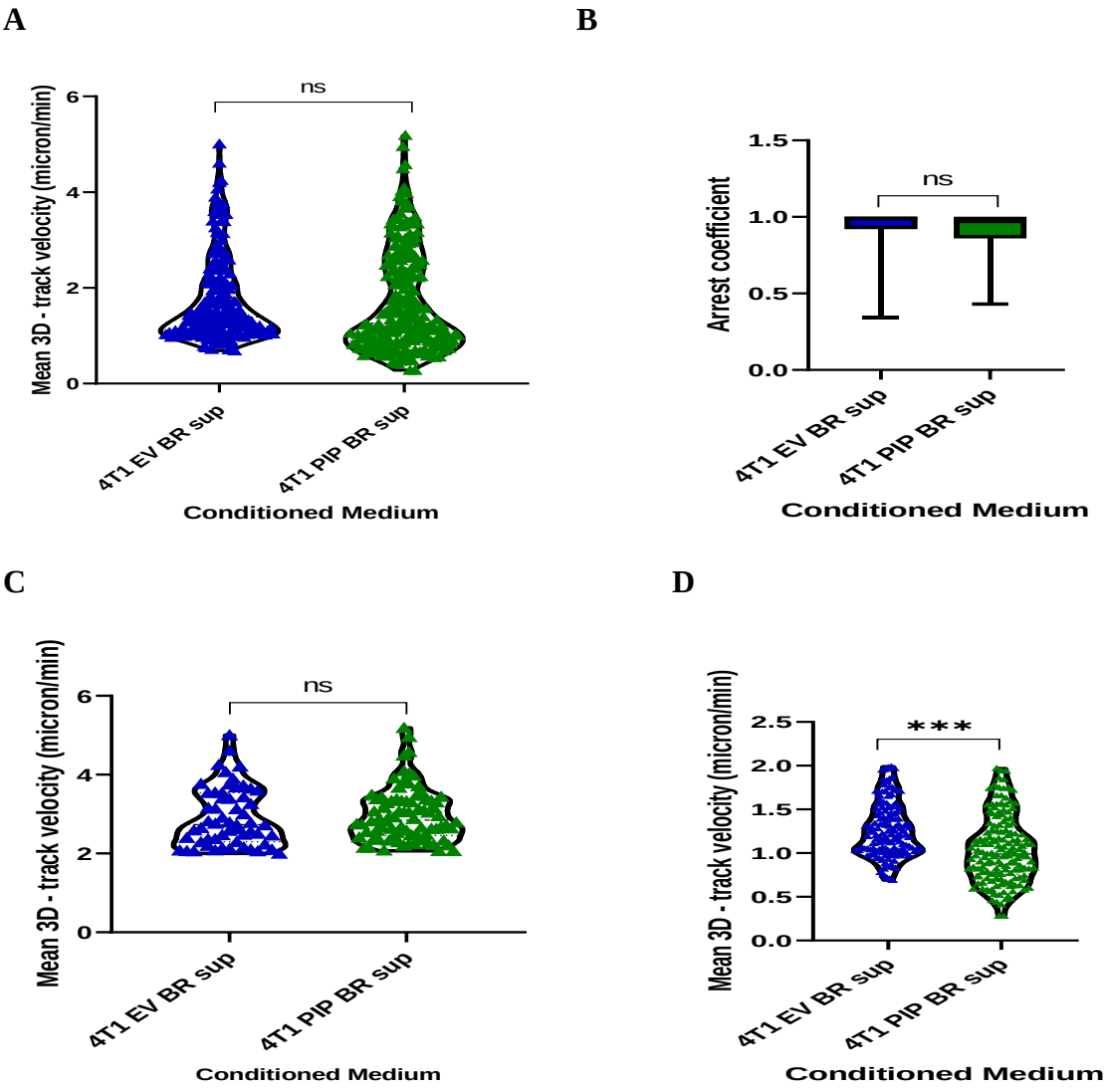


Fig S5. NK cells in conditioned media from PIP expressing 4T1 rederived from the breast showed lower arrest coefficient and higher mean 3D track velocity compared to conditioned media from EV control. Day 4 NK cells were used in a 3D collagen chamber to assess migration. (A). Showing result of overall mean 3D track velocity. (B). Showing result of overall arrest coefficient. (C) Arbitrary cutoff of tracking velocity by scores >/= 2. (D) Arbitrary cutoff of tracking velocity by scores <2. n-value = 1, ns = not significant, ***$p \leq 0.001$, unpaired t-test.

Supplementary Data 6

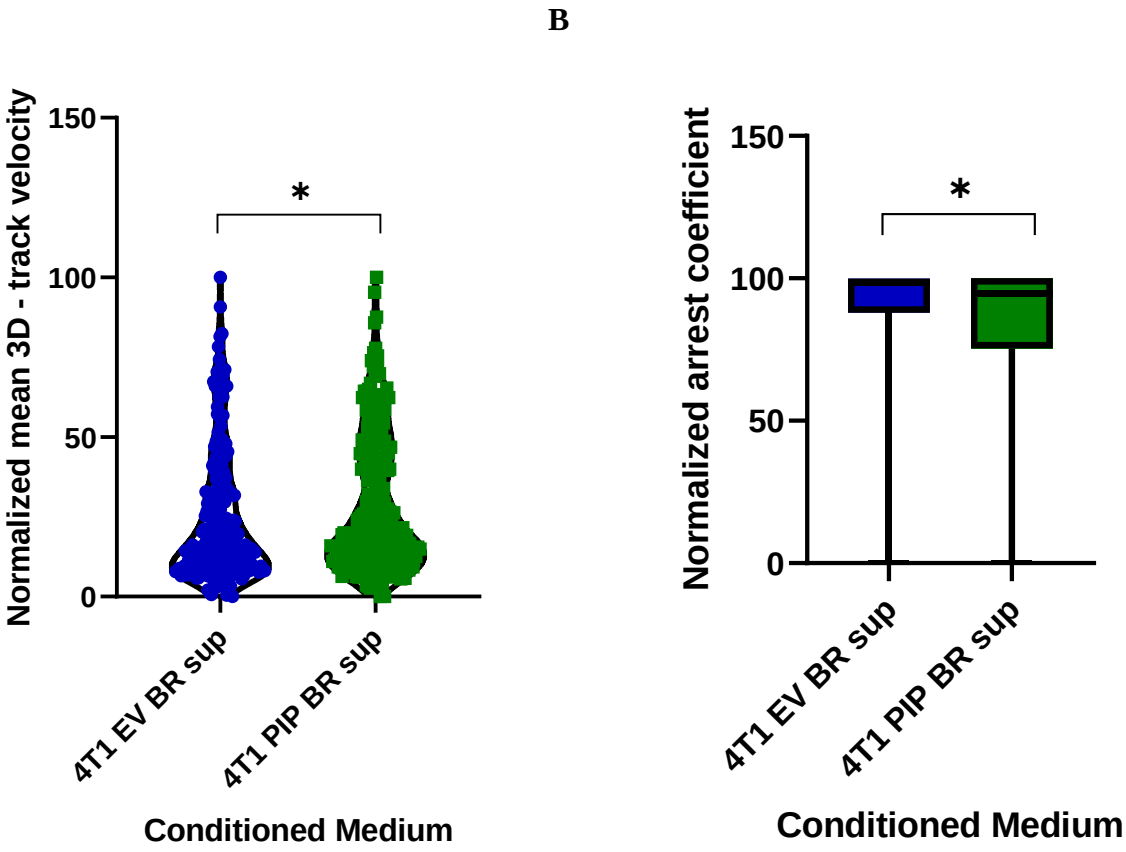
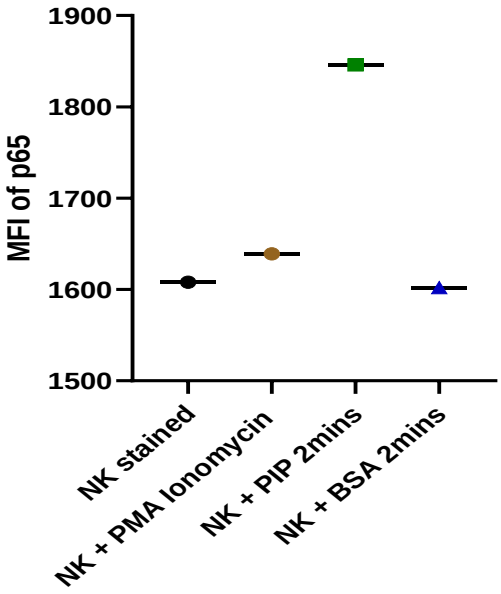


Fig S6. Normalized result of NK cell migratory behaviour in conditioned media from PIP or EV expressing 4T1 rederived from the breast showing lower arrest coefficient and higher mean 3D track velocity compared to conditioned media from EV control. Day 4 NK cells were used in a 3D collagen chamber to assess migration. (A). Showing result of normalized mean 3D track velocity. (B). Showing result of normalized arrest coefficient. n-value = 1, ns = not significant, *$p \leq 0.05$, unpaired t-test unpaired t-test.

Supplementary Data 7

A



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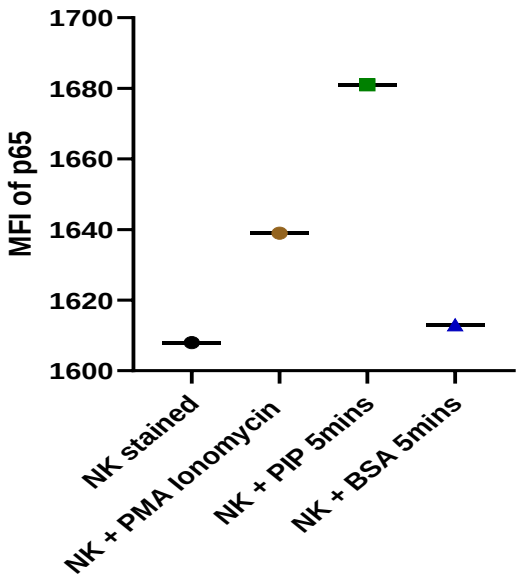


Fig S7. PIP phosphorylated the NF-kappa-B p65 subunit. Day 4 purified NK cells activated with IL2 were treated with different stimulants. (A). Showing mean fluorescence intensity of p65 phosphorylation at 2mins (B). Showing mean fluorescence intensity of p65 phosphorylation at 5mins.

Supplementary Data 8

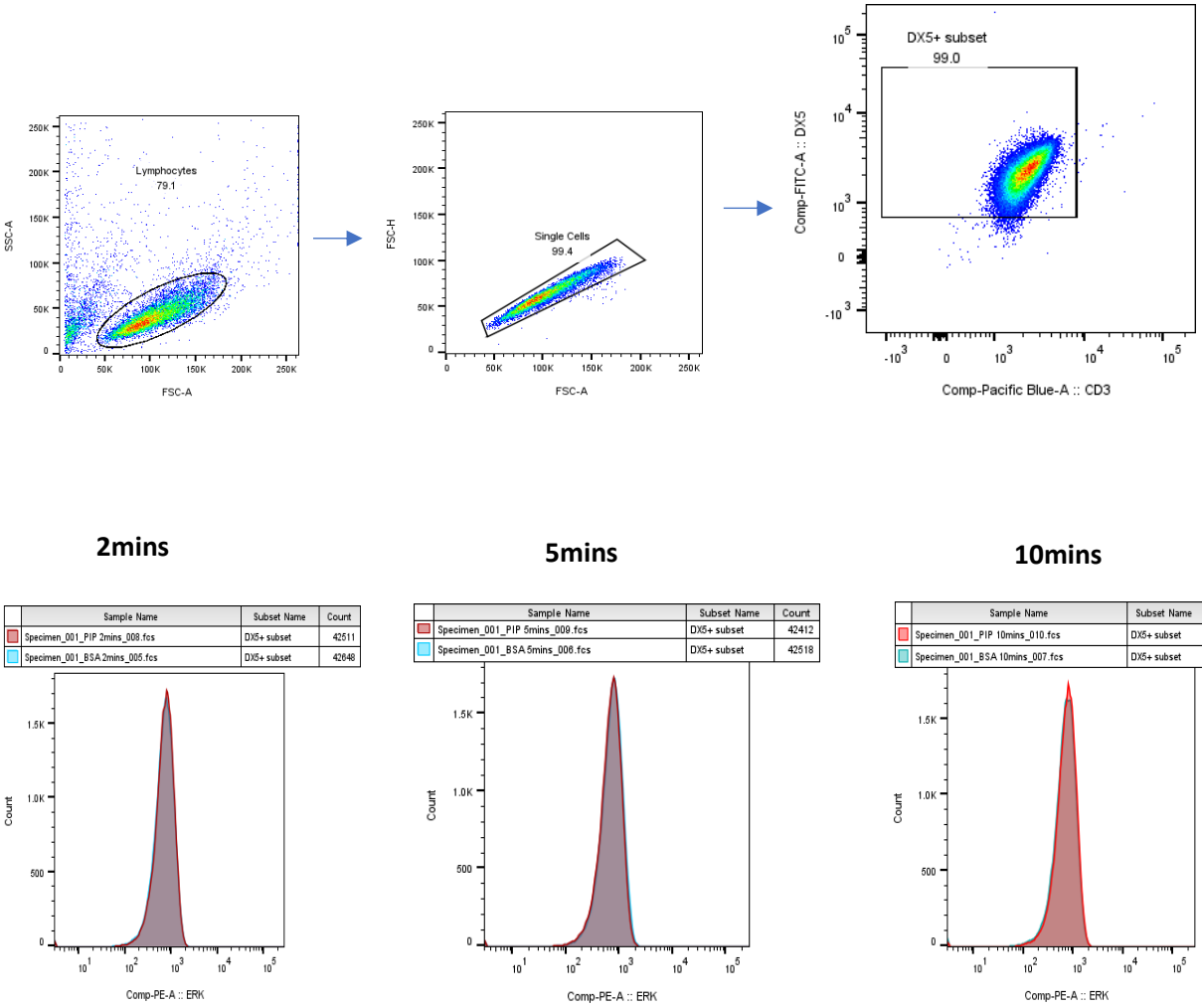


Fig S8: Representative figure of ERK expression on NK cells treated with PIP and BSA. Day 4 NK cells were treated with 1ug of PIP or BSA at different time points. PMA/ionomycin and untreated NK cells served as controls.

Supplementary Data 9

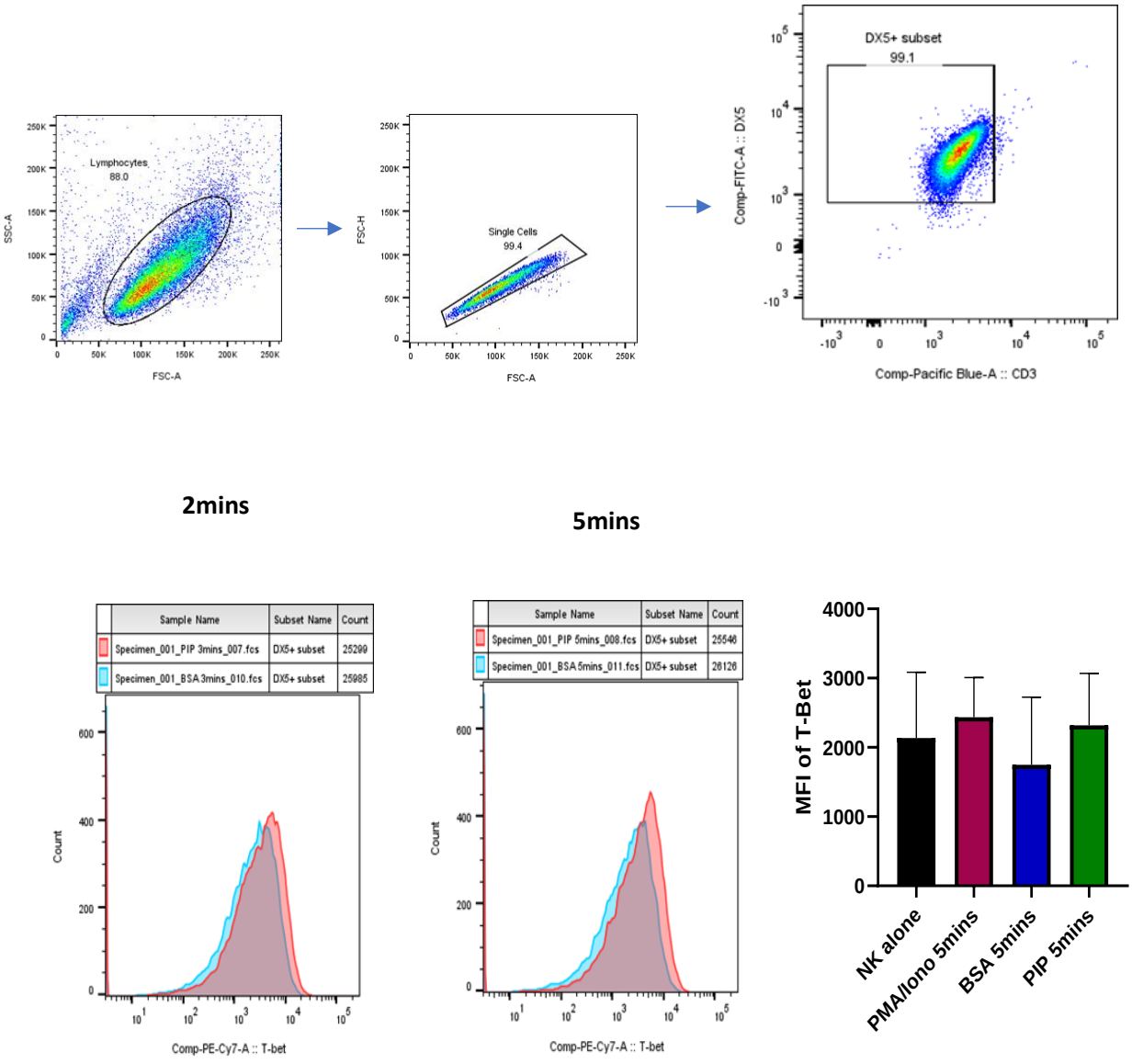


Fig S9: Representative figure of T-bet expression on NK cells treated with PIP and BSA. Day 4 NK cells were treated with 1ug of PIP or BSA at different time points. PMA/ionomycin and untreated NK cells served as controls.

Supplementary Data 10

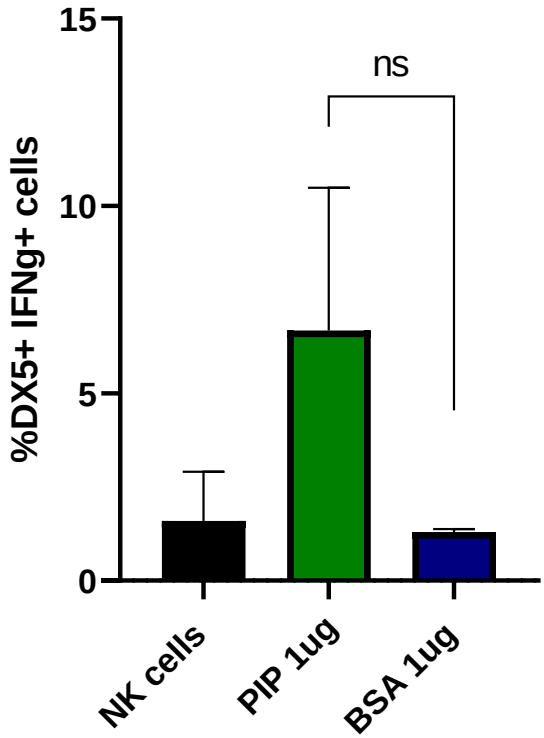


Fig S10. Direct PIP treatment of NK cells induced the production of IFN-γ compared to BSA treatment and untreated control. NK cells were treated with 1ug of PIP or BSA and cultured for 6hours and the percentage of IFN-γ producing NK cells was assessed by flow cytometry. N-value = 3, ns = not significant, paired t-test.

Supplementary Data 11

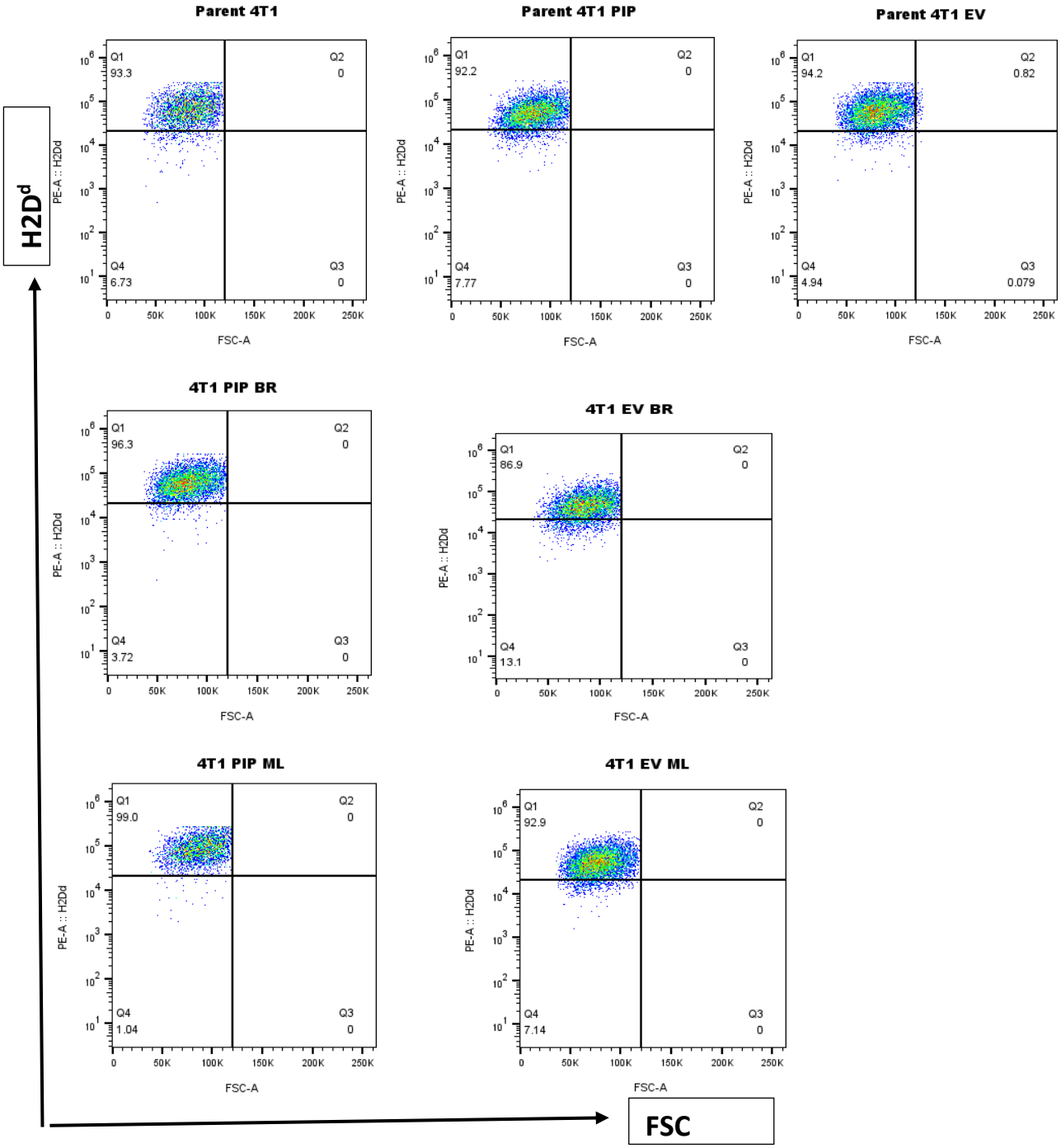
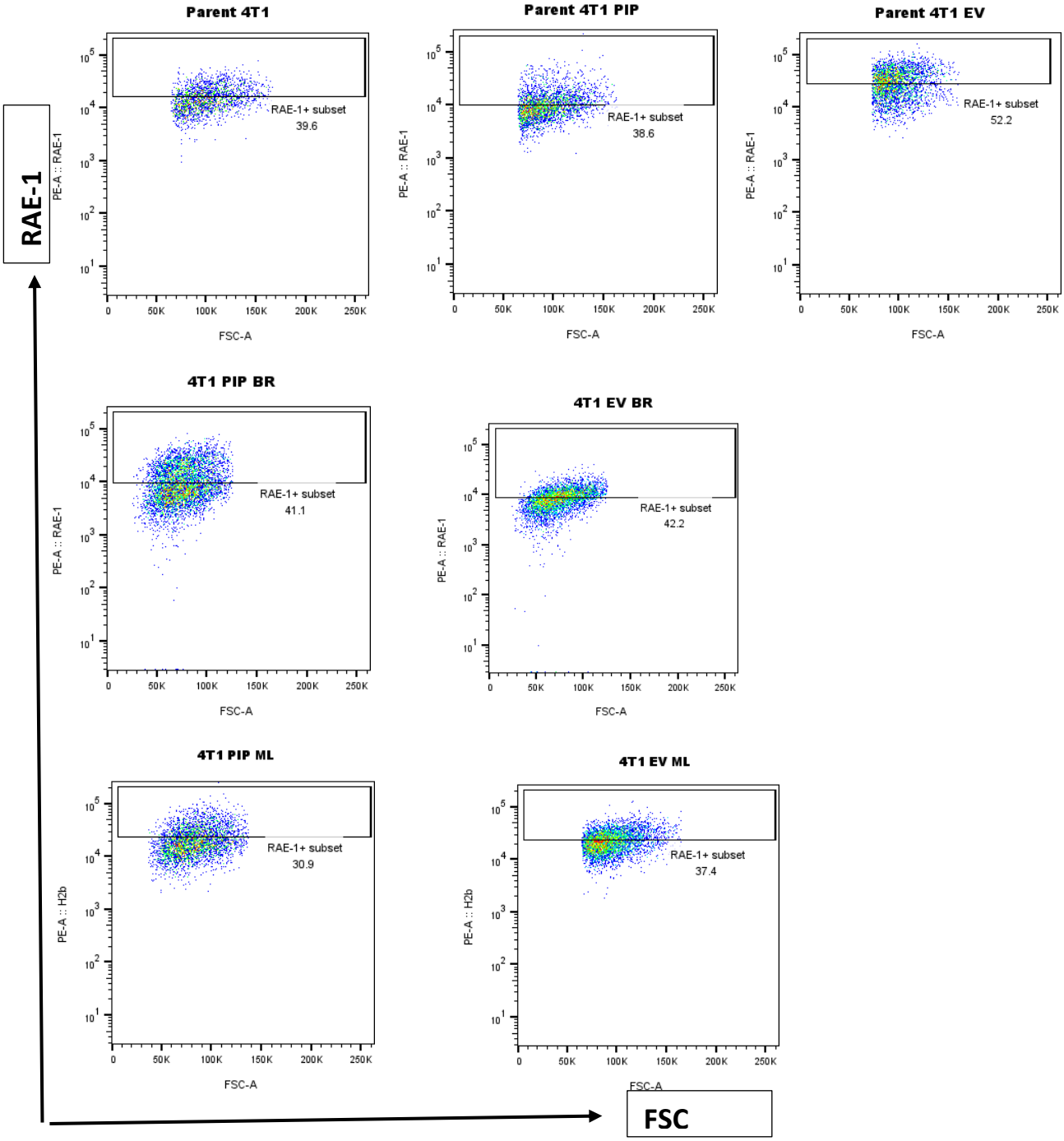


Fig S11. Representative figure of H2D^d expression by 4T1 cell lines. 4T1 cells were harvested and stained with antibody to detect for H2D^d (MHC class 1) by flow.

Supplementary Data 11



Suppl fig 11: Representative figure of RAE-1 expression by 4T1 cell lines. 4T1 cells were harvested and stained with antibody to detect for RAE-1 (a ligand for NKG2D) by flow.

Supplementary Data 12

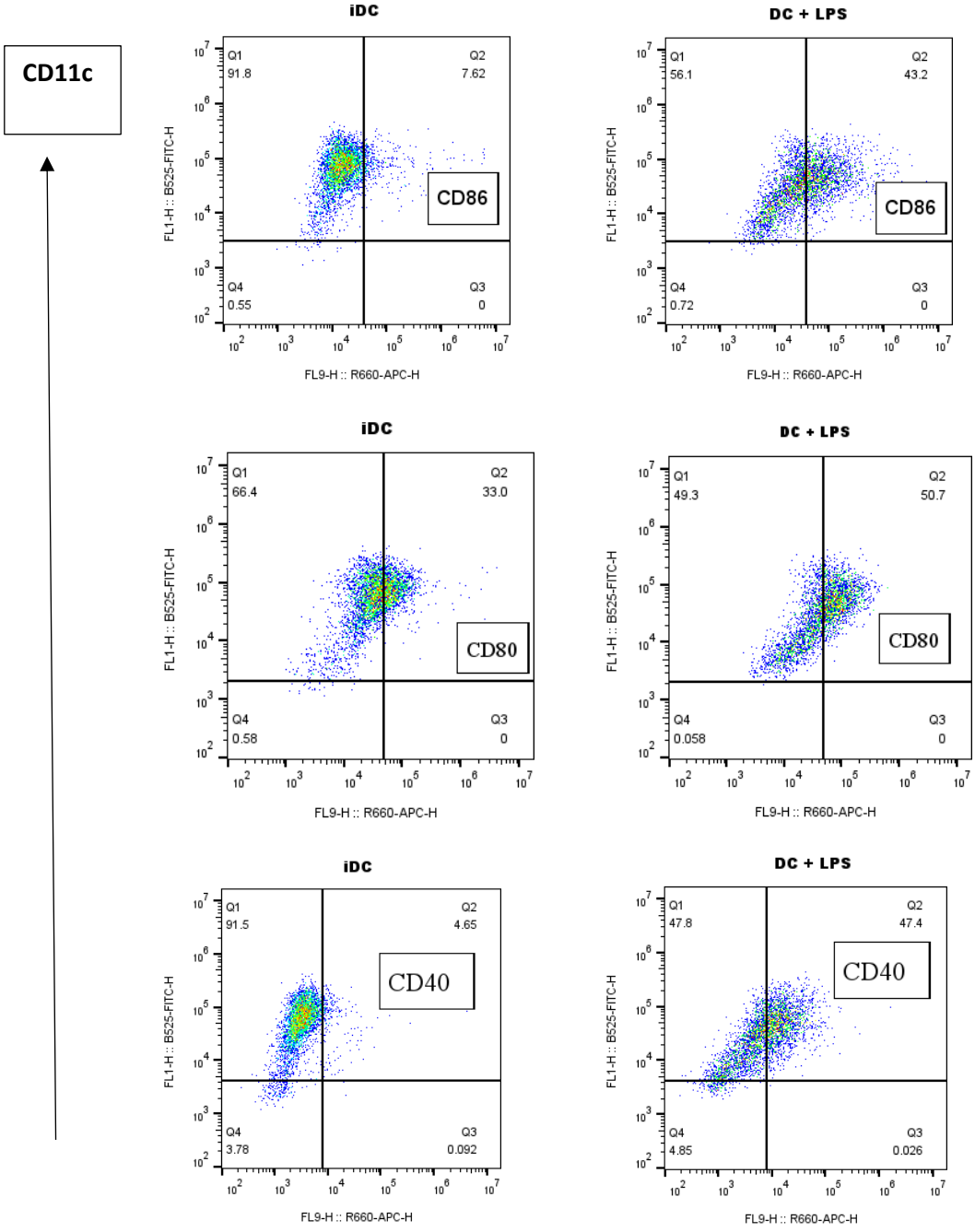


Fig S12: Representative Dendritic cells maturation analysis. BMDCs were cultured in GM-CSF medium. On day-8, lipopolysaccharide (LPS)- at 1µg/µl were introduced in the culture for 24-hours to acquire matured DC-phenotype. Surface staining was used to detect the expression of CD40, CD80 and CD86 surface markers, which represent matured-DC phenotype. In contrary to immature DCs (iDC), mature DCs with LPS up-regulated mature –DC marker.