

**Migratory Properties of IL-2 and IL-15 Activated Natural Killer
Cells in Dendritic Cells and tumor microenvironments**

by

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Abstract

Natural killer cells are bone marrow derived lymphocytes that play a key role in defense against infected or transformed cells. Upon direct target activation, NK cells mediate direct cytotoxic killing of target and production of cytokines and chemokines. NK activation and function can also be regulated by cytokines (such as IL-2, IL-12, IL-15 and IL-18) or Dendritic cells, DC, in the microenvironment. DC are professional Antigen Presenting cells, APC, that are important in linking the innate and adaptive immunity. Activated DC produces cytokines that specifically activate NK cells. Reciprocally, activated mature NK cells induce maturation and activation of immature DC. Such interactions are important in coordinating innate and adaptive immune response that shape anti-tumor and anti-microbial response in vivo. Impaired NK function and/or trafficking would therefore have a direct and/or indirect impact on the effective priming of anti-tumor immunity and cancer progression.

Recent work on Natural Killer (NK)-Dendritic cell (DC) crosstalk has focused on how NK-DC interaction can lead to NK activation, DC activation, apoptosis and effector functions. However, the way in which NK-DC interaction helps in NK cell recruitment has not been studied in detail. Good understanding of how NK-cell migratory properties are regulated in physiological and pathological microenvironments will provide further insights into the development of NK cell based therapeutic against cancer.

Using a conventional Trans-well assay and a microfluidic platform in vitro, we reported recently on the migratory properties of IL-2 activated NK cells in response to conditioned media, CM, from

immature or LPS-stimulated mature DC. Based on this report, I hypothesized that migration of cytokine activated NK cells is differentially regulated by the cytokine and/or DC activated by different Toll like Receptor Ligands (TLRLs) and tumor microenvironment.

Here, I compared the migratory properties of the IL-15 and IL-2-activated NK in the CM obtained from either immature or mature BMDC preparations that had been activated by different TLRLs (LPS, Pam3C, polyI:C, CpG) at its optimal concentration. I observed that IL-2 and IL-15 activated NK elicited weak chemotactic responses towards CM obtained from immature DC, iDC. Also, CM from all the tested TLRLs stimulated DC promoted chemotaxis of the IL-2 and IL-15 activated NK cells. However, CM from the CpG-activated DC elicit less NK chemotactic compared to the other TLRLs activated DC (Pam 3C-, LPS- or polyI:C activated DC). The CM from PIC stimulated DC elicit the strongest NK cell chemotaxis. Interestingly, I observed that the IL-15 activated NK migrated strongly towards the different CM compared to the IL-2 activated NK cells.

In summary, NK migration can be affected by DC activated with different TLR ligands and by the cytokine used to activate the NK cells.

Understanding the migration of cytokine activated NK cells in tumor settings will be relevant in the design of NK based immunotherapy for the treatment of cancer. So, I compared the migratory properties of the cytokine activated NK in tumor settings. I used CM from different tumor cell lines and compared the migration of the IL-2 NK with the IL-15 NK cells. I observed that IL-15 NK cells exhibited stronger chemotaxis response compared to the IL-2 NK cell in tumor settings. Using the MF platform, I found that IL-2 NK cells is repelled whilst IL-15 NK cells is attracted

towards the gradient of different tumor cell lines. I also observed that IL-2 NK migration is modulated indirectly by tumor cell lines via the NK-DC axis. Moreover, GM-CSF was identified as a factor involved in repelling IL-2 NK migration but not the IL-15 NK migration.

IL-15 activated NK cell may be a good candidate cell type in adoptive cell therapy of breast cancer or other malignancies.

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List of abbreviations

2-ME	2-Mercapthoethanol
4T1	Mouse 4T1 breast cancer model
Ab	Antibody
Ag	Antigen
AICD	Activation induced cell death
APC	Antigen Presenting Cell
B16	Mouse model for human melanoma
BMDC	Bone Marrow derived Dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CI	Chemotactic Index
CM	Conditioned Medium/Media
CpG	CpG oligodeoxynucleotides
DC	Dendritic Cell
dsRNAs	Double-stranded RNAs
E0771	Murine breast cancer cell line
ELISA	Enzyme linked immunosorbent Assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum

GM-CSF	Granulocyte macrophage colony stimulatory factor
HEV	High endothelial venule
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
Hsp	Heat shock protein
Ig	Immunoglobulin
IL	Interleukin
Ip-10	Interferon gamma induced protein
ITAM	Immunoreceptor tyrosine based activating motif
ITIM	Immunoreceptor tyrosine based activating motif
KIR	Killer cell immunoglobulin like receptor
LPS	Lipopolysaccharide
MHC	Major histocompatibility Complex
MMC	Mouse Mammary Cell
MF	Microfluidic
NCR	Natural cytotoxicity receptor
NK	Natural killer

NKG2	Natural killer group 2
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffer solution
PIC	Polyinosinic:Polycytidylic acid
PSG	Penicillin Streptomycin Glutamine
PRR	Pattern Recognition Receptor
ICAM-1	Intercellular adhesion molecule type 1
LIR	Leukocyte immunoglobulin like receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MIPs	Macrophage inflammatory proteins
miRNAs	MicroRNAs
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RANTES	Regulated and normal T cell expressed and secreted
RBC	Red blood cells

Rpm	Revolution per minutes
RPMI	Roswell Park Memorial Institute
shRNA	Short-hairpin RNA
siRNAs	Small interfering RNAs
SLAM	Signaling lymphocytic Activation Molecule
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor alpha
TLR	Toll like receptor
TLRL(s)	Toll like Receptor Ligand(s)
TW	Transwell
VCAM-1	Vascular adhesion molecule type 1
WAS(P)	Wiskott–Aldrich syndrome (protein)

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Chapter 1 Introduction

Multicellular organisms possess a variety of organ systems composed of cells, tissues and organs specialized to carry vital functions of the organism. One such vital organ system is the immune system. The immune system protects multicellular organisms against disease. The Immune system is divided into the innate and adaptive immune system (1, 2). Disorder in the immune system can result in pathological conditions such as immunodeficiency, cancer, autoimmune and inflammatory disease. The response from the Immune system can be classified into humoral or cell mediated immunity.

In the 60s and the early 70s, the established dogma on cell mediated immunity was that cell mediated immunity was due to sensitized T cells (3). At the time there were well defined and established methods to assay for cell mediated immunity. Work on in vitro cell mediated immunity consistently observed reactivity against cancer cells without prior sensitization (4-8). This contradicted with the established model on cell mediated response at the time. During that time, artifacts or limitations of the assays were suggested as possible reasons for this unexpected reactivity and these suggestions were later proven to be wrong (3). A novel cell type with natural cytotoxicity was born and was then identified to be the reason for the reactivity observed. These identified cells were named functionally because of their natural cytotoxicity towards target as Natural Killer, NK, cells. NK cells as a novel cell type of separate lineage was discovered. In addition to mice and humans, NK cells were also discovered in other species.

In the same decade, work by a different group from the Rockefeller University identified another novel cell type called Dendritic cell, DC (9).

1.1 Natural killer cells

NK cells are bone marrow derived large granular lymphocytes that are involved in immune response to microbial infection (or pathogen), tumor, autoimmunity and pregnancy (10-15). NK cells are the 3rd member of the lymphocytes family of immune cells (16). They are present throughout the body from lymphoid to non-lymphoid organs (17, 18). They are only a small population of cells. In the blood, NK cells make up about 10% of lymphocytes (19). NK cells are different from the other lymphocytes members. NK cells are fast responders to infection or tumor formation. Unlike B cells or T cells, NK cells express neither the B cell receptor, BcR, nor the T cell receptor, TcR (20). Also, B and T cells are adaptive lymphoid cells whilst NK cells are innate lymphoid cells. Nonetheless, NK cell cytotoxic function is similar to that of the cytotoxic T lymphocytes, CTL.

NK cells were identified and named about 40 years ago because of their natural ability to kill tumor cells without prior activation. Yet, there are fractions of NK cells from the spleen or blood that are not cytotoxic (21). The initial functional association of NK cell was killing but over the past 4 decades since NK cell discovery, the functions of NK cells have been expanded to not only include killing but to also include cytokine production, immune regulation, shaping of both arms of the immune response through crosstalk with other immune cells, tumor surveillance and pregnancy (22-24). Also, the classification of NK cells as innate cells is currently being challenged because of recently discovered properties of NK cells that are considered hallmarks of the adaptive immune response. For example, NK exhibition of memory like properties and NK expression of receptors that can specifically recognize components such as viral HA (25-28).

1.1.1 NK development

NK cells develop in the bone marrow in response to both intrinsic cues – genetic and extrinsic cues – cytokines (29). Ablation of the Bone Marrow, BM, environment has been shown to drastically affect NK development, homeostasis and function. Also, the BM is enriched in NK cell precursors and data supporting the acquisition of multiple NK cell properties such as NK cell receptors and NK education in the BM supports the BM as the site of NK cell development. Despite this, other site such as the thymus and the LNs has been suggested to play a role in NK development (30, 31). In a study with impaired T cell development in the thymus, a high number of NK was shown to develop in the thymus (31, 32). There are data supporting the presence of NK precursors in sites other than the BM. In addition, the liver contains immature NK that can undergo further maturation (33). But, these findings and other findings have suggested that NK cells can undergo further development and even education outside the BM but the BM is the primary site of NK cells development. NK cells precursors can egress from the BM and continue further development in the other sites.

NK cell development is a series of steps defined by various developmental stages involving the acquisition of effector functions, education, upregulation or downregulation of specific receptors, response to certain chemokines or cytokines and signaling (32). NK cells are derived from the CD34⁺ hematopoietic progenitor cells in the bone marrow (32, 34). The common lymphoid progenitor, CLP, is a precursor of NK cells. Another developmental stage in NK cell development is the NKP. Unlike the CLP, NKP is defined as a precursor with the potential to develop to NK cells but not to other lymphocytes. This stage in NK cell development is downstream of the CLP. These developmental stages lack classical NK cell identifying markers. For example, NKP from

C57BL/6 lack NK1.1 and CD49b (35). Further development of NK cell endows it with the typical NK cell markers. The acquisition of NK cells receptors occurs in a sequentially manner (36). Developing NK cells acquire other properties and subsequently develops into immature NK cell. The immature NK cell undergoes further development including education eventually resulting in mature NK cells with full functional competency. The mature NK cell exhibit a high level of cytotoxic and cytokine production potential. Immature NK cells were previously assumed to be non-functional but recent findings point to immature NK cell exhibiting some functional properties but this is less clear compared to the mature NK cell. Mature NK cells is not a homogenous population of cells but a heterogeneous population.

1.1.2 NK subtypes

NK cells are a heterogeneous population of cells that have been classified into different subtypes based on the expression of some cell surface receptors or on tissue localizations or other criteria. These NK cells subsets exhibit different homing, functional and phenotypic properties.

1.1.2.1 In Humans

Human NK cells have been classified into 2 major subtypes based on the surface expression of the neural cell adhesion molecule, CD56 (18, 23, 37). CD56 also called NCAM is a surface glycoprotein that is part of the Ig superfamily. CD56 is expressed on non-immune cells such as neurons and muscle cells and as well as on immune cells such as NK cells. The NK cell subtypes based on the CD56 classification are CD56^{bright} and CD56^{dim} subtypes (38). These subtypes possess

different phenotypic and functional properties and also exhibit different tissue localization or homing properties (18, 39).

CD56^{bright} subtype is the major population of NK in the lymph node, tonsils, uterus and lungs (18, 40). In the lymphoid tissues, the CD56^{bright} subtype have been shown to play a regulatory role in the initiation and shaping of the adaptive response. In the uterus, they play an important part in angiogenesis during pregnancy (41). CD56^{bright} NK cells are less efficient killers but they are more potent cytokine and chemokine producers (18). CD56^{bright} NK lack the expression of killer cell immunoglobulin-like receptor, KIR. They express the CCR7 that facilitate their preferential homing to the secondary lymphoid tissues, SLT.

CD56^{dim} subset is the major population of NK cells in the peripheral blood making up about 90% of the NK cell population (18, 37). CD56^{dim} NK are potent killers and mediate Antibody dependent cellular cytotoxicity, ADCC, via CD16. CD56^{dim} NK cell express a high level of KIR. They do exhibit a low proliferation potential compared to the CD56^{bright} subtype. They express CXCR1, CX3CR1, Chem23 that facilitate their recruitment to inflamed sites.

A controversial or unclear or debatable issue between these 2 NK cell subtypes is their developmental relationship (42, 43). Some studies have suggested that there is a developmental relationship between the 2 NK subtypes in which the CD56^{bright} NK cells are the less matured subtype and precursor to the CD56^{dim} NK cells (43-45). Conversely, the CD56^{dim} NK cells are the more matured subtype derived from the CD56^{bright} subtype. Romagnai et al. demonstrated that the CD56^{bright} NK cells can acquire the phenotypic signatures of the CD56^{dim} NK (43). It has been

reported that in an adoptive transfer experiment, the CD56^{bright} subset is first observed before the CD56^{dim} subset and moreover, the CD56^{bright} subtype decrease with time whilst the CD56^{dim} increase over time (42, 46). In addition, there is data showing that steady state LN NK cells are predominantly CD56^{bright} NK cells whilst inflamed lymph node, LN, has NK cells with phenotype of the CD56^{dim} subtype exiting the inflamed LN (43). Yet, other studies have suggested that these 2 NK cells subsets are not differentially related. The in-vitro activation of the CD56^{dim} with cytokines such as IL-2 increases the CD56 expression on this population. Cytokine activation of the CD56^{bright} can produce CD56^{bright} with potent cytotoxicity.

1.1.2.2 In Mice

Because mice lack the expression of CD56, murine NK cells classification is based on the CD27 surface expression instead of the CD56 expression (40, 47). On the other hand, human NK cells also express CD27 but there is a differential level of the CD27 expression between mice and humans. The CD27 expression has been recently used to classify human NK cells so as to correlate the murine NK classification with the human NK classification. Nevertheless, the major classification of human NK is still based on the CD56 expression. So the direct reconciliation of the mouse and human NK findings between these subtypes can be difficult (40). Yet, there are still similarities between the subtypes in mouse (based on the CD27 classification) and the subtypes in human (based on CD56 classification) (48). The major subtypes of NK cells in mice are the CD27⁺ and CD27⁻ (49, 50).

CD27+ is the major NK cell subtype in secondary lymphoid tissues, SLT. They exhibit high proliferative potential, high cytokine production but low cytotoxicity and low expression of inhibitory receptors.

CD27- is the dominant NK cell subtype in the peripheral blood and spleen. They express a higher inhibitory receptor and higher cytotoxic potential but are lower cytokine producers. They exhibit a more mature phenotype and a lower proliferation compared to the CD27+ subtype.

The CD27- NK subtype is a stable population after an in vivo transfer of CD27- NK cells. On the other hand, the transfer of CD27+ NK cells is followed by differentiation into the CD27- NK cells subtype. This suggest that the CD27- NK cell subtype is the more matured and terminally differentiated subtype (49).

In addition to the surface markers on NK cells that are employed in the classification of NK cells into the different subtypes, NK cells also express other surface receptors.

1.1.3 NK cell Receptor

Unlike the other lymphocytes derived from the common lymphoid progenitor that express either TcR (on T cells) or BcR (on B cells) that undergo somatic rearrangement and fine tuning, NK cell express a battery of redundant germline-encoded receptors that do not undergo somatic rearrangement (16, 20, 35). NK cell effector function is regulated by these germline-encoded receptors on NK cell surface (21, 51). These germline-encoded receptors on NK cells are classified

into either activating receptors or inhibitory receptors depending on whether they can deliver activating or inhibiting signals respectively (52). The expression of the inhibitory receptors on the NK cells is a stochastic event that can lead to difference in expression between individual NK cells within a given strain or between NK cells from the same individual (36, 47, 53). Some NK cells lack inhibitory receptor expression whilst other NK express single to multiple inhibitory receptors (53).

The Dynamic equilibrium hypothesis proposed to define and help understand how the activation of NK cell is regulated (18, 54). The functional fate of NK cell is determined by the integration of signals from both the activating and the inhibitory receptors (55). The integration could result in either NK activation or inhibition depending on which of the signals is dominant. For example, if signaling from the inhibitory receptor dominates, then the resulting response is the inhibition of NK effector function. On the other hand, if the signal from the activating receptors dominates then the resulting response is activation of NK effector function.

Activating receptors: Unlike T and B cells that rely on a single dominant antigenic receptor to determine their activation, NK cells do not rely on a single dominant receptor but instead rely on a vast array of activating receptors (56, 57). The activating receptors possess short cytoplasmic tail that interact via an electrostatic force with chains containing immunoreceptor tyrosine-based activation motif, ITAM or other activating motifs (52). The electrostatic force between the short cytoplasmic tail of the activating receptor and the intracellular activating motifs allows for the association of these 2 domains and this association fosters the initiation of downstream activating signals when the extracellular domain of the receptor is engaged by its cognate ligand (36).

Engagement of the activating receptor activates downstream signaling pathways through the associated activating motif. Ligands for activating receptors range from pathogenic molecules such as viral HA and m157 to stress induced ligands such as MIC A/B and ULBP (58, 59).

Inhibitory Receptors: This class of germline-encoded receptors expressed on NK cell prevents the attack of healthy self-cells by NK cells. The inhibitory receptors also play a role in NK cell education and NK cell reactivity. The inhibitory receptors recognize constitutively expressed molecules on self-cells such as the classical or non-classical MHC class I molecules. MHC class I molecules are expressed on all nucleated cells. The binding of the MHC class I on self-cell to the inhibitory receptors on the NK cell prevent NK cell activation and autoreactivity by NK cells. The loss of the MHC class I renders a cell susceptible to NK cell cytolysis. Inhibitory receptors signal through the ITIM in their long cytoplasmic tail. Engagement of the inhibitory receptors cause phosphorylation of the immunoreceptor tyrosine-based inhibition motif, ITIM, that result in phosphatase recruitment and the eventual downstream dephosphorylation of key signaling molecules involved in NK activation (56). For example, signaling through inhibitory receptors prevent the activation of Vav1 and consequently prevent NK activation.

Examples of these germline-encoded receptors expressed on NK cell surface are

- **Ly49** – Highly polymorphic homodimers that are part of the C-type lectin family (60, 61). They are present in mice but absent in humans except for one pseudogenic Ly49. They are the receptors for the classical MHC class I molecules. Ly49 have both activating and

inhibitory isoforms (61, 62). The activating members signal through ITAM whilst the inhibitory members signal through ITIM.

- **KIR** – Highly polymorphic group of receptors that are part of the Ig superfamily (63). They are present in humans but absent in mice. They recognize the MHC class I molecules. They are structurally different from the Ly49 in mice. Nevertheless, they are the functional homologs of the Ly49 in mice (60). Like the Ly49 family, the KIR family have both activating and inhibitory isoforms. The activating members signal through the DAP-12 associated with the cytoplasmic tail of the activating receptor. The inhibitory isoforms recognize classical HLA class I molecules (HLA-A, -B, -C) and signal through the ITIM on the cytoplasmic tail of the inhibitory receptor upon ligand binding. Part of the nomenclature of the KIR isoforms is also determined by the length of the cytoplasmic tail (64). For example, KIR with short tails have S incorporated into their names whilst those with long tails have L in their names. The long tails isoforms have ITIM in their cytoplasmic tails whilst the short tails isoforms do not have an ITIM but instead are associated with DAP-12.
- **NCR** – type I membrane proteins that belong to the Ig superfamily. These receptors include NKp46, NKp44 and NKp30 (65). They have a short cytoplasmic tail that associate with ITAM containing adaptor protein. The Engagement of the NCR receptor results in the phosphorylation of ITAM and subsequent downstream signaling cascade that activate the NK cell. NCR play a role in NK tumor lysis, controlling viral infection, cytokine

production and NK-DC crosstalk. Ligands for NCR range from tumor antigen to viral peptides.

- **CD94/NKG2** – part of the C-type lectin family and present in both mice and humans (66). Compared to the KIR or the Ly49, CD94/NKG2 have limited polymorphism. They are heterodimers made up of the CD94 subunit and one of either member of the NKG2 - NKG2A, NKG2B, NKG2C, NKG2E or NKG2H. Like the KIR and Ly49, the CD94/NKG2 family of receptors have both activating and inhibitory members. The activating members associate with DAP-12 in their cytoplasmic tail whilst the inhibitory members have an ITIM. Some ligands for these receptors are the non-classical MHC class I molecules. Examples are HLA-E in human and Qa-1 in mice. They have been suggested to play a part in transplantation.
- **CD16 (Fc-γ-RIII)** – binds with a low affinity to the Fc region of IgG and plays an important role in mediating ADCC by NK cells (37, 38, 67). Engagement of the CD16 and the Fc region of the Ab initiate downstream activation cascade. The relevance of this mechanism of cytotoxicity is being currently exploited to treat certain malignancies. For example, the use of Cetuximab (chimeric monoclonal Ab) in the treatment of colorectal cancer, head and neck cancer and other malignancies.
- **NKG2D** – Homodimer present in both mice and humans. NKG2D, a C-type lectin, binds to induced self-ligands that get upregulated during cellular transformation from a healthy to an unhealthy cell (66). Ligands for NKG2D are MIC A/B, ULBP etc. NKG2D

engagement signal through DAP10 or DAP12 (68, 69). They are important in NK control of some cancers - in the recognition and lysis of some tumors.

- **SLAM** – important in recognition of hematopoietic cells. They contain multiple ITSM that allows for the recruitment of either activating or inhibiting SH2 containing proteins (70). Examples are CD48, 2B4. They are important in the killing of hematopoietic cells by NK cells. Some members of this group of receptors such as 2B4 are co-receptors since they can only trigger NK response when other receptors are engaged (21).
- **ILT or LIR** – they are only being recently discovered. They are part of the Ig superfamily. Relatively unknown family of receptors. The ligands for this group of receptors is still been investigated but they are known to bind to MHC class I molecules.

In addition to these receptors, NK cells express different TLRs such as TLR9/3/2 that are important in the recognition of pathogen associated molecular pattern, PAMP (71, 72). In vitro pulsing of NK with TLR ligands induce NK cytotoxicity and IFN- γ production. PIC, a TLR3 agonist activates NK cells. Furthermore, NK cells also express other surface receptors such as the chemokine receptors. The chemokine receptors and chemokines is discussed in the Migration section.

1.1.4 NK cell education

NK cell education describes the sum total processes during NK cell development that eventually determines NK cells level of responsiveness or functionality. NK cell education allows formation

of NK cells that can execute a useful response against unhealthy cells but prevent response against healthy self-cells.

NK cell education is based on the ability of NK to recognize self MHC class I molecules (73, 74). Inhibitory receptor for self-MHC class I molecules was initially taught to be the sole determinant of NK cell functionality during NK education. But recent findings have shown that recognition of self MHC class I molecules is not the only determinant of NK education. Studies have shown that activating receptors such as NKG2D can play a role in NK education.

The **at least one model** was initially proposed to explain NK education. This model proposed that NK cells needs to express at least one inhibitory receptor to prevent reactivity against self-cells. But the existence of NK without self-MHC class I receptor and the hyporesponsiveness of those NK cells suggest the possibility of other mechanisms or models to explain NK education. Some models that have been put forward to explain NK education are the following.

- **Licensing Model** – this model was proposed by Yokoyama et al. and it claims that NK cell are inactive by default and gain full functional competency after engagement of the receptor for self-MHC class I molecules (75). State of NK cell full functional competency is endowed upon NK cells after the NK cells inhibitory receptors are engaged. NK cell from MHC class I deficient mice or NK cells lacking receptors specific for the MHC class I molecules are hyporesponsive (76, 77).

- **Disarming model** – Proposed by Rault and his group claims that by default, NK cells are active and the chronic or continuous stimulation of the activating receptors without simultaneous engagement of the inhibitory receptors renders the NK cells hyporesponsive (76). Full functional competency of NK cell is attained if there is inhibitory signaling to counteract the activating signal (76, 78).
- **Rheostat Model** – Proposed to account for the variation on NK cell responsiveness with inhibitory signaling. In both human and mice, it has been demonstrated that there is a correlation between NK cell reactivity and inhibitory signaling. NK cell responsiveness increase with an increase in number of inhibitory receptors and inhibitory signals and decrease with decrease in inhibitory signaling. In short, there is more functional response in NK cells with higher surface inhibitory receptor expression (79). In addition, there is a quantitative tuning of NK cell functionality – stronger inhibitory interaction is correlated with strong reactivity (18). This model incorporates the previously described models (Licensing and Disarming models) to explain NK cell education. The responsiveness of NK cells is not an all or nothing or an on or off mode. Both the licensing and the disarming model fail to account for this fact that NK cell response or Education is neither an “all or nothing” nor an on/off event.

There are other models that are been put forward to help explain and understand NK education – the cis/trans presentation of MHC class I and its receptors (18) – currently there is conflicting evidence on this model, role of activating receptors and the involvement of other cell types etc. Conversely, in a knockout study of some NK activating receptors, Sheppard and el., reported no

effect on the frequency or function of the NK cells (80). Better understanding of NK cell education will be useful in understanding NK biology and in devising a better NK cell based immunotherapy.

Another aspects of NK cell responsiveness or NK cell education is the dynamic nature and the ability to re-set NK cell responsiveness in response to change in the microenvironment (53, 77). It has been shown that the adoptive transfer of NK cell to a new environment with altered MHC class I alters NK cell responsiveness. For example, transfer of full functionally competent NK cell from a WT MHC class I sufficient host that had previously undergone NK cell education into a MHC class I deficient host will transform the NK cell to a hyporesponsive state. Conversely, adoptive transfer of uneducated hyporesponsive NK cell from a MHC class I deficient background to a WT host with sufficient MHC class I molecules will transform the NK cell to a full functionally competent state (81). Inflammatory cytokines during viral infections have also been shown to modify NK cell responsiveness.

In summary, NK cell education or responsiveness is not a static but a dynamic process that can be altered during and after development by factors such as MHC class I, environment and cytokines.

1.1.5 NK cell target recognition and activation

Most cases of cellular transformation due to malignancies or infection are associated with the down regulation of the MHC class I molecules. The loss of the MHC class I molecules on cell surface prevent the presentation of Ag to T cells that eventually allows for T cell evasion (18). This has been reported as an evasion strategy for certain viruses. But this method of T cell evasion allows

for susceptibility to NK cell attack. As mentioned above, NK cells express inhibitory receptors that engage the self MHC class I molecules that dampens NK cell activation. Loss of this interaction renders the target susceptible to lysis by NK cells (77).

1.1.5.1 Missing Self Hypothesis

To help guide how NK cells recognize their target and get activated by their target, Karre and colleagues proposed in the 90s the “Missing Self Hypothesis” (82-84). This principle proposed about a decade following the discovery of NK cells has significantly aided the understanding of NK recognition and activation by target. A pre-requisite for this principle is that NK cells express at least one receptor for the MHC class I molecules. All nucleated cells express MHC class I molecules that serve as a marker for self. The inhibitory receptors engage with these self-molecules to prevent reactivity against healthy self cells (83). But the loss of self (MHC class I) due to cellular transformation, stress or infection renders these infected or transformed cells susceptible to NK attack because of the loss of inhibition due to the lack of inhibitory receptors engagement.

Support for the missing self hypothesis ranges from the susceptibility of YAC-1 (lacks MHC class I) to NK cell lysis, the lysis of bone marrow cells that lack MHC class I expression because of mutation to the lysis of normal cells from MHC class I deficient background.

The application of this principle is currently in use in HSCT. Unlike autologous HSCT, in allogeneic HSCT, there is enhanced NK activity and tumor clearance because of KIR and MHC I molecules mismatch between donor and recipient.

The Hallmark of this principle rests on NK cell detecting something been absent and not on something been present to mediate NK cell activity. Advance in the understanding of NK cell activity has shown this model to be insufficient in accounting for NK cell activity. Other principles such as “induced self hypothesis” have been proposed to explain NK reactivity.

1.1.5.2 Induced Self Hypothesis

The missing self hypothesis fails to account for the inability of NK to lyse tumor cell lacking MHC class I molecules or normal self cells without MHC class I such as red blood cells (erythrocytes). These inadequacies lead to the proposition by Raulet and colleagues of the “Induced self hypothesis”. Transformation of healthy cells to unhealthy cells alters the expression of some self ligands that could be ligands for NK cell activating receptors (85). For example, healthy cell express no or low levels of MIC A/B but can increase the expression of MIC A/B under stress. MIC A/B are ligands for the NK activating receptor, NKG2D. These stress-induced ligands on stress cells interact with their receptors on NK cell. This interaction results in NK activation rendering these stress cells susceptible to NK cell killing.

Both these principles can compliment each other to help explain NK cell integration of signals from activating and inhibitory receptors in determining NK cell functional fate and subsequent NK cell response. A stress cell that lack MHC class I expression is susceptible to NK cell (missing self) and upregulates stress induced self ligands is susceptible to NK cell (induced self) (74).

1.1.5.3 NK immunological synapse

NK cell upon recognition of target forms, through a multistep process involving membrane bound receptors, signaling molecules and signaling pathways, a specialized signaling domain termed the Immunological Synapse, IS (86, 87). The IS allows for the directed and polarized exocytosis of cytotoxic granules containing lytic effector molecules such as granzymes and perforins towards the target (86). In some cases of NK killing that do not involve soluble molecules but involve membrane bound molecules, the IS allows for directed engagement of the target. This polarized directed release (or engagement of target) of lytic granules prevents the indiscriminate killing of normal bystander cells. Defects in formation of IS are associated with impaired NK cells function. It is reported that impaired ability of NK to form an immunological synapse is associated with altered NK cell function (88). For instance, in a *Was*^{-/-} mice, the NK cell showed an inability to form an IS with tumor cell after the mice were challenged with B16 melanoma. The impairment in the formation of immunological synapses in patients with WAS also demonstrate the importance of IS in NK functions (89, 90).

Important in NK-DC crosstalk is the formation of IS between the NK cell and DC. It has been demonstrated in a human study on the importance of IS in NK-DC crosstalk. For instance, in a human study conducted by Catucci et al., the WAS in DC was silenced using siRNA (91). WASP absence affected the interaction of NK cell and DC and in turn compromised tumor rejection by NK cell (87, 92). The IS also allows for selective diffusion between the NK cell and the target. The IS allow molecules of certain sizes to enter and prevent molecules above certain sizes to enter (93, 94). After formation of IS with target, NK can execute it directed effector function towards the target.

1.1.6 NK cell mode of killing

NK cell upon forming an IS with its target triggers the necessary machinery to execute its effector functions. NK cell effector functions include cytokine production and cytotoxicity. The defining predominant effector function associated with NK cells is cytolysis. Cytolysis of target by NK cell can occur in either a direct or indirect mechanism (18).

Direct killing is one in which the lysis is directly mediated by the NK cell and does not directly involve another effector cell. This mode of killing can be an active or an inactive process. In the active process, the apoptotic process is inherent in the NK cell and the effector molecules are directly derived from the NK cell whilst in the inactive process, the apoptotic mechanism is inherent in the target cell. In active NK cell lysis, NK cells release lytic granules containing granzymes and perforin (95). These granules diffuse through the IS onto the target. The perforin forms pores on the target membrane rendering the target permeable to granzymes and other lytic molecules (96, 97). The result is the eventual death of the target. In the inactive mode of killing, the target expresses inducible death receptors, DR, such as Fas and DR5 (21, 98). These death receptors bind to their ligands (e.g. FasL) on the NK cell resulting in the initiation of the inherent apoptotic signaling cascade in the target and the eventual death of the target.

Indirect killing is a form of killing executed by other effector cells but initiated by NK cell via production of mediators such as cytokines and chemokines. These mediators released by NK cell can promote the differentiation, activation and recruitment of other immune cells to target site resulting in the subsequent killing of the target (23, 98). For example, NK cells produce IFN- γ and TNF- α (99). IFN- γ and TNF- α and other molecules produced by NK cell induce maturation of DC

into a Th1 type polarizing DC capable of priming CD4 T cells into a Th1 responsive cell. IFN- γ produced by NK cell has been shown to increase sensitivity of target to killing (99). Also, IFN- γ and TNF- α produced by NK cells has been reported to work synergistically to upregulate ICAM-1 on target cell promoting susceptibility of the target (99). In addition, GM-CSF produced by NK cell promotes DC differentiation. Activated NK cells promoting priming of CD8 T cells to CTL is another indirect mechanism of NK killing.

Natural killer cells can be specifically activated to execute their effector functions by cytokines such as IL-15, IL-18 and the type I interferons produced by activated DC.

1.2 Dendritic cells, DC

Early initial notion on the development of an immune response was the requirement for lymphocytes and accessory cells. The cells typically considered accessory cells at that time were the macrophages (100, 101). Work by Steinman and Zhan on spleen cells from mice, identified a unique population of cells with an unusual probing morphology and adherent properties (9, 101). These cells were structurally different from the known accessory cells at the time. This population of cells had tree-like or branch-like processes. Further work showed that they were a unique population of cells and moreover, they were the main initiator of immune response (9, 102-106). The term dendron, the Greek word for tree, was used in naming these cells. They were called Dendritic cells because of their tree-like processes. These dendrites allow for probing of a larger area of the environment by the DC. Steinman was awarded the Nobel Prize in physiology or medicine for his significant contribution to DC biology.

1.2.1 Properties of DC

DCs are professional APC that act as key mediator between the innate and the adaptive arms of the immune system (107-111). Unlike other APC, DC can prime both naïve and memory T cells (112, 113). DCs are derived from hematopoietic cells in the bone marrow (109, 114). They are a heterogeneous group of cells that are present throughout the body from surfaces close to the external environment such as skin, lung, stomach, intestines and nose to organs entry points. They are also present in the blood, lymphoid and non-lymphoid tissues (115, 116). They are typically classified into two major groups - conventional DC (cDC) and plamacytoid DC (pDC). cDC express high levels of CD11c whilst the pDC do not express high levels of CD11c (108, 109). cDC are the type with features originally identified by Steinman – classical fingerlike projections. They are the class specialized for antigen processing and presentation. They express a variety of the TLRs (TLR-1, -2, -3, -4, -5, -7 and -8) (109). pDC express high TLR7 and TLR9 and are rapid large producers of type I IFNs (108, 109, 115, 117). pDC plays an important part in viral and bacterial immune response. They express low MHC class I and class II molecules.

DC can also be classified based on their origin, anatomical location (resident or migratory) or maturation status (116).

1.2.2 Maturation of DC

Immature DC are characterized by low MHC expression, low co-stimulatory molecules such as CD40 expression, high phagocytic activity and low migratory potential. iDC possess efficient mechanism for the recognition and uptake of pathogens. iDC through receptors such as TLRs

sample the environment for pathogens. Upon encountering the pathogen, DC internalized the Ag by either phagocytosis, pinocytosis or endocytosis. These internalization processes are aided by TLRs or other specialized receptors on the DCs. The DC then process the captured Ag into peptides and present it on the surface as a MHC – peptide complex (118).

During the capture and presentation of Ag, the immature DCs get activated into mature DCs characterized by high MHC molecules expression, low phagocytic activity, high co-stimulatory molecules expression, up-regulated expression of chemokine receptors like CCR7 and high cytokine production (115, 119). The DC then travel to the T cell zone of the lymph node - a region enriched with T cell - and then present the Ag to the T cells resulting in the priming of the T cells (115).

Maturation of DC can be induced by Toll like receptors, TLRs, ligands or by other immune cells such as B, NK cells.

1.2.2.1 Maturation of DC via TLRs

TLRs are member of the PRRs. TLRs are a type I membrane proteins with a leucine rich repeats (120). TLRs are important in innate immunity and in the recognition of conserved molecular patterns called PAMP associated with pathogens (121, 122). TLRs recognize components from bacteria, fungi and virus. They have been shown to recognize nonpathogenic molecules that are associated with danger called damaged associated molecular pattern, DAMP (123). For example, TLR can recognize heat shock proteins or more specifically, hsp70 binds to TLR 2 and 4.

TLR can be expressed either on the cell surface or on intracellular vesicles. Examples of TLRs expressed on cell surfaces are TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 whilst examples of TLRs expressed on intracellular vesicles are TLR3, TLR7, TLR8 and TLR9. The ligands for most of these TLRs have been identified. For example, TLR 4 binds LPS, TLR9 binds CpG DNA, TLR 3 binds dsRNA (PIC, a dsRNA mimic), TLR5 binds bacterial flagella, TLR7 and TLR8 binds ssRNA (111).

Binding of the TLR ligands to the TLRs initiates a cascade of intracellular signaling event. These signaling events can occur in a MyD88 dependent or independent manner (124, 125). These signaling events initiate transcriptional factors activation, up-regulation of transcription, production of cytokines, up-regulation of surface molecules and execution of DC effector functions.

1.2.3 Functions of DC

As mentioned above, DCs capture, process and present Ag and these DC functions initiate the adaptive response to pathogens and transformed molecules (103, 115). In addition, through the production of cytokines and cellular interactions with other immune cells, DC can shape the quality and quantity of both the innate and adaptive immune response.

In addition to priming T cell for a response, DCs also play a role in controlling immune response as evident in their role in both central and peripheral tolerance (126-129). Thymic DC have been

reported to play a role in the selection of T cells during T cell development in the Thymus. DC are also involved in peripheral tolerance through the induction of Treg. The presentation of Ag by iDC induce anergic or tolerogenic T cells.

The crosstalk between DC and other cells of the immune system has been shown to be important in immunity against virus and tumor. A well-studied and important crosstalk that DC engages in is the NK-DC crosstalk.

1.3 NK-DC crosstalk

The relevance of NK-DC crosstalk has been well demonstrated by multiple studies and the interaction has been shown to be important in their respective activation (130, 131). NK-DC crosstalk is a reciprocal and a bi-directional interaction. NK cell activate DC and DC reciprocally activates NK. For example, DCs express on its surface IL-15R α and trans present IL-15 to activate NK cells (132-134). Activated NK cell produce IFN- γ and the produced IFN- γ induce high expression of IL-15. A positive feedback loop is created between the IL-12 from the DC and the IFN- γ from the NK cells. NK-DC crosstalk is influenced by both a direct cell-to-cell contact and soluble mediators.

DC through the release of cytokines such as IL-12, IL-15, IL-18 and type I IFN affects NK functions ranging from NK activation, proliferation, survival, cytokine production to cytotoxicity (135-137). DC release IL-12 during maturation that influence the production of IFN- γ by the NK cells and as well as the cytotoxicity of NK cells. The IL-15 transpresented by the DC to the NK cells has been demonstrated to be important in varieties of NK cells functions and properties.

Proliferation of NK cell is affected when there is no physical contact between NK and DC, underscoring the importance of the DC presentation of IL-15 to NK cell. Li et al, showed that the expansion efficiency of NK cells can be increased when NK cells and DC are in direct contact with each other during co-culture (20). DCs do express ligands for the NK cells activating receptor NKG2D and the interaction of NK cell and DC via this receptor/ligand axis markedly upregulate the expression of CD69, an NK activation marker (138). Co-culture of DC with NK cell has also been shown to enhance the NK cell production of IFN- γ and NK cell cytotoxicity towards some cancer cells. NK cells effector functions have been shown to be enhanced by using matured DC stimulated with the TLR4 agonist, LPS. In an in vivo settings as well, transfer of DC to tumor site showed enhanced tumor clearance compared to the control and the tumor clearance was demonstrated to occur in an NK cell dependent manner via NK-DC crosstalk.

NK cells induce maturation and editing of DCs (129). NK cell through it activating receptor Nkp30 has been implicated in NK cell editing of DC and DC mediated NK cell activation (139-141). The editing of DC by NK cell allows for the selection of optimal DC for T cell priming and the elimination of hyporesponsive or tolerogenic DC (129). This DC editing eliminate sub optimal DCs that could prime T cells into tolerogenic or anergic T cells. The low expression of MHC molecules on immature DC render them susceptible to NK cell lysis whist mature DC because of their up-regulated MHC molecules expression provides the inhibition that endows them with resistant to NK cell lysis. In vitro co culture of NK cells with DCs can mature the DCs.

NK cells also produce GM-CSF that is important in the differentiation of precursor cells to DCs and the survival of the DCs (142). Soluble mediators such as IFN- γ and TNF- α produced by NK

cells affect DC activation and the subsequent priming of T cells. DC polarized by NK cell possessed an enhanced potential to produce IL-12p70 and induce Th1 type response.

1.3.1 NK-DC crosstalk in anti-tumor response

As self cells, tumor cells are poorly immunogenic. An important element in the activation of DC is danger signals. Activation of DC in the absence of danger signal can lead to priming of tolerogenic T cells by these DC. NK cell through lysis of the tumor cells can provide necrotic materials and danger signals for the maturation of DC. This enhances DC cross priming. Fay Chinnery et al. demonstrated the importance of NK-DC crosstalk in tumor rejection. By activating NK via the NKp46, Chinnery et al. observed an enhanced tumor rejection. They demonstrated this tumor rejection to be NK cell dependent and to also occur via NK-DC crosstalk (143). Furthermore, they showed the tumor rejection to be dependent on direct cell-to-cell contact and soluble factors such as IL-12. In another in vivo setting, transfer of DC to tumor site showed enhanced tumor clearance compared to the control. Moreover, this clearance was demonstrated to occur in an NK cell dependent manner via NK-DC crosstalk. Adoptive transfer of DC can enhance NK cell clearance of NK cell sensitive tumors via NK-DC crosstalk.

1.3.2 NK –DC crosstalk in microbial infection

There are viruses that can infect DC. The infected DC produce mediators that activate NK effector function resulting in the elimination or the control of the viral infection. NK cells express activating receptors that can recognize viral components. For example, Ly49H recognizes m157 from MCMV and NCR recognizes viral HA. This recognition can result in NK cell activation and NK-DC

crosstalk that result in the appropriate response. The killing of virally infected DCs by NK provide the appropriate mediators for adaptive response against the virus and also prevent the impaired priming of the adaptive immune response by the infected DC and promote the appropriate priming of the immune response.

The interaction between the activating receptors NKG2D and NCR on NK cells and their ligands on DC plays a role in immune response to influenza virus infection. Defect in receptor/ligand interaction between NK cell and DC impairs anti-viral response. In chronic HCV infection, low expression of the ligand for NKG2D on DC has been suggested as a possible reason for the reduced NK cell function and persistent infection. Depletion of DC in an MCMV infection affected the viral clearance by NK cells compared to the control group without DC depletion. Impaired NK/DC crosstalk induced by HIV Nef was suggested as a possible mechanism of immune evasion by the virus (130).

In addition to the soluble mediators produced by either the NK or the DC, membrane bound molecules have been reported to play a vital role in NK-DC crosstalk. Both NK and DC have been reported to produce chemokines whose receptors are expressed on the other cell types. This has been suggested to allow for the preferential recruitment of these cells to their site of interaction.

1.4 Cell Migration

Cell migration is an important cellular property essential in physiological and pathological processes such as immune response, cancer metastasis, embryogenesis, wound healing and development (144). Cell migration involves a complex network of signaling events involving

ligand, receptor, signaling molecules, signal transduction pathways and as well as cellular rearrangement and polarization. Cells can migrate in response to sensing chemical, electrical, mechanical and magnetic change.

1.4.1 Electrotaxis

Electrotaxis is the directional movement of cell in response to an electric field (144, 145). Immune and non-immune cells have been reported to migrate in response to electric field. Also reported are data showing that some cell types can move to either the positive or negative end of an applied electric field or voltage. The directional movement of PBMC under the influence of electric fields has been reported in both TW and MF device. For instance, in a modified TW device with a voltage created between the top and the bottom chamber, lymphocytes were shown to move from the top to the bottom when the electric fields were of a particular direction (146). But the reversal of the electric field direction abolished the directional migration. This demonstrate the directional movement of cells when current is applied. No migration was observed in the absence of the fields. In another migration assay, cells exhibit a direction movement towards the anode but migrated randomly in the absence of electric field (145)

Electrotaxis during wound healing has been used to elucidate electrotaxis and its importance in wound healing *in vivo* (144, 146). Endogenous wound healing can be altered by application and manipulation of the electric fields *in vivo*.

Some specific signaling pathways important in electrotaxis have been elucidated (145). The disruption of the PI3K decrease the electrotaxis of epithelial cells during wound healing. The polarized activation of Src has also been induced upon electrical stimulations.

1.4.2 Chemotaxis

Chemotaxis is the directional movement of cell in response to chemical gradient. Unlike chemokinesis, chemotaxis is not random but a directional movement in response to chemical signal. Chemotaxis involves a complex network of signaling events initiated after the binding of the ligand called chemokine to the chemokine receptor.

Chemokine

Chemokine is a type of cytokine that cause the kinesis (Greek word for movement) of cells. Chemokines are small proteins of about 70-80 amino acid residues with molecular weight between 8-10kDa. Chemokines are classified into 4 major subgroups based on the number and spacing of conserved cysteines residues. The main subclasses are C, CC, CXC and CX3C. Each subclass can still be further sub-classified. Chemokines can also be classified based on their functions into homeostatic and inflammatory or inducible (147).

Non-chemokines

Immune cells can migrate in response to chemical signal that are not part of the chemokine family. Cellular migration can be induced in response to factors such as chemerin or S1P during steady

state or inflamed conditions. Silvia Parolini et al. demonstrated the role of chemerin in the trafficking and colocalization of NK cell and DC to inflammatory sites (148). The role of CD62L in the recruitment of lymphoid and non-lymphoid cell via the HEV is another example of non-chemokine involved in cell migration. Integrins and other selectins are also involved in NK cell trafficking (149).

1.4.3 Migration Assays

There are lots of in vitro assays that have been developed to study cell migration in response to chemical gradients. For this study, I used the conventional TW and the MF device.

1.4.3.1 Transwell

The TW device designed in the early 60s by Dr. Stephen Boyden and formerly called Boyden or modified Boyden chamber allows for the assay of cell migration. The TW device consists of a multi wells (ranging from 12 to 96 wells) tissue culture plate. In the wells are cylindrical inserts with bottoms made of polycarbonate membrane. The polycarbonate membrane has defined pores of varying sizes. The inserts allow the compartmentalization of the wells into 2 chambers – top and bottom chambers. Cells are loaded into the top chamber whilst the bottom chamber is loaded with certain volume of the CM or the test medium or control medium. The volume of the medium used depends on the size of the wells used. During the assay, there should be no air space or bubble between the medium in the bottom chamber and the insert that separate the well into different chambers.

After the cells are loaded into the top chamber of the device and the medium loaded into the bottom chamber, the device is incubated for a certain period of time. After the incubation, the cells are counted to determine the number of cells that transmigrated from the top to the bottom chamber. The method of assessing the transmigrated cells is dependent on the adherent properties of the cells. If the cells are adherent, the membrane is stained and the cells attached to the membrane counted. But if the cells are non-adherent, the cells in either compartment can be collected and counted to determine the transmigrated cells.

The TW assay has some limitations despite it been a sensitive assay. The initial cell number seeded on the top chamber of the TW assay has to be within an optimal range – a very low cell number prevents detection of cell migration whilst a very higher cell number causes overcrowding of the cells on the membrane. A TW device insert with large pore size causes the spontaneous movement of the cells under the influence of gravity instead of a directed chemotactic movement from the top to the bottom chamber. TW with smaller pores prevents the squeezing of the cells through the membrane even when the cells sense a chemoattractant in the bottom chamber. A long incubation time can cause the equilibration of the media between the 2 compartments or chambers. Diffusion between the 2 compartments impact the formation of a stable chemical gradient over time.

In addition to these technical limitations with the TW assay, there are limitations on the parameters that could be measured with the TW assay. The TW assay cannot measure the speed, chemorepulsion and CI. Distinguishing chemokinesis (random migration) from chemotaxis (directed migration) can be difficult.

1.4.3.2 Microfluidic device

A more sensitive device to assay for cell migration is the Microfluidic, MF, device. The MF device allows us to overcome some of the limitations with the TW assay (150). The MF device consists of a main chamber or channel, fluid inlets and outlets. The main channel is coated with fibronectin to promote the adherence of the loaded cells (151).

The cells are loaded into the fibronectin coated main chamber and then allowed to adhere onto the chamber. With the aid of the inlets, a gradient is created in the main chamber by allowing fluids to flow at a very slow rate through the inlets. The inlets are connected to syringe pumps via polyethylene tubings. Fluids from the syringe flow via the tubing and inlets into the main chamber and out through the outlets. The temperature of the device is kept at 37°C. One of the inlets is used to load the medium with the 0.4% BSA into the main chamber whilst the other inlets is used to load the test medium or CM. FITC-dextran is added to the CM before being loaded into the main chamber. The FITC-dextran allows the visualization and monitoring of the created gradient and the stability of the generated gradient during the experiment. FITC-dextran has similar molecular weight with the soluble chemokine mediators in the CM. Hence, both are expected to diffuse at the same rate. The inlets and the main chamber together form a Y shape. A reason this device is specifically called the Y shaped MF device. There are other more complicated MF devices that allow generation of more complicated gradients.

Cell migration is visualized and recorded with the aid of the attached camera. The cells are tracked and migration data acquired using imageJ software. The data are exported and further analyzed to determine migration parameters such as speed, chemotactic index, CI.

We recently validated the use of the MF platform in NK migration assays and published our findings in the European Journal of Immunology.

1.5 NK migration

Upon development in the BM, the mature NK cells exit their site of development and traffic to lymphoid and non-lymphoid tissues throughout the body. NK cells are a widely distributed population of cells found in the lungs, blood, LN, spleen, uterus and gut (32). Their distribution in steady state in these various locations is not uniform but vary significantly between these different sites (32, 152). For example, NK cell is higher in the lungs than the liver whilst NK cell is higher in the spleen than in the thymus (32). The distribution and NK cell number can also change in some locations or organs due to conditions such as inflammation, infection and pregnancy (32, 153, 154). For instance, in both mice and humans, the uterine NK drastically increase during pregnancy. Uterine NK cells, CD56^{bright}, play a role in angiogenesis during pregnancy (153). The distribution of NK cells is subset specific. In an adoptive transfer of labeled splenic NK cell into mice, the NK cells distribute into the host in a subset specific manner. Moreover, the distribution pattern was similar to the host distribution pattern of the different NK cell subsets (32). The CD27^{high} NK cell was predominantly localized to the LN whilst the CD27^{low} NK was predominant in the blood. The trafficking of NK cell during steady state or inflammatory conditions is governed

by receptors, organ specific chemokines and other mediators that are not part of the chemokines or chemokine receptors superfamily.

During the development of NK in the BM, NK cells express CXCR4 that recognize the chemokine, CXCL12, expressed in the BM environment. The CXCR4/CXCL12 axis prevents the exit of the developing NK from the BM and promotes the retention of NK in the BM (155). The deficiency in either CXCR4 or CXCL12 affects NK distribution and the homing of NK in the BM. The administration of the CXCR4 antagonist, AMD3100, has been reported to cause increase in NK cells in the blood and spleen and affect NK localization in the BM (156). As NK develops further, there is an associated downregulation of the CXCR4 to promote egress of the NK from the BM. Mayol et al. showed that the downregulation of CXCR4 is associated with a subsequent upregulation of S1P₅ (157).

S1P₅ is one of 5 isoforms of the S1P receptor. The other isoforms of S1P receptors, S1P₁, functions in the chemotaxis of B and T cells. S1P is a secreted lysosphingolipid or a lipid mediator. S1P is less abundant in tissues fluid but more abundant in extracellular fluids such as the blood and lymph. This differential concentration creates a S1P gradient between the tissues and the extracellular fluids. The S1P receptor is a G protein coupled receptor that influence immune cells migration in response to S1P (158, 159). The matured NK cells with upregulated S1P₅ exit the BM into the blood in response to the gradient of the S1P between the BM and the blood. Walzer et al. demonstrated the role of S1P₅ in the mobilization of NK cell and the effect of the deficiency of S1P₅ in the tissue distribution of NK cells (160). Walzer et al. reported that in S1P₅ deficiency, the number of NK cell in the blood was drastically affected. The expression of S1P₅ on NK cells is

also correlated with the maturation of NK cell and on the localization of the different NK cell subsets (160). In the blood, the response of NK cells to S1P can decrease to facilitate the exit of NK cells from the blood to other sites. The trafficking is then mediated by other molecules that promote exit from the blood. S1P₅ has also been reported to play a part in the recruitment of NK to inflamed sites (160).

Certain subsets of NK express the CD62L, L-selectin, that facilitate the trafficking to the LN in response to the CD62 expressed on the HEV (149). The CCR7 promotes the trafficking of NK to the LN in response to the CCL19/21. Another non-chemokine important in migration of NK is chemerin (21, 148, 161). Parolini et al., reported the important in NK trafficking to non chemokine, chemerin (148).

Chemokine receptors such as CXCR4 and CCR7 are important in NK trafficking and homing in steady state. There are other chemokine receptors that play an important role in NK recruitment to inflammatory sites such as cancer environment or infection sites. Mice strains deficient in chemokine receptors such as CXCR3, CCR5, CX3CR1 do not show a significant alteration in NK distribution suggesting that these chemokine receptors are dispensable in steady state trafficking of NK. Yet, in inflammatory conditions, these chemokine receptors facilitate NK trafficking. Tumor bearing the ligand for CXCR3, CXCL10 or ip-10 had increased intratumoral infiltration compared to CXCL10-negative tumors. The increased infiltration in the CXCL10 producing tumor mice resulted in less tumor burden compared to the CXCL10 negative tumor mice. CX3CR1-deficient mice with experimental autoimmune encephalomyelitis (EAE) showed an impaired recruitment of NK to the inflamed CNS (162).

Rationale

Compared to NK cells effector functions (cytotoxicity and cytokine production), regulation of NK cells migration is less understood. The precise mechanisms regulating NK cells recruitment to tissues are poorly understood. The importance of NK migration is demonstrated in the association between NK infiltration to tumor microenvironment and tumor prognosis where it has been reported that low infiltration of NK cells to tumor microenvironments such as of Breast cancer is associated with poor prognosis. Overcoming NK cell infiltration by improving NK infiltration to tumor environment has been demonstrated to improve tumor prognosis. Moreover, in order for NK cell to form an immunological synapse with its target and execute its effector functions, NK ability to migrate to the target is indispensable. So, understanding NK cell migration may prove important to support better NK-based immunotherapy in cancer.

The migration of NK cell in the context of NK-DC crosstalk are often examined *in vivo*. However, NK migration in defined microenvironment has not been studied *in vitro*. Recent findings from the Kung's lab demonstrated that CM derived from LPS matured DC attracted IL-2 activated NK cell more significantly compared to immature DC (151). Different cytokine activated NK shows differential ability to recruit DC. However, it remained to be determined how the different modes of DC and NK activations regulate NK cell chemotaxis.

Hypothesis

Cytokine activated NK cell migration is differentially regulated by the cytokine, by DC activated by different toll like receptor ligands and by tumor microenvironment.

Objectives

1. Compare the migration of activated NK cell in CM of different TLRs stimulated DC
2. Compare the migration of different cytokines activated NK cells in various conditions
3. Understand the impact of CM obtained from tumor cell line in NK migration

Specific Questions

- I. Do the different TLRs pulsed DC recruit NK cells?
- II. Does the different cytokine activated NK cells migrate differently in different CM?
- III. Can expression of known chemokines and/or chemokine receptors account for the difference in migration?
- IV. Will the different cytokine activated NK cells differ in their migratory responses in cancer settings?

Chapter 2 Materials and Methods

2.1 Mice

4-8 weeks old female C57BL/6 mice were purchased from GMC through the Central Animal Care Services (CACS) of the University of Manitoba. Animals were housed and maintained in the CACS facility. All experiments with animals were conducted humanely and in very strict compliance with all recommended animal care protocols

2.2 NK cell purification, culture, activation and in vitro expansion

The spleens from euthanized C57BL6 mice were collected using sterile dissection kits. NK cells were isolated from the collected spleen(s) using the Easy Sep Mouse NK cells negative selection kit (Stem Cell Technology) following the isolation protocol as instructed by the manufacturer. The purified NK cells (purity > 80%) were cultured in supplemented RPMI 1640 (RPMI 1640 containing 10% FBS, 1% PSG and 500 μ L 2-ME/500 mL giving 1.6 mM 2-ME) containing either recombinant human IL-2 (1000 units/mL) or recombinant mouse IL-15 (50 ng/mL). The purity of the NK cells used in all experiments was > 90%. After 4 days of incubation in a tissue culture incubator (humidified 5% CO₂ at 37⁰C), the NK cells were harvested as either IL-2 activated NK cells or IL-15 activated NK cells respectively. These activated cells are used for further studies as cytokine activated NK cells or more specially IL-2 activated NK cells or IL-15 activated NK cells respectively. In some of the descriptions, I sometimes use IL-2 NK cells or IL-15 NK cells when referring to IL-2 activated NK cells or IL-15 activated NK cells respectively. During the NK cell culture, cells were constantly monitored for confluency and divided when cells were confluent;

medium color was as well monitored with old medium either completely or partially replaced with fresh medium. This replacement of medium is dependent on the color of the culture medium.

2.3 Bone Marrow Derived Dendritic Cells (BMDC)

The Tibiae and Femur from euthanized C56BL/6 mice were collected and cleaned using scissor and forceps to remove attached muscles. Bone marrow cells were flushed from the tibiae and femur using supplemented RPMI 1640 medium. The clumps were dispersed by passing the cell suspension several times through a syringe with 23-27 gauge needle. Cell clumps and debris were removed by flushing through a cell strainer (40 um Nylon cell strainer). The single cell suspension was centrifuged for 5 mins at 1500 rpm. Supernatant were discarded and cell pellet re-suspended in 5 mL Ack lysis buffer for 5 mins to lyse the RBC. After the 5 minutes, 10 mL of RPMI was added to the cell suspension to stop the lysis reaction and then centrifuged at 1500 rpm for 5 mins. Supernatant was discarded again and cells washed with supplemented RPMI 1640 to remove the Ack lysis buffer. After the washed, the pellet was re-suspended in supplemented RPMI 1640 containing 20 ng/mL of GM-CSF and cells counted using hemocytometer. 1×10^6 Cells in 1mL of the supplemented RPMI with 20 ng/mL GM-CSF were seeded in a 12 well tissue culture plate. The plates containing the cells were placed in the tissue culture incubator. On the 3rd day post cell culture, the medium (containing non-adherent cells) was replaced with 1ml of fresh supplemented RPMI 1640 medium containing the GM-CSF. When replacing the old medium, the wells containing adherent cells were kept slightly wet during the medium replacement to prevent drying of the wells. Cells culture was monitored every day for cell growth, confluency, medium color etc. Depending on the medium color, the old medium was either completely or partially replaced with fresh culture medium. I found similar results in my experimental platform whether I completely

or partially replace the old medium. The resulting DC are usually > 85% CD11c high but with low level of CD40, CD80/86 expression that can be up-regulated upon stimulation.

2.4 DC maturation

To generate mDC, the BMDC were stimulated for 24 hrs with optimal concentrations of the different TLRs (LPS, PIC, Pam3C or CpG). The un-stimulated BMDC was the iDC. The mDC or iDC were harvested and stained for key DC maturation markers and used in flow cytometry. At the same time, the media from the stimulated or un-stimulated DC were collected, spun at 1500 rpm for 5 mins to remove debris and kept at -80°C for further use

2.5 Conditioned Medium (CM) from cell line

Different tumor cell lines (4T1, E0771, B16 melanoma) were cultured in a T75 flask at 0.7×10^6 cells in 7 mL of the appropriate culture medium for 72 hrs. The flask were then placed in the humidified incubator. After 72 hrs, the culture media were collected, centrifuge for 5 mins at 1500 rpm to remove debris. The pellet discarded and the supernatant collected as CM. CM were aliquot in 1.5 mL eppendorf tubes and kept at -80°C for further use.

2.6 Flow Cytometry

To analyze the surface expression on either the DC or the NK cells, 0.1×10^6 cells were collected in labeled tubes and washed with FACS buffer. The supernatant was discarded and cell pellet re-suspended in 50 μ L of Fc blocker (eBioscience) and incubated in ice for 15 mins. The Fc blocker

prevents the non-specific binding of Antibody. After, 1 μ L of the appropriate fluorochrome conjugated Ab (CD11c, NK1.1, CXCR3 conjugated with either APC, PE or FITC) was added and then incubated in ice for another 30 mins. The cells were then washed with FACS buffer to remove unbound Ab. The corresponding isotype control Abs as recommended by the manufacturer were used as appropriate. Cells were then fixed in 200 μ L of 2% PFA until analysis with flow cytometry. The Cells' fluorescence intensity was measured using FACS Canto (BD) and analyzed using FlowJo software.

2.7 Conventional Trans-well Assay

0.2×10^6 of treated or untreated cytokine activated NK cells in 100 μ L migration medium (RPMI 1640 with 0.2% BSA) were seeded into the top chamber of the TW whilst 600 μ L of the appropriate CM was added to the bottom chamber of the TW (24 wells plate of 5.0 μ m polycarbonate membrane with 6.5 μ m insert purchased from VWR International). After 3 hrs of incubation in a tissue culture incubator (humidified 5% CO₂ at 37⁰C) except for the neutralization experiment where the incubation time was for only 45 mins, the NK cells that transmigrated from the top to the bottom chamber were harvested, re-suspended in a smaller volume and counted to get the total number of NK cell that transmigrated.

2.8 Migration Assay using Microfluidic device (Platform)

In a recent publication in the EJI from our lab (Mahmood et al, 2014), we validate the use of the MF device in NK migration assay. Using the syringe pumps of the Y-shaped Microfluidic device, a stable gradient of the appropriate CM medium was generated by allowing the media to flow at a

very slow flow rate of 0.4 $\mu\text{L}/\text{min}$. FITC-Dextran allows visualization of the created gradient at any time point (before, during or after the experiment). Between 0.4×10^6 to 0.5×10^6 NK cells were loaded and allowed to adhere onto the fibronectin coated chamber of the MF device. Using microscope and Camera, NK cells migration was measured and later analyzed using ImageJ Software. Speed, CI, angular histogram, cell track can be determined during the analysis.

2.9 ELISA

CM collected from the different conditions (BMDC, cell lines etc) and stored at -80°C were collected, thawed and used in ELISA. ip-10 or GM-CSF were quantified in the CM using ip-10 platinum ELISA kit or GM-CSF platinum ELISA kit (eBioscience) respectively according to the manufacturer's instruction. The concentrations then measured and analyzed using the appropriate software (Max190 system and SoftMax Pro software).

A multiplex assay on the CM was carried out to determine an array of cytokine/chemokine in the CM. I got help with this assay from Dr Ball's lab.

2.10 Neutralization Assay

To examine the effect of CXCR3 on NK migration, CXCR3 on NK cell surface was blocked with anti-CXCR3 Ab or its isotype control (Armenian Hamster IgG). 6 μL of the Ab (stock conc. 1.0 $\mu\text{g}/\mu\text{L}$) was added to the NK cell suspension (0.2×10^6 cells in 100ul PBS or serum free medium or 2% BSA) to give a final concentration of 0.6 $\mu\text{g}/\text{mL}$ Ab. I used equal final concentrations of anti-CXCR3 Ab or its isotype (0.6 $\mu\text{g}/\text{mL}$). The cells and the Ab were placed in a 1.5 mL eppendorf tubes. After, the tubes containing the cells were placed in a tissue culture incubator for 30 mins.

The tubes were removed from the incubator and gently mixed and placed back in the incubator every 10 mins. The treated cells (CXCR3 or its isotype control) were used in the migration studies. Neutralization of the CXCR3 was verified by staining for CXCR3 using APC conjugated CXCR3 (or isotype control) on the treated or untreated cells. Flow cytometry was used to determine the expression of neutralized or un-neutralized receptor on the NK cells. I tested 2 cases during the neutralization. First, after the blocking, I spun down the cells to remove unbound Ab and then suspended the cells using migration medium before using the cells in the TW assay. In the other case, I did not remove the Ab but instead just used the cells directly after the neutralization. I found no difference between these 2 cases.

2.11 Mouse Mammary Epithelial cell isolation, culture, MMC CM

The 4th mouse inguinal mammary glands from euthanized C57BL/6 mice were surgically excised under sterile conditions. The glands were chopped into smaller pieces and placed in a dissociation medium. The glands were dissociated mechanically and enzymatically for 2-3 hours. To obtain single cell suspension, the dissociated glands were first subjected for 5 mins to warm 0.05% trypsin-EDTA then another 5 mins on 1.5U/mL dispase and finally filtered through a cell strainer (0.45 um cell strainer). The number of viable cells were counted using trypan blue and the automated cell counter (BioRad). 2.1 million cells were plated on a T25 flask containing 7mL of culture medium. After an overnight incubation in a tissue culture incubator, the medium containing non-adherent cells was completely replaced leaving the adherent cells and another 7 mL of fresh medium added. After another 72 hrs, the medium was collected, centrifuged for 5 min at 1500 rpm to remove debris. The supernatant was collected as MMC CM and stored at -80⁰C for future use. The confluency of the cells after 72hrs culture was about 90%.

2.11 Statistical Analysis

For the NK cell migration, I reported the total number on NK cells that migrated except in few cases (in NK neutralization case) were I used mean or percentage. Data were analyzed using paired student t-test. P values < 0.05 was set as the threshold for statistical significance. NS represent $P > 0.05$. * represents $P \leq 0.05$. ** represents $P \leq 0.01$. *** represents $P \leq 0.001$. n values in all cases was at least equal to 3.

Chapter 3 Results

3.1 NK cell culture

The purity of the freshly isolated NK cells from the spleen of euthanized C57BL/6 mice was determined by flow cytometry. The NK cells were cultured for 4 days in supplemented RPMI containing either IL-2 or IL-15 to generate the respective activated NK cells. After the 4 days of culture, the purity of the activated NK cells was as well determined. Mouse NK cell are defined as NK1.1 positive and CD3 negative. I found the purity of the NK cells to be over 90% for both the IL-2 and the IL-15 activated NK cells. The starting purity was about 80% for the freshly isolated NK cells (Fig 1). This increase in NK purity could have been due to increase in proportion of NK cells due to proliferation of NK cells and death of non-NK cells.

Using the Evos inverted digital microscope, I checked for cell morphology at a 40X magnification. The shape of the freshly isolated NK cells was round and small on Day 0 but the size and shape changed drastically in the culture medium containing either the IL-2 or IL-15. I found the NK cells changed from small and round on Day 0 to large and elongated on Day 4 (Fig 2). The cell morphology in both IL-2 and IL-15 culture were large and elongated and similar to each other on Day 4.

3.2 DC characterization

The generated BMDC were phenotyped for CD11c expression. The BMDC were CD11c high (Fig 3). CD11c high is a property of conventional DC. Compared to unstimulated DC, DC stimulated

with TLRs like LPS and PIC up-regulate the expression of key co-stimulatory molecules such as CD40 and CD80. The Kung's lab has already established the optimal concentrations of LPS, PIC and CpG required to fully mature DC as defined by the up-regulation of key co-stimulatory molecules. For my studies, mDC were defined as DC with up-regulated CD40 whilst iDC were defined as DC with low CD40 expression. As a positive control, the already established conc. of LPS (1 μ g/mL) or PIC (10 μ g/mL) was used to stimulate the DC to full maturation. DC stimulated with LPS or PIC up-regulate the CD40 expression compared to the un-stimulated DC (Fig 3).

3.3 Titration of Pam3C or Flag for optimal DC maturation

To determine the optimal conc of Pam3C or Flag for DC maturation, the Day 7 generated DC were stimulated with different conc of either Pam3C or Flag for 24 hrs. On Day 8, the DC were harvested and phenotyped for the surface expression of CD40.

In the first titration trials, I used concentrations of 1 μ g/mL, 5 μ g/mL, 10 μ g/mL, 25 μ g/mL, 50 μ g/mL, and 100 μ g/mL of Pam3C (Top panel of Fig 4A). For Flag, I used concentrations of 10 μ g/mL, 20 μ g/mL 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 300 μ g/mL (Fig 4C). I observed a dose dependent increase in CD40 expression with DC activation using the Pam3C (Fig 4A top and bottom panels). I repeated the stimulation of DC with 25 μ g/mL, 50 μ g/mL and 100 μ g/mL of Pam3C and then measured the CD40 expression by FACS. These 3 concs showed a significant increase in CD40 expression. I observed similar CD40 expression between these 3 conc (Fig 4B). Based on the CD40 expression (Fig 4B), the search for the optimal conc was further narrowed to 50 μ g/mL and 100 μ g/mL Pam3C. The association between TLR signaling and cellular apoptosis in different cell types has been previously reported (163-165). Using Tryphan blue staining, I

determined and then compared the viabilities of the DC stimulated with the 50 $\mu\text{g/mL}$ to the 100 $\mu\text{g/mL}$ Pam3C. I found slightly more viable cells with the DC stimulated with 50 $\mu\text{g/mL}$ compared to the stimulation with the 100 $\mu\text{g/mL}$ Pam3C.

CD40 is just one of several key maturation markers and the up-regulation of CD40 expression on DC does not conclusively imply that the DC is functionally matured. I looked at the other co-stimulatory molecules such as CD80 and CD86 as well. Of all the key surface co-stimulatory molecules tested, CD40 was consistently up-regulated upon stimulation with the different TLR Ligands tested. So, for my studies, I used the CD40 expression as a marker for DC maturation and based my maturation definition on CD40 expression. As mentioned above, I defined mDC as DC with high CD40 expression and iDC as DC with low CD40 expression.

I compared the CD40 expression on DC stimulated with the already established optimal conc of LPS and PIC with the newly determined optimal conc (50 $\mu\text{g/mL}$) of Pam3C. Comparing the CD40 expression between the different TLRLs stimulated DC would give an indication of how the newly determined optimal concentration (50 $\mu\text{g/mL}$ of Pam3C) compares with the other already established optimal TLR ligands concentrations for DC stimulation. As shown in Fig 5, the CD40 expression on the different TLRL stimulated DC is comparable. The CD40 expression on the DC stimulated with 50 $\mu\text{g/mL}$ Pam3C is higher than the expression on the DC stimulated with the PIC but closer to the LPS stimulated DC.

I compared the viabilities of the DC stimulated with the different TLR ligands. I used Annexin V and Trypan blue. These are dyes that work on the principle that viable cells do not take up certain

types of dyes whilst their non-viable counterparts do take up certain dyes. I found the viabilities of the DC stimulated with optimal concs of the different TLRs to be similar (Fig 6A and Fig 6B). In both Annexin V and Trypan blue, the viabilities were comparable. The percentage of Annexin V negative cells were in all 3 cases over 90%. With the Trypan blue, the percentage of viable cells were all close to 80%.

In the case of DC stimulation with Flag, after lots of titration, I could not find an optimal conc of Flag that up-regulate the CD40 expression. As shown in Fig 4C, I did not see a CD40 up-regulation on the DC stimulated with the different conc of Flag compared to the iDC. Also, I did not find a Flag dose dependence increase in CD40 expression as previously observed with the Pam3C stimulation or as previously reported with the other TLRs. I also checked for the expression of other co-stimulatory molecules and found no difference between the iDC and the DC stimulated with the different conc of Flag (Data not shown).

Based on the above findings, I used the following optimal TLRs concentration - LPS (1 $\mu\text{g}/\text{mL}$), PIC (10 $\mu\text{g}/\text{mL}$), Pam3C (50 $\mu\text{g}/\text{mL}$) and CpG (25 $\mu\text{g}/\text{mL}$ - to stimulate DC for my future work and ignored the DC stimulation with Flag. These are the concs of the different TLR ligands used for all future stimulation of DC with these different ligands.

3.4 CM from different TLR Ligands stimulated DC recruit cytokine activated NK differently

In a recent publication from the Kung's Lab published in the EJI (Mahmood et al, 2014), we reported that CM from iDC and LPS mature DC elicit IL-2 NK chemotactic and also that the LPS

mature DC elicit more IL-2 chemotactic compared to the iDC (151). I used CM generated from either LPS matured DC or iDC to compare the migration of IL-2 activated NK cells. I confirmed our previous findings on the ability of LPS stimulated DC to recruit IL-2 NK better than iDC (Fig 7A). I found more IL-2 NK to be recruited to the bottom chamber of the TW containing the CM generated from LPS DC compared to the bottom chamber containing CM from the iDC.

The recruitment potential of CM generated from the different TLR ligands stimulated DC were compared using a particular cytokine activated NK cells (IL-2 or IL-15 NK). The CM obtained from DC stimulated for 24 hrs with the different TLRs at their respective optimal concentrations were used in a conventional TW assay. I observed that all the different TLRs induce strong IL-2 activated NK cells chemotaxis. I found that the different TLRs stimulated DC induce IL-2 activated NK cells chemotactic with different potentials. PIC stimulated DC induces the strongest chemotactic movement followed by the LPS stimulated DC and then Pam3C stimulated DC and finally CpG stimulated DC induces the least chemotactic movement of IL-2 activated NK cells (Fig 7B).

I repeated the comparison of the recruitment potential of the CM from the different TLR ligands stimulated DC with IL-15 activated NK cells instead of the IL-2 activated NK cells. With the IL-15 activated NK cells as well, I found stronger chemotaxis using CM from LPS stimulated DC compared to CM from iDC (Fig 8A). Both the iDC and the mDC elicited strong IL-15 NK chemotaxis compared to the medium control. The medium used as control was the media used to culture the DC. I observed the same trend in the recruitment potential of the different TLR ligands stimulated DC on IL-15 NK chemotaxis i.e. PIC DC > LPS DC > Pam3C DC > CpG DC (Fig 8B).

In all the comparison, there was a statistical significant difference between the recruitment potential of the CM from the different TLR ligands stimulated DC.

In summary, different TLR Ligands stimulated DC recruit cytokine activated NK differently. The CM from the PIC stimulated DC > CM from the LPS stimulated DC > CM from the Pam3C stimulated DC > CM from the CpG stimulated DC.

3.5 IL-15 activated NK cells are recruited strongly towards the different DC CM compared to IL-2 activated NK cells

In the previous migration experiments with the different DC CM, the variable was the CM from the different TLRs stimulated DC. I compared the recruitment potential of the different TLRs stimulated DCs. It has been reported that different cytokine activated NK cell recruit DC differently (163), but how the different cytokine activated NK cells migrate towards DC stimulated with different TLR agonists is unclear. I compared the migration of the different cytokine activated NK towards the CM generated from the different TLRs stimulated DC.

CM from the different TLRs stimulated DC was employed in the comparison of the migration of the IL-2 and IL-15 activated NK. Using the conventional TW assay, I observed that in all the different TLRs stimulated DC tested, more IL-15 activated NK cells were recruited towards the DC CM compared to the IL-2 activated NK cells (Fig 9). I found a statistical significant difference

in the recruitment towards the different DC CM of the IL-15 activated NK cells compared to the IL-2activated NK cells.

To validate the above findings, I employed a different migration device – MF device. In our EJI publication (Mahmood et al, 2014), we validated the use of the MF device to assay for NK migration. Using CM from LPS stimulated, a stable gradient was generated using the LPS stimulated DC CM. Then the migration of the IL-2 and IL-15 NK in the created gradient was assayed and compared. Unlike MF device in which a stable chemical gradient can be generated, it is difficult to create a stable chemical gradient in a TW assay. Similar to the observation in the TW assay, I observed using the MF platform that more IL-15 NK migration are recruited towards higher gradient of the DC CM compared to the IL-2 NK (Fig 10). Dark lines are the tracks of cells moving towards higher gradient of the DC CM whilst the light grey are tracks of cells moving away from the higher gradient towards the low gradient. As shown in Fig 15, there are more dark lines in the tracking of IL-15 NK (Fig 10B) compared to the tracking in the IL-2 NK (Fig 10A) – indicating that more IL-15 NK are recruited towards the DC CM gradient compared to IL-2 NK. At the end of the MF assay, majority of the cells were found to have migrated towards the end of the chamber with the highest DC CM gradient. These observations also confirm the previous findings or data that DC induce cytokine activated NK chemotaxis.

Having demonstrated that the IL-15 NK cells are more migratory than the IL-2 NK cells, I seek to account for the difference in the migration observed between the IL-15 and IL-2 NK cells.

I compared the level of activation between the IL-15 NK and IL-2 NK by assessing the proliferation. I also compared the viability of the IL-2 and IL-15 NK. Finally, I checked whether the difference in the IL-2 and IL-15 NK cell migration can be accounted for by the difference in expression of some known chemokines receptors.

3.6 Comparison of proliferation of IL-2 NK to IL-15 NK cells

After NK isolation, the cells were counted to give the NK cell number on Day 0. The cells were then seeded on a 48 well tissue culture plate in supplemented RPMI 1640 containing either IL-2 or IL-15. To check for NK cell proliferation, I monitored the number of cells over time by counting the number of viable cell everyday between Day 0 and Day 4. I found that both IL-2 and IL-15 activation of NK exhibited similar proliferation potential (Fig 11A). Also, they both followed almost a linear pattern. Between Day 0 and Day 1, there was a decrease in the cell count. But after Day 1, the cell count increase almost linearly.

The same number of cells were seeded in either the IL-2 or IL-15 culture and after 4 days of culture, the cells were harvested and counted. I then compared the cell count between the IL-2 and the IL-15 activation of NK cells. I found similar number and no statistical difference between the cell number in the 2 culture conditions (Fig 11B). Both cultures increase NK cells number similarly.

3.7 Comparison of viabilities of IL-2 to IL-15 activated NK cells

After 4 Days of activation of NK cells with either IL-2 or IL-15, the cells were harvested and their viabilities determined using Annexin V and Tryphan blue. Annexin V staining was carried out according to the manufacturer protocol and Annexin V binding determined using FACS. Using hemocytometer, the % of viable cells counted with Tryphan blue. As observed in Fig 12A and Fig 12B, there was similar viabilities between NK cells activated with either IL-2 or IL-15.

3.8 Can expression of known chemokines or chemokine receptor account for the difference in migration between IL-2 and IL-15 activated NK cells?

The expression of some chemokines receptors known to play a role in NK cells migration were tracked. I followed the expression of CXCR3, CCR5, CXCR4 and CX3CR1 from Day 0 to Day 4. As shown in Fig 13 and Fig 14, I observed a change in receptor profile on NK cells upon activation with either IL-2 or IL-15. For instance, I found the expression of CXCR3 to be up-regulated in both IL-2 and IL-15 activation of NK. But for CX3CR1, I found a decrease in expression in both IL-2 and IL-15 activation of NK. It has been previously reported in human studies that the activation of NK cells with IL-15 down regulate CX3CR1 expression (164). Another study reported that CX3CR1 expression on mouse NK is oppositely regulated by IL-2 and IL-15 (165). But my data shows that the expression of CXCR3 on splenic NK cell to be downregulated by both cytokines (IL-2 and IL-15).

The surface expression of the 4 chemokines receptors were compared between the Day 4 IL-2 and IL-15 activated NK cells. As shown in Fig 15, only CXCR3 is differentially expressed between

the IL-2 and IL-15 NK cells. The expression of the other 3 receptors was similar between the 2 cytokine activated NK cells.

It has been reported that there is an association between CD40 signaling and ip-10 production and also reported is the association between TLRs and ip-10 production (166). As mentioned above, CXCR3 was the only tested chemokine receptor that was upregulated with IL-2 or IL-15 activation. I checked for the production of the ligand for CXCR3 in the DC CM. CXCR3 has 3 known ligands: CXCL9, CXCL10 (IP-10) and CXCL11 (167). Our lab has reported on the role of the ip10/CXCR3 axis on NK migration towards LPS DC CM (151). In addition, of the 3 ligands for CXCR3, ip-10 is the most studied, so I checked for ip-10 production by the different DC using the DC CM. The amount of ip-10 in the different DC CM was quantified using the ip-10 platinum ELISA kit. The data shows that ip-10 is produced by the different TLR Ligands stimulated DC. There is differential production of ip-10 by the different TLR ligands stimulated DC (Fig 16). Fig 16 is a representative figure for the ip-10 ELISA data. I observed a similar trend in ip-10 production between the different TLR ligands in all ip-10 ELISA repeated. LPS DC produced more ip-10 compared to the other TLR ligands. The least amount of ip-10 was produced by the immature DC. For the stimulated DC, the least amount was produced by the CpG stimulated DC.

Having identified the ligand (ip-10) in the DC CM and its receptor (CXCR3) on the cytokine activated NK cells, I asked whether the CXCR3 receptor plays a role in activated NK migration towards the different TLRs stimulated DC's CM. I neutralized the CXCR3 receptor. I treated the cells with anti-CXCR3 mAb to block the CXCR3 receptor. As an isotype control, I used the Armenian Hamster IgG according to the manufacturer sheet. I then checked for the specific

binding of the anti-CXCR3 by staining for CXCR3 using the conjugated anti-CXCR3 Ab or its isotype control (anti-CXCR3 or IgG - APC) followed by flow cytometry. As shown in Fig 17, compared to either the non-neutralize or its isotype control, cells treated with anti-CXCR3 showed a significantly low available CXCR3 that can bind to the APC conjugated anti-CXC3. In summary, I observed a specific neutralization of the CXCR3.

The treated cells (anti-CXCR3 or isotype control treated cells) were used in a TW assay to determine the effect of CXCR3 on NK migration in the already established NK migration experimental platform. Neutralizing CXCR3 suppressed IL-2 and IL-15 NK migration towards the LPS and PIC stimulated DC CM (Fig 18A). In short, CXCR3 played a role in NK migration towards DC CM. I also quantified the NK migration of the treated NK cells towards the CM from the other TLR agonists and observed similar attenuation of NK migration upon blocking of the CXCR3.

I compared the suppression of NK migration between the IL-2 NK and the IL-15 NK migration. I found a stronger suppression of IL-15 NK cell migration towards the DC CM compared to suppression observed with the IL-2 NK cells (Fig 18B). The suppression in IL-15 NK migration was about 60% whilst the suppression in the IL-2 NK migration was about 20%. Finally, I compared the migration of the treated IL-2 NK cell to the migration of the treated IL-15 NK cells towards the CM of stimulated DC. I found similar migration and no statistical difference between the migration of the treated IL-2 and IL-15 NK cells (Fig 18C). Altogether, the data suggests that the difference in CXCR3 expression between IL-2 and IL-15 NK migration might account for the difference in migration observed between IL-2 and IL-15 NK towards the DC CM.

3.9 Migration of IL-2 and IL-15 activated NK cells in tumor settings

CM generated from different tumor cell lines was used in both the TW device and the MF platform to compare the migration of the IL-2 and the IL-15 activated NK cells.

With the TW device, I used the CM generated from the different tumor cell lines and compared the migration of the IL-2 and IL-15 NK migration towards the different tumor cell line CM. I found IL-15 activated NK cells to be strongly recruited towards the different tumor (4T1 and B16) or normal MMC CM compared to the IL-2 NK cells (Fig 23). CM from 4T1, B16 and MMC elicits a significantly stronger IL-15 NK cells chemotaxis compared to the IL-2 NK cells.

Because of the limitation of the TW, I employed the MF to validate my findings and as well measure other parameters. I used CM from E0771 and 4T1. I was expecting differential recruitment in the same direction between the IL-2 and IL-15 NK cells, but instead I surprisingly found that the IL-2 NK cells moved away from the gradient whilst the IL-15 NK cells moved towards the gradient generated from the different tumor cell line CM (Fig 24, Fig 25, Fig 26 and Fig 27). Fig 24 and Fig 25 are the images at the end of the migration and the figures show the last frame in the migration assay using the MF device. In Figures 24 and 25, I found more IL-15 NK cells were moving towards the higher gradient end of the chamber whilst more IL-2 NK were moving towards the lower gradient end of the chamber. There are more red tracks in the IL-15 NK migration indicating more cells moving towards the higher gradient. On the other hand, there were more green tracks in the IL-2 NK migration indicating more cells moving away from the gradient. The CI of IL-15 NK cells was positive indicating a net migration towards the gradient whilst the CI of IL-2 NK cells was negative indicating a net migration away from the gradient (Fig 27).

In the attached disc are videos of the migration in the MF device. These videos show live cell imaging data of the IL-2 and IL-15 NK migration in the CM gradient generated from different tumor cell line. E0771 and 4T1 are models of mouse breast cancer from C57BL/6 and Balbc background respectively. The tracks or the lines associated with the cells are colored to allow easier visualization of cells moving towards the gradient (red tracks) and cells moving away from the gradient (green tracks). The data shows that more IL-15 NK cells are moving towards the gradient generated from the different breast cancer cell lines CM. On the other hand, the data also shows that more IL-2 NK are moving away from the gradient generated from the different breast cancer cell lines CM.

Altogether, these data demonstrate that IL-15 NK cells are attracted towards the gradient generated from the CM of 4T1 and E0771 whilst the IL-2 NK are repelled away from the gradient generated from the CM of 4T1 and E0771.

Having shown that IL-2 NK cells are repelled by the CM generated from the 4T1, I seek to identify the factor(s) responsible for the repulsion of IL-2 NK by the 4T1 CM. In our recent publication, we showed that GM-CSF repels IL-2 activated NK migration and that neutralizing the GM-CSF by using anti-GM-CSF Ab enhance IL-2 NK migration (151). I used the same concentration of GM-CSF as in our previous study (20 ng/mL) in a MF device and assessed the migration of IL-15 NK in a gradient of GM-CSF (Fig 28). Unlike the IL-2 NK, IL-15 NK cells migrate randomly in the gradient of GM-CSF and the migration is similar to the migration in the control medium without the GM-CSF. In Fig 28, there is no net directional migration of the IL-15 NK in 20 ng/mL of GM-CSF. Similar number of green lines compared to the red lines. The videos show the random

migration of the IL-15 NK cell in both the GM-CSF and the control. This data indicates that GM-CSF at the tested concentrations (20 ng/mL) affects IL-2 NK cell migration but not IL-15 NK cell migration.

Using the CM from the different cell lines in an ELISA assay, I quantified the concentration of GM-CSF produced by the different cell lines. I found the CM from the 4T1 contained GM-CSF (Fig 29A). I also observed GM-CSF in the other tumor cell lines (Data not shown). After, I neutralized the GM-CSF in the 4T1 CM and checked the effect of the neutralization on the recruitment potential of the 4T1 CM. I found that neutralization of GM-CSF enhances IL-2 NK cells chemotaxis towards the 4T1 CM (Fig 30). Compared to the 4T1 alone and/or the GM-CSF isotype control, neutralization of GM-CSF significantly enhances IL-2 NK migration.

Taken together, this suggests that 4T1 produce GM-CSF that negatively affects IL-2 NK cells migration towards 4T1. Unlike IL-2 NK, GM-CSF does not affect IL-15 NK cells recruitment.

The previous findings showed that 4T1 and E0771 directly affect NK migration. A sub hypothesis I tested was whether tumor cells could affect NK migration indirectly via NK-DC crosstalk. It is well established that the tumor microenvironment affects immune cell functions in a positive or a negative way by either producing danger signals to boost the immune system or producing suppressive factors to suppress the immune system. The infiltration of NK cells to certain tumor environment is low compared to normal environment. We have shown that tumor cells can produce factors that can repel NK cells migration. We have also demonstrated elegantly that the stimulation of DC with different TLRs elicit a stronger cytokine activated NK cells chemotaxis compared to

the unstimulated DC. To test the sub-hypothesis, I used the CM from the different cell lines as the test condition and the CM from MMC as the control. I stimulated the DC with the different TLRs in the presence or absence of the CM from MMC or tumor cell lines. Then, I check for the maturation of the stimulated DC and the ability of these DC to recruit IL-2 activated NK. I assessed the expression of CD40 as marker for DC maturation.

As shown in Figures 31 B, 32 and 33, DC stimulated with the different TLR Ligands in the presence of the CM from the different tumor cell lines showed a lower CD40 expression compared to the DC stimulated with the different TLR Ligands in the absence of the CM. DC stimulated in the presence of the CM from the MMC showed a higher CD40 expression compared to the DC stimulated in the presence of CM from 4T1 or E0771 tumor cell line (Fig 33). The proportion of matured DC defined as the CD40 positive cells was lower in the LPS stimulated DC in the presence of either 4T1 or E0771 compared the absence or in the MMC treatment (Fig 33A). The MFI, a parameter that gives the intensity of the CD40 expression, in the treated condition with MMC was higher than the MFI in the condition with the tumor cell line. This data suggests that there is an impaired maturation of DC in tumor microenvironment compared to normal microenvironment. Fig 33 supports the findings that tumor microenvironment can negatively modulate immune response and specifically DC maturation compared to normal microenvironment.

I collected the CM from the treated DC and used it in a migration studies of IL-2 activated NK cells. I have already shown that LPS stimulated DC elicit strong IL-2 NK chemotaxis. The DC stimulated with LPS in the presence of 4T1 did not elicit a strong IL-2 NK chemotaxis compared to DC stimulated with LPS in the absence of 4T1 CM (Fig 31A). The iDC recruitment of IL-2 NK

migration was also impaired in the presence of the CM from tumor cell lines. This suggests that 4T1 also indirectly impair IL-2 NK chemotaxis through the NK-DC axis.

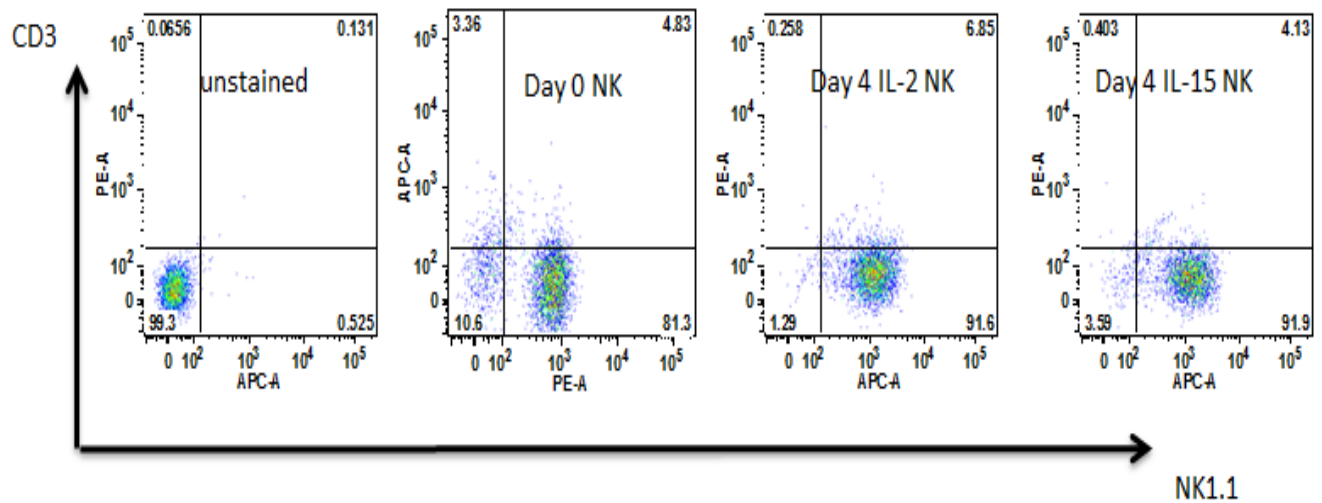
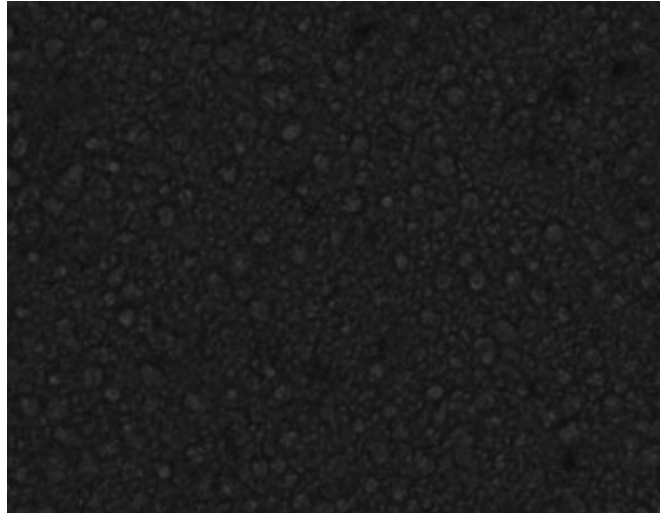
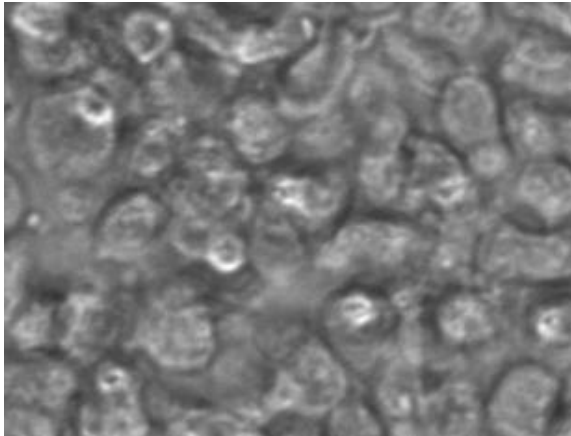


Figure 1: Representative figure on the purity of NK cells. The purity of the freshly isolated NK cells was determined immediately after isolation and 4 days post isolation using FACS by staining for CD3 and NK1.1. NK cells are defined as the CD3- and NK1.1+ population. NK cells were first isolated, counted and purity checked before being split for either the IL-2 or the IL-15 activation. The purity of the freshly isolated NK was about 80%. After the required days of activation (2, 3 or 4 days), cells were then harvested and phenotyped for NK1.1 and CD3 to determine cells purity. After the culture, we got purity of over 90%.

Day 0 NK



Day 4 IL-2 NK



Day 4 IL-15 NK

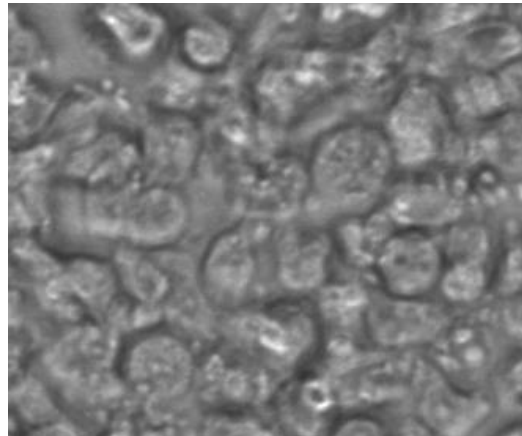


Figure 2: Activation of NK with cytokine changes NK cell morphology. IL-2 and IL-15 activated NK cells show similar morphology – large and elongated compared to the freshly isolated NK without the cytokine activation. Picture of the freshly isolated NK (Day 0) and the Day 4 NK cultured in either IL-2 or IL-15 was taken using the Evos inverted digital microscope. NK cell changes from small and round on Day 0 to large and elongated on Day 4. I used the same magnification for both the naïve and the cytokine activated NK cells (40×)

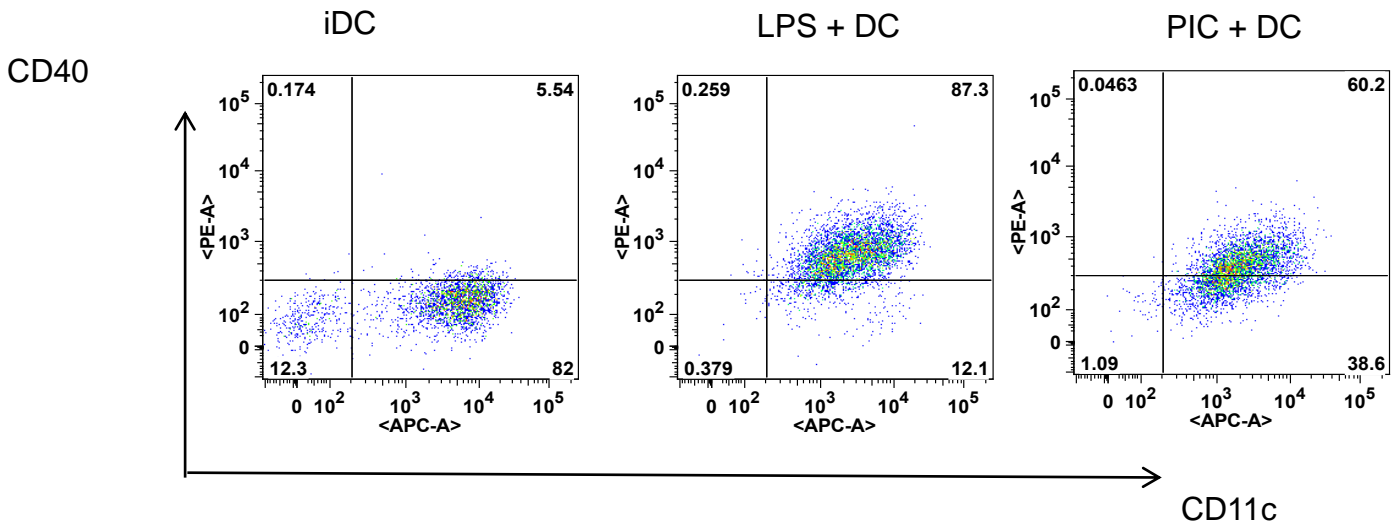


Figure 3: LPS and PIC mature DC express stronger CD40 compared to the unstimulated DC (iDC). The Day 7 generated BMDC were stimulated with optimal LPS conc (1ug/mL) or PIC conc (10ug/mL) for 24 hrs. The stimulated and un-stimulated DC were harvested and CD40 expression assessed using flow cytometry. The generated BMDC - the iDC and mDC- were CD11c high. Stimulation of DC with LPS or PIC increases the expression of CD40 and the percentage of CD40+ cell

Fig A

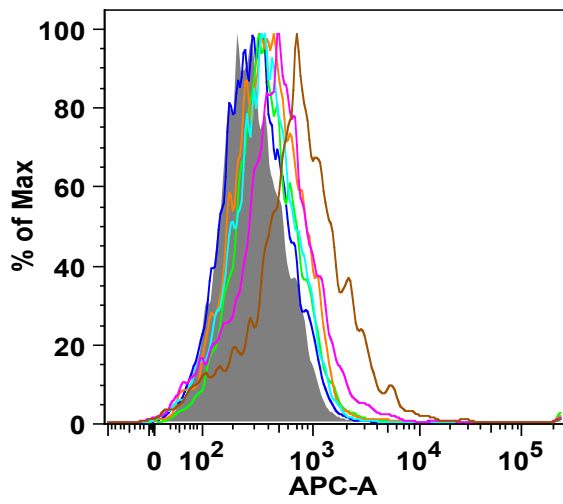
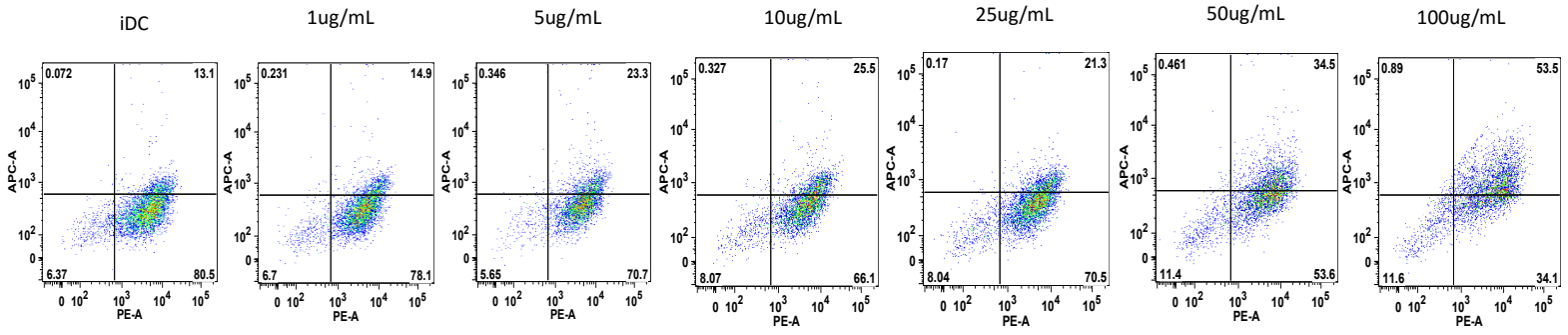


Fig B

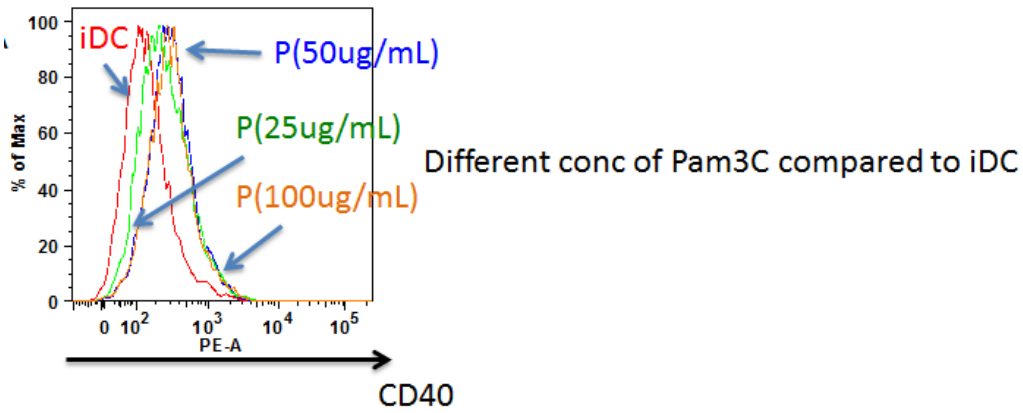


Fig C

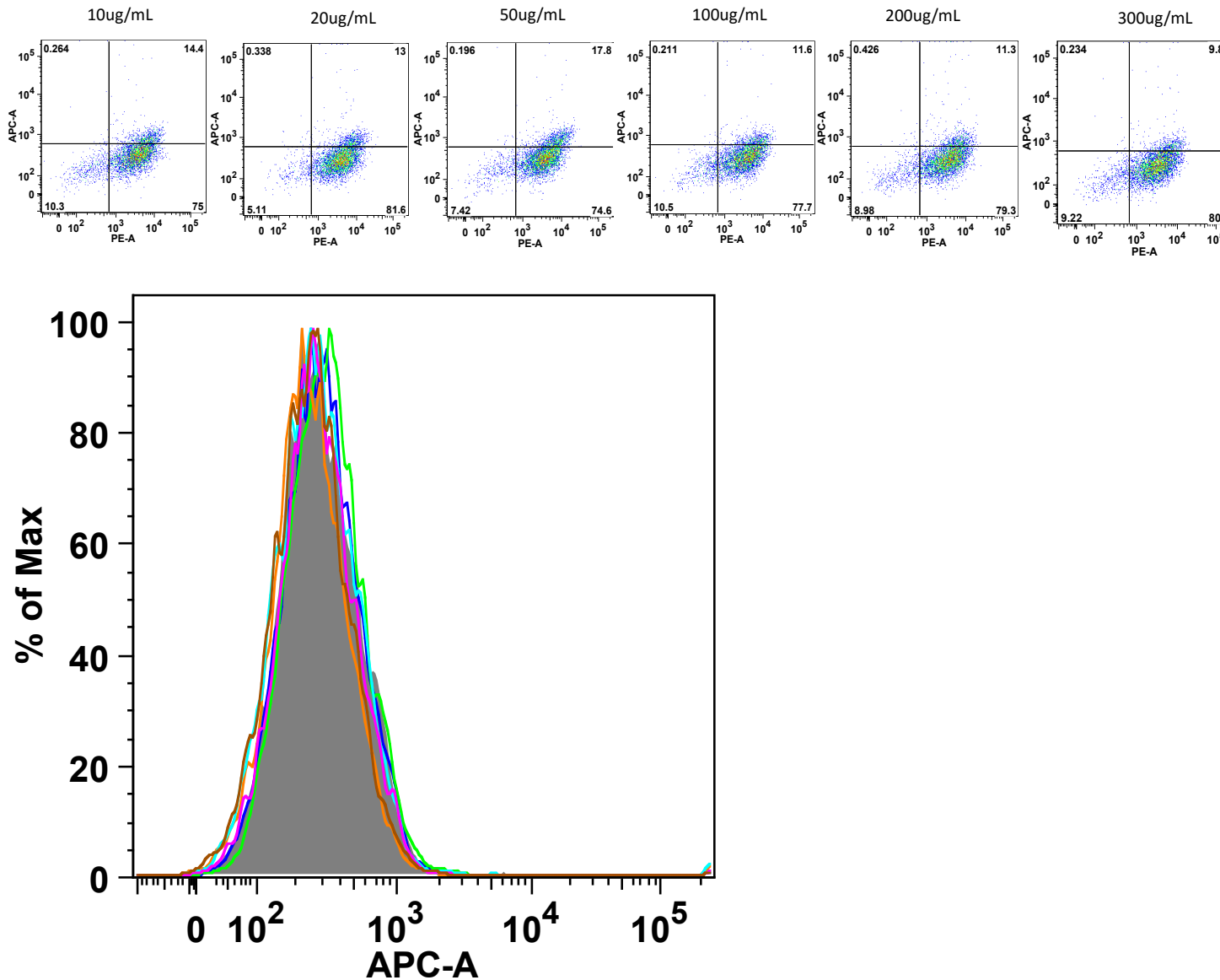


Figure 4: Titration of different concentration of Pam3C and Flagellin to obtain optimal concentration required for BMDC maturation. CD40 expression on DC stimulated with different concentration of Pam3C and Flagellin. **Fig A:** Stimulation of DC with Pam3C up-regulate CD40 expression compared to the unstimulated DC. DC were stimulated with either 1ug/mL, 5ug/mL, 10ug/mL, 25ug/mL, 50ug/mL or 100ug/mL of Pam3C for 24 hrs. Cells were harvested and phenotyped for CD40 using flow cytometry. The top and bottom panels of Fig A indicates the CD40 expression on DC stimulated with different Pam3C conc. **Fig B:** I repeated the titration with 25ug/mL, 50ug/mL or 100ug/mL of Pam3C instead of 6 different concs and phenotype for CD40 expression. **Fig C:** Stimulating DC with different Conc of Flag (10ug/mL, 20ug/mL, 50ug/mL, 100ug/mL, 200ug/mL and 300ug/mL) does not up-regulate CD40 expression compared to iDC. The concentration of Flag tested does not increase the CD40 expression and does not show a dose CD40 upregulation. APC gives the CD40 expression whilst PE is the CD11c

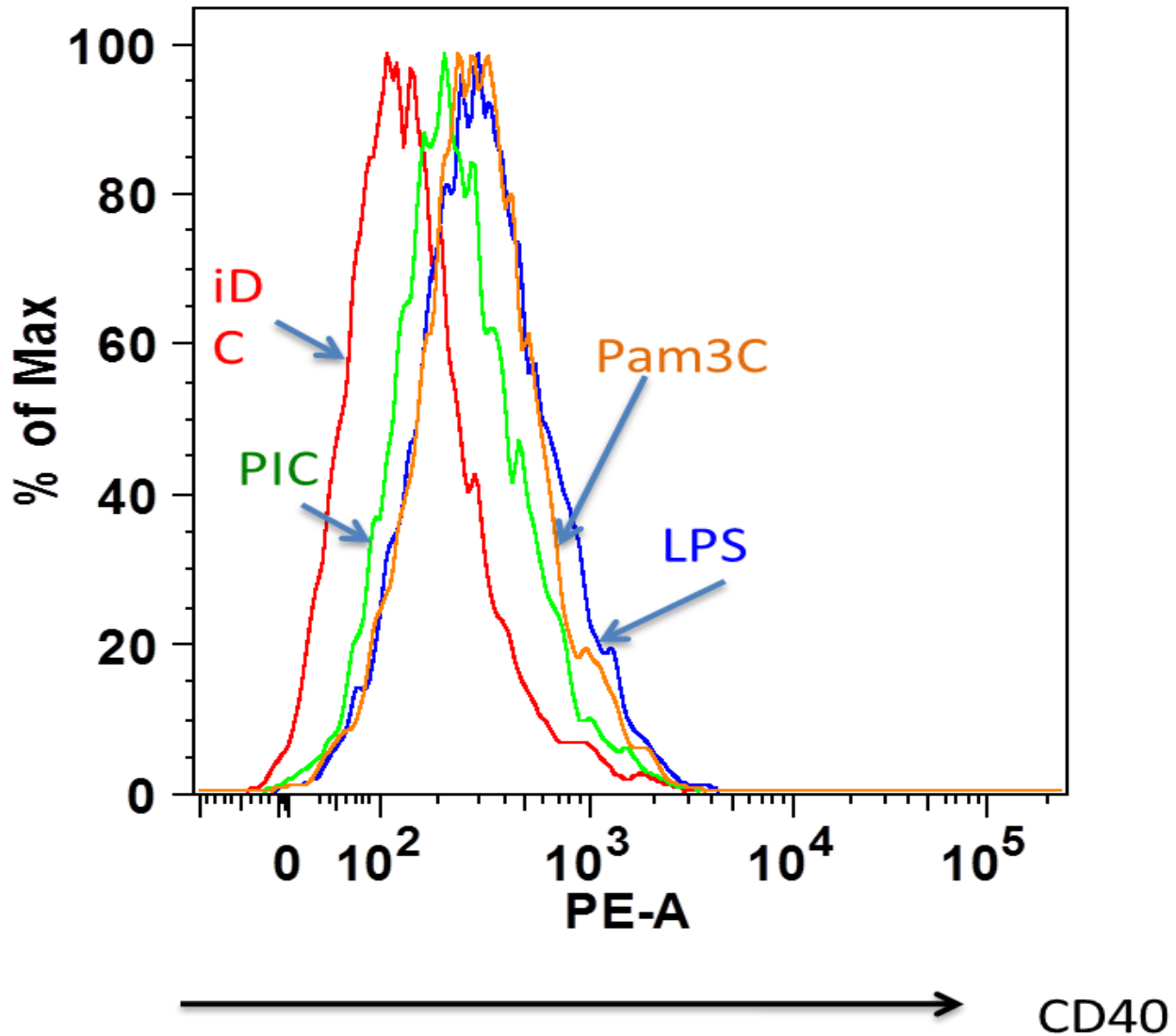


Figure 5: Comparison of CD 40 expression on DC stimulated with optimal conc of the different TLR Ligands. DC were stimulated with the optimal conc of the different TLR for 24 hrs. The DCs were harvested and stained and phenotyped for CD 40 expression using flow cytometry. Conc of the different TLR used were LPS (1ug/mL), PIC (10ug/mL) and Pam3C (50ug/mL). The optimal conc of the different TLR agonists upregulate the CD40 expression on DC compared to the immature DC.

Fig A

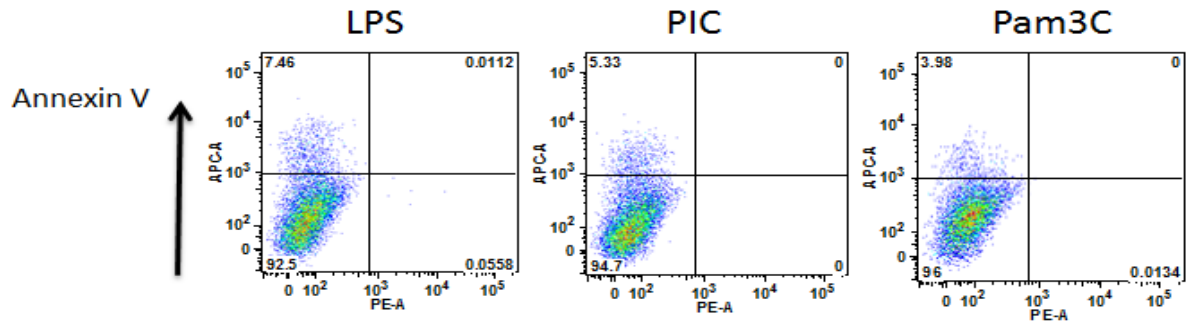


Fig B

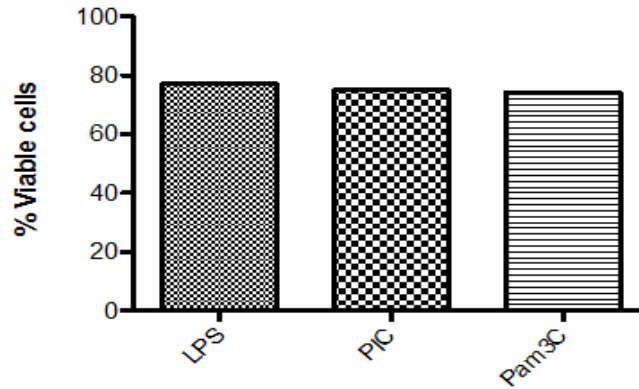


Figure 6: Comparison of Viability of DC stimulated for 24 hours with the different TLR using Annexin V and Tryphan blue. After 24 hrs stimulation with optimal conc of the different TLR, the DC were harvested and stained with Annexin V dye and the cell that do or do not take up the dye determined using flow cytometry (Fig A). Fig B: The harvested cells were counted with the hemocytometer to determine the percentage of viable cells using Typhan blue. Conc of TLR used to stimulate the DC were LPS (1ug/mL), PIC (10ug/mL) and Pam3C (50ug/mL).

Fig A

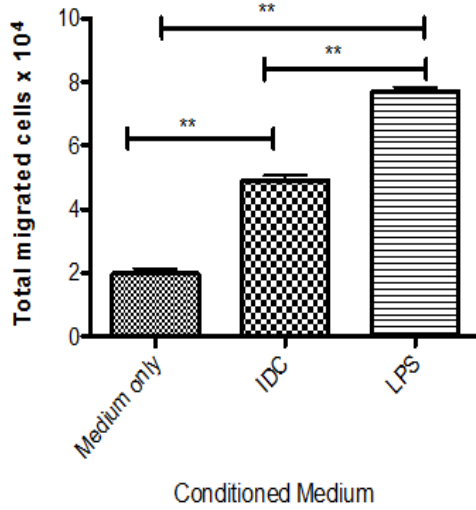


Fig B

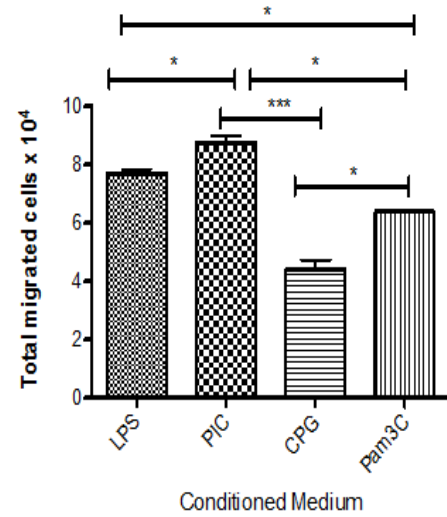


Figure 7: TLRL pulsed DCs induce migration of IL-2 activated NK cell. DC activated by different Toll like receptor (TLR) ligands at optimal concentration already determined. iDCs were generated using GM-CSF culture condition. On day 7, DCs were stimulated with either LPS (1ug/mL), Poly IC (PIC) (10ug/mL), Pam3C (5ung/mL) or CpG (5ug/mL) for 24 hrs. The conditioned medium from the DCs were collected and used in the trans-well experiment. **Fig A:** Compared to the medium control group, iDC as well as LPS stimulated DCs induced IL-2 activated NK cells. **Fig B:** The different TLRL pulsed DC recruit IL-2 activated NK differently, PIC > LPS > Pam3C > CpG.

Fig A

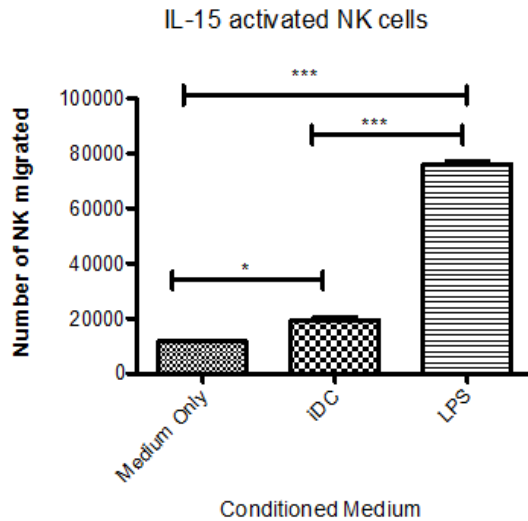


Fig B

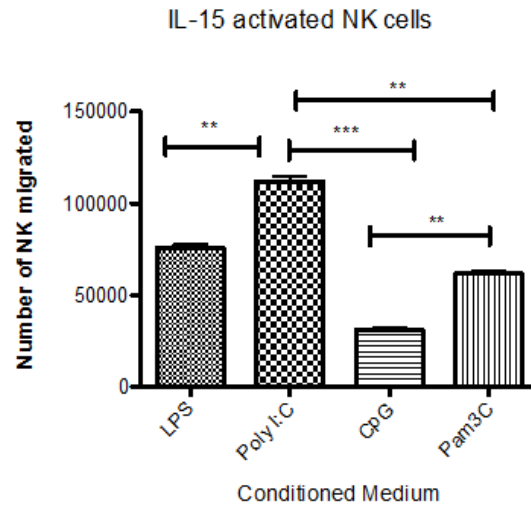


Figure 8: TLR pulsed DCs induce migration of IL-15 activated NK cell. IL-15 activated NK shows differential migration towards the CM of DC activated by different Toll like receptor (TLR) ligands at optimal concentration. iDCs were generated using GM-CSF culture condition. On day 7, DCs were stimulated with either LPS (1ug/ml), Poly IC (PIC) (10ug/ml) CpG (25ug/ml) or Pam3C (50ug/ml) for 24 hrs. The conditioned medium from the DCs were collected and used in the trans-well experiment whilst the expression of CD40, CD80 and CD86 on the DC were checked to ensure that the DC were stimulated. **Fig A:** DC CM enhance IL-15 NK cells migration with the LPS mature DC enhancing stronger chemotaxis compared to the immature DC. Also, compared to the medium control group, iDC as well as stimulated DCs induced chemotaxis in the IL-15 activated NK cells. **Fig B:** Different mature DC shows differential ability to recruit IL-15 activated NK cells. PIC shows stronger recruitment than LPS which in turn shows a stronger recruitment than Pam3C and CpG.

Fig A

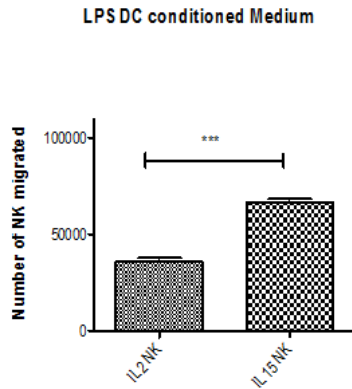


Fig B

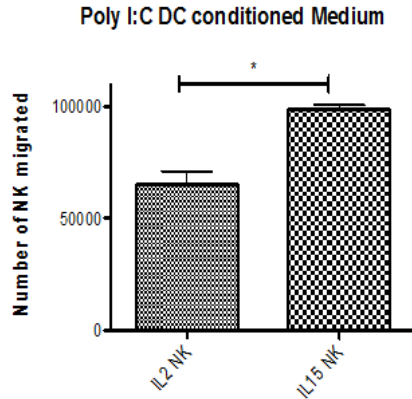


Fig C

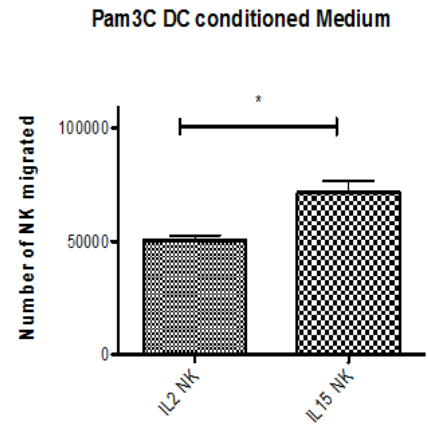


Figure 9: Migratory properties of different cytokine activated NK cells towards TLR Ligands stimulated DC. IL-15 activated NK migrate strongly towards the CM of the different TLR stimulated DC compared to IL-2 activated NK cells. 0.2 million cells in 100ul medium were placed in the top chamber of a conventional trans-well assay whilst the bottom chamber contained the CM from the TLR pulsed DC. **Fig A** LPS, **Fig B** PIC and **Fig C** Pam3C. After 3 hrs incubation, the cells that migrated from the top to the bottom chamber were harvested and counted to give the number of NK migrated.

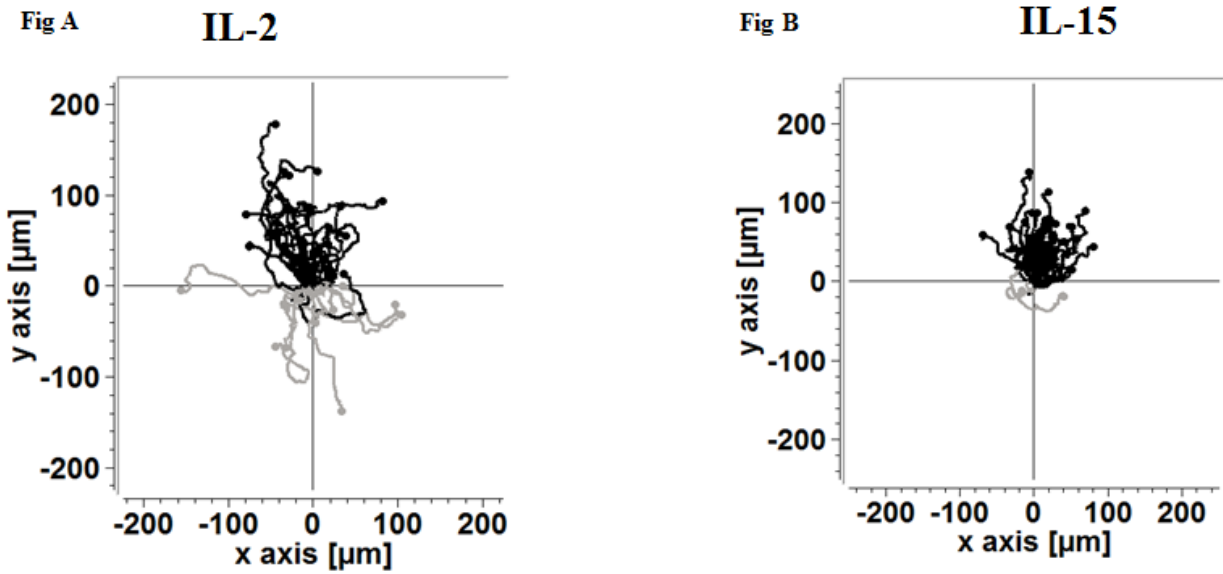


Figure 10: Comparison of the IL-2 NK and IL-15 NK cell migratory responses towards the CM of the LPS stimulated DC in the microfluidic system. Cell track analysis. IL-15 activated NK cells migrated strongly compared to IL-2 activated NK in a gradient established using CM from LPS activated DC in a microfluidic device. Cell tracks with light grey color show cells migrating away from the gradient whilst the black tracks show cells moving towards the gradient. 0.4 Million cells were loaded in the chamber. **Fig A** shows cell tracks of the IL-2 activated NK cells whilst **Fig B** shows the tracks for the IL-15 activated NK cells. The gradient of LPS CM increase from bottom to the top of the page.

Fig A

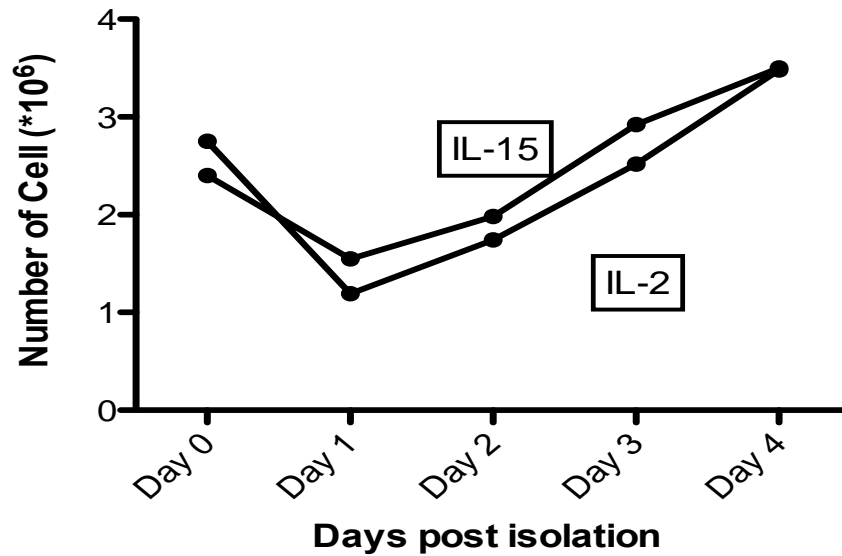


Fig B

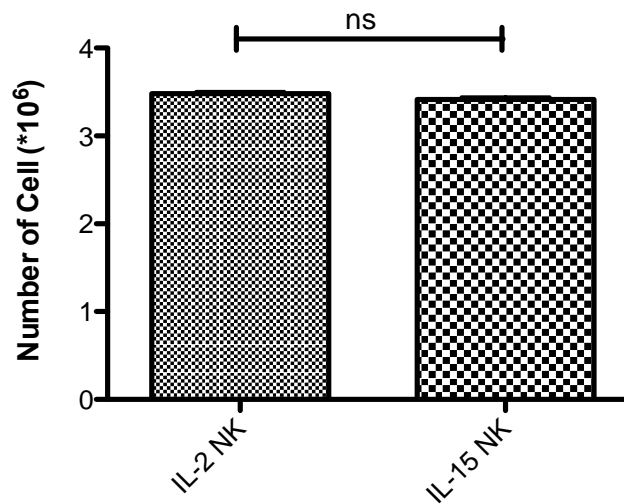


Figure 11: Comparison of NK cell proliferation by monitoring NK cell count during the 4 day culture in either IL-2 or IL-15 culture medium. After NK cell isolation, using typhan blue, the number of viable cells were counted and seeded in a 48 well tissue culture plate in supplemented RPMI 1640 containing either IL-2 or IL-15. Using typhan blue to get the number of viable cells, viable cells were determined and plotted against the day post culture. **Fig A:** Cell count vs Days post isolation. Viable cells initially decrease on first day of culture and later increase almost linearly. **Fig B:** 2 million cells of freshly isolated NK cells were cultured in either IL-2 or IL-15 for 4 days and the cells counted and compared. There was no statistical difference between the number of cells with IL-2 or IL-15.

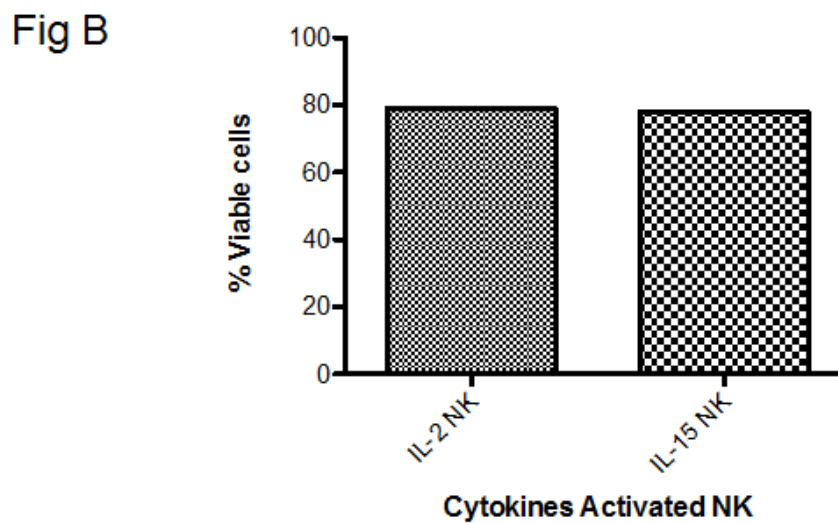
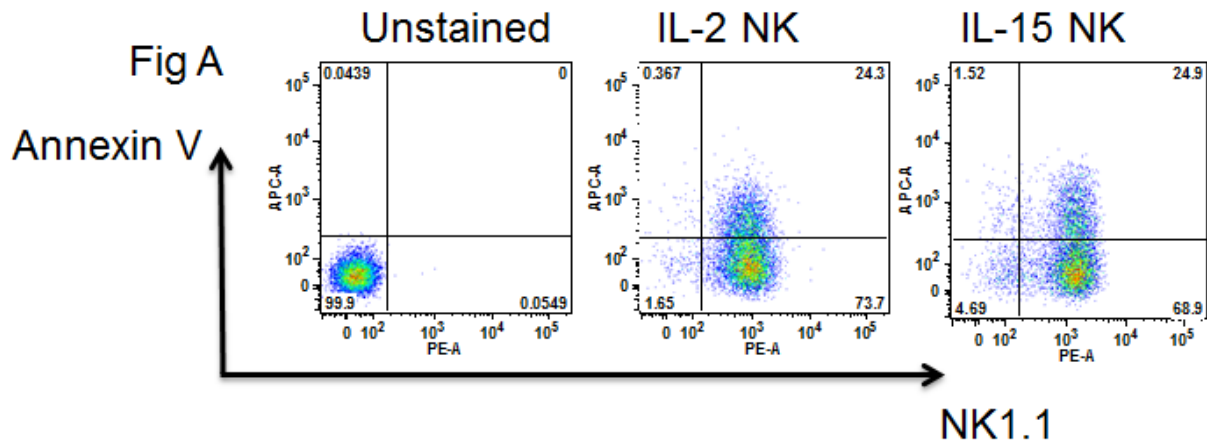


Figure 12: Viability of Day 4 IL-2 and IL-15 NK cell using Annexin V and tryphan blue. The Day 4 IL-2 and IL-15 NK were harvested and stained for cell viability using Annexin V staining as recommended by manufacturer's protocol. Also the number of viable cells counted using hemocytometer. **Fig A:** After Annexin V staining, flow cytometry used to determine the NK cells that do and do not take up the dye determined. Both activation showed similar annexin V staining. **Fig B:** The cells were counted using the hemocytometry in tryphan blue to determine % of live cells. Both IL-2 and IL-15 NK activation produced similar percentage of viable cells.

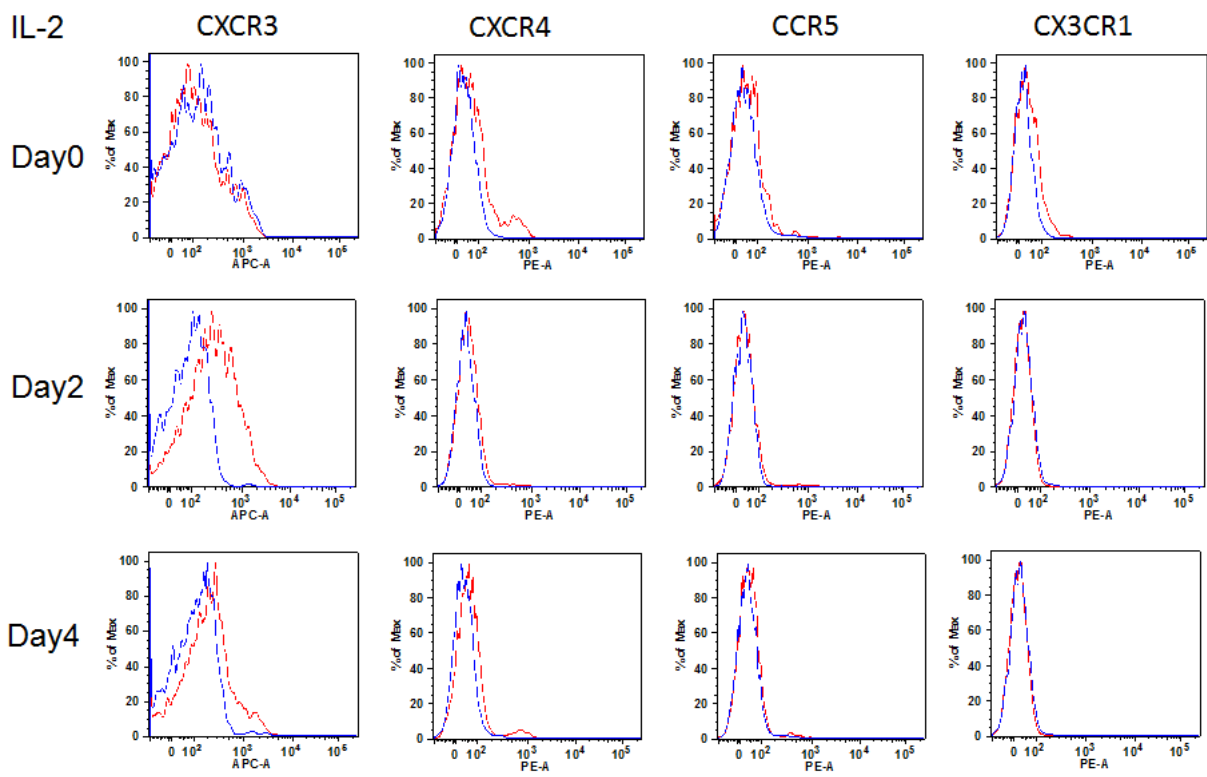


Figure 13: Chemokine receptor profile of NK cells upon IL-2 activation. The expression of CXCR3, CXCR4, CCR5 and CX3CR1 on the NK cell were determined using flow cytometry. The cells were harvested on different days and stained using the antibody for the respective receptor and its isotype control. The expression determined using flow cytometry. Blue curve is the curve for the isotype control whilst the red curve is the curve for the actual chemokine. Only CXCR3 showed a significant upregulation upon IL-2 activation.

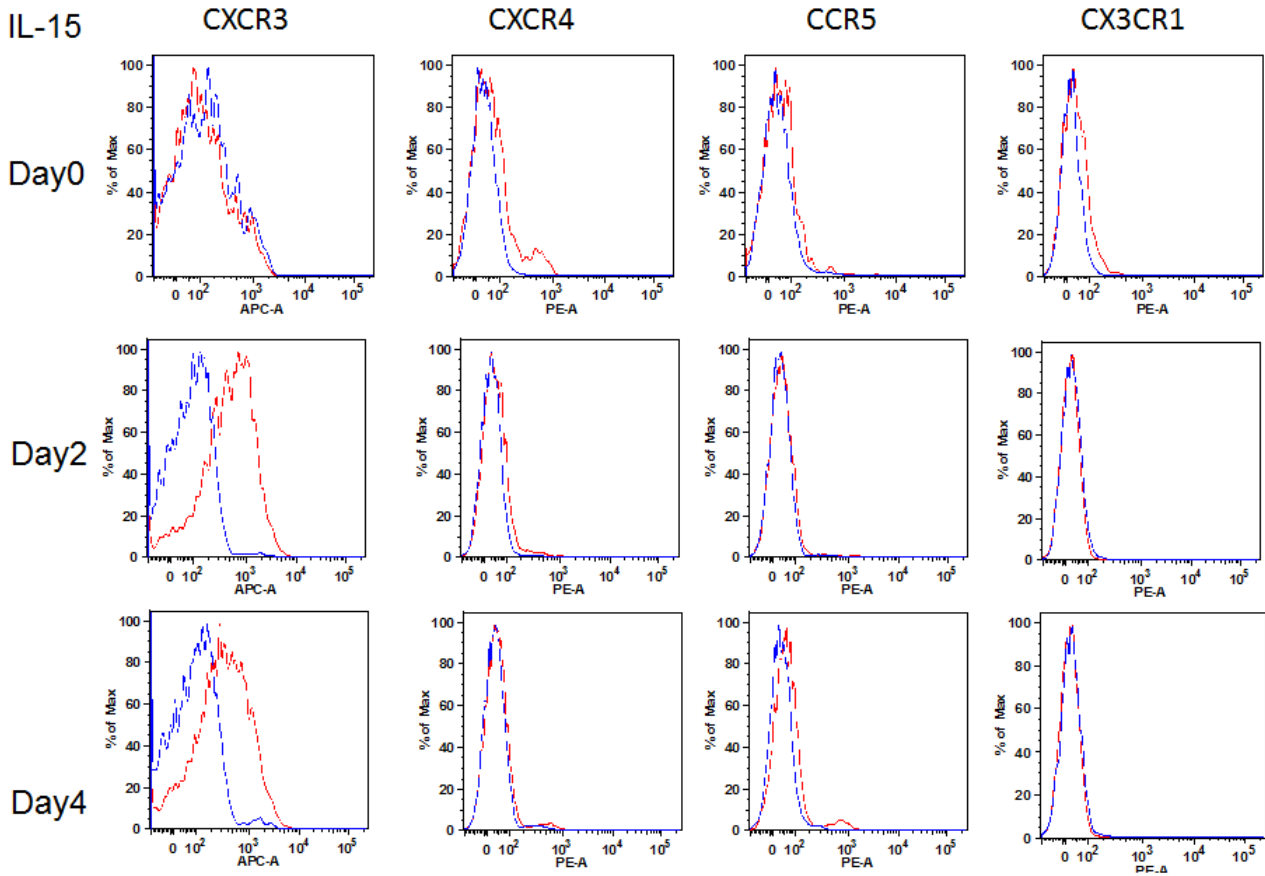


Figure 14: Chemokine receptor profile of NK cells upon IL-15 activation. The expression of CXCR3, CXCR4, CCR5 and CX3CR1 on the NK cell were determined using flow cytometry. The cells were harvested on different days and stained using the antibody for the respective receptor and its isotype control. The expression determined using flow cytometry. Blue curve is the curve for the isotype control whilst the red curve is the curve for the actual chemokine. Only CXCR3 showed a significant upregulation on the NK cells upon IL-15 activation.

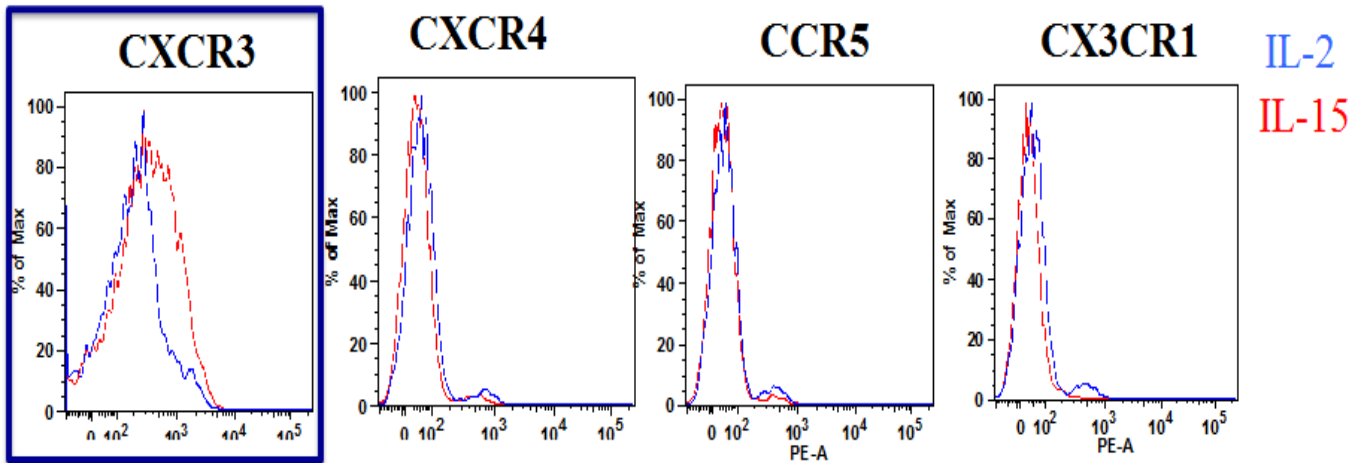


Figure 15: Comparison of known chemokine on Day 4 between the IL-2 and IL-15 activated NK cells. Flow cytometry data of some chemokine receptor expression on IL-2 and IL-15 activated NK cells. Day 4 activated NK were stained and phenotyped for the expression of CXCR3, CXCR4, CCR5 and CX3CR1 and the expressions compared between the cytokine activated NK. The expressions on the IL-2 and IL-15 NK were similar for all the tested chemokine receptors except for the CXCR3. IL-15 NK expressed stronger CXCR3 compared to the IL-2 NK cells.

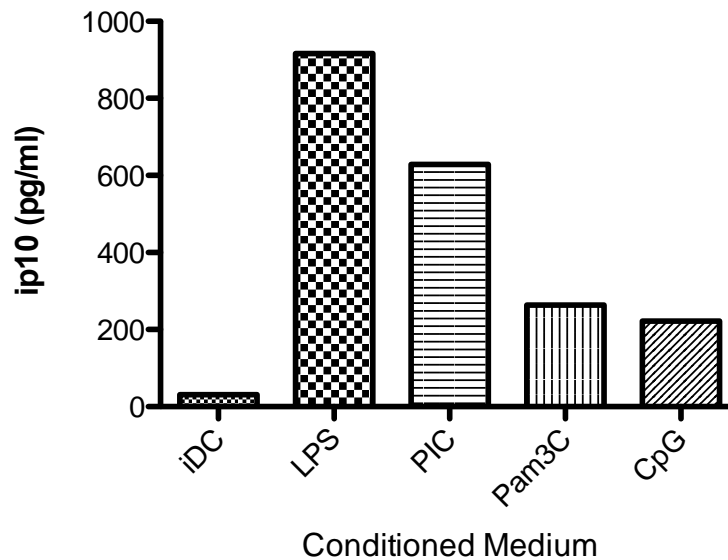


Figure 16: Elisa data quantifying the chemokine expression in the CM obtained from various TLR-stimulated DC. The amount of CXCL10 (ip10) in the CM from different TLR pulsed DC was measured using ELISA. Maturation of DC with TLR for 24 hours increased the production of ip10 compared to the immature DC. LPS stimulated DC produced greater amount of ip10 compared to PIC, Pam3C or CpG stimulated DC. LPS > PIC > Pam3C > CpG. CM collected after 24 hrs stimulation with the different TLR were assayed using ip-10 optimum ELISA kit according to the manufacture protocol

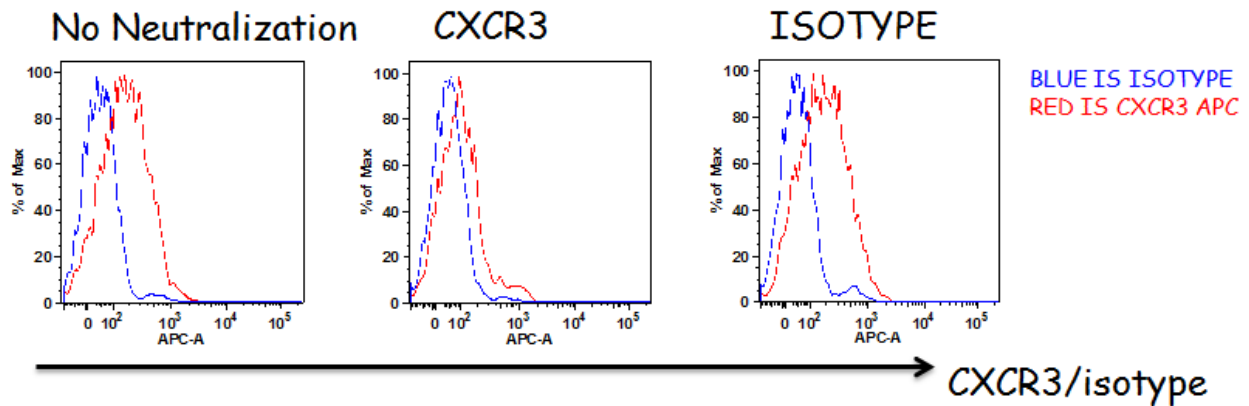


Figure 17: Confirmation of the neutralization of CXCR3 on activated NK. Flow cytometry data of neutralization of CXCR3 on cytokine activated NK. Activated NK cells were treated with Anti-CXCR3 or its isotype (Armenian Hamster IgG) and incubated for 30 mins in the humidified incubator. After the incubation, the cells were stained for CXCR3 to check whether the CXCR3 was blocked or not. Flow analysis of the stained cells showed CXCR3 neutralization and anti-CXCR3 Ab specificity.

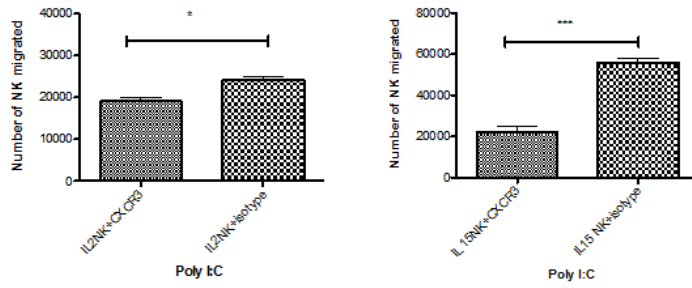
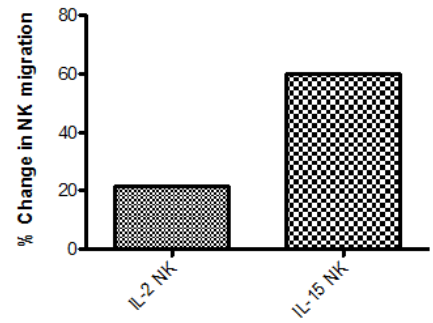
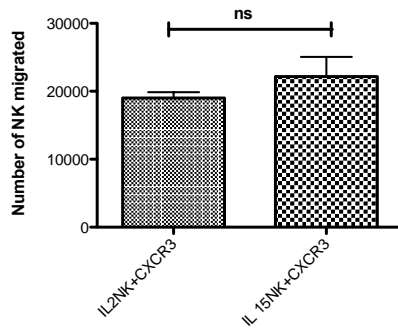
Fig A**Fig B****Fig C**

Figure 18: Role of chemokine receptors in mediating the differential migratory responses of cytokine activated NK cells. Fig A: Neutralizing CXCR3 attenuate IL-2 and IL-15 NK migration towards the CM of TLR pulsed DC. IL-2 and IL-15 activated NK cells were treated with Anti-CXCR3 or its isotype (Armenian Hamster IgG) and incubated for 30 mins. The treated cells were used in a conventional transwell assay with supernatant from DC pulsed with PolyI:C as the CM. After 45 mins incubation, the NK cells that migrated from the upper to the lower chamber were harvested and counted. **Fig B:** Percent suppression in NK migration after CXCR3 neutralization. The difference between the migration upon neutralization of the CXCR3 and the isotype control was expressed as percentage of the un neutralize. **Fig C:** Migratory response of IL-2 and IL-15 NK migration after neutralization of the CXCR3 receptor.

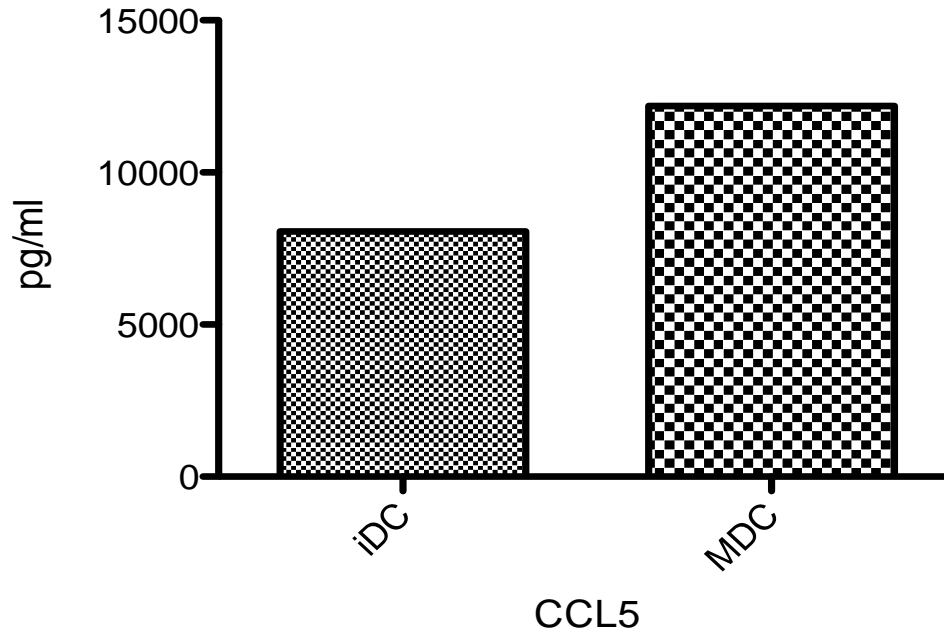


Figure 19: LPS matured DC secrete a higher amount of CCL5 compared to iDC. Quantification of CCL5 production using multiplex assay. The CM from the immature DC and LPS stimulated DC (MDC) were collected and used in a multiplex assay. The horizontal axis indicated whether the CM was obtained from i.e. whether it is from an immature DC or mature DC. The vertical axis indicates the conc of the CCL5 in the CM expressed in pg/mL.

IL-15 NK Migration

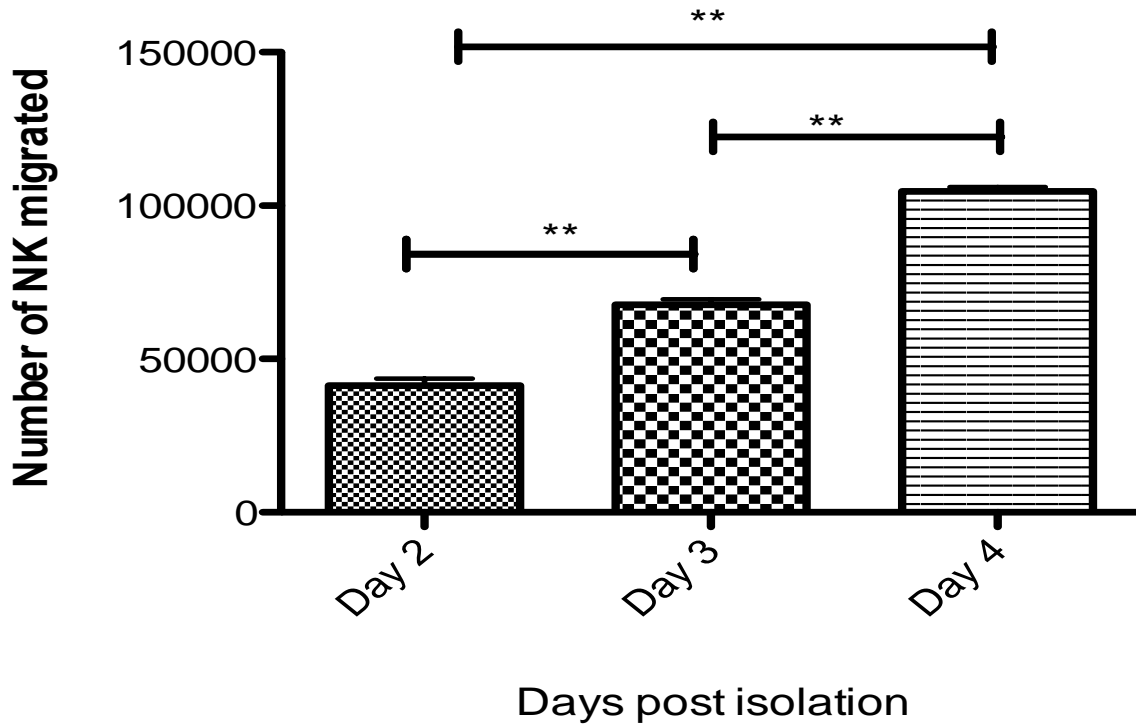


Figure 20: Different days cytokine activated NK migrate differently towards DC CM. The migration of NK cells activated with IL-15 for different period of time post isolation were conducted in a conventional TW assay. 0.2×10^6 of the different days activated NK cells were loaded into the top chamber of the TW and 600uL of DC CM placed in the bottom chamber. After 3 hrs of incubation, the transmigrated cells were harvested and counted. Day 4 cytokine activated NK are more migratory compared to Day3 and Day2. The horizontal axis indicates the number of days the NK cells were in the culture medium.

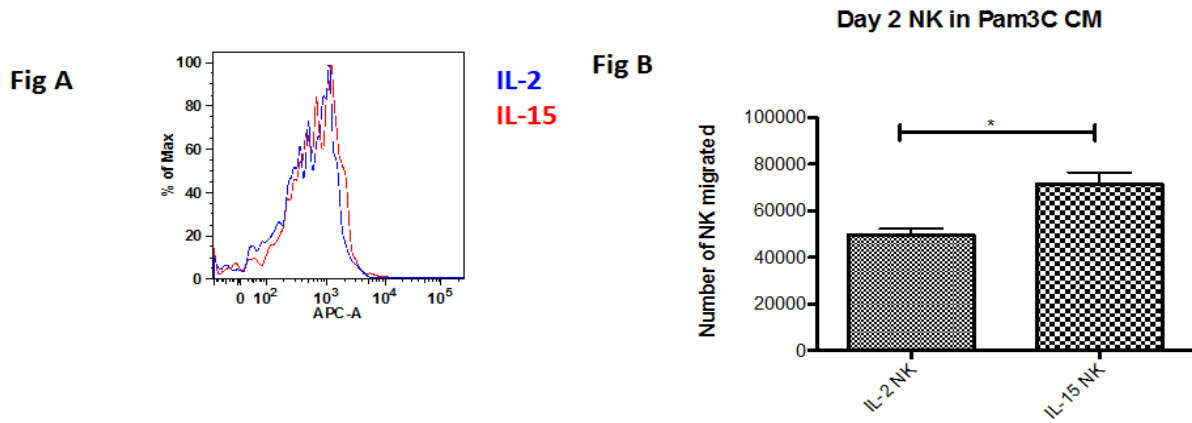
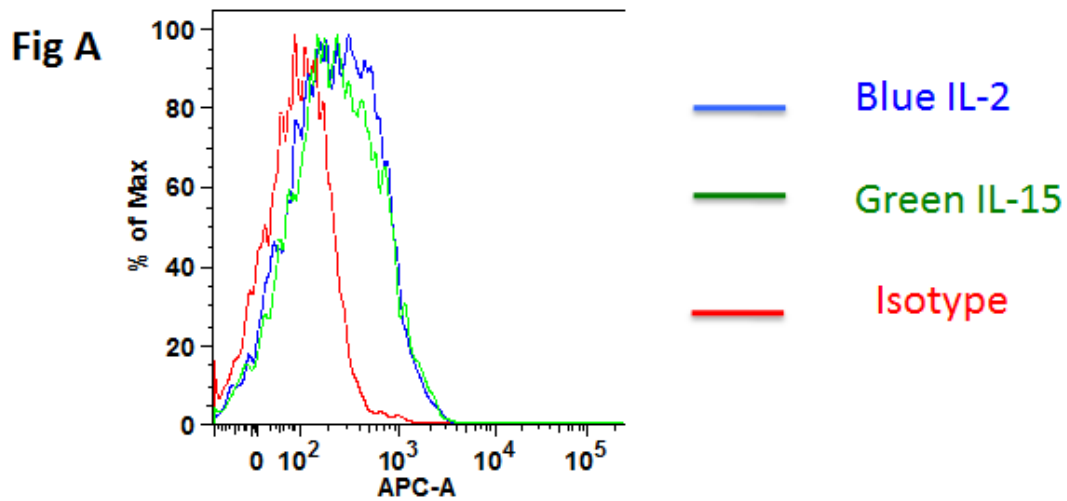


Figure 21: Day 2 IL-15 activated NK migrate strongly towards DC CM despite similar CXCR3 expression. Fig A: Comparison of CXCR3 expression on Day 2 IL-2 and IL15 NK cells. Day 2 IL-2 and IL-15 NK shows similar CXCR3 expression. The NK were phenotyped for CXCR3 expression using flow cytometry. **Fig B:** Comparison of the migration of the Day 2 IL-2 and IL-15 activated NK towards DC CM. NK migration was carried out in the TW device.



Day 3 NK migration in PIC CM

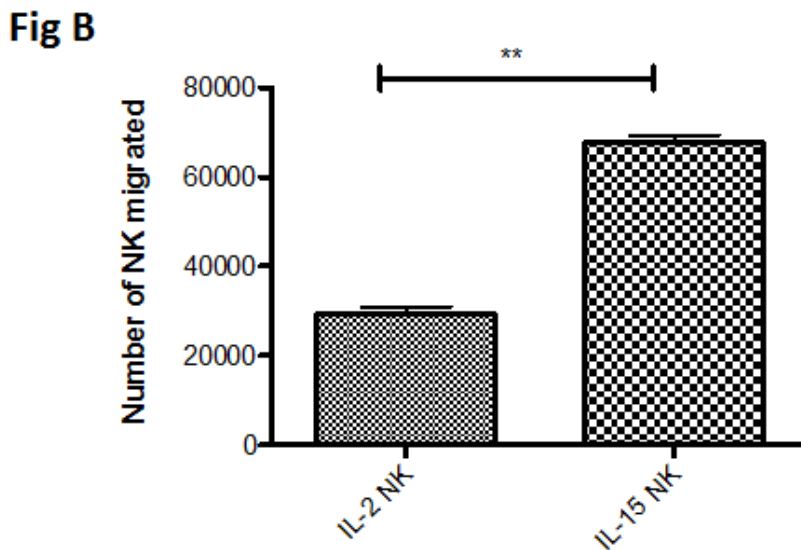


Figure 22: Day 3 IL2 and IL-15 activated NK shows different migration towards DC CM despite similar CXCR3 expression. The NK migration was assayed in a conventional TW device **Fig A:** Comparison of CXCR3 on Day 3 IL-2 and IL15 NK. Day 3 IL-2 and IL-15 NK shows similar CXCR3 expression. **Fig B:** Comparison of the migration of the different cytokine activated NK towards DC CM. Day 3 IL-15 NK migrate strongly towards DC CM compared to the Day 3 IL-2 NK. I employed the TW device.

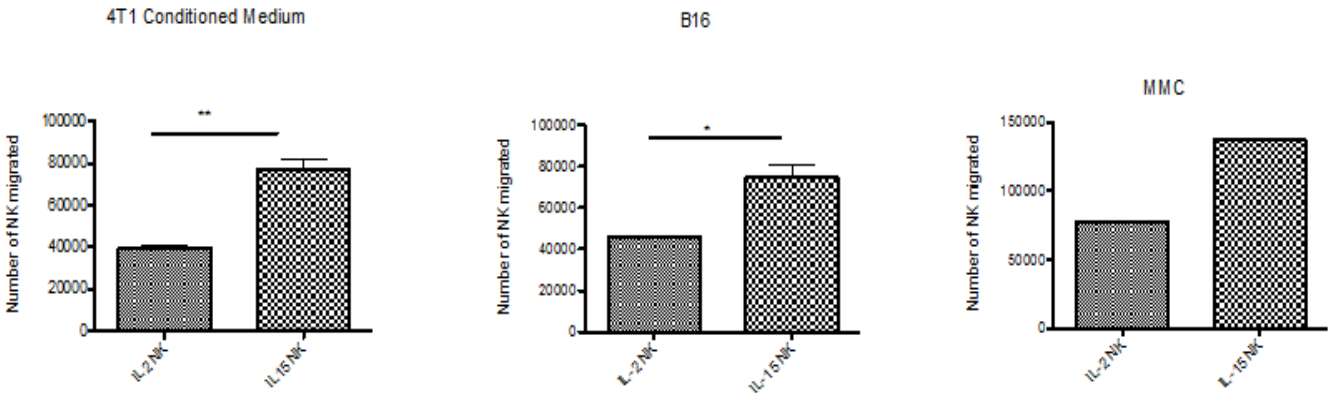


Figure 23: IL-15 activated NK migrate strongly towards the CM of different cancer cell line and normal mouse mammary cells (MMC) compared to IL-2 activated NK cells. CM was obtained from 4T1, B16 cell line. For the MMC, the 4th mouse mammary gland was isolated and cultured to obtain its CM (see materials and methods). CM were used in a conventional trans-well assay to compared the migration of IL-2 and IL-15 NK towards the different CM.

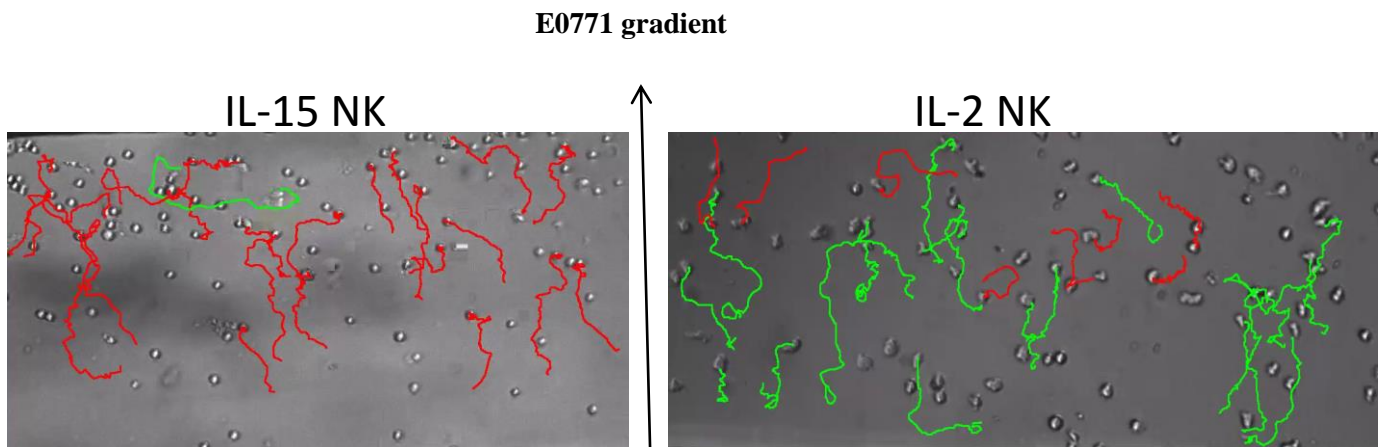


Figure 24: Live cell tracking of IL-2 and IL-15 activated NK cells in gradient of E0771 using a Microfluidic device. The last frame in the assay using the MF platform. IL-15 NK moves towards the gradient of E0771 whilst IL-2 NK moves away from the gradient. Red tracks are the tracks of cells moving towards the gradient whilst green tracks are the tracks of cells moving away from the gradient. 0.4×10^6 of either IL-2 or IL-15 activated NK cells were loaded into a chamber coated with fibronectin and the cells allowed to adhere to the fibronectin coated chamber. Cells are then allowed to move in the gradient and live cell images capture with camera and then analyzed with the appropriate software. More red tracks with the IL-15 NK compared to the IL-2 NK with more green tracks. Red tracks are for cell moving towards the gradient whilst the green tracks are for cells moving towards the gradient. See attached videos.

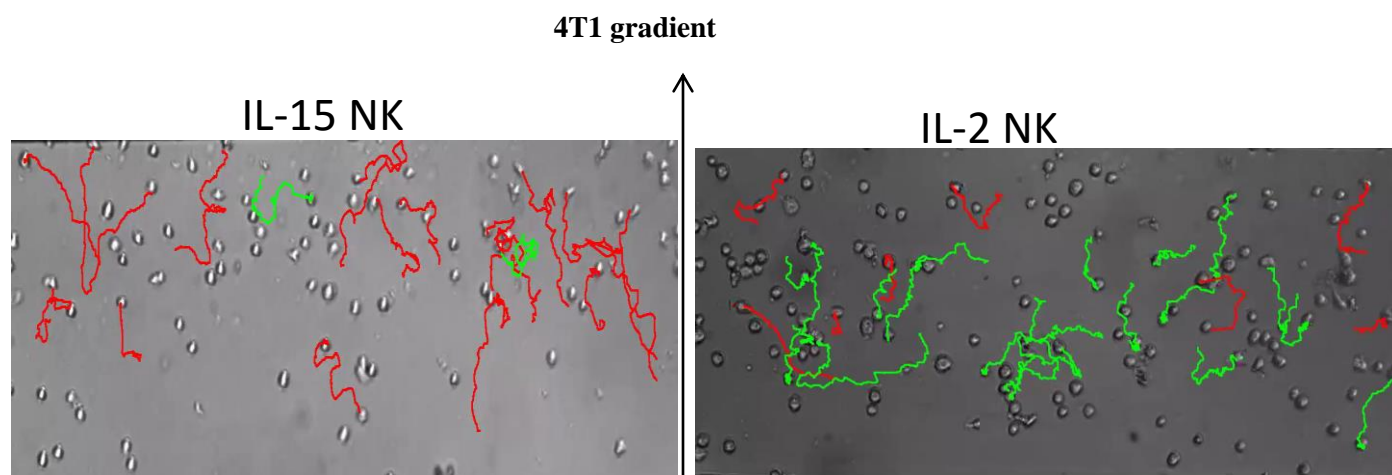


Figure 25: Live cell tracking of IL-2 and IL-15 activated NK cells in gradient of 4T1 using a Microfluidic device. The top is the high 4T1 gradient end whilst the bottom is the low 4T1 gradient end. IL-2 NK cells get repelled by the gradient whilst IL-15 are attracted to the gradient. More red tracks with the IL-15 whilst there are more green tracks with the IL-2 NK. The arrow shows direction of increase 4T1 gradient. See attached videos

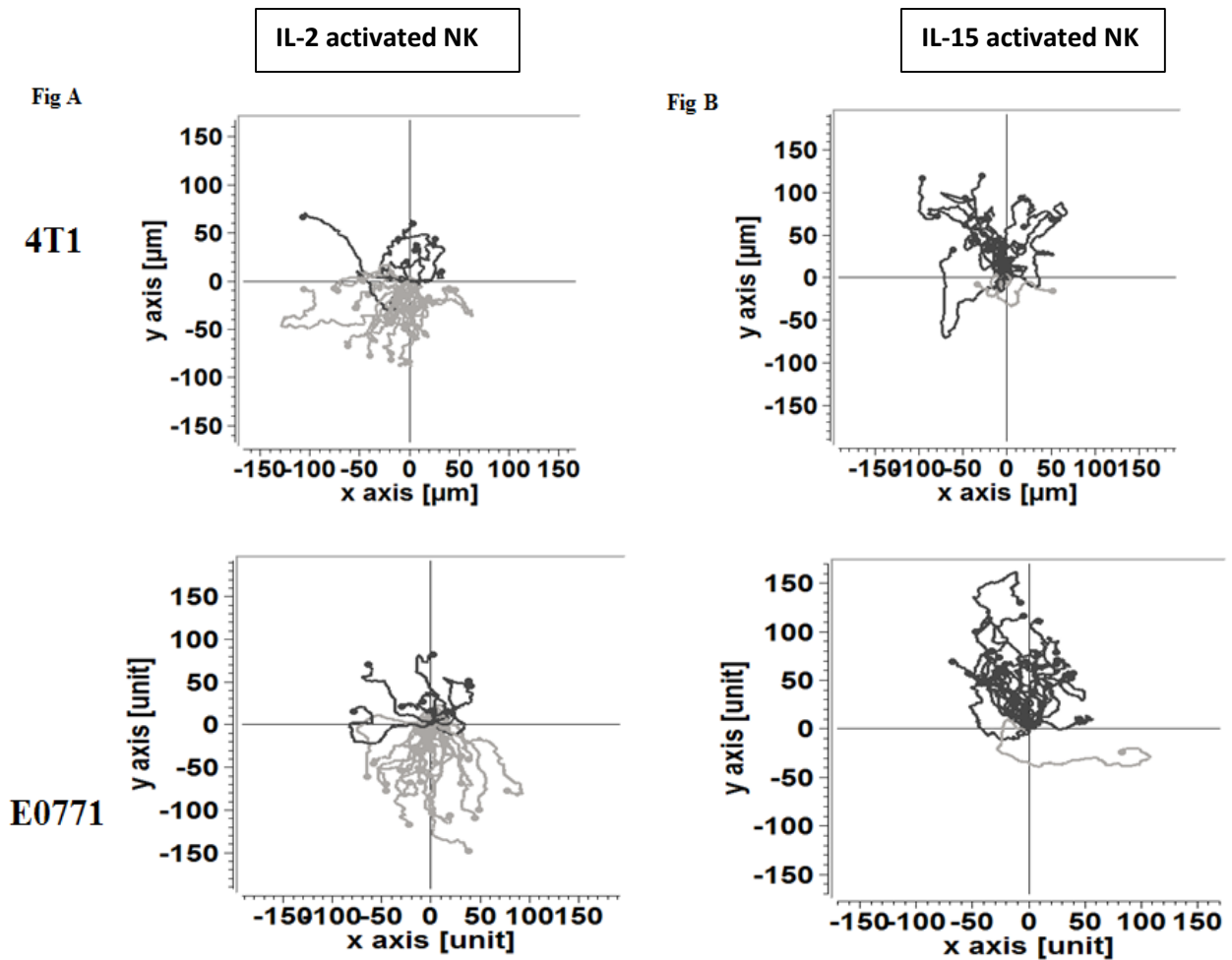


Figure 26: Cell tracking - Comparison of the IL-2 NK and IL-15 NK cell migratory responses towards the CM of the 4T1 and E0771 in the microfluidic system. IL-15 activated NK cells migrated towards the gradient of 4T1 and E0771 whilst IL-2 activated NK migrated away from the 4T1 and E0771 gradient in a microfluidic device. Cell tracks with light grey shows cells migrating away from the gradient whilst the dark grey shows cells moving towards the gradient. 0.4 Million cells were loaded in the chamber. **Fig A:** showing cell tracks of the IL-2 activated NK cells whilst **Fig B:** shows the tracks for the IL-15 activated NK cells. The gradient of 4T1 and E0771 CM increase from the bottom to the top. Left columns are the tracks for the IL-2 NK whilst the right columns are the tracks for the IL-15 NK.

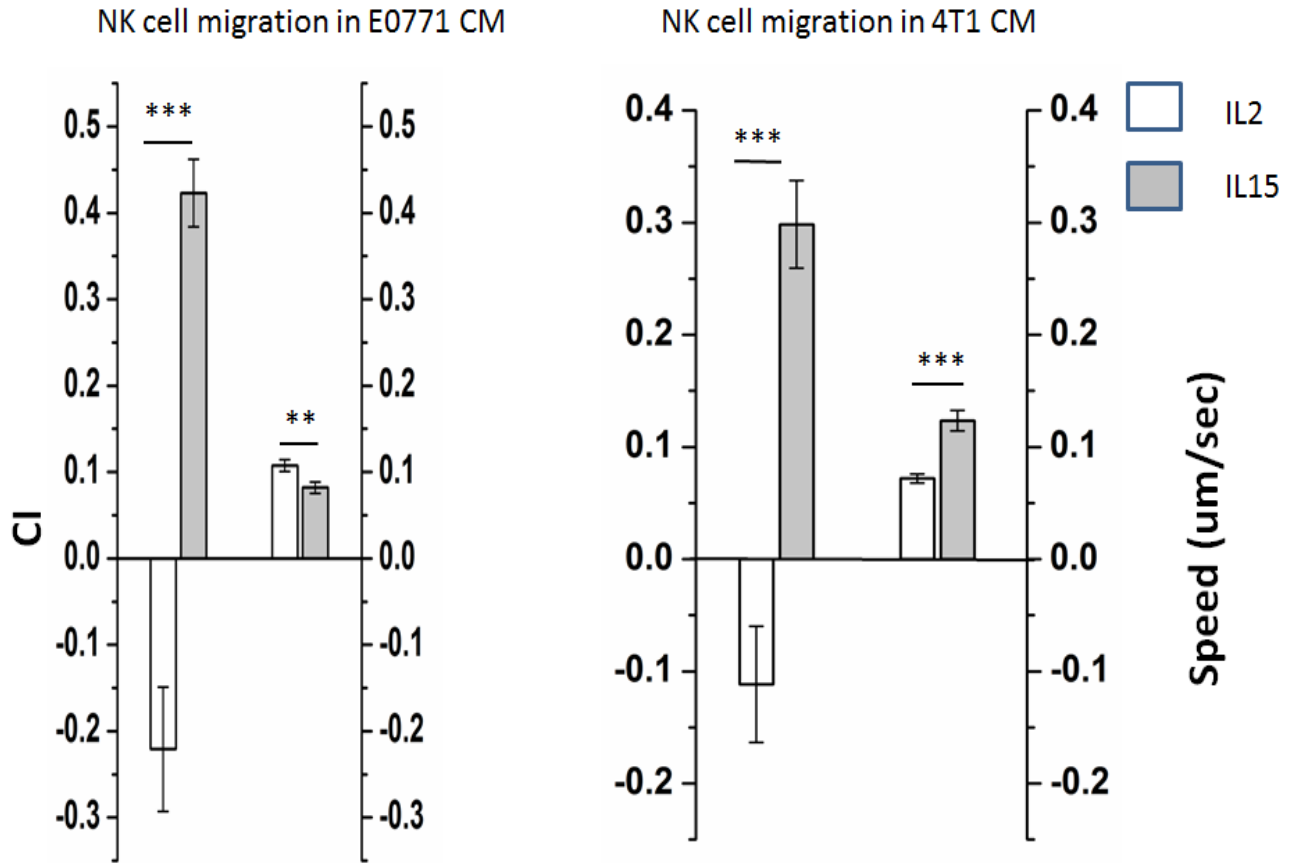


Figure 27: Comparison of the IL-2 NK and IL-15 NK cell migratory responses towards the CM of the 4T1 and E0771 in the microfluidic system. The CI of IL-15 activated NK in both 4T1 and E0771 is positive indicating a net migration towards the gradient whilst the CI of IL-2 activated NK in both CM is negative indicating a net migration away from the gradient. After capture of live cell imaging of the NK cell migrating in the CM gradient, the analysis was later done using imageJ software to determine speed and CI.

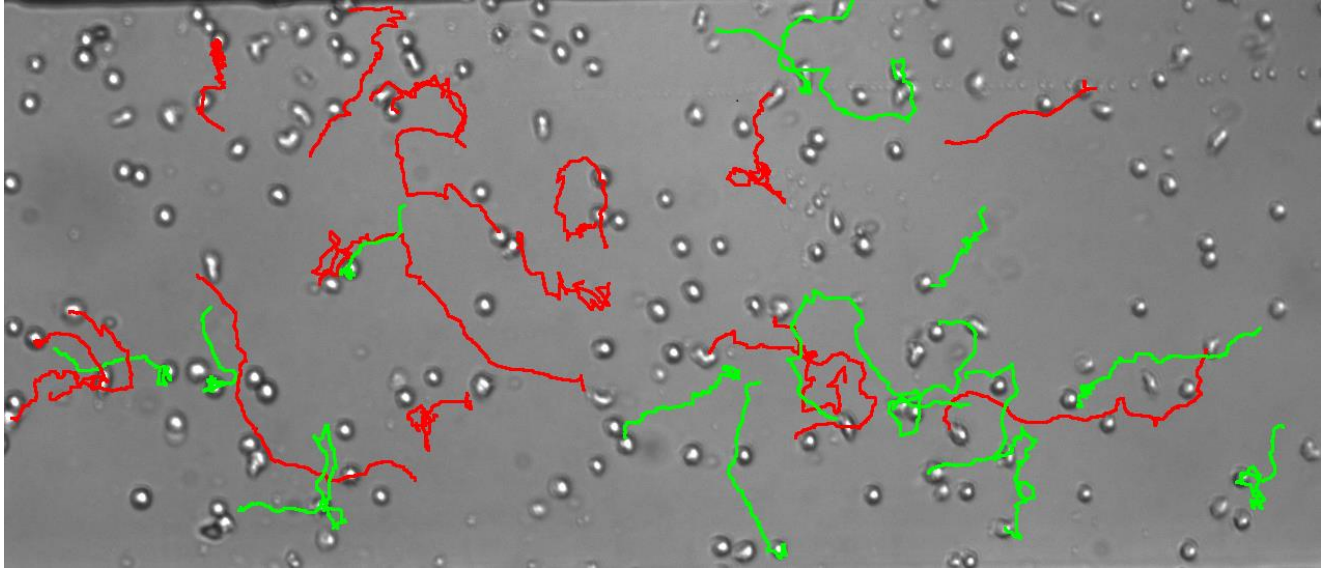


Figure 28: Live cell imaging of IL-15 NK cells in the gradient of 20ng/mL GM-CSF in a MF device. The last frame from the migration assay using the MF platform. A stable gradient from a medium containing 20ng/mL of GM-CSF was created and IL-15 NK cells migration in the created gradient monitored. As a negative control, gradient from medium without GM-CSF was as well created and IL-15 NK migration monitored. 20ng/mL of GM-CSF does not affect IL-15 NK migration. IL-15 NK migration was random. See attached videos.

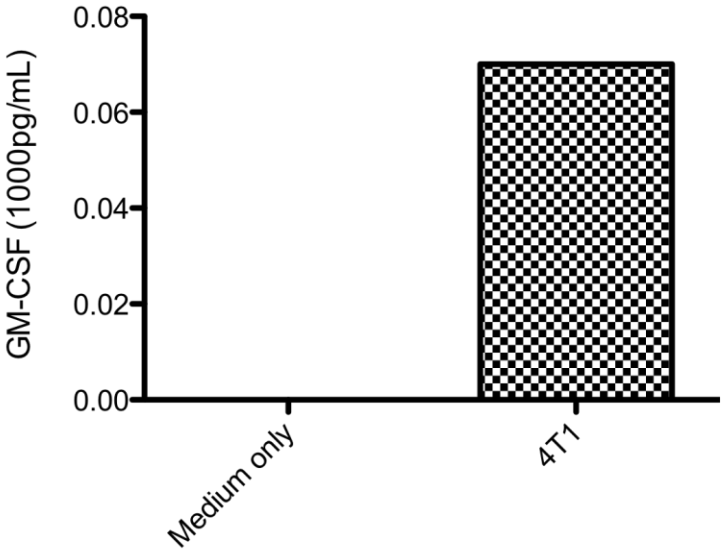


Figure 29: ELISA on GM-CSF production by the different tumor cell lines. The production of GM-CSF assayed using GM-CSF platinum ELISA kit (eBioscience). Elisa data of GM-CSF in the medium used to culture the 4T1 and the CM obtained after culturing 4T1 cell line.

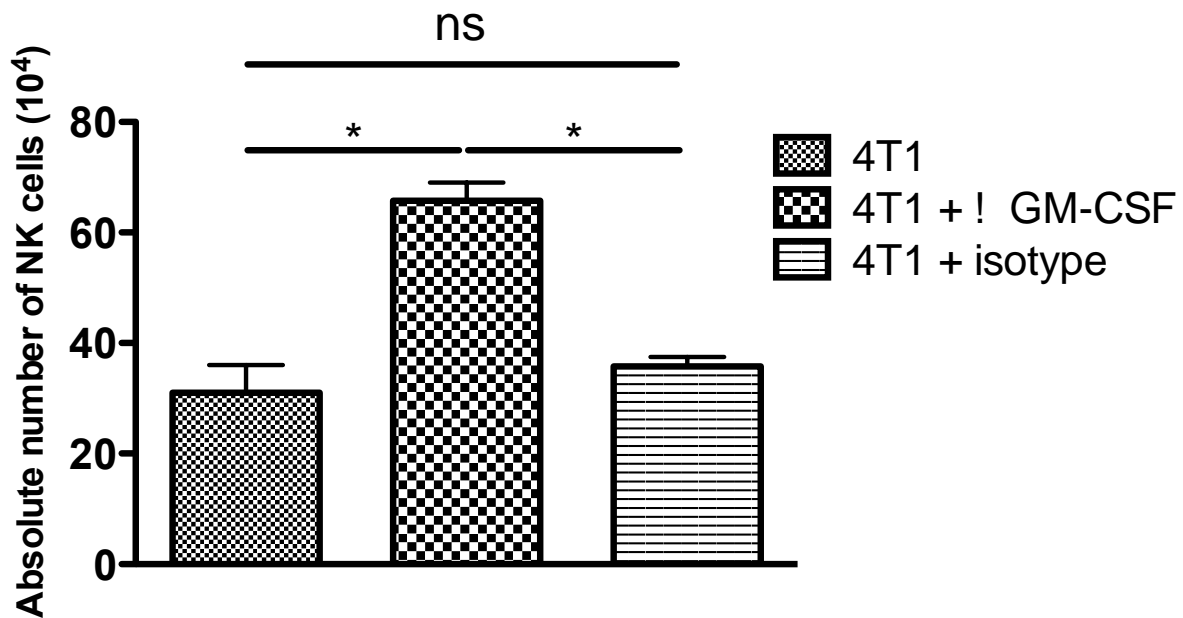


Figure 30: Neutralizing GM-CSF in 4T1 CM enhance IL-2 NK recruitment. 6ug of either anti-GM-CSF or its isotype control was added to 600uL of 4T1 CM to give a conc of 10ug/mL. The mixture was kept in ice for 30 mins. Every 10 mins, the mixture was mixed gently. This mixture was then added to the bottom chamber of the TW whose top chamber contained the IL-2 activated NK cells. After 3 hrs of incubation, the number of NK cells that transmigrated from the top to the bottom chamber was harvested and counted. Compared to the isotype or non-neutralize set up, neutralizing GM-CSF enhance IL-2 NK migration towards 4T1 CM

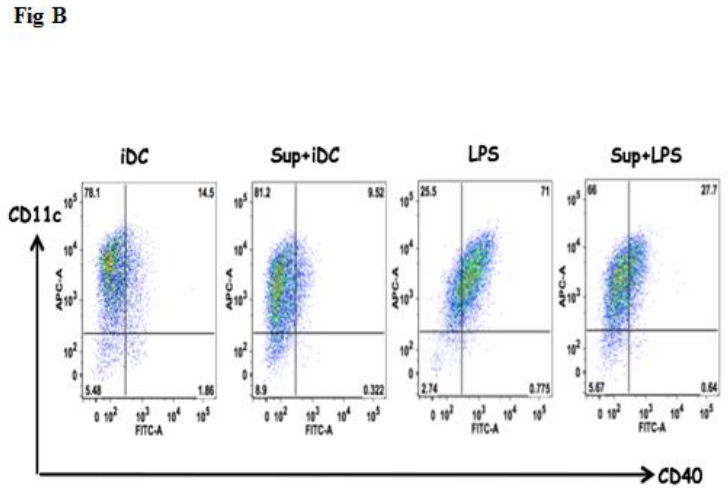
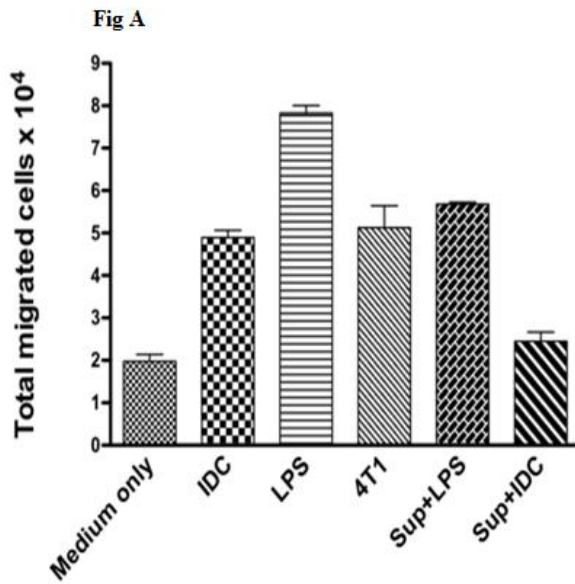


Figure 31: Conditioned medium of 4T1 altered DC maturation and its ability to promote NK migration in the trans-well system. Fig A: 4T1 CM alters DC ability to recruit IL-2 NK cells. 4T1 conditioned medium was collected from cultured 4T1 cell line and used for NK migration. Day 7 BMDC were divided into 4 batches. Batch 1 (iDC) with no treatment, in Batch 2 (LPS) the optimal amount of LPS (1ug/ml) was added, in Batch 3 (Sup +iDC) the medium was replaced by 4T1 sup and in Batch 4 (Sup + LPS) the medium replaced by 4T1 sup and pulsed with LPS (1ug/ml). After 24 hours, the conditioned medium from each batch was collected and used in the trans-well experiment. **Fig B:** 4T1 CM negatively modulate DC maturation. The DC were harvested, stained and phenotyped for maturation markers (CD40) by flow cytometry.

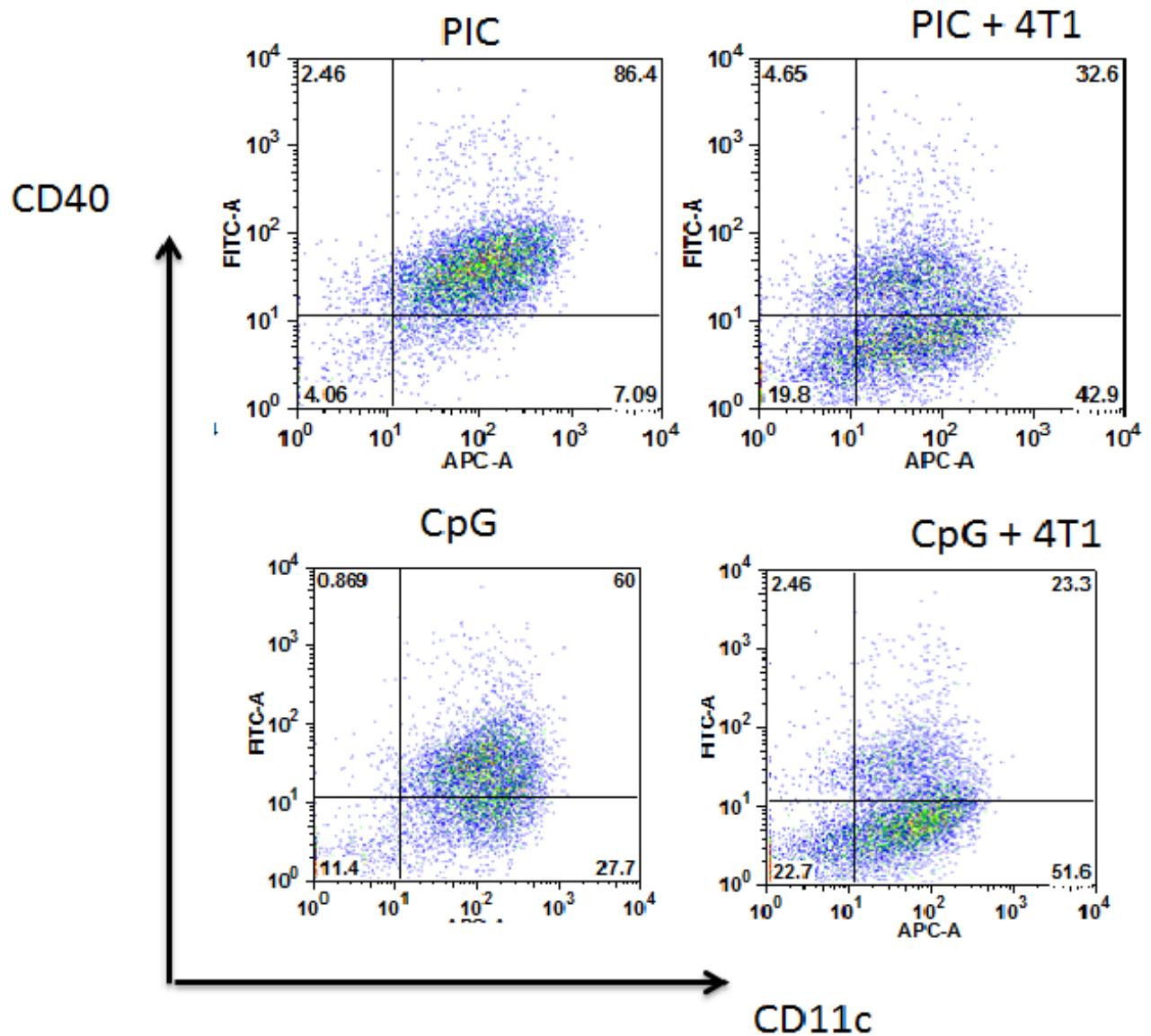


Figure 32: Conditioned medium of 4T1 altered DC maturation by different TLR ligands. Day 7 DC were stimulated with optimal conc of PIC or Pam3C or CpG in the presence or absence of 4T1 for 24 hrs and the DC harvested, stained and phenotyped for CD40 expression.

Fig A

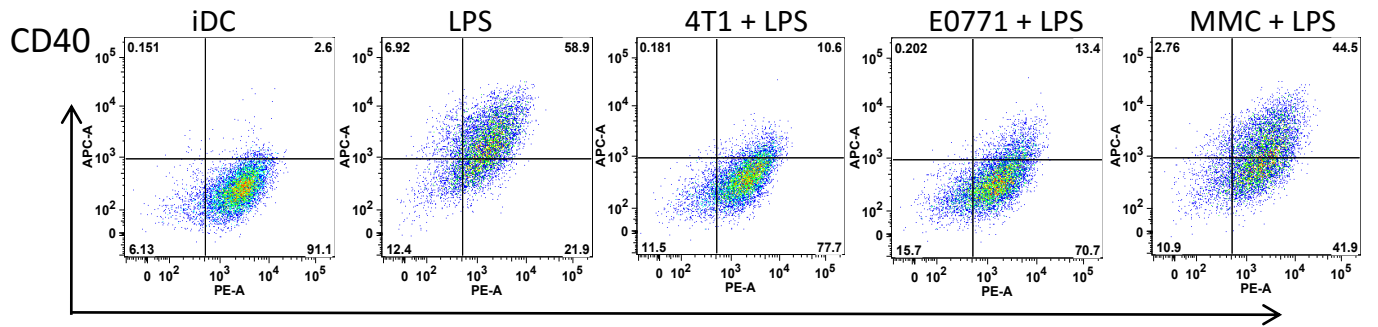


Fig B

Fig C

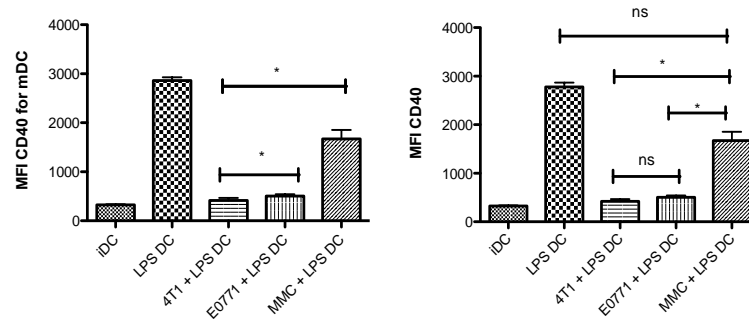


Figure 33: Conditioned medium of tumor cell lines negatively modulate DC maturation by TLR ligands compared to CM from normal cell from mouse mammary glands. Day 7 DC were stimulated with optimal conc of LPS in the presence or absence of 4T1, E0771 and MMC for 24 hrs. The DCs were harvested, stained and phenotyped for CD40 expression. DC stimulated in the presence of CM from 4T1 and E0771 expressed less CD40 on their surface compared to DC stimulated in the presence of CM from MMC. **Fig A:** Flow cytometry data on the LPS stimulated DC in 4T1, E0771 and MMC. **Fig B:** The MFI for the CD40 on all the DC including the CD40 + and CD40-. **Fig C:** MFI for the CD40 on the CD40 + cells. Both 4T1 and E0771 affect DC maturation both in terms of the number of matured DC and in terms of the intensity of the maturation.

Chapter 4 Discussion

NK cells are innate cytotoxic lymphoid cells that execute their effector function against their target – primarily killing- without prior sensitization. NK cell can kill its target either directly or indirectly. In order to directly kill its target, it is important that NK cell form an immunological synapse with its target so that there is a directed release of effector molecules towards the target. This prevents bystander killing and tissue damage. NK cells are highly motile cells and can migrate to target and form an IS with target. So, regulating NK migration is vital in the execution of NK effector function.

Only activated NK cell can execute effective killing function. Naïve NK cells are less cytotoxic compared to the activated NK cells. NK cell can be activated by either pro-inflammatory cytokines or by interacting with other immune cells. A critical interaction of NK cell with another immune cell is the interaction between NK and DC referred to as NK-DC crosstalk.

In NK-DC crosstalk, there is a bidirectional interaction involving not only multiple cytokines but also direct cell-cell interaction between the two cell types. When DC are stimulated by pathogens or the different TLR Ligands or other factors, the DC become a significant source for a number of cytokines such as IL-12, IL-18, IL-15. These cytokines specifically regulate NK cell effector functions such as cytokine production and cytotoxicity and as well as NK cell proliferation. On the other hand, NK also induce maturation of DC.

In addition to producing cytokines that reciprocally activate NK and DC, DC is shown to produce chemokines that recruit NK cells and NK cells as well can produce chemokines that recruit DC (168, 169). This has led to the coining of the concept of reciprocal NK-DC chemoattraction. DC produces a number of chemokines important in the recruitment of NK cell such as ip-10, CXCL8, 9, 11 (170) and whose receptors are expressed on NK (32). Meanwhile, NK produce chemokines such as CCL5 important in DC recruitment and whose receptor is expressed on DC. This reciprocal chemoattraction has been suggested to be important in the preferential recruitment and interaction between the NK and DC.

Compared to the bi-directional reciprocal crosstalk between NK-DC crosstalk, the reciprocal NK-DC chemoattraction is less understood. In our recent publication, we showed that LPS stimulated DC induce stronger IL-2 activated NK cell chemotaxis compared to the unstimulated DC (151). We identified the ip-10 produced by the DC and the CXCR3/ip-10 axis to play a partial role in the recruitment of the IL-2 NK cell by the LPS stimulated DC. To our knowledge, we are not aware of studies that had compared the recruitment potential of the different DC and the migratory potential of the different cytokine activated NK in an in vitro setting.

To expand on our previous findings, I looked at the migratory properties of the different cytokines activated NK and as well as the recruitment potential of the different TLRs stimulated DC.

My data shows that the different TLR ligands stimulated DC recruits both IL-2 and IL-15 NK cells differently (Fig 7 and Fig 8). PIC stimulated DC was the better recruiter of both the IL-2 and IL-15 activated NK cell. In a previous study conducted by a different group, they showed that

changing an infection site to mimic viral infection improves recruitment of immune cells like NK to the site of infection. PIC is a mimic of viral infection, whilst the other TLRs are mimics of bacterial infection. My findings might suggest that viral infection might strongly recruit NK cells. It will be interesting to compare the migration of NK cells to the different classes of pathogenic infection (virus, bacteria and fungi). Another *in vivo* study showed that the injection of LPS stimulated DC induced NK cell recruitment to the LN unlike the CpG stimulated DC that did not induce NK cell recruitment. This is in corroboration with my findings that LPS stimulated DC recruits NK better than CpG stimulated DC (Fig 7B and Fig 8B). This might serve as an important piece of information in the use of adjuvant in vaccine to help improve immune cells recruitment to infection or tumor sites. In DC based therapy and/or vaccines, adjuvants from microbial products have been used to boost the immune system by promoting immune activation. Moreover, having products that can improve recruitment of immune cells to inflammatory sites can further boost the immune response by recruiting more effector cells to the target site. Also, we know that NK cells are more wired or programmed to respond to viral infection than to bacterial infection. So the above findings and suggestions do not contradict that but support that.

Having shown difference in recruitment potential of the different TLR Ligands stimulated DC, I asked whether difference in the production of chemokine known to play a role in NK migration to this condition can account for this difference. Our lab and others have shown the importance of ip-10 in NK cell migration towards DC CM. In the ELISA data, I found the most ip-10 to be produced by the LPS stimulated DC compared to the other TLR Ligands stimulated DC. Had PIC produce more than LPS DC, I would have suggested that the difference in recruitment between the different TLRs stimulated DC might have been because of ip-10 production. Since the other trend in ip-10

production by the other TLR stimulated DC correlated with their recruitment potential. Nevertheless, this discrepancy between ip-10 produced by LPS and PIC DC and their recruitment does not discount the possibility that the differential amount of ip-10 produced accounts for the difference in the different DC recruitment potential. Possible explanations for the discrepancies are that both PIC and LPS DC produced the maximum amount of ip-10 to saturate the CXCR3 and that PIC DC is producing other chemokine(s) that is responsible for the difference in migration observed. Apart from ip-10, there are 2 other chemokines (CXCL9 and CXCL11) that act on CXCR3. I did not look at these other chemokines.

I looked at the production of the CCL5 in the LPS stimulated DC CM and found the LPS stimulated DC produced ip-10 more significantly compared to the unstimulated DC (Fig 19). I also looked at the expression of the CCL5 receptor on the different NK and found only a low expression on both the IL-15 and the IL-2 NK. I did not demonstrate the role of the CCR5/CCL5 axis in NK migration towards the DC CM or compare the CCL5 in the different DC CM to see if CCR5/CCL5 axis can account for the differential recruitment. CCR5 has been shown to be important in the trafficking of NK to infection sites (171). Another study has reported on the increased trafficking of NK in CCR5 deficiency (172). So, it will be interesting to compare the trafficking of these cytokine activated NK cells via the CCR5/CCL5 axis.

These 2 CXCR3 chemokines (CXCL9/CXCL11), some novel chemokine, some non-chemokines or other known chemokine that I did not consider or did not further consider might account for the difference in migration observed. I am currently working on using the discovery assay from Eve

technology to check for the expression of an array of chemokines and cytokines in the CM. It is a 32-plex assay that contains chemokines and cytokines that are of interest to me.

I used IL-2 and IL-15 to activate NK and compare their migration towards different conditions. IL-2 and IL-15 share many similar properties. IL-2 and IL-15 act through the same receptor and activate similar downstream signaling molecules on NK cells. NK cells activated by IL-2 and IL-15 exhibit similar in vitro functions such as similar cytotoxicity and cytokine production. However, the in vitro migration potential has not been compared to the best of my knowledge.

In Fig 7 and Fig 8, the data suggest that both cytokine activated NK cell showed similar migration pattern towards the different TLRs stimulated DC. But the next question was how the different cytokine activated NK migration compare to each other. I initially used the already established platform to do the comparison. In all the conditions comparing IL-2 vs IL-15 NK migration, I found IL-15 NK to exhibit stronger chemotaxis compared to IL-2 NK cells.

Since the downstream signaling pathways are similar in the IL-2 and IL-15 activation of NK, I assessed 3 parameters to see if the difference in migration can be accounted for based on these parameters. I checked for activation, viability and the chemokine receptor profile.

Can the activation state of the different cytokine activated NK cells account for the difference in migration between the IL-2 and IL-15 NK cells? Cytokine influence the activation and the phenotypic state of NK cells. As shown in Fig 13 and Fig 14, the phenotypic profile of NK cells can be altered upon stimulation with cytokine. Comparing the proliferation of the IL-2 and IL-15

NK to gauge for the activation of these NK cells. I found similar proliferation trend between the IL-2 and IL-15 NK cells (Fig 11) suggesting although not strongly that they exhibit similar activation state. Other studies have reported similar expression of activation markers between the IL-2 and IL-15 NK. I understand that this is not a sensitive approach to gauge for the activation state or the proliferation potential. I am interested in future studies to use more sensitive assay to compare the activation and proliferation of the IL-2 and IL-15 NK cells.

State of activation of cell alters their migratory properties. For instance, matured DC are more migratory compared to their immature counterpart. Also activated NK migrate strongly compared to naïve NK cell. In Fig 20, I compared the migration of IL-15 NK cells activated for different length of time. I assume that the activation of NK is correlated with the period of time the NK are in the activation milieu. During the tracking of cell morphology using the Evos inverted digital microscope, I observed the cells to be larger and more elongated the longer the cells were in the activation culture medium. Based on this, I assumed that the Day 4 NK are more activated. I found that Day 4 NK were more migratory compared to the Day 2 or Day 3 NK (Fig 20). The migratory difference was statistically significant between the NK activated for different days. This corroborate with reports that activated NK cells are more migratory. Since the activation state of the IL-2 and IL-15 NK are similar based on the criteria I used, the difference in the recruitment observed between the different cytokine activated NK cells might not be due to the level of activation.

The devices used to assay for the NK cell migration requires that the cell are healthy. In order for cells to transmigrate in a TW device, the cells must be able to squeeze through the membrane

insert. In the MF device as well, cells must first adhere to the fibronectin coated chamber and then detach themselves from the chamber in order to move towards or away from the gradient. In both cases, the assays require healthy cell to expend energy in order to undergo chemotaxis. For the TW, unhealthy cell might just clog the pores preventing the pores from been accessed by the healthy cells. In short, unhealthy cells can affect both assays.

IL-2 activation of NK has been reported to increase NK susceptibility to AICD. In a study involving the adoptive transfer of cytokine activated NK cells. The authors reported less recruitment of IL-2 NK and attributed this to increase susceptibility of IL-2 NK to AICD. In this study, I discount the cell viability as a possible explanation for differential migration, when I compared the viability of the cytokine activated NK cells and found them to be similar between the IL-2 and IL-15 activated NK cells (Fig. 12B).

Comparing the chemokine receptors known to play a role in NK cell recruitment and the role of the chemokine receptor, CXCR3, suggested that the differential expression of CXCR3 might be responsible or partially responsible for the differential migration between the cytokine activated NK cell. Stimulation of NK cells with either IL-2 or IL-15 activate downstream signaling pathway that results in the upregulation of the expression of CXCR3. But the CXCR3 is more upregulated with IL-15 stimulation compared to the stimulation with the IL-2. As shown, the CXCR3 was highly expressed on the IL-15 NK cells compared to the IL-2 NK cells. Engagement of the chemokine receptor, CXCR3, by it cognate ligand stimulates downstream signaling pathway involved in inducing NK cell migration. The detailed signaling pathway following the binding of CXCR3 by it ligand has not been fully elucidated. CXCR3 is a pertussis toxin, PT, sensitive G

protein coupled receptor that is part of the chemokine receptor family. Its engagement induces integrin activation, cytoskeletal rearrangement and intracellular Ca^{2+} levels increase.

Impairing the chemokine/chemokine receptor axis by neutralizing the chemokine or chemokine receptor has been shown to not completely abrogate migration but only partially abrogate cell migration. My data as well did not show a complete abrogation of the NK chemotaxis upon neutralization since in the neutralization experiment, I still found NK migration towards the CM. This is in agreement with other studies where neutralization of a chemokine/chemokine receptor only partially attenuates migration and not completely abrogates migration. This suggests that there are other factors in the CM that influence the migration of the NK cells.

But the differential effect between the IL-2 and IL-15 NK migration after neutralization of the CXCR3 and the data showing that IL-15 NK cells expressed stronger CXCR3 than the IL-2 NK cells suggest that it is the differential CXCR3 expression between these 2 different cytokine activated NK that is responsible for the differential migration towards the DC CM. Moreover, the similar migration of the neutralized CXCR3 on the IL-2 and IL-15 NK cells corroborate the suggestion that the differential expression of CXCR3 accounts for the differential migration observed.

During the staining for the chemokine receptors, I observed that at certain time points (Day 2 and Day 3), the IL-2 and IL-15 NK expressed similar CXCR3 (Fig 21A and Fig 22A). Despite this similarity in the CXCR3 expression and to my surprise, IL-15 NK cells still migrated strongly towards the tested CM compared to the IL-2 NK (Fig 22B and Fig 22B). This seems to contradict

the suggestion that day 4 cytokine activated NK cells differential migration can be accounted for by the differential expression of the CXCR3. Nevertheless, this does not discount the suggestion that the differential migration between IL-2 and IL-15 NK was due to the differential expression of CXCR3. I used NK cells activated for different time points. Majority of my studies was done on the Day 4 activated cells. In the neutralization experiment, I used NK activated for 4 days and not 2 or 3 days. It is possible that the Day 2 and Day 3 NK cells migration is influenced by other factors that account for the differential migration. This also suggest a support for the involvement of other factors in NK recruitment.

In summary, IL-15 NK are better recruited by DC stimulated with different TLRs compared to IL-2 NK, CXCR3 plays a role in cytokine activated NK migration and in the differential migration observed between the cytokine activated NK cells.

Implication

What is another relevance of comparing cytokine activated NK migration in vitro in addition to helping to understand NK cell biology? In addition, can the different cytokine activated NK cells shows differential migratory pattern in the CM collected from different tumor cells line? The CM from the tumor cell lines were used in both TW and MF platform to assess the effect of these CM on NK migration. I used the already established migration platform for this part of my study.

I used different TLRs to stimulate the DC in the presence or absence of CM for the following reasons. I wanted to mimic a pathological situation in an in vitro setting. The use of TLR agonists could provide me with different test conditions because of the number of different TLR ligands

that I could use to test as different conditions. Also, there are some self molecules that can bind to TLRs. For example, HSP binds to TLRs (173, 174). Hsp70 has been used as an adjuvant to induce NK cell activity in patients with cancer (141). The resulting activation of NK was shown to occur via the NK-DC crosstalk. The Hsp70 induced NKG2D on DC and promote NK activation via the NK-DC crosstalk.

A cell upon undergoing necrosis can release debris such as Hsps. Released debris can be picked up by DC and be presented by DC to prime T cell in a process called cross priming. This cross presentation of Ag by DC allows appropriate immune response to be mounted. Impairing DC cross presentation and maturation could be a tumor evasion mechanism. DC in solid tumor have been reported to be of the immature phenotype characterize by low co-stimulatory molecules expression whilst the peritumoral region contained the matured phenotype (98, 175). Cancer cells can suppress DC maturation and maintain the DC in an immature state with dysfunctional antigen presentation and other DC effector functions (123). Figures 31, 32 and 33 corroborate the report of DC impairment in tumor environment. So understanding mechanisms of tumor evasion will be helpful in devising better therapy to circumvent such tumor evasion mechanisms.

NK cell can crosstalk with DC and result in their respective reciprocal activation. I have shown that DC maturation can be impaired in a tumor microenvironment. So enhancing NK recruitment to tumor environment might serve as a mechanism of reversing the impairment of DC activation by the tumor environment since DC can be activated by NK cells.

High dose bolus IL-2 is a currently approved treatment for malignancies such as metastatic renal cancer. But there are associated toxicity such as capillary leakage, adverse effect on the heart with high dose IL-2 and the expansion of Treg with low dose (176-178). IL-15 is being considered as a possible better alternative. IL-15 selectively expands the more cytotoxic NK cell subset (CD56^{dim}) whilst low IL-2 selectively expands the less cytotoxic subset (CD56^{bright}) (18, 176). There are undergoing research into the tolerated dose and toxicity in the use of IL-15 for cancer treatment. IL-15 is a pro survival factors for some cells (NK and certain T cell subsets etc.) and as such it use could be detrimental to some malignancies. Also, there are other cells that can compete and utilize IL-15. IL-15 is being suggested to be detrimental in some auto immune diseases and some malignancies. It will be detrimental if suppressive cells such as myeloid derived suppressor cells, MDSC, are expanded by IL-15. Moreover, the use of cytokine to treat certain malignancies assumes that the recipient has a competent immune system.

Because of the above concerns in the use of cytokines for treatment of certain malignancies and to circumvent some of these limitations, there is growing research into the use of ex-vivo expanded NK cells for cancer treatment. The use of ex-vivo expanded NK cells has been relatively more successful in hematological malignancies compared to solid tumors. Studies have demonstrated that the hostile environmental of the TME negatively affect NK functions thereby affecting anti-tumor response (98, 179). In addition, there is correlation between NK cell infiltration to TME and prognosis. BC environment showed a lower NK cells infiltration compared to normal tissues. Improving NK cell infiltration into tumor environment has been shown to improve anti-tumor activity. This underscores the importance of improving infiltration of effector cells such as NK cells to tumor environment for anti-tumor immunity.

In summary, I demonstrated that IL-15 NK cells migrate better towards CM from different tumor cell lines compared to IL-2 NK cells. GM-CSF produced by 4T1 negatively affects IL-2 NK cells migration but not IL-15 NK cells migration. CM from tumor cell lines negatively modulate DC maturation with different TLR ligands and the stimulated DC ability to recruit IL-2 NK cell.

My findings suggest that NK cells infiltration can be improved by choosing the appropriate cytokine activated NK that will not be repelled or inhibited from infiltrating the TME. So far my findings suggest that IL-15 activated NK cells can better penetrate certain environment compared to the IL-2 activated NK cells.

Chapter 5 Conclusion and Future Directions

I have demonstrated that DC stimulated with different TLR ligands recruits IL-2 and IL-15 activated NK cells better than the unstimulated immature DC. Yet, the different TLR Ligands matured DC differentially recruits cytokine activated NK cells. The CXCR3/ip-10 axis plays a role in DC recruitment of NK cells. PIC stimulated DC recruits IL-2 and IL-15 NK cells better than the other TLRs stimulated DCs. CM from TLR agonists stimulated DC elicits stronger IL-15 NK chemotaxis compared to IL-2 NK chemotaxis. Differential expression of CXCR3 between the IL-2 and IL-15 NK cells might be a possible explanation for the differential migration between IL-2 and IL-15 NK cells.

In tumor settings, IL-15 NK cells migrate better than IL-2 NK cells. CM from tumor cell lines such as 4T1 and E0771 repels IL-2 NK but not IL-15 NK cells. These tumor cell lines can repel IL-2 NK directly by secreting GM-CSF. GM-CSF as a chemorepellent for IL-2 NK cell was recently reported by our lab. CM media from these cell lines also indirectly affect IL-2 NK cell migration via the NK-DC axis in addition to impairing the maturation of DC stimulated with the different TLR ligands.

Hence, IL-15 activated NK cell might be a better candidate for adoptive transfer of NK cell in NK cell based immunotherapy.

As mentioned above in study that switch the nature of the inflammation to a viral type, and the finding on the recruitment potential of the different TLR ligands stimulated DC. It will be interesting to compare the recruitment of NK cell towards the inflammatory conditions caused the

different class of pathogen. In a setting more relevant to tumor, it will be significant to look at how DC stimulated with different apoptotic tumor cell lines recruit the different cytokine activated NK cells. Additionally, how DC stimulated with Hsp recruit the different cytokine activated NK cells.

I am currently quantifying an array of chemokines and cytokines in the CM from the different TLR stimulated DC using the discovery assay from Eve Technology. After, I check whether the chemokine profile can account for the differential recruitment potential of the different TLRs stimulated DC. At the same time, check for the involvement of other non-chemokines in the migration of NK cells. The low expression of CCR5 on IL-2 and IL-15 activated NK does not rule out the involvement of this receptor on NK migration. It will be difficult to verify the neutralization of the receptor on the NK cell because of the low surface expression. Nevertheless, I could still neutralize the chemokine in the CM and then assess the migration of the NK cells.

Furthermore, I would like to do an in vivo comparison of the IL-2 and IL-15 NK cell migration. I will first culture the isolated NK in either IL-2 or IL-15 culture medium for 4 days. Then label the NK with dyes to allow me to track the transferred cells and then adoptively transfer the IL-2 and IL-15 NK cells. Alternatively, I could use NK cells from different mouse background that will allow me to track the transferred cells into the recipient. I will then assess the migration of the IL-2 and IL-15 NK to different LNs, spleens etc. I will also assess the trafficking of the different NK cell into tumor microenvironment. Based on my in vitro data, I expect IL-15 NK cells to be trafficked better than the IL-2 NK cells.

I have been generating DC from murine bone marrow cells in culture medium containing 20 ng/mL GM-CSF. I assumed that the generated cells are a highly homogenous DC population. Recently, a paper published in Cell reported that these generated cells are a heterogeneous population of Macrophages and DC (180). This finding has some implication in the interpretation of my data and as well on the title of my project.

My study did a lot of comparison and as such some of the interpretation are still valid even if the generated cells were a heterogeneous population. The study suggested that the generated cells should be better termed monocyte derived cells, MC (180). Even if we assume they are MC, the TLR Ligands stimulation was done on similarly generated cells. Also, the comparison of the migratory potential of the cytokines activated NK cells was done using CM from the generated cells and CM from tumor and non-tumor cells. So the variable in the comparison was either the TLR ligands or the cytokine activated NK cells.

In their study, on Day 3, the old medium was discarded and replaced with new medium. During my study, I employed several scenarios and compared the different scenarios to see if it might affect my comparison. On day 3, I either partially or completely replaced the old medium or I just added fresh medium to the old medium.

On Day 7 in my study, I completely replaced the old medium with fresh medium before the stimulation with the different TLR ligands. In another case, I just added the TLR ligands to stimulate the DC without changing the old medium. I found similar results whether I completely or partially replaced the old medium or even without replacing the old medium. I found that the

factors produced by the cells that elicit strong NK chemotaxis are produced upon stimulation with the different TLR ligands.

I will be looking to see if the generated DC is a homogeneous population or not. Since as the paper suggest, there are multiple factors that could affect the homogeneity or heterogeneity of the culture. Concurrently, I will be isolating splenic DC and repeating some of the migration study to see if I could observe similar findings as I reported. I do have preliminary result on the use of CM generated from splenic DC. I observed splenic LPS stimulated DC induce stronger NK chemotaxis compared to immature DC (Supplementary figures).

Chapter 6 References

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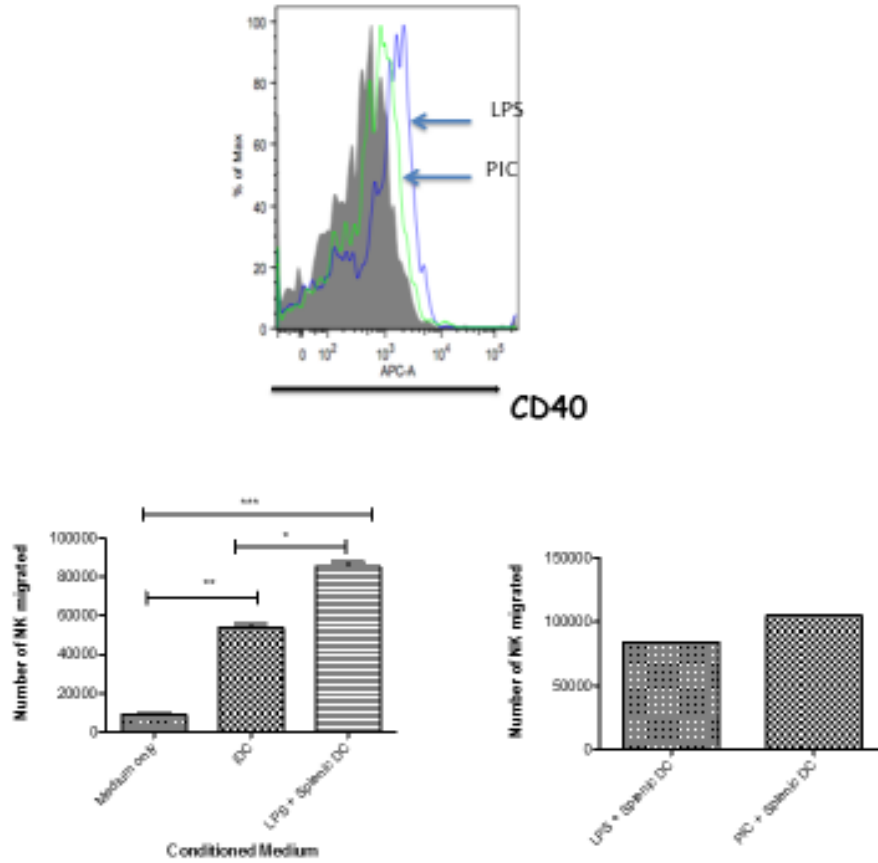
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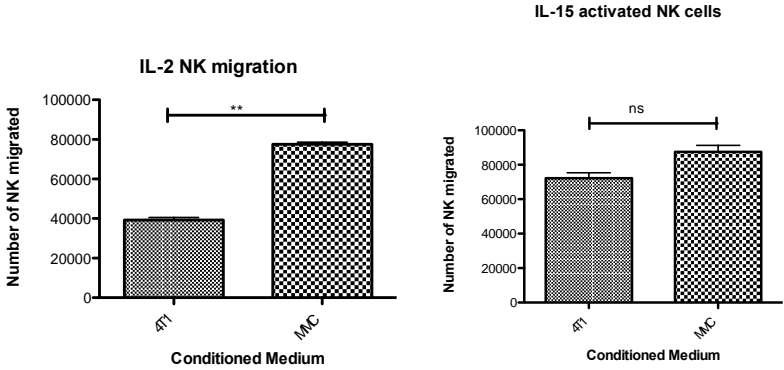
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Chapter 7: Supplementary figures

Splenic DC maturation and the recruitment of cytokine activated NK cells



Comparison of cytokine activated NK migration in CM of normal primary cells and tumor cell lines



Working model of findings

