9-27-2023

Regulation of natural killer cell responses by myeloid-derived suppressor cells in mouse mammary tumors

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By

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A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Integrative Biology and the Department of Biomedical Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2023

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ABSTRACT

Natural killer (NK) cells of the innate immune system play important roles in anti-cancer immunity. NK cell functions are regulated by inhibitory and activating receptors, which recognize specific ligands on the target cells. One example is the inhibitory NKR-P1B receptor which recognizes the ligand C-type lectin-related protein b (Clr-b). Work in our lab has shown NKR-P1B:Clr-b interactions are involved in mammary tumor immunosurveillance by NK cells. However, the cellular interactions and factors involved in modulating NK cell functions via this receptor:ligand system in the tumor microenvironment (TME) are not fully understood. This project aims to understand the role of myeloid-derived suppressor cells (MDSC) in regulating NK cell responses via the NKR-P1B:Clr-b axis in TME. Using MMTV-PyVT transgenic mouse model and E0771 mammary adenocarcinoma cells injected into wild-type (WT), NKR-P1B-deficient, and Clr-b-deficient mice to induce mammary tumors, we assessed MDSC recruitment and function in mammary tumors, and their effects on anti-cancer immune responses by NK cells. We have found that MDSCs express Clr-b, suggesting that they could potentially interact with NK cells via the inhibitory NKR-P1B receptor. However, MDSC differentiation, recruitment to tumors, and subset distribution in tumors is not affected by NKR-P1B:Clr-b axis. Our data demonstrates heterogeneity in MDSC recruitment and distribution, and inflammatory cytokine levels in MMTV-PyVT and E0771 mammary tumor models that could differentially impact NK cell responses in tumors. In *in vitro* co-culture assays, MDSCs induced downregulation of transcription factor EOMES, downregulation of integrins CD49a and CD49b, and upregulation of checkpoint receptor Lag-3 in NK cells, suggesting that MDSCs may induce NK cell dysfunction. Our data also show that Clr-b may have an inhibitory function in MDSCs, but this is independent of NKR-P1B receptor. These findings will advance our understanding of the functions of inhibitory receptor:ligand systems in cancer immune evasion.

ACKNOWLEDGMENTS

I would like to first thank my supervisor, Dr. Munir Rahim, for his many hours of help and support over the last two years. Dr. Rahim, your patience, guidance, and assistance over the last two years have truly made my graduate school career amazing. You have provided me with so much knowledge about lab techniques and research in general. You have given me the confidence to know that I am a truly capable scientist, and your guidance and assistance will benefit me in many of my future endeavors.

I want to thank my committee members, Dr. Otis Vacratsis and Dr. Dora Cavallo-Medved. Thank you for all of your support, encouragement, and guidance over the last two years.

I would like to thank my lab members; Mohamad Alkassab, thank you for all the support and help you have provided me over the last two years. You have always told me I am capable when I felt I couldn't get anything right. Karla Alnajm, thank you for always lending a listening ear when I was stressed; you always kept me laughing; thank you also for being my in-lab calculator and always coming to the rescue when my mind would blank. Fareeha Shaikh, thank you for being a constant beam of light in our lab. You somehow could cut through any stressful situation with a joke. Thank you also for always being a confidence boost and making me feel like I can do anything. Without you three, my lab experience would not have been the same, it has been an honor to share the last two years laughing, stressing, and collaborating with you guys, and I wish you all luck in your future endeavors.

I would like to thank my friends for supporting me throughout my graduate school career. You guys have been such a vital part of my career, whether forcing me to take a break and catch up with friends or reminding me to sleep. You have all been amazing.

To my family, thank you all. You have provided me with so much support and carried the weight of many other tasks for me so I can focus on my writing and research. You guys have always lent a listening ear and provided me with a laugh when you knew I needed it. You have been essential in pushing me to the finish line. Without your love and support, I could not have gotten here.

Finally, to my husband, thank you for staying up with me through all the late-night writing sessions, for listening to every presentation and pretending like my science rants made sense to you, and for always providing me with the confidence to know I am supposed to do this and can carry on even when I thought I could not. Your love and support have meant more to me over the last two years than I could explain.

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ACK	ammonium-chloride-potassium
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen-presenting cells
ARG1	arginase 1
BCL-2	B-cell lymphoma 2
BFA	brefeldin A
BME	β-mercaptoethanol
BRCA	breast cancer gene
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
CAA	cancer-associated adipocytes
CAF	cancer-associated fibroblast
CDMEM	complete Dulbecco's modified eagle medium
CRPMI	complete Roswell park memorial institute
CTL	cytotoxic T cells
DC	dendritic cells
DCIS	ductal carcinoma in situ
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
EDTA	etylenediamine tetra acetic acid
EV	extracellular vesicles
FASL	fas ligand
FBS	fetal bovine serum
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	human epidermal factor 2
HSC	hemopoietic stem cells
IDO	indoleamine 2,3-dioxygenase
IFN	interferons
IgG	immunoglobulin G
IgM	immunoglobulin M
ILC	innate lymphoid cells
iNOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LCIS	lobular carcinoma in situ
LMP2	latent membrane protein 2
LMP7	latent membrane protein 7

M-CSF	macrophage colony-stimulating factor
M-MDSCS	monocytic MDSCs
MDSC	myeloid-derived suppressor cells
MFI	mean fluorescence intensity
MHC-I	major histocompatibility complex 1
MHC-II	major histocompatibility complex 2
MMP	matrix metalloproteinases
NK	natural killer
NKC	natural killer gene complex
NKT	natural killer T cells
NO	nitric oxide
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein ligand 1
PMN-MDSCs	polymorphonuclear myeloid-derived suppressor cells
RCAS1	receptor binding cancer antigen
ROS	reactive oxygen species
RPMI	Roswell park memorial institute
SHP-1	protein tyrosine phosphatase-1
SHP2	protein tyrosine phosphatase-2
STAT	signal transducer and activators of transcription
TAMs	tumor associated macrophage
TANs	tumor associated neutrophils
TCR	T cell receptor
TGF-B	transforming growth factor beta
TILs	tumor infiltrating lymphocytes
TME	tumor microenvironment
TNBC	triple negative breast cancer
TNM	tumor, node, and metastasis
TRAIL	TNF-related apoptosis inducing ligand
VEGF	vascular endothelial growth factor
WT	wildtype

CHAPTER 1: INTRODUCTION

1. Cancer

Cancer is a worldwide disease; it is the second leading cause of death and is responsible for 10 million deaths worldwide [(1)]. Cancer is a complex disease that is very hard to treat because of its heterogeneous nature. There are variations in disease from person to person and even from one tumor to another [(2)]. Various factors can contribute to causing cancer, including mutations in tumor suppressor genes or oncogenes, carcinogen exposure, and chromosomal alterations [(3)]. These factors induce tumorigenesis, a multi-step process where normal cells become malignant and eventually resistant to cell death. Tumorigenesis induces cells to attain (1) sustained proliferative signaling and (2) evade growth suppressors. This will lead to (3) resistance to apoptosis, (4) rendering tumor cells virtually immortal. As the tumors grow, they induce (5) angiogenesis and (6) metastasize to other tissues. Tumor cells are also characterized by (7) genomic instability, (8) metabolic alterations, (9) pro-tumorigenic inflammation, and (10) the ability to evade anti-cancer immune responses. The 10 characteristics of tumor cells mentioned above are also known as the hallmarks of cancer [(2)]. Along with the tumor-causing factors, the tumor microenvironment (TME) plays a huge role in cancer development and tumor growth. It has supportive factors that create the perfect environment for cancer growth and to mute anti-tumor effects [(2)].

1.1 Breast Cancer

Breast cancer is the most common cancer among women. More than 1 in every 10 cancer diagnoses in women yearly is a breast cancer diagnosis. It also accounts for the second highest number of deaths in women annually worldwide. Breast cancer is often a silent killer as most early breast cancers are asymptomatic. Women who do not regularly go for mammograms or do

self-examination may not notice a change until cancer has spread outside of the breast. Breast tumors tend to spread lymphatically and hematologically; this leads to distant metastasis and often a poor prognosis for the patient [4]. As with all cancers, breast cancers can be broken down into stages; it is classified using tumor size (T), regional lymph node status (N), and distant metastasis (M); TNM classification system [5]. The prognosis of a patient with breast cancer relies on many factors; however, it is usually good with early detection and effective therapy. The 5-year survival rate for breast cancer has improved to 100% survival rate for stages 0 and I, and 93% and 72% survival rates for stage II and III patients, respectively. The 5-year survival rate for those in stage IV is still low at only 22% [5]. The most common breast cancer treatments include surgery, chemotherapy, radiation, and immunotherapy, which aim to reduce the chance of reoccurrence and limit the risk of metastatic spread [5].

1.2 Breast Cancer Classifications

Breast cancer can be classified either histologically or based on molecular characteristics. Regarding histological classification, breast cancer is divided into two main groups, in situ (non-invasive) carcinoma and infiltrating (invasive) carcinoma according to its relation to the basement membrane [5]. Non-invasive breast cancer can be divided into ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). LCIS is often harder to detect due to its ability to conform to the outline of the normal lobule; due to this, they are often hard to see on a mammogram and goes undetected [5]. DCIS is much easier to detect because it has discrete spaces filled with malignant cells. In addition, these cancers usually have a recognizable basal cell layer [5]. DCIS is more morphologically heterogenous than LCIS and is broken down into five categories: comedo, crib form, micropapillary, papillary, and solid, as shown in Figure 1 [6].

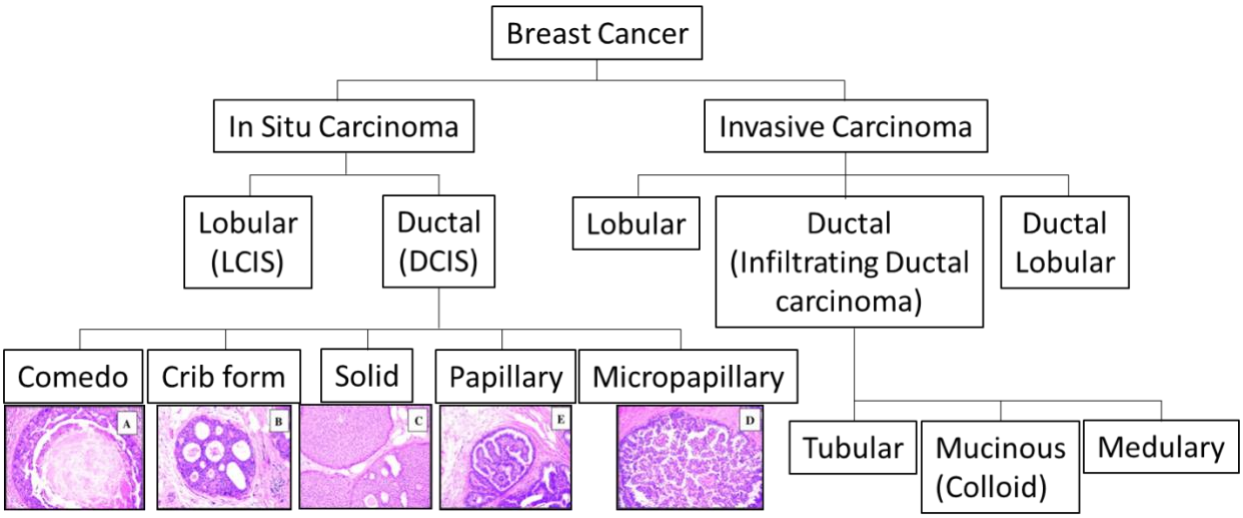

Figure 1: Different types of breast cancer. Schematic demonstrating the different subtypes of breast cancer. Images from Craig Allred D, 2010 with permission from Oxford University press[6].

Papillary and crib forms of DCIS are usually lower-grade lesions. They usually take longer to form into invasive cancer, while solid and comedo are higher grade, and if not treated, they will develop into invasive cancer [5]. Invasive ductal cancers grow as a cohesive mass, often detected on a mammogram, or as a small lump that can be felt in the breast. They make up 50-70% of all invasive breast cancer. Invasive lobular cancer is harder to detect on mammograms or by self-exam and accounts for 10% of breast cancers. In some cases, a mix of both invasive ductal and lobular cancers can occur.

Breast cancer can also be classified based on molecular characteristics, namely the expression of three general receptors: estrogen receptors, progesterone receptors, and human epidermal factor 2 (HER2) [7]. Breast cancer can be classified into 4 subtypes depending on the expression of these receptors. These include (1) luminal A and (2) luminal B, both of which are ER+; (3) HER2+, which is negative for estrogen and progesterone receptors but positive for HER2 receptors; and (4) triple-negative breast cancer (TNBC), which is negative for all these

receptors and is the hardest to treat [7]. ER+ breast cancers constitute over half of all breast cancers. Estrogen and progesterone receptors are normally expressed in breast tissue, as it depends on these two hormones for their proper development and function. As women go through different stages of their lives, the levels of estrogen and progesterone will rise and fall, and signaling from estrogen and progesterone receptors will promote cell growth and proliferation [7]. HER2+ tumors make up close to 20% of breast cancers. HER2 dimerization in malignant cells results in the autophosphorylation of tyrosine residues within the receptors cytoplasmic domain and the induction of various signaling pathways, leading to cell proliferation and tumorigenesis [7].

1.3 Risk Factors

Although breast cancer can occur in anyone, many risk factors can impact susceptibility to breast cancer, for example:

(1) Age: This is one of the most significant risk factors. A positive correlation exists between an increase in age and the likelihood of breast cancer. The incidence rate is significantly increased in women between 40-60 years of age [8].

(2) Family history: Women with family members who have a history of breast or ovarian cancer are more likely to get breast cancer themselves. A mutation in one or both BRCA genes, BRCA1 and BRCA2, predisposes women to breast cancer [9].

(3) Reproductive factors: Late menopause or early menarche, as well as late age of first pregnancy, can increase the risk of breast cancer. Each of these factors is associated with increased estrogen levels, which can elevate the risk of breast cancer. With each year of menopause delay, there is a 3% increase in breast cancer risk. Each one-year delay in menarche

and each birth that a woman gives decreases her risk by 5-10% [10]. This shows the direct relationship between breast cancer and estrogen levels.

(4) Exogenous hormone use: Women who take supplemental estrogen or progesterone, for example, birth control, are more likely to get breast cancer than those that do not.

(5) Personal history: If a woman has cancer in one breast, she is more than likely to get cancer in her other breast [5].

1.4 Tumor Microenvironment

For cancer to proliferate and metastasize, the tumor cells need help from other cells and factors in its vicinity. The tumor microenvironment (TME) is an ecosystem inside the body that surrounds the tumor. The TME plays a huge role in the tumor's ability to grow and proliferate; it consists of immune cells, extracellular matrix, blood vessels, and other cells like fibroblasts [11]. Tumor cells can communicate with the microenvironment very early in tumor development, and a dynamic relationship forms between the cancer cells and the components of the TME; this relationship provides the tumor with what it needs to grow, invade, and metastasize. The cells within the microenvironment affect tumor cells' growth, proliferation, and maturation.

The TME is constantly evolving and is extremely complex in its makeup. Each tumor type and each patient has a different TME composition. However, some hallmark features are the same throughout; these include the presence of immune cells, stromal cells, blood vessels, and the extracellular matrix (ECM). Without the TME, the tumor cells would not be able to grow the way that it does; the TME works as a backbone to the tumor [12] [13,14]. The natural environment within a tumor is hypoxic and acidic, which is not ideal for tumor cells. To get past this, the TME coordinates a program that restores the oxygen/nutrient supply and removes metabolic

waste [15]. The TME not only helps tumors grow and proliferate but has also been found to be a major player in making tumors drug-resistant. It does this through the acellular components of the TME, such as the ECM; they build physical barriers which affect tumor growth, among other functions [16].

1.5 Anti-cancer Immune Responses

The immune system is a sizeable interconnecting network of components that protect the body against invading pathogens. Each element in this network has a unique and individual function that it must execute to protect the body. Sir Frank Macfarlane first hypothesized the theory of immunosurveillance in 1975. He hypothesized that an immune response can be induced against tumor cell antigens. Since then, it has been found that patients with a deficient or compromised immune system have a higher chance of developing cancer and a worse prognosis when they do develop cancer [17] . The immune system can prevent tumor and cancer growth after detecting abnormal cells. Therefore, the patient's prognosis is better when more tumor-infiltrating lymphocytes (TILs) are found within the patient's tumor. A positive correlation exists between the number of TILs and patient survival [18].

The immune system is broken down into two arms, adaptive and innate, which work together to eliminate developing tumors. Activation of anti-cancer immune responses is a multistep process involving cells of the innate and adaptive immune systems. Activation of an adaptive anti-cancer immune responses begin by the release of cancer neoantigens from dead and dying cancer cells, and their transport to the nearest draining lymph node (DLN). Antigens are processed by dendritic cells (DCs) and presented to T cells through major histocompatibility class I and II molecules (MHC-I and MHC-II). T cells that recognize these antigens are cancer antigen-specific and become activated [19]. Activated T cells express cell adhesion molecules and

chemokine receptors that aid them in migration and infiltration into the tumor. In tumors, Cytotoxic T cells (CTL) will use their T cell receptor (TCR) to recognize and bind to MHC-I: antigen complex on cancer cells and kill them [19–22].

1.6 Anti-tumor Cells of the TME

While the TME assists heavily in the growth and proliferation of cancer cells, there are also anti-cancer immune cells within the microenvironment. These cells include:

(1) $CD8^+$ T cells: Naïve $CD8^+$ T cells differentiate into cytotoxic T cells (CTL) after activation. These cells are activated by mature dendritic cells (DC), which present antigens on MHC-I molecules. $CD8^+$ T cells infiltrate the TME with the aid of chemokines, such as CCL3, CCL4, CCL20, etc [23]. Tumors also release chemokines, such as CCL5 and CXCL9, which can attract lymphocytes to the TME [24]. CTLs can induce apoptosis in tumor cells through death receptor ligation by ligands, which include FASL and TRAIL, as well as by secreting apoptosis-inducing proteins granzyme B and perforin. As effective as $CD8^+$ T cells are, tumors can still evade them; they can do this by downregulating MHC-I and undergoing mutations that reduce antigen processing and presentation capacity. Tumors can also upregulate anti-apoptotic molecules such as BCL-2; they can also dysregulate expression of death receptors [25].

(2) $CD4^+$ T cells: $CD4^+$ T cells are also called helper T cells. When $CD4^+$ T cells encounter tumor antigens presented by antigen-presenting cells (APC), they can differentiate into different helper T cell types, which include Th1, Th2, Th9, Th17, Th22, and Tregs [26] [27]. $CD4^+$ T cells' main role is helping $CD8^+$ T cells in their antitumor immunity. They also secrete effector cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) that regulate the activity of other immune cells [28].

(3) Natural killer (NK) cells: NK cells are a member of the innate immune system and can target and kill diseased cells without prior exposure to antigens. NK cells' activation depends on a delicate balance of activating and inhibiting receptors that recognize ligands on target cells. When activating receptors are engaged on the NK cell by target cells, the NK cell can target the cell for destruction; When inhibitory receptors are engaged, the cell is seen as a self-cell and not killed. NK cell's main killing method is to release cytotoxic granules containing perforin and granzymes, which work in concert to kill the target cell. Perforin creates pores in the tumor cell membrane, which allow granzymes to enter the cell and induce apoptosis by activating the caspase cascade. Although NK cells are very important anti-cancer immune cells, tumors can perform immune escape by upregulating the expression of inhibitory ligands specific to the receptors on NK cells. This tricks the NK cell into seeing the tumor cells as healthy or self and not killing them [(29)].

(4) Natural killer T cells (NKT cells): NKT cells develop from lymphoid progenitors just as T and B cells do; these cells mature in the thymus. In humans, the number of NKT cells in the peripheral blood of cancer patients is very low. These cells play a role in eliminating tumor cells from the body, similar to NK cells and T cells. A positive correlation exists between the number of NKT cells in the patient's blood and cancer prognosis [(30)]. The presence of lipid antigens, which NKT cells recognize, possibly recruits NKT cells into the tumor. NKT cells may also be recruited into the tumor through the action of chemokines CCR2 and CXCR6 and leukocyte function-associated antigen-1 (LFA-1) [(31)].

(5) Eosinophils: Eosinophils are cells that mature in the bone marrow and migrate into the blood to eventually reach their target tissue [(32)(33)]. Eosinophils have been shown to play a role in killing cancer cells. These cells are found commonly in the TME. In colorectal cancer, eosinophils,

which are IFN-γ activated, could kill colorectal cancer cells [34]. However, a subset of these cells called regulatory eosinophils can promote tumor progression and suppress immune cells [35].

1.7 Pro-tumor Cells of the TME

In addition to antitumor immune cells, there are other cell types in TMEs that are pro-tumor, which means that they assist tumor growth and proliferation. These cells include;

(1) Tumor-associated macrophages (TAMs): There are two subtypes of TAMs; these are the pro-inflammatory M1 and the anti-inflammatory and pro-tumor M2 TAMs, which can inhibit the antitumor immune responses and promote tumor progression through the expression of cytokines and chemokines [36] [37] [38].

TAMSs develop from monocytes, which initially develop into functional macrophages, but then acquire various immunosuppressive functions at each stage of differentiation in TME. Monocytes circulating in the blood migrate into the TME in response to growth factors such as vascular endothelial growth factor (VEGF), cytokines, and chemokines released by different cells within the TME [39]. M2 TAMs promote tumor progression by releasing VEGF, IL-12, and MMP7, as well as high levels of IL-1 and TGF-β [39]. TAMs produce various chemokines, including CCL17, CCL18, and CCL22; these attract Tregs to the cancer site and inhibit T cells, allowing for a positive feedback loop between TAMs and Tregs [36]. TAMs can also promote angiogenesis through production of VEGF, TGF-β, and platelet-derived growth factor (PDGF) that can trigger tumor angiogenesis.

(2) Tumor-associated neutrophils (TANs): TANs can be either antitumor or pro-tumor depending on their acquired phenotype. TANs can be of two subsets, N1 neutrophils which are anti-tumor, and N2 neutrophils, which are pro-tumor [21]. Neutrophils develop in the bone marrow and

migrate in high numbers to inflammation sites in the early stages of cancer compared to the late stages. N1 TANs can aid T cells in killing tumor cells and cause cell apoptosis through TRAIL [38]. Neutrophil development into either subset depends on the presence of TGF-β. If there is an increase in TGF-β and an inhibition of IFN-γ production, the N2 phenotype is induced [21]. TANs can recruit macrophages along with Tregs into tumors to promote the growth and progression of tumors [38]. N2 TANs in TME secrete different molecules, which can promote angiogenesis via release of VEGF, promote tumor development and metastasis, and induce inflammation through cathepsin-g and release of IL-12 [40]. A previous study found that mice depleted of N2 TANs had reduced tumor growth and increased activation of $CD8^+$ T cells [41].

(3) Cancer-associated fibroblast (CAF): These fibroblasts are heterogenous cells, which include mesenchyme epithelial cells, fibrocytes, and epithelial-mesenchymal cells [42]. CAFs interact with other stromal cells and immune cells in TME [78], promoting cancer cell proliferation, invasion, and metastasis. CAFs support tumor invasion by releasing matrix metalloproteinase (MMP) that can degrade the extracellular matrix. CAFs also secret TGF-β and IL-10 to induce Tregs and immunosuppression in TME [43] [44].

(4) Cancer-associated adipocytes (CAA): These cells are fat cells that can store energy and support cancer cells through lipid accumulation [16] [45]. CAA plays a pivotal role in tumorigenesis, tumor growth, and metastasis. They can do this by overexpressing pro-inflammatory cytokine, matrix metalloproteinase, and insulin-like growth factor binding protein 2 [45] [46] [47]. CAAs also recruit myeloid cells into the TME, which can differentiate into immunosuppressive myeloid cells that play a role in maintaining the immunosuppressive milieu in the TME [48] [49].

(5) Tumor-associated endothelial cells: These cells form the blood vessels that play a critical role in the TME, such as angiogenesis, migration of immune cells, permeability, regulating fluidity, intravasation, and extravasation of tumor cells [50] [51] [52].

(6) Tumor-associated pericytes (TAP): These cells wrap around vascular endothelial cells and closely interact with one another in both physical and paracrine signaling [53]. These cells play a pivotal role in the TME by maintaining blood flow, modulating ECM remodeling, and participating in angiogenesis [54].

(7) Myeloid-derived suppressor cells (MDSCs): These are heterogeneous groups of immature myeloid cells with very high immunosuppressive activity and impaired function as antigen-presenting cells. These cells are plastic; they respond to microenvironment signals and can differentiate into macrophages, granulocytes, and dendritic cells *in vitro*. These cells change their function in response to cytokines and growth factors, which are present in the TME [55]. MDSCs in the TME have immunosuppressive functions; they can cause Treg cell differentiation and expansion, inhibit dendritic cell polarization, and induce macrophage differentiation to the M2 phenotype. One of the largest immunosuppressive roles they can play is to deprive T cells of amino acids. MDSCs also induce apoptosis in T cells by producing galectin-9, which binds to the Tim3 receptor.

1.8 How do Tumors Evade the Immune System

Although immune responses effectively fight off cancers, tumors have evolved ways to evade the immune response. There are several mechanisms that tumors use to evade immune responses. These include:

(1) Regulatory cells: Many types of immunosuppressive cells reside within the TME and can mediate immune escape. An example is regulatory T cells (Tregs) or other forms of regulatory cells [56]. It has been shown that Tregs, which are induced in tumors, have a higher suppressive effect than Treg cells induced outside the TME [57]. Tregs are attracted to the TME through tumor cell-mediated chemokine production [57]. Tumor cells also produce transforming growth factor (TGF-β), which causes $CD4^+$ T cells (helper T cells) to become Treg cells *in situ* [58]. In addition to immunosuppression, some immune cells can create an inflammatory environment in tumors, essential for tumors to grow and metastasize. One of the major players in this inflammatory environment is myeloid cells. These include but are not limited to myeloid-derived suppressor cells (MDSCs), modulated dendritic cells (DCs), and M1 and M2 macrophages, which also suppress immune responses [59].

(2) Defective antigen presentation: Tumors can evade immune surveillance by down-modulating antigen-presenting mechanisms. These mechanisms include the downregulation of proteins, such as MHC-I, latent membrane protein (LMP2 and LMP7), and TAP proteins, involved in antigen presentation [60] [61] [62] [63] [64]. This leads to tumors evading immune surveillance since the presentation of tumor antigens to cytotoxic T cells (CTL) is disrupted [65].

(3) Immune suppressive mediators: Immune suppressive cytokines released by cancerous or non-cancerous cells within the TME can cripple CTL function and allow tumors to evade their immune surveillance. These cytokines include TGF-β, Tumor necrosis factor-α (TNF-α), IL-1, IL-16, etc., which can all contribute to cancer growth [66] [67] [68,69] [70]. Some factors, such as vascular endothelial growth factor (VEGF), can negatively affect the differentiation of progenitor cells into DCs, thereby, affecting antigen presentation [71]. Studies have shown that factors such

as receptor-binding cancer-associated surface antigens (RCAS1) contribute to tumor progression by inducing apoptosis in tumor-infiltrating lymphocytes (TILs) [72] [73].

(4) Tolerance and immune deviation: Tumor cells can induce anergy in T cells, making them unresponsive to stimuli. Tumors can do this by expressing protein ligands normally expressed on healthy cells and binding to inhibitory receptors on immune cells, tricking them into thinking they have encountered a healthy cell. Tumor cells can also induce cell death in immune cells by direct interactions. An example is when tumors express program cell death ligand (PD-L1) to engage with the inhibitory receptor PD-1 expressed on activated T cells and induce them to undergo apoptosis [11].

2. Myeloid-derived suppressor cell (MDSC)

Myeloid-derived suppressor cells (MDSCs) are a population of heterogenous cells from the common myeloid lineage. These cells develop from hemopoietic stem cells (HSC) similar to lymphoid T, B, and NK cells [55]. Under homeostatic conditions, common myeloid progenitors differentiate into monocytes, granulocytes, macrophages, dendritic cells, erythrocytes, or megakaryocytes, dependent on the factors within their environment. Once the body is under stress, such as cancer, virus infection, or obesity, the frequency of MDSC population increases. These cells are extremely immunosuppressive and can assist the tumor in growth and proliferation while down-regulating the functions of immune cells such as NK and T cells [74]. The first time MDSCs were introduced into the literature was in 2007 when they were found in tumor-bearing mice. Since this discovery, MDSCs have become a hot topic in cancer research [75]. MDSCs are divided into two subsets; granulocytic or polymorphonuclear (PMN-MDSCs), which are morphologically similar to neutrophils, and monocytic MDSCs (M-MDSCS), which

are comparable in morphology to monocytes. In cancer, there is an increase in granulocyte-macrophage colony-stimulating factor (GM-CSF), which drives myelopoiesis. G-CSF stimulates granulocyte differentiation, while M-CSF induces monocyte differentiation. This overexpression may lead to MDSC development [54] [75]. Development and migration of MDSCs to the primary and metastatic sites is regulated by a combination of tumor-derived factors, which include: (1) Cancer cells-derived factor and trafficking signals to induce MDSC expansion and recruitment into the tumors, and (2) Tumor stroma- and T cell-derived factors similar to those used for neutrophils and monocyte recruitment to recruit MDSCs into the tumors [75] . The two subsets of MDSC have requirement for different factors to migrate to the tumor sites. M-MDSCs and monocytes are recruited to tumors through a chemokine cascade, which include CCL1, CCL2, and CCL5. This cascade is propagated by cancer cells and is retained in the primary tumor by CCL3. PMN-MDSCs and neutrophils are recruited to tumors through CCL2 and CCL3. In some studies, it is hypothesized that MDSCs first arrive to create a pre-metastatic niche to condition the organs for tumor invasion [76] [77]. The signal transducer and activator of the transcription (STAT) family (STAT1, STAT3, STAT6, and NFKB) have been shown to regulate MDSC activation and survival [78].

2.1 Phenotype and Activity

MDSCs are distinguished from other immune cells base on the expression of CD11b, a myeloid lineage marker, and Ly6G, Ly6C, and Gr1, all of which comprise the granulocyte marker [79]. The different expression of granulocytic markers Ly6G and Ly6C differentiates the two subsets of MDSCs. PMN-MDSCs carry the phenotype of $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$, which resemble the pro-tumor neutrophils [79]. M-MDSCs carry the phenotype of $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$. These cells also express CD115, CCR2, and CD49a; they differ from healthy

monocytes because they do not express CD11c and MHC-II [61]. MDSCs differ functionally from healthy monocytes and neutrophils due to their pro-tumorigenic phenotype [80]. A high MDSC number is an indicator of a poor prognosis because it inhibits the inflammatory response at the cancerous site and assists in tumor cell proliferation [80].

2.2 Immune Suppressive Mechanisms of MDSCs

Most of what we know about MDSCs' suppressive capabilities within the TME comes from studies of T cell suppression. A more recent area of study is the suppressive ability of MDSCs on other immune cells, such as NK cells. MDSCs have multiple immunosuppressive mechanisms (Figure 2), which include:

(1) Arginase and inducible nitric oxide synthase (iNOS) enzymes: One of the major ways in which T cells are inhibited by MDSCs is through the metabolism of L-arginine. L-arginine is a substrate for two enzymes, iNOS, and arginase, which generate nitric oxide (NO) and convert L-arginase into urea and L-orthinine, respectively [81]. L-arginine is essential for T cell proliferation; when arginase is increased in the TME due to increased numbers of MDSCs, it leads to enhanced L-arginine catabolism, which depletes the essential amino acid in TME. This inhibits T cell proliferation by decreasing their CD3 expression. Absence of CD3 signals prevents upregulation of cell cycle regulators, such as cyclin D3 [82]. NO can inhibit T cells in a variety of ways; this includes inhibitation of JAK3 and STAT5 signaling pathway, inhibition of MHC-II expression, and induction of apoptosis in T cells [83].

(2) Reactive oxygen species (ROS): Increased levels of ROS has become a main characteristic of MDSC in tumor-bearing mice and patients [54]. It has been reported that when ROS produced by MDSCs is neutralized, the suppressive capabilities of MDSCs were abrogated *in vitro* [84].

Superoxides, which are released by MDSCs, rapidly reacts with many molecules, e.g., H_2O_2, hydroxyl radical, hypochlorous acid, and peroxynitrite, forming ROS. ROS promotes apoptosis by damaging proteins, lipids, and nucleic acids [85].

(3) Peroxynitrite: A chemical reaction between NO and superoxide anions gives rise to peroxynitrite, which is one of the most powerful oxidants produced in the body [85]. Presence of MDSCs is associated with an increased level of peroxynitrite [54]. Peroxynitrite alters specific peptide-binding by T cells and renders them unresponsive to antigen stimulation [85].

(5) Cytokines: MDSCs also produce and release cytokines, such as TGF-β and IL-10, that inhibit T cells. IL-10 can inhibit T cells indirectly by impairing the expression of MHC and co-stimulatory molecules by antigen-presenting cells [86] [87].TGF-β can suppress T cell proliferation by inhibiting c-Myc expression and by inducing SMad3-Foxp1 interaction [88]. TGF-β can repress protein tyrosine kinase activities required for TCR signaling, this is done by inducing SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1), that can dephosphorylate many signaling proteins [89]. TGF-β can also reduce nuclear factors of activated T cells (NFAT) activation and nuclear translocation in effector and memory T cells through inhibiting IL-2 and its receptors [89] . Increased TGF-β within the TME leads to T cells that cannot mount an immune response against cancer cells and, therefore, cannot suppress a tumor growth [90].

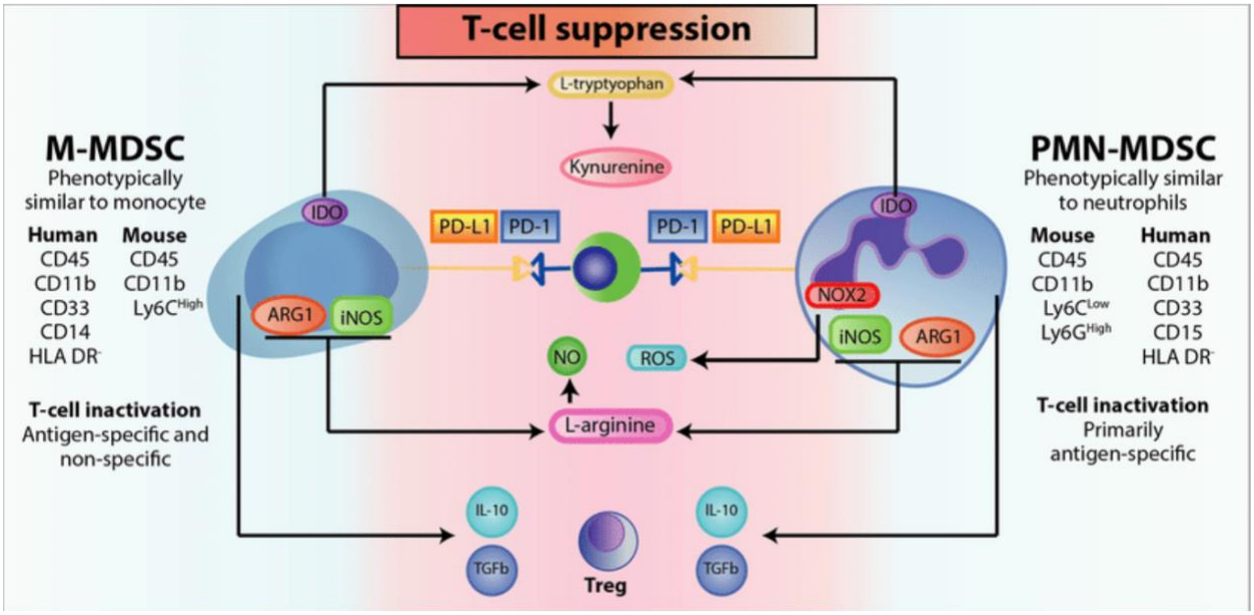

Figure 2: Phenotype and function of MDSC. M-MDSC and PMN-MDSC can be distinguished by their expression of markers Ly6C and Ly6G, respectively. Both subsets have multiple mechanism, for example expression of PD-L1, production of iNOS and ARG1 enzymes, and production of IL-10, and TGF-β cytokines. Image from Law AMK, 2020 with permission from MDPI [(91)]

2.3 Targeting MDSCs in Immunotherapy

Immunotherapy has been a breakthrough in cancer treatments; however, one of the biggest issues with immunotherapy has been the vast differences in response between patients. MDSCs have been found to play a pivotal role in promoting tumor growth and contributing to immunotherapy resistance. Recent studies have found that MDSCs can be therapeutic targets to alleviate their pro-tumor functions [(91)]. There are many ways in which MDSCs can be targeted. These include;

(1) Depletion of circulating and tumor-infiltrated MDSCs: It has been found that giving low doses of chemotherapeutic agents is effective in eliminating MDSC populations in tumor-bearing mice; treatments with certain chemotherapies such as 5-fluorouracil, paclitaxel, cisplatin, and gemcitabine depleted MDSCs and helped the immune system to fight off tumors [(91) (92) (93) (94)].

Another target has been the signaling cascades involved in MDSC expansion; this is targeted to reduce MDSC amplification [95].

(2) Prevention of MDSC recruitment and trafficking: When CCL2 and CCL5 levels are elevated in TME, MDSC recruitment occurs through chemokine receptor CXCR2 [96][97]. $CXCR2^{+}$ MDSCs can promote tumor expansion, metastasis, and T-cell exhaustion in breast cancer [98]. When CXCR2 was targeted, there was a decrease in the population of MDSCs as well as decreased metastasis. There was also a promotion of T-cell infiltration into tumors and an overall better survival found in patients with pancreatic cancer [99].

(3) Inhibition of MDSCs immunosuppressive functions: MDSCs have many ways by which they can suppress immune cells. A major way to suppress MDSC function is to suppress the immunosuppressive mechanisms they use. By suppressing MDSC function, T cell activity can be re-established, and immunotherapy success can be restored. [100]. An example is using a phosphodiesterase-5 (PDE-5) inhibitor to target the iNOS and ARG1. Administrating PDE-5 inhibitors has been shown to prolong survival [101].

(4) Differentiation of MDSC into a non-suppressive immune state: Another successful strategy used to reduce MDSC populations and eliminate the immunosuppressive functions is promoting immature myeloid cells to differentiate, so that they cannot become MDSCs [102]. In both mice and cancer patients, this has shown a reduction in the MDSC population and increased T-cell responses [103] [104].

3. Natural Killer Cells

Natural killer (NK) cells are part of the innate immune system and can kill a target cell without prior exposure to the invading cell. These cells belong to the group 1 innate lymphoid

cells (ILC1) but differ from other ILC1 cells due to their cytotoxic function [105]. NK cells circulate through nonlymphoid and lymphoid tissues. NK cells comprise 5-20% of circulating lymphocytes in humans and 2-5% of lymphocytes in spleen and bone marrow in mice [105] [106]. Of the many markers for NK cells, the first one discovered was CD16, a receptor for IgG, which triggers antibody-dependent cell-mediated cytotoxicity (ADCC) activity of NK cells. The antigen NK1.1 characterizes mouse NK cells. In addition, mouse NK cells express CD11b and CD27; NK cells that are positive for both, are considered mature and highly cytotoxic. In humans, NK cells are identified as lymphocytes that express CD56 surface protein. NK cells also express cell surface receptors such as NKG2A NKG2D, NKp46, etc. NK cells differ from T cells as they do not express CD3 or other components of the T cell receptor (TCR) [107].

3.1 NK Cell Functions

NK cells have two major functions: cytotoxicity and cytokine production [108] [109]. When NK cells target a cell for destruction, they release effector molecules, perforin, and granzymes, which work in concert to induce apoptosis in target cells. When perforin is lacking, apoptosis is reduced but not inhibited fully; this is because granzyme is still functional [110]. Granzymes are serine proteases that are packaged in cytotoxic granules with perforin. These granules are released when an NK cell targets a cell for destruction. Perforin binds to the target cell surface; it polymerizes in a Calcium (Ca)-dependent manner [111]. Ca^{2+}-ions bind to the phosphatidylcholine of the cell membrane, increasing binding affinity for perforin. After perforin has created pores in the cell membrane, granzyme diffuses passively through these pores, and apoptosis is initiated by activating caspases. Pores in the cell membrane can also induce apoptosis due to an altered state of homeostasis; this is because the pores disrupt cell membrane permeability [112]. NK cells have other methods of killing target cells, which do not

depend on perforin and granzyme. These involve TNF-related apoptosis-inducing ligand (TRAIL) and antibody-dependent cell mediated cytotoxicity (ADCC) [113]. TRAIL is expressed on NK cells and is a ligand for death receptors found on target cell; their binding induces apoptosis in the target cell. ADCC is a mechanism of immune defense where the NK cells recognize target cells coated with immunoglobulin molecule IgG [114]. NK cells can also activate other immune cells and release pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-10 [115] [116] [117].

3.2 NK Cell Receptors

NK cells have a vast array of receptors present on their surface. NK cell receptors survey host tissues, detect cellular abnormalities and respond to cellular stress and virally infected cells. Receptors interact with their respective ligands on a target cell; activation or inhabitation of NK cell activity is determined by the finite balance of inhibiting and activating signals from these receptors. Inhibitory receptors have a signaling motif known as immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain [118]. When an inhibitory receptor interacts with its ligand, the tyrosine residue of the ITIM becomes phosphorylated, creating a docking site for the intracellular protein tyrosine phosphatase, such as SHP-1 and SHP-2, which become localized near the cell membrane. These phosphatases inhibit NK cell activity through the dephosphorylation of tyrosine residues on other intracellular signaling molecules [119]. Unlike inhibitory receptors, activating receptors do not have a signaling motif in their cytoplasmic tail. Instead, activating receptors use adaptor proteins that contain an immunoreceptor tyrosine-based activation motif (ITAM) [120]. When activating receptors interact with their respective ligands, the ITAM located on the adaptor molecule becomes phosphorylated by protein tyrosine kinases

of the Src family, such as Lck and Fyn; this leads to downstream signaling and the release of cytotoxic granules to kill the target cell [121].

3.3 Cancer Immunosurveillance by NK cells

NK cells have an important role in controlling tumorigenesis; it has been shown that impaired NK cell functions or a deficiency of NK cells results in increased susceptibility to malignancy [122]. In mice, depletion of NK cells resulted in higher susceptibility to cancer induction [121] [123]. NK cells assist the immune system in suppressing tumor growth and progression and maintaining cell homeostasis [123]. NK cell levels have a direct correlation with cancer prognosis. Patients with a higher number of NK cells infiltrating the tumors have a better chance of survival and a better prognosis than those with low infiltrating NK cell numbers. In tumors, immune cells such as NK cells can become dysfunctional [124]. NK cells show reduced effector function in cancer patients than in healthy patients. This is demonstrated by a lower level of perforin, granzyme, TRAIL, and FasL expression. Other studies have shown a decrease in the production of perforin, granzyme B, CD107a, and IFN-γ compared to healthy individuals [125]. When NK cells develop immune exhaustion, they acquire a specific phenotype that differs from normal NK cells. NK cells may also differentiate into other types of innate lymphoid cells (ILC) which have reduced anti-tumor activity. Gill *et al*. demonstrated NK cell conversion into group 1 ILC (ILC1) cells in their study of the adoptively transferred NK cells in lymphoma mouse models. They found that healthy NK cells could rapidly accumulate at the tumor site but fail to attack the tumor due to their conversion into ILC1-like cells with reduced anti-cancer effector functions. ILC1-like cells also exhibited downregulation of transcription factors such as Tbet and EOMES [126]. In a study done by Al Olabi *et al*. NK cells in mice bearing mammary tumors exhibited an exhausted phenotype characterized by

upregulation of checkpoint receptors PD-1 and TIGIT, and the inhibitory receptors Lag-3 in addition to downregulation of EOMES expression [127]. This shows that NK cell population in tumors is plastic and may attain different phenotype and function, or become exhausted and dysfunctional.

NK cell receptors have been shown to play important roles in cancer immunosurveillance by NK cells. Mice deficient in activating NKG2D receptor were found to be more susceptible to tumorigenesis, demonstrating an important role of this receptor in controlling tumor growth in mice [128]. Tu *et al.* demonstrated a role for the inhibitory receptor Ly49 in breast cancer immunosurveillance [124]. Ly49 receptors bind to MHC-I proteins. Ly49-deficient mice were found to be more susceptible to mammary tumors induced by injection of MHC-I-deficient E0771 mammary adenocarcinoma cells or oncogene-driven mammary tumorigenesis (MMTV-PyVT transgenic mice). It was also observed that mammary tumors in Ly49-deficient mice had a lower expression of MHC-I, suggesting tumor immunoediting, which may be attributed to avoidance of $CD8^+$ T cells cytotoxic effects. This study demonstrated an important role for Ly49 receptors in tumor immunosurveillance and immunoediting [124].

3.4 NKR-P1 Receptors and Clr Ligands

NKR-P1 receptors are C-type lectin proteins and a type II transmembrane glycoprotein receptors [129]. The first NK cell marker discovered in mice was NK1.1 or NKR-P1C, which is an activating member of this receptor family [130]. There are five members of the NKR-P1 receptor family in mice (Figure 3); three of these are activating (NKR-P1A,-P1C,-P1F) and two are inhibitory (NKR-P1B/D and –P1G) [131] [132]. NKR-P1 receptors recognize C-type lectin-

related (Clr/Clec) protein family of ligands. In mice, there are 7 Clr ligands (Clr-a, -b, -c, -d, -f, -g, -h) (Figure 3). On the natural killer gene complex (NKC), the NKR-P1 receptor genes are interspersed with the Clr ligand genes; this means that their inheritance is linked, highlighting the importance of these receptor-ligand interactions in immunity [132]. There are currently three known receptor-ligand interactions between the NKR-P1 and Clr protein families (Figure 3); these include NKR-P1B:Clr-b, NKR-P1F: Clr-c,d,g, and NKR-P1G: Clr-d,f,g [133]. In humans there are three receptor:ligand pairs belonging to this family. NKp65:KACL (KLFR2:CLEC2A) and NKp80:AICL (KLRF1:CLEC2B) interactions are activating, while NKR-P1A interaction with LLT1 inhibits NK cell effector functions in humans [134] [135].

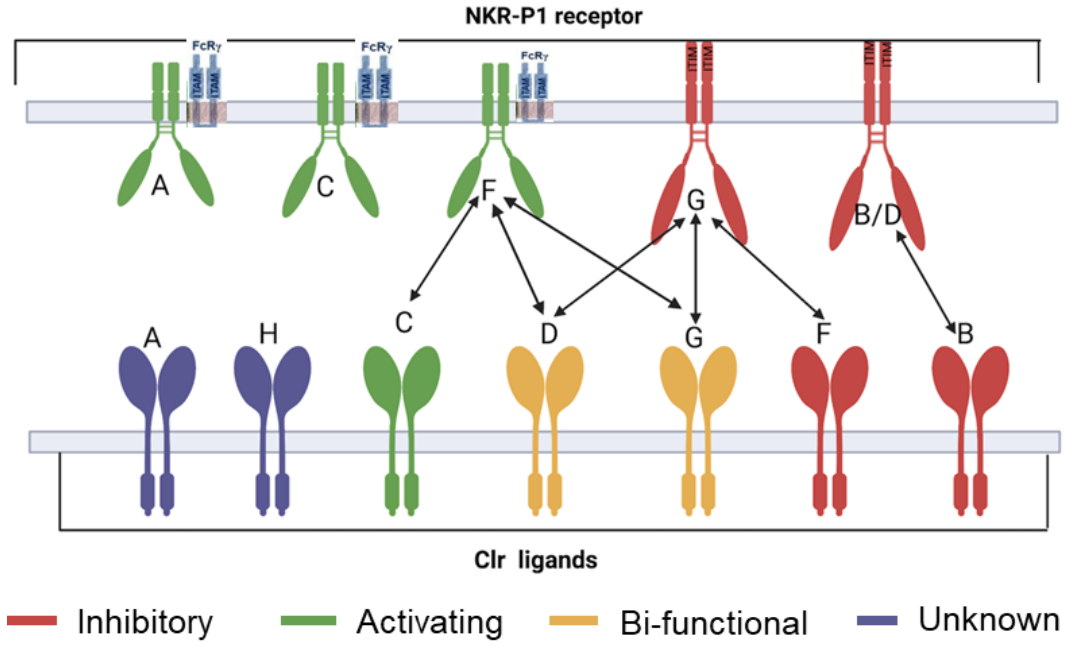

Figure 3: NKR-P1 receptor and Clr ligand families. The NKR-P1:Clr family is composed of 5 receptors and 7 ligand members. Specific receptor:ligand interactions are shown by arrows.

3.5 NKR-P1B:Clrb recognition axis

NKR-P1B is an inhibitory receptor that is found on 60% of NK cells in wild-type C57BL/B6 mice [136]. NKR-P1B recognizes the ligand Clr-b, which is expressed in many organs

and hematopoietic cells, as well as on stressed and infected cells [137]. NKR-P1B interactions with Clr-b induces NK cell inhibition (Figure 4). On the other hand, loss of expression of Clr-b on the target cell induces a 'missing-self' response, where NK cells recognize the absence of Clr-b expression through their NKR-P1B receptor and target the cell for destruction [137]. When NK cells are missing the NKR-P1B receptor, they can no longer have an efficient 'missing-self' response to a target cell that is not expressing Clr-b [137]. A study by Rahim *et al*. using Eμ-myc transgenic mice, which develop spontaneous B cell lymphoma due to the expression of the *c-myc* oncogene in B cells showed that myc-induced B cell lymphoma cells express Clr-b and can engage the NKR-P1B receptor to inhibit NK cells and escape immunosurveillance. It was also found in a study by Al Olabi *et al*. that the NKR-P1B: Clr-b inhibitory axis plays a role in the immunosurveillance of mammary tumors. They used MMTV-PyVT mice, which spontaneously form mammary tumors where most of the tumor cells do not express Clr-b. NKR-P1B knockout mice were found to be less resistant to mammary tumors than wild-type mice due to their defective 'missing-self' responses against tumor cells lacking Clr-b expression [127].

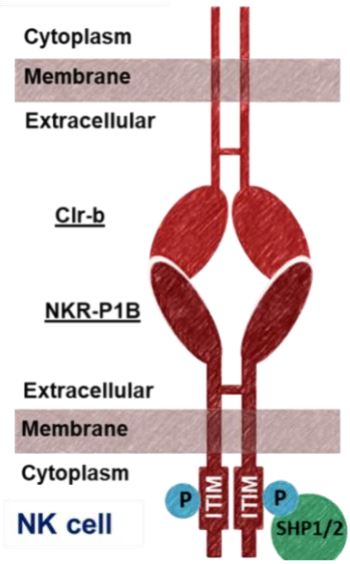

Figure 4: The interaction between NKR-P1B receptor and Clr-b ligand. NKR-P1B receptor interaction with Clr-b results in phosphorylation of tyrosine residue in the ITIM motif and recruitment of SHP-1 and -2 causing dephosphorylation other signaling molecules.

3.6 MDSC and NK Cell Interactions

Much of what is known about MDSCs' immunosuppressive abilities is known about the interaction between MDSCs and T cells. Recently the relationship between MDSCs and other immune cells, such as NK cells, has been explored. It has been shown that MDSCs can inhibit NK cell's cytotoxicity. NK cells and MDSCs interact with each other through three main methods (1) Cell-to-cell contact, (2) Secretion of soluble factors, (3) Release of extracellular vesicles (EV) [138]. The main way that MDSCs can inhibit NK cells is through cell-to-cell contact involving TGF-β. Membrane-bound TGF-β on MDSCs has been shown to induce NK cell anergy; this impairs the cytotoxic capabilities of NK cells, reduces NKG2D receptor expression, and inhibits production of IFN-γ [140]. In humans, MDSCs have been shown to inhibit NK cells through an inhibitor NKp30 receptor-dependent mechanism [141]. MDSCs also inhibit NK cells by expressing PD-1 ligand (PD-L1) that binds to the inhibitory receptor PD-1 on activated NK cells [142]. In addition to cell-to-cell contact, MDSCs can also inhibit NK cells by releasing inhibitory molecules such as NO and Indoleamine-pyrrole 2,3-dioxygenase (IDO). NO has been shown to inhibit both T and NK cells. NO inhibits NK cells by impairing Fc receptor-mediated killing and production of IFN- γ, TNF-α, and granzyme B. IDO can impair the development and activation of NK cells by decreasing the expression of NKG2D and DNAM1 receptors, and inhibiting IFN-γ expression [142].

Hypothesis and Research Objectives

Previously, our lab examined the role of NKR-P1B:Clr-b recognition axis in mammary tumor immunosurveillance using MMTV-PyVT mice [128]. NKR-P1B:Clr-b recognition axis was found to play a role in cancer immunosurveillance; NKR-P1B K/O mice developed mammary tumors earlier than their WT counterparts [128]. Additionally, it was found that tumor-infiltrating

NK cells lose expression of EOMES and become dysfunctional rapidly in NKR-P1B K/O mice compared to their WT counterparts [128]. This suggests a role for NKR-P1B:Clr-b interactions in NK cell functional homeostasis. More recently, using E0771 and Clr-b K/O E0771 mammary adenocarcinoma cell lines, our lab has found that NKR-P1B:Clr-b interactions involving tumor cells as well as other cells in the TME may be involved in NK cell mediated cancer immunosurveillance. The cellular interactions and factors involved in modulating NK cell responses in mammary tumors via NKR-P1B receptor are not fully understood. Clr-b is expressed at high levels in cells of the hematopoietic system. Hence, we hypothesize that MDSCs in the tumor microenvironment (TME) express Clr-b and can interact with NK cells through the NKR-P1B receptor to modulate their functions. Additionally, Clr-b expression on MDSCs may regulated their recruitment to tumors and their function in TME. This project aims to understand the role of NKR-P1B:Clr-b recognition axis in cross-talk between NK cells and MDSC in mammary tumors.

CHAPTER 2: METHODS

1. Mouse Models

Transgenic MMTV-PyVT (Strain: B6. FVB-Tg(MMTV-PyVT)634Mul/Lel1J) and C57BL/6 (B6) mice were obtained from The Jackson Laboratories. NKR-P1B deficient mice on a B6 background were previously generated by Rahim et al.[143]. Clr-b K/O mice have been described previously [144]. MMTV-PyVT mice develop spontaneous mammary tumors due to their expression of the polyoma virus middle T antigen; it is controlled by the mouse mammary tumor virus promotor [145]. Male MMTV-PyVT mice were bred with female NKR-P1B knockout mice. The NKR-P1B$^{+/-}$ progeny were positive or negative for the MMTV-PyVT transgene. The males which were MMTV-PyVT^{+} NKR-P1B$^{+/-}$ were bred with females which were NKR-P1B$^{+/-}$ to obtain MMTV-PyVT^{+} and either NKR-P1B $^{+/+}$, NKR-P1B $^{+/-}$, or NKR-P1B $^{-/-}$. Twice a week, the female MMTV-PyVT^{+} NKR-P1B$^{+/+}$ and MMTV-PyVT^{+} NKR-P1B$^{-/-}$ mice were monitored for tumor development. Tumor size was measured with a digital caliper. When tumors exceeded 10mm in diameter, mice were euthanized, and the tumors and spleen were dissected for analysis.

E0771 cells were provided by Dr. Andrew Makrigiannis from Dalhousie University. E0771 cell line originates from a spontaneously arising medullary breast adenocarcinoma in a C57BL/6 mouse [146]. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin referred to as complete DMEM or CDMEM. The cells were grown in an incubator at 37 °C and 5% CO_2. To induce mammary tumors using E0771 cell lines, groups of WT, NKR-P1B K/O, and Clr-b K/O female mice were injected with $5x10^5$ E0771 cells in the mammary fat pad. The injections were performed by first putting the mice under anesthesia with 2.5% isoflurane gas in 1 L/min O_2 for 2-3 min. Next, $5x10^5$ cells in 100 µl sterile PBS were injected into the fourth mammary

gland fat pad using a 28G insulin syringe. Mice were observed weekly post injections for palpable tumors. The diameter of the tumors was measured twice a week using a digital caliper. When tumors exceeded 10 mm in diameter, the mice were euthanized, and the tumors and spleen were dissected for analysis.

2. Tumor analysis

Tumors were chopped into small pieces with a razor blade and incubated at 37°C for 30 minutes in 5 mL digestion medium (5 mL serum-free RPMI + 200 µg/mL collagenase D + 20 ug/mL DNase1) with gentle mixing every 5 minutes. After the 30-minute incubation, digestion was terminated by adding EDTA at 1 mM concentration to each dissociated tumor. Tumors were then passed through a 70 µm cell strainer to obtain a single-cell suspension for analysis. Spleens from the respective mice were dissociated by crushing them between two glass microscope slides in phosphate-buffered saline (PBS), and the cell suspensions were collected in a tube. All cells were spun down in a centrifuge at 500xg for 5 min. Supernatant was discarded and cells were treated with 5 mL Ammonium-chloride-potassium (ACK) lysis buffer for 5 minutes to lyse red blood cells. After addition of 10 mL PBS, cells were spun down in a centrifuge at 500xg for 5 min and cell pellets were resuspended in 1xPBS. Tumor cells and splenocytes were counted using a hemocytometer, and $1x10^6$ cells were stained with antibodies for flow cytometry analysis.

3. Antibodies and flow cytometry

TILs and splenocytes were resuspended in PBS at $1x10^6$ cells/100 µl concentration. Approximately,100 µl of cell resuspension was then added to a 96-well plate, spun down at 500xg for 5 min, and supernatant was removed. To each well, 150 µl of FACS buffer was added and the plate was spun down at 500xg for 5 min again, and the supernatant was dumped. Surface

staining of cells was then performed in 50 µL FACS buffer containing primary antibodies at concentrations shown in Tables 1-6 at 4 °C in dark for 20 minutes. Cells were then washed with 150 µl of FACS and spun down again as before. Supernatant was dumped and cells were incubated in 50 µL FACS buffer containing secondary antibodies at concentrations shown in Tables 1-6 at 4°C in dark for 20 minutes. Approximately, 150 µl of FACS was added, samples were spun down, and the supernatant was dumped. Intracellular staining for TGF-β was done using fixation and permeabilization reagents (BD Biosciences) following the manufacturer's instructions. Unstained controls cells where maintained where required.

The following tables represent the antibody used to stain cells for flow cytometry analysis.

Table 1: Stain 1 - Surface stain, primary antibodies

Antibody	Fluorochrome	Dilution
CD45	AF700	1:200
CD11b	PerCP Cy5.5	1:100
Ly6C	PE 594	1:1000
Ly6G	PE Cy7	1:300
4A6 (Clr-b)	None	1:500
Fixable viability dye	APC-Cy7	1:500

Table 2: Stain 1 - Secondary antibodies

Antibody	Fluorochrome	Dilution
Anti-rat IgM	PE	1:200

Table 3: Stain 2 (secondary antibody control stain) - Surface stain, primary antibodies

Antibody	Fluorochrome	Dilution
CD45	AF700	1:200
CD11b	PerCP Cy 5.5	1:100
Ly6C	PE 594	1:1000
Ly6G	PE Cy7	1:300
Viability dye	APC-Cy7	1:500

Table 4: Stain 2 - Secondary antibodies

Antibody	Fluorochrome	Dilution
Anti-rat IgM	PE	1:200

Table 5: Stain 3 - Surface stain

Antibody	Fluorochrome	Dilution
CD45	AF700	1:200
CD11b	PerCP Cy 5.5	1:100
Ly6C	PE 594	1:1000
Ly6G	PE Cy7	1:300
Fixable viability dye	APC-Cy7	1:500

Table 6: Stain 3 – Intracellular stain

Antibody	Fluorochrome	Dilution
TGF-B	BV421	1:100
iNOS	PE	1:100

Data were acquired using the BD LSR Fortessa^{TM} X-20 analyzer and FACSDiva^{TM} software. Flow cytometry data was analyzed using FlowJo^{TM} software from BD Biosciences.

4. Co-cultures

To obtain NK cells, WT and NKR-P1B K/O mice with no tumors were sacrificed and spleens were collected. Splenocytes were prepared as described above and were resuspended in EasySep buffer (StemCell Technologies) to at 10^8 cells/100 µl. Splenocytes were then processed using an NK cell purification kit from Stem Cell Technologies to purify NK cells. A portion of the cells were stained using anti-NK1.1 and anti-TCR-β antibodies and analyzed by flow cytometry to determine NK cell percentage.

To obtain MDSCs, female WT and Clr-b K/O mice bearing E0771 tumors were sacrificed, and the spleens were dissected to prepare splenocytes as described above. MDSCs were purified using an MDSC purification kit from Stem Cell Technologies. A portion of

isolated MDSCs was stained with anti-CD11b, anti-Ly6G, and anti-Ly6C antibodies and analyzed by flow cytometry to determine MDSC percentage.

NK cells were co-cultured with MDSCs at a 1:2 ratio in 1 mL complete RPMI (CRPMI) supplemented with 1.5 µg/mL IL-2 and 1 µg/mL β-mercaptoethanol in a 24 well plate. These were left in the incubator at 37°C for 5 days. After 5 days cells were stimulated with 0.1 mg/µl of PMA and 1mg/mL of ionomycin. After 30 minutes, 5 µg/mL brefeldin A (BFA) was added to inhibit cytokine release from the cells. Cells were incubated for 4 hours in the incubator at 37°C. After the 4 hours, cells were stained with antibodies for flow cytometry as described above. Data were acquired using the BD LSR Fortessa™ X-20 analyzer and FACSDiva™ software. Flow cytometry data was analyzed using FlowJo™ software from BD Biosciences.The following tables show the antibody used to stain cells for flow cytometry analysis.

Table 7: Stain 1 - Surface stain, primary antibodies

Antibody	Fluorochrome	Dilution
NK1.1	PE 594	1:200
TCR-B	BV510	1:200
CD49b	PE-Cy7	1:100
CD49a	PE	1:100
PD-1	FITC	1:100
DNAM	PercP-Cy5.5	1:100
LAG-3	Biotinylated	1:100
Fixable viability dye	APC-Cy7	1:500

Table 8: Stain 1 - secondary stain

Antibody	Fluorochrome	Dilution
Streptavidin	BV650	1:100

Table 9: Stain 1 – Intracellular stain

Antibody	Flurochrome	Dilution
IFN-γ	BV605	1:100
EOMES	APC	1:200

5. Cytokine assay

To measure cytokines in tumor tissue, tumors were weighed and homogenized in the 1 mL of Hanks Balanced Salt solution using a tissue homogenizer. Tubes were then spun down at 500xg at 37°C for 5 minutes and supernatant collected. Appropriate volume of tumor tissue homogenate was placed into a 96-well plate. Biolegend LEGENDplex^{TM} 13-plex mouse inflammation kit was then used to prepare standard curves and measure cytokine concentration in extracts prepared form tumors following the manufacturer's instructions. Data was acquired using the BD LSR FortessaTM X-20 analyzer and FACSDivaTM Software. Data were analyzed using Biolegend software and cytokine concentrations were determine per gram of tumor tissue.

6. Statistical analysis

Statistical analyses were performed using GraphPad Prism Software. Statistical significance was determined by virtue of one-way or two-way ANOVA (with a cut-off *P* value of 0.05 [$P<0.05$(*), $P < 0.01$(**), *$P<0.001$(***)]*. Results which did not reach statistical significance were not designated.

CHAPTER 3: RESULTS

1. Tumor infiltrating lymphocytes, including MDSCs, express Clr-b

Tumor-infiltrating lymphocytes (TILs) reside within the TME; many are immunosuppressive and work in concert with tumor cells to assist with immunoevasion. MDSC is an immunosuppressive TIL that has been shown to interact with NK cells and inhibit their cytotoxic function and cytokine production. They can do this either directly through interacting with receptors on the NK cell surface or indirectly through releasing immunosuppressive cytokines. To understand if MDSCs can interact with NK cells directly via the NKR-P1B receptor, we first examined whether MDSCs express Clr-b in MMTV-PyVT and E0771 mammary tumors. Clr-b is the ligand for NKR-P1B receptor.

Firstly, we examined if TILs found in MMTV-PyVT and E0771 mammary tumors expressed Clr-b. To do this, we compared Clr-b expression on $CD45^+$ TILs and $CD45^-$ tumor cells by flow cytometry analysis. We found that in the MMTV-PyVT tumors, all TILs expressed Clr-b at high levels (Fig 5a), but only a proportion of tumors cells expressed Clr-b and at lower level than TILs (Fig 5a). In the E0771 mammary tumors, we found that both tumor cells and TILs expressed Clr-b (Fig 5b). This means that all TILs express the ligand for NKR-P1B receptor; however, depending on the tumor model, tumor cells may or many not express this ligand.

Next, we examined if MDSCs themselves are positive for Clr-b expression. We also examined if there were any differences in Clr-b expression in MDSC subsets from WT and NKR-P1B K/O mice. The two MDSC subsets were identified by the expression of Ly6C and Ly6G. Flow cytometry analysis showed that both MDSC subsets expressed Clr-b in tumors from

MMTV-PyVT mice (Fig 6a). Clr-b was also expressed highly in $Ly6C^+$ M-MDSCs from E0771 tumors (Fig 6b). Although, $Ly6G^+$ PMN-MDSCs from E0771 tumors appear to express Clr-b, we were not able to accurately quantify Clr-b expression in this subset of MDSC due to high background signal in control staining (Fig 6b).

In MMTV-PyVT mammary tumors, we found that the proportion of both PMN-MDSC and M-MDSC positive for Clr-b expression and the mean fluorescence intensity (MFI) of Clr-b staining, which indicates Clr-b expression level, remained unchanged across tumor sizes, large (L; >10 mm in diameter), medium (M; 5-10 mm in diameter), and small (S; <5 mm in diameter) tumors, and between WT and NKR-P1B K/O mice (Fig 6A). The same trends were seen in the MDSCs from the spleens of these mice (Fig 6C). Similarly, in both the spleen and tumors of mice bearing E0771 mammary tumors, we found no difference in the percentage of PMN-MDSCs and M-MDSCs positive for Clr-b and Clr-b expression level (MFI values) between the WT vs. NKR-P1B K/O mice (Fig 6B and 6D). These results together demonstrate that MDSCs in mammary tumors express Clr-b, which does not change whether NKR-P1B receptor expression is present or absent in the TME.

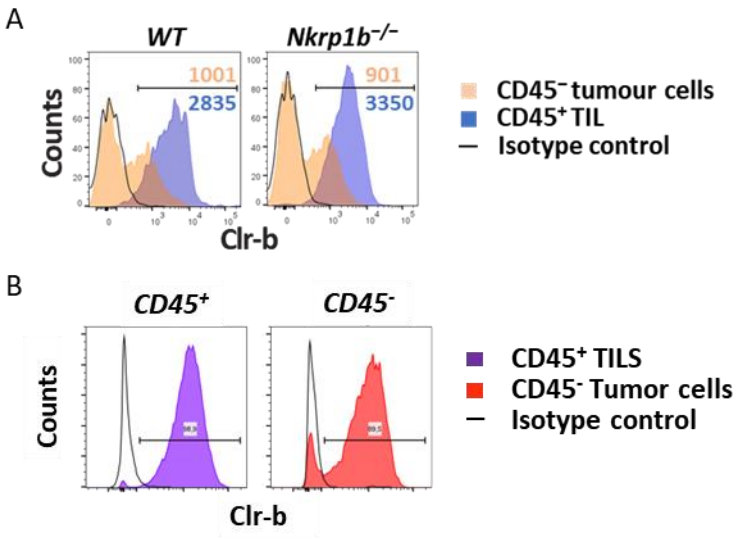

Figure 5: Clr-b expression on tumor-infiltrating lymphocytes (TILs) and mammary tumor cells. Cells prepared from mammary tumors were stained with antibodies against Clr-b and CD45 proteins and analyzed by clow cytomety.

A, Expression of Clr-b on $CD45^-$ tumor cells (orange histogram) and $CD45^+$ TILs (purple histogram) from for WT and NKR-P1B K/O MMTV-PyVT tumors. Mean fluorescence intensity (MFI) values are shown. Isotype control staining is represented by a solid line. B, Expression of Clr-b on $CD45^+$ TILs (purple histogram) and $CD45^-$ tumor cells (red histogram) in E0771 mammary tumors. Isotype control staining is shown by solid line.

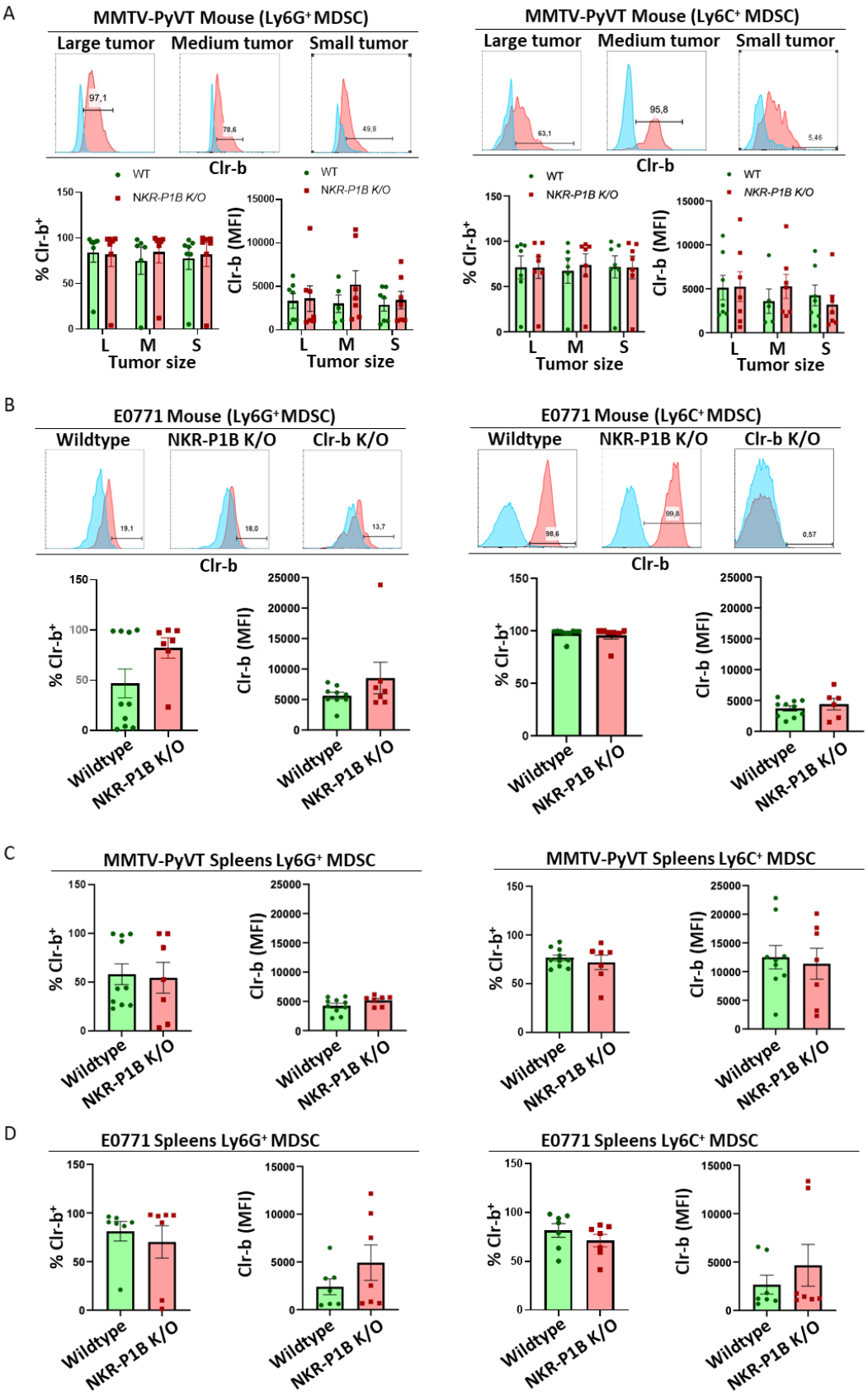
A
MMTV-PyVT Mouse (Ly6G+ MDSC)
Large tumor
Medium tumor
Small tumor
97,1
78,6
49,8
Clr-b
WT
NKR-P1B K/O
% Clr-b+
Clr-b (MFI)
L M S
Tumor size
MMTV-PyVT Mouse (Ly6C+ MDSC)
63,1
95,8
5,46
B
E0771 Mouse (Ly6G+MDSC)
Wildtype
NKR-P1B K/O
Clr-b K/O
19,1
18,0
13,7
E0771 Mouse (Ly6C+ MDSC)
98,6
99,8
0,57
C
MMTV-PyVT Spleens Ly6G+ MDSC
MMTV-PyVT Spleens Ly6C+ MDSC
D
E0771 Spleens Ly6G+ MDSC
E0771 Spleens Ly6C+ MDSC

Figure 6: Clr-b expression in MDSC subsets in the spleen and tumors from E0771 and MMTV-PyVT tumor-bearing mice. Flow cytometry analysis of Clr-b expression in MDSC subsets.

A, Clr-b expression on PMN-MDSC (left panel) and M-MDSC (right panel) in large (L; >10 mm in diameter), medium (M; 5-10 mm in diameter), and small (S; <5 mm in diameter) tumors from WT and NKR-P1B K/O MMTV-PyVT mice. Clr-b staining is shown in pink histograms and control staining, which only received the secondary antibody, in blue histogram. Both average frequency (%) of $Clrb^+$ MDSCs and expression level (MFI values) are shown in bar graphs. Clr-b MFI values are normalized to the control staining. Each symbol represents MDSCs from one tumor (n=6-7). B, Clr-b expression on PMN-MDSC (left panel) and M-MDSC (right panel) in E0771 tumors from WT and NKR-P1B K/O and Clr-b K/O mice. Clr-b staining is shown in pink histograms and control staining, which only received the secondary antibody, in blue histogram. Both average frequency (%) of $Clrb^+$ MDSCs and expression level (MFI values) are shown in bar graphs. Clr-b MFI values are normalized to the control staining. Each symbol represents MDSCs from one tumor (n=7-10) C, Graphical representation of average frequency (%) and expression level (MFI values) for Clr-b^+ PMN- MDSC (left panel) and M-MDSCs (right panel) in spleens from WT and NKR-P1B K/O MMTV-PyVT mice. Each symbol represents one spleen (n=7). Clr-b MFI values are normalized to the control staining. D, Graphical representation of average frequency (%) and expression level (MFI values) for Clr-b^+ PMN-MDSC (left panel) and M-MDSCs (right panel) in spleens from E0771 tumor-bearing WT and NKR-P1B K/O mice. Clr-b MFI values are normalized to the control staining. Each symbol represents MDSCs from one tumor (n=7).

2. NKR-P1B:Clr-b interactions are not required for MDSC recruitment into mammary tumors.

MDSCs are immature myeloid cells that are generated in the bone marrow. These cells migrate out of the bone marrow in immature phase when the body is under stress, such as cancer or other forms cellular stress, and gain an immunosuppressive function. We wanted to examine if there is any difference in the differentiation and recruitment of M-MDSC and PMN-MDSC into the tumors and spleens from WT, NKR-P1B K/O, and Clr-b K/O mice.

In MMTV-PyVT mice, we saw no difference in the recruitment of either subset of MDSC between the WT and NKR-P1B K/O tumors; this remained consistent throughout large, medium, and small tumors (Fig 7A). We saw large variation in MDSC recruitment due to the heterogeneous nature of mammary tumors in MMTV-PyVT mice. The same trend was seen in the spleens from tumor-bearing WT and NKR-P1B K/O mice (Fig 7B). In both the spleens and tumors from MMTV-PyVT mice, PMN-MDSC represented the larger proportion of total MDSCs than the M-MDSC (Fig 7A and B). In contrast, M-MDSC was the major MDSC subset in E0771 mammary tumors, but not spleens from these mice (Fig 7C and D). However, we saw no differences in the frequencies of PMN-MDSCs and M-MDSCs in tumors and spleens from E0771 mammary tumor-bearing WT, NKR-P1B K/O, and Clr-b K/O mice (Fig 7C and D). These results show that, although MDSC differentiation and recruitment varies between tumor types (MMTV-PyVT versus E0771 mammary tumors), it is independent of NKR-P1B:Clr-b interactions.

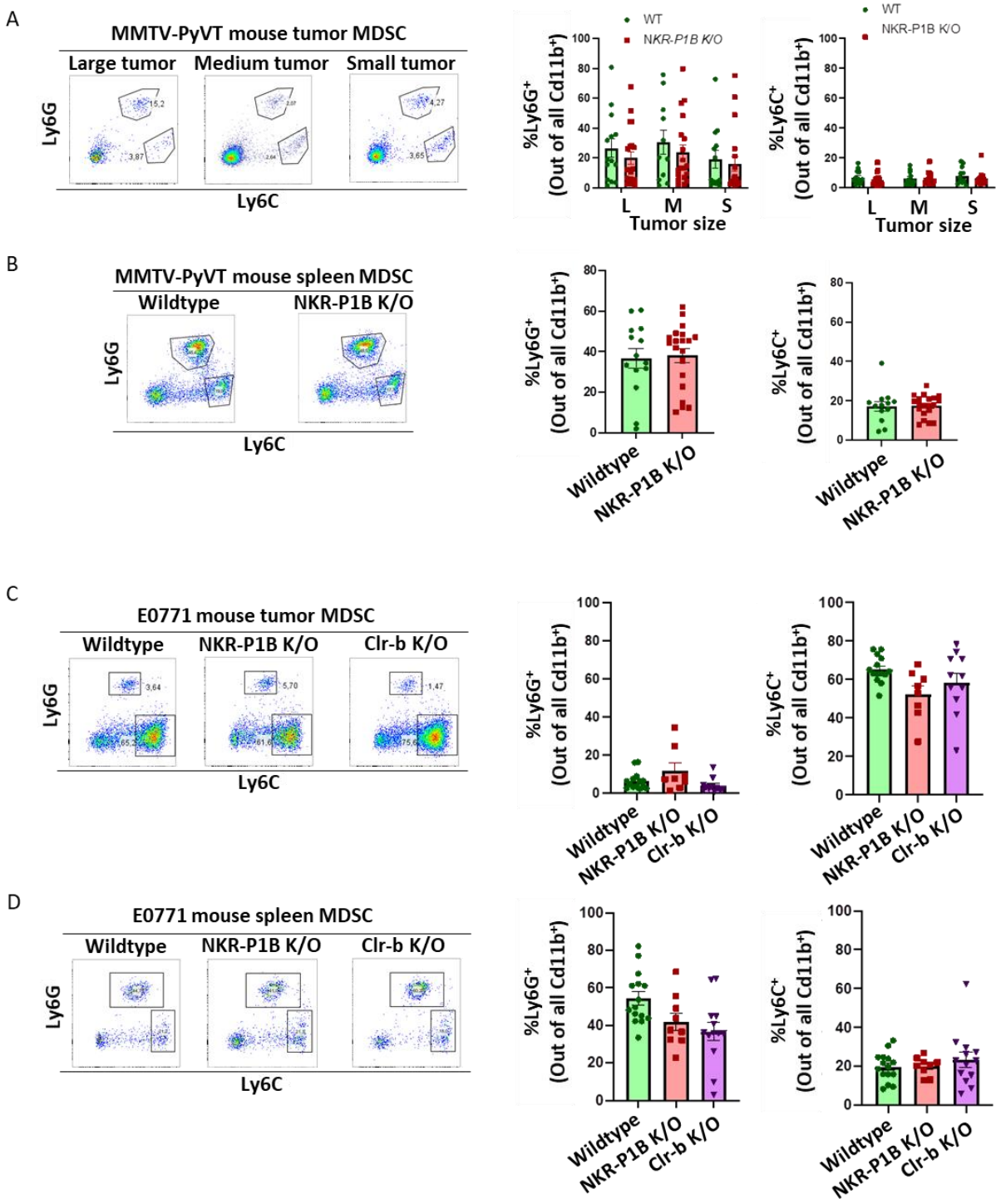
A
MMTV-PyVT mouse tumor MDSC
Large tumor
Medium tumor
Small tumor
Ly6G
Ly6C
15,2
3,87
2,07
2,64
4,27
3,65
WT
NKR-P1B K/O
%Ly6G+ (Out of all Cd11b+)
%Ly6C+ (Out of all Cd11b+)
L
M
S
Tumor size
B
MMTV-PyVT mouse spleen MDSC
Wildtype
NKR-P1B K/O
Ly6G
Ly6C
%Ly6G+ (Out of all Cd11b+)
%Ly6C+ (Out of all Cd11b+)
C
E0771 mouse tumor MDSC
Wildtype
NKR-P1B K/O
Clr-b K/O
Ly6G
Ly6C
3,64
5,70
1,47
%Ly6G+ (Out of all Cd11b+)
%Ly6C+ (Out of all Cd11b+)
D
E0771 mouse spleen MDSC
Wildtype
NKR-P1B K/O
Clr-b K/O
Ly6G
Ly6C
%Ly6G+ (Out of all Cd11b+)
%Ly6C+ (Out of all Cd11b+)

Figure 7: MDSC differentiation and recruitment in mammary tumors. Flow cytometry analysis of PMN-MDSC ($Ly6G^{+}$ $Ly6C^{low}$) and M-MDSC ($Ly6G^{-}$ $Ly6C^{high}$) subsets in tumors and spleens.

A, A comparison of the proportions (%) of PMN- and M-MDSCs out of total $CD11c^{+}$ TILs in large, medium, and small tumors from WT and NKR-P1B K/O MMTV-PyVT mice. Each symbol represents a single tumor. WT tumors: L, n=14; M, n=11; S, n=10. NKR-P1B K/O tumors: L, n=21; M, n= 19; S, n=19. B, A comparison of the proportions (%) of PMN- and M-MDSC in spleens from WT and NKR-P1B K/O MMTV-PyVT mice. Each symbol represents a single spleen. WT: n=14 NKR-P1B K/O: n=20 . C, A comparison of the proportions (%) of PMN- and M-MDSCs in E0771 tumors from WT, NKR-P1B K/O, and Clr-b K/O mice. Each symbol represents a single tumor. WT: n=14, NKR-P1B K/O: n=8, and Clr-b KO: n=11. D, A comparison of the proportions (%) of PMN- and M-MDSCs in spleens from E0771 tumor-bearing WT, NKR-P1B K/O, and Clr-b K/O mice. Each symbol represents a single spleen. WT: n=15 NKR-P1B KO: n=9, and Clr-b KO: n=13.

3. ***In vivo* expression of TGF-β by PMN- and M-MDSC is independent of NKR-P1B:Clr-b interactions.**

After demonstrating that MDSCs express Clr-b and are recruited into mammary tumors, we wanted to determine if their immunosuppressive activity is regulated by NKR-P1B:Clr-b interactions. TGF-β is a cell-bound immunosuppressive cytokine produced by MDSCs that can inhibit NK cells through cell-to-cell contact. It has been shown to induce NK cell anergy, impair cytotoxic activity of NK cells, and reduce activating NKG2D receptor expression. To examine if NKR-P1B:Clr-b interactions regulate TGF-β production by MDSCs, we analyzed TGF-β expression in MDSCs in spleens and mammary tumors from WT, NKR-P1B K/O, and Clr-b K/O mice.

In MMTV-PyVT mice, we found that both PMN- and M-MDSCs in all tumor sizes produced the TGF-β (Fig 4A). We saw no statistically significant differences in the proportion of TGF-β^+ MDSCs or TGF-β MFI values in the tumors or spleens of WT and NKR-P1B K/O mice (Fig 8A and C). Similarly, no statistically significant differences in the expression of TGF- β by MDSCs was observed in E0771 mammary tumors and spleens from WT, NKR-P1B K/O, and Clr-b K/O mice (Fig 8B and D). These results show that NKR-P1B:Clr-b interactions do not regulate TGB-β production by MDSCs *in vivo*.

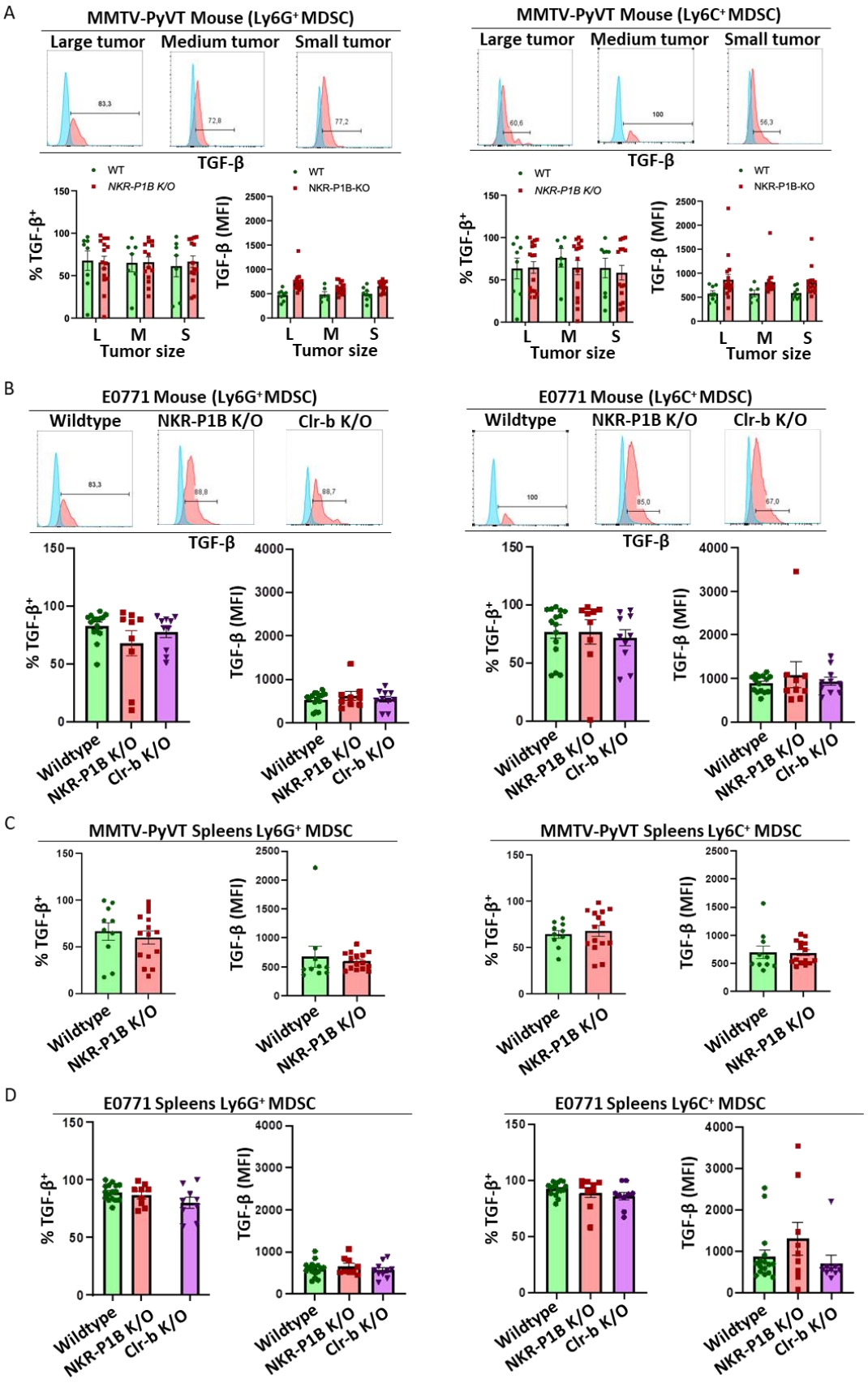
A
MMTV-PyVT Mouse (Ly6G+ MDSC)
Large tumor
Medium tumor
Small tumor
TGF-β
WT
NKR-P1B K/O
% TGF-β+
TGF-β (MFI)
NKR-P1B-KO
L
M
S
Tumor size
MMTV-PyVT Mouse (Ly6C+ MDSC)
B
E0771 Mouse (Ly6G+ MDSC)
Wildtype
NKR-P1B K/O
Clr-b K/O
E0771 Mouse (Ly6C+ MDSC)
C
MMTV-PyVT Spleens Ly6G+ MDSC
MMTV-PyVT Spleens Ly6C+ MDSC
D
E0771 Spleens Ly6G+ MDSC
E0771 Spleens Ly6C+ MDSC

Figure 8: TGF-β expression in MDSC subsets from tumors and spleens of mice bearing mammary tumors. Flow cytometry analysis of TGF-β expression in MDSCs from mammary tumors and spleens.

A, Histogram plots showing TGF-β staining (pink histograms) and isotype control staining (blue histogram) in PMN-MDSC (left panel) and M-MDSC (right panel) from large, medium, and small MMTV-PyVT tumors. Graphical representation of the proportions (%) and expression level (MFI values) of TGF-β^+ PMN-MDSC and M-MDSCs in WT and NKR-P1B K/O MMTV-PyVT tumors. MFI is normalized to isotype control. Each symbol represents one tumor. WT tumors: L, n=8; M, n=6; S, n=8. NKR-P1B K/O tumors: L, n=15; M, n=14; S, n=14. B, Histogram plots showing TGF-β staining (pink histograms) and isotype control staining (blue histogram) in PMN-MDSC (left panel) and M-MDSC (right panel) from E0771 tumors. Graphical representation of the proportions (%) and expression level (MFI values) of TGF-β^+ PMN-MDSC and M-MDSCs in E0771 tumors from WT, NKR-P1B K/O, and Clr-b K/O mice. MFI is normalized to isotype control. Each symbol represents a single tumor. WT: n=16 NKR-P1B K/O: n=9 Clr-b K/O: n=10. C, Graphical representation of the proportions (%) and expression level (MFI) of TGF-β^+ PMN-MDSC and M-MDSCs in spleens form WT and NKR-P1B K/O MMTV-PyVT mice. MFI is normalized to isotype control. Each symbol represents a single spleen. WT: n=10 NKR-P1B K/O: n=15. D, Graphical representation of the proportions (%) and expression level (MFI) of TGF-β^+ PMN-MDSC and M-MDSCs in spleens form E0771 tumor-bearing WT, NKR-P1B K/O, and Clr-b K/O mice. MFI is normalized to isotype control. Each symbol represents a single spleen. WT: n=16, NKR-P1B K/O n=9, Clr-b K/O: n=10.

4. Differences in inflammatory cytokine profiles reflects heterogeneity in the mouse mammary tumor models.

We detected high level of variability in MDSC frequencies within tumors from MMTV-PyVT mice possibly reflecting mammary tumor heterogeneity in this mouse model of breast cancer. As well, MDSC frequencies and subset distribution was different between MMTV-PYVT and E0771 mammary tumors, reflecting heterogeneity between different tumor models. MDSC differentiation and recruitment into tumors is influenced by the types of cytokines present in the TME. Therefore, we wanted to examine if there is a difference in the tumor cytokine profile between the mouse mammary tumor models and if it is influenced by NKR-P1B:Clr-b interactions in TME. We performed a flow cytometry-based multiplex cytokine assay comparing levels of 13 cytokines involved in inflammatory responses in MMTV-PyVT and E0771 mammary tumors from WT, NKR-P1B K/O, and Clr-b K/O mice.

We found that MMTV-PyVT and E0771 mammary tumors had distinct inflammatory cytokine profiles. IL-23 was present at higher concentrations in MMTV-PyVT tumors compare to E0771 tumors (Fig 9A). MCP-1, IL-6, GM-CSF, IFN-γ, IL-1α, IL-1β, IFN-β, and IL-12p70 were present at higher concentrations in E0771 tumors (Fig 9B) indicative of a highly inflammatory TME. Other cytokines, such as IL-10, IL-27, IL-17, and TNF-α were expressed at similar or very low concentration in both mammary tumor types (Fig 9C).

Next, we examined if there were any differences in inflammatory cytokine levels in mammary tumors from WT, NKR-P1B K/O, and Clr-b K/O mice. In the MMTV-PyVT tumors, cytokine levels were mostly unchanged between Wt and NKR-P1B K/O mice with a trend towards increased levels of IL-6, MCP-1, and IL-1α in tumors from NKR-P1B K/O mice (Fig 10A and B). IL-12, IL-17, and GM-CSF was undetectable in MMTV-PyVT tumors (Figure

10C). In the E0771 tumors, we saw a trend towards increased levels of TNF-α, GM-CSF, IL-1α, and MCP-1 in tumors from NKR-P1B K/O mice, but were not statistically significant (Fig 11A). No differences were observed in IL-6, IL-1β, IL-27, IL-10, IL12p70, and IFN-β between tumors from the WT and NKR-P1B K/O mice (Figure 11B), while IFN-γ, IL-23, and IL-17 appears to be expressed at lower levels in tumors from NKR-P1B K/O compared to WT mice (Fig 11C). The data together shows that while inflammatory cytokine expression profiles are distinct in each of the mouse mammary tumor models, NKR-P1B:Clr-b interactions in TME may have little to no influence on inflammatory cytokine production in tumors.

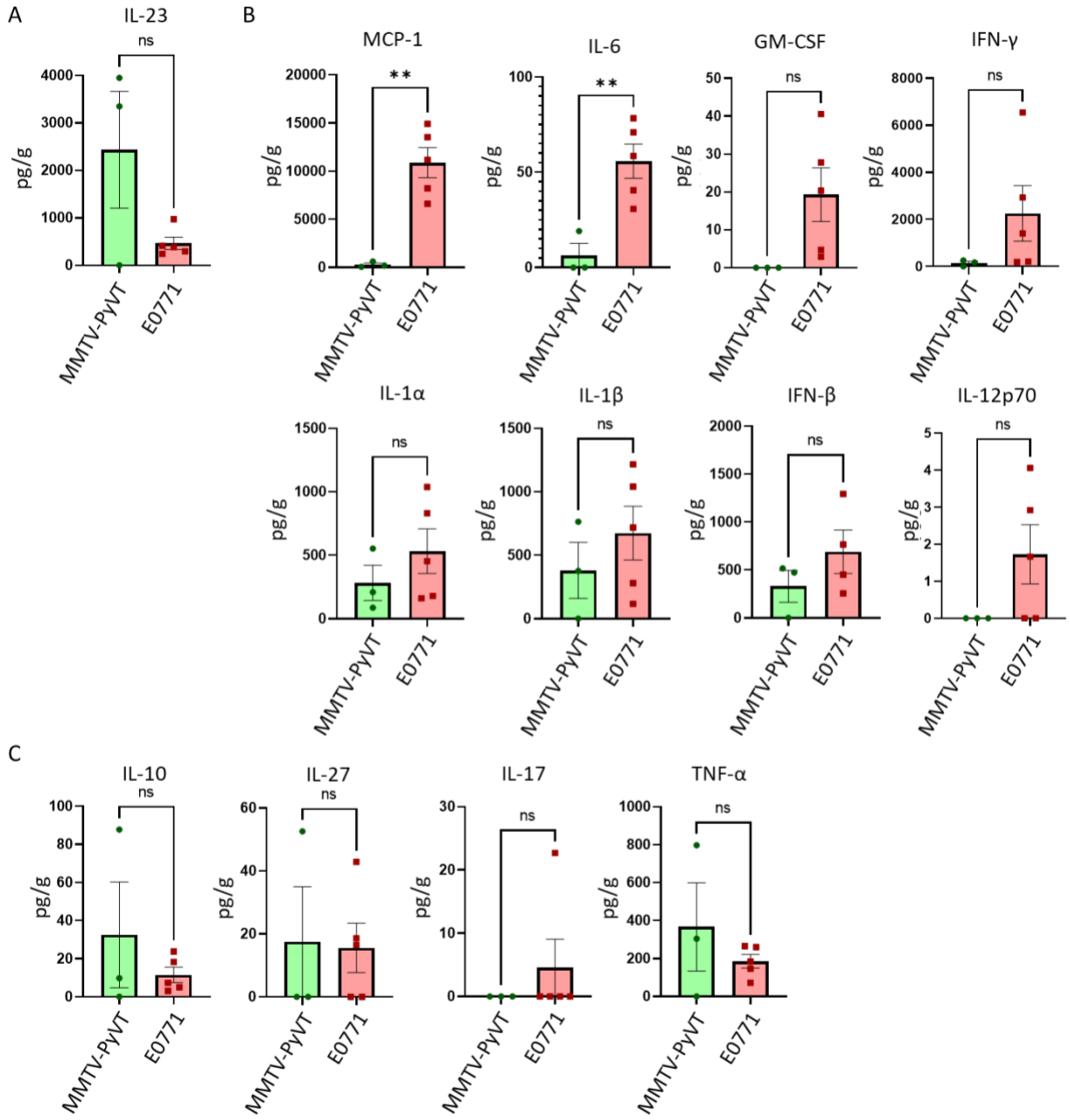

Figure 9: Heterogeneity in cytokine expression profile between MMTV-PyVT and E0771 mammary tumors. Cytokine levels were determined in tumors using a flow cytometry-based multiplex assay and concentrations are expressed as pictograms of cytokine per gram of tumor tissue.

A-C, Concentrations of IL-23 (A), MCP-1, IL-6, GM-CSF, IFN-γ, IL-1α, IL-1β, IFN-β, and IL-12p70 (B), and, IL-10, IL-27, IL-17, and TNF-α (C) in WT MMTV-PyVT and E0771 tumors.

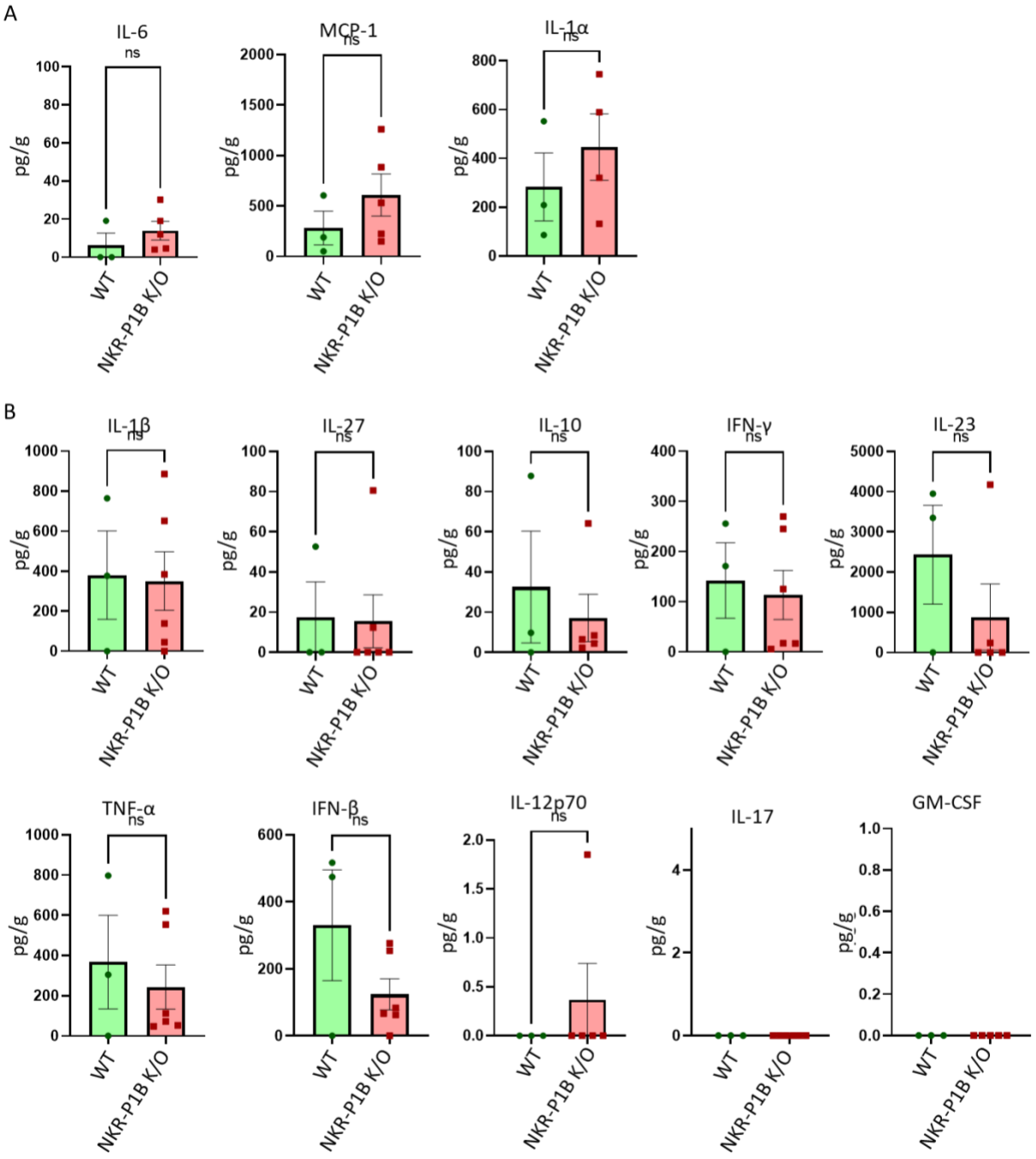

Figure 10: Comparison of tumor cytokine levels in MMTV-PyVT tumors from WT and NKR-P1B K/O mice. Cytokine levels were determined as described above and expressed as pg/g tumor tissue.

A and B, Concentrations of IL-26, MCP-1, and IL-1α, IL12p70, and GM-CSF (A), and IL-1β, IL-27, IL-10, IFN-γ, IL-23, TNF-α, IFN-α, and IL-17 (B) in MMVT-PyVT tumors from WT and NKR-P1B K/O mice.

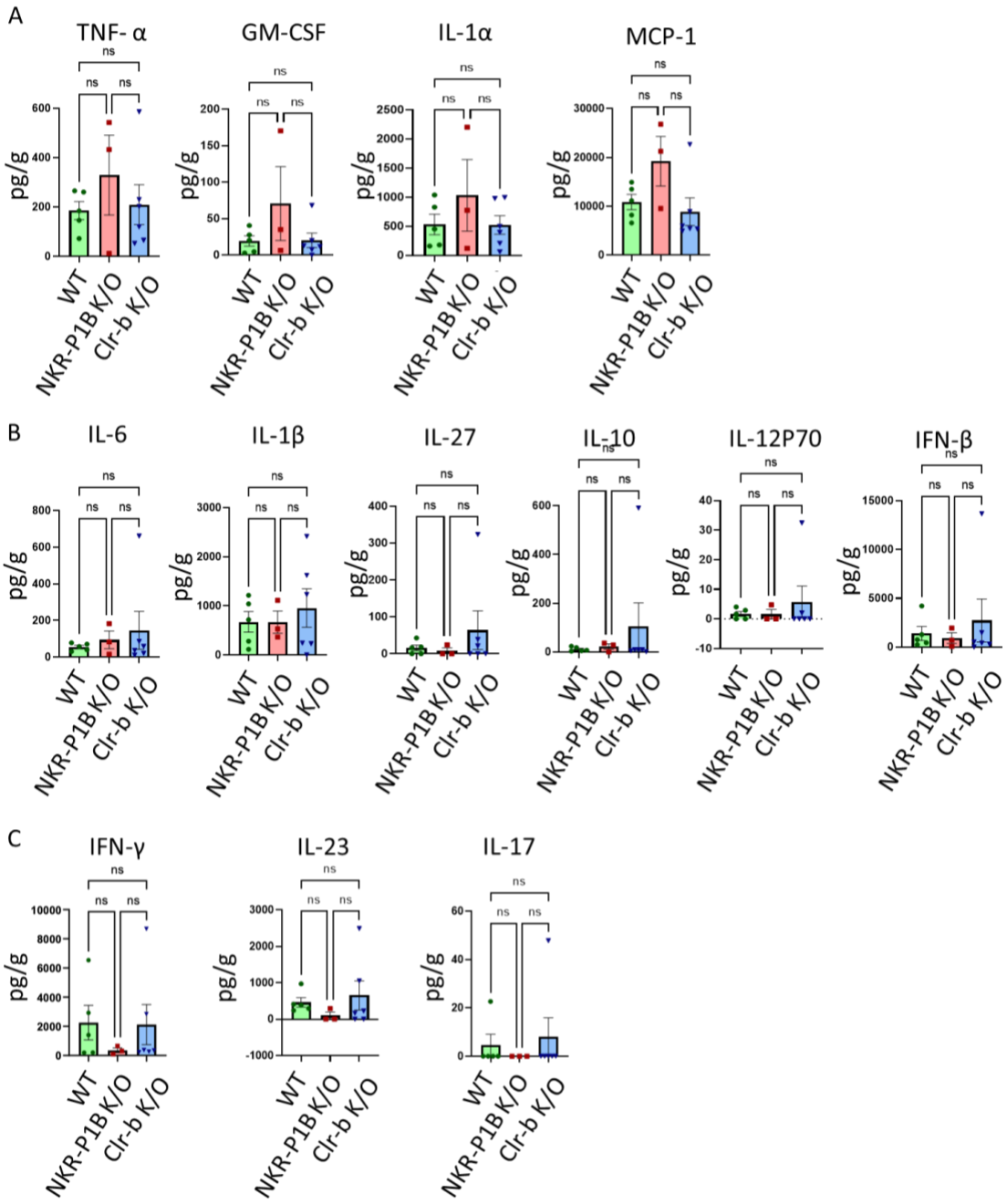

Figure 11: Comparison of cytokine levels in E0771 tumors from WT, NKR-P1B K/O, and Clr-b K/O mice. Cytokine levels were determined as described above and expressed as pg/g tumor tissue.

A-C, Concentrations of TNF-α, GM-CSF, IL-1α, and MCP-1 (A), IL-6, IL-1β, IL-27, IL-10, IL-12p70, and IFN-β (B), and IFN-γ, IL-23, and IL-17 (C) in E0771 tumors from WT, NKR-P1B K/O, and Clr-b K/O mice.

5. Clr-b K/O MDSCs are more effective in inducing NK cell phenotypic changes associated with NK cells dysfunction than WT MDSCs *in vitro*

To avoid TME affects on how MDSCs influence NK cell phenotype and function, we performed *in vitro* co-cultures of purified healthy splenic NK cells with MDSCs isolated from E0771 mammary tumor-bearing mice. Co-cultures of WT and NKR-P1B K/O NK cells with WT and Clr-b K/O MDSCs were performed to study the role of NKR-P1B:Clr-b interactions in MDSC:NK cell cross-talk.

Firstly, we examine expression of CD49a and CD49b molecules, which are cell-surface integrins expressed in mature NK cells. CD49b is expressed on all mature NK cells, while CD49a is mainly expressed on activated and tissue-resident NK cells. We and others have observed a loss of CD49a and CD49b expression in dysfunctional NK cells. In our co-culture's assays, both WT and Clr-b K/O MDSCs induced downregulation of CD49b expression equally in WT and NKR-P1B K/O NK cells (Fig 12 A-C). In contrast, only Clr-b K/O MDSCs induced downregulation of CD49a expression in in WT and NKR-P1B K/O NK cells (Fig 13 A-C), indicating that Clr-b could play a role in modulating MDSC responses in an NKR-P1B receptor-independent manner.

Next, we examined expression of EOMES transcription factor in NK cells co-cultured with MDSCs. EOMES is a T-box transcription factor that is necessary for the maturation of NK cells. EOMES is expressed throughout the life of an NK cell and is essential for its function. NK cells that lose expression of EOMES have dysfunctional NK cell responses. Four subsets of NK cells can be identified based on the expression of EOMES and CD49a after activation: $EOMES^+$ $CD49a^-$, $EOMES^+$ $CD49a^+$, $EOMES^-$ $CD49a^+$, and $EOMES^-$ $CD49a^-$. We and others have shown

that loss of EOMES expression correlates with NK cell dysfunction. We found that WT MDSC could induce loss of EOMES expression in NK cells resulting in a large population of $EOMES^{-}$ $CD49a^{+}$ NK cells compared to NK cells alone (Fig 14 B-C). We also found that Clr-b K/O MDSC caused a much more dramatic loss of EOMES expression in NK cells compared to WT EOMES with a significant population of NK cells exhibiting an $EOMES^{-}$ $CD49a^{-}$ phenotype (Fig 14 D-F). These effects where independent of NKR-P1B receptor expression in NK cells (Fig 14 D-F).

Finally, we examined expression of Lag-3, an inhibitory checkpoint receptor, on NK cells co-cultured with MDSCs. When NK cells become activated, Lag-3 is upregulated so that NK cells do not become overly activated. Lag-3 is also a marker for immune exhaustion. We found that WT MDSC, and Clr-b K/O MDSCs at a lesser degree, caused an increase in the percentage of NK cells expressing Lag-3 (Fig 15B and 15C). This effect was not different in WT and NKR-P1B K/O NK cell, meaning that Lag-3 expression induced by MDSCs is independent of the NKR-P1B receptor expression.

Together, the results show that MDSCs can induce phenotypic changes in NK cells resembling a dysfunctional NK cell phenotype. Interestingly, Clr-b KO MDSCs had a more profound effect on NK cells than WT MDSC, which was independent of NKR-P1B receptor expression on NK cells.

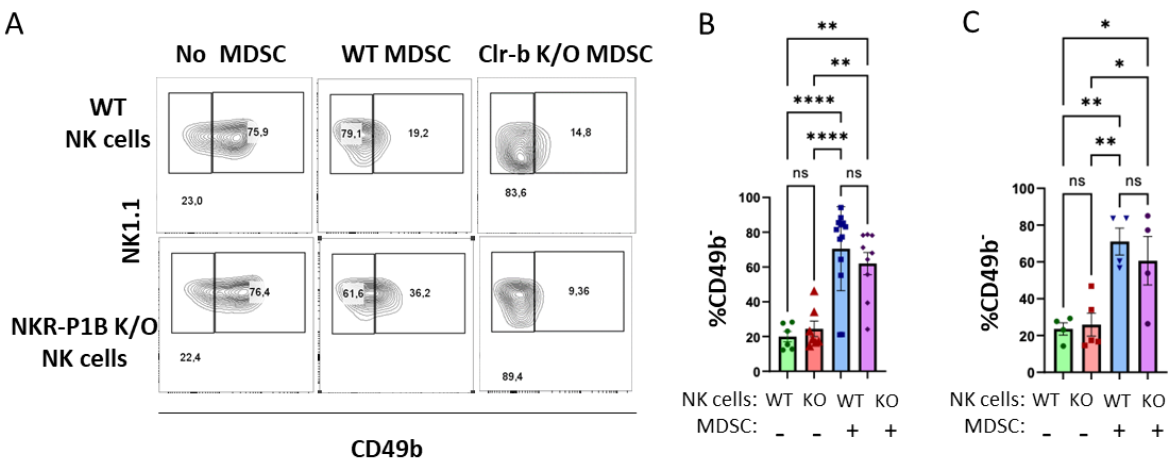

Figure 12: MDSC cause downregulation of the CD49b expression in NK cells.

A, Flow cytometry analysis of CD49b expression on WT and NKR-P1B K/O NK cells ($NK1.1^+$) co-cultured with WT and Clr-b K/O MDSCs isolated form E0771 mammary tumor-bearing mice. B and C, graphical representation of the increased frequencies (%) of WT and NKR-P1B K/O $CD49b^-$ NK cells after co-cultured with WT (B) and Clr-b K/O (C) MDSCs. Each symbol represents data from a single NK cell:MDSC co-culture. Bars represent mean±SEM. Statistical analysis was performed by two-way ANOVA. *: $p<0.05$, **: $p<0.005$, ****: $p<0.0001$.

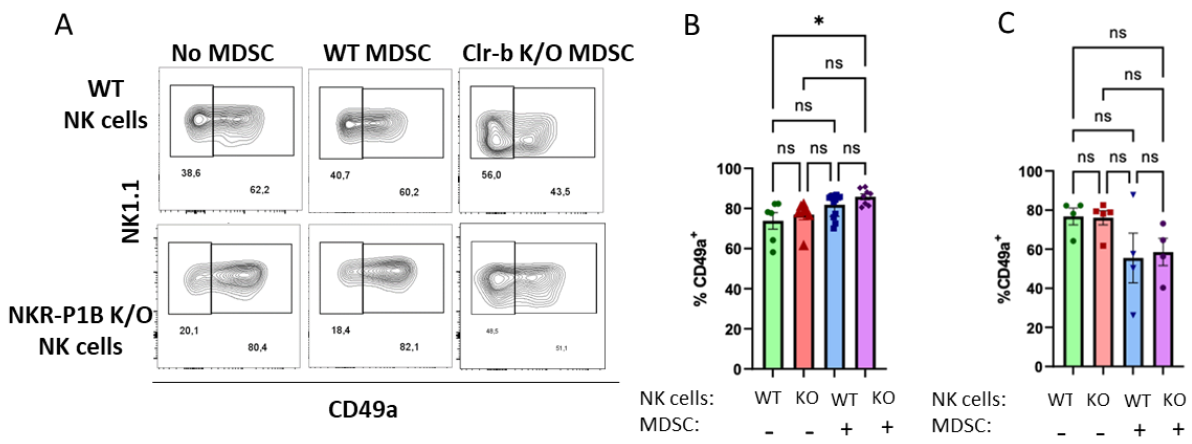

Figure 13: CD49a downregulation in NK cells induced by Clr-b K/O but not WT MDSC.

A, Flow cytometry analysis of CD49a expression on WT and NKR-P1B K/O NK cells ($NK1.1^+$) co-cultured with WT and Clr-b K/O MDSCs isolated form E0771 mammary tumor-bearing mice. B and C, Graphical representation of the frequencies (%) of WT and NKR-P1B K/O $CD49a^+$ NK cells after co-cultured with WT (B) and Clr-b K/O (C) MDSCs. Each symbol represents data from a single NK cell:MDSC co-culture. Bars represent mean±SEM. Statistical analysis was performed by two-way ANOVA. *: $p<0.05$.

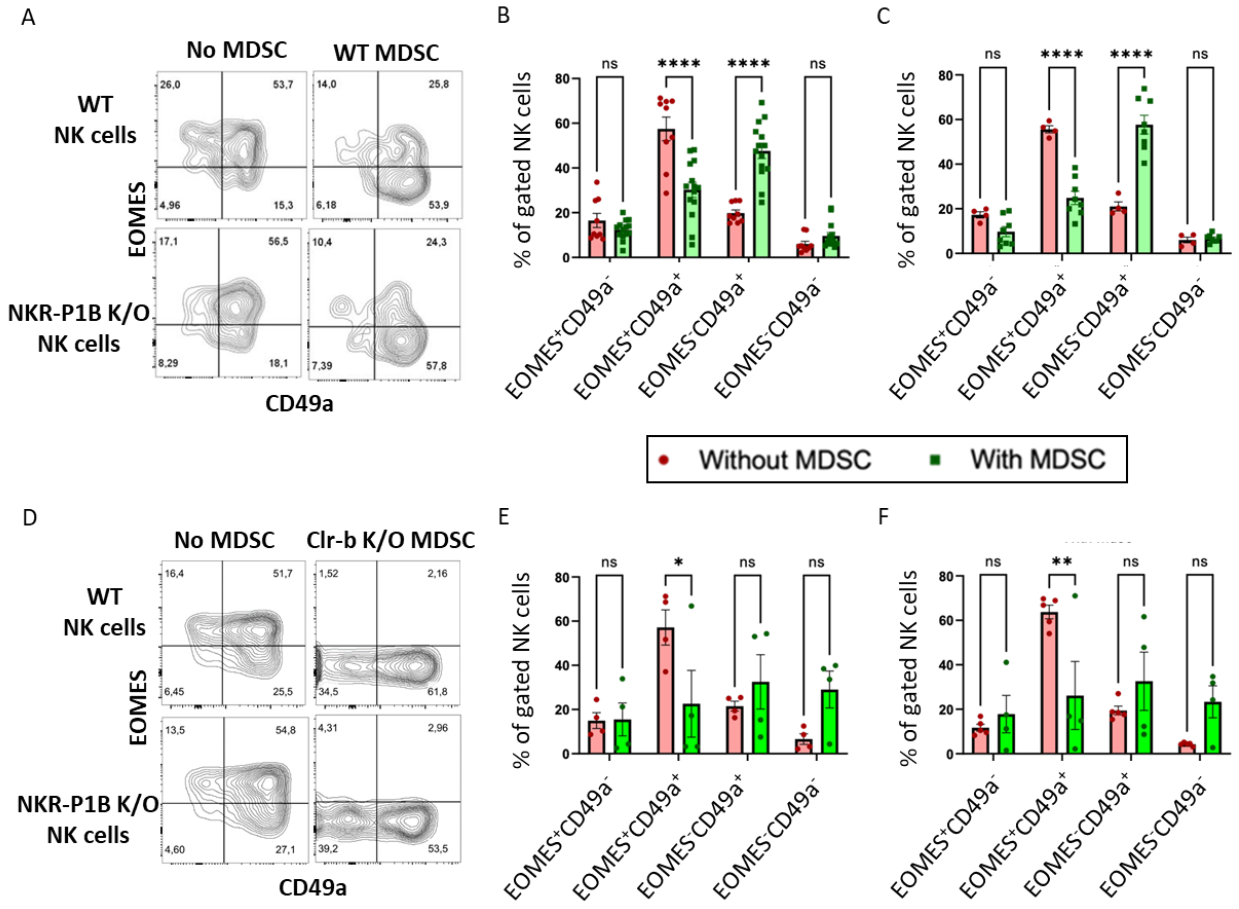

Figure 14: NK cells lose expression of EOMES in the presence of WT and Clr-b K/O MDSC.

A, Flow cytometry analysis of EOMES and CD49a expression in WT and NKR-P1B K/O NK cells with and without WT MDSC. Four NK cell subsets are identified: EOMES$^+$ CD49a$^-$, EOMES$^+$ CD49a$^+$, EOMES$^-$ CD49a$^+$, and EOMES$^-$ CD49a$^-$. B and C, Graphical representation of the frequencies of WT (B) and NKR-P1B K/O (C) NK cell subsets co-cultured with WT MDSCs. D, Flow cytometry analysis of EOMES and CD49a expression in WT and NKR-P1B K/O NK cells with and without Clr-b K/O MDSC. E and F, Graphical representation of the frequencies of WT (B) and NKR-P1B K/O (C) NK cell subsets co-cultured with WT MDSCs. Each symbol represents data from a single NK cell:MDSC co-culture. Bars represent mean±SEM. Statistical analysis was performed by two-way ANOVA. *: $p<0.05$, **: $p<0.005$, ****: $p<0.0001$.

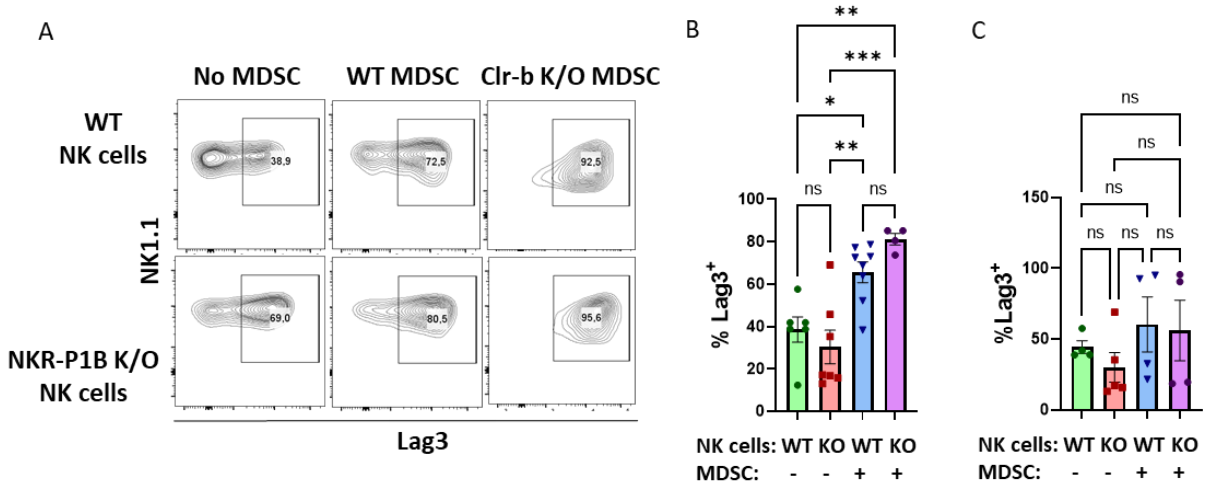

Figure 15: MDSCs induce expression of Lag-3 in NK cells.

A, Flow cytometry analysis of Lag-3 expression on WT and NKR-P1B K/O NK cells ($NK1.1^+$) co-cultured with WT and Clr-b K/O MDSCs isolated form E0771 mammary tumor-bearing mice. B and C, Graphical representation of the frequencies (%) of WT and NKR-P1B K/O Lag-3^+ NK cells when co-cultured with WT (B) and Clr-b K/O (C) MDSCs. Each symbol represents data from a single NK cell:MDSC co-culture. Bars represent mean±SEM. Statistical analysis was performed by two-way ANOVA. ****: $p<0.0001$.

6. MDSCs inhibit production of IFN- γ by NK cells.

One of the main functions of NK cells, when they become activated, is to produce IFN-γ. To determine the effect of MDSCs on NK cell function, we examined their IFN-γ production ability after activation. To do this, NK cells were co-cultured for 5 days with MDSC. On day 5, we stimulated NK cells with PMA and Ionomycin for 4 hours and stained them with an anti-IFN-γ antibody. This preliminary experiment showed that in the presence of MDSCs, fewer WT and NKR-P1B K/O NK cells produced IFN-γ (Fig 16A), indicating that NK cells lose their ability to produce IFN-γ in the presence of MDSC.

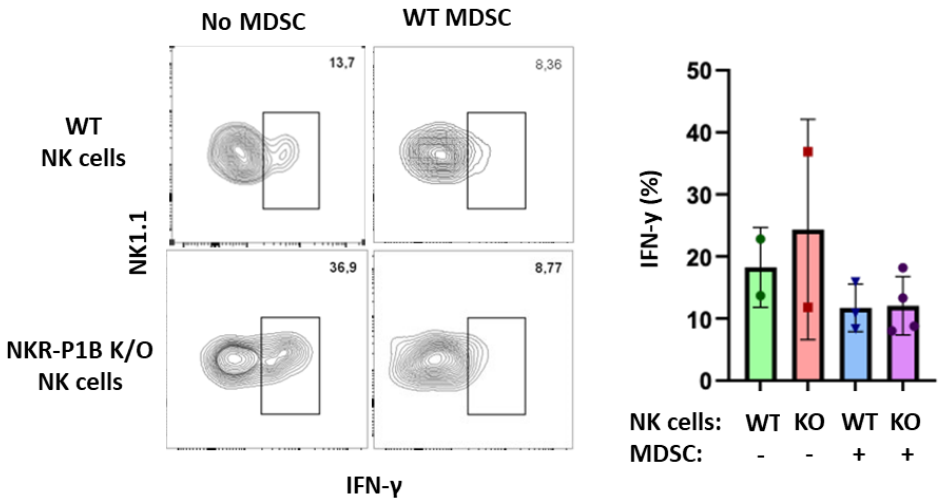

Figure 16: Wildtype MDSCs inhibit IFN-γ production from WT and NKR-P1B K/O NK cells. Flow cytometry analysis of IFN-γ production by WT and NKR-P1B K/O NK cells after stimulation with PMA and ionomycin in the presence or absence of WT MDSC. Graphical representation of the frequency (%) of total NK cells that produce IFN-γ. Each symbol representsdata from a single NK:MDSC co-culture. Bars represent mean±SEM.

CHAPTER 4: DISCUSSION

The NKR-P1B:Clr-b recognition axis has been found to play a role in mammary tumor immunosurveillance in the MMTV-PyVT mouse model mediated by NK cells [77]. NKR-P1B K/O NK cells have a defective 'missing-self' response against Clr-b-deficient target cells. Since MMTV-PyVT mammary tumors are composed of a large proportion of tumor cells lacking Clr-b expression, NKR-P1B K/O mice develop MMTV-PyVT tumors earlier than WT mice [77] . In the E0771 mammary tumor model, where all tumor cells express Clr-b, NKR-P1B K/O mice are more resistant to mammary tumor development since tumor cells are unable to inhibit NK cells through NKR-P1B:Clr-b interactions (unpublished data). Clr-b is also expressed on TILs (Fig 1), which include many cell types with immunosuppressive functions, but their role in inhibiting NK cells via NKR-P1B receptor is not known.

In this current study, we used WT, and NKR-P1B K/O MMTV-PyVT mice, as well as E0771 mammary tumors induced in WT, NKR-P1B K/O, and Clr-b K/O mice to determine if MDSC express Clr-b and can directly inhibit NK cell functions causing them to no longer be effective at mammary tumor immunosurveillance. MDSC are known inhibitors for NK cell activity and cytotoxic function [93]. In mice, MDSC can suppress NK cells through cell-to-cell contacts, involving cell-bound TGF-β. MDSCs can aid in tumor growth by inducing NK cell anergy, impaired cytotoxic functions, and inhibition of IFN-γ production [93]. Here we have shown that MDSCs in both mammary tumors and in spleens express Clr-b, therefore, they can potentially interact with NKR-P1B receptor on NK cells to inhibit their functions (Fig 6A-6D).

Previous studies have shown that tumors recruit MDSC to inhibit NK cells and other immune cells from targeting a tumor for destruction [94]. It has also been seen that different tumor

types can recruit MDSC at different rates [95]. We wanted to examine if MMTV-PyVT and E0771 tumors recruit MDSC, and if NKR-P1B receptor or its Clr-b ligand played a role in MDSC recruitment into mammary tumors. Each subset of MDSC is recruited into the tumor in different ways. M-MDSCs are often recruited into tumors at a higher rate than PMN-MDSCs, and they have a higher immunosuppressive capabilities [96]. PMN-MDSCs are more specific in their immunosuppressive capabilities and are often recruited into the tumor in lower numbers, but largely remain in the spleens and other lymphoid organs [96]. Our results show that both M-MDSC and PMN-MDSC are recruited in to MMTV-PyVT and E0771 tumors, but at very different rates (Fig 7A and 7C). Our results showed no difference in the frequency of MDSC recruited into mammary tumors between WT, NKR-P1B K/O, and Clr-b K/O mice, showing that MDSC recruitment into tumors is independent of the NKR-P1B:Clr-b axis. In our work, we found that MDSCs are recruited into E0771 tumors at much higher rate than in MMTV-PyVT tumors. In E0771 tumors M-MDSCs were the predominant MDSC subset, while PMN-MDSC were present in the spleens (Fig 7A and 7B). In the MMTV-PyVT mouse model, where the recruitment of MDSC was much less, there was no difference in M- and PMN-MDSC frequencies in tumors; both were recruited at very low levels. Again, PMN-MDSC were largely present in the spleens. These differences remained consistent across the mouse genotypes: WT, NKR-P1B K/O, and Clr-b K/O mice.

One of the main mechanisms that MDSC use to inhibit NK cells is by producing TGF-β, which can induce energy and impair cytotoxic capabilities in NK cells [93]. Therefore, we determined if NKR-P1B:Clr-b interactions have any role in TGF-β production by MDSCs in the mammary tumors. We found that both PMN- and M-MDSC from mammary tumors and spleens stain positive for TGF-β, however, no difference were observed between MDSCs from WT,

NKR-P1B K/O, and Clr-b K/O tumors, meaning that this is independent of the NKR-P1B:Clr-b axis (Fig 8A-D). Unfortunately, we were not able to assess other immunosuppressive functions of MDSCs, such as expression of arginase I and iNOS enzymes, and production of immunosuppressive IL-10 cytokine due to technical difficulties.

The differences in MDSC recruitment in E0771 and MMTV-PyVT described above, as well as differences in NK cell responses in the two mammary tumor models (unpublished data) could reflect the heterogeneity in the TME of mammary tumors. We observed large variability in MDSC phenotype and recruitment in MMTV-PyVT mammary tumors, which is demonstrated by large error bars in Figures 2-4, compared to E0771 tumors, reflecting the heterogeneous nature of oncogene-induced mammary tumors in MMTV-PyVT mice. In contrast, E0771 tumors are induced by injection of E0771 tumor cell line into mammary glands and are expected to be more homogenous, and hence lesser variability is seen in data from this tumor type.

Immune functions in TME are modulated by the inflammatory environment in tumors. Therefore, we examined whether the pro-inflammatory cytokine profiles of mammary tumors could account for the differences in MDSC recruitment and other immune functions in each the mammary tumor models. When comparing cytokine profiles, we observed clear differences between MMTV-PyVT and E0771 tumors (Fig 9A-C). E0771 tumors were found to be rich in pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, MCP-1, TGF-β, IFN-β, and IFN-γ. In contrast, MMTV-PyVT tumors contained higher levels of IL-23, which has been shown to inhibit NK cell responses. The relationship between the distinct cytokine profiles in TME and MDSC recruitment and function, as well as NK cell responses remain to be determined. Unfortunately, due to high variability in cytokine levels between tumors and small number of

samples tested, we could not draw any conclusion from differences in cytokine profiles between tumors from WT, NKR-P1B K/O, and Clr-b K/O mice.

Due to the complex nature of the TME, it is difficult to study direct interactions between MDSCs and NK cells *in vivo*. Therefore, we used an *in vitro* co-culture system to examine how MDSCs impact NK cell phenotype and function, and whether this involves NKR-P1B receptor and Clr-b ligand interactions. It is more feasible to isolate MDSCs from spleens than from tumors, therefore, we used spleen MDSCs from mammary tumor-bearing WT and Clr-b K/O mice to co-culture with NK cells isolated from spleens of healthy WT and NKR-P1B K/O mice. All mature NK cells express CD49b [(97)]. In contrast, CD49a is a marker for activated mature NK cells [(97)]. We found that in the presence of MDSC, there was a loss of both CD49a and CD49b expression in NK cells. Similarly, EOMES, which is a transcription factor needed for NK cell maturation and function [(76)] was downregulated in NK cells when co-cultured with MDSCs. These phenotypic changes in NK cells have been shown to be associated with reduced NK cell functions [(76)] [(77)]. Additionally, we observed that Clr-b K/O MDSCs induced a more potent loss of EOMES expression in NK cells than the WT MDSCs, suggesting an inhibitory role of Clr-b in MDSC function. Finally, we examined expression of inhibitory checkpoint receptors known to be expressed on dysfunctional immune cells in tumors and found that Lag-3 expression was upregulated in the presence of MDSCs compared to activation alone. Lag-3 upregulation along with the loss of EOMES, CD49a and CD49b expression, indicated that that MDSCs may induce a dysfunctional phenotype and cause NK cells to lose their function. Future studies should focus on revealing whether these phenotypic changes in the presence of MDSCs is associated with immune dysfunction in NK cells, such as reduced IFN-γ production and cytotoxic activity. While Clr-b K/O MDSCs appear to be more potent in inducing phenotypic changes in NK cells

described above, these changes occurred at similar levels in WT and NKR-P1B K/O NK cells, indicating that the receptor may not play a role in this process.

This study has provided details on the role of MDSC in modulating NK cell responses. It has also revealed differences between mouse tumor models that must be considered for experimental design and interpretation of results. There has been some limitations and challenges with the use of animal models of breast cancer. A major challenge with using MMTV-PyVT mouse model in this study was the heterogeneity in mammary tumors. Tumors can develop at multiple locations in mammary glands, at different times, and may also be composed of different mammary tissue types in MMTV-PyVT mice. Although, this is relevant as it more closely resembles breast cancer in humans, but it also adds variability in data from different mice and also between tumors from the same mouse. This heterogeneity made it difficult to accurately assess smaller differences and trends in data, requiring larger sample sizes. In the E0771 mouse model, the tumors are more homogenous since they are all generated from the injection of the same cell type, however, it may not be as physiologically relevant as the MMTV-PyVT mouse model. We also encountered differences between the two mammary tumor models in MDSC recruitment and distribution, as well as, pro-inflammatory cytokine profiles, that could differentially impact anti-cancer immune responses. Future experiments should consider these differences when evaluating anti-cancer immune response in mammary tumors from different mouse models. A limitation with our cytokine assay was the small sample size. Although we observed differences in cytokine levels between different tumor models and across different mouse genotypes, the small sample size and variability between tumors prevented differences to reach statistical significance. Future experiments should aim at increasing sample size for these assays. The TME is an extremely complex network of cellular and acellular components, making

if difficult to study specific cellular interactions, such as between MDSC and NK cells *in vivo*. Future studies can be performed with specific depletion of MDSC *in vivo* by injecting mice with anti-Gr-1 antibody. Analysis of anti-tumor immune response in tumors from these mice will provide a better understanding of how MDSCs affect NK cell responses in mammary tumors. Another way of studying MDSC and NK cell interactions specifically is to separate them from other components of the TME. This was done in vitro in MDSC:NK cell co-cultures. A limiting factor in these experiments was the number of MDSCs and NK cells that we could obtain from each mouse. Therefore, we begun optimizing an *in vitro* culture system to derive MDSCs from mouse bone marrow hematopoietic cells using GM-CSF and G-CSF (Data not shown). This system is capable of generating large numbers of MDSCs that will allow us to perform assay replicates with and without transwell chambers to distinguish between direct cell-to-cell interactions and the involvement of soluble factors produced by MDSCs. MDSCs also possess multiple immunosuppressive mechanisms that could mask direct affects of receptor:ligand interactions on NK cells functions. Future co-culture experiments can be performed to also include commercially available inhibitors for these immunosuppressive mechanisms, such as inhibitors of arginase and iNOS enzymes, antibodies to neutralize TGF-β, and catalase and superoxide dismutase enzymes to remove ROS generated in the co-cultures. The use of inhibitors will also be helpful in determine which specific function of MDSC is responsible for inducing NK cell dysfunction. Finally, future experiments should also include functional assay and further characterization of Clr-b K/O MDSC to explore the function of Clr-b protein in MDSCs.

In conclusion, our data suggest that MDSCs play a pivotal in modulating NK cell responses in mammary tumors. MDSCs may employ multiple mechanisms to inhibit NK cell responses (Fig 17). Through the expression of Clr-b, MDSCs could potentially engage NKR-P1B

receptor to inhibit NK cells. Alternatively, Clr-b may play a role in modulating MDSC functions. Our data provides evidence that MDSC induce change in NK cell phenotype resembling dysfunctional NK cells, which is elevated when Clr-b expression in MDSC is disrupted. However, this appears to be independent of NKR-P1B receptor expression in NK cells. Therefore, Clr-b may have an inhibitory function in MDSCs. Our data also shows differences in immune infiltration, MDSC numbers and distribution, and differences in the cytokine levels in different mouse mammary tumor models, that may influence the nature of the immune responses in each tumor model. This study advances our understanding of the immunosuppressive TME and modulation of NK cell responses via NKR-P1B:Clr-b axis to inform future studies targeting its human counterpart NKR-P1A receptor for cancer immunotherapy.

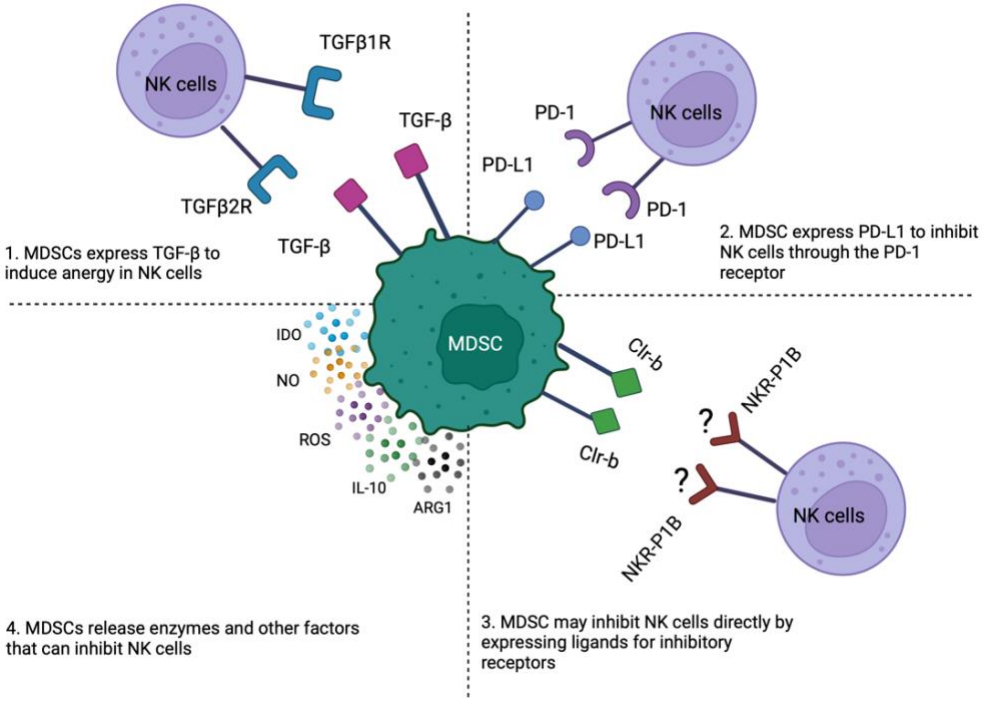

Figure 17: Mechanisms of inhibition of NK cells by MDSCs

(1) MDSCs express TGF-β which interacts with receptors TGFβ1R and TGFβ2R on NK cells to induce NK cell anergy. (2) MDSCs express PD-L1 the ligand for checkpoint inhibitory receptor PD-1 on NK cells to induce NK cell inhibition. (3) MDSCs express ligands for inhibitory NK cell receptors that could mediate NK cell inhibition. (4) MDSCs release enzymes and other factors such as IDO, NO, ROS, IL-10, and ARG1 to inhibit NK cell functions.

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