THE IMPACT OF MACROPHAGE POLARITY AND THE TUMOR MICROENVIRONMENT ON NK CELL PHENOTYPE AND FUNCTION

THE IMPACT OF MACROPHAGE POLARITY AND THE TUMOR MICROENVIRONMENT ON NK CELL PHENOTYPE AND FUNCTION

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ABSTRACT

NK cells play a pivotal role in tumor rejection; however, once present in the tumor microenvironment, they are characterized by decreased cytotoxicity and reduced expression of activating receptors. The mechanisms governing the inactivation of NK cells within tumors remain poorly understood. Since tumor associated macrophages (TAMs) are a highly abundant and suppressive cell type within tumors, we hypothesized that they are capable of altering the function of NK cells. Following the co-culture of alternatively activated macrophages (M2) or TAMs with NK cells we observed that the expression of the cytotoxic marker CD27 on NK cells was down-regulated as well as the ability of these cells to kill YAC-1 cells in a killing assay. We have demonstrated that the mechanism by which M2 cells inhibit NK cells is TGF-β dependent. Notably, the developmental stage of NK cells after interaction with TAMs was altered and the NK cells became phenoytpically mature and potentially exhausted ($CD27^{low}CD11b^{high}$). This prompted our interest in examining the developmental stage of NK cells from polyoma MT antigen (pyMT) transgenic mouse (MMTV-pMT) breast tumors. Interestingly, in contrast to the *in vitro* results, we have shown that NK cells isolated from pyMT tumors are developmentally immature; however maintain their maturity within the spleen. Their immature phenotype correlates well with their decreased expression of perforin and granzyme as well as NKp46. Future studies should investigate whether the tumor recruits immature NK cells or if the microenvironment alters the development of these cells once they arrive at the transformed site. In order to assess the plasticity of intratumoral NK cells, we treated pyMT tumors with IL-12 and anti-TGF-β in an effort to modify the

tumor microenvironment and shift the immune response towards tumor rejection. In the subcutaneous pyMT model, combination treatment was associated with extended survival compared to PBS and IL-12 alone. Assessing the phenotype of NK cells after combination treatment in the spontaneous pyMT model has demonstrated that there is an increase in the developmental status of NK cells and they possess the ability to change phenotype quickly (within one week). Both our *in vitro* studies with TAMs and our *in vivo* developmental studies using the pyMT model demonstrate that NK cells are altered by their surroundings. A better understanding of how NK cells are modified by the tumor microenvironment will help to develop strategies aimed at bolstering immune responses against tumors.

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LIST OF ABBREVIATIONS

ADCC = antibody dependent cellular cytotoxicity
AML = acute myeloid leukemia
ANOVA = analysis of variance
CFSE = carboxyfluorescein succinimidyl ester
CML = chronic mylogenous leukemia
CTLs = cytotoxic T lymphocytes
DC = dendritic cell
ELISA = enzyme linked immunosorbent assay
EpCam = epithelial cell adhesion molecule
FACS = fluorescent activated cell sorting
FMO = fluorescence minus one
FSC = forward scatter
GM-CSF = granulocyte macrophage colony-stimulating factor
H&E = hematoxylin and eosin stain
Her2 = human epidermal growth factor receptor 2
HRP = horseradish peroxidise
HSC = hematopoietic stem cells
i.p. = intraperitoneal
IDO = indoleamine 2 3 dioxygenase
IFN = interferon
IL = interleukin
IL-1ra = IL-1 receptor antagonist
KIR = killer immunoglobulin-like receptor
KLRG1 = killer-cell lectin-like receptor subfamily G, member 1
LAMP-1 = lysosomal-associated membrane protein-1
LPS = lipopolysaccharide
LTR = long terminal repeat
M1 macrophage = classically activated macriohage
M2 macrophage = alternatively activated macrophage
M-CSF = macrophage-colony stimulating factor
MDSC = myeloid derived suppressor cells
MHC = major histocompatability complex
MICA = MHC class I-chain related proteins
MIP-1= macrophage inflammatory protein-1
MMR = macrophage mannose receptor
MMTV = mouse mammary tumor virus
NCR = natural cytotoxicity receptor

NK cell = natural killer cell
NO = nitric oxide
NSCLC = non-small cell lung carcinoma
PAMP = pathogen associated molecular pattern
PBMC = peripheral-blood mononuclear cells
PD-L1 = programmed death ligand-1
PFA = paraformaldahyde
PGE_{2} = prostaglandin E2
PI = propidium iodide
PI3K = PI3 kinase
pyMT = polyoma MT antigen
qPCR = quantitative polymerase chain reaction
RAE -1 = retinoic acid early inducible-1 protein
RCC = renal cell carcinoma
ROI = reactive oxygen intermediates
SEM = standard error of the mean
SSC = side scatter
TAM = tumor associated macrophage
TG = transgenic
TGF-β = transforming growth factor-β
T_H1 = T helper 1
T_H2 = T helper 2
TIL = tumor infiltrating lymphocytes
TLR = toll-like receptor
TNF = tumor necrosis factor
TRAIL = TNF-related apoptosis-inducing ligand
VEGF = vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1 IMMUNE CELLS AND THE TUMOR MICROENVIRONMENT

The description of a tumor as a complex organ with a complex microenvironment has superseded the view that it is merely composed of homogenous transformed cells. It is now generally accepted that the many components of the tumor shape its biology. This includes cancer cells, but also cancer stem cells, endothelial cells, cancer associated fibroblasts, and immune cells (Hanahan and Weinberg 2011). Notably, cells of the innate and adaptive immune system are key components of this tumor microenvironment.

The idea that the immune system can control tumor growth gained attention with the proposition of the theory of cancer immunosurveillance (Dunn, Bruce et al. 2002). The theory argues that cancerous cells form on a regular basis within the body, however, these malignant cells can be recognized and eliminated by cells of the innate and adaptive immune system (Sengupta, MacFie et al. 2010). Evidence supporting the theory stemmed from animal models deficient in various components of the immune system. It was found that animals deficient in Natural Killer (NK) cells were more likely to develop spontaneous tumors and had higher growth rates of transplantable tumors in comparison to immunocompetent animals (Ostrand-Rosenberg 2008). Clinical evidence has also shown that there is a positive correlation between NK cell infiltration and survival in several cancer types (Ishigami, Natsugoe et al. 2000; Villegas, Coca et al. 2002). In contrast, animals deficient in macrophages were found to have lower rates of tumor development and decreased metastasis (Lin, Nguyen et al. 2001). Interestingly, these

immune cells have opposing roles in the context of tumor progression. While the NK cells are involved with tumor destruction the macrophages seem to promote tumor growth. This idea is analogous to the contrasting roles of the immune system: to kill malignant or pathogen infected cells and to subsequently invoke wound healing and clean up cellular debris (Hanahan and Weinberg 2011). When cells important in wound healing are recruited to the tumor, they may have detrimental effects for the host.

In addition to the recruitment of both killer and healer immune cells to the tumor, even those with purpose to kill may become altered or suppressed at the transformed site. This idea has become one of the emerging hallmarks of cancer proposed by Weinberg and Hanahan in 2011: evading immune destruction (Hanahan and Weinberg 2011). Although the immune system may initially eliminate transformed cells, some cancer cells may develop ways to escape immune attack (Dunn, Old et al. 2004). For instance, cancer cells may release immunosuppressive factors (such as TGF-β) in response to an influx of cytotoxic T lymphocytes (CTLs) and NK cells, thereby inhibiting their functions (Yang, Pang et al. 2010). Simultaneously, the tumor can recruit suppressive inflammatory mediators (such as myeloid derived suppressor cells) which act to suppress cytotoxic immune cells (Ostrand-Rosenberg and Sinha 2009). These observations also apply to the cancer immunoediting concept. During the final escape phase, cancer cells capable of circumventing immune attack expand uncontrollably in contrast to those not adapted to inhibit the immune response (Dunn, Bruce et al. 2002). A detailed understanding of the suppressive mechanisms imposed by the tumor microenvironment in the inhibition of cytotoxic immune cells is essential before approving their use in cancer therapies.

1.2 NATURAL KILLER (NK) BIOLOGY

1.2.1 Overview

NK cells, characterized as granular lymphocytes, are known for their ability to lyse virally infected or transformed cells without the recognition of a specific antigen (Srivastava, Lundqvist et al. 2008). They were first discovered based on this premise in 1975 and have since been famous for their involvement in the anti-tumor immune response (Kiessling, Klein et al. 1975). They encompass 10-15% of circulating lymphocytes and are able to extravasate into peripheral tissues that contain malignant or pathogen-infected cells (Fogler, Volker et al. 1996) (Glas, Franksson et al. 2000). Once in peripheral tissues, there is extensive evidence that they are important in tumor destruction. Their cytotoxic potential has been observed in transgenic models that lack NK cells or models which lack NK cell activation receptors (Kim, Iizuka et al. 2000). In spontaneous epithelial and lymphoid malignancies, absence of the NK cell activating receptor NKG2D resulted in defective tumor surveillance (Guerra, Tan et al. 2008). In the Her2/neu model of spontaneous mammary carcinoma, it was shown that NK cells delay tumor formation in a perforin dependent manner (Street, Zerafa et al. 2007). These studies have revealed the importance of NK cells in tumor suppression.

Their description as a large granular lymphocyte is fitting as NK cells utilize their granularity to perform their role as professional killers. This is conducted through the release of cytotoxic granules containing perforin and granzyme B, which perforate the target cell and cause apoptosis (Fehniger, Cai et al. 2007). NK cells also express CD16, a low affinity Fc receptor, allowing them to kill antibody coated cells through antibody

dependent cellular cytotoxicity (ADCC). Furthermore, NK cells express death receptor ligands on their surface such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand which bind to receptors on tumor cells and cause cell death (Takeda, Smyth et al. 2001). Indirect killing can occur through the secretion of cytokines such as IFN-γ, which promote the activation of DCs, $CD8^+$T cells, and classically activated macrophages (M1) (Mocikat, Braumuller et al. 2003). The release of IFN-γ can also stimulate effector T_H1 CD4+ T cells, which have been shown to be important in tumor cell clearance (Flavell, Sanjabi et al. 2010).

1.2.2 NK Cell Activation

NK cell activation requires priming from a number of factors, including interleukin-15 (IL-15). IL-15 is a key cytokine involved in the survival, development, and activation of NK cells (Vivier, Raulet et al. 2011). IL-15 signaling occurs by the trans-presentation of the cytokine and its receptor (IL15Rα) on antigen presenting cells to effector cells, such as NK cells (Vivier, Raulet et al. 2011). Other cytokines, such as IL-2, IL-12, and interferon (IFN)-α/β are also involved in the activation of NK cells (Waldhauer and Steinle 2008). The interaction between NK cells and the various components that produce these cytokines is vital in stimulating NK cells to protect against cancer.

NK cell recognition and subsequent destruction of tumor cells is controlled by a balance of activating and inhibitory receptors. These receptors fall into two major families: the immunoglobulin superfamily (KIR and NCR) and the C-type lectin superfamily (Waldhauer and Steinle 2008). Some of the main activating NK cell

receptors include NKp30, NKp44, NKp46, and NKG2D, which are all part of the natural cytotoxicity receptors (NCRs) group (Waldhauer and Steinle 2008). If ligands for activating receptors are over-expressed on tumor cells, they will be detected and killed by NK cells. As an example, the ligands for NKG2D in mice include retinoic acid early inducible-1 (RAE-1) proteins and MHC class I-chain related proteins (MIC-A and –B) in humans (Nausch and Cerwenka 2008). In general these ligands are not expressed on untransformed cells but are found to be up-regulated on tumor cells undergoing stress due to DNA damage (Gasser, Orsulic et al. 2005). In mice, the killer immunoglobulin-like receptor (KIR) Ly49 is an example of an inhibitory NK receptor (NKG2 in humans), in which the ligands include MHC class I molecules expressed on all nucleated cells of the body (Orr and Lanier 2011). NK cells detect and kill tumor cells which lack the expression of these molecules (Moretta, Bottino et al. 1996). This is the basis of the missing self hypothesis proposed by Klas Kärre which explains the method by which NK cells are able to differentiate between malignant and healthy cells (Karre, Ljunggren et al. 1986).

1.2.3 NK Cell Subsets

Both mouse and human NK cells can be divided into subsets based on their cytotoxicity. In humans, two distinct populations can be identified based on the cell surface marker CD56 (Sutlu and Alici 2009). The majority of NK cells (90%) are $CD56^{low}$ and these are the more cytotoxic subset (Cooper, Fehniger et al. 2001). On the other hand, $CD56^{high}$ NK cells represent an immunoregulatory population of NK cells which are the main producers of cytokines such as IL-10, IL-13, MIP-1α, GM-CSF, IFN-

γ, and TNF-α (Farag and Caligiuri 2006). Murine NK cells are similar to human NK cells in their ability to lyse tumor cells that are deficient in MHC class I molecule expression, however, they do not express CD56. Identifying murine NK cells from C57BL/6 mice by flow cytometry requires the use of NK1.1, an antigen for the activating receptor NKR-P1C as well as CD3, to exclude NKT cells (Walzer, Blery et al. 2007). However, not all NK cells from the same strains share these markers. NK cells isolated from BALB/c mice are identified based on the expression of CD49b, recognized by DX5 antibodies (Arase, Saito et al. 2001). Murine NK cells also express another cell surface marker CD27, used to identify functional subsets. $CD27^{high}$ NK cells produce larger amounts of cytokines and are highly cytotoxic in contrast to the $CD27^{low}$ subset, which are much more tightly regulated (Hayakawa, Huntington et al. 2006). In a cancerous environment, NK cell activation markers are altered, which significantly affects their cytotoxic potential.

1.2.4 NK Cell Development

As with all other cells of the immune system, NK cells are derived from hematopoietic stem cells (HSC) originating in the bone marrow (Colucci, Caligiuri et al. 2003). Based on phenotypic markers determined by flow cytometry, three main stages of NK cell development have been proposed: precursors, immature, and mature NK cells (Di Santo 2006). NK cell precursors have been characterized in the bone marrow as CD122+NK1.1-DX5-. These cells require the cytokine IL-15 for their continuous development and journey to maturation (Puzanov, Bennett et al. 1996). NK cells then progress to an immature subset characterized by the absence of mature NK cell markers, such as DX5 and CD11b. Immature NK cells also transiently express TRAIL (Tumor

necrosis factor (TNF)-related apoptosis-inducing ligand), an effector molecule used in NK cell lysis of target cells (Takeda, Cretney et al. 2005). Immature Trail+ NK cells can further develop into mature Trail- NK cells which gain the expression of phenotypic markers such as DX5 and CD11b as well as effector molecules such as perforin and granzyme in addition to their increased ability to secrete IFN-γ (Huntington, Vosshenrich et al. 2007). These cells are able to leave their site of generation and enter the blood and peripheral tissues, patrolling the body for virally infected and transformed cells. This model of NK cell maturation put forth by James DiSanto, is based upon the idea that the gradual progression and development of NK cells is paralleled by an increase in their effector functions (Huntington, Vosshenrich et al. 2007). However, Hayakawa and Smyth have proposed that the mature subset of NK cells can be further characterized into subsets based on the expression of CD27 and CD11b (Hayakawa and Smyth 2006). Their model describes the ability of NK cells to go through development beginning from the most immature subsets $CD11b^{low}CD27^{low}$ and $CD11b^{low}CD27^{high}$ to the most mature which include $CD11b^{high}CD27^{high}$ and $CD11b^{high}CD27^{low}$ NK cells. Phenotypically, they describe the $CD11b^{high}CD27^{high}$ subset as being the most potent effector NK cells while the $CD11b^{high}CD27^{low}$ subset expresses Ly49 inhibitory receptors and KLRG1 (killer-cell lectin-like receptor subfamily G, member 1) at increased levels (Hayakawa and Smyth 2006). KLRG1 is an inhibitory receptor found to be up-regulated after chronic stimulation and proliferation, giving rise to the idea that the $CD11b^{high}CD27^{low}$ subset is exhausted (Robbins, Nguyen et al. 2002).

1.3 TUMOR ASSOCIATED NK CELLS

Liu et al. have recently revealed the importance of activated NK cells during tumor destruction by observing their ability to eradicate large solid tumors (Liu, Engels et al. 2012). Destruction of tumors was dependent on the release of IL-15 by cancer cells, resulting in a highly proliferative, granular NK cell subset that mediates killing in a perforin dependent manner (Liu, Engels et al. 2012). Therefore, in the presence of IL-15, NK cells are mature, highly activated, and able to impart significant effects on established tumors. However, using non-transgenic tumor models, most studies have shown that NK cells do not kill established tumors. The difficulty lies in stimulating NK cells in an immunosuppressive environment where there are low levels of activating cytokines – such as IL-15.

NK cells can be altered depending on the microenvironment in which they reside. These alterations are associated with the cytokines, chemokines, or growth factors present in the microenvironment. I*n vitro,* the culturing of NK cells under various conditions have shown that IL-12 and IL-4 prime human NK cells differently, which result in divergent interactions with tumor cells (Marcenaro, Della Chiesa et al. 2005). IL-12 cultured NK cells are able to lyse tumor cells and induce DC maturation while IL-4 cultured NK cells are unable to do so, resulting in impaired Th1 priming and favoring of a Th2 type response (Marcenaro, Della Chiesa et al. 2005). Interestingly, although IL-2 activated NK cells can extensively lyse melanoma cells *in vitro,* Balsamo et al. have shown that this might not necessarily be the case *in vivo*. The effector/target-cell ratios utilized in several published *in vitro* killing assays do not correlate with the NK/target-cell ratios found in

histological evaluations of melanoma lesions (Balsamo, Vermi et al. 2012). Not only do the regulatory factors in the tumor microenvironment alter the ability of NK cells to kill target cells, they are also not present at high enough levels within tumors to impart meaningful effects on tumor destruction.

TGF-β is one example of an immunoregulatory factor present within the tumor microenvironment in which there is extensive evidence that it can modulate NK cell activity. NK cell function is most often measured by the levels of activating receptors and/or pro-inflammatory cytokines secreted by these cells. TGF-β can reduce both of these properties on NK cells (Laouar, Sutterwala et al. 2005). As an example, TGF-β inhibits the expression of activating receptors on human NK cells (NKp30 and NKG2D) thereby decreasing the cytotoxic ability of NK cells (Castriconi, Cantoni et al. 2003). TGF-β treatment of human NK cells also reduced the levels of the NKG2D adapter protein DAP10 at both the transcriptional and translational levels (Park, Choi et al. 2011). In addition to alterations in activating receptors, a decrease in IFN-γ secretion was found after chronic interactions of NK cells and autologous human tumor cells (Wilson, El-Jawhari et al. 2011). This decrease in IFN-γ was discovered to be dependent on TGF-β as use of a TGF-β inhibitor restored cytokine levels to those produced by NK cells in the absence of autologous tumor cells. Since IFN-γ is important in the promotion of an adaptive immune response, the ability of tumor cells to inhibit this factor helps them evade immune attack, a key feature of cancer progression.

It has also become apparent from clinical evidence that NK cells lose their cytotoxic capabilities as a tumor develops. The progression of human breast cancer has

been associated with a reduction in the function of tumor infiltrating NK cells in comparison to peripheral blood NK cells from the same patients (Mamessier, Sylvain et al. 2011). Several factors were found to downregulate NK cell activating receptors in these patients including levels of TGF-β1 found within the tumor microenvironment (Mamessier, Sylvain et al. 2011). Research conducted on patients with non-small cell lung carcinoma (NSCLC) has revealed that the local tumor microenvironment impairs NK cell function and renders them less tumoricidal in their ability to degranulate and produce IFN-γ (Platonova, Cherfils-Vicini et al. 2011). In addition, NK cells in intratumoral regions were found to have a reduced expression of NKp30, NKp80, CD16, DNAM1, and NKG2D compared to distant NK cells or blood NK cells from the same patients (Carrega, Morandi et al. 2008). Defective expression of activating receptors has also been a hallmark of metastatic melanoma (Konjevic, Mirjacic Martinovic et al. 2007) and acute myeloid leukemia (AML) (Costello, Sivori et al. 2002). Patients with breast and head and neck cancer have been shown to display a population of $CD56^{low}$ NK cells with reduced cytotoxic activity and at an early stage of apoptosis in comparison to the $CD56^{high}$ population (Bauernhofer, Kuss et al. 2003). Along with decreased cytotoxicity, NK cells have proven to be defective in proliferation and cytokine production in various cancers such as renal cell carcinoma (RCC) (Gati, Da Rocha et al. 2004) and chronic mylogenous leukemia (CML) (Pierson and Miller 1996). NK cells home to a tumor site to eliminate cells which lack MHC class I molecules (Algarra, Cabrera et al. 2000). However, once they arrive there, their cytolytic activity, cytokine secretion, and proliferation are hindered, giving the tumor the advantage to proliferate.

1.4 MACROPHAGE BIOLOGY

1.4.1 Overview

Macrophages are classified as mononuclear phagocytes, which includes bone-marrow derived precursor cells, peripheral-blood mononuclear cells (PBMCs), as well as mature macrophages found in tissues (Pollard 2009). Acting as the first line of defense in innate immunity, they are essential immune phagocytic cells and also orient the adaptive immune response through antigen presentation to T cells (Biswas and Mantovani 2010). Monocytes are recruited to peripheral tissues from blood vessels in response to chemotactic factors (Mosser and Edwards 2008). As they enter these sites they give rise to mature macrophages with varying degrees of functionality. In this regard they have been described as extremely heterogeneous cell types with contrasting levels of activation dependent on their environment (Mosser and Edwards 2008). Cytokine production by both other immune cells, as well as microbial products, can alter the activity of macrophages and their effects on tumor cells (Mantovani, Sozzani et al. 2002). This has led to a classification system of macrophages, described in the literature as classically activated (M1 macrophages) and alternatively activated (M2 macrophages). Polarization of macrophages can be described as a continuous spectrum of activation states, defined by up-regulation of specific markers by flow cytometry. General characterization of macrophages by flow cytometry is distinguished by the use of markers such as F4/80 and CD11b as well as the absence of Gr-1 (Zhang, Goncalves et al. 2008).

1.4.2 M1/M2 Polarization

Polarized macrophages can be delineated by their ability to produce differential cytokines and chemokines, their varying degrees of effector function, and receptor expression (Mantovani, Sozzani et al. 2002). In the presence of bacterial stimuli such as lipopolysaccharide (LPS) or the T_H1 cytokines IFN-γ, TNFα, as well as GM-CSF, macrophages are polarized to become M1 (Solinas, Germano et al. 2009). They produce a distinct profile of inflammatory cytokines such as IL-12, IL-15, IL-1β, and chemokines such as CXCL10 (Solinas, Germano et al. 2009). Additionally, they produce high levels of reactive oxygen intermediates (ROI), nitric oxide (NO), and express MHC class II molecules (Movahedi, Laoui et al. 2010). As a result of their increased expression of co-stimulatory molecules, M1 macrophages have increased antigen presentation capabilities and act to kill tumor cells efficiently (Solinas, Germano et al. 2009).

In the presence of IL-4 and IL-13, produced mainly by Th2 cells, mast cells, and basophils, macrophages are polarized to become M2 (M2a) (Varin and Gordon 2009). This cytokine profile is similar to one which would be found in the tumor microenvironment. Therefore M2 macrophages resemble tumor associated macrophages (TAMs) in their cytokine secretion, receptor expression, and ability to assist tumor progression. Other distinct types of M2 cells can be differentiated when stimulated with immune complexes and TLR ligands (M2b) as well as IL-10, glucocorticoids, and TGF-β (M2c) (Varin and Gordon 2009). M2a and M2b macrophages are responsible for type II immune responses and immunoregulatory functions while M2c macrophages suppress immune responses and are important in tissue remodelling (Solinas, Germano et al.

2009). The first description of an alternative macrophage occurred with the observation that IL-4 induced the up-regulation of the macrophage mannose receptor (MMR), which subsequently became a prominent M2 marker (Stein, Keshav et al. 1992). Another distinct feature of M2 macrophages is their down-regulation of iNOS and up-regulation of arg1 expression (Gordon and Martinez 2010). A decrease in NO correlates with the reduced ability of these cells to clear tumor cells (Martinez, Helming et al. 2009). Cytokine production by M2 macrophages is typified by the release of anti-inflammatory cytokines such as TGF-β, IL-10, and IL-1 receptor antagonist (IL-1ra) (Pollard 2009). These features of activation and cytokine secretion result in an increase in angiogenesis, tissue remodelling, and repair – a perfect environment for tumor growth (Pollard 2009).

Macrophages can be easily polarized *in vitro* with the addition of recombinant cytokines; however, the *in vivo* microenvironment is much more diverse. Tumor cells and infiltrating immune cells release a multitude of factors which can impact macrophage plasticity and activation status. Therefore, M1 and M2 macrophages are considered to be extremes along a spectrum of multiple phenotypes. Nonetheless, they constitute a useful paradigm allowing researchers to study altered macrophage activity in the context of tumor formation. Evidence suggests that although macrophages have the potential to impact tumor suppression, they are subverted to do so by mechanisms of tumor escape. For their own benefit, tumors create an environment where TAMs are unable to initiate effective immune responses against tumor cells or initiate adaptive immune responses towards tumor rejection.

1.5 TUMOR ASSOCIATED MACROPHAGES

Solid tumors recruit an abundance of immune cells into their microenvironment. In this complex tumor milieu, macrophages can account for up to 50% of the tumor mass (Solinas, Germano et al. 2009). Monocytes enter neoplastic tissues following a chemokine gradient of vascular endothelial growth factor (VEGF) and macrophage-colony stimulating factor (M-CSF). At the tumor site, they differentiate into TAMs which act in a regulatory fashion (Mantovani, Savino et al. 2010). They express properties similar to M2 polarized macrophages, although their functionality can vary throughout the tumor depending on the levels of hypoxia versus normoxia (Mantovani, Sozzani et al. 2002). Clinical evidence has revealed that the number and density of TAMs within the tumor correlates with poor prognosis in a number of human cancers (Bingle, Brown et al. 2002). When Lin *et al.* removed mature macrophages in a breast cancer mouse model by crossing pyMT mice with mice carrying a $Csf1^{op}$ mutation, they saw a lag in the development to malignancy and progression to metastasis (Lin, Nguyen et al. 2001). Furthermore, TAMs express many angiogenic factors including VEGF which are important in regulating the angiogenic switch in cancer development (Lin, Li et al. 2006). This increase in vascularization provides tumors with the oxygen and nutrients required for the development to malignancy. Utilizing the same pyMT tumor model, it was shown that CD4+ T cells which produce IL-4 are responsible for the increase in lung metastasis by promoting the polarization of an M2 macrophage phenotype (DeNardo, Barreto et al. 2009). Overall, these studies reveal that macrophages have a pro-tumoral role in cancer development.

1.6 MACROPHAGE NK CELL INTERACTIONS

Recently the interactions between macrophages and NK cells in the regulation of NK cell anti-tumor activity have been described (Zhou, Zhang et al. 2012). Poly I:C-treated macrophages increased NK cytotoxicity against tumor cell targets in a NKG2D-dependent manner (Zhou, Zhang et al. 2012). The increase in NKG2D ligands on activated macrophages increased the ability of NK cells to kill tumor targets, but not the macrophages themselves. Similarly, a separate group found that macrophages exposed to microbial products such as LPS or bacillus Calmette–Guérin can drive NK cells to up-regulate activation markers such as CD69, secrete IFN-γ, and consequently lyse tumor cells *in vitro* (Bellora, Castriconi et al. 2010). This study also demonstrated the plasticity of the macrophage phenotype by adding M1-polarizing signals to M2 macrophages. This rescued their regulatory role and increased their ability to activate NK cells (Bellora, Castriconi et al. 2010). Although these studies show that activated macrophages have the capacity to stimulate NK cells, macrophages within tumors do not have an activated phenotype. Macrophages within tumors are immunosuppressive cells with the capacity to promote tumor growth as well as facilitate angiogenesis and metastasis (Lin, Li et al. 2006; Qian, Deng et al. 2009). However, there is a lack of understanding and knowledge surrounding the ability of suppressive macrophages to alter NK cells, specifically in the context of tumor development - which is the focus of this thesis.

1.7 RATIONALE, HYPOTHESIS, AND OBJECTIVES

The mechanism of NK cell inhibition within tumors is not clear. Not only are soluble mediators such as TGF-β present within the tumor microenvironment, but NK cells can interact with multiple cell types such as TAMs or tumor cells to become modulated. The immunosuppressive TAMs may play a large role in altering NK cells at the tumor site, which has not been studied previously. Macrophages are the main regulatory population of myeloid cells in the tumor (Solinas, Germano et al. 2009). They are characterized by their ability to promote tumor cell proliferation and metastasis through the release of factors such as TGF-β, IDO, and PGE_2 (Pollard 2004). TAMs work in favour of the tumor and their interaction with NK cells may prevent effective anti-tumor NK cell responses.

In addition to the mechanism of NK cell inactivation within the tumor, thorough phenotypic studies of NK cells have not been carried out. Most studies focus on the defective expression of activating receptors or overexpression of inhibitory receptors on tumor associated NK cells in comparison to peripheral blood or splenic NK cells (Levy, Roberti et al. 2011). Thus, we want to further understand the development of NK cells in the murine pyMT breast cancer model. In particular we have investigated whether NK cells from tumors differ in their maturation status in comparison to NK cells found at a distant site to the tumors. As the title of this thesis suggests we are interested in the influence of the tumor microenironment on NK cells. This prompted us to hypothesize that **the phenotype and function of NK cells are altered after interaction with tumor associated macrophages and within the tumors of pyMT mice.**

1.7.1 Objective 1: To Investigate the Effects of Polarized Macrophages and TAMs on NK cell Phenotype and Function

In an attempt to understand how tumor associated macrophages alter NK cells; we first began to use macrophages polarized *in vitro* as a model for TAMs. M1 and M2 macrophages perform divergent functions and would be expected to have distinct effects on NK cell phenotype and activity. *In vitro* co-cultures were conducted between M2 cells and NK cells and the NK cells were subsequently isolated and analyzed for markers of activation by flow cytometry. To investigate a more relevant model to TAMs, we isolated tumor associated macrophages from the murine MMTV-pyMT breast cancer model. From this experiment we wanted to determine the impact that TAMs have on the ability of NK cells to lyse transformed cells.

1.7.2 Objective 2: To Investigate the Phenotype and Function of NK cells from pyMT Tumors

Throughout the course of the TAM-NK cell co-culture experiments, the development of NK cells after interaction with TAMs was altered. They were found to express a $CD27^{low}CD11b^{high}$ phenotype. We next sought to determine if NK cells isolated from the murine pyMT breast tumors had a similar developmental phenotype. It has been previously shown that in the bone marrow of tumor bearing hosts, there is a reduction in the number of mature NK cells (Richards, Chang et al. 2006). However, the development of NK cells within the tumor has not been evaluated. We were interested in understanding the effect of the tumor microenvironment on the maturation status of tumor infiltrating NK cells in comparison to peripheral NK cells at sites such as the spleen.

1.8 MODEL SYSTEM

The interaction between TAMs and NK cells within the tumor microenvironment will be studied in a model of breast cancer. Our lab utilizes the mouse mammary tumor virus (MMTV) polyoma MT antigen (MT) transgenic mouse (MMTV-pyMT) tumor model on a C57BL/6 background. In this model, expression of the MT antigen is driven by the MMTV long terminal repeat (LTR). The MT antigen is the main oncogene of the murine polyomavirus and acts by activating proto-oncogenes such as src, Shc, and PI3 kinase (PI3K) within the cell (Schaffhausen and Roberts 2009). It has been shown to cause the transformation of tissue culture cells and subsequent tumorigenesis in mouse models (Schaffhausen and Roberts 2009). The mice develop spontaneous multifocal mammary adenocarcinomas involving all the mammary glands that are able to metastasize to the lung (Vivier, Tomasello et al. 2008). It has been reported that this model is a good model of human mammary cancer (Guy, Cardiff et al. 1992). The tumors develop from normal cells in their natural environment with the immune system of the animal intact (Chen, Xia et al. 2003). As well, tumor formation is sequential and tumors form in 100% of animals by day 108 (Xia, Tanaka et al. 2003). Isolation of tumors from this model, will allow us to retrieve macrophages from within the tumor in order to do subsequent analysis on their interactions with NK cells. NK cells will also be isolated from the tumors and spleens of pyMT mice to investigate whether these NK cells differ in development.

CHAPTER 2: MATERIALS AND METHODS

2.1 NK CELL ISOLATION

2.1.1 DX5+ and NK1.1+ Selection of NK Cells

In order to isolate NK cells, spleens were harvested from C57BL/6 mice, homogenized with the stopper of a sterile syringe, and re-suspended in PBS. Red blood cells were lysed with 1mL of ACK lysis buffer for 2 minutes. Viable cell counts were conducted using trypan blue and cells were re-suspended at a concentration of 1 x 10^8 cells/mL in PBS containing 2% FBS. Cells were placed in a 5ml polystyrene round-bottom tube and a mouse panNK (CD49b/DX5) selection kit (Stem Cell Technologies, Vancouver, BC) was used according to the manufacturer's protocol. Briefly, DX5+ cells were labeled with a PE-conjugated antibody for 15 minutes at a concentration of 50µL/mL. EasySep magnetic nanoparticles were added at a concentration of 100µL/mL. Magnetically labeled cells were separated from unlabeled cells with the use of an EasySep magnet. Cells were washed thoroughly with 2% FBS PBS and placed in the magnet for 5 minutes three times to ensure minimal contamination of unlabeled cells. DX5+ cells were then re-suspended in 10% complete RPMI and counted for later use. In a similar fashion, an EasySep PE positive selection kit was used for the isolation of NK1.1+ cells (Stem Cell Technologies, Vancouver, BC). The kit was used according to the manufacturer's protocol, however, with the addition of an NK1.1-PE antibody (BD Pharmingen, San Jose, CA)

2.1.2 *In vitro* Culture of Murine NK Cells

After NK cell isolation with the mouse panNK (CD49b) selection kit, cells were plated in a 96-well round-bottom plate at a concentration of 1.5 $x10^5$ cells/200µL. Cells were cultured with 50ng/mL of recombinant mouse IL-15 (Peprotech, Rocky Hill, NJ) for 24 hours to maintain NK cell survival.

2.2 MACROPHAGE ISOLATION

2.2.1 Peritoneal Lavage

Macrophage extraction was conducted using a peritoneal lavage. C57BL/6 mice were anesthetized intraperitoneal (i.p) with a combination of ketamine-xylazine. A vertical incision was made in the skin of anesthetized mice and 8 mL of room temperature 10% complete RPMI was injected into the peritoneal cavity using a 10mL syringe with a 25 gauge needle. The mouse was tilted back and forth to ensure thorough mixing of media within the peritoneal cavity. A 10 mL syringe with an 18 gauge needle was then used to retrieve the RPMI from the mouse, taking care not to perforate any organs and obtain red blood cell contamination. The peritoneal fluid was kept on ice until further use. Cells were spun down at 1200 rpm at 4^0C for 10 minutes. In the case that there was any red blood cell contamination, all samples were ACK lysed (1mL for 2 minutes). Viable cell counts were conducted using trypan blue and cells were re-suspended in 10% RPMI.

2.2.2 M1/M2 Polarization

Peritoneal macrophages were isolated and stimulated *in vitro* with varying cytokines to obtain M1 and M2 phenotypes. Macrophages were stimulated with IFN-γ (Peprotech, Rocky Hill, NJ) and LPS (from Escherichia coli 026:B6; Sigma-Aldrich Life Sciences, St. Loiuis, MO) (20ng/mL and 200ng/mL respectively) to obtain an M1 phenotype and with IL-4 and IL-13 (Peeprotech, Rocky Hill, NJ) (40ng/mL and 100ng/mL respectively) to obtain an M2 phenotype. Approximately $10x10^6$ cells were placed in a 60mm tissue culture dish for 24 hours at 37^0C with the addition of the appropriate cytokines. Subsequently, any non-adherent cells were washed away and macrophages were scraped off of the tissue culture dish with a cell scraper and pelleted at 1200rpm for 10 minutes at 4^0C. Cells were thoroughly washed with PBS (2 times) to ensure there was no residual cytokine contamination. Viable cell counts were conducted using trypan blue and cells were re-suspended in 10% RPMI.

2.2.3 Macrophage Isolation from pyMT Tumors

All mammary gland tumors were removed from pyMT mice when they were at or near endpoint. Multiple tumors from individual mice were grouped together, weighed, and digested accordingly. A mixture of collagenase A (3mg/mL) and DNase (25µL/10mL) (Roche Applied Science, Nutley, NJ) was prepared in Hanks media and filter sterilized (0.22µM micropore). Sterile scalpel blades were used to dice tumors and 10mL of digestion mixture was added per gram of tumor. Tumors were digested for 45 minutes at 37^0C on a shaker. Cells were then filtered through 70µM and 40µM cell strainers, with the addition of Hanks media to deactivate the digestion mixture. The cells

were spun down for 7 minutes at 1200 rpm and red blood cells were lysed with 1mL of ACK lysis buffer for 2 minutes. Viable cell counts were conducted using trypan blue and cells were re-suspended at a concentration of 1 x 10^8 cells/mL in PBS containing 2% FBS. Cells were placed in a 5ml polystyrene round-bottom tube and a mouse CD11b positive selection kit (Stem Cell Technologies, Vancouver, BC) was used according to the manufacturer's protocol. Briefly, CD11b+ cells were labeled with a PE-conjugated antibody for 15 minutes at a concentration of 50µL/mL. EasySep magnetic nanoparticles were added at a concentration of 50µL/mL. Magnetically labeled cells were separated from unlabeled cells with the use of an EasySep magnet. Cells were washed thoroughly with 2% FBS PBS and placed in the magnet for 5 minutes three times to ensure minimal contamination of unlabeled cells. CD11b+ cells were then resuspended in 10% complete RPMI and counted for later use.

2.3 PHENOTYPIC ANALYSIS OF POLARIZED MACROPHAGES

2.3.1 ELISAs

Peritoneal macrophages were isolated from C57BL/6 mice and cultured as described to obtain M1 and M2 phenotypes. Any non-adherent cells were washed away and macrophages were thoroughly washed with PBS, counted in trypan blue and re-suspended in 10% RPMI. 2x10^5 M1 or M2 cells were then re-plated in a 96-well flat bottom plate and allowed to adhere and incubate for 24 hours at 37^0C. Supernatants were collected and stored at -80^0C until further use. IL-12 and anti-TGF-β ELISAs were conducted with the use of the DuoSet ELISA Development Kits (R&D Systems, Minneapolis, MN). The TGF-β ELISA kit measures both natural and recombinant mouse

TGF-β-1 from cell culture supernatants. To activate latent TGF-β to the immunoreactive form, 1N HCl was used for acid activation and 1.2N NAOH was used for neutralization. The ELISA was performed according to the manufacturer's protocol. Briefly, the capture antibody was diluted to a working concentration of 4µg/mL in PBS and 100µL was added per well. The plate was sealed and incubated overnight at room temperature. Each well was aspirated with wash buffer (0.05% Tween 20 in PBS) three times using an autowasher. The plates were blocked with 300µL of block buffer (5% Tween 20 in PBS) and incubated at room temperature for 1 hour. The wash step was repeated as previously described. 100µL of activated sample (1:2 dilution) or standard (serial dilutions in the range of 2000 pg/mL - 0 pg/mL) in reagent diluent (0.05% Tween 20 in PBS) was added to the appropriate wells. The plate was covered and incubated for 2 hours. Following incubation, the plate was washed, the detection antibody was added (200ng/mL), and incubated for 2 hours. After completion of a wash step, 100µL of streptavidin-HRP was added to each well and incubated in light sensitive conditions for 20 minutes. A final wash step was completed and a 1:1 mixutre of Colour Reagent A (H_20_2) and Colour Reagent B (tetramethylbenzidine) (BD Biosciences, San Jose, CA) was generated and added to the wells (100µL/well) for 20 minutes. 50µL of the stop solution (2N H_2S0_4) was added to terminate the reaction and the optical density of each well was measured immediately using a microplate reader set to 450nm with a correction wavelength of 540nm. For analysis, the average absorbance value for each set of duplicate standards was calculated. In excel, a standard curve was created by plotting the mean absorbance for each standard (x-axis) against the protein concentration (y-axis). The concentration of

protein in each particular sample was calculated by finding the average absorbance value for each sample, plugging it into the equation of the line generated from the standard curve, and solving for x by plugging in the average OD value as y. A similar protocol was followed for the mouse IL-12/IL-23 p40 ELISA with several minor differences. The detection antibody was diluted to a working concentration of 400ng/mL in reagent diluent (1% BSA in PBS). In addition, reagent diluent with 2% heat inactivated normal goat serum was prepared 1-2 hours prior to use and used for the dilution of the detection antibody.

2.3.2 Nitric Oxide (NO) Assay

An NO assay was performed to determine the indirect concentration of NO in M1 or M2 supernatants by the measurement of its breakdown product, nitrate ($N0^{2-}$). $2x10^5$ M1 or M2 cells were plated in a 96-well flat bottom plate and allowed to adhere and incubate for 24 hours at 37^0C. 100µL of supernatants were collected to be used immediately in an NO assay. Briefly, 2g of Greiss Reagent (Sigma-Aldrich Life Sciences, St. Louis, MO) was added to 50mL of autoclaved water and dissolved. 0.69g of $NaN0_2$ (Sigma-Aldrich Life Sciences, St. Louis, MO) was added to 100mL of RPMI and dissolved (100mM). Standards were prepared by adding 50µL of $NaN0_2$ into 50mL of RPMI to make a 1:1000 dilution (100µM). Serial dilutions of $NaN0_2$ were made at concentrations of 50, 40, 30, 20, 15, 10, 5, 2.5, and 1 µM. 100µL of serial dilution as well as sample was transferred to a 96-well flat bottom plate and 100µL of Greiss Reagent was then added to each well (RPMI added to the 0µM well included as a reference). The plate

was incubated at room temperature for 10 minutes and the optical density of each well was measured immediately using a microplate reader set to 550nm. For analysis, a standard curve was created in excel by plotting the mean absorbance for each standard (x-axis) against the $N0^{2-}$ concentration (y-axis). The concentration of $N0^{2-}$ in each particular sample was calculated by finding the average absorbance value for each sample, plugging it into the equation of the line generated from the standard curve, and solving for x by plugging in the average OD value as y.

2.3.3 Arginase Assay

$2x10^5$ M1 or M2 cells were plated in a 96-well flat bottom plate and allowed to adhere and incubate for 24 hours at 37^0C. The cells were subsequently washed with PBS and lysed with 100µL of 0.1% Triton-x-100 in PBS supplemented with a protease inhibitor (Roche Applied Science, Nutley, NJ). Samples were either stored at -80^0C or an arginase assay was conducted right away. The arginase assay was conducted as follows: the lysed cells were incubated at room temperature for 30 minutes on a shaker. 100µL of 25mM Tris-HCl (pH 7.5) was added to the samples and 100µL of this lysate was placed into 2 separate 1.5mL microtubes (a and b). 10µL of $MnCl_2$ in ddH_20 was added to the samples. The enzymes were then activated by heating the samples to 56^0C for 10 minutes. For a negative control, 100µL of 0.1% Triton-x-100 and 100µL of 25mM Tris-HCl were placed in a separate 1.5mL microtube (c). For arginine hydrolysis, 100µL of lysate (a) was added to 100µL of 0.5M L-arginine. As a control, 100µL of lysate (b) was added to 100µL ddH_20. All samples (a-c) were incubated at 37^0C for 30 minutes. A urea standard

was created by making a 200µL two-fold serial dilution in ddH_20 (20mM, 10mM, 5mM, and 1mM). The arginase reaction was stopped by adding 800µL of stop solution (1:3:7 $H_2S0_4/H_3P0_4/ddH_20$) to all tubes. 40µL of 9% α-isonitrosopropiophenone in 100% ethanol was added to each sample and samples were heated to 95-99^{0}C for 30 minutes. 200µL of samples, controls, and urea standard were then added to a 96-well microtiter plate and the optical density of each well was measured immediately using a microplate reader set to 540nm. For analysis, a standard curve was created in excel by plotting the mean absorbance for each standard (x-axis) against the arginase concentration (y-axis). The concentration of argianse in each particular sample was calculated by finding the average absorbance value for each sample, plugging it into the equation of the line generated from the standard curve, and solving for x by plugging in the average OD value as y.

2.4 NK CELL- MACROPHAGE CO-CULTURE

2.4.1 Contact Dependent Co-Culture

NK cells cultured in 50ng/mL of recombinant mouse IL-15 for 24 hours were thoroughly washed of the cytokine. M1 and M2 cells were also thoroughly washed with PBS (2 times) to ensure there was no residual cytokine contamination. Viable cell counts were conducted using trypan blue and cells were re-suspended in 10% RPMI. In other experiments, macrophages were isolated from pyMT mice using the CD11b+ selection kit (Stem Cell Technologies, Vancouver, BC) and counted using trypan blue. Macrophages (either polarized or TAMs) were added to the NK cells in contact in the 96-well round bottom plate at a ratio of 1:1. Wells containing NK cells stimulated with IL-15 were used

as controls. The cells were in contact for 24 hours at 37^0C. NK cells were stained and analyzed for NK cell markers by flow cytometry.

2.4.2 Transwell Co-Cultures

NK cells cultured in 50ng/mL of recombinant mouse IL-15 for 24 hours were thoroughly washed of the cytokine and counted in trypan blue. M1, M2 cells, or TAMs were prepared as previously described. Transwell co-cultures were conducted in 24-well plates with 0.4µm transwells. NK cells were placed on both the apical and basolateral surfaces to examine the effects that macrophages have on NK cell phenotype. The cells were incubated for 24 hours at 37^0C and then collected and stained for NK cell markers by flow cytometry.

2.4.3 TGF-β Antibody

A TGF-β antibody was obtained (Bio-X-Cell, West Lebanon, NH) and a working concentration of 10µg/mL was used *in vitro*. Prior to the co-culture of NK cells with macrophages, M2 macrophages were incubated for 1 hour at 37^0C with the TGF-β antibody. Contact dependent co-culture of NK cells and macrophages was conducted as previously described, with certain wells receiving the TGF-β antibody to reduce the levels released by M2 cells. The cells were in contact for 24 hours at 37^0C. NK cells were stained and analyzed for NK cell markers by flow cytometry.

2.5 FLOW CYTOMETRY ANALYSIS

2.5.1 Extracellular Staining

NK cells were stained after interaction with TAMs or after isolation from pyMT tumors. The following staining protocol was utilized: Cells plated in a 96-well round bottom plate were spun down at 1200rpm for 7 minutes and dumped of any remaining media. Fc block (anti-mouse CD16/32, e-Bioscience, San Diego, CA) was prepared at a 1:100 dilution with FACS buffer (0.2% BSA in PBS). 100µL of Fc block was added to each well, re-suspended, and incubated for 20 minutes. A master mix containing various anti-mouse fluorescently labelled antibodies to be used for flow cytometric analysis was prepared in FACs buffer as well as cocktail mixes for any FMOs (fluorescence minus one). Subsequently, 100µL of the appropriate antibody mixture was added to the samples and incubated for 30 minutes under light sensitive conditions. The plate was then washed with FACS buffer and the samples transferred into FACS tubes for immediate analysis or fixed with 1% PFA in PBS and run the following day. For immediate analysis, cells were kept on ice and in the dark and run on the BD LSR II or BD Canto (BD Biosciences, San Jose CA).

2.5.2 Intracellular Staining

Following the extracellular staining protocol, some samples were also stained for intracellular factors. 100µL of Cytofix/Cytoperm (BD Biosciences, San Jose, CA) was added to each sample and allowed to incubate in the dark for 20 minutes. Subsequently the samples were washed with 1x BD Perm/Wash (BD Biosciences, San Jose, CA) buffer

(diluted from 10x in sterile water). A master mix containing various intracellular anti-mouse fluorescently labelled antibodies was prepared in BD Perm/Wash as well as any FMOs required for the analysis. 100µL of the appropriate antibody mixture was added to the samples and incubated for 20 minutes under light sensitive conditions. The plate was then washed with FACS buffer and the samples transferred into FACS tubes for immediate analysis or fixed with 1% PFA in PBS and run the following day. For immediate analysis, cells were kept on ice and in the dark and run on the BD LSR II or BD Canto (BD Biosciences, San Jose CA).

2.5.3 Antibodies

Prior to antibody use for staining, they were titrated to obtain the correct dilution to be used at. The following antibodies (e-biosciences, San Diego, CA) were used according to the manufacturer's instructions: anti-mouse CD45 (Pacific Blue), anti-mouse NK1.1 (APC), anti-mouse NK1.1 (PE), anti-mouse CD3 (Alexa 700), anti-mouse F4/80 (APC), anti-mouse Gr-1 (Alexa 700), anti-mouse CD27 (PerCP-Cy5.5), anti-mouse CD107a (FITC), anti-mouse CD11b (PE-Cy-7), anti-mouse DX5 (APC), anti-mouse perforin (FITC), anti-mouse granzyme (PE-Cy-7), and anti-mouse NKp46 (PerCP-Cy5.5).

2.5.4 Data Acquisition and Analysis

Data acquisition was conducted on the BD LSR II or the BD Canto (BD Biosciences San Jose CA). Positive and negative beads (BD Biosciences, San Jose, CA)

were used to set up compensation prior to running any samples. Combeads are important in the optimization of fluorescence settings prior to flow cytometry analysis. The FlowJo software (Tree Star, Ashland, OR) was used for analysis of all FCS files. Gates were set according to an unstained sample as well as FMOs (fluorescence minus one).

2.6 CYTOTOXICITY ASSAYS

2.6.1 NK Cell-YAC-1 Killing Assay

To determine the ability of NK cells to directly lyse target cells under varying conditions, a killing assay was performed against YAC-1 cells. The YAC-1 cell line is a murine T-lymphoma cell line that is sensitive to killing by NK cells. NK cells isolated with the DX5+ selection kit (Stem Cell Technologies, Vancouver, BC) and stimulated with IL-15 for 24 hours were used as effectors for this assay. 1.5 x 10^5 polarized macrophages or TAMs were co-cultured with 1.5 x 10^5 NK cells for 20 hours at 37^0C in a 96-well round bottom plate. Cells were spun down at 1200rpm for 7 minutes and dumped of any remaining media. 1.5 x 10^5 CFSE (Sigma-Aldrich Life Sciences, St. Loiuis, MO) labeled YAC-1 cells were then added to the plate which was incubated at 37^0C for 4 hours to allow for NK cell mediated killing. A control was included to visualize the ability of macrophages to kill YAC-1 target cells alone. CFSE labeling of YAC-1 cells was performed by washing the cells two times with PBS, making CFSE at a 2.5μM concentration, and adding it to 20x10^6 cells. The cells were then incubated for 5 minutes at room temperature before being washed of CFSE two times with PBS. Following the killing assay, 5μL of 7-AAD (BD Biosciences, San Jose, CA) was added to each well and flow was performed on the BD Canto (BD Biosciences, San Jose, CA). To determine the

percentage of YAC-1 cells killed, cells were gated as CFSE+ (FITC) and 7-AAD+ (PerCP).

2.6.2 NK Cell Degranulation Assay (CD107a)

To determine the ability of NK cells to degranulate, a CD107a assay was performed. CD107a or lysosomal-associated membrane protein-1 (LAMP-1) has been described as a marker of NK cell degranulation and activity, correlating well with NK cell-mediated lysis of target cells. NK cells isolated with a DX5+ selection kit were co-cultured with either polarized macrophages for 24 hours. NK cells in co-culture with macrophages were used as effectors for this assay. YAC-1 cells were used as targets. 1.5 x 10^5 YAC-1 cells were added to 1.5 x 10^5 NK cells (1:1 ratio) in 180µL of 10% complete RPMI. Wells without targets were included as unstimulated controls resulting in the spontaneous release of CD107a. 1µL of anti-CD107a antibody was added to the samples (pre-titrated anti-mouse CD107a) and the plate was incubated at 37^0C for 1 hour. After 1 hour, Golgi Stop (BD Biosciences, San Jose, CA) was added to the wells (4µL of Golgi Stop in 596µL of 10% complete RPMI) at a volume of 20µL/well to ensure a total volume of 200µL. The plate was incubated for another 3 hours after which time it was spun down at 1200 rpm for 7 minutes. Cells were re-suspended in FACS buffer and subsequently stained for NK1.1 and CD3 (already DX5+). The samples were run on the BD Canto or LSR II. Flow cytometry analysis was conducted using the FlowJo software.

2.7 RNA ANALYSIS AND REAL-TIME PCR

Intratumoral and splenic NK cells were isolated with a NK1.1+ selection kit (Stemcell Technologies, Vancouver, BC). The purity of isolation was ascertained by flow cytometry and RNA was isolated using the RNeasy Mini Kit with the recommended on-column DNase treatment step (RNase-Free DNase set) according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). The RNA concentration and purity were determined by analysis of samples on the Thermo Scientific Nanodrop and stored at -80^0C until further analysis. 0.5µg of total RNA was used as the starting material for the RT^2 First Strand cDNA Synthesis Kit (SABiosciences, Frederick, MD). The finished First Strand cDNA Synthesis Reaction was held at -20^0C overnight before performing the real-time PCR. A 96-well Cyokines and Chemokines PCR Array was obtained from SABiosciences (PAMM-150Z) including the experimental cocktail containing the qPCR Master Mix. 1350µL of the qPCR Master Mix was added to 102µL of cDNA and 1248µL of H_20. 25µL of this mixture was then added to each well in the 96-well plate. The plate was sealed with 8-cap strips and run on the ABI 7900HT real time PCR instrument using a two-step cycling program as described by the manufacturer. Data analysis was performed using the ΔΔCt method on an excel-based template on-line (SABiosciences, Frederick, MD). Several house-keeping genes were used as controls including beta actin and GAPDH. The program analyzed genes up- and down-regulated on the PCR array between the test sample (pyMT tumor NK cells) and the control sample (splenic NK cells).

2.8 pyMT TUMOR CHALLENGE

2.8.1 Engraftment of Tumor Cells

6 week old C57BL/6 mice were anesthetized with isofluorene. The mice were shaved and injected subcutaneously with $1x10^5$ pyMT cells in the right flank. Care was taken to inject in the same spot in each mouse.

2.8.2 Tumor Monitoring and Palpation

Mice were palpated every other day until tumor formation. When tumors reached 2mmx2mm, tumor diameters were measured. Two diameters were measured using calipers, the length (longer) and the width (shorter). The following ellipsoid formula was used to calculate tumor volume: ($\pi/6 * L * W^2$) where L=length and W= width.

2.8.3 Isolation of Tumor Infiltrating Leukocytes

Tumors were removed from mice when they were at endpoint (10mm x 10mm). Tumors were weighed and digested accordingly. A mixture of collagenase A (3mg/mL) and DNase (25µL/10mL) (Roche Applied Science, Nutley, NJ) was prepared in Hanks media and filter sterilized (0.22µM micropore). Sterile scalpel blades were used to dice tumors and 10mL of digestion mixture was added per gram of tumor. Tumors were digested for 45 minutes at 37^0C on a shaker. Cells were then filtered through 70µM and 40µM cell strainers, with the addition of Hanks media to deactivate the digestion mixture. The cells were spun down for 7 minutes at 1200 rpm and red blood cells were lysed with

1mL of ACK lysis buffer for 2 minutes. Viable cell counts were conducted using trypan blue. Cells were stained and analyzed by flow cytometry. All tumor samples were run on the BD LSR II flow cytometer.

2.9 INTRATUMORAL TREATMENT OF pyMT TUMORS

2.9.1 IL-12 and anti-TGF-β Treatment in a Subcutaneous pyMT Tumor Model

6 week old C57BL/6 mice were injected subcutaneously with $1x10^5$ pyMT cells in the right flank. Tumor growth was monitored by palpation and when all tumors reached palpable sizes (2x2-4x4 mm), the mice were randomly distributed to 4 different treatment regimens (5 mice per group). The treatments included 50ng of IL-12 (Peprotech, Rocky Hill, NJ), 50µg of anti-TGF-β (Bio-X-Cell, West Lebanon, NH), a combination of the two as well as a PBS control. In order to impart a meaningful effect on the tumor infiltrating lymphocytes (TILs), treatments were administered intratumorally in a total volume of 25µL using a Hamilton syringe (Hamilton, Reno, NV). Injections were administered three times a week and mice were subsequently monitored for endpoint (10mmx10mm). At endpoint, tumors were isolated for analysis of tumor infiltrating immune cells. Cells were stained and analyzed by flow cytometry. All tumor samples were run on the BD LSR II flow cytometer.

2.9.2 IL-12 and anti-TGF-β Treatment in a Spontaneous pyMT Tumor Model

The spontaneous MMTV-pyMT transgenic mouse model is on a C57BL/6 background. The mice develop spontaneous multifocal mammary carcinomas in all mammary glands. Mice were palpated weekly to follow tumor growth. When at least 2 tumors reached 5mm x 5mm, intratumoral treatments were begun on each individual tumor. The treatments included 50ng of IL-12 and 50µg of anti-TGF-β as well as a PBS control. Treatments were administered intratumorally in a total volume of 25µL using a Hamilton syringe (Hamilton, Reno, NV). Injections were administered three times a week and mice were sacrificed on the following day in order to analyze the changes in the phenotype of the tumor associated NK cells. Tumors and spleens from pyMT mice were isolated and cells were stained and analyzed by flow cytometry. All tumor samples were run on the BD LSR II flow cytometer.

2.10 STATISTICAL ANALYSIS

Statistical analysis was performed with the use of Graph Pad Prism software (San Diego, CA). Any graphs which compared two conditions were analyzed using an unpaired student's T-test. In certain circumstances where comparing three conditions was required (ex. Comparing NK+IL-15 with NK+M1 and NK+M2), a one-way anova was utilized. Survival curves and % tumor free curves were compared using a logrank test. Error bars represent standard error of the mean (SEM) and a p value of less than 0.05 was interpreted to be statistically significant.

CHAPTER 3: RESULTS

3.1 NK CELL FUNCTION IS REDUCED AFTER CONTACT WITH M2 CELLS

3.1.1 The Generation and Characterization of M1 and M2 Macrophages

Since M1 and M2 macrophages were described as being functionally diverse, we asked whether they would have different effects on NK cell activity. In particular, we were interested in the alternatively activated subset of macrophages (M2) as they resemble macrophages found within tumors. To examine the effects of polarized macrophages on NK cells, peritoneal macrophages were isolated from C57BL/6 mice and stimulated in culture for 24 hours to become M1 and M2 cells. All M1 macrophages were stimulated with IFN-γ/LPS (20ng/mL and 200ng/mL respectively) and all M2 macrophages were stimulated with IL-4/IL-13 (40ng/mL and 100ng/mL respectively). It is established in the literature that M1 macrophages produce high levels of NO while M2 macrophages produce high levels of arginase (Mosser and Edwards 2008). L-arginine can be metabolized by inducible nitric oxide synthase (iNOS) to produce nitric oxide and L-citrulline or by arginase to produce ureum and L-ornithine (Chen et al., 2003). In conducting the NO and arginase assays, we observed that M1 macrophages produced elevated NO (20μM) while M2 macrophages produced elevated arginase (15mM urea) and essentially no NO unless they were re-stimulated with LPS (Figure 1). Cytokine analyses of M1 and M2 supernatants collected at 24 hours post stimulation were conducted by ELISA. Results revealed that M1 macrophages produced increased IL-12p40 (500 pg/mL) and M2 macrophages produced increased TGF-β (1000 pg/mL). As such, *in vitro* polarization of macrophages into M1 and M2 cells was effective.

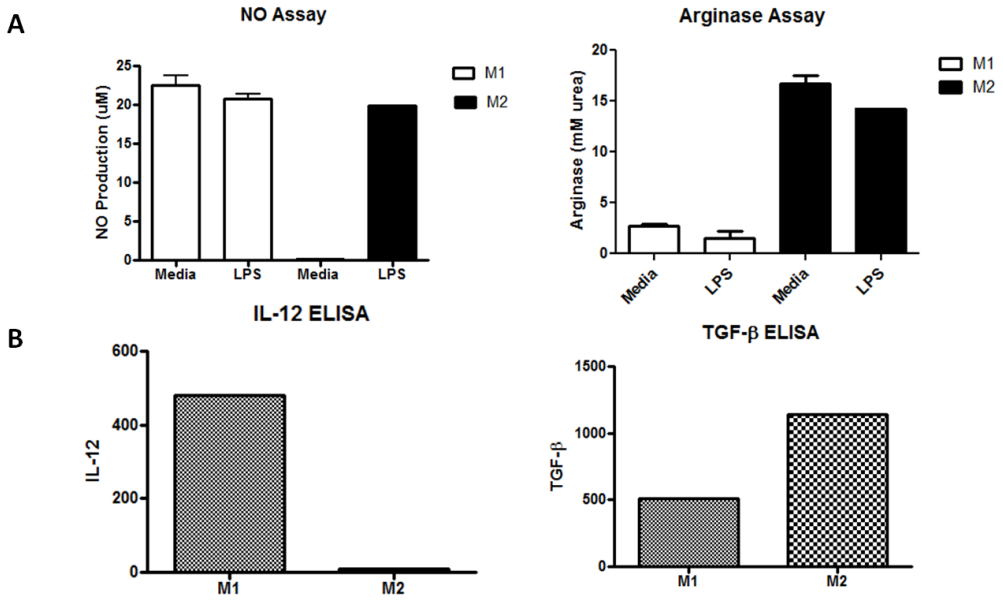

Figure 1

Phenotypic analysis of macrophages stimulated to become M1 and M2

Peritoneal macrophages were isolated from C57BL/6 mice treated for 24 hours with either LPS/IFNγ (M1) or IL-4/IL-13 (M2). The resultant cells were washed of the M1 and M2 stimulants and plated in a 96-well plate at a concentration of 2.0 x 10^5 cells/well for an additional 24 hours. (A) The supernatants were harvested for NO production and the cells were harvested for arginase. Each condition was repeated in duplicate. (B) IL-12p40 and TGF-β ELISAs were conducted (measured in pg/mL). The results were repeated two times with the same trends evaluated.

3.1.2 M2 Macrophages Inhibit NK Cell CD27 Expression

With the establishment of the M1 and M2 macrophage phenotype, the suppression of NK cell activity by M2 cells could be assessed. Splenic NK cells were isolated from C57BL/6 mice with the DX5 positive selection kit (Stemcell Technologies). NK cells were cultured with 50ng/mL of IL-15 for 24 hours to ensure survival of the NK cells in culture, which was repeated in all subsequent experiments. After 24 hours, the NK cells were thoroughly washed of the IL-15, thereby preventing any potential interference to the addition of macrophages to the co-culture. Polarized macrophages were added to the NK cells at a 1:1 ratio for 20 hours of co-culture after which cells were stained for CD3 and the NK cell activation marker CD27 and analyzed by flow cytometry. Since the DX5 selection kit labels any DX5+ cells with a PE label, it also allows for the simultaneous detection of DX5 via flow cytometry. The CD27+ NK cell population was based on a DX5+ CD3- gate and subsequently analyzed based on high and low SSC (granularity) (Figure 2). Highly granular NK cells (high SSC) are indicative of NK cells that are highly activated and have the potential to secrete granules of perforin and granzyme B. 10% of NK cells activated with IL-15 expressed $CD27^{hiSSC}$ while the addition of M1 macrophages increased the $CD27^{hiSSc}$ subset to 19.4%. With the addition of alternatively activated macrophages, the expression of the $CD27^{hiSSC}$ population drastically diminished to 3%. Essentially, the granular population of activated NK cells disappeared with the addition of M2 macrophages. This experiment was repeated a total of three times and the same trends were revealed. M2 macrophages have the ability to inhibit the expression of the cytotoxicity marker CD27, specifically on highly granular NK cells.

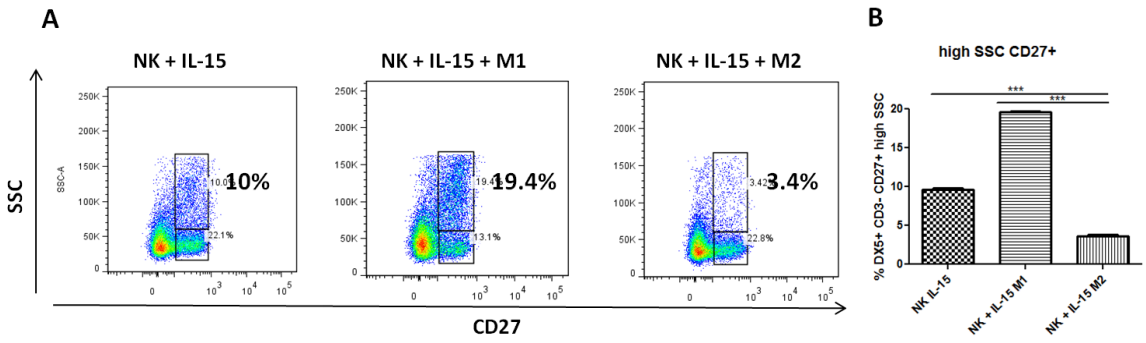

Figure 2

M2 Macrophages Inhibit IL-15 Activated NK Cell CD27 Expression

M1 and M2 cells were polarized with LPS/IFN-γ and IL-4/IL-13, respectively, for 24 hours. Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit (Stemcell Technologies). NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. Macrophages were added to the NK cells at a 1:1 ratio for 20 hours of co-culture upon which time cells were stained for CD3 and CD27. (A) CD27, based on a DX5+ CD3- population, was analyzed by FACS and (B) quantified (Results were analyzed by one-way ANOVA ***P<0.001, n=3).

3.1.3 M2 Macrophages Inhibit NK Cell Killing Activity

To further assess the role that alternatively activated macrophages have in inhibiting NK cell cytotoxicity, a killing assay was performed with YAC-1 cells as the target cell line. The YAC-1 cell line is a murine T-lymphoma cell line that is sensitive to killing by NK cells (Kiessling, Klein et al. 1975). Previous conventional NK cell killing assays have labeled target cells with ^{51}Cr, which can be quantified when released upon cell lysis. To avoid use of radioactive ^{51}Cr, we used a flow cytometric based technique. CFSE is used to label target cells whether alive or dead. All the target cells retain the label as it covalently links to free amines of cytoplasmic proteins and is not released from the cells (Xiao-Qi *et al*., 2005). 7AAD is then used as a dead cell marker. 7AAD differs from propidium iodide (PI) in its ability to be taken up by less advanced apoptotic cells (Invitrogen). In this assay, NK cells were added to polarized macrophages for 20 hours of co-culture after which a 4 hour killing assay was performed against CFSE labeled YAC-1 target cells. Incubation of NK cells with M1 cells increased the amount of 7AAD+ YAC-1 cells to approximately 20% (Figure 3) However, after co-culture with M2 cells, NK cytotoxicity was decreased to 11%. As controls, M1 and M2 macrophages were incubated with YAC-1 cells alone to ensure that they did not contribute to the killing of this cell line. The percentage of killing after addition of M1 or M2 cells was not higher than the percentage of dead YAC-1 cells alone, indicating that YAC-1 cells are specific NK cell targets, and can be used for this purpose (data not shown).

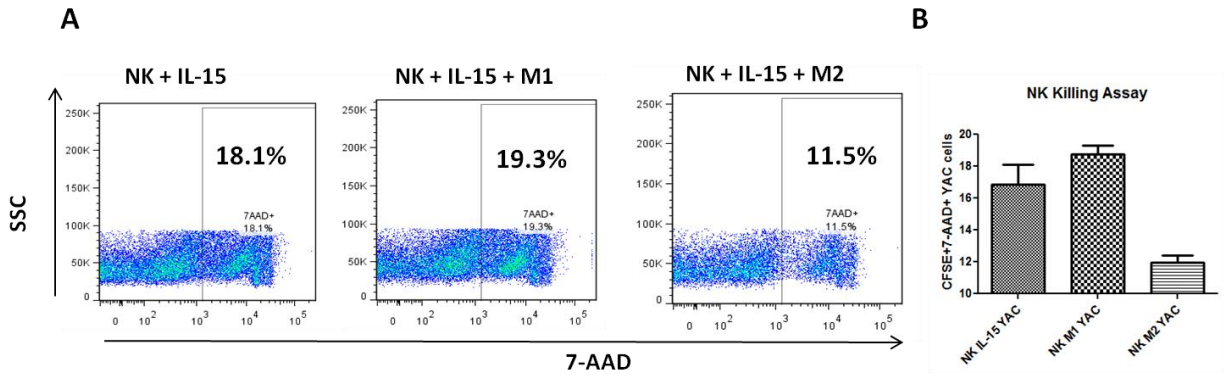

Figure 3

M2 Macrophages Inhibit NK Cell Killing Activity

M1 and M2 cells were polarized with LPS/IFN-γ and IL-4/IL-13, respectively, for 24 hours. Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit (Stemcell Technologies). NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. Macrophages were added to the NK cells for 20 hours of co-culture upon which time a 4 hour killing assay was performed against CFSE labelled YAC-1 target cells. All cells in the co-culture were subsequently labeled with the dead cell marker 7AAD. (A) CFSE+7AAD+ YAC cells analyzed by FACS and (B) quantified (n=2).

3.1.4 M2 Macrophages Inhibit NK Cell CD107a in a TGF-β Dependent Manner

The next step in examining the ability of alternatively activated macrophages to suppress NK cells was to identify the mechanism(s) by which M2 cells inhibited NK cell $CD27^{hiSSC}$ expression and killing ability. Based on information gathered from the literature, there were three possible candidates to evaluate: the expression of B7 molecules by macrophages, as well as the release of both PGE_2 and the immunosuppressive factor TGF-β. Analyzing the expression of B7 molecules and conducting a PGE_2 ELISA revealed that the expression of these factors was increased on M1 cells and not M2 cells (data not shown). This is consistent with data in the literature which suggests that B7 molecules such as PD-L1 as well as immunosuppressive factors such as PGE_2 are up-regulated in response to classical activation stimuli such as IFN-γ (Loke and Allison, 2003). Therefore, we decided to look more closely at TGF-β, a factor which we have shown to be released at higher levels by alternatively activated macrophages by ELISA (Figure 1).

TGF-β is an immunosuppressive cytokine which plays an important role in tumor development (Massague 2008). Both tumor cells as well as immune cells have been shown to produce TGF-β, which has the ability to promote cancer cell metastasis by inhibiting immune surveillance and increase cancer cell invasion into surrounding tissues (Wrzesinski, Wan et al. 2007). Researchers have shown that TGF-β inhibits the expression of activating receptors such as NKp30 and NKG2D on human NK cells, resulting in inhibition of NK mediated killing of immature DCs as well as certain tumor cell lines (Castriconi, Cantoni et al. 2003). M2 macrophages, as well as TAMs are known

producers of TGF-β (Bastos, Alvarez et al. 2002). Therefore, we wanted to determine if this was the mechanism by which M2 macrophages were using to suppress NK cell cytotoxicity and $CD27^{hiSSC}$ expression.

M2 macrophages were added to NK cells for 20 hours of co-culture. Some wells received the TGF-β antibody (Bio-X-Cell) to reduce the levels released or expressed by alternatively activated macrophages. After 20 hours of co-culture, a 4 hour CD107a assay was performed with YAC-1 target cells. CD107a or lysosomal-associated membrane protein-1 (LAMP-1) has been described as a marker of NK cell degranulation and activity, correlating well with NK cell-mediated lysis of target cells (Alter, Malenfant et al. 2004). Cells were subsequently analyzed by flow cytometry as DX5+ CD3- CD107a+. There was a reduction of CD107a with the addition of M2 cells. However, incubation with the TGF-β antibody increased the levels of CD107a close to those observed with IL-15 activated NK cells (Figure 4). Although inhibiting TGF-β restored NK cell levels of CD107a, it most likely will not activate the NK cells to become cytotoxic. As shown in Figures 2 and 3, M1 macrophages can stimulate the activation of NK cells as evidence by their increased expression of CD27 and cytotoxic activity. This could be potentially attributed to the release of IL-12 (measured by ELISA as 1000 pg/mL) by M1 macrophages. Therefore, when striving to activate NK cells to contribute towards tumor rejection, it is important to add a factor that will stimulate them in addition to suppressing the factor that inhibits their function.

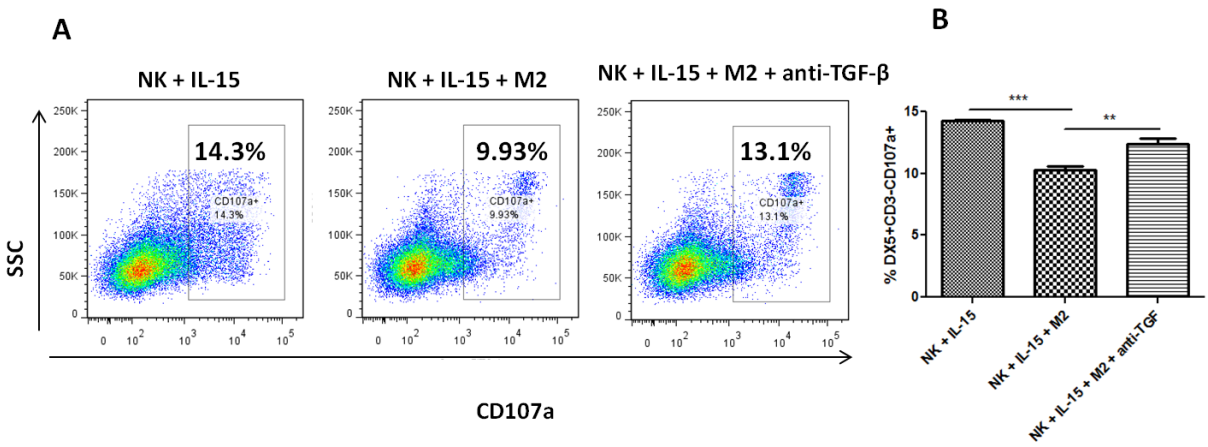

Figure 4

M2 Macrophages Mediate Suppression of NK Cell CD107a in a TGF-β Dependent Mechanism

Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit (Stemcell Technologies). NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. M1 and M2 macrophages were added to the activated NK cells for 20 hours of co-culture. In addition to M2 macrophages, anti-TGF-β was added at a concentration of 10 µg/mL. After incubation, a 4 hour CD107a assay was performed with YAC-1 target cells. Cells were subsequently stained for CD3 and CD107a. (A) CD107a, based on a DX5+ CD3- population, was analyzed by FACS and (B) quantified. (Results were analyzed by one-way ANOVA **P<0.01, ***P<0.001, n=3).

3.1.5 M2 Macrophages Mediate Suppression of NK Cells in Contact

To evaluate whether inhibition of NK cells by M2 macrophages is contact dependent, a transwell assay was performed. M2 cells were added to NK cells either in transwell (0.4μm) or in contact. In the results in Figure 5, the NK cells were added to the apical side; however, the experiment was repeated with NK cells added to the basolateral side with the same trends evaluated (data not shown). Under transwell conditions, the level of CD27 expression was similar to that of IL-15 cultured NK cells. Thus, it was confirmed that the inhibition of NK cells occurred in a contact dependent manner. To control for macrophage contamination in the contact dependent group, F4/80 staining was used to verify that DX5+ CD3- NK cells were also F4/80-. This was done to ensure the results from the NK co-culture with TAMs were not due to a flow cytometry phenomenon caused by the addition of macrophages to the wells. There was very minimal F4/80+ contamination after gating on NK cell markers, and this contamination did not alter the flow results (data not shown). In light of our previous observation that anti-TGF-β restored NK cell expression of CD107a, we can hypothesize that the mechanism by which this occurs is contact dependent requiring membrane bound TGF-β. A contact dependent phenomenon between NK cells and macrophages is similar to what others have reported. Bellora, F. *et al.* observed that M1 macrophages can stimulate NK cell expression of CD69 in a contact dependent manner (Bellora *et al*., 2010). Similarly, poly I:C stimulated macrophages promote NK cell IFN-γ and NKG2D expression through both cell-to-cell contact as well as cytokines secreted by poly I:C treated macrophages (Zhou *et al*., 2012).

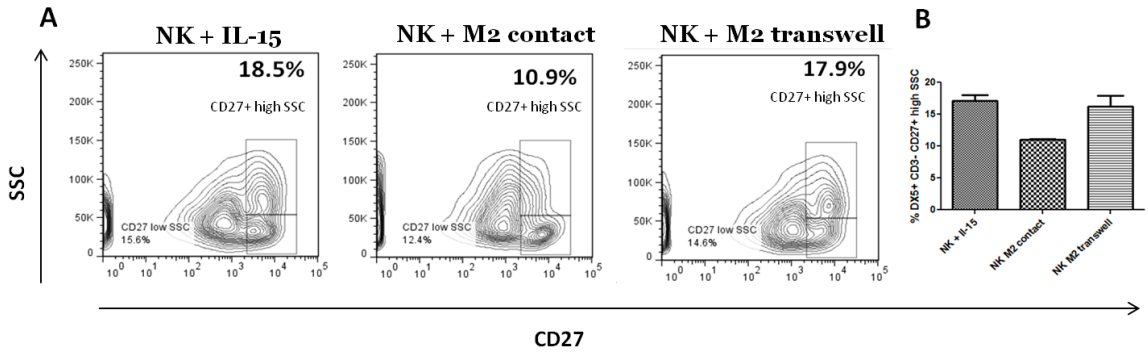

Figure 5

M2 Macrophages Mediate Suppression of CD27 on NK cells in a Contact Dependent Manner

M1 and M2 cells were polarized with the use of LPS/IFN-γ and IL-4/IL-13, respectively, for 24 hours respectively. Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit (Stemcell Technologies). NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. Macrophages were added to the NK cells for 20 hours of co-culture in a 24-well plate in contact or under transwell conditions (0.4µm). This experiment was repeated two times with the same trends observed when NK cells were placed on the basolateral or apical sides. (A) CD27, based on a DX5+ CD3- F4/80- population, was analyzed by FACS and (B) quantified (n=2).

3.2 NK CELL FUNCTION IS REDUCED AFTER CONTACT WITH TAMs

3.2.1 Isolation of Tumor Associated Macrophages from pyMT Mice

Alternatively activated macrophages are similar to tumor associated macrophages as they both display immunosuppressive properties and have been shown to promote tumor growth (Mantovani, Sozzani et al. 2002). Therefore, they are a good model to use to begin to delineate the mechanism by which macrophages in the tumor may impact NK cells. However, they are stimulated in an environment with only two factors present (IL-4 and IL-13). Therefore, this *in vitro* environment does not represent the much more complex tumor microenvironment. This prompted us to examine how tumor associated macrophages impact NK cell function and whether an altered NK cell phenotype exists similar to the effect of alternatively activated macrophages. First we had to ensure that we could successfully isolate TAMs from pyMT mice. Tumors were digested, and filtered to eliminate debris and produce a single cell suspension. Tumor associated macrophages were isolated using a CD11b+ selection kit. Flow analysis was conducted on the isolated cells to ensure the purity of macrophages used for subsequent assays. Percentages of CD11b+, F4/80+, and Gr-1- cells were analyzed by flow cytometry. It was imperative that macrophage purity be high as it would be difficult to assess the true impact of TAMs on NK cells with any contamination of suppressive tumor cells. In all tumors isolated, the purity of the CD11b+ cells was between 80-90% (Figure 6). In addition, approximately 85% of the CD11b+ cells were F4/80+ Gr-1- macrophages. This also confirmed the idea that the majority of the CD11b+ cells within the tumors are macrophages and not dendritic cells (DCs), neutrophils, or myeloid derived suppressor cells (MDSCs).

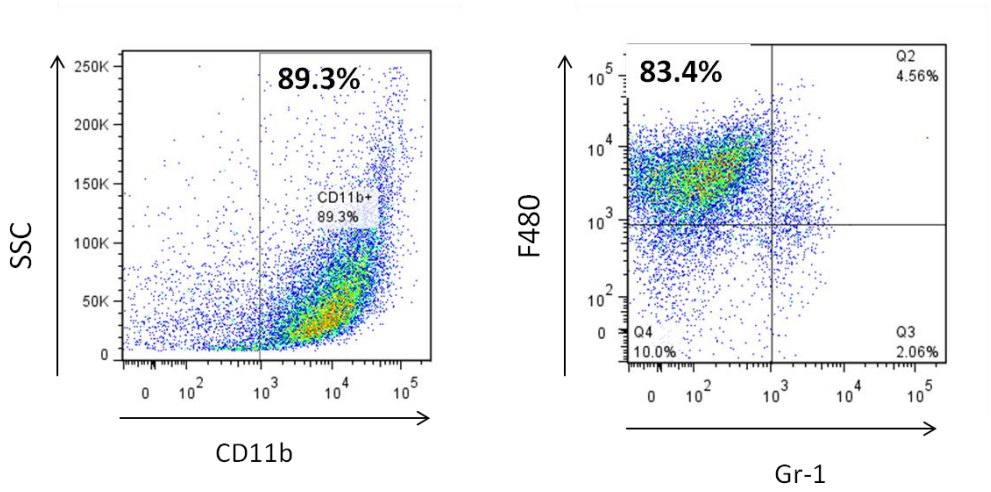

Figure 6

Isolation of Tumor Associated Macrophages

Tumors were isolated from pyMT mice, digested, and filtered to get rid of any debris. Macrophages were isolated using the CD11b+ selection kit (Stemcell Technologies). Flow analysis was conducted on the isolated cells to ensure the purity of macrophages used for subsequent assays. Percentages of CD11b+, F4/80+, and Gr-1- cells were analyzed by FACS. Figure reveals a representative flow plot of of the results observed from three pyMT mice.

3.2.2 TAMs Inhibit NK Cell CD27 Expression

In our lab, previous work using the pyMT tumor model has shown that NK cells isolated from these tumors express low levels of the NK cell activating receptor CD69 (Gillgrass, A – unpublished). The mechanism that mediates the suppression of these NK cells remains unknown. Once we were able to determine that isolation of CD11b+ cells from pyMT tumors resulted in a 90% pure macrophage population, we were then able to use these TAMs to determine the impact they have on NK cell CD27 expression. pyMT macrophages isolated with the CD11b+ selection kit were co-cultured at a 1:1 ratio with NK cells for 24 hours. The cells were then collected for flow staining and the NK cells were gated as DX5+ CD3- and the CD27+ population was analyzed based on high and low SSC (granularity). Similar to the results observed with alternatively activated macrophages, pyMT macrophages were able to suppress NK cell CD27 expression in comparison to IL-15 cultured NK cells (Figure 7). In particular the highly granular population which was present in IL-15 cultured NK cells was further decreased in the NK-TAM co-culture (from 15.3% to 7.3%). This experiment was repeated twice using macrophages isolated from the tumors of 6 pyMT mice with the same trend observed. Interestingly, in contrast to what was observed with the M2 co-culture, there was a CD27- NK cell population which was also highly granular when cultured with TAMs. The CD27-, but highly granular population was of particular interest to us as the phenotypic marker CD27, along with CD11b, has been used to depict stages of murine NK cell development.

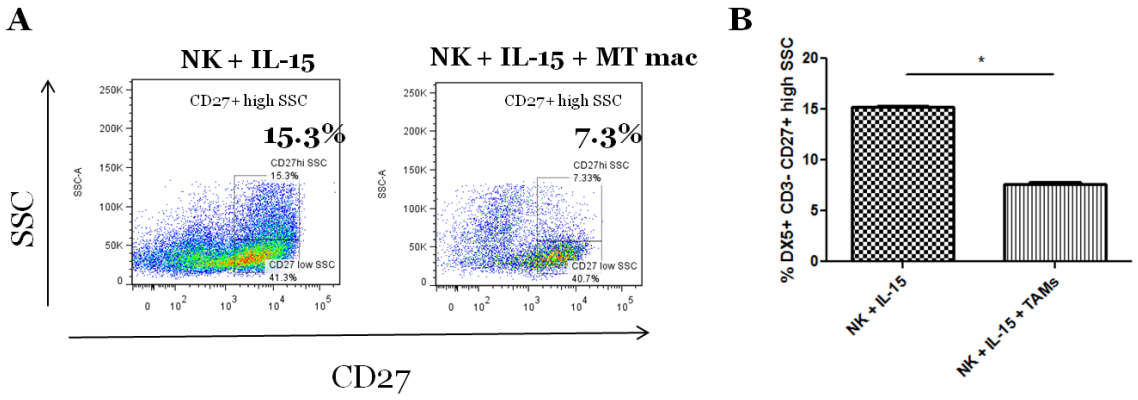

Figure 7

TAMs Inhibit IL-15 Activated NK Cell CD27 Expression

Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit. NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. Tumors were isolated from pyMT mice, processed, and macrophages were isolated using the CD11b+ isolation kit (Stemcell Technologies). Macrophages were added to the NK cells at a 1:1 ratio for 24 hours of co-culture upon which time the cells were stained for CD3 and CD27. (A) CD27, based on a DX5+ CD3- F4/80- population, was analyzed by FACS and (B) quantified. Repeated experiment twice for a total of 6 pyMT mice with the same trend. (Results were analyzed by student's t-test *$P<0.05$, n=3).

3.2.3 TAMs Inhibit NK Cell Killing Activity

Natural killer (NK) cells were first discovered due to their ability to kill tumor cells without any prior sensitization (Luci and Tomasello 2008). Therefore, a killing assay against tumor cells is the gold standard technique to examine NK cell functional activity. In addition to the phenotypic analysis of CD27 expression, we were also interested in determining if TAMs could decrease the ability of NK cells to kill target cells. The killing assay against YAC-1 cells, an NK cell target cell line, was described in section 3.1.3. In this scenario, CD11b+ cells were isolated from pyMT mice and added to NK cells for 24 hours of co-culture. CFSE was then used to label the target YAC-1 cells which were added to the culture of NK-TAMs for 4 hours. Subsequently, the cells were collected and stained for 7-AAD by adding 5µL of the antibody directly to the culture and analyzed immediately on the flow cytometer. Similar to previous results, incubation of NK cells cultured with IL-15 increased the percentage of 7AAD+ YAC-1 cells to approximately 20% (Figure 8). However, after co-culture with TAMs, NK cytotoxicity was decreased to 12%. As a control, TAMs were added to YAC-1 cells to ensure that they were unable to kill this cell line. The percentage of killing after addition of TAMs was not higher than the percentage of dead YAC-1 cells alone, indicating that YAC-1 cells are specific NK cell targets, and can be used for this purpose (data not shown). This outcome confirmed the idea that if macrophages from the tumor interact with NK cells, they are able to suppress their function, specifically killing, in order to favour tumor growth.

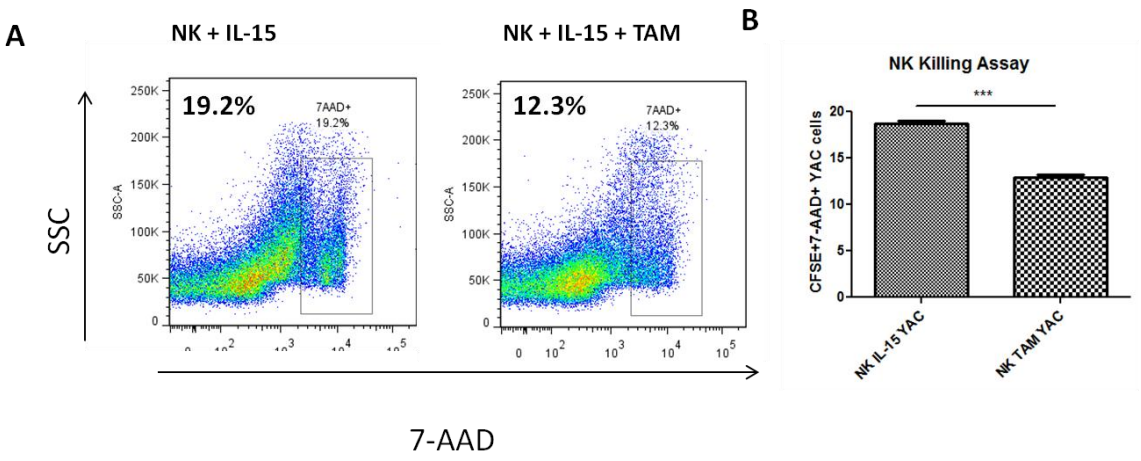

Figure 8

TAMs Inhibit NK Cell Killing Activity

Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5 positive selection kit. NK cells were cultured with 50ng/mL of IL-15 for 24 hours and subsequently washed thoroughly of the cytokine. Tumors were isolated from pyMT mice, processed, and macrophages were isolated using the CD11b+ isolation kit (Stem Cell Technologies). Macrophages were added to the NK cells at a 1:1 ratio for 24 hours of co-culture upon which time a 4 hour killing assay was performed on CFSE labelled YAC-1 target cells. (A) CFSE+7AAD+ YAC cells were analyzed by FACS and (B) quantified. (Results were analyzed by student's t-test *P<0.001, n=3).

3.3 NK CELLS IN CONTACT WITH TAMs DISPLAY A MATURE PHENOTYPE

3.3.1 Phenotypic Markers of Developing NK Cells

With the confirmation that NK cells have the capacity to be regulated by macrophages from the tumor, we wondered whether their developmental stage was altered after interaction with TAMs. Multiple stages of NK cell development have been proposed based on the phenotypic markers CD27 and CD11b. Immature NK cells are characterized as $CD11b^{low}CD27^{low}$ and are mainly found in the bone marrow and lymph nodes (Chiossone, Chaix et al. 2009). $CD11b^{high}$ NK cells are characterized as mature NK cells and occupy peripheral tissues. These cells can be further divided into $CD11b^{high}CD27^{high}$ cells which are found to be the most potent effector cells and $CD11b^{high}CD27^{low}$ cells which express Ly49 receptors at the highest frequency (Chiossone, Chaix et al. 2009). In contrast, other papers argue that the most mature subset, the $CD11b^{high}CD27^{low}$ population, exhibits the highest cytolytic function and therefore their activity is still up for debate (Fu, Wang et al. 2011). Other important markers of developing NK cells include CD122 and NKG2D, which are representitive of NK cell precursors (as they lack all other markers). Subsequently, immature NK cells gain NK1.1 expression and are also characterized by their transient expression of TRAIL. Immature NK cells are also DX5- and $CD11b^{low}$. These markers are gained as NK cells become mature (Di Santo 2006). The main stages of NK cell development are reviewed in Figure 9.

NK Precursors	Immature NK Cells	Mature NK Cells	Exhausted NK Cells?
CD122+	NK1.1+	NK1.1+	NK1.1+
NKG2D+	TRAIL+	DX5+	DX5+
	$CD11b^{low}$	$CD11b^{high}$	$CD11b^{high}$
		$CD27^{high}$	$CD27^{low}$

Figure 9

Phenotypic Markers of Developing NK Cells

NK cell development has been identified with the use of multiple cell surface markers. NK cell precursors are found in the bone marrow and express the markers CD122 and NKG2D (lack all other markers). Immature NK cells gain NK1.1 expression and are also characterized by their transient expression of TRAIL. Immature NK cells are also $CD11b^{low}$ and DX5-. These markers are gained as NK cells home to peripheral tissues, become mature, and gain the acquisition of CD27 expression and cytotoxic function. Exhausted NK cells are characterized as $CD11b^{high}$ but $CD27^{low}$. The develomental status and mechanism of maturation of NK cells within tumors is unknown.

3.3.2 NK Cells in Contact with TAMs Express a $CD27^{low}CD11b^{high}$ Phenotype

Due to the observation that NK cells that are in co-culture with TAMs become highly granular and are CD27- (Figure 7), we became interested in determining the NK cell developmental phenotype by examining the expression of both CD27 and CD11b simultaneously. pyMT macrophages isolated with the CD11b+ selection kit were co-cultured at a 1:1 ratio with NK cells for 24 hours. The cells were then collected for flow staining and the NK cells were gated as DX5+ CD3- and subsequently the CD27 and CD11b population were analyzed accordingly. After the interaction of NK cells with TAMs, NK cells exhibited a $CD27^{low}CD11b^{high}$ developmental phenotype (Figure 10). Furthermore, the histogram in Figure 10c shows that overall CD27 expression is decreased on NK cells after interaction with TAMs. To evaluate whether inhibition of NK cells by TAMs was contact dependent, a transwell assay was performed. TAMs were added to NK cells either in transwell (0.4μm) or in contact for 24 hours. It was confirmed that the inhibition of NK cells occurred in a contact dependent manner. To ensure that the $CD27^{low}CD11b^{high}$ population was not a FACS phenomenon due to the addition of F4/80+ macrophages, I only gated on F4/80- cells. It is unclear if this population is displaying an exhausted phenotype as described in the paper by Chiossone et al., but our data would suggest that it is due to the fact that this population of NK cells displays decreased killing ability in Figure 8 (Chiossone, Chaix et al. 2009). Since NK cells are crucial in the anti-tumor immune response, the regulation of these cells by TAMs alters their anti-tumoral role and has the potential to inhibit their function within a tumor.

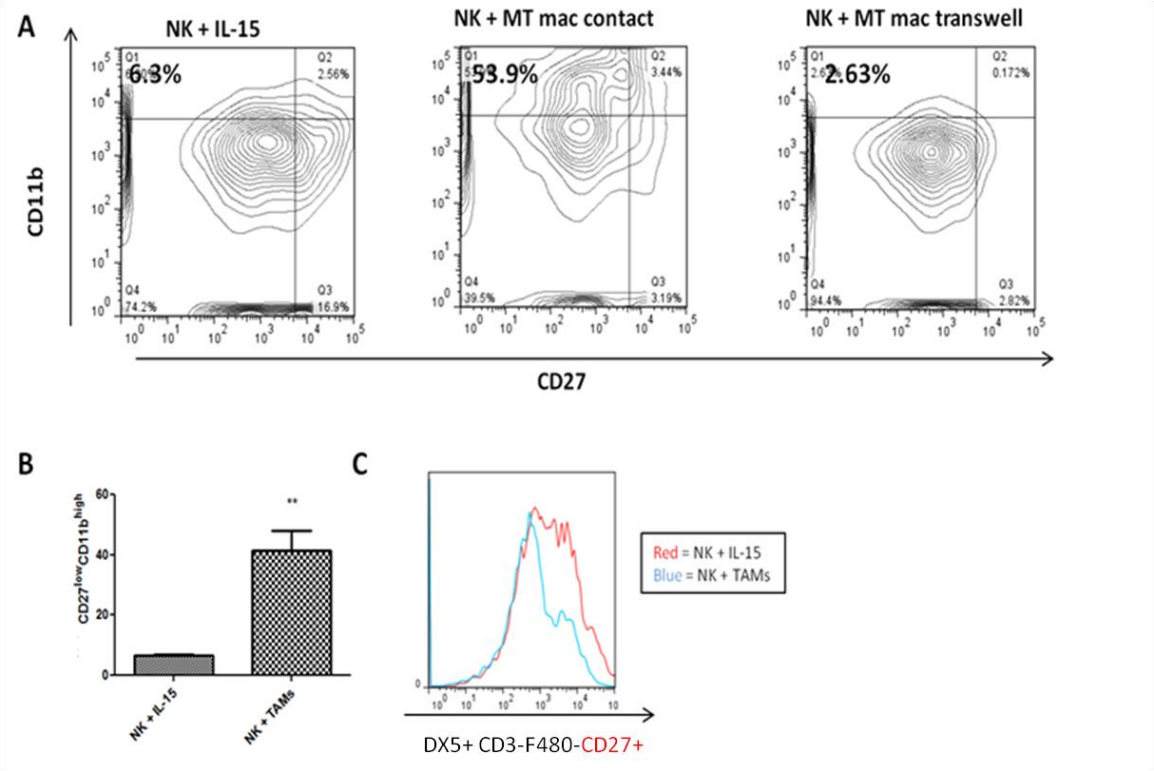

Figure 10

NK Cells in Co-culture with TAMs Express a $CD27^{low}CD11b^{high}$ Phenotype in Contact

Splenic NK cells were isolated from C57BL/6 mice with the use of the DX5+ selection kit. TAMs, isolated from pyMT tumors with a CD11b+ selection kit, were added to the NK cells at a 1:1 ratio for 24 hours of co-culture upon which time cells were stained for F4/80, CD3, CD27, and CD11b (A) CD27 and CD11b, based on a DX5+ CD3- F4/80- population, were analyzed by FACS. The third panel shows macrophages and NK cells under transwell conditions. (B) The results were quantified. Repeated experiment twice for a total of 6 pyMT mice in the NK + MT contact condition (C) The histogram shows the percentage of cells that are DX5+ F4/80- CD27+. Red represents NK + IL-15 while blue represents NK + TAMs. (Results were analyzed by student's t-test **P<0.01, n=3)

3.4 NK CELLS FROM pyMT TUMORS DISPLAY AN IMMATURE PHENOTYPE IN COMPARISON TO SPLENIC NK CELLS

3.4.1 NK Cells from pyMT Tumors Display an Immature $CD27^{low}CD11b^{low}$ Phenotype in Comparison to Splenic NK Cells

Examining NK cell markers *in vitro* revealed that co-culture of TAMs with NK cells lowered their expression of CD27, decreased cytotoxicity, and advanced their developmental stage to a $CD27^{low}CD11b^{high}$ phenotype. We wondered if the developmental stage we examined in our *in vitro* model matched the developmental stage of NK cells that infiltrate pyMT tumors. It was previously shown, in different subcutaneous tumor models, that the maturation of NK cells is halted at the final stage of NK cell development selectively in the bone marrow (Richards, Chang et al. 2006). However, the developmental stage of NK cells within tumors has not been evaluated. To analyze the differences between intratumoral NK cells and splenic NK cells, tumors and spleens were isolated from pyMT mice near endpoint. The tumors were digested, processed into a single cell suspension, and stained for CD45, NK1.1, CD3, CD27, and CD11b. NK cells were gated as CD45+ NK1.1+ CD3- (Figure 11). It was found that the phenotype of intratumoral NK cells is quite different from that of splenic NK cells. The majority of NK cells in the tumor exhibit an immature phenotype ($CD27^{low}CD11b^{low}$ – 60-70%) in contrast to the spleen where only 10% of NK cells are immature. In contrast, splenic NK cells are positive for markers of activation (CD27) and maturation (CD11b). The histogram (Figure 11b) also shows that overall expression of the cytotoxicity marker CD27 is decreased in NK cells from tumors compared to spleens.

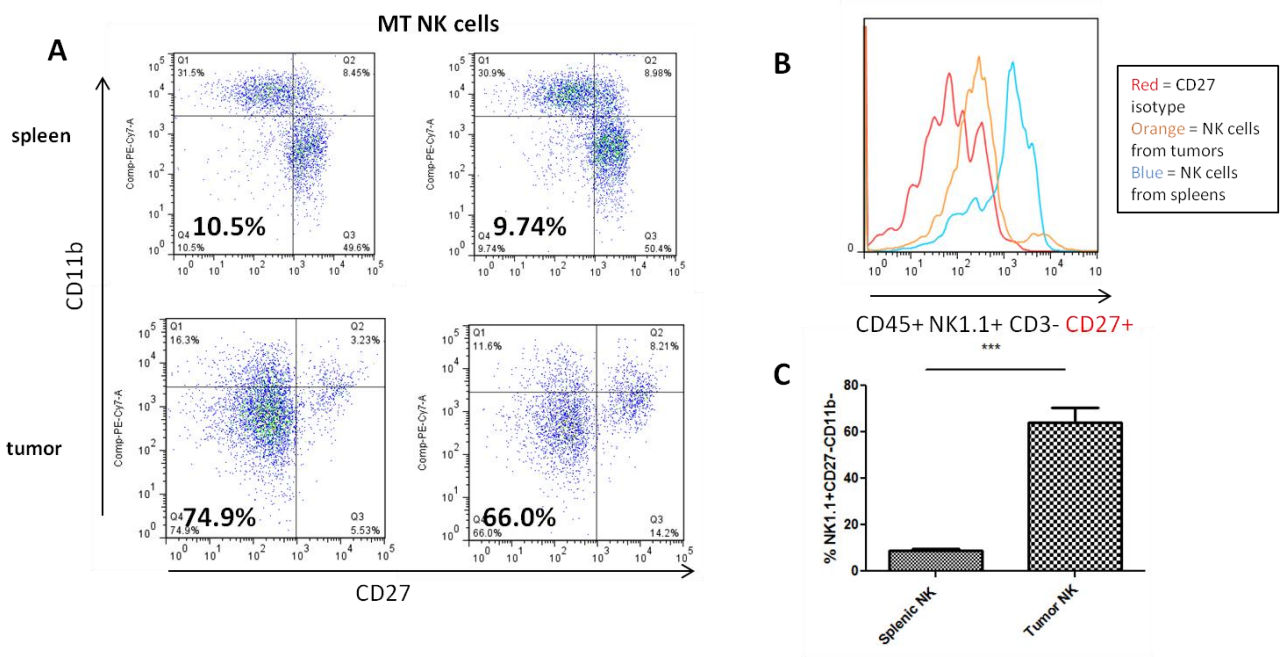

Figure 11

NK Cells from pyMT Tumors Display an Immature CD27^{low}CD11b^{low} Phenotype

Tumors (isolated from pyMT mice near endpoint) and spleens were isolated from pyMT mice, processed, and stained for CD45, NK1.1, CD3, CD27, and CD11b. Analysis was conducted on 5 mice. (A) CD11b and CD27, based on a CD45+ NK1.1+ CD3- population, were analyzed by FACS and (C) quantified. (Results were analyzed by student's t-test ***P<0.001, n=3). (B) The histogram shows the percentage of cells that are CD45+ NK1.1+ CD3- CD27+. Red represents a CD27 isotype control, orange represents CD27+ NK cells from tumors and blue represents CD27+ NK cells from spleens.

3.4.2 NK Cells from pyMT Tumors Display an Immature DX5- CD11b- Phenotype in Comparison to Splenic NK Cells

In addition to CD27 and CD11b, NK cell maturation can be defined by the acquisition of DX5 (Di Santo 2006). Therefore, in a separate experiment, to corroborate the developmental stage of intratumoral NK cells, tumors and spleens were isolated from pyMT mice near endpoint. The tumors were digested and processed into a single cell suspension, and stained for CD45, NK1.1, CD3, DX5, and CD11b. NK cells were gated as CD45+ NK1.1+ CD3- and analyzed using a quadrant plot for DX5 and CD11b (Figure 12). The expression of DX5+CD11b+ NK cells was greatly reduced on intratumoral NK cells (10%) in contrast to those from the spleen of the same mice (50%). This was further evidence that NK cells which are located in the tumor are of an immature phenotype. Whether they are recruited to the tumor as immature or are altered by their microenvironment is currently unknown. On the other hand, peripheral NK cells in the spleens of tumor bearing animals are not altered in the same way and maintain their mature activated status.

The phenotype of intratumoral NK cells is quite different from that observed after interaction of NK cells with TAMs *in vitro*. Although both observed phenotypes exhibited low levels of CD27 expression, intratumoral NK cells exhibited an immature phenotype *in vivo*, while the cells *in vitro* exhibited a mature phenotype. Therefore, the interaction of TAMs with NK cells in the tumor might not necessarily alter their developmental stage, but it does have the ability suppress their function, a characteristic of tumor associated NK cells.

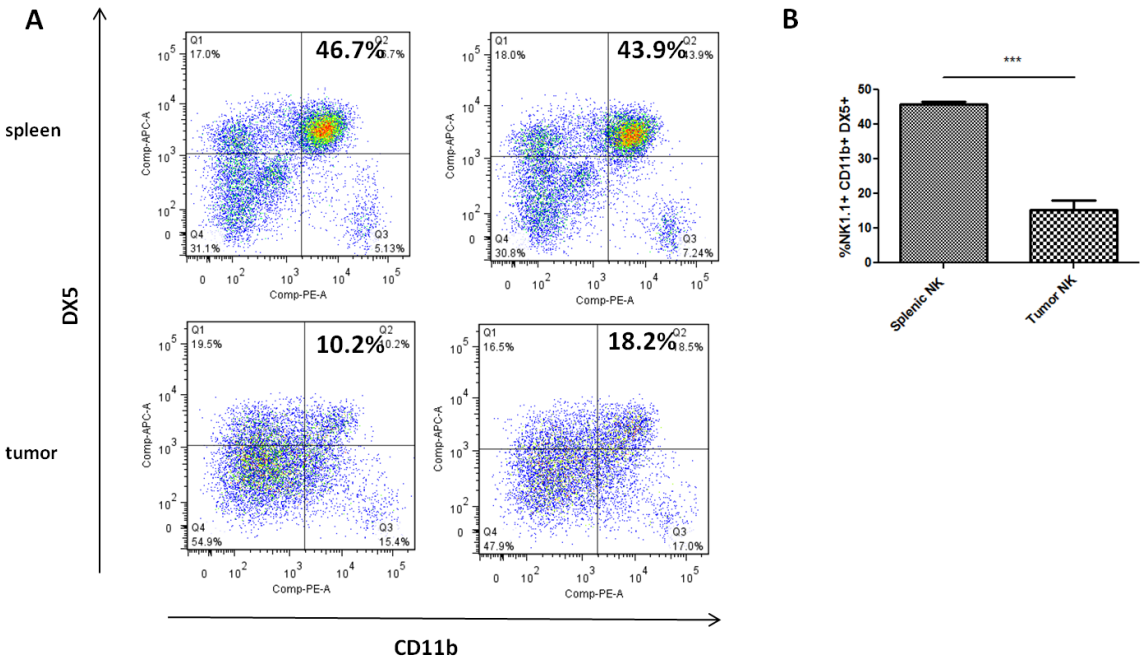

Figure 12

NK Cells from pyMT Tumors Display an Immature DX5- CD11b-Phenotype

Tumors and spleens were isolated from pyMT mice, processed, and stained for CD45, NK1.1, CD3, DX5, and CD11b. Analysis was conducted on 3 mice. (A) CD11b and DX5, based on a CD45+ NK1.1+ CD3- population, were analyzed by FACS and (B) quantified. (Results were analyzed by student's t-test ***P<0.001, n=3).

3.4.3 NK Cells from pyMT Tumors Have Low Expression of Perforin and Intermediate Expression of Granzyme

It has been shown that an increase in CD11b expression on NK cells increases IFN-γ production and cytotoxicity against NK cell targets (Kim, Iizuka et al. 2002). We wondered whether the developmentally immature NK cells we observed in the tumor were also non-functional. To answer this question we examined functional NK cell markers (perforin and granzyme) in both tumors and spleens of pyMT mice. NK cell killing of tumor cells occurs through their release of cytotoxic granules containing perforin and granzyme B. Once released, these granules perforate the target cell and cause apoptosis (Fehniger, Cai et al. 2007). Tumors and spleens were isolated from pyMT mice, processed, and stained for CD45, NK1.1, and CD3 extracellularly and perforin and granzyme intracellularly. NK cells were gated as CD45+ NK1.1+ and CD3-. Splenic NK cells, which were shown to be mature in Figures 11 and 12, also expressed high levels of perforin and granzyme B in Figure 13. On the other hand, immature intratumoral NK cells expressed these markers at a lower frequency. In particular, perforin expression was very low – only found in 10% of NK cells in tumors. Flow analysis of granzyme revealed that it was expressed at an intermediate level (50%) in intratumoral NK cells. Separate FMOs were created to draw the gates on intratumoral and splenic NK cells individually, as the autofluorescence from the tumor cells is quite high and can alter the gating strategy used in the flow analysis.

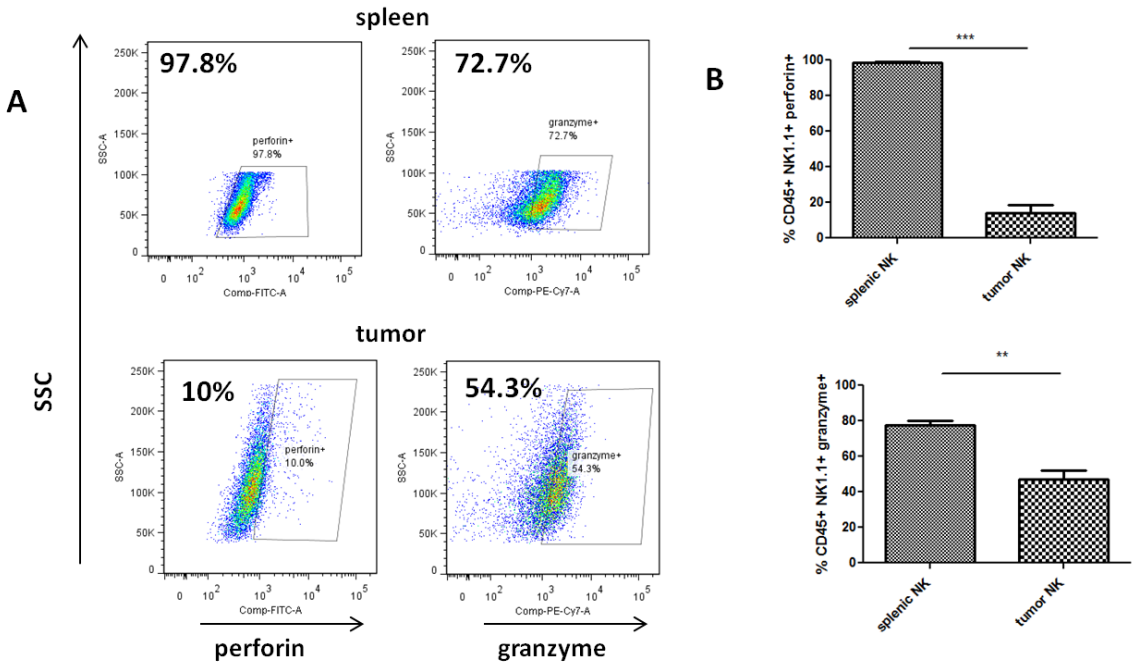

Figure 13

NK Cells from pyMT Tumors Have Low Expression of Perforin and Intermediate Expression of Granzyme

Tumors and spleens were isolated from pyMT mice, processed, and stained for CD45, NK1.1, and CD3 extracellularly and perforin and granzyme intracellularly. Analysis was conducted on 3 mice. (A) Perforin and granzyme, based on a CD45+ NK1.1+ CD3- population, were analyzed by FACS and (B) quantified. (Results were analyzed by student's t-test **P<0.01, ***P<0.001, n=3).

3.4.4 Immature NK Cells Express Low Levels of NKp46

The ability of NK cells to kill their target cells is controlled by their expression of activating and inhibitory receptors. If a target cell such as a cancer cell up-regulates more ligands for activating receptors than inhibitory receptors, then it will become prone to attack by NK cells. Some of the major activating NK cell receptors include: NKp30, NKp44, NKp46, and NKG2D, part of the natural cytotoxicity receptor (NCR) family (Waldhauer and Steinle 2008). Work on human NK cells has shown that human cervical tumors express decreased expression of NKp46 and NKp30, and as a result, are less cytotoxic displaying a reduced ability to produce IFN-γ (Garcia-Iglesias, Del Toro-Arreola et al. 2009). Our interest in examining the activating receptor NKp46 on tumor infiltrating NK cells was two-fold. We first wanted to ensure that the cells we were immunophenotyping from pyMT tumors were NK cells and not another cell type expressing the marker NK1.1. Although we had analyzed these cells by flow cytometry as NK1.1+CD3-, they were still $DX5^{low}$. Utilizing another NK cell marker such as NKp46 would help to confirm that these were indeed NK cells. The second was to examine if the immature DX5-CD11b- population displayed differential expression of the activating receptor NKp46 in comparison to the DX5+CD11b+ mature population.

Tumors and spleens were isolated from pyMT mice, processed, and stained for CD45, NK1.1, CD3, DX5, CD11b, and NKp46. NK cells were gated as CD45+ NK1.1+ and CD3-. A similar developmental phenotype was observed in this experiment as was noted in Figure 12. NK cells which infiltrate pyMT tumors are developmentally immature compared to their spleen counterparts. The spleen isolated from a tumor bearing mouse

displayed a consistent population of mature DX5+CD11b+ NK cells which was decreased on the NK cells isolated from pyMT tumors (from 33% to 12% respectively) (Figure 14). Gating on the mature NK cell population in the spleen revealed that the majority of these cells express the activating receptor NKp46 (92%). The level of NKp46 on the immature population (DX5-CD11b-) from the spleen, however, was drastically diminished (15%). Therefore, we concluded that an increase in NK cell development corresponds to an increase in the ability of these cells to express the activation receptor NKp46, an important factor in tumor cell clearance. In the context of a tumor, however, even the mature DX5+CD11b+ population displayed a decreased level of NKp46 in comparison to the mature NK cells found within the spleen. The same trend has been observed in both environments: the more immature the NK cell is, the less activated or functional it is. In the tumor this was seen as a decrease in NKp46 levels from 65% (on mature NK cells) to 24% (on immature NK cells).

We also wanted to determine if these cells were true NK cells and not a flow cytometry phenomenon, representing a cell type that is present in tumors that expresses NK1.1, but not elsewhere. To do this we used additional markers for phneotyping NK cells by flow analysis, such as NKp46. Although the cells isolated from pyMT tumors expressed NKp46 at differing levels, both the immature population as well as the mature population was NKp46 positive. This provided further proof that the cells we were analyzing as developmentally immature in pyMT tumors were indeed NK cells.

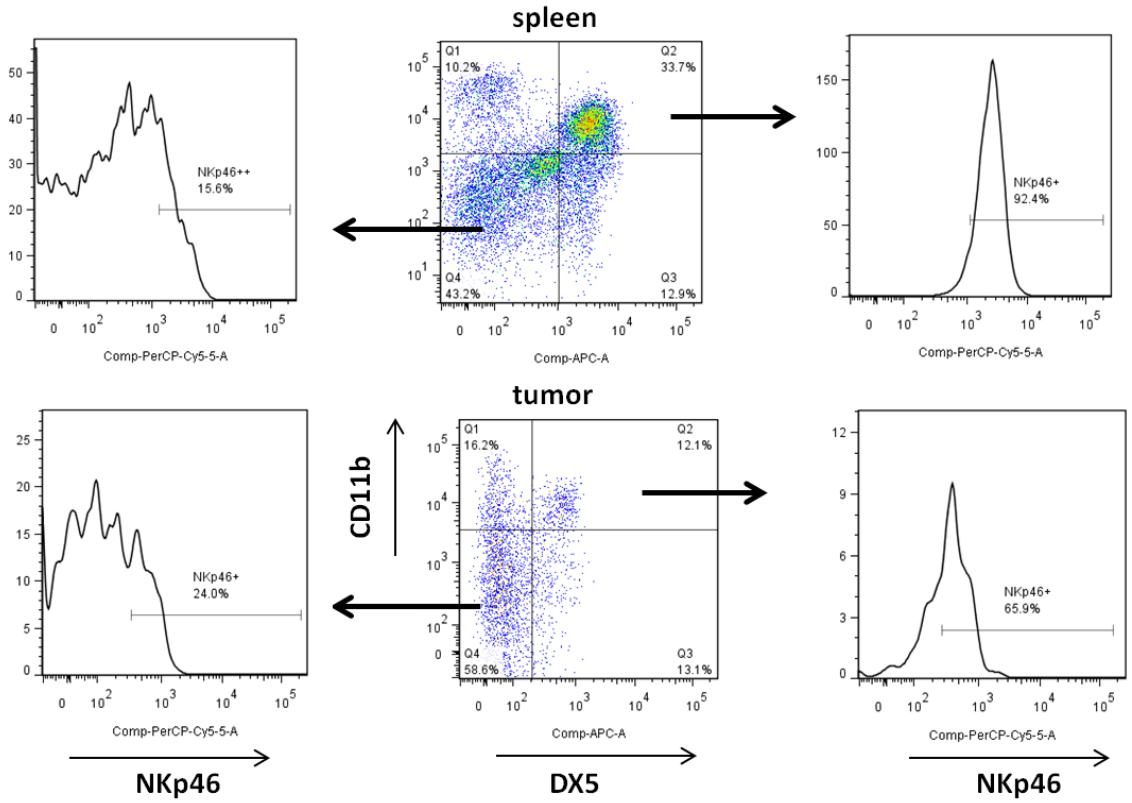

Figure 14

Immature DX5-CD11b- NK Cells from pyMT Tumors Have Low Expression of NKp46 in comparison to Mature DX5+CD11b+ NK Cells

Tumors and spleens were isolated from pyMT mice, processed, and stained for CD45 , NK1.1, CD3, and NKp46. NK cells were gated as CD45+ NK1.1+ CD3-. Subsequent analysis of NKp46 expression was conducted on either the mature DX5+CD11b+ or immature DX5-CD11b- phenotype. This experiment was repeated with the same results observed.

3.5 CHARACTERIZATION OF TUMOR ASSOCIATED NK CELLS

3.5.1 H&E Stain of NK1.1+ Cells Isolated from pyMT Tumors

All of the previous data indicated that NK cells at the tumor site are developmentally immature and do not express perforin, granzyme, and NKp46 at a high frequency. This data was acquired by flow cytometry assays and the NK1.1+ CD3- cells analyzed from the tumor were $DX5^{low}$ and $perforin^{low}$. Therefore, we wondered if they could be extraneous cells in the tumor environment that are not NK cells, but express NK1.1. Since tumors are known to have quite heterogeneous populations of cells with numerous markers being shared by multiple immune cell types, we wanted to guarantee that the cells we were examining by flow analysis were true NK cells.

pyMT tumors were isolated, digested, and processed into a single cell suspension. Cells were then re-suspended at a concentration of 100×10^6cells/mL (in 2% FBS PBS) and a PE selection kit was used with the addition of a PE-conjugated NK1.1 antibody. Four magnetic separations were used to isolate the cell type of interest and care was taken to wash the cells of any contaminants. 5.0×10^4 cells were cytospun onto a slide, fixed, and stained with H&E. Analysis of the slides on a Leica Microscope at 40x revealed that the cells resembled NK cells and were quite granular, with variations in size from 10-20μm (Figure 15). Therefore, we were able to confidently conclude that the results from our developental analysis (Figures 11-13) involved the phenotyping of true NK cells, not extraneous cells simply expressing NK1.1

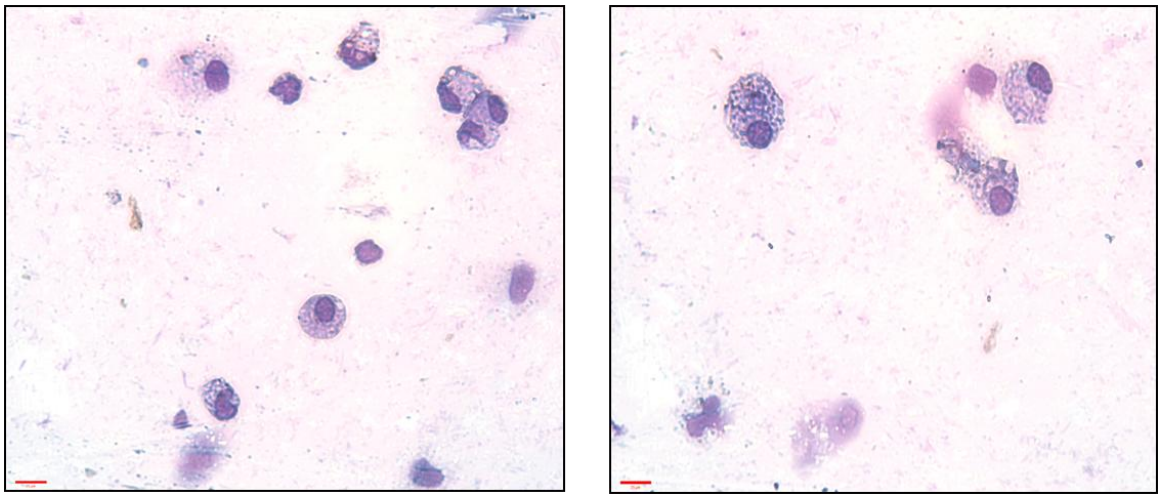

Figure 15

H&E Stain of NK1.1+ Cells Isolated from pyMT Tumors

Tumors were isolated from pyMT mice, processed, and NK cells were retrieved using a PE Selection Kit for NK1.1+ cells (Stem Cell Technologies, Vancouver, BC). NK cells were stained with H&E and images were taken on a Leica Microscope at 40x. Scale Bar = 25µm. The images display two representative areas on the slide.

3.5.2 Characterization of NK Cells from Tumors and Spleens of pyMT Mice Using a Cytokine and Chemokine PCR Array

Considering that we saw such a distinct developmental phenotype when examining tumor associated NK cells from pyMT mice in comparison to splenic NK cells from the same mice, this prompted us to explore the gene expression differences between these two subsets. We used a 96-well PCR array for analyzing the expression of a particular panel of genes (SABiosciences). In our case, we were interested in the cytokine and chemokine profile of genes induced in NK cells from pyMT tumors.

pyMT tumors and spleens were isolated, digested, and processed into a single cell suspension. Cells were then re-suspended at a concentration of 100 x 10^6cells/mL (in 2% FBS PBS) and a PE selection kit was used with the addition of a PE-conjugated NK1.1 antibody. Six magnetic separations were applied to isolate the cell type of interest and care was taken to wash the cells of any contaminants. Flow analysis was conducted on the isolated cells to ensure a high purity of NK cells used for subsequent assays (Figure 16a). After isolation, 90% of the cells expressed the marker NK1.1, and we proceeded to perform the RNA isolation and subsequent real-time PCR for the 96-well Cytokine and Chemokine PCR array (SABiosciences). Data analysis was performed using an excel-based program provided online by SABiosciences. The analysis was based on the ΔΔCt method with data normalized to a number of housekeeping genes including beta actin and GAPDH. Figure 16b shows the array data represented as a scatter plot revealing genes that were up- and down-regulated on the PCR array between the test sample (pyMT tumor NK cells) and the control sample (splenic NK cells). We observed that many genes

were altered between the two samples. To take a closer look at which cytokines changed expression, we graphed the data in Figure 16c. Immunoregulatory cytokines such as IL-10 and TGFβ2 were up-regulated on the array while immunostimulatory cytokines such as IL-12, IL-18, IFN-γ, and IL-2 were down-regulated. This correlates well with a paper published describing the expression of type I (IFN-γ) and type II (IL-13) cytokines at distinct stages of NK cell maturation (Loza, Zamai et al. 2002). For instance, IFN-γ is a factor that is acquired late during NK cell development and is therefore not highly expressed on immature NK cells.

It should be noted that since 90% of the cells isolated and used for RNA isolation were PE+, the remaining 10% were most likely tumor cells. Conducting a separate NK1.1+ isolation on pyMT tumors revealed that approximately 10-15% of the isolated cells expressed the epithelial marker EpCam (data not shown). Not only were many cytokines altered on the array, many growth factors and chemokines were up-regulated on intratumoral NK cells in comparison to splenic NK cells. This is likely a result of tumor cell contamination. Therefore, it is hard to delineate which factors on the PCR array can be attributed to differences between the NK cells or from tumor cell contamination. Nonetheless, we proved that drastic differences exist between intratumoral NK cells and splenic NK cells in pyMT mice.

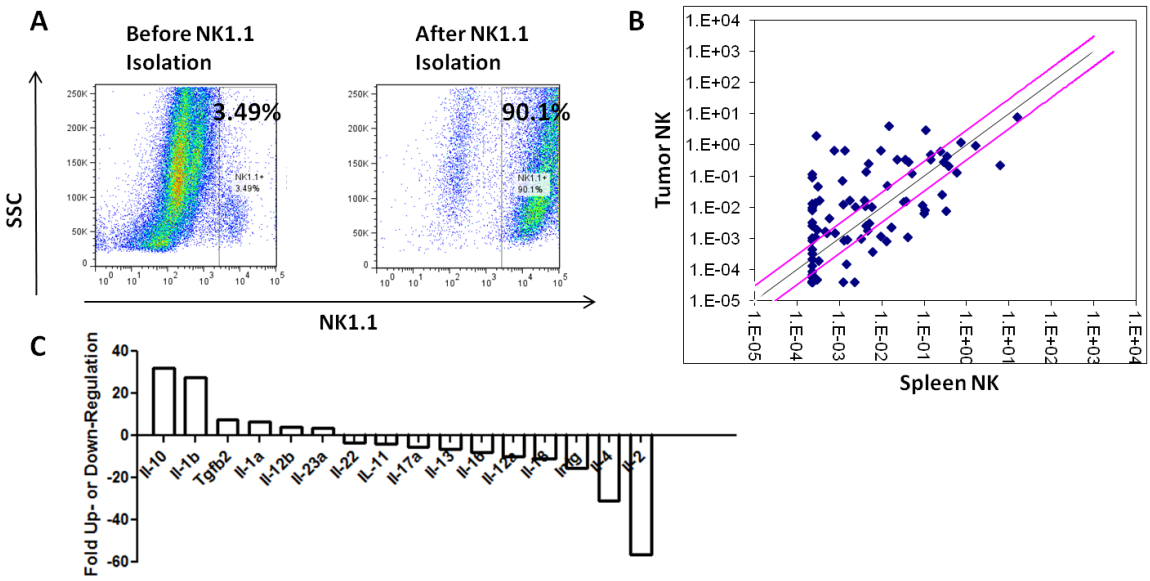

Figure 16

Gene Expression Data: NK Cells from Tumors and Spleens of pyMT Mice

Tumors and spleens were isolated from pyMT mice, processed, and NK cells were retrieved using a PE Selection Kit for NK1.1+ cells (Stem Cell Technologies, Vancouver, BC). 1 x10^6 NK cells were used for isolation of RNA and 0.5µg of RNA was used for cDNA synthesis using the RT^2 First Strand Kit. Real-time PCR was performed on a 96-well Cytokine and Chemokine PCR array. (A) FACS analysis - purity of PE selection kit. (B) The graph show genes up- and down-regulated on the PCR array in the form of a scatter plot. The differences between test (pyMT tumor NK cells)/control (splenic NK cells) are shown. (C) All of the cytokines on the array are graphed in C (n=1).

3.6 TREATMENT OF SUBCUTANEOUS pyMT TUMORS WITH IL-12 AND anti-TGF-β PROLONGS SURVIVAL

3.6.1 Treatment of Subcutaneous pyMT Tumors with IL-12 and anti-TGF-β

The importance of NK cells in the pyMT tumor model as effector cells that control tumor growth has been previously determined by our lab (Gillgrass, A - unpublished). Using the NK1.1 depleting antibody in the subcutaneous pyMT tumor, we have shown that this regimen decreases survival and the percentage of tumor free animals. Although NK cells are important in this model, they do not seem to fully control tumor growth. In order to be fully functional, NK cells need an environment that promotes their maturation. We aimed to change the tumor microenvironment in the pyMT model to one that would support NK cell maturation. In an environment which promotes tumor destruction and not growth, we hoped to alter the suppressive phenotype of pyMT NK cells. To our knowledge, the ability of NK cells to change their developmental stage within tumors after treatment has not been reported before.

In order to choose a treatment regimen that would alter the tumor microenvironment and shift the immune response towards tumor destruction, two factors were administered and/or targeted: IL-12 and TGF-β. We have shown that TGF-β expressed on M2 macrophages can down-regulate CD107a on NK cells (Figure 4). Therefore, inhibition of TGF-β will allow the cells to revert back to their activated state. In animal studies, it has been shown that use of an anti-TGF-β antibody restores NK cell activity and suppresses breast cancer metastasis (Arteaga, Hurd et al. 1993). Secondly administration of immunostimulatory factors would be vital to maximally activate NK

cells and alter the environment in which they reside. It has also been shown that IL-12 primes human NK cells to lyse tumor cells and induce DC maturation and macrophage polarization through the release of IFN-γ (Marcenaro, Della Chiesa et al. 2005). Previous treatment of tumors with IL-12 *in vivo* promoted anti-angiogenic activity and increased p53 activation, causing tumor suppression (Yuzhalin and Kutikhin 2012). We believe that the most potent therapy in modifying the tumor microenvironment and activating NK cells will involve a combination of the two factors (Figure 17).

The experiment was completed as follows: we began by injecting 6 week old C57BL/6 mice subcutaneously with 1 x 10^5 pyMT cells in the right flank. Tumor growth was monitored by palpation and when all tumors reached palpable sizes (2x2 - 4x4 mm), four different treatment regimens were started (5 mice per group) (Figure 17). A recent paper which described the major barriers regarding systemic administration of IL-12 and its inability to reach high enough local concentrations within tumors, led us to give intratumoral treatments in a total volume of 25µL (Kerkar, Goldszmid et al. 2011). The treatments included 50ng of IL-12, 50µg of anti-TGF-β, a combination of the two as well as a PBS control. Injections were administered three times a week and mice were subsequently monitored for endpoint (10x10mm). Combination treatment was associated with extended survival compared to PBS (** P<0.01s) and IL-12 alone (*P<0.05). Furthermore, IL-12 was associated with extended survival compared to PBS control (*P<0.05) (Figure 18). Combination treatment slowed the growth of subcutaneous pyMT tumors as measured by tumor volume over time but did not prevent tumor growth (Figure 19).

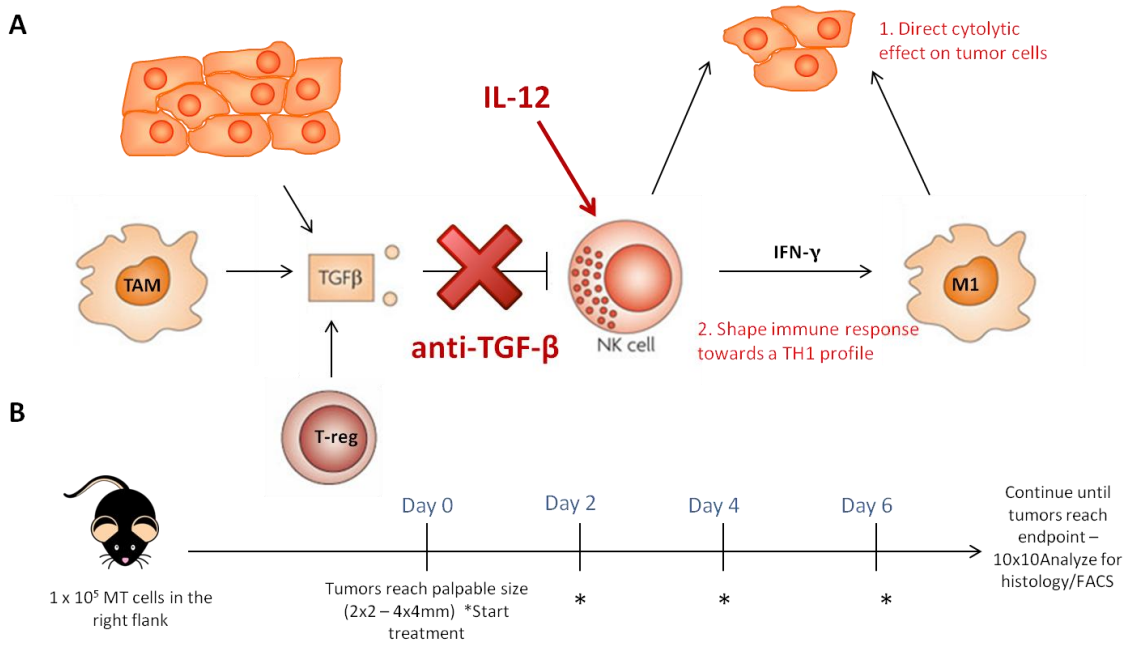

Figure 17

Treatment Schedule and Predicted Outcome of Intratumoral IL-12 and anti-TGF-β Treatment

In an effort to reverse the immature and non-cytotoxic phenotype of tumor associated NK cells a combination treatment of IL-12 and anti-TGF-β was chosen. IL-12 is a potent activator of NK cell function and administration of blocking antibodies against TGF-β could potentially inhibit the suppression of NK cells observed in pyMT tumors. (A) Diagram of expected changes to immune populations in the tumor after treatment (B) Treatment schedule of C57BL/6 mice subcutaneously injected with pyMT cells.

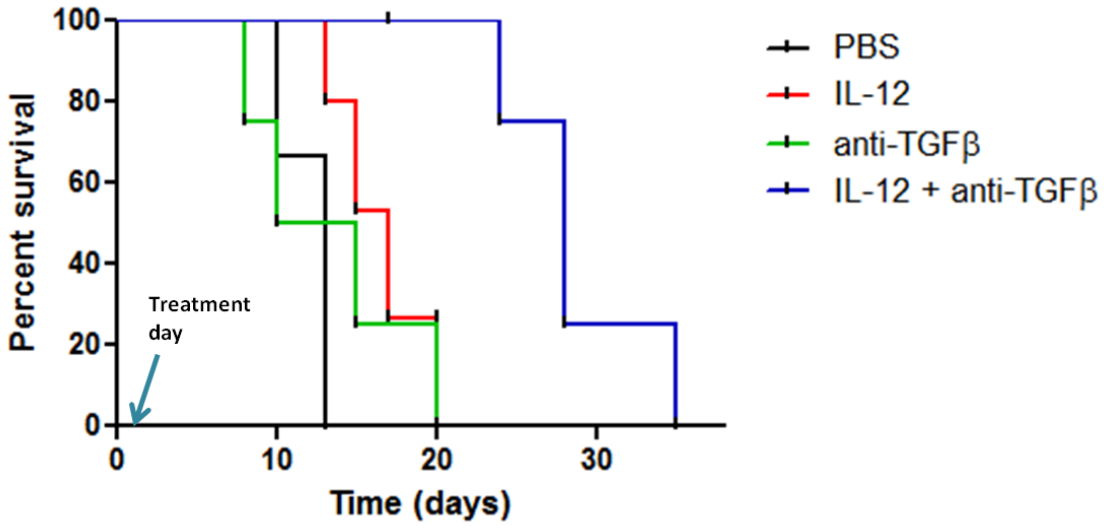

Figure 18

Combination Treatment with IL-12 and anti-TGF-β Prolongs Survival in a Subcutaneous pyMT Tumor Model

6 week old C57BL/6 mice were injected with 1 x 10^5 pyMT cells in the right flank. Tumor growth was monitored by palpation and when all tumors reached palpable sizes (2x2 - 4x4 mm), 4 different treatment regimens were started. The treatments included 50ng of IL-12, 50µg of anti-TGF-β, and a combination of the two as well as a PBS control. Injections were intratumoral in a total volume of 25µL administered three times a week. Mice were monitored for endpoint (10x10mm) and there were 5 mice per group. Combination treatment was associated with extended survival compared to PBS (** P<0.01s) and IL-12 alone (* P<0.05) and IL-12 was associated with extended survival compared to PBS (*P<0.05).

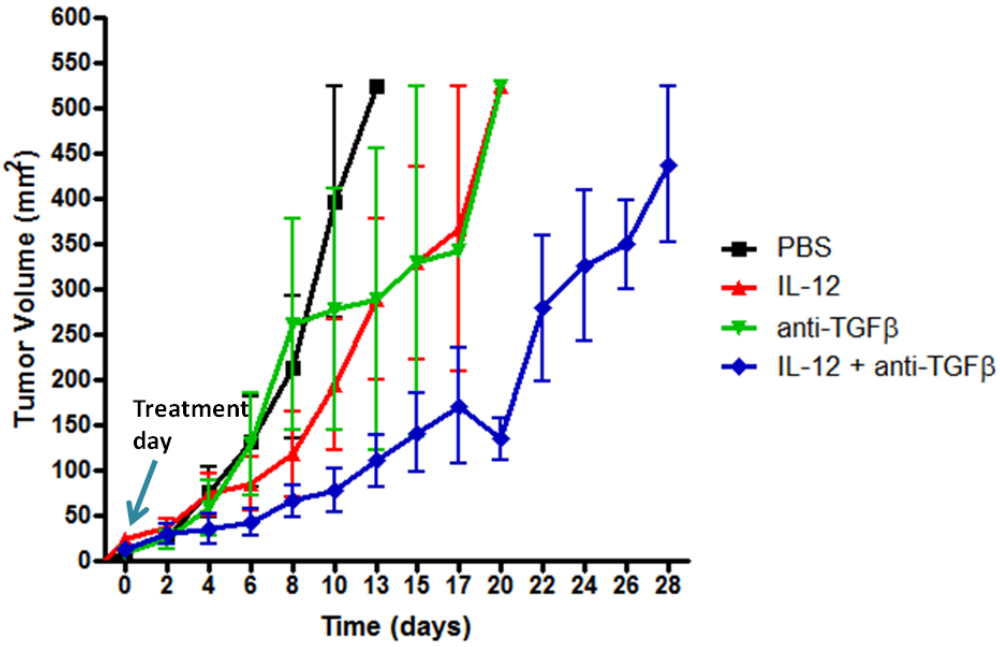

Figure 19

Combination Treatment with IL-12 and anti-TGF-β Slows Growth of Subcutaneous pyMT Tumors as Measured by Tumor Volume Over Time

6 week old C57BL/6 mice were injected with 1 x 10^5 pyMT cells in the right flank. Tumor growth was monitored by palpation and when all tumors reached palpable sizes (2x2 - 4x4 mm), 4 different treatment regimens were started. The treatments included 50ng of IL-12, 50µg of anti-TGF-β, and a combination of the two as well as a PBS control. Injections were intratumoral in a total volume of 25µL administered three times a week. Mice were monitored for endpoint (10x10mm) and tumor measurements were taken before every treatment. Three mice were used to calculate tumor volume in the PBS group and four in each other group. The data is not significant (results were analyzed by a one-way anova).

3.6.2 Combination Treatment Increases the Percentage of Lymphocytes but does not Change the NK Cell Phenotype in Subcutaneous pyMT Tumors

The goal of IL-12 and anti-TGF-β treatment was to modify the tumor microenvironment in the pyMT tumor and subsequently determine if the NK cells would mature and/or become activated in an altered environment. Mice with subcutaneous pyMT tumors were treated as described in Figure 17. At endpoint, the tumors were excised, digested, and stained for subsequent flow markers of immune cell infiltrate (Figure 20). The percentage of CD45+ cells in the tumors increased with combination treatment in relation to PBS control, indicating an increase in the infiltrating lymphocytes. In addition, although the percentage of macrophages (F4/80+ CD11b+ Gr-1-) in the tumors was not altered, the percentage of macrophages expressing the M1 marker MHC II was highly up-regulated on cells from tumors treated with IL-12 and the combination treatment in comparison to those treated with PBS or anti-TGF-β alone. Although the phenotype of the macrophages within the tumors had become M1 upon IL-12 and anti-TGF-β treatment, the phenotype and number of tumor infiltrating NK cells had not changed (data not shown). Perhaps the window of time during which the NK cells were highly activated and contributing to slow tumor growth occurred before endpoint and as the tumors got larger, the NK cells were once again suppressed. The treatment with the highest impact on survival in the subcutaneous pyMT model involved both inhibition of the immunoregulatory factor TGF-β and administration of the immunostimulatory factor, IL-12.

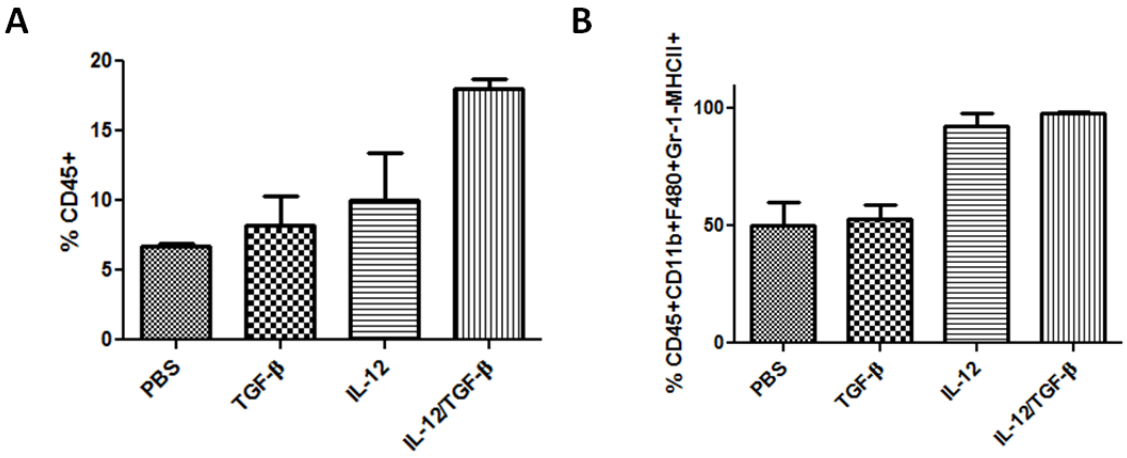

Figure 20

Combination Treatment with IL-12 and anti-TGF-β Alters the Phenotype of Infiltrating Immune Cells in a Subcutaneous pyMT Tumor Model

6 week old C57BL/6 mice were injected with 1 x 10^5 pyMT cells in the right flank. Tumor growth was monitored by palpation and when all tumors reached palpable sizes (2x2 - 4x4 mm), 4 different treatment regimens were started. The treatments included 50ng of IL-12, 50µg of anti-TGF-β, and a combination of the two as well as a PBS control. When mice reached endpoint (10x10mm), tumors were isolated for FACS analysis. (A) Percentage of CD45+ cells within the tumors (B) and the percentage of macrophages expressing the M1 marker MHC II (n=2).

3.7 TREATMENT OF SPONTENOUS pyMT TUMORS WITH IL-12 AND anti-TGF-β ALTERS THE PHENOTYPE OF TUMOR ASSOCIATED NK CELLS

3.7.1 Combination Treatment Promotes Maturation of NK cells in Spontaneous pyMT Tumors to Become $CD27^{low}CD11b^{high}$

To avoid the issue of NK cell suppression by large tumors at end point, we isolated NK cells from the spontaneous pyMT tumor model after three injections of IL-12 and anti-TGF-β. Two tumors from individual pyMT mice were injected with IL-12 and anti-TGF-β as well as a PBS control. pyMT mice were chosen for tumors between the sizes of 4x4mm and 6x6mm. Similar to the subcutaneous model, injections were intratumoral in a total volume of 25µL administered 3 times a week. At the end of the treatments, individual tumors were isolated from pyMT mice, processed, and stained for the markers CD45, CD3, NK1.1, CD27, and CD11b. The combination of IL-12 and anti-TGF-β was found to reverse the phenotype of intratumoral NK cells in the spontaneous pyMT model in comparison to the PBS control (Figure 21). Upon treatment, the NK cells became $CD27^{low}CD11b^{high}$, a characteristic of mature NK cells, in contrast to the immature phenotype observed in PBS injected mice. Although we did not continue treatment to follow the mice for survival, 3 intratumoral treatments were enough to alter the tumor microenvironment and drive the NK cells to a more mature status. We have also shown previously that the more mature an NK cell is the higher it expresses NKp46, a receptor important in tumor cell recognition (Figure 14). Future experiments should determine the levels of NKp46 before and after combination treatment to examine if an increase in maturation correlates with an increase in NKp46 expression.

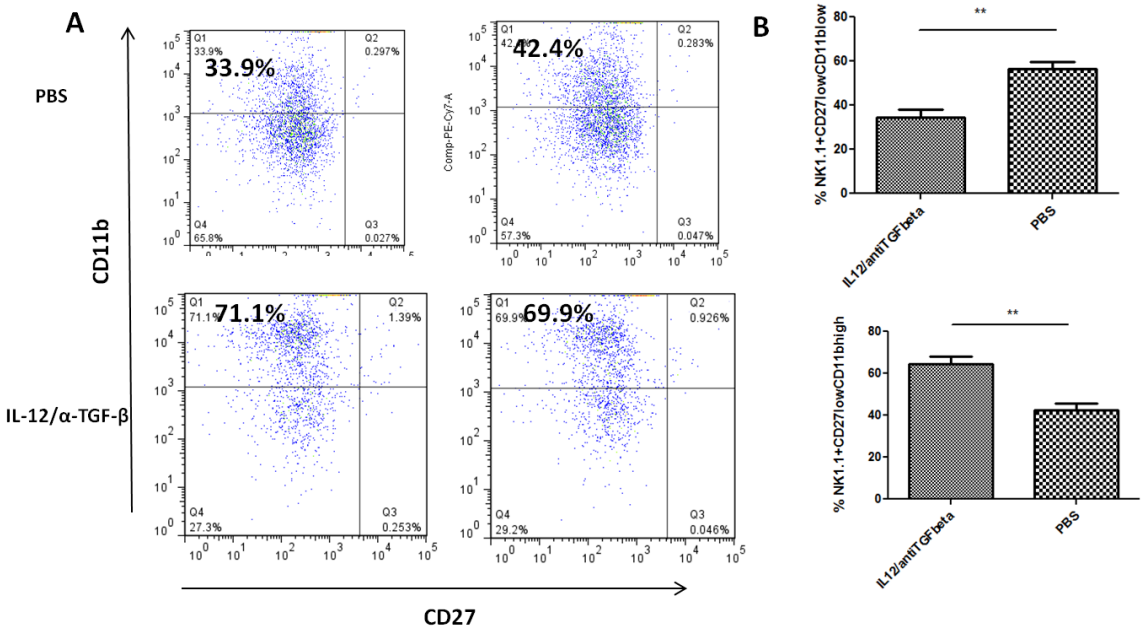

Figure 21

The Combination of IL-12 and anti-TGF-β Can Reverse the Phenotype of NK Cells in Spontaneous pyMT Tumors to Become Mature $CD27^{low}CD11b^{high}$ Cells

Two tumors from individual pyMT mice were injected with IL-12 and anti-TGF-β (50ng and 50µg respectively) or a PBS control. Injections were intratumoral in a total volume of 25µL administered three times a week. At the end of three treatments, individual tumors were isolated from pyMT mice, processed, and stained for the markers CD45, CD3, NK1.1, CD27, and CD11b. (A) CD27 and CD11b, based on a CD45+ NK1.1+ CD3- population, were analyzed by FACS and (B) quantified. (Results were analyzed by one-way ANOVA **P<0.01, n=3).

3.7.2 Combination Treatment Promotes Maturation of NK cells in Spontaneous pyMT Tumors to Become DX5+CD11b+

We were interested in examining if IL-12 and anti-TGF-β treatment could alter the tumor microenvironment in a pyMT tumor and subsequently promote NK cells to become DX5+. Four tumors from individual pyMT mice were injected with the combination IL-12 and anti-TGF-β as well as a PBS control. pyMT mice were chosen for tumors between the sizes of 4x4mm and 6x6mm. Similar to the subcutaneous model, injections were intratumoral in a total volume of 25μL administered three times a week. At the end of the three treatments, tumors and spleens were isolated from pyMT mice, processed, and stained for the markers CD45, CD3, NK1.1, DX5, and CD11b. Spleens were processed in order to verify if intratumoral treatments were local and only affected the tumor immune infiltrate or were systemic. There was an increase in the percentage of DX5+CD11b+ cells in the tumors after treatment compared to PBS control (12% to 25% respectively) (Figure 22). In addition, there was also an increase in the splenic NK cell population that expressed DX5 and CD11b from 15% to 30%. This led us to conclude that the intratumoral treatment of four tumors on one mouse creates both a local and systemic effect. If we could administer IL-12 and anti-TGF-β in the spontaneous pyMT tumor model continuously, we would hypothesize that it would extend survival, just as it had in the subcutaneous pyMT tumor model. The effect might be even more pronounced as the spontaneous pyMT tumors are not as aggressive and form naturally at a slower pace.

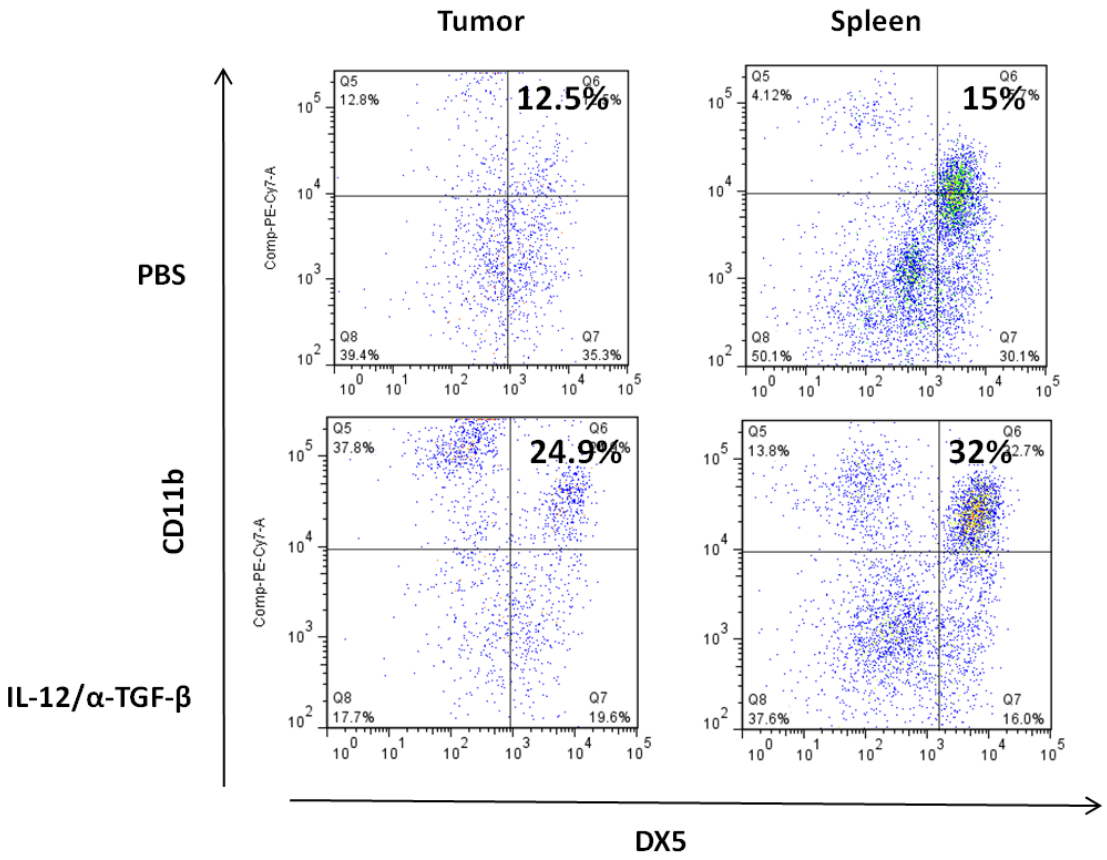

Figure 22

The Combination of IL-12 and anti-TGF-β Can Reverse the Phenotype of NK Cells in Spontaneous pyMT Tumors to Become Mature DX5+ CD11b+ Cells

Four tumors from individual pyMT mice were injected with the combination of IL-12 and anti-TGF-β (50ng and 50µg respectively) or a PBS control. Injections were intratumoral in a total volume of 25µL administered three times a week. At the end of three treatments, tumors were isolated, pooled and stained for the markers CD45, CD3, NK1.1, DX5 and CD11b (n=1). DX5 and CD11b, based on a CD45+ NK1.1+ CD3- population, were analyzed by FACS.

CHAPTER 4: DISCUSSION

4.1 M2 CELLS AND TAMs INHIBIT THE FUNCTION OF NK CELLS

Interestingly, the interaction between macrophages and NK cells has not been previously investigated in the context of tumor development. Two studies have shown that macrophages stimulated with poly I:C or LPS can stimulate NK cells to kill tumor cell lines *in vitro* (Zhou, Zhang et al. 2012); (Bellora, Castriconi et al. 2010). This allowed us to conclude that macrophages have the ability to interact with and influence NK cell functionality. However, what happens to macrophages that are stimulated in an environment devoid of pathogen associated molecular patterns (PAMPs) such as poly I:C or LPS? The tumor microenvironment is highly immunosuppressive, and over time, develops ways to escape immune attack (Zou 2005). Therefore, in this unique environment, we believe there is a role for macrophages in altering NK cell function.

From our NK cell co-culture with M2 macrophages or TAMs, we have shown that after these cells interact, the granular population of NK cells (based on SSC by flow cytometry) expresses CD27 at a lower frequency (Figure 2 and 7). Other NK cell markers for activation such as CD69, NKp46 or NKG2D should also be evaluated for their expression in the NK-TAM co-culture system. The levels of IFN-γ (evaluated by ELISA) from the supernatants collected from the NK and TAM co-culture were reduced in comparison to those cultured with IL-15 alone. However, the levels of IFN-γ were low (in the range of 50-150 pg) and therefore it was hard to delineate if they were true by ELISA. Intracellular staining of IFN-γ should therefore be considered as an alternate method to examine expression in NK cells after contact with TAMs. The most instrumental data

demonstrating the inhibitory activity of M2 cells or TAMs on NK cell activity was derived from the CFSE 7-AAD YAC-1 killing assays (Figures 3 and 8). These assays demonstrated that the interaction between TAMs and NK cells decreases the ability of NK cells to kill target cells. A reduction in NK cell cytotoxicity, one of the most important attributes of NK cells, results in less tumor destruction *in vivo*, and consequently, greater tumor growth. However, the 7-AAD stain only labels cells at later stages of cell death, which likely contributes to the low levels of killing observed in this assay. Annexin V is displayed on cells in the early stages of apoptosis and in subsequent assays should be used in conjunction with 7-AAD. In addition, a dose response killing assay would be a more reliable way to optimize the killing levels and observe greater differences in NK cell killing under various conditions.

To provide *in vivo* proof that macrophages impact NK cell activation, it would be relevant to deplete macrophages in the pyMT tumor model. Previous work in our lab has shown that depletion of macrophages in this model by clodronate liposomes delays tumor formation and increases survival (Gillgrass, A - unpublished). Similarly, others have shown that, animals deficient in macrophages (which have a null mutation in the CSF-1 gene) were found to have lower rates of tumor development and less metastasis (Lin, Nguyen et al. 2001). However, to our knowledge, NK cell activation markers and functionality have not been examined after macrophage depletion in these tumor models. It would be of great relevance to see if NK cell activity could be restored by the depletion of macrophages in the tumor microenvironment. Although this would not provide direct

proof that the two cells interact in the tumor microenvironment, it would allow us to speculate that macrophages have the potential to impact NK cells, *in vivo*.

To delineate the mechanism by which macrophages suppress NK cell activity, we examined transforming growth factor-β (TGF-β). TGF-β is an immunosuppressive cytokine which plays an important role in tumor initiation and progression (Massague 2008). It can be produced by both tumor cells as well as immune cells and has been shown to promote cancer cell metastasis by inhibiting immune surveillance and increasing cancer cell invasion (Wrzesinski, Wan et al. 2007). Researchers have shown that TGF-β inhibits the expression of activating receptors on human NK cells, such as NKp30 and NKG2D, which subsequently decreases NK cell killing ability (Castriconi, Cantoni et al. 2003). First, we determined if M2 cells or TAMs suppressed NK cells in a contact-dependent mechanism or under transwell conditions (0.4μm). Down-regulation of NK cell CD27 expression was only observed in contact, suggesting that this phenomenon required cell-to-cell interaction (Figures 5 and 10). Neutralization of TGF-β in the co-culture system restored CD107a expression on NK cells (Figure 4). In particular, this suggests that the interaction between M2 cells and NK cells could occur through a mechanism which requires membrane bound TGF-β. Similarily, others have shown that down-regulation of NK cell activity by myeloid-derived suppressor cells (MDSCs) in tumor bearing hosts is mediated by membrane-bound TGF-β1 (Li, Han et al. 2009). However, in order to validate this phenomenon in our system, flow cytometry analysis of membrane bound TGF-β or confocal microscopy should be conducted on M2 cells and TAMs from pyMT tumors to ensure that they do in fact express this form of TGF-β.

Since M2 cells and TAMs have the capacity to suppress the function of NK cells, we were interested in determining if they could also alter their development (Figure 10). Given that we observed a large population of granular CD27- NK cells after contact with TAMs, we performed flow cytometry to examine developmental markers on NK cells. Hayakawa and Smith have described maturation of NK cells using two markers: CD27 and CD11b. Phenotypically, they describe the $CD11b^{high}CD27^{high}$ subset as being the most potent effector NK cells while the $CD11b^{high}CD27^{low}$ subset expresses Ly49 inhibitory receptors and KLRG1 (killer-cell lectin-like receptor subfamily G, member 1) at increased levels (Hayakawa and Smyth 2006). After examining the expression of these markers by flow cytometry, we noticed that the NK cell population after interaction with TAMs had a high level of the $CD11b^{high}CD27^{low}$ subset. In the literature, this subset is described as being exhausted as it up-regulates KLRG1, an inhibitory receptor found to be up-regulated after chronic stimulation (Robbins, Nguyen et al. 2002). However, whether this $CD11b^{high}CD27^{low}$ subset correlates with exhausted NK cells in our system remains unclear. Further examination of this population of NK cells should be performed. Either the lack of activation markers or the gain of inhibitory markers by flow cytometry would allow us to better categorize this population as exhausted.

Although we have shown that macrophages isolated from pyMT tumors have the ability to downregulate cytotoxicity of NK cells *in vitro*, the *in vivo* relevance of this interaction still remains unknown. It has been shown that TAM density within human breast tumors is highest in hypoxic or anoxic areas (Murdoch, Giannoudis et al. 2004). Hypoxia within tumors has been shown to promote angiogenesis as well as metastatic

spread (Kalliomaki, McCallum et al. 2009). However, there is limited information available on the localization of tumor infiltrating NK cells and whether they also localize to hypoxic areas. Immunohistochemistry of tumor sections would reveal the location of NK cells within the tumor microenvironment and whether they co-localize with TAMs under these conditions. Even if NK cells and TAMs were found to co-localize within tumors, this is likely not the only mechanism of NK cell suppression that is relevant in the complex tumor microenvironment. There are many factors that can influence NK cells at the tumor site, including both cellular interactions and soluble factors released into the microenvironment. For instance, the co-culture of melanoma cells with NK cells inhibited their expression of NK activating receptors (NKG2D, NKp30, and NKp44), in part mediated by suppressive factors such as indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) (Pietra, Manzini et al. 2012). Tumor-associated stromal cells also have the ability to modulate the phenotype and cytotoxicity of NK cells (Balsamo, Scordamaglia et al. 2009). Fibroblasts and immune cells, such as T regulatory cells, were found to down-regulate IFN-γ secretion and cytotoxicity of NK cells (Smyth, Teng et al. 2006; Zhou, Chen et al. 2010). Therefore, it is important to keep in mind that although we are examining a novel mechanism of NK cell suppression, there may be multiple mechanisms which alter NK cells in the more complex tumor microenvironment.

4.2 TUMOR ASSOCIATED NK CELLS FROM pyMT TUMORS ARE DEVELOPMENTALLY IMMATURE

Tumor infiltrating NK cells are poorly characterized. Evidence suggests that tumor infiltrating NK cells are unable to carry out their role as 'natural killers'. Other than their lack of activity, (decreased IFN-γ secretion and down-regulation of activating receptors), there is little information regarding their stage of development within tumors. We observed that NK cells in co-culture with TAMs become potentially exhausted and displayed a $CD11b^{high}CD27^{low}$ phenotype (Figure 10). However, we know that in many cases, an *in vitro* observation might not carry over to an *in vivo* phenotype. As a result, we analyzed NK cells from pyMT tumors to observe if they had a similar developmental status. We found that tumor infiltrating NK cells display an immature developmental phenotype correlating with their functional incapability of producing high levels of perforin and granzyme (Figures 11-13). Others have also shown that cytolytic enzyme content (expression of perforin and granzyme) in NK cells is linked with cellular maturity (Chattopadhyay, Betts et al. 2009). In addition, the tumor infiltrating NK cells do not express NKp46, a finding in agreement with published results (Narni-Mancinelli, Chaix et al. 2011). On the other hand, peripheral NK cells in the spleens of tumor bearing animals were not developmentally altered in the same way and maintained their mature phenotype along with their expression of NKp46, perforin, and granzyme.

Interestingly, the developmental phenotype of intratumoral NK cells is quite different from that observed after the interaction of NK cells with TAMs *in vitro*. Although both phenotypes observed exhibited low levels of CD27 expression,

intratumoral NK cells exhibited an immature phenotype *in vivo*, while the cells exhibited a highly mature exhausted phenotype *in vitro*. The tumor microenvironment is much more complex and contains multiple immune cell types and factors which can be responsible for subverting NK cell maturation. It is difficult to replicate this *in vivo* environment in an *in vitro* experiment. Furthermore the *in vitro* experiments were conducted on NK cells isolated with the DX5+ selection kit (Stem Cell - the main commercial kit available). The use of the DX5 isolation kit has only allowed for the examination of the impact of TAMs on mature NK cells, as immature DX5- cells are not selected for. In contrast, we predominantly found immature NK cells within the tumor. Isolating cells based on NK1.1 expression would be a more specific way to examine all NK cell subsets.

Richards et al. have shown that a similar phenomenon occurs in the bone marrow of tumor bearing mice (Richards, Chang et al. 2006). Using multiple cancer cell lines (including breast, colon, and melanoma), they found that NK cells from the bone marrow of mice challenged with tumor cells had an immature $CD11b^{low}$ phenotype in comparison to non-tumor bearing mice. Specifically, these cells had impaired IFN-γ production but normal cytotoxicity as assessed by a YAC-1 chromium release assay. Since there was very little tumor-cell infiltrate in the bone marrow, they could conclude that this effect was not due to a tumor-cell mechanism. Instead they found that the defect was associated with a reduction in the percentage of cells expressing IL-15Rα in the bone marrow. They did not examine the phenotype of NK cells within the tumors of these mice. In our study we found that intratumoral NK cells are immature whereas splenic NK cells are mature in

the pyMT mouse model. In comparison to the subcutaneous tumor model used by Richards et al., the spontaneous pyMT tumor model allows us to study tumors which form naturally in a sequential fashion (Xia, Tanaka et al. 2003). Therefore we can examine NK cells within tumors, which is more relevant than NK cells isolated from the bone marrow of tumor bearing mice. In addition, from a single pyMT mouse, we were able to isolate enough NK cells from multiple tumors to analyze phenotypic differences by flow cytometry. The mechanism causing NK cell immaturity within our pyMT tumors, however, still remains unknown.

To identify a mechanism of altered NK cell development, it would be essential to examine the phenotype of NK cells within the bone marrow of pyMT mice. This would help to define if the phenomenon that we observed is tumor specific. We are further interested in understanding if the tumor microenvironment alters mature NK cells or if immature NK cells are recruited to the tumor. To answer this question in future studies, it would be important to adoptively transfer mature labeled NK cells isolated from C57BL/6 spleens, allow them to infiltrate the tumor and the spleen of pyMT mice, and subsequently isolate them to examine phenotypic changes in development. If the transferred NK cells are found to be mature in the spleen but immature in the tumors, we will be able to conclude that the local tumor microenvironment alters these cells. If there is no difference in the development of the transferred NK cells within the tumors, then we can conclude that the tumor recruits already immature NK cells, perhaps from the bone marrow. We would hypothesize that NK cells can rapidly change their phenotype and developmental status depending on the environment. Others have shown this in adoptive

transfer models of NK cells. For instance, Gill et al. have shown that adoptive transfer of NK cells in a murine model causes them to rapidly home to and accumulate within tumors, however, they are unable to deter tumor growth (Gill, Vasey et al. 2012). Once in the tumor, these NK cells down-regulate activating receptors, cytokine production, and cytotoxic factors. This exhaustive phenotype is accompanied by down-regulation of the transcription factors Eomesodermin and T-bet (Gill, Vasey et al. 2012). However, the authors did not examine the developmental status of the NK cells transferred into these tumors. Since the use of NK cells for adoptive transfer into cancer patients has been quite limited, further research needs to be conducted on the fate of these NK cells and how the tumor microenvironment shapes them before they can be widely used. In addition, examination of NK cells isolated from the tumors of IL-15TG/pyMT mice would allow us to determine if IL-15 plays a role in the altered NK cell development, as was described by Richards et al. If the mechanism is similar and requires IL-15 signaling, we would expect to see more mature NK cells in the tumors of IL-15TG/pyMT mice compared to the tumors of pyMT mice.

To our knowledge, a global scale of gene expression analysis of NK cells from tumors has not been conducted. Since we observed such a unique NK cell developmental phenotype within pyMT tumors, we wanted to examine gene expression differences between splenic and intratumoral NK cells of pyMT mice. Understanding the gene expression profile of tumor infiltrating NK cells would help us to further characterize this distinct subset. We used a 96-well PCR array for analyzing the expression of a diverse set of cytokines and chemokines (Figure 16). Immunoregulatory cytokines such as IL-10 and

TGFβ2 were up-regulated on tumor specific (immature) NK cells while immunostimulatory cytokines such as IL-12, IL-18, IFN-γ, and IL-2 were down-regulated. Others have also described that the expression of type I (IFN-γ) and type II (IL-13) cytokines is produced at distinct stages of NK cell maturation (Loza, Zamai et al. 2002). They described that IFN-γ is a factor that is acquired late during NK cell development while immature NK cells produce IL-13 and not IFN-γ. However, one major confounding factor in the PCR results is tumor cell contamination. Although, 90% of the cells isolated were NK1.1+CD3-, the remaining 10% expressed the marker EpCam, indicating that they were of epithelial origin. It is therefore hard to confidently state which factor on the PCR array is derived from the differences between the NK cells or is due to tumor cell contamination. For future analysis, isolation of NK1.1+ cells and subsequent flow sorting to eliminate EpCam+ cells, would ensure that the differences observed on the array were due to differences between the NK cell subsets themselves.

The next step in analyzing the immature NK cells within pyMT tumors was to address if altering the tumor microenvironment would affect their maturity. To modify the tumor microenvironment and shift the immune response towards tumor rejection, we chose to use IL-12 and anti-TGF-β in combination. We found that combination treatment was associated with extended survival compared to PBS (** P<0.01s) and IL-12 alone (*P<0.05) and IL-12 was associated with extended survival compared to PBS control (*P<0.05) (Figure 18). Combination treatment in our model increased the percentage of tumor infiltrating lymphocytes, as evidenced by flow cytometry analysis of CD45+ cells (Figure 20). To our knowledge, this combination of factors has not been tested for

efficacy in cancer models. However, in a model of *Leishmania major* infection, combined treatment with IL-12 and anti-TGF-β antibody was found to shift the balance of cytokine production from Th2- to Th1, revealing that TGF-β may influence the *in vivo* responses to IL-12 treatment (Li, Hunter et al. 1999).

In the subcutaneous pyMT tumor model we were unable to change the phenotype of the tumor infiltrating NK cells. Since this was such an aggressive model and we only examined tumors when mice were at endpoint, we most likely missed the time point at which they were altered and able to influence tumor growth. Therefore, we decided to examine NK cells isolated from the spontaneous pyMT tumor model after three injections of IL-12 and anti-TGF-β. The spontaneous model is less aggressive and we were able to isolate the cells before the mice reached endpoint. Using this model, we have shown that we can alter the NK cell developmental status to a more mature state – one where the cells express a $CD27^{low}CD11b^{high}$ phenotype as well as an increased expression of DX5. We are not necessarily interested in the direct impact of IL-12 and anti-TGF-β on NK cells, but rather the idea that if the environment changes, the NK cells can change with it. Plasticity of NK cells is a topic that is rarely described. While, on the other hand, macrophages are well known for their plasticity and can fall anywhere between the M1 and M2 spectrum characterized by distinct functional states (Biswas and Mantovani 2010). Within a week of modifying the tumor microenvironment with direct injections of IL-12 and anti-TGF-β, NK cells increased their developmental status, providing evidence of the plasticity of NK cells. Since we did not extend the study further, we cannot directly state whether this change in NK cell phenotype directly impacts survival or tumor growth.

However, mature NK cells display greater cytotoxic potential and therefore we could hypothesize that a change in NK cell development could indeed delay tumor growth and further examination of this fact would be beneficial.

CHAPTER 5: CONCLUSION

NK cells in our model and others play an important role in inhibiting the growth of tumors (Liu, Engels et al. 2012). Although this critical immune subset is initially effective at tumor destruction, over time the tumor develops its own defense mechanisms to evade immune attack. One of these mechanisms is the recruitment of monocytes which mature into tumor associated macrophages. Along with promoting tumor growth, these highly immunosuppressive cell types may play a role in augmenting the inhibition of NK cells at the tumor site. Our *in vitro* results revealed that M2 macrophages and TAMs isolated from pyMT tumors are able to inhibit NK cell killing and CD107a expression in a YAC-1 killing assay, in part mediated by their expression of TGF-β. Whether this interaction is relevant in an *in vivo* tumor setting has yet to be determined. Interestingly, in our experimental settings we noticed that the development of NK cells after interaction with TAMs was altered. The NK cells expressed a $CD27^{low}CD11b^{high}$ phenotype correlating with mature and potentially exhausted cells. For this reason, we began to investigate the developmental phenotype of NK cells from pyMT tumors.

In the *in vivo* context of a tumor microenvironment, we discovered that NK cells are characterized as immature based on phenotypic markers by flow analysis. In contrast, peripheral NK cells in the spleen maintained their maturation status. Indeed the majority of immature NK cells within pyMT tumors had decreased expression of perforin, granzyme, and NKp46 compared to their spleen counterparts. Future studies elucidating the mechanism of NK development and its impact on tumor progression are required. We believe that the different *in vitro* and *in vivo* results regarding the NK cell developmental

status is due to the fact that the tumor microenvironment is much more multifaceted and difficult to replicate in an *in vitro* culture system.

Although we have described NK cells in the tumor as immature and non-cytotoxic, it is beneficial that their phenotype can be reversed upon treatment of tumors. We chose treatment with IL-12 and anti-TGF-β and have shown that this combination of factors alters the tumor microenvironment and subsequently promotes the NK cells to a more mature state. Our results argue that NK cells are more adaptable than previously thought. Overall the data suggests that if we can promote the development of NK cells in tumors, they could have greater impact on tumor destruction. Finally, to gain a better understanding of tumor infiltrating NK cells, it would be beneficial to perform gene expression analysis on tumor associated NK cells in order to define their unique phenotype and their roles within tumors. Further experiments and pre-clinical trials are required before any therapies involving NK cell adoptive transfer or the activation of these cells using cytokines are used for therapeutic purposes.

CHAPTER 6: REFERENCES

Algarra, I., T. Cabrera, et al. (2000). "The HLA crossroad in tumor immunology." Hum Immunol **61**(1): 65-73.

Alter, G., J. M. Malenfant, et al. (2004). "CD107a as a functional marker for the identification of natural killer cell activity." J Immunol Methods **294**(1-2): 15-22.

Arase, H., T. Saito, et al. (2001). "Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2)." J Immunol **167**(3): 1141-1144.

Arteaga, C. L., S. D. Hurd, et al. (1993). "Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression." J Clin Invest **92**(6): 2569-2576.

Balsamo, M., F. Scordamaglia, et al. (2009). "Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity." Proceedings of the National Academy of Sciences of the United States of America **106**(49): 20847-20852.

Balsamo, M., W. Vermi, et al. (2012). "Melanoma cells become resistant to NK-cell-mediated killing when exposed to NK-cell numbers compatible with NK-cell infiltration in the tumor." Eur J Immunol.

Bastos, K. R., J. M. Alvarez, et al. (2002). "Macrophages from IL-12p40-deficient mice have a bias toward the M2 activation profile." J Leukoc Biol **71**(2): 271-278.

Bauernhofer, T., I. Kuss, et al. (2003). "Preferential apoptosis of CD56dim natural killer cell subset in patients with cancer." Eur J Immunol **33**(1): 119-124.

Bellora, F., R. Castriconi, et al. (2010). "The interaction of human natural killer cells with either unpolarized or polarized macrophages results in different functional outcomes." Proc Natl Acad Sci U S A **107**(50): 21659-21664.

Bingle, L., N. J. Brown, et al. (2002). "The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies." J Pathol **196**(3): 254-265.

Biswas, S. K. and A. Mantovani (2010). "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm." Nat Immunol **11**(10): 889-896.

Carrega, P., B. Morandi, et al. (2008). "Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells." Cancer **112**(4): 863-875.

Castriconi, R., C. Cantoni, et al. (2003). "Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells." Proc Natl Acad Sci U S A **100**(7): 4120-4125.

Chattopadhyay, P. K., M. R. Betts, et al. (2009). "The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression." J Leukoc Biol **85**(1): 88-97.

Chen, D., J. Xia, et al. (2003). "Immunotherapy of spontaneous mammary carcinoma with fusions of dendritic cells and mucin 1-positive carcinoma cells." Immunology **109**(2): 300-307.

Chiossone, L., J. Chaix, et al. (2009). "Maturation of mouse NK cells is a 4-stage developmental program." Blood **113**(22): 5488-5496.

Colucci, F., M. A. Caligiuri, et al. (2003). "What does it take to make a natural killer?" Nat Rev Immunol **3**(5): 413-425.

Cooper, M. A., T. A. Fehniger, et al. (2001). "The biology of human natural killer-cell subsets." Trends Immunol **22**(11): 633-640.

Costello, R. T., S. Sivori, et al. (2002). "Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia." Blood **99**(10): 3661-3667.

DeNardo, D. G., J. B. Barreto, et al. (2009). "CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages." Cancer Cell **16**(2): 91-102.

Di Santo, J. P. (2006). "Natural killer cell developmental pathways: a question of balance." Annu Rev Immunol **24**: 257-286.

Di Santo, J. P. (2006). "Natural killer cell developmental pathways: A question of balance." Annual Review of Immunology **24**: 257-286.

Dunn, G. P., A. T. Bruce, et al. (2002). "Cancer immunoediting: from immunosurveillance to tumor escape." Nat Immunol **3**(11): 991-998.

Dunn, G. P., L. J. Old, et al. (2004). "The three Es of cancer immunoediting." Annu Rev Immunol **22**: 329-360.

Farag, S. S. and M. A. Caligiuri (2006). "Human natural killer cell development and biology." Blood Rev **20**(3): 123-137.

Fehniger, T. A., S. F. Cai, et al. (2007). "Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs." Immunity **26**(6): 798-811.

Flavell, R. A., S. Sanjabi, et al. (2010). "The polarization of immune cells in the tumour environment by TGFbeta." Nat Rev Immunol **10**(8): 554-567.

Fogler, W. E., K. Volker, et al. (1996). "NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction." J Immunol **156**(12): 4707-4714.

Fu, B. Q., F. Y. Wang, et al. (2011). "CD11b and CD27 reflect distinct population and functional specialization in human natural killer cells." Immunology **133**(3): 350-359.

Garcia-Iglesias, T., A. Del Toro-Arreola, et al. (2009). "Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions." BMC Cancer **9**: 186.

Gasser, S., S. Orsulic, et al. (2005). "The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor." Nature **436**(7054): 1186-1190.

Gati, A., S. Da Rocha, et al. (2004). "Analysis of the natural killer mediated immune response in metastatic renal cell carcinoma patients." Int J Cancer **109**(3): 393-401.

Gill, S., A. E. Vasey, et al. (2012). "Rapid development of exhaustion and downregulation of eomesodermin limits the anti-tumor activity of adoptively transferred murine natural killer cells." Blood.

Glas, R., L. Franksson, et al. (2000). "Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype. An adaptive component of NK cell-mediated responses." J Exp Med **191**(1): 129-138.

Gordon, S. and F. O. Martinez (2010). "Alternative activation of macrophages: mechanism and functions." Immunity **32**(5): 593-604.

Guerra, N., Y. X. Tan, et al. (2008). "NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy." Immunity **28**(4): 571-580.

Guy, C. T., R. D. Cardiff, et al. (1992). "Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease." Mol Cell Biol **12**(3): 954-961.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hayakawa, Y., N. D. Huntington, et al. (2006). "Functional subsets of mouse natural killer cells." Immunol Rev **214**: 47-55.

Hayakawa, Y. and M. J. Smyth (2006). "CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity." J Immunol **176**(3): 1517-1524.

Huntington, N. D., C. A. Vosshenrich, et al. (2007). "Developmental pathways that generate natural-killer-cell diversity in mice and humans." Nat Rev Immunol **7**(9): 703-714.

Ishigami, S., S. Natsugoe, et al. (2000). "Prognostic value of intratumoral natural killer cells in gastric carcinoma." Cancer **88**(3): 577-583.

Kalliomaki, T. M., G. McCallum, et al. (2009). "Progression and metastasis in a transgenic mouse breast cancer model: effects of exposure to in vivo hypoxia." Cancer Lett **282**(1): 98-108.

Karre, K., H. G. Ljunggren, et al. (1986). "Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy." Nature **319**(6055): 675-678.

Kerkar, S. P., R. S. Goldszmid, et al. (2011). "IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors." J Clin Invest.

Kiessling, R., E. Klein, et al. (1975). ""Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype." Eur J Immunol **5**(2): 112-117.

Kim, S., K. Iizuka, et al. (2000). "In vivo natural killer cell activities revealed by natural killer cell-deficient mice." Proc Natl Acad Sci U S A **97**(6): 2731-2736.

Kim, S., K. Iizuka, et al. (2002). "In vivo developmental stages in murine natural killer cell maturation." Nat Immunol **3**(6): 523-528.

Konjevic, G., K. Mirjacic Martinovic, et al. (2007). "Low expression of CD161 and NKG2D activating NK receptor is associated with impaired NK cell cytotoxicity in metastatic melanoma patients." Clin Exp Metastasis **24**(1): 1-11.

Laouar, Y., F. S. Sutterwala, et al. (2005). "Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma." Nat Immunol **6**(6): 600-607.

Levy, E. M., M. P. Roberti, et al. (2011). "Natural killer cells in human cancer: from biological functions to clinical applications." J Biomed Biotechnol **2011**: 676198.

Li, H., Y. Han, et al. (2009). "Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1." J Immunol **182**(1): 240-249.

Li, J., C. A. Hunter, et al. (1999). "Anti-TGF-beta treatment promotes rapid healing of Leishmania major infection in mice by enhancing in vivo nitric oxide production." J Immunol **162**(2): 974-979.

Lin, E. Y., J. F. Li, et al. (2006). "Macrophages regulate the angiogenic switch in a mouse model of breast cancer." Cancer Res **66**(23): 11238-11246.

Lin, E. Y., A. V. Nguyen, et al. (2001). "Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy." J Exp Med **193**(6): 727-740.

Liu, R. B., B. Engels, et al. (2012). "Densely Granulated Murine NK Cells Eradicate Large Solid Tumors." Cancer Research **72**(8): 1964-1974.

Loza, M. J., L. Zamai, et al. (2002). "Expression of type 1 (interferon gamma) and type 2 (interleukin-13, interleukin-5) cytokines at distinct stages of natural killer cell differentiation from progenitor cells." Blood **99**(4): 1273-1281.

Luci, C. and E. Tomasello (2008). "Natural killer cells: detectors of stress." Int J Biochem Cell Biol **40**(11): 2335-2340.

Mamessier, E., A. Sylvain, et al. (2011). "Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity." J Clin Invest **121**(9): 3609-3622.

Mantovani, A., B. Savino, et al. (2010). "The chemokine system in cancer biology and therapy." Cytokine Growth Factor Rev **21**(1): 27-39.

Mantovani, A., S. Sozzani, et al. (2002). "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes." Trends Immunol **23**(11): 549-555.

Marcenaro, E., M. Della Chiesa, et al. (2005). "IL-12 or IL-4 prime human NK cells to mediate functionally divergent interactions with dendritic cells or tumors." Journal of Immunology **174**(7): 3992-3998.

Martinez, F. O., L. Helming, et al. (2009). "Alternative activation of macrophages: an immunologic functional perspective." Annu Rev Immunol **27**: 451-483.

Massague, J. (2008). "TGFbeta in Cancer." Cell **134**(2): 215-230.

Mocikat, R., H. Braumuller, et al. (2003). "Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses." Immunity **19**(4): 561-569.

Moretta, A., C. Bottino, et al. (1996). "Receptors for HLA class-I molecules in human natural killer cells." Annu Rev Immunol **14**: 619-648.

Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-969.

Movahedi, K., D. Laoui, et al. (2010). "Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes." Cancer Res **70**(14): 5728-5739.

Murdoch, C., A. Giannoudis, et al. (2004). "Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues." Blood **104**(8): 2224-2234.

Narni-Mancinelli, E., J. Chaix, et al. (2011). "Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor." Proc Natl Acad Sci U S A **108**(45): 18324-18329.

Nausch, N. and A. Cerwenka (2008). "NKG2D ligands in tumor immunity." Oncogene **27**(45): 5944-5958.

Orr, M. T. and L. L. Lanier (2011). "Inhibitory Ly49 receptors on mouse natural killer cells." Curr Top Microbiol Immunol **350**: 67-87.

Ostrand-Rosenberg, S. (2008). "Immune surveillance: a balance between protumor and antitumor immunity." Curr Opin Genet Dev **18**(1): 11-18.

Ostrand-Rosenberg, S. and P. Sinha (2009). "Myeloid-derived suppressor cells: linking inflammation and cancer." J Immunol **182**(8): 4499-4506.

Park, Y. P., S. C. Choi, et al. (2011). "Complex regulation of human NKG2D-DAP10 cell surface expression: opposing roles of the gammac cytokines and TGF-beta1." Blood **118**(11): 3019-3027.

Pierson, B. A. and J. S. Miller (1996). "CD56+bright and CD56+dim natural killer cells in patients with chronic myelogenous leukemia progressively decrease in number, respond less to stimuli that recruit clonogenic natural killer cells, and exhibit decreased proliferation on a per cell basis." Blood **88**(6): 2279-2287.

Pietra, G., C. Manzini, et al. (2012). "Melanoma Cells Inhibit Natural Killer Cell Function by Modulating the Expression of Activating Receptors and Cytolytic Activity." Cancer Research **72**(6): 1407-1415.

Platonova, S., J. Cherfils-Vicini, et al. (2011). "Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma." Cancer Res.

Pollard, J. W. (2004). "Tumour-educated macrophages promote tumour progression and metastasis." Nat Rev Cancer **4**(1): 71-78.

Pollard, J. W. (2009). "Trophic macrophages in development and disease." Nat Rev Immunol **9**(4): 259-270.

Puzanov, I. J., M. Bennett, et al. (1996). "IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells." J Immunol **157**(10): 4282-4285.

Qian, B., Y. Deng, et al. (2009). "A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth." PLoS One **4**(8): e6562.

Richards, J. O., X. Chang, et al. (2006). "Tumor growth impedes natural-killer-cell maturation in the bone marrow." Blood **108**(1): 246-252.

Richards, J. O., X. Chang, et al. (2006). "Tumor growth impedes natural-killer-cell maturation in the bone marrow." Blood **108**(1): 246-252.

Robbins, S. H., K. B. Nguyen, et al. (2002). "Cutting edge: inhibitory functions of the killer cell lectin-like receptor G1 molecule during the activation of mouse NK cells." J Immunol **168**(6): 2585-2589.

Schaffhausen, B. S. and T. M. Roberts (2009). "Lessons from polyoma middle T antigen on signaling and transformation: A DNA tumor virus contribution to the war on cancer." Virology **384**(2): 304-316.

Sengupta, N., T. S. MacFie, et al. (2010). "Cancer immunoediting and "spontaneous" tumor regression." Pathol Res Pract **206**(1): 1-8.

Smyth, M. J., M. W. L. Teng, et al. (2006). "CD4(+)-CD25(+) T regulatory cells suppress NK cell-mediated immunotherapy of cancer." Journal of Immunology **176**(3): 1582-1587.

Solinas, G., G. Germano, et al. (2009). "Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation." J Leukoc Biol **86**(5): 1065-1073.

Srivastava, S., A. Lundqvist, et al. (2008). "Natural killer cell immunotherapy for cancer: a new hope." Cytotherapy **10**(8): 775-783.

Stein, M., S. Keshav, et al. (1992). "Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation." J Exp Med **176**(1): 287-292.

Street, S. E., N. Zerafa, et al. (2007). "Host perforin reduces tumor number but does not increase survival in oncogene-driven mammary adenocarcinoma." Cancer Res **67**(11): 5454-5460.

Sutlu, T. and E. Alici (2009). "Natural killer cell-based immunotherapy in cancer: current insights and future prospects." J Intern Med **266**(2): 154-181.

Takeda, K., E. Cretney, et al. (2005). "TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver." Blood **105**(5): 2082-2089.

Takeda, K., M. J. Smyth, et al. (2001). "Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth." Cell Immunol **214**(2): 194-200.

Varin, A. and S. Gordon (2009). "Alternative activation of macrophages: immune function and cellular biology." Immunobiology **214**(7): 630-641.

Villegas, F. R., S. Coca, et al. (2002). "Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer." Lung Cancer **35**(1): 23-28.

Vivier, E., D. H. Raulet, et al. (2011). "Innate or adaptive immunity? The example of natural killer cells." Science **331**(6013): 44-49.

Vivier, E., E. Tomasello, et al. (2008). "Functions of natural killer cells." Nat Immunol **9**(5): 503-510.

Waldhauer, I. and A. Steinle (2008). "NK cells and cancer immunosurveillance." Oncogene **27**(45): 5932-5943.

Walzer, T., M. Blery, et al. (2007). "Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46." Proc Natl Acad Sci U S A **104**(9): 3384-3389.

Wilson, E. B., J. J. El-Jawhari, et al. (2011). "Human tumour immune evasion via TGF-beta blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity." PLoS One **6**(9): e22842.

Wrzesinski, S. H., Y. Y. Wan, et al. (2007). "Transforming growth factor-beta and the immune response: implications for anticancer therapy." Clin Cancer Res **13**(18 Pt 1): 5262-5270.

Xia, J., Y. Tanaka, et al. (2003). "Prevention of spontaneous breast carcinoma by prophylactic vaccination with dendritic/tumor fusion cells." J Immunol **170**(4): 1980-1986.

Yang, L., Y. Pang, et al. (2010). "TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression." Trends Immunol **31**(6): 220-227.

Yuzhalin, A. E. and A. G. Kutikhin (2012). "Interleukin-12: clinical usage and molecular markers of cancer susceptibility." Growth Factors **30**(3): 176-191.

Zhang, X., R. Goncalves, et al. (2008). "The isolation and characterization of murine macrophages." Curr Protoc Immunol **Chapter 14**: Unit 14 11.

Zhou, H., L. J. Chen, et al. (2010). "Foxp3-transduced polyclonal regulatory T cells suppress NK cell functions in a TGF-beta dependent manner." Autoimmunity **43**(4): 299-307.

Zhou, Z., C. Zhang, et al. (2012). "Macrophages Help NK Cells to Attack Tumor Cells by Stimulatory NKG2D Ligand but Protect Themselves from NK Killing by Inhibitory Ligand Qa-1." PLoS One **7**(5): e36928.

Zou, W. (2005). "Immunosuppressive networks in the tumour environment and their therapeutic relevance." Nat Rev Cancer **5**(4): 263-274.