THE ROLE OF IL-15 AND NK CELLS IN BREAST CANCER

THE ROLE OF IL-15 AND NK CELLS IN BREAST TUMOR FORMATION AND METASTASIS

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Abstract:

IL-15 is a cytokine that has effects on both innate and adaptive immune cells, including NK and CD8 T cells. The involvement of these cell types in tumor immunosurveillance and eradication has led to interest in IL-15 as an immunotherapy. In several engrafted models of tumor formation or metastasis, especially melanoma, it has proven efficacious. Its role in spontaneous solid cancers has not been studied thoroughly. Here, we have shown for the first time that IL-15 overexpression in a spontaneous breast cancer model, MMTV-polyoma Middle T antigen (MT), leads to increased survival, tumor destruction, and decreased metastasis (IL-15 TG/MT). In contrast, lack of IL-15 led to decreased survival (IL-15 KO/MT) and increased metastasis. Protection in IL-15 TG/MT mice was dependent upon the presence of highly activated NK1.1+ cells, but not dependent upon CD8 T cells. The cytokine environment found in IL-15 TG/MT tumors was capable of activating human NK cells to kill human triple negative breast cancer cells. IL-15 was also able to promote the formation of M1 polarized macrophages within the tumor. In a model of metastasis (intravenous MT cells), we found that overexpression of IL-15 protected from metastasis in a NK cell dependent manner. We also found that a lack of IL-15 promoted the polarization of CD4 T cells to a Th2 phenotype and they influenced polarization of macrophages to an M2 phenotype. M2 macrophages promote metastasis from the primary tumor, as well as help establish tumors at the metastatic site. Here we found that M1 polarized macrophages could prevent engrafted breast tumor formation, whereas M2 macrophages promoted it. Overall, IL-15 is an extremely promising immunotherapy that has more anti-tumor

effects on the immune system than were previously appreciated. Additionally, our data argues that it could be used to generate immune responses against both primary breast cancer and metastasis.

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Lastly, I would like to thank my family. My mother, who passed away during my PhD (but was very proud of me), my father and my sister, for supporting me through all the stages of what some might call a crazy dream. My husband, for not complaining about those really late night experiments and giving me pep talks when I needed them. And especially my children, for reminding me everyday what is really important in life.

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List of Abbreviations:

7-AAD- 7- amino actinomycin
aAPC- artificial antigen presenting cell
ACK- ammonium chloride potassium
ADCC- antibody-dependent cellular cytotoxicity
AML- acute myelogenous leukemia
ANOVA- analysis of variance
APC- antigen presenting cell
AREB- animal research ethics board
ARG- arginase
Blimp-1-B lymphocyte induced maturation protein (TF)
BM- bone marrow
cDNA- complementary DNA
CFSE- 5,6-carboxyfluorescein diacetate succinimidyl ester
CML- chronic myelogenous leukemia
CMV- cytomegalovirus
cNK- conventional NK cells
CSC- cancer stem cells
CSF-1- macrophage colony-stimulating factor
CTL- cytotoxic T lymphocytes
DC- dendritic cell
DNAM-1- DNAX accessory molecule-1
EGF- epidermal growth factor
ELISA- enzyme-linked immunosorbent assay
Eomes-eomesodermin (TF)
ER-estrogen receptor
FMO- fluorescence minus one
GM-CSF- granulocyte macrophage colony-stimulating factor
gMDSC- granulocytic MDSC
GMP- good manufacturing processes
H&E- haematoxylin and eosin
HER2- human epidermal growth factor receptor 2
HIV- human immunodeficiency virus
HLA- human leukocyte antigen
HSV- herpes simplex virus
Id2- inhibitor of DNA binding
IDO- indoleamine 2,3-dioxygenase
IFN- interferon
IL- interleukin
ILC- innate lymphoid cells
iNK- immature NK
IRF- interferon regulatory factor
IV- intravenous

KIRs- killer immunoglobulin-like receptors
KO- knockout (-/-)
LFA-1-leukocyte function associated antigen 1
LPS- lipopolysaccharide
Lti- lymphoid tissue inducer cells
mAb- monoclonal antibody
MCA- methylcholanthrene
MCMV- mouse cytomegalovirus
MCP-1- monocyte chemotactic protein
MDSC- myeloid derived suppressor cells
MHC- major histocompatibility complex
MIC-A/B- MHC Class I related
miRNA- micro ribonucleic acid
MMP- matrix metalloproteinase
MMTV-LTR- mouse mammary tumor virus long terminal repeat
mNK- mature NK
moMDSC- monocytic MDSC
mRNA- messenger ribonucleic acid
MULTI-1- murine UL16 binding protein like transcript
NCR- natural cytotoxicity receptor
NK- natural killer
NKG2D- NK group 2 D
NKP- NK progenitor
NO- nitric oxide
NSCLC- non-small cell lung carcinoma
PBMCs- peripheral blood mononuclear cells
PBS- phosphate buffered saline
PCNA- proliferating cell nuclear antigen
PCR- polymerase chain reaction
PD-1- programmed death 1
PDL-1- programmed death ligand 1
PFA- paraformaldehyde
PGE_2- prostaglandin E2
PI3'K- phosphatidyl- inositol 3' kinase
PIGF- placental growth factor
PLCγ- phospholipase C gamma-1
pMT- polyoma Middle T antigen
PP2A- protein phosphatase 2A
PR- progesterone receptor
RAE-1- retinoic acid early inducible protein
RAG- recombinase activating gene
RCC- renal cell carcinoma
rhIL-15- recombinant human IL-15
RNA- ribonucleic acid

ROS- reactive oxygen species
RPMI- Roswell Park Memorial Institute
SDF-1- stromal cell-derived factor
TAA- tumor associated antigens
TAMs- tumor associated macrophages
TF- transcription factor
TGF-β- transforming growth factor beta
TIL- tumor infiltrating lymphocytes
TNBC-triple negative breast cancer
TNF- tumor necrosis factor
TOX- thymocyte selection-associated HMG box factor
TRAIL- TNF related apoptosis-inducing ligand
TUNEL- terminal deoxynucleotidyl transferase dUTP nick end labelling
ULBP- UL16 binding protein
uNK- uterine NK cells
VEGF- vascular endothelial growth factor
VSV- vesicular stomatitis virus

Declaration of Academic Achievement:

The experiments presented within this thesis have been designed by Dr. A. Ashkar and A. Gillgrass. The animal experimentation and lab experiments were carried out by A. Gillgrass with a few exceptions. The initial NO assay on IL-15 TG, IL-15 KO and C57BL/6 mice was performed by Dr. Kiran Gil. Also the initial subcutaneous injection of the MT cell line in IL-15 TG, IL-15 KO and C57BL/6 mice was performed by Dr. A. Ashkar before I arrived in the lab. I repeated this with similar results. In addition, a co-op student, Artem Babian assisted with the quantitation of lung metastasis area. The CD8/CD4 immunohistochemistry was performed by our histology department. During large experiments, I received technical help from Marianne Chew, our Research Assistant.

The Role of IL-15 and NK cells in Breast Cancer Formation and Metastasis

Chapter 1.0: Introduction

1.1 Breast Cancer:

In 2013, 23 800 Canadian women will be diagnosed with breast cancer and 5000 will succumb to the disease (Canadian Breast Cancer Foundation). While the outlook for those diagnosed with breast cancer has improved over time, it is still the second most common cause of cancer-related deaths among women. Breast cancer is a very heterogeneous disease, but it can be classified using several different methods, including histological analysis/stage, biological classification and molecular classification. This classification system is constantly being further developed and no one scheme is universally used. The WHO has endorsed a histopathological classification scheme of breast cancer that has 20 major subtypes and 18 minor subtypes (Viale 2012). The two most common major subtypes in this scheme are invasive ductal carcinoma and invasive lobular carcinoma (70-80% of breast cancers)(Viale 2012). This scheme is not very clinically informative, as it lacks prognostic value and has low predictive capacities. Clinics also use a classification scheme wherein tumors are staged on a scale of 0-4, taking into account tumor size, metastasis and lymph node status. Recently, research has focused on promoting a biological classification system of breast cancer, thereby enabling clinicians to select interventions based on expression of specific receptors. Tumors would be evaluated via immunohistochemistry for estrogen receptor (ER),

progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) or lack of these receptors (Viale 2012). Examination of Ki67 status is also recommended as it assesses tumor proliferation. Using this biological classification method, ER or PR^+ tumors receive endocrine therapy and $HER2^+$ tumors receive trastuzumab etc...., as each specific receptor expression combination has been observed to respond to specific treatments (Viale 2012). Molecular classification is the most recently developed scheme, which was derived from cluster analyses of complementary DNA (cDNA) microarrays from breast tumors. This analysis was used to define luminal, basal-like, HER2+ and normal-like subgroups (Perou, Sorlie et al. 2000). In the most recent recommendations, a combination of the biological and molecular classification has emerged wherein tumor composition is examined using an immunohistochemical evaluation of ER, PR, HER2 and Ki67 expression. Tumors are then classified as luminal A (ER and/or PR+, HER2-, Ki-67 low), luminal B(ER and/or PR+, HER2+/-, Ki67 +), HER2+ (ER and PR-, Her2 overexpressed) and basal-like (ER-, PR-, HER2- (triple negative breast cancer- TNBC))(Goldhirsch, Wood et al. 2011). There are many treatment strategies currently being employed that have shown efficacy for specific tumor types. These include traditional treatments such as surgery, radiation and chemotherapy, but also hormone inhibitors (ER/PR+) and monoclonal antibodies (mAb) to HER2. While there is a greatly improved prognosis for certain tumor subtypes, not all patients respond to treatment and those with TNBC do not have effective therapeutic options. Thus, there is still an urgent need for the development of new innovative therapies.

As a tool in breast cancer research, researchers employ various mouse models of breast cancer formation. These models can either be engrafted, orthotopic, metastatic (inject intravenous (IV)) or spontaneous, however, spontaneous breast tumor models most closely mimic human breast cancer formation. There are many similarities between mouse mammary tumors and human tumors, making them an appropriate model for studying human breast cancer. For example, the mammary glands from both species have a similar structure and function and metastasis from primary tumors (mouse to lung mostly) occurs in both species. Additionally, many mouse models are multi-hit kinetic models, similar to human tumor development, which results in similar tumor pathology. Importantly, both species have immune systems that respond to tumor formation (Cardiff and Wellings 1999; Cardiff 2001). However, mouse models are not perfect mimics of human breast tumors as some models have one-hit kinetics and/or different pathology. Furthermore, some mouse tumors are hormone independent and have less inflammation and fibrosis in comparison to human breast tumors, which are frequently hormone dependent (Cardiff and Wellings 1999; Cardiff 2001).

A model that very closely mimics breast tumor formation uses the polyoma middle T antigen (pMT) under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR pMT)(Guy, Cardiff et al. 1992). Polyoma is a small double stranded DNA virus that is endemic in wild mice. The MT protein is associated with the membrane and while it has no enzymatic activity itself, it binds to host regulatory proteins and transforms cells. In the intracellular domain of MT, protein phosphatase 2A (PP2A) binds and creates a binding site for non-receptor tyrosine kinases

such as c-src. Once bound and activated, c-src phosphorylates MT on key tyrosine residues to generate a docking site for phosphatidyl- inositol 3' kinase (PI3-K), phospholipase C γ-1 (PLCγ) and shc (Guy, Muthuswamy et al. 1994; Webster, Hutchinson et al. 1998; Ichaso and Dilworth 2001). Without the activity of the tyrosine kinase c-src, MT induced transformation cannot occur (Guy, Muthuswamy et al. 1994). Ultimately, the pathways activated by MT lead to uncontrolled cell growth through MAPKinase and Akt -anti-apoptotic pathways, as well as the capability for cell migration through FAK/matrix metalloproteinases (MMP) pathways (Ichaso and Dilworth 2001). When Lin and Pollard performed an in-depth study comparing pMT tumors with human breast tumors, they observed that similar molecules are activated to cause transformation in both mice and human breast tumors (c-src, Ras and PI3'K)(Lin, Jones et al. 2003). As well, some pMT tumors express ER and PR, similar to human breast tumors (Lin, Jones et al. 2003). In addition, pMT tumor formation occurs through a multi-hit kinetics model. When outgrowth lines were generated from different pMT tumors, each one had a different tumor latency, metastatic potential and morphology, indicating that distinct pathways are initiated in pMT tumors (Maglione, Moghanaki et al. 2001; Maglione, McGoldrick et al. 2004). Further tumor examination revealed that they had variable expression of MT, ER, Ki67 and HER2, indicating that MT expression was not the only mechanism that contributed to tumor formation (Maglione, Moghanaki et al. 2001; Lin, Jones et al. 2003). Lastly, tumor formation in MMTV-pMT mice was a multi-stage sequential process, similar to that observed in human breast cancer. It begins with hyperplasia and progresses to adenoma, similar to ductal epithelial breast hyperplasia in

human. Malignant transformation then leads to early carcinoma (ductal carcinoma *in situ* in humans) and finally to late invasive carcinoma (invasive ductal carcinoma in humans) (Lin, Jones et al. 2003). While the etiology of the murine tumors varies, they are thought to be most similar to the luminal B tumors in humans. In the original mouse model, the MMTV-LTR directed expression of the MT transgene to the mammary gland of FVB mice, causing multi-focal tumor formation in the mammary gland 35 days after birth (Guy, Cardiff et al. 1992). The FVB strain has since been back-crossed onto a C57BL/6 background, which has much slower kinetics of tumor formation (92-108 days), thereby allowing for a more detailed analysis of tumor formation (Chen, Xia et al. 2003; Davie, Maglione et al. 2007). Lastly, because this is a spontaneous tolerized mouse model, it is difficult to find spontaneous CTL responses to the tumor, similar to what is observed in spontaneous human breast tumors (Xia, Tanaka et al. 2003). Therefore, spontaneous mouse models of breast cancer exist that closely mimic the steps and transforming events occurring in human breast cancer. These are invaluable tools for studying many aspects of tumor formation, especially any examination that involves immune responses.

1.2 Cancer and the Immune System:

The idea that the immune system can respond to cancer dates back to 1893 when William Coley observed that a patient with cancer went into remission after generating high fevers from 2 separate *Streptococcus pyogenes* infections (Wiemann and Starnes 1994). He subsequently injected Streptococcal cultures (subsequently called Coley's

toxin) into cancer patients with moderate success (Wiemann and Starnes 1994). Many years later, Burnet coined the theory of cancer immunosurveillance, postulating that tumors can be recognized and eliminated by the immune system (Burnet 1967). Since these early findings, research has come a long way in understanding how the immune system functions to fight or support tumor formation. In recent history, Robert Schreibers' group has proposed the theory involving the 3E's of cancer immunoediting: elimination (immunosurveillance), equilibrium and escape (Dunn, Old et al. 2004; Dunn, Old et al. 2004; Reiman, Kmieciak et al. 2007). In the elimination phase, immune cells (NK cells, M1 macrophages and cytotoxic CD8 T cells) and cytokines (interferon γ (IFN)) can recognize and promote the killing of tumor cells (Shankaran, Ikeda et al. 2001). If unsuccessful at eradicating the tumor cells, tumors proceed to the equilibrium phase which is basically a stand-off between the tumor and the immune cells, with neither able to fully eradicate the other (Koebel, Vermi et al. 2007). Ultimately, in the escape phase, the tumor has won the battle and the immune cells have been converted into cells that promote tumor growth, such as tumor associated macrophages (TAMs-M2 macrophages), myeloid derived suppressor cells (MDSCs) and T regulatory cells (Dunn, Old et al. 2004; Dunn, Old et al. 2004). With this large body of knowledge regarding immune system contributions to both tumor eradication and tumor promotion, we have reached a point at which it is possible to manipulate the immune system to generate more potent long-lasting anti-tumor immunity.

1.3 Natural Killer (NK) Cells:

The name "natural" killer cell was originally coined by Kiessling *et al*. upon discovery of a new cell type of lymphoid origin that could kill tumor cells without any prior sensitization (Kiessling, Klein et al. 1975). Since this initial observation, we have gained a plethora of knowledge regarding this unique cell type. They compose 10-15% of peripheral blood mononuclear cells (PBMCs) in humans and 4-6% in mice and can be found throughout the body. These cells have many roles in the body, including antiviral, tumor defence, a regulatory role in the placenta as well as homeostasis and interactions with the adaptive immune system. We will begin by discussing their development and activation in both mice and humans, followed by their various subsets and functions.

1.3.1 NK Cell Development:

Until recently, the NK cell developmental program remained a mystery. This year, several groups have published a scheme for the development of mouse NK cells (Huntington, Nutt et al. 2013; Narni-Mancinelli, Ugolini et al. 2013; Vosshenrich and Di Santo 2013). While they differ somewhat on the markers used for defining various developmental stages, they are very similar. First of all, NK cells begin with a common lymphoid progenitor in the bone marrow (BM) and differentiate into pre-pro NK progenitor (NKP) cells that express CD127. They further develop into NKPs or refined NKPs with loss of CD127 and gain of CD122, followed by expression of NK1.1 and

some activating receptors such as NKG2D to form early immature NK cells (iNK). Upon further development, NK cells then begin to express increased activation/inhibitory receptors (NKp46, Ly49) and DX5 to form late iNK cells. Lastly, they progress to a mature NK (mNK) cell that also possesses several stages of maturation. Initially, Hayakawa *et al*. defined the mNK subset as $CD11b^{high}$ (integrin) and subdivided the two stages as either CD27 ^{high} or ^{low} (tumor necrosis factor (TNF) receptor superfamily member, ligand is CD70) (Hayakawa and Smyth 2006). These two subsets were observed to have different functional capacities, with the $CD27^{high}$ group having a lower threshold of activation, increased migration, increased cytotoxicity and increased cytokine secretion in comparison to the CD27 ^{low} group (Hayakawa and Smyth 2006). Later, Chiossone *et al*. used these same receptors to define a four stage progressive development of NK cells (Chiossone, Chaix et al. 2009). The $CD11b^{low}CD27^{low}$ subset was defined as highly proliferative and likely corresponds to the iNK in other schemes. This is followed by the $CD11b^{low}$ $CD27^{high}$ subset that is more mature, but still highly proliferative with low frequency in organs. This progresses to the $CD11b^{high}CD27^{high}$ subset, followed by the terminally differentiated $CD11b^{high}CD27^{low}$. These last 2 stages are found more abundantly in organs and possess the highest effector potential (Chiossone, Chaix et al. 2009).

In human NK cells, the differentiation program has mostly been determined by *in vitro* experiments and the use of flow cytometry. It is quite similar to the mouse NK cell scheme with progression from hematopoietic stem cell (HSC) (CD34+) to NKP (CD117+CD161+,CD56-) to iNK (CD56+, gain NKp44,NKp46 and CD94/NKG2A) and

finally mNK (CD117-, KIRs+ (killer immunoglobulin-like receptors), CD16+/-, leukocyte function associated antigen 1 (LFA-1+))(Montaldo, Del Zotto et al. 2013) . Along this pathway, NK cells vary in their cytokine/chemokine secretion, with early cells producing immunoregulatory molecules, such as interleukin (IL)-13, granulocyte macrophage colony-stimulating factor (GM-CSF) and CXCL8, and progressing to expression of more classical type I cytokines such as IFNγ and TNFα (Montaldo, Del Zotto et al. 2013). In the peripheral blood and tissues, mNK subsets can be defined based upon expression of CD56 and CD16 (Farag and Caligiuri 2006; Montaldo, Del Zotto et al. 2013). CD56 is a neural cell adhesion molecule and while its functions are not entirely known, it can mediate homophilic adhesion and bind fibroblast growth factor 1 (Lanier, Chang et al. 1991; Chan, Hong et al. 2007). CD16, also known as FcγRIIIa, is a well known binding partner of IgG and can mediate cell death via antibody dependent cellular cytotoxicity (ADCC). $CD56^{bright}CD16^{dim}$ cells, which make up 10% of NK cells in PBMCs, are considered to be immunoregulatory (Farag and Caligiuri 2006; Montaldo, Del Zotto et al. 2013). Due to their high IL-2Rα expression, they are highly proliferative. They are capable of producing cytokines such as IFNγ, TNFα, M-CSF, IL-10 and IL-13, but they are poorly cytotoxic (low CD16, high inhibitory receptors)(Farag and Caligiuri 2006; Montaldo, Del Zotto et al. 2013). They are primarily found within lymphoid organs due to expression of CD62L and CCR7 (Farag and Caligiuri 2006; Montaldo, Del Zotto et al. 2013). This $CD56^{bright}$ $CD16^{dim}$ phenotype is thought to develop into a $CD56^{dim}CD16^{high}$ NK cell, which has different functional properties (Romagnani, Juelke et al. 2007). $CD56^{dim}CD16^{high}$ NK cells have been termed cytotoxic and constitute 90%

of NK cells in PBMCs. While they also possess the ability to produce cytokines, they can also potently kill other cells due to their low inhibitory receptor, high perforin and CD16 expression (Farag and Caligiuri 2006; Montaldo, Del Zotto et al. 2013). At the latest stages of $CD56^{dim}CD16^{high}$ differentiation, they appear to gain expression of CD57 and CX_3CR1, which correlates with the highest cytotoxic potential and the ability to home to inflamed tissues (Bjorkstrom, Riese et al. 2010; Montaldo, Del Zotto et al. 2013).

Great strides have been made in the last decade regarding the transcription factors (TF) involved in both the murine and human NK cell program of differentiation. This process is regulated by the sequential expression of transcription factors such as inhibitor of DNA binding (Id2), thymocyte selection-associated HMG box factor (TOX) and nuclear factor IL-3 regulated (NFIL3/E4BP4) (Montaldo, Del Zotto et al. 2013; Vosshenrich and Di Santo 2013). Other factors that are also involved include Ikaros, Helios, GATA3, Eomes (eomesodermin), Ets-1 and Blimp1 (B-lymphocyte induced maturation protein) (Vosshenrich and Di Santo 2013). Very recently it has been revealed that microRNA (miRNA) is also necessary for the development of NK cells, having found 200 unique miRNAs in human and mouse NK cells (Beaulieu, Bezman et al. 2013). These small non-coding regulatory RNA sequences can act as post-transcriptional repressors of messenger RNA (mRNA) (Beaulieu, Bezman et al. 2013). In complete miRNA knockout mice, there is a decrease in the mNK population, and similarly, overexpression of miRNA results in poor NK cell development (Bezman, Cedars et al. 2010; Thomas, Abdul-Wajid et al. 2012). Targeted deletion of individual miRNA species (mIR150, mIR-181, mIR-155, mIR-223) has shown that miRNA has a role in the

development, maturation and effector capacity of NK cells (Beaulieu, Bezman et al. 2013). Lastly, the survival and development of NK cells is also dependent on the key cytokine IL-15, which will be discussed in more detail at a later point (Carson, Fehniger et al. 1997; Ranson, Vosshenrich et al. 2003).

NK cells develop primarily in the BM of mice or humans. It is also possible that NK cells can differentiate elsewhere, as NK cell precursors have been found in sites other than the BM and can be induced to develop into mNK (Huntington, Vosshenrich et al. 2007; Huntington, Nutt et al. 2013). Thus far only conventional bone marrow derived NK cells (cNK) have been described. However, there are also thymic NK cells, fetal liver NK cells and uterine NK cells (uNK). Thymic NK cells are weakly cytotoxic and develop in the thymus and in the mouse they depend on IL-17 and the TF GATA3 for development instead of IL-15 and Id2 (cNK)(Huntington, Nutt et al. 2013). Fetal liver NK cells are present at E14 and have a slightly different TF profile without the need of Eomes (Hayakawa, Huntington et al. 2006). In the liver, these cells decrease over time and are gradually replaced by cNK (Hayakawa, Huntington et al. 2006). uNK are DBA+ (lectin) and release angiogenic factors, such as vascular endothelial growth factor (VEGF), to help induce blood vessel formation at the implantation site (Lima, Croy et al. 2012). Therefore, not all NK cells have the same function, depending on the signals they receive and their place in the body they can be vastly different.

Previously, it was believed that NK cells were the only cells, alongside T and B cells, that derived from a common lymphoid progenitor. Within the last few years, scientists have discovered several cell types that develop from the common lymphoid

progenitor, leading to the development of a new classification scheme. NK cells are the founding member of a new family labeled innate lymphoid cells (ILCs)(Bjorkstrom, Kekalainen et al. 2013; Vosshenrich and Di Santo 2013). By definition, ILCs are of lymphoid origin, do not have clonally rearranged antigen receptors (recombination activating gene (RAG) independent), require γ chain cytokines for development and require the Id2 transcription factor (Bjorkstrom, Kekalainen et al. 2013; Vosshenrich and Di Santo 2013). There are three ILC groups; ILC-1 group contains NK cells as well as other IFNγ secreting ILC (found in gut), ILC-2 produce type II cytokines (IL-4, IL-5, IL-9, IL-13) and depend on the TFs GATA3 and RORα for their development and function and lastly ILC-3 produce IL-17 or IL-22 and depend on the TF RORγt for development and function. The ILC-3 group is of interest as it includes lymphoid tissue inducer cells (LTi) that repair secondary lymph node tissues after infection, as well as what were previously called NK-22 cells or IL-22 NK cells that are characteristically found at mucosal barriers and are thought to play a role in protecting these sites (Colonna 2009; Bjorkstrom, Kekalainen et al. 2013). Another difference between NK cells and the non-NK ILCs is the requirement of IL-15 for NK cell development, whereas non-NK ILCs require IL-7 (Bjorkstrom, Kekalainen et al. 2013). Where these non-NK ILCs fit within the current understanding of NK cell development is unknown.

1.3.2 NK Cell Targets and Activation:

We have mentioned that NK cells are capable of killing cells, but not their targets or the mechanisms by which they do so. NK cells are capable of killing stressed, infected or transformed cells. Depending on the circumstances, induction of cell death involves perforin/granzyme release, death receptor ligation (TNF related apoptosis-inducing ligand (TRAIL), Fas/FasL), ADCC and cytokine release (IFNγ, TNFα). These mechanisms will be discussed in further detail in an anti-cancer capacity. While NK cells are capable of killing cells, this is not an unregulated process. In both mice and humans, NK cells possess a plethora of activating and inhibitory receptors that control their ability to kill cells. It is obvious that this must be a tightly controlled process or NK cells could equally kill both normal and aberrant cells. Early on in investigations into the mechanism by which NK cells killed, Ljunggren and Karre proposed the "missing self" theory (Ljunggren and Karre 1990). By observing differences in major histocompatibility complex I (MHC-I) expression among NK sensitive versus non-sensitive tumor cell lines, as well as the ability of NK cells to mediate rejection of mismatched MHC grafts, they proposed that NK cells do not kill cells expressing self MHC I molecules (Backstrom, Chambers et al. 2003). Much later, it was found that not all NK cells express inhibitory receptors for MHC genes, leading many to question this theory, as these NK cells would be unable to distinguish between self and non-self (Kim, Poursine-Laurent et al. 2005; Anfossi, Andre et al. 2006). Examination into this phenomenon created the NK cell "licensing" or "education" theory (Kim, Poursine-Laurent et al. 2005; Anfossi, Andre et

al. 2006). It was found that NK cells in mice or humans that do not possess the inhibitory receptor for self MHC Class I molecules are hyporesponsive to many stimuli (Kim, Poursine-Laurent et al. 2005; Anfossi, Andre et al. 2006). Therefore, if an NK cell does not possess the correct inhibitory receptor for the self MHC I, it will be “unlicensed” or “uneducated” and thus will not function correctly. By this method, NK cells that have not been "educated" are rendered unable to kill.

We now know that NK cell activation is controlled by the balance of signals it receives from both activating and inhibitory receptors and in addition, by the presence of cytokines such as IL-2, IL-15, IL-18, IL-21 and IL-12. The activating/inhibitory receptors in mouse and human NK cells are slightly different. Mouse NK cells have activating receptors such as natural cytotoxicity receptors (NCR- NKp46, NKp30, NKp44), c-lectin type family members (Ly49-D,H,P, NKgroup2D(NKG2D)), DNAX accessory molecule 1 (DNAM-1) and CD16 (Lanier 2008). It is usually necessary to stimulate combinations of activating receptors simultaneously to overcome the threshold and activate the NK cell (except for CD16) (Lanier 2008; Kwon and Kim 2012). In mice, there are several inhibitory Ly49 receptors (Ly49a,c,e,f,g,i,) as well as CD94-NKG2A (Lanier 2008). The main difference between humans and mice is that human do not have the Ly49 family, instead they have killer immunoglobulin like receptors (KIRs), some of which are activating (KIR2DS1, KIR3DS1) and others inhibitory (KIR2DL3, KIR3DL3)(Lanier 2008). There are many known ligands for the activating and inhibitory receptors, but this is still an active area of discovery and there are many receptors with unknown ligands. For example, NKG2D binds retinoic acid early

inducible protein (RAE)-1 and murine UL16 binding protein like transcript (MULT-1) in mice and in humans it binds UL16 binding protein (ULBP)1/2/3/4 and MHC Class I related (MIC) A or B (Nausch and Cerwenka 2008). In the Ly49 family, the inhibitory members generally bind self MHC I molecules, whereas an activating receptor called Ly49H binds the m157 protein that mouse cytomegalovirus (MCMV) infected cells produce (Schenkel, Kingry et al. 2013). Recently, several ligands for the NCR family have been discovered, including hemagglutinin (expressed by virally infected cells) for NKp46, proliferating cell nuclear antigen (PCNA- found on tumors) for NKp44 and B7-H6 (found on tumor cells) for NKp30 (Hudspeth, Silva-Santos et al. 2013). In humans, the inhibitory KIR family members recognize human leukocyte antigen (HLA) molecules (similar to MHC genes in mice)(Lanier 2008). Certain cytokines can control expression of these receptors on NK cells (Varchetta, Oliviero et al. 2013). For example, IL-15 up regulates NKp44 expression, whereas transforming growth factor β (TGF-β) decreases expression of NCRs (Hudspeth, Silva-Santos et al. 2013). In particular, the combination of IL-15 and IL-21 works well to increase NKp46 and CD16 on NK cells (Varchetta, Oliviero et al. 2013). Dendritic cells (DCs) also play a role in activating NK cells. Mature DC are capable of priming NK cell activation and survival by trans-presenting IL-15 or secreting other NK activating cytokines such as IL-12 and IL-18 (Koka, Burkett et al. 2004; Ullrich, Menard et al. 2008; Castillo, Stonier et al. 2009). Thus, if an NK cell receives more activating signals than inhibitory signals, which should only occur on altered cells (express more ligands for activation), it will be capable of killing that cell.

Until recently, NK cells were thought to be cells of the innate immune system that were instrumental as first responders against viral attack or tumor formation, but that did not possess memory like adaptive immune cells. This theory has been seriously reconsidered in the last several years and some suggest that NK cells are both innate and adaptive immune cells. The first paper to call this into question explored this possibility in a hapten-induced contact hypersensitivity model in RAG2 -/- mice (lack T and B cells, have NK cells)(O'Leary, Goodarzi et al. 2006). They found that "memory" to a particular hapten was present in the NK cell population for at least 4 weeks. Others began to look for this phenomenon in their system and it was soon discovered that NK cells possess the Ly49H receptor that is specific for the m157 protein expressed by cells infected with MCMV (Sun, Beilke et al. 2009; Vivier, Raulet et al. 2011). NK cell memory generated by MCMV infection could last several months. This NK cell activation was further studied and they found that the NK cells underwent a non-selective phase of activation and a specific phase for Ly49H positive cells (Fogel, Sun et al. 2013). Since then, the idea of memory like NK cells has expanded to other mouse and human systems including tuberculous pleurisy, vesicular stomatitis virus (VSV), human immunodeficiency virus (HIV-1), cytomegalovirus (CMV) and herpes simplex virus-2 (HSV-2)(Paust, Gill et al. 2010; Abdul-Careem, Lee et al. 2012; Foley, Cooley et al. 2012; Fu, Yang et al. 2013). Certainly such findings have started to revolutionize how people regard NK cells. Scientists have certainly come a long way from their initial description of NK cells as a large granular innate immune cell that kill tumors without prior exposure.

While the primary role of NK cells that we have focused on is to kill stressed/damaged cells, recently it has come to light that NK cells also have an immunoregulatory role and can affect other immune cells such as dendritic cells (DC) and T cells. It is now believed that NK cells can play different roles in different situations. For example, we know that NK cells have a strong link with adaptive immunity. In a certain situation an NK cell will see a MHC I low target tumor cell, become activated and make IFNγ. The cytokines produced by activated NK cells can prime mature DC responses so that they produce other pro-inflammatory cytokines like IL-12 and IL-15 (further activate NK and DC) and ultimately this can lead to a highly protective CD8 T cell response against the tumor (Mailliard, Son et al. 2003; Mocikat, Braumuller et al. 2003). In an alternate scenario, if an active NK cell makes contact with an immature DC, via interactions with its activating NKRs, it may kill that DC (Hayakawa, Screpanti et al. 2004; Sivori, Falco et al. 2004). Another interesting example is that NK cells may actually limit T cell responses to MCMV by producing IL-10 (Su, Nguyen et al. 2001; Lee, Kim et al. 2009). Lastly, NK cells in some cases are found to inhibit autoimmunity. In a mouse model of lupus, the loss of NK cells promoted the development of the disease, whereas increasing the number of NK cells delayed it (Takeda and Dennert 1993). Recently, in a mouse model of diabetes, they identified what they called immunoregulatory NK cells that were created in an IL-18 rich environment and were capable of killing activated, insulin specific CD8 T cells via a PD-PD-ligand method (Ehlers, Papewalis et al. 2012). A comprehensive review was recently published suggesting that the exact nature of the challenge determines the role a NK cell

will play in an immune response- killer, pro-inflammatory or regulatory (Crome, Lang et al. 2013).

1.3.4 NK Cells and Cancer:

The immune system is capable of performing immunosurveillance to detect and destroy tumor cells in the body and immune cells are an important part of the complex milieu that makes up the tumor microenvironment. Several studies, in both mice and humans have indicated that NK cells play a very important role in preventing tumor formation. In humans, an 11 year follow up study found that tumor incidence was associated with decreased cytotoxicity in PBMCs (Imai, Matsuyama et al. 2000). There is also a correlation between the presence of NK cells in various tumor types and a positive prognosis (gastric carcinoma (Ishigami, Natsugoe et al. 2000), colorectal carcinoma (Coca, Perez-Piqueras et al. 1997), lung carcinoma (Villegas, Coca et al. 2002)). In human breast cancer, it was recently found that patients with a better prognosis correlated with the expression of activating receptors such as NKG2D and NKp30 on NK cells (Ascierto, Idowu et al. 2013). In addition, the presence of activating ligands for NK cells, such as MICA/B or ULBP-2, on breast cancer tumor cells is linked to a beneficial outcome (de Kruijf, Sajet et al. 2012). It has also been shown *in vitro* that NK cells are capable of killing many human tumor cells lines (Pietra, Manzini et al. 2009; Kajitani, Tanaka et al. 2012). In human breast cancer cell lines, it has been shown

that NK cells used TRAIL, perforin and IFNγ to exert anti-tumor killing (Kajitani, Tanaka et al. 2012).

In mice, there is even more evidence that NK cells are important for the prevention of tumor formation. In studies using mouse models which lack functional NK cells, NK cells have been found to play a role in preventing tumor formation in methylcholanthrene (MCA) induced fibrosarcoma, B16 melanoma and colon cancer (Seaman, Sleisenger et al. 1987; Kim, Iizuka et al. 2000; Smyth, Crowe et al. 2001). In a spontaneous mouse model of breast cancer (Her2/neu transgenic) the depletion of NK cells or knockout of perforin, an effector molecule used by NK cells, led to delayed tumor onset and decreased tumors, although survival was not altered (Street, Zerafa et al. 2007). It has also been reported that the loss of NK cell activating receptors such as NKG2D or NKp46 leads to poor tumor immunosurveillance (Guerra, Tan et al. 2008; Hudspeth, Silva-Santos et al. 2013). Recently it was found that NK cells, when provided with IL-15 at the tumor site, can kill large solid established tumors (MC57 fibrosarcoma) using a perforin/granzyme method of cell death (Liu, Engels et al. 2012). In fact, perforin and IFNγ are a common method used by NK cells to kill tumor cells as depletion of these molecules in mouse models of B16 melanoma, T lymphoma and MCA induced fibrosarcoma lead to an increase in tumor formation (van den Broek, Kagi et al. 1996; Smyth, Thia et al. 2000). It has also been confirmed that TRAIL, as well as Fas/FasL interactions, between NK cells and tumor cells contribute to the ability of NK cells to kill tumor cells (Bradley, Zeytun et al. 1998; Takeda, Hayakawa et al. 2001).

Recently, it has been demonstrated that NK cells have the ability to kill cancer stem cells (CSCs). This is particularly important, as after traditional therapies such as chemotherapy and radiation, CSCs frequently persist and their survival can lead to relapse. In 4T1 mouse breast cancer, NK cells were capable of killing a chemotherapy resistant sub population of cells (Frings, Van Elssen et al. 2011). In a cell line derived from C57BL/6 polyoma MT breast cancer, NK cells were able to kill cells with CSC characteristics via ligation of death ligands (Li, Knight et al. 2012). There are also multiple examples in human CSC cell lines such as melanoma, breast cancer and primary oral squamous carcinoma that NK cells can target these cells with multiple methods including NCR and perforin and ADCC (Pietra, Manzini et al. 2009; Tseng, Arasteh et al. 2010; Diessner, Bruttel et al. 2013). Therefore, in a very tightly controlled manner, NK cells are capable of exerting many effects within the body, including tumor cell/CSC killing or conversely promoting/limiting T cell responses. While we have discovered a great deal about how NK cells kill, it is obvious that an increased understanding, particularly of the immunoregulatory role of NK cells, is necessary to fully develop strategies on how best to manipulate NK cells to get the desired response.

1.3.5 The Tumor Fights Back- How Tumors Effect NK Cell Phenotype:

While we have detailed how NK cells are capable of fighting cancer in multiple ways, are essential for immunosurveillance and are uniquely able to kill CSCs, it is obvious that not all tumors are prevented from forming. Tumors frequently proceed into

the equilibrium phase or the escape phase. What happens to NK cells at that point? The tumor has many strategies to prevent NK cell killing. These strategies include decreasing overall numbers of NK cells, changing the functions of NK cells and subverting their attack via secretion of soluble molecules. Understanding these strategies will be very important to designing immunotherapies's to prevent them.

First of all, there have been multiple reports of decreased numbers of NK cells in patients with various types of cancer including chronic myelogenous leukemia (CML), head and neck cancer, breast cancer and colorectal adenocarcinoma (Warren, Stembridge et al. 1985; Pierson and Miller 1996; Bauernhofer, Kuss et al. 2003). The reasons for this are not fully known, but it was reported that in cancer patients, $CD56^{dim}$ NK cells (cytotoxic) in circulation were targeted for apoptosis preferentially (Bauernhofer, Kuss et al. 2003). Similar findings of decreased numbers of NK cells were reported in multiple models of mice bearing tumors (Richards, Chang et al. 2006; Richards, McNally et al. 2008). In these models, NK cell development in the bone marrow was affected by tumor growth and the maturation of NK cells was impaired (Richards, Chang et al. 2006). Subsequently, it was found that there were reductions in common lymphoid progenitor cells that lead to the decreased numbers of NK cells in circulation (Richards, McNally et al. 2008). Regardless of the mechanism, we can conclude that NK cell numbers are decreased, particularly in advanced stages of cancer.

What has become the focus of a lot of research in the last few years is the fact that NK cells within tumors no longer function as killer NK cells. When an NK cell is recruited into a tumor, it may arrive ready to kill, but the tumor microenvironment can

fight back. In many cases, NK cells within tumors appear to have an altered phenotype in comparison to NK cells at other sites. When researchers began to analyze tumor NK cells in patients with a variety of tumor types, instead of just looking at PBMCs, they soon found that $CD56^{bright}CD16^{dim}$ NK cells predominated in the tumor (Carrega, Morandi et al. 2008; Liapi, Gogali et al. 2013). These NK cells had a decreased cytolytic capacity, and in some cancers, such as papillary thyroid cancer, were actually associated with disease progression (Carrega, Morandi et al. 2008; Konjevic, Jurisic et al. 2012; Liapi, Gogali et al. 2013). Mamessier *et al.* published several studies in breast cancer regarding NK cell phenotype in relation to disease progression (Mamessier, Sylvain et al. 2011; Mamessier, Sylvain et al. 2011; Mamessier, Pradel et al. 2013). Their detailed analysis revealed that as disease progressed to advanced stages, the function of both PB NK and tumor NK cells decreased and they saw decreased activating receptors (NKp30,NKG2D, DNAM-1, CD16) and increased inhibitory receptors (NKG2A)(Mamessier, Sylvain et al. 2011). Tumor NK cells had a much higher degree of impairment than PB NK cells (Mamessier, Sylvain et al. 2011). They also identified two new subsets of NK cells $CD56^{bright}CD16^{+}$ and $CD56^{dim}CD16^{-}$ that were prevalent in the PB of advanced breast cancer patients and were the main subsets found at the tumor site (Mamessier, Pradel et al. 2013). Where these fit into the classification of NK cell development remains to be determined. The decrease in activating receptors on tumor NK cells was also found in cases of cervical cancer and non-small cell lung carcinoma (NSCLC)(Garcia-Iglesias, Del Toro-Arreola et al. 2009; Platonova, Cherfils-Vicini et al. 2011). In several of these studies, the down regulation of NK cell effector function was

linked to the production of TGF-β in the tumor microenvironment (Mamessier, Sylvain et al. 2011; Bruno, Focaccetti et al. 2013). Recently, the markers more commonly used for NK cells in mice, CD27 and CD11b, were analyzed on the PB and tumor NK cells of NSCLC patients (Jin, Fu et al. 2013). They found that both in mouse models and in humans with lung cancer, $CD11b^{-}CD27^{-}$ immature NK cells predominated in the tumor and were correlated with tumor progression (Jin, Fu et al. 2013). The fact that the presence of these immature/noncytolytic NK cells in several tumor types is actually correlated with tumor progression is a key observation. Traditional NK cells, as we have presented them, would not "help" a tumor progress. Indeed, when they examined these tumors, NK cells that were $CD56^{bright}CD16^{-}$ were able to produce VEGF, placental growth factor (PIGF) and IL-8 (Bruno, Focaccetti et al. 2013). The production of these molecules from tumor NK cells led to angiogenesis (Bruno, Focaccetti et al. 2013). As mentioned earlier, it is also known that uNK cells are capable of producing cytokines such as these (Lima, Croy et al. 2012). Another study performed a comparative microarray analysis on tumor and non-tumor NK cell mRNA from NSCLC patients. While they did not see the same expression of pro-angiogenic factors in the tumor NK cells, they found they had the phenotype of exhausted memory NK cells and observed a slight decrease in activating receptor mRNA (Gillard-Bocquet, Caer et al. 2013). Thus, it appears that NK cells may be highly responsive to their environment and may change from killer to tumor supportive functions in the tumor microenvironment.

What tumor produced factors inhibit NK cell function? As mentioned, tumor cells play an important role in secreting molecules such as TGF-B that down regulate

NKR. When autologous tumor cells (breast cancer and other tumor types) are cultured *ex vivo* with NK cells from the PB of the same cancer patient, we see that the NK cells decrease their cytotoxic and cytokine activity, in part due to TGF-β secretion by those tumor cells (Mamessier, Sylvain et al. 2011; Wilson, El-Jawhari et al. 2011). Tumor cells are also capable of producing other molecules to decrease NK cell cytotoxicity and cytokine secretion (as well as T cell function). These include indoleamine 2,3-dioxygenase (IDO), adenosine ectoenzymes (CD39/CD73), prostaglandin E2 (PGE_2) and programmed death 1 receptor ligand (PDL-1)(Uyttenhove, Pilotte et al. 2003; Hoskin, Mader et al. 2008; Su, Huang et al. 2008; Yoshida, Ino et al. 2008; Benson, Bakan et al. 2010; Holt, Ma et al. 2011). Other immune cells within the tumor microenvironment, such as regulatory T cells and MDSCs also contribute to the production of some of these molecules and others (reactive oxygen species (ROS), control of available IL-2) that result in a decrease in NK cell cytotoxic activity (Liu, Yu et al. 2007; Li, Han et al. 2009; Gasteiger, Hemmers et al. 2013). Tumors cells are also capable of shedding soluble NKG2D-ligands (sNKG2D-L) into the blood in an attempt to subvert NK cell attack. In multiple tumor types, including gastric carcinoma, melanoma and leukemia, there are high levels of sNKG2D-L in the blood (Salih, Rammensee et al. 2002; Paschen, Sucker et al. 2009; Hilpert, Grosse-Hovest et al. 2012). These levels of sNKG2D-Ls were found to correlate with disease progression and poor survival (Paschen, Sucker et al. 2009; Hilpert, Grosse-Hovest et al. 2012). Interestingly, the effect of this is two- fold, it reduces expression of activating ligands on the tumor cells, but also decreases

systemically the level of NKG2D on NK cells so that they can't be activated when they reach the tumor environment (Hilpert, Grosse-Hovest et al. 2012).

As a result of all of these mechanisms, when IL-2 stimulated murine NK cells were transferred into tumor bearing hosts, NK cells could home to the tumors, but once there, they sequentially lost the ability to make IFNγ and then their cytotoxicity, and had little effect on tumor formation (Gill, Vasey et al. 2012). This change in NK cell phenotype was due to a decrease in the transcription factors Eomes and T-bet and the resultant NK cells displayed an "exhausted" phenotype (Gill, Vasey et al. 2012). Therefore, it is not enough to transfer NK cells to a patient with the expectation that they will kill tumors. We must transfer NK cells that are highly activated and resistant to the strategies of the tumor microenvironment and/or target other molecules/cell types that promote the inhibition of NK cells to allow efficient tumor cell killing.

1.4 CD8 T Cells- Role in Tumor Formation:

NK cells are not the only immune cells capable of anti-tumor activities. Studies in tumor immunosurveillance indicate that CD8 T cells have an important role in both elimination and equilibrium stages of tumor formation (Shankaran, Ikeda et al. 2001; Koebel, Vermi et al. 2007). It has been confirmed in multiple tumor types that the presence of CD8 T cells within the tumor is a positive prognostic factor (Marrogi, Munshi et al. 1997; Kawai, Ishii et al. 2008; Nelson 2008; Pages, Galon et al. 2010). In animal models, central memory CD8 T (CD44+CD62L+) cells have been shown to be

particularly effective at targeting tumors (Klebanoff, Gattinoni et al. 2005). In addition, CD8 T cells engineered to recognize known immunogenic tumor associated antigens (TAA) are capable of causing tumor regression in pre-clinical models (Yang, Wall et al. 2007). There have also been successes in the clinic with tumor infiltrating lymphocyte (TIL) therapies that expand CD8 T cells specific to TAA and re-infuse them back into the patient (Wu, Forget et al. 2012). Unfortunately, even if a tumor specific cytotoxic T lymphocyte (CTL) response is seen, patients are often unable to eliminate the tumor (Appay, Douek et al. 2008; Perret and Ronchese 2008). The reasons for this are multiple. First, the same methods employed by tumors to down regulate NK cell activity can also be effective with CD8 T cells (IDO, PDL-1). It has also been found that most TAAs are self antigens, and thus self-reactive T cell clones to these TAAs will be deleted during negative selection in the thymus (Nair, Jong et al. 2006; Rizzuto, Merghoub et al. 2009; Srinivasan and Frauwirth 2009). If some self-reactive T cells do escape this central tolerance, peripheral tolerance may render the CD8 T cell anergic. This occurs when a naive CD8 T cell binds an antigen bound in MHCI on a cell that is unable to provide co-stimulation. That T cell will be deleted, or become functionally hyporesponsive (anergic) (Huang, Shah et al. 2007; Srinivasan and Frauwirth 2009). Additionally, if improper co-stimulation occurs, it is also possible to generate CD8 T regulatory cells that will produce cytokines like IL-10 to suppress immune cytotoxicity responses (Kiniwa, Miyahara et al. 2007; Wang 2008). Even if an efficient CD8 T cell response is generated, once in the tumor microenvironment, the T cell may become exhausted due to constant antigen exposure and exhibit decreased ability to respond to targets (Srinivasan and Frauwirth

2009; Kim and Ahmed 2010). CD8 T cells also participate in the immunoediting of tumors and thus those tumor cells that have high levels of MHC I expressing TAA will be killed, but others with low levels of MHC I may escape attack (Knutson, Lu et al. 2006; Reiman, Kmieciak et al. 2007). Thus, while CD8 T cells are capable of killing tumor cells, tumors have many methods to evade their attack.

1.5 IL-15:

1.5.1 IL-15- The Basics:

IL-15 is a pleiotropic cytokine that has many effects on both innate and adaptive immune cell types such as NK cells and memory CD8 T cells. IL-15 mRNA is transcribed in many cell types, but its protein expression is limited to macrophages, dendritic cells, fibroblasts and epithelial cells due to tight regulation of IL-15 during transcriptional, translational and intracellular trafficking (Bamford, DeFilippis et al. 1998; Waldmann and Tagaya 1999). In cases of infection, production of IFNs or the binding of TLR ligands to their receptors leads to NF-κB and interferon regulatory factor (IRF) response element driven up regulation of IL-15 expression (Waldmann and Tagaya 1999; Mattei, Schiavoni et al. 2001). IL-15 is a gamma chain cytokine and member of the 4α helix bundle cytokine family that includes other family members such as IL-2, IL-4 and IL-7 (Waldmann and Tagaya 1999). IL-15 signals through IL-2 Rβ (CD122) and the common γ chain (γc), along with its own high affinity IL-15Rα (Castillo and Schluns

2012). At first, IL-15 was thought to bind to a trimeric IL-15 Rα/IL-2Rβ/γc on the cell of interest. It was soon discovered that IL-15 Rα was not required on the NK cell or CD8 T cell for IL-15 to signal (but IL2-Rβ and γc on the NK/CD8 cell are required)(Koka, Burkett et al. 2003; Burkett, Koka et al. 2004). Since then there have been several proposed methods by which IL-15 can signal, the most commonly accepted being trans-presentation and cis-presentation. Trans-presentation is when one cell type presents IL-15 bound to IL-15 Rα to another cell type that possesses IL-2Rβ and the γ chain. Several seminal papers using mixed bone marrow chimeras and IL-15 Rα knockout (KO) mice were critical in proving that trans-presentation was the dominant form of IL-15 action *in vivo* (Koka, Burkett et al. 2003; Burkett, Koka et al. 2004). Further studies on IL-15 presentation went on to reveal that IL-15 and IL-15 Rα need to be co-expressed by the same cell to get IL-15 to the surface to be trans-presented (Duitman, Orinska et al. 2008; Mortier, Woo et al. 2008). IL-15 was complexed with IL-15 Rα in the ER/Golgi before it could be shuttled to the cell surface (Duitman, Orinska et al. 2008; Mortier, Woo et al. 2008). So IL-15 Rα expression is not required on NK or CD8 T cells, but what cell type does present IL-15 to NK cells or CD8 T cells? It was soon confirmed using conditional knockouts of IL-15 Rα in selected cell types, that the major cells that are capable of trans-presenting IL-15 to NK or CD8 T cells are DCs and/or macrophages/monocytes (Mortier, Woo et al. 2008; Castillo, Stonier et al. 2009; Mortier, Advincula et al. 2009). The other model of activation via IL-15 is cis-presentation in which a cell type expresses IL-15 Rα that binds IL-15 and presents it to an IL-2Rβ/γ chain complex on the same cell. While this mechanism appears to be functional *in vitro*, its importance *in vivo* has been

questioned (Castillo and Schluns 2012). Binding of IL-15 (complexed with IL-15 Rα) to IL-2Rβ and γ chain, results in the activation of JAK1/JAK3, STAT3/STAT5 pathways, Syk kinase, PLCγ, Lck kinase and Shc leading to the activation of PI3K/AKT and Ras/Raf/MAPK signalling cascades (Steel, Waldmann et al. 2012). There have been reports that on myeloid cells, IL-15 can signal in an alternative way other than trans- or cis-presentation. In this case, in myeloid cells such as macrophages, IL-15 can also induce a signal via IL-15 Rα, independent of the γ chain and IL-2 Rβ (Chenoweth, Mian et al. 2012). It has also been found that IL-15 can complex with IL-15 Rα and be found circulating in the blood of both mice and humans and this has sometimes been called paracrine signalling (Bergamaschi, Bear et al. 2012). This free IL-15/IL-15Rα complex has likely been cleaved from the surface of an antigen presenting cell (APC) that co-expressed IL-15 and Rα (Mortier, Bernard et al. 2004). The biological role this may play in signalling has yet to be determined.

While IL-15 was originally described as a T cell growth factor (Bamford, Grant et al. 1994; Grabstein, Eisenman et al. 1994), it soon became apparent that it was the key cytokine involved in NK cell development, survival and proliferation (Carson, Fehniger et al. 1997; Cooper, Bush et al. 2002; Ranson, Vosshenrich et al. 2003). IL-15 is a required cytokine for the formation of NK cell precursors in the BM (Leclercq, Debacker et al. 1996). In addition, IL-15 promotes the survival of NK cells by increasing expression of anti-apoptotic molecules like Bcl-2 and suppressing pro-apoptotic molecules like Bim (Minagawa, Watanabe et al. 2002; Huntington, Puthalakath et al. 2007). Lastly, IL-15 also promotes the cytotoxic and cytokine secreting functions of NK

cells by increasing NKRs and promoting the formation of mNK cells (Gays, Martin et al. 2005; Narni-Mancinelli, Ugolini et al. 2013). Therefore, it is not surprising that in mice that lack IL-15 (IL-15 KO) the main effect is the absence of NK cells (Kennedy, Glaccum et al. 2000). In contrast, when IL-15 is overexpressed, a major increase in total number and activation of NK cells is observed (Fehniger, Suzuki et al. 2001). In regards to NK cells, IL-15 Rα KO mice have similar deficiencies to IL-15 KO mice, thus indicating that this receptor plays a very important role in IL-15 signalling (Lodolce, Boone et al. 1998). As mentioned, CD8 T cells are also highly responsive to IL-15, in particular CD8 memory T cells. IL-15 is in control of the proliferation and survival of CD8 memory T cells (Judge, Zhang et al. 2002). In fact, in IL-15 TG mice, there are large increases in $CD44^{hi}$ memory CD8 T cells (Marks-Konczalik, Dubois et al. 2000; Fehniger, Suzuki et al. 2001). We see the opposite effect in IL-15 KO mice, as they have a reduction of mostly $CD44^{hi}$ memory CD8 T cells (Kennedy, Glaccum et al. 2000). It is thought that IL-15 promotes survival in CD8 T cells via similar mechanisms to that of NK cells. It promotes the expression of anti-apoptotic Bcl-2 and decreases pro-apoptotic Bim/Bax (Oh, Perera et al. 2008; Inoue, Unsinger et al. 2010). Central memory CD8 T (CD44+CD62L+) cells are particularly responsive to IL-15 as they more highly express IL-15 receptors (Klebanoff, Finkelstein et al. 2004). These central memory CD8 T cells have been identified as being particularly efficacious for anti-tumor responses (Klebanoff, Gattinoni et al. 2005). Others report that IL-15 can expand both effector and memory CD8 T cells (Lu, Giuntoli et al. 2002; Melchionda, Fry et al. 2005). IL-15 has several other important effects on CD8 T cells including rescue of tolerized T cells

(Teague, Sather et al. 2006), circumventing the need for CD4 "help" to activate CD8 T cells (Oh, Perera et al. 2008) and preventing activation-induced cell death (Marks-Konczalik, Dubois et al. 2000). There have been reports that IL-15 treatment may also up regulate PD-1 and IL-10 production in CD8 T cells, a phenotype that is reminiscent of exhausted or CD8 T regulatory cells (Kim and Ahmed 2010; Yu, Steel et al. 2010). Another unique cell type that is promoted by chronic IL-15 overexpression is NK1.1+CD8+CD3+ T cells (Ohta, Hiroi et al. 2002; Terabe, Tagaya et al. 2008). These cells are reported to have high IFNγ secreting properties as well as an ability to degranulate quickly (Ohkawa, Seki et al. 2001; Ohta, Hiroi et al. 2002; Terabe, Tagaya et al. 2008).

While the role for IL-15 in NK cell and CD8 T cell function in well defined, much less is known about its effect on other immune cells such as macrophages, DCs and CD4 T cells. In terms of DCs, we have discussed their ability to both produce IL-15 and trans-present IL-15 to other cell types, but they also respond to IL-15 (have IL-2 Rβ/γc). IL-15 has been shown to increase maturation of DCs by increasing co-stimulatory molecules (CD86/CD40/MHCII) and IFNγ secretion (Mattei, Schiavoni et al. 2001; Dubsky, Saito et al. 2007). Therefore there is a large degree of cross talk between NK cells and DCs, whereby DCs can trigger NK cells responses (activate with cytokines), and NK cells can kill or promote the maturation of DCs. Ultimately, DCs induced with IL-15 are also able to prime efficient CTL responses (Dubsky, Saito et al. 2007). In terms of the effect of IL-15 on macrophages, the results are less clear. It was observed that IL-15 treatment of peritoneal macrophages induced high levels of both pro-

inflammatory and anti-inflammatory cytokines in response to LPS (Alleva, Kaser et al. 1997; Ruckert, Brandt et al. 2009). There have also been reported increases in co-stimulatory molecules (CD80/86) and MHC II as well as increased NO production in IL-15 treated macrophages (Liu, Zhai et al. 2004; Ruckert, Brandt et al. 2009). Thus, it appears that overall, IL-15 is able to promote the maturation of APCs. The effect of IL-15 on CD4 T cells is an area of controversy. Initially, it was reported that IL-15 primed CD4 T cells for a Th1 biased response and that IL-15 could induce proliferation of effector memory CD4 T cells (Seder 1996; Geginat, Sallusto et al. 2001; Purton, Tan et al. 2007). In fact, IL-15 TG mice have increased CD4 memory T cells and lack of IL-15 was found to decrease the ability of CD4 T cells to respond in a mouse model of collagen induced arthritis (Yoshihara, Yamada et al. 2007). It is likely that in normal conditions, IL-15 may be involved in CD4 T cell homeostasis and that the approximately normal numbers of CD4 T cells in IL-15 KO mice is due to compensatory mechanisms (Purton, Tan et al. 2007). Later, it was shown that IL-15 could induce proliferation of CD4 T cells and potentially enhance Th1 or Th2 differentiation, depending on what other cytokines were present (Niedbala, Wei et al. 2002). In recent studies, IL-15 has been found to promote the formation of T regulatory cells, but at the same time increase responses of CD4 and CD8 T cells to a degree that the T regulatory cells were unable to inhibit them (Ben Ahmed, Belhadj Hmida et al. 2009; Benito-Miguel, Garcia-Carmona et al. 2009). More research needs to be conducted to determine the exact effects of IL-15 on immune cells other than NK cells or CD8 T cells.

1.5.2 IL-15 and Cancer Immunotherapy:

It is not surprising that due to IL-15's ability to stimulate NK cell and CD8 T cell function and subsequently their ability to kill tumor cells, that there would be intense interest in this cytokine as an immunotherapy. In fact, expression of IL-15 in certain tumor types has been correlated with a positive prognosis (Blum, Koyama et al. 2008). There have been many pre-clinical studies that have attempted to determine the effect of IL-15 on cancer progression. IL-15 has been used to treat NK and CD8 T cells *ex vivo* and either transfer them back into tumor bearing hosts or test their cytotoxicity on tumor cell lines (Mueller, Schweier et al. 2008; Morales, Kmieciak et al. 2009; Szczepanski, Szajnik et al. 2009). Both NK cells and CD8 T cells treated in this manner appear to be quite efficacious at killing tumor cells (Mueller, Schweier et al. 2008; Morales, Kmieciak et al. 2009; Szczepanski, Szajnik et al. 2009). *In vivo*, the majority of studies have employed engrafted models of tumor formation and metastatic models to investigate the role of IL-15. In an early study using BLl6 melanoma cell lines that did or did not express MHC I, it was found that in IL-15 TG mice, MHC^+ cells were killed by CTL responses, whereas MHC^- cells were killed by NK cells (Yajima, Nishimura et al. 2002). In another study where the colon carcinoma cell line MC38 was injected intravenously into IL-15 TG mice to model metastasis, NK cells were able to inhibit tumor growth (Kobayashi, Dubois et al. 2005). In a subcutaneous engrafted lung adenoma model, daily treatment with recombinant human IL-15 (rhIL-15) was capable of eradicating tumors, although the mechanism was not examined (Tang, Zhao et al. 2008). In an interesting

study by Ugen *et al*., subcutaneous B16 melanoma was established and then treated intra-tumorally with a plasmid expressing IL-15 (Ugen, Kutzler et al. 2006). After *in vivo* electroporation, tumor regression was seen in 39% of animals (Ugen, Kutzler et al. 2006). In a xenograft model of mouse breast cancer, injection of a recombinant adenovirus-associated vector expressing human IL-15 prophylactically led to delayed tumor onset and increased survival (Yu, Wei et al. 2010). Unfortunately, they did not examine which cell types contributed to this effect.

It was soon discovered that the biological activity of IL-15 was greatly improved when given complexed with IL-15 Rα (Rubinstein, Kovar et al. 2006; Bergamaschi, Rosati et al. 2008). Complexing with IL-15Rα resulted in the stabilization of IL-15, an increase in its half life and led to even higher increases in NK and CD8 T cells numbers (Rubinstein, Kovar et al. 2006; Bergamaschi, Rosati et al. 2008). After this discovery, a plethora of studies exploring the ability of the complex to stimulate anti-tumor immune responses were initiated. The combination treatment of IL-15 and IL-15 Rα was found to induce more protection in a B16 melanoma metastasis model than IL-15 alone (Stoklasek, Schluns et al. 2006; Epardaud, Elpek et al. 2008). In a model of spontaneous pancreatic tumors, complex injection led to a reduction in the size of the tumor and prolonged survival in a CD8 T cell dependent manner (Epardaud, Elpek et al. 2008). Researchers soon began to produce fusion proteins with IL-15 and IL-15 Rα sushi domains and this was found to have NK cell dependent anti-tumor effects in both B16 melanoma lung and liver metastasis, as well as in a human colon carcinoma model in nude mice (Bessard, Sole et al. 2009). Recently, this technology has progressed to

include other molecules to target the complex to tumors. For example, an antibody fusion protein with IL-15/IL-15 Rα sushi domain/ antibody to tumor stromal fibroblast activation protein was created that had significant anti-tumor effects in a lung tumor mouse model (Kermer, Baum et al. 2012).

IL-15 immunotherapy has also shown efficacy when combined with other therapies including chemotherapy (Chapoval, Fuller et al. 1998), radiofrequency thermal ablation (Habibi, Kmieciak et al. 2009) and multi cytokine administration (Comes, Di Carlo et al. 2002; Kaspar, Trachsel et al. 2007). The addition of these factors all improved results over IL-15 alone. A few studies have looked at IL-15 in combination with other factors and breast cancer. For example, the study by Habibi et al. involved radiofrequency thermal ablation in combination with intra-tumoral IL-15 and IL-7 in mice injected with Her2 (N202) mouse breast tumor cells (Habibi, Kmieciak et al. 2009). They found that all three treatments together led to a delay in tumor growth (Habibi, Kmieciak et al. 2009). Lastly, recent usage of IL-15 in animal models has included IL-15 with antibodies to multiple immune system inhibitory checkpoints. Yu *et al*. found that IL-15 treatment was effective at treating IV colon carcinoma, but that on CD8 T cells there was still an increase in the inhibitory molecule PD-1 and increased IL-10 production, which could prevent effective anti-tumor responses (Yu, Steel et al. 2010). They went on to show that combining IL-15 with anti-PD-L1 and anti-CTLA-4 antibodies led to better CTL killing and survival in murine metastatic colon carcinoma and a prostate tumor model (Yu, Steel et al. 2010; Yu, Steel et al. 2012). Unfortunately, they did not examine the role of NK cells in this model. It is likely that future studies,

using the IL-15/IL-15 Rα complex as well as blockade of immune inhibition will be even more efficacious. From these pre-clinical models, it appears that IL-15, at least in engrafted models of tumor formation, is capable of promoting either NK or CD8 T cell based anti-tumor responses.

Obviously the next step for IL-15 immunotherapy would be to extend pre-clinical work to human clinical trials. Before this goal could be accomplished, IL-15 needed to be tested in nonhuman primate models. Initial tests indicated that IL-15 was better than IL-2 in generating long lived CD8 memory in rhesus macaques (Villinger, Miller et al. 2004). Further tests found that IL-15 could be safely administered with minimal toxicity, that it expanded memory CD8 and CD4 T cells as well as NK cells and only minimally expanded T regulatory cells (Berger, Berger et al. 2009; Lugli, Goldman et al. 2010). IL-2 has been the prototypic cytokine that was approved for immunotherapy due to its effects on NK cells and CD8 T cells. While IL-15 and IL-2 share some receptor subunits, studies have indicated that IL-15 is superior to IL-2 for immunotherapy for many reasons including: decreased toxicity (Rosenberg, Lotze et al. 1987; Berger, Berger et al. 2009), does not induce high levels of T regulatory cells (Zhang, Chua et al. 2005; Smyth, Teng et al. 2006), does not induce activation induced cell death (Marks-Konczalik, Dubois et al. 2000) and promotes stronger anti-tumor effects in NK cells and memory CD8 T cells (Ozdemir, Ravindranath et al. 2005; Ozdemir and Savasan 2005). Unfortunately, IL-15 studies in humans are still in their infancy due to difficulties in producing good manufacturing practice (GMP) level hIL-15. Several studies using PBMCs from healthy volunteers or cancer patients indicate that *in vitro*, IL-15 can promote CD8 and NK cell

proliferation and cytotoxic responses (Ozdemir, Ravindranath et al. 2005; Szczepanski, Szajnik et al. 2009). IL-15 has also shown promise for the expansion of activated NK cells from hNK samples (Decot, Voillard et al. 2010). IL-15 is now available for clinical trial testing and there are currently several Phase I/II clinical trials listed at the NIH with either IL-15 alone (NCT01727076, NCT01021059, NCT01572593) as a treatment or in a combination treatment with either NK cell/total lymphocyte infusions (NCT01385423, NCT0136988, NCT01337544). While the majority of the trials concentrate on treating known NK cell sensitive cancers such as acute myelogenous leukemia (AML) and melanoma, some will also examine solid tumors. Not much work has been done using the aforementioned complex of IL-15 and IL-15Rα in humans. Recently, Chertova *et al*. were able to make a stable heterodimer of human IL-15/IL-15Rα that shows similar effects on NK and CD8 T cells in mice and could make transfer of this complex to the clinic swift (Chertova, Bergamaschi et al. 2013). Results from these trials are eagerly awaited, although if what has been found in mouse models holds true, it may be in combination with other therapies that the most profound effects of IL-15 will be observed.

Not all expression of IL-15 is positive. Intense stimulation of NK cells and CD8 T cells with IL-15 can lead to both the progression of certain types of cancer (leukemia's in particular) as well as autoimmune diseases. In fact, IL-15 TG mice can develop large granular lymphocyte leukemia at later stages of life (Fehniger, Suzuki et al. 2001; Fehniger, Suzuki et al. 2001; Yokohama, Mishra et al. 2010). In some human cancers, such as multiple myeloma, it has been reported that IL-15 can promote survival of the

tumor cells (Tinhofer, Marschitz et al. 2000). In addition, renal cell carcinoma is unique in that is possesses a membrane bound form of IL-15 (Wittnebel, Da Rocha et al. 2007). Upon ligation with IL-15 Rα, a reverse signal can be triggered in the cancer cell that promotes epithelial to mesenchymal transition (Khawam, Giron-Michel et al. 2009). As of yet, this has only been reported in this particular system. In terms of autoimmune diseases, IL-15 has proven to be a negative prognostic factor in rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and celiac disease (Di Sabatino, Calarota et al. 2011). In these cases, blockade of IL-15 signalling is being attempted with antibodies to IL-15 or IL-2R/β and have shown some improvements in disease states (Di Sabatino, Calarota et al. 2011). Thus it is apparent that treatment with IL-15 must be undertaken only in the right circumstances and patients should be monitored for any adverse effects.

Several other cytokines that also target NK/CD8 T cells are being investigated for usage as an immunotherapy, some of which have already proceeded to clinical trials. The most promising molecules include IL-12 and IL-21. IL-12 is a cytokine that is known to activate NK cells and promote the generation of Th1 type T cells (Manetti, Parronchi et al. 1993). Due to its many successes in pre-clinical models, it quickly proceeded to clinical trials, but while it showed some promise, it also displayed high cytotoxicity (Robertson, Cameron et al. 1999; Weiss, Subleski et al. 2007). Thus, to reduce toxicities and lower required doses, researchers have moved to examine ways to combine IL-12 with other cytokine therapies or to direct the effects to the tumor rather than have systemic expression. For example, Jahn *et al.* recently published an interesting pre-

clinical study where IL-2 and IL-12 are fused with an antibody to a tumor antigen present on Hodgkin's lymphoma cells (CD30). In a mouse model with a CD30+ tumor, this complex was capable of inducing tumor regression (Jahn, Zuther et al. 2012). IL-21 is another cytokine that is involved in the proliferation and maturation of NK cells and T cells (Parrish-Novak, Dillon et al. 2000). It also appears to have less toxicity than IL-2 in terms of its' ability to cause vascular leak syndrome (Sivakumar, Garcia et al. 2013). In cancer patients, IL-21 administration causes immune activation of NK cells as well as CD8 T cells (Frederiksen, Lundsgaard et al. 2008) and in a Phase II trial enough efficacy was observed to warrant further investigation (Petrella, Tozer et al. 2012). Initial reports indicate that the combination of IL-21 with other cytokines or ADCC may be a future area of success (Tian, Yuan et al. 2011; Steele, Anthony et al. 2012). It has been discovered that IL-21 is also very potent in the expansion of NK cells from PBMCs due to its ability to promote lengthening of telomeres, which prevents NK cell senescence (Denman, Senyukov et al. 2011). Now that IL-15 has become available for clinical usage, combinations of these cytokines may be a potent area to explore. In a pre-clinical model, treatment of mouse or human NK cells with IL-12/15/18 creates "memory-like" NK cells that produce very high levels of IFNγ and are capable of persisting in hosts and maintaining their ability to produce IFNγ (Cooper, Elliott et al. 2009; Ni, Miller et al. 2012; Keppel, Yang et al. 2013). The potential for this therapy is exciting as perhaps combination cytokine treatments will allow us to overcome the effects that the tumor microenvironment promotes in NK cells.

1.6 Macrophages:

1.6.1 Macrophage Classification:

Macrophages are innate immune cells that play a role in both tumor immunosurveillance and tumor progression. Typically, macrophages are defined as innate immune cells that are capable of phagocytosis and are important for microbe clearance as well as setting the stage for future immune responses. The reality of this is much more complicated. Circulating monocytes can be recruited to tissues via chemokines such as CCL2/MCP1 (monocyte chemotactic protein), CSF-1 (macrophage colony-stimulating factor) and CXCL12/SDF-1 (stromal cell-derived factor)(De Palma and Lewis 2013). Once at a tissue, it is the combination of signals present within this microenvironment that will determine the phenotype of that macrophage. Mantovani's group was the first to coin the phrase M1" classically" vs M2 "alternatively" activated macrophage (Mantovani, Sozzani et al. 2002). These definitions are simplifications that can be used as a framework, but in reality there is likely a much more diverse group of macrophages. A classically activated macrophage would evolve in an environment rich in Th1 cytokines such as IFNγ and TNFα or TLR agonists (ie. lipopolysaccharide-LPS)(Mantovani 2006). For example, IFNγ could be expressed by an activated NK cell at the site of infection and that will promote M1 polarization. M2 macrophages may evolve in cases where Th2 cytokines such as IL-4 or IL-13, as well as immune complexes and possibly IL-10 are present (Mantovani 2006; Martinez, Sica et al. 2008). The

reported functions of M1 and M2 macrophages are vastly different. M1 macrophages release pro-inflammatory cytokines like IL-12, IL-15, IL-1β, TNF-α, as well as reactive oxygen species (nitric oxide-NO) and possess higher levels of MHC II molecules (Martinez, Sica et al. 2008). These molecules allow the macrophage to kill and phagocytose pathogens, kill tumor cells, support a Th1 response and present antigen (Martinez, Sica et al. 2008). M2 macrophages differ significantly in their function. M2 macrophages produce anti-inflammatory cytokines like IL-10, produce arginine and VEGF and have up-regulated scavenger receptors (CD206) (Martinez, Sica et al. 2008; De Palma and Lewis 2013). This allows them to reduce immune responses, promote tissue repair and promote tumor formation (angiogenesis). So while this describes two ends of the spectrum, in normal healthy tissues macrophages often express a phenotype that is mixed M1/M2 (De Palma and Lewis 2013). It is more often in pathological conditions that we observe more extreme polarization.

1.6.2 Macrophages in the Tumor:

Macrophages have been associated with increased angiogenesis and poor prognosis in a multitude of human tumors (Clear, Lee et al. 2010; Daurkin, Eruslanov et al. 2011; Heusinkveld and van der Burg 2011). In mouse tumor models, macrophage depletion or depletion of molecules involved with macrophage recruitment have revealed that removal of these cells from tumors actually inhibits tumor formation and angiogenesis (Lin, Nguyen et al. 2001; Zeisberger, Odermatt et al. 2006). Tumor

associated macrophages (TAMS) have a different mRNA profile than macrophages at other sites such as the spleen. In a mouse model of breast cancer, TAMs possess genes that suppress immune responses as well as mediators of tumor angiogenesis and matrix remodelling (Ojalvo, King et al. 2009). This gives a strong hint as to their functions within the tumor. When TAMs were further examined, they were said to be of the M2 phenotype (Mantovani, Sozzani et al. 2002). This phenotype is promoted by the microenvironment within the tumor which includes high levels of molecules such as PGE_2, IL-10 and TGF-β that can be produced by tumor cells themselves or T regulatory cells (Mantovani, Allavena et al. 2008; Eruslanov, Daurkin et al. 2011; Lee, Lee et al. 2013). Recently, in a spontaneous breast cancer model, CD4 T cells expressing IL-4 were implicated in tumor cell invasion and metastasis from the primary tumor due to their promotion of a M2 phenotype in TAMS (DeNardo, Barreto et al. 2009). In this case, removal of CD4 T cells led to decreased metastasis due to a change in TAM phenotype.

Within established tumors, macrophages have multiple roles. The following three shall be addressed: to support tumor growth via angiogenesis, promote immunosuppression and promote the intravasation of primary cells into the blood for metastasis. The most direct link between TAMs and tumor progression stems from the role of TAMs in angiogenesis. Many studies have shown that TAMs produce VEGF and other factors that contribute to angiogenesis in the tumor (Leek, Lewis et al. 1996; Fujimoto, Sakaguchi et al. 2000; Leek, Hunt et al. 2000; Tsutsui, Yasuda et al. 2005). Tumor macrophages have also been linked to suppression of the immune system. For example, TAMs recruit T regulatory cells to the tumor (Curiel, Coukos et al. 2004;

Kryczek, Wei et al. 2007). TAMs also mediate immunosuppression by inducing T cell apoptosis via ARG1, NOS2 and peroxynitrite (Gabrilovich, Ostrand-Rosenberg et al. 2012). In fact, in established tumors the higher the number of TAMs, the lower the number of CD8 T cells (DeNardo, Brennan et al. 2011). We have also found that TAMs can contribute to altering NK cell phenotype to a less cytotoxic state (Krneta, T., Gillgrass, A., Ashkar, A. unpublished data). Wyckoff *et al*. found that TAMs make epidermal growth factor (EGF), a molecule that can bind to cancer cell EGF receptor to increase the invasiveness and migration of the cancer cell (Wyckoff, Wang et al. 2004). In addition, it has been found using multiphoton intravital imaging, that in a mouse model of breast cancer, tumor cells only invaded blood vessels where perivascular TAMS were located (Wyckoff, Wang et al. 2007). Lastly, in several human cancers the progression to metastatic disease is linked with increasing TAMs (Robinson, Sica et al. 2009; Kang, Chen et al. 2010). Therefore, macrophages play key roles in many aspects of primary tumor growth and metastasis.

While the majority of reviews present this as the whole story, it is not really this simple. A few human tumor types, such as NSCLC, have reported macrophage infiltration into the tumor as a positive prognostic factor (Forssell, Oberg et al. 2007; Ohri, Shikotra et al. 2009). In some cases this has been associated with a more M1 macrophage phenotype (Ohri, Shikotra et al. 2009). In fact, it seems that at early stages of tumor formation or in regressing tumors, TAMS can be M1 skewed (Wang, Li et al. 2011). There are also indications that TAMS are capable of ADCC mediated killing of mAb targeted tumors (De Palma and Lewis 2013). Within mouse mammary tumors, M2

(MHC^{low}) macrophages are found in hypoxic regions, increase in number while the tumor grows and are pro-angiogenic, whereas M1 (MHC^{high}) macrophages are present elsewhere in the tumor (Movahedi, Laoui et al. 2010). Thus, we cannot say globally that macrophage presence in tumors is pro-tumorogenic and we need to be cautious when targeting them.

The good news is that as mentioned, macrophages are highly responsive to the signals they receive in their environment. Thus it should be possible to reprogram TAMs towards a M1 phenotype that will kill tumor cells. Several researchers have found that it is possible to convert macrophages of a M2 phenotype into that of a M1 phenotype via treatment with cytokines and vice versa (Stout, Jiang et al. 2005; Mylonas, Nair et al. 2009). Even in tumor models such as breast cancer, it was found that injection of GM-CSF into the tumors was capable of reprogramming the tumor macrophages to become anti-tumor (Eubank, Roberts et al. 2009). In mice bearing subcutaneous Lewis lung carcinoma tumors, treatment with IL-12 was capable of changing the functional profile of the TAMs to a more M1 phenotype (Watkins, Egilmez et al. 2007). In bladder cancer, bacillus Calmette-Guerin (BCG) administration has been shown to elicit a change in the macrophages towards an M1 phenotype so that they are capable of killing tumor cells via TNFα, IFNγ and NO production (Luo and Knudson 2010). It has been demonstrated that NF-κB is necessary for maintaining the immunosuppressive phenotype of TAMS and that this pathway can be targeted to cause a shift to a more M1 phenotype ($MHCII^{high}$, IL-12^{high})(Hagemann, Lawrence et al. 2008). Targeting this pathway specifically in macrophages led to a decrease in tumor burden in ovarian cancer (Hagemann, Lawrence

et al. 2008). If reprogramming proves difficult, other strategies have been aimed at removing macrophages altogether. Luo *et al.* published an interesting study where they targeted legumain, a stress protein overexpressed by TAMs, with a DNA vaccine and stimulated a CD8 response against TAMs (Luo, Zhou et al. 2006). This led to suppression of tumor angiogenesis, growth and metastasis in models of breast, colon and NSCLC and 75% of animals given a lethal tumor challenge survived (Luo, Zhou et al. 2006).

1.6.3 Macrophages at the Pre-metastatic Niche:

While we have detailed the important role that macrophages play in the primary tumor and how they encourage metastasis from the primary tumor, they also have an important role in establishing metastasis at distant sites. This is an area that has only recently received attention. Once tumor cells have exited the primary tumor and entered the blood, they have a long road to follow to form a met. They must survive in the blood where they may meet competent immune cells, they need to lodge at the distant site, extravasate into the tissue and then become established and grow. Macrophages play a key role during many aspects of this process. Kaplan *et al.* established that pre-metastatic niches exist prior to the arrival of metastatic tumor cells (Kaplan, Riba et al. 2005). In mice with established primary Lewis lung carcinoma or B16 F10 melanoma tumors, there are pre-metastatic sites that contain clusters of VEGFR1+ hematopoietic bone marrow cells that are necessary for metastasis to occur, as well as increased fibronectin deposits

(Kaplan, Riba et al. 2005). Interestingly, conditioned media from different types of tumor cells (Lewis lung carcinoma vs B16 melanoma) were able to induce pre-metastatic niches in different tissues (lung and liver only vs many sites throughout the body), indicating that soluble factors from tumors could promote the formation of these niches and confer tissue specificity (Kaplan, Riba et al. 2005). It was further established in a model that metastasized to the lung that secretion of TNFα, TGF-β and VEGF1 from primary tumors induced the expression of S100A8 and S100A9 (inflammatory chemo attractants) in lung macrophages and endothelial cells (Hiratsuka, Watanabe et al. 2006). Expression of S100A8/A9 attracts CD11b+ myeloid cells to the pre-metastatic niche and indirectly promotes primary cell migration (Hiratsuka, Watanabe et al. 2006). Primary tumor cells also secrete lysyl oxidase (LOX), which co-localizes with the fibronectin at the pre-metastatic site and also increases the ability of CD11b+ myeloid cells to bind to the lung site (Erler, Bennewith et al. 2009). These studies are exciting as the identification of factors necessary to metastasize to certain sites may lead to specific therapies that could potentially block metastasis to particular organs. To further examine the factors involved in establishing metastasis at distant sites, many researchers have employed intravenous injection of tumor cells. Pollard's group has done some seminal research using the intravenous injection of mouse mammary tumor cells (polyoma MT tumor cells)(Qian, Deng et al. 2009; Qian, Li et al. 2011). They found that to establish metastasis in the lung, tumor cells need to directly interact with macrophages to allow extravasation and without this interaction metastatic seeding is very inefficient (Qian, Deng et al. 2009). These macrophages were not lung resident macrophages and were

CD11b+F4/80+VEGFR+GR1-CD11c- (Qian, Deng et al. 2009). The macrophages were also necessary for growth of the metastatic lesion (Qian, Deng et al. 2009). They went on to further these findings with an examination of how inflammatory monocytes (Gr1+) could also be recruited via CCR2 (CCL2 expressed by tumor cells) to metastatic sites where they become Gr1- (Qian, Li et al. 2011). While the exact specifics of all the players involved in establishing metastatic sites remains under investigation, it is clear that macrophages play a role in this process. It will be interesting to determine the exact phenotype/functions that macrophages require to fulfill this role.

1.6.4 Myeloid Derived Suppressor Cells (MDSCs):

MDSCs have recently become an intense area of interest in terms of tumor immunology. They are a heterogeneous population of cells that can differentiate in certain circumstances from immature myeloid cells. When an immature myeloid cell is recruited to a tumor, it receives many signals that can promote it to become a DC, macrophage or MDSC. In mice, there are 2 main populations of MDSC, granulocytic (gMDSC)($CD11b^{+}CD11c^{-}$ $Ly6C^{low}Ly6G^{+}$) and monocytic (moMDSC) ($CD11b^{+}CD11c^{-}$ $Ly6C^{high}Ly6G^{-}$)(Gabrilovich, Ostrand-Rosenberg et al. 2012). While the exact molecules necessary to create MDSC *in vivo* have not been completely identified, it appears as if tumor or T cell secreted GM-CSF, VEGF, IL-6, IL-1β, IL-10, IL-4, IL-13 and S100A8/A9 may play a role (Gabrilovich, Ostrand-Rosenberg et al. 2012). MDSCs have many roles within the tumor including the immunosuppression of T cells/NK cells via depletion of nutrients like L-arginine, causing oxidative stress with reactive oxygen

species (ROS), interfering with lymphocyte trafficking and activating or expanding T regulatory cells (Gabrilovich, Ostrand-Rosenberg et al. 2012). For example, Movahedi *et al.* found that both moMDSC and gMDSC are capable of suppressing antigen specific T cell responses in T cell lymphoma models (Movahedi, Guilliams et al. 2008). It is thought that mo vs gMDSCs employ different mechanisms to exert these effects; moMDSCs use NO and ARG1, both use IFNγ, and gMDSCs use ROS (Movahedi, Guilliams et al. 2008; Lindau, Gielen et al. 2013). Depletion of MDSC in a lung cancer model led to increased numbers and activity of NK and CD8 T cells in tumors as well as decreased angiogenesis and tumor growth (Srivastava, Zhu et al. 2012). In models of virus infection, MDSCs have also been shown to suppress NK cell activation and function (Fortin, Huang et al. 2012; Zhu, Huang et al. 2012). In breast cancer patients, the number of MDSCs in PBMCs has been found to increase with disease progression (Markowitz, Wesolowski et al. 2013).

With their tumor supportive roles in mind, researchers have attempted to target MDSCs with therapies. It has been found that activated NK cells can render T cells resistant to the effects of MDSCs in a mouse model of breast cancer (Kmieciak, Basu et al. 2011). In addition, it has been reported that MDSCs can mature into TAMs (Gabrilovich, Ostrand-Rosenberg et al. 2012). If this is the case, then aforementioned treatments with molecules that promote M1 macrophage formation may be effective for reprogramming MDSCs. Other strategies being used include decreasing the numbers of MDSCs (sunitinib) and inhibiting MDSC function (PDE-5 inhibitor)(Najjar and Finke 2013). Several of these strategies are in clinical trials and have had promising results,

although perhaps the most promising results would be when used in conjunction with other immunotherapies. One of the ways that MDSCs promote tumor formation is by production of free radicals that nitrate MHC I on CD8 T cells and chemokine/cytokines, which prevents their ability to fight tumors (Markowitz, Wesolowski et al. 2013). Recently, a Phase II study on Stage II-IIIc breast cancer combined traditional chemotherapy treatments with an inhibitor of free radical formation, NOV-002, prior to surgery (Montero, Diaz-Montero et al. 2012). They found that the addition of NOV-002 increased complete response rates and those patients with decreased MDSCs had a greater chance of complete response (Montero, Diaz-Montero et al. 2012). Thus, by removing the suppressive effects of MDSCs, therapies aimed at generating effective NK and T cell responses will be much more effective.

1.7 NK Cell Immunotherapy in the Clinic:

We have outlined the evidence that NK cells play an important role in tumor defence and destruction in both mice and humans. It is no wonder then that this has led to interest in using NK cells as an immunotherapy. Cancer patients demonstrate a decrease in both NK cell numbers and activation. If we could increase the number of NK cells and activate them correctly, would we be able to cure cancer/prevent metastasis? There have been many barriers to enacting this idea, the first being that in the past scientists have been unable to generate large numbers of activated NK cells. Currently, technological advances and increased knowledge regarding NK cell biology have made

the generation of highly activated good manufacturing practice (GMP) quality NK cells a reality. In the most recent NK amplification systems, a variety of groups have devised what has been termed artificial APCs (aAPCs). These are composed of a modified K562 cell line that expresses either membrane bound IL-15 or IL-21, in addition to other co-stimulatory molecules like 41BB-ligand, CD86 and FCγRI that are cultured with human PBMCs either from patients or healthy volunteers (Fujisaki, Kakuda et al. 2009; Denman, Senyukov et al. 2011; Lapteva, Durett et al. 2012; Liu, Wu et al. 2013). Expansion methods using IL-21 were found to work the best, as IL-21 promotes proliferation by increasing telomere length so that the NK cells do no become senescent as they do with IL-15 alone (Fujisaki, Kakuda et al. 2009; Denman, Senyukov et al. 2011). It has been found that in only 3 weeks, the IL-21 aAPCs expand NK cells by $5X10^5$ fold (Denman, Senyukov et al. 2011). In addition, expanded NK cells had increased activation markers such as NKG2D, DNAM-1 and CD16 and were capable of killing tumors in xenograft models (Liu, Wu et al. 2013). These, and other studies, have also established that NK cells could be frozen and still maintain their functionality, which greatly facilitates the technology transfer into the clinic (Lapteva, Durett et al. 2012; Liu, Wu et al. 2013). Lastly, what to grow these NK cells in to maintain GMP quality and for ease of use has been another issue. Options thus far include tissue culture flasks, cell culture bags and bioreactors. A study that compared these technologies found that one of the best options was for NK cells to be grown in large-scale fully automated bioreactors, as it was less work intensive and good cytotoxicity levels were maintained (Sutlu, Stellan et al. 2010). Thus, major barriers to the growth of high numbers of activated NK cells have been

removed. Now the question is what type of NK cells should be transferred and should they be combined with additional treatments? The types of adoptive NK cell transfer can be classed into autologous and allogeneic. The possible combinations that could be useful alongside NK cell delivery include mAb therapy, systemic cytokine therapy and therapies to block inhibition of NK cells.

Adoptive therapy with NK cells can be autologous (from patient) or allogeneic (non-patient or cell lines). Autologous transfer of NK cells has not shown very much efficacy in clinical trials thus far. Early trials involved treatment of patients with IL-2, followed by lymphocyte harvest and *ex vivo* culture with IL-2, before re-infusion back into the patient and further systemic IL-2 (Phillips, Gemlo et al. 1987; Rosenberg, Lotze et al. 1987; Sparano, Fisher et al. 1994). These trials had very disappointing results and also had some deaths due to IL-2 treatment (Phillips, Gemlo et al. 1987; Rosenberg, Lotze et al. 1987; Rosenberg, Lotze et al. 1993; Sparano, Fisher et al. 1994). Unfortunately, these studies involved prolonged usage of IL-2, which is now known to increase T regulatory cells and is likely not the ideal cytokine for systemic treatment (Zhang, Chua et al. 2005). Later trials used new methods for generating NK cells such as *ex vivo* expansion of T cell depleted PBMCS, using irradiated autologous PBMCs as feeder cells, IL-2, and OKT3 (anti-CD3 Ab), but these too were clinically unsuccessful at clearing melanoma or RCC (Parkhurst, Riley et al. 2011). It was found that while the NK cells were cytolytic *in vitro*, they did not survive in the host for more than a week and quickly lost their cytotoxic ability (Parkhurst, Riley et al. 2011). Perhaps with the

aAPCs-mbIL-21 expansion system described above, there will be better proliferation and activation of the NK cells and they will survive longer *in vivo*.

Allogeneic transfer of NK cells has been more successful in clinical trials. This approach is based on the idea that NK cells are inhibited due to MHC I/HLA expression on target cells, therefore if the donor NK cells transferred have Ly49s/KIRs that don't match the MHC I/HLA molecules expressed by the tumor, they should be able to kill it. The seminal paper that explored this possibility focused on acute myelogenous leukemia (AML) patients, and found that when patients were given NK cells with a KIR/HLA mismatch, 60% of patients survived for 5 years without relapse, whereas when they were given those that had identical KIR/HLA only 5% survived (Ruggeri, Capanni et al. 2002). Several trials followed in patients with AML, lymphoma or solid tumors, examining the linkage between KIR/HLA mismatch versus complete match and found that mismatch was a major determinant of a successful response (Miller, Soignier et al. 2005; Leung, Handgretinger et al. 2007). Since then, trials have used IL-15 to culture allogeneic NK cells to treat NSCLC (Iliopoulou, Kountourakis et al. 2010), IL-2 treated NK cells with CD3 depletion for ovarian and breast cancer (Geller, Cooley et al. 2011) or even freshly harvested NK cells with no activation in high risk leukemia/highly malignant solid tumors after stem cell transplant (Stern, Passweg et al. 2013), but unfortunately these have not had a high degree of success. Again, it will be interesting to see if delivery of NK cells expanded using IL-21 expressing aAPCs will be more promising in allogeneic transplants. Another possible method for allogeneic NK cell transfer is the use of NK cells lines. If this work wells, it would be the most cost effective and easy to use

alternative as the cells lines can be expanded indefinitely, are readily available and can be manipulated easily. The most well known NK cell line is NK-92, which was established from a patient with non-Hodgkins lymphoma (Gong, Maki et al. 1994). It is quite cytotoxic, requires IL-2 for growth and is CD56+CD16-KIR- (Gong, Maki et al. 1994; Tonn, Becker et al. 2001). NK-92 has shown promise in xenograft models of melanoma (Tam, Miyagawa et al. 1999) and in a Phase I clinical trial of melanoma and RCC had mild toxicity and some positive responses (Arai, Meagher et al. 2008). Also of note, these cell lines can be genetically modified to increase their efficacy. For example, they can be transduced with retroviral vectors to encode chimeric antigen receptors specific to tumor antigens (CD20 in B cell lymphoma, disialoganglioside (GD_2) in neuroblastoma) so that they can kill targets that are normally NK resistant (Muller, Uherek et al. 2008; Esser, Muller et al. 2012).

Monoclonal antibodies (mAb) are approved for use in several tumor types, the most well known being herceptin (trastuzumab) that is commonly used against $HER2^+$ breast cancer. While the link between mAb and NK cell therapy may not seem obvious, recent discoveries indicate that the success of mAb is intimately entwined with NK cells. As mentioned, NK cells express the molecule CD16, also known as FcγRIII, and are capable of performing ADCC (Sulica, Morel et al. 2001). At first, treatment with mAbs showed promising results as immunotherapeutics, but their exact mechanism of action was unknown. Revetch's group was the first to determine that NK cells were involved in mAb therapy (Clynes, Towers et al. 2000). They found that mice lacking activating Fc receptors like FCγRIII, were not protected by herceptin from HER2 expressing tumors

(Clynes, Towers et al. 2000). This led to other studies that revealed that NK cell activity in metastatic breast cancer patients receiving trastuzumab is correlated with progression free survival (Beano, Signorino et al. 2008) and others found that ADCC was the most important method of cell death contributing to the success of mAb therapy (Weng and Levy 2003). Several studies went on to confirm that adding activated expanded NK cells to mAb therapy *in vitro* or in xenograft models increased tumor cell death via ADCC (Deng, Terunuma et al. 2012; Gottschalk, Kimmig et al. 2012; Roberti, Rocca et al. 2012). Very recently, researchers began to attempt to improve the ability of antibodies to bind both the tumor antigen and the NK cell by creating tetravalent, bispecific antibodies or by modifying the protein backbone of the antibody to better bind Fcγ activating receptors (Kellner, Zhukovsky et al. 2013; Reiners, Kessler et al. 2013). Both proved to be an improvement and have led to possible clinical trials. It has now been realized that mAb therapy will be extremely effective when combined with other therapies targeting NK cell activity. There is an ongoing clinical trial that is testing the combination of donor NK cells with mAb epratuzumab (targets CD22 Ag on B cells) and IL-2 in acute lymphoblastic leukemia (NIH-NCT00941928). The results for this will be interesting, although IL-2 may not be the best cytokine to combine with the therapy.

As has been mentioned, another method used to increase NK cell number and activity is to employ systemic cytokines. We have discussed the potential of IL-15 for this purpose, as well as the pitfalls of IL-2. Another interesting cytokine that has been introduced is IL-21. IL-21 also plays a role in the proliferation and maturation of NK cells (Parrish-Novak, Dillon et al. 2000). In comparison with IL-2, it inhibits T

regulatory cell differentiation and is less toxic (Tian, Yuan et al. 2011; Sivakumar, Garcia et al. 2013). In Phase I/II trials, it has proven safe, and it can increase NK cell anti-tumor activity (Frederiksen, Lundsgaard et al. 2008; Schmidt, Brown et al. 2010; Petrella, Tozer et al. 2012). It was used in conjunction with the mAb cetuximab (anti-EGFR) in a recent Phase I trial and showed promising results (Steele, Anthony et al. 2012). It is also likely that it will be a combination of NK cell activating cytokines that will work best at producing long-lived activated NK cells. It has been demonstrated that *in vitro* treatment of NK cells with an IL-15/IL-12/IL-18 cocktail promotes the formation of "memory-like" NK cells that secrete large amounts of IFNγ, and when transferred into immunocompromised mice, persist for 4 weeks in alymphoid hosts and 12 weeks in NK-competent hosts (Cooper, Elliott et al. 2009; Keppel, Yang et al. 2013). When injected into an engrafted model of lymphoma and combined with radiation therapy, the IL-15/IL-12/IL-18 treated NK cells were able to proliferate and persist in the tumor in high numbers (Ni, Miller et al. 2012). These very potent NK cells are likely moving into clinical trials at the present moment.

While producing activated NK cells will be important, blocking the inhibition of NK cells may be a key step in NK cell immunotherapy. It is obvious that the adoptive transfer of un-activated NK cells alone may be ineffective, as the tumor microenvironment can quickly change their phenotype even to the point that they promote tumor growth. We have already mentioned strategies to decrease or inhibit MDSCs as well as alter M2 TAMs to a more anti-tumor phenotype (target NF-κB, IL-12). There are also strategies to reduce T regulatory cells that in cancer promote tumor

immunosuppression (anti-CD25 mAb)(Rech, Mick et al. 2012). A strategy we have not mentioned that is relatively new is to block KIR activity on NK cells with anti-KIR antibodies. In pre-clinical models, this anti-KIR Ab allowed lysis of tumor cells while normal cells remained un-lysed and *in vivo* it promoted tumor cell rejection (Romagne, Andre et al. 2009). This antibody has proceeded to a Phase I trial (IPH2101) in multiple myeloma patients and was found to be safe and tolerable (Benson, Hofmeister et al. 2012).

It appears to be the beginning of a golden age for NK cell based immunotherapy. We are armed with knowledge regarding how NK cells kill and how tumors can subvert this attack. The future of NK cell based immunotherapy is definitely bright as combinations of the described therapeutic strategies will lead to increased clinical efficacy. As the novel therapies move into the clinic, we will see if pre-clinical data can be successfully translated into improved survival. If so, a much higher percentage of many cancers may be effectively cured.

1.8 Research Proposal Outline:

1.8.1 Rationale

IL-15 is a very important cytokine that can influence cells of both the innate and the adaptive immune system. Thus far, the majority of studies investigating the effect of IL-15 on cancer have focused on engrafted models of tumor formation or metastatic lung

cancer models. The positive outcomes observed in such studies often do not translate to the clinic as it is much easier to induce an immune response against a tumor that is seen as foreign. There is also a lack of studies performed on the effects of IL-15 on solid epithelial cancers. Several lines of evidence indicate that NK cells may be important effectors against breast cancer. Animal studies in breast cancer models revealed that a lack of NK cells led to faster tumor formation (Street, Zerafa et al. 2007). Recent human studies on breast cancer patients have indicated that the phenotype of NK cells are altered to a decreased state of cytotoxicity as the disease progresses (Mamessier, Sylvain et al. 2011; Konjevic, Jurisic et al. 2012; Mamessier, Pradel et al. 2013). These studies indicate that immunotherapies involving NK cells may be effective against breast cancer. Therefore, we feel that examination of the effect of IL-15 absence and IL-15 overexpression in a spontaneous model of breast cancer (MMTV-pMT) will be informative as to its efficacy in human breast cancer. To investigate this, we have crossed IL-15 KO mice and IL-15 TG mice with MT mice to create IL-15 KO/MT and IL-15 TG/MT mice. In addition, we feel that a more thorough investigation into the role of IL-15 during the establishment of breast cancer metastasis will be informative. IL-15 is thought to be an important factor that affects the phenotype of many immune cells that positively or negatively contribute to metastasis, including NK cells, CD8 T cells, macrophages and CD4 T cells. Early studies have only examined the contribution of NK cells and/or CD8 T cells. Therefore in an IV model of breast cancer metastasis we will examine the factors involved in promoting or preventing establishment of tumors at the metastatic site. We will inject a cell line established from pMT mice into IL-15 TG and

IL-15 KO mice, and examine the phenotypes/contributions of immune cells. Lastly, IL-15 has been reported to affect macrophage phenotype, possibly directly and/or indirectly. Thus we want to determine if the polarization of TAMs in IL-15 KO/MT or IL-15 TG/MT has been altered. We will investigate this, as well as the possibility that either M1 or M2 polarized macrophages will affect tumor formation in an engrafted model of breast cancer. These studies will increase our knowledge regarding how IL-15 can best be used in the clinic against breast cancer.

1.8.2 Hypothesis:

IL-15, via effects on cells of the innate and adaptive immune system, promotes anti-tumor responses in a model of spontaneous breast cancer as well as metastasis.

1.8.3 Objectives:

1) To determine the effect of IL-15 on tumor formation in a spontaneous model of breast cancer and the mechanism(s) which contributes to this effect.

2) To investigate the role of IL-15 in a model of breast tumor metastasis.

3) To delineate the impact of macrophages, in an altered NK cell/IL-15 environment, in the formation of mammary carcinoma and whether this can be utilized to alter breast tumor formation.

Chapter 2.0: Materials and Methods

2.1 Animal models: Mice were bred and maintained in the McMaster Central Animal Facility in "clean" rooms with a 12 hour day/night schedule and standard temperature controls. Procedures performed herein were approved by the McMaster Animal Research Ethics Board (AREB) and comply with the guidelines set out by the Canadian Council on Animal Care. Breeding pairs of MMTV-MT mice were provided by Dr. Gendler (Mayo Clinic, AZ) and IL-15 TG mice were provided by Dr. Caligiuri (Ohio State University, School of Medicine, Colombus, OH). IL-15 KO mice were purchased from Taconic (Germantown, NY). IL-15 KO mice and IL-15 TG mice were mated to MT mice to generate IL-15 KO/MT and IL-15 TG/MT mice (single copy of transgene for MT and IL-15 TG) (all on a C57BL/6 background). C57BL/6 control mice were purchased from Charles River (Quebec, Canada).

2.2 Subcutaneous tumor model: A cell line established from a MMTV-polyoma middle T (MT) C57BL/6 spontaneous breast tumor (Mayo Clinic) was grown in Roswell Park Memorial Institute media (RPMI)(10% FBS,1% penicillin/streptomycin, 1%

HEPES, 1% L-glutamine). After brief trypsinization, cells were washed in phosphate buffered saline (PBS) and resuspended at $1X10^5$ per 100 µl of sterile PBS and injected subcutaneously in the right flank of mice. Mice were monitored 3 times per week to check for tumor formation and endpoint (tumors >10X10mm).

2.3 Spontaneous tumor formation: In the spontaneous model, mice that possessed the IL-15 TG or MT transgene or lack IL-15 were identified via standard genotyping methods and published primers. Namely, DNA was extracted from tail clippings and run via standard PCR conditions using PCR Supermix (Invitrogen). Identified IL-15 KO/MT, MT and IL-15 TG/MT mice were palpated weekly from 6 weeks of age for tumor formation and endpoint (tumors >10x10mm). To examine metastasis, lungs from each group of mice were harvested at 120 days of age. Lungs were perfused with 2% paraformaldehyde (PFA), embedded and sectioned 2 times, 100 µM apart. Both sections were examined for the presence of lung metastasis and scored as positive or negative.

2.4 Metastasis model: The same MT cell line utilized for the subcutaneous model was washed in PBS and resuspended at $0.5X\ 10^6$- $1X\ 10^6$ per 400 µl of sterile PBS. Cells with <5% cell death were injected via the tail vein of IL-15 KO, C57BL/6 or IL-15 TG mice. Mice were monitored for endpoint and 13 days post injection, mice were euthanized and lungs were harvested. Lungs were fixed in 2% PFA for 48 hours. Sections were then stained with H&E for analysis of lung tumor metastasis. The level of metastasis was assessed while blinded in ImageJ. In brief, in an entire lobe of lung, per

each experimental animal, a threshold algorithm was applied to assess tumor area (manually checked for accuracy). Total lung area (with lumen areas subtracted) was also assessed and percent tumor area was calculated by the following formula:

Percent tumor area= (tumor area/total lung area)*100

2.5 Histology/immunohistochemistry: In the spontaneous model, tumors were excised from multiple mice per group (MT, IL-15 KO/MT, IL-15 TG/MT) and embedded in Tissue-Tek® OCT (Sakura) or fixed in 2% PFA. Sections were stained with haematoxylin and eosin (H&E)(n>10 per group). In addition, immunohistochemistry was performed on snap frozen tissues in Tissue-Tek® OCT (Sakura) for CD8α (PharMingen- #550281; 1:50) and CD4 (PharMingen- #550278; 1:50) using a Rat on Mouse Kit (Biocare Medical- #RT517H). Colour was developed using an AEC Chromogen substrate solution (Sigma). For quantitation, samples were blinded and 5 random fields of view per section were counted in 5 mice per group. A terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (ApopTag In Situ Apoptosis Detection Kit- Millipore) was also performed as per manufacturer's instructions on 3 size matched tumors per group.

2.6 Flow cytometry on tumors and spleens: To generate single cell suspensions suitable for analysis, tumors were excised, digested (3 mg/ml Collagenase A, 0.025 mg/ml DNase I (Roche) - 45 min., 37^0C, with shaking), and filtered sequentially through 70 µm and 40 µM filters. Spleens were also collected and a single cell suspension was

created by squishing. Red blood cells were removed with ammonium-chloride-potassium (ACK) lysis buffer. Cells were incubated with anti-mouse CD16/32 (eBioscience- #14-0161-86) (1 in 100, 15 min, 4ºC) and then stained for markers including: NK1.1, CD69, NKG2D, NKp46, CD8, CD4, CD3, CD44, PD-1, CD27, CD62L, F4/80, CD11b, Gr1 and IFNγ/Perforin (intracellular flow using BD-cytoperm/cytofix) (eBiosciences/BD Biosciences). For tumor immune cell analysis, the first gate was made on singlets followed by a gate on CD45+ cells (leukocytes) to remove tumor cells. NK cells were identified as NK1.1+CD3-. CD8 T cells were identified as CD8+CD3+. Macrophages were identified as F4/80+CD11b+Gr1-. Fluorescence minus one (FMOs) controls were performed for all experiments to determine appropriate gates. Samples were run on the BD LSRII or CANTO flow cytometer and FlowJo (Tree Star, Ashland, OR) was used for analysis.

2.7 Non-specific T cell stimulation: Cells were isolated from spleens and tumors as described above and CD8 T cells were further isolated using a CD8 T cell selection kit (Stem cell- #18753). Purity was assessed by flow cytometry analysis and was >80% for tumors and >90% for spleens. CD4 T cells were isolated from the spleens of IL-15 KO, C57BL/6 and IL-15 TG mice using a CD4 T cell enrichment kit (Stem Cell). Purity of CD4 T cells after enrichment was > 75% via flow cytometry. 96 well plates were coated overnight with purified 1 μg/ml anti-CD3 (eBioscience- #16-0031-82) and 5 μg/ml anti-CD28 (eBioscience- #16-0281-82) antibodies at 4 ºC. The next day, plates were washed thoroughly and $5X10^5$ CD8 or CD4 T cells/well were added in triplicate. Uncoated wells

were included as a control. Supernatants were collected 48 hours later for cytokine analysis. In cases where intracellular flow was performed, the same process was followed, but after plating for 12 hours, GolgiStop (BD Biosciences) was added for 10 hours prior to flow staining.

2.8 Enzyme-linked immunosorbent assay (ELISA)/Cytokine analysis: IFNγ (DY485), TNFα (DY410), IL-10 (DY417), IL-13 (DY413) and IL-12 p40 (DY499) ELISAs were performed using Murine DuoSet Kits from R&D (Minneapolis, MN). ELISAs were performed as per manufacturer's instruction. For IL-18 levels, 3 tumors per group (size matched) were homogenized and pooled in an equal volume and sent to Rules Based Medicine (RBM) for multi-analyte protein analysis (RodentMAP® version 2.0).

2.9 NK/Tumor cell killing assay: Mouse: NK cells were isolated from the spleens of C57BL/6 mice (Stem cell- PE selection Kit #18551 with PE NK1.1, 2 μg/ml). The MT cell line was labelled with 5-6-carboxyfluorescein diacetate succinimidyl ester (CSFE, 5 μM). The NK cells and the CFSE labelled MT cell line were put together at various Effector:Target ratios (1:1, 5:1, 10:1) for 5 hours. MT cells were incubated alone to determine basal cell death. Following 5 hours of incubation, 7-amino actinomycin D (7-AAD, BD, 5 μl/tube) was added to identify dead cells and flow cytometry was performed to quantify the percentage of CFSE and 7-AAD positive cells (dead MT cells). Specific

lysis was calculated via the following formula (Garcia-Iglesias, Del Toro-Arreola et al. 2009):

$$\% \text{ specific lysis} = \frac{100 \text{ X } (\% \text{ sample lysis} - \% \text{ basal lysis})}{100 - \% \text{ basal lysis}}$$

Human: NK cells were isolated from PBMCs (Ficoll-Paque Plus, StemCell, #07907- to isolate mononuclear cells) with a human NK cell enrichment kit (StemCell, #19055, >90% purity of CD56+CD3- NK cells). The resultant NK cells were cultured for 16 hours in IL-2 (100 U/ml) or in IL-12 (10ng/ml)/IL-15 (20ng/ml)/IL-18 (100 ng/ml) (Peprotech). Cells were washed 4X before being cultured with CFSE (5µM) labelled MDA-231 (NCI-60 panel) cells at various E: T ratios (1:1, 5:1, 10:1) for 5 hours. MDA-231 cells were incubated alone to determine basal cell death. After incubation, cells were stained with CD45-PE (BD) antibody and before flow cytometry, 7-AAD (BD, 5 µl/tube) was added to identify dead cells. FMO controls were included in each experiment. Flow analysis was performed to determine PE-CFSE+7AAD+ cells and the above formula was applied.

2.10 Adoptive transfer: Recipient mice were given 3 mg cyclophosphamide (Sigma- #C7397-1G) intraperitoneally to induce lymphopenia. Spleens and tumors were removed from tumor bearing or control mice, processed, CFSE stained (5 µM) and CD8 T cells were isolated using a CD8 T cell selection kit (Stem cell- #18753). 24 hours after

cyclophosphamide treatment, $5X10^6$ (spleen) or $1X10^6$ (tumor) CFSE labelled CD8 T cells were injected intravenously to the recipients. 24 hours after mice had received adoptive transfer of CD8 T cells, mice were challenged with 0.5 $X10^6$ freshly isolated MT primary cells. The MT primary cells were prepared by amalgamating the tumors from 2 endpoint MT mice, processing to single cell level and then performing epithelial cell enrichment (Stem Cell- #19758) to remove immune cells. Challenged mice were followed for tumor formation and endpoint. As well, several mice were checked for engraftment 8 days post tumor challenge. CFSE+ CD8 T cells were found in the spleen of all mice examined and were 4-9% of the total CD8 T cell population (data not shown).

2.11 Short term antibody depletions: For the subcutaneous model or metastasis model, mice were given 2 injections, one day apart of either anti-CD4 (GK1.5; ATCC) (100 ug), anti-CD8 (mouse IgG, clone 2.43; ATCC) or anti-NK1.1 (200 ug/dose) (PK136 mouse IgG2, hybridoma HB191; ATCC) antibody intraperitoneally. Antibody was given on day T-4, T-3 and then on T0. On T0 MT cells were injected in the tail vein or subcutaneously. CD4 and CD8 depletion was carried out once per week and NK1.1 depletion was every 3-4 days to maintain the depletion for the course of the experiment. Efficiency of depletion was checked via flow cytometry. Spleens from each group were analyzed for CD4+CD3+ T cells, CD8+CD3+ T cells or NK1.1+ cells (data not shown).

2.12 Long term antibody depletions: In the spontaneous model, IL-15 TG/MT mice were given two doses of 200 μg anti-NK1.1 mouse IgG antibody intraperitoneally one

day apart starting at 4 weeks of age. Antibody depletion was continued every 3-4 days at 200 μg doses. For CD8 depletion, 100 μg of anti-CD8α mouse IgG (clone 2.43; ATCC) antibody was administered intraperitoneally in 2 sequential doses one day apart and then continued once per week for the duration of the experiment. After tumor formation, animals were sacrificed and spleens/tumors were examined for efficient removal of NK1.1/CD8 positive cells. Tumors were fixed (2% PFA) and H&E sections were prepared to examine tumor formation. Several spleens/tumors were analyzed to determine the efficiency of depletion.

2.13 Collection of peritoneal macrophages: Mice were anaesthetized with ketamine (150 mg/kg)/xylazine (10 mg/kg). 10 ml of room temperature RPMI media was injected intraperitoneally and left in the mouse for 5 minutes with movement. Liquid was then collected with an 18 gauge needle and put on ice. Sample was then washed, resuspended in media and plated for 3 hours. Adherent cells were collected for further experimentation.

2.14 Nitric Oxide (NO) Assay: $1X10^5$ or $2X10^5$ peritoneal macrophages were plated in 96 well plates with RPMI (10% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% L-glutamine) or media supplemented with LPS (100 ng/ml; Sigma). Assay was performed in triplicate. At 24 hours and 48 hours, media was collected and combined with Greiss reagent (0.04 g/ml)(Sigma). Absorbance was measured at 450nm and compared to a standard curve of sodium nitrate.

2.15 Arginase assay: The arginase activity of the macrophages was determined using a protocol that has been previously outlined (Chan, Pek et al. 2011). In short, 2X10^5 cells were lysed in 100 uL 0.1% Triton X-100 in PBS (with protease inhibition- Complete Mini: Roche). After 30 minute room temperature incubation, an equal volume of 25 mM Tris-HCL (pH7.5) was added, as well as 10 ul of 10 mM $MnCl_2$. Samples were heated to 56 °C for 10 min. 100 uL of this solution was then combined with 100 uL of 0.5M L-arginine (pH 9.7) at 37 °C for 1 hour to allow hydrolysis of L-arginine. To stop the reaction, 800 uL of stop solution was added (H_2SO_4(96%)/H_3PO_4(85%)/H_2O [1:3:7, v/v/v]) and 40 uL of 9% α-isonitrosopropiophenone. Samples were heated to 99 °C for 30 min and then absorbance was assessed at 540 nm on a microplate reader. A standard curve solution was used to determine concentration. One unit of arginase activity was defined as the amount of enzyme that catalyzed the formation of 1 umol of urea per minute at 37 °C.

2.16 Isolation of cells and ribonucleic acid (RNA) from lungs and PCR Array in MT metastasis model: IL-15 KO mice or C57BL/6 mice were injected with 5X10^5 MT tumor cells IV. 2 days post injection, lungs were perfused, harvested and one lobe was used for RNA isolation and the other for cell isolation. For cell isolation, lungs were minced and digested in 150U/ml collagenase I (Gibco) in HANKS for 1 hour at 37ºC with shaking. Cells were then filtered and incubated with anti-mouse CD16/32 (eBioscience- #14-0161-86) (1 in 100, 15 min, 4ºC), followed by staining for CD45, F4/80, CD11b, CD11c, GR1 or CD45, CD4, CD3 (BD or eBiosciences). CD45+ cells

were gated on to begin leukocyte analysis. To extract RNA, TRIzol® (Invitrogen) was used initially, followed by Qiagen RNeasy® Mini Kit extraction and on column DNase digestion (RNase-Free DNase Set #79254, Qiagen). Subsequently, 1 µg of RNA per sample was used to create cDNA as per manufacturers' instruction (RT^2 First Strand Kit #3304011, Qiagen). The cDNA was then used in a RT^2 Profiler PCR Array for Mouse Cytokines and Chemokines as per manufacturers' instruction (RT^2 SYBR Green ROX™ PCR Mastermix was utilized, SABiosciences/Qiagen). This 96 well plate includes housekeeping genes, genomic DNA controls, reverse transcription controls and positive controls. The plate was run on the Applied Biosystems 7900 RT machine. Results were analyzed using the SABiosciences analysis tool and all samples used passed the quality controls inherent in these assays. Results are reported as fold up or down regulation, with 2 samples per group included in the analysis.

2.17 Flow sorting and qRT PCR of tumor macrophages: Tumors from 2 MT, IL-15 TG/MT or IL-15 KO/MT mice were pooled, digested (as previously described) and macrophages were selected with a CD11b Positive Selection Kit (Stemcell). Resultant cells were stained with F4/80 (eBioscience) and 7-AAD (BD) was added just before flow sorting (BD-FACSAria™ III). Live cells that were CD11b+ and F4/80+ were collected and RNA was extracted using an RNeasy Mini Kit (Qiagen). After use of a DNA free™ kit (Ambion), 150 ng of RNA was used to synthesize cDNA (Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT); Invitrogen). The resultant 20 ul of cDNA was diluted in dH_2O to 200 ul and 5 uL was used for each reaction. Taqman® Universal PCR

Master Mix (Applied Biosystems/Roche), Taqman® Gene Expression Assays primer/probe for either GAPDH or NOS2 and cDNA were combined and in triplicate run on Applied Biosystems 7900 RT machine. GAPDH was used as the endogenous gene and iNOS as the target gene to normalize the data. MT was chosen as the control sample, and other samples were compared to this. Initial analysis was performed in SDS2.2.1.

2.18 Preparation of M1 or M2 macrophages: Peritoneal macrophages from C57BL/6 mice were isolated as described. They were then cultured in 10 ng/ml IFNγ and 100 ng/ml LPS (M1) or 20 ng/ml IL-4 and 50 ng/ml IL-13 (M2)(cytokines from Peprotech, LPS from Sigma) for 24 hours. Cells were washed 3 times prior to any further experiments.

2.19 Injection of macrophages and tumor cells: Macrophages were isolated from C57BL/6, IL-15 TG or IL-15 KO mice as outlined. In other experiments, M1 or M2 macrophages were prepared as described. Right before subcutaneous injection into the right flank of mice, they were mixed with the MT cell line at 2:1 or 5:1 ratios (ex. $2X10^5$ macrophages: $1X10^5$ tumor cells).

2.20 Macrophage depletion: 200 ul of Clodronate liposomes (clodronateliposomes.org, Amsterdam, The Netherlands) were administered via intraperitoneal injection to deplete macrophages. These were given T-3, T-2, T0 (MT tumor cell injection) and every 3-4

days following until endpoint. The depletion scheme was tested by flow cytometry for CD11b and F4/80 in the spleen (data not shown).

2.21 Statistical analysis: Statistics were performed in GraphPad Prism. Either T tests or One-way analysis of variance (ANOVA) were performed, depending on the number of groups to be compared (T-test for 2, one way ANOVA ≥3). Bonferonni's post test was used in the case of ANOVAs. Survival curves were analyzed with the Log Rank test (Mantel-Cox).

Chapter 3.0: Results/Figures

3.1 IL-15 Stimulated NK cells cause tumor destruction in a spontaneous breast tumor model:

3.1.1 IL-15 TG mice are resistant to subcutaneous MT tumor formation, whereas IL-15 KO mice are susceptible. NK cells are key contributors to this effect. To determine if the presence or absence of IL-15 had an impact on the formation of subcutaneous breast tumors, C57BL/6, IL-15 KO and IL-15 TG mice were injected with $1X10^5$ MT cells (previously established from a MT breast tumor) subcutaneously in the right flank. Mice were subsequently palpated and tumors were measure 3 times/ week to determine when tumors formed and when they reached endpoint size (10X10mm). IL-15 TG mice were resistant to tumor formation and had 80% survival (n=5/group) (Figure 1A). In addition, IL-15 KO mice proceeded to endpoint more quickly than IL-15 TG mice. Since IL-15 mediated tumor protection is NK cell mediated in several models, we utilized anti-NK1.1 antibodies to deplete NK cells from C57BL/6 control mice to determine whether or not NK cells were important in this model (Yajima, Nishimura et al. 2002; Kobayashi, Dubois et al. 2005). NK cell depletion promoted tumor formation (data not shown) and decreased survival (Figure 1B). To further explore the role of NK cells, we tested whether or not NK cells were capable of killing MT tumor cells. When NK cells were cultured with labelled MT cells for 5 hours, they were capable of killing the MT cells (Figure 1C). At an effector (E): target (T) ratio of 10:1, we saw 47% specific lysis of MT cells. This data confirmed that NK cells play a role in

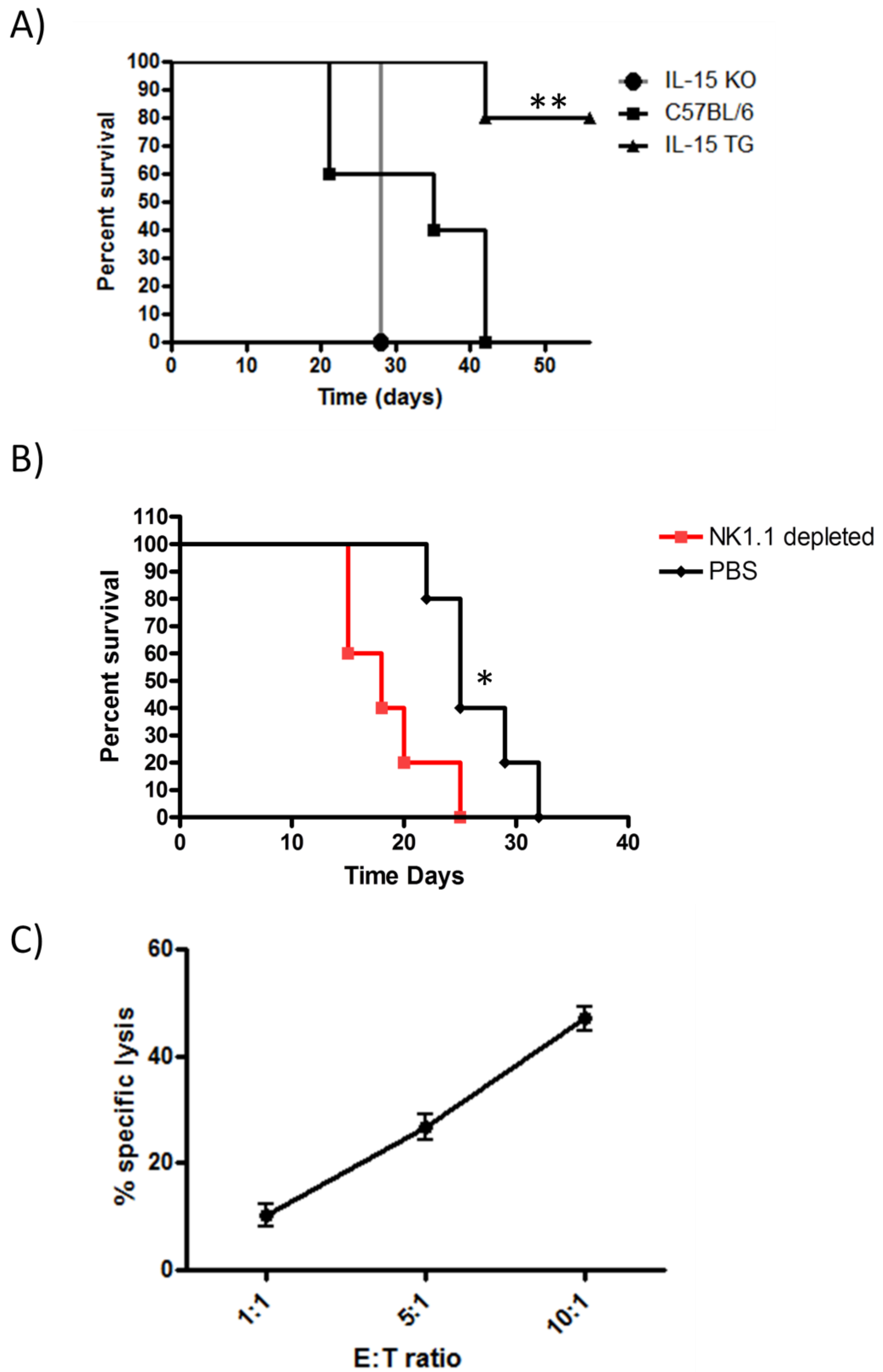


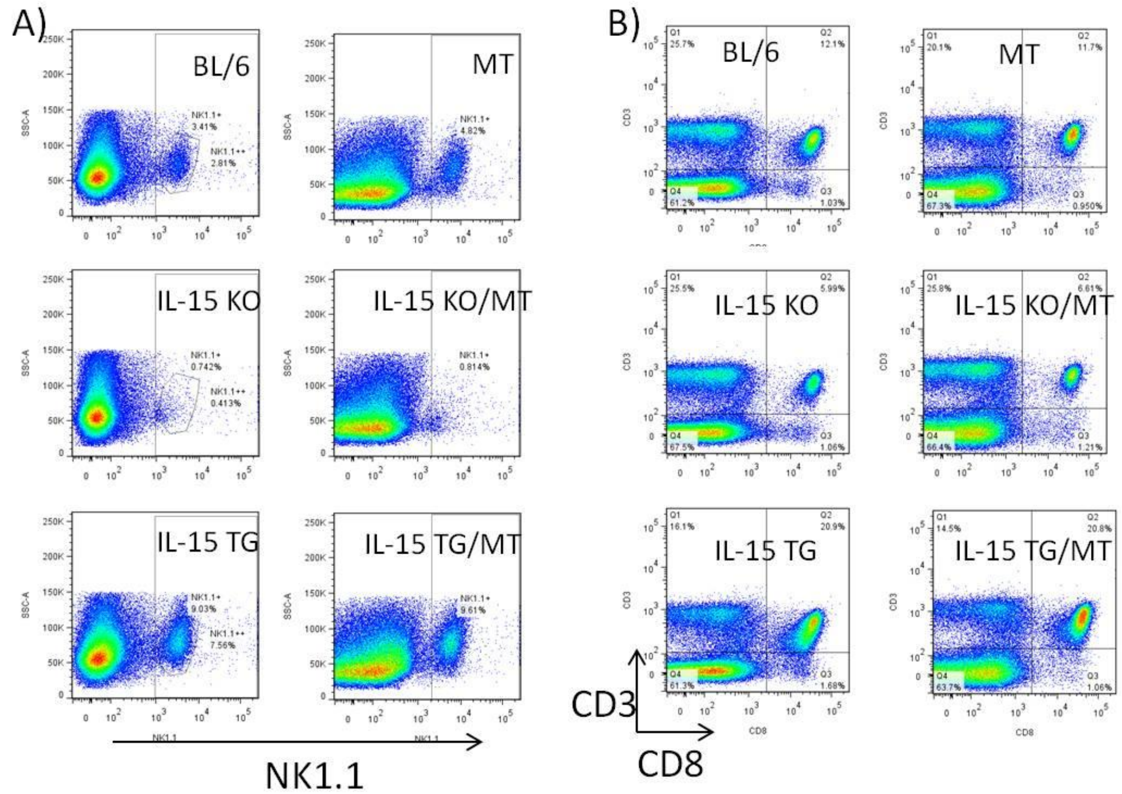
Figure 1

3.1- Figure 1: IL-15 TG mice are resistant to subcutaneous breast tumor formation and IL-15 KO mice are susceptible. NK cells are important to increased survival. (A) $1X10^5$ MT cells were injected subcutaneously into IL-15 KO, C57BL/6 and IL-15 TG mice. IL-15 TG mice were resistant to tumor formation in comparison to either the IL-15 KO or C57BL/6 mice. (B) Depletion of NK cells in C57BL/6 mice via NK1.1 antibody led to decreased survival. (C) NK cells isolated from the spleen of C57BL/6 mice (E-Effector) are capable of killing MT tumor cells (T-Target), in a killing assay. *Statistically significant

protection from this breast tumor subcutaneous model and are capable of killing MT cells specifically. We next sought to determine if this would extend to a spontaneous breast tumor model in which tumors form more similarly to those in humans (Lin, Jones et al. 2003).

3.1.2 IL-15 TG/MT mice have increased NK and CD8 T cells, whereas IL-15 KO/MT mice lack NK cells and have reduced CD8 T cells compared to control mice. To establish the role of IL-15 in spontaneous breast tumor formation, MT mice were crossed to IL-15 KO and IL-15 TG mice to create IL-15 TG/MT and IL-15 KO/MT mice. To confirm that the IL-15 KO and IL-15 TG phenotype is maintained in IL-15 KO/MT and IL-15 TG/MT mice, spleens from 6-8 week old IL-15 KO, IL-15 KO/MT, BL/6, MT, IL-15 TG and IL-15 TG/MT were analyzed by flow cytometry for NK cells and CD8 T cells. IL-15 KO and IL-15 KO/MT mice lack NK cells and also had decreased CD8 T cells (6% of total lymphocytes) (Figure 2). In addition, IL-15 TG and IL-15 TG/MT mice both had similar amounts of increased NK cells (9%) and increased CD8 T cells (21%). In control C57BL/6 and MT mice there were 4-5% NK cells and 12% CD8 T cells. Therefore, the addition of the MT transgene does not alter the NK and CD8 T cell percentages in the IL-15 KO or IL 15 TG mice.

3.1.3 IL-15 TG/MT tumors have a drastically different phenotype than IL-15 KO/MT tumors. IL-15 TG/MT mice form very small tumors in comparison to IL-15 KO/MT mice (Figure 3A). While IL-15 KO/MT mice display several very large tumors



3.1- Figure 2: The IL-15 KO and IL-15 TG phenotype is maintained in mice that also possess the MT transgene. Spleens from 6-8 week old IL-15 KO, IL-15 KO/MT, BL/6, MT, IL-15 TG and IL-15 TG/MT were analyzed by flow cytometry for NK cells and CD8 T cells. Both IL-15 KO and IL-15 KO/MT mice lack NK cells and have a reduced proportion of CD8 T cells. In contrast, IL-15 TG and IL-15 TG/MT mice both have an equal increase in NK cells and CD8 T cells proportions.

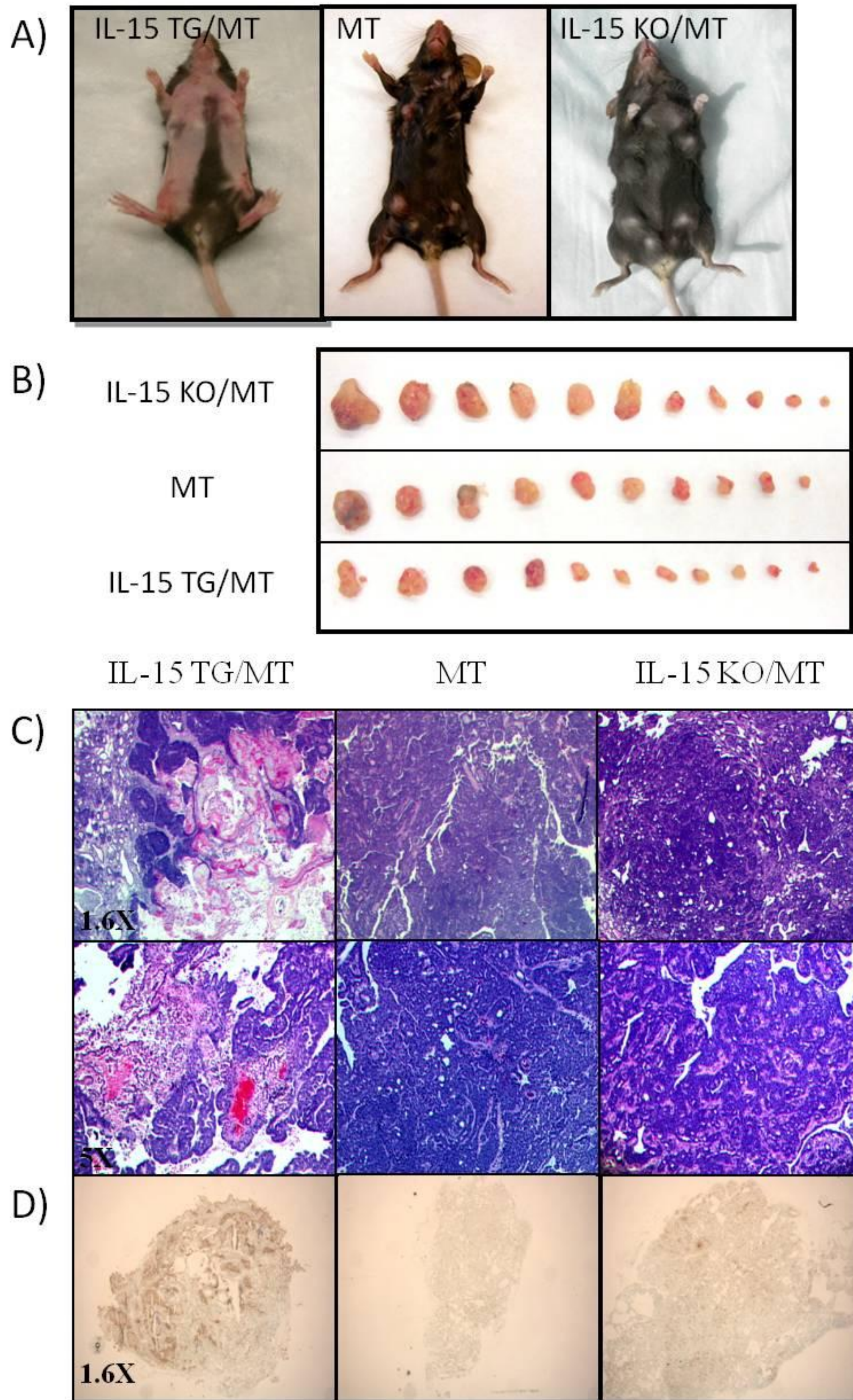


Figure 3

3.1- Figure 3: Visualization of the tumor. (A) Picture of representative IL-15 KO/MT, MT and IL-15TG/MT mice at endpoint. (B) All tumors excised from one endpoint mouse in each group. (C) H&E staining of IL-15 KO/MT, MT and IL-15 TG/MT tumors. Representative images from n=10 per group (1.6X and 5X). (D) TUNEL stain (ApopTag In Situ Apoptosis Detection Kit- Millipore) on cryosections from representative IL-15 KO/MT, MT and IL-15 TG/MT tumors (n=3/group) (Apoptosis=dark brown).

at endpoint, the majority of IL-15 TG/MT tumors remain very small and are hard upon palpation (Figure 3B). To visualize the effect of IL-15 on tumor formation, tumors of all sizes were excised from IL-15 KO/MT, MT and IL-15 TG/MT mice and were sectioned for H&E staining (Figure 3C). IL-15 KO/MT tumors were solid, well vascularised tumors that had very little cell death. In contrast, IL-15 TG/MT tumors had very little healthy tumor growth and TUNEL stain revealed cell death throughout (Figure 3D). In addition, there appeared to be a large lymphocytic infiltration of cells in IL-15 TG/MT tumors. This effect was seen in multiple tumors excised at various sizes (n>10).

3.1.4 IL-15 affects the speed of tumor formation, the progression to endpoint and metastasis in a spontaneous breast tumor model. The extensive histological differences between IL-15 TG/MT and IL-15 KO/MT mice indicated that IL-15 may play a role in preventing spontaneous breast tumor formation and progression to endpoint. In the MMTV-polyoma MT antigen (MT) mouse model (C57BL/6 strain), multifocal mammary adenocarcinomas developed in 100% of mice and tumors developed at a median of 95 days (Figure 4A). For these MT mice, the median age of endpoint was 153 days (Figure 4B). IL-15 KO/MT tumors formed faster (median= 78 days) than either MT or IL-15 TG/MT tumors ($p<0.0001$) (Figure 4A). In addition, IL-15 KO/MT (median=136) mice progressed to endpoint more quickly than MT or IL-15 TG/MT mice ($p<0.01$) (Figure 4B). IL-15 TG/MT mice had increased survival time when compared to MT mice ($p<0.01$) (Figure 4B). This indicates that a lack of IL-15 speeds tumor formation and promotes progression, whereas overexpression of IL-15 extends survival.

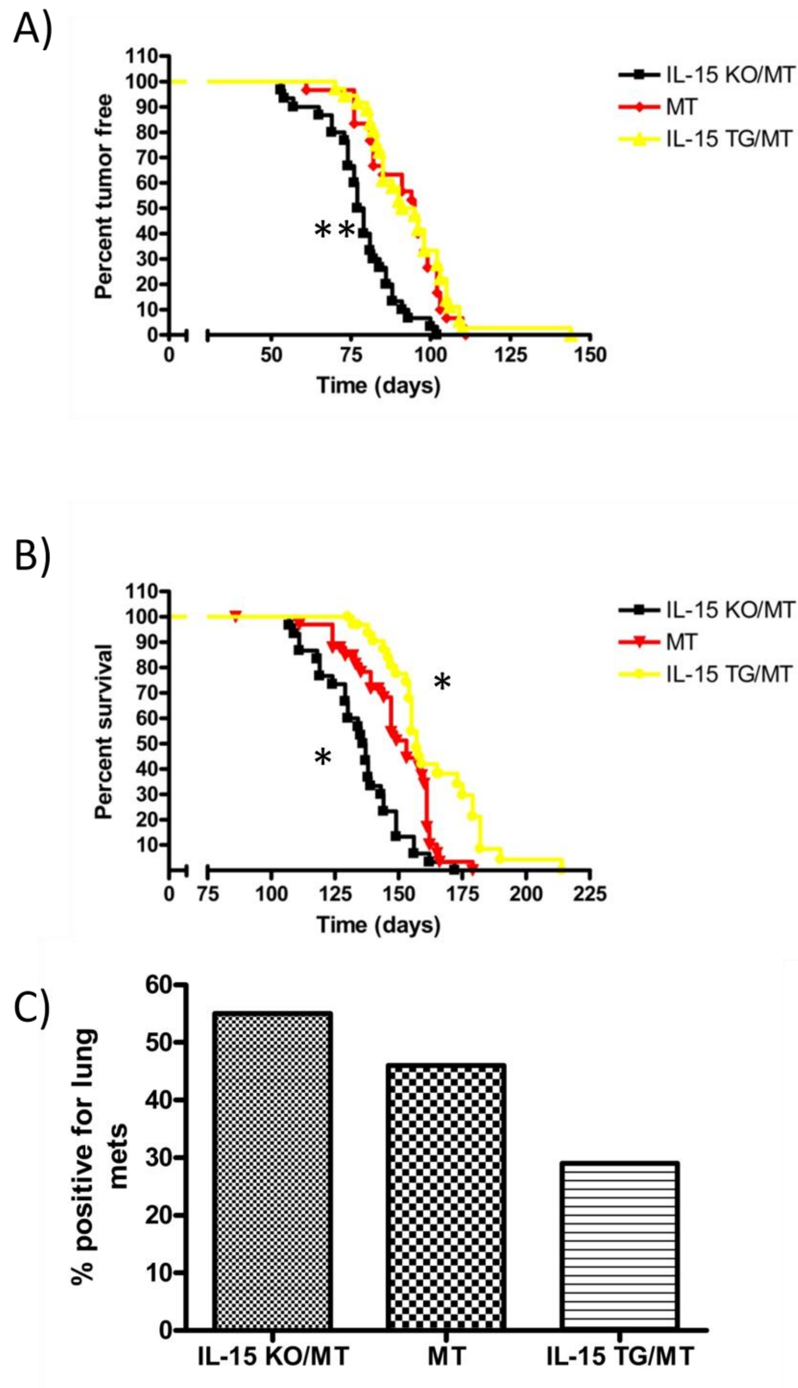


Figure 4

3.1- Figure 4: Percent tumor free (A), survival curve (B) and percent with lung metastasis (C) for MT, IL-15 KO/MT and IL-15 TG/MT mice. Mice were palpated and tumors were measured once per week to determine date of first palpation or endpoint. (A) The median age of tumor palpation and number of mice per group was as follows; MT 95 (n=30), IL-15 KO/MT 78 (n=30) and IL-15 TG/MT 93 days old (n=36). There is a statistical difference between the IL-15 KO/MT mice and the MT or IL-15 TG/MT mice ($p<.0001$). (B) The median age of endpoint and number of mice per group was as follows; MT 153 (n=30), IL-15 KO/MT 136.5 (n= 30) and IL-15 TG/MT 157 days old (n=28). In comparison to MT mice the IL-15 TG/MT mice have increased survival ($p=.0051$), whereas IL-15 KO/MT mice have decreased survival ($p=.002$). (C) Percentage of mice with lung metastasis at 120 days of age (MT n=13, IL-15 KO/MT n=11 and IL-15 TG/MT n=14).

The MT mouse model tumors have been shown to metastasize to the lung (Guy, Cardiff et al. 1992). To examine metastasis in the absence or overexpression of IL-15, mice from each group were analyzed for lung tumor metastasis at 120 days of age. We found that 29% of IL-15 TG/MT mice (n=14), 46 % of MT mice (n=13) and 55% of IL-15 KO/MT mice (n=11) had detectable metastasis at this time point (Figure 4C). This indicates that the overexpression of IL-15 decreases spontaneous breast tumor metastasis, whereas lack of IL-15 promotes it.

3.1.5 IL-15 TG/MT tumors have higher proportions of activated NK cells. IL-15 is known to promote the differentiation, survival and activation of NK cells (Carson, Fehniger et al. 1997; Cooper, Bush et al. 2002; Ranson, Vosshenrich et al. 2003; Farag and Caligiuri 2006). Therefore, we examined the status of NK cells within MT and IL-15 TG/MT tumors. IL-15 KO/MT mice do not possess NK cells and thus were not included in this analysis (Kennedy, Glaccum et al. 2000). Tumors were excised, digested and stained for flow markers to determine the proportion as well as the activation of NK cells (Figure 5). To analyze leukocyte populations alone, sample analysis was conducted on CD45+ cells. NK cells were defined as NK1.1+CD3-. The IL-15 TG/MT tumors had a higher proportion of NK1.1+ cells than MT tumors ($p<0.01$) (Figure 5A). While there was an increase in the proportion of NK cells, if these cells are not activated they are unable to function. It has been shown that tumors possess or secrete several factors that can decrease NK cell activation (Della Chiesa, Carlomagno et al. 2006; Waldhauer and Steinle 2008; Flavell, Sanjabi et al. 2010). 80% of NK cells within the IL-15TG/MT

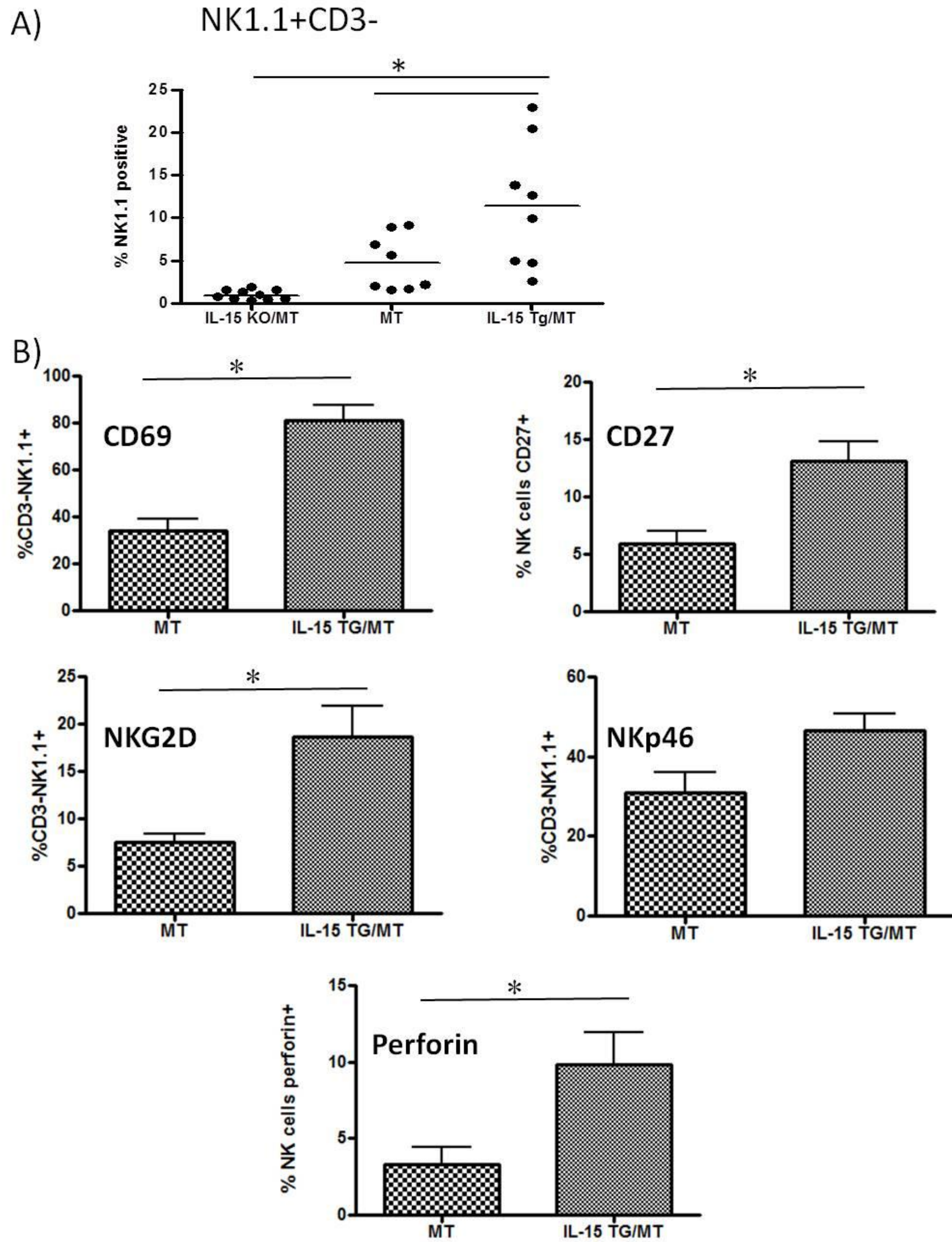
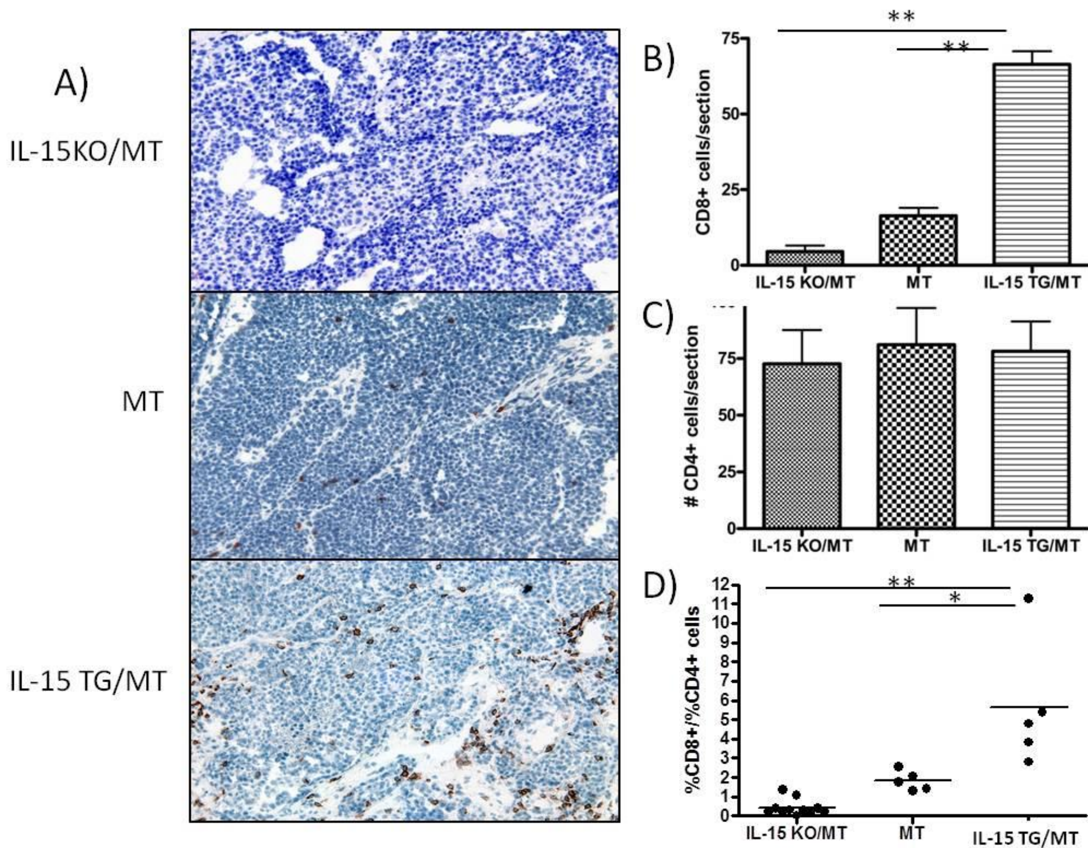


Figure 5

3.1- Figure 5: IL-15 TG/MT tumors possess increased activated NK cells. Tumors from MT, IL-15 KO/MT and IL-15 TG/MT mice were digested and stained for flow cytometry. (A) Of CD45+ cells in the tumor, there was a higher proportion of NK1.1+ cells in the IL-15 TG/MT tumors than in the MT or IL-15 KO/MT tumors (n= 10- IL-15 KO/MT, 8- MT or IL-15 TG/MT). (B) In the IL-15 TG/MT tumors, the majority of the NK cells possessed the early activation marker CD69. In addition, a higher percentage of NK cells in IL-15 TG/MT tumors possessed CD27, a marker of highly cytotoxic NK cells. Lastly, a higher percent of the NK cells in IL-15 TG/MT tumors possessed the activation markers NKp46 and NKG2D, as well as perforin (n=3 to 9). * Statistically significant

tumors possessed the early activation marker CD69, whereas <40% of MT NK cells were CD69+ (Figure 5B). In addition, more NK cells in IL-15 TG/MT tumors were CD27 positive and perforin positive (Figure 5B), which indicates an increased cytotoxicity and activation potential (Hayakawa and Smyth 2006). Lastly, the NK cells within IL-15 TG/MT tumors had higher levels of the activating receptors NKp46 and NKG2D (Figure 5B).

3.1.6 IL-15 TG/MT tumors possess increased CD8 T cells and a high CD8 to CD4 T cell ratio. IL-15 is also able to control the proliferation and survival of naive and memory CD8 T cells (Kanegane and Tosato 1996; Judge, Zhang et al. 2002). Therefore, we wanted to determine if we had altered the T cell environment within the tumors when we altered the IL-15 status. Thus, we took tumor sections from IL-15 KO/MT, MT and IL-15 TG/MT mice and stained them for CD8α (Figure 6 A/B). There were higher numbers of CD8 T cells within IL-15 TG/MT tumors in comparison to either IL-15 KO/MT or MT tumors ($p<0.001$ for both). The amount of CD4 T cells was also determined by immunohistochemistry, and there were approximately equal numbers of CD4 T cells in all of the tumor groups (Figure 6 C). The proportion of CD4 and CD8 T cells in the tumors was also analyzed using flow cytometry. It has been previously shown that a high CD8/CD4 ratio is a positive prognostic factor in tumors (Nelson 2008). The CD8 to CD4 ratio was much higher in IL-15 TG/MT (mean=5.63) tumors than in IL-15 KO/MT tumors (mean= 0.43) (Figure 6D).



3.1- Figure 6: IL-15 TG/MT tumors have increased CD8 T cells. (A) CD8 T cell immunohistochemistry on representative IL-15 KO/MT, MT and IL-15 TG/MT tumors. Levels of CD8 T cells were highest in IL-15 TG/MT tumors and lowest in IL-15 KO/MT mice (brown staining = CD8+, 20X) (B) Quantitation of CD8 T cells in tumors (n= 5 mice/group). (C) Quantitation of CD4 T cells in tumors assessed by immunohistochemistry (n= 5 mice/group). (D) Tumors from MT, IL-15 KO/MT and IL-15 TG/MT mice were digested and stained for flow cytometry. The CD8+/CD4+ T cell ratio was altered drastically in the presence or absence of IL-15. *Statistically significant

3.1.7 CD44+CD62L+/- CD8 T cells in IL-15 TG/MT spleens and tumors produce increased IFNγ/TNFα. IL-15 is thought to stimulate the production of long lived anti-tumor immunity via the production of anti-tumor memory CD8 T cells (Judge, Zhang et al. 2002; Lu, Giuntoli et al. 2002; Klebanoff, Finkelstein et al. 2004; Melchionda, Fry et al. 2005). To determine if there were increased memory CD8 T cells in IL-15 TG/MT mice, we examined CD44 and CD62L expression. CD8 T cells in the majority of IL-15 TG/MT tumors possessed a $CD44^{+}CD62L^{high}$ central memory population (>2 times higher than CD8 T cells of MT mice) (Figure 7A). While the presence of increased numbers of CD8 T cells in IL-15 TG/MT mice indicated that those cells may be mounting an immune response to the tumor, the CD8 T cells themselves may be either exhausted, regulatory or non-functional (Huang, Shah et al. 2007; Kiniwa, Miyahara et al. 2007; Wang 2008; Ahmadzadeh, Johnson et al. 2009; Mumprecht, Schurch et al. 2009; Srinivasan and Frauwirth 2009). To test this, CD8 T cells were isolated from the spleens or tumors of IL-15 KO/MT, MT and IL-15 TG/MT mice, and stimulated non-specifically (with antibody to CD3 and CD28) for 48 hours. The CD8 T cells from IL-15 TG/MT spleens (Figure 8) and tumors (Figure 7B) both produced high levels of IFNγ and TNFα. In contrast, the spleen and tumor CD8 T cells from MT mice produced much lower levels of IFNγ (Figure 7B, Figure 8). IL-15 KO/MT spleen or tumor CD8 T cells produced little to no IFNγ (data not shown). This indicates that in IL-15 TG/MT tumors, but not in IL-15 KO/MT tumors, the CD8 T cells are more Th1 biased and are capable of cytotoxic responses. We further examined the CD8 T cells via flow cytometry to determine which CD8 T cell populations were producing IFNγ (Figure 7C-E). We

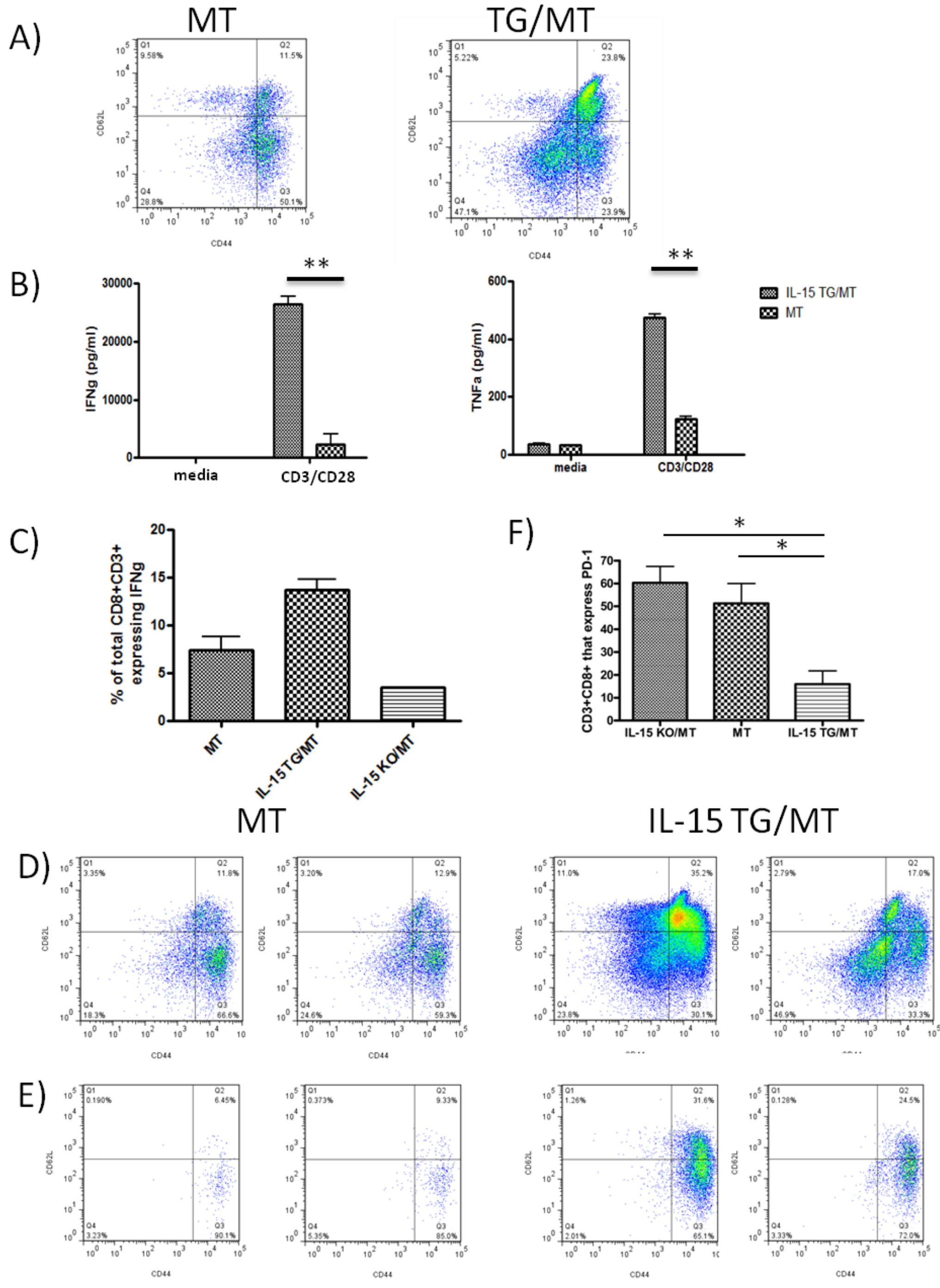


Figure 7

3.1- Figure 7: CD8 T cells from the tumors of IL-15 TG/MT mice have markers of highly functional CD8 T cells. (A) CD3+CD8+ cells from tumors of IL-15 TG/MT mice had a higher percentage of CD62L+CD44+ central memory T cells (Representative of n=3/group) (B-D) CD8 T cells were isolated by positive selection from tumors of mice and stimulated non-specifically. (B) After 48 hours, supernatant IFNγ/TNFα levels were highest in IL-15 TG/MT mice (Representative of 3 experiments). (C-E) In another set of experiments, after 12 hours of stimulation, Golgi Stop was added to determine via flow cytometry which cells were capable of producing IFNγ. (C) A high proportion of IL-15 TG/MT CD8+CD3+ cells produced IFNγ in comparison to MT CD8 T cells. (D) After stimulation, IL-15 TG/MT CD8 T cells had a higher proportion of CD44+CD62L+ (central memory) cells. (E) Of CD8+CD3+ T cells, it was the CD44+, both CD62L positive or negative, cells that were capable of producing IFNγ. (F) Fewer CD8+CD3+ T cells in IL-15 TG/MT mice expressed the exhaustion marker PD-1. *Statistically significant

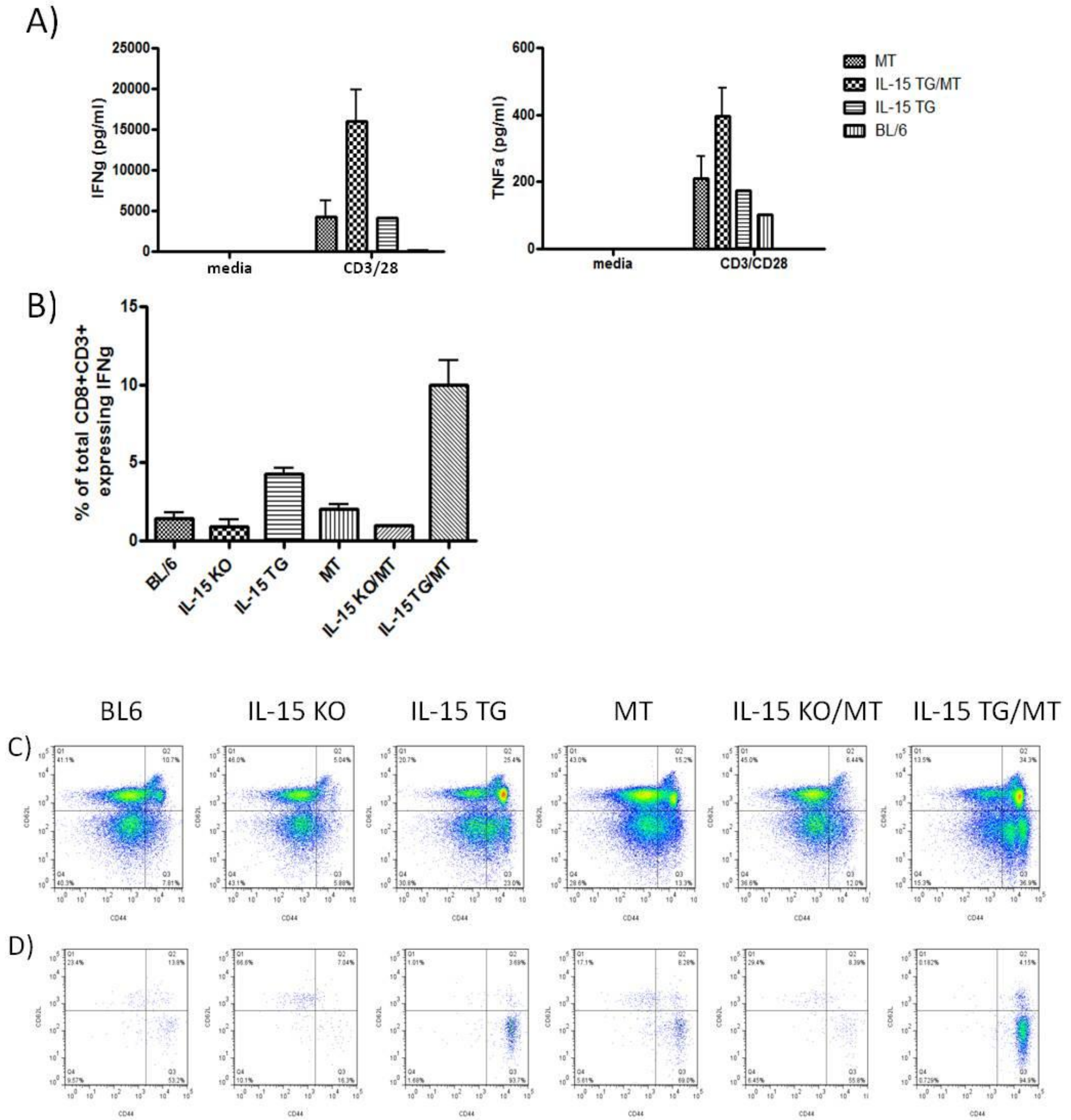


Figure 8

3.1- Figure 8: CD8 T cells from the spleens of IL-15 TG/MT mice are highly functional. CD8 T cells were isolated by positive selection from spleens of mice and stimulated non-specifically. (A) After 48 hours, supernatant IFNγ/TNFα levels were highest in IL-15 TG/MT mice. CD3+CD8+ T cells were identified in tumors by flow cytometry and examined for various markers. (B-D) In another set of experiments, after 12 hours of stimulation, Golgi Stop was added to determine which cells are capable of producing IFNγ. (B) A high proportion of IL-15 TG/MT splenic CD8+CD3+ cells produce IFNγ in comparison to MT CD8 T cells. (C) After stimulation, IL-15 TG/MT CD8 T cells have higher proportion of CD44+ CD62L- (effector/effector memory) and CD44+CD62L+ (central memory) cells. (D) It is the CD44+ cells that are capable of producing IFNγ. Representative of multiple experiments.

isolated CD8 T cells and performed non-specific stimulation again, but in the presence of GolgiStop (BD) to prevent protein secretion. Using this method, a higher percentage of CD8 T cells from IL-15 TG/MT tumors produced IFNγ (Figure 7C). Additionally, we examined CD3+CD8+ T cells for their CD62L and CD44 status (Figure 7D). After stimulation, the majority of the MT and IL-15 TG/MT CD8 T cells were CD44+ (effector/memory), but the IL-15 TG/MT tumor CD8 T cells had a higher proportion of CD44+CD62L+ central memory cells. After back gating on CD8 T cells that were capable of producing IFNγ, we determined that these cells were all CD44+, and in the case of IL-15 TG/MT, many were also CD62L+ (Figure 7E). Therefore, IL-15 TG/MT mice have higher numbers of polyfunctional CD8 T cells that are increased in the central memory phenotype. To ascertain if the CD8 T cells were exhausted, we examined them for the marker programmed death (PD)-1. This marker is increased on exhausted CD8 T cells in a variety of models (Ahmadzadeh, Johnson et al. 2009; Mumprecht, Schurch et al. 2009). PD-1 was high on intra-tumoral IL-15 KO/MT and MT CD8 T cells, but low on IL-15 TG/MT CD8 T cells (Figure 7F).

3.1.8 IL-15 TG/MT tumors contain CD3+CD8+NK1.1+ cells. While phenotyping the CD8 T cells from these mouse strains, it was found that a large proportion of the IL-15 TG/MT tumor CD8 T cells also expressed NK1.1 (Figure 9A) (small amount seen in the spleen- data not shown). These CD8 T cells that express NK1.1 were absent in IL-15 KO/MT tumors and much lower in MT tumors (Figure 9A). Previously, this population of NK1.1+CD8 T cells has been found to be highly cytotoxic and to produce high levels

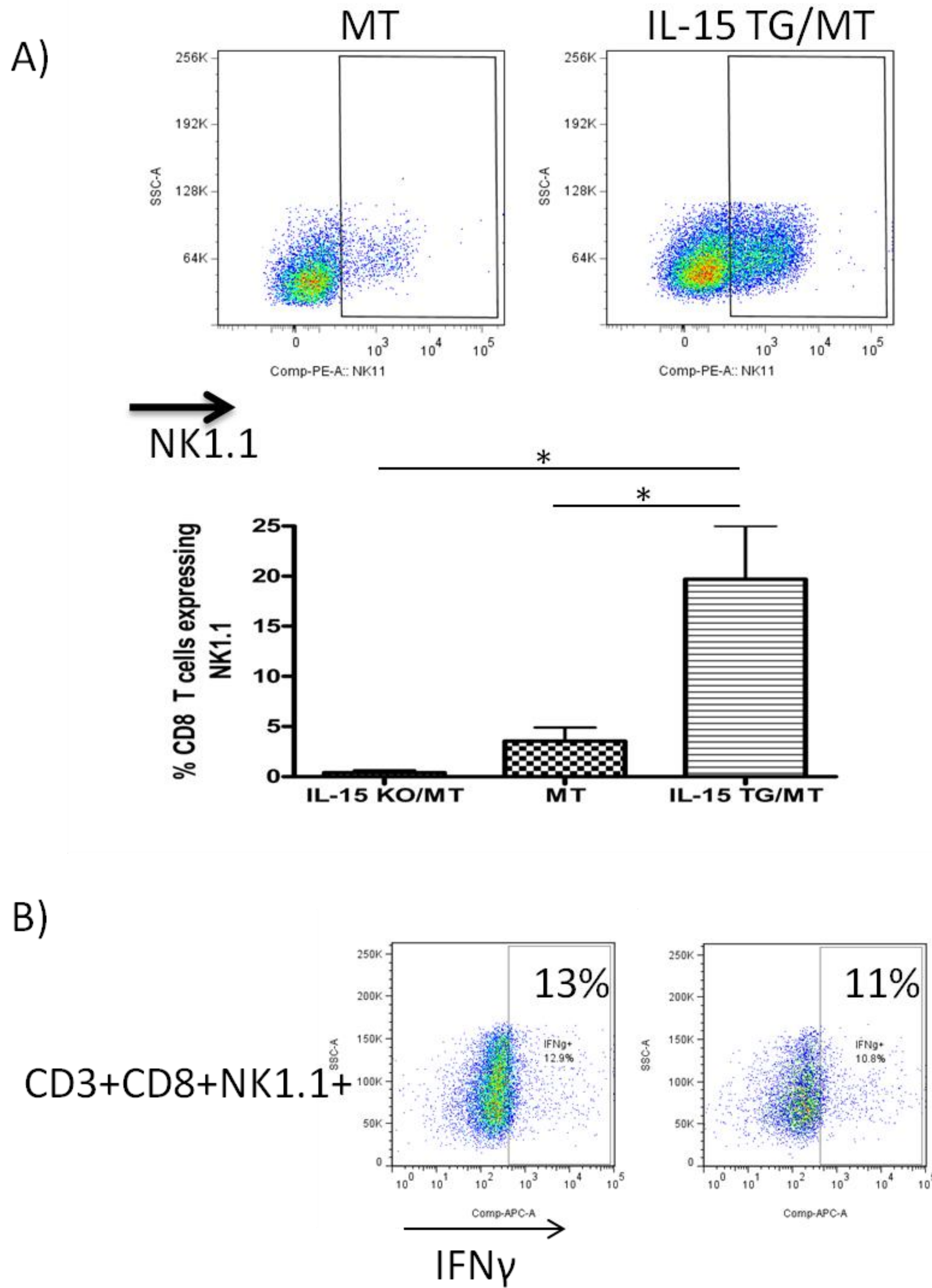


Figure 9

3.1- Figure 9: IL-15 TG/MT tumors possess high levels of CD8+CD3+NK1.1+ T cells. (A) Tumors were digested and stained for CD45, CD8, CD3, and NK1.1 for flow cytometric analysis. IL-15 TG/MT tumors possessed high levels of unique CD8+CD3+NK1.1+ T cells (B) When CD8 T cells were isolated from IL-15 TG/MT tumors and stimulated non-specifically with CD3/CD28 antibodies in the presence of GolgiStop, 11-13% of these cells were found to produce IFNγ. *Statistically significant

of IFNγ (Terabe, Tagaya et al. 2008; Correia, Costa et al. 2011). To determine if these cells produce high levels of IFNγ, we isolated NK1.1+ cells from IL-15 TG/MT tumors and performed non-specific stimulation (CD3/28) in the presence of protein secretion inhibitors. 11-13% of the CD3+CD8+ NK1.1+ T cells were able to secrete IFNγ (Figure 9B). This is a similar percentage to the total amount of CD8 T cells secreting IFNγ (Figure 7C). Therefore, while these cells were capable of producing IFNγ, in our model, we found that they did not have a higher IFNγ positive percentage than other CD44+ CD8 T cells.

3.1.9 Cells expressing NK1.1 are responsible for tumor destruction in IL-15 TG/MT mice. We have established that IL-15 TG/MT mice have extended survival, increased tumor destruction as well as increased activated NK and CD8 T cells within their tumors. To determine which cell types were responsible for these effects, we performed long term antibody depletion experiments with the use of anti-NK1.1 and anti-CD8α antibodies in IL-15 TG/MT mice. NK1.1 depletion resulted in a lack of NK1.1+ cells within the spleen and tumor (Figure 10A). Mice were palpated once per week to determine date of tumor formation and endpoint (Figure 10B). IL-15 TG/MT mice that were depleted of NK1.1 cells formed tumors faster and proceeded to endpoint more quickly than IL-15 TG/MT mice ($p<0.001$, 0.0001, respectively) (Figure 10B). In fact, tumor formation followed closely what was observed in IL-15 KO/MT mice. In addition, histological analysis revealed that the tumor destruction in IL-15 TG/MT mice was absent when these NK1.1+ or NK1.1+ CD8 T cells were lacking (Figure 10C). To specifically examine the

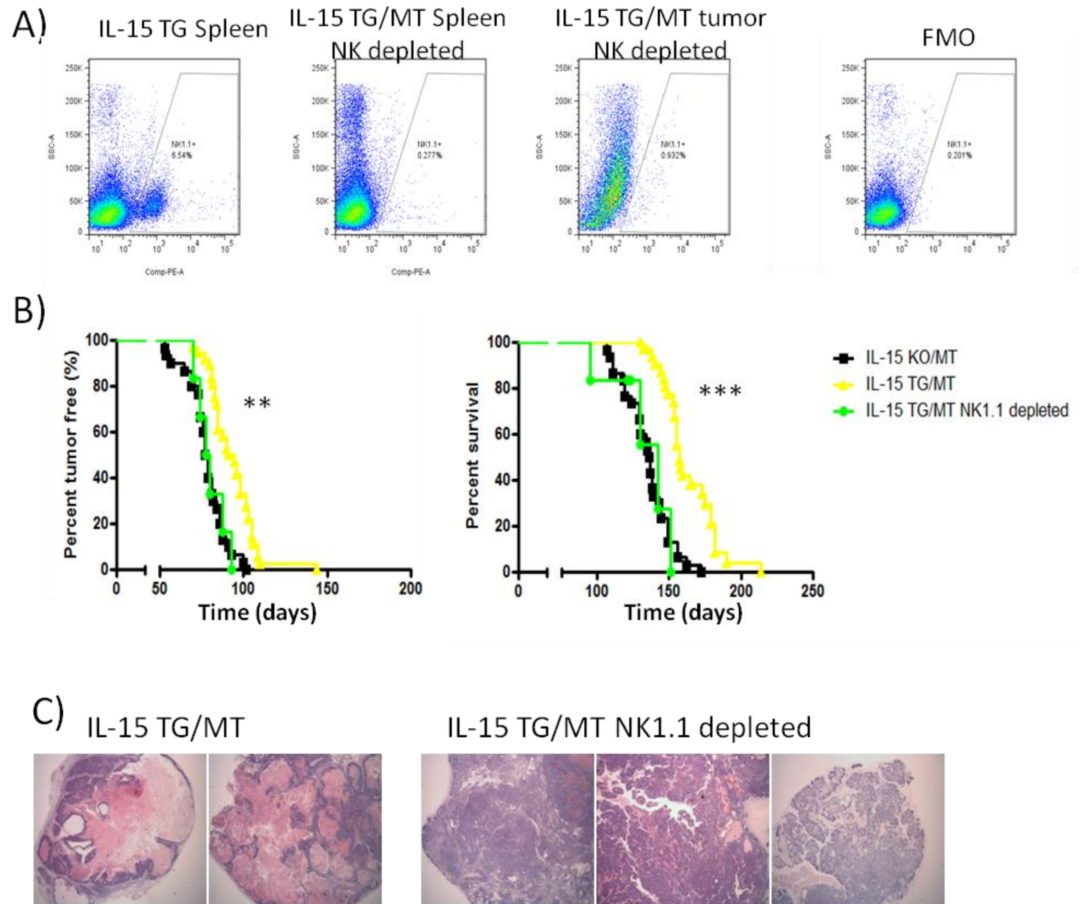


Figure 10

3.1- Figure 10: Depletion of NK1.1 positive cells promotes tumor formation. (A-C) IL-15 TG/MT mice were depleted with anti-NK1.1 antibody long term starting at 4 weeks of age. Long term depletion efficiently removed NK1.1 positive cells from both the spleen and tumor (A). (B) In comparison to the tumor formation and survival curves for IL-15 TG/MT mice, NK1.1 depleted IL-15 TG/MT mouse tumors formed and progressed to endpoint more quickly. IL-15 TG/MT NK1.1 depleted mice were not different from IL-15 KO/MT tumor mice. (C) Tumors that formed in IL-15 TG/MT NK1.1 depleted mice did not show the extensive destruction seen in tumors of untreated IL-15 TG/MT mice. *Statistically significant

role of CD8 T cells within this tumor model, we performed long term depletion of CD8 T cells with anti-CD8α antibodies. This antibody effectively removed CD8 expressing cells from the spleen of IL-15 TG/MT mice, but only partially from the tumor (Figure 11A). The proportion of tumor CD8 T cells was decreased (at least by two-thirds ie. 18% to 6 %). The majority of the CD3+CD8+ T cells that were left in the depleted tumor were NK1.1+ (Figure 11B). This indicates that these cells were resistant to this depletion method in this model. The mice were followed for tumor formation and endpoint, but there were no statistically significant differences between the IL-15 TG/MT and the IL-15 TG/MT CD8 depleted mice (Figure 11C). Lastly, the tumors that formed in IL-15 TG/MT CD8 depleted mice were very similar to tumors in untreated IL-15 TG/MT mice and there was still destruction of the tumor (Figure 11D). This data indicates that NK1.1+ cells play a major role in the tumor destruction and extension of survival in IL-15 TG/MT mice, whereas CD8 T cells, although activated phenotypically, play less of a role.

3.1.10 Adoptive transfer of CD8 T cells from IL-15 TG/MT mice does not lead to protection from tumor challenge. To confirm that the CD8 T cells from IL-15 TG/MT mice would not have a large impact on survival, we performed CD8 T cell adoptive transfers (Figure 12). C57BL/6 recipient mice were treated with cyclophosphamide to induce lymphopenia and allow for better engraftment of the transferred cells. 24 hours later CD8 T cells were isolated from the spleens and/or tumors of MT, IL-15 TG/MT and IL-15 TG mice, labelled with CFSE ($5X10^6$ spleen NK cells, $1X10^6$ tumor NK cells) and

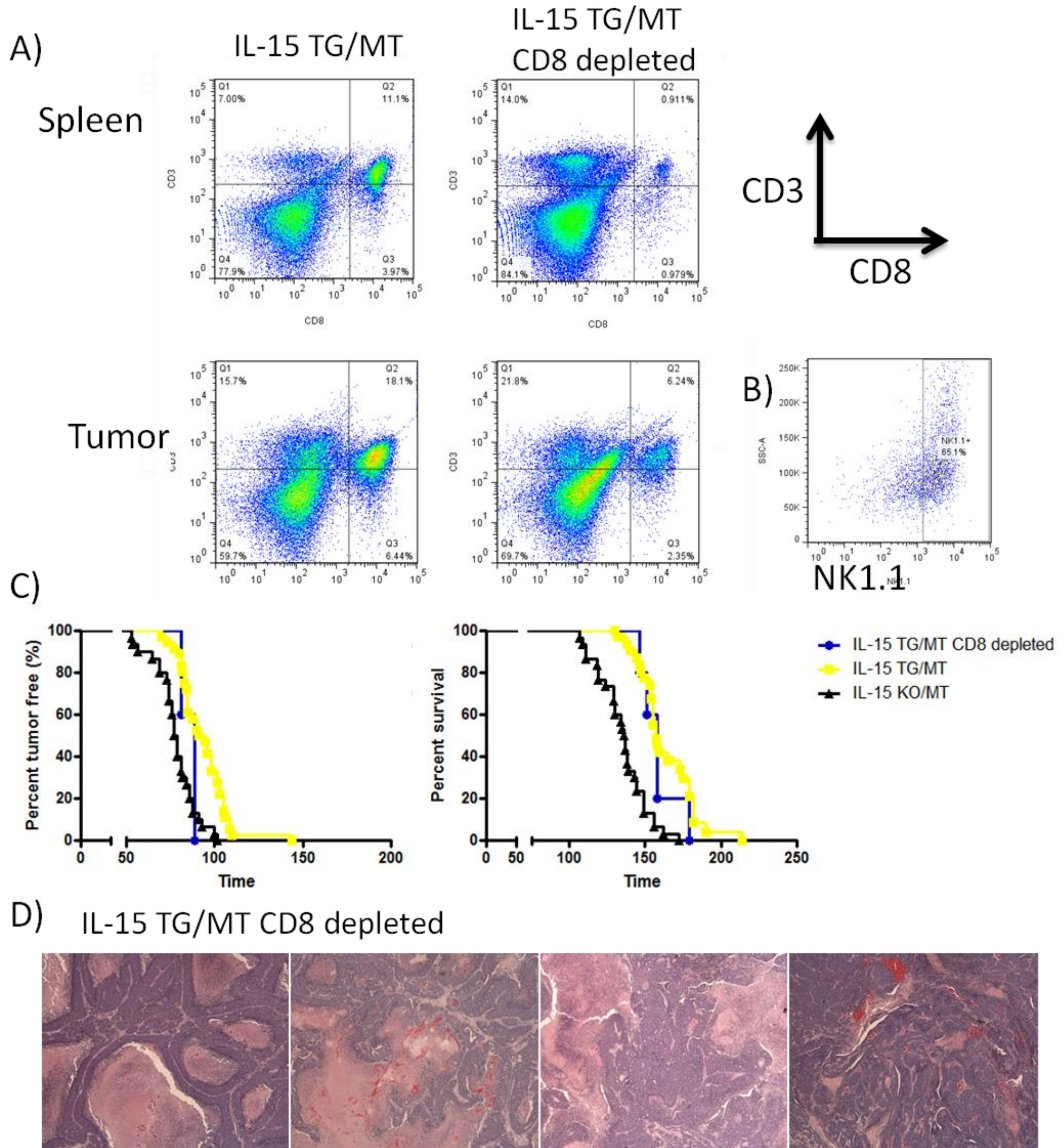


Figure 11

3.1- Figure 11: Depletion of CD8α positive cells does not promote tumor formation. (A-C) IL-15 TG/MT mice were depleted with anti-CD8α antibody long term starting at 4 weeks of age. Long term depletion efficiently removed CD8 positive cells from the spleen, but not completely in the tumor (A). (B) Of the CD8+CD3+ cells that remained in the tumor, the majority were NK1.1+. (C) In comparison to the tumor formation and survival curves for IL-15 TG/MT mice, CD8 depleted IL-15 TG/MT mouse tumors formed and progressed to endpoint at a similar rate. (ns. Log-rank test). (D) Tumors that formed in IL-15 TG/MT CD8 depleted mice still had the tumor destruction seen in normal IL-15 TG/MT tumors. * Statistically significant

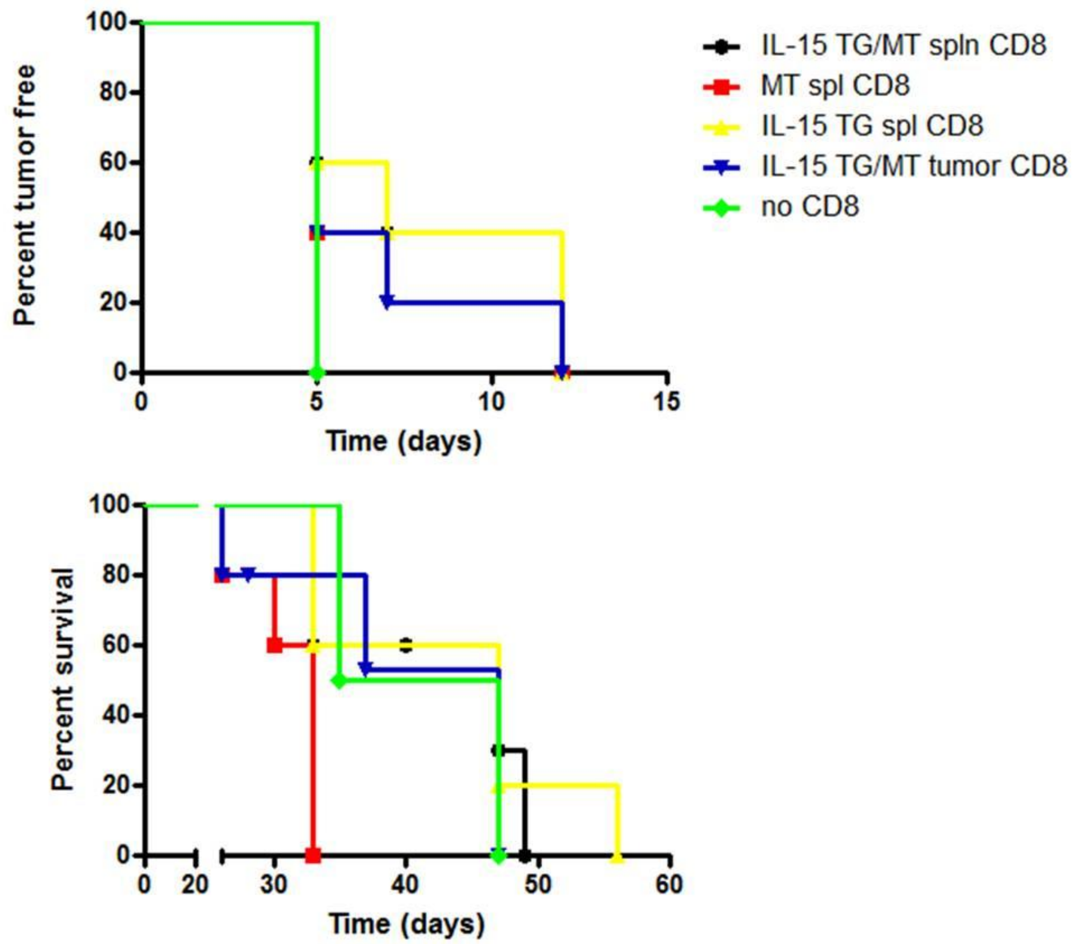


Figure 12

3.1- Figure 12: Adoptive transfer of CD8 T cells from IL-15 TG/MT, MT or IL-15 TG does not protect from tumor formation. CD8 T cells were isolated from the spleens of IL-15 TG/MT, MT, IL-15 TG or tumors of IL-15 TG/MT mice. $5X10^6$ spleen CD8 T cells and $1X10^6$ tumor CD8 T cells were transferred into lymphopenic hosts. 24 hours later, mice were challenged with $0.5X10^6$ fresh primary MT cells subcutaneously. Resultant mice were followed for tumor formation (A) and endpoint (B). No statistically significant differences were found (n=5 for all groups except those receiving no CD8, n=2).

injected IV into cyclophosphamide treated mice. 24 hours after transfer, mice were challenged with a subcutaneous dose of freshly isolated primary MT tumor cells from which immune cells had been removed. Mice were then followed for tumor formation and endpoint. 8 days after challenge, several mice were sacrificed to ensure that the adoptive transfer was successful (4-8 % of total CD8 T cells were CFSE positive - data not shown). There were no statistically significant differences in tumor formation or endpoint between control mice that received no CD8 T cells and mice that received CD8 T cells from MT, IL-15 TG/MT or IL-15 TG spleens or IL-15 TG/MT tumors (Figure 12). Therefore, CD8 T cells were unable to protect from tumor challenge.

3.1.11 Human NK cells, cultured in a similar cytokine environment as found in IL-15 TG/MT tumors, are highly capable of killing a human breast cancer cell line. As has been discussed, the challenge in creating anti-tumor NK cells is to create an environment in which their anti-tumor activities are supported and they will not be altered to perform a pro-tumoral role. IL-15 overexpression in MT breast tumors may have created an environment where several cytokines that affect NK cell activation and proliferation were altered. We examined the expression of these cytokines in IL-15 TG/MT tumors vs MT or IL-15 KO/MT tumors and found that in addition to IL-15, both IL-12 and IL-18 were increased within IL-15 TG/MT tumors (Figure 13). To determine if exposure to these three cytokines simultaneously would affect the ability of NK cells to kill breast tumor cells, we isolated NK cells from human PBMCs and stimulated them in an IL-15/IL-12/IL-18 rich environment for 16 hours. We then co-incubated them with a

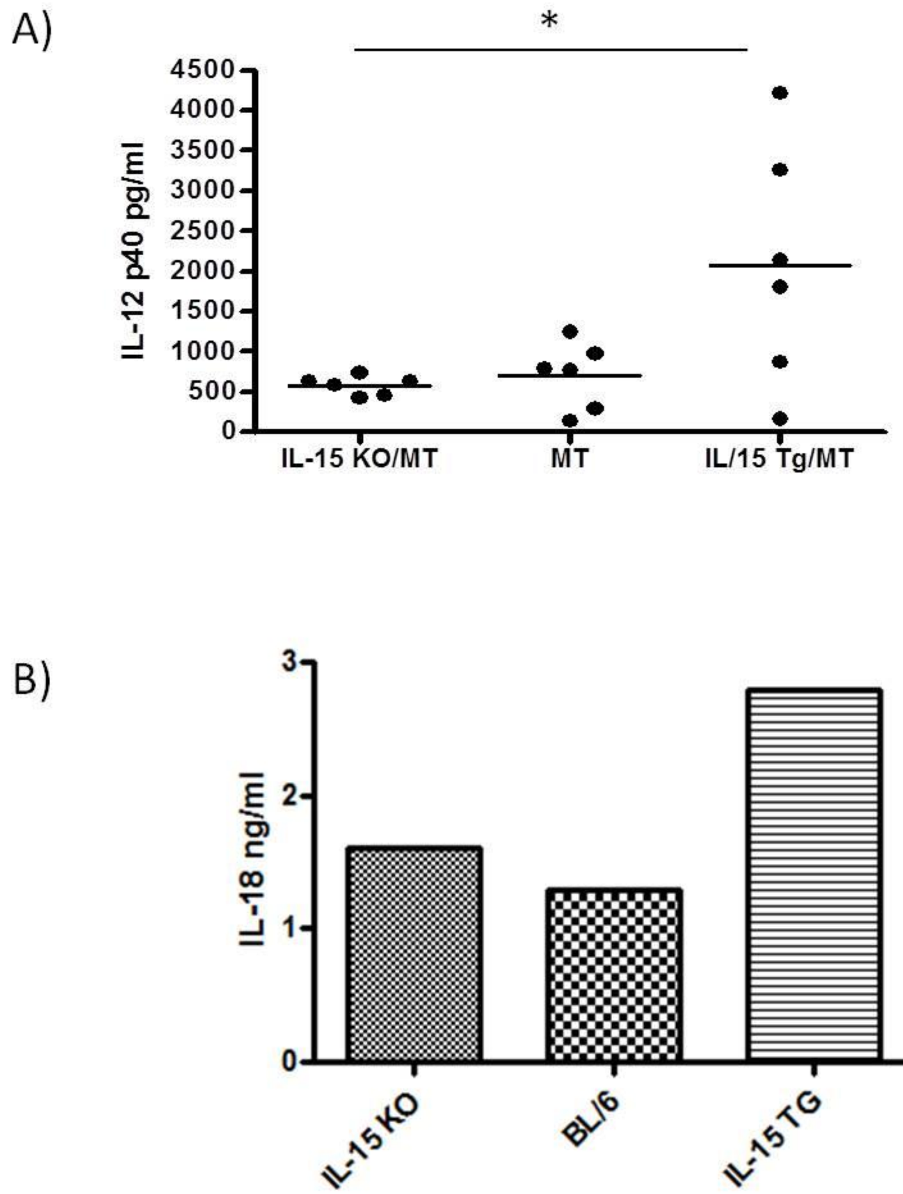


Figure 13

3.1- Figure 13: IL-12 and IL-18 are increased in IL-15 TG/MT tumors. A) IL-12 ELISAs were performed on tumor homogenates from 6 size matched tumors per group. B) 3 tumors from each group were pooled (size matched between groups) and assayed for various cytokine levels via a RBM protein array.

labelled MDA-231 human breast cancer cell line at various E:T ratios for 5 hours to determine their killing ability. In contrast to NK cells cultured in IL-2 alone, NK cells cultured in IL-15/IL-12/IL-18 were highly cytotoxic toward this breast cancer cell line and at E: T ratio of 10:1 reached specific lysis levels of 52% (Figure 14A/B, Figure 15).

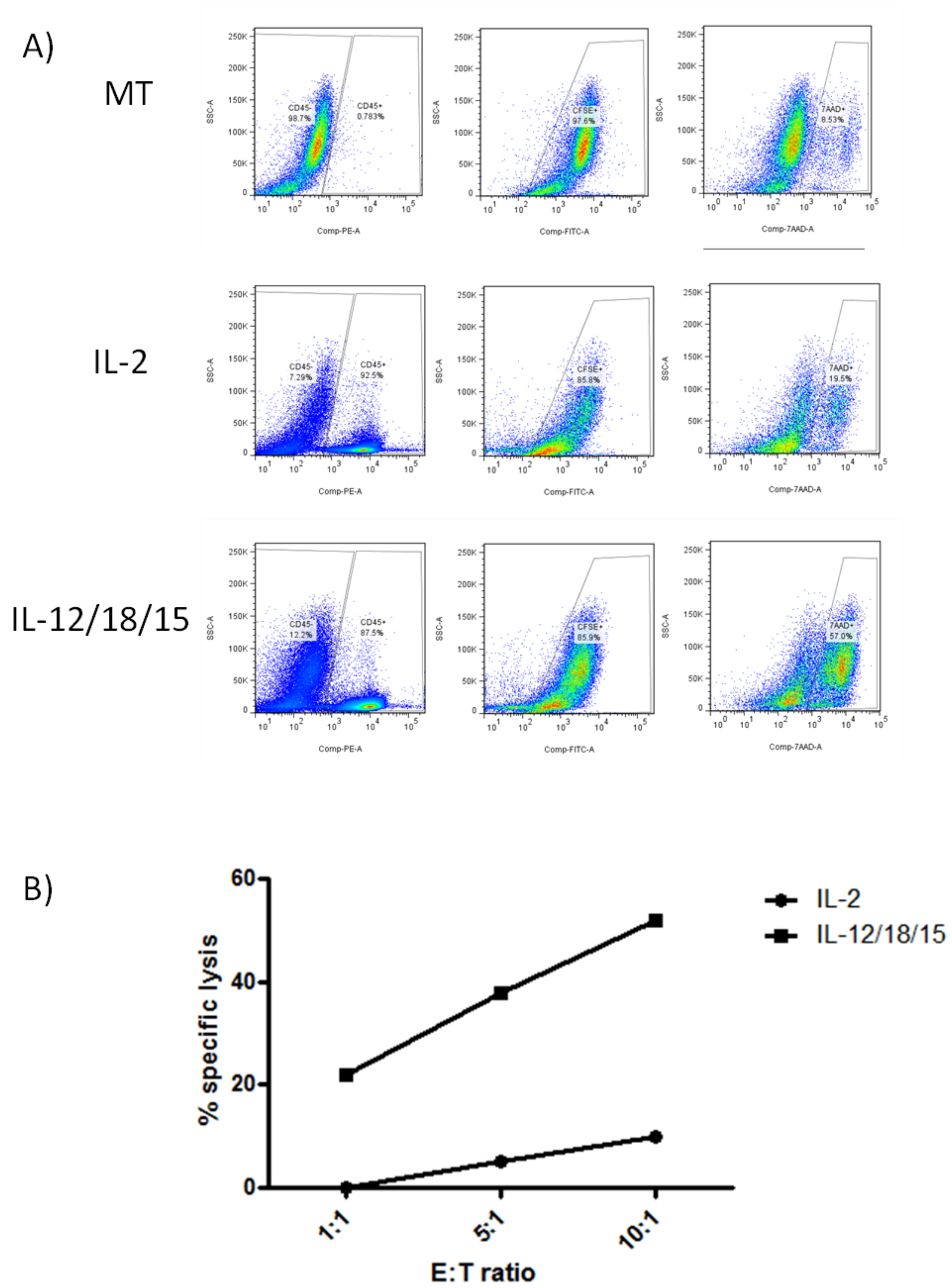
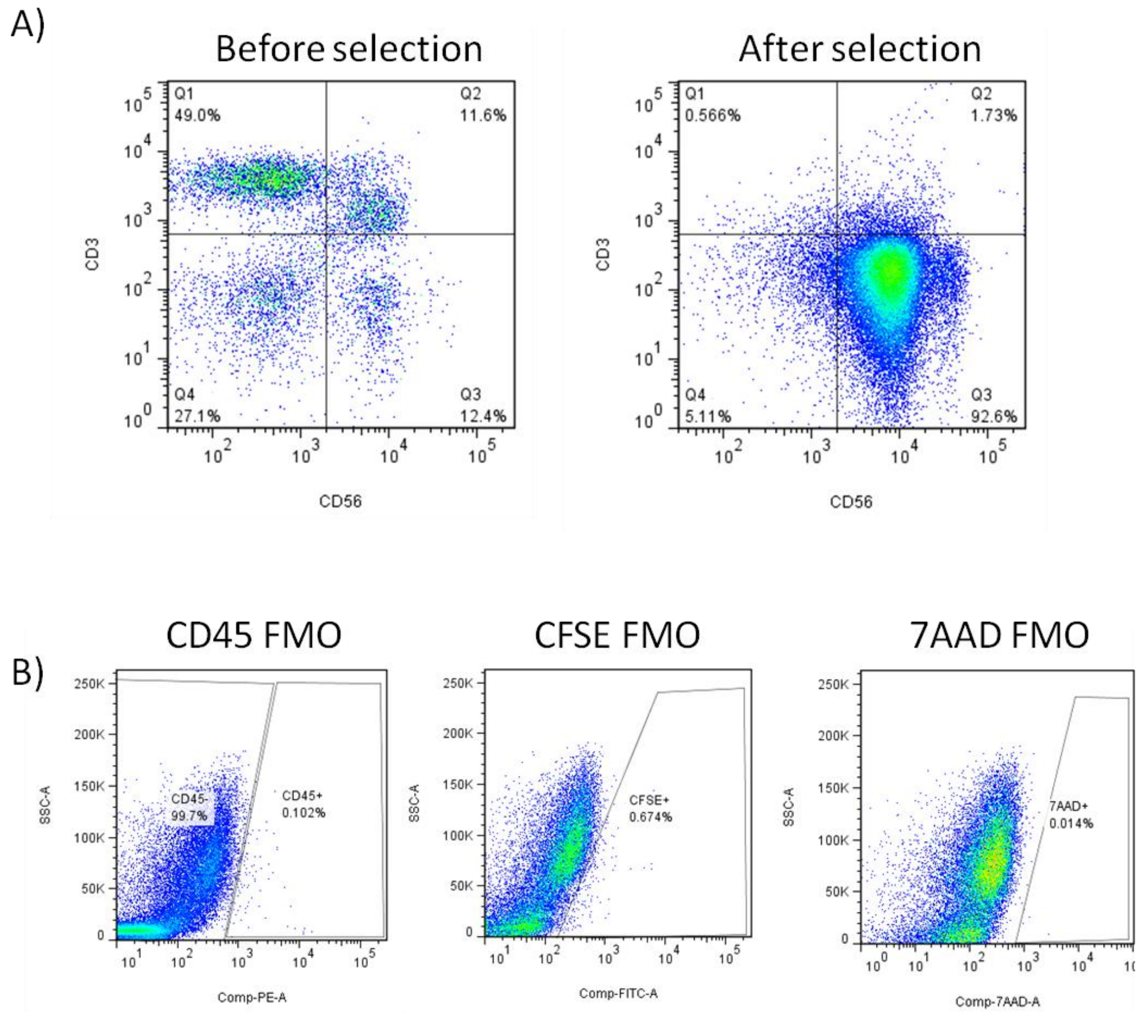


Figure 14

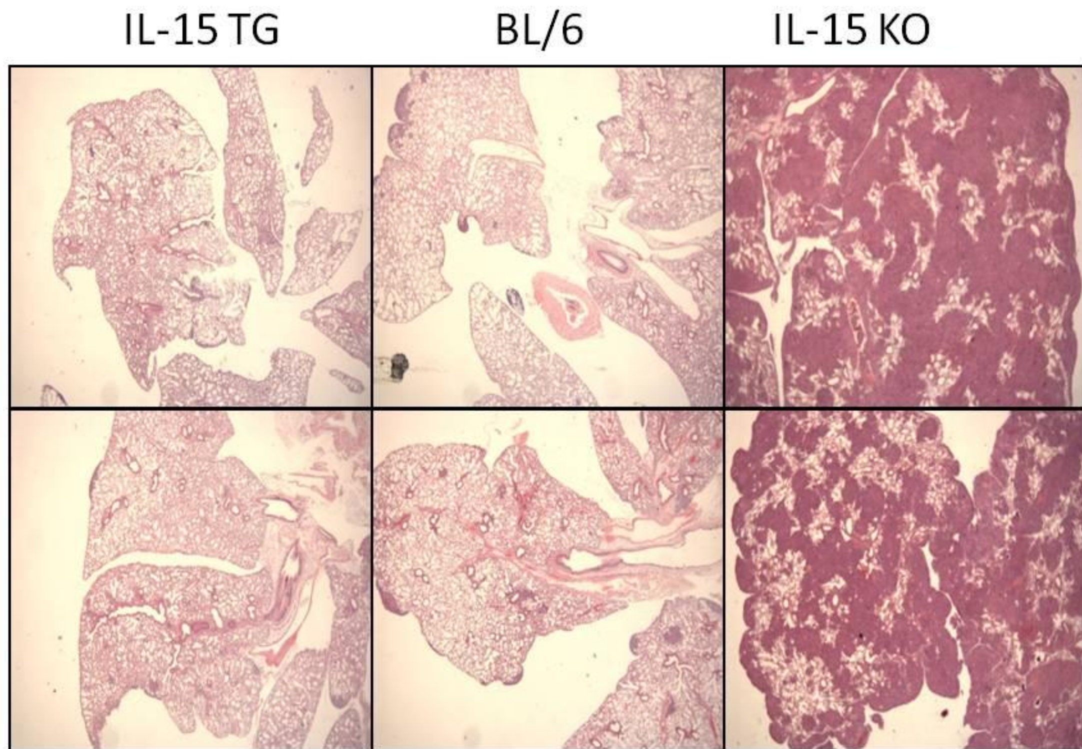
3.1- Figure 14: Human NK cells, exposed to a similar cytokine environment as found in IL-15 TG/MT tumors, are capable of killing human breast cancer cells. A) NK cells isolated from human PBMCs were cultured in IL-2 or IL-15/IL-12/IL-18 for 16 hours before being incubated with CFSE labelled MDA-231 cells for 5 hours in a killing assay. 7AAD was added to distinguish dead cells. Results at an E:T ratio of 1:10 are displayed (E:T= NKcell:MDA-231). B) Percent specific lysis of MDA-231 in the killing assay at various E:T ratios. Representative of 2 experiments.



3.1- Figure 15: Controls for Figure 14. A) Purity of NK cells after selection was greater than 92% B) FMO controls for kill assay.

3.2 The absence or overexpression of IL-15 alters breast cancer metastasis via effects on NK cells, CD4 T cells and macrophages:

3.2.1 IL-15 KO mice have increased lung metastasis, whereas IL-15 TG mice have decreased lung metastasis in comparison to control. IL-15 can impact, both directly and indirectly, many cell types that are thought to be important in the establishment of metastasis. To determine if the absence or overexpression of IL-15 was able to affect the formation of metastases, we utilized a mouse model mimicking breast tumor metastasis. IL-15 KO, C57BL/6 and IL-15 TG mice were injected IV with $5X10^5$ MT cells (cell line established from a MMTV-polyoma middle T (MT) mouse breast tumor). The IL-15 KO mice had lungs with extensive metastasis (Figure 1). At the same time point, there was greatly reduced metastatic tumor burden in control mice and metastasis was virtually absent in IL-15 TG mice (Figure 1). The difference between IL-15 KO and C57BL/6 or IL-15 TG mice was statistically significant. To explore the susceptibility of IL-15 KO mice, we reduced the amount of tumor cells injected to $1X10^4$ (Figure 2A). Tumors began to form in IL-15 KO mice at a dose as low as $5X10^4$, whereas they did not form in C57BL/6 mice until a dose of $5X10^5$. To delineate the differences between control and IL-15 TG mice, we increased the amount of tumor cells injected ($2X10^6$, $1X10^6$) (Figure 2B). In this scenario, metastasis in the control mice was much higher and was still absent in IL-15 TG mice (Figure 2B).



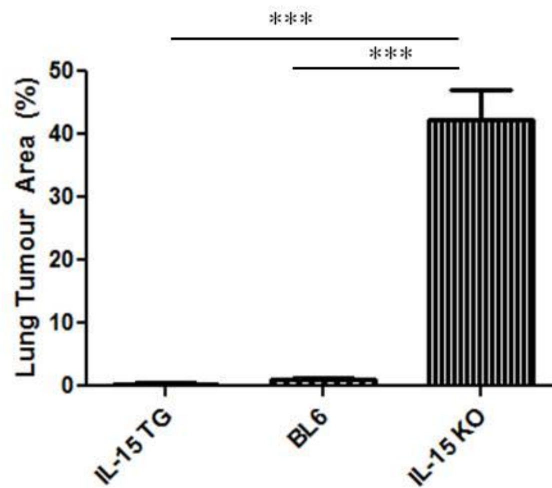
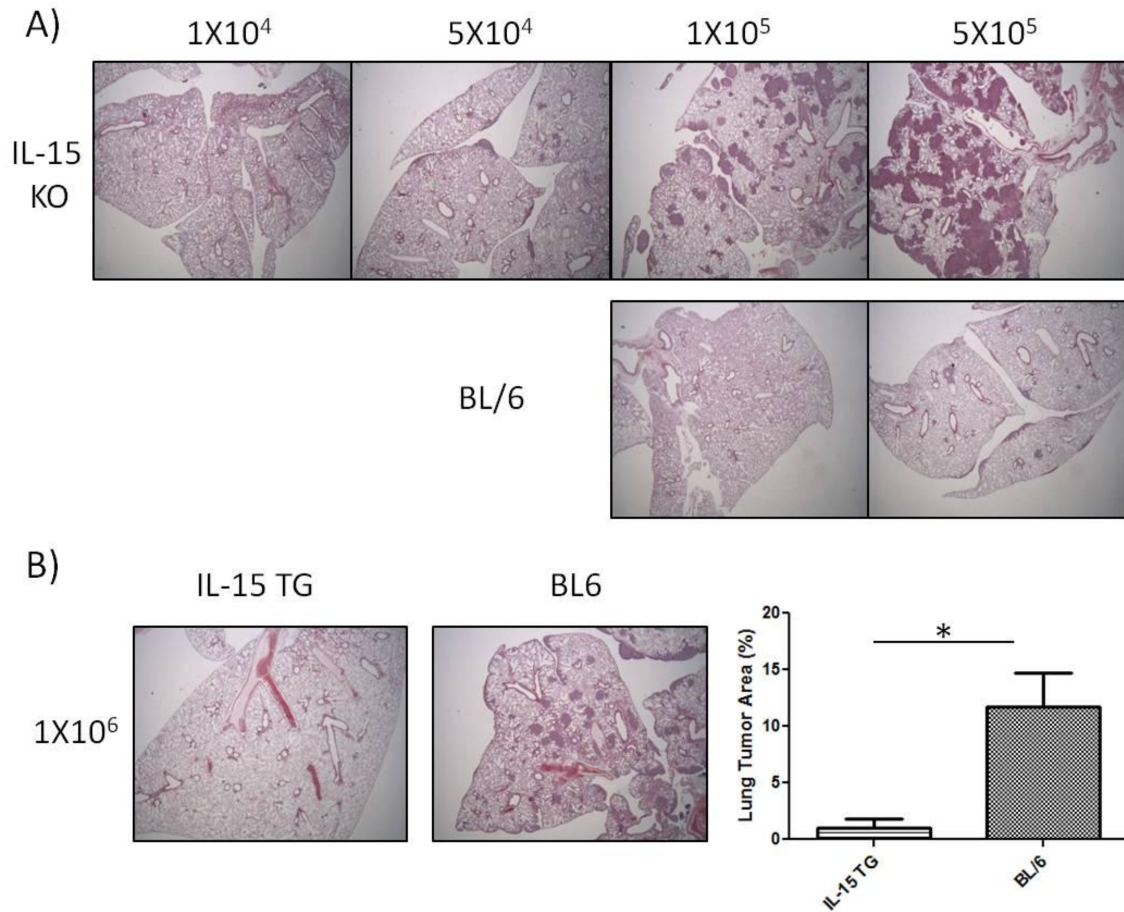


Figure 1

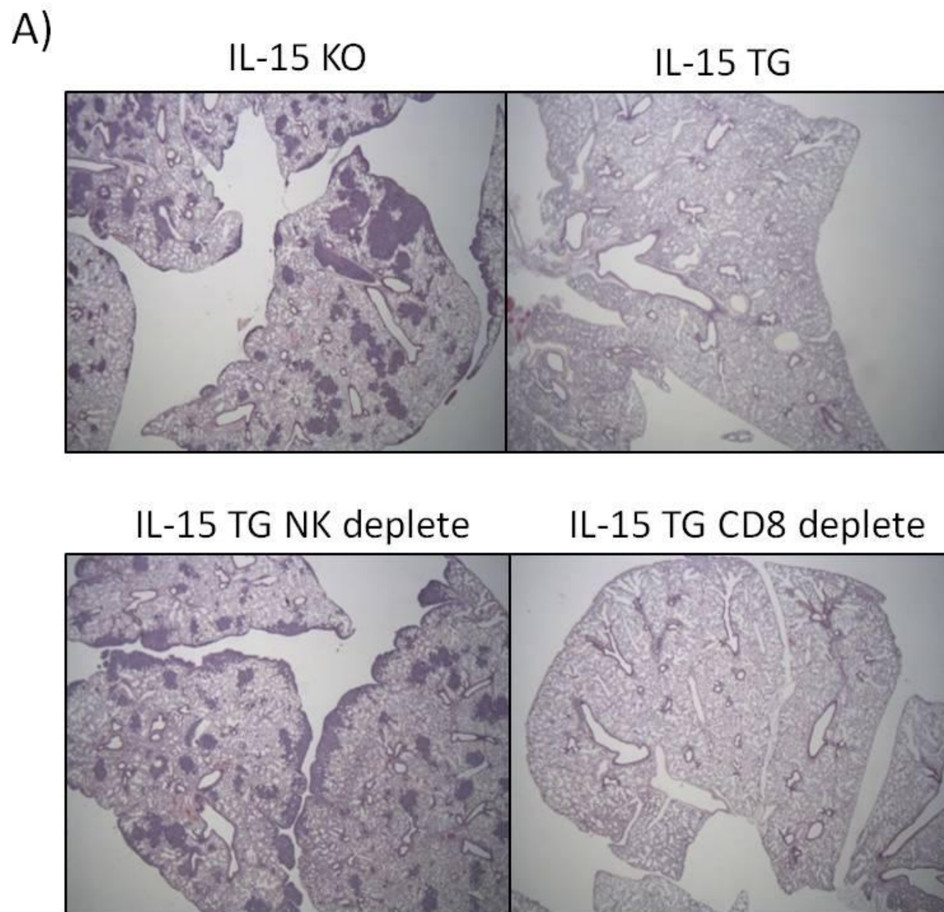
3.2- Figure 1: IL-15 KO mice have increased lung metastasis. IL-15 KO, C57BL/6 and IL-15 TG mice were injected with $5X10^5$ MT cells intravenously (IV). After 13 days, lungs were harvested, fixed, sectioned and stained for H&E (A). Percent lung area occupied by metastasis is reported in (B) (n=5/group). Representative of 3 experiments. * Statistically significant



3.2- Figure 2: IL-15 KO mice are 10 X more susceptible than control mice, whereas IL-15 TG mice are 10X more resistant. IL-15 KO, C57BL/6 (A) and IL-15 TG (B) mice were injected with $1X10^4$ to $1X10^6$ MT cells IV. After 13 days, lungs were harvested, fixed, sectioned and stained for H&E. Percent lung area occupied by metastasis is reported in (B)

3.2.2 The resistance to metastasis in the IL-15 TG mice is NK cell dependent. IL-15 has very important effects on cells of both the innate and the adaptive immune system. It is known that IL-15 KO mice lack NK cells, whereas IL-15 TG mice have increased NK cells and CD8 T cells (Kennedy, Glaccum et al. 2000; Fehniger, Suzuki et al. 2001; Srivastava, Lundqvist et al. 2008). NK cells and CD8 T cells function in tumor immunosurveillance and are able to kill tumor cells (Srivastava, Lundqvist et al. 2008). To determine if NK cells or CD8 T cells were important to the resistance seen in IL-15 TG mice, we depleted IL-15 TG mice with anti-NK1.1 or anti-CD8α antibodies prior to and throughout the experiment (Figure 3). Depletions with these antibodies were effective (data not shown). $5X10^5$ MT tumor cells were injected IV. It was found that the depletion of NK1.1+ cells increased metastasis to levels similar to those seen in IL-15 KO mice, whereas depletion of CD8 T cells had no effect on metastasis (Figure 3). Therefore, NK cells are key mediators of the protection seen in IL-15 TG mice.

3.2.3 NK depletion in control mice increases metastasis, but not to the same level observed in IL-15 KO mice. To determine if the lack of NK cells in IL-15 KO mice was the factor that promoted increased metastasis in these mice, control mice were depleted of NK cells before being injected with $5X10^5$ MT cells. Depletions were maintained for the duration of the experiment. We found that while depletion of NK cells in control mice did promote metastasis, the trend in 2 separate experiments was that there was still less metastasis than was observed in IL-15 KO mice (Figure 4). Thus, it seemed likely that there were other factors involved that promoted metastasis in IL-15 KO mice.



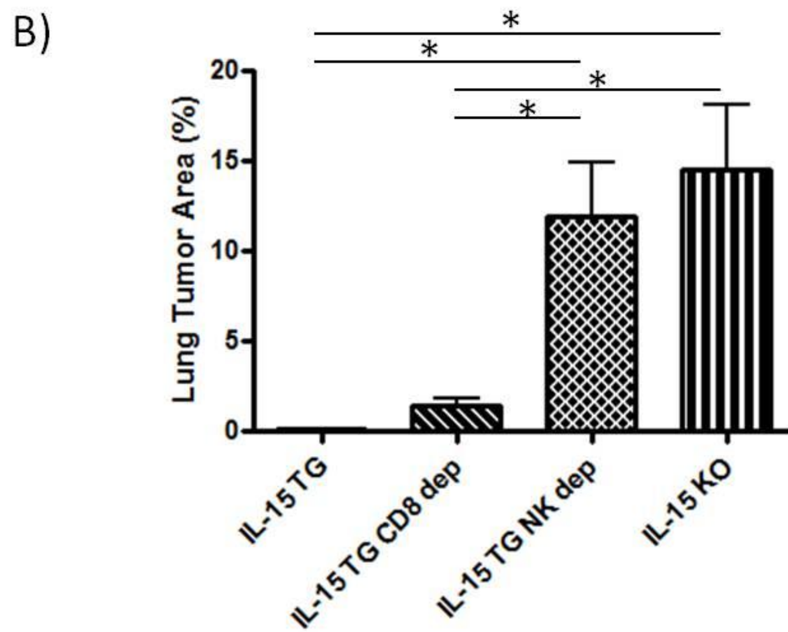
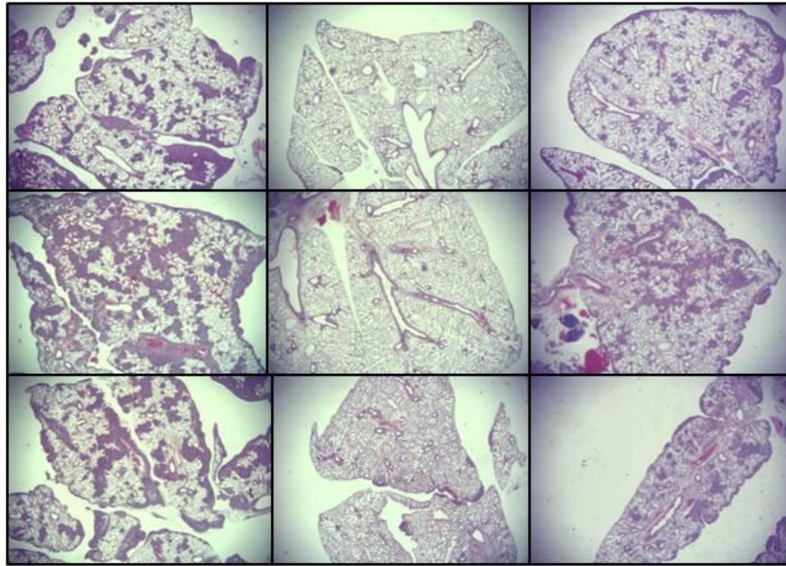


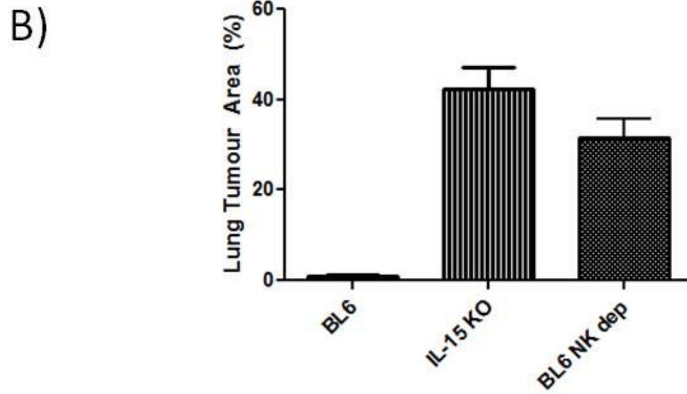
Figure 3

3.2- Figure 3: Depletion of NK cells in IL-15 TG mice promotes metastasis to a similar level as in IL-15 KO mice. (A) & (B) IL-15 TG mice were depleted of NK cells or CD8 T cells prior to injection with $5X10^5$ MT cells. Depletion of CD8 T cells had no effect on metastasis, whereas depletion of NK cells promoted tumor formation (representative pictures shown, n=5/depleted group, n=4 IL-15 KO, N=3 IL-15 TG). (B) Percent lung area occupied by metastasis is reported.

A)



B)



C)

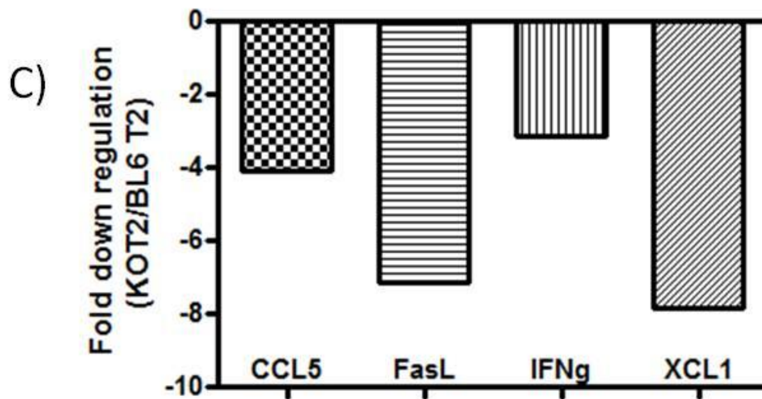


Figure 4

3.2- Figure 4: NK cells decrease metastasis in C57BL/6 mice and contribute to Type 1 cytokines in the lung environment, but do not fully recapitulate the IL-15 KO phenotype. (A) C57BL/6 were depleted of NK cells (anti-NK1.1 antibody) or treated with PBS prior to injection with $5X10^5$ MT cells IV. Depletion was continued throughout the experiment. After 13 days, lungs were harvested and fixed for H&E sectioning (n=5/group). (B) Percent lung area occupied by metastasis is reported. C) Lungs were isolated from IL-15 KO or C57BL/6 mice two days post tumor cell injection ($5X10^5$). RNA was extracted, cDNA was synthesized and a PCR Array was performed for cytokines and chemokines (n=2/group). Fold down regulation in IL-15 KO vs C57BL/6 is reported.

We also assessed the differences in an IL-15 KO lung versus a control C57BL/6 lung two days post tumor cell injection to examine global changes in cytokines in the lung environment. It was found that major differences in these lungs involved a decrease in CCL5 (RANTES), Fas ligand (FasL), interferon γ (IFNγ) and XCL1 (lymphotactin) in IL-15 KO mice (Figure 4B). CCL5, IFNγ and XCL1 have all been identified as being expressed when NK cells are activated in response to infections such as Listeria or murine cytomegalovirus (Dorner, Scheffold et al. 2002; Dorner, Smith et al. 2004).

3.2.4 CD4 T cells in IL-15 KO mice promote metastasis. Recently, several reports have indicated that CD4 T cells play a role in promoting metastasis (DeNardo, Barreto et al. 2009; Tan, Zhang et al. 2011; Wang, Cui et al. 2012). IL-15 is known to affect CD4 T cell phenotype, but conclusions regarding this have been mixed. To determine if CD4 T cells impacted metastasis *in vivo*, IL-15 KO mice were depleted of CD4 T cells before being injected IV with $5X10^5$ MT tumor cells (Figure 5). Depletion of CD4 T cells from IL-15 KO mice decreased tumor formation greatly in comparison to IL-15 KO mice (Figure 5 A&B). Therefore, CD4 T cells in IL-15 KO mice promoted tumor formation. It was also found that CD4 T cells were a higher proportion of CD45+ leukocytes in the lung of IL-15 KO mice two days post tumor cell injection (Figure 5 C). Thus, they were a highly prevalent immune cell present in the IL-15 KO lung microenvironment.

3.2.5 IL-15 KO mice have M2 polarized macrophages, whereas IL-15 TG mice have M1 polarized macrophages. TAMS or M2 macrophages are an important cell type for

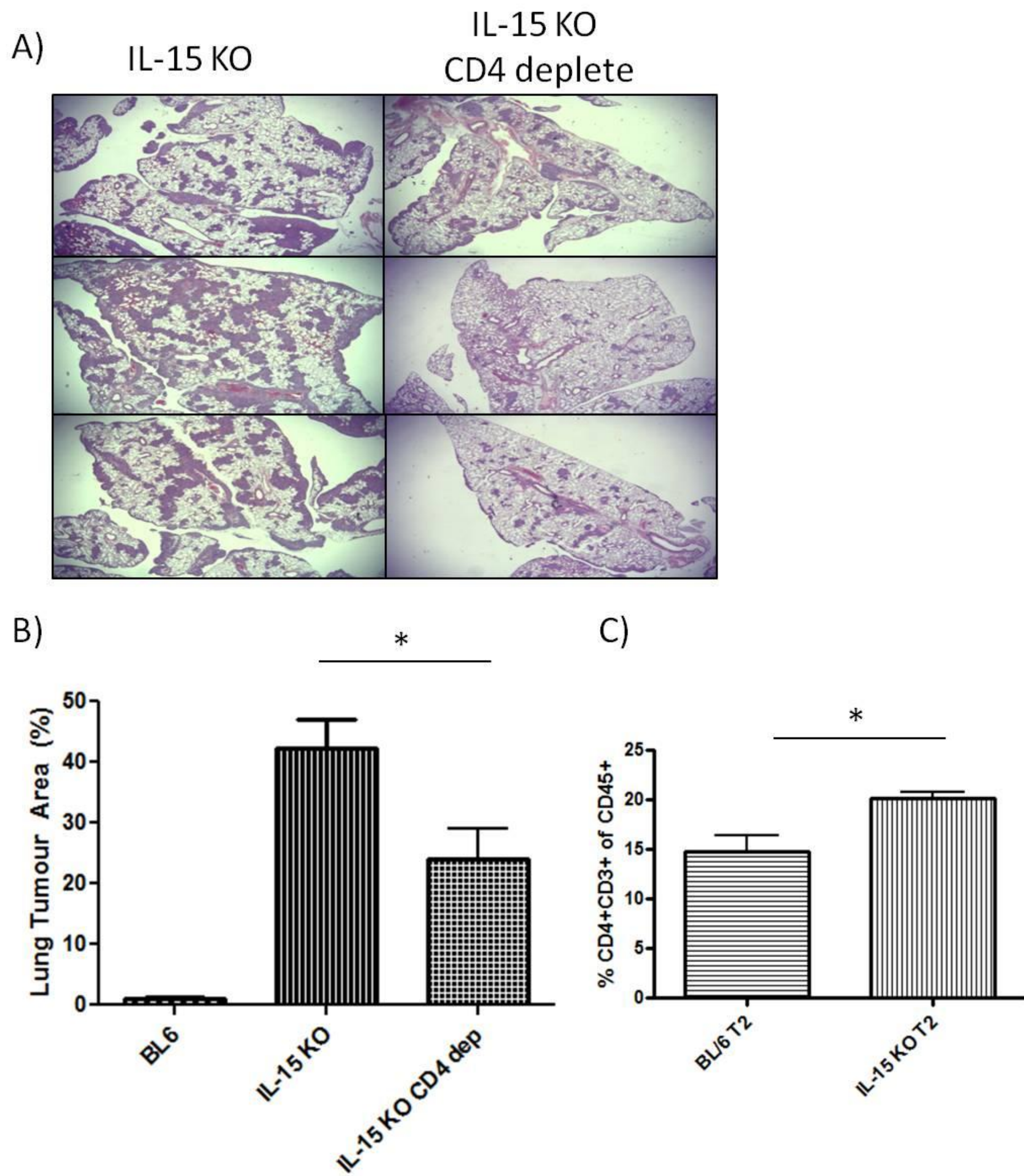


Figure 5

3.2- Figure 5: In IL-15 KO mice, CD4 T cells promote metastasis. IL-15 KO mice were depleted of CD4 T cells (anti-CD4α antibody) or treated with PBS prior to injection with 5X 10^5 MT cells IV. Depletion was continued throughout the experiment. After 13 days, lungs were harvested, fixed, sectioned and stained for H&E (A). Percent lung area occupied by metastasis is reported in (B) (n=5/group). (C) Lungs were isolated from IL-15 KO or C57BL/6 mice two days post tumor cell injection ($5X10^5$). Lungs were digested and the resultant cells were analyzed via flow cytometry for CD45, CD3 and CD4. Results are reported as percentage of CD45+ cells in the lung.

promotion of metastasis (Qian and Pollard ; Qian, Deng et al. 2009). Many studies have shown that at the primary site, TAMs promote both invasion and metastasis and are correlated with poor prognosis (Joyce and Pollard 2009). It has been previously found in the same model of metastasis used here, that genetic or chemical ablation of macrophages decreases both metastatic seeding and growth (Qian, Deng et al. 2009). Research has revealed that macrophages of a M2 phenotype support tumor formation, whereas those of a M1 phenotype may prevent it (Martinez, Sica et al. 2008). To determine if IL-15 affects the polarization of macrophages, we analyzed the ability of peritoneal macrophages from IL-15 KO, control and IL-15 TG mice to produce nitric oxide (NO) in response to LPS (Figure 6A). IL-15 TG macrophages produced the highest amounts of NO, which indicates a M1 phenotype, whereas IL-15 KO mice produced the lowest amount of NO. We also examined the monocyte population present in the lungs of IL-15 KO and C57BL/6 mice at two days post tumor cell injection (Figure 6B). Cells in the lung were stained for F4/80 to identify monocytes and various markers to delineate myeloid derived suppressor cells (MDSC- CD11b+, Gr1+), lung resident macrophages (CD11b-CD11c+) or recruited macrophages (CD11b+Gr1-)(Connelly, Barham et al. 2011). In IL-15 KO mice, there was an increase in recruited macrophages brought into the lung (Figure 6B). Since the macrophages in IL-15 KO mice have an M2 polarization, it is likely that these macrophages would assist in the formation of metastasis. In prior examinations of this metastasis model, recruitment of macrophages to the site was very important for efficient metastatic seeding and growth (Qian, Deng et al. 2009; Qian, Li et al. 2011).

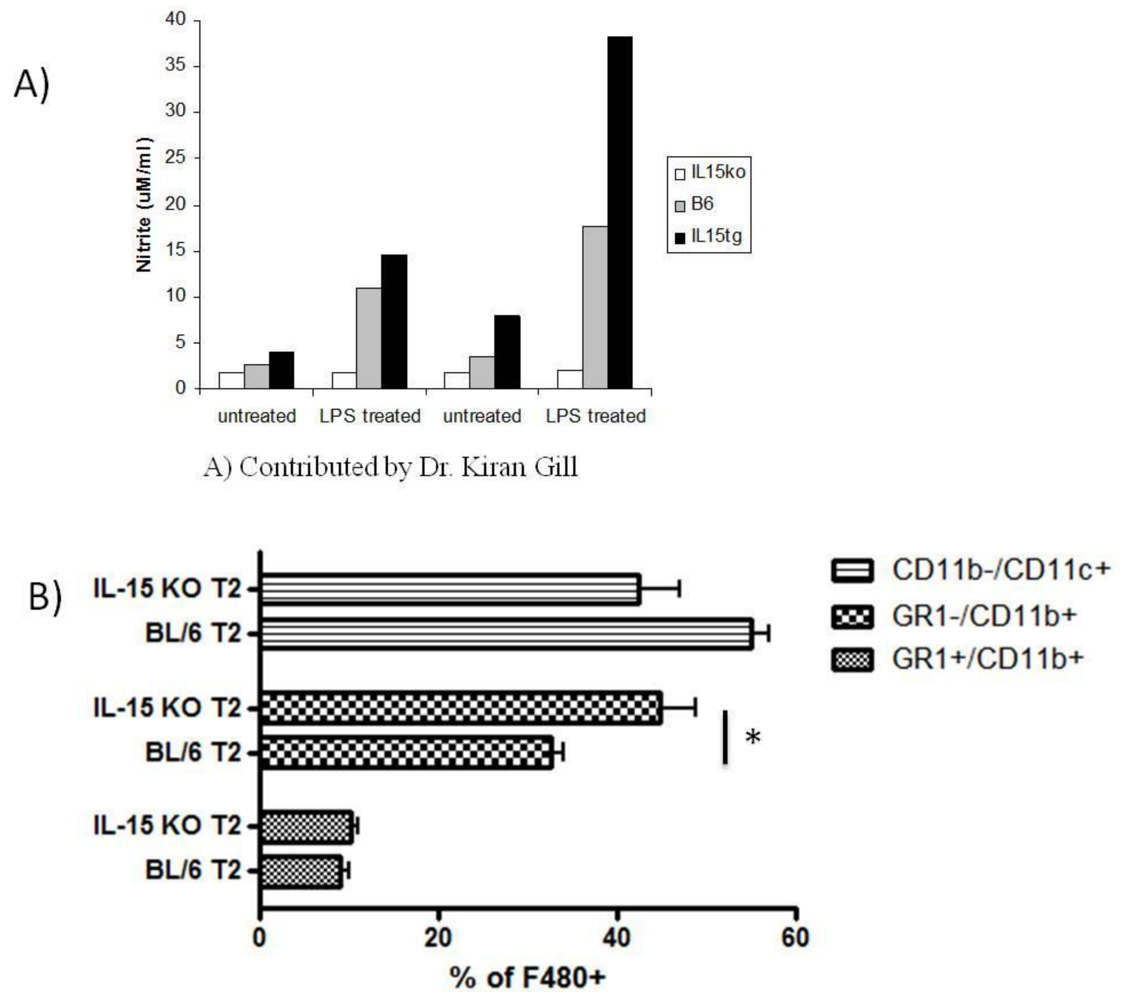


Figure 6

3.2- Figure 6: IL-15 affects macrophage polarization and recruitment. (A) Peritoneal macrophages were isolated from C57BL/6, IL-15 KO and IL-15 TG mice. Cells were assessed via a nitric oxide (NO) assay for their ability to respond to LPS by producing NO at 24 and 48 hours. IL-15 TG mice consistently had higher levels of NO production in response to LPS. Representative of 4 experiments. (B) Lungs were isolated from IL-15 KO or C57BL/6 mice two days post tumor cell injection ($5X10^5$). Lungs were digested and the resultant cells were analyzed via flow cytometry for CD45, F4/80, CD11b, CD11c and Gr1. Results are reported as percentage of F4/80+ cells.

3.2.6 In IL-15 KO mice, CD4 T cells have a Th2 phenotype and contribute to metastasis by altering macrophage polarization. To determine the phenotype of the CD4 T cells in our model, CD4 T cells were isolated from the spleen of IL-15 KO and control mice and stimulated non-specifically for 48 hours (Figure 7A). The CD4 T cells were >80% pure (data not shown). When analyzed for cytokine production, IL-15 KO CD4 T cells produced higher levels of both IL-10 and IL-4 (Figure 7A). This indicated that IL-15 KO CD4 T cells were more Th2 type than control CD4 T cells. To determine if the CD4 T cells in IL-15 KO mice were contributing to the M2 macrophage phenotype in these mice, peritoneal macrophages from IL-15 KO or CD4 depleted IL-15 KO mice were analyzed for their ability to produce NO. Although not significant, in several experiments it was found that CD4 depletion increased the ability of these macrophages to make NO to close to control levels (Figure 7B). Therefore, CD4 T cells in IL-15 KO mice promote tumor metastasis, possibly via their effects on macrophage polarization.

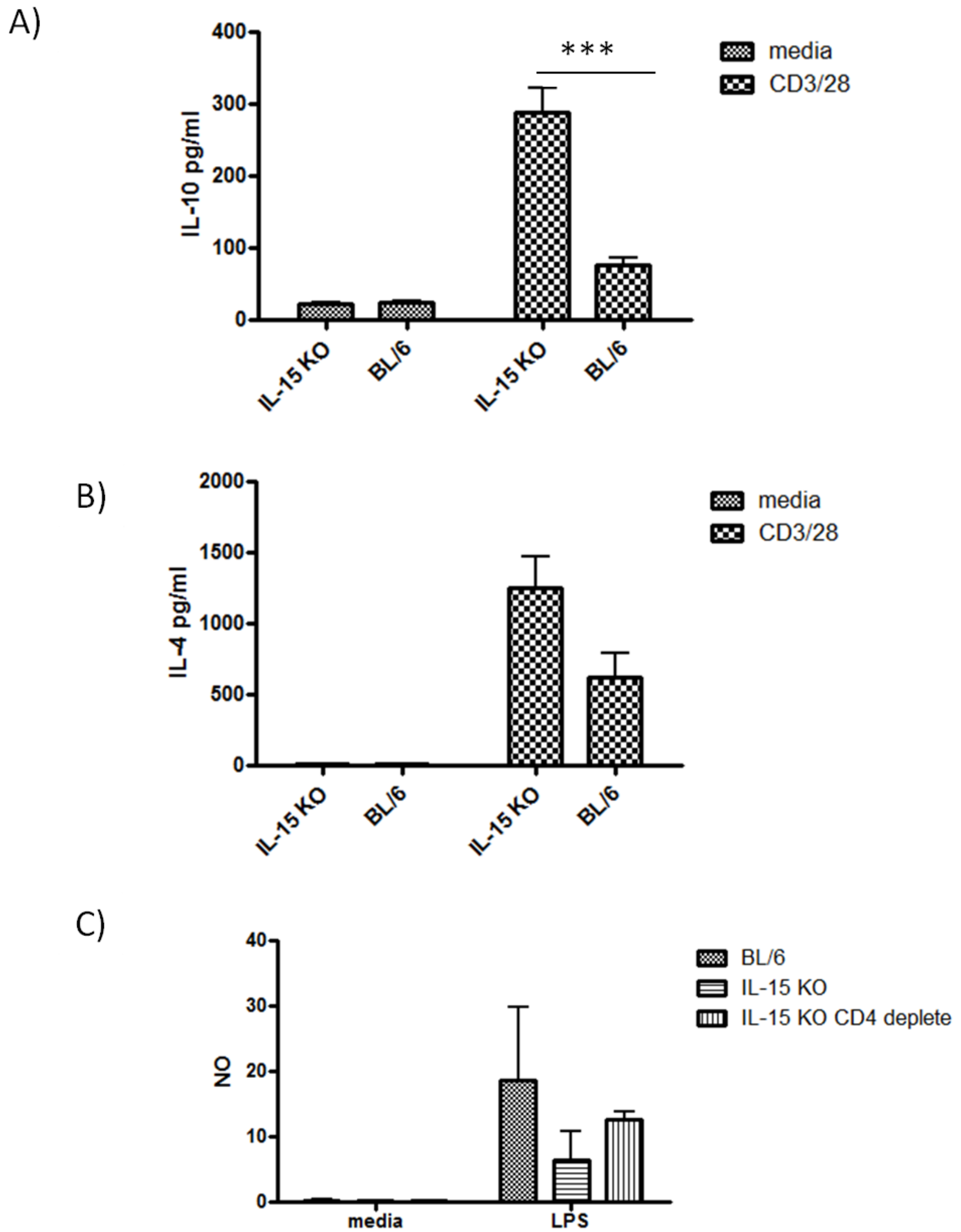


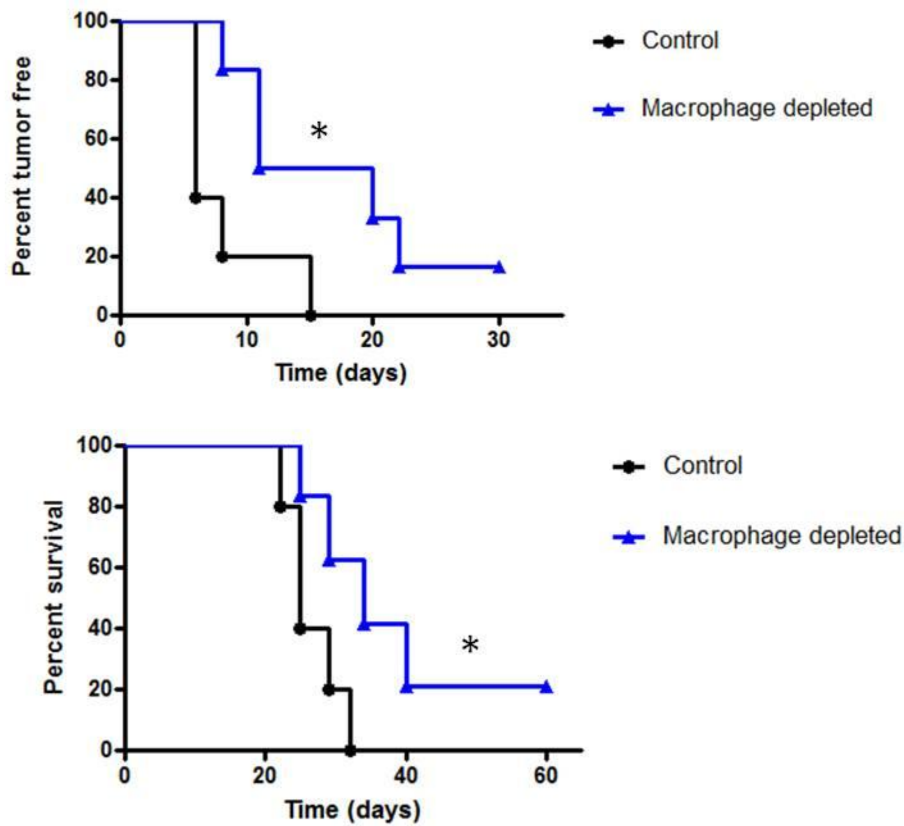
Figure 7

3.2- Figure 7: In the absence of IL-15, CD4 T cells are of a more Th2 phenotype and promote M2 macrophage polarization. (A/B) CD4 T cells were isolated from the spleen of IL-15 KO and C57BL/6 mice and stimulated non-specifically (CD3/CD28) for 48 hours. Supernatants were collected and assessed for IL-10 and IL-4 production via ELISA. (C) Peritoneal macrophages from C57BL/6, IL-15 KO and IL-15 KO CD4 depleted mice were harvested, plated and tested for production of NO.

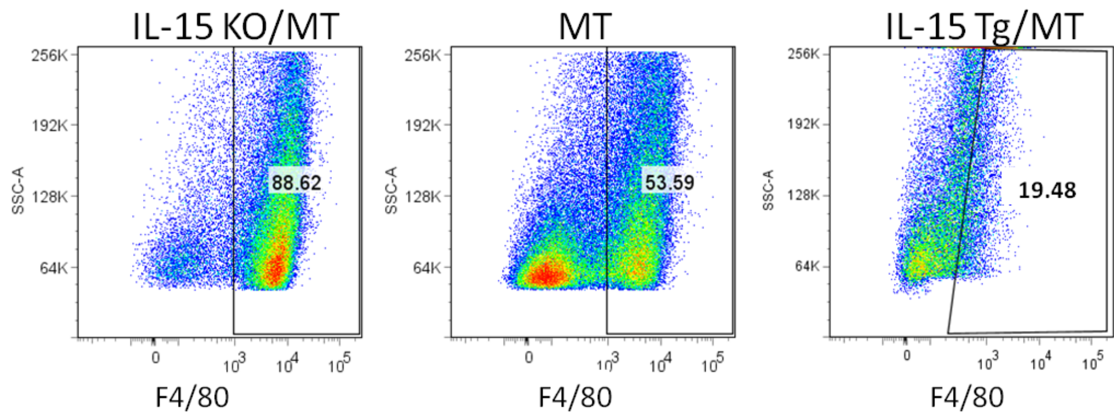
3.3 Macrophage Polarization and its effect on breast tumor formation:

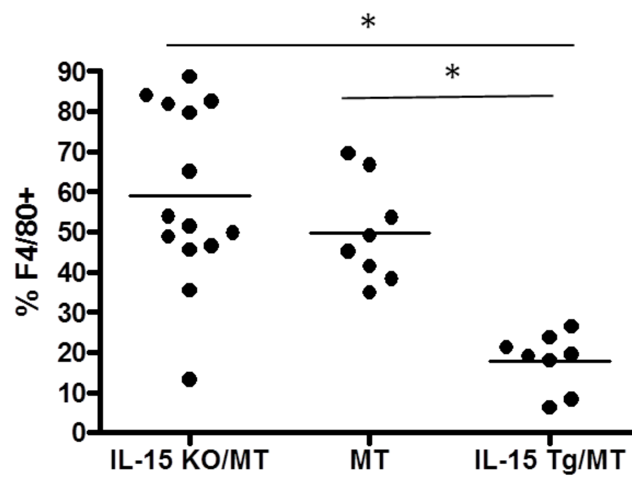
3.3.1 Macrophages play an important role in the formation of subcutaneous breast tumors. Within many models, macrophages have been found to play a pro-tumoral role (Lin, Nguyen et al. 2001; Zeisberger, Odermatt et al. 2006; Heusinkveld and van der Burg 2011). To delineate the role of macrophages in subcutaneous breast tumor formation, we depleted macrophages using clodronate liposomes before injection of the MT cell line subcutaneously into the right flank (Figure 1). We continued the depletion throughout the experiment and efficient depletion was confirmed via flow cytometry (data not shown). Mice were palpated 3 times per week for tumor formation and endpoint (10X10mm). Upon the removal of macrophages, tumors took longer to form and proceeded to endpoint more slowly (Figure 1). This confirms that in this model, macrophages do play a pro-tumoral role.

3.3.2 The presence or absence of IL-15 affects the proportion of macrophages found within spontaneous MT tumors, as well as their polarization. As an initial examination of the effects of IL-15 on macrophages we looked at the proportion of macrophages within IL-15 KO/MT, MT and IL-15 TG/MT tumors (Figure 2). We found that in the presence of high levels of IL-15, the proportion of CD45+ cells that were also positive for the macrophage marker F4/80 was much lower than that in either IL-15 KO/MT or MT tumors. While macrophages appeared to compose a much lower percentage of the leukocytes within IL-15 TG/MT tumors, we were also very interested



3.3- Figure 1: Macrophages promote tumor formation and progression in a subcutaneous MT model. 1X10^5 MT cells were injected into mice that had been depleted of macrophages using clodronate liposomes or control mice and observed for tumor formation (A) and endpoint (B). Clodronate injections were continued for the entire experiment. Macrophage depletion was confirmed in the spleen and tumor (data not shown)(Control group n=5, Macrophage depleted group n=6). * statistically significant difference via log-rank test

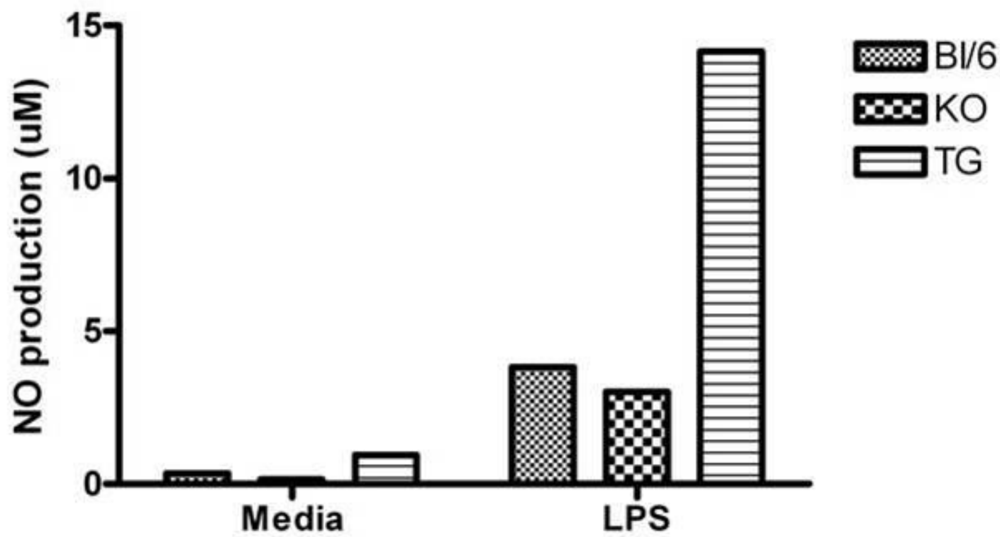




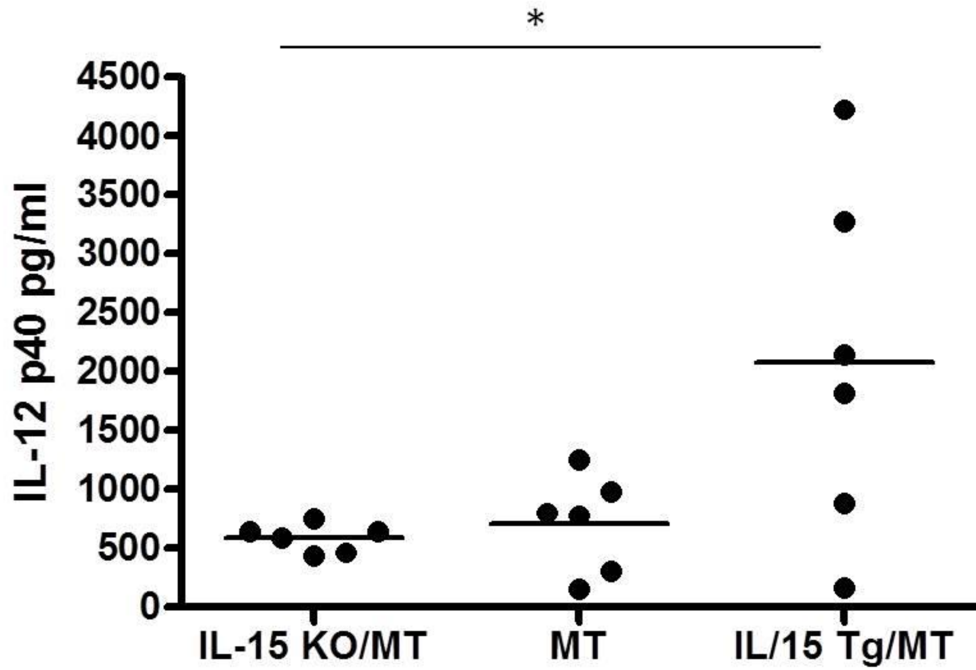
3.3-Figure 2

3.3- Figure 2: Macrophages distribution in IL-15 KO/MT, MT and IL-15 TG/MT tumors. Tumors were digested and stained via flow cytometry for CD45 (pan-leukocyte marker) and F4/80 (macrophage marker). Here, we report the percentage of CD45 cells within the tumor that are F4/80 positive. (A) Representative flow plots and (B) proportion of F4/80+ cells from a collation of multiple tumors (IL-15 KO/MT n=14, MT and IL-15 TG/MT n=8). * statistically significant difference via One Way ANOVA

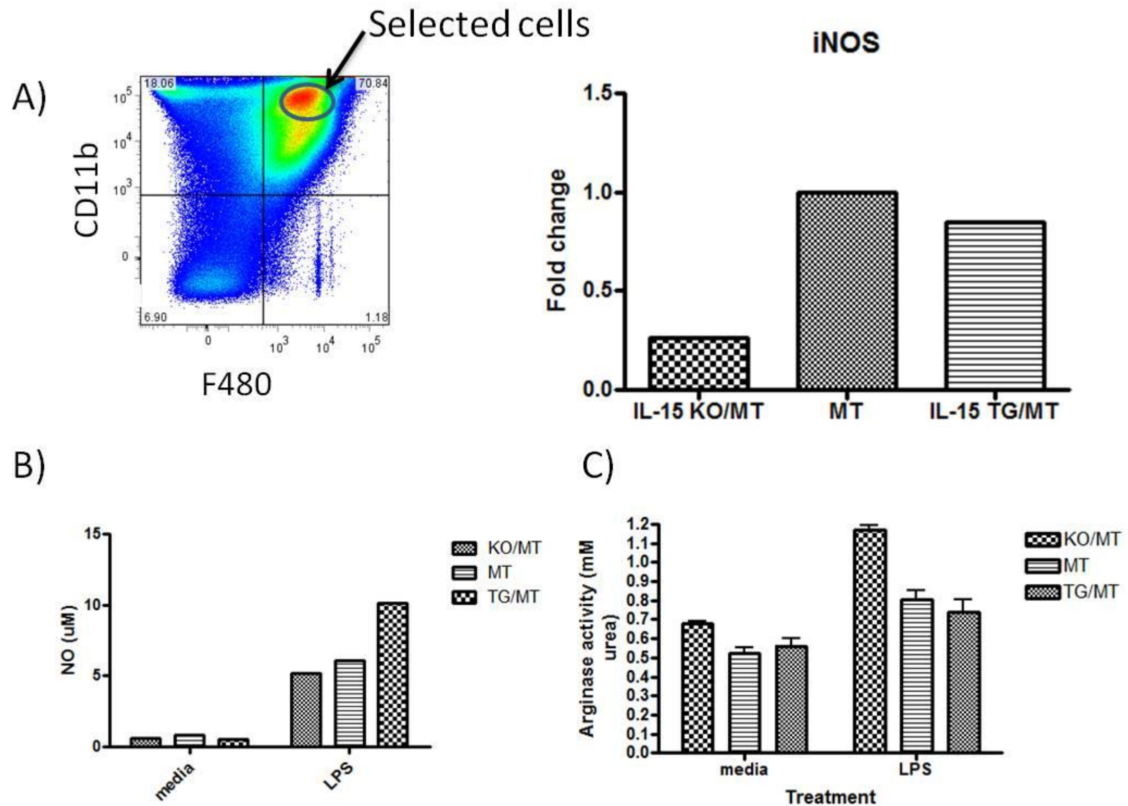
in whether or not IL-15 had effects on the polarization of macrophages in the tumor. Macrophages within tumors are reported to be of a more M2 phenotype and these play a pro-tumor role (Mantovani, Sozzani et al. 2002). In contrast, M1 macrophages may potentially play an anti-tumor role. As a first step, peritoneal macrophages were taken from IL-15 KO, IL-15 TG and C57BL/6 mice and analyzed for their ability to produce NO in response to LPS, an important functional marker of M1 macrophages (Martinez, Sica et al. 2008). Macrophages from IL-15 TG mice produced much higher levels of NO, whereas IL-15 KO mice macrophages produced low levels (Figure 3). This experiment has been repeated over 3 times with very similar results. Therefore, overexpression of IL-15 promotes the formation of M1 macrophages. To determine if this polarization is maintained in a tumor microenvironment, we first examined tumor homogenates from IL-15 KO/MT, MT or IL-15 TG/MT mice for the presence of IL-12 (Figure 4). IL-12 is a cytokine that is frequently produced by M1 macrophages, but not M2 macrophages (Martinez, Sica et al. 2008). IL-12 was found at much higher levels in IL-15 TG/MT tumors than in IL-15 KO/MT tumors, indicating that macrophages in IL-15 TG/MT tumors may maintain their M1 polarization. To further address this issue we also assessed NO and arginase activity in macrophages from IL-15 KO/MT, MT and IL-15 TG/MT tumors (Figure 5). Tumors were digested, filtered and underwent positive selection for CD11b (Stem cell) (Figure 5). In 5A, cells were flow stained and to achieve high purity, only cells expressing both CD11b and F4/80 were selected via flow sorting. RNA was extracted from the resultant cells and the expression of inducible nitric oxide synthase (iNOS2), a molecule that is critical for NO production, was assessed via



3.3- Figure 3: IL-15 affects macrophage polarization. Peritoneal macrophages were collected from IL-15 KO, IL-15 TG and BL/6 mice and plated in media or media with LPS. Nitric Oxide (NO) production was assessed after 48 hours. IL-15 TG mice have peritoneal macrophages with high NO production, indicating a more M1 macrophage phenotype in these mice. In contrast, IL-15 KO mice have very low levels of NO production (representative of 3 experiments).



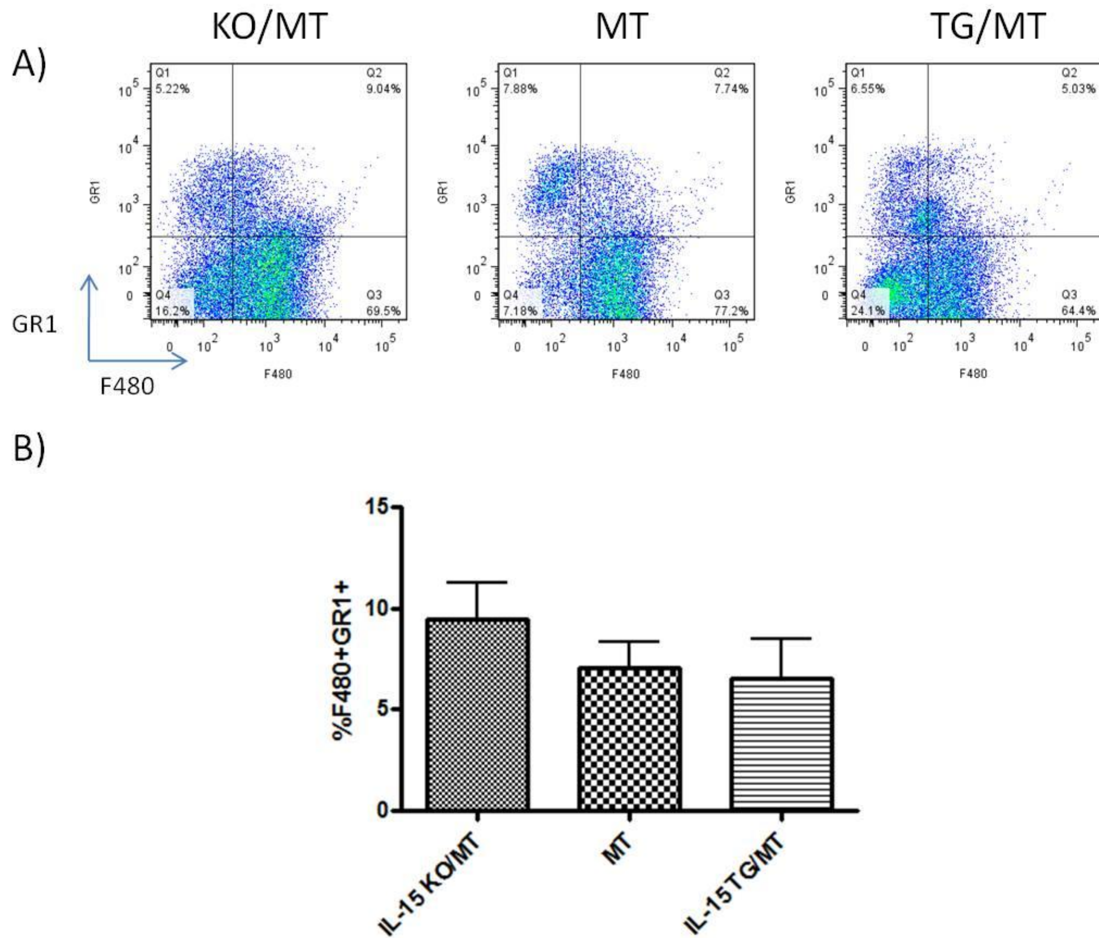
3.3- Figure 4: IL-12 production varies with IL-15 status. Homogenates of tumors from IL-15 KO/MT, MT and IL-15 TG/MT mice were assessed via ELISA for the presence of IL-12 p40 (n=6/group).* statistically significant difference via one-way ANOVA



3.3- Figure 5: IL-15 KO/MT tumor macrophages have characteristics of M2 macrophages, whereas IL-15 TG/MT tumor macrophages are more M1 like. A) All tumors were digested from a MT, IL-15 KO/MT or IL-15 TG/MT mouse, underwent CD11b+selection and then flow sorting to select for F4/80 and CD11b high macrophages. $2X10^5$ cells were taken for RNA extraction, cDNA synthesis and qRTPCR was performed for iNOS (n=1/group). B) CD11b+ cells were isolated via positive selection from digested tumors and then plated in the presence or absence of LPS for 24 hours to determine ability to produce NO. C) Cell lysates were then assayed for arginase activity (IL-15 KO/MT n=3, MT and IL-15 TG/MT n=2).

quantitative PCR (normalized to GAPDH expression). The lowest expression was found in IL-15 KO/MT macrophages, indicating that they are less M1 polarized. This was confirmed in a functional NO assay in which IL-15 TG/MT tumor macrophages had higher NO production (Figure 5B) (not performed with flow sorted macrophages, CD11b positively selected macrophages due to poor cell yield and survival during flow sorting). Arginase activity has been reported to be a good marker for M2 macrophages and is pro-tumoral (Martinez, Sica et al. 2008; De Palma and Lewis 2013). Therefore, we also performed arginase assays on the same cells and found that while the overall levels were low, IL-15 KO/MT macrophages had higher levels of arginase activity than either MT or IL-15 TG/MT macrophages (Figure 5C). Therefore, IL-15 is capable of altering macrophage polarization towards M1, both globally in the mouse and within the tumor microenvironment.

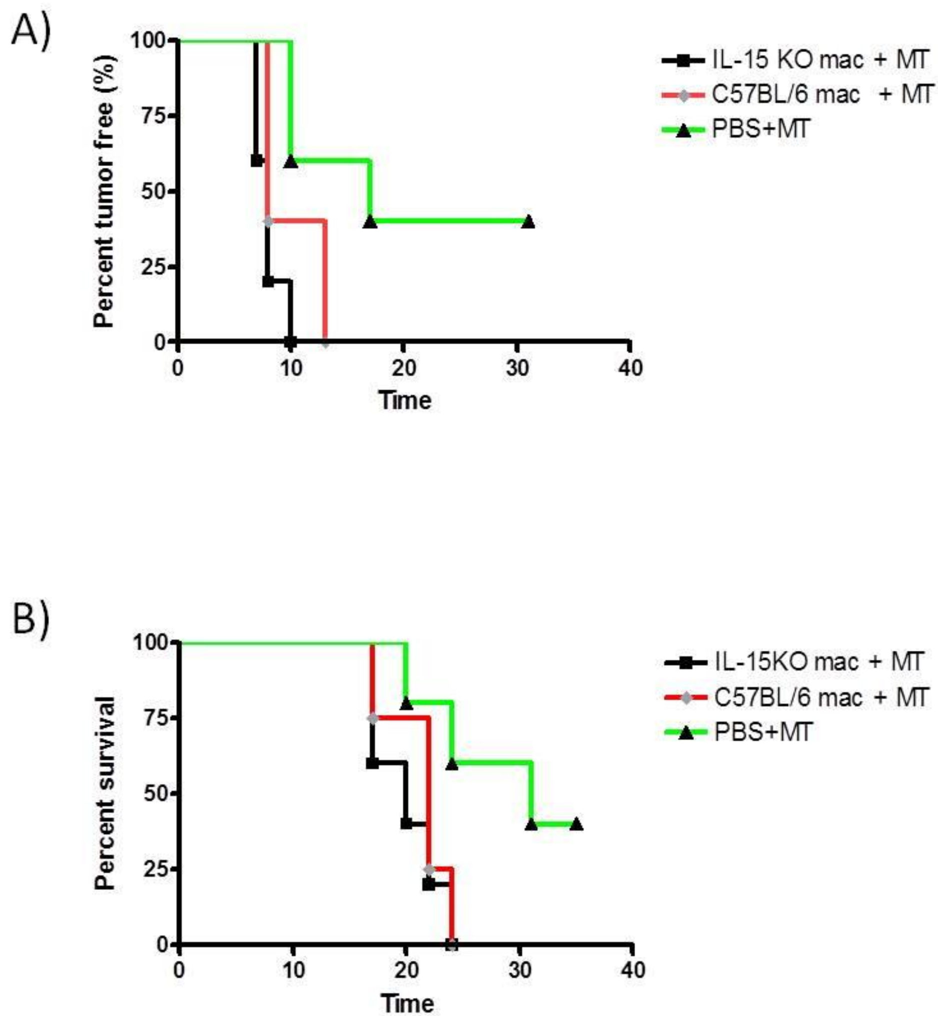
3.3.3 Myeloid Derived Suppressor Cells (MDSCs) are found within IL-15 KO/MT, MT and IL-15 TG/MT tumors. MDSCs have become a cell type of interest in cancer immunology as they are extremely pro-tumoral (Gabrilovich, Ostrand-Rosenberg et al. 2012). To determine if IL-15 effects their distribution within the tumor, we used flow cytometry and the markers CD45, CD11b, F4/80 and GR1 to identify them in IL-15 KO/MT, MT and IL-15 TG/MT tumors (Figure 6). MDSCs usually express all of these markers (CD45+CD11b+F4/80+GR1+), whereas other macrophages lack GR1 (CD45+CD11b+F4/80+GR1-). We found MDSCs made up less that 10% of the CD45+CD11b+ cells within the tumor and this was very similar among the tumor types.



3.3- Figure 6: Proportion of MDSC within IL-15 KO/MT, MT and IL-15 TG/MT tumors is approximately equal. Tumors from IL-15 KO/MT, MT and IL-15 TG/MT mice were digested and stained for flow cytometry. CD45+CD11b+ cells were further assessed for F4/80 and GR1. MDSCs are expected to be GR1+F4/80+. A) Representative flow cytometry plots. B) Graphical representation (MT n=4, KO/MT and TG/MT n=3). Representative of 2 separate experiments.

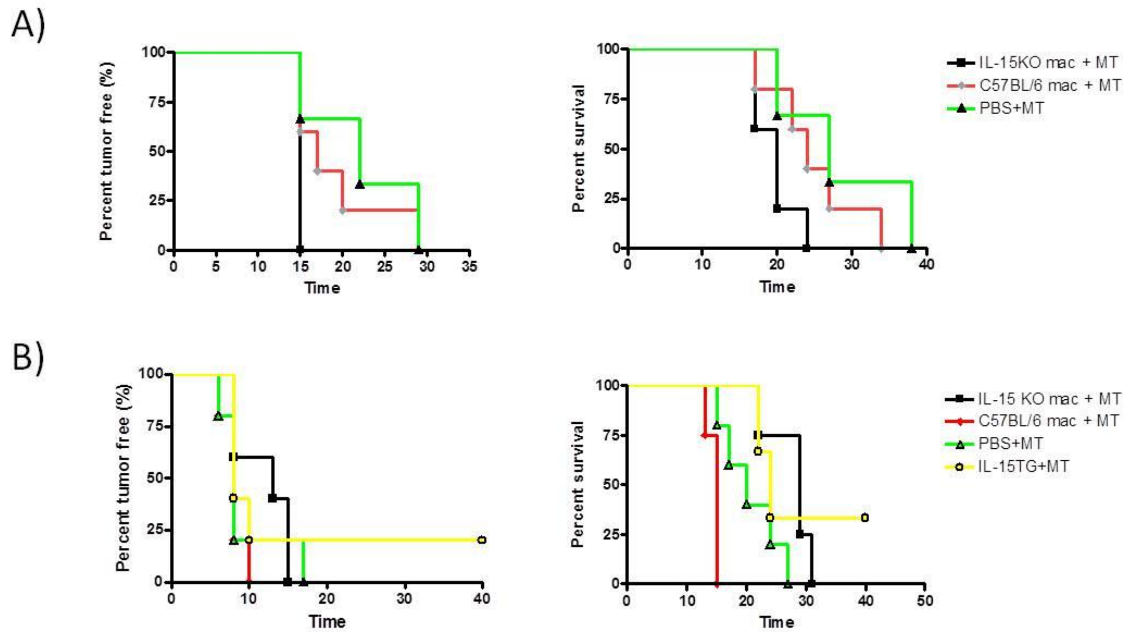
While it was similar among the tumor types, we do know that IL-15 KO/MT and MT tumors had a much higher proportion of macrophages (Figure 2). Therefore, overall, there are more MDSC in the IL-15 KO/MT tumors than in the IL-15 TG/MT tumors.

3.3.4 The addition of M2 or C57BL/6 macrophages promotes tumor formation, whereas M1 macrophages decrease tumor formation, in a subcutaneous breast cancer model. We have found that macrophages are important to tumor formation in a subcutaneous breast cancer model (Figure 1). Subsequent data indicated that IL-15 affects macrophage polarization towards a more M1 phenotype. To determine if the presence of polarized macrophages at the time of subcutaneous breast cancer formation could affect tumor formation, we isolated peritoneal macrophages from IL-15 KO and C57BL/6 mice and injected them at the same time as MT tumor cells in a 5:1 ratio (macrophage:tumor cell, $5X10^5$:$1X10^5$) (Figure 7). As previously mentioned, IL-15 KO macrophages express a M2 phenotype and C57BL/6 macrophages a more intermediate phenotype. We found that injection of this number of macrophages, regardless of their source, appeared to promote the speedy formation of tumors (ns, but met logrank criteria for trend). Several mice that just received tumor cells did not even form tumors, but all mice that received macrophages as well as tumors proceeded quickly to tumor formation. At this dose of macrophages, no real difference between IL-15 KO and control macrophages was seen, despite the fact that IL-15 KO mice had macrophages that were more M2 than control. To ascertain if polarized macrophages from IL-15 KO, C57BL/6 or IL-15 TG mice can differentially affect tumor formation, we decided to lower the dose

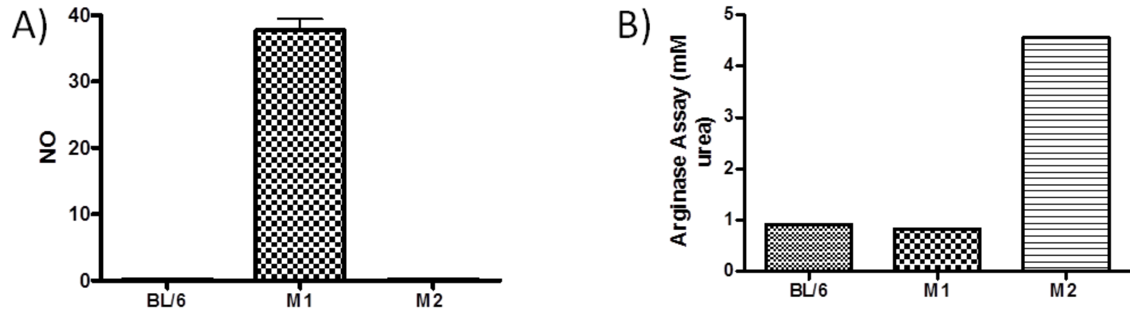


3.3- Figure 7: The addition IL-15 KO or C57BL/6 macrophages promotes subcutaneous breast tumor formation. Peritoneal macrophages were isolated from C57BL/6 and IL-15 KO mice. These were injected subcutaneously along with $1X10^5$ MT cells at a 5:1 ratio ($5X10^5$ macrophages: $1X10^5$ tumor cells). Mice were followed for date of 1st tumor palpation (A) and survival (B) (n=5/group).

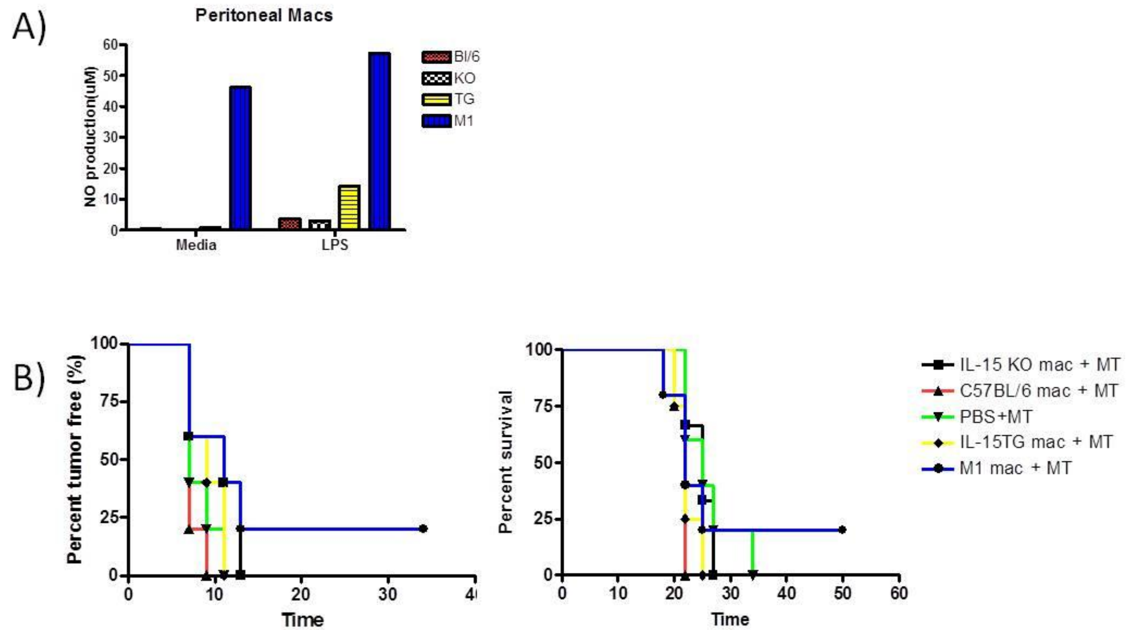
of macrophages to a 2:1 ratio (macrophage:tumor, $2X10^5$:$1X10^5$) (Figure 8). While the results were not statistically significant, mice that received IL-15 KO macrophages were fastest to form tumors and reach endpoint (Figure 8A). The addition of IL-15 TG macrophages did lead to survival in one mouse (Figure 8B). The results for this study were variable so to attempt to reduce this variability we moved toward the *in vitro* polarization of C57BL/6 macrophages towards M1 and M2 macrophages. Several studies have found that M1 and M2 polarized macrophages can be created by an altered cytokine environment (Stout, Jiang et al. 2005; Mylonas, Nair et al. 2009). We used a 24 hour exposure to IL-13/IL-4 to promote M2 macrophages and IFNγ and LPS to promote M1 macrophages (Figure 9). The resultant M1 macrophages had high NO activity and low arginase activity, whereas the M2 macrophages had low NO activity and high arginase activity (Figure 9). This was seen consistently over many experiments and therefore these were thought to be prototypic representatives to test the differential ability of M1/M2 macrophages to affect breast tumor formation. In addition, especially in comparison to IL-15 TG macrophages, the M1 macrophages produced even higher levels of NO (Figure 10A). We repeated the above experiment with the addition of M1 polarized macrophages, but unfortunately, in this experiment tumors formed very quickly and no difference was observed among the groups (Figure 10B). One mouse survived that had received M1 macrophages. To simplify, we switched to using M1 or M2 and C57BL/6 macrophages as intermediates (Figure 11). As can be seen, we consistently saw increased survival in mice that received M1 macrophages and tumor formation was promoted by either C57BL/6 or M2 macrophages.



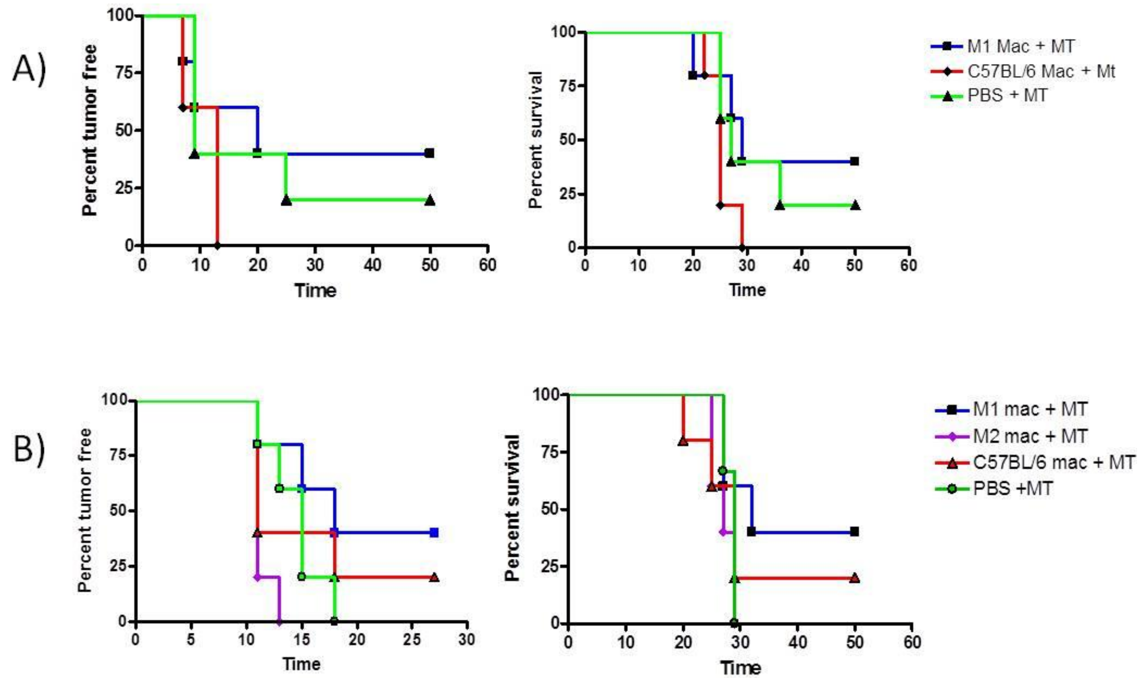
3.3- Figure 8: When injected with tumor cells, C57BL/6, IL-15 KO and IL-15 TG peritoneal macrophages affect tumor formation differentially. A) and B) Peritoneal macrophages were isolated from C57BL/6, IL-15 KO and IL-15 TG mice. These were injected subcutaneously along with $1X10^5$ MT cells at a 2:1 ratio ($2X10^5$ macrophages: $1X10^5$ tumor cells). Mice were followed for date of 1st tumor palpation and survival (n=5/group).



3.3- Figure 9: Polarization protocol results in the production of M1 macrophages that produce nitric oxide (NO) and M2 macrophages with arginase activity. Peritoneal macrophages were isolated from C57Bl/6 mice and cultured in IFNγ 10 ng/ml and LPS 100 ng/ml (M1) or IL-4 20 ng/ml and IL-13 50 ng/ml (M2) for 24 hours. Media was harvested for an NO assay (A) and cells for an Arginase assay (B). Representative of 3 experiments.



3.3- Figure 10: M1 macrophages produce more NO than IL-15 TG macrophages and can prevent tumor formation. Peritoneal macrophages were isolated from C57BL/6, IL-15 KO and IL-15 TG mice. A portion of peritoneal macrophages from C57BL/6 mice were treated with the M1 polarization protocol. (A) Cells were cultured in the presence or absence of LPS and a NO assay was performed on the 48 hour supernatants. (B) Peritoneal macrophages were injected subcutaneously along with $1X10^5$ MT cells at a 2:1 ratio ($2X10^5$ macrophages: $1X10^5$ tumor cells). Mice were followed for date of 1st tumor palpation and survival (n=5/group).



3.3- Figure 11: The effect of polarized macrophages on tumor formation. A) and B) Peritoneal macrophages were isolated from C57Bl/6 mice and cultured in IFNγ and LPS (M1) or IL-4 and IL-13 (M2) for 24 hours or untreated. These macrophages were injected subcutaneously along with $1X10^5$ MT cells at a 2:1 ratio ($2X10^5$ macrophages: $1X10^5$ tumor cells). Control mice were given PBS. Mice were followed for date of 1st tumor palpation and survival (n=5/group).

Chapter 4.0: Discussion

4.1 IL-15 Stimulated NK cells lead to tumor destruction in a spontaneous breast tumor model:

IL-15 is an exciting cytokine that has been under investigation as an immunotherapeutic for the last decade. It has the ability to promote NK cell survival, proliferation and activation, as well as to promote the formation of activated/memory CD8 T cells (Bamford, Grant et al. 1994; Grabstein, Eisenman et al. 1994; Carson, Ross et al. 1995; Cooper, Bush et al. 2002; Ranson, Vosshenrich et al. 2003). Since these have both been identified as key cell types in the defense against cancer, it is no wonder that IL-15 has garnered such interest. There have also been reports that IL-15 can have anti-tumor effects on other immune cells, although this has been investigated less thoroughly (Alleva, Kaser et al. 1997; Mattei, Schiavoni et al. 2001; Niedbala, Wei et al. 2002; Liu, Zhai et al. 2004; Ben Ahmed, Belhadj Hmida et al. 2009). The majority of tumor models that have been used to study the effect of IL-15 on tumor formation have involved engrafted or metastatic models. Unfortunately, these models do not closely mimic what occurs in spontaneous human tumors. Thus, while these systems are useful, they are a rather artificial system in which it is much easier to develop immune responses as they are foreign tumors. In addition, there is a lack of studies that examine the role of IL-15 in solid epithelial tumors such as breast cancer. Therefore, to address this, we examined the role of IL-15 in a spontaneous model of breast cancer formation. The MMTV-pMT

model was utilized due to its reported value as a good approximation of what occurs during human breast tumor formation (Lin, Jones et al. 2003). We crossed MT mice with mice that lack IL-15 (IL-15 KO) or mice that overexpress IL-15 (IL-15 TG), to create IL-15 KO/MT and IL-15 TG/MT mice.

Our initial examination of the role of IL-15 in tumor formation revealed that in a subcutaneous MT breast cancer model, IL-15 overexpression is protective and a lack of IL-15 promotes tumor formation. In this model, NK cells were protective and were capable of killing MT tumor cells directly. This led us to believe that this would be an interesting model to use to investigate the impact of IL-15. When we progressed to the spontaneous model, we also saw that IL-15 TG/MT mice proceed to endpoint more slowly than either IL-15 KO/MT or MT mice. In addition, IL-15 KO/MT mice formed tumors more quickly and proceeded to endpoint more quickly. These results are similar to the anti-tumor effects of IL-15 that have been observed in other subcutaneous and metastatic models of melanoma and colon cancer (Yajima, Nishimura et al. 2002; Kobayashi, Dubois et al. 2005; Ugen, Kutzler et al. 2006). Therefore, the overexpression of IL-15 is capable of protection from tumor formation or extending survival in both subcutaneous and spontaneous breast cancer models.

Tumors that form in IL-15 KO/MT mice have a very different phenotype than those that form in IL-15 TG/MT mice. Upon visual and histological examination, we observed that IL-15 TG/MT tumors were small, hard and dense and exhibited very few areas of healthy tumor cell growth and large amounts of immune cell infiltration. In addition, there was more cell death occurring in IL-15 TG/MT tumors than in either MT

or IL-15 KO/MT tumors. Thus, it appeared as if immune infiltration could possibly lead to extensive cell death in IL-15 TG/MT tumors. These observations encouraged us to look further at what was going on in the tumor microenvironment. Since IL-15 has been reported to effect NK and CD8 T cells primarily, we concentrated on these cell types. IL-15 TG/MT tumors had higher proportions of NK cells within their tumor environment. In several human cancers, it has been found that higher NK cell infiltration in tumors is associated with a better prognosis (Coca, Perez-Piqueras et al. 1997; Ishigami, Natsugoe et al. 2000; Villegas, Coca et al. 2002). That is similar to what we observe here. Additionally, many reports have indicated that NK cells in established tumors have a different phenotype than when found in the spleen/tissue of healthy individuals (Carrega, Morandi et al. 2008; Mamessier, Sylvain et al. 2011; Liapi, Gogali et al. 2013; Mamessier, Pradel et al. 2013). For example, the cells lose markers of maturity and activation. Here, we found that tumor IL-15 TG/MT NK cells had increased levels of the early activation marker CD69 as well as increased levels of activating receptors such as NKG2D and NKp46. Ligands for these activating receptors have been found on human breast tumors and these activating receptors have been described as important for NK cells to kill these tumor targets (Mamessier, Sylvain et al. 2011). In addition, more NK cells in IL-15 TG/MT tumors expressed CD27, a marker of highly mature and cytotoxic NK cells (Hayakawa, Huntington et al. 2006). Lastly, perforin, a key cell death molecule used by NK cells to kill tumor cells, was expressed by more IL-15 TG/MT intra-tumoral NK cells (Kajitani, Tanaka et al. 2012). Overall, examination of the NK cell phenotype

revealed that overexpression of IL-15 promotes the formation of tumors containing more NK cells in an activated state, and thus would be more capable of killing tumor cells.

The amount of CD8 T cells was increased in IL-15 TG/MT tumors in comparison to IL-15 KO/MT or MT tumors. In contrast, the number of CD4 T cells remained stable in all groups. We also examined the proportions of T regulatory cells in the tumor types and found no consistent differences (data not shown). The infiltration of tumors with CD8 T cells has been implicated as a predictor of positive outcome in human tumors (Marrogi, Munshi et al. 1997; Kawai, Ishii et al. 2008; Nelson 2008; Pages, Galon et al. 2010). In addition, a common predictor of disease outcome is the CD8 to CD4 ratio- a ratio higher than 1 is a positive predictor of disease outcome (Nelson 2008). In IL-15 TG/MT tumors, the average value is over 5, whereas in IL-15 KO/MT tumors it is well below 1. This indicates that there is a higher chance of survival for IL-15 TG/MT mice, which is what we observed. CD8 T cells within tumors can be anergic and/or exhausted (Huang, Shah et al. 2007; Kim and Ahmed 2010). Thus we examined the expression of the exhaustion marker PD-1 as well as the ability of the CD8 T cells to produce cytokines when stimulated non-specifically. CD8 T cells from IL-15 KO/MT and MT tumors have much higher expression of PD-1 on their surface than IL-15 TG/MT CD8 T cells. Therefore, exhaustion of CD8 T cells doesn't seem to play much of a role in IL-15 TG/MT mice, in fact overexpression of IL-15 prevents exhaustion. This is in contrast to another report that found IL-15 treatment increased PD-1 on CD8 T cells (Yu, Steel et al. 2010). This contrasting study was performed in an IV murine colon carcinoma model where IL-15 was given for 3 weeks post tumor cell injection and CD8 T cell expression

of PD-1 was examined in the spleen (Yu, Steel et al. 2010). It is likely either the short term administration of IL-15 or the tumor model system used that accounts for the discrepancies between our observations. IL-15 has been reported to increase memory CD8 T cells (CD44+CD62L+) that can be extremely efficient at anti-tumor responses (Klebanoff, Finkelstein et al. 2004; Klebanoff, Gattinoni et al. 2005). Our data supported this as we found that the majority of IL-15 TG/MT tumors and spleens had higher percentages of CD8 T cells with markers of memory. Functionally, the CD8 T cells from IL-15KO/MT CD8 T cells did not produce IFNγ or TNFα (data not shown). In contrast, IL-15 TG/MT CD8 T cells were capable of producing high amounts of both IFNγ and TNFα. The cells responsible for the production of IFNγ were the CD44+ CD8 T cells (effector and/or memory). Since MT tumors form in a tolerized immune environment, this indicates that IL-15 overexpression may be able to rescue T cells that have been tolerized and are anergic. This has been reported previously in a model of leukemia (Teague, Sather et al. 2006).

We also found a unique cell type in the IL-15 TG/MT tumors that are NK1.1+ CD8 T cells. This cell type has been previously identified in situations of IL-15 overexpression. This subset was found to be highly cytotoxic, able to produce large amounts of IFNγ, protected from activation induced cell death and highly expressed perforin/granzyme (Ohta, Hiroi et al. 2002; Terabe, Tagaya et al. 2008; Sugita, Tanaka et al. 2010; Correia, Costa et al. 2011). Here, we found that approximately the same percentage of NK1.1+ CD8 T cells were capable of making IFNγ as that of the total CD8

T cell population. Thus, it appears that these cells are not the major source of IFNγ in our model although they do contribute to its production.

From examination of IL-15 TG/MT tumors, there appeared to be both highly activated NK cells as well as CD8 T cells. While both cell types were present, the cell type involved in extending survival in IL-15 TG/MT mice was unclear. In previous immunotherapies involving IL-15, the protective effects had been NK cell or CD8 T cell dependent (Yajima, Nishimura et al. 2002; Kobayashi, Dubois et al. 2005; Epardaud, Elpek et al. 2008) To test this, long term depletion studies were carried out. Depletion of NK1.1+ cells (includes NK cells, NKT cells and NK1.1+ CD8 T cells) from IL-15 TG/MT mice revealed that these cells were key to the extended survival and tumor destruction in IL-15 TG/MT mice. We also carried out long term depletion of CD8α+ cells (CD8 T cells, NK1.1+CD8 T cells) in IL-15 TG/MT mice. There were no significant differences in survival between the IL-15 TG/MT CD8 depleted and not depleted mice, indicating that CD8 T cells did not play a major role in extending survival in these mice. In addition, the majority of CD8 depleted IL-15 TG/MT tumors still exhibited the typical IL-15 TG/MT histology. While depletion in the spleen of these mice was effective, in the tumors of this group some CD8+ cells were left and the majority were NK1.1+CD8 T cells. This indicated that the NK1.1+CD8 T cells were resistant to depletion by this method. Due to their continued presence in CD8 depleted mice, we can't rule an important role for this cell type in tumor destruction. Previously, in another spontaneous mouse model of breast tumor formation (Her2/neu), it was also found that depletion of NK cells, but not of CD8 T cells, was capable of promoting tumor

formation (Street, Zerafa et al. 2007). Our results also indicate that NK cells, not CD8 T cells, are important to the effects we see on tumor formation. Thus, it is the NK1.1+ cells (NK cells or NK1.1+CD8 T cells) that are contributing to survival and tumor destruction in IL-15 TG/MT mice.

The lack of contribution from the CD8 T cells was surprising due to their observed phenotype in IL-15 TG/MT mice. Thus, to further investigate this we performed adoptive transfer experiments with CD8 T cells from the spleen of IL-15 TG, IL-15 TG/MT, MT and C57BL/6 mice or IL-15 TG/MT tumor CD8 T cells. We then challenged the mice with subcutaneous primary MT cells (not the cell line). While the CFSE labeled CD8 T cells were present in the spleen and tumors of mice at endpoint (data not shown), there was no difference in the speed of tumor formation or survival. There are several interpretations of this data. It is possible that this aggressive tumor model formed too fast for the transferred CD8 T cells to have an impact. Additionally, it has been reported that each MT tumor that forms is slightly different and may express different tumor antigens, some even lose expression of MT (Maglione, Moghanaki et al. 2001; Lin, Jones et al. 2003; Maglione, McGoldrick et al. 2004). To account for this, multiple MT tumors from multiple MT mice were pooled for injection, and CD8 T cells were pooled from multiple mice. It is still possible that the tumor antigens expressed by the collection of MT primary cells used did not match with the antigen specific CD8 T cell pools that were present. It is also possible that the tumor rapidly lost expression of MHCI due to immunoediting via CD8 T cell attack. In fact, in several tumors examined at endpoint, there were very few MHC I + tumor cells (<2%) (data not shown). A similar

phenomenon may be occurring in our spontaneous model, which may explain why CD8 T cells do not have as much of an impact as NK1.1+ cells, that do not require MHC I expression for killing. Lastly, it has also been reported that IL-15 overexpression may stimulate non-specific proliferation of CD8 T cells instead of tumor specific responses (Ramanathan, Gagnon et al. 2008). Thus, there could be a great deal of functional CD8 T cell expansion in IL-15 TG mice, but it may not be specific to tumor antigens.

We observed that IL-15 TG/MT mice had decreased lung metastasis in comparison to IL-15 KO/MT or MT mice. This observation, in a spontaneous model of breast cancer and metastasis is extremely important. It indicates that IL-15 has the ability to prevent metastasis as well as extend survival. Previously, most studies have examined the role of IL-15 in models of metastasis, but rarely in spontaneous metastasis. This fact may be extremely useful in a clinical setting as immunotherapies may be particularly efficacious after surgery in cases where minimal primary tumor is present, but when metastasis may be highly likely and a significant cause of mortality. The role of IL-15 in metastasis will be discussed more thoroughly in a later section.

The cytokine environment in IL-15 TG/MT tumors has been altered. In addition to increased IL-15, there are also increases in other cytokines that promote NK cell activation such as IL-12 and IL-18. Both of these are being investigated as immunotherapies in their own right, but the combination of IL-15/IL-12/IL-18 has been reported to promote the formation of highly activated "memory-like" NK cells that efficiently attack tumors (Cooper, Elliott et al. 2009; Ni, Miller et al. 2012). To determine if the cytokine environment present in IL-15 TG/MT tumors could promote the

ability of human NK cells to kill tumor cells, we isolated NK cells from human PBMCs and investigated their ability, when exposed to different cytokine milieus, to kill a human breast cancer cell line (MDA-231). MDA-231 is a triple negative breast cancer cell line, and thus proves difficult to treat with current therapies. We found that treatment of human NK cells with IL-15, IL-12 and IL-18, in contrast to IL-2 alone, greatly increased their ability to kill MDA-231 cells. If IL-15 is capable of increasing these cytokines *in vivo*, it may promote formation of these effective "memory-like" NK cells. An additional benefit of these "memory-like" NK cells is that they last long term in the host and maintain their ability to produce IFNγ (Keppel, Yang et al. 2013). Future studies examining the therapeutic potential of these cells should be forthcoming.

Recently, several studies have indicated that NK cells can target breast cancer cells. In particular, human breast cancer cells frequently express NKG2D and other activating receptor ligands, as well as death receptors that NK cells can use to identify the correct cells to kill (Mamessier, Sylvain et al. 2011; de Kruijf, Sajet et al. 2012; Kajitani, Tanaka et al. 2012). In addition, NK cells can also kill cells with characteristics of CSCs (Li, Knight et al. 2012). Lastly, it was recently shown that NK cells can eradicate established fibrosarcoma, a solid epithelial cancer (Liu, Engels et al. 2012). Here we have found that IL-15 can promote NK cell mediated tumor cell death in a spontaneous model of breast cancer and that human NK cells, when exposed to a similar cytokine environment as is present in IL-15 TG/MT tumors, are capable of killing TNBC. Our data, in addition to these studies, strongly argues that NK cells are a good target for immunotherapy, even in solid epithelial cancers. The aforementioned findings have led

to even more interest in IL-15 as an immunotherapeutic. While results with other cytokines such as IL-2 have thus far been poor, IL-15 appears to be superior as it has lower toxicity, does not increase T regulatory cells to the same degree and induces better NK and CD8 immune responses (Villinger, Miller et al. 2004; Mueller, Petrovas et al. 2005; Berger, Berger et al. 2009). Based on the success of animal models for melanoma in particular, several clinical trials have begun (NCT01727076, NCT01021059, NCT01572593). We feel that IL-15, likely in combination with other therapies, will also be a prime candidate for treatment of solid epithelial cancers such as breast cancer.

4.2 The absence or overexpression of IL-15 alters breast cancer metastasis via effects on NK cells, CD4 T cells and macrophages:

The ability of IL-15 to decrease tumor formation and metastasis through increased anti-tumor NK cell and CD8 T cell responses has been extensively studied in several tumor models, particularly melanoma (Yajima, Nishimura et al. 2002; Ugen, Kutzler et al. 2006; Dubois, Patel et al. 2008) . Few studies have addressed the role of IL-15 in breast cancer metastasis and its effects on immune cells other than NK or CD8 T cells. IL-15 has reported effects on both macrophages and CD4 T cells, both of which have been implicated in facilitating metastasis (DeNardo, Barreto et al. 2009; Qian, Deng et al. 2009). To investigate this, we have utilized a model of breast cancer metastasis in which a MT cell line established from a MMTV-pMT tumor was injected intravenously. This mimics the point at which metastatic cells have entered the blood stream and can

establish tumor formation in other tissues, such as the lung. Utilizing this model, we found that mice that lack IL-15 (IL-15 KO) are extremely susceptible to metastatic growth, whereas mice that overexpress IL-15 (IL-15 TG) are resistant to metastatic growth in comparison to control C57BL/6 mice. The protection observed in IL-15 TG mice was dependent upon NK cells, not CD8 T cells. In fact, depletion of NK cells created mice that had a similar susceptibility to metastasis as IL-15 KO mice. This agrees with earlier studies in which the effects of IL-15 in metastatic models of colon carcinoma or melanoma are NK cell dependent, not CD8 T cell dependent (Kobayashi, Dubois et al. 2005; Bessard, Sole et al. 2009) .

These observations led us to examine changes in cytokine expression within the lungs upon loss of IL-15 expression. Two days after tumor cell injection, we observed a decrease in the expression of specific cytokines, such as IFNγ, CCL5 (RANTES) and XCL1 (lymphotactin), in IL-15 KO mice. In the literature, these cytokines have been described as Th1 promoting cytokines that are expressed by activated NK cells in certain infections (Dorner, Scheffold et al. 2002; Dorner, Smith et al. 2004). However, it is possible that these molecules could be expressed by Th1 CD4 or CD8 T cells, as well as macrophages (Dorner, Scheffold et al. 2002; Muller, Bischof et al. 2003; Dorner, Smith et al. 2004). These molecules can affect many cell types that can aid in the prevention of metastasis. For example, in a model of melanoma metastasis, production of IFNγ by lung NK cells was critical for resistance to metastasis (Takeda, Nakayama et al. 2011). In addition, IFNγ is known to promote the formation of M1 macrophages and Th1 T cells (Mantovani 2006). Chemokines such as CCL5 and XCL1 have been found to play a role

in anti-tumor defense by recruiting immune cells including NK cells, Th1 CD4 T cells and CD8 CTLs to the site of expression (Emtage, Xing et al. 2002; Huang, Li et al. 2002; Lapteva and Huang 2010). Due to the significant susceptibility of IL-15 KO mice to metastasis, it is likely that the lack of NK cells in these mice could be a significant contributor to susceptibility. Interestingly, when we depleted NK cells from control mice it did not fully recapitulate the degree of metastasis seen in IL-15 KO mice. While just missing statistical significance, the experiment was repeated twice with the same results. This indicates that the absence of IL-15 had a greater impact on metastasis than the loss of NK cells alone. IL-15 has a plethora of effects on other factors that are important in promoting metastasis. Indeed, it has been previously suggested that IL-15 has anti-tumor activities that are not dependent on either NK cells or CD8 T cells (Davies, Reid et al. 2010).

It has recently been discovered that macrophages not only have pro-angiogenic and metastatic roles at the primary tumor, but that they are also very important for the establishment of metastasis (Qian, Deng et al. 2009; Qian, Li et al. 2011). They contribute both to the extravasation of the tumor cells and growth at the metastatic site (Qian, Deng et al. 2009; Qian, Li et al. 2011). The macrophages that have been found to aid in tumor formation and metastasis are of a variety of subtypes, but they mostly exhibit characteristics of M2 macrophages (Mantovani, Sozzani et al. 2002; Joyce and Pollard 2009; Qian, Li et al. 2011). There have been reports that IL-15 can influence macrophage phenotype by promoting increased expression of MHC II and the secretion of pro-inflammatory cytokines as well as NO production (Alleva, Kaser et al. 1997; Liu,

Zhai et al. 2004; Ruckert, Brandt et al. 2009). Therefore, we were interested in determining if the presence or absence of IL-15 had an impact on the macrophage phenotype in our model. We found that IL-15 KO mice had macrophages that consistently produced lower levels of NO, whereas IL-15 TG mice produced higher levels of NO compared to control mice. NO production is increased in macrophages with a M1 polarization and is a method by which macrophages can kill tumor cells (Albina and Reichner 1998; Bhaumik and Khar 1998). Therefore, our results suggest that IL-15 promotes the formation of M1 macrophages, whereas lack of IL-15 alters macrophages to a M2 phenotype. The mechanism by which IL-15 polarizes macrophages could either be direct effects or a result of an indirect effect on other cell types. We also examined the myeloid cell content within the lungs of IL-15 KO and control mice two days post tumor cell injection to determine if different subsets were homing to the tumor environment. The only significant difference was observed in the recruitment of macrophages from the circulation (F480+CD11b+GR1-). The lungs from IL-15 KO mice were capable of recruiting an increased proportion of macrophages to the lung environment. Due to their key role in metastatic extravasation and growth, an increase in the recruited macrophage population could aid in metastatic establishment. In fact, it has been shown that recruited macrophages, and not lung resident macrophages (CD11b+CD11c+Gr1-), aid in the extravasation and establishment of lung metastasis (Qian, Deng et al. 2009). Levels of MDSC (CD11b+Gr1+) were similar between IL-15KO and control mice. Therefore, lack of IL-15 promotes the recruitment of macrophages to the lung tumor environment.

It has been suggested that CD4 T cells play a role in macrophage polarization and in promoting metastasis (Mantovani, Allavena et al. 2008; DeNardo, Barreto et al. 2009). In a study by Denardo *et al*., it was found that CD4 T cells secrete Th2 cytokines that promote the polarization of macrophages towards M2 in the primary tumor of a spontaneous breast cancer model (MMTV-pMT)(DeNardo, Barreto et al. 2009). They further observed that knockout of CD4 T cells promoted the formation of M1 TAMS and subsequently decreased the amount of metastasis. In our experiments using this model of metastasis, we were only able to observe the effects of CD4 T cells or macrophages at the metastatic site, not at the primary tumor. We have confirmed that CD4 T cells are present in the lungs of control and IL-15 KO mice and they are a slightly higher proportion of leukocytes in IL-15 KO mice. There are many contrasting reports regarding the effect of IL-15 on CD4 T cells. IL-15 has been reported to induce proliferation of CD4 T cells (naive and/or memory) and to promote either the formation of T regulatory cells or Th1 CD4 T cells (Seder 1996; Niedbala, Wei et al. 2002; Benito-Miguel, Garcia-Carmona et al. 2009). This lack of agreement between studies is likely due to the different *in vitro* conditions under which they investigated this effect. It is likely that *in vivo*, the effects of IL-15 on CD4 T cells are modified by other cytokines that are present in the microenvironment. To determine if the absence of IL-15 had an effect on CD4 T cell polarization, we non-specifically stimulated CD4 T cells isolated from IL-15 KO or control mice and found that IL-15 KO CD4 T cells secreted Th_2 cytokines, IL-10 and IL-4, at a higher level than control CD4 T cells. This indicates that when IL-15 is absent, CD4 T cells are of a more Th_2 phenotype. As mentioned previously, Th_2 CD4 T cells are

able to polarize macrophages to an M2 phenotype in a primary tumor environment (DeNardo, Barreto et al. 2009). Furthermore, macrophages in IL-15 KO mice are of a more M2 phenotype and produce low levels of NO. To determine if CD4 T cells are promoting this macrophage polarization, we depleted CD4 T cells from IL-15 KO mice and examined macrophage polarization. In 2 separate experiments, we observed that removal of CD4 T cells from IL-15KO mice led to a recovered ability to produce control levels of NO from macrophages. Thus, lack of IL-15 promotes the formation of Th_2 polarized CD4 T cells, which in turn promotes the polarization of macrophages to a M2 phenotype. Lastly, to determine if CD4 T cells were contributing to the increased susceptibility of IL-15KO mice *in vivo*, we depleted CD4 T cells from IL-15 KO mice. Removal of CD4 T cells from IL-15 KO mice decreased the amount of metastasis. Therefore, in the absence of IL-15, CD4 T cells do play an important role in promoting metastasis, likely via their effects on macrophage polarization.

As with every study, there are inherent limitations. It is now known that signals from the primary tumor help to set up a pre-metastatic niche to establish metastasis. For example, cells from the primary tumor secrete soluble factors such as TNFα, TGF-β, LOX and VEGF1 to aid in efficient establishment of metastasis (Kaplan, Riba et al. 2005; Hiratsuka, Watanabe et al. 2006; Erler, Bennewith et al. 2009). Many of these signals are to promote the ability of macrophages to move to the pre-metastatic niche (Hiratsuka, Watanabe et al. 2006; Erler, Bennewith et al. 2009). Thus, since we have no primary tumor in this model, we cannot examine these interactions. While this is a limit, many

other prominent researchers have used IV models of metastasis similar to ours to examine the later stages of metastasis.

In this model of metastasis, IL-15 is protective from breast cancer metastasis, whereas the absence of IL-15 promotes metastasis. The protective effect of IL-15 is due to both its effects on NK cells and other immune cells such as macrophages and CD4 T cells. Within the clinic, metastasis is a significant contributor to mortality. This study indicates that, for many reasons, IL-15 may be an ideal therapy to prevent tumor recurrence and spread. It has effects on immune cells within the tumor environment that are even more wide spread than previously appreciated.

4.3 Macrophage Polarization and its effect on breast tumor formation:

Macrophages within tumors are associated with increased angiogenesis and poor prognosis in many human tumor types (Clear, Lee et al. 2010; Daurkin, Eruslanov et al. 2011; Heusinkveld and van der Burg 2011). Additionally, in mouse tumor models, depletion of macrophages leads to inhibited tumor formation and angiogenesis (Lin, Nguyen et al. 2001; Zeisberger, Odermatt et al. 2006). Macrophages within tumors are usually of the M2 phenotype and promote tumor formation via increasing angiogenesis, promoting immunosuppression in the tumor and promoting metastasis (Mantovani, Sozzani et al. 2002). In contrast, M1 macrophages can kill tumor cells or promote tumor cell death via production of NO, IFNγ and TNFα (Luo and Knudson 2010). IL-15 has

been reported to have direct effects on macrophages, but it also has effects on other cells types such as NK cells and CD4 T cells that may push macrophage polarization toward M1 or M2 (Alleva, Kaser et al. 1997; Liu, Zhai et al. 2004; Gays, Martin et al. 2005; DeNardo, Barreto et al. 2009; Ruckert, Brandt et al. 2009). At the same time, tumors are powerfully immunosuppressive and produce molecules to promote M2 polarization. Therefore, we were interested in examining if IL-15 overexpression or loss would impact macrophage polarization in a spontaneous model of breast cancer.

As a first step, we confirmed that in a subcutaneous engrafted model of breast tumor formation, using a cell line established from our spontaneous model, that macrophages promote the formation of tumors and the progression to endpoint. We progressed to examine the macrophages within IL-15 KO/MT, MT and IL-15 TG/MT tumors. One of the first discoveries we made was that in tumors from IL-15 KO/MT and MT mice, macrophages made up a much higher percentage (almost 3X higher) of the total tumor leukocyte population than in IL-15 TG/MT mice. It is interesting that similar to human studies, in our model, the mouse with the highest proportion of macrophages was also that with the worst prognosis. We had previously found that using increased production of NO as a marker of M1 macrophages, that peritoneal macrophages from IL-15 KO mice had a more M2 polarization, whereas IL-15 TG mice had a more M1 polarization. Thus we wanted to determine if the overexpression of IL-15 or the absence of IL-15 in the spontaneous MT tumors was able to affect macrophage polarization within the tumor. In the tumor microenvironment, tumor cells/immunosuppresive immune cells would still be capable of producing increased TGF-β, PGE_2 and IL-10,

which would promote M2 macrophage polarization (Mantovani, Allavena et al. 2008; Eruslanov, Daurkin et al. 2011; Lee, Lee et al. 2013). Is IL-15 enough to counteract these influences?

Multiple strategies have been utilized to successfully reprogram macrophages from an M2 to M1 phenotype within the tumor environment. These include cytokine based therapies like GM-CSF or IL-12, as well as targeting pathways such as NF-κB, that are thought to be necessary for maintaining TAM M2 polarization (Watkins, Egilmez et al. 2007; Hagemann, Lawrence et al. 2008; Eubank, Roberts et al. 2009). Therefore, macrophages can be altered by changing the signals in the tumor microenvironment. As was seen in Objective 1, IL-15 absence or overexpression vastly alters the tumor microenvironment and the immune cell types that are present within the tumors. Since these cell types are known to produce many cytokines, it is likely that there would be differences in the cytokine environment. One of the first cytokines we examined was IL-12. IL-12 can be produced by M1 macrophages, but it is also a cytokine that has been reported to reprogram M2 macrophages to M1 (Watkins, Egilmez et al. 2007; Martinez, Sica et al. 2008). We found that within the tumor groups, there was higher expression of IL-12 in IL-15 TG/MT tumors. The major producers of IL-12 are either M1 macrophages or DCs (Watford, Moriguchi et al. 2003) and it is known that IL-12 can act directly on the macrophage, but can also stimulate IFNγ production from NK and T cells (Manetti, Parronchi et al. 1993). IFNγ is a major inducer of M1 macrophages (Mantovani 2006). The presence of higher levels of IL-12 in IL-15 TG/MT tumors

would be a push towards M1 macrophage polarization or an indication that the macrophages are already M1.

To directly examine the macrophage status within the IL-15 KO/MT, MT and IL-15 TG/MT tumors, we attempted to flow sort these macrophages. Unfortunately, this was not very successful and very few viable macrophages were recovered, even when starting with a large number of tumors. We were able to get enough cells to extract RNA and synthesize cDNA. NO production is typical of M1 macrophages, whereas arginase activity is typical of M2 macrophages (Martinez, Sica et al. 2008; De Palma and Lewis 2013). We used this fact to determine if we had altered macrophage polarization. In the flow sorted macrophages, we did observe lower expression of iNOS (enzyme that produces NO) in IL-15 KO/MT tumors. We also isolated CD11b+ cells using magnetic bead separation to gain higher numbers of live macrophages (although less pure) and we performed functional assays for NO and arginase. From pooled tumors, there were higher levels of NO produced by the IL-15 TG/MT tumor macrophages and higher levels of arginase in macrophages from IL-15 KO/MT tumors. Unfortunately, the arginase assay did not prove very reliable. We also tried to assess other molecules that are reported to be up regulated on either M1 (MHC) or M2 (MMR) macrophages, but were unable to achieve consistent staining via flow cytometry (data not shown). Overall, we were able to get some indication that the macrophages in IL-15 TG/MT tumors may be more M1 polarized and those in IL-15 KO/MT tumors are more M2 polarized. It is possible that we were unable to get complete re-polarization and this led to the variability in the data. It has been reported that a spectrum of M1-M2 macrophages exists in most

situations and that frequently, macrophages in different locations in the tumor may be of different polarization (Movahedi, Laoui et al. 2010; De Palma and Lewis 2013). It is also possible that the markers used to assess the M1/M2 status were not consistently altered in this model.

A limit to this study is that we did not identify how IL-15 altered macrophage status. As mentioned, IL-15 has been reported to have direct effects on macrophages, but these have been poorly characterized and contradictory. Some have found that IL-15 increased molecules involved in antigen presentation and NO production, whereas others reported increased secretion of both pro- and anti-inflammatory proteins from macrophages (Alleva, Kaser et al. 1997; Liu, Zhai et al. 2004; Ruckert, Brandt et al. 2009). It seems unlikely that IL-15 is working solely via direct effects on macrophages. As has been shown in Objective 1, in IL-15 TG/MT tumors there are very high levels of infiltrating activated NK cells. There is a lot of cross talk between NK cells and macrophages (Michel, Hentges et al. 2012). IL-15 or IL-12 (both of which are present in IL-15 TG/MT tumors) activated NK cells produce IFNγ, which leads to increased M1 macrophage polarization (Lapaque, Walzer et al. 2009). In addition, it has been reported that M1 polarized macrophages can promote cytotoxicity in NK cells via expression of NKG2D ligands and IFNγ (but are protected from being killed themselves due to high MHC and other inhibitory ligand expression)(Bellora, Castriconi et al. 2010; Zhou, Zhang et al. 2012). In contrast, others find that TAM (M2) macrophages decrease NK cell function (Wu, Kuang et al. 2013). Lastly, we have found that CD4 T cells, in the absence of IL-15, are of a more Th2 phenotype. In a spontaneous breast cancer model,

Th2 polarized CD4 T cells promote the formation of M2 macrophages in the primary tumor (DeNardo, Barreto et al. 2009). Thus, there are many mechanisms by which the absence or overexpression of IL-15 may alter macrophage polarization.

MDSCs are known to promote tumor formation mostly by helping to create an immunosuppressive environment (Gabrilovich, Ostrand-Rosenberg et al. 2012). While it is unknown exactly how MDSCs are induced in the tumor, it appears as if various cytokines/chemokines play a role (Gabrilovich, Ostrand-Rosenberg et al. 2012). Thus, we were interested in determining if the different immune environment in IL-15 KO/MT and IL-15 TG/MT tumors would alter the formation of MDSCs in the tumor. We found that while a slightly higher proportion of CD11b+ cells were also GR1+ in IL-15 KO/MT tumors, this difference was not statistically significant. Since there are higher levels of macrophages in IL-15 KO/MT tumors in general, we can confidently say that there are a higher proportion of MDSCs in the IL-15 KO/MT tumors. Interestingly, there are still MDSCs present in the IL-15 TG/MT tumors. Since tumors were not eradicated in IL-15 TG/MT mice, it is possible that MDSCs would be a good target to enhance the effects of IL-15.

After these observations, we began to wonder if the polarization of macrophages would have much of an effect on tumor formation in our engrafted MT model. Would the presence of M1 or M2 macrophages at the time of tumor formation have an impact? In initial experiments, we took peritoneal macrophages from either IL-15 KO (M2) or control (M1/M2 intermediate) mice and injected them at the same time as MT tumor cells. At a ratio of 5:1, macrophages to tumor cells, the trend was that either IL-15 KO or

C57BL/6 macrophages promoted swift tumor formation. To examine the differences among groups more clearly, we reduced the amount of macrophages added to a 2:1 ratio. At this ratio, we did see a similar trend where the addition of IL-15 KO macrophages promoted tumor formation. We repeated this experiment with the addition of IL-15 TG (M1) macrophages and they did protect one mouse from tumor formation, but there were no statistically significant differences among groups. One of the issues was that the tumors formed very quickly in this particular experiment and likely obscured any difference we may otherwise have observed. Also, peritoneal macrophages taken from the various mouse models did have some degree of variability in their polarization (data not shown).

To decrease the variability in our *in vivo* M1/M2 polarized macrophages we instead began to polarize the macrophages with cytokines *in vitro*. Several researchers reported that cytokine polarization of macrophages to either M1 or M2 states was reliable and consistent (Stout, Jiang et al. 2005; Mylonas, Nair et al. 2009). Using IFNγ and LPS to polarize M1 macrophages and IL-4 and IL-13 to polarize M2 macrophages, in 24 hours we were able to consistently create M1 macrophages high in NO and low in arginase activity and M2 macrophages high in arginase activity and low in NO activity. We repeated the same co-injection studies performed above with the addition of M1 and M2 macrophage groups. Unfortunately, we again saw no major differences in tumor formation, besides survival of one mouse in the M1 group. We repeated these experiments multiple times and each time saw a similar trend, with the addition of M1 macrophages leading to survival in some mice and M2 macrophages promoting fast

tumor formation. Tumors form very quickly in the subcutaneous MT model (some as early as 7 days post injection). To slow down tumor formation, we did attempt to decrease the dose of MT cells given (to 10^4 cells), but no tumors formed at all (data not shown). It is possible that either the addition of macrophages does not have enough of an effect for us to see or that the macrophages are rapidly repolarized. The few mice that did survive consistently received M1 polarized macrophages, indicating that these can have anti-tumor effects. It would be interesting to examine the mechanism of escape in the mice that did survive. Did the M1 macrophages directly kill the tumor cells, or did they help create a Th1 environment via IFNγ secretion that subsequently activated NK cells and Th1 responses?

Here we show that IL-15 overexpression promotes the formation of M1 macrophages, whereas lack of IL-15 promotes the formation of M2 macrophages. In spontaneous breast tumors, the polarization of macrophages due to the overexpression/lack of IL-15 appears to be maintained. In addition, while addition of M2 macrophages to an engrafted model of breast cancer leads to faster tumor formation, addition of M1 macrophages can prevent tumor formation. These studies indicate that macrophages are good targets to manipulate within the tumor microenvironment and that differential polarization can effect tumor formation. It is also possible that in addition to the effects of IL-15 on NK cells and CD8 T cells, altering macrophage polarization, either directly or indirectly, may be one of the mechanisms by which IL-15 promotes tumor cell death.

4.4 Overall Summary

We have investigated the impact of IL-15 absence or overexpression in a spontaneous model of breast cancer as well as in a model of breast cancer metastasis. IL-15, via its effects on NK cells, was capable of increasing survival in spontaneous breast cancer. In IL-15 TG/MT mice, the tumor NK cells expressed markers of activation including CD69, NKG2D and perforin. In addition, many changes were observed in the tumor microenvironment. IL-15, either directly or indirectly, was capable of changing the polarization of macrophages in IL-15 TG/MT tumors towards M1. It was found that M1 macrophages were able to slow/prevent tumor formation in an engrafted breast cancer model. In contrast, M2 macrophages promoted tumor formation. In terms of metastasis, both in the spontaneous and IV model, we found that overexpression of IL-15 promoted resistance to metastasis, whereas lack of IL-15 promoted metastatic spread. In the IV model, lack of IL-15 promoted the formation of Th_2 polarized CD4 T cells that in turn altered macrophage polarization towards a M2 phenotype. Both the lack of NK cells and the Th2 polarized CD4 T cells contributed to metastatic formation in IL-15 KO mice. In IL-15 TG mice, NK cells mediated protection from metastasis. Lastly, human NK cells, when exposed to a similar cytokine environment as was seen in IL-15 TG/MT tumors, were capable of killing TNBC.

These findings, in conjunction with several recent findings regarding NK cells, breast cancer and other solid epithelial cancers, indicate that IL-15 is a strong candidate for immunotherapy in breast cancer (Mamessier, Sylvain et al. 2011; de Kruijf, Sajet et

al. 2012; Kajitani, Tanaka et al. 2012; Li, Knight et al. 2012; Liu, Engels et al. 2012). Clinical trials have begun using recombinant human IL-15 (rhIL-15) (5 currently), and although the majority are examining IL-15 in metastatic melanoma or AML, there is one study that is more general and is designed for adults with advanced metastatic cancers (NCT01572493). In this Phase I trial, patients will receive 10 days of continuous rhIL-15, every 42 days. They will examine the immune and tumor responses. This is definitely a step in the right direction, and our study indicates that cancers other than melanoma or leukemia's would benefit from IL-15 immunotherapy. These studies are still in their infancy and unfortunately may not show as much efficacy as may be expected. As mentioned, IL-15 in conjunction with its receptor (IL-15/IL-15Rα) has shown much more efficacy in animal models than IL-15 alone (Stoklasek, Schluns et al. 2006; Epardaud, Elpek et al. 2008). Additionally, due to the new knowledge we have on how to activate NK cells, how NK cells kill and molecules/cell types that block their activity in the tumor, we can now hypothesize that the most effective therapies involving IL-15 will be of a multi-pronged approach. One Phase I/II trial is attempting to utilize several factors in conjunction with IL-15 to treat metastatic melanoma (NCT 01369888). Here they are giving chemotherapy before injections of tumor infiltrating lymphocytes (TILs) that have been expanded *in vitro*, followed by systemic rhIL-15 administration. This attempts to address the fact that NK cells/CD8 T cells in cancer patients often display decreased functionality, thus by delivering high numbers of lymphocytes that are given the activation signal from the IL-15, more effective tumor cell killing may result (Bauernhofer, Kuss et al. 2003; Kiniwa, Miyahara et al. 2007; Kim and Ahmed 2010;

Mamessier, Sylvain et al. 2011). It is likely that in the near future, especially once the safety trials are completed, we will see a large influx of trials involving IL-15.

It seems probable that it will be possible to combine IL-15 treatment with many NK cell immunotherapies. Here we argue that the application of IL-15 treatment doesn't need to be limited to known NK cell sensitive tumors like AML and melanoma. For example, mAb therapy is now known to be mediated by NK cell ADCC (Clynes, Towers et al. 2000; Weng and Levy 2003). To increase the effectiveness of Herceptin against breast cancer, it could be combined with an autologous or allogeneic NK cell transfer (perhaps activate the NK cells *in vitro* with IL-15/IL-12/IL-18) and then give IL-15 systemically, to maintain the activation state. In a trial such as this, we would overcome the decreased number and poor functionality of NK cells in patients with advanced cancers (Bauernhofer, Kuss et al. 2003; Mamessier, Sylvain et al. 2011). In addition, we could deliver highly activated NK cells that have been shown to maintain their activity *in vivo* (Ni, Miller et al. 2012; Keppel, Yang et al. 2013), and then follow this with IL-15 administration to provide signals to promote their proliferation and survival (Carson, Fehniger et al. 1997; Cooper, Bush et al. 2002). We could expand the NK cells using the new aAPCs expressing mbIL-21 that have been shown to increase telomere length in NK cells and thus prevent their senescence (Fujisaki, Kakuda et al. 2009; Denman, Senyukov et al. 2011). Lastly, we could also target tumor produced molecules such as TGF-β or IDO or immune cells such as MDSC or T regulatory cells that can decrease the immune response generated against a tumor. The possibilities to improve NK cell immunotherapy with IL-15 are many. We are limited, however, by the toxicity of some of these

immunotherapies. Thus far, IL-15 appears to be less toxic in monkeys than either IL-2 or IL-12, but when in combination with other treatments the effects cannot be fully predicted (Berger, Berger et al. 2009; Lugli, Goldman et al. 2010). Another possibility to consider and be wary of is the potential for autoimmunity. As we know, too much IL-15 can contribute to autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease (Di Sabatino, Calarota et al. 2011).

Of course, in all these therapies IL-15 will also act on other immune cells like macrophages and CD4 T cells to promote their anti-tumor effects. The ability of IL-15 to effect CD4 T cell and macrophage polarization is not trivial. Macrophages are key components in the tumor microenvironment and if macrophages are not helping the tumor (M2) they can be harming it (M1) (Martinez, Sica et al. 2008). As we have seen here, macrophage polarization can impact the formation of metastasis and may also contribute to primary tumor formation and metastasis in our spontaneous model. In addition, CD4 T cells polarized to Th2 may promote tumor metastasis, a major cause of mortality.

In the near future, IL-15 may become an important component in immunotherapy against many tumor types. Perhaps the effects of IL-15 on innate immune cells as well as on adaptive immune cells, and IL-15's potent anti-tumor actions have not been fully appreciated. Certainly there are still caveats to the use of IL-15 and several hurdles to get over, but we eagerly await the results of current clinical trials. Basic research still needs to be performed to find the perfect combination of treatments/immunotherapies that will likely involve boosting immune responses against the tumor as well as decreasing

immunosuppression. With the expansion of knowledge regarding NK cell biology, IL-15 biology and the tumor microenvironment, the time is right for the realization of the potential of this technology.

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