

THE ROLE OF INTERLEUKIN-12 ON MODULATING MYELOID-
DERIVED SUPPRESSOR CELLS

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For my beautiful daughter, Lyla Jette Tomaszewski.

Thank you for starting my mornings with a smile.

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Abstract

Catherine E. Steding

The Role of Interleukin-12 on Modulating Myeloid-Derived Suppressor Cells

More than 200,000 American women are diagnosed with breast cancer each year. Although therapies effective in treating metastatic breast cancer currently exist, each year approximately 40,000 women die from this disease. Current evidence indicates that anti-cancer immune responses can be induced by vaccination in situ to the growth of metastasis and protect patients from the tumor recurrence. However, induction of anti-cancer immune responses may be limited in their efficacy due to immune suppression mechanisms induced by the developing cancer. Myeloid-derived suppressor cells are one population of immune regulators comprised of immature cells of myeloid origin with important roles in blocking immune activation and promoting tumor progression. Elimination or maturation of these cells has been found to promote enhanced anti-tumor effects and improve overall survival. This thesis identifies a new role for interleukin-12 as a modulator of myeloid-derived suppressor cell activity. Interleukin-12 was found to promote up-regulation of cell maturation markers on the surface of myeloid-derived suppressor cells with an accompanying decrease in factors responsible for conferring suppressive activity such as nitric oxide synthase 2 and arginase I. The alterations in myeloid-derived suppressor cells were observed following both *in vitro* and *in vivo* treatment with interleukin-12. Further analysis of the anti-tumor efficacy of interleukin-12 revealed that at least part of its suppression of tumor growth can be linked to reductions in myeloid-derived suppressor cell populations in the tumor microenvironment and an influx of active CD8+ T cells into the tumor microenvironment. The findings outlined in this thesis show that interleukin-12 alters the suppressive function of myeloid-derived

suppressor cells leading to significant immune infiltration and activation resulting in increased overall survival and a reduction in metastasis.

Chinghai Kao, Ph.D., Chair

Table of Contents

List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xiii
Introduction	1
Materials and Methods.....	20
Results	26
Discussion.....	87
Future Directions.....	93
References.....	96
Curriculum Vitae	

List of Tables

Table 1	Summary of IL-12R β 1 and IL-12R β 2 expression on the surface of Gr-1/CD11b double positive cells 35
Table 2	Summary of the expression of surface markers after <i>in vitro</i> analysis 45
Table 3	Summary of the expression of surface markers after <i>in vivo</i> analysis 63
Table 4	AdIL-12 alters tumor growth and improves overall survival 76

List of Figures

Figure 1	Effects of IL-12 on MDSC	19
Figure 2	Gr-1/CD11b double positive cells are expanded in the spleens of tumor-bearing animals and are present in the tumors	27
Figure 3	The population of tumor-induced Gr-1/CD11b double positive cells includes both monocytes and PMN	28
Figure 4	Gr-1/CD11b double positive cells derived from C3L5 tumor-bearing C3H/HeJ animals are suppressive MDSC	30
Figure 5	Gr-1/CD11b double positive cells derived from 4T1 tumor-bearing Balb/c animals are suppressive MDSC	31
Figure 6	Expression of IL-12R β 1 on the surface of Gr-1/CD11b double positive cells.....	33
Figure 7	Expression of IL-12R β 2 on the surface of Gr-1/CD11b double positive cells.....	34
Figure 8	IL-12 alters the suppressive activity of C3H/HeJ MDSC.....	37
Figure 9	IL-12 alters the suppressive activity of Balb/c MDSC	38
Figure 10	Surface marker expression of naïve C3H/HeJ Gr-1/CD11b double positive cells does not change following <i>in vitro</i> treatment with IL-12	41
Figure 11	Changes in surface marker expression following <i>in vitro</i> treatment of Gr-1/CD11b double positive cells derived from the spleen and tumor of C3H/HeJ C3L5 tumor-bearing animals	42
Figure 12	Surface marker expression of naïve Balb/c Gr-1/CD11b double positive cells does not change following <i>in vitro</i> treatment with IL-12.....	43
Figure 13	Changes in surface marker expression following <i>in vitro</i> treatment of Gr-1/CD11b double positive cells derived from the spleen and tumor of Balb/c 4T1 tumor-bearing animals with IL-12	44
Figure 14	<i>In vitro</i> treatment with IL-12 reduces Nos2 mRNA expression	47
Figure 15	<i>In vitro</i> treatment with IL-12 reduces IFN- γ mRNA expression.....	48
Figure 16	<i>In vitro</i> treatment with IL-12 reduces Arg1 mRNA expression	50
Figure 17	<i>In vitro</i> treatment with IL-12 reduces IFN- γ protein expression in MDSC.....	52

Figure 18	<i>In vitro</i> treatment with IL-12 reduces IL-10 mRNA expression	54
Figure 19	<i>In vitro</i> treatment with IL-12 reduces IL-10 protein expression from MDSC	55
Figure 20	<i>In vitro</i> treatment with IL-12 induces TNF- α mRNA expression.....	56
Figure 21	<i>In vitro</i> treatment with IL-12 induces TNF- α protein expression from MDSC	57
Figure 22	Serum IL-12 expression 24 hours after injection with AdLuc or AdIL-12	58
Figure 23	<i>In vivo</i> treatment with AdIL-12 results in the up-regulation of surface markers on C3H/HeJ C3L5 tumor-bearing animals spleen- and tumor-derived Gr-1/CD11b double positive cells	60
Figure 24	<i>In vivo</i> treatment with AdIL-12 results in the up-regulation of surface markers on Balb/c 4T1 tumor-bearing animals spleen- and tumor-derived Gr-1/CD11b double positive cells	62
Figure 25	<i>In vivo</i> treatment with AdIL-12 reduces Nos2 mRNA expression	65
Figure 26	<i>In vivo</i> treatment with AdIL-12 reduces IFN- γ mRNA expression....	66
Figure 27	<i>In vivo</i> treatment with AdIL-12 reduces Arg1 mRNA expression	67
Figure 28	<i>In vivo</i> treatment with AdIL-12 reduces IL-10 mRNA expression	68
Figure 29	<i>In vivo</i> treatment with AdIL-12 induces TNF- α mRNA expression...	69
Figure 30	AdIL-12 treatment induces serum expression of IL-12 and IFN- γ ...	71
Figure 31	C3L5 tumor growth following AdLuc and AdIL-12 treatment of C3H/HeJ tumor-bearing animals.....	73
Figure 32	4T1 tumor growth following AdLuc and AdIL-12 treatment of Balb/c tumor-bearing animals	74
Figure 33	Overall survival of C3H/HeJ and Balb/c tumor-bearing animals following treatment with AdLuc and AdIL-12.....	75
Figure 34	C3H/HeJ C3L5 Tumor Histology.....	78
Figure 35	Balb/c 4T1 Tumor Histology.....	79
Figure 36	Gr-1/CD11b double positive cells are suppressed long-term following treatment with AdIL-12.....	81
Figure 37	<i>In vivo</i> treatment with IL-12 increases CD8+ T Cell infiltration into the tumor microenvironment	83

Figure 38	<i>In vivo</i> treatment with IL-12 increases the percentage of CD8+ T cells that also express IFN- γ in the tumor microenvironment.....	84
Figure 39	<i>In vivo</i> treatment with IL-12 decreases the number of metastases..	85
Figure 40	<i>In vivo</i> treatment with IL-12 decreases the number of lung metastases.....	86

List of Abbreviations

ABI	Applied BioSystems Inc
AdIL-12	Adenoviral Vector Expressing Recombinant Mouse Interleukin-12
AdLuc	Adenoviral Vector Expressing Luciferase
APC	Allophycocyanin
ArgI	Arginase I
ATRA	All Trans Retinoic Acid
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA2	Breast Cancer Susceptibility Gene 2
CCL2	Chemokine (C-C Motif) Ligand 2
CCR2	Chemokine (C-C Motif) Receptor 2
CD	Cluster of Differentiation
CD3	Component of the T Cell Receptor
CD4+	T Cell Expressing CD4 (Glycoprotein)
CD8+	T Cell Expressing CD8 (Transmembrane Glycoprotein)
CD28	T Cell Co-Stimulatory Molecule
CD45	Protein Tyrosine Kinase Expressed on All Hematopoietic Cells
CD80 (B7-1)	T Cell Co-Stimulatory Molecule on Antigen Presenting Cells
CD86 (B7-2)	T Cell Co-Stimulatory Molecule on Antigen Presenting Cells
CD11b	α_m Integrin

cDNA	Complementary Deoxyribonucleic Acid
C/EBPbeta	CCAAT Enhancer Binding Protein Beta
CFSE	5-(and 6-)Carboxyfluorexsein Diacetate Succinimidyl Ester
COX-2	Cyclooxygenase 2
CTX	Cyclophosphamide
DC	Dendritic Cells
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
ERBB2	Erythroblastic Leukemia Viral Oncogene Homolog 2
ER	Estrogen Receptor
F4/80	Glycoprotein; Member of Epidermal Growth Factor Transmembrane 7 Family
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
Gr-1	Myeloid Lineage Differentiation Antigen
H & E	Hematoxylin and Eosin Stain
Her2/neu	Human Epidermal Growth Factor Receptor 2

HNSCC	Head and Neck Squamous Cell Cancers
IFN- γ	Interferon- γ
IgG	Immunoglobulin G
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interlukin-10
IL-11	Interleukin-11
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-18	Interleukin-18
IL-23	Interleukin-23
IL-1 β	Interleukin-1 β
IL-4R α	Interleukin-4 Receptor α
IL-12R	Interleukin-12 Receptor
IL-12R β 1	Interleukin-12 Receptor β 1
IL-12R β 2	Interleukin-12 Receptor β 2
IP-10	Interferon-gamma Inducible Protein 10

JAK-STAT	Janus Kinase-Signal Transducers and Activators of Transcription
LPS	Lipopolysaccharide
MDSC	Myeloid-Derived Suppressor Cells
MMP-9	Matrix Metalloproteinase 9
MRI	Magnetic Resonance Imaging
MHCII	Major Histocompatibility Complex Class II
mRNA	Messenger Ribonucleic Acid
NHEJ	Non-Homologous End-Joining
NK	Natural Killer Cells
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
OCT	Optimum Cutting Temperature
PBS	Phospho-Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PECy-7	Tandem of R-Phycoerythrin Conjugated to Cyanine
PGE2	Prostaglandin E2
PgR	Progesterone Receptor
PMN	Polymorphonuclear Cells
RNA	Ribonucleic Acid

ROS	Reactive Oxygen Species
STAT1	Signal Transducer and Activator of Transcription 1
STAT3	Signal Transducer and Activator of Transcription 3
STAT4	Signal Transducer and Activator of Transcription 4
TCR	T Cell Receptor
TGF- β	Transforming Growth Factor β
Th1	T Helper Cell Subset 1
TNF- α	Tumor Necrosis Factor- α

Introduction

Breast Cancer and Clinical Significance

Out of every 1000 women as many as 125 of them will develop breast cancer in their lifetime. More than 200,000 American women are diagnosed with breast cancer each year. The chance of a woman diagnosed with breast cancer succumbing to the disease is 1 in 33 (1). Although the death rates for breast cancer have exhibited a steady decline, predominantly due to improvements in early screening and detection, it remains one of the most diagnosed cancers in women worldwide and the second leading cause of cancer-related death in American women (2). Understanding the nature of breast cancer in terms of both growth and progression is crucial to combating this deadly disease.

Breast cancer is a complex and heterogeneous cancer of the tissues in the breast. As a heterogeneous disease, different morphologies, molecular profiles, and responses to treatments exist. A classification system based on the differences in gene expression profiles reinforces what was found from pathological analysis of tumors and has led to a better definition of individual breast cancers. The classification system has even generated a better understanding of which treatment protocol has the most potential for optimal clinical outcomes for a given type of breast cancer. Better classification of breast cancers also helps in defining the overall prognosis for an individual patient beyond those provided by traditional assessments of tumor grade.

There are several types of breast cancer based on tissue type. The majority of breast cancers are ductal in origin (3). Ductal carcinoma in situ is a cancer that remains confined to the ducts of the breast. In addition to cancers of the ducts of the breast are cancers of the lobules of the breast. Lobular carcinoma *in situ* despite its name is not a cancer but does define an increased risk for developing cancer. In addition to defining the type of cancer based on tissue type, the next stage in pathologic classification defines whether the cancer has remained within the confines of the individual tissue or has spread to adjacent breast tissue and become invasive carcinoma (3). Invasive ductal carcinoma is a cancer that is initiated in the ducts but that has spread through the wall into the breast tissue. Invasive lobular carcinoma is a cancer that starts in the lobules of the breast and spreads to other tissues. A third type of breast cancer based on tissue type is also invasive and is a cancer that involves the ducts, lobules, and

lymphatics of the skin surrounding the breast. This form of breast cancer is called inflammatory breast cancer but is relatively rare.

Although these differences in tissue type are important, the genetic and molecular profile-based classification scheme further defines breast cancer into groups based on differences in gene characteristics. There are four groups of breast cancer based on the molecular profiling: luminal, ERBB2, normal breast, and basal-like (4). High expression of many genes expressed by breast luminal cells is a feature of the luminal group. Of particular importance is the expression of the Estrogen Receptor (ER). ER positive breast cancers are sensitive to the hormone estrogen in that estrogen causes the cancer of this type to grow while ER negative breast cancers do not exhibit increased growth in the presence of estrogen. The second subgroup also expresses ER but at low levels. It is mainly characterized by overexpression of the oncogene ERBB2 or Her2/neu protein and bears that name.

Similar to the second group, basal-like tumors do not express ER and the associated genes. These tumors are also referred to as the triple-negative breast tumors. The reason basal-like breast cancers are often referred to as triple-negative breast cancers is because they lack the expression of ER, the progesterone receptor (PgR), and ERBB2 (Her2/neu protein). These triple-negative breast cancers are often associated with younger age onset, larger tumor sizes, and higher rate of lymph node involvement (4). No matter the type of breast cancer, improvements in early diagnosis as well as developing treatments with enhanced therapeutic efficacy are crucial to continuing the positive trends in death rates and improving not only overall survival but also quality of life for patients.

Risk Factors and Diagnosis

There are several risk factors associated with increased probability of developing breast cancer. Heredity is perhaps the most well defined risk factor studied to date. Inheritance of specific gene mutations in both oncogenes and tumor suppressor genes has been well characterized for many cancers including breast cancer. In fact, there are several inherited mutations associated with increased risk of breast cancer. For example, BRCA1 and BRCA2 are two well characterized breast cancer susceptibility genes (5).

BRCA1 is a tumor suppressor gene with a role in cell cycle surveillance and repair of DNA damage specifically through association with other factors resulting in activation of homologous recombination or non-homologous end-joining (NHEJ) (5, 6). BRCA2 has been found to bind BRCA1 and participate in the DNA damage response particularly in terms of activation of homologous recombination and double-strand break repair (5, 7). Mutations in these genes are variable in distribution but are consistent with a role in hereditary breast cancer. Mutations can cause a loss of function of these genes resulting in a decreased ability of the cell to repair damaged DNA (5, 6). Cells that cannot repair damaged DNA exhibit genomic instability that often involves loss of significant regions of DNA (8). Hereditary breast cancers do differ from sporadic breast cancer in characteristics so understanding the individual risks of inheriting certain genes is crucial. Not only are BRCA1 and BRCA2 markers of hereditary breast cancer risk but they are also important in defining treatment and prognosis.

In addition to family history, age, a history of benign proliferative breast disorders, and endocrine factors are also major contributors to overall breast cancer risk. Recent studies have also linked an increased risk of breast cancer with obesity, diet, alcohol intake, and smoking (9-13). The importance of each of these factors in overall lifetime risk of developing breast cancer is still being evaluated. It is clear thus far that each factor alone is not sufficient to result in breast cancer but combined can lead to substantial risk for developing the disease.

Establishing the family history and overall risk of an individual developing breast cancer is important to determining when to begin screening the individual for cancer. Early detection and diagnosis are crucial to generate improved prognosis and continue the steady decline in deaths. Breast cancer is diagnosed using a combination of tools including: breast exam, mammography, ultrasound, breast magnetic resonance imaging (MRI), and biopsy of suspected lesions (1). In recent years genetic testing has also been a valuable tool especially for defining overall risk in hereditary cancers. Diagnosis via profiling protein markers is currently under investigation and could help in continuing early detection as well as providing a chance for better assessment of risk or even serve as markers for predicting cancer (14). These detection tools combined with a risk assessment are essential to defining the stage, prognosis, and treatment options for a particular patient.

Current Treatments and Therapies for Breast Cancer

Treatment of breast cancer initially involves surgery. Surgical intervention can involve either partial or simple/total mastectomy to remove the affected tissue (1, 3). Based on the surgical options there are two general surgical approaches to treating breast cancer. The first, breast conservation therapy, requires a small enough ratio of tumor size to breast to ensure a successful removal of the entire tumor without significant damage to the appearance of the breast. This form of intervention attempts to eliminate the entire tumor while maintaining the overall appearance of the breast. Follow up to this form of surgery is whole breast irradiation.

The second surgical approach is mastectomy. There are several forms of mastectomy based on patient desires for reconstruction of the breasts. Radical mastectomy involves complete removal of the breast without preservation of muscles. A modified radical mastectomy involves complete removal of the breast but the underlying musculature is not removed. In both cases lymph nodes are removed. An alternative to radical mastectomy, simple mastectomy, still involves removal of the breast; however, in simple mastectomy the lymph nodes are not removed. In addition to these types of mastectomy there are versions that attempt to spare the nipple and areola depending on the desires of the patient in terms of reconstruction. Although many of these specific surgical options are based solely on the desires of the patient in terms of aesthetics, it is important to note that the two general approaches to treating breast cancer do not differ in overall survival (3).

Some breast cancers are advanced at the time of diagnosis and require additional intervention. It is therefore often important to provide adjuvant therapy to patients following surgery. Adjuvant and/or additional treatment options for breast cancer currently involve radiation therapy, hormone therapy and chemotherapy (1). Radiation therapy is widely used to supplement surgical intervention. Although there are multiple types of radiation therapies though external beam radiation, treatment involving a focused beam of radiation from an external source directed at the cancer cells, is the most common. Hormone therapy targets hormone responsive cancers and attempts to lower the hormone levels or block the effects of estrogen. Several drugs are available that target estrogen including aromatase inhibitors and tamoxifen (Nolvadex[®]) (1, 15).

These therapies act to stop the body from making estrogen or block its effects. Additional treatment for both localized and metastatic diseases involves chemotherapy.

Chemotherapy utilizes medications to stop cell growth either through growth arrest or through direct cell killing. There are several chemotherapeutic drugs available with varying degrees of efficacy in breast cancer. These drugs are designed to target a wide range of cellular metabolic processes including but not limited to: microtubule formation, DNA repair, and telomerase activity (16, 17). Although there are significant positive outcomes associated with these therapies several limitations to these types of interventions exist. Chemotherapeutic strategies often have extensive side-effects and there are several instances of cancer developing resistance to the treatments (18, 19).

It is important to note that due to recent advances in early detection, the majority of breast cancers can be cured by surgery; however, 10 to 40 percent of women whose axillary lymph nodes are clear of metastases at the time of surgery will develop distant recurrence (20). Even when surgery is followed by radiation treatment, 10 to 15 percent of women will develop recurrence within 10 years (3, 21). Given the risk of recurrence and issues of metastasis, continued research in treatments for advanced disease are essential. Studies have demonstrated that targeted therapies can be successful at increasing survival for patients. Therapies that target specific proteins such as the oncoprotein *Her-2/neu* or prevent blood vessel formation have promise as potential agents against metastatic disease (1). For example, *Her-2/neu* is found to be amplified in some cancers with an excess of this protein resulting in faster growth and spread of the cancer. A few agents have been developed to combat this increased growth. The monoclonal antibody, Trastuzumab (Herceptin), targets *Her-2/neu* by directly binding the protein and blocking its ability to trigger cancer cells to grow. Although these anti-*Her-2* therapies are effective in treating metastatic diseases for *Her2/neu+* patients, each year, approximately 40,000 women in general still die from this disease making the development of new treatments critical.

It is well known that the tumor and host immune system interact throughout tumor growth and cancer progression. Breast cancer in particular has been shown to be subject to immunosurveillance as evidenced by enhanced survival in patients exhibiting lymphocyte infiltration as well as the observed down-regulation of MHC molecules in up

to 50% of primary tumors and cell lines (22-26). These findings indicate that breast cancer could be treated using factors that promote anti-tumor immunity.

Tumor Immunology

Previous studies have demonstrated that tumor-associated antigens can be used to induce protective anti-tumor immunity capable of eliminating metastatic events, thus preventing or curing disease (27, 28). Additionally, cytokine therapy studies performed in animal tumor models have demonstrated potent therapeutic effects in eliminating tumors (29). However, translation of those studies to clinical practice requires more investigation. Current criticism of clinical immunotherapy includes the inability to effectively overcome self-tolerance as well as failure to adequately stimulate anti-tumor immunity. Nevertheless, β -interferon and interleukin-2 (IL-2) have been used in combination with tamoxifen (Nolvadex[®]) to treat hormone-dependent breast cancer patients with distant metastases and this regimen has demonstrated a survival benefit (30, 31). This observation indicates that anti-cancer immune responses can be induced by vaccination *in situ* to inhibit the growth of metastasis and protect patients from tumor recurrence. Other immune modulators have also been explored and may prove to illicit potent anti-cancer effects against metastatic disease; however, initiation of anti-tumor immunity alone may not suffice. The presence of immunosuppressive cells must also be considered.

Tumor infiltration by immune suppressive cells coupled with T cell non-responsiveness is critical in tumor-associated immune evasion. The presence of cells capable of blocking immune activation both in the tumor and systemically limits the application of therapies meant to promote anti-tumor immunity. The overall immunosuppressive phenotype of the tumor microenvironment limits the efficacy of therapies that target immune activation (32). Understanding the role of suppressive cells in tumor progression and immunotherapy is essential to enhancing the effectiveness of therapies that target immune modulation. This is especially important in studies of cancer vaccination and attempts to promote a cell-type specific response (33). One population of immune modulators capable of suppressing T cell activation of particular interest in recent years is Myeloid-Derived Suppressor Cells (MDSC) (34-37).

Myeloid-Derived Suppressor Cells

MDSC are a population of bone marrow-derived cells that express both the myeloid lineage differentiation antigen, Gr-1, and the α_m integrin, CD11b, and exhibit the ability to suppress antigen-specific T cell activation. Gr-1/CD11b double positive cells represent approximately 20-30% of normal bone marrow cells, 2-4% of nucleated splenocytes, and are nearly absent in lymph nodes of healthy animals. These generic double positive cells differ from MDSC since they lack the ability to inhibit T cell activity (38-41). MDSC have been found to be expanded in a variety of carcinoma patients (42-45). Although some markers such as CD49d and interleukin-4 receptor α (IL-4R α) have been implicated as markers of cells with suppressive function, these markers may not be limited to the suppressive Gr-1/CD11b double positive cells in all mouse models (44, 46).

MDSC are a functionally suppressive, heterogeneous population of Gr-1/CD11b double positive cells composed of polymorphonuclear (PMN) cells and monocytes at early stages of myeloid differentiation. Several subpopulations have been identified; although the ratios of these distinct subpopulations of MDSC vary from strain to strain in the mouse models studied to date, there are unifying themes for MDSC (45, 47-51). All MDSC are of myeloid origin, are cells in an immature state, and exhibit a suppressive phenotype. It is clear from both the literature and the data contained within this dissertation that in order to be classified as an MDSC the cells must exhibit the ability to suppress T cell activation. The findings outlined in this dissertation also further demonstrate that this suppressive function is coupled with significant differences in overall phenotype.

MDSC have been found to accumulate in the spleens, tumors, and lymph nodes of tumor-bearing animals and function to promote tumor progression as well as block immune responses through both antigen-specific and nonspecific mechanisms (52). Recently, MDSC have been isolated from the liver, with this site serving as a potential location for secondary expansion (53, 54). MDSC have a role in the regulation of autoimmune effector cells; however, in tumor progression, these cells act to suppress immune system activation and effectively block anti-tumor responses (55-57). MDSC have even been found to correlate with cancer stage and tumor burden (42, 58). Although MDSC are well characterized in terms of their immunosuppressive properties

and their role in cancer progression, angiogenesis, and metastasis, several other activities for these cells also exist (59).

Recent studies have begun to define a role for MDSC in the following processes: regulation of immune responses during infection, acute and chronic inflammation, traumatic stress, surgical sepsis, chronic contact eczema, kidney allograft tolerance/transplantation, vessel formation post injury to muscle, inflammatory bowel disease, and demyelinating diseases (55, 56, 60-80). Now that both positive and negative roles for MDSC have been found, scientists have a better understanding of the importance of these cells in normal metabolic processes. The regulatory role these cells play in normal processes may be vital to controlling both innate and adaptive immune responses. Induction of these cells during tumor progression may be one of the more significant mechanisms through which tumors avoid challenge by the host immune system.

MDSC Induction and Recruitment

Profound alterations in myelopoiesis are associated with tumor growth. Induction and recruitment of MDSC through the promotion of myeloproliferation is a critical component to tumor progression (81). Factors produced by tumors are capable of triggering myeloid precursors to divide and sustain a level of immaturity associated with MDSC. MDSC accumulation in tumor-bearing animals occurs in response to a multitude of factors, especially those associated with chronic inflammation (56, 82, 83).

Vascular endothelium growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-6 (IL-6), and prostaglandin E2 (PGE2), in particular, have been shown to be inducers of MDSC accumulation with GM-CSF, G-CSF and IL-6 capable of acting on precursor cells in both humans and mice to generate MDSC (39, 71, 84-89). Interleukin-5 (IL-5) was recently discovered to be essential for MDSC recruitment to malignant pleural effusions, even though the actual production of IL-5 was found to be from the host cells rather than the cancer cells themselves (90). The tumor microenvironment promotes MDSC accumulation as evidenced by the production of cytokines and factors that cause their recruitment. Production of interleukin-1 β (IL-1 β) by tumors potently induces MDSC

accumulation as does tumor cell expression of c-kit ligand (86, 91-93). Interferon regulatory factor-8 has been shown to modulate the development of MDSC as well (94). Thus far, all studies indicate that these factors promote MDSC accumulation by promoting MDSC recruitment and proliferation from the bone marrow and myeloid precursors (95-97). It is important to note that stem-cell factor was shown to be vital for the accumulation of MDSC, though how it acts to induce suppressive cells remains to be determined (98).

In addition to the other factors involved in inflammation, the proinflammatory S100 proteins, S100A8 and S100A9, have been shown to have a role in MDSC accumulation (86, 99, 100). The role of these proinflammatory molecules in MDSC accumulation likely involves several mechanisms including direct cell signaling and chemoattraction. In fact, at least one chemoattracting pathway has been identified as having a role in MDSC recruitment: the CCL2/CCR2 pathway has been found to mediate the recruitment of MDSC though much about its involvement remains to be determined (101-103). The role of inflammation in tumor progression through the induction of MDSC is not only indicated by the effects of these proinflammatory cytokines, but also through studies that indicate a correlation between inflammation and the level of MDSC induction. Reductions in inflammation have been found to correlate with reduced MDSC accumulation (104). Though much has been determined in recent years regarding factors that induce MDSC recruitment, the mechanisms and pathways through which these factors promote MDSC proliferation and accumulation are still largely unknown.

Many of the factors that induce MDSC proliferation also have a role in the activity of MDSC after their induction and recruitment to the tumor. For example, MDSC and M2 macrophages (tumor-associated macrophages that exhibit a suppressive phenotype) recruited into the tumor microenvironment, up-regulate COX-2 expression and therefore exhibit enhanced PGE2 metabolism leading to enhanced production of immune modulators by the MDSC (105). This positive feedback mechanism may serve to not only promote the suppressive environment but may also be critical for tumor progression. The mechanisms through which MDSC influence the tumor microenvironment to evade anti-tumor immunity are of particular interest to defining how treatments might affect MDSC activity and immunosuppressive function.

MDSC Activity and Tumor Progression

MDSC have extensive roles in immune regulation and tumor progression. MDSC modulate immune surveillance of the tumor, inhibit the production of interferon-gamma (IFN- γ) by CD8⁺ T cells, induce apoptosis of CD4⁺ T cells, disrupt signaling through the T cell receptor (TCR), block T cell activation, subvert immunity toward a type 2 suppressive response, regulate Th1 immunity, cause T-cell dysfunction, suppress T cell activation and expansion, and induce CD8⁺ T cell tolerance (72, 91, 106-117). These cells can also inhibit natural killer (NK) cell activity and NK cell utilization of IL-2 even in some cancer patients (118-121). There is evidence indicating that MDSC can suppress T cell activity in the tumor draining lymph node though additional experimentation is required to fully understand the roles of these cells in the lymph nodes (122). Although the focus thus far has been on the role these cells play directly on immune activators, there are several other activities exhibited by these cells that are important in cancer research.

In addition to a role in immune regulation, MDSC are incorporated into the vascular endothelium and have been shown to promote tumor vascularization and progression (123-125). One mechanism through which they are known to induce angiogenesis is through the production of matrix metalloproteinase 9 (MMP-9) (126, 127). MDSC have also been shown to be important for tumor metastasis (128, 129). Although several activities have been identified much remains to be determined to further define the diverse roles of MDSC.

There are several known mechanisms through which MDSC exert their suppressive activity including the production of reactive oxygen species, production of nitric oxide, triggering apoptosis of antigen-activated T cells, depletion of L-arginine via production of arginase, and sequestration of cysteine (82, 111, 130-140). An indirect mechanism through which MDSC suppress immune activation also exists. MDSC are capable of inducing T regulatory cell development though this mechanism may be specific to only a subset of animal models or tumor-types (110, 141-143). How these mechanisms are regulated and how that regulation might translate into use as a therapeutic target has been of particular interest in recent years. Treatment of MDSC with LPS and IFN- γ has been shown to activate the suppressive function of these cells implicating IFN- γ in the regulation of MDSC activity (144). Recent studies have also demonstrated that MDSC

suppressive activity is dependent on the C/EBP β transcription factor, which indicates that certain transcription factors may be targets for altering MDSC suppressive function (71). Altering the suppressive activities of MDSC through depletion or differentiation of MDSC has been shown to result in restored T cell activation and stimulation of anti-tumor immune responses making targeting these cells ideal for tumor immunologists (98, 145-150).

Manipulation of MDSC activity stands as promising adjuvant to immunotherapeutic agents (36, 151, 152). The ability of a single-cytokine based therapy to work alone or in combination with an agent that alters MDSC accumulation or function has the potential to exhibit enhanced therapeutic efficacy through both the elimination of immune suppression and the activation of an anti-tumor immune response. Reductions in MDSC levels correlated with the induction of active macrophages but overall the alterations of MDSC had limited effects unless it was coupled with immune activation (40, 145, 147, 153-158). This immune activation was typically via secondary means rather than a consequence of treatment with the primary agent. Studies have shown a reduction in metastasis coinciding with reductions in MDSC number or function, though, once again, these effects were associated with limited alterations in overall tumor growth unless immune activation was also induced (129, 145, 153, 154, 159). Treatment of tumor-bearing animals as well as renal cell carcinoma patients with the tyrosine kinase inhibitor, sunitinib, was found to reverse MDSC accumulation and generate immune activation with significant therapeutic benefits (146, 160-162). Also, reductions in MDSC numbers in patients coupled with immune activation was also observed in a study involving GM-CSF therapy against melanoma (163). These studies imply that optimal therapeutic benefits can be obtained provided that the therapy target both reversing immune suppression and promoting immune activation simultaneously.

Besides reductions in the number of MDSC, inhibition of function has shown promise as a therapeutic target. This was particularly true when peptide inhibitors were used to block transforming growth factor β (TGF- β) (164). Several factors are known to act directly on MDSC to alter their suppressive function; however, the overall therapeutic efficacy was limited (40, 98, 147, 148, 165). All Trans Retinoic Acid (ATRA), for example, was found to differentiate MDSC into mature myeloid cells by neutralizing their production of reactive oxygen species (ROS) (148, 166). However, in another study

involving ATRA, the effects were limited even when administered as a combination therapy with a chemotherapeutic agent, cyclophosphamide (CTX), unless the therapy was administered during an optimal therapeutic timeframe (167). It is therefore of great importance to define the role that agents with proven therapeutic efficacy play in terms of MDSC in order to design improved therapies.

Proinflammatory cytokines have potential as cancer immunotherapy agents, with interleukin-12 (IL-12), in particular, showing significant promise and effectiveness. The role of IL-12 in the tumor microenvironment is diverse and most likely involves the activation of multiple cell types including NK cells, T cells, and macrophages. Although IL-12 has been extensively studied on its own; however, the specific mechanism behind the ability of IL-12 to promote tumor regression, including why differences in treatment efficacy exist, remains to be fully understood. Defining the mechanisms of IL-12 activity and its ability to reverse immune suppression within the tumor microenvironment will continue to be of interest in future studies.

Interleukin-12

IL-12 is a disulfide-linked heterodimeric cytokine with potent anti-tumor activity. The biologically active 74 kDa protein is generated by the association of the p35 light chain and p40 heavy chain subunits. The p35 subunit is structurally similar to IL-6 and G-CSF while the p40 subunit is homologous to the exogenous region of the IL-6 receptor and is shared with interleukin-23 (IL-23) (168-172). The genes encoding mouse p35 and p40 are located on chromosomes 6 and 11, respectively, while in humans, the p35 and p40 genes are located on chromosomes 3 and 5, respectively (172). Given that the genes are located on separate chromosomes, their protein expression is regulated independently from one another with the p40 subunit capable of being produced as a free monomer or homodimer.

IL-12 Production and the IL-12 Receptor

IL-12 is produced by dendritic cells (DC), macrophages, monocytes, neutrophils, and to a lesser extent by B cells (173-176). The p40 subunit is expressed only in phagocytic cells that are also capable of producing the active heterodimer. The p35 subunit,

however, is constitutively and ubiquitously expressed at low levels. Production of the active IL-12 heterodimer occurs through both T cell-mediated and toll-like receptor mechanisms. The T cell-mediated mechanism involves engagement of receptor CD40L on T cells with CD40 on antigen presenting cells thus inducing IL-12 while the other means of production involves Toll-like receptor signaling induced by infection (172, 177-180). Multiple pathogenic organisms and their products, including LPS, induce both the p40 subunit and the active heterodimer.

IL-12 exerts its biological activity through the IL-12 receptor. The IL-12 receptor is composed of two subunits designated $\beta 1$ and $\beta 2$. The subunits are structurally related to the type I cytokine receptor superfamily and are homologous to glycoprotein 130 (gp130), the IL-6 signal transducing chain (181-183). Although expression of one component of the receptor can be sufficient to trigger a response, expression of both subunits is essential for the generation of high affinity binding sites in humans (182). The IL-12R $\beta 1$ subunit associates with the p40 subunit of IL-12 while IL-12R $\beta 2$ associates with the p35 subunit and is the signal transducing chain.

The IL-12R is expressed on activated T cells, NK cells, and DC (172, 184-187). It is important to note that resting T cells do not express the IL-12R; however, the receptor is induced upon T cell activation. This difference in IL-12R expression could explain why only activated T cells can proliferate in response to IL-12. The mechanism of this difference in IL-12 mediated stimulation of activated T cells may involve the T cell receptor (TCR). Signals through the TCR may play a role in instructing the up-regulation of the IL-12R (188). In addition to the differences between activated and resting T cells, Th1 cells express both IL-12R $\beta 1$ and IL-12R $\beta 2$ subunits while Th2 cells only express the IL-12R $\beta 1$ subunit (189, 190). This implicates the $\beta 2$ subunit as a potential determinant of Th phenotype differentiation.

Although there are multiple factors that can induce IL-12 expression, there are also several cytokines that have inhibitory effects including: interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-13 (IL-13), and transforming growth factor- β (TGF- β) (191-193). These cytokines not only inhibit protein secretion but also prevent the accumulation of p35 and p40 mRNA in stimulated antigen presenting cells. The role of IL-4 in blocking IL-12 production is somewhat controversial. Some studies have

demonstrated decreases in IL-12, while others indicated an increase in IL-12 (194-196). IL-4 has been demonstrated to block IL-12R β 2 which could explain the reduction in IL-12 activity as having been the result of IL-12R down-regulation (190, 197, 198). IL-10, on the other hand, is known to inhibit IFN- γ production from T cells and NK cells. The mechanism of this activity appears to be through the inhibition of IL-12 production by accessory cells (191).

Biological Activity of IL-12

IL-12 has several important biological functions. It plays a critical role in the interaction between innate and adaptive immunity. IL-12 has a role in innate immunity through its ability to promote IFN- γ production. IL-12 induces IFN- γ production predominantly in NK cells and both CD4⁺ T cells and CD8⁺ T cells (199). IFN- γ stimulates phagocytic cells promoting their bacteriocidal activity. IL-12 has a role in not only inducing IFN- γ production from cells but also in polarizing naive T cells into T helper cells (Th1). Th1 cells are essential in the response against intracellular pathogens and through the production of IFN- γ and promotion of cell-mediated immunity. In addition to the production of IFN- γ , IL-12 stimulates T cell proliferation and enhances their cytolytic activity but only in activated T cells. This regulation of the adaptive immune response is a major function of IL-12.

It is important to note that the induction of IFN- γ can result in a potent positive feedback loop whereby IL-12 induces IFN- γ that in turn induces macrophages to generate more IL-12 (200). These mechanisms to increase IL-12 production enable IL-12 to enhance the generation and activity of cytotoxic lymphocytes and promote cell-mediated immunity resulting in a potent inflammatory response. IL-12 is also capable of inducing further expression of the IL-12R. Binding of IL-12 to the receptor initiates JAK-STAT signaling (Janus Kinase-Signal Transducers and Activators of Transcription) through the tyrosine residues located on the β 2 subunit (201-204). STAT1, STAT3, and STAT 4 have all been implicated in IL-12 signaling, though STAT4 appears to be the major factor in signal transduction (202-204). In addition to JAK-STAT signaling, IL-12 stimulation can also activate Lck (a Src family protein tyrosine kinase) and p38 (a mitogen-activated protein kinase/MAPK) (205-207).

Consequences of IL-12 Deficiency

Mice deficient in either subunit of IL-12 or the IL-12R share similar phenotypes without obvious developmental abnormalities. Secretion of IFN- γ , NK cytolytic activity, and Th1 differentiation are impaired in these animals, while Th2 development and IL-4 appear to be enhanced (208-210). Mice deficient in the p40 subunit of IL-12 are also deficient in IL-23 and its associated functions, making these mice more immuno-compromised than p35-deficient mice (211). In addition to the loss of IL-12 activity, deficiencies in the IL-12R exhibit a loss of STAT4 phosphorylation. The importance of STAT4 to IL-12 signaling is supported by the analysis of STAT4 knockout animals. These mice exhibit defective IL-12 responses including IFN- γ production and Th1 differentiation (212). Deficiencies in STAT4 also result in impaired long-term CD8⁺ T cell responses (213). Treatment with exogenous IL-12 in some studies cannot reverse the loss of IFN- γ production, particularly in animals deficient in IL-12R. Deficiencies in IL-12 have also been shown to be important in cancer, as indicated by the correlation with greater risk to skin tumors as well as increased chance of malignant progression of the tumors (214, 215).

IL-12 and Cancer Immunotherapy

IL-12 has been studied as a potential anticancer therapeutic agent in many different cancer types including: breast cancer, renal cancer, and prostate cancer (216, 217). The potential for IL-12 in cancer therapeutics is supported not only through past studies of efficacy but also in studies of the tumor microenvironment. Higher levels of intratumoral IL-12 expression in head and neck squamous cell cancers (HNSCC) and ovarian cancer was found to be associated with better survival (218-220). While increased expression is associated with better therapeutic outcomes, reductions in IL-12 expression in the tumor microenvironment have also been observed. MDSC, in particular, have been shown to suppress macrophage production of IL-12 within the tumor microenvironment through production of IL-10 (108). These findings indicate an important role for IL-12 as a potential anti-tumor agent with multiple activities. This importance of the expression of IL-12 in the tumor microenvironment is perhaps best illustrated by studies of the anti-tumor activity of treatment with exogenous IL-12.

Studies in IL-12 have indicated it has the potential to be the most effective single-cytokine therapy capable of inducing solid tumor regression (221). IL-12 has also demonstrated the ability to inhibit the cell growth of leukemia (222, 223). The ability of IL-12 to affect multiple tumor types makes it an ideal therapeutic agent for additional studies. In particular, IL-12 has significant promise as a treatment for breast cancer. Generating high localized levels of IL-12 has been shown to result in tumor regression in as many as 75% of treated murine breast tumors with 30% of animals exhibiting complete tumor regression (224). Complete tumor regression is also associated with immunity to subsequent challenge with fresh tumor cells. Several other studies utilizing gene therapy to induce tolerable levels of IL-12 have shown consistent reductions in tumor growth (216, 225-231).

While therapies utilizing IL-12 alone have shown significant promise, IL-12 has also been studied in combination with chemotherapeutic agents as well as other proinflammatory cytokines. Studies of IL-12 in combination with Paclitaxel showed improved therapeutic efficacy including a reduction in metastasis without increased toxicity (232, 233). Combination of the chemokine fractalkine with IL-12 increased the activation of tumor infiltrating lymphocytes compared to fractalkine alone resulting in enhanced anti-tumor responses (234). IL-12 has also been tested with cyclophosphamide, IL-2, interleukin-15 (IL-15), interleukin-18 (IL-18), caspase 3, and IFN- γ inducible protein 10 (235-241). These studies indicated a variety of therapeutic results including enhanced IFN- γ expression and improved CD8⁺ T cell activation.

While the specific mechanisms associated with positive clinical outcomes remain undefined, several studies have illuminated the role of IL-12 in both activating the immune system within the tumor microenvironment and fundamentally altering the tumor microenvironment itself. IL-12 is known to increase lymphocyte infiltration, inhibit tumor angiogenesis, and protect against metastasis (242-245). Rejection of metastatic disease has been associated with therapies that both promote immune responses and reverse immune suppression (246). IL-12 has proven to be one such factor. It is probable that the combination of IL-12 anti-tumor activity combined with the induction of active T cells results in the significant reductions in tumor growth and metastasis.

IL-12 not only increases lymphocyte infiltration it can also activate CD4+ Th1 cells, CD4+ Effector/Memory T cells, and CD8+ Cytotoxic T cells within the tumor microenvironment leading to potent antitumor effects (247-251). The effects of IL-12 on CD8+ T cells in particular are interesting in terms of cancer therapeutics. IL-12 has been shown to enhance CD8+ T cell cytotoxicity generating T cell-mediated antigen-specific antitumor activity (247, 252). CD8+ T cell based immunotherapies have even been applied in combination with IL-12 as a means to enhance the overall induction of endogenous anti-tumor responses (247, 253). IL-12 not only promotes immune activation but is also known to be capable of acting directly to reverse immune suppression. It accomplishes this through either the activation of T cells even in the presence of suppressive T regulatory cells or through inducing suppressor cell apoptosis (248, 254). In addition to these indirect mechanisms of antitumor activity, IL-12 is also capable of inducing direct tumor cell apoptosis through the induction of Fas and Fas Ligand (FasL) (255). Although IFN- γ is essential to promoting many of the activities of IL-12, some of the antitumor effects of IL-12 are IFN- γ independent (235, 243). For example, IL-12 has a role in macrophage polarization and can alter the functional profile of both tumor-associated and tumor-infiltrating macrophages turning suppressive macrophages into inflammatory macrophages (256, 257). Overall, the ability of IL-12 to generate a transition in the tumor microenvironment from a suppressive environment to an activating one is essential to the overall effectiveness of IL-12-based therapies.

Despite all of the benefits to IL-12 based therapies there are limitations. One limitation often associated with recombinant cytokines such as IL-12 is systemic toxicity. Systemic expression or delivery of IL-12 indicated a risk of toxicity to the liver and peripheral organs; however, Phase I and Phase II clinical trials have shown that the concentration of IL-12 used and the treatment regimen may have been the critical factors in the observed toxicity (224, 258, 259). In addition to following careful regimens while giving the therapy, limiting the systemic expression of IL-12 to the tumor microenvironment could abrogate the potential toxic effects of the cytokine, though if levels remain below tolerable doses, this is an unnecessary precaution. Several studies of IL-12 in murine models have demonstrated limited toxicity at levels of IL-12 capable of inducing significant therapeutic effects particularly when IL-12 is administered via recombinant adenovirus (224, 225, 258, 260).

Adenovirus and Its Therapeutic Applications with IL-12

Direct delivery of a gene expression cassette into a target cell by a viral vector is the ideal means through which expression of the gene can target a specific cell and achieve enhanced therapeutic efficacy with limited toxicity. To date, the most commonly used virus for human gene therapy clinical trials is adenovirus. Adenovirus is a double-stranded DNA, non-enveloped, icosahedral virus. It stands as a potential gene therapy agent due to its safety, gene capacity, ability to infect multiple human cell types, and ability to infect cells regardless of cell cycle status. In terms of generating therapeutic vectors, adenovirus is also an attractive virus in that it is easily manipulated, can generate high titers, and is relatively inexpensive to generate.

Adenovirus has been used as a vector to express many different cytokines to be utilized as cancer therapies. Of interest in these studies is the use of adenovirus to express IL-12. In situ expression of IL-12 via adenoviral vectors has the potential to induce a potent anti-tumor immune response. Several studies indicated that adenovirus containing IL-12 alone could eliminate an established tumor via intra-tumor injection of the virus. Bramson et al. treated 36 tumors with one single intra-tumor injection of adenovirus containing IL-12 and observed that 11 completely regressed (31%) and 17 partially regressed (224). Animals with complete tumor regression had protective immunity against re-challenging with fresh tumor cells. High levels of IL-12 expression were observed both in tumors (600-800 ng per tumor) and serum (40 to 60 ng/ml) 24 hours after virus injection without apparent systemic toxicity (224). However, this study only analyzed IL-12 expression in 2 animals from each treatment group, and did not attempt to correlate IL-12 expression with the therapeutic effect nor explain why some animals exhibited a complete response and others only partially responded or failed to respond. Another study performed by Gambotto et al. demonstrated moderate levels of serum IL-12 expression (8 ng/ml) via intra-tumor injection of adenovirus containing IL-12, resulting in eliminating 80% of treated tumors (261). Chen et al. was able to eliminate all but one tumor with intra-tumor injection of adenovirus containing IL-12; however, they did not report the concentration of IL-12 in the serum (262). In contrast, the majority of studies indicated that adenovirus-mediated IL-12 was not very effective in eradicating a tumor burden by itself, and required cooperation with another immune modulator, such as CD137 (4-1BB) (263), CD80 (B7.1) (264), or GM-CSF (265). Most of those studies either did not investigate serum levels of IL-12 expressed by the treatment or observed low

serum levels (0.6 to 2.5 ng/ml) and did not correlate those serum levels with the therapeutic effect (260, 266).

Dissertation Overview

The role of tumor associated immune regulation through the presence of immunosuppressive cells is relatively well established. However, the effects of IL-12 on immunosuppressive cells such as MDSC remain to be fully understood. I hypothesized that IL-12 is capable of reversing the suppressive effects of MDSC while simultaneously promoting immune activation. The first aim of this dissertation was to identify whether IL-12 acts directly on MDSC to alter their suppressive function. The second aim of this dissertation was to characterize the effect of IL-12 on MDSC and the mechanism of IL-12 activity. The third aim of this dissertation was to determine the therapeutic efficacy of treatment with AdIL-12 and determine a potential role for the IL-12 effect on MDSC as a contributing factor to the therapeutic efficacy of the treatment. The findings are summarized in figure 1.

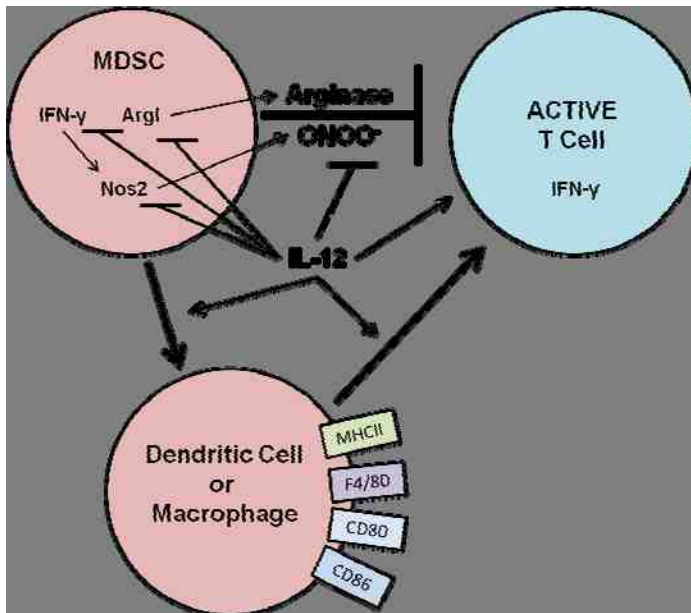


Figure 1. *Effects of IL-12 on MDSC.* MDSC are known to alter T cell activity through induction of apoptosis or T cell anergy. MDSC exert this activity through the production of reactive nitrogen species and arginase. I hypothesized that IL-12 can act to alter the production of IFN-γ, Nos2, and Arg1 thus altering the activity of the MDSC. In addition, I hypothesized that IL-12 promotes MDSC maturation.

Materials and Methods

Mice and Cell Culture

C3H/HeJ and Balb/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Murine breast cancer cell lines C3L5 (Dr. P. K. Lala, The University of Western Ontario, Canada) and 4T1 (ATCC, Manassas, VA) were maintained in RPMI 1640 and DMEM, respectively. All media was supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Cells were fed fresh growth media three times per week and maintained at 37°C in a 5% CO₂ incubator.

Generation of Gr-1/CD11b Double Positive Cells for Analysis

2.5×10^5 C3L5 cells and 1×10^5 4T1 cells were injected into the mammary fat pads of C3H/HeJ and Balb/c mice, respectively, to induce mammary tumors. Gr-1/CD11b double positive cells were found to expand in the tumors and spleens. Animals were sacrificed once tumors reached a size of 500 mm³. Tumor specimens were surgically removed and processed by cutting into small pieces and digested with 5 U/ml collagenase (Roche, Germany) and 5 µg/ml DNase I in RPMI 1640 (10% FBS, 1% Penicillin/Streptomycin) at 37°C for 1.5 hours. The resulting mixture was filtered through 40 µm nylon mesh. The cells were washed with fresh RPMI and red blood cells (RBC) were lysed via culture in 1X ACK/RBC lysis buffer at room temperature for 3 minutes. Cells were washed and maintained in RPMI 1640 (10% FBS, 1% Penicillin/Streptomycin). Spleens were also surgically removed, mashed, and RBC were lysed using the same process as with the digested tumor.

Isolation of Gr-1/CD11b Double Positive Cells

Whole splenocytes and cells from the digested tumors were stained using standard protocols with fluorochrome-conjugated antibodies, anti-Gr-1 (BD Bioscience, San Diego, CA) and anti-CD11b (BD Bioscience). Briefly, cells were washed with 1X PBS. Antibody was added at a concentration of 2 µg/ml of fluorochrome-conjugated antibody in 1X PBS with 1% bovine serum albumin (BSA) or 2 µg/ml mouse IgG (blocking agents; BD Bioscience) for 20 minutes at 4°C (BD Bioscience). Cells were washed with 1X PBS,

resuspended in RPMI supplemented with 1% FBS and 1% Penicillin/streptomycin, and sorted via flow cytometry at the Flow Cytometry Core Facility (IUPUI, Indianapolis, IN).

Analysis of the expression of both subunits of the IL-12R

Sorted GR-1/CD11b double positive cells along with whole splenocytes and digested tumor cells were stained with fluorochrome-conjugated anti-IL-12R β 1 antibody and a combination of anti-IL-12R β 2 primary antibody with fluorochrome-conjugated secondary antibody (BD Bioscience) in combination with fluorochrome-conjugated anti-Gr-1 and anti-CD11b antibodies. Each antibody was used at a concentration of 2 μ g/ml. Cells were blocked with 1% BSA or 2 μ g/ml mouse IgG (BD Bioscience). NK cells were stained with 2 μ g/ml fluorochrome-conjugated pan NK cell marker clone DX5 antibody (eBioscience, San Diego, CA), fluorochrome-conjugated anti-IL-12R β 1 antibody, and the combination of 2 μ g/ml anti-IL-12R β 2 primary antibody and 2 μ g/ml fluorochrome-conjugated secondary antibody as a positive control. Naïve CD4⁺ T cells were stained with 2 μ g/ml fluorochrome-conjugated anti-CD4 antibody (eBioscience), 2 μ g/ml fluorochrome-conjugated anti-IL-12R β 1 antibody, and the combination of 2 μ g/ml anti-IL-12R β 2 primary antibody and 2 μ g/ml fluorochrome-conjugated secondary antibody as a negative control. Analysis of the expression of the IL-12R was performed via flow cytometric analysis using a FACScalibur (Becton Dickinson, Mountain View, CA).

5-(and 6)-Carboxyfluorexsein diacetate succinimidyl ester(CFSE) labeling of cells

Cells were labeled with CFSE according to manufacturer's protocols (Cell Trace CFSE Cell Proliferation Kit, Invitrogen, Eugene, OR). Briefly, CFSE is diluted in DMSO at a stock concentration of 5 mM which is freshly diluted to a working concentration of 10 μ M. Whole splenocytes were suspended at a concentration of 1 x 10⁷ cells/ml in PBS containing 0.1% BSA and combined with the 10 μ M working concentration of CFSE. Following a 10 minute incubation period at 37°C, the CFSE labeling was stopped by adding 5 times volume of ice cold RPMI (10% FBS, 1% penicillin/streptomycin) and incubating on ice for 5 minutes. The CFSE labeled cells were washed 3 times with ice cold fresh RPMI (10% FBS, 1% penicillin/streptomycin) and resuspended to the desired concentration.

In vitro stimulation of T cells

CFSE labeled cells were plated in 96-well plates at a density of 1×10^6 cells/ml in RPMI (10%FBS, 1% penicillin/streptomycin) and incubated with or without 5 μ g/ml soluble anti-CD3 (eBioscience) and anti-CD28 (BD Bioscience) antibodies alone or in combination with sorted Gr-1/CD11b double positive cells in a 1:1 ratio for a total cell density per well of 2×10^5 cells. For analysis of the effect of IL-12, labeled cells and sorted Gr-1/CD11b double positive cells were incubated with 10 ng/ml recombinant IL-12 separately for 24 hours at 37°C, washed, and then co-cultured. Cells were incubated for 4 days at 37°C. After this incubation period, cells were harvested and labeled with fluorochrome-conjugated anti-Gr-1 antibody for identification and elimination of the Gr-1+/CD11b+ subset using a FACScalibur (Becton Dickinson). A small sample of cells was also labeled with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies (eBioscience) to identify the profile of lymphocytes for gating and analysis of lymphocyte activation using a FACScalibur (Becton Dickinson). One hundred thousand total events from triplicate wells were collected and analyzed for lymphocyte activation based on loss of CFSE intensity.

Analysis of changes in surface markers

Sorted Gr-1/CD11b double positive cells from C3H/HeJ and Balb/c naïve spleens, tumor-bearing spleens (C3L5 and 4T1 respectively), and digested tumor were incubated with or without 10 ng/ml recombinant IL-12 for 24 hours. Cells were stained with antibodies at a concentration of 2 μ g/ml in 1X PBS containing 1% BSA or 2 μ g/ml mouse IgG for 20 minutes at 4°C. Cells were washed with 1X PBS and fixed in 1% paraformaldehyde. Antibodies were all fluorochrome-conjugated and included anti-CD86, anti-CD80, anti-F4/80, and anti-MHCII antibodies (eBioscience). Analysis for expression of these markers compared to whole splenocytes positive controls and CD4+ T lymphocytes negative controls was performed via flow cytometry using the FACScalibur (Becton Dickinson).

Real Time Polymerase Chain Reaction (PCR)

Sorted Gr-1/CD11b double positive cells from naïve and tumor-bearing animals were treated for 24 hours with 10 ng/ml IL-12. Cells were lysed and RNA was extracted using

the RNeasy kit (E.Z.N.A. homogenizing columns and E.Z.N.A. RNA Extraction Kit, Quanta Biosciences Inc., Gaithersburg, MD). RNA was converted to cDNA via PCR reaction with the qScript cDNA Supermix (Quanta Biosciences Inc., Gaithersburg, MD). The RT step protocol was performed on the PTC-100 (MJ Research Inc., Gaithersburg, MD) thermocycler and involved the following 3 steps: 5 min at 25°C, 30 min at 42°C, and 5 minutes at 85°C. Taqman primer probes against Nos2, IFN- γ , Arg1, IL-10, and tumor necrosis factor (TNF) were purchased from Applied Biosystems (ABI, Carlsbad, CA). The concentration of RNA was obtained via analysis of OD260 versus OD280 readings and cDNA was diluted in EB buffer so that total concentrations were equal for all samples. Using the PCR master mix also obtained from ABI, semi-qualitative real time PCR was performed (ABI). The real time PCR was performed on the ABI 7500 Real Time PCR Machine using the following protocol: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C followed by 1 minute at 65°C (data recorded during this 65°C stage).

Generation of Recombinant Adenovirus

Adenovirus vectors were generated using methodology previously described though modified to contain the desired genes and without prostate specificity (267). Adenovirus containing a luciferase expression cassette (AdLuc) or a recombinant IL-12 expression cassette (AdIL-12) was used as a control for virus effect and to generate IL-12, respectively. Briefly, plasmids containing IL-12 or luciferase under control of CMV promoters are packaged into virus using packaging cells. Virus is then amplified and purified prior to use.

Enzyme-linked immunosorbent assay (ELISA)

Serum from AdLuc or AdIL-12 treated animals was harvested for analysis of IL-12 and IFN- γ . Sorted Gr-1/CD11b double positive cells were treated with 10ng/ml IL-12 for 24 hours. Media was harvested for the analysis of protein concentration with and without IL-12 treatment. Concentrations of proteins were determined via ELISA kits for IL-12, IFN- γ , IL-10 and TNF- α (BD Bioscience). Briefly, ELISA plates are coated with a capture antibody overnight. The capture antibody is blocked for 1 hour with assay diluent (1X PBS with 10% FBS), washed with wash buffer (1X PBS with 0.05% Tween-20), and then incubated with samples and standards for 2 hours. Wells are washed and treated with

detection antibody combined with streptavidin-horseradish peroxidase for 1 hour. Cells are washed again and treated with substrate solution (BD Bioscience). The reaction is stopped with 2N H₂SO₄ and read using the plate reader at 450nm.

In vivo Analysis of IL-12 Effect on MDSC

Tumors were generated as described previously and allowed to grow to roughly 300 mm³ in volume. Prior to treatment, serum was harvested to establish baseline levels of IL-12 in the tumor-bearing animals. 1 x 10⁹ adenovirus particles suspended in 40 µl of 1X PBS were injected intramuscularly into the animals. Twenty-four hours after treatment, serum, tumors, and spleens were harvested for further analysis. Serum was used to demonstrate IL-12 production while digested tumors and spleens were analyzed for changes in the Gr-1/CD11b double positive cells as described for the *in vitro* experiments.

In Vivo Treatment of Established Tumors

Tumors were also allowed to grow to approximately 65 mm³ as described previously and treated with intramuscular injections of 1 x 10⁹ adenovirus particles suspended in 40 µl 1 x PBS of either AdIL-12 or AdLuc (268, 269). Tumors were measured twice weekly using calipers until tumors grew to approximately 500 mm³. Animals were humanely sacrificed and tissues were harvested for further analysis as described previously. Frozen sections of tumor were prepared. Tumors were analyzed for histology via hematoxylin and eosin (H&E) staining as well as for changes in the Gr-1/CD11b double positive cell populations via flow cytometry. Single cell suspensions of tumors were stained with anti-Gr-1, anti-CD11b, anti-CD8, anti-IFN-γ, and anti-CD45 (BD Biosciences) antibodies and analyzed via flow cytometry. Lungs were stained in Bouin's Fixative (LabChem Inc., Pittsburg, PA) and metastases counted.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as a *p* value < 0.05 with

the actual p values indicated where appropriate. Analysis was performed on at least 3 individual experiments as indicated.

Results

Gr-1/CD11b double positive cells are expanded in the tumors and spleens of tumor-bearing animals and vary in morphology

Tumor progression promotes an increase in Gr-1/CD11b double positive cells in all murine breast cancer models studied to date; however, the type of cells induced during tumor progression has been found to vary based on animal strain and tumor type (45-52). Differences in the composition of the cells can factor into the overall activity and response to treatment. Gr-1/CD11b double positive cells can be either monocytic or PMN in nature though in both instances functionally suppressive Gr-1/CD11b double positive cells (MDSC) are immature versions of these cell types. Analysis of the Gr-1/CD11b double positive cells in the animal models of interest is essential to generating a complete understanding of differences in response to treatment that may occur.

Although it has been determined that Gr-1/CD11b double positive cells are expanded in the tumors and spleens following tumor progression for the murine models studied thus far, it was necessary to confirm this in the murine models of interest for these studies as well as for our experimental conditions. C3H/HeJ and Balb/c mice were selected as models for this study due to their potential for differences in the composition of Gr-1/CD11b double positive populations. C3L5 and 4T1 murine cell lines were injected into the mammary fat pads of C3H/HeJ and Balb/c animals, respectively. Figure 2 demonstrates that these Gr-1/CD11b double positive cells are expanded in the spleens and can be found in the tumors of both models following tumor development. The expression of Gr-1/CD11b double positive cells in the spleens of animals bearing tumors averaging approximately 500 mm³ in volume significantly increases to an average of 20% of all splenocytes in C3H/HeJ animals bearing C3L5 tumors and 25% in Balb/c animals bearing 4T1 tumors compared to less than 5% in naïve animals of both strains. In terms of the concentration of Gr-1/CD11b double positive cells in the tumor, an average of approximately 12% of the entire tumor was observed to be Gr-1/CD11b double positive cells. In addition to the slight differences in levels of induction, the composition of the Gr-1/CD11b double positive cells in C3H/HeJ animals was found to differ in percentages of cell type compared to from those isolated from Balb/c animals (Figure 3).

Figure 3 represents the findings regarding the morphology of Gr-1/CD11b double positive cells. C3H/HeJ-derived Gr-1/CD11b double positive cells were found to be predominantly monocytes while Gr-1/CD11b double positive cells from Balb/c animals were found to be composed of a ratio of approximately 2:3 PMN to monocytes. By selecting two models that differ in MDSC composition, comparisons in responsiveness to the same treatment can be performed, enabling a better overall understanding of the nature of functionally suppressive Gr-1/CD11b double positive cells.

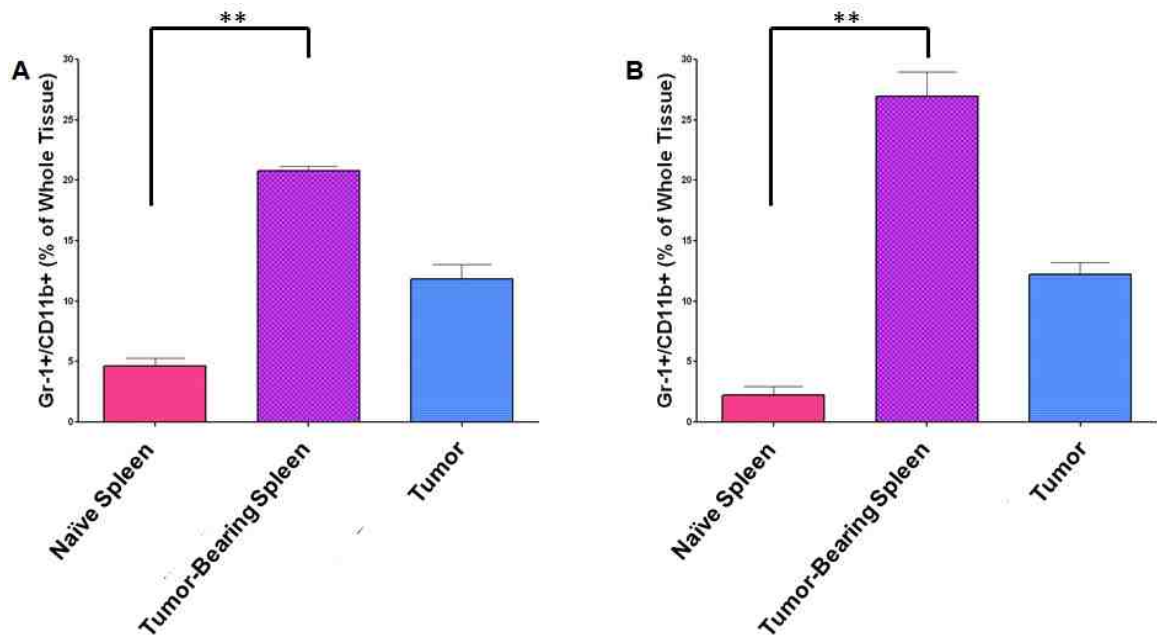


Figure 2. *Gr-1/CD11b* double positive cells are expanded in the spleens of tumor-bearing animals and are present in tumors. C3H/HeJ (A) and Balb/c (B) animals were injected with 2.5×10^5 C3L5 and 1×10^5 4T1 cells, respectively. Tumors were allowed to grow to 500 mm^3 in volume and tissues were harvested and single-cell suspensions obtained. Cells were stained with fluorochrome-conjugated anti-Gr-1 and anti-CD11b antibodies at a concentration of $2 \mu\text{g/ml}$. Cells were then analyzed for Gr-1/CD11b double positive cell populations in tumors and spleens as compared to spleens of naive animals from both strains via flow cytometry. The concentration of Gr-1/CD11b double positive cells was determined as a percentage of the whole tissue analyzed. Graphs represent percentages of cells obtained from 10 animals per group ($n = 10$; $**p < 0.01$).

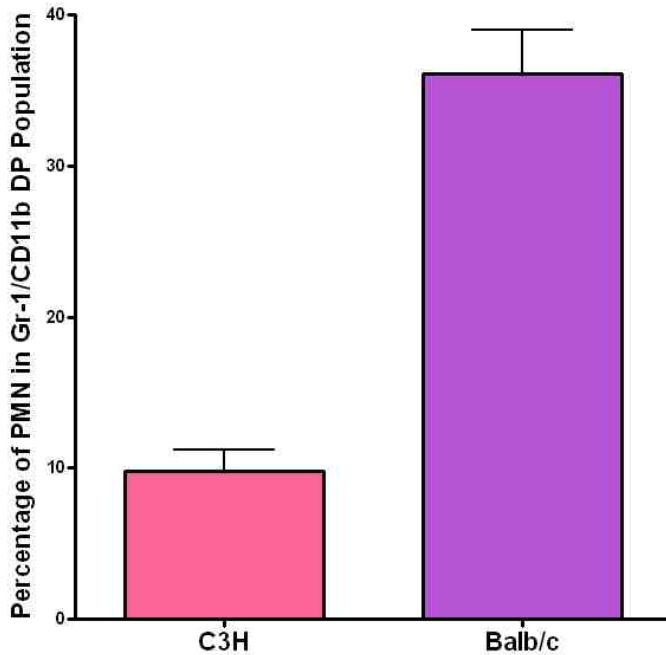


Figure 3. *The population of tumor-induced Gr-1/CD11b double positive cells includes both monocytes and PMNs. Morphological analysis of Gr-1/CD11b double positive cells from tumor bearing animals was performed. Single cell suspensions from the spleens of tumor-bearing animals were stained with fluorochrome-conjugated anti-Gr-1 and anti-CD11b antibodies and sorted for*

double positive cells via flow cytometry. Cells were placed onto slides via the cytopsin procedure and stained using the Diff Quick staining protocol. Analysis was performed via microscope analysis of cell morphology on triplicate slides (n = 3). The graph represents the percent of PMN cells.

Gr-1/CD11b double positive cells from tumor bearing animals are functionally suppressive MDSC

In order to assess the nature of Gr-1/CD11b double positive cells in terms of their suppressive activity, the ability of these cells to suppress CD3-mediated T cell activation was defined. CD3 is a component of the TCR and suppression of this type of signaling can be used to demonstrate an ability to suppress T cell responses. Anti-CD3 antibody can bind to the TCR/CD3 complex and induce cell signaling resulting in T cell activation in the same manner as antigen presentation from antigen-presenting cells (APC). When stimulation through the TCR is combined with stimulation through the co-receptor optimal activation can be observed. Using this principle, cells were treated with a combination of anti-CD3 and anti-CD28 antibodies and analyzed via T cell activation assays.

T cell activation assays measure the amount of activation by demonstrating a change in CFSE concentration as cellular division occurs. CFSE is a cytoplasmic dye that is equally divided along with the cytoplasm during cell division. As a T cell is stimulated, it is signaled to proliferate and this proliferation can be measured by a dilution of the CFSE cytoplasmic stain with each division. Suppression of T cell activation was determined via flow cytometric analysis of CFSE dilution following treatment of cells with anti-CD3 and anti-CD28 antibodies for 4 days.

Gr-1/CD11b double positive cells were stained with fluorochrome-conjugated antibodies and isolated from the spleens of naïve animals as well as the tumors and spleens of tumor-bearing animals. These cells were then cultured with CFSE-stained whole splenocytes at a 1:1 ratio. Results for C3H/HeJ spleen- and tumor-derived Gr-1/Cd11b double positive cells are shown in figure 4 while Balb/c spleen- and tumor-derived cells are shown in figure 5.

As figures 4 and 5 demonstrate, co-culture of T cells with Gr-1/CD11b double positive cells at a 1:1 ratio resulted in suppression of T cell activation but this was dependent on the origin of the Gr-1/CD11b cells. The figures are representative of 3 individual experiments of cells assayed in triplicate. Gr-1/CD11b double positive cells from tumor-bearing animals significantly suppressed T cell activation regardless of whether they were harvested from the spleen or tumor ($p < 0.01$). Percent activation from the positive controls was found to involve up to 60 percent of the total CD4⁺ T cell population. Co-culture with MDSC was found to suppress this activation to as little as 7 percent of the CD4⁺ T cell population. It is essential to note that Gr-1/CD11b double positive cells from naïve animals were not capable of significantly suppressing T cell activation ($p > 0.05$). Although there does appear to be low levels of suppression, the levels of activation for the CD4⁺ T cells remain within the range of activation levels observed in the positive control treatment group. This difference in ability to suppress T cell activity supports the concept that critical differences exist between functional MDSC and cells that are merely Gr-1/CD11b double positive cells. It also demonstrates that the method of study and analysis outlined in this dissertation does not cause the Gr-1/CD11b double positive cells to become suppressive. Gr-1/CD11b double positive cells derived from naïve animals are not suppressive; therefore, suppressive activity is a factor of a specific subtype of Gr-1/CD11b double positive cells, not a factor of the experimental conditions.

Functionally suppressive Gr-1/CD11b double positive cells will be described as MDSC for the remainder of the dissertation.

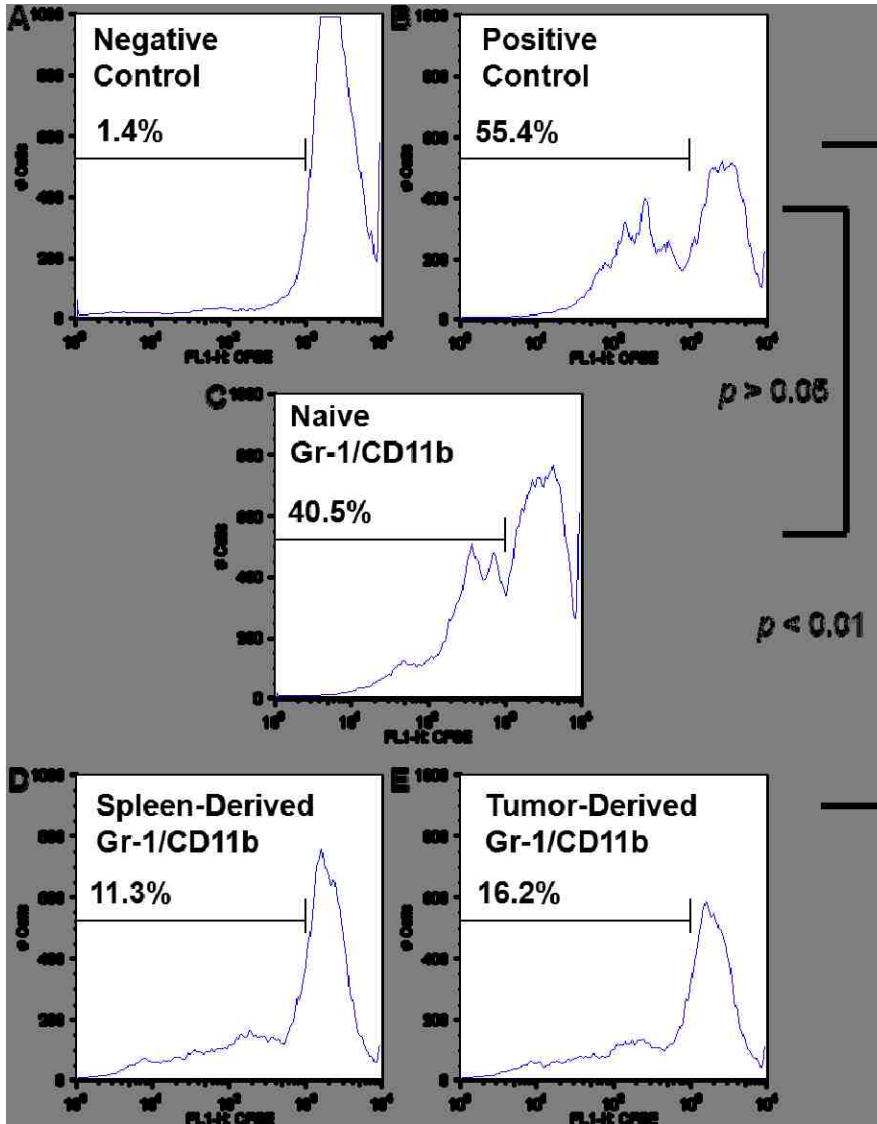


Figure 4. *Gr-1/CD11b* double positive cells derived from C3L5 tumor-bearing C3H/HeJ animals are suppressive MDSC. Whole splenocytes from C3H/HeJ animals were stained with 10 μ M CFSE and co-cultured with sorted Gr-1/CD11b double positive cells at a 1:1 ratio for a total of 2×10^5 cells per well. Cells were treated with 5 μ g/ml anti-CD3

and anti-CD28 antibodies in 96 well plates for 4 days. The cells were harvested and stained with anti-Gr-1 and anti-CD4 antibodies to isolate only CD4+ T cells for analysis. Cells were gated for CD4+ T cells only and analyzed for dilution of CFSE via flow cytometry. A negative control of unstimulated splenocytes (A) and a positive control of stimulated splenocytes only (B) were compared to splenocytes activated in the presence of Gr-1/CD11b double positive cells from the spleen of naïve animals (C) and Gr-1/CD11b double positive cells from the spleen (D) and tumor (E) of tumor-bearing

animals. Graphs are representative of three independent experiments. Percentages indicate percent activation.

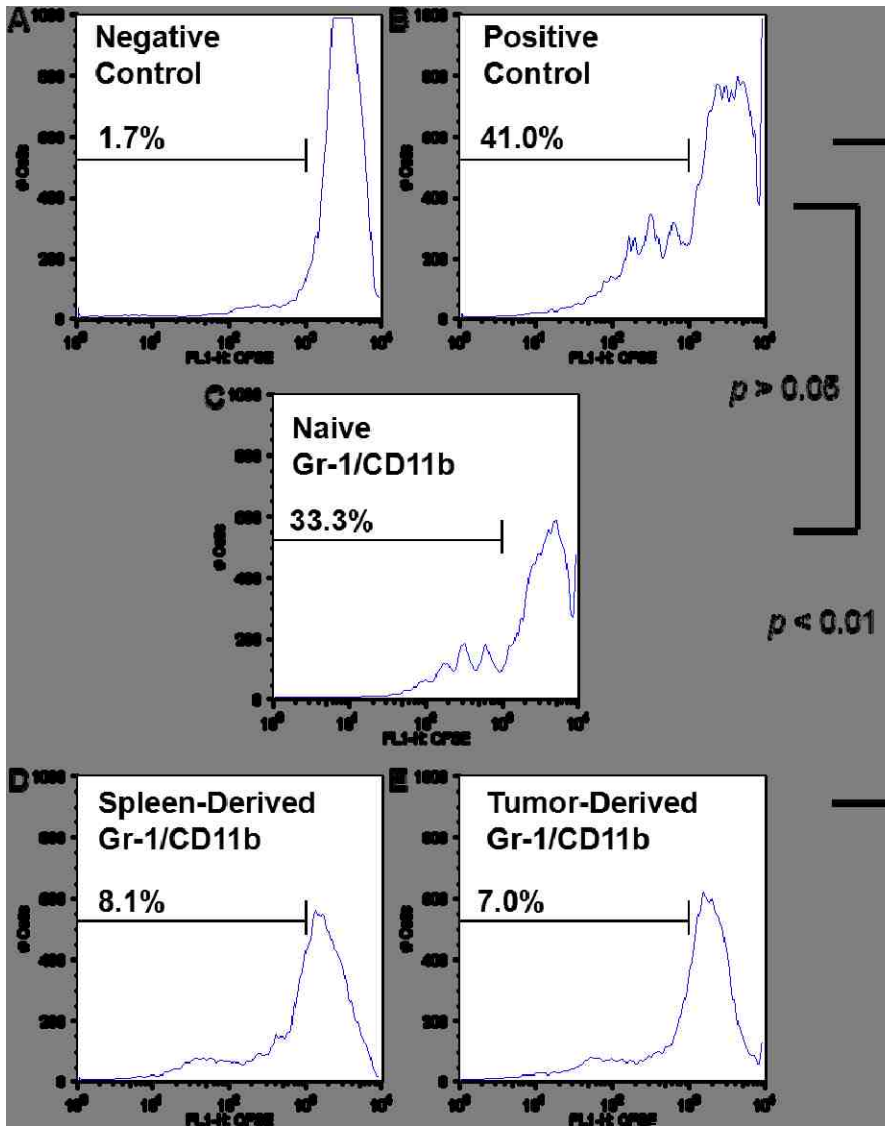


Figure 5. *Gr-1/CD11b double positive cells derived from 4T1 tumor-bearing Balb/c animals are suppressive MDSC. Whole splenocytes from Balb/c animals were stained and cultured as described previously (Figure 4). A negative control of unstimulated splenocytes (A) and a positive control of stimulated*

splenocytes only (B) were compared to splenocytes activated in the presence of Gr-1/CD11b double positive cells from the spleen of naïve animals (C) and Gr-1/CD11b double positive cells from the spleen (D) and tumor (E) of tumor-bearing animals.

Graphs are representative of three independent experiments. Percentages indicate percent activation.

MDSC express IL-12R β 1 and IL-12R β 2

Treatment of MDSC with factors known to alter their suppressive activity has shown some promise in terms of therapeutic efficacy (40, 145, 147, 153-158). As discussed previously, several cytokines have been shown to alter MDSC activity but with limited therapeutic effect (154-157). The effects of IL-12 on MDSC activity remains to be defined. IL-12 is of particular interest in tumor studies due to its demonstrated efficacy and potential to activate anti-tumor immune responses. In order to determine whether IL-12 could impact MDSC directly rather than through the activation of secondary cells, I first determined whether these cells exhibit the necessary receptor for IL-12-mediated activity.

Using fluorochrome-conjugated antibodies, I tested whether Gr-1/CD11b double positive cells express the IL-12 receptor. NK cells stained with NK marker antibodies, NK1.1 or NK clone DX5, served as a positive control for IL-12R expression while naïve, unstimulated CD4+ T cells served as a negative control. I determined whether Gr-1/CD11b double positive cells from naïve animals as well as MDSC express either or both of the IL-12R β 1 and IL-12R β 2 subunits (Figures 6 and 7; Table 1). Figure 6 represents the histogram analysis of IL-12R β 1 expression for Gr-1/CD11b double positive cells and figure 7 represents the histogram analysis of IL-12R β 2 expression for Gr-1/CD11b double positive cells. Naïve C3H/HeJ spleen-derived Gr-1/CD11b double positive cells do not express the IL-12R β 1 subunit but they do express the IL-12R β 2 subunit (Figure 6 A and Figure 7 A). Naïve Balb/c spleen-derived Gr-1/CD11b double positive cells express both of the receptor subunits (Figure 6 D and Figure 7 D). MDSC derived from tumor bearing animals of both strains were also studied. Figure 6 B and C represents IL-12R β 1 expression of C3H/HeJ C3L5 tumor-bearing animals' spleen-derived and tumor-derived cells, respectively. Figure 6 D and E represents IL-12R β 1 expression of Balb/c 4T1 tumor-bearing animals' spleen-derived and tumor-derived cells, respectively. Figure 7 B and C represents IL-12R β 2 expression for C3H/HeJ C3L5 tumor-bearing animals' while D and E represents the expression of IL-12R β 2 for Balb/c 4T1 tumor-bearing animals' spleen-derived and tumor-derived MDSC respectively. MDSC were found to express both subunits (Figure 6 B, C, E, and F and Figure 7 B, C, E, and F). Changes were regardless of whether the MDSC are derived from tumors (Figures 6 and 7 E and F) or spleens (Figures 6 and 7 C and D) of the tumor bearing animals. The results are summarized for whether the marker is expressed (+) or not (-) in table 1.

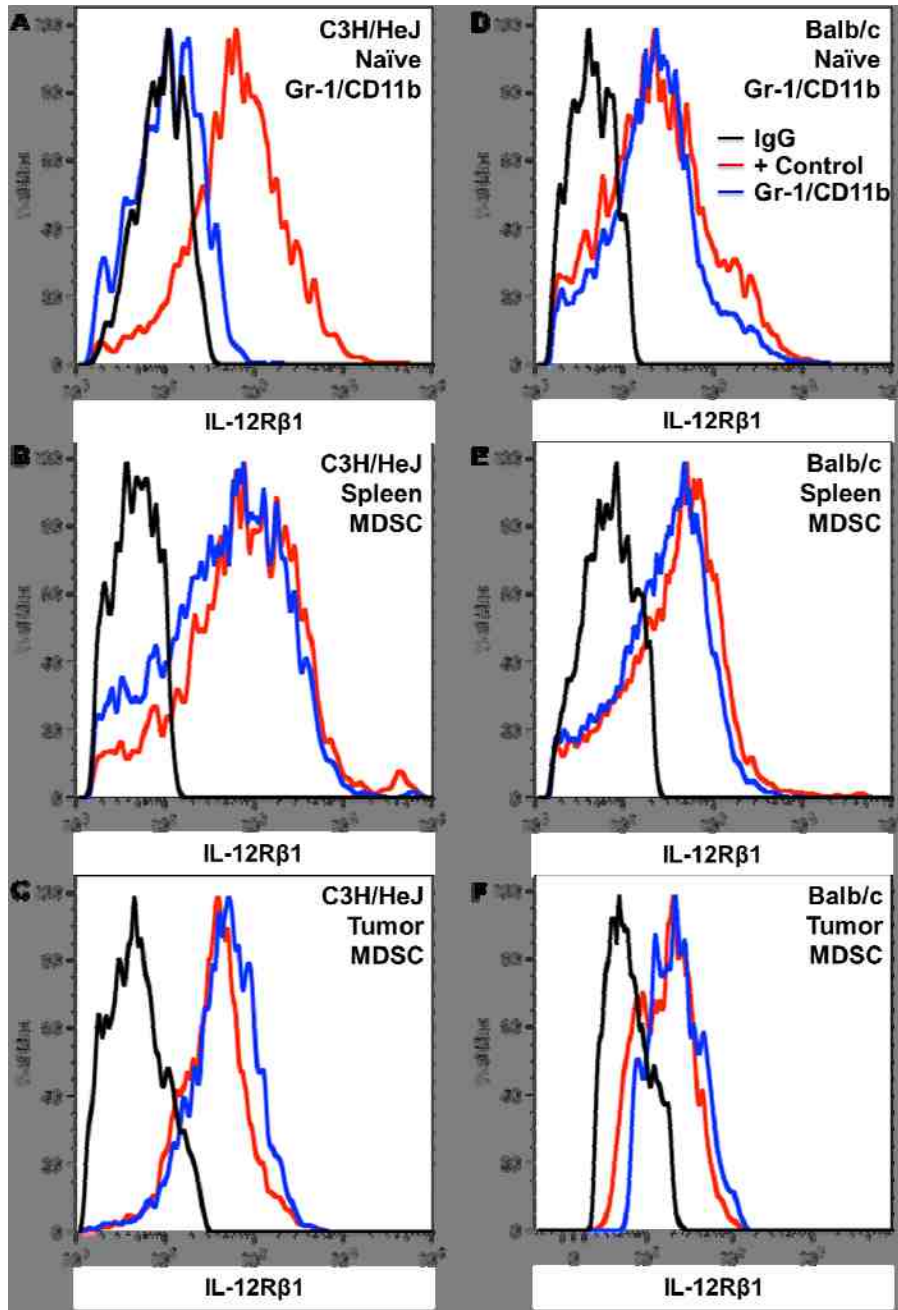


Figure 6. Expression of *IL-12Rβ1* on the surface of *Gr-1/CD11b* double positive cells. Single cell suspensions of tissues from tumor-bearing and naïve animals were obtained and stained with fluorochrome-conjugated anti-IgG, anti-*Gr-1*, anti-*CD11b*, anti-NK1.1 (Balb/c), anti-NK clone DX5 (C3H/HeJ), and anti-*IL-12Rβ1* antibodies at a concentration of 2 µg/ml. Cells

were analyzed for expression of the receptor above anti-IgG control antibody expression (black line) via flow cytometry. The red line represents the positive control NK cells while the blue line represents the *Gr-1/CD11b* double positive cells. C3H/HeJ-derived cells from the spleens of naïve animals (A) spleens of tumor-bearing animals (B) and tumors (C) are represented. Also represented are cells derived from the spleens of naïve Balb/c animals (D), spleens from tumor-bearing animals (E), and tumors (F).

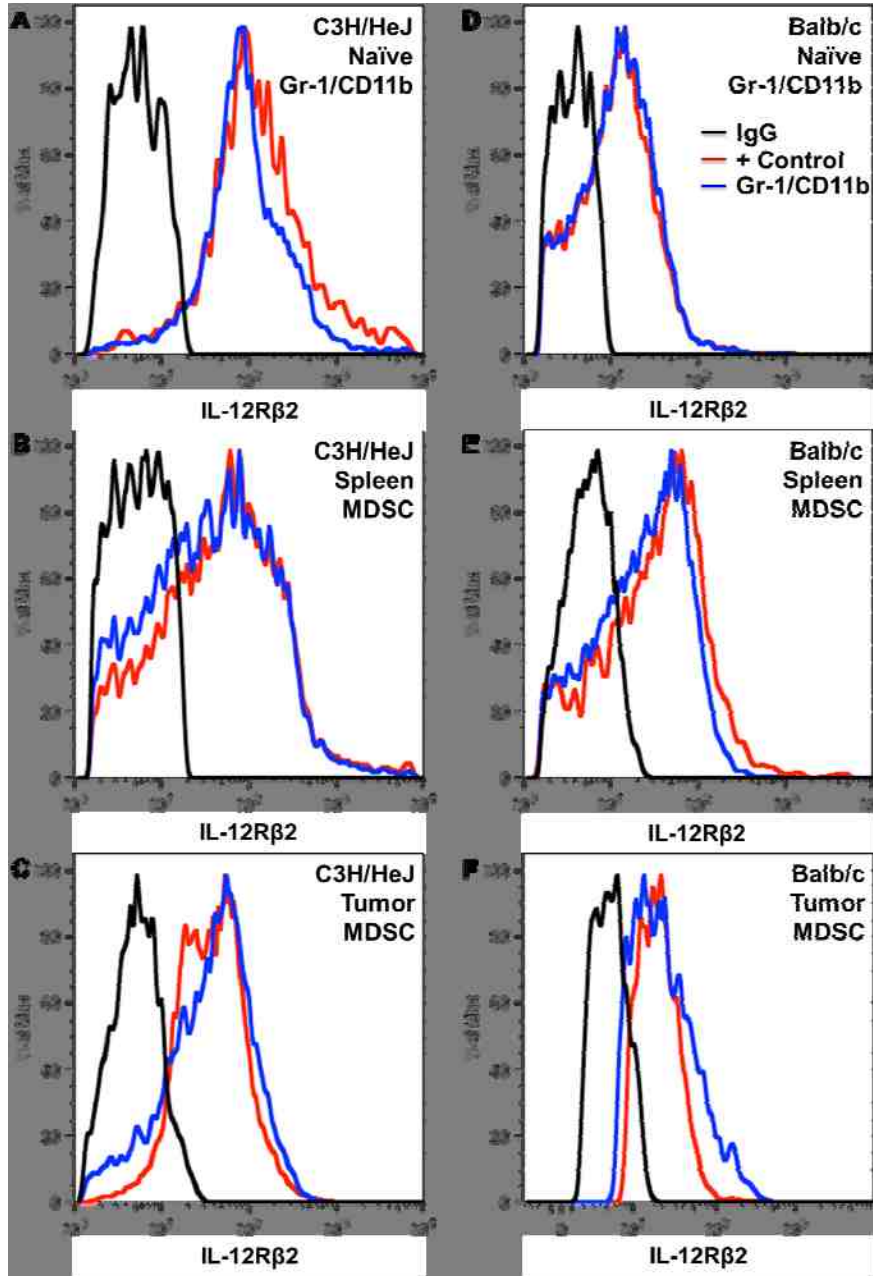


Figure 7.
Expression of IL-12R β 2 on the surface of Gr-1/CD11b double positive cells.
 Single cell
 □□□□□□□□□□
 □□□□□□□□□□
 tumor-bearing and naïve animals were obtained and stained with fluorochrome-conjugated anti-IgG, anti-Gr-1, anti-CD11b, anti-NK1.1 (Balb/c), and anti-NK clone DX5 (C3H/HeJ), antibodies at a concentration of 2 μ g/ml. IL-12R β 2 was also stained via anti-IL-12R β 2

primary antibody followed by fluorochrome-conjugated anti-IgG secondary antibody both at a concentration of 2 μ g/ml. Cells were analyzed for expression of the receptor above anti-IgG control antibody expression (black line) via flow cytometry. The red line represents the positive control NK cells while the blue line represents the Gr-1/CD11b double positive cells. C3H/HeJ-derived cells from the spleens of naïve animals (A) spleens of tumor-bearing animals (B) and tumors (C) are represented. Also represented are cells derived from the spleens of naïve Balb/c animals (D), spleens from tumor-bearing animals (E), and tumors (F).

Cells	IL-12Rβ1	IL-12Rβ2
C3H/HeJ Naïve Spleen Gr-1/CD11b Double Positive	-	+
C3H/HeJ Tumor MDSC	+	+
C3H/HeJ Tumor-Bearing Spleen MDSC	+	+
Balb/c Naïve Spleen Gr-1/CD11b Double Positive	+	+
Balb/c Tumor MDSC	+	+
Balb/c Tumor-Bearing Spleen MDSC	+	+

Table 1. Summary of IL-12R β 1 and IL-12R β 2 Expression on the Surface of Gr-1/CD11b Double Positive Cells. In the table, (+) designates expression of the receptor; (-) designates that the receptor is not expressed.

IL-12 alters the suppressive function of MDSC

After determining that MDSC from tumor-bearing animals are functionally suppressive and express the IL-12R on their plasma membranes, we sought to define the role that IL-12 could have on these cells. We sought first to determine whether IL-12 had any effects on the cells in terms of altering MDSC suppressive function. If IL-12 could reduce or even induce MDSC suppressive activity it would demonstrate a novel role for IL-12 in modulating MDSC and therefore immune activation during cancer progression.

In order to determine whether IL-12 could directly affect MDSC activity, MDSC were sorted and pretreated with 10 ng/ml recombinant mouse IL-12 for 24 hours at 37°C. Cells were washed and co-cultured with CFSE stained whole splenocytes and stimulated with 5 μ g/ml anti-CD3 and anti-CD28 antibodies for 4 days. Whole splenocytes were also pretreated with IL-12 for 24 hours and co-cultured with MDSC and stimulated with anti-CD3 and anti-CD28 antibodies as a control. Cells were harvested and stained with fluorochrome-conjugated anti-CD4 antibody and gated during analysis for CD4+ T Cells. Cell activation was determined by CFSE dilution of CD4+ T cells via flow cytometry.

As figures 8 and 9 demonstrate, pretreatment of whole splenocytes with IL-12 does not alter the ability of MDSC to suppress T cell activation with as few as 1 percent of the

cells capable of being activated (Figures 8 D and 9 D). This was equivalent to the levels of suppression exhibited by the untreated MDSC control (Figures 8 C and 9 C). MDSC pretreated with IL-12 for 24 hours, however, lose the ability to suppress T cell activation even at the strong 1:1 ratio (Figures 8 D and E and 9 D and E). As much as 65 percent of the T cells were activated following pretreatment of MDSC with IL-12. The loss of suppressive function is seen in both spleen-derived and tumor-derived MDSC (Figures 8 E and F and 9 E and F, respectively). This finding was not mouse strain-specific as the loss in suppressive function was observed in both C3H/HeJ (Figure 8) and Balb/c (Figure 9) mouse strains. This loss in suppressive function when MDSC are pretreated with IL-12 is an impressive indication that IL-12 not only can act directly on MDSC but that it also alters the activity of the cells.

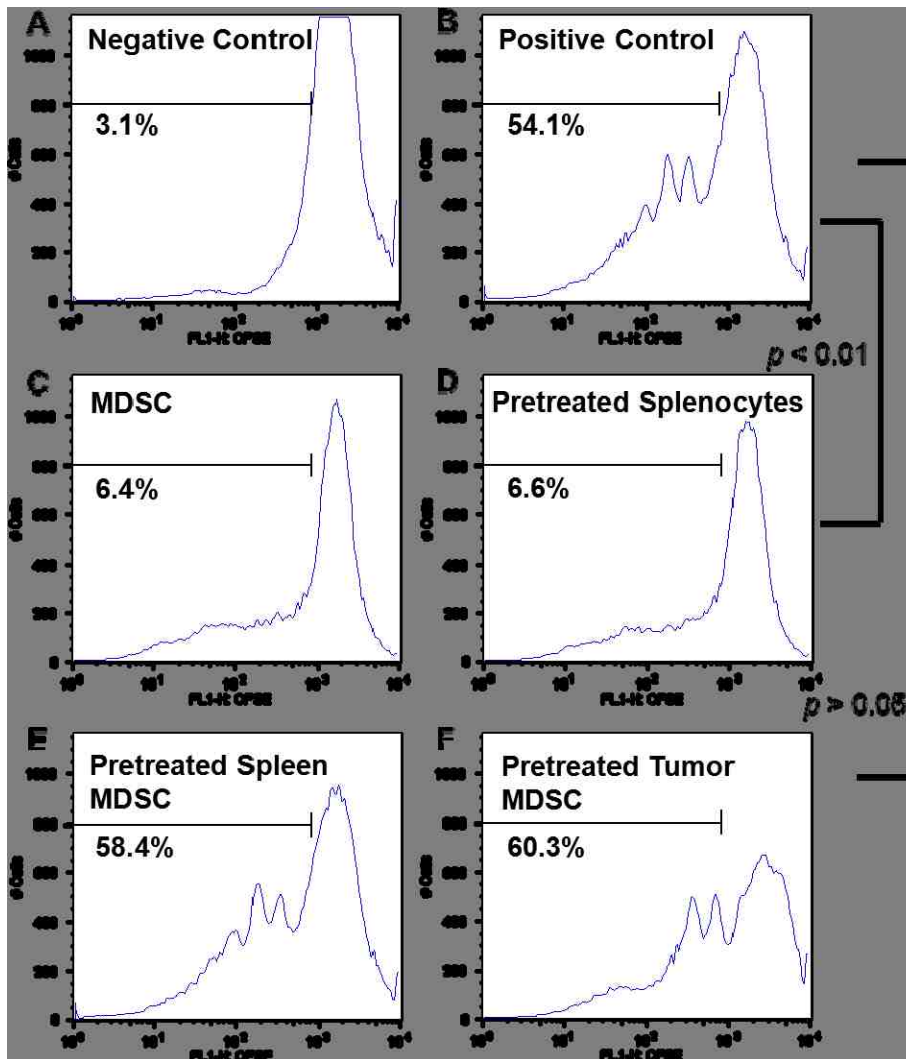


Figure 8. *IL-12 alters the suppressive activity of C3H/HeJ MDSC.* C3H/HeJ naive whole splenocytes were stained with 10 μ M CFSE and co-cultured with C3H/HeJ MDSC sorted from digested tumors and spleens of C3L5 tumor-bearing animals. MDSC

were pretreated with or without 10 ng/ml IL-12 for 24 hours. Cells were then co-cultured in 96 well plates at a 1:1 ratio of whole splenocytes to MDSC for a total of 2×10^5 cells per well stimulated with anti-CD3 and anti-CD28 antibodies for 4 days. After the 4 day incubation, cells were stained with fluorochrome-conjugated anti-CD4 and anti-Gr-1 to isolate only the CD4+ T cells for analysis. Using gating and flow cytometric analysis, CD4+ T cells from untreated cells (negative control) (A) positive control anti-CD-3 and anti-CD-28 treated cells (B) and untreated MDSC control (C) were compared to CD4+ T cells pretreated with IL-12 prior to co-culture with untreated MDSC (D) CD4+ T cells co-cultured with IL-12 pretreated spleen-derived MDSC (E) and IL-12 pretreated tumor-derived MDSC (F). Percentages indicate percent activation.

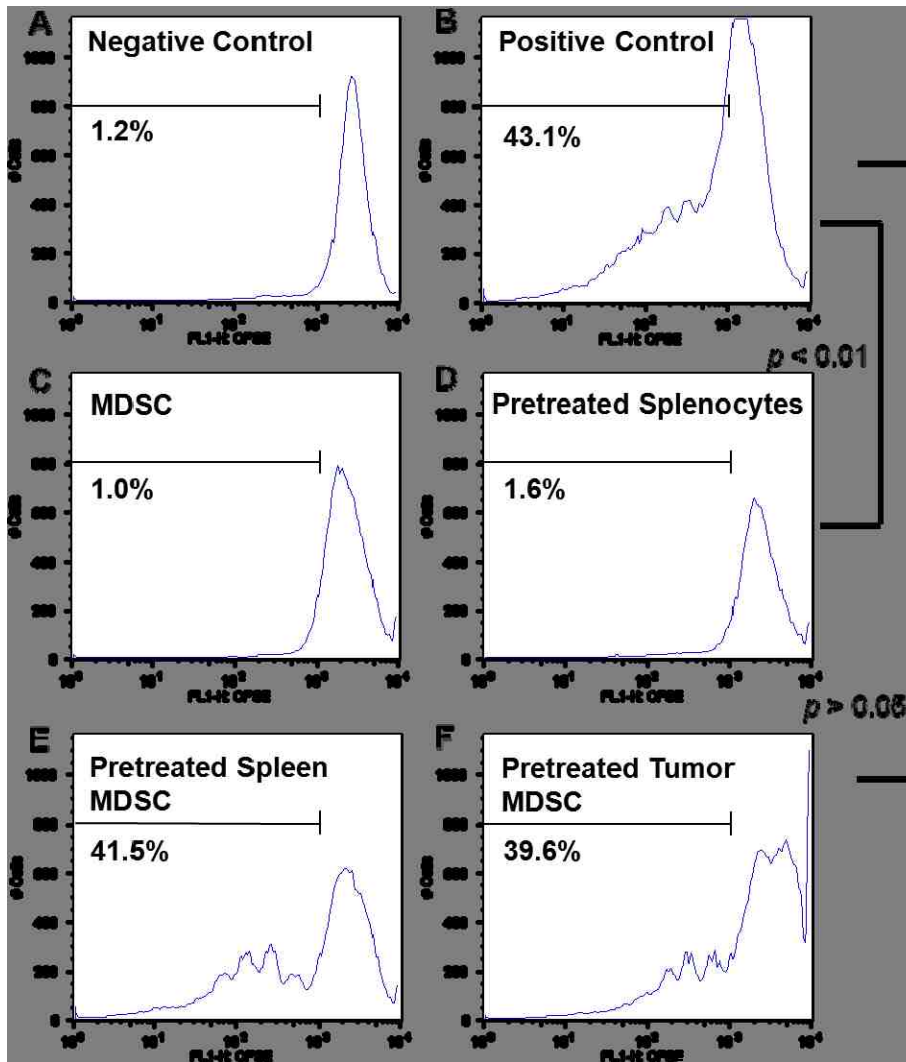


Figure 9. *IL-12 alters the suppressive activity of Balb/c MDSC.* Balb/c naive whole splenocytes were stained and cultured as described previously (Figure 7). Using gating and flow cytometric analysis, CD4+ T cells from untreated cells (negative control) (A)

positive control anti-CD-3 and anti-CD-28 antibody treated cells (B) and untreated MDSC control (C) were compared to CD4+ T cells pretreated with IL-12 prior to co-culture with MDSC (D) CD4+ T cells co-cultured with IL-12 pretreated spleen-derived MDSC (E) and IL-12 pretreated tumor-derived MDSC (F). Percentages indicate percent activation.

IL-12 induces MDSC up-regulation of maturation markers in vitro

It is clear from the data thus far that treatment of MDSC with IL-12 alters the suppressive function of the cells. The mechanism through which IL-12 alters MDSC activity remains to be determined. In order to assess how MDSC change following IL-12 treatment, several stains for surface markers were performed and analyzed. It has been proposed that Gr-1/CD11b double positive cells can be induced to mature into dendritic cells (DC) or macrophages, so we focused on markers known to be up-regulated on mature DC and macrophages: CD80, CD86, F4/80, and major histocompatibility complex class II (MHCII) (270). Up-regulation of these markers on MDSC could indicate a phenotypic change, that when coupled with the change in suppressive function, would indicate potential maturation. Comparisons between the functionally suppressive MDSC and naïve Gr-1/CD11b double positive cells were also performed.

The flow cytometric analysis for changes in surface marker expression for naïve untreated and IL-12 treated Gr-1/CD11b double positive cells for C3H/HeJ and Balb/c murine models are outlined in figures 10 and 12, respectively. The flow cytometry analysis for spleen-derived and tumor-derived MDSC before and after IL-12 treatment is represented in figures 11 and 13. The changes in expression are summarized in table 2.

The data show that Gr-1/CD11b double positive cells from naïve animals already express maturation markers for DC and Macrophages, supporting the findings that these cells are not only functionally but also phenotypically distinct from MDSC (Figures 10 and 12; Table 2). Spleen-derived and tumor-derived MDSC do not express the DC and macrophage maturation markers prior to treatment with IL-12 (Figures 11 and 13; Table 2). This distinguishes MDSC from general Gr-1/CD11b double positive cells and potentially from other macrophage types especially in the tumor microenvironment. The MDSC studied in this dissertation do express CD11b but do not express F4/80 unlike previously studied MDSC populations indicating that using CD11b as the sole marker for MDSC in previous studies did not evaluate at least one subpopulation of the MDSC populations present in the tumor.

Following IL-12 treatment all markers were found to increase on the surface of spleen-derived MDSC (Figures 11 A, B, C, and D and 13 A, B, C, and D). This up-regulation of surface markers indicates that these cells are potentially capable of maturation into cells

such as DC and macrophages and that IL-12 might be capable of inducing that maturation. Tumor-derived MDSC are distinct from spleen-derived MDSC in terms of IL-12 response. Upon stimulation with IL-12, tumor-derived MDSC *in vitro* up-regulate only F4/80 and MHCII surface markers. Gr-1/CD11b double positive cells from naïve animals express several markers of mature cells and do not change the expression of these markers following treatment with IL-12. This data supports the definition of MDSC as a distinct population of cells from naïve Gr-1/CD11b double positive cells.

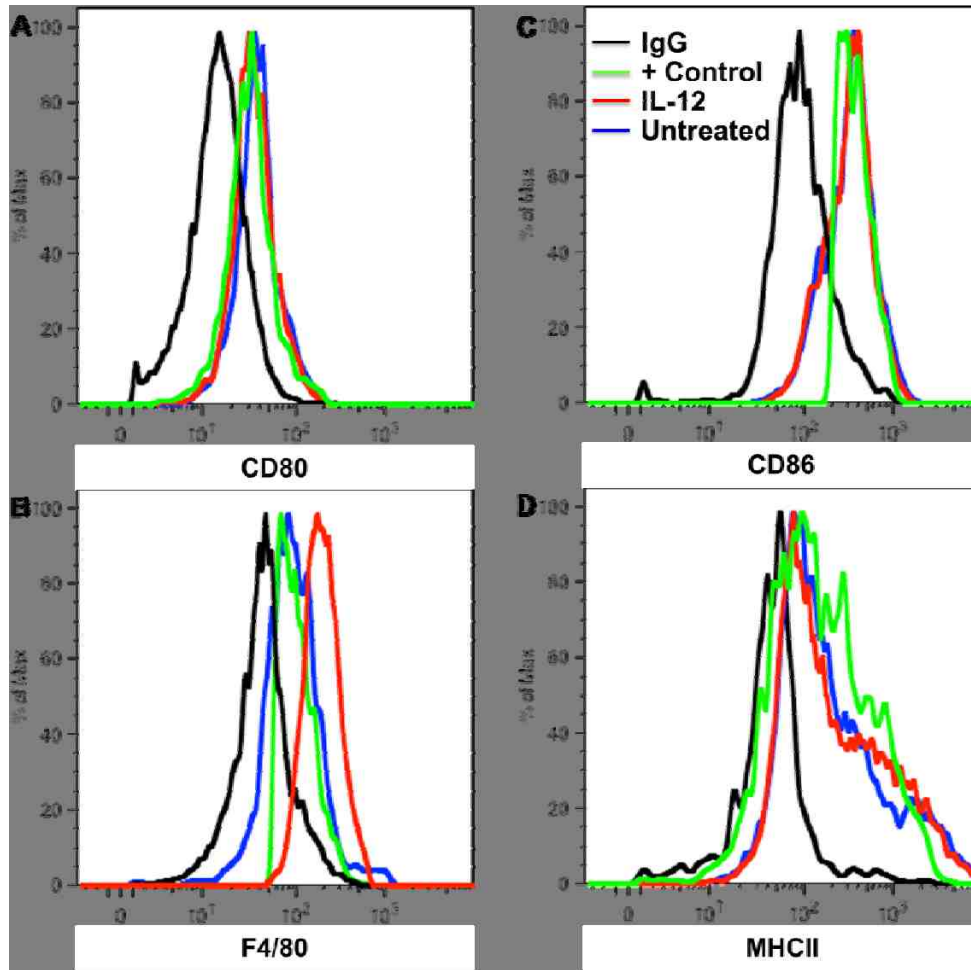


Figure 10. Surface marker expression of naïve C3H/HeJ Gr-1/CD11b double positive cells does not change following *in vitro* treatment with IL-12. Single cell suspensions of naïve splenocytes were stained with 2 µg/ml fluorochrome conjugated anti-Gr-1 and anti-CD11b antibodies and sorted via flow cytometry. Sorted double positive cells were treated with 10 ng/ml recombinant mouse IL-12 for 24 hours. Cells were stained with 2 µg/ml fluorochrome-conjugated anti-IgG, anti-CD80 (A), anti-F4/80 (B), anti-CD86 (C), and anti-MHCII (D) antibodies. Surface expression of the marker was determined by flow cytometry. Histogram analysis of whole splenocyte positive controls (green line), IgG stained as negative controls (black line), untreated Gr-1/CD11b double positive cells (blue line) and IL-12 treated Gr-1/CD11b double positive cells (red line) are shown. Results are summarized in table 2.

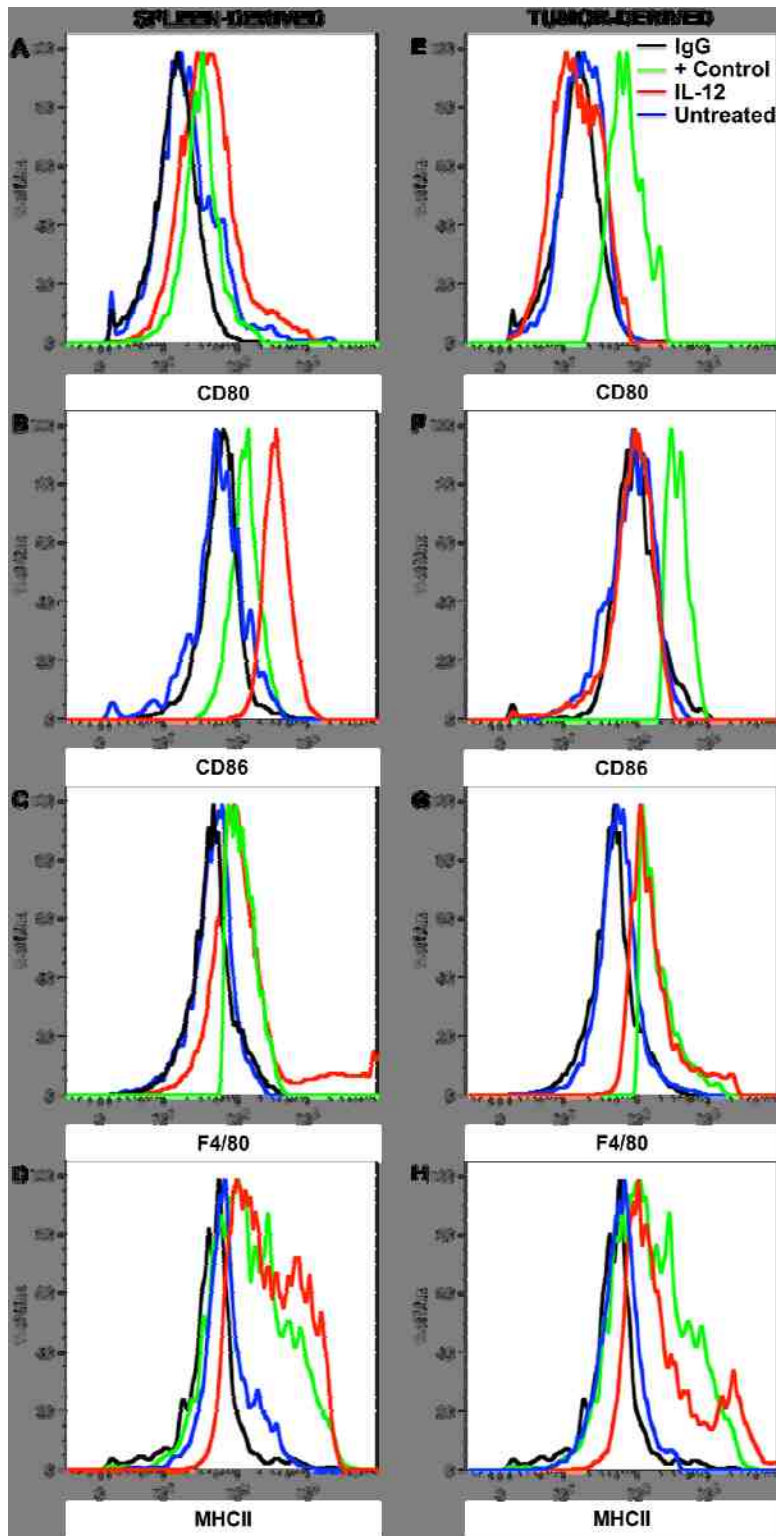


Figure 11. Changes in surface marker expression following *in vitro* treatment of Gr-1/CD11b double positive cells derived from spleen and tumor of C3H/HeJ C3L5 tumor-bearing animals with IL-12. Single cell suspensions of spleens and tumors from tumor-bearing animals were obtained and stained with 2 μ g/ml fluorochrome conjugated anti-Gr-1 and anti-CD11b antibodies and sorted via flow cytometry. Sorted double positive cells were treated with 10 ng/ml recombinant mouse IL-12 for 24 hours. Cells were stained with 2 μ g/ml fluorochrome-conjugated anti-IgG, anti-CD80, anti-F4/80, anti-CD86, and anti-MHCII antibodies. Surface expression of the marker was determined by flow cytometry. Histograms representing

the analysis of 3 experiments stained for the expression of CD80 on spleen-derived (A) and tumor-derived (E), F4/80 on spleen-derived (B) and tumor-derived (F). CD86 on spleen-derived (C) and tumor-derived (G), and MHCII for spleen derived (D) and tumor-

derived (H) Gr-1/CD11b double positive cells are shown. The histogram analysis includes expression of each marker on the surface of positive controls (green line), IgG stained negative controls (black line), untreated MDSC (blue line) and IL-12 treated MDSC (red line). Results are summarized in table 2.

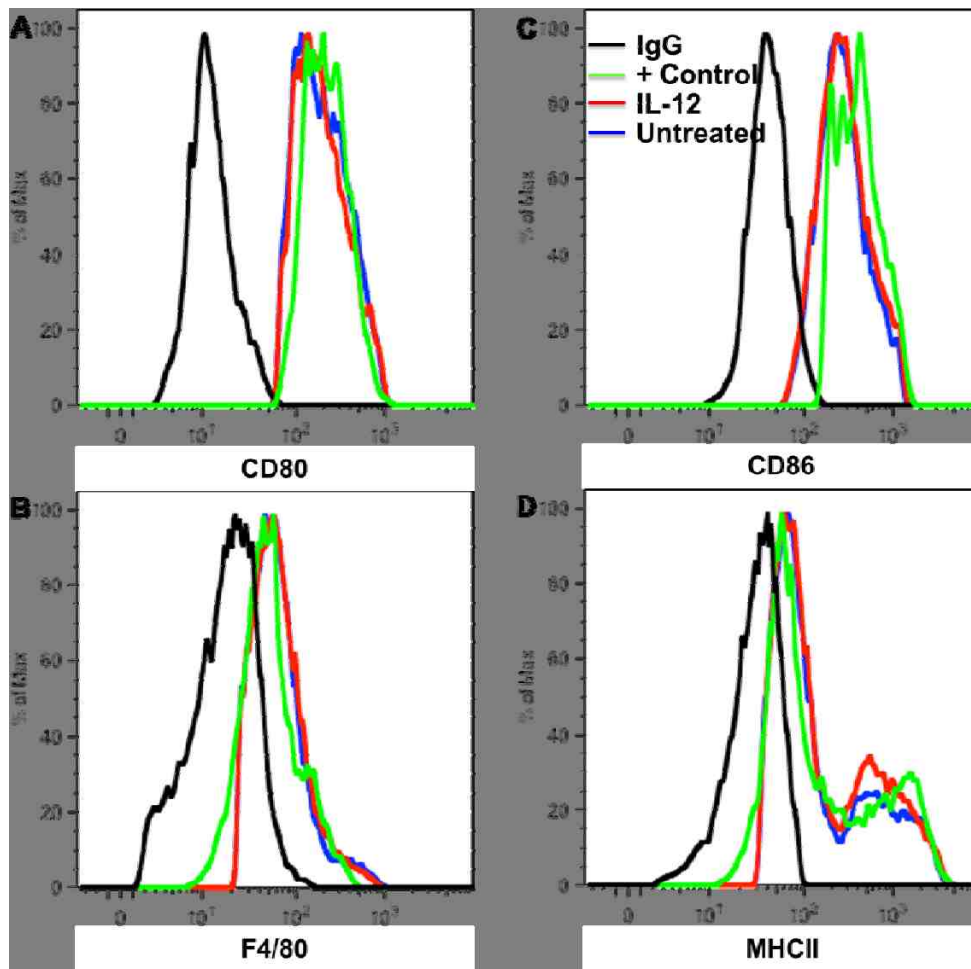


Figure 12. Surface marker expression of naive Balb/c Gr-1/CD11b double positive cells does not change following *in vitro* treatment with IL-12. Cells were sorted and stained as described previously (Figure 9). Results of staining with anti-CD80 (A), anti-F4/80 (B), anti-CD86 (C), and anti-MHCII (D) antibodies are shown. Histogram analysis of whole splenocyte positive controls (green line), IgG stained as negative controls (black line), untreated Gr-1/CD11b double positive cells (blue line) and IL-12 treated Gr-1/CD11b double positive cells (red line). Results are summarized in table 2.

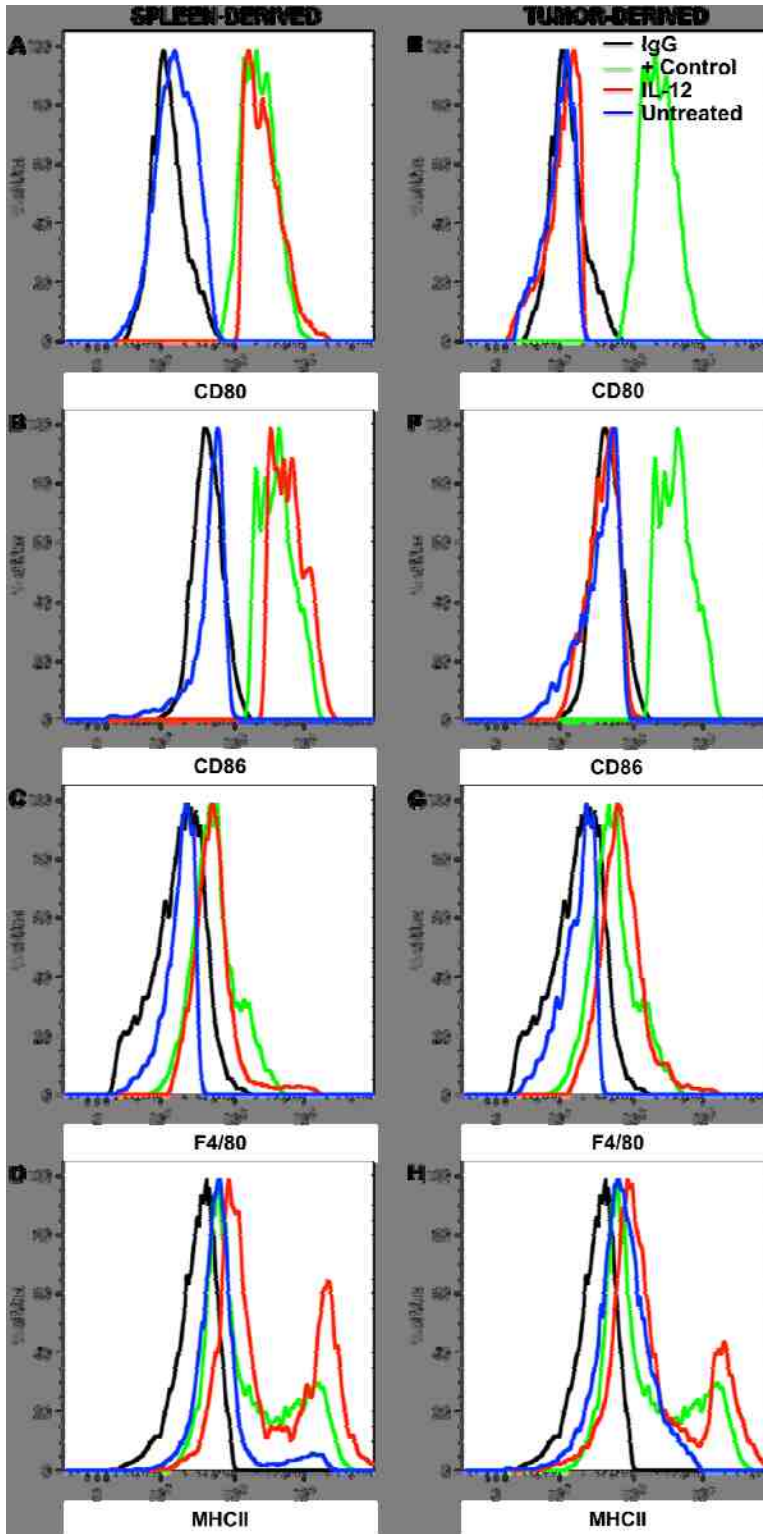


Figure 13. Changes in surface marker expression following *in vitro* treatment of Gr-1/CD11b double positive cells derived from spleen and tumor of Balb/c 4T1 tumor-bearing animals with IL-12. Cells were stained as described previously (Figure 10). Histograms representing the results of 3 experiments stained for the expression of CD80 on spleen-derived (A) and tumor-derived (E), F4/80 on spleen-derived (B) and tumor-derived (F), CD86 on spleen-derived (C) and tumor-derived (G), and MHCII for spleen derived (D) and tumor-derived (H) Gr-1/CD11b double positive cells are shown. The histogram analysis includes expression of each marker on the surface of whole splenocyte positive controls (green line), IgG stained negative controls (black line), untreated MDSC (blue line) and IL-12 treated MDSC (red line). Results are summarized in table 2.

Cell Type	CD80	CD86	F4/80	MHCII
C3H/HeJ				
Naïve Gr-1/CD11b	++	++	++	++
Naïve Gr-1/CD11b + IL-12	++	++	+++	++
Tumor-Bearing Spleen MDSC	-	-	-	-
Tumor-Bearing Spleen MDSC + IL-12	++	+++	++	+++
Tumor MDSC	-	-	-	-
Tumor MDSC + IL-12	-	-	+	+
Balb/c				
Naïve Gr-1/CD11b	++	++	++	++
Naïve Gr-1/CD11b + IL-12	++	++	++	++
Tumor-Bearing Spleen MDSC	-	-	-	-
Tumor-Bearing Spleen MDSC + IL-12	++	++	++	+++
Tumor MDSC	-	-	-	-
Tumor MDSC + IL-12	-	-	++	+

Table 2. Summary of the expression of surface markers after *in vitro* analysis. In this table, (-) designates not expressed, (+) designates expressed at levels below positive controls, (++) designates expressed at levels equal to positive controls, and (+++) designates expressed at levels exceeding positive controls.

IL-12 treatment reduces the expression of Nos2, IFN- γ and Arg1 mRNA in MDSC

Although this up-regulation of maturation markers is sufficient to demonstrate and begin to define a change on MDSC induced by IL-12, how that change is actually correlated with the loss of suppressive function needs to be determined. It is known that one of the critical mechanisms for MDSC suppressive activity is the production of reactive nitrogen species mediated by the expression of nitric oxide synthases such as Nos2. Reductions in Nos2 mRNA and protein have been shown to correlate to a reduction in suppressive activity both *in vitro* and *in vivo* (271). In addition to studying Nos2, it can be essential to study regulation of Nos2 to better define the effects of a given treatment. Nos2 is inducible by IFN- γ expression regardless of whether it is utilized from intracellular production or extracellular sources. Studies performed on MDSC after IL-12 treatment

using ELISA did not indicate any extracellular IFN- γ production (data not shown); however, it is possible that all of the cytokine was produced and utilized internally. In order to define whether changes in Nos2 and IFN- γ may be playing a role in these cells, RNA was extracted and mRNA levels analyzed using real-time polymerase chain reaction (PCR). Figure 14 represents relative Nos2 mRNA while figure 15 represents relative IFN- γ mRNA. The figures demonstrate levels of expression as normalized and compared to naïve Gr-1/CD11b double positive and graphed as a fold change. C3H/HeJ derived cells (Figures 14 A and 15 A) and Balb/c derived cells (Figures 14 B and 15 B) are representative of 3 individual experiments and real-time PCR assays.

Expression of Nos2 and IFN- γ mRNA were increased in MDSC compared to Gr-1/CD11b double positive cells from naïve animals regardless of tissue type. The levels of Nos2 and IFN- γ expressed by tumor-derived MDSC were, however, higher than the levels expressed in spleen-derived MDSC. Tumor-derived MDSC from C3H/HeJ animals exhibited up to 4 fold increase in Nos2 and 18 fold increase in IFN- γ mRNA expression over that from naïve Gr-1/CD11b double positive cells (Figures 14 A and 15 A). This is compared to approximately 1.5 fold and 4 fold increase over the expression in naïve cells for the spleen-derived MDSC. The levels for Balb/c animals were slightly lower than C3H/HeJ with tumor-derived cells exhibiting 2.5 fold and 4 fold increases over the expression in naïve cells of Nos2 and IFN- γ , respectively. Spleen-derived MDSC from Balb/c animals exhibit approximately 1.5 fold increases in Nos2 and slightly less than 1.5 fold in IFN- γ (Figures 14 and 15 B). Lower levels of IFN- γ expression in the spleen-derived MDSC may account for differences in ability to differentiate in response to IL-12 *in vitro* and is evidence that these cells may be distinct cells with potentially different activity than their tumor-derived associates in terms of overall suppressive activity *in vivo*. It is essential to note that the finding that both spleen-derived and tumor-derived cells expressed Nos2 at levels exceeding the naïve Gr-1/CD11b double positive controls supports the *in vitro* evidence of suppressive function for both cell types.

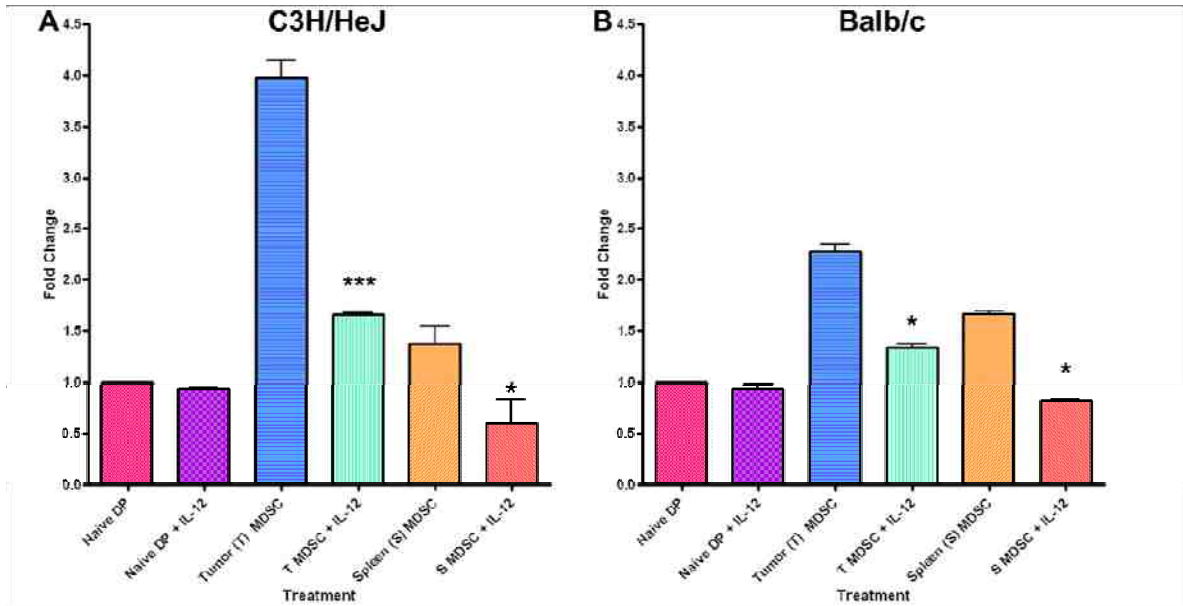


Figure 14. *In vitro* treatment with IL-12 reduces *Nos2* mRNA expression. Gr-1/CD11b double positive cells were stained using fluorochrome-conjugated antibodies and sorted from the spleens of naïve animals as well as the spleens and tumors of tumor-bearing animals. Sorted MDSC were treated for 24 hours with 10 ng/ml recombinant mouse IL-12. RNA was extracted from sorted cell populations, converted to cDNA and analyzed for mRNA expression via Real Time PCR. The expression levels for *Nos2* were normalized to expression from naïve double-positive cells and graphed as a fold change. The expression of *Nos2* in C3H/HeJ (A) and Balb/c (B) are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 ($n = 9$; * $p < 0.05$; *** $p < 0.001$).

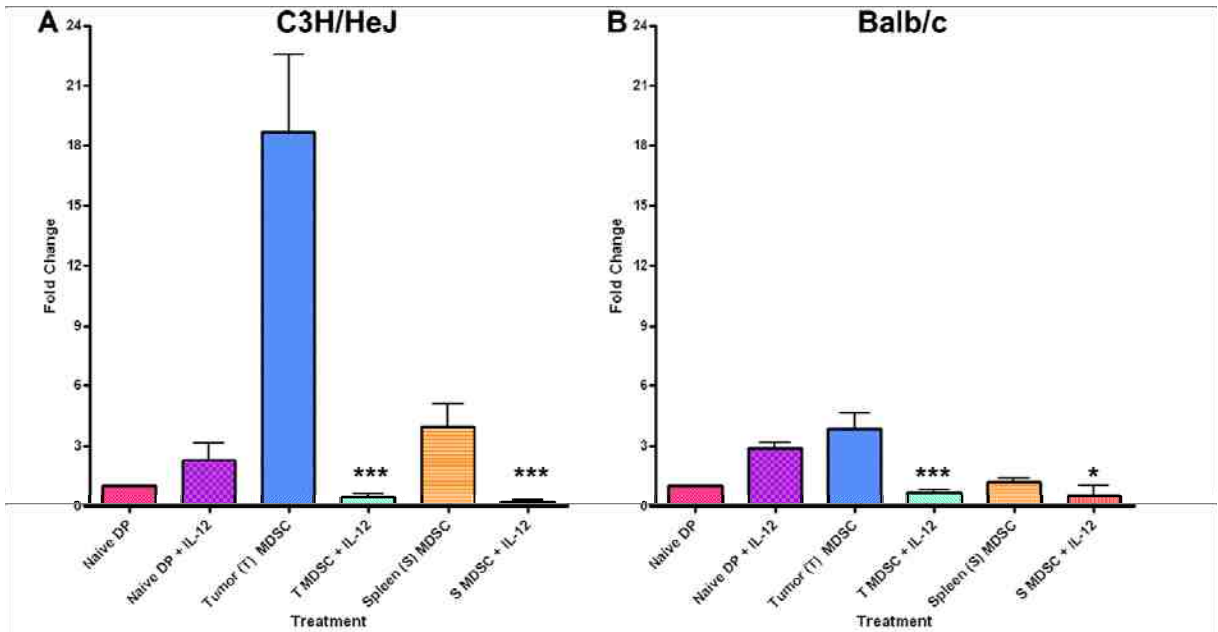


Figure 15. *In vitro* treatment with IL-12 reduces IFN- γ mRNA expression. Cells were stained and RNA extracted as described previously (Figure 14). The expression levels for IFN- γ were normalized to the expression levels from naïve double-positive cells and graphed as a fold change. The expression of IFN- γ in C3H/HeJ (A) and Balb/c (B) are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 ($n = 9$; * $p < 0.05$; *** $p < 0.001$).

In addition to Nos2, arginase expression in MDSC mediated by mRNA expression of Arg1 is also responsible for conferring the suppressive activity of MDSC. Reductions in Arg1 have been shown to abrogate the suppressive effects of MDSC (272). It has been suggested that in order to reverse suppressive function, treatment must induce a reduction in both Nos and Arg1. In order to determine whether arginase was also involved in conferring suppressive function, I analyzed mRNA expression of Arg1 in Gr-1/CD11b double positive cells and MDSC. Figure 16 represents mRNA expression of Arg1 normalized and compared to naïve Gr-1/CD11b double positive cells.

The pattern of mRNA expression for Arg1 mRNA in both C3H/HeJ derived cells (A) and Balb/c derived cells (B) was similar to the pattern of expression for both Nos2 and IFN- γ mRNA. Arg1 was increased in both tumor-derived and spleen-derived MDSC compared to naïve Gr-1/CD11b double positive cells. Arg1 expression was present in both spleen-derived and tumor-derived MDSC with approximately 3 fold increases in tumor derived MDSC and 2.5 fold increases in spleen-derived MDSC above mRNA levels in naïve Gr-1/CD11b double positive cells from C3H/HeJ animals (Figure 16 A). Higher levels of Arg1 expression were found in Balb/c MDSC with approximately 8 fold higher levels and 4 fold higher levels of mRNA expression above naïve Gr-1/CD11b double positive cells for tumor-derived and spleen-derived MDSC, respectively.

Treatment with IL-12 was found to significantly reduce Arg1 expression in both the spleen-derived and tumor-derived MDSC. IL-12 altered Arg1 mRNA expression to levels approximately equal to the levels in naïve Gr-1/CD11b double positive control cells. The positive finding of Arg1 mRNA expression in the spleen-derived MDSC also supports the results that these are functionally suppressive cells. Although similar expression patterns were found, these Arg1 studies indicate a key difference between the C3H/HeJ and Balb/c derived MDSC *in vitro*. C3H/HeJ derived MDSC exhibit higher levels of Nos2 expression while Balb/c derived MDSC exhibit higher levels of Arg1 expression. This reciprocal difference in expression levels could be representative of the differences in cell types that comprise the MDSC of these two murine models but further experimentation is required to define how distinct subpopulations may differ in terms of response to IL-12.

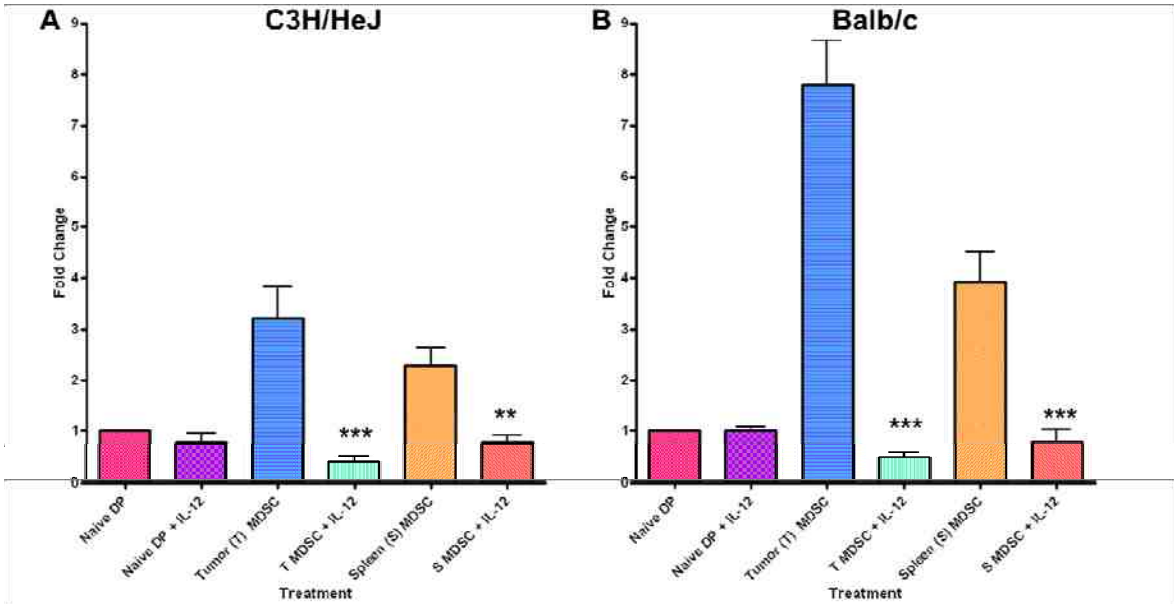


Figure 16. *In vitro* treatment with IL-12 reduces *Arg1* mRNA expression. Cells were isolated and RNA extracted as described previously (Figure 14). The expression levels for *Arg1* were normalized to the expression levels from naïve double-positive cells and graphed as a fold change. The expression of *Arg1* in C3H/HeJ (A) and Balb/c (B) IL-12 treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. (n = 9; **p < 0.01; ***p < 0.001).

In vitro treatment with IL-12 reduced intracellular IFN- γ in MDSC

In order to confirm that reduction in IFN- γ mRNA expression following *in vitro* IL-12 treatment correlates with a reduction in IFN- γ cytokine levels, the expression of intracellular IFN- γ was determined. As discussed previously, much of the IFN- γ required for the suppressive function of these cells is expressed internally. Decreases in the protein level serve as an indicator that the reductions in IFN- γ mRNA observed in these studies have physiological relevance to the suppressive function of the MDSC.

Sorted Gr-1/CD11b double positive cells from naïve and tumor bearing C3H/HeJ (A) and Balb/c (B) animals were studied following 24 hours *in vitro* treatment with IL-12 (Figure 17). Approximately 65 percent of the tumor-derived MDSC and 45 percent of the spleen-derived MDSC from C3H/HeJ animals exhibited IFN- γ intracellular cytokine expression (Figure 17 A). The percentage of MDSC expressing intracellular IFN- γ in Balb/c animals was slightly lower than in C3H/HeJ with approximately 50 percent of the tumor-derived MDSC and 35 percent of the spleen-derived MDSC testing positive (Figure 17 B). Treatment with IL-12 was found to significantly reduce the percentage of cells that express intracellular IFN- γ . IL-12 reduced IFN- γ expression to levels at or below naïve cells for both models. This is regardless of whether the cells were derived from the tumors or spleens of the animals. This confirmation of the mRNA results emphasizes the importance of IFN- γ in these cells and is consistent with findings that IFN- γ priming is critical to MDSC suppressive function.

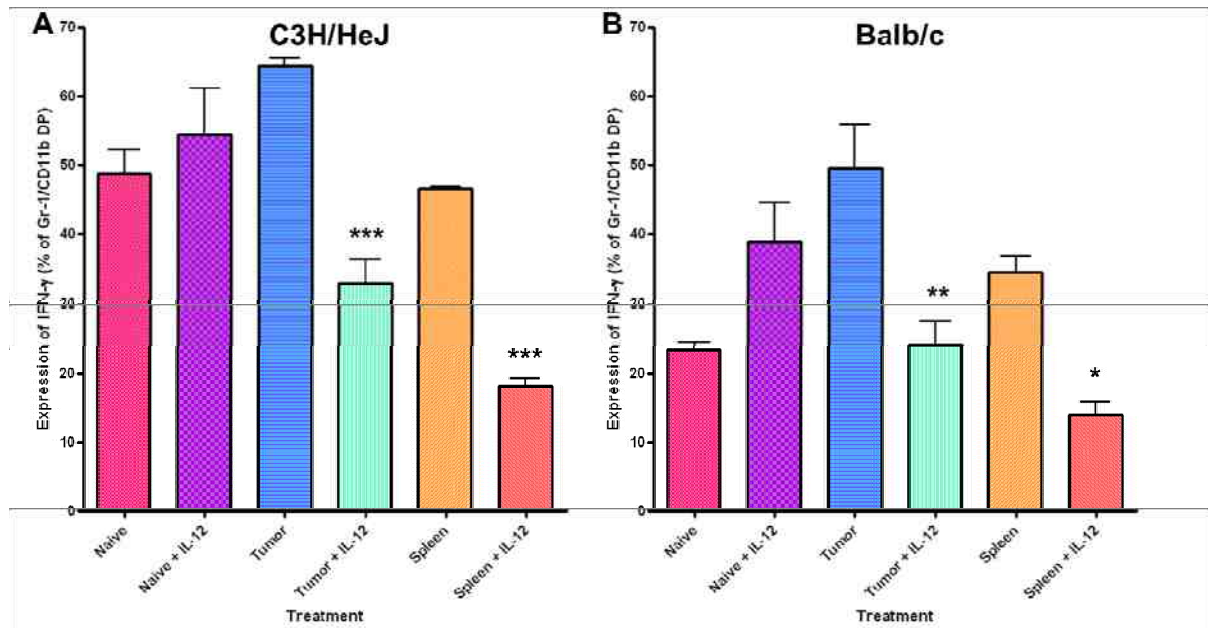


Figure 17. *In vitro* treatment with IL-12 reduces IFN- γ protein expression in MDSC. Gr-1/CD11b double positive cells were stained using 2 μ g/ml fluorochrome-conjugated antibodies and sorted from the spleens of naïve animals as well as the spleens and tumors of tumor-bearing animals. Sorted MDSC were treated for 24 hours with 10 ng/ml recombinant mouse IL-12. Cells were then permeabilized and stained using 2 μ g/ml fluorochrome-conjugated anti-IFN- γ antibody. The expression of IFN- γ in C3H/HeJ (A) and Balb/c (B) IL-12 treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. Results represent 3 individual experiments stained in triplicate ($n = 9$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

IL-12 reduces IL-10 production and increases TNF- α production predominantly from tumor-derived MDSC

Although the expression pattern of the surface markers of the tumor-derived MDSC defined in this dissertation are not equivalent to those expected for macrophages, the fact that MDSC have been shown to potentially function as macrophages in other studies means that further characterization of the cells in these studies is necessary to fully define their activity. Tumor-infiltrating macrophages are associated with the potential to be either activating (M1) or suppressive (M2) (273-276). Although there are limitations in the studies that determined MDSC could behave as suppressive macrophages, the similarities between the two populations is enough to suggest a possibility for similar cytokine activity (277). MDSC have been shown to express IL-10 while depleting IL-12 from macrophages, therefore the potential role for IL-12 on MDSC cytokine production needs to be defined (278). Since suppressive MDSC and M2 macrophages in the tumor microenvironment are known to express IL-10 while M1 activating macrophages express TNF- α , we determined the expression pattern for both of these molecules with and without treatment of IL-12 for 24 hours.

Gr-1/CD11b double positive cells from naïve animals and MDSC from tumor-bearing animals were sorted and treated with IL-12 for 24 hours. Cell conditioned media was harvested for analysis of protein expression while RNA was extracted from the cells for analysis of mRNA expression. mRNA expression for IL-10 (Figure 18) and TNF- α (Figure 20) was determined via quantitative real-time PCR while IL-10 (Figure 19) and TNF- α (Figure 21) protein concentration was determined via ELISA.

MDSC were found to express IL-10 mRNA and protein both of which were significantly reduced following treatment with IL-12. IL-10 mRNA expression was approximately 7.5 to 9 fold higher in tumor-derived MDSC compared to naïve Gr-1/CD11b cells for both Balb/c animals (Figure 18 B) and C3H/HeJ animals (Figure 18 A), respectively. Spleen-derived MDSC express 3 to 5 fold higher levels of expression of IL-10 mRNA over naïve Gr-1/CD11b double positive cells for Balb/c (Figure 18 B) and C3H/HeJ animals (Figure 18 A). Protein expression from both tumor-derived cells compared to spleen-derived cells was increased compared to naïve cells though they were not significantly different from each other for the 24 hour *in vitro* study regardless of whether they were from C3H/HeJ animals (Figure 19 A) or Balb/c animals (Figure 19 B). MDSC were found to

express higher levels of IL-10 protein which was significantly decreased following treatment with IL-12 for both tissue types and models (Figure 19).

The opposite pattern of expression from IL-10 was observed for TNF- α . Expression of TNF- α mRNA and protein was found to significantly increase following treatment with IL-12. MDSC from tumor and spleen express similar levels of TNF- α as naïve Gr-1/CD11b double positive cells; however, following treatment with IL-12 significant increases in TNF- α mRNA and protein were observed for both C3H/HeJ (Figures 20 A and 21 A) and Balb/c (Figures 20 B and 21 B). This was true for both tumor-derived and spleen-derived MDSC. These findings indicate potential similarities exist between the cytokine profile of the MDSC analyzed in these studies and suppressive M2 macrophages. Following treatment with IL-12, the cytokine profile of the MDSC analyzed in these studies changes to resemble at least one of the cytokines important in M1 activating macrophages.

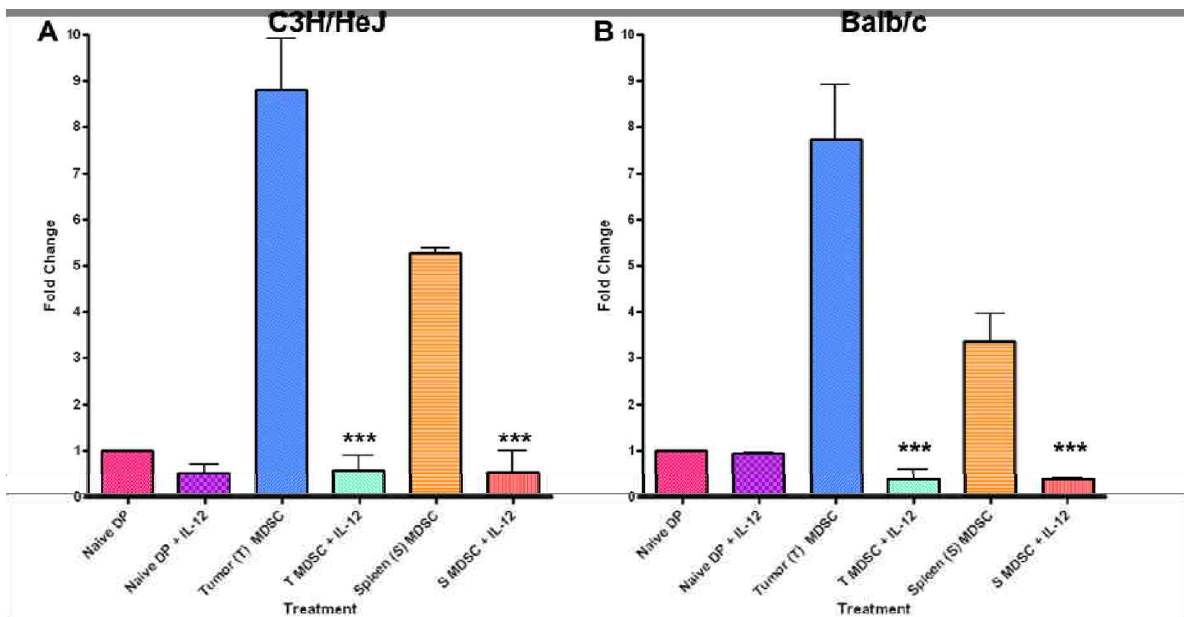


Figure 18. *In vitro* treatment with IL-12 reduces IL-10 mRNA expression. Cells were isolated and RNA extracted as described previously (Figure 14). The expression levels for IL-10 were normalized to the expression levels from naïve double-positive cells and graphed as a fold change. The expression of IL-10 in C3H/HeJ (A) and Balb/c (B) IL-12

treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. (n = 9; ***p < 0.001).

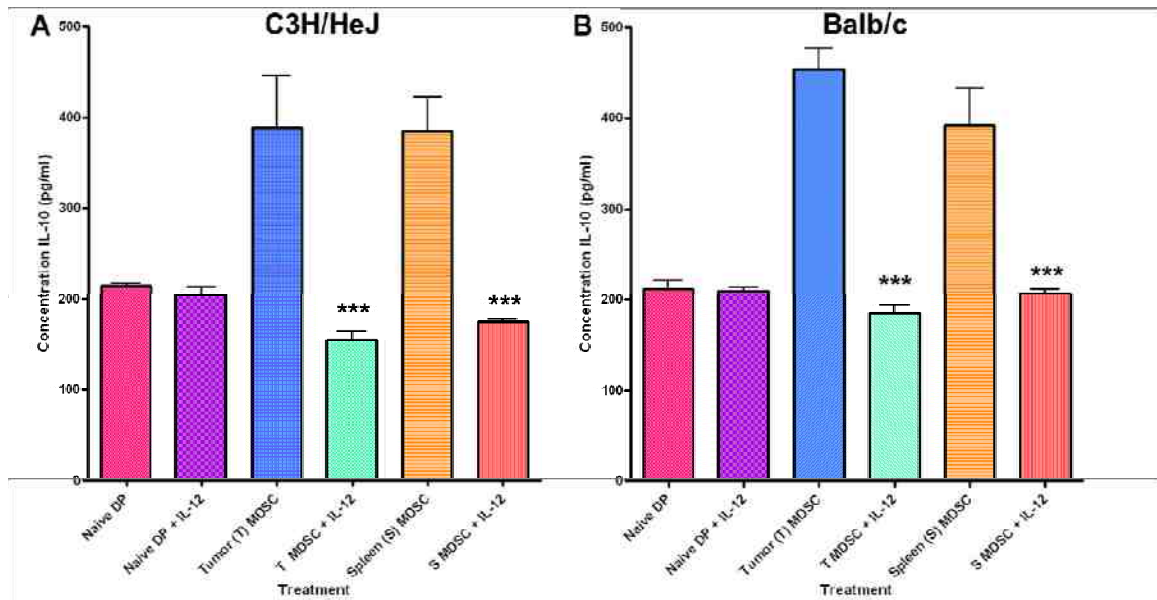


Figure 19. *In vitro* treatment with IL-12 reduces IL-10 protein expression from MDSC.

Gr-1/CD11b double positive cells were stained using fluorochrome-conjugated antibodies and sorted from the spleens of naïve animals as well as the spleens and tumors of tumor-bearing animals. 1×10^6 sorted MDSC were incubated alone or with 10 ng/ml recombinant mouse IL-12 for 24 hours. Media was harvested and analyzed for the expression of cytokine via ELISA. The expression of IL-10 in C3H/HeJ (A) and Balb/c (B) IL-12 treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown (pg/ml). (n = 9; ***p < 0.001).

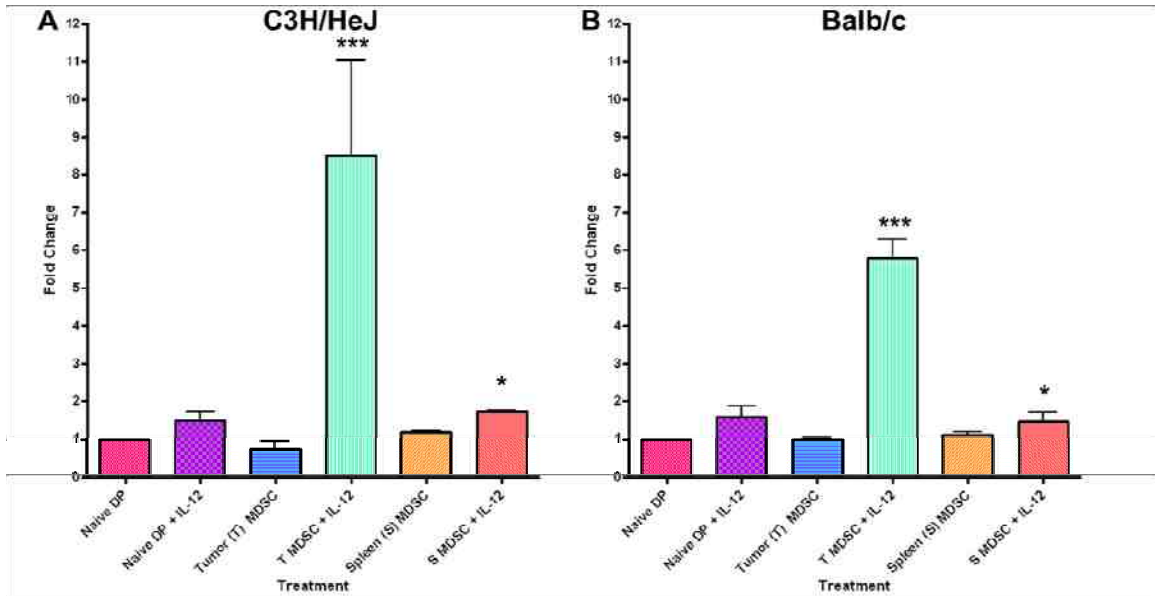


Figure 20. *In vitro* treatment with IL-12 induces TNF- α mRNA expression. Cells were stained and RNA extracted as described previously (Figure 14). The expression levels for TNF- α were normalized to the expression levels from naïve double-positive cells and graphed as a fold change. The expression of TNF- α in C3H/HeJ (A) and Balb/c (B) IL-12 treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. (n = 9; * p < 0.05; *** p < 0.001).

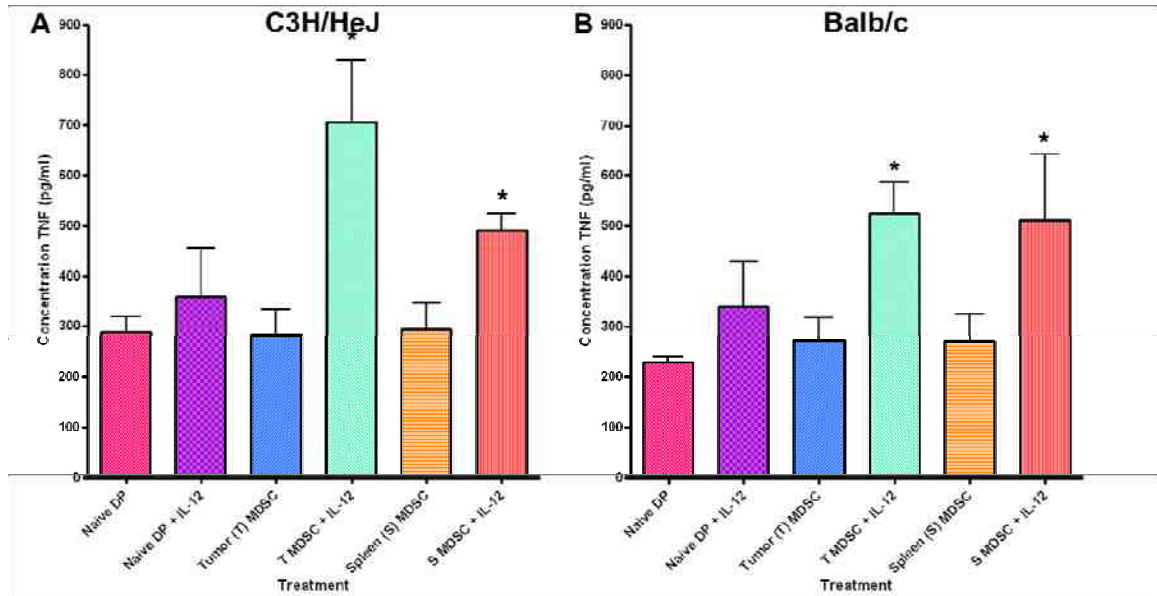


Figure 21. *In vitro* treatment with IL-12 induces TNF- α protein expression from MDSC. Cells were obtained and treated as described previously (Figure 19). The expression of TNF- α in C3H/HeJ (A) and Balb/c (B) IL-12 treated and untreated Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown (pg/ml). (n = 9; *p < 0.05).

IL-12 induces MDSC up-regulation of maturation markers in vivo

In order to determine whether all of these results correlate with an *in vivo* response to IL-12 treatment, analysis of *in vivo* treated cells was performed. C3L5 and 4T1 tumors were generated in the mammary fat pads of C3H/HeJ and Balb/c animals respectively. Once tumors grew to an average of 300 mm³, intramuscular injections with adenovirus expressing either luciferase or recombinant IL-12 were performed. Twenty-four hours after injection, tumors and spleens were harvested and single cells suspensions obtained for further analysis. Serum analysis was used to confirm IL-12 expression. Figure 22 represents IL-12 expression prior to and following treatment with the viruses. Only animals treated with adenovirus expressing recombinant IL-12 demonstrated a systemic increase in IL-12 expression. These expression levels on the average were similar to the levels used *in vitro* though with considerably more variation than the *in vitro* studies.

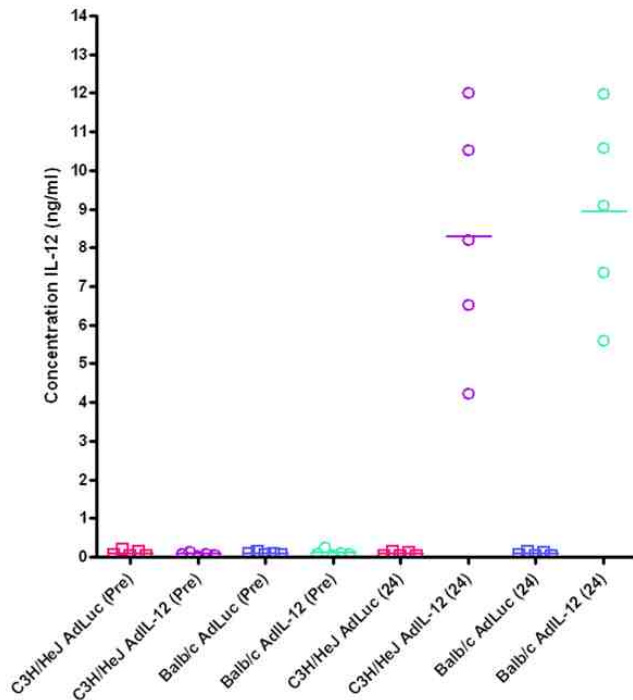


Figure 22. Serum IL-12 expression 24 hours after injection with AdLuc or AdIL-12. C3H/HeJ and Balb/c animals were injected with 2.5×10^5 C3L5 and 1×10^5 4T1 cells respectively. Once tumors reached an average size of 300 mm³ animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a

luciferase reporter gene (AdLuc) or recombinant mouse IL-12 (AdIL-12) (5 animals per group). Serum was harvested immediately prior to and 24 hours after injection for analysis of IL-12 expression. The concentration of IL-12 expressed in the serum of adenovirus-treated animals was determined via ELISA.

Using identical staining protocols as those used for the *in vitro* studies, treatment with IL-12 was found to be capable of altering the expression of all 4 markers studied regardless of whether they were spleen-derived or tumor-derived MDSC (Table 3). The ability to see changes in CD80 and CD86 expression for the tumor-derived cells *in vivo* indicates a discrepancy between treatments that can be explained by the additional *in vivo* cellular interactions. It is possible that there may be alterations of the cells caused by *in vitro* culturing but it is more probable that the presence of other cells capable of responding to IL-12 altered the overall effects of the treatment. For example, T cells can have co-stimulatory effects on macrophages and dendritic cells and they also respond to IL-12 when previously activated by antigen. It is probable that IL-12 is acting not only on the MDSC but also on stimulated T cells to promote further maturation and proinflammatory activation of the MDSC.

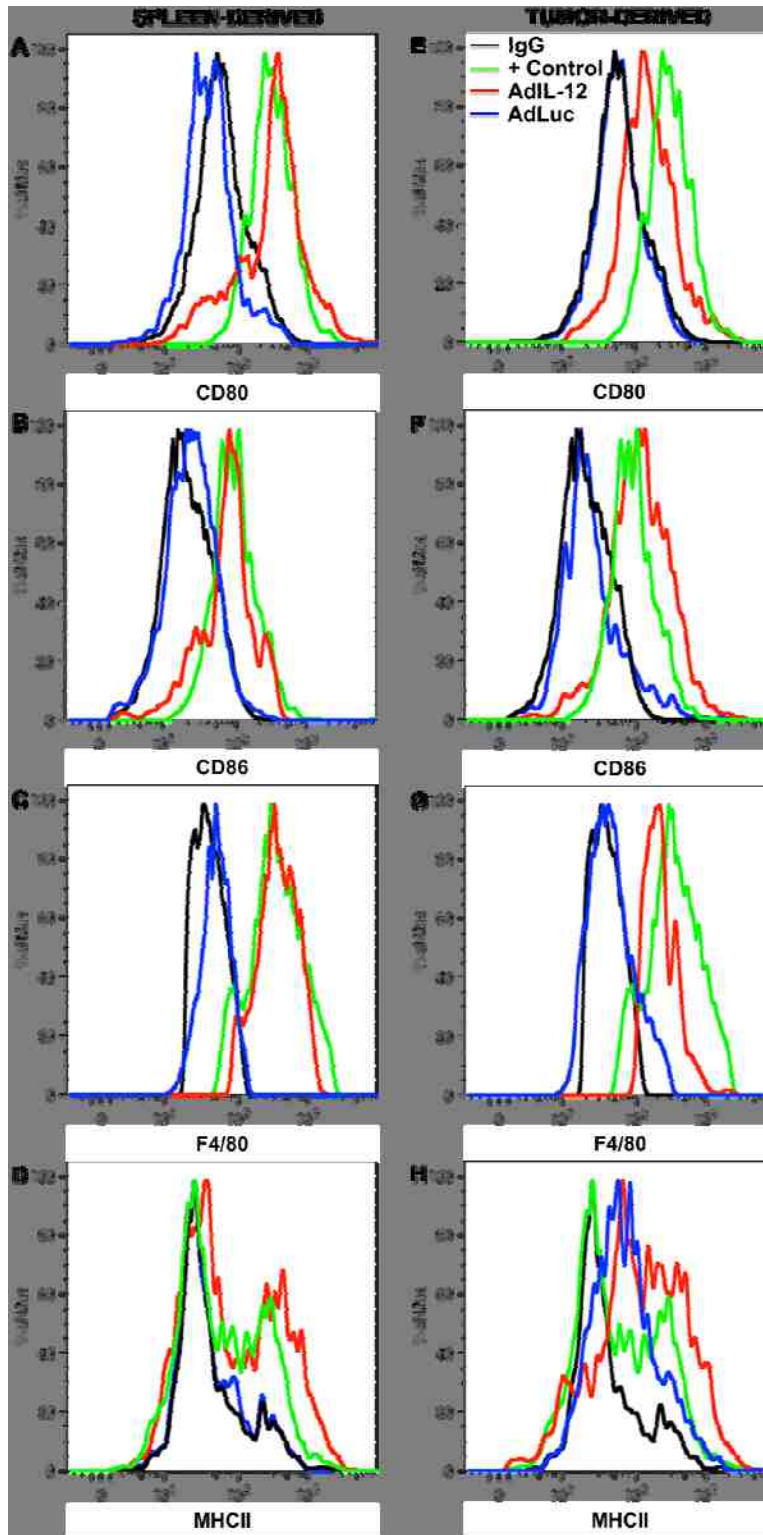


Figure 23. *In vivo* treatment with AdIL-12 results in the up-regulation of surface markers on C3H/HeJ C3L5 tumor-bearing animals spleen- and tumor-derived Gr-1/CD11b double positive cells. C3H/HeJ animals were injected with 2.5×10^5 C3L5 cells. Once tumors reached an average size of 300 mm^3 the animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) or IL-12 (AdIL-12) (5 animals per group). 24 hours after the injection animals were euthanized and tissues were harvested for further analysis. Single cell suspensions of spleens and tumors from adenovirus-treated tumor-bearing animals were obtained. Cells were stained with $2 \mu\text{g/ml}$

fluorochrome-conjugated anti-IgG, anti-Gr-1, anti-CD80, anti-F4/80, anti-CD86, and anti-MHCII antibodies. Surface expression of the marker was determined by flow cytometry. Histograms representing triplicate staining for the expression of CD80 on spleen-derived

(A) and tumor-derived (E), F4/80 on spleen-derived (B) and tumor-derived (F). CD86 on spleen-derived (C) and tumor-derived (G), and MHCII for spleen derived (D) and tumor-derived (H) Gr-1/CD11b double positive cells are shown. The histogram analysis includes expression of each marker on the surface of whole splenocyte positive controls (green line), IgG stained negative controls (black line), AdLuc treated Gr-1/CD11b double positive cells (blue line) and AdIL-12 treated Gr-1/CD11b double positive cells (red line). The graphs are representative of three individual experiments performed in triplicate (n = 9). Results are summarized in table 3.

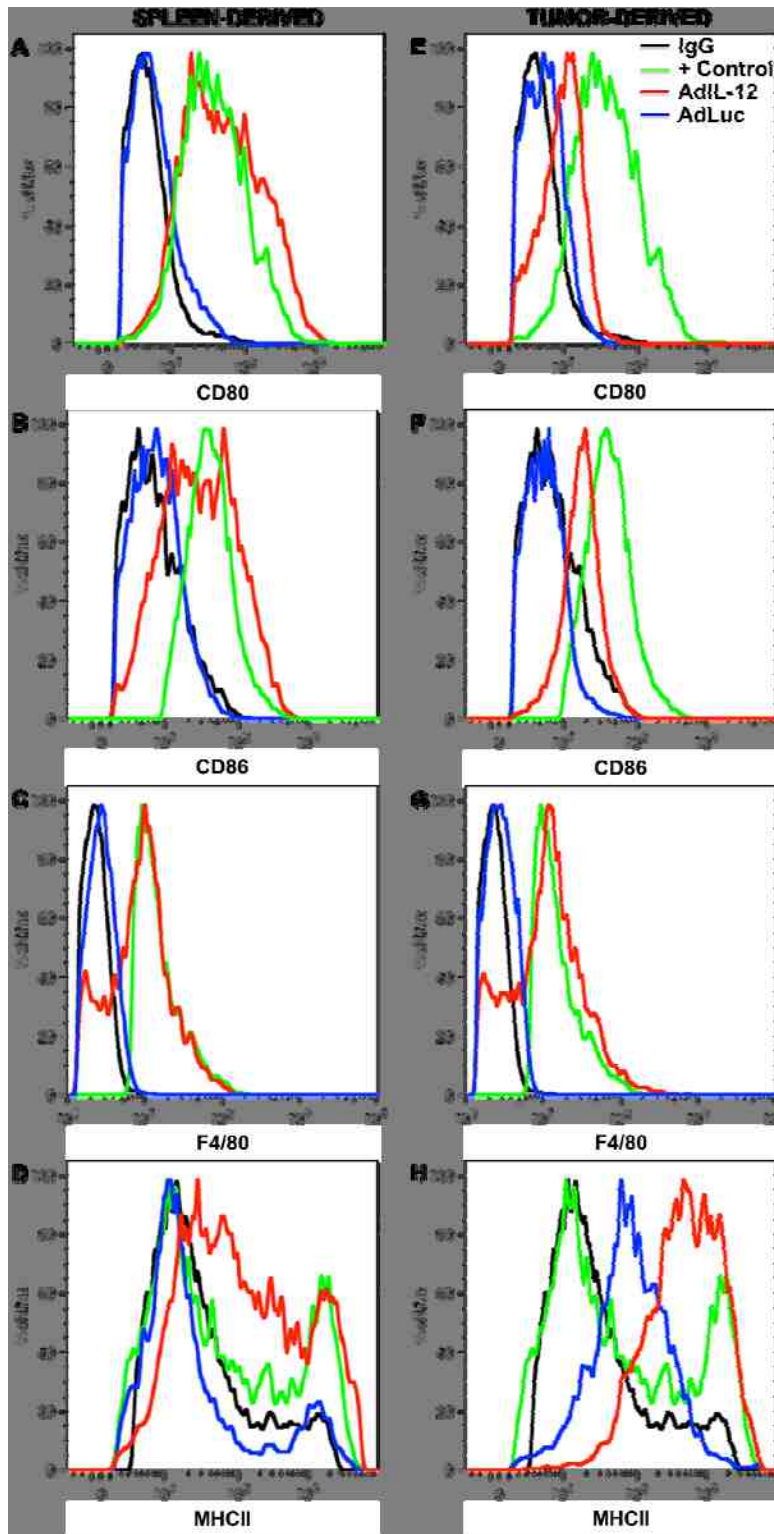


Figure 24. *In vivo* treatment with AdIL-12 results in the up-regulation of surface markers on Balb/c 4T1 tumor-bearing animals spleen- and tumor-derived Gr-1/CD11b double positive cells. Balb/c animals were injected with 1×10^5 4T1 cells. Animals were treated and cells harvested as described previously (Figure 22). Cells were stained with 2 $\mu\text{g}/\text{ml}$ fluorochrome-conjugated anti-IgG, anti-Gr-1, anti-CD80, anti-F4/80, anti-CD86, and anti-MHCII antibodies. Surface expression of the marker was determined by flow cytometry. Histograms representing triplicate staining for the expression of CD80 on spleen-derived (A) and tumor-derived (E), F4/80 on spleen-derived (B) and tumor-derived (F), CD86 on spleen-derived (C) and tumor-derived (G), and MHCII for spleen derived (D) and tumor-derived (H) Gr-1/CD11b double positive cells are shown. The histogram analysis includes expression of each marker on the surface of whole splenocyte positive

controls (green line), IgG stained negative controls (black line), AdLuc treated Gr-1/CD11b double positive cells (blue line) and AdIL-12 treated Gr-1/CD11b double positive cells (red line). The graphs are representative of three individual experiments performed in triplicate (n = 9). Results are summarized in table 3.

<u>Cell Type</u>	<u>CD80</u>	<u>CD86</u>	<u>F4/80</u>	<u>MHCII</u>
C3H/HeJ				
Tumor-Bearing Spleen MDSC + AdLuc	-	-	-	-
Tumor-Bearing Spleen MDSC + AdIL-12	++	++	++	+++
Tumor MDSC + AdLuc	-	-	-	+
Tumor MDSC + AdIL-12	+	++	++	+++
Balb/c				
Tumor-Bearing Spleen MDSC + AdLuc	-	-	-	-
Tumor-Bearing Spleen MDSC + AdIL-12	++	++	++	+++
Tumor MDSC + AdLuc	-	-	-	+
Tumor MDSC + AdIL-12	+	+	++	+++

Table 3. Summary of the expression of surface markers following *in vivo* analysis. In this table, (-) designates not expressed, (+) designates expressed at levels below positive controls, (++) designates expressed at levels equal to positive controls, and (+++) designates expressed at levels exceeding positive controls.

***In vivo* AdIL-12 treatment reduces the expression of Nos2, Arg1, and IFN- γ mRNA in MDSC**

In order to confirm whether the other significant internal changes in MDSC can be replicated *in vivo*, MDSC from AdLuc and AdIL-12 treated animals as well as Gr-1/CD11b double positive cells from naïve animals were sorted using the same protocols for the *in vitro* studies. Following RNA extraction and conversion into cDNA, quantitative real-time PCR analysis was performed.

The expression of Nos2 (Figure 25), IFN- γ (Figure 26), and Arg1 (Figure 27) mRNA were found to be significantly higher in MDSC compared to naïve Gr-1/CD11b double positive cells. The mRNA for all three molecules was found to decrease following *in vivo* treatment with AdIL-12. The expression patterns were consistent with the *in vitro* studies though the fold increase in MDSC expression of Nos2 and IFN- γ mRNA above that of naïve Gr-1/CD11b double positive cells was found to be higher *in vivo* for the Balb/c animals than in the *in vitro* studies (Figures 25 B and 26 B). Balb/c tumor-derived MDSC exhibit approximately 9 fold higher expression of Nos2, 20 fold higher expression of IFN- γ and 8 fold higher expression of Arg1 than naïve Gr-1/CD11b double positive cells (Figures 25 B, 26 B and 27 B). C3H/HeJ tumor-derived MDSC and spleen derived exhibit comparable levels of mRNA *in vitro* and *in vivo* with the *in vivo* studies indicating approximately 4 fold and 1.5 higher expression of Nos2, roughly 15 fold and 3 fold higher expression of IFN- γ and, 3 fold and 2.5 fold higher expression of Arg1 compared to naïve Gr-1/CD11b double positive cells (Figures 25 A, 26 A, and 27A). The changes in mRNA and surface marker expression observed in both the tumor-derived and spleen-derived MDSC harvested from the AdIL-12 treated animals implies a systemic effect of IL-12 is possible in cases of intramuscular injection. This consistency between *in vivo* and *in vitro* treatment with IL-12 indicates a new role for IL-12 in terms of its ability to induce up-regulation of maturation markers on the surface of MDSC, decrease key regulators of MDSC activity, and modulate immune suppression.

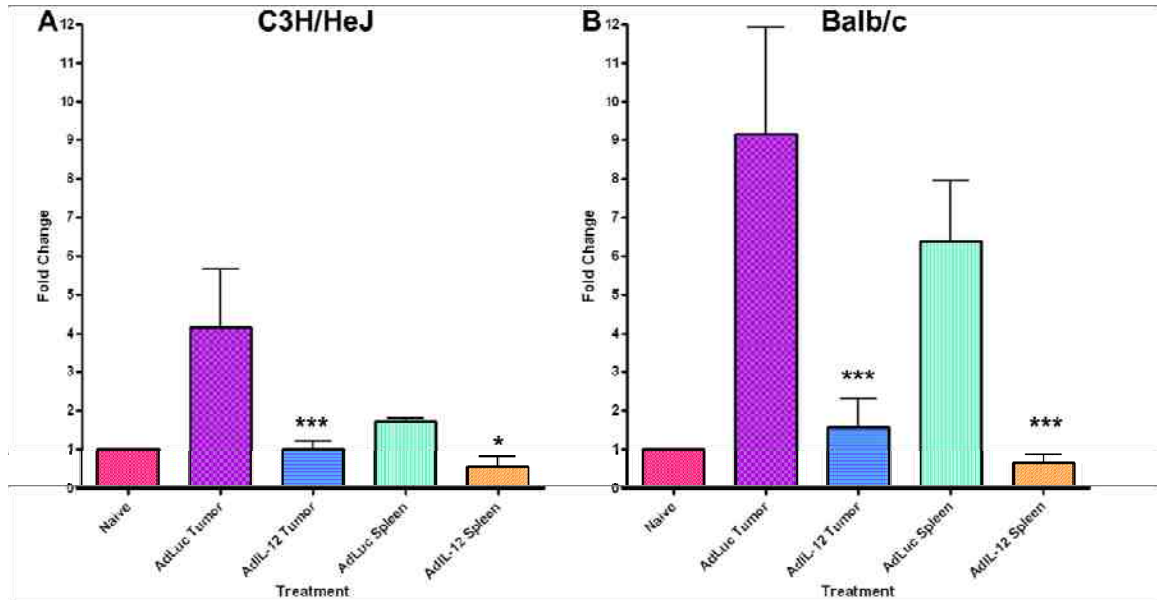


Figure 25. *In vivo* treatment with AdIL-12 reduces *Nos2* mRNA expression. C3H/HeJ and Balb/c tumor-bearing animals were treated with intramuscular injections of 1×10^9 adenovirus particles of either AdLuc or AdIL-12. Twenty-four hours after inoculation with virus, tumors were harvested and digested to obtain single cell suspensions. Gr-1/CD11b double positive cells were stained using fluorochrome-conjugated antibodies and sorted from the spleens and tumors of AdLuc or AdIL-12 treated animals. RNA was extracted from sorted cell populations, converted to cDNA and analyzed for mRNA expression via Real Time PCR. The expression levels for *Nos2* and $\text{INF-}\gamma$ were normalized to expression from naïve double-positive cells and graphed as a fold change. The expression of *Nos2* in C3H/HeJ (A) and Balb/c (B) and expression of $\text{INF-}\gamma$ in C3H/HeJ (C) and Balb/c (D) are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 (* $p < 0.05$; *** $p < 0.001$).

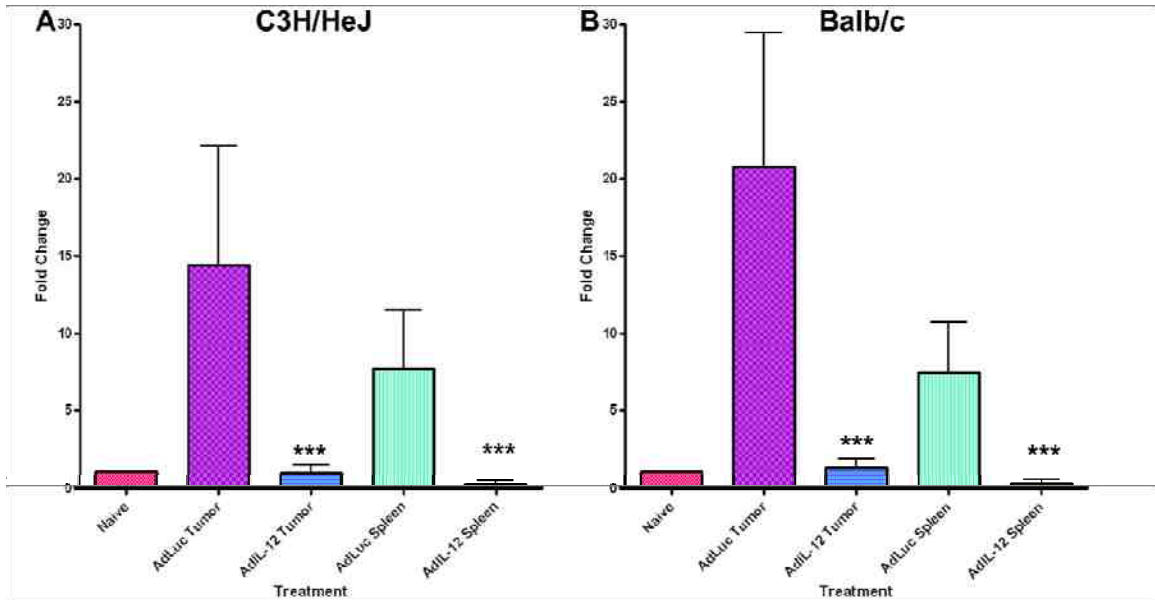


Figure 26. *In vivo* treatment with AdIL-12 reduces IFN- γ mRNA expression. Cells were generated, isolated and RNA extracted as described previously (Figure 25). The expression of IFN- γ mRNA in C3H/HeJ (A) and Balb/c (B) Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 (***) ($p < 0.001$).

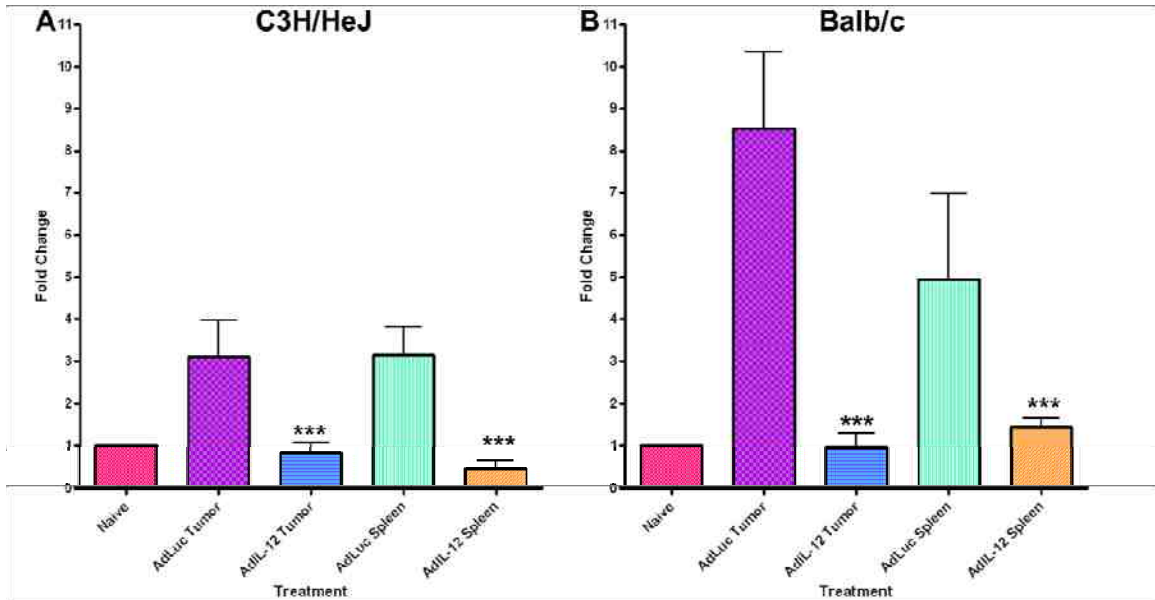


Figure 27. *In vivo* treatment with AdIL-12 reduces Arg1 mRNA expression. Cells were generated, isolated and RNA extracted as described previously (Figure 25). The expression of Arg1 mRNA in C3H/HeJ (A) and Balb/c (B) Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 (** $p < 0.001$).

***In vivo* AdIL-12 treatment reduces IL-10 production and increases TNF- α production predominantly from tumor-derived MDSC**

In order to continue to define the role that *in vivo* treatment of MDSC with IL-12 has on overall MDSC activity, we sought to determine whether the same M2 suppressive cytokine, IL-10, and M1 activating cytokine, TNF- α , were also altered following treatment with AdIL-12. Using the same experimental conditions as was used to study Nos2, IFN- γ , and Arg1, we determined the changes in expression of IL-10 (Figure 28) and TNF- α (Figure 29) mRNA following treatment with AdIL-12. As expected from the *in vitro* results, MDSC isolated from tumor-bearing animals treated with AdIL-12 exhibited decreased expression of IL-10 mRNA and increased expression of TNF- α mRNA. This was regardless of whether the cells were isolated from C3H/HeJ (Figures 28 and 29 A) or Balb/c (Figures 28 and 29 B) treated animals.

C3H/HeJ MDSC animals treated with AdLuc were found to express 7 fold higher and 4 fold higher levels of IL-10 in the tumor-derived and spleen-derived MDSC, respectively, which was reduced to the levels exhibited by naïve Gr-1/CD11b double positive cells if the cells received treatment with AdIL-12 (Figure 28 A). Balb/c AdLuc treated MDSC were found to exhibit 6 fold and 3 fold higher levels of IL-10 in tumor-derived and spleen-derived MDSC, respectively (Figure 28 B). These levels were also reduced to the levels of expression for naïve Gr-1/Cd11b double positive cells following treatment with AdIL-12. Similar to *in vitro* studies MDSC from AdLuc treated tumor-bearing animals did not express TNF- α at levels above those of naïve Gr-1/CD11b double positive cells regardless of tissue type or whether they were derived from C3H/HeJ (Figure 29 A) or Balb/c (Figure 29 B) animals.

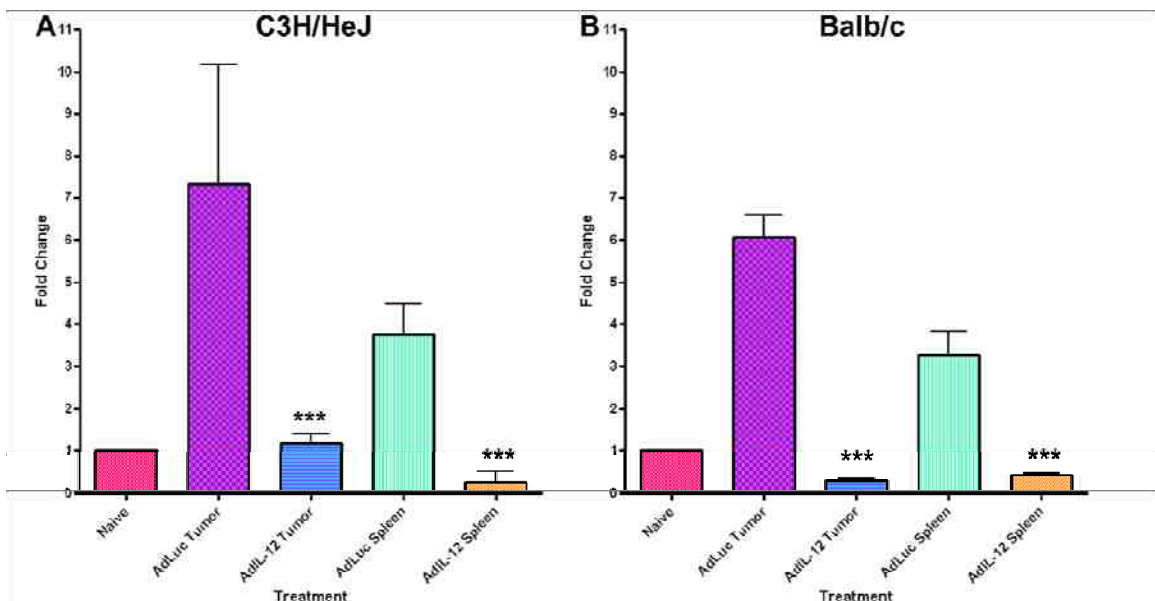


Figure 28. *In vivo* treatment with AdIL-12 reduces IL-10 mRNA expression. Cells were generated, isolated and RNA extracted as described previously (Figure 25). The expression of IL-10 mRNA in C3H/HeJ (A) and Balb/c (B) Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 ($***p < 0.001$).

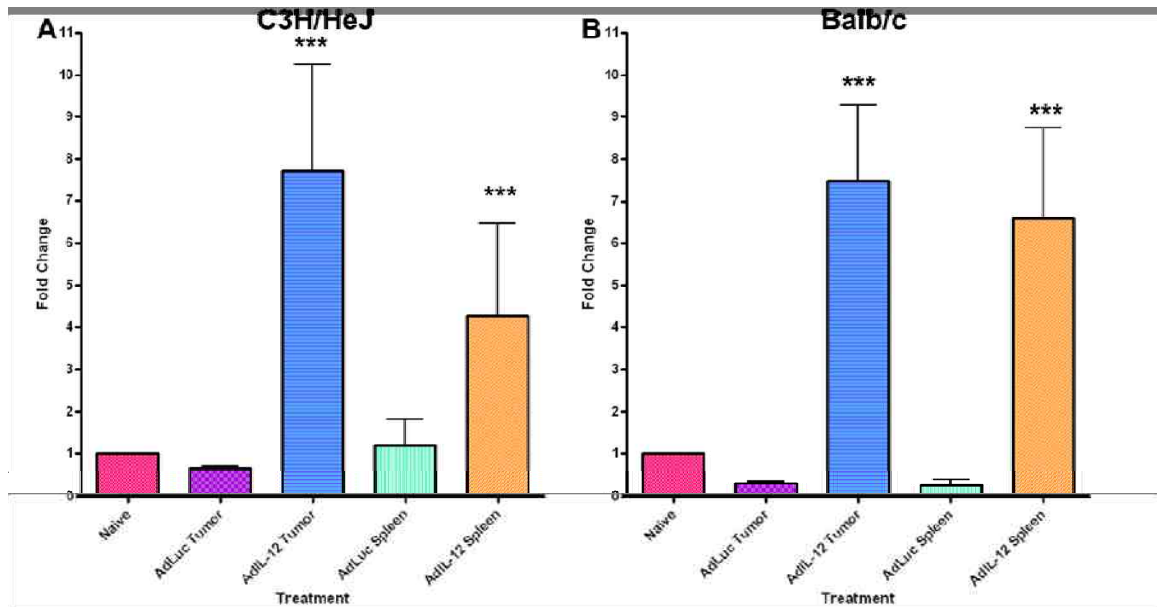


Figure 29. *In vivo* treatment with AdIL-12 induces TNF- α mRNA expression. Cells were generated, isolated and RNA extracted as described previously (Figure 25). The expression of TNF- α mRNA in C3H/HeJ (A) and Balb/c (B) Gr-1/CD11b double positive cells from spleens of both naïve and tumor-bearing animals as well as tumor-derived cells are shown. Statistically significant reductions in mRNA expression were observed following treatment with IL-12 (** $p < 0.001$).

Treatment of C3L5 and 4T1 tumor-bearing animals with AdIL-12 results in a reduction in tumor growth and enhanced overall survival

The studies performed thus far defined a new activity for IL-12 to act directly on immune suppressor cells altering their activity. These findings indicate that IL-12 reverses immune suppression which may permit immune activation in the tumor leading to suppression of tumor growth. In order to determine what effect the alteration of MDSC could have in terms of breast cancer therapeutics, we studied the anti-tumor therapeutic efficacy of treatment with AdIL-12. Throughout the *in vitro* and *in vivo* studies of the effects of IL-12, orthotopic murine breast cancer models were used to both generate the cells of interest and to study the *in vivo* effects on the MDSC. These models were generated by direct injection of the cells into mammary fat pads. In the previous *in vivo* studies, tumors were allowed to develop to above 300 mm³ so that there would be significant MDSC levels for analysis. In the following studies we were interested in

overall anti-tumor efficacy and long-term effects so tumors were injected at an average size of approximately 65 mm³.

C3L5 and 4T1 tumor-bearing animals were treated with AdIL-12 or AdLuc via intramuscular injections of 1×10^9 virus particles per animal (10 animals per group). Serum was extracted immediately prior to injection with the virus as well as 24, 48, and 72 hours after injection. One C3H/HeJ animal was lost due to overdose of ketamine prior to injection with virus and was eliminated from the study. Although the *in vivo* experiments were performed multiple times, there was a tendency to lose animals during serum extractions. Serum protein expression of both IL-12 and IFN- γ were determined via ELISA (Figure 30). Only AdIL-12 treated animals were found to express increased levels of IL-12 expression following injection with the recombinant adenovirus. This demonstrates that the expression of IL-12 is specific to the IL-12 expression cassette and not an effect of anti-viral immune activation.

Expression of IL-12 was found to peak 24 hours after treatment with AdIL-12 and return to basal levels by 72 hours after treatment for both C3H/HeJ (Figure 30 A) and Balb/c (Figure 30 B) tumor-bearing animals. Expression of IFN- γ was not observed above basal levels until 72 hours after treatment for both C3H/HeJ (Figure 30 C) and Balb/c (Figure 30 D) AdIL-12 treated, tumor-bearing animals. IFN- γ levels were found in past experiments to peak at 96 hours and return to basal levels by 7 days, though this was not determined for these experiments (data not shown). The levels of IL-12 from the *in vivo* experiments on the average were around the levels used for all the *in vitro* studies. It is important to note that these are relatively low levels compared to other IL-12 *in vivo* studies that quantified serum IL-12 levels described previously (224, 260-266). Analysis of animal weight over time and examination of livers did not indicate a significant difference between AdLuc and AdIL-12 treated animals (data not shown). This implies a decrease in risk of toxic side-effects from this adenovirus-mediated IL-12 expression compared to those observed following treatment with IL-12 in previously discussed studies.

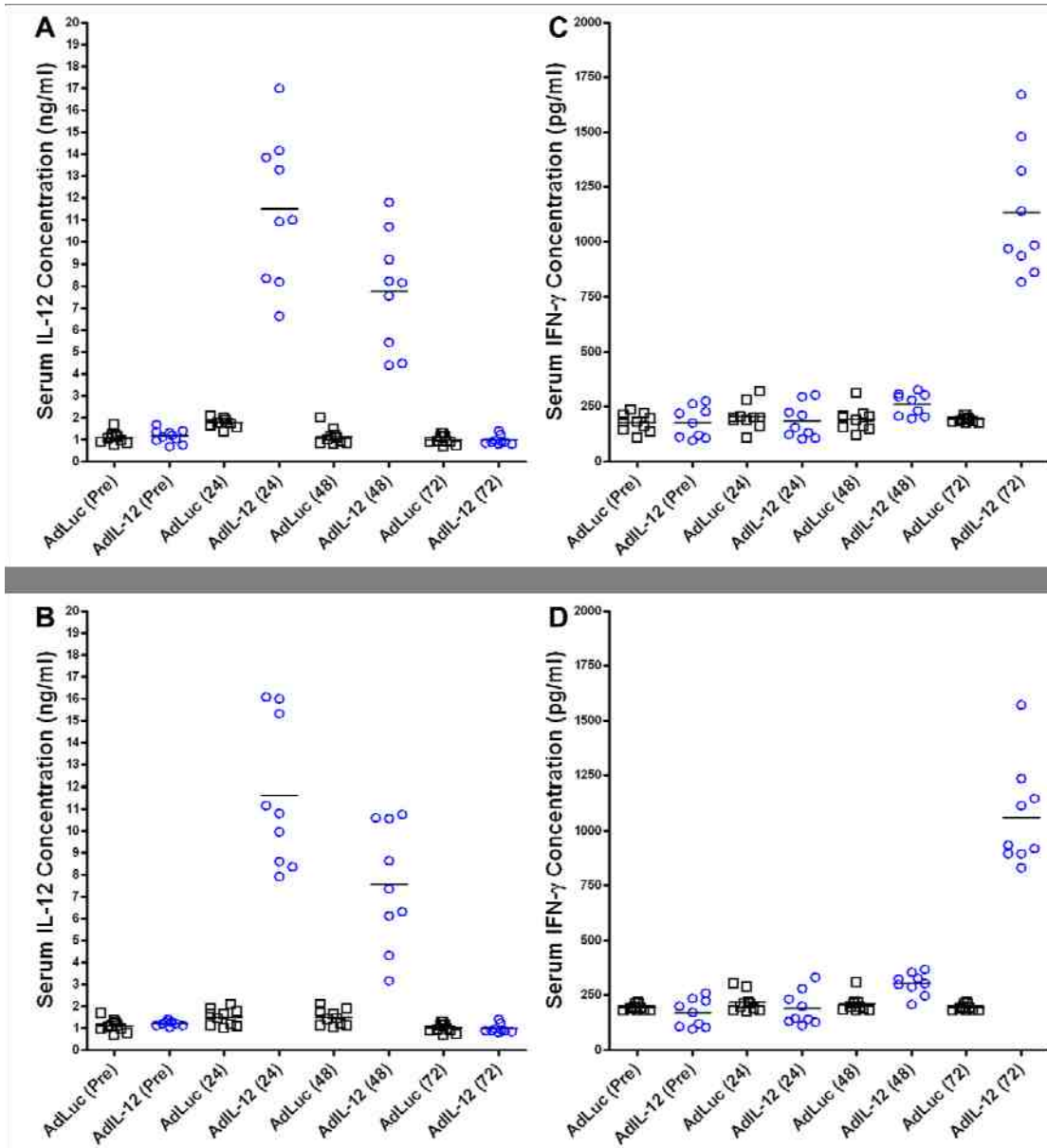


Figure 30. *AdIL-12 treatment induces serum expression of IL-12 and IFN- γ .* C3H/HeJ and Balb/c animals were injected with 2.5×10^5 C3L5 and 1×10^5 4T1 cells respectively. Once tumors reached an average size of 65 mm^3 animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) or recombinant mouse IL-12 (AdIL-12) (9 or 10 animals per group). Serum was harvested immediately prior to injection as well as 24, 48 and 72 hours after injection for analysis of IL-12 and IFN- γ expression via ELISA. One C3H/HeJ animal received an accidental overdose prior to injection with AdIL-12 and was therefore eliminated from the study. The concentration of IL-12 expressed in the serum of

C3H/HeJ (A) and Balb/c (B) adenovirus-treated animals was determined via ELISA and is represented in ng/ml. The concentration of IFN- γ expressed in the serum of C3H/HeJ (C) and Balb/c (D) adenovirus-treated animals was also determined via ELISA and is represented in pg/ml.

In order to determine whether this exogenous expression of IL-12 had an impact on tumor growth, tumors were measured twice weekly until they grew to a volume of approximately 500mm³. Tumor growth for C3H/HeJ C3L5 tumor-bearing animals are presented in figure 31 while tumor growth for Balb/c 4T1 tumor-bearing animals is presented in figure 32. Treatment with AdIL-12 was found to suppress tumor growth (Figures 31 and 32 B) with a significant increase in overall survival for both animal models studied (Figure 33). C3H/HeJ C3L5 tumor-bearing animals were found to have a better overall response to treatment with AdIL-12 including one animal that demonstrated complete tumor regression as evidenced by the normal appearance of the mammary fat pad (Figure 31 and Figure 33 A; data not shown). This difference in treatment response could be due to differences in overall aggressiveness of the C3L5 tumors compared to the 4T1 tumors. The effects of treatment with AdIL-12 are specific to IL-12, as AdLuc treatment did not suppress tumor growth (Figure 31 A and Figure 32 A). Overall, a single-dose, intramuscular treatment with AdIL-12 is capable of altering tumor growth leading to significant improvements in overall survival. Results are summarized in table 4.

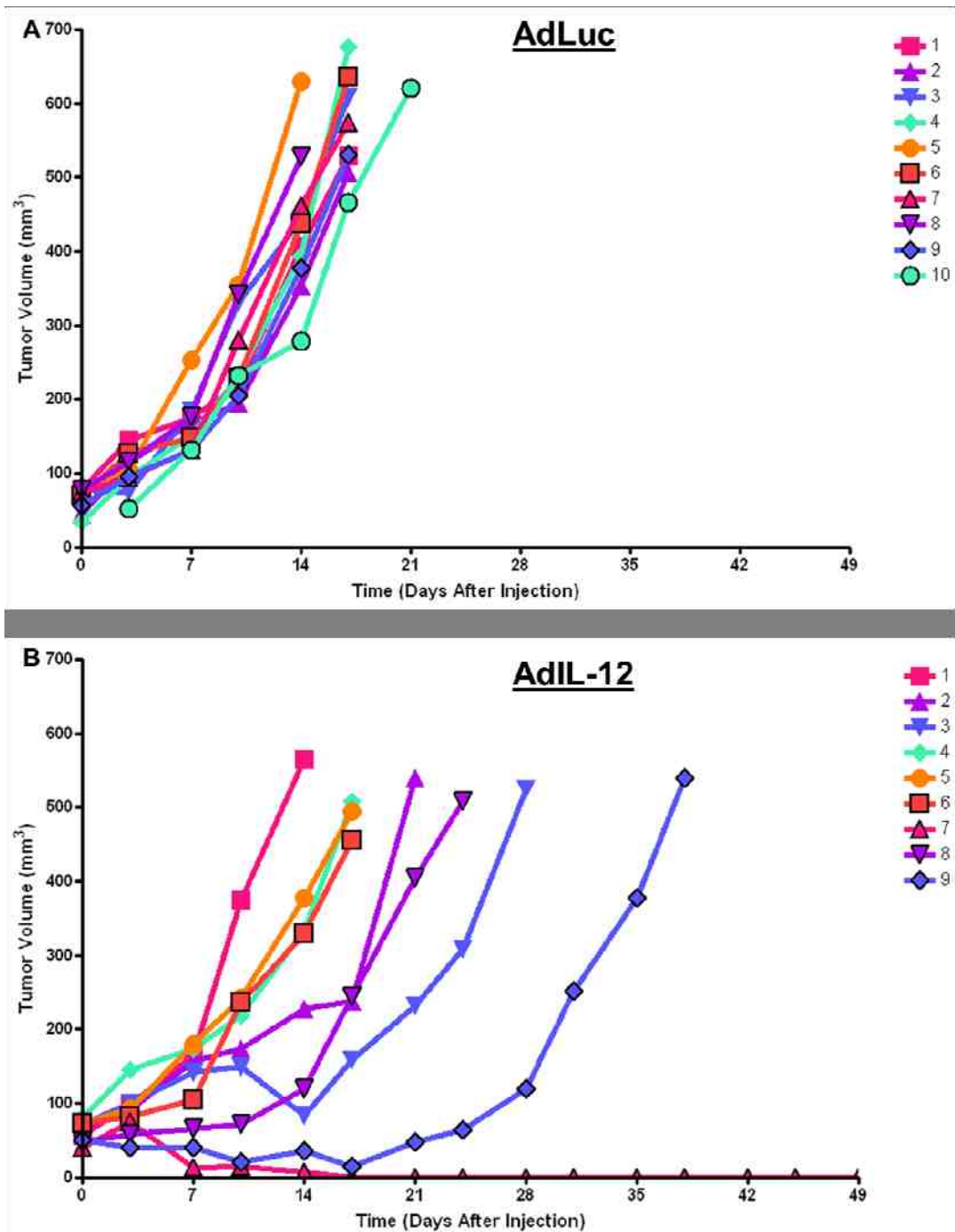


Figure 31. C3L5 tumor growth following AdLuc and AdIL-12 treatment of C3H/H3J tumor-bearing animals. C3H/HeJ murine fat pads were injected with 2.5×10^5 C3L5 cells. Once tumors reached an average size of 65 mm^3 animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) (A) or recombinant mouse IL-12 (AdIL-12) (B). Tumor growth was measured twice weekly.

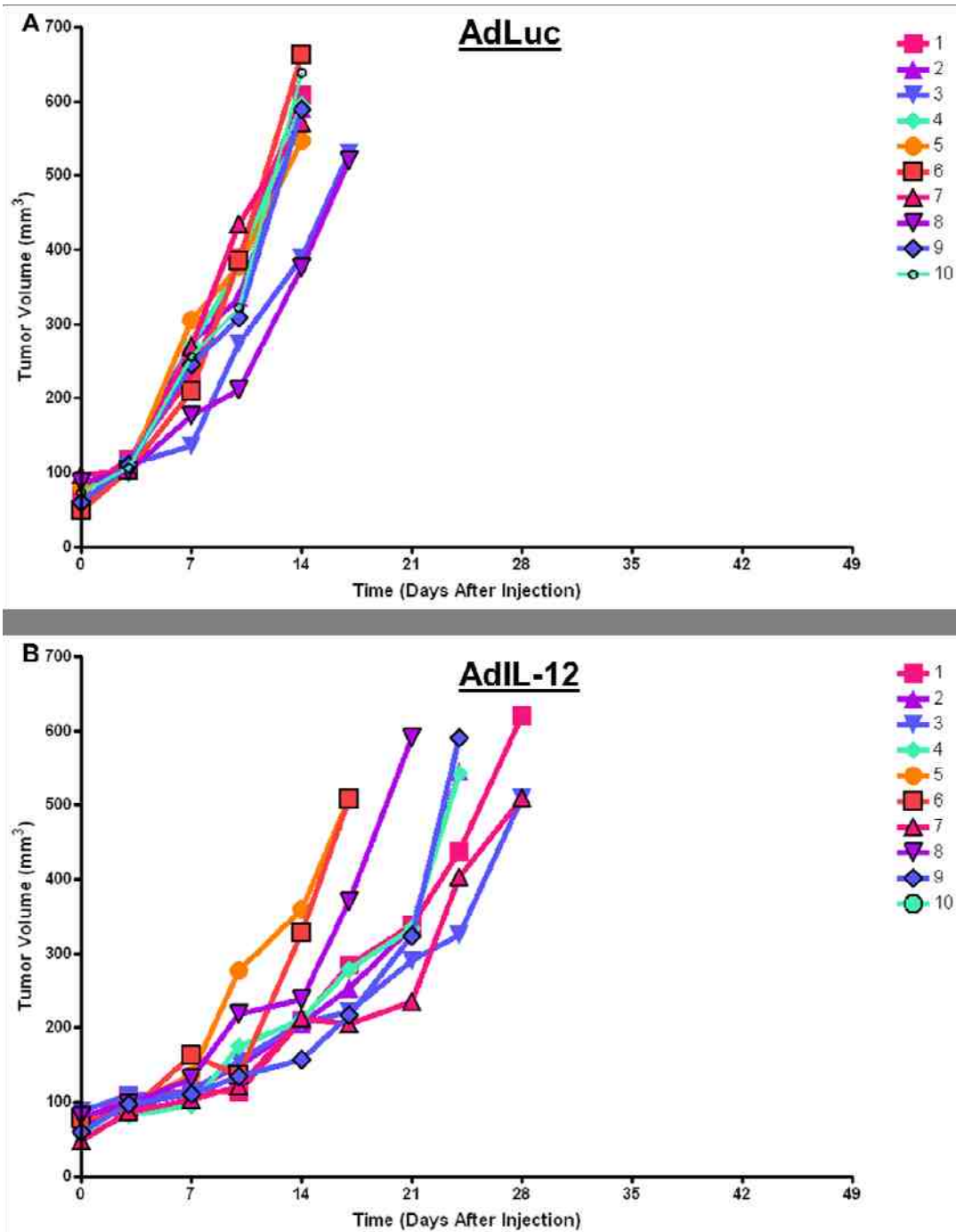


Figure 32. 4T1 tumor growth following AdLuc and AdIL-12 treatment of Balb/c tumor-bearing animals. Balb/c murine fat pads were injected with 1×10^5 4T1 cells. Once tumors reached an average size of 65 mm^3 animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) (A) or recombinant mouse IL-12 (AdIL-12) (B) (10 animals per group). Tumor growth was measured twice weekly.

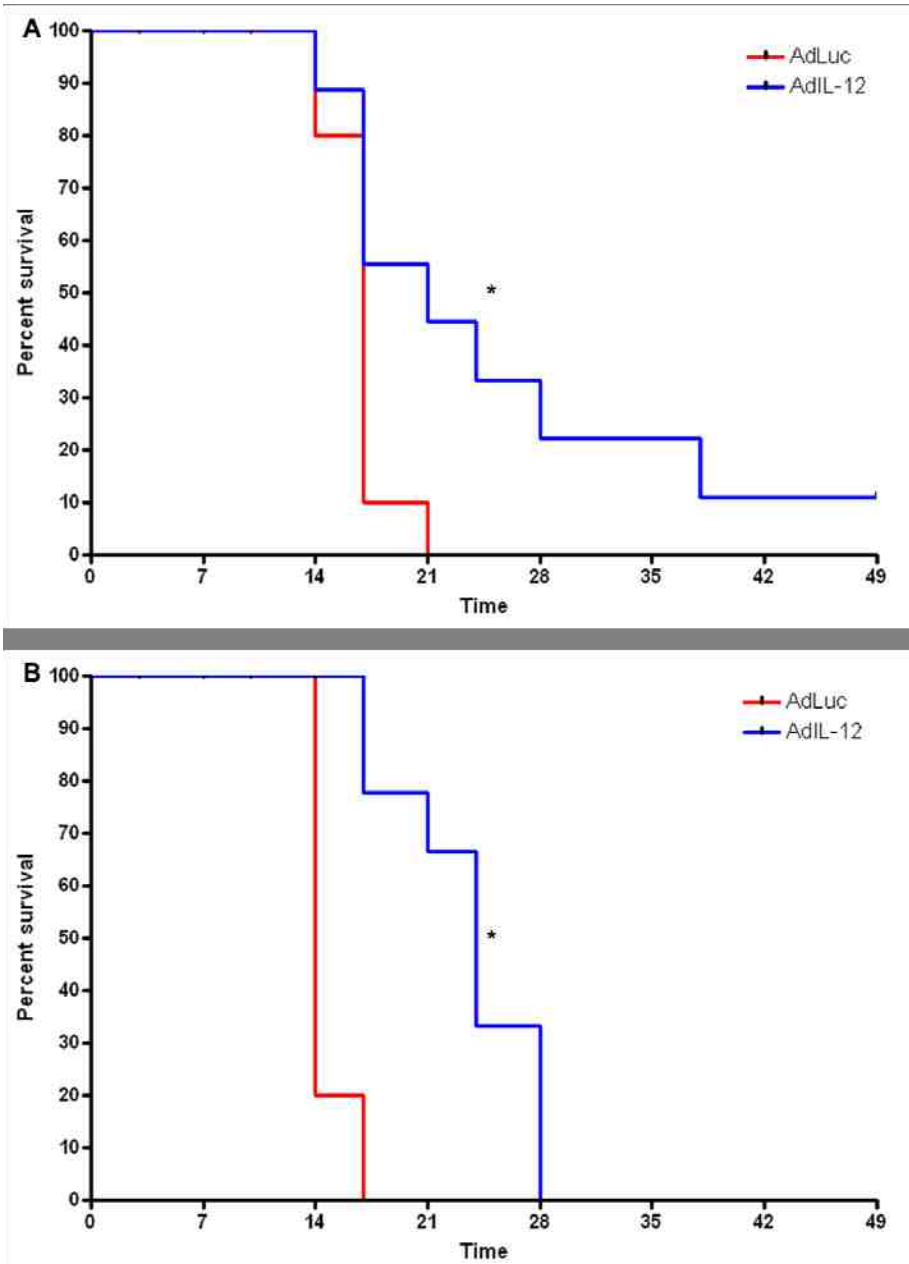


Figure 33. Overall survival of C3H/HeJ and Balb/c tumor-bearing animals following treatment with AdLuc and AdIL-12. C3H/HeJ (A) and Balb/c (B) animals in figure 31 and 32, respectively, were analyzed for overall survival with tumor volumes of 500 mm³ as the endpoint and counted as deaths (no actual deaths occurred) (**p* < 0.05).

	#/Response	Tumor Volume ~ 65 mm ³	Tumor Volume ~ 350 mm ³	Tumor Volume ~ 500 mm ³	Euthanized
C3H/HeJ AdLuc	2/NR	10	20	24	24
C3H/HeJ AdLuc	7/NR	10	20	27	27
C3H/HeJ AdLuc	1/NR	10	24	31	31
C3H/HeJ AdIL-12	1/NR	10	20	24	24
C3H/HeJ AdIL-12	3/NR-PR	10	24	27	27
C3H/HeJ AdIL-12	4/PR	10	27, 27, 34, 45	31, 34, 38, 48	31, 34, 38, 48
C3H/HeJ AdIL-12	1/CR	10	n/a	n/a	59
Balb/c AdLuc	8/NR	10	20	24	24
Balb/c AdLuc	2/NR	10	24	27	27
Balb/c AdIL-12	2/NR	10	24	27	27
Balb/c AdIL-12	1/NR-PR	10	27	31	31
Balb/c AdIL-12	4/PR	10	31	34	34
Balb/c AdIL-12	4/PR	10	31, 34, 34	38	38

Table 4. *AdIL-12 alters tumor growth and improves overall survival.* This table summarizes data in figures 31 and 32. It indicates days after inoculation with C3L5 and 4T1 cells in C3H/HeJ and Balb/c animals, respectively. (NR designates no response; PR designates partial response; CR designates complete response)

Treatment with AdIL-12 results in a long-term increase in leukocyte infiltration into the tumor microenvironment compared to AdLuc treatment

Treatment of tumor-bearing animals with AdIL-12 delayed tumor growth, but the mechanism of that delay and its relationship with alterations in immune suppression remain to be determined. The range of effect following treatment with AdIL-12 could be due to differences in IL-12 expression though these differences do not account for the large variations in tumor growth observed in C3H/HeJ C3L5 tumor-bearing animals. Although this study does not seek to answer this question directly, an inference can be made from observations of the changes in both the composition of lymphocytes in the tumor microenvironment and MDSC populations following treatment with AdIL-12.

In order to determine whether treatment with AdIL-12 was altering the tumor microenvironment in general, histological analysis of tumor sections was performed. Tumors from AdLuc and AdIL-12 treatment groups were embedded as frozen tissue, sectioned, and attached to slides for histological analysis. General light microscopy was used to generate images of the tumor histology following staining procedures for visualization of structures. C3H/HeJ C3L5 AdLuc (Figure 34 A and B) and AdIL-12 (Figure 34 C and D) treated tumors were analyzed. AdIL-12 treatment was found to exhibit an increase in leukocyte infiltration into the tumor compared to AdLuc controls. This finding was consistent with Balb/c 4T1 AdIL-12 treated tumors (Figure 35 C and D) compared to AdLuc treated tumors (Figure 35 A and B) as well. In order to quantify the results obtained from the analysis of tissue sections, single cell suspensions were obtained separately and cells were stained for the general hematopoietic cell marker, CD45. The percentage of CD45 positive cells infiltrating the tumor was found to increase following treatment with AdIL-12 for both tumor models (Figure 33 B and D; Figure 34 B and D).

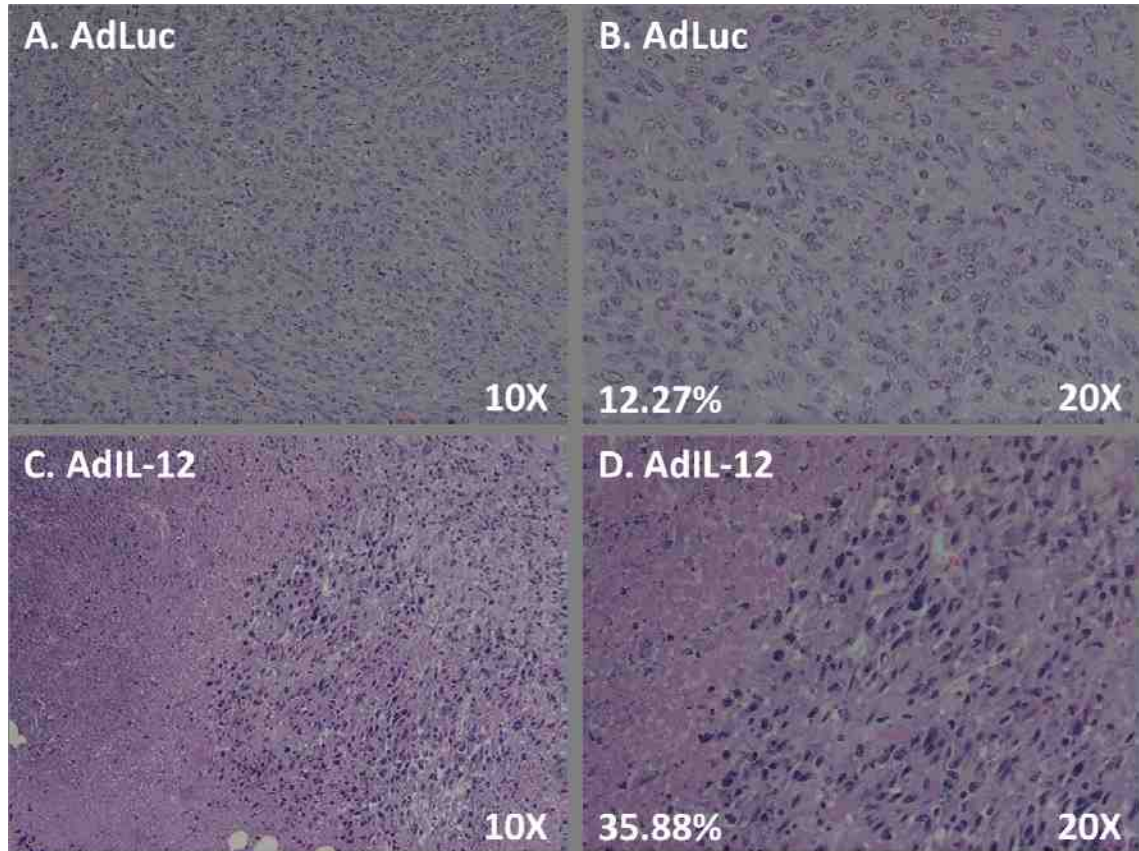


Figure 34. C3H/HeJ C3L5 Tumor Histology. C3H/HeJ murine fat pads were injected with 2.5×10^5 C3L5 cells. Once tumors reached an average size of 65 mm^3 animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) (A 10X; B 20X) or recombinant mouse IL-12 (AdIL-12) (C 10X; D 20X). Tumor growth was measured twice weekly. Once tumors reached a volume of approximately 500 mm^3 animals were humanely euthanized and tissues were harvested. Tumors were cut into three sections with one section preserved in OTC embedding compound and frozen. A second portion was digested and single cell suspensions were obtained. The frozen sections were sectioned onto slides and stained with H & E for analysis of histology. From the digested portion of tumor, single cell suspensions were stained with fluorochrome-conjugated anti-CD45 antibody and analyzed via flow cytometry. Percentages represent average percent of tumor that is CD45 positive (representative of 9 or 10 animals).

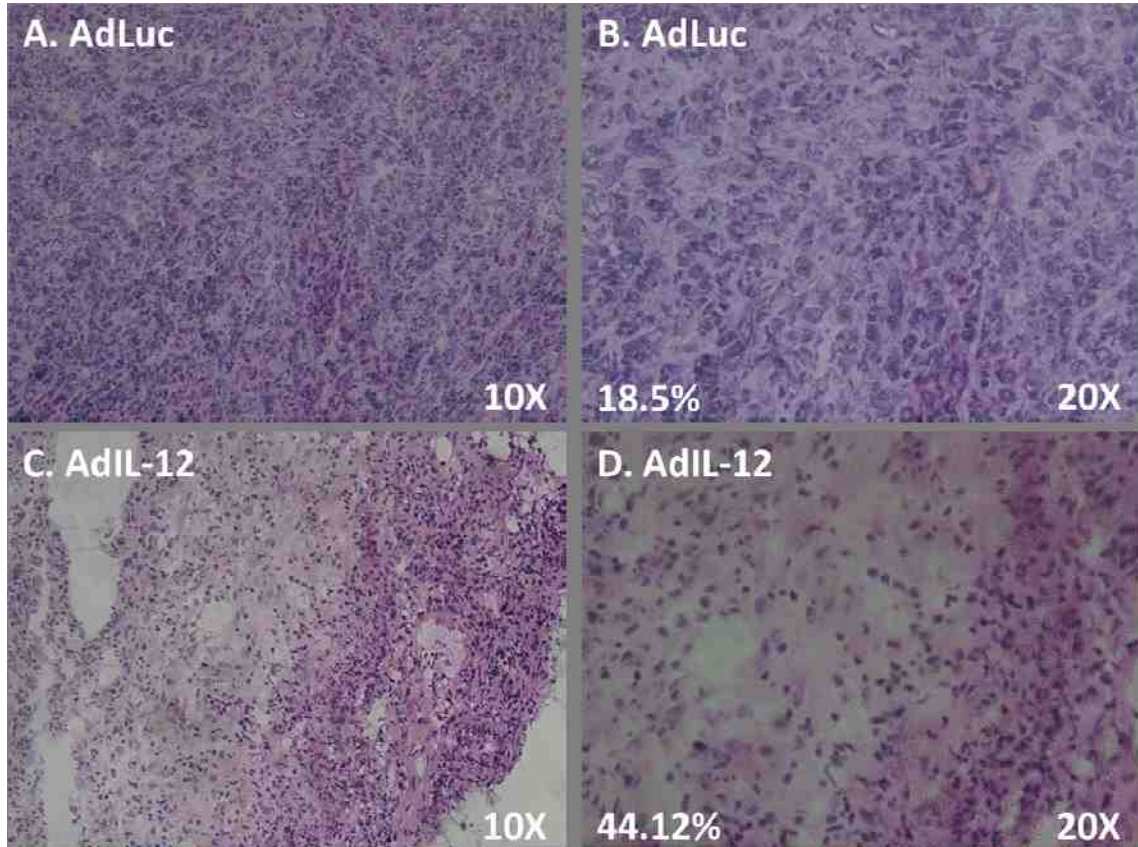


Figure 35. *Balb/c 4T1 Tumor Histology.* Balb/c murine fat pads were injected with 1×10^5 4T1 cells. Once tumors reached an average size of 65 mm^3 animals were intramuscularly injected with 1×10^9 virus particles of recombinant adenovirus expressing a luciferase reporter gene (AdLuc) (A 10X; B 20X) or recombinant mouse IL-12 (AdIL-12) (C 10X; D 20X). Tumor growth was measured twice weekly. Once tumors reached a volume of approximately 500 mm^3 animals were humanely euthanized and tissues were harvested and analyzed as described previously (Figure 34). Percentages represent average percent of tumor that is CD45 positive (representative of 10 animals).

IL-12 has long-term effects on MDSC levels in tumor-bearing animals

Although a general change in tumor architecture and leukocyte infiltration was observed what correlation that has if any with MDSC alterations remains to be determined. Thus far, we have demonstrated that even *in vivo* treatment with IL-12 can affect MDSC, but what correlation those changes have if any with tumor growth suppression long-term remains to be defined. It has been established that reductions in MDSC directly correlate with long-term effects on the tumor microenvironment and an increase in infiltration with T cells (279). In order to define a relationship between AdIL-12 treatment, tumor growth suppression, and MDSC in our system, we isolated single cell suspensions from AdLuc and AdIL-12 treated tumors and analyzed them for Gr-1/CD11b double positive cell content at the end of the tumor growth study.

Figure 36 represents the percentage of Gr-1/CD11b double positive cells in the tumor microenvironment as a percentage of CD45+ cells. CD45 can be used as a general marker for lymphocyte/myeloid populations and serves as a better indicator of cell infiltration than comparing the presence of cells as a percentage of the entire tumor. Treatment with AdIL-12 was found to have a significant effect on the percentage of tumor-associated Gr-1/CD11b double positive cells present in the tumor at the end of the study. C3H/HeJ C3L5 tumors (Figure 36 A) and Balb/c 4T1 tumors (Figure 36 B) were both found to exhibit decreased levels of tumor-infiltrating Gr-1/CD11b double positive cells. All tumors were harvested at approximately equal volumes so differences in size alone cannot account for the significant decrease in Gr-1/CD11b double positive cells. Treatment with AdIL-12 suppresses Gr-1/CD11b double positive populations but does not eliminate them. The retention of these cells in combination with the variations in IL-12 expression could explain the variations in tumor growth suppression though analysis of their overall function against T cell activation is necessary to define this further.

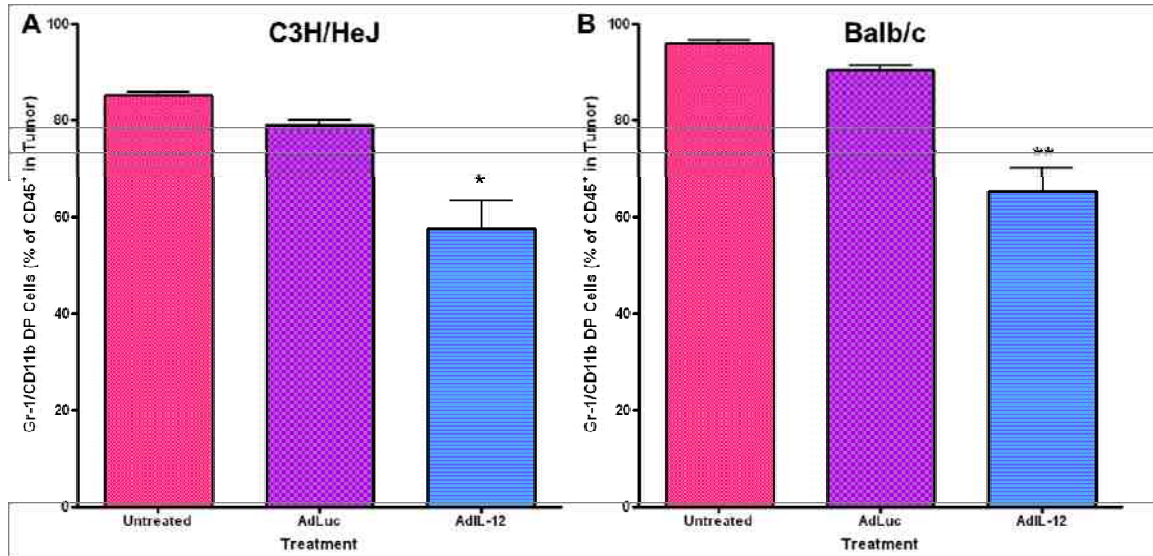


Figure 36. *Gr-1/CD11b* double positive cells are suppressed long-term following treatment with *AdIL-12*. C3H/HeJ and Balb/c animals were inoculated with 2×10^5 C3L5 and 1×10^5 4T1 cells, respectively. Once tumors reached an average size of 65 mm^3 , intramuscular injections of 1×10^9 adenovirus particles of *AdLuc* or *AdIL-12* was performed. Once tumors reached a volume of 500 mm^3 , tissues were harvested and single cell suspensions obtained. *Gr-1/CD11b* double positive cells were stained as a set of CD45+ cells using fluorochrome conjugated anti-*Gr-1*, anti-*CD11b* and anti-*CD45* antibodies. The percentage of *Gr-1/CD11b* double positive cells in C3H/HeJ (A) and Balb/c (B) tumors as a percentage of CD45+ cells was determined. Statistically significant decreases in *Gr-1/CD11b* double positive cells were observed following treatment with *AdIL-12* (* $p < 0.05$; ** $p < 0.01$).

Treatment with AdIL-12 results in an increase in activated CD8+ tumor-infiltrating T Cells

In order to determine whether *AdIL-12* treatment was having an impact not only on the type of cells that infiltrate the tumor but also the activity of the cells, the presence of tumor infiltrating CD8+ T cells as well as their expression of IFN- γ was determined. Single cell suspensions of digested tumors were stained with fluorochrome-conjugated antibodies and analyzed via flow cytometry as a percentage of CD45+ cells as described previously.

Figure 37 represents the infiltration of CD8+ T cells into C3H/HeJ C3L5 tumors (A) and Balb/c 4T1 tumors (B). Significant increases in the infiltration of CD8+ T cells occurred only after treatment with AdIL-12. Treatment with AdLuc did not significantly increase the infiltration of CD8+ T cells indicating that this is a direct effect of IL-12. The expression of IL-12 returns to basal levels after only 72 hours so this is a long-term effect of brief IL-12 expression. Since Gr-1/CD11b double positive cells were still observed in the tumors of the AdIL-12 treated animals, infiltration alone is not likely to be sufficient to promote immune activation long-term.

In order to determine whether these cells were active at the tumor even weeks after treatment with AdIL-12, the expression of intracellular IFN- γ was determined. Figure 38 represents the percentage of tumor infiltrating CD8+ cells that were also IFN- γ positive. The percentage of IFN- γ expressing, tumor infiltrating CD8+ T cells were increased long-term following treatment with AdIL-12 for both C3H/HeJ C3L5 (A) and Balb/c 4T1 (B) tumor models. This long-term immune activation indicates a significant potential for treatment utilizing IL-12. Although the percentage of IFN- γ positive CD8+ T cells is increased in the AdLuc treated animals the total percentage of CD8+ T cells coupled with the relatively unaltered Gr-1/CD11b double positive cell populations makes this result indicating a potential virus effect less impressive in minimizes the effect of the adenovirus vector in terms of potential therapeutic consequences.

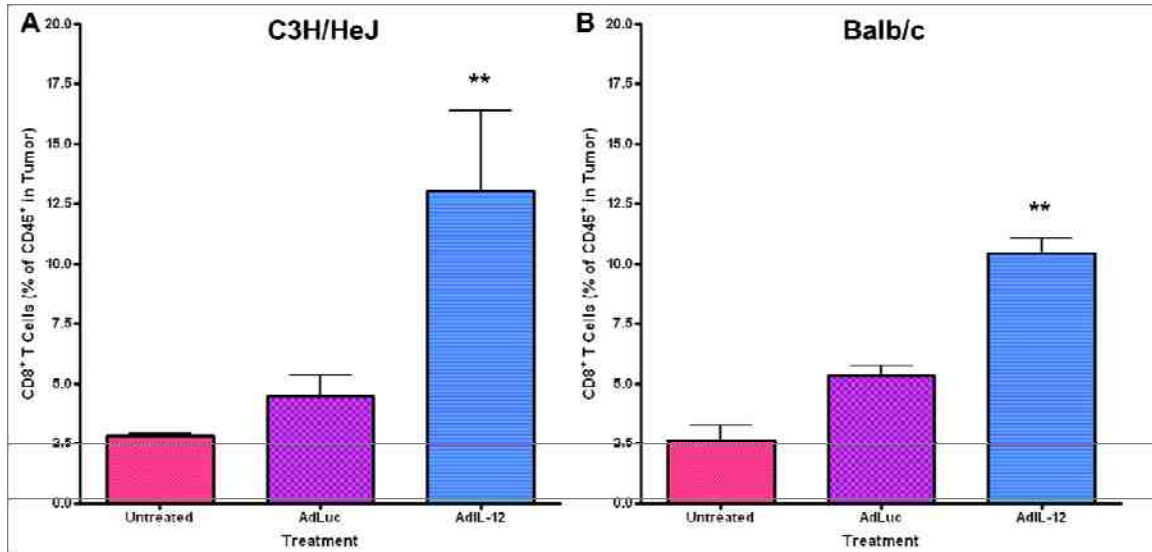


Figure 37. *In vivo* treatment with IL-12 increases CD8+ T Cell infiltration into the tumor microenvironment. C3H/HeJ and Balb/c animals were inoculated with 2×10^5 C3L5 and 1×10^5 4T1 cells, respectively. Once tumors reached an average size of 65 mm^3 , intramuscular injections of 1×10^9 adenovirus particles of AdLuc or AdIL-12 was performed. Once tumors reached a volume of 500 mm^3 , tissues were harvested and single cell suspensions obtained. CD8+ T cells were stained using fluorochrome-conjugated anti-CD8 and anti-CD45 antibodies and analyzed via flow cytometry. The percentage of CD8+ T cells in C3H/HeJ (A) and Balb/c (B) tumors are also shown. Statistically significant increases in CD8+ T cells were observed following treatment with AdIL-12 (** $p < 0.01$).

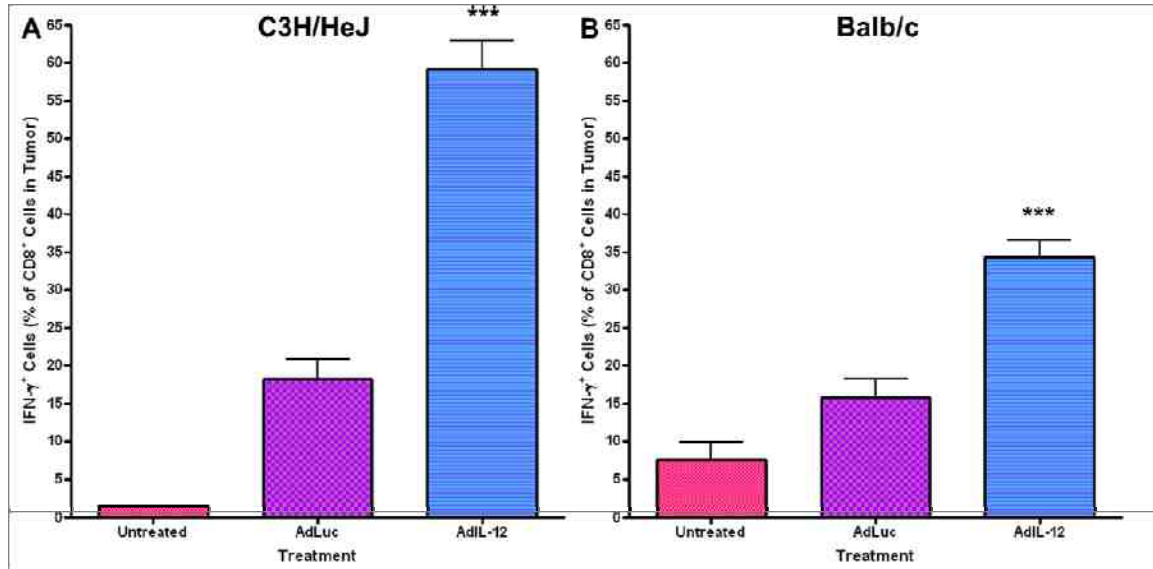


Figure 38. *In vivo* treatment with IL-12 increases the percentage of CD8+ T cells that also express IFN- γ in the tumor microenvironment. C3H/HeJ and Balb/c animals were inoculated with 2×10^5 C3L5 and 1×10^5 4T1 cells, respectively. Once tumors reached an average size of 65 mm^3 , intramuscular injections of 1×10^9 adenovirus particles of AdLuc or AdIL-12 was performed. Once tumors reached a volume of 500 mm^3 , tissues were harvested and single cell suspensions obtained. CD8+ T cells were stained using fluorochrome-conjugated anti-CD8 and anti-IFN- γ antibodies and analyzed via flow cytometry. The percentage of CD8+ T cells that also express IFN- γ in C3H/HeJ (A) and Balb/c (B) tumors are also shown. Statistically significant increases in active IFN- γ + CD8+ T cells were observed following treatment with AdIL-12 (** $p < 0.001$).

Treatment with AdIL-12 significantly reduces metastasis

It has been well established that IL-12 has anti-angiogenic and anti-metastatic properties (242-245). In addition to direct IL-12-mediated effects on metastasis, changes to MDSC populations have been shown to reduce metastases (28, 145, 153, 280). Given that AdIL-12 treatment results in an increase in potentially active CD8+ T cells in the tumor microenvironment and a decrease in Gr-1/CD11b double positive cells, what other long-term effects the therapy might have were of significant interest. Lungs from tumor-bearing animals were harvested at the point of tumor harvest and stained using Bouin's fixative. The number of metastases from untreated tumor-bearing animals as well as AdLuc and AdIL-12 treated animals was determined and is represented in figure 39.

Stained naïve and tumor-bearing C3H/HeJ lungs with metastases indicated are represented in 40.

Treatment with AdIL-12 was found to significantly reduce the number of metastases in both C3H/HeJ C3L5 (Figure 39 A) and Balb/c 4T1 (Figure 39 B) tumor-bearing animals. In the C3H/HeJ model, no lung metastases were found following IL-12 treatment. This significant reduction in metastasis may be the consequence of treatment with IL-12 or it could be a consequence of altering the overall immune suppression of the tumors. Although reductions in metastases have been linked to the influx of active T cells, how those cells can be active in the presence of suppressive cells needs to be considered. This dissertation begins to define the role of IL-12 as both an immune activator and an agent that alters immune suppression. In order to fully define the correlation between reversing immune suppression and promoting a T cell response in terms of reducing metastases, further experimentation is required.

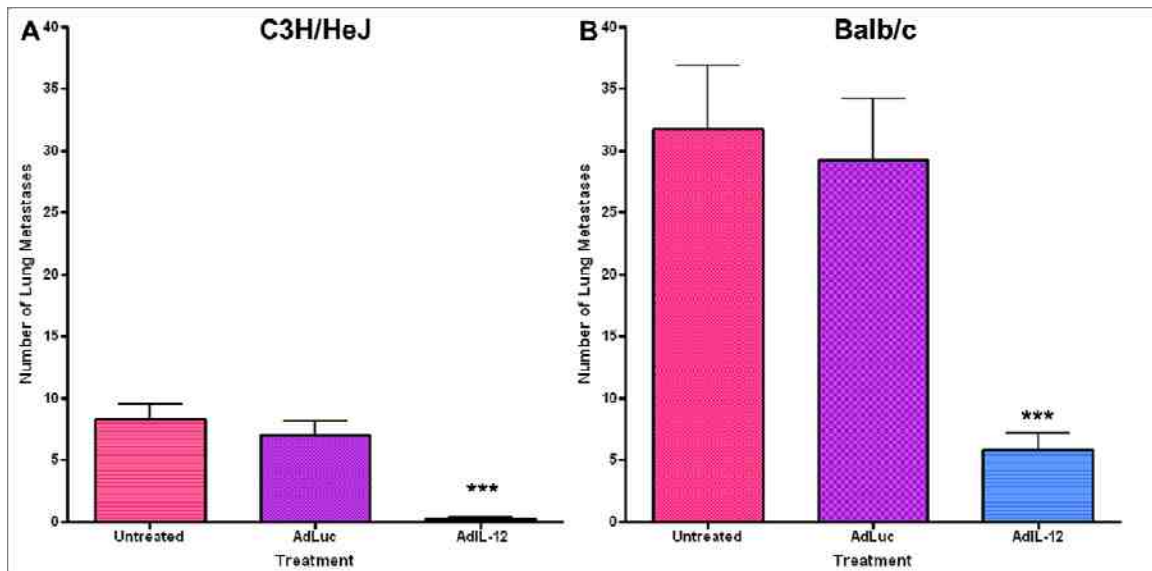


Figure 39. *In vivo* treatment with IL-12 decreases the number of metastases. C3H/HeJ and Balb/c animals' mammary fat pads were inoculated with 2×10^5 C3L5 and 1×10^5 4T1 cells, respectively. Once tumors reached an average size of 65 mm^3 , intramuscular injections of 1×10^9 adenovirus particles of AdLuc or AdIL-12 was performed. Once tumors reached a volume of 500 mm^3 , lungs were harvested for analysis of metastasis. Lungs were fixed in Bouin's fixative and metastases were counted. Analysis of

metastases from C3H/HeJ (A) and Balb/c (B) are also shown. Statistically significant reductions in metastasis were observed following treatment with AdIL-12 ($***p < 0.001$).



Figure 40. *In vivo* treatment with IL-12 decreases the number of lung metastases. C3H/HeJ animals' mammary fat pads were inoculated with 2×10^5 C3L5 cells. Once tumors reached an average size of 65 mm^3 , intramuscular injections of 1×10^9 adenovirus particles of AdLuc or AdIL-12 was performed. Once tumors reached a volume of 500 mm^3 , lungs were harvested for analysis of metastasis. Lungs were fixed in Bouin's fixative and images captured (images are not to scale). Findings are summarized in figure 39.

Discussion

MDSC are a population of cells of great interest to immunologists and cancer researchers alike. These cells have a wide range of impacts in terms of immune modulation, cancer progression, and metastasis. While MDSC are known as Gr-1/CD11b double positive cells, not all cells expressing both of these markers are MDSC. In order to be classified as an MDSC the cell must be functionally suppressive; specifically, these cells must be capable of suppressing T cell activation. I hypothesized that IL-12 can alter the immunosuppressive activity of MDSC leading to potent anti-tumor effects. These studies confirm a critical difference between MDSC and cells that are just Gr-1/CD11b double positive in terms of function, but more importantly, identify a new role of IL-12 in immune modulation that is specific to MDSC.

The Gr-1/CD11b double positive cells expanded during tumor growth for both of the tumor models studied were found to be functionally suppressive MDSC. Treatment with IL-12 was found to alter the suppressive function of these cells by inducing the up-regulation of markers associated with mature dendritic cells and macrophages. This implies a potential new role for IL-12 as an inducer of MDSC cell maturation. According to the results of the *in vitro* studies, tumor-derived MDSC do not up-regulate all markers studied. This limitation, however, is not reproduced in the results from the *in vivo* treatments with AdIL-12. There are several key distinctions between the two studies. The *in vivo* studies performed do not isolate the activity of IL-12 to MDSC and do not separate interactions between MDSC and other cells. It is probable that the observed difference in responsiveness between *in vitro* and *in vivo* treatments is due to interactions with other immune activators affected by either virus treatment or IL-12, or both. The improved response to IL-12 *in vivo* indicates potential for the potent MDSC effects to correlate with anti-tumor activity.

Several key distinctions exist for the MDSC in these studies verses other studies performed previously. The MDSC studied in this dissertation do express CD11b, but were found to lack F4/80 expression. As mentioned previously, many of the previous studies of MDSC in the tumor microenvironment were performed on cells based solely on CD11b expression. Studies of cells from the tumor microenvironment that rely solely on surface expression of CD11b and either ignore Gr-1 expression or specifically study

cells lacking Gr-1 expression do not separate actual MDSC from the CD11b+/Gr-1 negative macrophages also present in the tumor microenvironment (281-284). The studies outlined in this dissertation indicate that the Gr-1/CD11b double positive cells are fundamentally different from the tumor-infiltrating macrophages studied previously and should be evaluated as a unique population of cells. Although it is possible that some of the Gr-1/CD11b double positive cells differentiate upon reaching the tumor microenvironment resulting in the down-regulation of Gr-1, the fact that Gr-1/CD11b double positive cells remain present in the tumor as well means that no matter what changes some of these cells may undergo a population of undifferentiated immature MDSC remains. This population of cells should not only be considered as a separate population but studied further as there is potential for these cells to suppress T cell activation and block anti-tumor immune responses. The studies in this dissertation focus on the Gr-1/CD11b double positive cells and identify them as functionally suppressive, immature cells expressing at least one of the cytokines expressed by M2 suppressive macrophages. Further analysis of the cells is required to completely define the differences and similarities between MDSC and other tumor-infiltrating populations such as M2 macrophages.

After identifying and beginning to define key differences between MDSC and other tumor-infiltrating populations, these studies identify critical effects of treatment with IL-12 on MDSC activity. In addition to altering the expression of markers indicative of maturation, these studies demonstrate that IL-12 alters a few of the internal mechanisms of MDSC responsible for conferring their suppressive function. The findings in this dissertation demonstrate that IL-12 decreases the expression of Nos2, IFN- γ , and Arg1 mRNA in MDSC. The presence of all of these factors in the spleen-derived and tumor-derived Gr-1/CD11b double positive cells supports the findings that the cells from both tissue types are suppressive MDSC. Reciprocally, the relatively low expression of these factors in naïve spleen-derived Gr-1/CD11b double positive cells supports the findings that these cells are not suppressive MDSC. IL-12 is capable of reducing the production of each of these factors, demonstrating that the cells have been both phenotypically and fundamentally altered and those alterations correlate with the loss in suppressive function. The significant difference in overall levels of mRNA expression of Nos2 and IFN- γ in Balb/c MDSC for the *in vivo* study compared to the *in vitro* study is important to consider. This increase in overall expression *in vivo* could be due to the lack of *in vitro*

culturing for 24 hours. Immediate sorting and RNA extraction from the cells instead of 24 hour culturing may provide a more accurate analysis of overall mRNA levels. It is possible that *in vitro* culturing has an impact on overall MDSC activity, though further experimentation would be required to define what effect (if any) *in vitro* culturing has on these cells. *In vitro* culture might enhance MDSC activity, but suppressive function is not induced by *in vitro* culture. Since naïve Gr-1/CD11b double positive cells do not suppress CD3-mediated T cell activation (Figures 4 C and 4 C), it is likely that the suppressive activity is not artificially induced but rather the consequence of true MDSC activity. Although suppressive activity conferred by naïve populations was not observed, it is possible that the *in vitro* culturing reduces overall suppressive function in a manner that is not observed at the strong 1:1 ratio of MDSC to whole splenocytes. Although there was no loss in suppressive function observed in studies of a 1:2 ratio of MDSC to whole splenocytes (data not shown), differences may become apparent at a 1:4 or even 1:8 ratio.

The findings outlined in this dissertation indicate that following treatment with IL-12, MDSC are phenotypically altered leading to down-regulation of factors required for conferring their suppressive function. A direct correlation between IL-12 signaling and down-regulation of any of these factors has not been established. The fact that IFN- γ was found to decrease following treatment with IL-12 was particularly interesting since it would imply a new role for IL-12 in controlling IFN- γ signaling within certain cell populations. Although these data imply a correlation, it is far more likely that treatment with IL-12 fundamentally alters the cells resulting in the decreased IFN- γ production rather than IL-12 signaling directly turning off IFN- γ production. Further experimentation is necessary to define this result and determine specifically how treatment with IL-12 alters the production of factors necessary for conferring immune suppression.

Although these findings clearly demonstrate both a phenotypic and functional change in MDSC induced by IL-12, whether the IL-12-mediated alterations of these cells have made them merely no longer suppressive or whether the cells have been activated remains to be determined. If the cells have become activated, then not only would they no longer suppress anti-tumor immunity, but they would also be capable of promoting an anti-tumor immune response. It has been established that MDSC are capable of being converted into immunogenic antigen-presenting cells; however, whether IL-12 has the

ability to cause this remains unknown (285). The findings that treatment of the functional MDSC with IL-12 causes a decrease in IL-10 production and an increase in TNF- α production implies that their activity has potentially been switched from blocking immune activity to promoting anti-tumor immunity. This appears to be due to an IL-12-induced phenotypic change in the cells though it could also be due to direct IL-12-mediated signaling. It may be interesting to further define the effect of IL-12 by determining whether these cells respond to stimulation by producing M1 or M2 cytokines.

Challenging the Balb/c-derived MDSC with lipopolysaccharide (LPS) and IFN- γ should support the findings of unstimulated cells. LPS stimulated, IL-12-pretreated MDSC should respond to immune challenge by up-regulating production of M1 cytokines and/or down-regulating the production of M2 cytokines, if they have been converted into a population of cells capable of activating immune responses. Further studies will need to be performed to identify whether tumor MDSC exhibit additional M2 or M1 macrophage characteristics before and after IL-12 treatment in order to fully define the overall activity exhibited by MDSC.

Twenty-four hour treatment with AdIL-12 was sufficient to alter the MDSC *in vivo* but defining what role those alterations had on anti-tumor efficacy and long-term immune activation is of importance when applying IL-12-based therapies to breast cancer. Studies performed on tumor growth and lymphocyte infiltration into the tumor microenvironment demonstrated that treatment with AdIL-12 is capable of suppressing tumor growth with significant alterations in lymphocyte infiltration. Specifically, AdIL-12 treated animals exhibited a significant decrease in MDSC and a significant increase in tumor-infiltrating CD8⁺ T lymphocytes. Although significant increases in leukocytes were observed, MDSC were found to still be present in the tumors of AdIL-12 treated animals; therefore, the activity of the CD8⁺ T cells needed to be determined. The production of intracellular IFN- γ by CD8⁺ T cells can be used as a marker of activation. Studies defining the expression of IFN- γ (as a percentage of CD8⁺ T cells) performed on the CD8⁺ T cells harvested from AdIL-12 treated animals demonstrated that a larger percentage of these cells exhibit IFN- γ expression compared to those from AdLuc treated animals. The time point for these studies was weeks after injection with the virus implying a long-term effect on immune activation for the therapy. This finding coupled with the decrease in MDSC and prolonged overall survival implies that, at least in part, IL-12 exerts some of its therapeutic effect through reduction of immune suppression

coupled with activation of anti-tumor immunity. Based on the results outlined in this dissertation, another role for IL-12 as a modulator of immune suppression has been defined as well as a potential target for combination therapies involving IL-12.

Therapies that target immune activation as a means to reject established tumors are limited by the presence of cells capable of suppressing immune activity. The ability of T cells to be active while in the presence of MDSC and other suppressive populations is critical for the immune system to be able to promote an anti-tumor immune response (36). The idea that single cytokine treatments can not only promote immune responses but also overcome immune suppression is very attractive to researchers as it targets multiple aspects of tumor growth and progression. Defining the mechanism of IL-12 activity on MDSC is essential to understanding the therapeutic applications of IL-12. It is well established that IL-12 acts to suppress tumor growth in many tumor models; however, why a wide range of responses to IL-12 treatment exists has not yet been fully explained. It is clear that IL-12 promotes anti-tumor immune responses but why these changes in the tumor microenvironment result in such as dramatically varied responses in terms of tumor-growth suppression needs to be explored. This study provides a possible explanation for the discrepancy in IL-12-mediated therapeutic response. Since IL-12 fundamentally alters the suppressive function of MDSC through potentially inducing MDSC maturation then it is possible that differences in MDSC recruitment and/or incomplete reversal of MDSC activity by IL-12 can explain why a wide range of tumor responses have been observed in many studies. The potential role for IL-12 in modulating MDSC recruitment or accumulation should be explored. It is possible that treatment with IL-12 alters MDSC as well as tumor-induced cytokine expression long-term resulting in the decreased MDSC populations observed in these studies. Utilizing the enhanced understanding of IL-12 activity on a suppressive cell population obtained from these studies could go a long way toward enhancing the therapeutic efficacy of combination therapies. For example, treatment with docetaxel was found to alter MDSC as well as affect tumor cell growth (286). Combination therapy of docetaxel with IL-12 may not provide the synergistic or improved therapeutic effects as there is significant overlap in activities. Therefore, generating effective combination therapies with significant, synergistic anti-tumor activities requires an understanding of all activities associated with a given agent prior to combining it with another agent.

Treatment of both C3L5 and 4T1 tumor-bearing animals with AdIL-12 resulted not only in a suppression of tumor growth but also in an overall reduction in metastasis as measured by a reduction in total numbers of lung metastases. The data in this dissertation does indicate that the effects of IL-12 on MDSC may be essential to permitting T cell activity to reduce metastases. This reduction in metastasis demonstrates yet another long-term benefit to treatment with AdIL-12. Whether the reduction in metastases is the result of the altered MDSC or a general effect of IL-12 remains to be determined; however, this finding signals the potential of treatments of this type to be useful against advanced disease.

In summary, the findings outlined in this dissertation not only define a previously unknown role for IL-12 but also provide a rationale for determining which types of combination therapies could have the most therapeutic efficacy. Since IL-12 alone was shown to induce significant up-regulation of maturation markers and alter the suppressive function of MDSC, combination of it with other MDSC modulators may not provide significant improvements over treatment with IL-12 alone. It would instead most likely provide optimal therapeutic efficacy to treat tumor-bearing animals with IL-12 in combination with agents capable of inducing tumor cell death. Optimal therapeutic efficacy would include an increase in complete responders coupled with coinciding reductions in metastasis even in cases of advanced disease. Induction of cancer cell apoptosis coupled with the IL-12 effects demonstrated here could result in stronger suppression of tumor growth and a greater number of animals exhibiting complete tumor regression with protection from recurrence.

Future Directions

Although these studies have characterized the mechanism through which IL-12 alters MDSC and a potential means through which this alteration results in a therapeutic effect, several questions remain unanswered. For instance, while this dissertation outlines a role for IL-12 in potentially inducing MDSC maturation with accompanying reductions in factors responsible for conferring immune regulation, though the actual signaling pathway or pathways involved in these changes have not been defined. IL-12 signaling through the IL-12R is mediated by both STAT3 and STAT4 while STAT3 is essential to MDSC function (202-204, 287-291). Changes in the phosphorylation states and ratio of STAT3 to STAT4 would demonstrate that signaling is in fact occurring down the same pathway as for traditional IL-12-mediated responses. Disrupting signaling through STAT3 is important to consider regarding the findings outlined in this dissertation. Loss of STAT3 or changes in STAT3 signaling may be responsible for the down-regulation of factors necessary to confer MDSC suppressive function observed in these studies.

In addition to STAT signaling, MDSC function has been shown to occur through MyD88 signaling (287, 292). Analysis of gene activity for IL-12 responsive genes and transcription factors would provide a direct link between the changes observed in this dissertation and IL-12 signaling pathways. For example, Arg1 production in MDSC has been shown to be promoted by cyclooxygenase-2 (COX-2) (130). Alterations in COX-2 following treatment with IL-12 would establish a direct link between IL-12 signaling and the observed reduction in Arg1 expression. Many other pathways mediated by the tumor microenvironment may also be of interest. For example, new evidence has implicated a role for the IL-17 pathway in MDSC mobilization and activity (293, 294). What effect IL-12 has on this pathway in the tumor microenvironment and regarding MDSC remains to be defined. Defining the signaling pathway involved could implicate IL-12 as not only a factor that alters MDSC function but also a factor that mediates other MDSC activities.

Treatment of MDSC has been shown to not only reduce suppressive function but to also promote a transition of the cells into active antigen-presenting cells (295). The potential for IL-12 to not only reduce suppression but also to induce maturation, means that IL-12 could potentially activate these cells. Increased potential to present antigen is implied by the up-regulation of MHCII, but whether these altered cells also have the potential for

phagocytic activity or the ability to produce proinflammatory cytokines in response to stimuli is of interest in the future. In addition to further characterizing the role of IL-12 in terms of the mechanism of its effect on MDSC activity, several specifics regarding IL-12-mediated changes in both MDSC activity and in the tumor microenvironment remain undefined.

The studies in this dissertation demonstrate that cells expressing both Gr-1 and CD11b markers are present in the tumor microenvironment and that they are suppressive MDSC. This dissertation further characterizes these cells by demonstrating that they are a unique cell population lacking expression of the macrophage maturation marker, F4/80. Characterizing the complete cytokine and gene expression profile of MDSC compared to tumor-infiltrating macrophages should be performed to obtain a complete understanding of the activity of these cells in the tumor microenvironment and how they differ. Determining the differences between the IL-12-mediated responses of these two cell populations is of particular interest in that it could help further define how IL-12 acts on immature myeloid cells versus mature myeloid cells. Gaining a better understanding of how IL-12 alters MDSC in the tumor and systemically will yield a better understanding of how alterations in the tumor microenvironment lead to suppression in tumor growth, but also often allow for tumor persistence and regrowth. Defining whether IL-12 has the ability to alter MDSC recruitment, for example, will help to define why certain tumors respond better than others. If the effects of IL-12 can be better understood, then improved therapies can be developed.

The activity of IL-12 as both an immune modulator and an anti-tumor therapeutic agent remains of interest for tumor immunologists and will continue to be of interest as long as IL-12 continues to show promise as a treatment for cancer. Studies that attempt to improve the overall therapeutic efficacy of IL-12 with a reduction in toxic side-effects through either combination therapies or improvements in the IL-12 single-cytokine therapies could lead to significant implications for treatment of not only breast cancer but also other metastatic cancers. The ability to reduce metastasis is an essential ability for any treatment aiming to treat advanced carcinomas. As mentioned previously, combination of IL-12 with other therapeutic agents must take into account the effects of IL-12 single-cytokine therapy in order to determine which agents will provide the best therapeutic benefits. Since this dissertation defines a role for IL-12 in potentially inducing

MDSC maturation, combination therapies involving other agents that induce MDSC maturation or differentiation may not have added benefit. For example, combining IL-12 with ATRA most likely will not enhance the anti-tumor effects of IL-12 as both factors affect MDSC in a similar fashion. Combining IL-12 with agents capable of inducing tumor cell necrosis or apoptosis will most likely provide optimal therapeutic benefit and should be studied.

It is important to note that an additional complication for IL-12-based therapies may exist in regards to timing of the therapy. One study determined that blocking MDSC activity had to occur early in tumor growth in order to affect overall progression and reduce metastatic disease (296). Defining the late vs. early effects of treatment with IL-12 could lead to a better understanding of how this treatment as well as alterations in MDSC combats disease progression and metastasis. Combination therapies have already been shown to reverse tolerance and promote anti-tumor immunity so any studies that attempt to determine how IL-12 can be combined to further its effects on these processes are of crucial importance to optimizing therapeutic efficacy (297, 298). Better characterization of the effects of IL-12-mediated therapy will allow for an enhanced understanding of which agents have the most promise for use as combination cancer therapies and how alterations in MDSC function factor into promoting the effects of cancer immunotherapy.

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Denver DR, Feinberg S, **Steding C**, Durbin M, and Lynch M. 2006. The relative roles of three DNA repair pathways in preventing *Caenorhabditis elegans* mutation accumulation. *Genetics* 2006; 174:5765.

Baer CF, Shaw F, **Steding C**, Baumgartner M, Hawkins A, Houppert A, Mason N, Reed M, Simonelic K, Woodard W, and Lynch M. Comparative evolutionary genetics of spontaneous mutations affecting fitness in rhabditid nematodes. *Proc Natl Acad Sci U S A* 2005; 102:5785-5790.

BOOK CHAPTERS:

Xiong L, **Steding C**, Jimenez JA, Gardner TA, Kao C. Development of prostate-restricted replication competent adenoviral vectors. In *Cancer and Gene Therapy*. Transworld Research Network. Kerala, India. 2007.

ABSTRACTS/PRESENTATIONS:

Steding C, Wu S, Zhang Y, Elzey B, Jeng MH, Kao C. The Effects of IL-12 on MDSC Activity. Tumor Immune Suppression Meeting. July, 2010.

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