Myeloid-Derived Suppressor Cells in Tumor Immunology

DISSEPTION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2011

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Abstract

A class of immune suppressor cells termed myeloid-derived suppressor cells (MDSC) is known to be elevated in many solid tumor settings. MDSC are a heterogeneous population of early myeloid cells that expand from the bone marrow during tumor progression and have the ability to inhibit both innate and adaptive immunity. Previous work from our laboratory and others indicated that patients with cancer exhibited reduced activation of signaling pathways when stimulated with Type I and II IFN as compared to normal donors. This reduced immune response could be a barrier to successful clinical outcomes, since ability to respond to IFN is known to be important for anti-tumor immunity and efficacy of immune-based therapies. We hypothesized that MDSC could inhibit an immune response to the growing tumor by reducing the ability of immune cells to respond to IFN stimulation. C26 tumor-bearing mice had significantly elevated levels of GR1^+/CD11b^+ MDSC as compared to control mice, and splenocytes from these mice exhibited reduced phosphorylation of STAT1 in response to Type I and II IFN. Depletion of MDSC in C26 bearing mice restored splenocyte IFN responsiveness. Spleens from C26 tumor-bearing animals displayed elevated levels of iNOS and nitric oxide (NO), which was associated with increased levels of nitration on STAT1. *In vitro* treatment of splenocytes with a nitric oxide donor led to a decreased STAT1 IFN response. Finally, splenocytes from tumor-bearing iNOS knockout mice
exhibited a significantly elevated IFN-response as compared to WT tumor-bearing mice. This demonstrated a novel mechanism of MDSC inhibition and created a direct link between increased nitric oxide and reduced IFN responsiveness.

We further investigated an association between pro-inflammatory cytokines, subsets of MDSC, and reduced IFN responsiveness in patients with GI cancer. Plasma IL-6 was correlated with CD33^+HLADR^CD15^+ MDSC, while IL-10 correlated with CD33^+HLADR^CD15^- MDSC. The percentage of multiple MDSC subsets were inversely correlated with IFN-α-induced STAT1 phosphorylation in CD4^+ T cells, while co-culture with in vitro generated MDSC led to reduced IFN-α-responsiveness in both PBMC and the CD4^+ T cells from normal donors. These data support our findings that MDSC reduce IFN responsiveness and demonstrate that this also occurs in patients with cancer.

We also hypothesized that psychological stress could lead to changes in factors that modulate MDSC. It was discovered that patients with high cancer-specific stress had significantly elevated salivary cortisol and IL-1Rα levels. Interestingly, significantly increased MDSC levels were observed in patients with lower cancer-specific stress. Additional long-term stress analyses of the number of stressful events that affected these patients revealed that patients with more stress overall trended toward elevations in MDSC. This study indicated the existence of a complex relationship between stress and immune function in breast cancer patients.
Taken together, these data demonstrate a novel mechanism of MDSC action and provide evidence that environmental factors, such as psychological stress, can lead to alterations in MDSC in patients with cancer.
For my family.
Acknowledgments

I would first like to thank my advisor, Dr. William Carson, for accepting me into his lab mid-way through my graduate school career. Thank you for taking a chance, and hopefully I have proven to be a worthy student. You gave me the opportunity to continue and jump into a new area of science, and your lab quickly became a new home for me. I would also like to thank Dr. Greg Lesinski for being available for daily consultation and guiding my development. You pushed me to be a better scientist and look at things from many different angles.

I would next like to acknowledge all of the members of the Carson Lab, past and present for their tireless support, and for reminding me that research can actually be fun. Thanks for being there all of the times we had something to celebrate, and especially for being there when the hours were long and the work was hard. To Cristina, you were my best partner in crime and my faithful co-worker no matter what crazy things I asked you to help me with. Thank you to Valerie as well, the three of us together was always a dangerous combination. I would like to thank all of those along the way who have taught me the lessons I learned in graduate school. Although some lessons were good, others were hard, but each experience has created a better scientist and person than the one who started this process six years ago.
I also am very grateful for the assistance of my committee, Brian Kaspar, Cheryl London, and Tatiana Oberyszyn. They have been a constant source of advice and have been supportive of my goals and projects. Thank you for pushing me to achieve more and put the extra effort into becoming a productive scientist.

I finally thank my family, for unrelenting support of this process. Despite the long journey, you have always supported my goals and listened to whatever new topic I was excited about, whether you understood or not. Thank you to my parents, who had faith in me always. Last of all, I would like to thank my husband. Kevin, you went through this process yourself and never complained along the way. Thank you for your patience and your calm presence in the storm of my ever-hectic life.
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Mundy-Bosse, BL., Young, G., Bauer, T., Binkley, E., Bloomston4, M., Matthew A. Bill, M., Bekaii-Saab, T., Carson, WE 3rd, Lesinski, GB. Distinct Myeloid Suppressor Cell Subsets Correlate with Plasma IL-6 and IL-10 and Reduced Interferon-alpha Signaling in CD4+ T cells from Patients with GI Malignancy. Cancer Immunology, Immunotherapy. In Press.


Guenterberg, KD.; Grignol, VP.; Raig, ET.; Zimmerer, JM.; Chan, AN.; Blaskovits, FM.; Young, GS.; Nuovo, GJ.; Mundy, BL.; Lesinski, GB.; Carson WE 3rd (2010). Interleukin-29 binds to melanoma cells inducing Jak-STAT signal transduction and apoptosis. Molecular Cancer Therapeutics 9.2 (2010), 510-20.


Fields of Study

Major Field: Integrated Biomedical Science Program
# Table of Contents

Abstract ................................................................................................................................. ii  
Acknowledgments ................................................................................................................ vi  
Vita ........................................................................................................................................ viii  
Publications .......................................................................................................................... ix  
Fields of Study ....................................................................................................................... x  
Table of Contents .................................................................................................................. xi  
List of Figures ....................................................................................................................... xiv  
List of Tables ........................................................................................................................ xvi  
List of abbreviations ............................................................................................................ xvii  
Chapter 1 ............................................................................................................................. 1  
    Introduction to Cancer Immunology ................................................................................ 1  
    Interferons in Cancer ........................................................................................................ 3  
    Immune Cell Populations and the Tumor Microenvironment ........................................ 7  
    Myeloid-Derived Suppressor Cell Biology ....................................................................... 11  
    Myeloid-Derived Suppressor Cell Modulation ............................................................... 21
Conclusions ................................................................................................................. 26

Chapter 2: Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice ........................................................................................................ 27

Abstract ......................................................................................................................... 27

Introduction ...................................................................................................................... 29

Materials and Methods ................................................................................................. 31

Results ............................................................................................................................ 37

Discussion ......................................................................................................................... 44

Figures ............................................................................................................................. 49

Chapter 3: Distinct Myeloid Suppressor Cell Subsets Correlate with Plasma IL-6 and IL-10 and Reduced Interferon-alpha Signaling in CD4\(^+\) T cells from Patients with GI Malignancy .................................................................................................................. 66

Abstract ......................................................................................................................... 66

Introduction ...................................................................................................................... 68

Materials and Methods ................................................................................................. 71

Results ............................................................................................................................ 76

Discussion ......................................................................................................................... 80

Acknowledgments .......................................................................................................... 85

Figures ............................................................................................................................. 86
Chapter 4: Psychological Stress is Associated with Altered Levels of Myeloid-Derived Suppressor cells in Breast Cancer Patients

Abstract

Introduction

Materials and Methods

Results

Discussion

Acknowledgements

Figures

Discussion

Summary of Studies

Relevancy to the field of cancer biology

References
List of Figures

Figure 1.1: Interferon Signaling Pathway ................................................................. 5
Figure 1.2. MDSC Inhibit Multiple Immune Cell Functions ................................. 21
Figure 2.1. Decreased interferon response in tumor-bearing mice ..................... 49
Figure 2.2. Attenuated interferon-stimulated gene expression in splenocytes from C26-bearing mice ................................................................. 51
Figure 2.3. MDSC are elevated in tumor-bearing mice and are associated with decreased IFN responsiveness in vitro. ................................................................. 52
Figure 2.4. Reduction in MDSC with gemcitabine or anti-GR1 restores IFN responsiveness........................................................................................................... 53
Figure 2.5. Elevated levels of nitric oxide (NO) in tumor bearing mice inhibits IFN responsiveness in immune cells ......................................................... 57
Figure 2.6. Reduced CD34 and Ki67 expression in tumors from iNOS knockout mice. 58
Figure 2.7. Reduced P-STAT1 in response to IFN-alpha stimulation in spleen and lymph nodes of C26 tumor-bearing mice ......................................................... 59
Figure 2.8. Splenic MDSC increased over time and lymph nodes of tumor-bearing mice exhibit reduced IFN response ................................................................. 60

xiv
Figure 2.9. Expression of IFN responsive genes, iNOS expression, and nitration on STAT1 in normal and tumor-bearing mice................................................................. 62

Figure 2.10. Treatment of tumor-bearing mice with gemcitabine or anti-GR1 leads to a restoration of P-STAT1 protein levels................................................................. 63

Figure 2.11. Intra-tumoral MDSC are inhibited by treatment with gemcitabine or an anti-GR1 antibody................................................................. 64

Figure 2.12. Direct effects of IL-6 on interferon-responsiveness.......................... 65

Figure 3.1. Phenotypic analysis of MDSC subsets in patients with GI malignancies ..... 87

Figure 3.2. MDSC subsets are elevated in all types of GI malignancies............... 89

Figure 3.3. Plasma levels of IL-6 and IL-10 and the association with MDSC in patients with GI malignancies................................................................. 90

Figure 3.4. Inverse correlation between MDSC and IFN-α induced STAT1 phosphorylation................................................................. 93

Figure 4.1. IES scores are associated with alterations in stress hormone levels. .... 115

Figure 4.2. Patients with higher IES scores exhibit elevations in pro-inflammatory cytokines................................................................. 116

Figure 4.3. Acute stress is associated with decreased levels of myeloid-derived suppressor cells................................................................. 117

Figure 4.4. Chronic stress and surgery are associated with elevations in myeloid-derived suppressor cells................................................................. 118

Figure 4.5. MDSC and immune cell function post-surgery.............................. 120

Figure 5.1. NK ADCC................................................................. 137
List of Tables

Table 1. Patient Characteristics................................................................. 94
Table 2. Hazard Ratios for Univariable Models........................................ 96
Table 3. Hazard Ratios for Multivariable Models....................................... 97
Table 4. Patient Characteristics.................................................................. 121
List of abbreviations

ACTH  Adrenocorticotropic hormone
ADCC  Antibody-dependent cell-mediated cytotoxicity
APC   Antigen presenting cell
ARG1  Arginase I
ATRA  All-trans retinoic acid
Bel-xl B-cell lymphoma extra large
CD    Cluster of differentiation
ConA  Concanavalin A
COX2  Cyclooxygenase 2
CSF-1R Colony stimulating factor 1 receptor
DC    Dendritic cell
ERK   Extracellular signal-regulated kinase
GAS   Gamma activating sequence
G-CSF Granulocyte colony stimulating factor
GM-CSF Granulocyte- macrophage colony stimulating factor
GVHD  Graft versus host disease
HIF-1α hypoxia inducible factor 1 alpha
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary axis</td>
</tr>
<tr>
<td>IES</td>
<td>Impact of events scale</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon alpha receptor</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IFNGR</td>
<td>Interferon gamma receptor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iMC</td>
<td>Immature myeloid cell</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-inducible protein 10</td>
</tr>
<tr>
<td>IRF9</td>
<td>Interferon-regulatory factor 9</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon-stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon responsive elements</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immune –receptor tyrosine activating motif</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
</tr>
<tr>
<td>MCA</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MIC</td>
<td>MHC-class-I related antigen</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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MyD88 Myeloid differentiation primary-response gene 88
NADPH Nicotinamide adenine dinucleotide phosphate
NFXB Nuclear factor kappa B
NK Natural killer
NO Nitric oxide
NOS2 Inducible nitric oxide
NRF2 Nuclear factor-erythroid-2-related factor
PDE5 Phosphodiesterase type 5
PHA Phytohemagglutinin
PIAS Protein inhibitor of activated STAT
PI3K Phosphatidyl inositol 3-kinase
PBMC Peripheral blood mononuclear cells
PDGFR Platlet derived growth factor receptor
PGE2 Prostaglandin E2
RAGE Receptor for advanced glycation and products
ROS Reactive oxygen species
SNS Sympathetic nervous system
SOCS Suppressor of cytokine signaling
STAT Signal transducer and activator of transcription
TA Tumor antigen
TAM Tumor associated macrophage
TCR T cell receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TDF</td>
<td>Tumor derived factors</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>ULBP</td>
<td>U16 binding protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Chapter 1

Introduction to Cancer Immunology

Cancer has emerged as one of the deadliest diseases worldwide. Despite massive efforts into research and development over the past 50 years, there are few complete successes in solid tumor treatment. In the United States, cancer mortality is second only to heart disease (1, 2). Previously, therapeutics focused mainly on killing the fast-growing cancer cells, while sparing the normal tissue to minimize morbidity. While chemotherapy brought some success to specific cancers with this strategy, high recurrence rates coupled with the discovery of cancer stem cells that have a lower rate of division highlighted the need for improved targeted therapies. In addition, the normal cells of the body, such as stromal and immune cells, have been shown to assist in tumor growth and escape of treatment. These key advancements have set the stage for the development of new therapies designed to directly kill tumor cells while also removing the supportive environment and reactivating the immune response to aberrantly growing cells.

While Paul Ehrlich predicted the immune system acted as a barrier against the development of cancers in the host in the early 1900s, it was Drs. Macfarlane Burnet and Lewis Thomas who brought growing recognition to this theory nearly 50 years later (3-
While this theory was adamantly debated for the next 30 years, more recent studies indicate a clear involvement of the immune system in all stages of tumor development and progression (8). The process of immune involvement in cancer development and progression has been recently termed “immunoediting” (9). The theory can be divided into three steps. The first step is cancer elimination (also called immunosurveillance). At this stage, the adaptive and innate immune systems actively protect the host from developing tumors. This is achieved through multiple populations of immune cells, including T cells, natural killer (NK) cells, B cells, and macrophages, utilizing Type I and II interferons, and perforin as well as other factors which will be discussed in more detail later (10). The next step in immunoediting is referred to as equilibrium. At this point, the cancer cells still exist, but the immune system keeps the developing tumor in check. Finally, due to multiple potential mechanisms (additional genetic alterations, immune inhibition or immune cell anergy), the tumor progresses to the escape phase of immunoediting. During the escape phase, tumor cells that have a low level of immunogenicity advance and become clinically detectable masses, and have acquired all of the hallmarks of cancer (10, 11). These include the ability to grow independent of growth signals, the insensitivity to anti-proliferative signals, evasion of the normal apoptotic death pathway in aberrantly growing cells, limitless self-renewal, self-sustained angiogenesis, and ultimately escape from anti-tumor immune responses (9, 10).
Interferons in Cancer

Previous studies from our laboratory and others have demonstrated that peripheral blood mononuclear cells (PBMC) from patients with cancer exhibit inhibited responsiveness to interferon stimulation in immune cells when compared to normal donor PBMC (12-14). Interferons (IFNs) are a class of cytokines known to have anti-viral, anti-proliferative, and anti-angiogenic effects in vivo (15). Expansive studies support a role of IFNs as mediators in multiple stages of immunoediting and key factors in anti-tumor immunity (10). There are two types of IFNs, Type I (IFN-α/β/δ/ε/κ/τ/ω) and Type II (IFN-γ) (16). Type I IFNs all bind to a common receptor termed the interferon type I receptor, while IFN-γ binds to the type II IFN receptor. The Type I IFN receptor is composed of the IFN alpha receptor 1 (IFNAR1) subunit and the IFN alpha receptor 2 (IFNAR2) subunit, which are associated with members of the Janus Activated Kinase (JAK) family of proteins (16, 17). The IFNAR1 is associated with Tyrosine Kinase 2 (TYK2), while IFNAR2 is associated with JAK1 constitutively. The type II receptor is also composed of 2 subunits, termed IFN gamma receptor 1 and 2 (IFNGR1 and IFNGR2). IFNGR1 is associated with JAK1, while IFNGR2 is associated with JAK2. Upon ligand binding to either the Type I or II receptors, the associated kinases auto-phosphorylate and become active. Tyrosine residues on signal transducer and activator of transcription (STAT) proteins become phosphorylated by the active JAK and TYK kinases and create homodimers or heterodimers with other STAT family members. These complexes then translocate into the nucleus where they bind to IFN responsive
genes. Type I IFNs can activate STAT1, STAT2, STAT3, and STAT5 across all cell types, while STAT4 and STAT6 induction have been described under certain conditions (16, 18-20). Type I IFNs can activate STAT1-STAT2 heterodimers, which bind to interferon-regulatory factor 9 (IRF9) and form a gene activating complex termed interferon-stimulated gene factor 3 (ISGF3). The ISGF3 complex binds to interferon-responsive elements (IRSEs) in promoter regions of IFN-responsive genes. Other STAT homodimers and heterodimers can be activated by both Type I and Type II IFNs and these complexes bind to interferon-gamma-activated sequences (GAS) located in promoter regions of additional IFN stimulated genes. Both Type I and II IFNs can have a large range of downstream genes, with hundreds of promoters known to contain ISREs or GAS elements (see Figure 1.1 below) (16).
Interferon Signaling Pathway

Studies performed with interferon-gamma (IFN-γ) deficient mice support a role for interferons as important in immunosurveillance. Sarcomas induced by 3-methylcholanthrene (MCA) grew more aggressively when injected into mice treated with a neutralizing antibody specific to IFN-γ (21). Also, mice lacking the ability to produce IFN-γ specifically in γδ T cells were more susceptible to MCA treatment (22), indicating one source of endogenous IFN-γ. These studies were confirmed in additional models by treating interferon gamma receptor I (IFNGR1) or STAT1 knockout mice with
MCA and observing an increased rate of sarcoma development as compared to wild-type control treated animals (23, 24). The importance of IFN-γ in immunosurveillance has also been demonstrated with the use of spontaneous cancer models in mice. Specifically, mice lacking the tumor suppressor gene p53 were crossed with mice lacking IFNGR1 or STAT1, which would inhibit transduction of IFN-γ signaling. The p53−/− / IFNG1−/− mice developed a larger number of spontaneous tumors as compared to p53 null mice with functioning IFN-γ signaling pathways (23).

Type I IFNs have also been associated with immunosurveillance against cancer development. Initial studies aimed at neutralizing IFN-α indicated its potential involvement in anti-tumor immunity (15, 25). Mice deficient in the primary receptor for Type I IFN signaling, IFNAR1, treated with MCA developed more tumors, as well as tumors with increased aggressiveness as compared to wild-type treated mice (26). Additionally, tumors from mice lacking IFNGR1 or IFNAR1 are reported to be more immunogenic and elicit improved anti-tumor responses and ultimately rejection of the tumor when transplanted into naïve mice (23, 27). The main source of Type I IFNs endogenously are plasmacytoid dendritic cells (pDC), although most other cell types, including conventional DC, can produce small amounts of Type I IFN as well (28). Type I IFN in the tumor environment can assist DC in becoming fully activated and enhance tumor antigen (TA) uptake and presentation to T cells, driving a more robust TH1 anti-tumor response (29). Type I IFNs also enhance CD8+ T cells and NK cells through enhanced production of IL-15, which increases NK proliferation and survival while
assisting with formation of memory responses in T cells (28, 30, 31). Critical for direct tumor cell lysis, Type I IFN has been demonstrated to increase perforin and granzyme release from NK and CD8+ T cells which allows for improved cytotoxicity (32, 33).

Immune Cell Populations and the Tumor Microenvironment

With the mounting evidence of immune-involvement in tumor promotion, Dvorak took this one step further in the mid-1980s when he described the concept of tumors as inflammatory-rich environments, which were essentially “wounds that do not heal” (34). The tumor microenvironment does resemble a wound, with its complex cellular and vascular makeup. The tumor is composed of tumor cells, infiltrating immune cells, stromal fibroblasts, as well as a vascular network supplying nutrients that support growth and removing waste products (35, 36). Each of these components plays a role in tumor progression and immune evasion.

Multiple types of infiltrating immune cells are present in tumors, and this variety is determined by the tumor type as well as the specific milieu of the tumor. T cells have been one of the most extensively studied immune cells involved at the tumor site. Both CD3+/CD4+ and CD3+/CD8+ T cells have been discovered infiltrating both murine and human tumors (35). TAs and tumor-derived factors (TDFs) attract dendritic cells (DC) as well as other cell types to the site of the tumor. Functionally mature DCs take up antigens, travel to the draining lymph node, and present the TA to T\textsubscript{H}1 CD4+ T cells. The
T cells in turn produce IFN-γ, which assists in the expansion and function of CD8+ cytotoxic T cells that can lyse tumors cells through the use of perforin and granzyme B production. CD4+ T cells have the potential to recognize TAs presented in the context of major histocompatibility complex (MHC) class II by the tumor itself in addition to other antigen presenting cells (APC) and mount an immune response against the tumor cells. CD8+ T cells have the ability to directly lyse tumor cells by their cytotoxic function through recognition of altered MHC class I expression. However, both CD4+ and CD8+ T cells are impaired at the tumor site by multiple mechanisms of suppression.

Innate immune cells are also involved in anti-tumor immunity. Macrophages are present in most tissues of the body and serve a critical role in immune defense against invading pathogens, while maintaining homeostasis by engulfing cellular debris and apoptotic cells (37). Classically activated M1 macrophages become activated by the presence of IFN-γ and tumor necrosis factor (TNF). Macrophages can also become activated through toll-like receptor (TLR) stimulation in response to a foreign pathogen. Ligand binding to specific TLR on macrophages initiates a signal cascade through the myeloid differentiation primary-response gene 88 (MyD88) pathway, which induces transcription of TNF. IFN-γ produced by activated NK or T cells cooperates with the autocrine TNF to activate macrophages. Additional cell-independent pathways of IFN-β production have also been demonstrated directly from TLR stimulation, providing a mechanism of self-activation for macrophages (37). Cytokines produced in response to classical activation include interleukin (IL)-12 and IL-23, along with the
pro-inflammatory cytokines IL-1β, IL-6, and TNF. M1 macrophages also produce reactive oxygen and nitrogen species through the induction of inducible nitric oxide synthase (iNOS). The term M2 encompasses a broad range of macrophages thought to be activated under T_H2 conditions, specifically through the presence of IL-4 and IL-13 (38-40). M2 macrophages do not typically produce IL-12, but can produce alternative suppressive cytokines, such as IL-10, and have different receptor expression profiles as compared to M1 macrophages (40). In addition, M2 macrophages are thought to support tissue remodeling through the up-regulation of matrix metalloproteinases (MMPs) that can have important roles in tumor progression. A third population of macrophages termed tumor-associated macrophages (TAM) are considered part of the M2 phenotype, although they exhibit a combination of effector mechanisms attributed to both M1 and M2 macrophages (41).

In addition to CD8\(^+\) T cells, NK cells also possess the ability to kill tumor cells through altered recognition of MHC class I on tumor cells. NK cells have a number of activating and inhibitory receptors on the cell surface, and triggering a reaction requires a sufficiently strong activating signal that is able to overcome the inhibitory stimuli present to protect normal cells from NK cell attack. Aberrantly growing cells often express stress signals that provide additional activating simulation to elicit a response from NK cells. These ligands include highly polymorphic MHC-class-I-chain related A and B antigens (MICA and MICB) and U16-binding proteins (ULBP) in humans, which bind to the NK cell activating receptor NKG2D. In mice, the ligands retinoic acid early inducible 1
(RAE-1) and the minor histocompatibility antigen H60 (both members of the ULBP family of proteins) bind to NKG2D (42, 43). The NKG2D receptor is a type II disulphide-linked dimer with a lectin-like extracellular domain and is expressed on NK cells, as well as some subsets of T cells, and has been demonstrated to be important in anti-tumor immunity (44-47). NKG2D, like most NK receptors, has a short intracellular domain and requires additional adaptor molecules for signaling. Activation of NKG2D involves recruitment of associated proteins, including DAP-10 or DAP-12 adaptor molecules that allows for cell surface expression of NKG2D. DAP-10 signals through the phosphatidyl inositol 3-kinase (PI3K) pathway, which begins a cascade of intracellular signaling involving the extracellular-signal-regulated kinase (ERK) and AKT pathways. This ultimately stimulates the induction of NK-mediated cytotoxicity by the release of perforin and granzyme from NK cells (48, 49). DAP-12 signaling occurs through immune-receptor tyrosine based activating motifs (ITAMs), by the phosphorylation of signaling proteins leading to the generation of effector cytokines (such as IFN-γ) (50, 51). Cytokine production and direct cytotoxicity are the key effector functions that make NK cells important in anti-tumor surveillance. The importance of NKG2D expression in this process was demonstrated in a model system where human tumor cells were generated to over-express MICA, which sensitized these cells to NK-mediated cytotoxic killing \textit{in vitro} (47). The expression of NKG2D is not required for NK killing of tumor cells, but studies indicate the engagement of NKG2D enhances NK-mediated anti-tumor immunity \textit{in vivo} (44, 45, 52). Another class of receptors termed FCγ Receptors (FCγR) are expressed on NK cells, macrophages, and other
leukocytes have a key role in anti-tumor immunity. FCγR can recognize the Fc portion of immunoglobulin (IgG), which can accumulate in tumor environments and on tumor cells. There are four groups of FCγR that can be activating or inhibitory: FCγRI (CD64), FCγRII (CD32), FCγRIII (CD16), and FCγRIV (53). FCγRIIIα is primarily relevant for NK and macrophage antibody-dependent cytotoxicity cell mediated (ADCC) activity and signals through intracellular ITAM upon ligand binding (54). This receptor contributes to the success of antibody-based immunotherapies by allowing increased recognition of tumor cells by the innate immune system (55).

Myeloid-Derived Suppressor Cell Biology

The major components of innate and adaptive responses to tumors have been described previously, although the presence of suppressor cell populations represents a major hurdle to anti-tumor immunity. Classes of bone-marrow-derived cells demonstrating suppressive function have been described in tumor literature and other pathologic conditions for many years (56). Originally, these cells were called natural suppressor cells, and were termed “null” cells because they lacked mature lineage markers, as well as lacking cytotoxic functions that NK or macrophage cells possess. Upon further characterization, these cells were determined to be of myeloid origin, and they were then referred to as immature myeloid cells (iMC). iMC were initially examined in murine models, and were discovered to co-express GR1 and CD11b on their surface (GR1⁺/CD11b⁺). Antibodies against GR1 recognize the lymphocyte antigen 6
complex, both locus C and G (Ly6C and Ly6G) (56, 57). Under normal conditions, in both humans and mice, iMC would be generated in the bone marrow, and then quickly differentiate into mature myeloid cells, such as granulocytes, macrophages, and DC. In the setting of a tumor, the iMC appear to be recruited from the bone marrow, but are then arrested in their differentiation and these cells remain in an immature state. The field now refers to GR1⁺/CD11b⁺ iMC as myeloid-derived suppressor cells (MDSC) to clarify their origin and function (58).

MDSC are composed of a heterogenous population of early myeloid cells, including macrophages, granulocytes, dendritic cells, and immature myeloid cells (57). While MDSC represent 30-40% of bone marrow cells, less than 4% are found in circulation or in secondary lymphoid organs of normal mice. Immature myeloid cells with the same phenotype can be found in the bone marrow, however these cells do not have the ability to suppress immune cell function (56). MDSC with suppressive function can expand up to 50% in spleens of tumor-bearing animals, and are associated with increased tumor burden in patients with cancer (59, 60). MDSC are also elevated in non-cancer settings, such as graft versus host disease (GVHD), encephalomyelitis (EAE), diabetes, trauma, sepsis, and acute inflammation (61-66).

In mice, GR1⁺/CD11b⁺ cells are divided into additional morphological and functional categories. There are two major categories of murine MDSC which are defined by the expression of Ly6G versus Ly6C, and these populations are referred to as
granulocytic or monocytic based on this expression difference. Ly6G\(^+\)/Ly6C\(^{\text{low}}\)/CD11b\(^+\) granulocytic cells have been shown to produce elevated levels of reactive oxygen species (ROS) and arginase (Arg I), which can lead to increased reactive oxygen and nitrogen products. Ly6C\(^{\text{high}}\)/Ly6G\(^-\)/CD11b\(^+\) MDSC exhibit more monocytic morphology, and are thought to produce iNOS which subsequently leads to up-regulated nitric oxide (NO) in the environment (56, 57). While some studies suggest the location of MDSC dictates the generation of specific subsets, most studies phenotype for GR1\(^+\)/CD11b\(^+\), which includes a mixture of both monocytic and granulocytic MDSC in mice (67-73).

MDSC in humans are still being phenotypically characterized. In general, all MDSC are thought to express the common myeloid lineage marker CD33, with absent or low expression of MHC-class II (as would be found on more differentiated myeloid cells). Additional markers have been described, and could potentially represent different stages of MDSC development or completely independent populations of myeloid suppressor cells. An increase in CD33\(^+\)/CD34\(^+\)/CD15\(^+\)/lineage\(^-\) cells was reported initially in patients with head and neck cancer (74). This was further expanded to include CD11b expression, and Zea et al. demonstrated that this population produced high levels of the enzyme arginase and regulated L-arginine availability (75). Recent studies have also demonstrated the presence of a CD14\(^+\) subset of MDSC in patients with melanoma and hepatocellular carcinoma, and this subset appears to inhibit immune cell function through the actions of L-arginine regulation (76-78). Both murine and human subsets of MDSC have also been shown to express IL-4R\(\alpha\), which contributes to the expression of
factors such as transforming growth factor-beta (TGF-β), iNOS, and arginase (79). While there is still phenotypic debate regarding which antigens are critical for the definition of MDSC, the hallmark of each of these subsets is the ability to inhibit immune cell responsiveness. MDSC are known to inhibit T and NK cell function by multiple different antigen-specific and non-specific mechanisms that will be described in more detail in the following sections.

MDSC generation and expansion from the bone marrow is thought to be modulated by multiple tumor-derived or tumor-stromal-derived factors. Activated immune cells may also be a source of these MDSC-activating factors (56, 57). The most prevalent factors in the literature are granulocyte/macrophage-colony stimulating factor (GM-CSF) and IL-6 (69, 80-82). These factors have been described as sufficient to induce myeloid cells exhibiting suppressive capacity from normal PBMC (83). Other factors including cyclooxygenase 2 (Cox2), vascular endothelial growth factor (VEGF), prostaglandins (PGE2), and colony-stimulating factor-1 (CSF-1) have also been associated with MDSC generation or recruitment and function (56, 57, 84-86).

While multiple factors are involved with MDSC function, the majority of these activate signaling cascades in MDSC converge with JAK phosphorylation followed by STAT activation (57, 87, 88). MDSC in murine models exhibit elevated levels of STAT3 phosphorylation at baseline when compared to bone-marrow derived cells in naïve mice (89). Further studies have demonstrated that inhibiting STAT3 expression through the
use of pharmacological inhibitors, or using mice with STAT3 knocked out only in hematopoietic cells, led to reversal of T cell suppression with decreased expansion of MDSC outside of the bone marrow (89-91). STAT3 induction has been shown to be prevalent in many tumor and immune cells in the tumor microenvironment (92). Under normal physiological conditions, STAT3 would become phosphorylated in response to ligand binding (such as IL-6), and would then be rapidly inactivated by negative regulators of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) family of proteins (93-95). In tumor settings, however, STAT3 can remain active and promote the induction of genes supporting cell proliferation, survival (cyclin D1/D2, survivin, and BCL-X\textsubscript{L}), and angiogenesis (MMP2/9, and HIF-1\textalpha) (57, 67, 96).

STAT3 activation has also been shown to induce the expression of S100 calcium-binding proteins A8 and A9, and multiple studies have demonstrated that receptor expression for these proteins are found on the surface of MDSC (97, 98). S100 proteins are elevated in circulation during periods of inflammation and can bind to the receptor for advanced glycation and products (RAGE) as well as other carboxylated N-glycan receptors (99, 100). Elevated production of S100A8 and A9 has also been associated with tumor-infiltrating cells (100). Studies by Sinha et al. demonstrated that MDSC not only produce S100A8 and A9 but also bind these proteins, creating an autocrine feedback loop that further enhances MDSC-mediated suppression and accumulation at the tumor site (97, 98).
Multiple alternative mechanisms of suppression have been attributed to MDSC in addition to STAT3-dependent functions. The production of iNOS and arginase by MDSC has been shown in both human and murine models, and represents a major mechanism of immune suppression. It has been suggested that under certain conditions, activation of nuclear-factor-κB (NFκB) in addition to STAT can lead to an increase in both iNOS and arginase in MDSC (65). Unlike macrophage production of iNOS and arginase, where the two factors are not typically produced by the same cell, iNOS and arginase over-expression are both co-expressed in MDSC (101). iNOS and arginase generation has been demonstrated in GR1+/CD11b+ murine MDSC. However, this function is attributed primarily to the monocytic population of MDSC (Ly6C\textsuperscript{high}/Ly6G\textsuperscript{-}/CD11b\textsuperscript{+}), and has been demonstrated to require STAT1 activation for production (88).

Nitric oxide synthases generate nitric oxide (NO) from l-arginine, NADPH, and oxygen. In addition to MDSC, iNOS can also be up-regulated by other cells in the tumor environment or secondary lymphoid organs (102). There are two alternative forms of NOS, termed neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), and while these are constitutively active, they typically produce lower levels of NO (103). iNOS can be activated by IFN-γ, as well as hypoxia-inducible factor-1 alpha (HIF-1α), which have both been demonstrated to be present in many tumor settings (57, 67). In turn, iNOS can stimulate up-regulation of HIF-1α and subsequently increase VEGF formation, creating an additional positive activation loop within MDSC (104). When arginase is co-expressed with iNOS in MDSC, a reduced level of L-arginine can
lead to the production of superoxide (O$_2$) and NO, which can also combine to form peroxynitrite that can non-specifically nitrate cysteine and tyrosine residues in surrounding cells (105, 106). Interestingly, Nagaraj et al. demonstrated antigen-specific CD8$^+$ T cell inhibition, and discovered that MDSC production of peroxynitrite interfered with the T cell receptor (TCR)-CD3 complex in CD8$^+$ T cells when antigen was presented by MDSC, leading to inhibited signal transduction. This effect was short-lived, and appeared to be specific to the TCR interacting with MDSC, as other TCR-CD3 complexes on the same cell could respond to APCs loaded with the appropriate peptides and transduced signals normally (107).

MDSC also have additional non-antigen-specific suppressive mechanisms. In addition to the pathway described above, iNOS and arginase can also act together to deplete L-arginine from the environment. This non-essential amino acid is important for T cell proliferation and signal transduction (108-110). In addition to L-arginine depletion, Srivastava et al. also demonstrated that MDSC take up cystine from the environment and sequester it within the cell, and reduce cystine to cysteine for their own use. MDSC lack the ASC amino acid transporter which normally would export reduced cysteine. This leads to a reduction of cysteine in the environment, which is required by T cells during activation (73).

MDSC have also been linked to the generation of additional suppressor cell populations. Several studies have reported conflicting evidence for the role of MDSC in
promoting regulatory T cells (T\(_{\text{reg}}\)). CD4\(^+\)/CD25\(^+\)/forkhead box P3 (FOXP3\(^+\)) T\(_{\text{reg}}\) are known to be elevated both at the tumor site and systemically in patients with cancer (111). While T\(_{\text{reg}}\) are important in normal healthy individuals to protect against autoimmune reactions, T\(_{\text{reg}}\) promote tumor growth and progression through the generation of suppressive factors in the tumor setting (111). In a murine lymphoma model, MDSC presenting tumor antigens induced T\(_{\text{reg}}\) expansion by an arginase-dependent mechanism (112). In addition, GR1\(^+\)/CD11b\(^+\) cells treated with G-CSF and Flt3 ligand were able to suppress graft versus host disease (GVHD) through the induction of IL-10- producing T\(_{\text{reg}}\), while mature dendritic cells expanded with the same regimen had no effect (113). Huang et al. provided more of a direct link by demonstrating that when MDSC isolated from tumor-bearing mice were co-cultured with CD4\(^+\) T cells, there was an increase in the population of CD4\(^+\)/CD25\(^+\)/FOXP3\(^+\) T\(_{\text{reg}}\) cells, coupled with a suppression in CD4\(^+\)/CD25\(^-\) T cell activity (114). Contrasting studies indicate no involvement of T\(_{\text{reg}}\) expansion with MDSC (115, 116). These conflicting studies could be indicative of the complex nature of the interaction between these suppressor cell populations. While MDSC may induce T\(_{\text{reg}}\) in some settings, this may not occur in all settings. There could also be common development or stimulation pathways involved that drive the generation and expansion of both of these populations simultaneously.

Similar to the studies with T\(_{\text{reg}}\) cells, MDSC have also been linked to a population of suppressive macrophages termed TAM found in the tumor microenvironment. TAM are located at the tumor site, have primarily an M2 phenotype, but can also possess M1
MDSC co-cultured \textit{ex vivo} with TAM derived from tumor-bearing mice led to an increase in IL-10 production by MDSC and a reduction in IL-12 production by TAM (71). It has been suggested that MDSC represent an earlier stage of differentiation and that MDSC may differentiate into TAM at the tumor site (67). Indeed, data by Corzo \textit{et al.} suggested that HIF-1α expression could be responsible for the differentiation of MDSC into TAM (67). In support of this concept, MDSC can be differentiated \textit{in vitro} into mature macrophages (118). Whether MDSC have the potential to differentiate into TAM \textit{in vivo} has yet to be definitively determined. It is evident, however, that both populations are found at the tumor site, suggesting that cytokine cues or environmental stimuli alone are not likely to be driving MDSC to differentiate into TAM.

In addition to the multiple mechanisms of suppression previously described, MDSC have been shown to produce immunosuppressive cytokines that support not only their effector functions but also other cells in the tumor environment. Young \textit{et al.} demonstrated that MDSC produce TGF-β, IL-10, PGE2, and NO. TGF-β and NO acted together to inhibit T cell proliferation in response to CD3 stimulation (119). This was also supported by work by Terabe \textit{et al.} who demonstrated that IL-13 stimulation of myeloid cells led to TGF-β secretion and inhibited cytotoxic T cell function (120, 121). MDSC are also known to inhibit NK cell cytotoxicity through the action of membrane-bound TGF-β in a murine hepatic tumor model (122). In addition, MDSC have been shown to secrete MMPs (MMP2, MMP9, MMP13, and MMP14), which are
involved with tissue remodeling and can facilitate extravasation and metastasis of tumor cells (102, 123). Yang et al. demonstrated that co-injection of lewis lung carcinoma cells with MDSC derived from MMP9\(^{-/}\) mice led to smaller, less-vascularized tumors with the MMP9\(^{-/}\) MDSC as compared to tumors from mice co-injected with MDSC derived from WT mice. This study also reported higher levels of VEGF when MDSC were co-injected with tumor cells as compared to tumor cells alone, and demonstrated this increase was due to the elevated secretion of MMP9 from MDSC (124).

Multiple mechanisms of MDSC-inhibition have been described above. T cell function is inhibited both in an antigen-specific and antigen non-specific manner (increased iNOS, arginase, and ROS; cysteine depletion). Innate immune responses are also inhibited through multiple mechanisms (TGF-\(\beta\) production; decreased NKG2D expression; decreased cytokine production, increasing TAM function), and MDSC also decrease the generation of mature dendritic cell populations capable of processing TA. Taken together, these suppressive abilities create a major barrier to anti-tumor immunity (see Figure 1.2 below).
With all of the ways that MDSC can negatively affect immune function, there has been recent exploration into multiple classes of therapeutics to target MDSC and either drive differentiation, inhibit expansion from the bone marrow, or eliminate specific suppressive functions of MDSC (125). Nefedova et al. demonstrated, in both humans and murine models, that all-trans retinoic acid (ATRA), a derivative of vitamin A, could
drive MDSC maturation into differentiated myeloid cells, including granulocytes, DCs, and macrophages (126). While the association between ATRA and MDSC was evaluated clinically, the use of IL-2 as a combinatorial therapy potentially interfered with the ATRA effect. However, in patients that had high serum levels of ATRA, there were decreased circulating MDSC and improved allogeneic T cell responses (127). It has also been shown that increased vitamin D3 has an effect on myeloid differentiation, increasing T cell blastogenesis in patients with head and neck cancer (128).

MDSC are known to be expanded from the bone by many factors, and many of these factors converge on common signaling pathways, primarily the STAT pathway. STAT3 in particular has been demonstrated to be critical for multiple MDSC suppressive mechanisms (57). In addition, STAT3 has already been the target of multiple therapeutic strategies due to its over-expression in many types of cancer (129). Pharmacological inhibitors have been designed to attack multiple facets of STAT3 signaling, such as blocking STAT3 recruitment to activated tyrosine kinases, and preventing dimerization and subsequent DNA binding. One type of inhibitor that has been created utilizes peptides that bind to the SH2 domain of STAT3 and inhibits interaction with the engaged receptor (130). However, this type of peptide inhibitor can have low cell permeability rates, and are susceptible to protease digestion, therefore alternative peptidomimetics are being developed (131, 132). Additional non-peptide molecules have been discovered through screening approaches that have anti-STAT3 activity. These inhibitors have been shown to block STAT3-mediated gene regulation, and represent promising initial steps.
towards future therapeutic development (133-136). Oligonucleotide-based therapy against STAT3 has also shown promise in *in vivo* models (137). Intriguingly, Kortylewski *et al.* demonstrated that siRNA against STAT3 synthetically linked to a CpG oligonucleotide against toll-like receptor 9 (TLR9) resulted in STAT3 silencing in TLR9+ cells, including dendritic, B, and myeloid cells *in vivo* (138). Another class of STAT3 inhibitors includes natural product-derivatives that demonstrate STAT3 targeting abilities. These include curcumin, cucurbitacin, resveratrol, galiellalacone, and indirubin (139-143). Nefedova *et al.* used the inhibitor JSI-124 (cucurbitacin I) *in vivo* and demonstrated reduced numbers of immature myeloid cells and increased efficacy of DC-based vaccination (89).

There are several drugs already in clinical use that were recently discovered to have anti-STAT3 activity and modulate MDSC. Sunitinib is a tyrosine kinase inhibitor (TKI) that also modulates the STAT3 pathway in renal cell carcinoma (144). This TKI also has broad-spectrum effects including inhibition of signaling through VEGFR, platelet derived growth factor receptor (PDGFR), stem cell factor receptor (c-ki), and the CSF-1R (145). Ko *et al.* demonstrated that patients and mice being treated with sunitinib had decreased levels of circulating or splenic MDSC, as well as reversal of T cell inhibition and decreased T$_{reg}$ (144). Interestingly, sunitinib appears to have less effect on MDSC numbers and function at the site of the tumor, which may represent a mechanism of immune escape at the tumor site (146).
Alternative therapies in addition to STAT regulation have also demonstrated efficacy in modulating MDSC. Nagaraj et al. demonstrated that treatment of tumor-bearing mice or pancreatic cancer patients with a synthetic triterpenoid compound (CDDO-Me; RTA 402) led to a reduction in reactive oxygen species and significantly improved immune responses in vivo. Triterpenoids act by increasing expression of the nuclear factor-erythroid 2-related factor 2 (NRF2) transcription factor, which activates multiple antioxidant genes (superoxide dismutase, catalase; thioredoxin; heme oxygenase) and can lead to reduced intracellular levels of ROS (147, 148). Treatment with the anti-VEGF monoclonal antibody bevacizumab (Avastin) in renal cell carcinoma patients led to a reduction in MDSC in circulating PBMC (149). Gemcitabine, a nucleoside analog, reduces the accumulation of MDSC in spleens of tumor-bearing mice and is already approved for clinical use in pancreatic, breast, ovarian, and non-small cell lung cancer (150-152). Amino bis-phosphonate use has also been implicated in the reduction of MDSC from the bone marrow and in the periphery, through an MMP9-mediated mechanism (153). While these therapies have not achieved success as single-line agents clinically, they may represent good candidates for combinatorial therapy in future trials.

Additional MDSC suppressor pathways that have been targeted involve the modulation of iNOS and arginase. The enzyme cox-2 has been shown to increase both arginase and iNOS from MDSC (110). Cox-2 inhibitors act by decreasing prostaglandins which can regulate arginase and iNOS activity (84, 85, 110, 154). Phosphodiesterase
type 5 inhibitors (PDE-5), (sildenafil, tadalafil, vardenafil) have been shown to decrease arginase and iNOS, which resulted in restoration of T cell responsiveness in tumor-bearing mice (78). Chemical inhibitors of iNOS (L-N\textsuperscript{G}-Nitroarginine methyl ester or L-NAME) and arginase (N-hydroxy-L-arginine or NOHA), have been demonstrated to modulate MDSC function but not numbers in murine models (109) GR1 has also been targeted with depleting antibodies in mice in an attempt to deplete MDSC directly. While short-term studies appear to be effective, long-term treatment with an anti-GR1 antibody \textit{in vivo} or a low serum level of antibody can result in a resurgence of GR1\textsuperscript{+}/CD11b\textsuperscript{+} cells as compared to control mice. In some model systems, this can result in mice with even higher splenic levels of MDSC than in tumor-bearing control animals (125, 155). In addition, while this is the most direct option for MDSC depletion currently available, the GR1 antibody is not mutually exclusive to MDSC, it also recognizes mature granulocytes.

Each of these methods of modulating MDSC has targeted either a particular mechanism of suppression or was designed to remove these cells from the environment. While there has been some success with modulating MDSC and improving immune responses in murine models, unfortunately this has yet to translate directly to the clinic. Recent studies demonstrating differences between MDSC modulation in secondary lymphoid organs, in circulation, and at the site of the tumor may indicate a reason for only partial therapeutic success (67, 146). Finally, while removing these cells appears
important for restoring local and systemic immunity, there may be additional stimuli required to activate an immune response to an already established tumor.

Conclusions

The relationship between immunity and cancer development and progression has been described, along with the pro-tumorigenic and anti-tumorigenic components of this dynamic system. MDSC play a major role in shifting this balance towards pro-tumorigenic and allow tumors to progress largely un-checked by innate and adaptive immune responses. The studies outlined in this document are designed to better understand additional mechanisms by which MDSC inhibit normal immune function, as well as what part cytokines and environmental factors might play in this process. These studies were conducted in an effort to further the understanding of MDSC biology and to determine how more effective therapeutic options might be developed in order to improve anti-tumor immune responses in the cancer setting.
Abstract

Our group and others have determined that immune effector cells from patients with advanced cancers exhibit reduced activation of IFN signaling pathways. We hypothesized that increases in immune regulatory cells termed myeloid derived suppressor cells (MDSC) could interfere with the host immune response to tumors by inhibiting immune cell responsiveness to interferons. The C26 murine adenocarcinoma model was employed to study immune function in advanced malignancy. C26-bearing mice had significantly elevated levels of GR1+CD11b+ MDSC as compared to control mice, and splenocytes from tumor bearing mice exhibited reduced phosphorylation of STAT1 (P-STAT1) on Tyr 701 in response to IFN-alpha or IFN-gamma. This inhibition was seen in splenic CD4+ and CD8+ T cells as well as NK cells. In vitro co-culture experiments revealed that MDSC inhibited the IFN responsiveness of splenocytes from normal mice. Treatment of C26-bearing mice with gemcitabine or an anti-GR1 antibody led to depletion of MDSC and restored splenocyte IFN responsiveness. Spleens from C26 bearing animals displayed elevated levels of iNOS protein and nitric oxide (NO). In vitro treatment of splenocytes with a nitric oxide donor led to a decreased STAT1 IFN

response. The elevation in NO in C26 bearing mice was associated with increased levels of nitration on STAT1. Finally, splenocytes from iNOS knockout mice bearing C26 tumors exhibited a significantly elevated IFN-response as compared to control C26 tumor-bearing mice. These data suggest that NO produced by MDSC can lead to reduced interferon responsiveness in immune cells.
Introduction

Previous studies demonstrated that the endogenous production of interferon is essential for immunosurveillance against developing tumors (9, 23). Specifically, it has been determined that mice lacking either the IFNγ receptor (IFNγR1) or the major IFN signal transducer STAT1, developed tumors at a faster rate than wild type mice following exposure to the carcinogen 3-methylcholanthrene (MCA). In addition, mice lacking IFNγR1 or STAT1 as well as the tumor suppressor p53 developed a wider range of spontaneous tumors as compared to wild type mice lacking p53 only (23). Studies by Dunn et al. have also demonstrated the distinct importance of IFN-α/β sensitivity in cells of the hematopoietic lineage for immunoeediting and anti-tumor immunity in vivo (27). Interferons are now accepted as critical mediators of immunosurveillance and are important in both innate and adaptive anti-tumor immune responses. A functional immune system in patients with cancer is also critical for the success of immune-based therapies, such as exogenously administered cytokines, vaccines and targeted antibodies via the induction of type I interferons (e.g. IFN-α, IFN-β) and type II IFNs (IFN-γ). Our group and others have determined that immune effector cells from patients with advanced cancers exhibit reduced activation of IFN-induced signaling pathways (12, 13). One potential mechanism for the immune inhibition seen in tumor bearing hosts is the presence of increased numbers of immune suppressor cells.
Myeloid-derived suppressor cells (MDSC) are a heterogenous population of early myeloid cells that accumulate in the blood and tumors of patients with cancer. Their numbers correlate with tumor burden (57). These cells arise from myeloid precursors in response to tumor-derived growth factors and pro-inflammatory cytokines (57, 156). MDSC are described phenotypically in murine models as GR1⁺CD11b⁺, with subsets expressing IL-4Rα (59, 156). MDSC have been shown to reside in the peripheral blood, lymphoid tissue, and tumor tissue of mice in a number of experimental models (57, 87, 157-160). Prior studies have demonstrated that MDSC can inhibit the effector function of NK and T cells in tumor-bearing animals through multiple mechanisms, including the release of immune-suppressive cytokines, the generation of nitric oxide and reactive oxygen species, and the depletion of arginine or cystine from the tumor microenvironment (57, 73, 107). Studies in murine models indicate that disruption of MDSC function can reverse immune tolerance to tumor antigens, stimulate anti-tumor immune responses and markedly inhibit tumor growth (57, 156).

We hypothesized that elevated numbers of MDSC present in the setting of advanced malignancy would inhibit the response of immune cells to type I and II interferons. The results of our experiments demonstrated that mice bearing C26 adenocarcinoma tumors exhibit elevated numbers of MDSC which led to increased nitration on STAT1 and impaired responsiveness of immune effector cells to interferons.
Materials and Methods

*Cytokines and Reagents.* Recombinant murine IL-6 and IFN-γ were purchased from R & D Systems, Inc. (Minneapolis, MN). Recombinant Universal Type I Interferon (IFN-A/D) was purchased from PBL Biomedical Sciences (Piscataway, NJ). S-nitroso-N-acetylpenicillamine (SNAP) was purchased from Molecular Probes (Eugene, OR).

*Murine Tumor Models.* Male 4-6 week old BALB/c X DBA/2F1 (CD2F1) mice were used (Harlan, Indianapolis, IN). Colon 26 (C26) tumor cells (10^6 cells) were injected subcutaneously into the flank of CD2F1 mice (161). Tumor size was monitored over time. Tumor or splenic tissue for histologic analyses were obtained upon sacrifice and fixed in formalin or frozen in OCT (Sakura, Torrance, CA). Male Balb/c By and iNOS knockout mice were injected with C26 tumor cells (10^6 cells) for nitric oxide studies (4-6 weeks, Jackson Labs). Female BALB/c mice (4-6 weeks, Harlan) were injected in the mammary fat pad with 5x10^4 4T1 tumor cells.

*Intracellular Flow Cytometry.* Levels of P-STAT1 and STAT1 were measured via flow cytometry in cells derived from murine splenocytes or lymph nodes following *in vitro* stimulation with PBS, IFN-A/D or IFN-γ as described previously (12). Data were expressed as specific fluorescence (Fsp = Ft – Fb), where Ft represents the median value.
of total staining and Fb represents the median value of background staining with an isotype control antibody (12, 162, 163). CD4, CD8, and CD49b antibodies were used for surface staining of immune subsets (BD Biosciences). For nitration flow cytometry, splenocytes were processed as described above and co-labeled with anti-STAT1-PE and anti-nitrotyrosine-alexafluor-488 antibody (BD Biosciences and Millipore). Percent total represents the percentage of cells staining positive for STAT1 and nitrotyrosine above that of the appropriate isotype controls for each antibody.

Real Time PCR. Following TRIzol extraction (Invitrogen) and RNeasy purification (Qiagen), total RNA was quantitated and reverse transcribed as previously described (164). The resulting cDNA was used to measure gene expression by Real-Time PCR using pre-designed primer/probe sets and 2x TaqMan Universal PCR Master Mix with 18s rRNA as an internal control (Applied Biosystems).

Immunoblot Analysis. Lysates were prepared from splenocytes of C26-bearing mice or age matched CD2F1 mice without tumors following in vitro stimulation with PBS, IFN-A/D or IFN-γ and assayed for the expression of Jak-STAT proteins by immunoblot (164). For the detection of nitrated STAT1, samples were immunoprecipitated with STAT1 (BD Biosciences) and then probed with an anti-nitrotyrosine antibody (Invitrogen).
Flow Cytometric Analysis of Myeloid Derived Suppressor Cells. Analysis of the MDSC in splenocytes from mice was conducted as previously described (59). Briefly, 10⁶ splenocytes were labeled with fluorochrome-labeled Ab targeting murine CD11b, GR1, IL-4Rα or appropriate isotype control Ab (BD Biosciences) for 1hr at 4°C, washed, resuspended in 1% formalin, and analyzed via flow cytometry.

Immunofluorescent staining of MDSC. Tumors were fixed in 4% paraformaldehyde overnight, dehydrated in sucrose, and embedded in OCT and frozen. Frozen sections (5 microns) were probed for MDSC with anti-GR1 alexafluor 488 (Invitrogen) and anti-CD11b alexafluor 647 (BD Bioscience) antibodies, or appropriate isotype control. Sections were also labeled with DAPI nuclear stain. Slides were analyzed on an Olympus FV1000 Spectral Confocal microscope.

Isolation of MDSC from C26-bearing mice. Spleens were harvested aseptically from tumor-bearing mice (163), filtered through 70 µM cell strainers, washed with PBS, and resuspended in RPMI 1640 with 10% FBS (complete media). GR1⁺/CD11b⁺ MDSC were isolated on a magnetic column using anti-GR1 biotinylated beads (Miltenyi Biotec, Auburn, CA) with purity > 95% by flow cytometry. Isolated MDSC were co-cultured with splenocytes at a 1:3 ratio. This ratio recapitulates the number of MDSC present within the spleen of a C26-bearing mouse. After 24 hours, splenocytes were harvested from culture and stimulated with IFN-A/D (10⁴ U/mL) for 15 minutes. IFN-induced levels of P-STAT1 were measured by flow cytometry as described.
**MDSC depletion with GR1 antibody.** On day 20 post-tumor inoculation with C26, mice were treated with either anti-GR1 (BD Biosciences) or isotype control antibody (Sigma) at a concentration of 0.25mg/mouse in 200 µl PBS. This allowed for comparable tumor sizes in the antibody treated and control animals. Depletion was verified by flow cytometry staining for GR1 in splenocytes after 24 hours. Intra-tumor MDSC depletion was verified by immunofluorescent staining of frozen tissue sections.

**Gemcitabine treatment of C26-bearing mice.** Two days after C26 tumor inoculation, mice received twice weekly i.p. injections of gemcitabine (Gemzar®, Eli Lilly) at a dose of 75µg/g of body weight. This agent has been previously validated as an effective means of reducing MDSC numbers (69, 150, 151). For 24 hour depletion studies, mice were injected with 75µg/g gemcitabine 24 hours prior to animal harvest. MDSC depletion was also verified in the tumor by immunofluorescent staining of frozen tumor sections.

**Measurement of nitric oxide by DAF-FM fluorescence.** Spleens obtained from C26 tumor bearing mice were washed with an ice-cold PBS buffer and embedded in OCT. The frozen segments were cut into 6 µm thick sections and incubated with 10 µM DAF-FM diacetate (Molecular probes, D-23842) for 30 min at 37°C. The images of the tissue sections were obtained using a fluorescence microscope (Nikon). The fluorescence
intensity was quantitatively determined using MetaMorph image analysis software (Molecular devices, CA).

**Immunohistochemistry of tumors.** Tumors obtained from wild type and iNOS knockout mice bearing C26 tumors were fixed in formalin overnight, washed with PBS, paraffin embedded, sectioned into 4-μm slices using a standard microtome, attached to lysine-coated slides, and stained with H&E as previously described (165). Replicate sections were de-paraffinized in xylene (two times, 10 min each at room temperature) and rehydrated by stepwise washes at decreasing ethanol/H$_2$O ratios. Endogenous peroxidase activity was blocked with dH$_2$O (diH$_2$O) containing 3% hydrogen peroxide for 5 min, followed by repeated rinses in diH$_2$O. Ag retrieval was achieved in Dako's target retrieval solution (Dako S1699) by heating slides in a steamer at 94°C for 30 min and cooling at room temperature for 15 min. After rinsing in diH$_2$O, slides were incubated for 60 min with Abs specific for CD34 (Abcam; clone MEC14.7), and Ki67 (Thermo; clone SP6). Detection was achieved with the Vectastain Elite ABC system and Novared Chromogen (Vector, Burlingame, CA). All samples were examined in a blinded fashion by an experienced pathologist (K.M.L.) using an Olympus BX45 light microscope with an attached DP25 digital camera (B & B Microscopes limited, Center Valley, PA). The binding of each Ab to target Ag was determined in viable areas of the tumor based on the following semiquantitative scoring system: 0, none; 1, few (<25%); 2, many (25–75%); 3, majority (>75%). Additionally, automatic quantification of immunoreactivity was performed on whole slide images scanned at 40x magnification (ScanScope XT, Aperio.
Technologies, Vista, CA) using the Positive Pixel Count Algorithm in the ImageScope software (Aperio Technologies).

Statistical Analysis. Two-sample t-tests were used to compare outcomes (e.g. IL-6, Ifit2, P-STAT1) between groups. If necessary, outcomes were log-transformed to meet the assumptions of normality and constant variance for the test. Pearson correlation coefficients were used to quantify the relationship between outcomes. Tumor growth was assumed to be log-linear and a mixed effects model with a random intercept and slope for each animal was used to estimate the rate of increase. P-values less than 0.05 were considered statistically significant.
Results

*Decreased interferon response of immune cells from tumor-bearing mice.* We hypothesized that immune effector cells in tumor-bearing mice would have an altered response to cytokines that promote anti-tumor immunity as is seen in patients with advanced malignancy. Of particular interest were the type I and type II IFNs, as these cytokines mediate tumor immunosurveillance, and reductions in immune cell IFN responsiveness have been shown to exist in cancer patients (9, 10, 23, 27). The C26 model was chosen for this study due to its rapid tumor growth and ability to mimic advanced clinical disease by the development of a muscle wasting syndrome and decreased immune function. The ability of IFN-α or IFN-γ to activate the STAT1 transcription factor (by phosphorylation at Tyr701) was measured by intracellular flow cytometry in splenocytes from normal and C26-bearing mice (Figure 2.1 and representative histogram Supplemental Figure 2.7A). Induction of P-STAT1 following a 15 minute stimulation of splenocytes with IFN-A/D ($10^4$ U/mL) was significantly reduced at day 22 in C26-bearing mice (mean MFI P-STAT1 = 18.8; range = 4.0 – 33.4) as compared to control mice (mean MFI P-STAT1 = 37.9; range = 27.0 – 50.0; Figure 2.1A; p<0.0001). Induction of P-STAT1 was also significantly decreased in splenocytes from C26-bearing mice following stimulation with 10 ng/ml IFN-γ (Figure 2.1B; p = 0.003). MDSC (approximately 15-20% of total splenocytes) were excluded from this analysis by gating to ensure that the lymphocyte populations of normal and
tumor-bearing animals were of comparable composition. These results were validated by immunoblot analysis (Supplemental Figure 2.7B). The decrease in IFN responsiveness was also observed in cells derived from lymph nodes (Supplemental Figure 2.7C). To evaluate the effect of the C26 tumor on IFN responsiveness in vivo, normal or C26 tumor-bearing animals were injected with IFN-A/D (2x10^4 U) and splenocytes were harvested 2 hours later for analysis. The level of P-STAT1 was evaluated by intracellular flow cytometry as described previously (Figure 2.1C). As expected the P-STAT1 response was reduced in C26-tumor bearing mice both at baseline and in response to exogenous administration of IFN. An analysis of IFN responsiveness in lymphocyte subsets by dual parameter intracellular flow cytometry revealed that the induction of P-STAT1 was inhibited in CD4, CD8, and CD49b (NK cells) (Figure 2.1D, CD4 p = .009; CD8 p = .002; NK, p = .02). We next analyzed the induction of the IFN-α stimulated gene (ISG), Ifit2 and the IFN-γ stimulated gene, interferon-regulatory factor (Irf1) by Real Time PCR following a 4 hour in vitro treatment of splenocytes from C26-bearing or normal mice with IFN-A/D (10^2 U/mL or 10^4 U/mL). The expression of Ifit2 was significantly reduced in splenocytes obtained from C26-bearing mice as compared to controls (n = 5 mice per group) at both doses of IFN-A/D (Normal = 92.7 ± 24.5 vs. C26 36.4 ± 12.6, p = 0.0018 at 10^2 U/mL; Normal = 202.2 ± 21.7 vs. C26 99.9 ± 17.2, p = .0059 at 10^4 U/mL; Figure 2.2A). The expression of Irf1 was also significantly reduced in splenocytes from C26-bearing mice (n = 5-6 mice per group) versus controls following a 4 hr stimulation with IFN-γ (Figure 2.2B; p<0.05). We also evaluated
expression of the interferon-inducible genes ISG15, OAS1, and IRF8 and observed similar trends (Supplemental Figure 2.9A–B and data not shown).

MDSC levels are elevated in tumor-bearing mice, and are associated with decreased IFN response in vitro. Flow cytometric analysis demonstrated a significantly higher percentage of MDSC in splenocytes from C26-bearing mice as compared to splenocytes from control animals (Figure 2.3A-B) (166). Histologic analysis of splenic tissue revealed extramedullary hematopoiesis and abundant myeloid precursors resulting in extensive splenomegaly (data not shown). Time course studies revealed that an increase in MDSC occurred as early as 7 days post-tumor inoculation (Supplemental Figure 2.8A). To test whether MDSC can directly inhibit IFN responsiveness of immune cells, a series of in vitro studies was initiated in which GR1⁺CD11b⁺ MDSC were isolated from splenocytes of C26-bearing mice and co-cultured with immune cells from tumor-naïve animals. Splenocytes isolated from normal mice and co-cultured with MDSC had a reduced level of IFN-stimulated P-STAT1 as compared to normal splenocytes cultured for the same time period (Figure 2.3C; mean MFI P-STAT1 = 22.87 vs. 33.61; p = 0.0004). P-STAT1 was measured in the lymphocyte population only, excluding MDSC from analysis by gating. We also showed that elevated MDSC levels were associated with concomitant decreases in IFN responsiveness in the 4T1 murine epithelial cancer model (Supplemental Figure 2.8B) which supported the association of MDSC with reduced generation of P-STAT1 in response to IFN-α.
Reduction of MDSC leads to restored IFN-responsiveness in C26-bearing mice.

C26-bearing animals were treated with the drug gemcitabine, a nucleoside analog, which has been previously employed by other groups to deplete MDSC (69, 150-152). This treatment regimen led to a decrease in the percentage of MDSC present within splenic tissues (Figure 2.4A; 10.1% vs. 3.7%; p = 0.0010). We also treated mice with a single dose of gemcitabine 24-hours prior to spleen harvest, and saw comparable levels of MDSC depletion in splenocytes from C26 tumor-bearing animals. As predicted, long-term gemcitabine treatment reduced tumor growth by approximately 90%, while the 24-hour treatment resulted in no change in tumor volume. Splenocytes obtained from C26-bearing mice treated both long term and for 24-hours with gemcitabine exhibited a restoration of interferon responsiveness as measured by intracellular P-STAT1 flow cytometry and western blot analysis (Figure 2.4B and Supplementary Figure 2.10). Notably, when we evaluated the effects of gemcitabine directly on C26 tumor cells in vitro, only 15% of C26 cells were apoptotic when using an equivalent dose of gemcitabine (data not shown). C26-bearing animals were also treated with an anti-GR1 antibody 24-hours prior to spleen harvest (day 20 post-tumor inoculation). This short-term depletion strategy was utilized to evaluate the direct effects of MDSC on IFN response in the presence of fully developed tumors. The reduction in MDSC levels (77% decrease) was verified by flow cytometry (Figure 2.4C). Splenocytes from tumor-bearing mice treated with one dose of anti-GR1 exhibited a significantly higher IFN response compared to the splenocytes of tumor-bearing mice treated with isotype control as evaluated by intracellular P-STAT1 flow cytometry and western blot analysis.
(Figure 2.4D, 42% increase in mean MFI P-STAT1 at 10^4 U/mL p = 0.002; Supplemental Figure 2.10). GR1+CD11b+ MDSC were also evaluated in the tumor after treatment with gemcitabine or anti-GR1. All treatments led to a reduction in intra-tumor MDSC as compared to tumor-bearing control animals (Supplemental Figure 2.11).

**Elevated nitric oxide and IFN-responsiveness.** We hypothesized that soluble factors produced from the abundant MDSC present in the splenic tissue could contribute to the reduced IFN-response in tumor-bearing mice. We evaluated two of the major mechanisms through which MDSC are known to exert their suppressive effects, and discovered that while arginase I transcript levels were not significantly different in tumor-bearing splenocytes as compared to splenocytes from normal mice, iNOS mRNA levels were dramatically elevated (data not shown and Supplemental Figure 2.9C). DAF-FM and iNOS staining and fluorescent microscopic analysis were employed to test whether decreased IFN-responsiveness might be associated with increased levels of NO in the splenic tissue. These data confirmed a dramatic increase in the level of iNOS protein and NO in splenocytes from C26-bearing mice as compared to spleens from control animals (Figure 2.5A). Pre-treatment of splenocytes from normal mice with the NO donor S-nitroso-N-acetylpenicillamine (SNAP; Molecular Probes, Eugene, OR) led to a decreased phosphorylation of STAT1 in response to IFN-α stimulation (Figure 2.5B; mean MFI P-STAT1 = 35.7 vs. 16.4). STAT1 was immunoprecipitated from equal numbers of splenocytes from normal and C26 tumor-bearing mice and probed with an anti-nitrotyrosine antibody to detect nitration of STAT1 protein. We performed a
Student’s t-test analysis on the densitometry results and determined that the ratio of nitrated STAT1 to total STAT1 in tumor bearing mice is statistically higher than in normal mice, demonstrating that C26-bearing animals exhibited significantly elevated nitration of STAT1 as compared to control mice (p=0.03; Figure 2.5C). In addition, we validated the level of nitrated tyrosine on STAT1 by flow cytometry. Splenocytes from normal and C26 tumor-bearing mice were labeled with antibodies against total STAT1 and nitrotyrosine. Splenocytes from tumor bearing and normal mice had equal levels of total STAT1. There was significantly more nitrated STAT1 in splenocytes from tumor-bearing animals as compared to splenocytes from normal mice (36 ± 2.0% vs. 17 ± 0.3%; p<0.001; Supplemental Figure 2.9D). This result provides direct evidence that nitration on STAT1 was enhanced in C26-bearing mice. These data support a role for NO in dampening the response of immune effector cells to stimulation with IFN in tumor bearing animals. To determine the role of nitric oxide in interferon responsiveness in vivo, iNOS knockout mice were inoculated with C26 tumor cells and tumors were allowed to grow for 21 days. At the end of the study, tumor sizes were comparable between iNOS knockout mice and wild type control mice bearing C26 tumors. Splenocytes from iNOS knockout mice exhibited a significantly elevated induction of P-STAT1 in response to IFN stimulation as compared to immune cells from wild type mice (IFN 10^4 U/mL p = 0.01; Figure 2.5D). Quantitative histological analysis of iNOS-deficient tumors and tumors from WT mice revealed a significant decrease in Ki67 staining (34.24 ± 0.4% vs. 11.2 ± 2.85%; p = 0.01) as well as CD34 expression (29.93 ±
2.25% vs. 6.55 ± 2.77%; p = 0.02) expression in iNOS-deficient mice, indicating a reduced proliferation rate as well as decreased vascularity (Figure 2.6).
Discussion

In the present report, we have demonstrated that immune cells from tumor-bearing mice display a significantly reduced capacity for IFN-induced signal transduction and gene induction via the STAT1 pathway. This inhibitory effect was observed in CD8+ and CD4+ T cells, as well as in NK cells and occurred following both in vivo and ex vivo IFN stimulation. C26 tumor-bearing mice exhibited enlarged spleens as compared to normal controls and displayed a concomitant increase in the level of GR1+CD11b+ myeloid derived suppressor cells, and depletion of MDSC in tumor-bearing mice led to a restoration of IFN-responsiveness in immune cells. Consistent with these observations, in vitro co-culture of splenocytes with MDSC reduced their ability to respond to IFNs. Splenocytes from C26-bearing mice had elevated levels of iNOS protein, NO production, and STAT1 tyrosine nitration. Importantly, splenocytes from iNOS-deficient mice exhibited improved IFN responsiveness as compared to splenocytes from tumor-bearing wild type mice. These results indicate that MDSC contribute to the blunted responsiveness of immune cells to IFN-α and IFN-γ via their ability to produce reactive nitrogen species, which leads to reduced IFN-induced signal transduction in immune cells.

Our group and others have determined that immune cells from patients with advanced cancers exhibit reduced activation of IFN-induced signaling pathways,
although the mechanisms underlying this observation are still under investigation. We utilized the C26 model to recapitulate the complex interactions that take place between immune cells and cancer cells \textit{in vivo}. The IFN response of lymphocytes from C26 tumor-bearing mice (as measured by STAT1 phosphorylation and regulation of IFN-stimulated genes) was significantly reduced when compared to normal mice. It was also noted that the spleens of these mice were enlarged and filled with myeloid precursor cells. Phenotypic characterization revealed that these precursor cells were MDSC, and we hypothesized that they were responsible for the reduced activation of STAT1 in response to IFN-\(\alpha\). Previous work by Mazzoni \textit{et al.} showed that co-culture of immortalized murine MDSC with T cells in an \textit{in vitro} non-tumor model led to an inhibition of the T cell response to IL-2 (167). We subsequently demonstrated that the presence of primary MDSC in tumor-bearing animals was associated with altered IFN signal transduction and that removal of this cell population led to restoration of IFN responsiveness. Thus, MDSC appear to be an important regulator of the downstream response to IFNs in tumor-bearing mice. This is the first report in which MDSC have been implicated as a mechanism for the reduced activation of immune cells following IFN stimulation.

While other tumor or MDSC-derived factors may contribute to reduced IFN responsiveness, our data suggests that the production of NO by MDSC is a major mechanism in this model. Increased levels of iNOS and NO in the spleen lead to the nitration of STAT1 on immune cells, which is the proposed mechanism of inhibition of
IFN signaling. Importantly, splenocytes from iNOS deficient tumor-bearing mice had a significantly improved interferon response as compared to splenocytes from wild type tumor-bearing mice. This finding provides a direct *in vivo* link between excess nitric oxide and decreased interferon responsiveness in the setting of malignancy. In support of these observations, Brito *et al.* have demonstrated that peroxynitrite treatment of PBMCs led to increased nitration of tyrosine residues, which inhibited transcription factor phosphorylation following CD3 stimulation, while Llovera *et al.* showed that LPS-treated macrophages had an impaired *in vitro* immune cell response to IFN-γ due to an increase in nitric oxide in the culture (168, 169). We observed that tumor-bearing mice exhibited elevated splenic levels of iNOS protein and treatment of normal splenocytes with SNAP, a nitric oxide donor, suppressed the P-STAT1 response of the splenocytes to IFN-α stimulation. Nitration on STAT1 in primary lymphocytes from tumor-bearing mice has not previously been demonstrated in an *in vivo* murine model. These findings support a role for nitric oxide in the inhibition of IFN signal transduction.

In the studies with iNOS-deficient mice, interferon responsiveness in splenocytes from tumor-bearing animals was not fully restored to levels observed in normal animals. This indicates that other mechanisms may exert an effect on the interferon response in the tumor microenvironment. We evaluated alternative mechanisms that could lead to this inhibition, such as increased levels of arginase I or IL-6. Up-regulation of arginase I has been identified as a mechanism of immune suppression that is employed by MDSC (72, 170, 171). While previous studies in macrophages have demonstrated an inverse
relationship between iNOS and arginase I levels, it has been shown that in a tumor setting it is possible for iNOS and Arg I to be co-expressed (59, 69, 109, 172). However, splenic levels of this enzyme were not significantly elevated in the C26 model. While arginase could still be playing a role in the tumor environment, our data in iNOS-deficient mice suggests that iNOS is a driving factor for the decreased IFN response in immune cells in this model. Despite the observation that immune cells from iNOS-deficient mice exhibited improved responsiveness to IFN, it is important to note that C26 tumors from these knockout mice were similar in size those from control mice. We anticipated that enhancement of the immune cell IFN response might lead to improved growth control of tumor cells in iNOS-deficient mice and reduced overall tumor volume. However, there was no significant reduction in external tumor size at the end of the study. It is important to note that in this model, the nitric oxide deficiency was present in all host tissues, rather than just the MDSC compartment, and nitric oxide has effects on multiple biologic processes (tumor cell invasion/proliferation, and angiogenesis). A careful quantitative histologic analysis of tumors revealed significantly decreased levels of Ki67 (proliferative marker) and CD34 (expressed on vascular endothelium) in iNOS-deficient mice as compared to those from WT mice (Figure 2.6). These findings indicate that the iNOS deficiency resulted in biological differences at the site of the tumor. Additional studies would be needed to directly assess the long-term impact of improved IFN response and iNOS-deficiency on survival and anti-tumor immunity.
The presence of tumor can also induce elevations in pro-inflammatory cytokines that have been associated with MDSC generation or function. In the C26 model, IL-6 levels were elevated in tumor-bearing mice as compared to normal mice. However, *in vitro* treatment of splenocytes with IL-6 did not alter the P-STAT1 response to IFN-α and administration of exogenous IL-6 was similarly incapable of inhibiting the splenocyte response to this cytokine (Supplemental Figure 2.12). Another potential explanation for our results relates to possible changes in levels of Jak-STAT signaling intermediates. However, a careful analysis revealed no significant difference in the protein expression of STAT1, IFNAR, or Jak1 in splenocytes obtained from tumor-bearing as compared to normal control mice (data not shown). As seen in Figure 5C, there was some variability in total STAT1 protein between individual mice, but this variability was not associated with the presence or absence of tumor. Previous work by Brito *et al* demonstrated that increased NO did not lead to changes in total STAT1 levels in immune cells, and this was confirmed in the present model when it was shown that STAT1 protein levels were similar in splenocytes from normal and tumor-bearing mice despite high levels of NO (168).

The observation of decreased IFN-responsiveness in immune cells has important implications for the clinical course of disease in cancer patients. Type I and II IFNs play a critical role in mediating the response to immune-based therapies (10). Therefore, conditions that inhibit IFN-induced signal transduction and gene transcription are likely to dampen the responsiveness of the host immune system to immune-based treatments.
and the host’s ability to recognize and eliminate established tumors (15, 173-176). Reports have also demonstrated that type I and II IFNs are functionally important in mediating tumor immunosurveillance against methylcholanthrene-induced sarcomas and the progression of tumors in p53 null mice (9, 23). Previous studies from our group and others have shown that lymphocytes from patients with metastatic melanoma and other solid malignancies have an inherent reduction in basal and/or IFN-induced STAT1 phosphorylation as compared to normal donors (12-14). It is important to note that these studies focused on the IFN response in T cells and NK cells, but did not evaluate the responsiveness of dendritic cells or other cell subsets that have been previously shown to play a role in IFN mediated anti-tumor immunity. These subsets could potentially be impaired by nitrated residues as was observed in other lymphocyte populations. Reduced immune surveillance resulting from NO-dependent inactivation may therefore be important in the process of tumorigenesis and/or tumor progression.

To our knowledge this is the first in vivo report to demonstrate that elevated numbers of MDSC are associated with a decreased cellular response to IFN-stimulation in the setting of cancer. Further experiments suggest that increased nitration of STAT1 on tyrosine residues is responsible for the diminished IFN response in splenocytes. Therefore, therapeutic strategies targeted at reducing MDSC or upstream factors could potentially serve to restore IFN-responsiveness in tumor-bearing hosts.
Figure 2.1. Decreased interferon response in tumor-bearing mice. P-STAT1 was measured in splenocytes from control (Control; n = 19) or day 22 C26 tumor-bearing mice (C26 Tumor; n = 19) by intracellular flow cytometry following a 15 minute stimulation with (A) IFN-α (10^4 U/mL) or (B) IFN-γ (10 ng/mL). Y-axis is specific fluorescence (Fsp) of staining for P-STAT1. (C) P-STAT1 was measured in splenocytes from untreated normal mice and mice bearing C26 tumors, or from mice stimulated with 2x10^4 U IFN-A/D in vivo and harvested 2 hours later. (D) P-STAT1 was measured following IFN stimulation in splenocytes that were co-labeled with antibodies against CD4, CD8, and CD49b.
Figure 2.2: Attenuated interferon-stimulated gene expression in splenocytes from C26-bearing mice. (A) Total RNA was isolated from splenocytes of control or C26-tumor bearing mice following stimulation of the cells for 4 hours with IFN-A/D or PBS, converted to cDNA and analyzed by real-time RT-PCR for Ifit2. (B) Irf1 expression was evaluated in a similar manner following stimulation of splenocytes with PBS or IFN-γ (10 ng/mL). Real-time RT-PCR data were obtained in triplicate and are expressed as the mean fold increase relative to the level of 18s mRNA. Each symbol represents the fold change of an individual animal.
Figure 2.2. Attenuated interferon-stimulated gene expression in splenocytes from C26-bearing mice.
Figure 2.3. MDSC are elevated in tumor-bearing mice and are associated with decreased IFN responsiveness in vitro. (A) Splenocytes from day 22 C26 mice were evaluated for MDSC using antibodies targeting GR1, IL-4Rα, and CD11b. (B) Splenocytes from day 22 C26 mice were evaluated for MDSC using antibodies targeting GR1 and CD11b. MDSC percentage in splenocytes from day 22 C26 tumor-bearing mice (C26; n = 19) or controls (Normal; n = 22) as described above. (C) MDSC were isolated from C26 splenocytes by magnetic labeling with anti-GR1 beads. After 24 hours, P-STAT1 was measured in splenocytes obtained from control mice (n = 10) or normal splenocytes that had been co-cultured with MDSC at a 1:3 ratio (n = 10).
Figure 2.4: Reduction in MDSC with gemcitabine or anti-GR1 restores IFN responsiveness. (A) Splenocytes obtained from day 22 C26 tumor-bearing were evaluated by flow cytometry for the presence of MDSC. (B) P-STAT1 was measured in splenocytes obtained from control mice (Normal; n = 5), Day 22 mice bearing C26 tumors and treated with PBS (C26; n = 5), Day 22 mice bearing C26 tumors and treated with gemcitabine (C26+gemcitabine; n = 6), and Day 22 mice bearing C26 tumors and treated with gemcitabine 24-hours prior to harvest (C26+gemcitabine 24hr; n=5) by intracellular flow cytometry following a 15 minute stimulation with IFN-A/D (10^4 U/mL). Data are expressed as the mean specific fluorescence (Fsp) of staining for P-STAT1. (C) Splenocytes obtained from Day 21 control mice (Normal), mice bearing C26 tumors and treated with isotype control (C26+IgG; n = 6), or mice bearing C26 tumors and treated with anti-GR1 (C26+GR1; n = 7) were evaluated for MDSC by flow cytometry and (D) P-STAT1 was measured in splenocytes obtained from normal or day 21 tumor-bearing mice and treated with isotype control (C26+IgG; n = 6), or mice bearing C26 tumors and treated with anti-GR1 (C26+GR1; n = 7) by intracellular flow cytometry following a 15 minute stimulation with IFN-A/D (10^4 U/mL).
Figure 2.4. Reduction in MDSC with gemcitabine or anti-GR1 restores IFN responsiveness.
Figure 2.5. Elevated levels of nitric oxide (NO) in tumor bearing mice inhibits IFN responsiveness in immune cells. (A) IF staining for iNOS protein (Fitc) and DAPI nuclear counterstain (left two panels). DAF-FM nitric oxide staining of snap frozen splenic tissue from normal or C26 tumor-bearing mice. DAF-FM Fitc staining for nitric oxide and DAPI nuclear counterstain. (B) Splenocytes from normal mice were pre-treated with the nitrogen donor SNAP for 16 hours, stimulated for 15 minutes with PBS or IFN-A/D (10⁴ U/mL) and evaluated for P-STAT1 by flow cytometry. (C) Protein lysates from splenocytes of normal or C26-bearing mice were immunoprecipitated with STAT1 antibody and then probed for nitrotyrosine. Lysates were also run on a separate gel and probed with an anti-STAT1 antibody (lower panel). Numbers represent densitometry values for nitrotyrosine and STAT1, and the ratio of nitrotyrosine/STAT1 (p=0.03). (D) P-STAT1 was measured in splenocytes obtained from day 21 iNOS knockout and control mice with and without C26 tumors by intracellular flow cytometry following a 15 minute stimulation with IFN-A/D (10⁴ U/mL).
Figure 2.5. Elevated levels of nitric oxide (NO) in tumor bearing mice inhibits IFN responsiveness in immune cells.
Figure 2.6. Reduced CD34 and Ki67 expression in tumors from iNOS knockout mice. Formalin-fixed, paraffin-embedded tumor tissue from C26 tumors in wild type mice as compared to iNOS KO mice. Each panel represents an individual mouse tumor section stained with (A) CD34, a vascular endothelial marker (p=.02) or (B) Ki67, a proliferative marker (p=0.01).
Figure 2.7. Reduced P-STAT1 in response to IFN-alpha stimulation in spleen and lymph nodes of C26 tumor-bearing mice. (A) A representative scatter plot and histogram demonstrating the parameters used to measure levels of phosphorylated STAT1 levels. The gray shaded region is the isotype control, the dashed peak indicates baseline P-STAT1 fluorescence, and the solid peak indicates IFN-stimulated P-STAT1. (B) Representative immunoblot of splenocytes harvested from normal and 21 day C26 tumor-bearing mice (n=2) that were treated with PBS or IFN-alpha (10^4 U/ml) for 15 min and then processed for analysis using an antibody specific for P-STAT1. Levels of β-actin were measured to control for loading. (C) Lymph node cells were harvested from normal and 21 day C26 tumor-bearing mice then treated with 10^3 U/ml or 10^4 U/ml IFN-alpha for 15 min and analyzed for the presence of phosphorylated STAT1 by flow cytometry. Gemcitabine treatments were used to deplete mice of MDSC.
Figure 2.8. Splenic MDSC increased over time and lymph nodes of tumor-bearing mice exhibit reduced IFN response. (A) Time course analysis of total MDSC in splenocytes following injection of tumor cells. Data are derived from n > 6 mice and expressed as the mean percent positive staining for GR1, IL-4Rα, and CD11b. (B) P-STAT1 was measured in splenocytes from control mice (Normal; n = 5) or day 22 mice bearing 4T1 tumors (4T1; n = 5) following a 15 minute stimulation with IFN-A/D (10^4 U/mL).
Figure 2.8. Splenic MDSC increased over time and lymph nodes of tumor-bearing mice exhibit reduced IFN response.
Figure 2.9. Expression of IFN responsive genes, iNOS expression, and nitration on STAT1 in normal and tumor-bearing mice. Splenocytes from normal and 21 day C26 tumor-bearing mice were treated with (A) IFN-alpha (10^4 U/ml) or (B) IFN-γ (10 ng/ml) for 4 hr, processed for total RNA, and then analyzed for ISG15 transcript expression relative to PBS treatment by Real-Time PCR. All Real-time RT-PCR data were normalized to the level of 18s. (C) Total RNA was isolated from splenocytes of day 7 or day 23 C26 tumor-bearing, or age-matched normal control mice (Normal). RT-PCR analysis was used to detect expression of iNOS relative to PBS treatment as indicated above (n=5 mice/group). (D) Splenocytes from normal and 21 day C26 tumor-bearing mice were evaluated for expression of total STAT1 and nitrotyrosine. Intracellular flow cytometry analysis was used to determine the level of nitration on total STAT1.
Figure 2.10. Treatment of tumor-bearing mice with gemcitabine or anti-GR1 leads to a restoration of P-STAT1 protein levels. Splenocytes from normal and 21 day C26 tumor-bearing mice were treated with PBS or IFN-alpha (10^4 U/ml) for 15 min and then processed for immunoblot analysis using an antibody specific for P-STAT1. Mice were depleted of MDSC via 21 day or 24 hour treatment with gemcitabine (left panel) or 24 hour treatment with an anti-GR1 Ab.
Figure 2.11. Intra-tumoral MDSC are inhibited by treatment with gemcitabine or an anti-GR1 antibody. Tumors from C26 tumor-bearing mice were harvested, fixed with paraformaldehyde, snap frozen in OCT, and analyzed for the presence of GR1 and CD11b positive cells by immunofluorescence microscopy. Cell nuclei were counterstained with DAPI. MDSC were depleted via 24 hour depletion with gemcitabine (75 mg/kg) or an anti-GR1 antibody (0.25 mg/mouse).
Figure 2.12. Direct effects of IL-6 on interferon-responsiveness. (A) Splenocytes from normal mice were pre-treated with 10 ng/mL IL-6 for 24 hours, stimulated for 15 minutes with PBS, IFN-A/D (10^3 U/mL), or IFN-γ (10 ng/mL) and evaluated for P-STAT1 by flow cytometry. (B) CD2F1 mice were given i.p. injections of recombinant murine IL-6 (500 ng/day) for twenty consecutive days. Splenocytes were harvested, stimulated for 15 minutes with PBS or IFN-A/D (10^4 U/mL) and analyzed for levels of P-STAT1 by flow cytometry.
Chapter 3: Distinct Myeloid Suppressor Cell Subsets Correlate with Plasma IL-6 and IL-10 and Reduced Interferon-alpha Signaling in CD4⁺ T cells from Patients with GI Malignancy²

Abstract

Interferon-alpha (IFN-α) promotes anti-tumor immunity through its actions on immune cells. We hypothesized that elevated percentages of myeloid-derived suppressor cells (MDSC) and increased pro-inflammatory cytokines in peripheral blood would be associated with impaired response to IFN-α in patients with gastrointestinal GI malignancies. This study evaluated relationships between plasma IL-6, IL-10, circulating MDSC subsets and IFN-α-induced signal transduction in 40 patients with GI malignancies. Plasma IL-6 and IL-10 were significantly higher in patients versus normal donors. CD33⁺HLADR⁺CD11b⁺CD15⁺ and CD33⁺HLADR⁻/lowCD14⁺ MDSC subsets were also elevated in patients versus normal donors (p<0.0001). Plasma IL-6 was correlated with CD33⁺HLADR⁺CD15⁺ MDSC (p=0.008) and IL-10 with CD33⁺HLADR⁻CD15⁻ MDSC (p=0.002). The percentage of CD15⁺ and CD15⁻ but not CD14⁺ MDSC subsets were inversely correlated with IFN-α-induced STAT1 phosphorylation in CD4⁺ T

² This chapter was reprinted with kind permission from Springer Science+Business Media: Cancer Immunology, Immunotherapy. 2011. Mundy-Bosse, BL., Young, G., Bauer, T., Binkley, E., Bloomston, M., Matthew A. Bill, M., Bekaii-Saab, T., Carson, WE 3rd, Lesinski, GB. Distinct Myeloid Suppressor Cell Subsets Correlate with Plasma IL-6 and IL-10 and Reduced Interferon-alpha Signaling in CD4⁺ T cells from Patients with GI Malignancy. Cancer Immunology, Immunotherapy. In Press. License # 2679371012653

66
cells, while co-culture with in vitro generated MDSC led to reduced IFN-α-responsiveness in both PBMC and the CD4^+ subset of T cells from normal donors. Exploratory multivariable Cox proportional hazards models revealed an increased percentage of the CD33^+HLADR^-CD15^- MDSC subset was associated with reduced overall survival (p=0.049) while an increased percentage of the CD33^+HLADR^-/lowCD14^+ subset was associated with greater overall survival (p=0.033). These data provide evidence for a unique relationship between specific cytokines, MDSC subsets and IFN-α responsiveness in patients with GI malignancies.
Introduction

Gastrointestinal (GI) malignancies represent more than 15% of yearly cancer diagnoses and were responsible for an estimated 135,830 deaths in the United States in 2009 (2). One promising approach to treat this group of cancers includes immune-based therapy (177, 178). Numerous strategies are being developed to overcome the profound systemic immunosuppression in GI cancer patients, as this represents a major factor which limits the potential benefits from immunotherapy (74, 125). An altered balance of circulating cytokines is thought to drive this immunosuppression. Systemic levels of pro-inflammatory cytokines including interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are elevated and elicit multiple downstream effects. These cytokines have been recognized as key regulators of immunosuppression and poor quality of life in patients with advanced cancer (57, 179-183).

Many of the same cytokines are also known to play an important role in regulating the generation and function of myeloid derived suppressor cells (MDSC) (57, 69, 184). This heterogeneous population of early myeloid cells represents a major barrier to the success of immune-based therapy. MDSC are also thought to promote cancer progression by virtue of their ability to suppress cytotoxicity and cytokine production by natural killer (NK) cells and CD8\(^+\) T cells (107, 122, 144). MDSC can deplete arginine
and cysteine from the microenvironment, and also produce high levels of nitric oxide, which results in nitration of signal transduction intermediates within CD8$^+$ T cells, and can induce oxidative stress via the production of reactive oxygen species (73, 107, 156, 167, 185). Although great insight into the ability of MDSC to inhibit anti-tumor immunity has been gained from murine studies (69, 71, 72, 74, 85, 88, 185-187), the biologic properties of these cells in human cancer patients are only beginning to be defined (60, 74-76, 144, 147, 188). Emerging evidence suggests that unique phenotypic MDSC subsets exist in human cancer patients. MDSC have been minimally defined as having a CD33$^+$HLADR$^-$ phenotype and can be further divided into granulocytic (CD15$^+$) or monocytic (CD15$^-$) subsets (115, 144). The prevalence of a CD33$^+$HLADR$^{low}$CD14$^+$ subset of MDSC has also been reported in patients with various malignancies including hepatocellular carcinoma, ovarian carcinoma and melanoma (38, 76, 77, 189, 190). Few studies have explored potential relationships between MDSC and the cellular response to clinically-relevant cytokines in human cancer patients.

Interferon-alpha (IFN-α) is a cytokine that plays a key role in promoting anti-tumor immunity via its ability to activate the STAT1 signal transduction pathway, promote NK and T cell cytotoxicity (191, 192) and enhance the clonal expansion or cross-priming of T cells (164, 193, 194). Our group and others have shown that patients with advanced malignancy display reduced STAT1 phosphorylation (12, 14). In addition, using the C26 murine model of adenocarcinoma, we have shown that MDSC can directly inhibit IFN-α-responsiveness by nitrating tyrosine residues on the STAT1 pathway.
protein (155). Therefore, we hypothesized that increased pro-inflammatory cytokines in patients with GI malignancies would be associated with elevated circulating percentages of MDSC and an impaired IFN-α responsiveness. This study provides evidence for unique relationships between systemic IL-6 or IL-10 levels with individual MDSC subsets and MDSC with reduced IFN-responsiveness in patients with GI malignancies.
Materials and Methods

Patients. Peripheral blood samples were obtained from 12 healthy adult blood donors (source leukocytes, American Red Cross, Columbus, OH) and from 40 patients with a confirmed diagnosis of gastrointestinal malignancy at The Ohio State University Comprehensive Cancer Center (Columbus, OH) from 2006 – 2009 under an Institutional Review Board-approved protocol (#2006C0046; PI: Lesinski, G.B.). Plasma from normal donors (n = 20) was purchased from Innovative Research, Inc., (Novi, MI). Plasma was obtained from all forty patients prospectively enrolled in this study. Cryopreserved peripheral blood mononuclear cells (PBMC) of sufficient quantity for flow cytometric analysis were available from thirty-one of the patients enrolled. Overall survival data was obtained from the time of diagnosis on 24 patients. The total number of systemic cancer therapies at any time point (before or after study blood draw) were also recorded.

Blood Procurement. PBMC were isolated from peripheral venous blood via density gradient centrifugation with Ficoll-Paque, (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (12). Plasma samples were snap frozen and stored at -80°C until analysis.
Measurement of Cytokines. For quantitative detection of IL-6, IL-10, IL-1β and TNF-α in plasma, commercially available enzyme-linked immunosorbent assays (ELISA) were used according to manufacturer’s specifications (R & D Systems, Inc.). All samples were run in batches to minimize inter-assay variability, assayed in duplicate and quantitated using a standard curve.

Flow cytometry for myeloid-derived suppressor cells. Cryopreserved PBMC from each normal donor or patient were suspended at a concentration of 1x10⁷/mL in flow staining buffer (PBS plus 1% FBS). Cells were incubated with fluorochrome-labeled antibodies at 4°C. Specific antibodies include CD15 FITC (eBioscience), CD33 PE (BD Biosciences), HLA-DR PERCP-Cy5.5 (eBioscience), CD11b APC (BD Biosciences), and CD14 Pacific Blue (BD Biosciences). PBMC were also labeled with the appropriate isotype control antibodies for each fluorochrome to use as negative controls. Cells were then washed with flow buffer, fixed with 1% formalin, and stored at 4°C until analysis. All samples were run on a BD LSR II flow cytometer, and were subsequently analyzed with FlowJo software (Tree Star, Inc.). MDSC were defined as cells positive for CD33, and lacking HLA-DR with subsets expressing CD15, CD14 and CD11b as discussed in the legends.

Analysis of STAT1 phosphorylation by flow cytometry. Levels of intracellular Tyr⁷⁰¹-phosphorylated STAT1 (P-STAT1) within total PBMC or individual cell subsets were measured by flow cytometry as previously described (12). Cryopreserved patient
cells were thawed, resuspended in fresh RPMI-1640 media containing 10% Human AB serum (Sigma Aldrich, St. Louis MO) and stimulated for 15 minutes with $10^4$ U/mL recombinant, human IFN-α2b (Schering-Plough). This dose and time point has been previously shown by our group to induce maximal IFN-α-mediated STAT1 phosphorylation at the Tyr$^{701}$ residue (12, 162). Following stimulation, cells were fixed and permeabilized (Fix&Perm Permeabilization Kit; Invitrogen), washed in PBS containing 5% fetal bovine serum, and then stained for 30 minutes at room temperature with fluorochrome-labeled antibodies against pSTAT1 Ab (BD Biosciences, San Diego, CA) in combination with antibodies targeting extracellular markers (CD4, CD8, CD56). After a final wash, samples were fixed in 1% formalin and analyzed using a BD FACScalibur flow cytometer using at least 10,000 cells in the lymphocyte gate based on light scatter properties. Data were expressed as specific fluorescence ($F_{sp} = F_t - F_b$), where $F_t$ represents the median value of total staining and $F_b$ represents the median value of background staining with an isotype control antibody (162).

In vitro generation of MDSC. PBMC were isolated from normal donor Red Cross leukopaks via density gradient centrifugation with Ficoll-Paque, (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (12). A protocol for in vitro generation of MDSC was adapted from Lechner et al. (83). Briefly, cells were plated at a concentration of $5 \times 10^5$ cells/mL in complete media (RPMI media supplemented with 10% fetal bovine serum and antibiotics). GM-CSF, IL-6, and IL-10 were added to the media at a concentration of 10 ng/mL. Cells were cultured at 37°C for 7 days, with
media and cytokine replacement every 2-3 days. After 7 days, suspension and adherent cells were harvested and myeloid cells were isolated from culture using the Easy Sep Myeloid Isolation Kit (Stem Cell Technologies). Cells were labeled with anti-CD33/66b magnetic microbeads and positively selected using an Easy Sep magnet. Isolated cells were washed twice prior to further studies. PBMC isolated from the same donor but not treated with cytokines were used as a control. *In vitro* generated MDSC were phenotypically evaluated for expression of CD33, HLA-DR, CD11b, and CD15 by flow cytometry. Consistent with prior studies, they were found to express CD33 and CD11b, with variable levels of HLA-DR and CD15.

*Co-culture of MDSC and PBMC.* *In vitro* generated MDSC were co-cultured with autologous PBMC that were thawed and rested in complete media one day prior to co-culture. MDSC were plated at a 1:1 ratio with PBMC in complete media and incubated for 24 hrs at 37°C. After 24 hours, non-adherent cells were harvested and stimulated with $10^3$ or $10^4$ U/mL IFN-α for 15 minutes at 37°C as previously described (12). This dose and time point has been shown to induce robust STAT1 phosphorylation as previously described by our group (12, 162). P-STAT1 staining and analysis was conducted as described in the same manner as the patient samples described above. Co-culture protocol was adapted from previous studies conducted by Nagaraj et al. (147).

*Statistical Methods.* Pearson correlation coefficients were used to evaluate the relationship between measures. Comparisons of MDSC percentages and the plasma
concentration of cytokines between GI cancer patients and normal donors were made using the Wilcoxon rank sum test. Exploratory univariable and multivariable Cox proportional hazards regression models were used to evaluate the effect of plasma cytokine levels, the percentage of MDSC subsets and number of systemic cancer therapies on overall survival. Because we were primarily interested in the potential contribution of MDSC subsets to overall survival, we used a backward elimination procedure (195) (with 0.1 as the p-value criterion for removal) that considered only the three MDSC subsets. In this manner, we explored the multivariable relationship between the three MDSC subsets (CD15+, CD15− or CD14+) and survival. Once a final multivariable model was obtained based on MDSC subset, three additional indicators (pancreatic cancer, >1 systemic cancer therapy, stage of disease) were added to the model individually to assess their effect on the hazard ratio for each subset. This approach was utilized due to the large number of potential predictors compared to the limited sample size. Plasma cytokine concentrations and MDSC levels (as percentage of total cells) were log-transformed after a small positive adjustment to account for zero values. This transformation improved their normality for the Pearson coefficients and their linearity in the Cox models.
Results

Patient Demographics. The demographics of patients in this study are illustrated in Table I. Blood from 27 of the patients was obtained within three months of initial cancer diagnosis and none of these individuals had prior therapy. Of the 13 patients diagnosed over three months prior to obtaining blood, 11 had prior therapy. Thirteen patients (32.5%) had prior surgical resection. In all patients, blood draws were obtained a minimum of 2 weeks following their last therapy or surgical procedure to minimize the effects of treatment on the profile of circulating cytokines or MDSC.

Myeloid derived suppressor cell subsets are elevated in patients with various GI malignancies. The level of individual CD33⁺HLADR⁻CD11b⁺CD15⁺ or CD33⁺HLADR⁻/lowCD14⁺ MDSC subsets were significantly elevated in patients elevated as compared to normal healthy donors (p<0.0001; Figure 3.1A-D), and widely distributed across patients with various GI malignancies (Figure 3.2A-B). The analysis of immature myeloid cells with a CD33⁺HLADR⁻ phenotype was further sub-classified by the presence of CD15⁻ (CD33⁺HLADR⁻CD11b⁺CD15⁻, CD33⁺HLADR⁻CD11b⁺CD15⁻) or CD33⁺HLADR⁻/lowCD14⁺ MDSC subsets. Although the CD15⁺ and CD14⁺ MDSC were more prevalent in patient PBMC (Figure 3.2C), there were significant co-linear relationships between the each of the MDSC subsets (CD15⁺ vs. CD15⁻ Pearson r= 0.580,
Expression of pro-inflammatory cytokines in plasma from GI cancer patients.

Systemic levels of pro-inflammatory cytokines important for MDSC expansion and function were evaluated in this cohort of GI cancer patients (57, 144). Plasma levels of IL-6 and IL-10 were significantly elevated in all patients as compared to normal controls (Figure 3.3A, p<0.0001, p<0.0001). Plasma levels of IL-1β and TNF-α were up-regulated in only 11 and 8 patients, respectively (data not shown).

Relationship between MDSC subsets and plasma cytokines in patients with gastrointestinal malignancy. Analysis of cytokines and MDSC subsets revealed a strong correlation between IL-6 and the percentage of CD15⁺ MDSC (Pearson r=0.468; p=0.008; Figure 3.3B), but not the percentage of CD15⁻ MDSC (Pearson r=0.267; p=0.146) or CD14⁺ MDSC (Pearson r=0.245; p=0.184). In contrast, IL-10 strongly correlated with the CD15⁻ MDSC subset (Pearson r=0.534; p=0.002; Figure 3.3C) but not the level of CD15⁺ MDSC (Pearson r=0.197; p=0.288) or CD14⁺ MDSC (Pearson r=0.114; p=0.542).

MDSC are inversely correlated with reduced IFN-α responsiveness in CD4⁺ T cells. The relationship between the percentage of MDSC and IFN-α responsiveness of
immune effector cells was evaluated in this cohort of patients. We observed an inverse correlation between the percentage of CD15\(^+\) or CD15\(^-\) MDSC and the level of IFN-\(\alpha\)-induced pSTAT1 in CD4\(^+\) T cells (Figure 3.4A-B). This relationship was strongest for the CD15\(^+\) MDSC subset (Figure 3.4A; Pearson \(r=\) -0.55; \(p=0.002\)), and the CD15\(^-\) MDSC subset (Pearson \(r=\) -0.388, \(p=0.034\)). In contrast, there was no relationship between the CD14\(^+\) MDSC subset and IFN-\(\alpha\)-induced pSTAT1 in CD4\(^+\) T cells (data not shown; Pearson \(r=\) -0.139, \(p=0.46\) for CD14\(^+\) MDSC). No significant relationships observed between the percentage of MDSC and pSTAT1 within CD56\(^+\) (NK) cells or CD8\(^+\) T cell subsets (data not shown).

**MDSC reduce IFN-\(\alpha\) responsiveness in vitro.** PBMC co-cultured with *in vitro* generated MDSC had a significantly reduced level of pSTAT1 (at Tyr\(^{701}\)) following IFN-\(\alpha\) treatment at both \(10^3\) and \(10^4\) U/mL as compared to PBMC alone (\(p = 0.0017\) and \(p = 0.0062\); Figure 3.4C). Similar to the patient data, the CD4\(^+\) T cell subset also had a reduced response to IFN-\(\alpha\) treatment when co-cultured with MDSC (Figure 3.4D; \(10^3 p = 0.049\) and \(10^4 p = 0.186\)). PBMC were also co-cultured in parallel with non-cytokine treated autologous PBMC as a control. These cells did not inhibit IFN-\(\alpha\)-responsiveness as compared to PBMC cultured alone (not shown).

**Relationship between cytokines, MDSC subsets and overall survival.** The data were analyzed to identify whether the percentage of each MDSC subset was related to the clinical course of disease. Univariable Cox proportional hazards models indicated the
hazard of death was not significantly related to the level of any plasma cytokine or the percentage of any individual MDSC subset (CD14\(^+\) p=0.233; CD15\(^+\) p=0.343; CD15\(^-\) p=0.323; Table II). As expected, a diagnosis of pancreatic cancer was associated with an increased risk of death when compared to cancer originating from other organ sites [Hazard Ratio (HR) = 4.36, p = 0.001]. To evaluate potential relationships between overall survival and the three MDSC subsets, an exploratory backwards elimination procedure was used to select predictors for a multivariable model. The final model included the CD15\(^-\) and CD14\(^+\) subsets, revealing an increasing hazard of death with increasing CD15\(^-\) MDSC and a decreasing hazard with increasing CD14\(^+\) MDSC (HR for 2-fold increase in CD15\(^-\) = 1.42, p=0.049; HR for 2-fold increase in CD14\(^+\) = 0.69, p=0.033, Table III). Addition of an indicator for pancreatic malignancy into this model influenced significance of both MDSC subsets (pancreatic cancer p = 0.01, CD15\(^-\) p = 0.32, CD14\(^+\) p = 0.114). However, adding stage of disease or number of cancer therapies into the model did not meaningfully alter the effect or significance of MDSC subsets on overall survival (not shown).
Discussion

MDSC represent a key cellular mediator of immune suppression in both pre-clinical models and in patients with advanced malignancy (144, 146, 147). These cells represent an important barrier that likely limit the full potential of immune-based cancer therapies or endogenous host responses to developing tumors. Immunotherapy with recombinant cytokines, antibodies and vaccine approaches continue to emerge as a promising treatment approach for many solid tumors, and is also garnering increased interest in a number of GI malignancies (177, 178, 196). For example, recent trials utilizing allogeneic pancreatic cancer cells that secrete GM-CSF as a vaccine in combination with cyclophosphamide have demonstrated a disease control in a number of patients with refractory cancer (92, 197). These data indicate that a better understanding of systemic immunosuppression such as the interactions between MDSC, systemic cytokines and their signaling pathways could lead to new strategies for augmenting the host immune response against tumors.

The present study examined relationships between plasma cytokines associated with immune suppression, MDSC levels and IFN-α-induced signal transduction in peripheral blood of patients with various GI malignancies. We have demonstrated that IL-6, IL-10 and multiple MDSC subsets were significantly elevated in patients when compared to normal donors. A highly significant correlation between plasma IL-6 with the CD15+ MDSC subset and IL-10 with the CD15− MDSC subset was also observed, despite the heterogeneity of the patients included in the study. These data suggest that
the systemic profile of pro-inflammatory cytokines may be associated with specific phenotypic subsets of circulating MDSC. Consistent with prior murine studies by our group, an inverse correlation between circulating CD15\(^+\) or CD15\(^-\) MDSC subsets and IFN-\(\alpha\)-responsiveness of CD4\(^+\) T cells was also observed (155). Interestingly, co-culture with \textit{in vitro} generated MDSC led to reduced IFN-\(\alpha\)-responsiveness of total PBMC and the CD4\(^+\) T cell compartment. Finally, multivariable Cox proportional hazards models revealed that levels of individual MDSC subsets differentially influenced the hazard of death in this heterogeneous patient population. These data reveal novel relationships between individual MDSC subsets and systemic immunologic parameters that may regulate disease biology and progression.

Phenotypic subsets of MDSC in humans are continually being defined. This study has characterized what appear to be novel relationships between CD15\(^+\) and CD15\(^-\) phenotypic sub-populations of MSDC and systemic cytokine profiles in patients with GI malignancy. The CD15 marker is expressed on circulating granulocytes and some monocytes, but is absent on lymphocytes. This protein is believed to play a role in phagocytosis, stimulation of degranulation, and chemotaxis. Consistent with other studies of MDSC in cancer patients (147), the majority of CD33\(^+\)HLA-DR\^-CD15\(^+\) MDSC were also positive for CD11b (integrin \(\alpha\)M\(\beta\)2) expression. Our data suggested that the overall balance between systemic IL-6 and IL-10 may influence the percentage of individual MDSC subsets. In addition, these data suggest the presence of the CD15
marker may represent a particular stage of MDSC differentiation. However, the cytokine profile driving this process is likely complex and not limited to IL-6 and IL-10.

Multivariable Cox proportional hazards models were used to evaluate potential relationships between MDSC subsets and overall survival. These exploratory modeling approaches showed that increased levels of CD15− MDSC and decreased levels of CD14+ MDSC were associated with reduced overall survival in this cohort of patients. These data indicate that MDSC subsets may play some role in influencing the outcome of patients with GI malignancy. Although these results are consistent with other studies documenting a prognostic role for MDSC in advanced cancer (60, 76), we caution over-interpretation of this data due to the heterogeneity of this patient population. Notably, patients diagnosed with pancreatic cancer had a significantly increased risk of death independent of MDSC level, which reflects the aggressive nature of this malignancy. Future studies are likely to resolve the question of whether individual MDSC subsets may be more prevalent in patients with a particular type of GI malignancy. Indeed, prior studies showed that melanoma patients had significantly elevated CD33+HLA-DR−lowCD14+ MDSC but not LinHLA-DRCD33+ MDSC as compared to normal donors (77). This CD14+ MDSC subset has also been noted in other types of cancer including hepatocellular and ovarian carcinoma (198). These data highlight the importance of studying individual MDSC subsets and indicate that disease biology likely influences the phenotypic properties of MDSC subsets.
The strengths of this study are the fact that the majority of patient samples were obtained soon after cancer diagnosis. This is particularly advantageous since data from most patients was obtained prior to surgery or adjuvant therapy such as chemotherapeutic drugs and radiation which could alter suppressor cell populations or function. Also, this represents the first report linking human MDSC with reduced IFN-α-responsiveness of immune effector cells. One factor that might influence these results is the use of Ficoll-Paque separation. Previous studies have indicated that this separation technique can reduce the yield of CD15\textsuperscript{+} MDSC (60). This was controlled for in the comparison to normal donor samples, as both were processed in the same manner. However, our interpretation of the relative contribution of each subset to the biology and outcome of disease could be influenced by this technical factor. A second limitation of this study was the fact that the limited number of patient cells available permitted only phenotypic analysis of individual MDSC subsets and an analysis of IFN-α-induced signal transduction. We are in the process of conducting prospective studies to evaluate whether human MDSC confer reduced IFN-α-responsiveness via reactive nitrogen intermediates as shown in our prior murine studies. We are also interested in studying whether the ability of MDSC subsets to inhibit IFN-α-responsiveness is related to their ability to suppress T cell proliferation or NK cell cytotoxicity (57, 122). Finally, we plan to explore the phenotypic differences of MDSC isolated from both primary and metastatic tumor sites. Indeed MDSC at the tumor site have been shown to be more suppressive, and therefore the relative level of CD15 expression could also play a
different role in this environment (144). These questions will be best answered in the context of a prospective study in a more homogeneous patient population.

This study suggests the specific cytokine milieu in the periphery of cancer patients is associated with unique subsets of MDSC populations that impact the cellular response to clinically relevant cytokines. Likewise this report is the first to demonstrate that human MDSC lead to reduced IFN-α-responsiveness in immune effector cells. These data support MDSC as a relevant target that could be modulated to enhance the response to immune-based therapy in patients with advanced malignancies.
Acknowledgments

We thank Dr. Susan Geyer for critical review of this manuscript. We thank the OSU CCC Analytical Cytometry Shared Resource. We would also like to thank the following agencies for grant support: The Valvano Foundation for Cancer Research Award (to G.B. Lesinski), National Institutes of Health (NIH) Grants T32 GM068412 (to B. Mundy), CA84402, K24 CA93670 (to W.E. Carson), K22 CA134551 (to G.B. Lesinski), and The Samuel J. Roessler Memorial Scholarship at The Ohio State University College of Medicine (to E. Binkley).
Figure 3.1. Phenotypic analysis of MDSC subsets in patients with GI malignancies. Peripheral blood mononuclear cells (PBMCs) were obtained from n = 31 GI cancer patients for whom peripheral blood was available or normal donors (n = 12) and stained for MDSC using flurochrome-labeled antibodies targeting CD33, HLA-DR, CD15, CD14 and CD11b or the appropriate isotype controls. MDSC levels were evaluated by flow cytometry based on a minimum of 20,000 live events and presented as the percentage of total cells, listed in box R2 in figure. (A) Representative scatter plots from two GI cancer patients as compared to PBMC obtained from a normal control. PBMCs were labeled with CD33, HLA-DR, CD15, and CD11b, red box indicates gated population, arrows indicate sequence of gating. The first dot plot depicts forward scatter (FSC) by side scatter (SSC) properties of PBMC for determining initial live cell gate. (B) Representative scatter plots from two GI cancer patients as compared to PBMC obtained from a normal control. PBMCs were labeled with CD33, HLA-DR, and CD14, red box indicates gated population, arrows indicate sequence of gating. (C) CD33+HLA-DR-HLADR-CD11b+CD15+ MDSC and (D) CD33+HLA-DR-/lowCD14+ MDSC and compared to levels found in normal control PBMCs.
Figure 3.1. Phenotypic analysis of MDSC subsets in patients with GI malignancies.
Figure 3.2. MDSC subsets are elevated in all types of GI malignancies. (A-B) The levels of each MDSC subset, as determined by flow cytometry, were evaluated by type of GI malignancy and compared to normal control PBMC. The number of patients with cancer at each organ site is listed on the plot. Patients designated as ‘other’ consist of one patient each with cancer of the gallbladder, appendix, hepatocellular carcinoma or cholangiocarcinoma. (C) Early myeloid cells (CD33+HLADR−) were evaluated in a similar manner from all 31 patients to determine the percentage positive and negative for CD15 or positive for CD14.
Figure 3.2. MDSC subsets are elevated in all types of GI malignancies.
Figure 3.3 Plasma levels of IL-6 and IL-10 and the association with MDSC in patients with GI malignancies. (A) IL-6 and IL-10 were measured by ELISA in plasma isolated from the peripheral blood of patients with GI malignancies (n = 40) and plasma obtained from normal donors (n = 20). The levels of IL-6, IL-10 were significantly elevated in patients with GI malignancy as compared to normal controls. All samples were assayed in duplicate and quantitated using a standardized protein curve. (B-C) MDSC levels were obtained as described previously from n = 31 patients and compared to plasma cytokine levels from the same patient. (B) The percentage of CD33⁺HLADR⁻CD15⁺ MDSC and plasma levels of IL-6 and (C) the percentage of CD33⁺HLADR⁻CD15⁻ MDSC and plasma levels of IL-10.
Figure 3.3. Plasma levels of IL-6 and IL-10 and the association with MDSC in patients with GI malignancies.
Figure 3.4. Inverse correlation between MDSC and IFN-α induced STAT1 phosphorylation. MDSC levels were obtained as described previously from n = 31 patients and compared to levels of IFN-α induced STAT1 phosphorylation (P-STAT1). (A-B) The percentage of both CD33+HLADR−CD15+ MDSC and CD33+HLADR−CD15− MDSC inversely correlated with STAT1 phosphorylation following ex vivo stimulation with IFN-α (10^4 U/mL for 15 minutes) in CD4+ T cells from GI patients. (C) Normal PBMC co-cultured with in vitro generated MDSC exhibited reduced IFN-α induced STAT1 phosphorylation. In vitro generated MDSC expressed CD33 and CD11b, with variable, donor-dependent levels of HLA-DR and CD15, and showed no difference in CD15 expression in the presence or absence of IL-10 (data not shown). (D) CD4+ T cells from normal donors have reduced IFN-α induced P-STAT1 when co-cultured with autologous, in vitro generated MDSC. Error bars represent standard deviation obtained from separate experiments using PBMC and autologous, in vitro generated MDSC from four unique donors.
Figure 3.4. Inverse correlation between MDSC and IFN-α induced STAT1 phosphorylation.
Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
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<tbody>
<tr>
<td>Number of patient ((n))</td>
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</tr>
<tr>
<td>Mean age in years (range)</td>
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<tr>
<td>Median age in years</td>
<td>64</td>
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<tr>
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<tr>
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<tr>
<td>Female</td>
<td>13 ((32.5%))</td>
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</tr>
<tr>
<td>Esophageal</td>
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<tr>
<td>Colon</td>
<td>7 ((17.5%))</td>
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<tr>
<td>Gastric</td>
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<td>Cholangiocarcinoma</td>
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<tr>
<td>Gallbladder</td>
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<td>Appendix</td>
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<tr>
<td>Hepatocellular</td>
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<td>II</td>
<td>7 ((17.5%))</td>
</tr>
<tr>
<td>III</td>
<td>7 ((17.5%))</td>
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<td>IV</td>
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<tr>
<td>2</td>
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Table 2. Hazard Ratios for Univariable Models.

<table>
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<th>Factor</th>
<th>Hazard ratio* (95% CI)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>1.08 (0.89 - 1.32)</td>
<td>0.441</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.23 (0.93 - 1.62)</td>
<td>0.141</td>
</tr>
<tr>
<td>More than 1 prior systemic therapy</td>
<td>0.62 (0.25 – 1.53)</td>
<td>0.303</td>
</tr>
<tr>
<td>Stage III-IV Disease</td>
<td>1.83 (0.68 – 4.94)</td>
<td>0.232</td>
</tr>
<tr>
<td>Pancreatic malignancy</td>
<td>4.36 (1.80 - 10.55)</td>
<td>0.001</td>
</tr>
<tr>
<td>CD15⁺ MDSC</td>
<td>0.90 (0.72 - 1.12)</td>
<td>0.343</td>
</tr>
<tr>
<td>CD15⁻ MDSC</td>
<td>1.11 (0.90 - 1.37)</td>
<td>0.323</td>
</tr>
<tr>
<td>CD14⁺ MDSC</td>
<td>0.86 (0.67 - 1.10)</td>
<td>0.233</td>
</tr>
</tbody>
</table>

* HRs computed for a 2-fold increase in the biomarker (IL-6, IL-10, CD15⁺ MDSC Subset, CD15⁻ MDSC Subset, CD14⁺ MDSC Subset), more than 1 prior therapy vs. 1 or fewer, pancreatic malignancy vs. other sites, and stage III-IV disease vs. stage I – II.
Table 3. Hazard Ratios for Multivariable Models.

Multivariable model containing all MDSC subsets.

<table>
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<th>Hazard ratio* (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15(^-) MDSC</td>
<td>1.5 (1.03 - 2.17)</td>
<td>0.033</td>
</tr>
<tr>
<td>CD15(^+) MDSC</td>
<td>0.83 (0.55 - 1.27)</td>
<td>0.400</td>
</tr>
<tr>
<td>CD14(^+) MDSC</td>
<td>0.77 (0.49 – 1.21)</td>
<td>0.259</td>
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Multivariable Model with CD15\(^+\) MDSC subset removed.

<table>
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<tr>
<th>Factor</th>
<th>Hazard ratio* (95% CI)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>CD15(^-) MDSC</td>
<td>1.42 (1.00 - 2.02)</td>
<td>0.049</td>
</tr>
<tr>
<td>CD14(^+) MDSC</td>
<td>0.69 (0.49 - 0.97)</td>
<td>0.033</td>
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* HRs computed for a 2-fold increase in the biomarker (CD15\(^+\) MDSC Subset, CD15\(^-\) MDSC Subset, CD14\(^+\) MDSC subset).
Chapter 4: Psychological Stress is Associated with Altered Levels of Myeloid-Derived Suppressor cells in Breast Cancer Patients

Abstract

Our group has shown in a randomized clinical trial that psychological intervention to reduce stress in patients with stage II and III breast cancer led to enhanced immune function, fewer recurrences and improved overall survival. We hypothesized that patients with high levels of stress would have alterations in myeloid-derived suppressor cells (MDSC) compared to patients with lower stress. PBMC from 16 patients with high stress (n = 8) or with low stress (n = 8) after surgery as measured by the Impact of Event Scale (IES) questionnaire were evaluated for the presence of MDSC. Patients with higher IES scores had significantly elevated salivary cortisol levels (P = 0.013; 13 μg/dl vs. 9.74 μg/dl). Levels of IL-1Rα were also significantly elevated in the higher IES group (45.09 pg/mL vs. 97.16 pg/mL; P = 0.010). IP-10, G-CSF, and IL-6 were all higher in the high stress group although not to a significant degree. Flow cytometric analysis for CD33+/HLA-DR-neg/CD15+/CD11b+ MDSC revealed increased MDSC in patients with lower IES scores (P = 0.009). CD11b+/CD15+ cells constituted 9.4% of the

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This chapter was reprinted with permission from Cellular Immunology. Mundy-Bosse, BL., Thornton, LM., Hae-Chung, Y., Andersen, BL., Carson, WE. Psychological stress is associated with altered levels of myeloid-derived suppressor cells in breast cancer patients. Cellular Immunology. In Press.
CD33+/HLA DR-neg cell population in patients with high IES, versus 27.3% in patients with low IES scores. Additional analyses of the number of stressful events that affected the patients in addition to their cancer diagnosis revealed that this type of stress measure correlated with elevated levels of MDSC (P = .064). These data indicate the existence of a complex relationship between stress and immune function in breast cancer patients.
Introduction

The negative impact of stress on immune function is well established. Multiple studies have demonstrated that stressful events activate the hypothalamic-pituitary-adrenal axis which increases the production of hormones such as epinephrine, norepinephrine, and cortisol. Immune cells express receptors for these hormones and their function can be markedly altered by chronic exposure to these factors (199-201). T cell cytokine production is shifted from T_{H1} towards a T_{H2} profile, and T cell proliferation and NK cell lysis is inhibited after chronic exposure of the organism to stress (199, 202). Stress can also lead to alterations in systemic levels of interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and interleukin-1 (IL-1), cytokines which are thought to be involved with tumor progression (203-207). Additionally, these pro-inflammatory cytokines have been associated with the induction of suppressor cell populations that can further inhibit immune responses (57).

Myeloid-derived suppressor cells (MDSC) are a class of immune suppressor cells that are comprised of a heterogenous population of early myeloid cells. They are produced in the bone marrow from hematopoietic stem cells and represent less than 1% of circulating cells in normal individuals. MDSC are generally defined phenotypically as being positive for CD33+/CD11b+ (the common myeloid lineage and macrophage markers, respectively) and negative for other lineage markers (57). Some investigators
have further characterized subsets of MDSC as being negative for CD14, and positive for CD15 and IL-4Rα (156, 208, 209). The apparent variety of MDSC subsets now being described could represent various stages of differentiation or functionally distinct cellular compartments.

MDSC are known to accumulate in patients with cancer and their numbers seem to correlate with tumor burden (144, 160, 188, 210). The generation of MDSC in the bone marrow is believed to be enhanced in response to tumor-derived factors and these substances also promote the migration of MDSC from the bone marrow to the tumor site (57). The available evidence strongly suggests that immune suppressor cells are able to blunt the ability of the innate and adaptive immune system to eliminate the developing tumor. The depletion of MDSC in murine models leads to a reduction in tumor growth and enhances the anti-tumor effects of immunotherapeutic regimens (59, 150, 157, 184, 211). MDSC use multiple different mechanisms to induce immune suppression. They produce nitric oxide which can inhibit immune cell signal transduction and they release arginase which depletes L-arginine from the tumor microenvironment, thus crippling T cell function (57, 157, 167, 209, 210). In addition, they are known to produce reactive nitrogen and oxygen species that can further impair immune cell function (57, 87, 98, 101, 122, 158, 167, 209).

MDSC may represent a link between stress and cancer progression. It is known that stress leads to elevations in pro-inflammatory cytokines, and many of these stress...
induced factors are associated with the generation and/or expansion of MDSC (202). However, the relationship between MDSC and stress has not been previously explored. A randomized clinical trial conducted by investigators at The Ohio State University found that a psychological intervention to reduce life stressors in surgically-treated breast cancer patients led to a significant reduction in recurrence, and an increase in overall survival (212). Further, these positive outcomes were associated with improved function of immune cells as measured by NK cell cytotoxicity and T cell proliferation (213). Based on these observations, we hypothesized that high levels of chronic stress would be associated with increased levels of stress hormones and cytokines that in turn would stimulate the generation and accumulation of MDSC. These suppressor cells are known to negatively impact immune function and could theoretically have led to the decrease in breast cancer survival as was seen in the clinical trial.

To evaluate this hypothesis, we examined patients with high or low stress as measured by the Impact of Event Scale (IES) to determine the relationship between cancer stress, stress hormones, pro inflammatory cytokines, and MDSC levels in stage II and III breast cancer patients who were post-surgery and awaiting the start of adjuvant therapy.
Materials and Methods

Patients. Patients with stage II or III breast cancer were recruited for a randomized trial studying the effects of a structured psychological intervention on breast cancer recurrence and overall survival. Blood and saliva samples were taken from patients post surgery and prior to the initiation of adjuvant therapy. This study describes baseline data, prior to any psychological intervention for a subset of trial participants (N=16). All samples were obtained under an Institutional Review Board-approved protocol (IRB No. 1992C0350).

Stress Groups. Two groups were defined based on the Impact of Event Scale (IES), which is a self-report measure of symptoms of traumatic stress (intrusive thoughts and avoidance behaviors) related to a specific event (214). In this study, questions were worded to reflect the cancer diagnosis specifically. Women reported the frequency of these symptoms during the previous week using a 4-point Likert scale (not at all = 0, rarely = 1, sometimes = 3, and often = 5). Items were summed for a total score which can range from 0 to 75 with higher scores reflecting greater cancer related stress. Scores of 30 and higher indicate clinically significant stress symptoms suggestive of post-traumatic stress disorder (215). We have previously shown that higher scores on the IES questionnaire correlate with decreased immune function in newly diagnosed breast cancer patients (213, 216). The majority of patients on the trial reported an IES score of 20-30, therefore patients with scores outside of this normal range were chosen. We
identified a High Stress group of 8 patients who had IES ≥ 30 at approximately one month after surgery. IES scores for the High Stress group ranged from 30 to 46 (mean=37.1, SD=6.3). For comparison, we selected 8 patients who had low stress at the same time period post surgery and had sufficient samples for analysis. IES scores for the Low Stress group ranged from 0 to 21 (mean=13.5, SD=8.8). These two groups were significantly different with respect to IES scores (p<.001).

Stressful Life Events. Patients also filled out a Life Event Scale that was adapted from the Women’s Health Initiative study (217, 218). Patients were asked whether they had experienced each of 5 different significant life events in the past year (death of a friend or family member, financial difficulty, divorce or separation from a family member or friend, major conflict with children or grandchildren, robberies or accidents). The total number of different events reported (range 0-5) was analyzed. This scale was used to objectively measure stressful events that occurred in the patient’s life in addition to a cancer diagnosis.

Plasma Cytokine Analysis. Plasma was procured via centrifugation of peripheral blood samples of study patients and stored at -80° until analysis. For cytokine profiling, plasma samples were thawed at room temperature and then analyzed in duplicate with the premixed Bio Plex Pro Human Cytokine Array (Bio-Rad) according to manufacturer’s instructions. Plasma samples were available for n=14 patients.
**Immune Function Studies.** Natural killer cell cytotoxicity assays (NKCC) were performed using a standard chromium release assay as described (219). Briefly, K562 target cells were labeled with chromium and incubated with NK cells at effector: target (E:T) cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and 3.13:1 (in triplicate). T cell proliferation was evaluated by stimulating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) and Concanavalin A (Con A) at 2.5, 5.0, and 10.0 μg/mL, and using a standard MTT assay (219).

**Flow Cytometry for Myeloid-Derived Suppressor Cells.** PBMC were suspended at a concentration of 1x10^7/mL in flow buffer (PBS plus 1% FBS). Cells were incubated with fluorochrome labeled antibodies at 4°C. Specific antibodies included CD15 FITC (eBioscience), CD33 PE (BD Biosciences), HLA-DR PERCP-Cy5.5 (eBioscience), CD11b APC (BD Biosciences), and CD14 Pacific Blue (BD Biosciences). Cells were then washed with flow buffer, fixed with 1% formalin, and stored at 4°C until analysis. All analysis was conducted on a BD LSR II flow cytometer (BD Biosciences) (57, 144, 188).

**Salivary Cortisol.** Cortisol content was determined using the Cortisol Coat-A-Count RIA (Diagnostic Products Corp., Los Angeles, CA). Sensitivity was 0.025 μg/dl. Intra-assay variation was 4.3% and inter-assay variation was 5.2% (218).
**Plasma Catecholamines (Norepinephrine, Epinephrine).** Determinations were made by HPLC with ElectroChemical Detection using Standards and Chemistry from ChromSystems (Thermo-Alko, Beverly, MA), and C-18 Columns from Waters Corporation (Milford, MA). Intra-assay variation for norepinephrine and epinephrine was 3% and 6%; inter-assay variation was 6% and 13%; and sensitivity was 15 pg/ml and 6 pg/ml, respectively (218). Catecholamine samples were available for n=15 patients.

**Adrenocorticotropin Hormone.** An Immulite 1000 device was used with reagents manufactured specifically for this instrument (Diagnostic Products Corp., Los Angeles, CA). Intra assay coefficient of variation was 5.6% and inter-assay coefficient of variation was 7.8%. Sensitivity was 9 pg/ml (218).

**Statistics.** The Mann-Whitney U test was used to compare high and low stress groups on MDSC populations, plasma cytokine levels, and hormone levels. The Mann-Whitney Wilcoxon test is a nonparametric analogue of the Student’s t-test and is appropriate for these data due to the sample size. We also tested for relationships between MDSC populations and immune cell function (NK cell cytotoxicity, T cell blastogenesis) using Spearman’s rank correlations. Follow-up analyses tested for relationships between MDSC populations and patient background information (number of pre-cancer stressful life events and surgery type) using Spearman correlation or Mann-Whitney U tests, as appropriate.
Results

_Hormones, Pro-inflammatory Cytokines and Stress._ Chronic stress is associated with increases in cytokines which are known to have effects on MDSC generation and expansion. To delineate two different patient stress groups, patients with higher or lower than the average cancer-specific stress IES score (20-30) were chosen for study (Table IV; n=16; P = 0.001). Stress hormone levels were evaluated in the high and low IES groups to determine the relationship between these two factors. Salivary cortisol levels were significantly elevated in the high stress group (13 μg/dl vs. 9.74 μg/dl, P = 0.013, Figure 4.1A), while epinephrine and levels were moderately increased in this group as compared to the low IES group (31 pg/mL vs. 22 pg/mL, P = 0.118, Figure 4.1B). Adrenocorticotropic hormone (ACTH) levels were moderately decreased, and norepinephrine levels were comparable (P > 0.1 Figure 4.1C and 1D). A panel of pro-inflammatory cytokines was also evaluated. Interleukin-1 receptor alpha (IL-1Rα), interferon-inducible protein-10 (IP-10), and granulocyte-colony stimulation factor (G-CSF) were all higher in the high stress group (P = .010, .317, and .662 respectively; Figure 4.2A-C). Interleukin-6 (IL-6) levels were comparable in both groups of patients (3.06 pg/mL in the high IES group vs. 2.01 pg/mL in the low IES group, P > 0.1; Figure 4.2D). Monocyte chemotactic protein-1 (MCP-1), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFNγ), platelet derived growth factor (PDGF), and interleukin-8 (IL-8) were comparable between the two groups (P values > 0.1; data not shown). Plasma levels of vascular endothelial
growth factor (VEGF) were elevated in patients with low IES but not to a significant degree (P > 0.1; data not shown).

*Myeloid-Derived Suppressor Cells and Cancer-Specific Stress.* Patients treated surgically for Stage II or III breast malignancy were evaluated for the presence of myeloid-derived suppressor cell (MDSC) populations by flow cytometry (Figure 4.3A). We evaluated multiple subsets of MDSC that have been associated with immune suppressive function in previous studies to determine if these subsets were affected by stress (57, 144, 188). Interestingly, patients who self reported low levels of stress related to their cancer diagnosis exhibited higher levels of MDSC as compared to patients with lower IES scores. Patients in the low stress group had elevated numbers of CD33+/HLA-DR- MDSC (3.51% vs. 1.17%; P = 0.005) and CD33+/CD14- MDSC (2.05% vs. 0.7%; P = 0.013; Figure 4.3B-C). Further phenotypic characterization revealed that patients with lower stress had elevated levels of the CD33+/HLA-DR/CD15+/CD11b+ MDSC population (27.28% vs. 9.4%; P = 0.009; Figure 4.3D).

*Myeloid-Derived Suppressor Cells, Life Stress, and Surgery.* In addition to the IES stress measure, patients were also asked to indicate if they had experienced any of five stressful life events during the previous year (212, 214, 218, 219). The Life Events test evaluates a longer time period of stress as compared to the IES questionnaire which focuses on the relatively acute time since cancer diagnosis. Interestingly, patients that scored higher on the Life Events test exhibited higher levels of MDSC (rho = 0.385,
P = 0.064; Figure 4.4A). These results indicate that the relationship between MDSC and stress could be dependent on both the type and duration of the stressor. Of note, patients who underwent more aggressive surgical treatment (total or modified radical mastectomy) exhibited lower levels of CD33+/HLA-DR-/CD15+/CD11b+ cells as compared to patients that received less extensive surgery (breast conservation therapy or partial mastectomy; P = 0.030) (Figure 4.4B).

**MDSC and Immune Function.** Increased levels of MDSC levels were associated with decreases in the number of NK cells (Spearman’s rho = -0.493, P = 0.062). While there was no significant relationship between the percent of MDSC and NK cell cytotoxicity (P values > 0.3) or T cell proliferation (P values > 0.1), there was a trend towards low MDSC levels being associating with increased T cell proliferation (Figure 4.5A-B).

**Stress and Survival.** In the high-stress group, four patients experienced breast cancer recurrence (at 23 to 71 months) and four are currently disease-free after a minimum of 88 months of follow-up (range 88 to 104 months). In the low-stress group, one patient recurred at 42 months, and seven are currently disease-free after a minimum of 88 months of observation (range 88 to 93 months).
Discussion

This study was initiated to evaluate the effects of cancer-specific stress on MDSC levels in post-surgical breast cancer patients. Patients at the two extreme ends of the IES scale were selected for evaluation. Those with below average scores were designated as the low IES group while those with elevated scores were designated as the high IES group. Cortisol and IL-1Rα were significantly elevated in the high IES group as compared to the low IES group. Low IES patients had significantly elevated levels of CD33+/HLA-DR-/CD15+/CD11b+ MDSC, which is the converse of the anticipated relationship. Interestingly, when the same patients were evaluated for objective, longer-term stressors by the Life Events measure of stress, the inverse relationship between stress and MDSC was observed: Patients with higher stress levels exhibited higher baseline numbers of MDSC. MDSC levels at baseline did not correlate with measures of NK or T cell function. Levels of MDSC correlated with the extent of surgery, as patients who underwent more extensive surgical procedures had significantly lower levels of MDSC. Importantly, the current study is the first to specifically measure MDSC levels in post operative breast cancer patients who were considered to be macroscopically disease-free.

Previous studies have demonstrated that stress can lead to elevations in pro-inflammatory cytokines and stress hormones in cancer patients. Some of these same factors, such as IL-6 and VEGF are associated with the development and/or promotion of
immune suppressor cell populations (57, 69, 86, 144, 156, 205, 211, 220). Since stress induces alterations in multiple factors associated with MDSC accumulation and generation, stress may very well lead to alterations in MDSC levels and/or function. The induction of MDSC by stressful events could therefore lead to reduced immune function. Data from our collaborators and others indicates that stress is associated with decreased immune function (202, 204, 205, 212). Data from our groups’ randomized clinical trial of stress reduction demonstrated that the psychological intervention to reduce stress correlated with improvements in immune function at 4 months post-surgery (219). We proposed that stress and the associated immune suppressive effects could lead to reduced tumor surveillance and increased risk of recurrence. Indeed, in our previous studies, a multivariate comparison of survival using the Cox proportional hazards analysis showed that patients in the Intervention arm had a reduced risk of breast cancer recurrence and death as compared to patients in the Assessment only arm. From this work, it was hypothesized that patients with high levels of cancer specific stress after surgery might exhibit higher levels or increased activity of MDSC and that this cell compartment could be responsible for the reduced immune function that was observed in the Assessment only arm. As the IES is a subjective measure of stress specific to cancer, it was expected that high IES would correlate with high levels of MDSC. Instead, the opposite relationship was observed. Patients with low IES scores actually had higher levels of MDSC at baseline as compared to patients with high IES scores. While there are fewer MDSC, the direct suppressive effects of these cells has not been fully evaluated. It is possible that with stress, there are fewer MDSC in peripheral blood, but these MDSC
could be more active. In addition, the manner in which stress is measured could represent a source of variability.

At this point, we examined other factors that could be affecting patients at this stage of treatment. The patients in this study were post-surgery and had minimal tumor burden. In addition, while the IES measures subjective stress associated with a cancer diagnosis, this does not take into account other stressful events that could have occurred in their lives. In this cohort of patients, the IES questionnaire represents an acute measure of stress since it specifically looks at the time period since cancer diagnosis (< 2 months). When these same patients were classified by an objective measure of long-term stress, the Life Events Scale, it was found that patients with high stress had elevated levels of MDSC, as had been predicted. It has long been recognized that the duration and intensity of a stressor affects its impact on immune function (221). A recent meta-analysis compared immune function in healthy volunteers who had either experienced a short period of stress (i.e. short stressful events similar to the stress of a cancer diagnosis) or a longer period of stress (e.g. caring for a spouse with dementia). This study found that only chronic stress was associated with dysfunction of the innate and adaptive immune cell compartments. Chronically stressed participants exhibited significantly decreased NK cell lysis, decreased proliferation of T cells in response to PHA and ConA and reduced levels of interleukin-2 secretion by PBMCs (202). These data suggest that chronic life stress could promote MDSC generation, while acute stress might have the opposite effect on MDSC levels. Further prospective studies with larger
patient populations will be needed to prove a direct connection between immune function, MDSC, and chronic stress.

When the patients in this study were evaluated according to surgical procedure received (breast conservation therapy or mastectomy), it was found that patients who underwent the more extensive surgical procedure had significantly fewer MDSC. Of note, surgery type was not associated with self-reported stress levels in this sample. These results indicate that the extent of surgery could have an impact on the generation or survival of MDSC. A more extensive surgery could be associated with increased trauma and/or pain for the patient. These factors could lead to additional psychological changes that might alter MDSC numbers. The use of analgesic medications in the post-operative period could also affect MDSC accumulation. Given the temporal proximity of the surgical procedure to the stress measurement, it is necessary to consider possible influences of this intervention on factors that can initiate and support MDSC expansion (222-224).

Examination of a larger sample of patients in a prospective manner will further our understanding of the connection between stress, stress hormones and pro-inflammatory cytokines, and immune suppressor cells in breast cancer. Future studies will also evaluate the direct effects of MDSC on NK and T cell function and how stress alters these interactions.
Acknowledgements

These projects have been funded by the following granting agencies: National Institutes of Health (NIH) Grants T32 GM068412 (to B. Mundy), P01 CA95426, K24 CA93670 (to W.E. Carson), American Cancer Society (PBR-89, RSGPB-03-248-01-PBP; PF-07-169-01-CPPB), Longaberger Company-American Cancer Society Grant for Breast Cancer Research (PBR-89A), U.S. Army Medical Research Acquisition Activity Grants (DAMD17-94-J-4165; DAMD17-96-1-6294; DAMD17-97-1-7062), National Institutes of Mental Health (R01 MH51487), the National Cancer Institute (K05 CA098133; R01 CA92704), the General Clinical Research Center (M01-RR0034), and The Ohio State University Comprehensive Cancer Center (P30 CA16058).
Figure 4.1. IES scores are associated with alterations in stress hormone levels. Sixteen patients with higher or lower than average IES scores were evaluated for (A) salivary cortisol, (B) epinephrine, (C) ACTH, and (D) norepinephrine. Hormone levels were compared to individual IES scores for each patient.
Patients with higher IES scores exhibit elevations in pro-inflammatory cytokines. Plasma samples obtained from sixteen patients were evaluated for multiple cytokines by ELISA: (A) Interleukin-1R alpha (IL-1Ra), (B) interferon response protein 10 (IP-10), (C) granulocyte-colony stimulating factor (G-CSF), and (D) interleukin-6 (IL-6) levels are shown. Cytokine levels were compared to individual IES scores for each patient.
Figure 4.3. Acute stress is associated with decreased levels of myeloid-derived suppressor cells. Peripheral blood mononuclear cells (PBMCs) were obtained from breast cancer patients post-surgery and stained for multiple subsets of MDSC. (A) Representative scatter plots from a breast cancer patient as compared to PBMC obtained from a normal control. PBMCs were labeled with CD33, HLA-DR, CD15, and CD11b, red box indicates gated population, arrows indicate sequence of gating. PBMCs were harvested from sixteen patients with higher than average or lower than average IES scores and labeled with markers to evaluate early myeloid cells. (B) CD33 and HLA-DR, (C) CD33 and CD14. Graphs represent the percent of total live cells that express each marker. (D) MDSC subset analysis, percent positive represents the number of CD15+/CD11b+ cells of the CD33+/HLA-DR- early myeloid population.
Figure 4.4. Chronic stress and surgery are associated with elevations in myeloid-derived suppressor cells. (A) PBMCs were obtained from breast cancer patients post-surgery and stained for CD33+/HLA-DR-/CD15+/CD11b+ MDSC. These results were compared to the Life Events measure of stress for each patient. (B) Patients were grouped according to surgical procedure: breast conservation therapy or mastectomy. These surgical groups were then compared with levels of MDSC.
Figure 4.5 MDSC and immune cell function post-surgery. PBMCs were obtained post-surgery from patients and were evaluated by flow cytometry for the expression of CD33, HLA-DR, CD15, and CD11b. NK cells were isolated from each patient and evaluated for the ability to lyse K562 chromium-labeled target cells, and T cell proliferation was measured in response to 72 hr Concavalin A stimulation. CD33+/HLA-DR-/CD15+/CD11b+ MDSC levels were compared to (A) NK cell cytotoxicity and (B) T cell proliferation within the same patient.
Figure 4.5. MDSC and immune cell function post-surgery.
Table 4. Patient Characteristics

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Discussion

Summary of Studies

The relationship between cancer and immunology is becoming increasingly complex as additional mechanisms of interaction are discovered. The involvement of MDSC in tumor progression has been described by our group as well as others. The collection of studies presented in this manuscript is focused on the evaluation of precise mechanisms by which MDSC effect immune cell responsiveness and how this can lead to changes in outcome in patients with cancer. This work began with the study of murine models of advanced cancer, and progressed into evaluations of MDSC and correlations with clinical outcomes and measures. Each of these studies contributes a novel piece of MDSC biology and furthers the understanding of how these suppressor cells function to inhibit immune responses.

Original studies by our laboratory, as well as others, indicated that patients suffering from cancer exhibited inhibited interferon responsiveness in circulating PBMC. These observations were taken back to the research setting to evaluate what mechanisms might be involved in this lack of response. We first verified that our model system
recapitulated clinical studies by evaluating IFN responsiveness in splenocytes from a colon adenocarcinoma murine model. Response to both Type I and II IFNs was inhibited in splenocytes, as well as immune cells from lymph nodes, of tumor-bearing mice as compared to normal control animals. Additional studies demonstrated that this inhibition was seen in all immune cell compartments evaluated, indicating a non-antigen specific mechanism was leading to the reduced signaling.

The splenic environment was examined and an increase of myeloid precursor cells was discovered in tumor-bearing mice. MDSC had been previously demonstrated to increase in many murine tumor models and were known to have multiple mechanisms of immune cell inhibition. Flow cytometric analysis of GR1+/CD11b+ cells was employed to verify that MDSC were elevated in the spleens of tumor-bearing mice. To determine if MDSC played a direct role in the decreased IFN responsiveness observed in tumor-bearing mice, MDSC were isolated and co-cultured ex vivo with normal splenocytes. Splenocytes co-cultured with MDSC had significantly reduced levels of STAT1 phosphorylation in response to IFN stimulation. These data indicated that MDSC directly impacted the IFN response of naïve splenocytes. To our knowledge, this is the first time MDSC have been associated with reduced IFN signaling both in murine models and patients.

In addition to increased levels of MDSC, elevated levels of both iNOS and nitric oxide were observed in spleens from tumor-bearing animals. Since the production of
reactive nitrogen and oxygen species had previously been associated with MDSC, we hypothesized that MDSC could be inhibiting IFN responsiveness through increased generation of nitric oxide in the splenic environment. A direct link between MDSC and inhibited IFN responsiveness was already established, therefore it was next determined if nitric oxide alone could inhibit splenocyte response to IFN stimulation. Splenocytes isolated from naïve mice were treated overnight with the nitric oxide donor SNAP, stimulated with IFN, and evaluated for STAT1 phosphorylation by flow cytometry. It was discovered that splenocytes treated with nitric oxide had significantly lower levels of P-STAT1, as compared to vehicle control splenocytes. These data supported a direct link between nitric oxide and decreased IFN response.

While MDSC had been shown to produce iNOS in other models, this had not been previously examined in the context of IFN signaling. Increased nitric oxide can lead to the formation of reactive nitrogen species, such as peroxynitrite. This formation can lead to non-specific labeling of tyrosine residues in proximity to the reaction. Therefore, it was next hypothesized that the observed reduction in IFN response of splenocytes resulted from nitration of key proteins. This could result in reduced phosphorylation of these proteins, leading to reduced signal transduction. To test this hypothesis, IFN responsiveness was measured by phosphorylation of key tyrosine residues on STAT1 as previously described. To determine if STAT1 was also nitrated, total STAT1 protein was immunoprecipitated from splenocytes of normal and tumor-bearing mice and an anti-nitrotyrosine antibody was then used to evaluate levels of
nitrated STAT1 to total STAT1 protein. The levels of nitrated STAT1 were also validated by dual parameter flow cytometry. While the total level of STAT1 protein was comparable between groups, there were significantly higher levels of nitrated STAT1 in tumor-bearing animals as compared to normal controls. These data indicated that STAT1 protein is nitrated in splenocytes, which has been shown to interfere with other signal transduction pathways such as IL-2 signaling in T cells (168).

The next step of these studies focused on validating the specific effector mechanism that could be driving this increased nitric oxide production. iNOS knockout mice and wild-type mice were inoculated with C26 tumor cells, and tumors were allowed to progress to late stage disease. At this stage of growth, the splenocytes were harvested and stimulated ex vivo with IFN. Splenocytes from tumor-bearing iNOS knockout mice had a significantly higher response to IFN stimulation as compared to splenocytes from tumor-bearing wild-type mice, and a decreased level of STAT1 nitration was also observed in these iNOS knockout mice as compared to tumor-bearing control mice. The level of response was not completely restored to that of the non-tumor-bearing controls, indicating that while iNOS plays a major role in IFN responsiveness, there could be additional factors involved. The tumors were also examined histologically. While there were no gross differences in tumor size at the end of the study, there was significantly less Ki67 staining in iNOS knockout mice, indicating a lower proliferative rate in these tumors. In addition, the tumors were evaluated for CD34 expression, which is a measure of tumor vascularity. It was determined that iNOS knockout tumors also had
significantly less vascular staining than the wild-type tumors. This IHC data indicated that tumors from iNOS knockout mice were biologically different, and appeared less aggressive as compared to tumors from wild type mice. Taken together, these data support a role for iNOS in regulating IFN responsiveness in immune cells of tumor-bearing mice.

As a possible next direction for the studies outlined above, it would be interesting to determine which residues on STAT1 are being nitrated. The previous work evaluated levels of nitration on total STAT1 protein due to the lack of specific STAT1 nitrotyrosine antibodies. This could be achieved through the use of mass spectrometry to isolate STAT1 from primary splenocytes and evaluate specific residues known to be important for STAT1 signal transduction. In addition, creating a transgenic mouse model that allows for conditional knockout of MDSC, or knockout of iNOS production in only the MDSC compartment, would further solidify the link between MDSC, IFN response, and tumor progression.

While MDSC were being evaluated in murine models, there was still little existing information on MDSC in humans. We chose to evaluate a cohort of patients with gastrointestinal (GI) malignancies to determine if MDSC were elevated in this broadly defined setting. Patient PBMC were evaluated by multi-color flow cytometry for the presence of extracellular surface markers associated with MDSC. Since there is still much debate about which markers minimally define MDSC, we evaluated multiple
subsets previously demonstrated to be altered in patients with cancer (57, 127, 144, 147). These included CD33+/HLA-DR+/CD11b+/CD15+, CD33+/HLA-DR+/CD11b+/CD15−, and CD33+/CD14+/HLA-DR−/low subsets of MDSC. The CD15+ population has been described as granulocytic in morphology, while the CD15− is thought to be more monocytic. These populations are roughly comparable to the murine Ly6G (granulocytic) and Ly6C (monocytic) populations, although this has not been definitely determined at this point. The levels of each subset of MDSC were compared to normal control PBMC, and it was discovered that all of these populations are elevated in GI patients. Since this cohort of patients had multiple different types of GI malignancies, each specific type of cancer was evaluated to determine if there were differences among malignancies. While the numbers were very small per tumor subset, MDSC were elevated in all types of cancer as compared to normal controls. There appeared to be a trend towards increased levels of MDSC in pancreatic patients as compared to other cancers, however these patients also tended to have higher tumor burden which is known to be associated with elevations in MDSC (60).

Since MDSC expansion and function have been associated with elevations in pro-inflammatory cytokines, several cytokines previously shown to be elevated in GI malignancies were evaluated in this cohort of patients (180-182). It was discovered that the GI patients had significantly elevated plasma levels of IL-6 and IL-10 as compared to normal donors. Interestingly, IL-6 was associated with elevations in the CD15+ granulocytic population of MDSC, while IL-10 was associated with elevations in the
CD15⁺ monocytic population of MDSC. Neither cytokine alone was associated with levels of CD14⁺ MDSC. It is not known if the specific cytokine environment might be driving a particular phenotype, or if the predominant MDSC subset drives production of one cytokine over the other. Further functional studies would be needed to distinguish this interaction.

The next step was to evaluate if particular subsets of MDSC were associated with decreased IFN responsiveness in PBMC from patients, as was observed in the murine studies. PBMC isolated from patients were stimulated with IFN ex vivo and evaluated for the level of P-STAT1 by flow cytometry. Patients with elevated levels of either CD15⁺ or CD15⁻ MDSC had reduced levels of IFN responsiveness in CD4⁺ T cells. A negative trend between MDSC and IFN responsiveness was also seen with CD8⁺ T cells, although this did not reach statistical significance. In addition, the CD14⁺ population of MDSC was not associated with IFN responsiveness.

To directly evaluate the link between human MDSC and decreased IFN responsiveness, MDSC were generated in vitro and co-cultured with normal autologous PBMC overnight. Previous work by Epstein et al. demonstrated that isolating myeloid cells from a seven day in vitro culture of normal PBMC treated with GM-CSF and IL-6 yields myeloid cells with similar inhibitory characteristics to tumor-derived MDSC (83). Our studies utilized this in vitro system for MDSC generation, and thawed autologous PBMC for co-culture at the end of seven days. Total PBMC populations, as well as
CD4+ T cells, had inhibited response to IFN stimulation after co-culture with these MDSC as compared to PBMC alone. These studies directly correlated with the results observed in the murine models, indicating that the mechanism of inhibition discovered in the murine model could potentially translate into the patient setting. Additional studies would be required to determine if iNOS and nitric oxide play a role in the human system as well. Prospective studies evaluating nitric oxide levels and iNOS production by human MDSC in circulation and in the tumor would provide insight into the mechanism of action in patients.

The study with GI patients represents a first look into the relationship between MDSC and response to IFN stimulation in a clinical setting. There were several areas which could be strengthened by additional work. First, the sample size of 40 patients is a sufficient population for a pilot study, but this cohort was composed of mixed tumor types. Further studies will be conducted on more homogenous populations of patients. There is also some indication that PBMC isolated through the use of ficoll-hypaque could result in a loss of certain suppressor cell populations. However, this was controlled for in these studies by isolating samples from normal donors by the same protocol; although, using whole blood for phenotyping would eliminate this concern. A larger cohort of patients would also be required to draw conclusive information on the relationship between MDSC and survival. While some MDSC subsets were correlated with decreased survival in this cohort, further validation studies should be conducted.
The third chapter examined MDSC in a different context than the other works. Previous studies with collaborators demonstrated that psychological intervention to reduce stress in breast cancer patients could lead to improved immune function and decreased recurrence rates (212, 219). In addition, modulation of the hypothalamic pituitary axis (HPA) and sympathetic nervous system (SNS) by both acute and chronic stressors has been demonstrated (225, 226). These effects are modulated directly through the presence of stress receptors on immune cells, as well as indirectly through the modulation of stress hormones and cytokines which can lead to changes in additional cell populations in the environment (227, 228). For example, studies have demonstrated that stress can reduce the number of circulating lymphocytes, as well as dampen T cell response to IL-2 stimulation, and decrease antibody production \textit{in vivo} (201, 202, 228). The reported capabilities of stress and stress hormones warrants further investigation into the exact components that are acting in the body to effect cancer patient treatment and outcomes.

We hypothesized that these previously demonstrated effects on the immune system could be related to the generation, or promotion, of MDSC and that patients with abnormal levels of stress could have altered levels of circulating MDSC. Some of the same factors that are dysregulated with stress have been associated with the generation and/or function of MDSC. In previous studies, elevated levels of IL-1β, IL-6, IL-10, TNFα, VEGF, and MMP-9 have all been associated with increased stress and MDSC (228-230). This report is the first to examine MDSC in the context of psychological
stress, and represents an important initial look at the complex interaction between stress and immune suppressor cell populations.

The manuscript presented here evaluated a cohort of sixteen breast cancer patients. These patients were evaluated for their level of cancer-specific stress by a self-reported measure called the Impact of Events Scale (IES) questionnaire. In this survey, patients described the level of intrusive thoughts and avoidance behavior directly related to their cancer diagnosis. The patients answered a series of questions and the sum of these was totaled, for a score total ranging from 0-75. The patients were divided into two groups for the study, one group with extremely high levels of stress (>30 IES) and the other with extremely low levels of stress (<20 IES). The majority of patients fell between 20-30 IES, so these patients represented the extreme ends of the stress spectrum. The timepoint that was evaluated in this cohort of patients was post-surgery, less than 2 months after initial diagnosis.

Patients in this study were evaluated for the levels of stress-associated hormones and this was compared to the level of cancer-specific stress. Cortisol, epinephrine, norepinephrine, and ACTH levels have been demonstrated to be altered with stress when compared to normal control samples (226). In this cohort of patients, salivary cortisol levels were significantly associated with elevated levels of IES stress, while epinephrine and norepinephrine were similar regardless of IES level. Interestingly, ACTH trended toward negative association, with high levels of ACTH associated with low levels of
stress, although this did not reach statistical significance. These results indicated that while some association between stress hormones and IES existed, all hormones were not consistently elevated with stress in this cohort of patients. A panel of pro-inflammatory cytokines was also evaluated in plasma of this cohort of patients to determine if there were correlations between cytokines and IES levels. There were multiple plasma circulating cytokines that were associated with IES levels, including IL-1Rα, IP-10, and G-CSF, however only IL-1Rα reached statistical significance. Serum levels of IL-6 were comparable between low and high IES groups. These results indicated that a larger cohort of patients would be required to draw conclusive information on the relationship between IES and serum cytokines.

Multiple populations of MDSC were evaluated in this study, including total levels of early myeloid cells, CD33⁺/HLA-DR⁻ cells, as well as CD33⁺/HLA-DR⁻/CD11b⁺/CD15⁺ and CD33⁺/CD14⁻ MDSC. At the time these studies were initiated, these populations represented the common markers associated with MDSC in human studies. Interestingly, it was discovered that patients with high levels of stress, as measured by the IES, actually had reduced levels of all populations of MDSC. While this result was unexpected, it is of note that there were significant differences between the high and low IES groups. This indicated that cancer-specific stress correlated with alterations in levels of MDSC in patients at the timepoint assessed, although the relationship is complex and not easily understood with the initial experiments.
When it was discovered that MDSC were altered by cancer-specific stress, we decided to evaluate if longer-term stressors would have a similar relationship to MDSC. These same patients were also evaluated by the Life Events Scale of stress, which is a measure of five different significant life events in the past year (death of a friend or family member, financial difficulty, divorce or separation from a family member or friend, major conflict with children or grandchildren, robberies or accidents). The Life Events measure is both more objective and covers a longer period of time than the IES questionnaire in this study. By this measure, the exact inverse relationship was observed. Here, as would be expected, patients with a greater number of stressful events in their lives had elevated levels of MDSC as compared to patients who had fewer stressful events.

These results are intriguing and bring to light several issues with studying stress and immunity. First, the duration and type of stress appear to have a significant impact on the cell populations being studied. This indicates that improved markers for both short-term and long-term stress should be determined to standardize studies and outcomes. While differences in immune effects between acute and chronic stress have been known for a long time, translating these studies into clinical therapeutics can be challenging. The issue of objectivity is also important. The IES is a self-reported questionnaire about emotions and behavior as a result of a cancer diagnosis. There is a large degree of subjectivity to this measure, and these emotions may be less connected to the release of stress factors in some individuals as compared to others. The Life Events
scale measures concrete events that occurred in the lives of the patient, which eliminates some ambiguity. In addition, the Life Events scale takes into account a longer period of stress. While all patients are suffering some level of stress associated with a cancer diagnosis, the individuals with high levels of life stress, in addition to their cancer, may have an elevated risk of immune suppressor cells persisting after surgery.

Surgery is also an additional aspect of this study to consider. While the high and low stress groups had no difference in the amount of surgery they received overall, a trend existed for patients that had more extensive surgery (mastectomy as compared to lumpectomy) to have reduced levels of circulating MDSC. All of these patients were considered macroscopically disease free at the time of MDSC evaluation, which makes these findings intriguing. This indicates that MDSC persist above levels found in normal individuals a month post-surgery, and that removal of additional tissue surrounding the tumor mass may reduce the numbers of MDSC in circulation. To verify this work, additional studies would have to be conducted with a larger cohort of patients, and it would also be interesting to track the patients over time to determine if this alteration in MDSC levels observed after surgery persists and relates to the risk of relapse.

The level of circulating MDSC was also compared to immune function in this cohort of patients. MDSC were associated with reduced numbers of circulating NK cells and this trend approached statistical significance. There was also a trend for reduced T cell blastogenesis in response to ConA stimulation. These results are consistent with
previous studies indicating that MDSC levels correlated with decreased immune cell responsiveness, although more robust cohorts of patients studied in a prospective manner would more completely address this relationship. In addition, multiple clinical studies have demonstrated alterations in MDSC function with various therapeutic treatments, but these did not all change the number of circulating MDSC (147). Functional studies along with measures of cytokines and stress hormones would assist in developing a more complete understanding of how stress effects factors associated with MDSC, and therefore how stress effects MDSC function in cancer patients.

Relevancy to the field of cancer biology

Each of the studies described were designed to further the understanding of the important factors that mediate MDSC, and how MDSC may lead to inhibited immune function in cancer patients. The first two studies discovered a novel mechanism of MDSC inhibition, and demonstrated that a major component of anti-tumor immunity could be impaired in the presence of MDSC. The third study evaluated an environmental component and provided the first indication that stress in cancer patients could lead to alterations in levels of MDSC. Each of these may have an impact on the design of additional studies and the implementation of therapeutic options for clinical treatment of patients.
Immune suppressor cells represent a massive blockade in the efficacy of immune-based therapies. While vaccination strategies are focusing on improved tumor antigen presentation and trafficking to tumor sites, and monoclonal antibody therapies are being employed to label tumor cells and inhibit growth, each of these components can be largely ineffective *in vivo* with barriers like MDSC. Both in circulation and at the tumor site, MDSC have the ability to completely dysregulate the immune system and shut down both innate and adaptive cellular responses. Investigating the mechanisms by which MDSC can achieve this inhibition, and the factors that influence MDSC expansion and recruitment, are critical to designing effective therapies to overcome this hurdle.

Both Type I and II IFNs have been proven to be important for anti-tumor immunity, and are also important for immune-based therapies. Without normal IFN signaling, immune surveillance could be impaired, and function of immune cells would be inhibited. Dendritic cells have recently been demonstrated to be important in anti-tumor immunity, and IFN assists with maturation and effective antigen presentation (29). IFNs have also been shown to modulate CD8$^+$ T cells and NK cell cytotoxicity, and CD4$^+$ T cell recruitment (28, 30, 31). The studies outlined in the first two chapters demonstrate this key mediator is less effective in the presence of MDSC. Understanding how MDSC effect signaling pathways could lead to specific targets for novel drug design. The data discovered in these studies demonstrated a direct relationship between MDSC and reduced signaling *in vivo*, and identified a potential therapeutic target for restoration of this response.
Nitration of tyrosine residues, as observed with this model, could also impact additional signaling pathways. Only STAT1 was investigated for levels of nitration, however other key signaling proteins important for immune function, such as the MAPK/ERK pathway, should also be evaluated. Future directions for this work include evaluation of NK cell function, since NK cells have the ability to directly kill tumor cells and increasing NK cell cytotoxic killing holds promise therapeutically. NK cells are known to be important for efficacy of antibody-based therapies, and understanding how MDSC modulate their activity could improve therapies already used clinically (231). Preliminary data from our lab suggests that depletion of MDSC in vivo with gemcitabine can improve NK antibody-dependent cell-mediated cytotoxicity (ADCC), allowing improved killing of tumor cells (Figure 5.1).

Further studies will be aimed at understanding the mechanisms by which MDSC inhibit NK cell ADCC. If NK cell signaling pathways are nitrated in vivo, combinatorial therapies aimed at eliminating iNOS upregulation and subsequent nitric oxide production

Figure 5.1. NK ADCC. NK cells isolated from normal mice, tumor-bearing mice (4T1 NK), or tumor-bearing mice depleted of MDSC with gemcitabine (4T1+Gem NK) were co-cultured with Cr-51 labeled tumor cells and percent ADCC was determined.
could be added prior to commencement, or included during the duration of treatment, of antibody-therapies such as herceptin. Continual depletion or modulation of MDSC by multiple different suppressive pathways may be required during the course of treatment to prevent MDSC from developing resistance.

While immune-based therapy has been used clinically, the drive to increase use of these treatments as well as improve efficacy and patient applicability is encouraging. Our bodies possess the natural ability to fight cancer, and harnessing this potential represents one of the few opportunities to obtain an actual cure for cancer. Until the body recognizes aberrantly growing cells and is able to effectively eliminate cells before they become vascularized tumor masses, oncologists will be continually vigilant for relapses and cancer will become a chronic disease.

From promising preclinical data, immune-based therapies have been integrated into the clinic as new options and an alternative to traditional treatment. While there have been some successes, such as the use of the monoclonal antibody herceptin, immune-based therapies have been successful only in limited settings and certain situations (55, 232-234). There is a need for immune-based therapies that can be effective in a broader range of patients, and MDSC as well as other negative regulatory mechanisms should be addressed in the clinic along with additional treatment. Data in mice and with MDSC-modulating agents in humans indicates that removal of MDSC, or removing a single effector-mechanism of MDSC alone, will not be sufficient to
effectively treat patients. Instead, the utility of MDSC modulation could be in multi-modal therapy where the first line of treatment would be aimed at removing MDSC, while a second line immune-stimulatory therapy is employed to then active the immune system.

Cancer treatment has largely focused on the use of chemotherapy and semi-targeted drugs to somewhat selectively kill tumor cells. While this is an important component of anti-tumor treatment, within the past decade the concept of exploiting the patient’s immune system to also attack the tumor while sparing normal cells has been actively translated into clinical practice. Activation of an immune response that would ultimately eliminate both existing tumors and prevent relapses is the pinnacle goal of cancer therapy. Accomplishment of this single idea would move cancer from a deadly disease into a manageable disorder. It would give patients the opportunity for long-term success, and lessen the psychological impact of a cancer diagnosis and the life-long burden of knowing a relapse could occur at any time. These are some of the hardest aspects of cancer, it becomes a life-long disease for patients that achieve survival beyond the initial diagnosis. Reaching this goal can be achieved through better understanding of the level of complexity of the pro-tumorigenic environment in cancer patients. Studying MDSC biology, function, and modulation is an important component of this progression.
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150


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160


