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Reprogramming of Myeloid Compartments Supporting Tissue Repair During Dss-Induced Colitis Recovery

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REPROGRAMMING OF MYELOID COMPARTMENTS SUPPORTING TISSUE REPAIR DURING DSS-INDUCED COLITIS RECOVERY

by

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Under the Direction of Yuan Liu MD, PhD

ABSTRACT

Myeloid-derived suppressor cells (MDSC), emerging during tumor growth or chronic inflammation play a critical role in regulating T cell function. However, mechanisms governing the generation of these cells remain unclear, and need to be further defined. Using a DSS-induced colitis and recovery model, we characterized the dynamic changes within myeloid compartments and the emergence of MDSC during active and resolution phases of inflammation. We show that the immature myeloid compartment expands in bone marrow (BM) specifically at the resolution phase of inflammation during colitis transition to recovery. Additionally, we found enhanced levels of IL-17 in the serum of colitis mice tightly correlates with expansion of the IMC compartment, and is likely the factor responsible for expansion of these cells. Our study also determined that the expanded population of myeloid cells underwent a functional reprogramming event. In particular, two major functional changes occurred when colitic mice were allowed to recover: 1) $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells in bone marrow and spleen acquired T cell suppressive functions, and 2) acquired the ability to enter into circulation from BM, confirming previously reported characteristics of MDSC. Additionally, we determined that

acquired migratory capability in the low density myeloid cells isolated from resolution time points was due to enhanced surface expression of chemokine receptor CXCR2. Furthermore, we determined that after mobilization of MDSC from the bone marrow, these cells collected in the T cell-rich spleens, where they effectively functioned to suppress T cell proliferation. Through these acquired functions, our study determines a protective role for MDSC during the recovery phase of post-acute inflammation during persistent DSS-induced colitis.

INDEX WORDS: MDSC, Colitis, IBD, Inflammation, Tissue Repair, Immunosuppression

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DEDICATION

This work is dedicated to my incredible family, both by blood and by choice. Mom, Dad, Mare, Kyle, and Jon there is no way I could have accomplished this feat without your constant love and support. I appreciate all of your patience and encouragement, and for believing in me even when I didn't believe in myself. Thank you for always teaching me to reach for the stars. I love you all; this work is dedicated to you.

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LIST OF ABBREVIATIONS

ACT-Adoptive cell therapy

ADAM17-A disintegrin and metalloproteinase domain 17

Arg-Arginase

BM-Bone Marrow

C/EBP-CAAT-enhancer binding proteins

CART-Chimeric antigen receptor T cell

CCR-CC chemokine receptor

CD-Chron's disease

CD-Cluster of differentiation

CDK-Cyclin-dependent kinase

CFSE-Carboxyfluorescein succinimidyl ester

CLP-Common lymphoid progenitor

CMP-Common myeloid progenitor

CTLA-Cytotoxic T-lymphocyte antigen

CXCL-CXC chemokine

CXCR-CXC chemokine receptor

DAI-Disease activity index

DC-Dendritic cell

DSS-Dextran Sulfate Sodium

EAE-Experimental autoimmune encephalitis

ECM-Extracellular matrix

EGF-Epidermal growth factor

ELISA-Enzyme-linked immunosorbent assay

EMT-Epithelial/mesenchymal transition

FACS-Fluorescence activate cell sorting

FGF-Fibroblast growth factor

G-CSF-Granulocyte colony-stimulating factor

G-MDSC-Granulocytic MDSC

GAL9-Galectin 9

GALT-Gut-associated lymphoid tissue

GM-CSF-Granulocyte/Macrophage colony-stimulating factor

GPCR-G protein-coupled receptor

GTPase-Guanosine triphosphatase

HRP-Horseradish peroxidase

HSC-Hematopoietic stem cell

IBD-Inflammatory bowel disease

ICAM-Intercellular adhesion molecule

IFN-γ-Interferon gamma

IFX-Infliximab

IgSF-Immunoglobulin superfamily

IL-Interleukin

IMC-Immature myeloid cells

ITIM-Immunoreceptor tyrosine-based inhibition motif

iNOS-Nitric oxide synthase

JAK-Janus kinase

LCN/NGAL-Lipocalin/neutrophil gelatinase-associated lipocalin

LPS-Lipopolysaccharide

M-MDSC-Monocytic MDSC

MAPK-Mitogen-activated protein kinase

MCP-1 Monocyte chemotactic protein

MDSC-Myeloid-derived suppressor cells

MHC-Major histocompatibility complex

MMP-Matrix metalloproteinase

MPO-Myeloperoxidase

MΦ-Macrophage

NF-κB-Nuclear factor kappa B

NK-Natural killer cell

NKT-Natural killer T cell

NO-Nitric oxide

PAMP-Pathogen-associated molecular pattern

PD/PDL-Programmed death receptor/ligand

PDGF-Platelet-derived growth factor

PECAM-Platelet endothelial cell adhesion molecule

PGE-Prostaglandin E

PMN-Polymorphonuclear leukocyte/neutrophil

PRR-pathogen recognition receptor

PS-Phosphatidylserine

PSGL-1-P selectin glycoprotein-1

RBC-Red blood cell

RNS-Reactive nitrogen species

ROS-Reactive oxygen species

RTK-Receptor tyrosine kinase

SEFIR-Similar expression to FGF receptor/IL-17R

SH2-SRC homology 2 domain

SMA-Smooth muscle actin

STAT-Signal transducer and activator of transcription

TAM-Tumor-associated macrophage

TAP-Transporter associated with antigen processing

TCR-T cell receptor

TGF-β-Transforming growth factor beta

TIGIT- T cell immunoreceptor with Ig and ITIM domains

TIM3-T-cell immunoglobulin and mucin domain containing-3

TIR-Toll/IL-1 receptor

TNF-α-Tumor necrosis factor alpha

TRAIL-TNF-related apoptosis-inducing ligand

Treg-Regulatory T cell

UC-Ulcerative colitis

VEGF-Vascular endothelial growth factor

VLA-1-Very late antigen-1

1 INTRODUCTION

1.1 The Inflammatory Response

Inflammation is the immune system's protective response to any disruption of tissue homeostasis, and serves as the first line of host defense against invading pathogens [1]. The immune system uses the inflammatory process to detect and eliminate "foreign" cells. The inflammatory process of the immune system serves as the first level of protection against harmful agents to the host. The terms "foreign" and "alien" refer to many potential sources of danger for the host, including invading pathogens (bacteria, fungi, toxins), foreign cells (transplanted, incompatible tissue), and abnormal cells (tumor, apoptotic, and necrotic cells) [1]. The immune system has many methods of detecting and eliminating these threatening stimuli; the primary method of removal of most for these threats is the inflammatory response.

Inflammation is described by five hallmark characteristics: heat, swelling, redness, pain, and in extreme cases, loss of function. These characteristics are all consequences of increased blood vessel permeability, which allows for the robust infiltration of circulating inflammatory leukocytes into the tissue. These leukocytes, primarily monocytes and neutrophils, are the first effector cells that respond to the initial detection of danger signals. Once in the tissue, these cells remove sources of tissue damage and pathogens [1]. Functional deficiency in any of the cells that are responsible for coordinating the inflammatory response has profound consequences on the host. For example, loss of function in neutrophil-mediated digestion of pathogen leads to chronic granulomatous disease, characterized by the formation of pus-filled abscesses, and inability to remove pathogens [2]. Decreased inflammatory function often leads to increased susceptibility to infection for the host, while disorders that increase activity of the inflammatory response have similar detrimental outcomes for the host. One example of this type of inflammatory

dysregulation is represented by a wide variety of diseases, including rheumatoid arthritis [3], inflammatory bowel disease [4], and atherosclerosis [5]. There are three major goals of the inflammatory response: 1) The recognition and removal of pathogen and other sources of threatening stimuli, 2) removal of the damaged and "alien" cells within the tissue, and 3) the initiation of the tissue repair process, which is responsible for terminating the inflammatory program and returning the tissue to homeostasis [1]. Beginning first in the locally insulted tissues, the inflammatory response gradually expands to include systemic signaling molecules that result in the robust influx of effector innate and adaptive immune cells [3].

1.2 Initiation of Inflammation, Cytokine Signaling, and Immune Cell Recruitment

The first step in the inflammatory response is the recognition of the source of tissue damage. These sources include two distinct types. The first type of tissue injury is referred to as sterile injury because the tissue's protective barrier layer remains intact [6]; therefore no pathogen is able to enter the wound. Sources of insult in the sterile injury include necrotic and apoptotic cells, and are typically inflicted by trauma [6]. The second type of injury occurs when a protective barrier, such as the skin, is breached, which allows entry of pathogen into the wound. In the case of non-sterile injury, pathogens are recognized by the resident immune cells, including macrophages and dendritic cells [1]. These cells have unique cell-surface pathogen recognition receptors (PRRs), which recognize non-specific conserved pathogen-associated molecular patterns (PAMPs), including bacterial peptides lipopolysaccharide and flagellin, and viral DNA and RNA antigens [7]. Once the pathogens have been recognized, an immune response is mounted with the purpose of removing any sources of tissue damage and/or pathogen, and commences the tissue repair process [3]. The following describes in detail how the inflammatory response accomplishes these goals.

1.2.1 Early Inflammatory Response

The immediate initiation of the inflammatory response functions primarily through activation of innate immune cells, including macrophages, dendritic cells, and neutrophils, which are responsible for rapid, broad identification and clearance of pathogen [1, 3, 6]. Upon recognition of PAMPs through PRRs, the immune cells undergo activation of signal transduction pathways [6]. Consequences of these activation events result in the dynamic alteration of many cell functions, including modification of transcriptional programs. After local tissue resident immune cells recognize the sources of tissue insult they undergo transcriptional activation of the inflammatory program for the purpose of recruiting leukocytes to the site of insult [7]. The alteration of the transcriptional programs leads to generation of pro-inflammatory mediators including

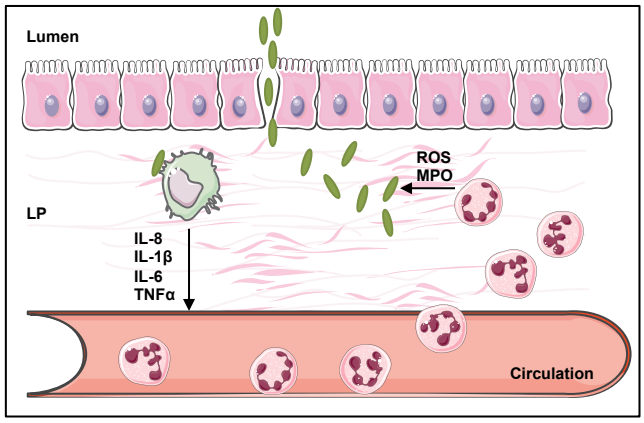

Figure 1-1 The early inflammatory response.
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chemokines, cytokines, and lipid mediators, which are then secreted into the local environment and diffuse into circulation [8]. The generation of secreted chemokines acts as a gradient to direct the infiltrating leukocytes into the tissue. Some of the cytokines produced by the tissue immune cells, such as IL-1β and TNFα, function to induce changes in the endothelium, such as enhanced expression of adhesion molecules and loosening of the junctions between adjoining cells, thereby

increasing vascular permeability, which helps facilitate extravasation of circulating leukocytes [1, 7].

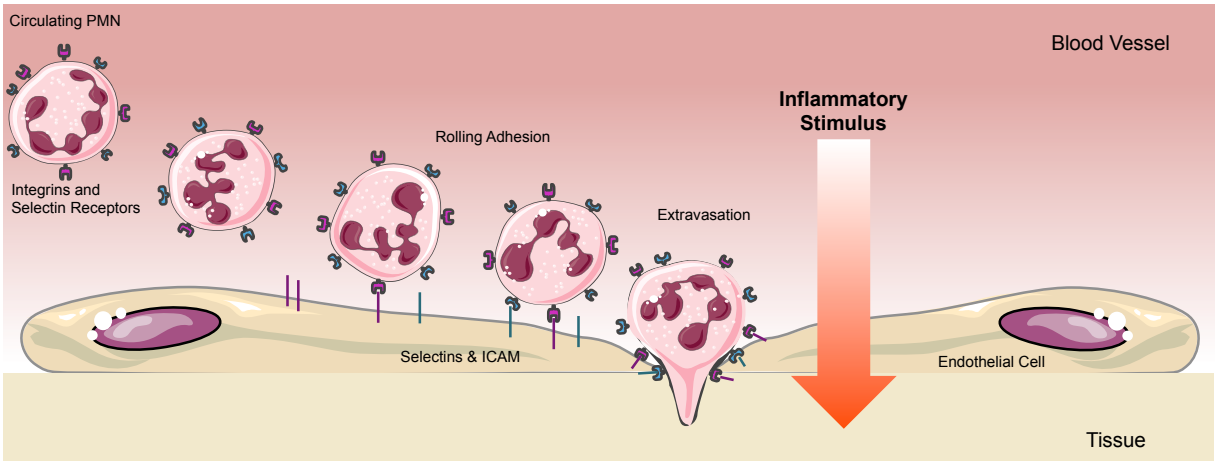

Figure 1-2 Rolling adhesion and diapedesis.
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Meanwhile, naive myeloid leukocytes circulate in the bloodstream waiting to sense inflammatory signals that will trigger activation. Soluble cytokines produced primarily by immune cells in the local insulted tissue bind to extracellular receptors on the circulating monocytes and polymorphonuclear leukocytes (PMN) to induce powerful changes (Fig. 1-2). These changes can be characterized as a series of processes known as rolling adhesion and diapedesis [9]. First, the leukocytes increase L-selectin and VLA-1 surface molecules to leave the sweeping flow of the blood stream and begin tethering to the endothelial walls. Next, the leukocytes increase their presentation of more adhesion molecule, including p-selectin glycoprotein ligand-1 (PSGL-1). These molecules begin the process of rolling adhesion, a transient process of weak adhesion and dissociation with counter-receptors on the endothelium, that helps the leukocytes bind the endothelium more steadily and further decreases the speed of the circulating cells (Fig. 1-2). After the leukocyte rolling begins to slow down, the cells become activated by the locally diffused cytokines and chemokines emanating from the area of insult [9].

The binding of cytokines and chemokines to their leukocyte receptors induces signaling cascades within the leukocytes that leads to activation of the leukocyte surface integrins, in a process known as “inside-out” signaling [1, 9].

Leukocytes use activated integrins to form tighter associations with the endothelial counter-receptors, further enhancing the binding avidity between the leukocytes and the endothelium. It is important to note that the endothelium at the site of insult has undergone several complimentary changes as a results of inflammatory cytokine and chemokine secretion that will help the leukocytes identify the area of interest and facilitate extravasation from the circulation into the tissues, such as increased vascular permeability and increased expression of surface adhesion molecules (ICAM, PECAM) [9]. Once tight adhesion is established, the leukocytes begin the process of extravasation from the endothelium. To do this, the leukocytes must use the chemokine gradient as a directional guide to crawl along the endothelial lumenal surface. The myeloid cells use the CD11b/CD18 integrin pair to interact with ICAM-1 on the endothelial surface to facilitate movement toward areas of concentrated chemokines, usually found near the endothelial cell borders. It is here that the leukocytes will undergo transmigration or diapedesis across the endothelium to reach the insulted tissues [9]. Once the cellular junctions are reached, the leukocytes flatten against the endothelium and extend a cytoskeletal protrusion between the cell junctions. The leukocytes then use a PECAM to pull themselves through the meeting cells. Once the leukocytes reach the basement membrane, PMN must undergo degranulation to release proteolytic enzymes, including gelatinase and collagenase to digest the basement membrane [10]. Upon entering the tissue, the leukocytes use the chemokine gradient (primarily IL-8) to migrate toward the injury site [10].

1.3 Mechanisms of Pathogen Elimination

Upon entry into the insulted tissue, PMN undergo further sequential degranulation steps necessary for the destruction of microbial pathogens present at the wound site. During this phase, the PMN are fully activated and quickly mobilize microbiocidal factors into the tissue including many proteases, complement proteins, and defensins via release of intracellular granules. PMN also produce high quantities of hydroxy radicals H_2O_2 and O_2^- in an effort to kill microbes present within the wound by producing an environment rich in oxidative stressors [10, 11]. These molecules are primarily meant to induce DNA breakage and protein and lipid modifications in invading pathogens; however, because the secretion of these reactive pro-oxidant species is non-specific, DNA damage and signaling molecule modification accumulates in the host tissue as well [11].

1.3.1 Oxidative Stress

Although intended only to cause harm to present pathogens, reactive oxygen and nitrogen species secreted during the influx of leukocytes function in a non-specific way, and can also cause harm to the host tissue. This process is known as oxidative stress, and describes the accumulation of pro-oxidant molecules in the inflamed tissue [6, 12]. These molecules are powerful reductants, meaning they donate electrons to other molecules in the environment. Pro-oxidants can be grouped as radical pro-oxidants or non-radical pro-oxidants [12]. The radical group of these reactive oxygen and nitrogen species possess an unpaired electron in their outermost electron shell making them highly volatile, and very reactive. The non-radical group of pro-oxidant reactive oxygen species do not possess such an unpaired electron; however, these compounds are highly reactive and have a tendency to donate electrons to other molecules in order to reduce their energy state [12]. Consequences of this electron donation event include three major types of

oxidative damage: 1) lipid peroxidation, 2) protein modifications, and 3) DNA damage [12]. Lipid peroxidation is the process by which free radicals react with lipids in the plasma membrane resulting in the production of more reactive peroxide products and membrane degradation [12]. While this consequence of oxidative stress is indeed harmful to the host, the second and third outcomes of oxidative damage are directly transformative; meaning the modifications of signaling proteins and direct DNA damage are capable of transforming healthy tissue cells into neoplastic pre-tumor cells [13]. Protein modifications due to oxidative damage come in a variety of different versions, including cross-linking, misfolding, and peptide fragmentation. These types of modifications often result in functional changes in the proteins [12]. Cellular DNA damage is the third target of oxidative damage, and can result in the accumulation of gene mutations within the cell. If these gene mutations occur in oncogenes or tumor suppressor genes, the cell may be able to escape cell cycle regulation and become a neoplasm. For these reasons, inflammation-induced oxidative stress is defined as a powerful inducer of cancer [13].

1.3.2 Microbiocidal Agents & PMN Degranulation

In addition to producing large quantities of reactive oxygen and nitrogen species, the influx of leukocytes to the area of insult also induces release of many more specific molecules that help to rid the system of invading pathogen, and restore the tissue to homeostasis. In addition to production of oxidative stressors, the innate immune system functions through an additional two pathogen elimination strategies: 1) the complement system, and 2) PMN degranulation events. Both of these approaches to pathogen elimination are more specific than the alternative oxidative stress induction pathway, and are somewhat less harmful to the host [10]. Although complement is an essential mechanism of eliminating pathogen from the wounded tissue, it is not the focus of our study, therefore for the purposes of this discussion, PMN degranulation and direct

phagocytosis of pathogen will be the primary focus. Indeed, complement system and PMN degranulation are more specific than widespread production of detrimental reactive oxygen and nitrogen species, these pathways are general enough to be effective against a wide spectrum of pathogens without inducing damage to the host tissue. These molecules are secreted specifically for the elimination of microorganisms either by direct puncture of the cells, destruction of key biological molecules, and by direct labeling for destruction by immune cells. The following is a detailed description of how pathogen elimination is accomplished in a more specific manner than respiratory burst.

1.3.2.1 Primary (azurophil) granules

Primary granules are first to be released during migration into the site of inflammation. These granules, also called azurophilic granules contain many microbiocidal agents that function differently to eliminate a wide range of pathogens [10]. MPO is one such enzyme secreted from primary granules that functions to produce many antimicrobial compounds from the peroxide generated during respiratory burst [2, 10]. Like most antimicrobial molecules, MPO is produced in myeloid leukocytes, primarily neutrophils, and are sequestered in primary granules [2]. Upon immune activation, leukocytes undergo the process of degranulation, which results in release of MPO into the extracellular space, or during phagocytosis, primary granules can fuse with the phagocytosis vesicle [12]. This fusion results in the total destruction of the pathogen within the phagosome. The MPO enzyme functions as a potent antimicrobial enzyme by producing hypohalous acids, predominantly hypochlorus acid from the reactants H_2O_2 and chloride ions present in the periphery [2]. The MPO enzyme works in conjunction with the NADPH oxidase complex, as the NADPH oxidase enzymes produce the peroxide reactants that are used in the MPO reaction (Fig. 1-2). Due to the highly reactive nature of the hypochlorus acid, excess

degranulation of myeloid leukocytes during periods of inflammatory overstimulation results serious pathological conditions; many of which are due to increases in oxidative stress [14]. Conversely, lack of the MPO enzyme, or defects in MPO enzyme function does not typically present any characteristic immunodeficiency as seen in NADPH oxidase enzyme defects [14]. This is due to the redundant nature of the microbiocidal agents present in leukocyte granules [12]. Primary granules also contain a large quantity of the α-defensin proteins. These small peptides are cationic in nature and have potent microbiocidal functions through creation of large pore-forming transmembrane complexes within the pathogenic cells [10]. This group of proteins also includes bactericidal/permeability-increasing protein (BPI). Additionally, α-defensins function as chemoattractants for innate immunes cells, including monocytes and macrophages as well as adaptive immune T cells. The third major component of azurophilic granules is the serprocidins. Standing for serine proteases with microbiocidal activity, this group of proteins includes cathepsin G, elastase, and proteinase-3, all of which possess proteolytic function on extracellular matrix proteins [10]. The function of these proteases is to facilitate digestion of the basement membrane to allow the migration of activated PMN into the tissue. The release of azurophilic granules must be tightly regulated to reduce the amount of unnecessary tissue digestion, as this can lead to degradation of tissue structure and function. For this reason, many anti-serprocidin proteins are secreted into the plasma constitutively, as a method of restraining excess ECM damage by proteases [10].

1.3.2.2 Secondary (specific) and tertiary granules

The second and third types of neutrophil granules are secondary (or specific) and tertiary, which primarily contain microbiocidal substances and digestive enzymes. These granules are peroxidase negative, meaning they do not produce peroxide as a major antimicrobial substance.

They do, however, produce large amounts of lactoferrin, hCAP-18, lipocalin-2/neutrophil gelatinase-associated lipocalin (LCN2/NGAL), and lysozyme [10, 15]. These molecules are potent antimicrobial proteins that target a wide variety of microbial pathogens through multiple mechanisms. The most common function of these antimicrobial proteins is to sequester precious limited metals from bacterial uptake [16]. Tertiary granules contain mostly digestive enzymes that function to digest the basement membrane and extracellular matrix to facilitate migration of PMN through the tissue [10]. Because of the harmful nature of these enzymes, it is imperative for their release to be stringently controlled, as dysregulation of this system leads to extreme consequences, particularly tissue damage, within the host.

1.3.2.3 Monocyte recruitment and differentiation

Initially, monocytes entering the site of tissue injury function similarly to activated PMN, secreting proteolytic enzymes and rapidly taking up pathogen via phagocytosis through PRRs; however, unlike PMN, recruited monocytes undergo further differentiation into macrophages and dendritic cells after reaching the wound site [1]. This differentiation is mediated by cytokines, chemokines, growth factors, and other secreted molecules in the local wound microenvironment. Because monocytes are the first responders of the immune system during inflammation, and initially establish a non-specific response to inflammation, they use this differentiation opportunity to transform themselves into more wound type-specific sentinels [5]. From a single population of monocytes develops a highly plastic spectrum of professional antigen presenting cells (macrophages and dendritic cells) capable of recognizing and phagocytizing a wide variety of targets. The utility of this transformation lies in the ability for the innate immune system to communicate with the adaptive immune system about antigens that have entered the body [5]. This system, bridging the innate immune system with the adaptive immune system, allows the

host to become educated about encountered pathogens, helping to initiate a sustained, life-long immunity to regularly contacted antigens such as bacteria and viruses, and is responsible for inducing tolerance to regularly-encountered, non-pathogenic antigens [3].

1.4 Removal of Target Cells Via Phagocytosis

Phagocytosis is the process of direct uptake of antigenic targets primarily by professional phagocytes, including dendritic cells, macrophages, and PMN. The phagocytic targets can be pathogenic or commensal microbes (alive or dead), alien cells, and misplaced or damaged host cells. Depending on the target cell being taken up, this process can stimulate the activation of the inflammatory response, or can function as a method of inducing immune tolerance. Phagocytosis is an absolutely essential process for sampling antigen, and maintaining the appropriate immune response within the tissues, and significantly contributes to maintenance of homeostasis.

1.4.1 Phagocytosis

As previously stated, the first and second major functions of the inflammatory response are the removal of pathogen and damaged tissue cells from the insulted tissue. Phagocytosis is the process used to accomplish these tasks. Removal dead and dying cells, and pathogens from the tissue are eliminated from the tissue by professional phagocytic cells, such as macrophages, neutrophils, dendritic cells, and B cells, however other, non-professional phagocytic cells can undergo changes that allow them to perform phagocytosis [17]. The process of phagocytosis involves three steps: 1) sensing target cells, 2) determining whether the target is labeled for destruction, and 3) engulfment of the target [17, 18]. Sensing of the phagocytic target is carried out using chemoattractant gradients. These can be cytokines or chemokines, or as in the case of pathogens, can be microbial PAMPs [6, 17]. The second step in phagocytosis is to determine whether the target cell is labeled for destruction. Many tags, or labels can be used to induce

activation of phagocytosis of the target cells, and these tags are collectively referred to as pro-phagocytic "eat me" signals [17]. "Eat me" signals include a wide variety of pro-phagocytic molecules, including exposure of PS by apoptotic cells, complement proteins, and antibody tags. Conversely, a range of anti-phagocytic "don't eat me" signals can also be displayed on cells, which work to oppose the activation signaling of the "eat me" signals [18]. These cellular indicators include markers that define cellular health, such as MHC molecules and CD47 [18]. Upon contact with a potential target, the phagocyte searches the cell for these pro- and anti-phagocytic molecules using counter receptors. In most cases, both pro- and anti-phagocytic signals are received by the phagocyte; however, the prevailing signal determines the outcome of the phagocytosis event. If pro-phagocytosis signals overpower the anti-phagocytic signals, then activation of phagocytosis is achieved, and the target will be taken up by the phagocytic cells. If anti-phagocytosis signals prevail, then the target cell will not be removed from the tissue by the phagocyte. Therefore, target cells maintain the delicate balance of expression of phagocytic signals, and have some control over their ability to either survive in the tissue, or to be removed [17]. The third step of phagocytosis is the actual act of target engulfment by the phagocyte. This is highly controlled process that involves the activation of cytoskeletal rearrangement and coordination of engulfment and digestion machinery [19].

1.4.1.1 Necrosis vs. apoptosis

Removal of damaged cells is dependent on detection of damage-associated molecular patterns, or DAMPs. These DAMPs are specific to the type of cell death occurring within the tissue. Two major types of cell death processes can be observed in the eukaryotic tissue: necrosis and apoptosis. Necrosis, which is best described as "unplanned" cellular death [19]. This type of cell death typically results from cellular injury, and is a far messier process than apoptosis, or

"programmed" cell death, which is an extremely tightly coordinated and controlled process [19]. During necrosis, the cells have been suddenly damaged, and do not undergo the clean, self-contained process of tidy cell death, as occurs in apoptosis. For this reason, necrosis is referred to as passive cell death, and apoptosis is described as active cell death.

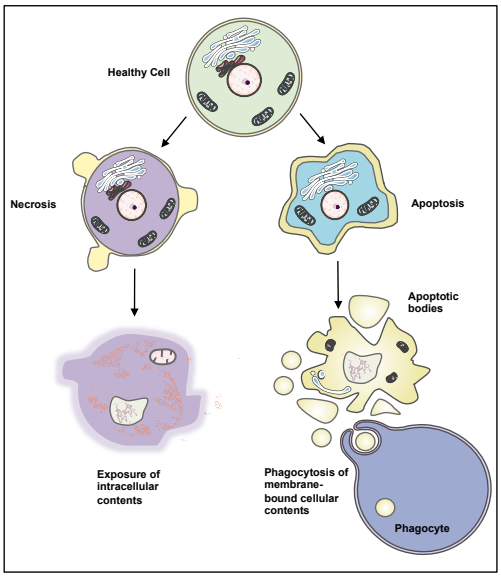

Figure 1-3 Necrosis versus apoptosis.
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Apoptosis occurs when the cell has sensed inducible death signals, lack of resources, or intracellular pathogen, and progresses through a series of tightly regulated protein cleavage cascades using the caspase proteases [19]. Once activated, the caspases promote internal destruction of cellular components, including cellular proteins. Additionally, release of intracellular calcium from the mitochondria induces changes to the plasma membrane, including exposure of inner leaflet phosphatidyl serine (PS) to the extracellular environment [18]. PS is a pro-apoptotic marker that signals the act of cell death to patrolling professional phagocytes, thus marking the cell for removal. Unlike necrosis, apoptosis is a unique, tidy method of controlling exposure of self-antigen, which can go on to cause autoimmune disease through activation of B

cells (Fig. 1-3). Because the intracellular contents of the apoptotic cell remain sequestered within the plasma membrane of the dying cell, until uptake via phagocytosis, risk for inducing an autoimmune reaction remains low. This prevents any self cellular antigens from leaking out into the extracellular space, where they can be taken up by patrolling B cells [18]. Limiting the activation self-reactive B and T cells is crucial to avoiding autoimmunity in the host. Necrosis, on the other hand, is not the preferred method of cell death, as it does not guarantee the sequestration of self antigen within the plasma membrane, and can therefore, induce the activation of autoimmunity [17].

1.5 Progression to the Adaptive Immune Response

During the innate immune response, many foreign antigens are taken up through the phagocytosis pathway. Dendritic cells and macrophages are the two major populations of innate phagocytes that present antigens during inflammation [18]. Of these two populations of phagocytes, macrophages primarily function as transducers of inflammation, whereby the phagocytosis pathway stimulates these cells to produce cytokines that work to recruit other leukocytes to the area [6]. Unlike macrophages, dendritic cells are antigen presenting cells whose primary purpose is to bridge communication between the innate and adaptive components of the immune system. In other words, they collect and process antigen with the express purpose of presenting the antigen to adaptive immune cells [18]. This process educates adaptive immune cells and initiates the adaptive immune response, involving activation of lymphocyte-derived cells, such as T cells, B cells, and NK cells, which coordinate development of the memory response, and development of T cell-mediated immunity.

As tissue resident dendritic cells take up antigens through the phagocytic pathway, they become activated, a process which induces maturation of dendritic cells (Fig. 1-4). The process of

activating maturation in DCs is characterized by the changes in morphology of the cells, upregulation of antigen presenting machinery, and increased cellular mobilization [20]. First, antigen-loaded MHC molecules become upregulated on the cell surface. Correspondingly, DCs undergo transcriptional upregulation of T cell co-stimulatory molecules, including CD40, CD80, and CD86 [21, 22]. Coordination of actin polymerization induces changes to the DC cytoskeleton to increase production of dendrites. These dendrite protrusions extend out from the smooth cell surface and serve to increase the surface area of the plasma membrane [23]. Additionally, DCs augment the expression of chemokine receptor CCR7, which allows them to migrate into local T cell rich lymphoid tissues, where they present the antigens to T cells. [22]

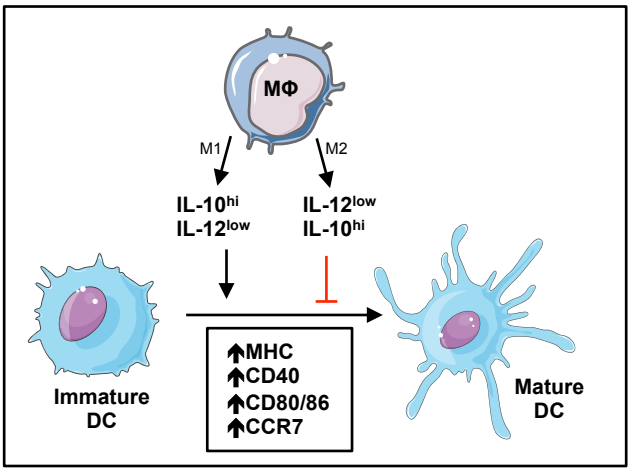

Figure 1-4 Dendritic cell maturation.
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Following maturation and migration of DCs into lymphoid tissue, such as the lymph nodes, antigen presentation commences. The major consequence of antigen presentation is activation of T cells. Briefly, this process takes place between antigen-loaded MHC molecules displayed on the surfaces of DCs, and matching, antigen-specific T cell receptors expressed on the naïve T cells present in the lymphoid organs [24]. Additionally, many co-receptors are utilized to strengthen the signal produced upon binding of MHC to the TCR. This process is collectively referred to as

formation of the immunological synapse [24, 25]. It is important to distinguish the MHCI-restricted antigen pathway from the MHCII-restricted antigen pathway. Although MHCI and MHCII are both similar molecules that function to present antigens to TCRs, MHCI molecules generally present endogenous peptide antigens, whereas MHCII molecules generally present exogenous peptide antigen. The type of MHC molecule that is most relevant in for responding to non-sterile injury is the MHCII molecule because DCs are primarily concerned with removal of exogenous pathogen [24]. Therefore, the following discussion will focus mainly on the MHCII:TCR immunological complex formation; however, activation of T cells via the endogenous MHCI presentation pathway is absolutely essential for recognition of aberrant tissue cells, and is used as then primary mechanism for recognition and removal of tumor cells.

1.6 Formation of the Immunological Synapse

Although a necessary component of T cell activation, ligation of the TCR with antigen-loaded MHC molecules is not sufficient for sustaining substantial T cell activation or proliferation. It is through the formation of the immune synapse that sufficient activation of the T cell is achieved [25]. The immunological synapse centers around the initial TCR:MHC complex, and forms a “bull’s eye” shaped protein cluster around the complex (Fig. 1-5). On the T cell side of the assembly, the TCR complex is positioned at the very center of the synapse [26]. Following activation, this receptor complex becomes surrounded by costimulatory receptors, including CD4 or CD8, and CD28. The costimulatory molecule CD28 binds it’s counter receptor CD80 (also termed B7) on the antigen presenting cell, which results in the activation of many intracellular signaling pathways, including JNK kinase, Cdc42, and PI3K. Activation of these signaling molecules results in enhanced transcription of cytokines, restructuring of the cytoskeleton, and stimulation of growth and proliferation signals within the T cell [25]. The costimulatory

molecules function to ensure the activation and proliferation signals persist, and result in the proliferation and maturation of antigen-responsive T cells.

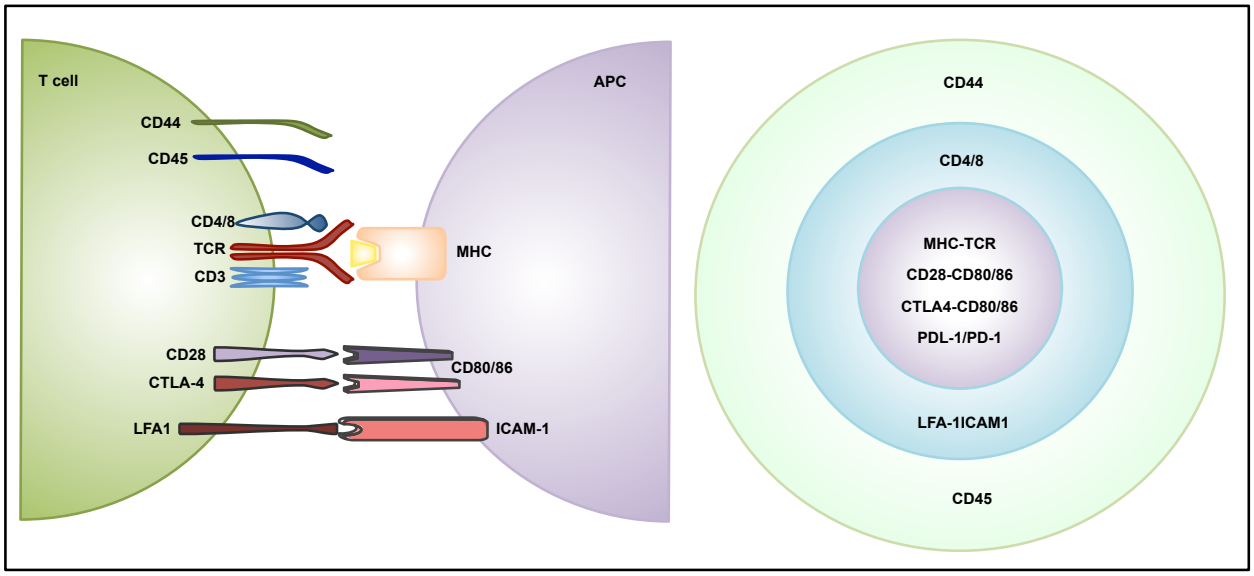

Figure 1-5 Formation of the immunological synapse.
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The immunological synapse is also comprised of co-inhibitory molecules. Perhaps the most significant, well-characterized pair of co-inhibitory molecules within the immunological synapse are CTLA-4 and PD-1 [27]. These two molecules induce T cell exhaustion upon ligation to their counter receptors CD80/CD86 and PDL-1, respectively. These inhibitory receptors function to limit the activation of T cells during the adaptive immune response in order to avoid any undesired chronic activation of the immune response during inflammation by inducing T cell exhaustion [27]. TIGIT, another representative of the IgSF family of co-inhibitory molecules, induces inhibition of T cell proliferation, NK cell activation, and promotes the secretion of IL-10 at the expense of IL-12 in lymphocytes upon ligation with any of its three counter receptors CD155, CD112, and CD113 [27]. Like many inhibitory IgSF receptors, TIGIT carries out this inhibition through the phosphorylation of its intracellular ITIM domains [27]. Induction of the

adaptive immune response is an incredibly important process, which ensures the defense of the host from infection, and allows for clearance of damaged, or unhealthy cells from the tissue; however, equally as important is the ability to reduce the adaptive immune response when the source of inflammation is removed. All of these co-inhibitory receptors play a vital role in inhibiting the immune response via induction of T cell exhaustion [27]. As will be discussed later, under certain conditions, these non-pathogenic co-inhibitory molecules can become the dominant species within the immunological synapse, resulting in the inappropriate inhibition of the adaptive immune response, and defining a pathogenic role for these receptors.

1.7 Late Inflammatory Response and Wound Healing

Although onset of the inflammatory response is crucial for the elimination of pathogen and damaged tissue, the resolution of this process is equally as important for the maintenance of tissue homeostasis. In fact, if this hostile environment is allowed to persist, the tissue becomes the source of chronic wounding, leading to a state of constant inflammation. Fortunately, the recruitment of leukocytes into the tissue functions through a self-limiting process that initiates the third function of the inflammatory response: the tissue repair process. Leukocytes orchestrate the wound healing program via secretion of signaling molecules and growth factors that stimulate tissue growth, proliferation, and angiogenesis. It is also very important that this tissue repair response is terminated quickly, as prolonged, unresolved tissue repair programming can lead to tissue fibrosis, and in extreme cases, leads to the creation of an immunosuppressive environment which can help neoplastic cells escape the anti-tumor immune response.

1.7.1 Late Inflammatory Phase

The first step in resolution of inflammation is to stop the recruitment of circulating leukocytes into the injured tissue [3]. Persistence of leukocyte recruitment only functions to

further promote the inflammatory response and create a more detrimental environment for the host tissue. Under these circumstances, the wounded tissue cannot begin to recover from the damage inflicted by the initial insult and the active PMN. Therefore upon entry into the tissue, the leukocytes begin a self-limiting feedback mechanism of lipid mediator secretion [28-30]. By generating large quantities of protectins and resolvins through lipid mediator class switching, inflammatory leukocytes (primarily PMN and macrophages) limit their own recruitment into the tissues (Fig. 1-6). Through binding of GPCR receptors on immune cells, resolvins block the transmigration of circulating monocytes and PMN into the wound [28]. Specifically, resolvin E1 directly antagonizes the BLT1 GPCR on PMN, a receptor that is activated upon binding of its agonist leukotriene B4. This antagonism results in the inactivation of NF-kB transcription factor pathways in migrating PMN, thereby turning off the activity of the prevailing inflammatory leukotriene pathways [30]. Resolvin E1 also functions to increase MAPK signaling activity in monocytes, and reduces dendritic cell migration and pro-inflammatory cytokine production through activation of the ChemR23 receptor [30]. Protectin D1 serves a similar purpose to curb inflammation and promote the initiation of wound healing. Protectin D1 inhibits recruitment of leukocytes, decreases TNFα and IFNγ production, and aids in the induction of T cell apoptosis at the wound site [30, 31]. All of these mechanisms help to reduce the influx of inflammatory cells, and help reduce the production of pro-inflammatory mediators by resident inflammatory cells. It is clear from this information that the initiation of the inflammatory response also induces the termination signals required to limit the tissue damage induced by the acute inflammatory response [29].

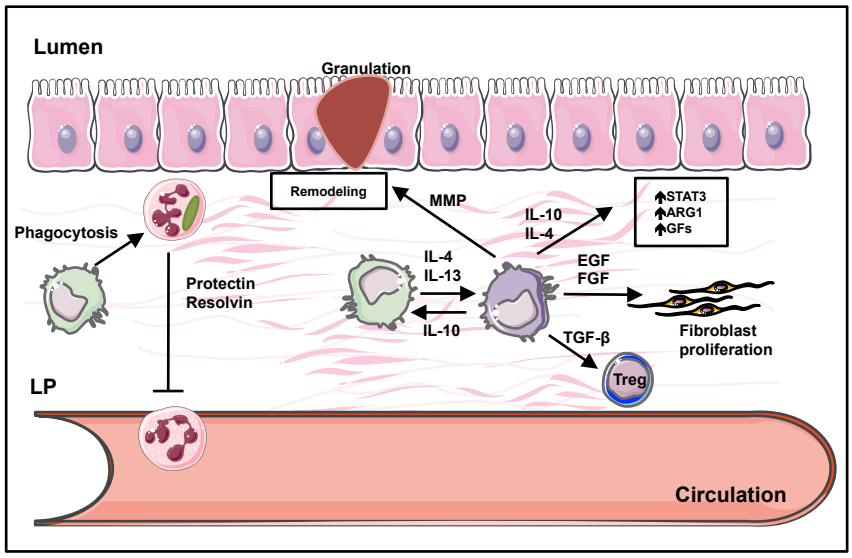

Figure 1-6 Late inflammatory phase.
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As the inflammatory response progresses, initially inflammatory leukocytes build up in the wound site and continue their differentiation and phenotypic changes into more specialized effector cells, such as macrophages and dendritic cells [3]. As a result, these cells add to the wave of secreted factors building up in the wound microenvironment. Some of these factors, such as TGF-β, EGF, FGF, and collagenase are very important for beginning the transition to the tissue reparative wound healing program [32]. These secreted factors function to establish a shift in the tissue microenvironment away from the proinflammatory milieu and toward the tissue repair-supportive setting. These factors induce the microenvironment to promote cell proliferation, angiogenesis, and ECM remodeling to return the tissue to its previous, undisrupted function. At this late stage of inflammation, cytokines and chemokines function to recruit lymphocytes, mainly T cells to the wound site, and promote transition to the adaptive immune phase of wound healing, which promotes memory and tolerance functions of the immune response [3].

1.7.2 Stages of Wound Healing

1.7.2.1 Fibroblast proliferation and migration

The initial response to wounding also induces the deposition of granulation tissue, which serves as a plug that prevents infiltration of microbes and acts to tether the edges of the wound together to prevent tearing of the tissue (Fig. 1-6). Granulation tissue is composed mainly of extracellular matrix components such as collagen. Generation of the granulation tissue is carried out by fibroblasts. Following the decrease in the immediate inflammatory response, and establishment of the granulation tissue, fibroblasts at the wound site undergo extreme proliferation, and migrate into the wound as a result of increased secretion of growth factors [33]. Specifically, PDGF, EGF, and FGF function to enhance proliferation, and TGF-β and PDGF act as chemoattractants to aid in recruitment of the newly proliferated fibroblasts into the wound [32, 33]. Once in the wound, the fibroblasts continue to proliferate and begin to produce large quantities of extracellular matrix components including fibronectin and collagen, which helps establish tissue repair [33].

1.7.2.2 Angiogenesis

Another consequence of growth factor secretion is the stimulation of new blood vessel growth, a process known as angiogenesis, within the wound. This process is key to reestablishing the blood supply to the injured tissue. Growth factors FGF, VEGF, PDGF, TGF-α and β act on remaining endothelial within the wound to promote their proliferation. In turn, new blood vessels are formed within the wound site, which helps the tissue return to its uninjured state [33].

1.7.2.3 Remodeling

Concurrent with the restoration of blood flow to the wound site during angiogenesis, the final step of the wound healing process is the remodeling stage [33]. This process involves secretion of proteolytic enzymes by PMN, fibroblasts, and tissue macrophages that digest the newly deposited ECM, as well as redeposition of collagen and fibronectin within the wound [32, 33]. Many of the same growth factors necessary for the other stages of wound healing are also required for the remodeling stage including PDGF, FGF and TGF-β.

1.7.3 Changes in Microenvironment During Wound Healing

Although inhibiting the influx of inflammatory leukocytes is a very important, necessary step in the resolution of inflammation, it is not only the absence of inflammation that defines the wound healing program, but other characteristic changes to the tissue microenvironment. As changes in the microenvironment are known to have profound effects on the functions of effector immune cells and the processes they carry out, it is important to discuss the cells that are responsible for inciting the changes within the tissue microenvironment, and how shifting the microenvironment toward pro- or anti-inflammatory conditions regulates the functions of nearby immune cells.

1.7.3.1 M1 vs M2 polarized macrophages

One example of these characteristic microenvironmental changes is the polarization of recruited inflammatory monocytes to the alternatively activated M2 phenotype, instead of the classically activated proinflammatory M1 phenotype [3]. The major consequence of M2 polarization is the transcriptional downregulation of inflammatory mediators such as TNFα, IL-1β, IL-12, and IL-8 [34]. The loss of these proinflammatory cytokines also contributes to the switching of the microenvironment towards a tissue repair permissive state. Macrophage

polarization is crucial for tuning the tissue microenvironment away from the inflammatory state and toward the wound healing. Many different cell types respond to cues via secreted factors within the local environment, and macrophages are the primary sources of such factors. M1, or "classically activated" macrophages, are termed thus because they respond directly to pathogen-associated molecular patterns (PAMPs), or to local tissue response [34]. As a result of danger signal detection, these cells undergo transcriptional alterations, resulting in increased expression or proinflammatory cytokines. As discussion previously, this action sets off the recruitment of inflammatory effector cells (monocytes and PMN) into the tissue, and the inflammatory response progresses through until resolution of the insult. Conversely, M2, or "alternatively activated"

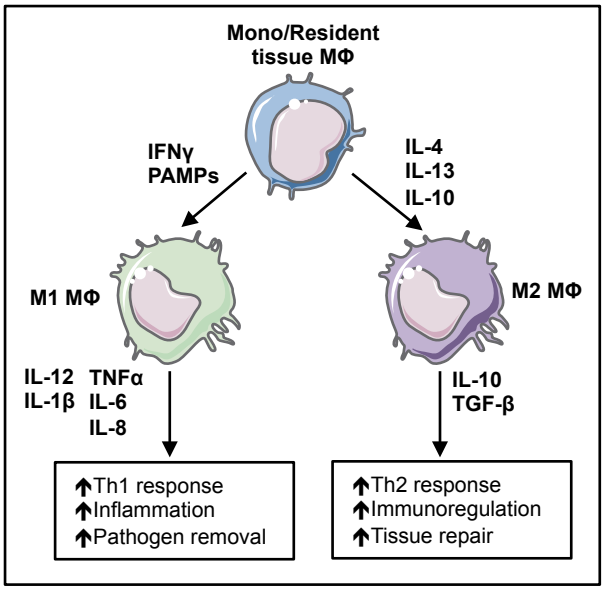

Figure 1-7 Macrophage polarization.
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macrophages are polarized under very different conditions from M1; the polarization of M2 macrophages is carried out via signaling through tissue repair cytokines, specifically IL-4 and IL-13 [34] (Fig. 1-7). These characteristic anti-inflammatory cytokines induce transcriptional changes within the macrophages, thus promoting production of tissue repair supporting

molecules, including cytokines, growth and angiogenic factors. These transcriptional changes support the tissue repair process, and also function to further inhibit the inflammatory immune response. Furthermore, a second consequence of M2 polarization is the transcriptional downregulation of inflammatory mediators such as TNFα, IL-1β, IL-12, and IL-8 [34].

M2 macrophages transform the microenvironment to support tissue repair through a number of mechanisms. The first is that they secrete massive amounts of IL-4, which results in conversion of any non-activated tissue macrophages to M2 polarization [5]. IL-4 secretion induces a positive feedback system, whereby the initial production of IL-4 induces alternative polarization of M1 macrophages toward the M2 phenotype. This polarization event converts any present macrophages to the M2 tissue repair phenotype [5, 34]. Consequences of upregulated IL-4 secretion also include further polarization of M2 cells, and inhibition of pro-inflammatory cytokine expression [5]. Furthermore, IL-4 is a powerful stimulator of fibroblast functions, including proliferation and collagen deposition. Consequences of excess IL-4 production include the accumulation of fibrotic lesions due to hyperstimulation of the tissue repair process [35]. IL-4 has also been shown to induce the production of other anti-inflammatory cytokine including IL-10 [5]. This second mechanism of M2 macrophage-induced tissue repair uses a subtype of M2 macrophages, termed “deactivated” macrophages, which exclusively secrete large amounts of IL-10 and TGF- β [34]. These two anti-inflammatory cytokines further attenuate any expression of inflammatory mediators by the tissue and/or immune cells [5]. IL-10 expression also stimulates the activation of the STAT3 transcription factor, which increases the expression of factors that further drive the tissue repair process [36]. Additionally, IL-10 reduces NF-κB activity [37], which further reduces the expression of pro-inflammatory cytokines and results in the reduction of MHC molecules in antigen presenting cells [38].

One consequence of increased IL-10 and TGF-β secretion is the tuning of activated T cells toward the immunomodulatory phenotype [6]. These cells are known as regulatory T cells, or Tregs, which promote tissue repair through inhibition of localized immune effector cells. As mentioned previously, Treg cells work to induce immunomodulation directly through decreasing $CD4^+$ helper T cell function, and indirectly by secreting cytokines, which contribute to maintaining the skewed polarization of immune cells toward immunosuppressive phenotypes within the microenvironment [38]. Tregs use many co-inhibitory molecules within the immunological synapse to induce T cell exhaustion pathways in helper T cells. Specifically, Treg cells use the PD1-PDL-1 and CTLA4-B7 interactions to induce inhibition of helper T cell activity. This directly inhibits the activity and proliferation of activated helper T cells within the tissue repair microenvironment [27]. Treg cells also secrete high quantities of immunosuppressive cytokines, IL-10 and TGF-β, which further promote the polarization of infiltrating immune cells toward immunosuppressive Treg and M2 phenotypes [38]. Collectively, these cytokines condition the tissue repair microenvironment to inhibit the inflammatory response and support the wound healing response. In addition to tissue repair cytokine accumulation, the tissue repair-supporting microenvironment is also rich in angiogenic and growth factors, which work to drive the proliferation and migration of fibroblasts for the generation of new tissue [33]. These factors facilitate the production of new tissue cells, as well as promote the establishment of new blood vessels via stimulation of angiogenesis. The process of tissue repair also induces deposition of extracellular matrix components such as collagen, and activates proteases that coordinate remodeling of the tissue and the extracellular matrix. These pathways all work in concert to restore the tissue to homeostasis [33].

1.8 Dynamic Regulation of Hematopoiesis

The process of inflammation is not restricted to the local tissue environment, but intimately associated with the production of responsive immune cells in the distal bone marrow. This function allows the immune system to specifically adapt production of immune cells in order to appropriately respond to the type of insult that is present in the tissue. Hematopoiesis is the process of forming the cellular components of the blood [39]. This process occurs in the bone marrow, and follows a hierarchical flow of development and cellular differentiation. Several stem cell subtypes and progenitor cells undergo proliferation and fate commitment steps to produce the terminal effector cells of both myeloid and lymphoid lineages, which go on to perform the necessary functions of the immune system [39]. Tight regulation of hematopoiesis is critical in controlling the immune response, and any dysfunction of this system can result in disruptions to immune function. For example, overstimulation of the inflammatory process can result in extreme production of inflammatory leukocytes, and deficiency in hematopoiesis can result in extreme neutropenia and increased susceptibility to infection [40]. Regulation of hematopoietic development is critical to maintaining the population of inflammatory effector cells, and multiple levels of regulation exist to maintain dynamic flexibility of the process of hematopoiesis. Two major levels of regulation are of note: 1) basal maintenance or steady-state, and 2) emergency, or demand-adapted hematopoiesis [40].

1.8.1 Hematopoiesis Process

Hematopoiesis is a tightly regulated process that involves several proliferation and commitment steps that are arranged in a hierarchical pattern (Fig. 1-8). The hierarchy begins with the hematopoietic stem cells (HSC), which are multipotent, self-renewing stem cells that give rise to all of the cells of the blood system. Due to the short-lived, terminally differentiated nature of

hematopoietic effector cells, HSC undergo many rounds of self-renewal and differentiation events to keep up with the constant loss, and subsequent replacement demand of effector cells [41]. Therefore, by replenishing the pool of HSC, the sources of all hematopoietic progenitors is replenished resulting in the constant supply of new effector cells to replace the outgoing hematopoietic cells. The next level of differentiation involves a certain subset of HSC from the self-renewing pool to differentiate into the two major progenitor lineages: The common lymphoid progenitors and the common myeloid progenitors. Like the HSC they are derived from, these two progenitor cell types retain their multipotent differentiation ability, but lose self-renewal capability [39]. This loss of self-renewal function highlights the importance of maintaining the pool of HSC, as HSC are the sole source of all the terminally differentiated hematopoietic effector cells. Without the HSC population, the entire population of effector immune cells, and components of the blood system collapses [41].

The multipotent common lymphoid progenitor cells are distinguished by the dramatic upregulation of the *ikaros* gene; a zinc-finger transcription protein that helps program the fate of the HSC into lymphoid-like cells. Mice deficient in *ikaros* do not produce thymic dendritic cells, B cells, NK cells, γδ T cells, and display dysfunctional αβ T cells [42]. In addition to impairments of the lymphoid arm of hematopoiesis, *ikaros* null mice also display a dramatic reduction in the number of mature PMN within the bone marrow compartment, indicating that *ikaros* also plays an important role in granulopoiesis [42]. In parallel, other cells from the HSC pool may alternatively undergo transcriptional enhancement of transcription factor PU.1 [43]. This event commits the cells to a myeloid progenitor fate. Following irreversible commitment of HSC to either the lymphoid, or myeloid progenitor fate, cells undergo a number of transcriptional programming changes that further differentiate them into oligopotent progenitors. Although still capable of

differentiating into all the cell types of the blood system, oligopotent progenitor cells are no longer able to revert to multipotent progenitors, and are committed to producing either lymphoid or myeloid subsets of terminally differentiated effector cells.

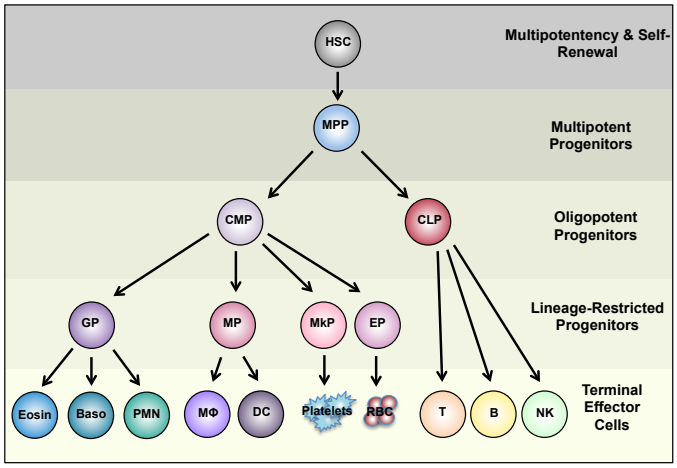

Figure 1-8 Hematopoiesis process.
Hierarchy of hematopoiesis. Self-renewing multipotent hematopoietic stem cells undergo several rounds of differentiation to become lineage-restricted progenitor cells, eventually producing all the effector components of the blood system. HSC, hematopoietic stem cell; MMP, multipotent progenitor; CMP, common myeloid progenitor ($CD11b^+$ population of BM cells); GP, granulocyte progenitor; MP, monocyte progenitor; MkP, megakaryocyte progenitor; EP, erythrocyte progenitor; Eosin, eosinophil; Baso, Basophil; PMN, polymorphonuclear leukocytes; MΦ, macrophage; DC, dendritic cell; RBC, red blood cell; CLP, common lymphoid progenitor; TNK, T cell-natural killer cell progenitor; T, T cell; NK, natural killer cell; B cell.

Once formed, oligopotent progenitor cells from both the common lymphoid and common myeloid progenitor pools that continue to diverge transcriptionally, and thus develop into different types of cells [39, 43]. The next stage of differentiation results in the generation of lineage restricted progenitors [43]. These cells form the final level of precursor cells before final differentiation is achieved; the lymphoid progenitors go on to develop into B, T, and NK cells, while the myeloid progenitors produce monocytes, and granulocytes [39]. Of particular note, the development of the effector cells from the common myeloid progenitors is carefully orchestrated through a series of cytokine signals, which induce transcriptional alterations within the cells, silencing genes that contribute to oligopotency, and activating genes that contribute to specific effector functions of the particular cell type [39]. Monocytes can further differentiate into

macrophages and dendritic cells, and granulocytes develop into three major types of granulocytic cells: basophils, eosinophils, and neutrophils. As with the early stages of hematopoiesis, development of these terminally differentiated cells occurs through cytokine-induced transcriptional regulation [39]. It has been determined that bone marrow resident macrophages are responsible for regulating this process of hematopoiesis, at least in part through production and secretion of cytokines and growth factors [44].

1.8.1.1 Hematopoietic signaling is carried out using secreted cytokines & growth factors

Progression of cells through the hematopoiesis process requires stimulation events that are carried out by secreted cytokines [39] (Fig. 1-9). Cytokines are secreted by the stroma and the BM resident macrophages, and bind to cytokine receptors on some or all of the progenitor cells found in the bone marrow to incite intracellular kinase activity to complete one of two major functions: 1) to promote growth and survival of progenitor cells, or 2) to induce lineage commitment differentiation and/or maturation [44]. These two functions together maximize the production of hematopoietic effector cells, and are easily adapted to the needs of the system.

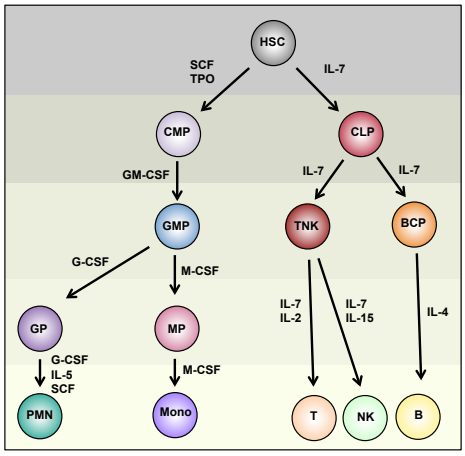

Figure 1-9 Cytokine-driven hematopoiesis.
Cytokines drive hematopoiesis. Growth factors and cytokine drive progression of hematopoiesis through multiple stages of differentiation and lineage commitment steps. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; GP, granulocyte progenitor; MP, monocyte progenitor; PMN, polymorphonuclear leukocytes; Mono, monocyte; CLP, common lymphoid progenitor; TNK, T cell-natural killer cell progenitor; BCP, B cell progenitor; T, T cell; NK, natural killer cell; B cell.

Secreted factors responsible for controlling hematopoiesis fall into four major categories: 1) interleukins (ILs), 2) interferons (IFNs), 3) colony stimulating factors (CSFs), and erythropoietin and thrombopoietin growth factors [39]. Each factor induces either a growth and proliferation signal or a differentiation/maturation event in the cell type(s) expressing the specific cytokine receptor(s). These receptors function as the signal transducer for the cell. Once activated through cytokine-receptor binding, activation of effector molecule signaling is achieved generally though a cascade of phosphorylation events carried out by a number of potential tyrosine kinases [39, 45]. The type of non-receptor tyrosine kinase that will disseminate the cytokine-induced activation signal cascade is determined by the type of receptor that recognizes the cytokine, and more specifically, the arrangement of amino acid residues that make up the intracellular domain of the receptor [45].

There are two classifications of cytokine signaling receptors, type 1 and type 2 [39] (Fig. 1-10). Type 1 receptors are made up of two signaling chains and can be either homodimeric, or heterodimeric. The heterodimeric type 1 receptors can be further differentiated by their shared, or conserved signaling chain. Although these cytokine share a common signaling chain, specific cytokine-receptor interactions are carried out through a cytokine-specific α chain, that interacts directly with the cytokine, and subsequently recruits the conserved signaling chain. There are three families of shared signaling chain [39]. The first type of shared signaling chain is the gp130 subunit. This is the common signaling protein used by IL-6 cytokine family members, including IL-6, LIF, and IL-11 [45]. The second type of shared cytokine receptor signaling chain is the β chain family. Cytokines that use this signaling chain include GM-CSF, IL-3, and IL-5 [45]. The third shared receptor chain family is the γ chain. Many cytokines use this type of signaling,

including IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, and IL-21 [45]. Although type 1 cytokines signal using various shared chain peptides, and the consequence of each cytokine signal is different, the general mechanism of signal transduction is conserved in all type 1 receptors. The accepted model for type 1 cytokine signaling is that the cytokines first bind the specific cytokine receptor subunit (termed the α subunit) with high affinity [45]. Binding of the cytokine induces a conformational change in the intracellular domain of the α receptor chain, which induces oligomerization, or recruitment of the shared chain (gp130, β, or γ) [45]. Only then can activation of the signal pathway occur. Not unlike the type 1 receptors, type 2, or single chain receptors often follow a similar pattern of activation; however, single chain receptors may form homodimers after cytokine binding to propagate the signal, instead of using a shared signaling receptor. Additionally, type 2 cytokine receptors possess all the necessary domains for recruiting non-receptor tyrosine kinases that will disseminate the signal [45]. Examples of type 2 cytokine receptors include IFNs and the IL-10 family [46].

1.8.1.2 Dissemination of cytokine signaling is mediated by non-receptor tyrosine kinases

Although receptor binding is the first level of cytokine signaling, and receptor expression determines the ability of the cell to respond to cytokines and other growth factors, this is simply the first step of the signaling cascade. Membrane-bound receptors are responsible for transmitting the signal of cytokine binding into the cell, where non-receptor tyrosine kinases (RTKs) function to activate the cellular machinery, thus inducing the changes to the cell that result in either enhanced growth, survival, and proliferation, or in changes to the transcriptional program [45]. There are many families of non-RTKs that carry out the intracellular cytokine signaling cascades. The type of non-RTK responsible for disseminating the cytokine signal is highly dependent on the

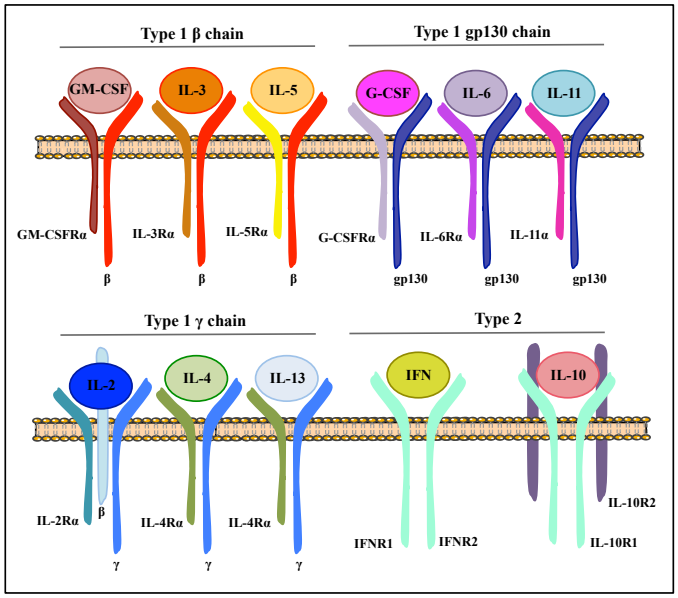

Figure 1-10 Cytokine signaling families.

type of receptor that receives the initial stimulus, and the domains that are phosphorylated upon binding of the external cytokine stimulus [45]. There are a number of domains that are conserved in the intracellular signaling domains of cytokine receptors chains, and recognized by downstream signaling proteins. The first, perhaps most common type of intracellular receptor domain is the SRC homology type 2 (SH2) domain [45]. This conserved sequence of amino acids is present in nearly every cytokine receptor signaling subunit, and, once phosphorylated, this domain functions as a docking site for non-RTKs that also possess SH2 domains, including Janus kinases (Jaks), Src family kinases, feline sarcoma virus kinase (Fes/Fps), Tec/Btk, and Syk family kinases [45]. Although each of these kinase families have different signaling pathway targets, they all have a conserved method of disseminating the cytokine signal, that is that all become phosphorylated after docking to the activated receptor via the SH2 domains, and are then able to "pass the phosphorylation" to the next target molecules. These phosphorylation cascades modulate the

target protein in either a positive, or negative manner, thereby activating or inhibiting the function of the target protein [39, 45].

It is important to note that although each cytokine receptor functions through a different mechanism of activation, and use different non-RTKs to activate signaling cascades, many of these pathways converge at the effector molecule level [45]. The advantage to using different methods of signaling is that each pathway is subject to individual methods of regulation, therefore bestowing flexibility of regulation of each cytokine signaling pathway.

1.8.1.3 Cytokine signaling directs hematopoiesis

It would be impossible to discuss the entire list of possible cytokine signaling pathways identified during hematopoiesis; however, because later discussions will address the importance of regulating myelopoiesis, three major cytokine signaling pathways that are critical to myelopoiesis will be presented here. The first major type of SH-2 mediate signaling pathway occurs through the activation of Jak kinases [45]. These kinases, as in most signaling models, binds to the phospho-tyrosine residues in the SH2 domains of either type 1 or type 2 receptors (including IL-6) [46]. Once bound, individual Jak monomers phosphorylate each other in a *trans* manner, and recruit monomers of the signal transducer and activator of transcription (STAT) proteins to dock at the SH2 domain. Once STATs bind, they are also phosphorylated, which allows for the dimerization of the phospho-STATs, and translocation of this complex to the nucleus, where it acts as a transcription factor to drive differentiation of the cell [46]. The Jak/STAT mechanism is highly variable, as each protein family consists of multiple members, and varying the combinations of Jaks and STATs alters the specific gene targets that ultimately become transcribed as a result of the pathway activation. The second and third major types of SH2-mediated signaling pathways involve the activation of the small GTPases. Most families of

tyrosine kinases are capable of activating guanine nucleotide exchange factors, which regulate activity of small GTPase effector proteins. In these signaling pathway methods, tyrosine kinases either directly activate guanine nucleotide exchange factors, or adaptor proteins first, which is followed by the rapid activation of small GTPases, including Rac, Cdc42, and Grb2 [45]. These activated proteins can go on to complex with scaffolding proteins, or effector molecules. Typically, this is where cytokine activation pathways converge, in many cases resulting in activation of mitogen-activated protein kinase (MAPK), or other transcription factors that go on to regulate gene expression or cell growth and proliferation [45].

1.8.2 Steady-State Hematopoiesis

Steady-state hematopoiesis refers to the generation and maintenance of hematopoietic cells during a state of relative homeostasis. In other words, steady-state hematopoiesis occurs continuously within the body of the host that is not under a "stressed" condition (i.e. no active inflammation, infection, or trauma) [44]. Conversely, emergency, or demand-adapted hematopoiesis occurs during times of stress, including systemic inflammation and infection [40, 44]. This concept will be addressed in more detail in the following section. Steady-state hematopoiesis is the process of continuously replenishing terminally differentiated hematopoietic cells from both the lymphoid and myeloid lineages by stimulating the self-renewal of the multipotent HSC population, which results in a "trickle-down" effect for the increased production of the progenitors and the terminally differentiated effector cells. During steady-state hematopoiesis resident BM macrophages, stromal, and endothelial cells are responsible for secreting the necessary cytokines for maintaining pools of hematopoietic effector cells [44]. Furthermore, BM macrophages provide secreted factors that drive the differentiation of stem cells to lineage-committed progenitors, and eventually effector cells. In addition to maintaining the

generation of effector cells, BM macrophages also control the mobilization of effector cells into the periphery through maintaining appropriate expression of tethering molecules, which serve to keep effector cells sequestered in the bone marrow until it appropriate for them to be released, such as a period of infection or wide-scale tissue damage [40]. For example, tethering, or anchorage of effector leukocytes in the bone marrow occurs through binding of the CXCR4 chemokine receptor on the leukocytes to the CXCL12 chemokine, which is highly expressed in the bone marrow [40]. Upon stimulation of leukocytes with G-CSF, a cytokine potently upregulated during periods of infection and inflammation, leukocytes reduce their expression of CXCR4, and thus, as the tethering molecule expression decreases, the interaction between the tether and anchor is also reduced, releasing the effector cells into circulation [40, 44]. Macrophages can also regulate the egress of mature effector cells from the bone marrow by directly increasing G-CSF production, thereby promoting egress of mature leukocytes into circulation for immediate response to insult [44].

1.8.3 Demand-Adapted Hematopoiesis

Unlike steady-state hematopoiesis, emergency, also known as demand-adapted hematopoiesis, occurs when there is a harmful stimulus detected by the immune system, or directly by the resident BM macrophages, or the HSC themselves [40]. During a systemic infection, pathogen recognition receptors (PRRs), including toll-like receptors, present on innate immune cells and HSC detect the presence of pathogen-associated molecular patterns (PAMPs), such as flagellin, LPS, or peptidoglycan. This detection results in the

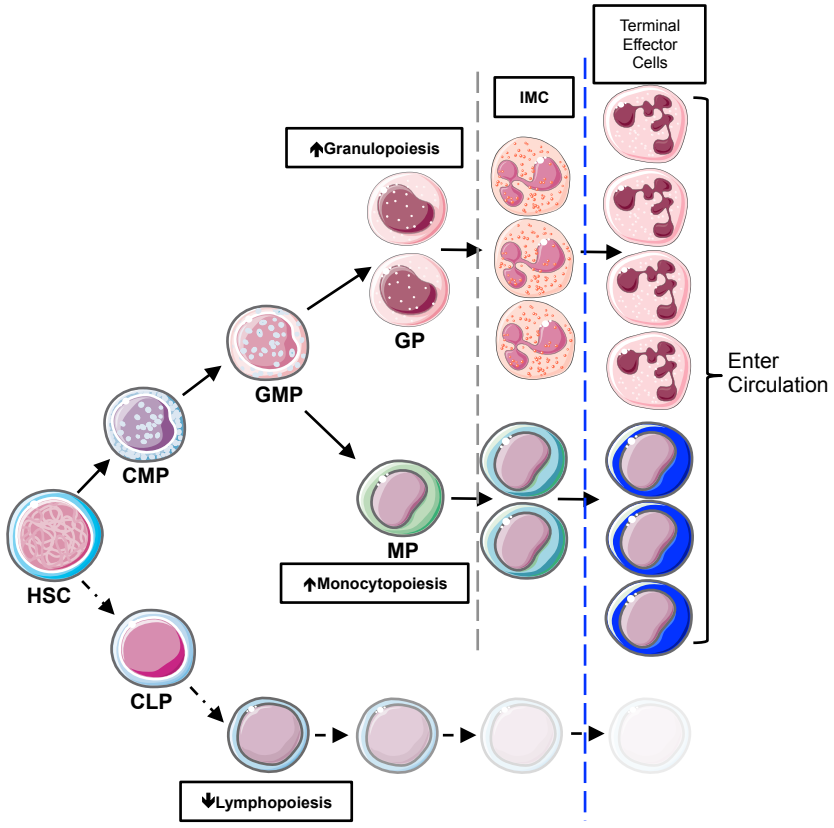

Figure 1-11 Emergency hematopoiesis.
Emergency hematopoiesis. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; GP, granulocyte progenitor; IMC, immature myeloid cells (heterogeneous group of immature monocytes and immature granulocytes); MP, monocyte progenitor; CLP, common lymphoid progenitor. During emergency hematopoiesis IMC population expands, and acquire migratory function. Production of myeloid cells increases as production of lymphoid cells decreases. Generated using "Blood and Immunology" licensed under CC BY 3.0.

production of inflammatory mediators [40]. If the pathogen stimulus is detected directly by PRRs present on HSC, the HSC undergo a process of rapid self-renewal in anticipation of the need for replenishing neutrophils that will be mobilized to the site of infection. This evolutionary response mechanism allows the HSC to sense the threat, anticipate the need for augmented generation of immune cells, and response accordingly by directly initiating the self-renewal process, thereby replenishing the pool of multipotent cells that will resupply the effector immune cells [40]. Furthermore, the production of lymphocyte cells becomes reduced during emergency hematopoiesis, demonstrating yet again that hematopoiesis is an extremely adaptable process

capable of mounting a tailored response to the specific nature of the insult. As in the steady state, demand-adapted hematopoiesis is also controlled by resident BM macrophages [44]. As the pool of self-renewing HSC proliferates in response to threatening stimuli, BM macrophages secrete cytokines that induce differentiation of newly formed HSC, so as the pool of progenitor cells increases, the cells are bathed in differentiation-inducing cytokines [44]. This system allows for rapid production of effector cells, while remaining dynamically adaptive to the particular insult (Fig. 1-11). The ability to sense and quickly adapt to the changing inflammatory needs of the host is a crucial system for maintaining the leukocytes effector cell populations for the response to inflammatory stimuli. In addition to increasing the pools of available leukocyte progenitors, cytokines produced during demand-adapted hematopoiesis also mediate cell release mechanisms [40, 44]. Therefore, cytokines secreted by BM macrophages during demand-adapted hematopoiesis not only stimulate cell production, but they also stimulate the migration of leukocytes into circulation.

1.9 The Importance of Cytokine Signaling in Inflammation

Cytokines are important immunoregulators that coordinate both the onset and resolution of the inflammatory response. As small, secreted molecules, cytokines function as the primary method of communication between cells of the immune system; however communication is not their only function [7]. These secreted factors are often effector molecules that result in regulation of function in their target cells. This functional regulation occurs at many levels, including both transcriptional and post-translational modifications. Thus, cytokines act as effector response molecules that control cellular function via a multitude of methods.

1.9.1 Coordinating the Inflammatory Response

Proinflammatory cytokines specifically control the onset of the inflammatory response. The

three most notable inflammatory cytokines involved in inflammation are IL-1β, TNFα, and IL-6 [7]. These cytokines work together to mount the innate immune response during tissue injury. IL-1β is primarily secreted by monocytes, macrophages, and non-immune endothelial cells and fibroblasts during wounding [32]. IL-1β is secreted as a consequence of pathogen-associated molecular patterns being recognized by the pathogen recognition receptors present on the surface of tissue resident macrophages. IL-1β serves as a method of signal amplification in the inflammatory response. Through upregulation and secretion of active of IL-1β as a result of pathogen recognition, several other proinflammatory mediators become transcriptionally upregulated and the proinflammatory signal cascade is initiated [47]. The IL-1β pathway is critical to the host immune response as it is the first "domino" to fall in the series of inflammatory signals required for orchestrating the removal of pathogen from the tissue. IL-1β accomplishes its immunostimulatory functions by mimicking activation of toll-like receptors and signaling through an intracellular TIR domain to activate MAPK and NF-κB transcription factors [48].

IL-1β is a key proinflammatory cytokine responsible for coordinating inflammation. In fact, it is so efficient at coordination inflammation, it is known to be the key factor in the generation of autoimmunity [47]. IL-1β is upregulated when tissue senses danger signals by many different cell types, including resident immune cells and fibroblasts. Once in the tissue, IL-1β drives further activation of the inflammatory response in many different cells types, through many different mechanisms. For instance, IL-1β has been shown to drive production of master regulators of inflammation, such as IL-6 and TNF-α, and induction of monopoiesis and granulopoiesis at the expense of erythropoiesis [47]. Furthermore, IL-1β drives the activation of many immune cells, including helper T cells, Th17 cells, macrophages, dendritic cells, NK cells, and B cells. IL-1β also induces changes to endothelial cells, which facilitates leukocyte infiltration, a core function

of the inflammatory response [1].

The second key inflammatory cytokine required for mounting a successful immune response is TNFα [7]. This cytokine has been demonstrated as a key regulator of the inflammatory response, often gaining recognition for its role in chronic auntoimmune disorders, including inflammatory bowel disease, psoriasis, and arthritis[49]. Like IL-1β, TNFα signaling also results in the activation of NF-κB, MAPK, and AP-1 transcription factors to induce upregulation of inflammatory genes, and is also produced by macrophages following binding of pathogen-derived antigens to PRRs [49]. Unlike IL-1β however, following secretion of this cytokine, TNFα binds a unique set of receptors that signal through death domains to induce activation of transcription factors and their targeted genes or to induce activation of the apoptotic pathway [50].

Like IL-1β, TNFα induces proinflammatory changes within the tissue through a number of different mechanisms. First, TNFα increases vascular permeability in the insulted tissue by increasing cyclooxygenase 2 (COX2) enzyme expression in epithelial cells. This leads to an increase in the production of proinflammatory lipid molecules, such as PGE_2, which subsequently acts to induce vasodilation in the endothelium [49]. Vasodilation of the blood vessels allows for increased blood flow, and thus, increased leukocyte infiltration into the insulted tissue. TNFα also contributes to the initiation of clotting in wounded tissue through inducing expression of coagulation proteins [49]. At the same time, TNFα works to decreases the expression of anticoagulation factors. These two functions stimulate clotting at the injury site, a very important function of the inflammatory response. TNFα has also been shown to play a key role in septic shock, whereby neutralizing TNFα was shown to inhibit detrimental activities of the host immune system, such as stress hormone release, and organ dysfunction [49]. In addition to being a key

cytokine for the orchestration of the inflammatory response, TNFα is also a potent inducer of apoptosis [50]. It is for this reason that tumor necrosis factor α (TNFα) acquired its name; however, unlike its name implies, TNFα does not kill most of its target cells, but paradoxically promotes survival signals through induction of NF-κB [50].

The third, major cytokine involved in coordinating the inflammatory response is IL-6. IL-6, like IL-1β and TNFα also stimulates the activation of the NF-κB pathway, resulting in the expression of survival signals, and inhibition of pro-apoptotic pathways within the target cells[51]. Additionally, IL-6 induces the activation of the STAT3 transcription factor, through the gp130-JAK1/2 pathway. Activation of STAT3 induces the transcriptional upregulation of many genes that allow the cells to survive, and resist apoptotic signals within the wound environment [51]. In addition to directly inducing transcription changes within its target cells, IL-6 is also a powerful inducer of other proinflammatory effector cytokines and chemokines that help to drive inflammation, such as MCP-1 [51]. IL-6 not only functions as a localized coordinator of the inflammatory response, but also exerts its power over peripheral tissues, such as the bone marrow, where this cytokine is a powerful mediator of hematopoiesis[44].

These cytokines, in addition to many others, work in combination to produce both system and localized environments the promote the inflammatory response, and without these small molecules, cells of the innate immune response would be unable to effectively remove invading pathogen from the tissues, thus rendering the host susceptible to environmental contaminants and life-threatening infection. In contrast to proinflammatory cytokines, the anti-inflammatory cytokines function to limit the immune response, and to inhibit the proinflammatory cytokines from inducing excess tissue damage during response to infection and injury.

1.9.2 Orchestrating Immune Suppression

Because the inflammatory response results in the production of a radical, hostile environment in response to injury or infection, coordination of resolution of the inflammatory response is key to limiting excess damage to the host. Although much is known about the onset of inflammation, and the characteristics of the tissue repair process, the mechanisms governing transition from active inflammation to the resolution phase of the immune response are incompletely understood. The general consensus regarding the transition to the resolution phase of inflammation is that the wound must first inhibit the influx of inflammatory cells, and then must orchestrate the generation of a localized area of immune suppression [3]. Induction of the immunosuppressive pathways is an absolutely crucial component of the wound healing program. As with all mechanisms that govern homeostasis in the body, the inflammatory response has methods to both promote the immune response when necessary, and of course, to inhibit the immune response when appropriate. Many types of immune cells are used to orchestrate immunosuppression, therefore it can be said that immune cells have dual functionality. Immune cells can either enhance or inhibit the inflammatory response, through the generation of specific cellular subtypes. These subtypes are created through polarization events, which are primarily driven through cytokine signals. The immunosuppressive effector cell subtypes use many different methods to control the immune responses, including cell-cell contact through counter receptor ligation, and secretion of soluble factors, such as lipid mediators and cytokines [3]. Like the onset of inflammation, suppressive functions of the immune system are frequently orchestrated by secreted cytokines. Of all the soluble mediators that regulate inflammation, the three major cytokine contributors to resolution of inflammation are IL-4, IL-10, and TGF-β [35, 38, 52]. The following section will discuss, in detail, the major functions of these

immunosuppressive cytokines, and their role in establishing localized immune suppression that supports tissue repair during inflammation resolution, as the third function of the inflammatory response.

1.9.2.1 IL-10 coordinates the inhibition of active inflammation in the wound site

IL-10 is powerful anti-inflammatory cytokine that plays a vital role in the tissue repair process [38]. IL-10 is secreted by many cells in response to inflammatory stimuli[36]. This cytokine works through many different functions to quell the active inflammatory response in favor of promoting the tissue repair process. The first method in which IL-10 suppresses inflammation in favor of tissue repair is by limiting the production of inflammatory cytokines produced by various cells in the insulted tissue. IL-10 has been show to reduce the production of many inflammatory cytokines, including but limited to TNFα, IL-1β, IL-6, and IL-12 [38]. A second function of IL-10-mediated inflammation inhibition is the ability for this cytokine to drastically reduce the production and secretion of chemoattractants, such as IL-8 and MCP-1 [38]. This reduction enhances the inhibition of leukocyte recruitment process that has already been established by lipid mediator secretion by the infiltrating neutrophils. Thus, IL-10 helps stem the flood of inflammatory leukocytes into the insulted tissue, which allows for the transition of the tissue out of the inflammatory condition, and into the tissue repair process. Furthermore, IL-10 conditions the microenvironment toward a tissue repair-promoting milieu, and in so doing, induces the skewing of macrophage and T cell polarization toward immunosuppressive M2 and Treg phenotypes [5]. Once polarization of these immune subtypes is induced, they continue to promote immunosuppressive conditioning of the microenvironment through secretion of immunosuppressive cytokines, including IL-4, IL-13, TGF-β, and of course, additional IL-10 [34]. IL-10 is also a powerful inducer of STAT3, which is a major transcription factor responsible

for driving tissue repair processes, such as cell survival, growth and division, and angiogenesis [53]. Through induction of these tissue repair pathways, and by inhibiting the further accumulation of inflammatory cells and cytokines within the insulted tissue, IL-10 is a potent inhibitor of inflammation in the wound site.

1.9.2.2 IL-4 and IL-13 coordinate tissue repair processes

Like IL-10, IL-4 is a pleiotropic cytokine show to have a profound role in orchestrating the tissue repair response [54]. Secreted mainly by M2 macrophages and Th2 polarized T cells, IL-4 functions to inhibit the inflammatory response by inhibiting the polarization of classically activated M1 macrophages in favor of tissue repair-supportive M2 cells [5]. Because IL-4 and IL-13 are secreted by M2 and Th2 cells, and induce further M2 and Th2 skewing, it can be said that M2 and Th2 cells promote further generation of their own phenotype. Furthermore, IL-4 and IL-13, both considered signature cytokines secreted by M2 and Th2 cells, go on to promote the expression of tissue repair genes, such as MMP12, EGF, and IL-10, and Arg1 [55]. MMP12, also known as macrophage elastase, promotes wound healing by enabling the degradation of extracellular matrix components to stimulate tissue remodeling after inflammatory insult. Epidermal growth factor (EGF) is a growth factor necessary for promoting many functions of the wound healing response. For example, EGF promotes the deposition of fibronectin, induces the synthesis of various angiogenic factors, and increases the activity of collagenase [56]. EGF uses these pro-repair functions to actively coordinate tissue repair and remodeling of wounded tissue. Furthermore, increased secretion of IL-10 works in concert with IL-4 and IL-13 to maintain the generation of suppressive phenotype immune cells within the injured tissue. Additionally, it has been shown, through the use of various tissue repair animal models, that ablation of the IL-4Rα chain results in impaired wound healing [35]. Not only is IL-4 able to stimulate positive effectors

of tissue repair, but it is also able in inhibit the proinflammatory effects of classically activated immune cells [55]. IL-4 potently suppresses the generation of Th1 and Th17 inflammatory responses by increases the activity of transcriptional repressors of IFNγ and IL-17, both cytokines that stimulate the inflammatory response [6]. Furthermore, IL-4 plays and indispensible role in limiting the inflammatory response by enhancing the production of arginase, which depletes arginine in the local environment [35]. Depletion of this essential amino acid potently impairs the activation of proliferation in responding T cells, thereby limiting the immune response in favor of promotion of tissue repair. Because IL-4, and its shared cytokine signaling partner IL-13, exert both tissue repair stimulating activities, and suppress proinflammatory immune cell activities, these cytokines are absolutely essential to the coordination of the tissue repair process[35].

1.9.2.3 TGF-β facilitates return to homeostasis

Transforming growth factor beta, or TGF-β is another cytokine/growth factor that is key to the wound healing process. TGF-β is secreted by many cells in the insulted tissue microenvironment, including platelets, and macrophages [52]. Its secretion occurs throughout the inflammatory and tissue repair processes, and coordinates many different aspects of the healing process. The first major goal of TGF-β is to stimulate the formation of the granulation tissue (a temporary layer of extracellular matrix components that acts to plug the wound to keep out infection) [52]. TGF-β does this by stimulating the production of connective tissue factors, including collagen and fibronectin [52]. At the same time, TGF-β acts to inhibit the production of MMP molecules that contribute to digestion of the ECM [52]. Next, TGF-β, along with other growth factors, acts to stimulate fibroblast proliferation and migration, and stimulates activation of angiogenesis to increase oxygenation to support the new tissue growth [33, 52]. As mentioned previously, TGF-β, in conjunction with IL-6, and IL-10 works to polarize T cells toward a

regulatory subset (Tregs and Th2 subsets) [6]. These cells actively secrete more TGF-β and IL-10, which pushes the microenvironment toward an immunosuppressive phenotype by maintaining the M2 polarization of the local macrophages [34]. TGF-β has been shown to have pathogenic functions as well. During periods of sustained tissue repair signaling, such as chronic inflammation and tumorigenesis, excess production TGF-β leads to generation of tissue fibrosis. TGF-β has also been demonstrated to support growth and development of tumors [52].

1.10 Inflammation is a "Double-Edged" Sword

As previously stated, the goals of inflammation are to remove harmful stimuli from host tissues, in an effort to restore the area to homeostasis; however, production of antimicrobial substances by the inflammatory cells, although necessary, also results in many undesirable outcomes for the host. This harmful outcomes range from increased local tissue damage, to complete loss of function and development of cancer. For these reasons, it is imperative that the inflammatory response be tightly controlled in order to limit the tissue damage caused as a result of inflammation.

1.10.1 Acute vs. Chronic Inflammation

There are two distinct types of inflammation: acute and chronic inflammation. It is very important to distinguish the characteristics of each type of inflammation, as the consequences of these cellular processes are vastly different. For example, acute inflammation is usually transient in nature, lasting from minutes to hours before undergoing resolution and tissue repair [57]. The primary objective of acute inflammation is to flood the insulted tissue with phagocytic neutrophils, which are in turn removed by tissue resident macrophages after the insult/pathogen has been cleared [6]. On the other hand, chronic inflammation is a prolonged process that can persist for years if left unattended. Chronic inflammation is also distinct from acute inflammation

because of the mononuclear nature of the exudate. In contrast to the exudate of acute inflammation, which primarily consists of polymorphonuclear leukocytes, inflammatory exudate from chronic inflammation is characterized as being predominantly mononuclear, including monocytes, macrophages and lymphocytes [6].

1.10.1.1 Acute inflammation

The process of acute inflammation is characterized by the quick onset of neutrophil recruitment, followed by swift removal of pathogen and/or tissue insult followed by clearance of inflammatory leukocytes, and return to homeostasis [3]. This process is very short-lived due to the self-limiting nature of acute inflammation, resolving within minutes to hours following the initial tissue insult. Acute inflammation is quite a conspicuous phenomenon exhibiting some, if not all five hallmarks of inflammation: heat, redness, swelling, pain, and in extreme cases, loss of function. Neutrophils are the predominant cells recruited to the injury site during acute inflammation. Once in the tissue, neutrophils degranulate, releasing many cytotoxic factors to kill invading pathogen [3]. Additionally, neutrophils take up pathogens and necrotic cells by recognizing PAMPs and cell death molecules through initiating phagocytosis. As the tissue insult is eliminated, leukocytes secrete lipid mediators that work to inhibit leukocyte recruitment, and eventually stops the recruitment of circulating leukocytes [28]. After neutralization of the tissue insult, neutrophils are either phagocytized by resident macrophages or are recycled back into circulation [58]. Once active inflammation is neutralized, the resident tissue cells initiate the wound healing program as discussed above. As with the onset of inflammation, the wound healing program persists only as long as is necessary to restore the tissue to homeostasis. Although the exact mechanisms governing transition to the resolution phase of inflammation are not well understood, the necessity for tight regulation of inflammation and resolution is very

important in guarding against adverse side effects of the inflammatory response. These consequences of dysregulation of the inflammation resolution process can be severe, and will be discussed in the following section.

1.10.1.2 Chronic inflammation

In contrast to acute inflammation, chronic inflammation is, as its name suggests, a more persistent form of inflammation, lasting from weeks to years in duration [6]. This type of inflammation fails to transition to the resolution phase, thus becomes a persistently active inflammatory condition. There are many sources that contribute to the onset of chronic inflammation, including persistent infection, prolonged exposure to toxins, and autoimmune disorders. Although many types of tissue insults may initiate the chronic inflammatory response, striking differences between acute and chronic inflammatory characteristics are evident. First, the type of leukocytes recruited to the injury site in chronic inflammation are predominantly mononuclear, composed of mainly monocytes, macrophages, and lymphocytes [1]. This is in stark contrast to the polymorphonuclear neutrophils that comprise the leukocytes exudate during acute inflammation. Second, the onset of the inflammatory response is markedly slower during chronic inflammation [6]. During acute inflammation, neutrophils are recruited, kill pathogen and are quickly removed by the resident macrophages, however during chronic inflammation many additional changes within the tissue microenvironment take place. One such change is described as macrophage polarization [6]. These cells, along with their monocyte precursors dominate the leukocyte exudate during chronic inflammation. Upon entering the tissue, monocytes can further differentiate into macrophages, which can then subsequently undergo further polarization steps. Macrophage polarization occurs through either of two pathways: classical or alternative [36]. During classical activation, macrophages are polarized to provide inflammatory signals either

through pathogen recognition or via IFNγ stimulation [5]. These classically activated macrophages secrete high levels of antimicrobial factors including ROS and NO [59]. Additionally, this polarization serves to perpetuate the inflammatory response by increasing the expression and secretion of pro inflammatory cytokines IL-1β, IL-12, and IL-23 [34]. The second type of macrophage polarization, alternative activation, is brought about via the presence of cytokines in the local tissue microenvironment. These cytokines are often secreted by Th2 cells, and function to stimulate the production of "wound healing" cytokines such as IL-10 and TGF-β, which serve to polarize the microenvironment toward protected phenotype. These cytokines are thought to play a role in coordination of wound repair and tissue resolution [34]. Alternatively activated macrophages also secrete growth factors that promote fibroblast proliferation, stimulates the generation of new tissue at the wound site [60]. In the case of chronic inflammation, monocytes and macrophages are constantly recruited into the insulted tissues, and continue to cause damage to the tissue, and eventually undergo polarization events that lead to a persistence of leukocytes recruitment and inflammatory mediator secretion followed by cell proliferation and extracellular matrix remodeling. Although presence of anti-inflammatory cytokines and growth factors during the dysregulation of the inflammatory response seem like they would aid in limiting the extent of inflammation-driven tissue damage, during chronic inflammation the tissue is unable to reduce the influx of leukocytes to the wound site, inhibiting the transition to the tissue repair program [6]. Instead of coordinating tissue repair, alternatively activated macrophages produces growth factors that induce a persistent wound healing program by promoting wild proliferation of fibroblasts and deposition of extracellular matrix components, that are then digested by newly recruited leukocytes and ECM remodeling enzymes. This type of perpetual, unresolved cycle of inflammation and wound healing leads to an prevailing immunosuppressive

microenvironment rich in DNA damaging ROS and NO, and an abundance of proliferation-inducing growth factors and cytokines. Taken together, this type of environment is conducive for initiating and supporting tumorigenesis [1].

1.10.2 IL-17 Drives Chronic Inflammation

The proinflammatory cytokine IL-17 has been shown to drive chronic inflammation in many diseases, including psoriasis and rheumatoid arthritis, however has also been demonstrated to elicit a protective capability in colitis models. Il-17 in mainly produced by activated $CD4^+$ Th17 cells. In psoriasis and rheumatoid arthritis, IL-17 perpetuates the activity of the inflammatory response by stimulating the production of proinflammatory cytokines, chemokines and growth factors. Specifically, IL-17 has been shown to drive the production of IL-6, IL-1β, TNFα, GM-CSF, G-CSF, IL-8 and CCL20 in a multitude of cell types. Additionally, IL-17 can synergize with the proinflammatory activities of TNFα and IL-1β produced from the initial inflammatory insult. Enhanced expression of the proinflammatory cytokines IL-6, IL-1β, and IL-8 work to sustain the recruitment of inflammatory leukocytes, such as neutrophils and monocytes into the wound, where the secrete toxic compounds that destroy tissue cells. Furthermore, IL-17 drives the enhanced expression of CCL20, which serves as the major chemokine for the recruitment of T cells. Accumulation of activated Th17 cells within the wound site further contributes to the generation of IL-17, which perpetuates chronic inflammation within the wound. IL-17 also stimulates enhanced expression of the growth factors GM-CSF and G-CSF. These growth factors drive the proliferation of new tissue within the wound, but also function as a stress signal. Although incompletely understood, it is possible that through enhanced production of IL-17, induces upregulation of GM-CSF and G-CSF within the bone marrow, which drive the emergency hematopoiesis program.

1.10.3 Chronic Inflammation Increases Cancer Risk

For some time, chronic inflammation has been closely linked to, and is now considered a risk factor for the development of cancer in many tissues. Research by many groups suggests that unresolved inflammation increases the probability of tumor formation through two general pathways [61]. First, chronic inflammation results in the unabated recruitment of inflammatory leukocytes. As previously discussed, the infiltrating leukocytes enter the tissue and secrete cell-damaging substances. Neutrophils release superoxide radicals non-specifically into the affected tissue, with the intention of causing damage to the DNA of invading pathogens. Because this process is not directed toward pathogen in particular, but is rather a more general process, the host tissue is also at risk for DNA damage [61]. Any breakages in the DNA of surviving cells will be repaired by the DNA breakage repair enzymes; however, as a result of this process, point mutations are introduced into the cell's genome. If introduced into the DNA of an oncogene, such as a growth factor receptor or cell cycle kinase, then these mutations can result in unchecked cell growth leading to the development of neoplastic cells that can grow into tumors [61, 62]. In addition to introducing genetic mutations in the host tissue cells, chronic inflammation also results in the prolonged production and secretion of proinflammatory cytokines both locally and systemically, which continue to promote the recruitment of damaging leukocytes [61]. Proinflammatory cytokines are the first step in an inhibitory feedback loop that drives the tissue repair process, therefore promotion of proinflammatory mediator generation also encourages the production of tissue repair molecules, such as growth factors and remodeling enzymes.

The second pathway that is thought to contribute to chronic inflammation's role in tumor formation is the prolonged activation of the wound healing program [61]. As mentioned previously, the wound healing program is initiated by the onset of inflammation, and during acute

inflammation is the self-limiting, terminal process of the inflammatory response. Unlike acute inflammation, the wound healing program during chronic inflammation begins anew following each new wave of inflammatory cell infiltration. This process, when tightly controlled is absolutely necessary to inhibit further recruitment of inflammatory leukocytes and thus, limits further unnecessary damage to the tissue. The wound healing process is designed to inhibit further inflammation and protect the inflamed environment by creating a local temporary immunosuppressive, proliferation supporting microenvironment [33]. During chronic inflammation, this activation of the tissue repair-promoting process is prolonged and sustained, which results in an environment rich in anti-inflammatory and proliferative growth factors [6]. This results in a decrease in immunosurveillance in a tissue environment that is rapidly growing and dividing. Taken together with the increase in the DNA mutation rates from the free radicals generated by the inflammatory leukocytes, chronic inflammation works to create an environment that is well suited to initiate and sustain neoplastic cell formation and tumor immune escape.

In the process of chronic inflammation the inflammatory stimuli persist, which results in the unlimited activation of the inflammatory response. As described previously, the initiation of the inflammatory process also induces the activation of the tissue repair process. As part of this process, the tissue cells, inflammatory leukocytes, and adaptive immune cells work in concert to inhibit further inflammatory function, and to stimulate fibroblasts to proliferate and secrete factors that help to restore the tissue to homeostasis [61]. During chronic inflammation, this system is dysregulated, which results in what has been described as an “unhealing” or “overhealing” wound [60]. The “unhealing” wound is described as a region of inflammation that does not resolve to homeostatic conditions [60]. Dysregulation in the transition between inflammation and tissue repair often results in both systems being active simultaneously [61]. Activation of the early

inflammatory response and wound repair process is dangerous because it creates an environment rich in both inflammation stimulatory factors, including IL-6, IL-1β, and TNF-α, and rich in immunosuppressive factors, including IL-4, IL-10, and TGF-β. These "mixed signals" result in a wound that constantly initiates the tissue repair process, but is unable to reduce the proinflammatory leukocytes infiltration. Thus, immune cells are recruited to the site of inflammation, damage the tissue, then become transformed into cells that drive wound healing, including M2 and Th2 phenotype effector cells [57].

1.10.3.1 Wound healing environment establishes localized immunosuppression

In order for newly established neoplastic cells to survive, and eventually grow into a tumor mass, they must first escape detection and removal by the immune system. One such mechanism of immune escape is closely tied with the hallmark characteristics of the tissue repair response [60]. In order to stem the flow of inflammatory leukocytes into the site of inflammation, many immunosuppressive factors are secreted into the local environment. These factors include anti-inflammatory cytokines, such as IL-10, IL-4, and TGF-β [63], and lipid mediators, including protectins, resolvins, and lipoxins [30]. These soluble factors have many different targets, but all function in concert to produce a microenvironment is protected from aggressive, proinflammatory cells and supports cell proliferation and return to homeostasis. One such example is the secretion of anti-inflammatory mediators is IL-10, which causes tissue macrophages to polarize into type 2 macrophages [57]. These cells produce soluble factors including IL-4, IL-13, IL-10, TGF-β, PDGF, and VEGF that promote tissue growth and development, while also inhibiting the inflammatory immune response [34]. These factors have many different targets including the promotion of further M2 polarization, the differentiation of activated T cells into Tregs, and the promotion of proliferation and angiogenesis [34, 64]. Tregs decrease the proliferative ability of

both $CD4^+$ and $CD8^+$ T cells, as well as convert professional antigen presenting dendritic cells into T cell killing cells [65]. These mechanisms decrease the likelihood that antigen presenting cells will present tumor antigen to the local T cells, thus increasing the survival capability of the neoplastic cell. With every neoplastic cell division, the tumor will become more difficult to eliminate, and will exert greater influence over the immune system both locally and systemically. Many mechanisms supporting immune evasion exist, however chronic inflammation drastically increases the chances of neoplasm survival by combining increased risk of abnormal cell generation with immune suppressive systems in a predominantly proliferative microenvironment [61].

1.11 Inflammatory Bowel Disease

Colitis is a prime example of a chronic inflammatory disorder that often proceeds to the development of cancer as a result of sustained tissue damage. Through many studies conducted in both animal models and in the clinical setting, it is widely acknowledged that colitis-associated cancer is heavily dependent on the initiation of sustained colon inflammation, and that curbing such inflammation is absolutely crucial to limit the incidence of colon cancer in the clinic.

1.11.1 IBD: UC and CD

Affecting 1.4 million Americans each year, inflammatory bowel disease (IBD) is comprised of two distinct, yet related types of chronic gut inflammation: Ulcerative colitis and Chron's disease[66]. While symptomatically similar, these two varieties of chronic bowel inflammation differ tremendously upon closer inspection. Ulcerative colitis is characterized as inflammation of primarily the colon, whereas Chron's disease is typically not limited to the colon tissue, but rather describes inflammation of all parts of the intestinal tract [67]. Additionally, Chron's disease patients can be described as having multiple areas of inflammation throughout the intestine in a

"patchy" pattern, while ulcerative colitis is usually a more uniform inflammation of the whole colon, even extending to the rectum [67]. Furthermore, ulcerative colitis generally describes inflammation that is limited to the colonic mucosa, whereas Chron's disease often presents as transmural, spanning the entirety of the intestinal wall[67, 68]. Although not yet entirely elucidated, many studies have determined that host-microbial interactions play a large role in the presentation of inflammatory bowel disease, however these mechanisms differ with regard to the type of IBD.

Over the course of many years using both human and animal studies, researchers have determined that there are a number of factors that govern inflammatory states of the bowel. Studies have defined that the intestinal epithelium relies heavily on commensal gut microbiota for digestion of food, production of essential nutrients, and development of the immune system, primarily induction of immune tolerance [4]. These microbes, although essential to the function of the digestive system also play a role in the induction of inflammatory bowel disease, as demonstrated by experiments performed in germ-free mice. These mice do not possess gut microbiota, and therefore cannot be used as a model for human colitis, as they do not develop colitis even after treatment with established colitis-inducing chemicals such as DSS [69]. The types and diversity of commensal microbiota present in the intestine have been demonstrated to influence the inflammatory state of the bowel. For example, patients presenting with either type of IBD tend to lack microbiota from the phyla Bacteroidies and Firmicutes in their intestinal epithelium, and usually recover from IBD flare ups when these organisms are re-introduced to the intestine through a procedure known as fecal transplant [4].

The onset of IBD has also been attributed to defects in the epithelial cell junctions leading to increased gut permeability [69, 70]. Goblet cells of the intestinal epithelium are responsible for

secreting mucus that acts as a natural barrier between the microbiota and the epithelium. In addition to this transient barrier, the epithelial cells function as a physical barrier between the microbe-exposed lumen and the antigen-responsive immune cells residing in the lamina propria. Tight junctions present at the apical side of the lumen-exposed epithelial cells prevent microorganisms, and their pathogen-associated molecular antigens (LPS, peptidoglycan, flagellin) from breaching the sterile lamina propria [70]. Furthermore, resident dendritic cells extend dendrites between the epithelial tight junctions into the lumen and uptake antigen in a process called "antigen sampling." Once the antigens have been taken up by the dendritic cells, they are presented to the gut-associated lymphoid tissues (GALTs) [6]. This process does not however result in an inflammatory response, but rather works to induce tolerance of commensal microbes and reduces the inflammatory immune response to commensal microbiota. In contrast, if the tight junctions between epithelial cells become compromised, influx of pathogen-associated molecular antigens come in contact with resident immune cells in the lamina propria, and the inflammatory response begins. Although meant to inhibit the invasion of pathogen into the sterile LP, inflammation only serves to exacerbate the permeability of the epithelium, leading to persistence of the inflammatory insult [67].

Genetic factors have also been shown to play a role in regulating the onset of inflammatory bowel disease, although genetic mechanisms of IBD are usually confined to Chron's disease [67]. For this reason, Chron's disease has a much higher degree of familial association as compared to ulcerative colitis [67]. Polymorphisms that govern the CD are found in a multitude of genes of varying function. One such gene contributing to CD is a mutation within the prostaglandin E4 (PGE4) receptor. PGE4 is a small lipid molecule that is responsible for promoting repair of epithelial barrier function. In both humans and mice, mutations or complete deletion of this

receptor renders the host susceptible to CD [67]. Genetic deficiencies in mucin protein have also been identified as regulators of gut inflammation. Many other genetic polymorphisms contributing to IBD exist, however not all of them are directly related to maintenance of the epithelial barrier. Some polymorphisms alter the immune response to antigen sampling. Instead of producing a tolerogenic outcome, changes to the cytokine signaling network receptors such as IL-23 and IL-12b can result in the lack of inflammatory inhibition [67]. If cells become unable to recognize cytokine mediators that are responsible for inhibiting the immune response, then accumulation of immune cells in the lamina propria indirectly results in altered barrier function and enhanced permeability [70]

1.11.2 Current Treatment Strategies for IBD

1.11.2.1 Drugs

Many drug-centered therapies currently exist for the treatment of IBD, both CD and UC. These drugs range typical anti-inflammatory non-steroidal anti inflammatory drugs (NSAIDs) to corticosteroids, and even antifungals and anti-anaerobic antibiotics [71]. The typical progression of IBD treatment is to begin with aminosalicylate therapy, which are aimed at minimizing the inflammatory response, thus these drugs are ideal for reducing CD and UC flare ups, but should be noted that this category of therapeutic drug has had much more success in treating patients with UC [71]. Although their exact mechanism of action is unknown, many have proposed that aminosalicylates decrease the production of leukotrienes and IL-1 [72]. This treatment technique may also be combined with probiotic therapies to maintain the diversity of the gut microbiota. If aminosalicylate treatment is unsuccessful, additional drug-based therapies may be attempted. The next suggested therapy is corticosteroid therapy. This strategy targets inhibition of the inflammatory response in favor of tissue repair [71]. Although effective, corticosteroid treatments

have some significant drawbacks. Because they work to inhibit immune function, corticosteroids cannot be used as a maintenance therapy, and often result in unwanted side effects such as endocrine dysfunction, osteoporosis, and psychosis [71]. Therefore it is important that other, more specific types of therapies be used as a method for controlling gut inflammation. Other methods of treatment include drugs that limit the colonization of certain types of gut bacteria using antimicrobial pathways.

1.11.2.2 Management of microbiota diversity

If initial treatments using drug-based therapies are unsuccessful, then more creative methods must be used to control the insulting gut inflammation. Recently, regulation of microbiota diversity has presented itself as a potential target for IBD therapy [4]. There are two methods that are used to control the microbiotic diversity. First, is the use of antibiotics to limit the many species of gut bacteria known to specifically exacerbate inflammation, and second is the use of probiotic suspensions to increase the population of bacteria known to drive tolerogenic effects within the colon environment [4, 71]. In either case, the goal is the same, to shift the diversity of microbial flora present in the colon to a tolerance-inducing phenotype. Additionally, commensal bacteria that are tolerated by the host typically help their human host with specific tasks, such as digestion, which, presumably is why they do not induce an inflammatory response in the host [4]. Additionally, maintaining the numbers of commensal microbiota helps the entire microbiotic system outcompete the pathogenic microorganisms. This is helpful to the human host because opportunistic pathogens can only exert their virulence if they are allowed to attach to the intestinal mucosa [4]. Maintaining high levels of non-pathogenic bacteria in the gut reduces the risk for attachment of pathogens because bacteria directly compete with one another for space and resources [4]. Clinical trials using probiotic therapy has yielded profound results for patients

suffering from Chron's disease [4]. These results show a potent reduction in relapses compared to placebo treated patients, reducing the number of relapses from 100% to just 15% in 9 months [73]. This type of therapy has been less studied in ulcerative colitis patients, however clinical trials are in progress. Probiotic therapy is an extremely favorable method of IBD treatment due to its relatively low cost, ease of administration, and availability to the consumer. Often, these probiotics can be found in bacterially cultured foods such as yogurt and fermented foods, making them easily accessible to the public [73]. A new method of introducing tolerogenic microbes into the colon is fecal transplant. This is the process by which donor feces (containing the total microbiotic suspension) are transplanted into a recipient either by oral or rectal routes [71]. This treatment is typically reserved for patients who have stopped responding to, or are resistant to antibiotic and probiotic therapies. Because antibiotic treatments reduce the helpful commensal bacteria, as well as the pathogens, fecal transplant is often used in patients who suffer from *Clostridium difficile* infections brought on by antibiotic treatments [4]. Although in early stages of development, this method of microbiota management has become a promising therapeutic avenue to be pursued for the treatment of IBD.

1.11.2.3 Immunomodulators to control immune response

Perhaps one of the most important developments made in the treatment of chronic inflammatory diseases is the blockade of proinflammatory cytokines. Since their initial application, cytokine inhibitors have become widely prescribed method of controlling hyperstimulation of the immune response during many chronic inflammatory disorders, including RA, psoriasis, and IBD [50, 74]. The major therapeutic immunomodulator group is the TNFα inhibitor family, which include three related, albeit subtly different therapeutic molecules. The first anti-TNFα molecule is Infliximab (IFX) [71, 75]. This molecule is a chimeric

(mouse/human) antibody that works by binding to excess TNFα, thereby neutralizing its pro-inflammatory ability [75]. IFX has been shown to greatly reduce the occurrence of IBD symptoms in both UC and CD, and has also proven to limit the number of relapsing episodes in patients with IBD [75]. The second type of anti-TNFα biologic molecule is adalimumab (Humira) [75]. This human recombinant monoclonal antibody is most well known as a rheumatoid arthritis treatment, but has also been approved as therapeutic treatment for Chron's disease, psoriatic arthritis, and plaque psoriasis [75]. Both IFX and adalimumab are capable of neutralizing both soluble and membrane-bound forms of TNFα, functioning to repress the proinflammatory, and apoptosis-inducing activities TNFα exerts on its target tissues [75]. A third type of anti-TNFα antibody is unique due to its pegylated region. Instead of possessing the usual Fc conserved antibody region, which can be recognized by Fc receptors present on phagocytic cells, certolizumab pegol is a monoclonal antibody that instead contains a polyethylene glycol polymer attached to the antigen binding domains [75]. Because this antibody does not contain the Fc domain, it is effectively unrecognizable to the immune system, which increases its viability within the tissue. Collectively, these anti-inflammatory, TNFα-neutralizing treatments have been extremely successful for the treatment of Chron's disease and ulcerative colitis.

As IL-17 is the major proinflammatory cytokine upregulated during both Chron's disease and ulcerative colitis, blockade of IL-17 has been developed and investigated as a treatment for IBD [74]. Many pre-clinical experiments were carried out to determine whether blocking IL-17 would significantly impact the severity of colitis symptoms in mice. Interestingly, blockade of IL-17 using antibodies or small IL-17 drug inhibitors in many different colitis models was either ineffective or even worsened the disease activity in colitic animals [74]. Similarly, clinical trials using IL-17 receptor antibodies secukinumab and brodalumab proved ineffective at reducing

disease activity in these patients, even to the point of aggravating the colitis symptoms in Chron's disease patients. The small chemicals vidofludimus and tofacitinib, however, proved effective at limiting colitis symptoms in both UC and CD [74]. These data suggest that although IL-17 undoubtedly plays a role in regulating the inflammatory response during colitis, blockade of IL-17 is not enough to inhibit the disease activity in IBD patients, and that the role of IL-17 in colitis, and other chronic inflammatory conditions is incompletely understood.

1.11.2.4 Future of IBD treatment

Many of the factors that are responsible for inducing, and sustaining the inflammatory response during colitis are currently unknown. More studies are being conducted to determine the role of IL-17 in the inflammatory response, and seek to understand why blockade of this important inflammatory regulator does not inhibit the immune response in a clinical setting. Additionally, it is important to strive for improvement within the current anti-inflammatory biologics, specifically anti-TNF-α therapies, as reducing activity of such master regulators of the inflammatory response can often lead to undesirable side effects, including susceptibility to infection as certain types of cancer [50]. By adding to the information the scientific community understands about the mechanisms that govern colitis, and the dysregulation of the inflammatory immune response, we can hope to improve the efficacy of anti-inflammatory immune therapies [76].

1.11.3 Animal Models of IBD

1.11.3.1 Introduction to various models

Since the identification of inflammatory bowel disease, and the recent increase in disease prevalence, predominantly in western society, many models of ulcerative colitis and Chron's

disease have been generated using a wide variety of animal species [77]. The most commonly used being the rat and mouse models. Additionally, each animal model has been generate to mimic the specific pathophysiological presentations of either ulcerative colitis or Chron's disease. Many approaches have been taken to induce colitis in these models, and accordingly, each model presents advantages and drawbacks. These model approaches include various methods of colitis induction including chemical, microbial, and transgenic manipulation of the model animals to establish the specific characteristics of inflammatory bowel disease to be studied [77]. The following discussion will explore the characteristics, advantages, and drawbacks of several commonly used colitis models, and will discuss how each model is tailored to specifically investigate the mechanisms of disease progression and treatment strategies for both types of inflammatory bowel disease.

1.11.3.2 Chemical inducers of colitis

Many chemicals are able to induce colitis symptoms in mice and rats, such as oxazolone, acetic acid, and TNBS; however, many of these chemicals must be administered rectally and therefore present difficulty of use to the researcher [77]. Therefore, this portion of the introduction will focus solely on the chemical DSS, which is administered orally, and will be used as the basis of our experimental model.

1.11.3.3 Dextran sulfate sodium (DSS)

Among the most commonly used methods of chemically induced colitis is DSS, which is typically administered orally in mice and rats. These sulfated sugar chain molecule acts like a detergent in the colon, inducing chemical injury in the epithelium of the intestinal mucosa, thereby facilitating introduction of microbial pathogen into the lamina propria of the colon. Due to the prevalence of antigen-detecting resident immune cells in the sterile environment of the

lamina propria, the inflammatory response is quick to follow epithelial injury [77]. This response is characterized by markedly enhanced production of inflammatory mediators such as IL-1β, TNFα, IL-6, and IL-8 [69], which results in recruitment of circulating inflammatory monocytes, macrophages, and most predominantly PMN. This model can be used as a method of assessing both acute and chronic colitis; however, chronic colitis induction requires multiple rounds of DSS administration, or continuous, low dose administration [77, 78]. Characteristics of DSS-induced colitis include rapid weight loss, loss of colon epithelial tissue, crypt architecture disruption, bloody stool, and increased fecal lipocalin [79]. The DSS colitis model is a rapid, and effective method of colitis induction, and is self-limiting. Once the DSS is removed, the inflammatory response abates quickly in favor of tissue recovery [77]. Colitis-associated disease characteristics are easily measured, and most of them do not require euthanasia of the animals. This presents a significant advantage because it reduces the expense of the study and allows this model to not only be applied to studies of active inflammation during colitis, but can also be used to address periods of colitis recovery. DSS is routinely administered in the drinking water, and because of the nature of preparation and administration is an easy model to work with when assessing colitis. The DSS model of colitis is also advantageous due to the ease of dosage control [78]. Although DSS administration is a preferred method of inducing colitis in mice and rats due primarily to ease of use, there are significant drawbacks to consider when selecting a colitis model. The first disadvantage to the DSS model system is the large variability in susceptibility and disease progression between test subjects. Additionally, sex of the animals is also cause for consideration with use of DSS, as male mice are more susceptible to colitis-associated pathophysiology in this model [77]. Strain of the animals used can also greatly impact the results of a DSS-induced colitis study due to the mechanism of action of DSS-induced colitis. Mice that exhibit alterations to their

innate and/or adaptive immune cells may have delayed colitis onset, and in come cases may render the animals resistant to colitis [78]. Additional factors that pose disadvantages to the DSS-induced colitis model concern the effectiveness of the DSS itself. The DSS chemical differs widely among manufacturers, as well as between lots/batches. The variations in chemical formulations available also range in purity and size of the DSS molecules [78]. The facility that houses the experimental animals also impacts the susceptibility and severity of the colitis model. For example, the specific commensal and pathogenic microorganisms that are present within the animal housing facility can greatly impact the results of the colitis study, and can influence the severity and presentation of the colitis [77]. Another drawback of the DSS colitis model is expense. DSS is a more expensive method of inducing colitis than other chemical methods. It is for these reasons that other colitis models have been developed for examining colitis mechanisms.

1.11.3.4 Microbial pathogen inducers of colitis

In addition to chemical inducers of colitis, many models of colitis use bacteria to directly induce inflammation of the colon. Some bacteria that are capable of directly inducing colitis are *Salmonella typhimurium*, *S. dublin* and adherent-invasive *E. coli* [77]. Because these methods do not use chemical means to disrupt the colonic epithelium, some researchers argue that these techniques result in colitis that more closely resembles the probable onset of human colitis. However, these systems use gavage administration methods, and often lead to complications such as septicemia in the experimental animals [77].

1.11.3.5 Transgenic and knockout mice as inducers of colitis

In addition to both chemically- and microbial-induced methods of experimental colitis, several transgenic and knockout mice models have also been established to assess the mechanisms underlying the onset and maintenance of colitis *in vivo*. Two examples of these

animal models are the IL-2 and IL-10 knockout strains of mice. Both of these strains of mice spontaneously develop colitis [77]. Interestingly, for these mice to develop colitis, both of these experimental model animals must be housed in the presence of commensal bacteria, as germ-free mice do not develop colitis symptoms, highlighting the importance of the microbiotic component during colitis [77]. Because animals used in these models are genetically modified to have specific immune cell deficiencies, they are typically used for more targeted studies of the immune system during inflammation, and therefore will not be used as the basis for our study.

1.11.3.6 Justification for DSS-induced model choice

In this particular study, we found that using the DSS model of colitis was the most appropriate choice for examining the inflammatory mechanisms of wound healing during colitis. In addition to its ease of delivery (drinking water), DSS-induced colitis offers many other attractive advantages. First, this model mimics ulcerative colitis that can be reversed upon elimination of the DSS administration. Because this study examines the role of tissue repair in the generation of MDSC, recovery from colitic inflammation is a necessary attribute the model. The DSS colitis model also offers ease of inflammatory phenotype measurement. In this study we endeavored to establish a timeline of inflammation and tissue repair, therefore it was a requirement that the model used in our study would yield easily quantifiable symptoms of disease progression and severity. The DSS method of colitis induction produces observable symptoms of inflammation, that can be traced over time, such as body weight loss, stool consistency, blood in feces, colon tissue loss, and fecal lipocalin levels. Furthermore, because DSS-induced colitis is established using a highly controllable method of chemical administration, this model will afford us the ability to establish a recovery period, and thus allow us to trace the inflammation recovery process, an impossible task for other models of colitis. It is for these reasons that the DSS-induced

model of colitis was chosen for our study.

1.12 Lipocalins as Diagnostic Markers of Inflammation

Lipocalins are small lipophilic proteins secreted by bone marrow-derived cells and luminal epithelial cells. Members of the lipocalin family are typically very small proteins composed of eight strands of anti-parallel β-barrels, surrounding a core pocket of hydrophobic amino acid side chains [80]. This hydrophobic core can be either very selective for substrate, or very promiscuous, binding to many lipophilic molecules, including prostaglandins, fatty acids, retinoids, and cholesterol-based hormone/steroid-like molecules [80]. Members of this family of proteins have a wide variety of functions, including lipophilic molecule transport, immunomodulation, and regulation of cell senescence. Lipocalins are capable of forming large secreted macromolecular complexes, and are often secreted as covalent complexes with other proteins [80]. The underlying mechanism for this covalent linkage between molecules is not entirely understood, however studies using lipocalin 2 (NGAL) and matrix metalloproteinase 9 (MMP-9) suggest a regulatory role for the lipocalin, which has been found to limit the gelatinase function of MMP-9 when in complex. Lipocalins can therefore, be thought of as multifunctional secreted proteins; at their core, lipocalins primarily function as transporters of hydrophobic molecules, but also play additional roles in modulating cell function [80].

Neutrophil gelatinase-associated lipocalin is one member of the lipocalin family. This protein, also called NGAL and lipocalin, is 25 kDa in size, and forms various associations with itself, and other secreted proteins, and has been demonstrated as a useful clinical marker of inflammation [16]. Originally isolated as a covalently bound protein to matrix metalloproteinase 9 (MMP-9), human NGAL has since acquired many other names including, uterocalin, and oncogene 24p3. This 25kDa protein was found to exist as a monomer, a homodimer, and as a

heterodimer in complex with MMP-9. Soon after identification of the human protein NGAL, the murine homologue was discovered. Possessing 62% sequence homology, LCN2 is considered the murine form of NGAL, and also forms these dimeric associations in mice.

NGAL protein, and its murine homologue LCN-2, are primarily expressed in bone marrow-derived cells, specifically neutrophils, and are inducible in epithelial cells of luminal tissues. In neutrophils, NGAL/LCN-2 are generated and packaged into specific granules [81]. This packaging indicates that neutrophils tightly regulate the secretion of these molecules, only when the neutrophils become activated by some inflammatory stimulus (i.e. transmigration into inflamed tissues). Additionally, only epithelial cells that are constantly exposed to microorganisms secrete NGAL/LCN-2, and unlike neutrophils, these cells constitutively secrete basal levels of NGAL/LCN-2 into the microbe-exposed lumen. One known function of lipocalins is to bind small, hydrophobic peptides shed by bacterial or fungal pathogens [81]. These hydrophobic molecules have been identified as siderophores: small, bacterially produces iron-scavenging molecules. The ability for NGAL/LCN2 to act as a sponge for these bacterial survive molecules illustrates an antimicrobial function for this protein [15].

In recent studies, additional functions have been proposed for NGAL/LCN2. Perhaps most obviously, NGAL/LCN2 has been shown to be markedly upregulated during persistent infections, such as sepsis, chronic inflammation, and cancer [16]. In contrast to the tightly regulated and limited release of NGAL/LCN2 from inflammatory PMN during acute inflammation, this enhanced production of NGAL/LCN2 during persistent inflammation and infection is attributed to induced expression primarily in the luminal epithelium via stimulation by inflammatory cytokines, specifically the IL-1, and IL-17 cytokine families [16, 81]. Stimulation of epithelial cells with IL-1β results in activation of the TIR domains within the IL-1 receptor, resulting in

activation of downstream transcription factor NF-κB, which leads to enhanced transcription of *lcn2* [16]. In addition to activation of transcription via IL-1β, induction of NGAL/LCN2 transcription in epithelial cells can be accomplished at later stages of inflammation by IL-17 [16]. Although the IL-17 receptor complex lacks a true TIR domain, it does possess a similar signaling domain SEFIR. Activation of the IL-17 receptor similarly initiates activation of NF-κB the SEFIR domain, which increases transcription of *lcn2* [16]. IL-17 also stimulates the activation of C/EBPβ and δ through activation of the CBAD domain on IL-17RC [82]. Activation of C/EBP molecules results in two contrasting functions. At early IL-17 stimulation time points, C/EBPβ becomes activated, travels to the nucleus, and robustly enhances *lcn2*; however, in later IL-17 stimulation time points, C/EBPβ becomes hyperphosphorylated by ERK and GSK3β, resulting in sequestration in the cytosol [83]. This pathway appears to function as a method of feedback inhibition to limit the transcription of *lcn2* and thereby limiting the amount of secreted NGAL/LCN2.

It is important that NGAL/LCN2 expression and secretion be regulated in epithelial cells, as this protein is a potent immunomodulator [16]. In addition to being a sink for collection of metal scavenging molecules, NGAL/LCN2 functions to limit the immune response by inhibiting secretion of proinflammatory cytokine from M1 macrophages and by transforming classically activated M1 macrophages into immunomodulatory M2-like "deactivated" macrophages [84]. A study performed by Zhang et al. in 2008 demonstrated that treatment of classically activated macrophages with LCN2 and LPS resulted in a complete inhibition of production of inflammatory factors IL-1β, IL-6, MCP-1, TNFα, GM-CSF, and iNOS 20 hours post LPS stimulation [85]. These data demonstrate a powerful anti-inflammatory tissue protective function for NGAL/LCN2 during inflammation. In addition to this staggering finding, Warszawska et al. have since

demonstrated that LCN2 is highly expressed in “deactivated” macrophages-a subtype of alternatively activated macrophages [84]. In addition to this finding, the authors also reported a new function of LCN2, the induction of “deactivated” macrophage polarization. Upon treatment of bone marrow-derived WT or *Lcn2*$^{-/-}$ macrophages with classically activating stimuli (*S. pneumoniae), Lcn2*$^{-/-}$ macrophages produced significantly more proinflammatory cytokines including, KC, TNFα, and IL-6 than the WT macrophages. Furthermore, the authors reported increased expression of IL-10 in these “deactivated” macrophages [84]. This data suggests that LCN2 plays a role in skewing the polarization of macrophages toward an immunosuppressive, tissue repair-supporting, protective phenotype. These data taken together support the hypothesis that NGAL/LCN2 has multiple functions in governing the immune response. The first function is to inhibit the growth of microorganisms by limiting their access to freely available iron [16]. The second function is to protect the host from the detrimental effects of the sustained inflammatory response during an infection [84].

Enhanced NGAL/LCN2 expression has also been observed in cancer patients, and has even been proposed as a potential diagnostic marker[16]. Increased expression of LCN2 has been documented in many cancer presentations including, prostate, breast, ovarian, brain, lungs, liver, stomach, and pancreatic cancers[16]. In addition to being widely expressed by tumor tissue, LCN2 may play a tumor-supportive role during cancer progression. Like other immunomodulatory systems, many hypothesize that LCN2 may become coopted by neoplastic cells to support growth and serve to generate an environment that is protected from immunosurveillance [81, 86, 87]. In support of this theory, LCN2 overexpression studies in breast cancer models have demonstrated that LCN2 induces transformation of neoplastic cells to a more aggressive phenotype, which is characterized by the loss of contact inhibition and progression to

the epithelial-mesenchymal transition (EMT) [16]. This allows the neoplastic cells to invade and metastasize to other tissues [87]. Studies performed in colon cancer models corroborate the theory that high expression of LCN2 contributes to the invasiveness of neoplastic cells, and that levels of LCN2 expression correspond well with the clinical stage of tumor diagnosis [88] The type of molecular complex LCN2 forms also seems to determine its function in tumor progression. For example, in glioblastoma brain cancer patients, high levels of secreted LCN2 are found almost exclusively complexed with MMP-9, which serves as a faithful biomarker for measuring the severity of disease [89].

The idea that NGAL/LCN2 is a good biomarker candidate for measuring disease progression is not limited to cancer. This protein has also been used as a diagnostic marker for sepsis and chronic inflammation, particularly colitis [15, 79] Although LCN2 can be used as a non-invasive biomarker in clinical application settings, it is perhaps most frequently used as a criterion for assessing disease progression in animal models of inflammation such as inflammatory bowel disease. In contrast to other methods of assessing disease progression, measurement of fecal LCN2 is a more sensitive, quantitative test for determining colitis-associated inflammation in animals [79]. Traditional inflammation assessment methods are often more subjective, or are based on vague disease activity index scoring criteria such as body weight and stool consistency. Other criteria for assessing disease activity often involve collection of body tissues, which requires sacrifice of the animal in order to determine the full scope of the disease activity. Another advantage to testing of LCN2 in body fluids is that it is a less invasive technique, and does not require sacrifice of the animal. Additionally, this means that animals may be monitored as “repeated measures” subjects. The advantage to using this method in animal models is that one can observe the disease progression of a single animal over time, and this

allows the researcher to determine the disease state of the animal in real time, without having to sacrifice an animal only to determine that the predicted disease activity index is contrary to the disease activity index determined by terminal necropsy. LCN2 is also an incredibly stable molecule [79], making it an ideal candidate for simple *ex vivo* quantification. LCN2 is a reliable marker of disease which presents in most body fluids, as it is inducibly expressed and secreted by luminal epithelia, thus LCN2 can be measured in a multitude of body fluids, including feces, urine, and sputum. Because of these traits, LCN2 is an ideal candidate for use as a biomarker for several human and animal model pathologies.

1.13 Cancer

1.13.1 Overview of Cancer and Tumorigenesis

Because the process of tissue repair and the onset of cancer share many defining characteristics, and it has long been established that chronic, unresolved inflammation contributes to the generation of tumors, it is our goal to understand how the transition from active inflammation to the resolution phase and tissue repair process coincides with the initiation of tumorigenesis. The following section will discuss the hallmark characteristics that describe cancer cells. By outlining the changes seen during tumor generation, we hope to further define similarities between tumorigenesis and the changes that occur during the transition to the resolution of inflammation.

Cancer is a broad term used to describe a number of diseases caused by the dysregulation of cell division. This disruption of tightly regulated cell division can occur in virtually any tissue in the human body, and results in the rapid, unchecked proliferation of cells that results in an abnormal growth of tissue known as a neoplasm [13]. The neoplastic mass of cells is often referred to as a tumor. Cancer affects millions of people worldwide each year, and eradication of

this group of diseases has been the focus of many research foundations, including the National Cancer Institute at the NIH. Improvements to current treatments for cancer have been forthcoming; however, because of the diverse nature of this group of diseases, no one treatment strategy can be expected to be effective for all cancer types. Thus, researchers must strive to understand the more complicated underlying mechanisms of these diseases to bring about an end to their dominance.

Cancer has, thus far, been described using six hallmarks: 1) sustaining proliferative signaling, 2) evasion of growth suppressors, 3) resistance of cell death programs, 4) enabling replication immortality, 5) induction of angiogenesis, and 6) activation of invasion and metastatic signals [13]. Each one of these hallmarks contributes to the ability of the neoplastic mass to grow, divide, and perhaps most importantly, resist detection and clearance by the host's immune system. Transformation of healthy cells into neoplastic unregulated dividing cells is often twofold. That is to say the DNA must often be damaged in at least two distinct places: a proto-oncogene and a tumor suppressor gene [13]. This process often begins when the cell undergoes a DNA breakage event, resulting in repair of the DNA, perhaps incorrect repair of the DNA sequence. This first mutation is usually a "gain-of-function" point mutation within the proto-oncogene and is necessary to promote the growth signals within the effected cell [13]. These mutations are often in proteins that participate in growth factor receptor signaling cascades, such as Ras and Wnt [90]. These proto-oncogenes must be mutated in such a way that they are constitutively active, and therefore support unregulated cell growth and proliferation. Although a powerful type of mutation, the enhanced activity of the proto-oncogenes is usually not enough to transform the cell into an unchecked neoplastic cell because the cell has a protective group of genes known as the tumor suppressor genes. These gene products function to sense dysregulated cell division and

inhibit the cell from progressing though further replication cycles [13]. To achieve total unregulated cell division, the cell requires a mutation within one of its tumor suppressor genes, most notably retinoblastoma (RB) and p53. This second mutation is usually a "loss-of-function" mutation, resulting in the loss of the detection and inhibition of unregulated cell division [13]. When these two types of mutations occur in the same cell, it confers a new ability on the cell: complete unchecked cell division, resulting in the formation of an exponentially dividing population of cells [90].

It is important to note that many of the hallmarks that characteristically define cancer also define the tissue repair process [60]. It is because of these similarities, that understanding the transition from active inflammation to the tissue repair process is critical to exposing the mechanisms that drive establishment of the tumor microenvironment. Although the regulation of inflammation is a topic frequently studied within the scientific community, investigation of the mechanisms governing transition from active inflammation to the tissue repair processes have been largely ignored in favor of investigations that focus on the onset of the inflammatory response. In order to understand the mechanisms by which tumor cells co-opt existing immunosuppressive functions of the immune response to facilitate their own growth and survival, more attention must be paid to the mechanisms that drive transition to the resolution phase of inflammation and eventually to the tissue repair process.

1.13.2 The Six Hallmarks of Cancer

1.13.2.1 Sustained proliferative signaling

Due to mutations in proto-oncogenes, cancerous cells exhibit dysregulated cell cycle control, which results in sustained proliferative signaling capability. The mutations that make this signaling possible are "gain-of function" mutations in proteins that participate in growth factor

signaling cascades [13]. Mutations render these proteins that drive cell division constitutively active. Under healthy circumstances, these proteins are subject to regulatory post-translational modifications that inhibit their activity, and thus the proliferative ability of the cell. Once a gain-of-function mutation has occurred in an oncogenic protein, it becomes constitutively active, which often results in rendering the cell prone to unregulated cell division. This makes the mutated cells able to proliferate *independently* of the availability of resources and cell density signals that, under healthy conditions, modulate cell proliferation. This first type of DNA mutation is absolutely necessary for the production of neoplastic cells, thus sustained proliferative signaling is the first hallmark of cancer [13].

1.13.2.2 Evasion of growth suppressors

Although "gain-of-function" mutations are critical for the production of cancer cells, they are not sufficient for the total conversion of healthy cells to fully neoplastic cells. As mentioned previously, transformation of healthy cells to cancerous cells requires not one, but two mutation events. The first mutation event primes the cells for unchecked cell division, but can be overcome by the activation of tumor suppressor genes. Therefore mutation of the tumor suppressor genes to a "loss-of-function" phenotype is the second required step in cell transformation. The function of tumor suppressor genes is to control cell proliferation, and generally including checkpoint proteins including: cyclin-dependent kinase inhibitors, hormone receptors, proteins that promote cellular apoptosis, and DNA repair proteins [13]. Mutations in these genes render the proteins inactive, making them unable to participate in proliferation checkpoint regulations and DNA repair coordination [13]. After overcoming this final obstacle in checkpoint regulation, the newly transformed cells are capable of proliferating unchecked until they can be recognized and cleared by the immune system.

1.13.2.3 Resistance of cell death programs

The third hallmark of cancer is the ability for the cells to resist programmed cell death. Apoptosis is a critical pathway for regulating cell damage and overall health. Without apoptosis, cells lose their ability to respond to 'danger' signals, and gain the ability to escape removal by the immune system. The accumulation of mutations in oncogenes resulting from the functional loss of the tumor suppressor proteins results in the buildup of apoptotic stresses. In healthy cells, the collection of apoptotic stresses set off the apoptotic cell death cascade, ultimately resulting in the activation of pro-caspases 8 and 9 [13]. Activation of these molecules leads to widespread proteolytic activity, which signals the exposure of apoptotic signals on the outer leaflet of the cell membrane, and eventual uptake by phagocytes. A defining characteristic of cancer cells is that they evolve ways to escape succumbing to the apoptotic pathway [13]. Many proteins within the cell act as sensors to detect signs that the cell is in trouble: DNA damage, viral infection, killer lymphocyte recognition, etc. These signals function to trigger the onset of apoptosis by "inhibiting the inhibitor." In other words, once the alarm is tripped and the cell senses that there is a problem, it enables activity of the apoptotic program, which results in a shift toward cellular controlled cell death [13]. The ability to escape the apoptotic pathway is an especially important function of tumor cell immune evasion, as many immune cells use activation of death domain-containing receptors to trigger apoptosis as a way to eliminate tumor cells from the tissue [91].

1.13.2.4 Enabling replication immortality

Neoplastic cells undergo cell replication paying no deference to the health of the cell or the resources available to the cell. As mentioned previously, the ability to resist cell death programs is partially responsible for continued cell division, however there are other pathways that, when corrupted, add to this division ability [13]. For example, activation of telomerase activity can

circumvent the onset of senescence in neoplastic cells. Through elongation of chromosomes, the cell can avoid signaling senescence. Similarly, apoptosis is avoided through many different methods. One such method is the downregulation of apoptotic death receptors in neoplastic cells [91]. External death receptors are triggered by immune cells, which externally recognize the abnormality of the neoplastic cell and attempt to induce apoptosis. The tumor cells often inhibit expression of death receptors to avoid immune cell-induced apoptosis.

1.13.2.5 Induction of angiogenesis

Cells require a steady influx of nutrients and oxygen and a way to transport waste products away from themselves in order to survive and replicate. Tumor cells are no different in this regard, and therefore must acquire the ability to stimulate the formation of new blood vessels through a process termed angiogenesis [13]. Through a number of mechanisms, tumor cells are able to stimulate the nearby vasculature to grow new vessels and branches, which work to supply the tumor cells with precious nutrients, such as glucose and oxygen, and also act as a waste receptacle for metabolic wastes [13]. Additionally, tumor cells themselves often secrete growth factors, cytokine, and chemokines that drive changes in the immune system. In order to induce systemic changes using these secreted molecules, the tumor must recruit branches of the circulatory system so that the secreted molecules have an entry point into major circulation. This is important because it allows tumors to signal to peripheral tissues, thereby conditioning other tissues of the host to support tumor survival. Additionally, formation of new blood vessels increases the risk for tumor cell invasion and migration [13].

Tumor cells induce angiogenesis by stimulating the nearby endothelial cells to proliferate through the secretion of growth factors. One major growth factor known to induce angiogenic changes in endothelial cells during cancer is vascular endothelial growth factor-A (VEGF-A) [5,

13]. Angiogenesis occurs early on in tumorigenesis, and is absolutely critical for the survival of the neoplastic cells. Bone marrow-derived cells are also responsible for contributing to growth factor secretion that leads to angiogenesis. Macrophages, neutrophils, and even myeloid progenitor cells are able to infiltrate the tumor microenvironment, where they accumulate at the margins between neoplastic cells and untransformed cells [5]. Here, they function to induce angiogenesis in previously unaffected endothelial cells, and maintain the secretion of angiogenic factors to sustain the angiogenic process in the vascular tissue.

1.13.2.6 Activation of invasion and metastatic signals

Transformation of neoplastic to invasive tumor cells requires many cellular changes that are described as the epithelial-mesenchymal transition (EMT). This process is coordinated by a group of transcription factors that work together to facilitate the loss of contact inhibition and result in the ability for cancer cells to become migratory. Taken together with the ability for tumor cells to condition distal tissue to be immunosuppressive, acquisition of migratory capability allows tumor cells to establish new colonies in foreign tissue, without the risk of immediately being recognized and eliminated by the local immune response.

1.13.3 Tumor Survival is Dependent on Immune Evasion

Tumors are very strange cells, and they often express surface molecules that mark them as such, identifying them as aberrant cells. Surface markers that label cells as "foreign" or unhealthy come in many varieties [91]. The best example of cell surveillance is the carried out using the self-antigen presentation molecule MHCI. This protein is present on all cells, and functions to display peptides to surveying $CD8^+$ T cells [92]. Mutated peptides (not present in healthy cells) displayed via MHCI are identified as a marker of cellular damage by the TCR on cytotoxic T cells, which subsequently triggers the destruction of the cell. These markers make tumor cells

antigenic to the immune system; however tumor cells are derived from healthy tissue cells, therefore they also express healthy markers of self making them somewhat tolerated by immune cells. Elimination of tumor cells by the immune system is entirely dependent on the signals received by the surveying immune cells [91]. Tumor cells typically express "foreign" markers which, upon ligation to counter receptors on immune cells, results in pro-apoptotic, or phagocytic pathways that ultimately lead to elimination of the tumor cell from the tissue; however, tumor cells also develop ways of concurrently enhancing markers of self that inhibit these elimination strategies [91]. These strategies often function by camouflaging tumor cells with healthy self cell markers, which allows these cells to hide within the tissue and efficiently evade clearance by immune cells.

1.13.3.1 Impaired antigen presentation and tolerance

Survival of neoplastic cells is absolutely dependent on evasion of elimination by the immune system, therefore tumor cells develop many mechanisms to escape immune surveillance. The first method used by tumor cells to evade detection by T cells is through the downregulation of antigen presentation. This can be achieved through a number of mechanisms. The first is simple, many tumor cells decrease the surface expression of peptide-loaded MHCI molecules [92]. This is a very effective strategy for avoiding detection by T cells, however NK cells use cellular MHCI levels to determine if the cells are healthy, therefore total inhibition of MHCI is an ineffective method of immune escape[91]. To avoid elimination by NK cells due to lack of MHCI expression, tumor cells can alter the function of the peptide-loading enzymes LMP2, LMP7, TAP, and tapasin [91]. This mechanism of immune evasion preserves the expression of MHCI molecules on the tumor cell surface, so they cannot be eliminated by NK cells, but inhibits the self antigen presentation to T cells [91]. Another mechanism of immune escape used by tumors is

via the induction of tolerance. Just as in the ignorance mechanism, tumor cells often downregulate T cell costimulatory molecules, including B7, the counter receptor for CD28. Ligation of the TCR in the absence of costimulatory support induces anergy in the T cells [27]. Anergic T cells cannot elicit their cytotoxic functions, and are thus eliminated as a threat to the tumor cell.

1.13.3.2 Apoptosis resistance and induction of T cell death

Two more methods that enable tumor cells to evade immune cell-mediated clearance are the ability for tumor cells to acquire resistance to apoptotic pathways, and the ability for tumor cells to directly induce T cell death [91]. These two mechanisms are somewhat related because these new abilities generally conferred through the development of changes in surface-expressed receptors as a function of mutations gained within the tumor cells. First, the development of apoptosis resistance by tumor cells is acquired by changing the levels of apoptosis-inducing signaling molecules on the surface of the tumor cells, primarily by members of the TNF family [91]. Two examples of apoptosis-inducing receptors are CD95L expressed on killer lymphocytes, which interacts with CD95 death receptor on target tumor cells, and the TRAIL protein expressed in killer lymphocytes, which targets both TRAIL-R1 and -R2 death receptors on target cells [91]. Under healthy circumstances, these apoptosis-inducing proteins bind to their counter receptor on either a T cell, or a natural killer cell, which results in downstream activation of caspase cleavage cascades that ultimately lead to apoptosis of the target cell. Unfortunately, as is the case with tumor cells, downregulation of the death-inducing surface molecules, or disruption of any part of the receptor signaling can lead to a loss of function in the extrinsic apoptotic pathway. Alteration of the apoptotic signaling pathway often leads to the apoptotic resistant phenotype observed in many cancer types. Other studies have reported that tumor cells often secrete soluble death receptors, such as CD95, which act as decoy receptors to limit the interaction of CD95L on the

lymphocytes with ligation of transmembrane CD95 on target cells [91]. These are some of the most detrimental mutations for cancer patients, as they effectively render the immune system incapable of clearing tumor cells through apoptosis, and limits the T cell-mediated clearance of tumor cells strictly to granzyme B- and perforin-mediated elimination. Furthermore, some tumor cell types have also demonstrated dramatic upregulation of PI-9, a protease inhibitor that specifically targets granzyme B and perforin [91, 93]. Thus, many mechanisms contribute to the evasion of immune cell-mediated apoptosis, but all mechanisms of apoptotic resistance highlight the necessity T cells for effective tumor clearance[91] .

The activation of apoptosis through CD95-CD95L ligation can be reversed, and used for the benefit of the tumor. In addition to being a method for resisting T cell-mediated apoptosis, tumor cells have also demonstrated the ability to directly induce the killing of T cells. CD95L expression has also been reported to be upregulated in tumor tissue as well as in immune privileged sites, and has been shown to profoundly increase the viability of allograft transplanted tissue by reducing the activity of CD95-sensitive infiltrating lymphocytes [91]. By overexpressing CD95L, tumor cells employ a counter-attack to the cytotoxic lymphocytes, thereby inducing apoptosis in the anti-tumor immune cells. CD95, however, is not the sole example of this type "self-defense" system used by tumors to evade clearance and prolong survival. In fact, a large group of these molecules have been recently termed checkpoint blockade proteins, and have been extensively studied as targets for drug development to promote immune clearance of tumor cells [94]. Perhaps the most well described, and clinically relevant set of molecules that induce T cell death are PDL-1 and CTLA4. PDL-1 is a transmembrane protein highly expressed by tumor cells, and is the ligand for PD1, or programmed death receptor 1, which is expressed on cytotoxic lymphocytes. Ligation of these molecules results in inhibition of T cell proliferation through

increased SH2 phosphatase activity within the T cells [94]. This method of inhibition is used by the tumor to disrupt the expansion of activated T cells, thereby preventing clearance of tumor cells by cytotoxic T cells. The second checkpoint blockade molecular interaction used to inhibit T cell-mediated clearance of tumors uses cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), an inhibitory receptor present on the surface of T cells [94]. CTLA4 has two binding partners: CD80 and CD86. Interestingly, these two counter receptors also bind CD28 on T cells, and upon ligation between these ligands and CD28 produce a positive stimulation of T cell activation. Unlike CD28-CD80/86 ligation, binding of CTLA4 to CD80/86 results in potent inhibitory signaling within the T cells [94]. So, depending on the specific binding partner, CD80/86 molecules induce divergent signaling within T cells, and CTLA4 appears to outcompete CD28 for binding to the counter receptors. Both CTLA4 and PDL-1 have been identified as targets of immunotherapeutic treatment techniques, which have been hugely successful in the treatment of many cancers. In these therapies, antibody infusions specifically targeting the interactions between PDL-1, CTLA4, and their counter receptors interrupt the binding of these molecules with their counter receptors (expressed by tumor cells), which allows the T cells to circumvent inhibitory signaling by the tumors [94]. By "inhibiting the inhibitor" T cells retain their proliferation capability, which leads to improved clearance of tumor cells by activated T cells [94].

1.13.3.3 Expression of immunosuppressors and immune deviation

Tumors can also avoid immune detection by co-opting the immunosuppressive functions of the immune system. The first way tumor cells accomplish this task is by inducing expression of soluble cytokines and growth factors [91]. As discussed previously, overexpression of immunomodulatory cytokines, including IL-4, IL-10, and TGF-β can induce "immune deviation" resulting in the polarization of immune cells toward the tissue repair response [91]. Deviation

away from the Th1 response results in the further conversion of immune cells to the tumor supportive phenotypes M2 and Tregs, which contribute to the creation and maintenance of the immunosuppressive microenvironment surrounding the tumor cells [91]. Through increased expression of IL-10 and TGFβ, the tumor microenvironment becomes conditioned to drive the polarization of immunosuppressive immune cells subtypes, including M2 macrophages and Treg cells. One such subset of immunosuppressive cell types that has been powerfully associated with facilitating immune escape of tumor cells are the myeloid-derived suppressor cells (MDSC). Notably, these cells have also been observed during periods of chronic inflammation and persistent infection [95]. Furthermore, tumor cells have been shown to directly induce the generation, and recruitment of these suppressive cells, with the sole purpose of supporting their growth and survival [96]. Because the tumor microenvironment shares many of the same defining characteristics as the tissue repair process during resolution of the inflammatory response, the key to unlocking the initiation of immune suppression during tumorigenesis is to first define a mechanism for the onset of the tissue repair response. Our study will focus on the role of MDSC in modulating the transition from chronic inflammation to tissue repair. Understanding of the non-pathogenic role of these immunosuppressive cells will allow the scientific community to limit their co-optation by tumor cells, and to increase their suppressive abilities to control unresolving inflammation.

1.14 Myeloid-Derived Suppressor Play a Key Role in Immunosuppression

1.14.1 Historical Overview and Significance of MDSC

Myeloid-derived suppressor cells (MDSC) were first shown to have a powerful correlation with tumor incidence over a hundred years ago in the early 1900s [97]. At the time these cells could only be characterized as myeloid in nature, and were found to be greatly expanded in the

circulation of cancer patients. This phenomenon was described as extramedullary hematopoiesis resulting in extreme neutropenia, or extreme deprivation of neutrophils [97]. That is to say, cancer patients presented with extremely high numbers of circulating myeloid cells, however these cells were not mature monocytes or neutrophils, which under healthy conditions are the prevailing populations of circulating myeloid cells. Although of myeloid lineage, these cells were termed "null" cells because they lacked distinct cell surface markers that distinguish them from other myeloid cells and because their primary function was unknown [97]. Recently, many functions have been described for these previously uncharacterized cells, such as potent suppressive and migratory capabilities [64]; however, MDSC remain morphologically indistinguishable from their non-suppressive counterparts.

1.14.2 Nature of MDSC

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of $CD34^+$ bone marrow-derived cells that resemble immature monocytes and granulocytes, and share common acquired functions not found in their non-suppressive immature myeloid cell counterparts [98]. Unlike immature myeloid cells, MDSC are greatly expanded and perform additional immune suppressive functions during active inflammation, infection, and cancer [97]. At first glance, MDSC appear morphologically identical to IMC, expressing Gr-1 and CD11b surface markers, corresponding to the myeloid subsets of monocytes and neutrophils [97]. These cells can further be broken down into monocyte-like and granulocyte-like subsets; cells expressing $Ly6C^{high}Ly6G^-$ surface marker pattern are defined as monocytic myeloid-derived cells, and $Ly6C^{low}$ $Ly6G^{high}$ surface marker pattern are defined as granulocyte myeloid-derived cells [97]. Since the initial discovery of MDSC, researchers have focused their attention on the role of these cells in cancer models. Studies using mouse tumor models have revealed the expansion of this population of

cells not only in the circulation, but also in the spleen, and within the tumor microenvironment; however it is unknown whether these cells expand outside of the bone marrow, or whether the

Table 1-1 MDSC arise during many conditions

Condition	Model Observed	Functions
Cancer		
	Breast Colon Melanoma Pancreas Lung	Increased MDSC in peripheral blood correlates with poor prognosis
Persistent infection		
Bacterial	*Pseudomonas aeruginosa* Polymicrobial sepsis	Host protection
Viral	HIV Hepatitis C	Host protection
Chronic inflammation		
	IBD RA	Host protection
	MS	Conflicting evidence
Pregnancy		
	Human/mice	Protection from fetal rejection
Transplantation		
	Human/Rat renal allograft Human skin allograft	Protection from tissue rejection

expansion occurs within the bone marrow, and is followed by recruitment of the cells into the extramedullary tissues. Other models examining autoimmunity have determined a potential role for MDSC [98]. Specifically, researchers using the EAE model for autoimmune encephalitis reported that MDSC arise from the overstimulation of the immune response, and hypothesize the

acquisition of immunosuppressive functions is a mechanism of control to limit excessive damage to the host from overstimulation of the inflammatory response [98]. As seen in table 1-1 expanded MDSC populations have been identified in a number of different pathological conditions, including cancer, sepsis, chronic inflammation, and even parasitic infections [98].

Since the initial identification of MDSC as potent suppressors of immune function, many studies have been performed to determine the precise mechanisms of immune suppression used by MDSC. Perhaps the most obvious method of MDSC immune regulation is the inhibition of T cell proliferation. Each subset of MDSC contributes to this phenomenon using multitude of mechanisms. Because the MDSC potently inhibit T cell proliferation and function, it is likely that these cells have an alternative function in tumor-bearing hosts: to promote tumor survival by disrupting immunosurveillance and crippling the adaptive immune response's ability to remove aberrant self cells [99]. Furthermore, MDSC not only reduce T cell function, but interact with most immune cells to further suppress the immune response, often inducing the polarization of immune cells such as macrophages toward tissue repair supporting phenotypes [64, 100]. The specific mechanisms governing MDSC-mediated immunosuppression will be further discussed in the following sections, and are summarized in table 1-2.

1.14.2.1 Monocytic MDSC-specific characteristics

As previously mentioned, two subpopulations of MDSC exist, the monocytic MDSC, and the granulocytic MDSC. Although both considered "immature" myeloid cells, each one of these subsets adopts its own set of cell type-specific characteristics. Specifically, the monocytic MDSC (M-MDSC) express surface markers consistent with immature monocyte cells, expressing a $CD11b^{+}$, $Ly6C^{high}$, $Ly6G^{-}$ phenotype [98]. Unlike their non-suppressive immature monocyte counterparts, M-MDSC are capable of rapidly catabolizing the amino acid L-arginine through two

pathways. The first is the arginase pathway, whereby the substrate L-arginine is converted into L-ornithine and urea products via the arginase enzyme [101]. The second method of L-arginine depletion is through uptake by the nitric oxide synthase (iNOS) enzyme. In this process, iNOS breaks down L-arginine into nitric oxide (NO), a free radical molecule. Interestingly, these two enzymes are inducible under STAT3-mediated transcription [102].

1.14.2.2 Granulocytic MDSC-specific characteristics

The second type of MDSC most closely resemble neutrophils, and for that reason are termed granulocytic MDSC (G-MDSC) or PMN-MDSC [97]. These cells, like neutrophils have multi-lobed nuclei, and form intracellular granules. These cells also share surface molecule expression patterns with neutrophils, and can be described as $CD11b^{+}$, $Ly6C^{-}$, $Ly6G^{+}$cells [98]. Like neutrophils, these cells express NADPH oxidase, the enzyme complex responsible for the production of reactive oxygen species (ROS), also know as the respiratory burst reaction [98]. This process is regulated by a multi-subunit complex with both membrane and cytosolic components. Interestingly, expression of Gp91, the primary component of the membrane-associated respiratory burst machinery is unregulated by the STAT3 transcription factor in MDSC [103]. Unlike mature neutrophils, these G-MDSC do not produce vast quantities ROS, but, when coupled with nitric oxide release, MDSC use ROS as an effective T cell proliferation inhibitor. Alone, ROS secretion does not function as an effective inhibitor of T cell proliferation; however, used in conjunction with NO to form peroxynitrite radicals, MDSC limit the proliferative capability of T cells most potently [98].

1.14.2.3 Transcriptional regulation MDSC function

In addition to functional studies, researchers have attempted to determine characteristic that define MDSC as a separate cell type from immature myeloid cells. Although many studies

have been carried out to identify specific cells surface markers that categorically define each population of MDSC, none have been identified. Therefore, researchers have relied on transcriptional signature changes to classify MDSC. As mentioned previously, M-MDSC express high levels of arginase and iNOS. While G-MDSC produce enhanced gp91, these transcriptional changes can be attributed to corresponding increases in upstream phosphorylated transcription factors STAT3 and STAT6 [98]

1.14.2.4 The STAT5-STAT3 suppression axis in hematopoiesis

One hypothesis that may explain, at least in part, the mechanism development of MDSC during emergency hematopoiesis is supported by the observation of STAT5 activity over STAT3 activity during steady state hematopoiesis, but not during emergency hematopoiesis [104]. During steady-state hematopoiesis, GM-CSF is the predominant growth factor regulating maturation and lineage commitment of myeloid-derived effector cells. Signaling through GM-CSF receptor results in potent activation of the STAT5 transcription factor, among other pathways [104]. The transcription factor STAT5 has been shown by researchers to be the dominant transcription pathway under steady-state hematopoiesis, whereby its activation potently suppresses other STAT activation, specifically STAT3. Cohen et al found that cells cultured *in vitro* under GM-CSF stimulation used this STAT5 dominant pathway of regulation, to produce healthy populations of mature myeloid-derived effector cells, including monocytes, granulocytes, and dendritic cells; all of which exhibited restricted proliferative capability, and did not display any suppressive functions [104] (Fig. 1-12). In contrast, addition of STAT3 activators, including IL-6 at the expense of STAT5 activation resulted in an alternative myeloid cell programming pathway. In the absence of STAT3 silencing by STAT5, STAT3 activation prevailed, and produced a vastly proliferative, lineage negative population that were able to differentiate into mature professional

antigen presenting dendritic cells [104]. MDSC were found to be responsive to both STAT3 and STAT5 programming signals; however using STAT5 knockout experiments, it was determined that MDSC likely arise through insufficient exposure to STAT5 stimulators, including GM-CSF, thereby allowing STAT3 activation to become the predominant programming transcription factor *[104]*.

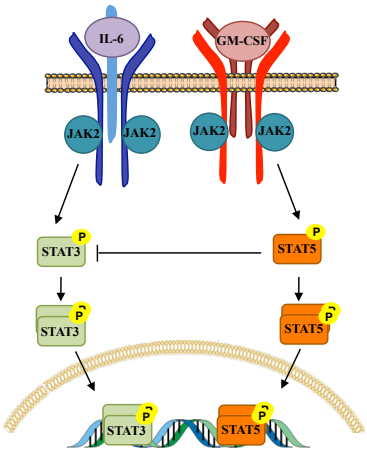

Figure 1-12 STAT5 suppresses STAT3 signaling.
GM-CSF induces STAT5 signaling, which suppresses STAT3 signaling during hematopoiesis.

1.14.2.5 Inducers of inhibitory function

As mentioned previously, many cytokines and growth factors induce changes in the transcriptional programming of immune cells. This process is carried out by activation of surface receptors, which function to activate downstream signaling pathways using post-translational modifications to their intracellular signaling domains. The most common modification utilized by signaling receptors is the activation phosphorylation. Members of the JAK/STAT signaling family, including IL-4/IL-13, IL-6, and IL-10, induce activation of signaling pathways that result in activation of STAT transcription factors [46]. Some of these STAT transcription factors drive the expression of genes that confer inhibitory function in the MDSC. For example, IL-4 induces the activation of STAT6, which, like STAT3 drives expression of arginase in MDSC, M2

macrophages, and TAMs [105]. IL-10 stimulates the activation of STAT3, which has also been proven to increase expression of arginase and nitric oxide synthase. IL-6 also induces changes to the transcriptional program via activation of STAT3 [105]

C/EBPβ is another well-known transcription factor that has been implicated in the generation of MDSC [106]. In a study carried out using C/EBPβ knockout mice, it was found that IMC derived from tumor-bearing animals exhibited no suppressive function [106]. Many cytokines and growth factors have been demonstrated to use activation of C/EBPβ to confer suppressive function within myeloid cells. IL-10, IL-1β, IL-6, and IL-17 are all cytokines that have been shown to induce activation of C/EBPβ [107-109]. This transcription factor has also been show to regulate emergency hematopoiesis, making it a potential factor responsible for switching the transcriptional program away from healthy hematopoiesis and toward an adapted, immunosuppressive program [106].

1.14.3 MDSC mechanisms of immunosuppression

MDSC are powerful suppressors of the immune system, functioning through several mechanisms affecting both innate and adaptive components. MDSC cause widespread immune suppression in cancer and several other pathological conditions. The presence and expansion of these cells, since there discovery have been noted in chronic inflammation, persistent infection, and cancer, therefore it is no surprise that successful immunotherapy techniques seek to subvert the immunosuppressive mechanisms exerted by MDSC in these maladies.

1.14.3.1 MDSC suppress T cell activity

Both subpopulations of MDSC participate in the suppression of immune cell function, often coupling their different capabilities to produce a stronger suppressive phenotype than either subtype alone [101]. Perhaps the most profound method of immune suppression is the inhibition

of both T cell activation and proliferation. When in close proximity to either $CD4^+$ or $CD8^+$ MDSC exert potent inhibition of T cell function through several mechanisms [98] (Fig. 1-13). The first is through the depletion of L-arginine by arginase and iNOS enzymes [98]. L-arginine is a critical factor for T cell

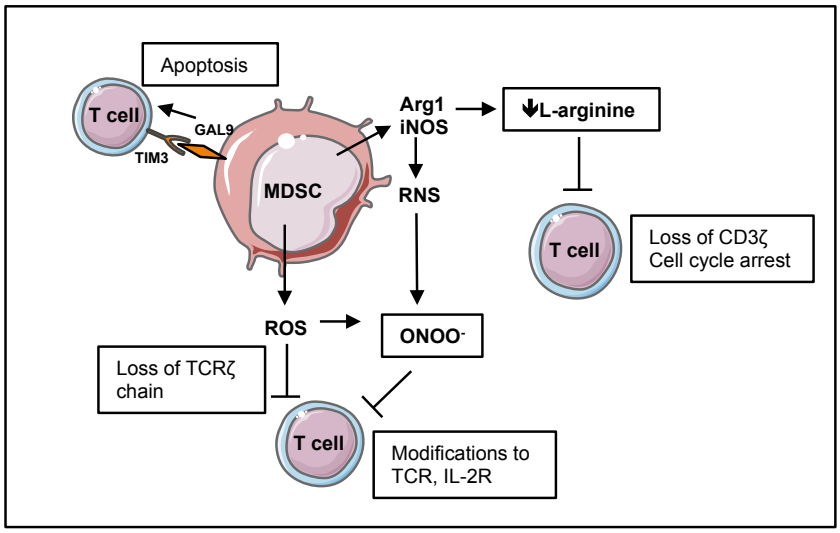

Figure 1-13 Mechanisms of MDSC-T cell suppression.
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proliferation, as it acts as a sensor of cell health within the T cell to gauge resource availability. When L-arginine is low, the T cell senses that there is a limit to the cellular resources, and subsequently downregulates proliferation machinery cyclin D3 and cyclin-dependent kinase 4 (CDK4) [98]. This effectively inhibits T cell proliferation by keeping the T cells in the G0/G1 cell cycle phase. Although T cell activity and proliferation are inhibited by the rapid uptake of L-arginine by MDSC, activation is also inhibited by the products of the nitric oxide synthase reaction. Once L-arginine is used as a substrate for the iNOS enzyme, production of NO can further suppress the activation and proliferation through inducing posttranslational nitration modifications on signaling molecules within the T cells [98]. Build up of NO in the

microenvironment causes T cells to decrease depression of the T cell receptor signaling molecules.

CD3ζ, thereby reducing the ability for the T cells to be activated through binding of the

Table 1-2 MDSC suppress immune cells.

Interacting Cell Type	MDSC Function	Consequence
Innate Immune Cells		
Macrophages	↑ IL-10	Drives M2 polarization
	↑ PGE2	Suppresses inflammatory function
	↓ IL-12	Inhibits M1 polarization
Dendritic Cells	↑ IL-10	Inhibits DC maturation
Adaptive Immune Cells		
Natural Killer Cells	↓ IFNγ production	Limits cytotoxicity
Natural Killer T Cells	↓ NKG2D	Limits activation
T cells	↓ Available arginine	Cell cycle arrest
	↑ RNS	Nitration of TCR, costimulatory molecules; limits activation
	↑ ROS	Loss of TCRζ chain; limits activation (RNS+ROS=OONO-) nitrosylation of IL-2R; limits proliferation
	↑ ADAM17	Cleavage of L-selectin; loss of trafficking

	↑ GAL9	Direct induction of apoptosis
	↑ TGF-β	Treg polarization

counter receptor MHCI/II [98]. Since the CD3ζ chain is required for T cell activation during antigen presentation, downregulation of this protein results in T cells that cannot respond to presented antigen, thereby rendering them non-functional [110]. NO has also been shown to decrease the activity of the Jak3/Stat5 signaling pathway, and has demonstrated the ability to directly induce T cell apoptosis by stimulating release of cytochrome C from the mitochondria and thus, triggering the intrinsic apoptotic pathway [98]. Nitration of signaling molecules results in inhibition of activation-inducing phosphorylation modifications that regulate transduction of activation stimuli, including cytokines and other mitogens. Since IL-2 signaling and secretion are essential to the induction of T cell proliferation, inhibition of this signal pathway results in limited T cell expansion [111]. NO can also combine with superoxide and H_2O_2 produced using the respiratory burst reaction in G-MDSC to produce peroxynitrite ($ONOO^-$) [98]. This highly reactive radical nitrite is capable of modifying many more signaling molecules involved in the T cell activation and proliferation pathways. Peroxynitrite functions as a powerful oxidizer that both nitrates and nitrosylates four amino acids: tryptophan, cysteine, methionine, and tyrosine; tyrosine being one of the most common and important substrates involved in cell signaling [98]. This modification of the cellular amino acids changes their chemical nature and can drastically change their molecular functions. In particular, addition of nitrogen groups to amino acids negatively impact the ability for protein signaling cascades, which can render the T cell non-functional, and eventually apoptotic. Therefore, high levels of peroxynitrite induce widespread protein modifications within T cells, rendering them unable to transduce activation and proliferation

signals [98]. MDSC also produce large quantities of the cleavage enzyme ADAM17. This enzyme reduces T cell activity by cleaving L-selectin, thereby limiting T cell trafficking to lymph nodes [64]. MDSC also directly induce T cell apoptosis through enhanced expression of galectin-9 (GAL9). GAL9 interacts with TIM3 on the T cells to induce apoptosis in the T cell [64].

1.14.3.2 MDSC induce suppressive polarization of immune cells

It is well known that MDSC cause a shift toward the TGFβ- rich immunosuppressive microenvironment [99]. One consequence of the abundant production of TGFβ is the development Treg cells. These cells develop under TGFβ dominant conditions, and much like the M1/M2 polarization paradigm, Treg cells are functionally distinct from the inflammatory Th1 and Th17 subtypes [35]. Treg cells secrete large amounts of IL-10, which serve to perpetuate the IL-10 environmental dominance initiated by the MDSC-macrophage interaction [35]. These cytokines form a feed-forward mechanism of environmental immune suppression, polarizing macrophages to the M2 phenotypes, and T cells to the Treg phenotype [112]. Additionally, IL-10 is necessary for maintaining the activity of the distinctive Treg transcription factor Foxp3 [113]. Tregs further compound the immunosuppressive state of the environment by reducing the proliferative ability of Th1 effector cells, effectively reducing antigen presentation and inducing a tolerogenic environment.

In addition to influencing the types of T cell subsets present in the tissue, MDSC also directly control the activity of innate immune effector cells, such as macrophages, dendritic cells, and natural killer cells [100]. The following sections will address the specific interactions between MDSC and innate effector immune cells.

1.14.3.3 MDSC-MΦ interactions

MDSC also regulate the immune response by interacting directly with macrophages. This

interaction results in alteration of macrophage cytokine production. The major overall change that occurs as a result of the MDSC-macrophage interactions in the tissue microenvironment is the shift from a highly inflammatory environment characterized by powerful expression of IL-6 and IL-12 to a tissue repair-supportive environment rich in IL-10, and poor in IL-12 [100]. The relative amounts of these two cytokines dictate whether the microenvironment will become immunocompetent or immunosuppressed. The IL-12 dominant environment favors NK, Th1, and M1 inflammatory type immune cell differentiation, while the IL-10 dominant environment results in the development of Treg and M2/TAM suppressive type immune cells [100]. In this process, MDSC work to inhibit the production of IL-12 secreted by macrophages, thus lowering the T cell proliferation stimulus in the tissue. This inhibitory action is achieved by direct MDSC-macrophage contact, and is thought to occur through MDSC production and secretion of IL-10 [100, 114]. This cell-cell contact also induces polarization of macrophages to the M2, or alternatively activated phenotype. There is bi-directional crosstalk that occurs during this time, whereby as the macrophages begin to polarize toward the M2 phenotype, they stimulate MDSC to produce more IL-10 creating a positive feed-forward loop [100]. This cytokine loop mechanism establishes a local immunosuppressive microenvironment, which results in a ripple effect that contributes to the alternative polarization of other immune cell types, and further exacerbating the changes to the microenvironment. Excess IL-10 results in the polarization of T cells toward the Treg subtype, and thereby decreases the development of the immunocompetent $CD4^+$ and $CD8^+$ T cells further supporting immunosuppression at the expense of immunosurveillance.

MDSC in the microenvironment also support the polarization of tumor-associated macrophages (TAMs); however, not only do MDSC promote the polarization of macrophages toward the TAM phenotype, but M-MDSC can further differentiate into TAMS [100]. Another

consequence of MDSC-macrophage crosstalk is the downregulation of MHCII expression by the macrophages [114]. Although not the primary antigen presenting cells, macrophage-mediated antigen presentation is vital to the induction of cytotoxic $CD8^+$ T cell activation. Elimination of this pathway precludes activation of the Th1 and cytotoxic T cells within the local tissue microenvironment.

Chronic inflammation further intensifies the creation of an immunosuppressive environment via the MDSC-macrophage interaction through the secretion of IL-1β. During periods of chronic inflammation, large amounts of IL-1β are secreted by resident tissue macrophages [99]. Monocytes and MDSC are recruited into the tissue, where due to the persistence of inflammatory stimuli, they collect in large numbers. Following recruitment, monocytes differentiation into macrophages, whereupon they respond to IL-1β by secreting IL-6. The IL-6 secreted by the newly differentiated macrophages drives production of IL-10 by tissue MDSC. In addition to IL-6 induced mechanisms of IL-10 upregulation, PGE_2 has also been shown to drive IL-10 production in MDSC in the presence of macrophages [99]. IL-10 functions to shift the prevailing environmental conditions away from active inflammation, and toward an immunosuppressive state [38, 99, 114]. It is presumed that this mechanism is meant to limit the damage caused by the inflammatory response, and to push the environment into state suitable for supporting the tissue repair process [100].

1.14.3.4 MDSC-DC interactions

MDSC also interact with other innate immune cells such as the professional antigen presenting dendritic cells (Fig. 1-14). These cells are myeloid-derived cells that are primarily responsible for antigen sampling and presentation to T cells. Dendritic cells isolated from tumor-

bearing hosts have been demonstrated to be dysfunctional [99]. Specifically, it was found that MDSC inhibit the maturation of dendritic cells in the microenvironment, which prohibits their T cell activation function by inhibiting the antigen presentation function of the DCs. Additionally, MDSC inhibition of DC maturation also reduces the migratory capabilities of DC, thereby limiting their interactions with T cells in the lymph nodes. MDSC induce DC dysfunction through a multitude of processes, including hypoxia, and build up of adenosine and lactic acid. In addition to contributing to DC dysfunction, production of MDSC leads to decreased total production of

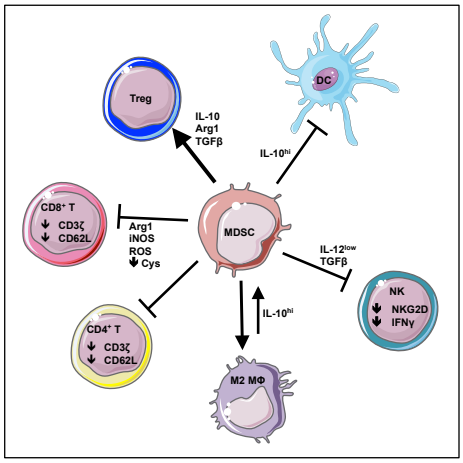

Figure 1-14 MDSC regulate immune function.
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dendritic cells, proportionately [99, 100]. MDSC-mediated enrichment of IL-10 at the expense of IL-12 production further limits the mature DC population. IL-10 precludes DC maturation, whereas IL-12 drives DC maturation. By shifting the production of IL-12 in favor of IL-10, MDSC induce the accumulation of immature DCs, that are unable to present antigen to activate the adaptive immune response [98].

1.14.3.5 MDSC-NK and MDSC-NKT interactions

MDSC have been show to suppress natural killer cell effector functions. Specifically, MDSC inhibit the cytotoxic effect of natural killer (NK) cells in a cell-cell contact-dependent

manner by limiting their ability to produce IFNγ [100]. MDSC also act to suppress NK cell expression of the NKG2D activation receptor. Much like in T cell activation, ligation of the NKG2D to it's counter receptors (produced on target cells as an "abnormality" signal) is absolutely essential for activation of NK cell cytotoxic function [100]. Natural killer T cells (NKT) cells activities are also subject to regulation by MDSC. Two subtypes of NKT cells exist, type I, and type II NKT cells [115]. Type I NKT cells are primarily responsible for identification and elimination of harmful or aberrant cells. These cells are extremely important for the removal of unhealthy cells in the context of anti-tumor immunity. Conversely, type II NKT cells support function as tissue repair supportive cells. NKT type II cells have been most frequently studied in tumor models, where they have been shown to support the immunosuppressive environment through the secretion of IL-13 [100]. These two subsets of NKT cells and their opposing functions further demonstrate immune counterbalancing techniques similar to those seen in macrophages (M1 vs. M2) and helper T cells (Th1 vs. Th2). Therefore it is no surprise that type I NKT cells inhibit the collection of MDSC within the tissue, while type II NKT cells promote the recruitment of MDSC into the affected tissue.

1.14.3.6 MDSC function as immunomodulators of the inflammatory response

Since the advent of their discovery, and notable correlation with tumor incidence and predicted survival outcome, perturbations of the myeloid compartment have also been described to a lesser degree in many other conditions, both pathogenic and non-pathogenic. In addition to being found in pathogenic conditions (chronic inflammation, persistent infection, and cancer), MDSC have also been shown to modulate the immune response during non-pathogenic conditions such as pregnancy, and for inducing tolerance in allograft transplantation systems [3].

The role of MDSC in nonpathogenic systems has been proposed to be immunoregulatory.

MDSC have been proposed to be the myeloid homologous counterpart to the lymphoid Tregs, meaning MDSC regulate the potentially harmful functions of the inflammatory response quite like Tregs regulate many proinflammatory consequences of immune function. It is easy to understand why MDSC could be considered immunoregulatory cells because their populations are often expanded during overstimulation of the immune system. Examples of this include persistent infection and chronic inflammation. Another, perhaps more important example of a non-pathogenic immunosuppressive role for MDSC is observed during pregnancy [116]. Expanded MDSC populations can be seen throughout the entire gestation period in both humans and mice [117]. This immunosuppressive expansion is also observed in Treg populations during pregnancy [117]. In the case of Tregs, to protect the allogenic fetus from rejection by the mother, a state of global immunosuppression is employed. It is thought that MDSC contribute to this type of necessary immunosuppression. Additionally, it has also been found that during pregnancy, the host is far more immune tolerant of cancer as found in a study by Mauti et al. that showed implantation of B16 melanoma in pregnant mice results in higher metastasis rates regardless of tumor cell types [117].

Pregnancy, however, is not the only example of the immune system employing immunosuppressive cells to induce immune tolerance that is beneficial to the host. A second example of this type of acquired immune tolerance is demonstrated in tissue allograft transplantation [118]. Here, immunosuppressive cells such as Tregs and MDSC are absolutely crucial for limiting allograft rejection mechanisms in transplant recipients [118]. Although both pregnancy and organ transplantation are both rarely encountered conditions where potent inhibition of inflammation is absolutely essential to induce a positive outcome in the host, these cells may also play a role in the regulation of the inflammatory response at the post-acute phase of

inflammation. It has been proposed that the non-pathogenic function of MDSC may be to help orchestrate the transition from chronic inflammation into the tissue repair process, the first step of which is to reduce inflammatory signals and functions in order to promote wound healing. Because MDSC only arise during inflammation that is persistent, and because MDSC exhibit such a powerful ability to inhibit the inflammatory functions of most immune cells, it can be said that these cells act as a layer of protection against inflammation-driven host tissue damage, which is absolutely essential for wound healing. On the other hand, activation of this wound repair process must be tightly regulated, as overstimulation of the tissue repair process can lead to unwanted pathologies, such as the generation of fibrotic tissue [36, 52].

1.14.4 The Functional Significance of MDSC in Suppressing Anti-Tumor Immunity

Since originally observing MDSC and their extreme extra medullary expansion during cancer in the early 1900s, more information about these interesting cells has been forthcoming. The functional significance of MDSC in many states has recently been addressed. Since the advent of their discovery, and notable correlation with tumor incidence and predicted survival outcome, this perturbed myeloid compartment has also been described in many other conditions, both pathogenic and non-pathogenic [97]. Additional studies aimed at further defining this perturbed population of cells have found that MDSC potently inhibit T cell proliferation, both *in vitro* and *in vivo,* and are even found to induce T cell apoptosis [98]. These findings, along with the observation that MDSC are found to be drastically increased in the circulation of cancer patients points to the theory that MDSC function to promote tumor survival by enhancing tumor immune evasion.

The major type of cells responsible for recognizing and eliminating tumor cells are cytotoxic T cells [92]. Therefore suppression of these cells by MDSC renders the immune

response to cancer cells insufficient, thereby allowing immune escape and further tumor cell survival. MDSC are the major obstacle impeding current immunotherapeutic approaches for the removal of tumors in the clinic [119]. Although many therapies have been developed to increase the efficacy of T cell-mediated tumor clearance, the exhibition of potent T cell functional inhibition is powerful enough to override the therapeutic activities of altered T cells *in vivo*. Furthermore, once MDSC expansion is established in the tumor-bearing host, the M-MDSC subtypes can further differentiate into tumor-supportive macrophages (TAMs), which then act to sustain the production of more MDSC [99]. Furthermore, these TAMs are polarized not only to protect the tumor from immune cell-mediated clearance, but to directly support the growth, survival, and metastatic capabilities of the altered cells. This includes production of growth and survival factors, and stimuli that confer loss of adhesive molecules and promote the intravasation of tumor cells into circulation [99]. Additionally, there is some evidence that the tumor-supporting molecules secreted by TAMs and fibroblasts in the tumor microenvironment can become systemic, where they can condition other tissues to be immunosuppressed [99]. This conversion of healthy tissue into immunosuppressive tissue aids in the establishment of migratory tumor cells from the initial site of tumorigenesis into new tissue, thereby facilitating the generation of new metastatic tumor sites. Although T cells are the major cell type inhibited by MDSC, it is also important to remember that MDSC inhibit nearly every immune cell responsible for tumor cell clearance. In addition to this function, these cells also function to promote the generation of many immunosuppressive cell types, including Tregs, and suppressive macrophages and dendritic cells [64].

One potential method of limiting the immunosuppressive capabilities of MDSC is to inhibit their egress from the bone marrow. Because MDSC must be in close proximity to T cells to exert

their suppressive functions, inhibiting the migration of MDSC into T cell-rich zones may promote the efficacy of T cell-mediated immunotherapeutic techniques on tumor elimination [98]. Unfortunately, not enough is yet understood about MDSC tethering in the bone marrow to develop such a therapeutic system. It is likely, however, that MDSC egress is mediated by similar tethering molecule interactions that control the release of mature inflammatory monocytes and PMN into circulation. If this hypothesis is correct, it further limits the design of therapeutic targets because limiting MDSC egress will likely also limit mature leukocyte recruitment to the periphery. Such a system that inhibits the recruitment of mature leukocytes to the periphery is not ideal, posing undesirable side effects, such as the inability to respond to bacterial or fungal infection. Therefore it is much more desirable to limit the generation of MDSC altogether as a treatment strategy, because this method of MDSC inhibition is less likely to alter the generation and egress of mature leukocytes.

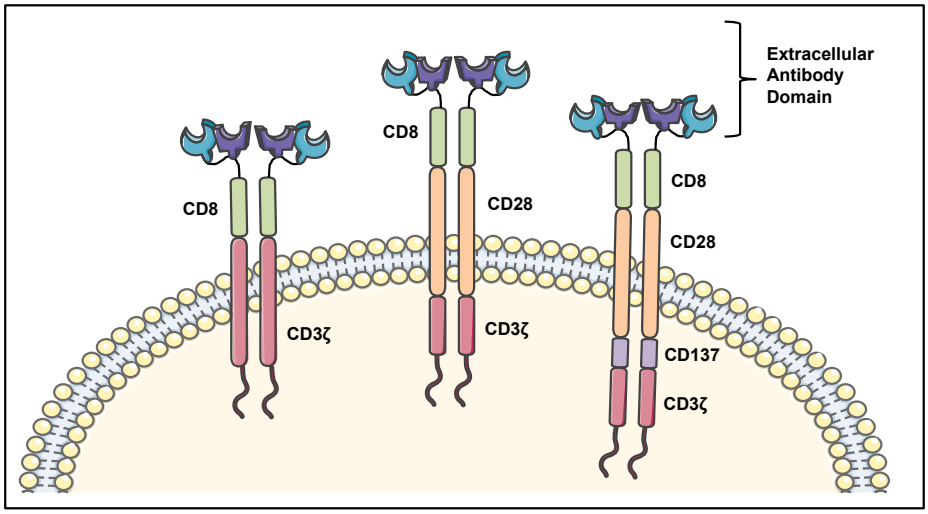

Figure 1-15 CAR T cell receptors.
Antigen-recognition domains are engineered to be covalently bound to T cell co-activation domains. Generated using "Cell Membrane" licensed under CC BY 3.0

MDSC are so effective at inhibiting immune cell function, that they are acknowledged as the primary obstacle impeding the efficacy of anti-tumor immunotherapy techniques. Many anti-

tumor immunotherapies are tailored specifically to each patient. One such immunotherapy technique, known as adoptive cell transfer (ACT) uses a process that requires harvest of host-generated T cells, introduction/education of the T cells with the specific tumor antigen, and reinfusion of the activated clones for the eventual T cell-mediated destruction of tumor cells [119]. This method has proven to be extremely effective in blood cancers, and to a lesser degree in solid tumors; however, because MDSC limit the infiltration of T cells into the tumor microenvironment, and condition the tumor microenvironment to an IL-10/TGFβ-rich milieu, T cells that do eventually traffic into the tumor risk being polarized toward a tolerogenic phenotype. Another method that also uses host T cells, to eradicate tumors is the CART cell therapy method [98]. Unlike ACT, Chimeric antigen receptor (CAR)-T cell therapy uses modified host T cells. These CART cells are artificially modified to contain "super active" TCRs specific to the tumor antigen [119]. These TCRs often have costimulatory activation domains attached directly to the TCR ζ chain (Fig. 1-15). This method induces extreme activity in the modified T cells, which act directly to eliminate the tumor cells, while limiting damage to other cells. The downside to this treatment is that activation of so many cytotoxic T cells results in a cytokine storm, producing extremely high fever and sepsis-like symptoms in patients treatment recipients [119]. This method is thought to be capable of overcoming MDSC-mediated inhibition because of its ability to powerfully induce a substantial inflammatory response capable of overcoming the anti-inflammatory signals used by the MDSC. Other methods of immunotherapy include checkpoint blockade, whereby neutralizing antibodies toward receptors that induce T cell exhaustion are given to the patient. This method has been proven to be very effective with less severe side effects including formation of necrotic tissue at the tumor site [119]. Checkpoint blockade functions by limiting the interactions between T cells and their inhibitory counter-receptors, thereby

prolonging the lifespan and activity of the anti-tumor T cells. Unfortunately, because MDSC limit T cell trafficking of the tumor infiltrating lymphocytes, and use additional methods of T cell inhibition, MDSC are able to overcome the immunotherapeutic effects of checkpoint blockade [98].

1.14.4.1 MDSC are co-opted by tumors to facilitate the immunosuppressive environment

In addition to being a major obstacle in the battle against cancer, MDSC generation is also directly induced by tumors [64]. The process by which tumors create MDSC for their own support is called co-optation, or less formally "hijacking." Tumor cells use the host-evolved protective response of MDSC generation not to limit immune function for the purpose of turning down inflammation, but instead for the pathogenic function of supporting tumor growth and survival. In fact, tumors can directly stimulate the creation of these cells through secretion of growth factors and cytokines, and/or through stimulation of cytokine/growth factor expression from surrounding fibroblasts [64]. These secreted factors not only work to condition the microenvironment to become generally immunosuppressive, but also skew hematopoiesis away from the steady-state, and toward a demand-adapted phenotype. By artificially inducing an emergency inflammatory response the tumor is able to shift leukocyte production away from the steady-state, and toward the emergency hematopoiesis program. Although the exact mechanism of this induction is unknown, some proposed factors that may act to drive the hematopoietic shift from a healthy to an altered state are IL-6, GM-CSF, and G-CSF, as these cytokine have been shown to induce MDSC generation *in vitro* [64]. Upon generation, the newly formed MDSC are recruited into the tumor microenvironment, where the M-MDSC rapidly mature into M2 macrophages and TAMs. Once in the microenvironment, MDSC operate as a shield to protect the tumor from detection by T cells, and support tumor growth and development through secretion of

pro-tumor cytokines and growth factors. For these reasons, MDSC are extremely important to the maintenance of the tumor microenvironment, and limiting MDSC generation during tumorigenesis decreases the ability for tumor cells to persist within the tissue. Many inhibitors of MDSC have been developed for use in cancer treatment, and are primarily concerned with reducing the suppressive nature of these cells. Additional methods seek to inhibit generation and recruitment of MDSC to the tumor site. Many of these drugs, however are not specifically designed to inhibit MDSC, but have been developed for other targets, and many of the mechanisms of action are still unknown. Therefore, understanding the precise mechanisms govern generation of MDSC will allow us to produce better inhibitors of these powerfully immunosuppressive cells.

2 MATERIALS & METHODS

2.1 Mice & Disease Models

C57BL/6 mice (6-10 weeks old, 18-20g) originally obtained from The Jackson Laboratory (Bar Harbor, ME) were bred and housed in a specific pathogen-free facility with free access to food and water. Georgia State University's Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures performed in the mice. To induce colitis in mice, 2% dextran sulfate sodium (DSS, MP Biomedicals) was freshly dissolved into pure drinking water. Mice were treated with DSS for 9 consecutive days, followed by up to 21 days of untreated drinking water to allow for colitis recovery and tissue repair. Mice were examined daily to assess the level of stress, onset of colitis-associated symptoms, and recovery from inflammation. Body weight, stool consistency, and rectal bleeding were monitored daily to determine the disease activity index (DAI) of each animal. DAI was calculated using an average of three criteria: 1) percentage body weight loss (0, none; 1, 1-5%; 2, 5-10%; 3, 10-15%; 4, >15%), 2) stool

consistency (0, solid stool; 2, loose stool; 4, diarrhea), and 3) presence of blood in stool (0, negative; 2, positive; 4, gross bleeding) as shown in previous colitis studies [120] . Stool was also collected daily and tested for the concentration of fecal lipocalin-2 by ELISA. Mice were euthanized at three day time points (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30) during DSS treatment and recovery. Serum, spleen, colon, and bone marrow tissues were collected from the euthanized mice and analyzed for inflammatory markers (Fig. 2-1).

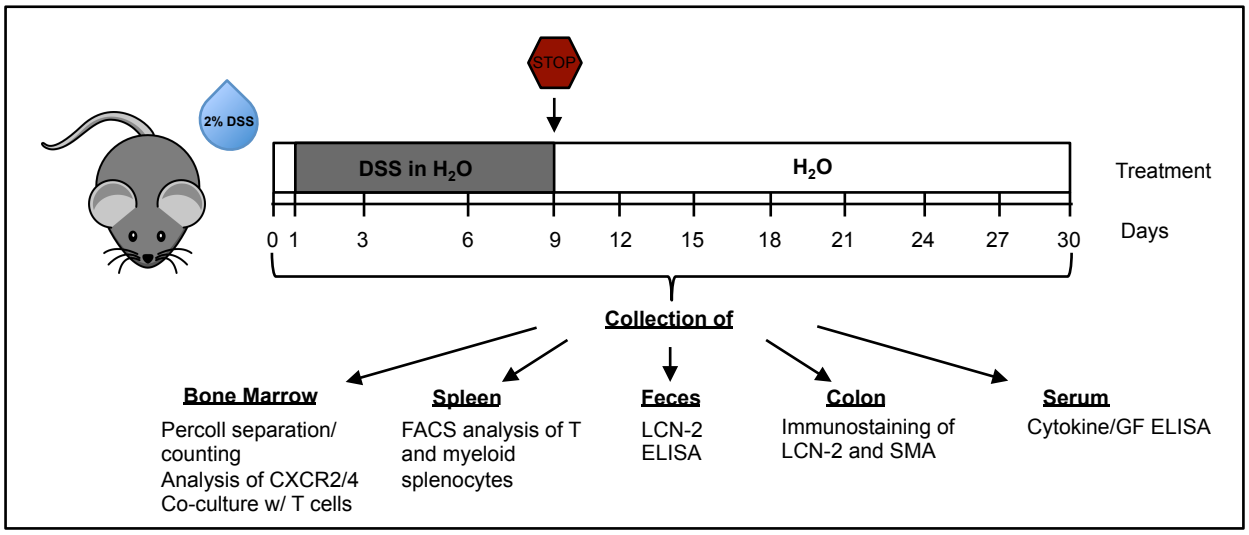

Figure 2-1 Treatment model of DSS colitis and collection of tissues for analysis.
C57BL/6 mice are treated with 2% DSS via drinking water for 9 days. DSS is removed from drinking water at day 9, and mice are allowed to recover from colitis. Tissues were collected at 3 day intervals and analyzed.

2.2 Measurement of Serum Cytokines

Whole blood was collected from mice without the use of anti-coagulation additives, and centrifuged at low speed to isolate the serum fraction. Isolated serum was assayed for cytokines using standard sandwich ELISA technique. Briefly, a 96-well flat-bottom ELISA plate was coated with purified antibodies against IL-6, IL-17,and GM-CSF. Following washing, serum samples collected from mice were incubated in the antibody-coated wells, each sample in duplicate. Following incubation, the wells were washed with 0.005% Tween-20 PBS, and biotinylated and HRP-conjugated streptavidin antibodies were added to each well. Finally, the HRP substrate, o-

phenylenediamine dihydrochloride (OPD, Sigma) was added to each well, and color change was detected at wavelength 450nm. Purified and biotinylated antibodies against all cytokines were purchased from BD Biosciences and BioLegend. Recombinant cytokines used for standards were purchased from PeproTech. Data was analyzed using SoftMax Pro microplate data software.

2.3 Measurement of Fecal LCN2

Control, and mice treated with DSS were removed from cage mates using isolation collection chambers. Fecal pellets were collected from isolation chambers daily for 30 days. Once collected, fecal pellets were weighed and homogenized at a concentration of 100mg/ml in 0.1% Tween-20 PBS. Following homogenization, fecal suspensions were vortexed vigorously for 20 minutes, followed by centrifugation at 12,000 rpm, 4°C for 10 minutes. Supernatants were collected and stored at -20°C before analysis. Fecal lipocalin-2 levels were determined using the commercial LCN2/NGAL ELISA DuoSet (R&D Systems). Samples were diluted (1:20 for untreated and 1:200 for DSS treated mice) using 1% BSA PBS, and evaluated using standard sandwich ELISA technique. Each sample was prepared in duplicate. HRP-conjugated streptavidin in conjunction with OPD were used for color development at wavelength 450nm. Results were recorded using SoftMax Pro microplate data software.

2.4 Measurement of Bone Marrow Cell Populations

Bone marrow cells were harvested from the femora and tibiae of each mouse by flushing the bone cavities with Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+} ($HBSS^{-}$, Corning). Following RBC lysis, cells were loaded onto a discontinuous Percoll® density gradient (40%, 50%, 60% and 70% Percoll® concentrations) and centrifuged at 1,500 RPM at 18°C for 45 minutes. Following centrifugation, cells aggregated at the interface of each Percoll® density layer, resulting in four discrete bands. Each band of cells was collected using a syringe. Cells were

centrifuged and washed three times using $HBSS^-$ to remove residual Percoll®. Cells were counted using a Nexcelom Cellometer Auto 2000 (Lawrence, MA). The sum of all fractions was used to calculate the total number of isolated BM cells. Each cell fraction was divided by the total number of isolated cells to determine the percentage contribution of each fraction to the total.

2.5 Measurement of Chemokine Receptors

Following Percoll® fractionation, fraction 3 and 4 BM cells were labeled with fluorophore-conjugated anti-mouse Ly6C, Ly6G, and CXCR2, CXCR4 chemokine receptor antibodies, followed by washing. Stained cells were analyzed by FACS (LSR Fortessa, BD Biosciences) to determine changes in surface receptor expression. All antibodies were obtained from BioLegend. FACS analysis was performed using FlowJo software.

2.6 FACS Analysis of Splenocytes

Whole spleens were excised from euthanized mice, and emulsified in RBC lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA). After RBC lysis, the purified splenocytes were suspended in PBS and labeled with two combinations of fluorescence-conjugated antibodies (anti-mouse CD4 and CD8, or CD11b, Ly6C, and Ly6G) and analyzed by flow cytometry (LSR Fortessa, BD Biosciences). All antibodies were obtained from BioLegend. FACS analysis was performed using FlowJo software.

2.7 T cell Proliferation Assay

Isolated splenocytes were labeled by incubation with carboxyfluorescein succinimidyl ester (CFSE) followed by several washes, and then resuspended in complete RPMI (1% glutamine, 10% heat inactivated FBS, 0.1% β-mercaptoethanol) cell culture media. A 96 well cell culture plate was coated with anti-CD3e antibodies (BioLegend), followed by washing. Splenocytes were

loaded into each coated well. For co-culture experiments MDSC were added to the splenocytes in each well at a ratio of 1:8. Soluble anti-CD28 (BioLegend) was added to each sample to induce

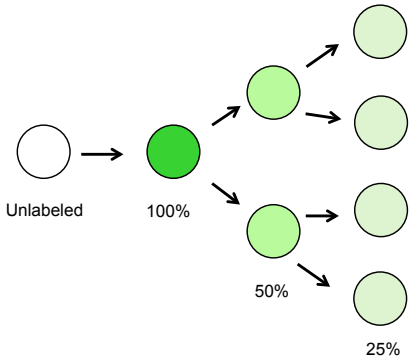

Figure 2-2 T cell proliferation schematic.
T splenocytes are labeled with CFSE, and activated with anti-CD3 and -CD28 ligation antibodies to induce activation. During proliferation, intracellular CFSE will become more dilute through each round of cell division.

proliferation through T cell ligation. Cells were incubated at 37°C, 5% CO_2 for 4 days. Cells were collected and labeled with fluorescent anti-CD4, and -CD8 antibodies (BioLegend) and analyzed by flow cytometry. As T cell proliferated, total CFSE per cell decreases, therefore low CFSE levels in T cells isolated from the final day of the experiment indicates strong proliferation, while high levels of CFSE per T cells indicates little to no proliferation (Fig. 2-2). Percent proliferation was measured using FlowJo analysis software.

3 RESULTS

Because the specific mechanisms governing transition from the onset to the resolution phase of inflammation remain undefined by immunologists, and because understanding these elusive mechanisms will contribute greatly to the understanding of inflammatory regulation, our study endeavored to determine the mechanisms governing this transition. Because MDSC are potent suppressors of the immune system, have been shown to coordinate the dramatic proliferation of tissue cells, and arise during chronic inflammation, we propose that immature myeloid cells

become transcriptionally reprogrammed to MDSC during the post-acute phase of colitis for the purpose of suppressing the overstimulated immune response.

3.1 Establishing a Colitis Model to Assess Wound Healing

We first needed to establish a mouse model to evaluate the changes in the myeloid compartments during inflammation and resolution phases of colitis. To accomplish this, we used the already established murine dextran sulfate sodium (DSS) colitis model to develop inflammation to the post-acute phase, followed by removal of the chemical insult to establish

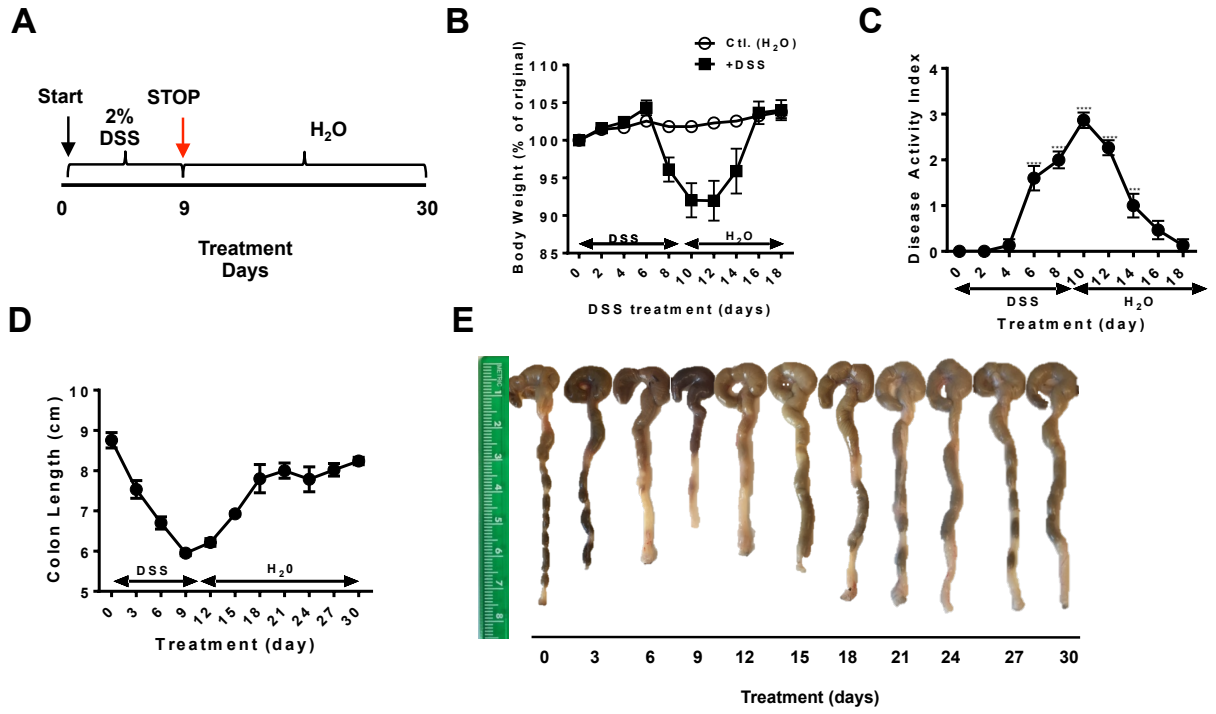

Figure 3-1 Establishment of inflammation and recovery during DSS-induced colitis.
A) Model of DSS treatment used to induce colitis and recovery. B) Percent body weight loss in DSS-treated colitis and control mice. C) Disease activity index was calculated in colitis mice. Disease score represents the average of three disease scoring criteria: body weight loss, stool consistency, and bloody stool. D) Colon length was assessed at various time points during colitis progression. Colon length was dramatically shortened from 3d-12d, followed by an increase in length after stopping DSS treatment. All averages represent n=5 mice/group ± SEM. E) Representative colon length comparisons of colons taken from mice at each time point during DSS+ water administration cycle. While colon length returned to baseline untreated levels by day 18, colon diameter remained dilated through 12d. Images are representative of at least 3 independent experiments.
***$p < 0.0001$, ** $p < 0.01$ versus non-treated control mice.

inflammation recovery. Mice were treated for 9 days with 2% DSS administrated in drinking water, after which the DSS water was replaced with untreated water, as seen in the schematic in figure 3-1A. All animals were monitored for percentage body weight loss, disease activity, colon tissue loss, and fecal lipocalin-2 during treatment. Mice were sacrificed at three day time points and key organs, including spleen, serum, bone marrow, and colon tissue were harvested and analyzed for symptoms of inflammation as well as to determine changes within the myeloid and lymphoid compartments. As seen in figure 3-1B, mice treated with DSS rapidly lost weight from day 6 through day 9, however steadily recovered from this weight loss after stopping DSS treatment (days 12-18), and returned to the baseline value by day 18. Disease activity index was also determined during the treatment and recovery phases of DSS administration (Fig. 3-1C). As seen in the body weight curve, mice treated with DSS showed increased disease activity scores starting at day 6 and peaking between days 9-10, after which the mice recovered through day 18. Other criteria used to determine the relative disease condition of the DSS-treated mice included colon length and fecal LCN-2, a marker of intestinal inflammation [79] (Figs. 3-1 D&E and 3-2A). Significant loss of colon tissue length coupled with maximum weight loss and DAI scores, indicated that mice reached peak inflammation 1-2 days post removal of DSS from drinking water. It should also be noted that loose and bloody stool can be seen during peak colitis at day 12 (Fig. 3-1D&E), illustrating the severe tissue damage acquired during onset and progression of the inflammatory response. Recovery following this phase of persistent inflammation was gradual, and colon tissue did not return to the pre-inflammatory state until nearly 21-24 days after stopping DSS treatment (Fig.3-1E). Although all common, observable indicators of active inflammation returned to baseline levels by day 18, fecal LCN-2 peaked at day 17, and is slow to return to

baseline levels demonstrating that the inflammatory process continues beyond the day 18 (Fig. 3-2A). Because LCN-2 is factor that is secreted first by degranulating neutrophils during active inflammation, and second by epithelial cells during tissue repair, we believe that the overwhelming increase in LCN-2 at day 17 is due to the initiation of the resolution phase of inflammation.

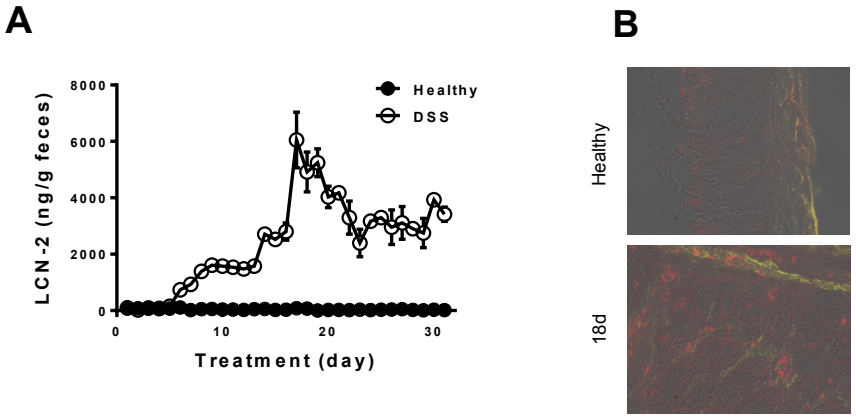

Figure 3-2 LCN-2 increases during colitis resolution.
A) ELISA of fecal lipocalin-2 (LCN2), a marker of inflammation in DSS-treated and control mice. LCN-2 produces a biphasic expression pattern, peaking at day 17. Data represents average fecal LCN-2 ± SEM 5 mice/group. B) Immunofluorescent staining of colon tissue; red, SMA; green, LCN-2. Co-expression of SMA and LCN-2 in colon tissue at day 18 indicates LCN-2 is produced during the tissue repair process. Images are representative of tissue sections taken from 3 independent experiments.

LCN-2 has previously been reported to control the immune response during inflammation by inducing the polarization of macrophages toward the tissue repair-supportive M2 phenotype. To determine if the initiation of the tissue repair process correlates with increased expression of LCN-2 at day 17, we performed immunofluorescent staining of colonic epithelium harvested at this time point. Smooth muscle actin (SMA), a marker of the tissue repair process, was stained in conjunction with LCN-2 to illustrate that LCN-2 is released from colonic epithelium as a function of the tissue repair process (Fig. 3-2B).

As seen in figure 3-2A, colonic epithelial LCN-2 increases during a period of active inflammation recovery starting at days 12-13 and peaking at day 17. Taken together these data

confirm the establishment of a DSS-induced colitis and inflammation recovery model to be used for determining changes to the immune system during inflammation recovery. With this model, we will determine the dynamic changes in the myeloid compartments, to define the role of myeloid cells in the coordinating the transition to the resolution phase of inflammation.

3.2 IL-17 Drives Expansion of MDSC in the Bone Marrow

MDSC are present during chronic inflammation and are potent suppressors of the immune response. Furthermore, prevailing conditions during cancer directly induce the generation of MDSC. MDSC populations have been demonstrated to expand during chronic inflammation and are known to acquire at least two defining functions. To determine when during the inflammatory process this population of cells expand, and to determine when IMC acquire additional functions, thus defining them as MDSC, we examined changes in the dynamic regulation of hematopoiesis during chronic inflammation. To achieve this, we examined the myeloid cell populations within the bone marrow and spleens of mice during our recovery and treatment model. We asked three questions: 1) when during the inflammatory process do IMC expand, 2) when do IMC acquire migratory function, and 3) when do IMC become functionally reprogrammed to become fully suppressive MDSC?

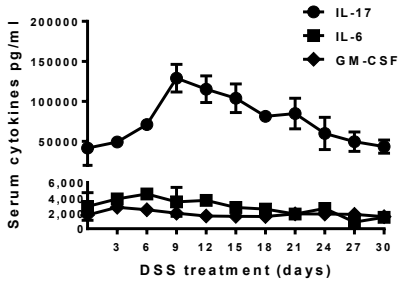

Figure 3-3 IL-17 increases during colitis.
ELISA of proinflammatory cytokines from serum from mice treated with colitis recovery model of DSS administration. IL-17 increases steadily during DSS treatment, and slowly returns to baseline levels by 30d. No significant increases seen in IL-6 of GM-CSF serum levels. Data represents the mean ± SEM of 5 mice/group.

To address when IMC populations expand, whole bone marrow populations were collected at each time point during the inflammation and recovery model. To specifically determine changes within the BM myeloid populations, we applied a novel density gradient centrifugation technique using Percoll®. This technique allowed for the simultaneous separation of IMC from

Table 3-1 Dynamic changes in BM populations during colitis.

Treatment Day (n)	Bone Marrow Fractions (Percoll interface) (% total BM cells isolated ± SEM)			
	Fraction 1 (0-40%)	Fraction 2 (40-50%)	Fraction 3 (50-60%)	Fraction 4 (60-70%)
0 (n=5)	16.79 ± 1.33%	8.00 ± 1.41%	37.52 ± 2.69%	37.71 ± 0.96%
3 (n=6)	22.88 ± 0.95%	6.58 ± 0.94%	39.60 ± 1.74%	30.74 ± 1.73%
6 (n=5)	27.86 ± 4.12%	8.55 ± 1.03%	40.70 ± 5.78%	23.00 ± 4.47%
9 (n=5)	16.35 ± 1.90 %	11.41 ± 0.89%	**61.61 ± 4.24%**	10.61 ± 2.29%
12 (n=5)	11.01 ± 1.52%	10.18 ± 2.03%	**67.59 ± 1.75%**	11.23 ± 1.14%
15 (n=5)	8.36 ± 0.90%	4.66 ± 0.79%	**68.33 ± 1.66%**	18.66 ± 2.33%
18 (n=5)	11.88 ± 1.767%	5.84 ± 1.7-%	**40.71 ± 6.35%**	41.46 ± 7.43%
21 (n=5)	10.40 ± 0.98%	4.70 ± 0.41%	37.90 ± 4.23%	46.57 ± 4.48%
24 (n=6)	9.45 ± 1.60%	5.07 ± 0.97%	51.29 ± 4.78%	34.30 ± 5.29%
27 (n=5)	15.21 ± 1.47%	5.33 ± 0.77%	39.19 ± 2.93%	40.24 ± 3.38%
30 (n=5)	12.95 ± 1.307	7.59 ± 1.21%	42.88 ± 2.94%	36.46 ± 4.28%

mature myeloid cells and other bone marrow cell types. Separation of whole bone marrow using this density gradient technique resulted in the formation of four density fractions. Through flow cytometry analysis, we defined fraction 1 as stromal cells, fraction 2 as mostly B cells, with ~15% myeloid cells, fraction 3 as a heterogeneous population of $CD11b^+$ myeloid cells, and fraction 4 as mature PMN (data not shown). Dynamic changes occurred in all fractions over though course

of colitis and recovery; however, the most dramatic changes were seen in fractions 3 and 4, which were the myeloid-saturated fractions. Figure 3-8A illustrates positive fractionation of whole bone marrow. Low density $CD11b^+$ IMC were enriched in the third fraction, specifically at the 50-60% Percoll® interface, whereas mature myeloid cells ($CD11b^+$, Gr-1^{high}) were enriched in the fourth fraction (the 60-70% Percoll® interface). Tracking the dynamic changes within the bone marrow over the course of inflammation and recovery revealed that the bone marrow IMC population (fraction 3) expands beginning 9 days into the inflammatory process, and continues to expand after the removal of the inflammatory stimulus, peaking at day 15, followed by reduction at day 18 (Tbl. 3-1). This population of cells stabilized to the baseline healthy levels at the late stages of the colitis model. Contrastingly, mature myeloid cell populations (fraction 4) steadily declined over the course of inflammation. This steady decrease is due to recruitment of PMN to the site of active inflammation, a phenomenon characterized by our lab in previous studies. The decrease in BM IMC at the post acute phase of colitis suggests one of two possibilities, either a decrease in IMC production, or as with the mature leukocytes, recruitment into circulation. Furthermore, the percentage perceived "decrease" in fraction 3 cells may also be due to the increase of other cell populations within the bone marrow.

To determine if the decrease in bone marrow myeloid cells at day 18 was due to the acquisition of migratory capabilities, we assessed changes to the myeloid cell populations within the spleen. Changes to both the myeloid and lymphoid splenocyte populations were tracked over the course of the inflammatory process. Interestingly, $CD11b^+$ myeloid splenocytes increased slightly during active inflammation as days 3-9 exhibited only a minimal increase (11.33 to 13.35%), but increased dramatically from days 12-21, peaking at day 18 (Fig. 3-4 and Tbl. 3-2). Because the increase in myeloid splenocytes occurs at the time point corresponding with the

sudden decrease in IMC populations observed within the bone marrow, and because MDSC have previously been shown to acquire migratory function, this data suggests that IMC first expand in the bone marrow, and then acquire migratory function during inflammation resolution at day 18, where they are released into circulation and accumulate in the spleen.

In an effort to identify soluble factors that drive the dynamic changes in hematopoiesis during inflammation and resolution, we measured serum cytokine levels at each time point during treatment. As previously reported by our lab and others, IL-17 is the primary cytokine upregulated during colitis, and our model recapitulates this finding. Within our model, we observed baseline healthy serum IL-17 levels at 40ng/ml, however this cytokine increased nearly 3 fold at the peak of colitis (days 9-12, Fig. 3-3). Interestingly, cytokines known to directly drive the generation of MDSC, IL-6 and GM-CSF, were not observed at significantly enhanced levels within the serum during colitis, remaining in the low pg/ml range. Although these cytokines were not significantly elevated in the serum, they may indeed play a role in the expansion of IMC during inflammation. Because IL-17 is known to stimulate production of both IL-6 and GM-CSF in macrophages, systemic upregulation of IL-17 may induce local bone marrow macrophages to produce these cytokines, thereby driving changes to hematopoiesis locally, rather than systemically.

In our colitis inflammation and recovery model, serum IL-17 steadily increased during active colitis, peaking at day 9. After removal of the inflammatory stimulus, IL-17 remained elevated in the serum. Additionally, the height of IL-17 production directly preceded the peak of IMC expansion in the bone marrow. This data, coupled with the ability for IL-17 to drive IL-6

Table 3-2 Inverse relationship between myeloid and T splenocytes during colitis.

Treatment Day	Percentage Splenocytes		
	$CD4^+$	$CD8^+$	$CD11b^+$
0	10.72 ± 1.31% (n=4)	6.89 ± 0.26% (n=2)	11.63 ± 0.58% (n=6)
3	6.82 ± 1.75% (n=4)	7.92 ± 0.49% (n=4)	11.33 ± 1.31% (n=4)
6	7.61 ± 0.11% (n=2)	8.34 ± 1.89% (n=2)	14.04 ± 0.92% (n=5)
9	8.62 ± 0.36% (n=4)	9.64 ± 0.40% (n=5)	13.35 ± 1.07% (n=4)
12	4.70 ± 0.98% (n=3)	5.13 ± 0.94% (n=3)	15.98 ± 2.03% (n=3)
15	5.36 ± 0.14% (n=5)	5.67 ± 0.42% (n=5)	15.75 ± 1.24% (n=5)
18	4.17 ± 0.38% (n=4)	4.29 ± 0.25% (n=4)	**29.73 ± 4.42**% (n=3)
21	3.06 ± 0.38% (n=6)	4.50 ± 0.42% (n=6)	**27.73 ± 5.11%** (n=4)
24	7.91 ± 0.57 (n=3)	7.04 ± 0.67% (n=4)	**27.56 ± 2.62%** (n=5)
27	6.89 ± 1.33% (n=4)	6.67 ± 0.982% (n=5)	22.55 ± 3.36% (n=4)
30	8.06 ± 1.50% (n=3)	6.83 ± 1.09% (n=3)	19.86 ± 4.44% (n=5)

and GM-CSF production, and the known associations between IL-17, chronic inflammation and MDSC suggests that IL-17 drives expansion of IMC during inflammation.

3.3 Myeloid Cells Deplete T Cells in the Spleen During Inflammation Resolution

From the gradient density experiments, we observed that the immature, low density myeloid cell population was dramatically expanded during the post-acute phase of colitis, particularly at the early stages of inflammation resolution through a process likely coordinated by enhanced systemic IL-17. In addition to characterizing the dynamic regulation of hematopoiesis during colitis inflammation and recovery, we also attempted to further define the nature of the accumulated myeloid splenocytes. To achieve this goal, FACS was used to determine if myeloid cells accumulating in the spleen tissues represented known characteristics of MDSC, including

heterogeneity and T cell suppressive qualities. Using specific monocyte and granulocyte cell type markers Ly6C and Ly6G respectively, it was observed that the enhanced population of $CD11b^+$ myeloid cells found within the spleens during inflammation were composed of a heterogeneous

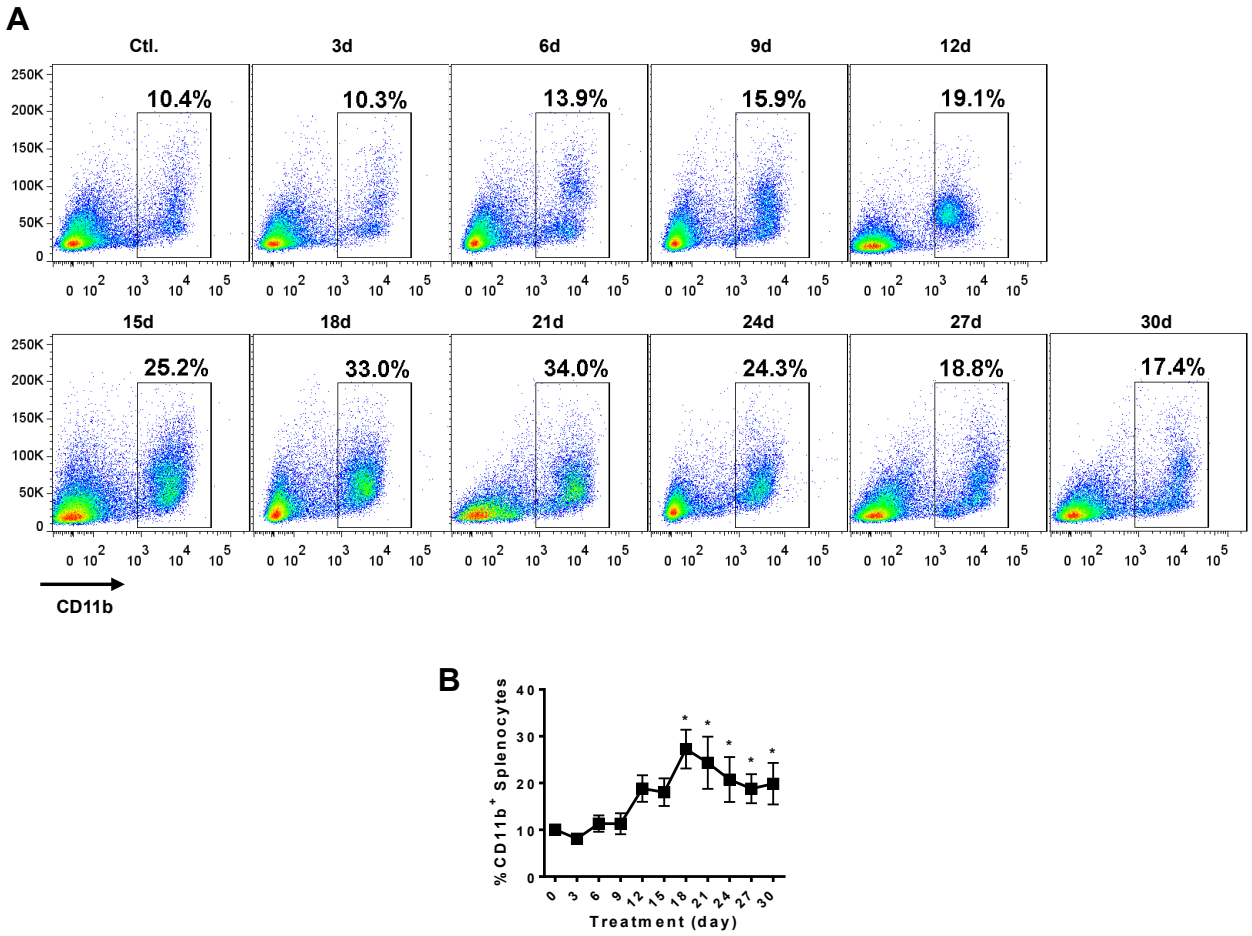

Figure 3-4 Myeloid cells accumulate in spleens of colitis mice.
A) FACS of whole spleens from DSS-treated colitis mice. Percentage of myeloid cells significantly increases in the spleen during inflammation resolution time points, most dramatically at day 21. Quantification of $CD11b^+$ splenocyte populations n=4 mice/time point. FACS scatter plots representative of at least 4 individual experiments.

mixture of monocyte- and granulocyte-like cells (Fig. 3-5). This observation supported the notion that the enhanced myeloid population seen in the spleen during inflammation were likely MDSC, as these cells are characteristically composed of a mixed population of monocytic and

granulocytic subpopulations. As seen in figure 3-5, both subpopulations of myeloid cells were increased over the course of colitis treatment and recovery, peaking at days 18-21.

To further characterize the physiological purpose of the myeloid cell accumulation in the spleen, we evaluated the T lymphocyte populations within the spleen during inflammation and recovery. Because MDSC are known to potently suppress T cell proliferation, we examined both $CD4^+$ and $CD8^+$ splenocyte populations over the course of inflammation and colitis recovery. We

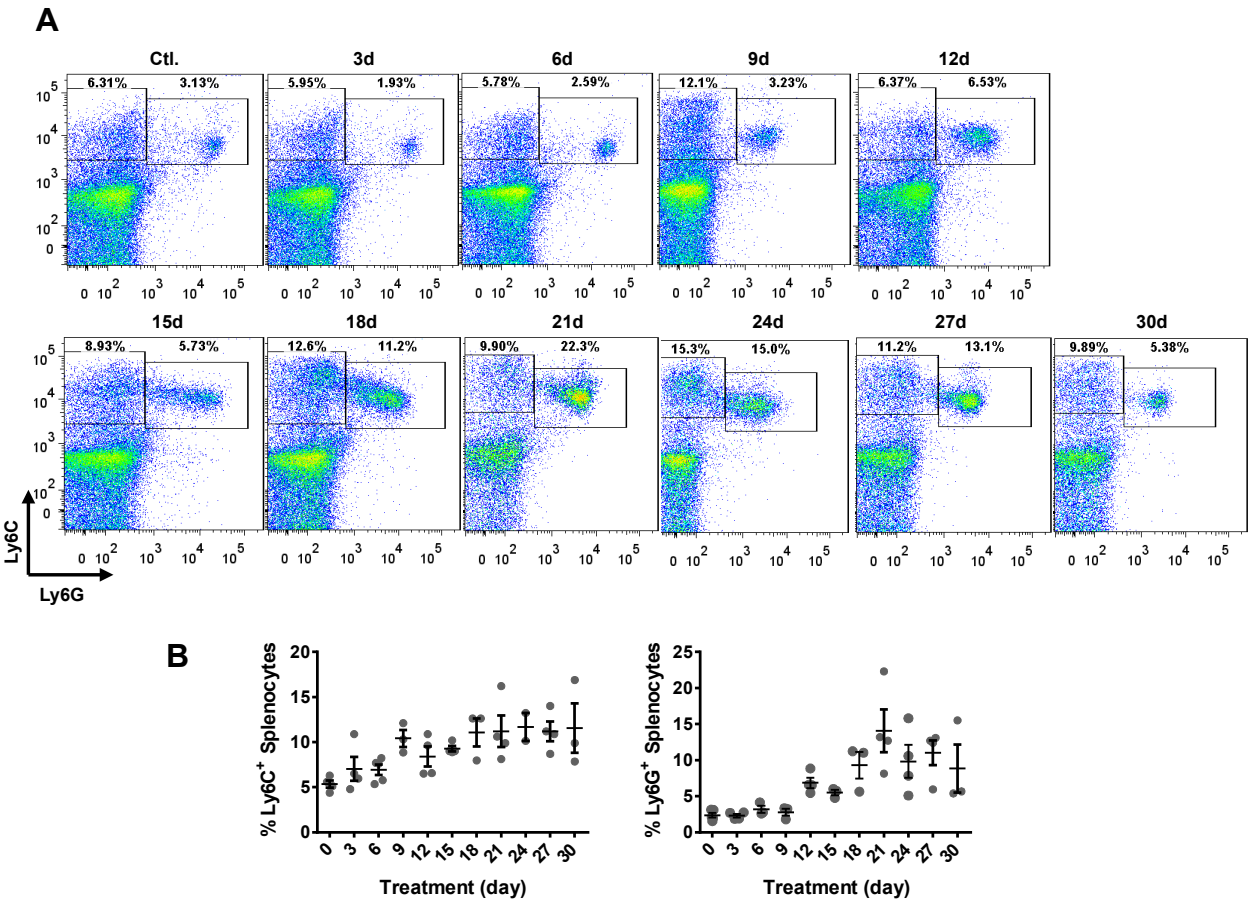

Figure 3-5 Expanded $CD11b^+$ splenocytes represent a heterogeneous population.
A) FACS of $CD11b^+$ myeloid splenocyte subpopulations taken from whole spleens of colitis mice. Ly6C and Ly6G were used markers of monocyte-like and granulocyte-like cells, respectively. FACS scatter plots representative of at least 4 individual experiments. B) Quantification of individual myeloid splenocyte subpopulations. Both myeloid subpopulations increase during inflammation recovery. Data represented as the mean ± SEM

anticipated that IMC were undergoing a functional reprogramming event to MDSC during inflammation, and that observed accumulation of myeloid cells within the spleen was functioning to inhibit the immune response during inflammation by dampening T cell activation and proliferation. Supporting this theory, we found myeloid and T splenocyte populations exhibited an inverse relationship during the inflammatory process (Fig. 3-6). As spleen myeloid populations increased over the course of inflammation from days 3-21, both $CD4^+$ and $CD8^+$ T splenocyte populations correspondingly decreased (Fig. 3-6, and Tbl. 3-1). Furthermore, this inverse relationship reached its climax at the 18 day time point. Here, it was observed that

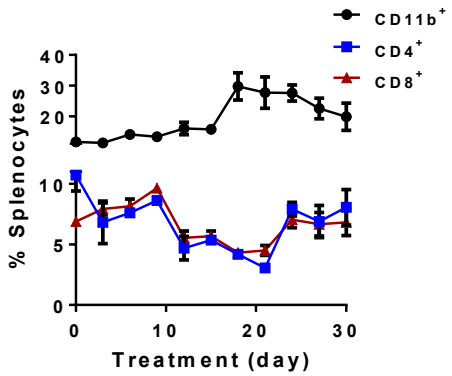

Figure 3-6 Inverse relationship between myeloid and T splenocytes.
As $CD11b^+$ myeloid splenocytes increase, $CD4^+$ and $CD8^+$ splenocytes decrease during the post-acute phase of colitis, peaking at days 18-21.

myeloid cells constituted a staggering 29.73% of the total spleen cells, while at the same point the total $CD4^+$ and $CD8^+$ T splenocyte percentages decreased to 4.17 and 4.29% respectively; levels nearly half those of their healthy counterparts. At later time points however, as the myeloid splenocyte population waned (days 27-30) the T cell populations steadily recovered to healthy levels (Table 3-2). From these data, it was observed that accumulated myeloid cells in the spleens of colitis mice were of a heterogeneous nature, representing both monocytic and granulocytic subpopulations, and that accumulation of these cells in the spleens of treated mice was inversely

proportionate to the T splenocyte populations. These data suggest that increased, heterogeneous myeloid cell populations within the spleen exhibited T cell suppressive capability, and that these characteristics are congruent with characteristics describing MDSC. Therefore, we proposed that IMC expand in the bone marrow during persistent inflammation, acquire suppressive function, and are recruited to the T cell-rich spleen for the purpose of dampening the immune response in favor of the resolution of inflammation and promotion of tissue repair.

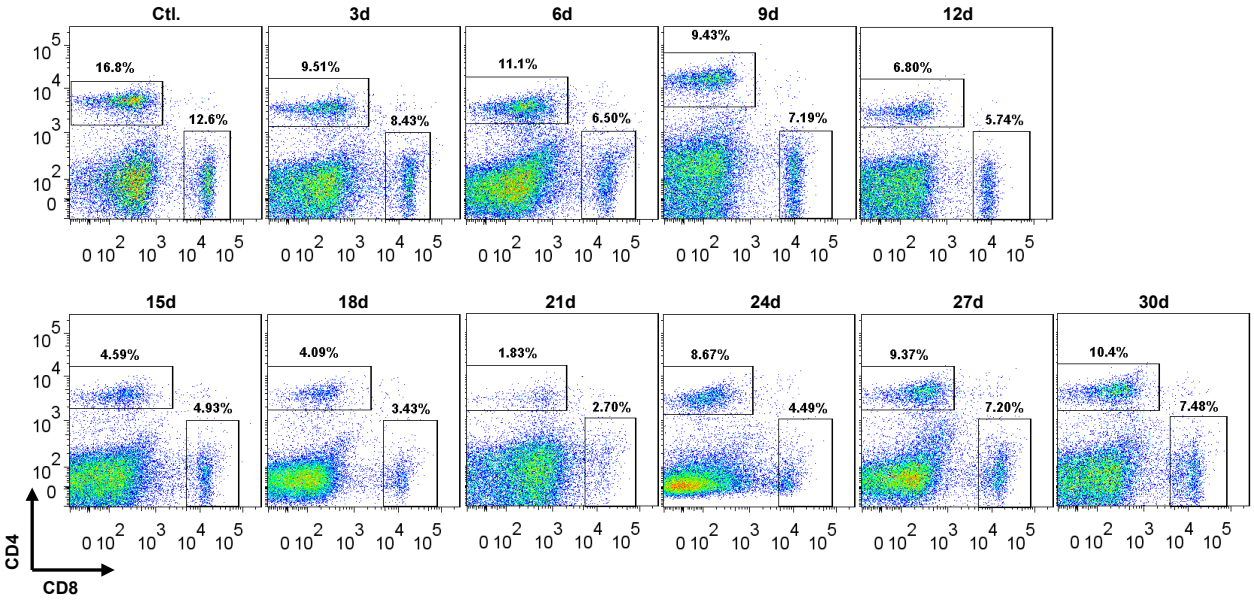

Figure 3-7 T splenocytes decrease during colitis.
FACS of $CD4^+$ and $CD8^+$ T splenocytes. T cell populations decrease as the myeloid population increases within the spleen. FACS scatter plots representative of at least 4 individual experiments.

3.4 Increased CXCR2 Expression Allows MDSC to Enter Circulation

To determine the mechanism responsible for driving the acquisition of migratory function in MDSC, we evaluated surface expression of the chemokine receptor CXCR2 using FACS. Observation of increased CXCR2 chemokine receptors present in the IMC fraction confirmed that IMC gain migratory functional capability during the post-acute phase of colitis. Interestingly, M-MDSC did not increase CXCR2 expression, but the G-MDSC subpopulation did exhibit increased

CXCR2 surface expression (Fig. 3-8). Observation of enhanced expression of CXCR2 over the course of colitis substantiates our claim that the decrease in BM IMC seen at day 18 is, in part due

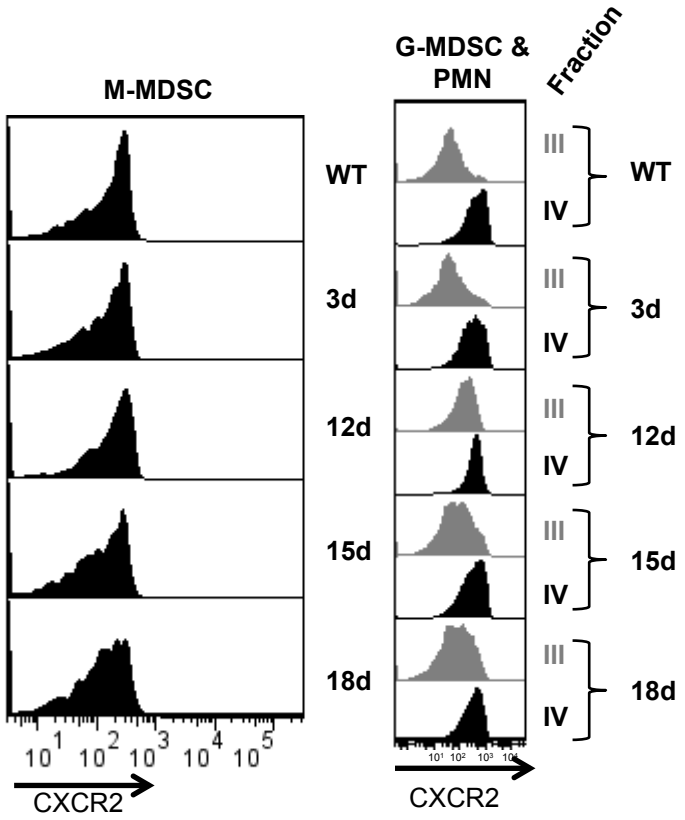

Figure 3-8 Increased expression of CXCR2 in MDSC.
M-MDSC do not show any increased CXCR2 expression over the course of colitis, however, G-MDSC increase CXCR2 surface expression most evidently at the post-acute phase of colitis, but do not reach maximum levels of CXCR2 expression as seen in mature PMN.

to the acquisition of migratory function, and not because of decreased generation of these cells. Because both M-MDSC and G-MDSC subpopulations increase within the spleen during colitis, further investigation is needed to determine the chemokine receptor that is responsible for coordinating the migration of M-MDSC. As with mature inflammatory leukocytes (PMN), this method of acquired migratory capability appears to be the major mechanism governing the entry of IMC into circulation. Enhanced CXCR2 in IMC allows these cells to respond to the major inflammatory recruitment chemokine IL-8 that is dramatically upregulated in inflamed tissue.

Although not explicitly determined in this study, it is easy to imagine that recruitment of IMC into circulation via the IL-8-CXCR2 axis not only results in accumulation of IMC in the spleen, but also at the site of inflammation.

3.5 Immature Myeloid Cells Become Functionally Reprogrammed to Suppress T cell Proliferation

We have demonstrated that during active inflammation, and the early phases of resolution, the immature myeloid cell compartment expands in the bone marrow, and these cells are recruited to the T cell-rich environment of the spleen, where they accumulate in large numbers. Additionally, as the myeloid cell population increases in the spleen, the T splenocyte population correspondingly decreases. To verify that within our model of inflammation and resolution, myeloid cells do, in fact acquire T cell suppressive capability, and that spleen myeloid cells inhibit the activation and proliferation of T cells leading to a reduction of T splenocyte populations, we assessed T cell inhibitory capability using an *in vitro* T cell proliferation model.

To measure the suppressive activities of these cells, IMC were isolated from the bone marrow during inflammation and recovery, and were subjected to co-culture with activated T splenocytes at a ratio of 1:8 (IMC:splenocytes). Following incubation, $CD4^+$ and $CD8^+$ T cells were analyzed for proliferation via FACS. As shown in figure 3-8B immature myeloid cells isolated from the bone marrow began to inhibit T cell proliferation as early as 3 days into DSS treatment, and became more potently suppressive of T cell proliferation during the resolution phase of experimental colitis. IMC isolated during early inflammation resolution time points (days 9-18) exerted the most significant T cell suppressive function (Fig. 3-8B) where T cell proliferation was almost undetectable for both $CD4^+$ and $CD8^+$ T cells. Remarkably, the immature myeloid cells isolated from days 12-18 exhibited the most potent T cell suppression *in vitro*; a

quality that recapitulates the inverse relationship between myeloid and T splenocytes gathered from the *in vivo* data. These data support the hypothesis that T splenocyte depletion corresponding with myeloid cell accumulation is due to active suppression of T cell proliferation by MDSC *in vivo.* This data supports the notion that IMC are functionally reprogrammed to MDSC during the late phase of inflammation and may support the transition to the early phases of inflammation resolution.

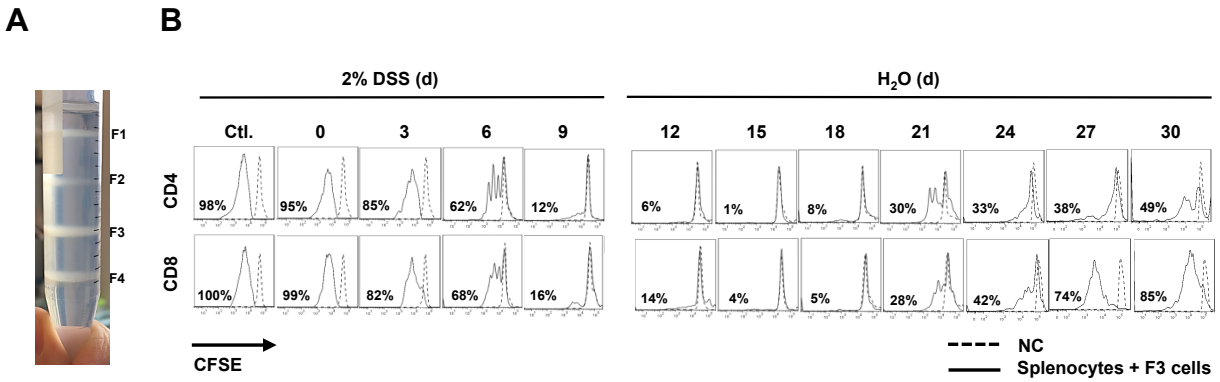

Figure 3-9 MDSC inhibit T cell proliferation.
Immature myeloid cells from inflammation recovery time points inhibit T cell proliferation. A) Example of discontinuous Percoll gradient following separation of whole BM. Four resulting fractions, F1-4. B) Assessment of immature myeloid cells (F3) inhibitory function using T cell proliferation assay. Fraction 3 myeloid cells were co-cultured with healthy activated, CFSE-loaded T splenocytes at a ratio of 1:8. Proliferation was determined by FACS for both $CD4^+$ and $CD8^+$ T cells. T cells cultured with F3 from untreated mice exhibited no reduction in T cell proliferation when compared to the positive control. T cell proliferation was greatly inhibited in all DSS-treated mice, but most notably at the inflammation recovery time points. BM immature myeloid cells acquire T cell suppressive function during inflammation recovery. Histograms are representative of three independent experiments performed in duplicate.

4 DISCUSSION

As discussed previously, MDSC are potent suppressors of the immune response that have been established in many models, including chronic inflammation, and cancer. Additionally, these cells have been shown to have non-pathogenic, protective functions during allograft transplantation and pregnancy. In previous studies using models of post-acute/chronic inflammation in our lab, we determined functional changes to inflammatory leukocytes, which

resulted in the potentiation of inflammatory function, specifically in PMN [121]. This study characterized additional functional changes within the myeloid cell compartment; however, these changes did not support the inflammatory response, instead the changes we observed were suppressive, and functioned to counteract the inflammatory response in favor of resolution and tissue repair. Our findings defined a non-pathogenic role for MDSC in the coordination of inflammation resolution, and support the growing idea that soluble factors secreted as a result of post-acute inflammation induce functional reprogramming of the myeloid compartment, which further regulates the inflammatory response.

4.1 DSS Colitis is a Useful Model for Studying Inflammation Resolution

DSS-induced colitis has been a long-used, predictable, easily-administered model of inflammation; however, until now, DSS was used mainly as a method to examine the mechanisms responsible for the onset of inflammation, and the individual relationships between cells of the immune system and affected tissue cells. A tremendous amount of extremely useful information about the immune system and inflammatory response has been gleaned from DSS colitis studies; although, our study went a step further, and sought to use the DSS-induced colitis model to examine the resolution of inflammation, and the onset of immunomodulatory functions within the effector immune cells. In addition to the commonly measured markers of disease activity such as body weight loss, bloody stool, and colon tissue loss, our study also examined the non-lethal disease activity index measurement of fecal LCN-2. LCN-2, a prevailing clinical marker of inflammation was found to be associated with both weight loss and disease activity index curves, all demonstrating a steady onset of colitis activity from days 1-9 post initial DSS administration, followed by a steady decrease to basal levels. Astonishingly, unlike the weight loss and disease activity indexes, LCN-2 levels remained significantly elevated through day 30, reaching 3 fold

induction compared to their untreated counterparts. This novel characterization of the DSS-induced colitis model suggests that although animals visibly seem to return to a basal level of health, as if inflammation had never been induced, these animals are far from completely recovered. LCN-2 levels confirm our other findings, which collectively determine that DSS-treated mice continue to undergo inflammatory processes long after they return to healthy body weight and disease activity index. This data is consistent with the previously described notion that the inflammatory response has three functions: 1) removal of pathogen, 2) removal of damaged/alien tissue cells, and 3) initiation of the tissue repair process. Often in inflammation research, the third function of the inflammatory response is ignored, but we can see from our study, that tissue repair is a persistent, long-lived function of the inflammatory response that deserves as much, if not more attention from researchers, as tissue repair induces significant changes within the immune system, and these changes can be mimicked, or co-opted by tumor cells to support their own functions.

4.2 LCN-2 Participates in Coordinating Resolution of Inflammation

LCN-2, secreted first by inflammatory PMN, and second by epithelial tissue cells and MΦ, has previously been demonstrated as a modulator of the inflammatory response, capable of reducing generation of inflammatory mediators through conversion of MΦ to the M2-like phenotype [84]. A study conducted by Warszawska et al. found that $LCN2^{-/-}$ macrophages produced significantly more proinflammatory mediators including IL-8, IL-6, and TNF-α than unaltered MΦ, in response to activation with *S. pneumoniae.* Furthermore, it was discovered that these MΦ also produced significantly more IL-10 that WT MΦ. In this study, researchers were able to reduce proinflammatory mediator secretion and rescue IL-10 secretion in activated LCN2 knockout MΦ by supplementing media with recombinant LCN-2 [84].

As previously reported by others, our study found that LCN-2 is induced during infection and during periods of persistent inflammation. Additionally, *in vitro* analyses of the role of LCN-2 during inflammation have confirmed an immunomodulatory role for this protein; however, an exact mechanism for LCN-2 immunomodulatory regulation has yet to be described. In the previously described study, it was found that LCN-2 potently inhibits bacterial clearance by alveolar MΦ through an IL-10-dependent mechanism. When discussed in the context of this study, our data appears to confirm a role for LCN-2 in transforming the insulted microenvironment toward a more suppressive state, perhaps through the conversion of inflammatory M1 MΦ toward an M2-like polarization. This immunomodulatory function of LCN-2 may help to establish a protective response during times of extreme inflammation to limit tissue damage acquired by the constant influx of inflammatory leukocytes. Our study also confirms that LCN-2 and augmented production of smooth muscle actin (SMA) occur concurrently within the insulted colon tissue, establishing that the second wave of LCN-2 production by epithelial cells is tightly associated with the onset of tissue repair. This data, taken with the known immunomodulatory function of LCN-2 previously established by Warsawska et al. suggests that LCN-2 likely plays a role in orchestrating the transition from active inflammation to inflammation resolution and tissue repair.

Our study recapitulates the previously reported immunomodulatory function of LCN-2 and illustrates the tight association between LCN-2 production and onset of the tissue repair process during colitis, confirming *in vivo* that LCN-2 likely aides the establishment of immunosuppression during tissue repair.

4.3 MDSC Arise as a Result of Persistent Colitis

Our study identified significant expansion of the BM IMC population during colitis. Expansion of these cells began early on in the inflammatory process (9d) and continued to expand even after removal of the inflammatory stimulus (DSS). The immature, low density myeloid cell population reached a maximum level of expansion at day 15, which was followed by a sudden, dramatic decrease of these cells at day 18. We believe that this sudden decrease was due to the acquisition of migratory function in the IMC, leading to egress from the bone marrow, and entrance into circulation. Using this model of post-acute DSS-induced colitis, we were able to show that colitis stimulates the generation of expanded IMC in the bone marrow. These data illustrate that hematopoiesis is dynamically regulated by inflammatory processes occurring in distant tissues. Previous data from another study by our lab illustrated a similar phenomenon, whereby many models of persistent inflammation, including STZ-induced diabetes, LPS-induces bronchitis, and DSS-induced colitis resulted in functional changes in bone marrow-derived PMN [121]. Furthermore, many studies have collectively suggested cytokine-driven emergency hematopoiesis is a powerful method of dynamically changing the types of effector immune cells produced in the bone marrow to suit the needs of the host during conditions of chronic infection. These findings led us to hypothesize that soluble factors (cytokines and growth factors) secreted at the site of colitis, travel through circulation to induce changes to hematopoiesis in the distant bone marrow, and that these changes either potentiate or diminish the inflammatory capabilities of bone marrow-derived immune cells to suit the need of the host.

Our study found that indeed, additional changes occur within the bone marrow myeloid compartment as a result of persistent colitis inflammation. We determined that in addition to potentiating PMN inflammatory activity during inflammation, IMC expand and acquire at least

two additional functions: migratory capability and T cell suppression. The acquisition of these two additional functions in the heterogeneous population of IMC were consistent with previous reports describing MDSC. Furthermore, our study establishes a role for MDSC in the coordination of the resolution of persistent inflammation. As in our previous study, we determined that IL-17, the major cytokine upregulated during the post-acute stage of colitis corresponded with the expansion and acquisition of new functions in development of MDSC.

4.3.1 IL-17 Drives MDSC Expansion

By treating mice with a chemical inducer of colitis (DSS) to the post-acute phase of inflammation, we were able to demonstrate significant, systemic upregulation of the cytokine IL-17. Consistent with our earlier studies and others, we identified IL-17 as the most prominently enhanced serum cytokine during colitis. Furthermore, we found that enhanced IL-17 serum expression was associated with the expansion of IMC in the bone marrow. This finding was not surprising, as other have reported that IL-17 plays a significant role in hematopoiesis, and is a well-characterized hallmark of chronic inflammation in many autoimmune maladies [122, 123]. Additionally, pilot studies in our lab confirmed the expansion of IMC by direct i.v. injection of IL-17 (data not shown). This experiment confirmed that IL-17 dramatically increases production of myeloid-derived cells within 24 hours of injection. In a previous study by our lab, we reported that IL-17 produced as a result of post-acute inflammation induced functional changes in mature PMN, potentiating their inflammatory functions. Specifically, we showed that IL-17 enhanced respiratory burst and phagocytosis capacity, and accelerated PMN migratory capabilities [121]. Because many studies have defined roles for IL-17 in hematopoiesis and myeloid cell functional alteration, it is not surprising that our study suggests a role for IL-17 in MDSC generation during colitis inflammation.

Previous studies seeking to determine a mechanism for IL-17-driven enhanced hematopoiesis discovered that IL-17 is only able to increase hematopoiesis in the presence of bone marrow fibroblasts [124], but not in the absence of these cells. This finding suggests that IL-17 alters hematopoiesis through an indirect mechanism, whereby IL-17 acts on the fibroblast intermediaries, which in turn, secrete additional cytokines and growth factors that promote hematopoiesis. Because IL-17 has been demonstrated to enhance myelopoiesis, and has been shown to alter the function of PMN at the post-acute/chronic stage of inflammation, and because IL-17 expression directly correlates with the acquisition of immunosuppressive capabilities by IMC, we believe IL-17 is also responsible for driving the generation of MDSC during the post-acute phase of colitis. In a previous study by Lechner et al., and confirmed by our lab, *in vitro* generation of MDSC was achieved by adding IL-6 and GM-CSF to an *in vitro* culture of whole bone marrow [95]. In our current study, we did not observe any significant increase of either IL-6 or GM-CSF in the serum of colitis mice; however we did observe extreme enhanced systemic IL-17 levels. Because IL-17 induces increased myelopoiesis through fibroblast intermediaries, and IL-17 has been shown to induce expression of both IL-6 and GM-CSF, we believe IL-17 drives MDSC generation by stimulating bone marrow fibroblasts and bone marrow macrophages to produce IL-6 and GM-CSF locally within the bone marrow milieu.

Although incompletely understood, STAT3 is the major characteristic transcription factor associated with MDSC. STAT3 activation promotes the transcriptional induction of many of the genes that confer suppressive capabilities specifically within the M-MDSC population, including *arg1*, *inos,* and *cxcr2*. IL-6 is a powerful inducer of STAT3, therefore it is no surprise that IL-6 has been defined as a factor that induces the generation of MDSC; however, GM-CSF is an activator of STAT5, a potent suppressor of STAT3 activity. The finding that GM-CSF is

necessary to promote *in vitro* generation of MDSC is somewhat unexpected. In fact, Cohen et al demonstrated that absence of GM-CSF is necessary to elicit STAT3-dependent myeloid differentiation [104]. It remains unclear exactly how these two factors drive transcriptional alteration during MDSC generation, however it appears that activation of the STAT3 transcriptional program is indispensible for the activation of suppressive genes that define the suppressive MDSC phenotype.

4.4 MDSC Accumulate in the Spleen to Inhibit T Cell Proliferation

Our data illustrated that immature myeloid cells expanded in the bone marrow until day 15, after which this population of cells sharply decreased. We hypothesized that this drastic reduction in bone marrow IMC was the result of two possible mechanisms: the first, was through the dramatic decrease in production of IMC, and the second was through the acquisition of migratory function. If the drastic reduction in bone marrow IMC was due to decreased production, we would expect the kinetics of this depletion to more gradual than sudden. Additionally, if loss of IMC was due to promotion of maturation of monocytes and granulocytes, we would expect the mature monocyte/granulocyte population to increase accordingly; however, we do not see this phenomenon. Instead, we postulated that the sudden, sharp decrease in bone marrow IMC was due to the acquisition of migratory capability in the IMC. As the IMC become recruited into circulation by inflammatory signals produced within the inflamed colon, IMC at day 18 are able to respond to the call from the wound site, and enter the circulation, much like the mature inflammatory leukocytes.

4.4.1 MDSC Develop Migratory Function

MDSC did not acquire migratory function until the post-acute stage of inflammation. As mentioned previously, unlike the pattern of steady mature PMN mobilization from the bone

marrow, onset of migratory capability in IMC was observed as a more sudden pattern of acquired function. We observed that bone marrow IMC populations expanded as a result of inflammation in the colon, and sharply decreased at day 18. Furthermore, we determined that the expanded IMC spleen population was likely due to release from the BM. This accumulation of myeloid cells in the spleen peaked at days 18-21. Although acquisition of migratory function has been established as a defining characteristic of MDSC, most studies investigating the functional capabilities of MDSC have been performed in tumor models, and as a result, have determined that MDSC develop migratory function for the purpose of migrating into tumor tissue. Our study, for the first time, establishes a different migratory location for MDSC, the spleen. By homing to the spleen during persistent inflammation, we propose that MDSC are limiting the expansion of activated T cells for the purpose of inhibiting the immune response.

Although it is unclear what mechanisms specifically drive acquisition migratory function, using tumor models, other studies have proposed that MDSC increase expression of chemokine receptor CXCR2, which enables them to respond to mobilization signals from the site of inflammation, or tumor. Specifically, Katoh et al. found that CXCR2 knockout MDSC are unable to promote colitis-associated tumorigenesis [125]. To determine if MDSC generated under inflammatory conditions exhibited similar mobilization mechanisms, we tested the expression of CXCR2 on fraction 3 IMC from bone marrow samples. This data revealed steady upregulation of surface expression of CXCR2 peaking at day 18, but not before this time point. This data confirms that functional changes are acquired within the bone marrow, before the cell enters circulation, and that increased expression of CXCR2 is likely one mechanism explaining acquisition of IMC migratory capability, at least in G-MDSC. More studies must be carried out to investigate how M-MDSC may enter circulation.

Furthermore, it has been shown in previous studies that CXCR2 is subject to expression under STAT3 regulation [126], a pathway that has been shown to be dominantly activated during the generation of MDSC in tumor models [101]. This pathway is inducible by many factors produced during persistent inflammation, including IL-6 and G-CSF, two cytokines upregulated by bone marrow macrophages in the presence of IL-17 [124]. This information, along with the discovery that IL-17 drives the expansion of IMC during persistent colitis raises the obvious question: does IL-17 drive both the expansion and functional alteration of IMC to MDSC, specifically promoting the acquisition of migratory function? Although our study does not specifically answer this question, IL-17 appears to play a key role in regulating at least the expansion of IMC, and therefore further examination of this hypothesis certainly has merit.

4.4.2 MDSC Inhibit T Cell Function in the Spleen

One consequence of the acquisition of migratory capability in MDSC was the observed accumulation of MDSC in the spleens of mice at the post-acute phase of inflammation. Therefore, we examined if accumulation of MDSC in the T cell-rich spleen environment contributed to the loss of T splenocytes *in vivo*. Indeed, our study confirmed that myeloid cells that accumulated in the spleens of mice during the post-acute phase of colitis corresponded with the depletion of both $CD4^+$ and $CD8^+$ T splenocyte populations. Although a trend diplaying an inverse relationship between myeloid and T splenocyte populations was established, the decline of T splenocytes slightly predated the expansion of myeloid splenocytes. We believe this may be due to parallel inhibitory functional acquisition by the extramedullary myeloid cells within the spleen, and that this function change occurs in a similar fashion to the functional changes in the myeloid population of the BM. Because both maximum myeloid cells accumulation and T splenocyte depletion peak at days 18-21, we believe the total myeloid cell population exerts maximum

inhibitory function over T splenocytes at these time points. These time points are also consistent with the BM IMC exhibiting very inhibitory profiles. From these data, we proposed that collection of MDSC in the spleens of colitis mice suppressed the proliferation of activated T splenocytes, and that this inhibition of T cell response directly contributed to the resolution of inflammation in the colon. We therefore, assessed the suppressive functions of bone marrow-derived IMC at all time points during colitis *in vitro* to ensure that the MDSC were directly responsible for depleting T splenocyte populations.

4.4.3 MDSC From Inflammation Resolution Phase Potently Inhibit T Cell Proliferation

To further ensure that MDSC directly developed T cell inhibitory properties as a result of persistent inflammation, we assessed IMC isolated from the bone marrow of colitis mice and subjected them to co-culture with activated healthy T splenocytes. As seen from our data, IMC developed T cell inhibitory properties as early as 3d, and the effectiveness of acquired inhibitory function steadily increased through the entire inflammatory DSS treatment, peaking at the early phase of inflammation resolution. Perhaps, most striking was the complete T cell proliferation suppression in the artificial-activation system mediated entirely by MDSC isolation from inflammation recovery time points (12d-21d). This suppression was seen for both $CD4^+$ and $CD8^+$ T cells, and mirrors the *in vivo* data, demonstrating most significant T cell suppression during the same time points. These data confirms that T splenocyte populations are indeed being reduced as a direct function of MDSC accumulation *in vivo*, and that MDSC are most potent inhibitors of T cell function during the tissue repair process. Taken together, these data supports the immunomodulatory role for MDSC at the adaptive immune effector cell level.

T cell inhibitory function within the spleen can be seen from our *in vivo* FACS data, which shows as MDSC collect in the spleen, T splenocyte populations dramatically decrease.

Interestingly, MDSC formation begins very early during the inflammatory response, before the insult moves to the post-acute phase of colitis; however, these suppressive MDSC are unable to mobilize into the circulation. Thus, the immunosuppressive capabilities of MDSC are reserved for times of truly persistent, unresolving inflammation. This system allows the immune system to develop a suppressive subtype of myeloid cells, which are only able to exert their suppressive function during times of persistent inflammatory activation, but not during acute, resolving inflammatory conditions. If inflammatory signals subside quickly, MDSC are not able to migrate out into circulation, and thus are unable to inhibit the immune response; however, if inflammatory signals persist to a point where potently suppressive myeloid cells are required to modulate immune function for the protection of the host, then suppressive myeloid cells gain the ability to enter circulation and carry out suppression of the immune response.

4.5 MDSC Function as Inhibitors of the Inflammatory Response During Chronic Inflammation

In the past, MDSC have been discussed primarily as a harmful, immunosuppressive subtype of cells responsible for enabling tumor immune escape. As seen from our study, we determined a suppressive, non-pathogenic role for MDSC during colitis. As described in our study, we determined that MDSC first acquire T cell suppressive functions that peak at the post-acute stage of colitis inflammation, and that these cells remain sequestered away from T cells in the bone marrow until inflammation persists to the post-acute phase. Our study, in conjunction with other studies, demonstrated that soluble factors produced at the site of inflammation drive these functional changes within the bone marrow. Additionally, previous studies by our lab have described a role for IL-17 in the potentiation of PMN function. In this study, we propose IL-17 also functions to induce the generation of an immunosuppressive subtype of IMC which function

to coordinate resolution of persistent colitis inflammation by inhibiting the expansion of activated T cells. Our model describes a non-pathogenic evolutionary function of MDSC for the control of hyperactivation of the inflammatory response.

Since their discovery in tumor-bearing hosts, MDSC have primarily been studied in the context of cancer models that predominantly examine the specific methods MDSC use to induce immunosuppression, but these studies tend to neglect the naturally-evolved, non-pathogenic role of MDSC; however, expanded MDSC populations have also been reported as essential for the control of immune clearance of foreign tissue during allograft transplantation and pregnancy. During allograft transplantation, MDSC colonize the transplanted tissue and induce tolerance of the allograft, leading to decreased risk of rejection by the immune system of the recipient [127]. Similarly, during pregnancy, MDSC protect the "non-self" fetal tissue from clearance by the type I immune response by inducing tolerance, and helping to generate regulatory T cells [101]. Using the limited information regarding the non-pathogenic functions of MDSC during necessary immune suppression, we proposed that MDSC develop suppressive capabilities as a result of prolonged inflammation as a protective immune inhibitory feedback mechanism. As in pregnancy, MDSC-mediated non-pathogenic inhibition of the immune response is meant to protect the inflamed tissue from additional damage brought on by continuous activation of the inflammatory response. As mentioned in the introduction, recruitment of mature inflammatory effector leukocytes to the site of inflammation results in secretion of many toxic, cell-damaging factors. The infiltration of PMN during inflammation secretes these cytotoxic factors in an effort to eliminate pathogen from sites of non-sterile injury, however these functions are non-specific to pathogen and result in the accumulation of damaged cells within the tissue. During progression to the post-acute phase of colitis, the accumulation of epithelial cell damage results in loss of gut

barrier function, which induces more inflammation that is unable to resolve in the tissue. In this system, inhibition of the type I immune response to pathogen is essential for the resolution of inflammation. Generation of anti-inflammatory, immunosuppressive myeloid cells function to inhibit the inflammatory response to stop the recruitment of tissue-damaging leukocytes, and to promote the transition toward inflammation resolution and tissue repair

4.6 Generation of MDSC Promotes Survival of Tumor Cells

Since their initial discovery, MDSC have been shown to enhance tumor cell survival, even functioning as a clinical marker of cancer severity. Many studies have been performed to elucidate the intricate mechanisms MDSC use to promote tumor survival. As previously discussed many of these mechanisms inhibit T cell immune functions, limiting the ability for T cells to identify and eliminate transformed, pre-tumor cells. Furthermore, these studies have also uncovered the ability for tumors to directly induce MDSC generation through the secretion of cytokines. Our study confirms that the process of altering hematopoiesis through production of secretion of cytokines is a mechanism evolved by the host to combat the overstimulation of the immune response. Under tumor conditions, this protective mechanism for inhibiting excessive tissue damage due to an advanced inflammatory process is co-opted to support the tumor. Indeed, many tumors have demonstrated the ability to either directly increase secretion of cytokines, or to stimulate surrounding fibroblast tissue to perform this function. As a result, hematopoiesis is altered in such a way that mimics the response to advanced inflammation. Bone marrow cells respond to cytokine signals produced by the tumor to develop expanded, suppressive MDSC, which are recruited to the tumor. Once in the tumor tissue, MDCS create a “shield” around the tumor cells, which limits the surveillance of tumor cells by T cells. By inactivating and/or

suppressing any approaching T cells, MDSC circumvent many immunotherapeutic approaches to

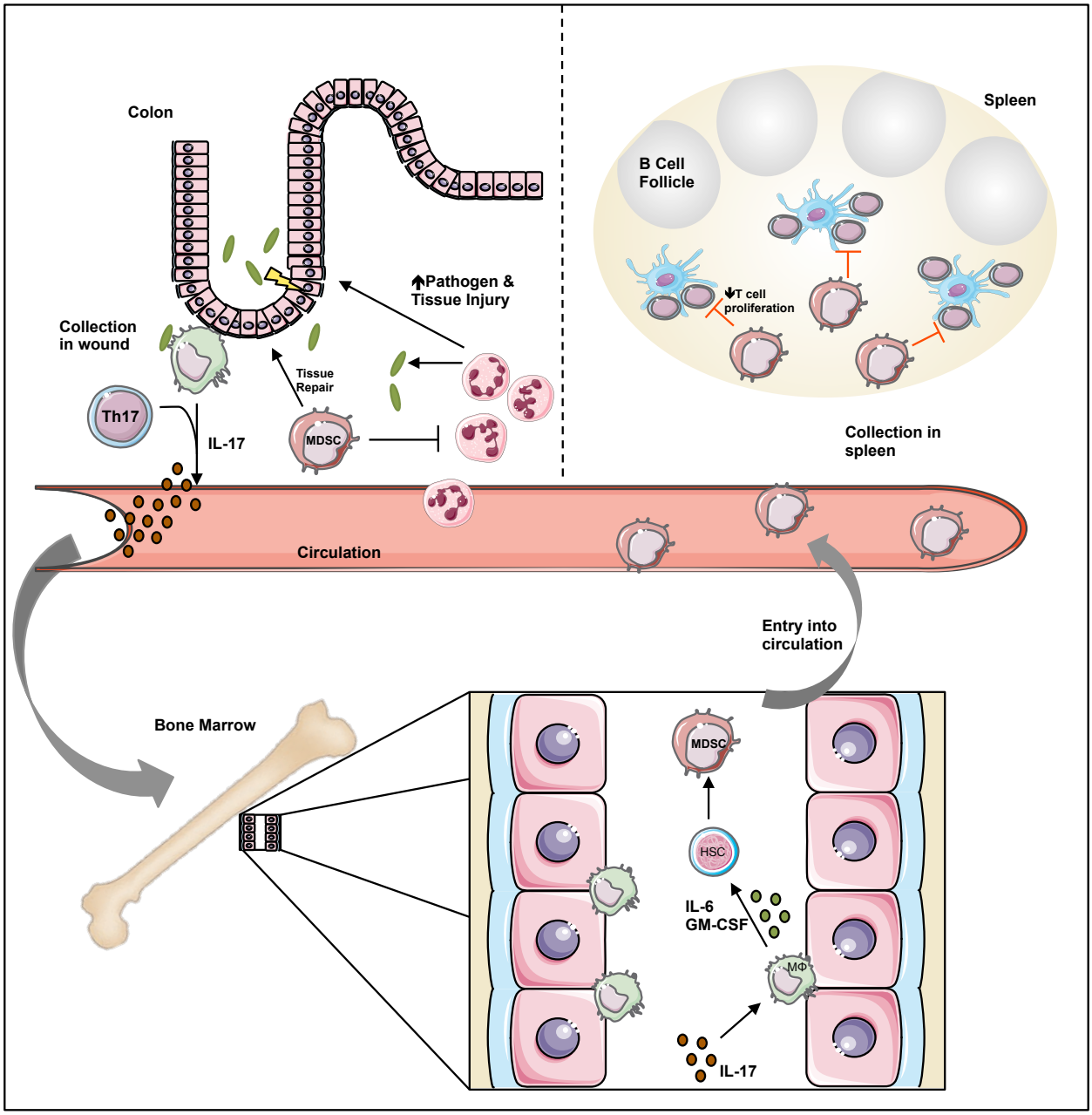

Figure 4-1 MDSC arise during chronic inflammation through increased production of IL-17.

Model of *in vivo* MDSC generation during DSS-induced colitis and recovery. Post-acute colitis results in the upregulation of IL-17 from Th17 cells and other subsets of immune cells. High levels of IL-17 enter circulation, where it acts on BM MΦ, inducing the localized increased expression of IL-6 and GM-CSF. These cytokines induce changes to the transcriptional programming during emergency hematopoiesis leading to generation and expansion of suppressive MDSC. MDSC develop increased CXCR2 expression, which reaches a critical threshold at day 18, resulting in release of MDSC into circulation. After entry into circulation, MDSC collect in the T cell-rich environment of the spleen, and presumably at the site of inflammation, where they function to suppress T cell activation and proliferation, thereby limiting the immune response in favor of tissue repair. Generated using Blood and Immunology" and "Tissues" licensed under CC BY 3.0.

cancer treatment, which focus on bolstering the effectiveness of tumor recognition and clearance by immune cells.

Once in the tumor tissue, MDSC continue to drive the conversion of the microenvironment toward an immunosuppressive phenotype, driving polarization of local immune cells toward suppressive phenotypes, and limiting activation of T cells by inhibiting DC maturation. Although these functions likely occur during MDSC-mediated transition of inflammation toward tissue repair, our data did not investigate the suppressive functions of MDSC on any other immune cells except T cells. In addition to shielding the tumor from detection by the immune system, MDSC also directly promote tumor growth by stimulating secretion of growth and angiogenic factors. We believe, that MDSC that arise from chronic inflammatory conditions promote transition from active inflammation to tissue repair and resolution through a similar mechanism, whereby MDSC inhibit proinflammatory functions of the immune system in favor of tissue repair functions, including reepithelialization and establishment of new blood vessels. MDSC are generated by both chronic inflammation and cancer conditions, and our study demonstrates that MDSC arise during the post-acute phase of inflammation to restrain the immune system from causing further damage to the inflamed tissue. Therefore, it is easy to imagine that MDSC arise during periods of persistent, damaging inflammation, and that inhibition of T cell immune surveillance by MDSC protects any transformed, neoplastic tissue cells from clearance. We believe that this is a possible mechanism of action responsible for the observed tight correlation between chronic inflammation and cancer.

4.7 Targeting MDSC to Control the Immune Response

MDSC have major significance as a therapeutic target for treatment of both autoimmune disease and cancer. As discussed previously, MDSC have been demonstrated as indispensible

cells for inducing necessary immune suppression during pregnancy, and high numbers of MDSC have been shown to correlate with the successful tolerance of transplanted allograft tissue. Furthermore, our study and others illustrate that MDSC, arising during both chronic inflammation and cancer, coordinate immunosuppression. This ability appears to contribute to promotion of inflammatory resolution during persistent inflammation, and drives immune escape of tumor cells during cancer. For these reasons, control of MDSC generation and function present unique therapeutic opportunities for the treatment of both autoimmune disorders and cancer treatment.

By developing therapies that target augmentation of generation of MDSC, scientists can hope to alleviate autoimmune-acquired tissue damage due to overstimulation of the inflammatory response. Rheumatoid arthritis and IBD are two specific disorders that may be improved by selective augmentation of MDSC generation.

Although future therapies that target augmentation of MDSC generation could potentially work to assuage autoimmune disease symptoms, these therapies must be applied carefully, as uncontrolled generation of MDSC has the potential to induce neoplastic cell immune escape. In this case, limitation of MDSC generation would benefit the host.

4.8 Current Perspective for MDSC: The Role of Immunotherapeutics in Treatment of Autoimmune Diseases and Cancer

Currently, many obstacles exist in the treatment strategies of many autoimmune diseases and cancer. These two types of human clinical ailments represent two sides of the same coin, that is overactivation of the immune response leads to conditions of autoimmunity, whereas overinhibition of the same immune effector functions result in failure to clear neoplastic cells. In an effort to correct dysfunction of the immune response, often treatment of one ailment results in

pressure on the immune system to swing too far the other direction. For this reason, it is essential that further studies be conducted with the purpose of understanding the intricate mechanisms that govern each specific immune dysregulation, so that more targeted therapies can be produced, with less risk further compromising the immune responses.

One example of this can be seen in the current treatment strategies of autoimmune inflammatory disorders RA, psoriasis, and IBD. Currently, two major categories of inhibitory antibody therapies have been developed: anti-TNFα and anti-IL-17. While anti-TNFα strategies have been a very successful treatment strategy across all of these disorders, anti-IL-17 has only been successful in RA and psoriasis, but not in IBD. In fact, neutralization of IL-17 has been shown to worsen clinical symptoms of IBD; however no studies have determined why IL-17 promotes inflammation in RA and psoriasis, but is necessary for stabilizing the system in IBD. Through our study, we revealed that IL-17 may play a central role in establishing a feedback mechanism that inhibits continuation of the inflammatory response in the bowel through promoting MDSC generation, however more studies must be conducted to confirm our theory. If IL-17 does coordinate acquisition of inhibitory immune function as our study suggests, then this information would certainly why treatment of IBD patients with anti-IL-17 worsens the clinical presentation.

Treatment with biologic neutralization antibodies, while very promising treatments for limiting the detrimental effects of the immune response, also drastically increase the patients' risk for developing cancers and increasing susceptibility to infections. Such broad inhibition of major pro-inflammatory cytokines has powerful ramifications, which for limited treatment may be necessary, but must be used with caution as overuse of these biologics severely limit many aspects of the immune response.

On a cautiously optimistic note, it seems that overcoming MDSC-mediated immune cell inhibition in conjunction with treatments that boost T cell function is a promising avenue for clinical application in the removal of cancer cells for those tumors that are unable to be removed surgically. Furthermore, as more information about MDSC generation is gathered, it may become possible to manage when MDSC are produced. One such example of this type of treatment strategy is for treatment of cancer. Following surgical or radiological removal of cancer cells, the risk for developing a more aggressive relapsing cancer is always a concern for clinicians. Because MDSC likely coordinate the growth of new tumor cells, immediate treatment with MDSC inhibitors may significantly reduce the risk for relapse, thereby improving survival in these patients.

In conclusion, the more information that is gained from studies on MDSC generation during many different conditions will contribute significantly to new therapeutic approaches for treatment of many diseases of the immune system. Targeting these very special cells either for the promotion of immune inhibition, or for limitation of immune inhibition may hold the key for tailoring immune function, although we must do so cautiously.

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