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ADAM10 overexpression dysregulates Notch signaling in favor of myeloid derived suppressor cell (MDSC) accumulation that differentially modulates the host response depending on immune stimuli and interaction with mast cells.

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

By

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DEDICATION

This dissertation is dedicated to my family for their unconditional love and support throughout my life and academic pursuits. I am especially grateful for my parents, Jamal and Sheren Saleem. As refugees, they had the courage to start anew in United States and strived to provide us the opportunities to shape our future. Their determination and persistency in overcoming adversity provided the direction and motivation I needed to be a successful individual both in and outside of the classroom. My brother, Botan Saleem, whose ongoing battle with mental illness has allowed me to remain committed and focused to scientific exploration. My sister, Khoshe Saleem, whose sense of responsibility and commitment to my family, afforded me the flexibility to embark on my PhD training. My sister, Chawan Saleem, for despite being much younger, has proved to be a source of inspiration and exemplary figure for helping me tackle the social norms of my society and transform into a confident Kurdish-American woman. My brother, Choman Saleem, for helping me find the courage to push the boundaries of science and cultivate a sense of humor while do so. Finally, my uncle, Sherwan Saleem, whose his friendship has served as a significant support system since the initial stages of my graduate training.

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LIST OF ABBREVIATIONS

-/-	Homozygous deletion of a gene
2.4G2	mAb recognizing the stalk region of murine CD23
ADAM	A disintegrin and metalloproteinase
APC	Allophycocyanin
APP	Amyloid plaque protein
Arg1	Arginase-1
AT	Adoptive transfer
B220	mAb recognizing murine CD45R; mouse B cell marker
BAC	Bacterial artificial chromosome
BM	Bone marrow
BMP	Bovine metalloprotease
C57BL/6	Inbred mouse strain
CD	Clusters of differentiation
cDNA	Complementary DNA
CLP	Common lymphoid progenitor
CLMP	Common lymphoid-myeloid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
DLL	Delta-like Notch ligand
DMSO	Dimethyl sulfoxide
DN	Double negative

DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
ECD	Electrochemical detector or PE/Texas-Red
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ES	Embryonic stem cells
FACS	Fluorescence active cell sorting
FBS	Fetal bovine serum
FcERI	The high affinity IgE receptor
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte-colony stimulating factor
GI254023X	ADAM10 selective inhibitor
GM-CSF	Granulocyte macrophage-colony stimulating factor
G-MDSCs	Granulocytic-myeloid derived suppressor cells
GSI	γ -secretase inhibitor
GvHD	Graft vs. host disease
HA	Hemagglutinin
HDC	Histidine decarboxylase
Hes	Hairy enhancer of split
HGF	Hepatocyte growth factor
HNSCC	Head and neck squamous cell carcinoma

HRs	Histamine receptors
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
IBD	Inflammatory bowel disease
ICD	Intracellular domain
IFNs	Interferons
ILs	Interleukins
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
i.v.	Intravenous
kDa	Kilodalton
kuz	Kuzbanian
Lag-3	Lymphocyte activated gene 3
L-Arg	L-arginine
Lin	Lineage
LM	Littermate
LSK	Lineage ⁻ SCa ⁺ c-Kit ⁺ bone marrow cells
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MCs	Mast cells

M-CSF	Macrophage-colony stimulating factor
MDSCs	Myeloid derived suppressor cells
MEFs	Mouse embryonic fibroblasts
METs	B16 tumor nodule metastases
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MEP	Myeloid erythroid progenitors
M-MDSCs	Monocytic myeloid derived suppressor cells
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MZB	Marginal zone B cells
Nb	<i>Nippostrongylus brasiliensis</i>
NF- κ B	Nuclear factor kappa B
NICD	Notch intracellular domain
NO	Nitric oxide
NRR	Negative regulatory region
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PIR-A/B	Paired immunoglobulin-like receptors A and B

RASV	<i>Salmonella enterica</i> Serovar <i>Typhimurium</i> vaccine
RBP-J κ	Canonical Notch transcription factor
RCC	Renal cell carcinoma
RIP	Receptor intramembrane proteolysis
RNA	Ribonucleic acid
ROR A/C	Orphan nuclear receptor A or C
ROS	Reactive oxygen species
s.c.	Subcutaneous
SCI	Spinal cord injury
SDF-1 α	stromal cell derived factor 1 α
SHP-1	SH ₂ -homology-containing protein-tyrosine phosphatase-1
TACE	TNF- α converting enzyme
T-ALL	T cell acute lymphocytic leukemia
TCR	T cell receptor
TGF- β	Transforming growth factor - Beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TLRs	Toll-like receptors
TMEV	Theiler's murine encephalomyelitis
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WPG	Whole β -glucan particle

WT

Wild-type

ABSTRACT

ADAM10 dysregulates Notch signaling in favor of myeloid derived suppressor cell accumulation (MDSC) that differentially modulates the host response depending on immune stimuli and interaction with mast cells.

By Sheinei J. Saleem, PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Director: Daniel H. Conrad, Professor, Department of Microbiology and Immunology

Although the physiological consequences of Notch signaling in hematopoiesis have been extensively studied, the differential effects of individual notch cleavage products remain to be elucidated. Given that a disintegrin and metalloproteinase 10 (ADAM10) is a critical regulator of Notch and that its deletion is embryonically lethal, we generated transgenic mice that overexpress ADAM10 at early stages of lymphoid and myeloid development (A10Tg). ADAM10 transgene expression alters hematopoiesis post-hematopoietic Lineage⁻Sca-1⁺c-kit⁺ (LSK) subset differentiation but prior to lineage commitment of progenitor populations. This results in delayed T cell development, abrogated B2 cell development, and dramatic expansion of functionally active myeloid derived suppressor cells (MDSCs) in A10Tg mice. Given ADAM10's role in Notch signaling, we hypothesized that the observed hematopoietic alterations may be a consequence of perturbed Notch signaling. In fact, blockade of ADAM10 (S2) rescues B cell development and reduces myeloid cells in A10Tg LSKs. Inhibition of γ -secretase (S3) in wild type (WT) LSKs results in enhanced myelopoiesis, mimicking the

phenotype of A10Tg mice. Collectively, these findings indicate that the differential cleavage of Notch into S2 and S3 products regulated by ADAM10 is critical for hematopoietic cell-fate determination.

Albeit arising in a tumor-free host, A10Tg MDSCs are functionally and phenotypically analogous to tumor-derived MDSCs. A10Tg MDSCs inhibit T cell activation *in vitro*, and inhibit adoptive immunotherapy (AIT) of metastatic melanoma *in vivo*, which can be reversed with MDSC depletion. Intriguingly, A10Tg mice are resistant to parasitic infection upon inoculation of *Nippostrongylus brasiliensis*. However, depletion of MDSCs abrogates this response, while adoptive transfer (AT) of MDSCs into WT mice increases their resistance. This polarized activity of MDSCs is heavily dependent upon interaction with mast cells (MCs). In fact, B16 melanoma cells metastasize more rapidly in WT mice infused with MDSCs when compared to MC-deficient mice (*Kit^{Wsh/Wsh}*), with or without MDSC AT. Parallel to B16 progression, the ability of MDSCs to promote anti-Nb immunity is significantly diminished in MC-deficient (*Kit^{Wsh/Wsh}*) mice even with MDSC AT. This augmentation of MDSC activity in the presence of MCs is further corroborated by *in vitro* co-culture assays that demonstrate a synergistic increase in cytokine production. Furthermore, MDSCs preferentially migrate to the liver in a MC-dependent manner. This interaction is mediated by MC-released histamine. In fact, MDSCs express histamine receptors (HR) and histamine induces MDSC survival, proliferation, and activation. We demonstrate that MDSC activity is abrogated with histamine blockade. Moreover, in humans, allergic patients present with an increase in MDSC population, and MDSCs purified from a stage

I breast cancer patient exhibit increased survival in the presence of histamine. Taken together, our studies indicate that MCs and MC-released histamine are critical for the observed functional duality of MDSCs, ranging from immunosuppressive to immunosupportive, depending on the disease state.

INTRODUCTION

I. ADAM10 is critical for hematopoietic cell differentiation.

Disintegrin and metalloproteinases (ADAMs) regulate cell signaling pathways by cleaving the extracellular domains of membrane-bound receptors and ligands. Consequently, these proteins serve as initiators for signaling pathways that require regulated intramembrane proteolysis (RIP) of receptor:ligand complexes. The shedding of membrane anchored proteins releases soluble fragments into the extracellular milieu that can subsequently modulate signaling events. Although ectodomain shedding is thought to occur constitutively, RIP requires the binding of ligands expressed on adjacent cells. Numerous receptors including Notch undergo regulated proteolysis of the receptor: ligand complex to release their intracellular domains (ICD), that subsequently translocates to the nucleus and alters gene expression. Mutations in the negative regulatory region (NRR) of ADAM substrates can cause ligand-independent intramembrane proteolysis, resulting in excessive ICD signaling and numerous pathological conditions(1-5).

The prototypical ADAM contains an inhibitory pro-domain, a highly conserved metalloprotease domain, a disintegrin domain conferring substrate specificity, a cysteine-rich region, a transmembrane portion, and a cytoplasmic tail capable of binding SH3 domains(6, 7). Among the 38 ADAMs identified to date, the most studied are ADAMs 8, 9, 10, 12, 15, 17, and 33, that contain the conserved zinc-binding consensus motif which confers proteolytic activity to the protease domain. Within this subset, ADAM10 has emerged as an important mediator of ectodomain shedding and RIP of multiple substrates

including epidermal growth factor (EGF), Fas-ligand, CD23, and most notably Notch. This proteolytic processing is critical for appropriate cellular processes and its dysregulation results in the pathogenesis of multiple disease states, including Alzheimer's, cancer, and inflammation. Thus, there is growing interest in elucidating ADAM10 as well as its inhibition for pharmacologic treatment. However, determination of the physiologic consequences of ADAM10-mediated cleavage events has been limited by lethality of ADAM10-null murine embryos. These same studies highlight the important role of ADAM10 in hematopoietic cell development mediated via regulation of Notch signaling(8, 9).

Many studies have indicated the importance of Notch signaling in lymphocyte development. The Notch signaling pathway is highly conserved, consisting of four families of receptors (Notch1-4) that interact with ligands (Jagged and Delta) expressed by neighboring cells(10-12). Following ribosomal synthesis, the Notch receptor undergoes a furin-mediated maturation at site 1 (S1) in the Golgi apparatus prior to trafficking to the cell surface. At the surface, Notch is expressed as an integral membrane protein, consisting of both extracellular (NEXT) and intracellular domains (NICD). Once engaged with its ligand, the extracellular domain undergoes an ADAM10-mediated cleavage at site 2 (S2). This event generates a substrate for the γ -secretase complex to perform a final cleavage of Notch at site 3 (S3), releasing the transcriptionally active NICD(3). Several studies have reported the accumulation of intact receptor and the S2 product as a result of ADAM10 and γ -secretase blockade, respectively(2, 4)-CITATION_IS_EMPTY. Although inhibition of both enzymes prevents NICD activation, the

consequences of accumulation of these different cleaved products on hematopoiesis remains to be determined.

A. Discovery of ADAM10 as main sheddase of Notch1.

ADAM10 has been characterized after extensive study of its drosophila homolog, Kuzbanian (kuz). Rooke *et al.* initially identified Kuz in 1996 from the generation of kuz deficient embryos. The authors revealed its essential role in lateral inhibition required for development of peripheral and central nervous systems. Cloning and sequencing analysis of kuz demonstrated the presence of disintegrin and metalloproteinase domains that exhibited a 43% amino acid identity to its mammalian homolog, bovine metalloprotease (BMP)(1-6). This was isolated from myelin and shown to cleave myelin basic protein. For this reason, BMP, which was later named ADAM10, was initially well characterized in the cleavage of amyloid plaque proteins and Alzheimer's disease(7-9). Later, by overexpressing dominate negative (DN) mutants of kuz in drosophila and *Xenopus*, Pan *et al.* demonstrated the requirement for kuz to initiate RIP of Notch (10-12). The contribution of ADAM10 is further supported by the generation of ADAM10-deficient embryos and conditional knockout mice. These studies, which mostly focused on Notch 1, indicated impaired development in the absence of ADAM10 and consequent impaired Notch signaling(6,13-16). As mentioned earlier, ADAM10-mediated S2 cleavage generates a substrate for γ -secretase complexes to perform the S3 cleavage of the receptor, releasing NICD that subsequently translocates to the nucleus and induces transcriptional activation (Fig. 1). The NICD complexes with the transcription factor

RBP-J κ , and induces transcription of Notch target genes, including Hairy enhancer of split (Hes1), Hes5, and Deltex-1(4,9).

Although ADAM10 is now considered the main sheddase of the Notch1 Receptor, it was initially considered to be highly controversial(3-5,12). Several groups reported that ADAM17 (TNF- α converting enzyme, TACE) conducted the S2 cleavage of Notch1 receptor(3,5,13,15,16). In fact, loading cell membrane fragments from Notch-transfected HeLa cells onto RED-TSK columns resulted in co-elution of Notch receptor cleavage products with ADAM17, rather than ADAM10. Moreover, an *in vitro* monocytic cell differentiation experiment demonstrated ADAM17's ability to cleave Notch1 and direct Notch-dependent monocyte differentiation(5). Thus, ADAM17 is often referenced in the literature as the relevant proteinase that initiates Notch signaling. Although these findings were in direct contrast to studies of kuz in drosophila, the authors could not rule out ADAM10-directed Notch cleavage. They suggest that ADAM10 and ADAM17 may be functionally redundant *in vivo*. It is plausible that in the absence of ADAM17, ADAM10 can serve a compensatory role and vice versa. In support of this, several studies have demonstrated that ADAM10 can cleave many ADAM17 substrates from ADAM17^{-/-} murine embryonic fibroblasts (MEFs)(17). However, the exclusive contribution of ADAM10 in Notch activation became more acceptable following the generation of ADAM10-deficient mouse embryos that displayed many features observed in nonviable Notch1^{-/-} embryos(12,18). In contrast, embryonic loss of ADAM17 did not result in a Notch1^{-/-} phenotype(19). Following these observations, two groups utilizing ADAM10^{-/-} MEFs reported that while multiple

proteases can perform ligand-independent proteolysis of Notch1, ADAM10 is required for ligand-dependent cleavage(16,20). Thus, ADAM10 may play a more critical role in Notch signaling than earlier *in vitro* studies predicted. Additional studies are required to further elucidate ADAM10-mediated cleavage events in hematopoietic development but are limited by in utero lethality of ADAM10-null embryos.

B. ADAM10 in lymphocyte development.

Notch1 signaling is essential for the development of thymocyte precursors. This is best illustrated by the presence of thymic B cells in mice that lack Notch1 expression in common lymphoid progenitors (CLPs)(21). Additionally, enforced Notch1 signaling in BM progenitors expressing the constitutively active NICD promotes T cell development(22). In fact, human mutations in the NRR surrounding the S2 cleavage site of Notch1 results in ligand-independent proteolysis and excessive Notch1 activation, ultimately causing T cell acute lymphocytic leukemia (T-ALL). This mutation accounts for approximately 50 percent of all T-ALL cases(4). Multiple groups have reported effective use of γ -secretase inhibitors (GSIs) in limiting T cell development *in vitro* and in mouse models of T-ALL(23,24). However, GSIs cause gastrointestinal disease and must be coupled with potent anti-inflammatory drugs, such as dexamethasone, in T-ALL mouse models(23). Thus, there has been great interest in elucidating the role of ADAMs in Notch1-mediated thymocyte development. Manilay *et al.* circumvented the limitation of ADAM10^{-/-} embryo lethality by generating transgenic mice that overexpress the dominant negative form of ADAM10 (dnKuz) under control of the T cell-specific

promoter, *lck*(25). *dnKuz* expression caused a partial block in thymocyte development between the double negative (DN) and double positive (DP) stages as indicated by decreased TCR β expression and premature down-regulation of CD25. While these findings were also observed in *Notch1*-deficient thymocytes, there were discrete differences in thymocyte development and gene expression between *dnKuz* and *Notch1* deficient mice(26). *dnKuz* mice have reduced levels of DN thymocytes and $\gamma\delta$ T cells, whereas conditional *Notch1*-deficient mice do not, suggesting that ADAM10 may regulate early thymocyte development by processing other substrates in addition to *Notch1*. *Notch* ligand, DLL-1, rescued thymocyte development in *dnKuz* mice in a non-cell autonomous manner, indicating that ADAM10 may regulate thymocyte development by processing DLL-1 expressed on adjacent cells. This is supported by studies of MEFs in which ADAM10 was demonstrated to be the main sheddase of DLL-1(27). Further examination of ADAM10's role in thymocyte development awaited the production of ADAM10-floxed mice, which has resulted in significant progress toward elucidating the physiologic impacts of ADAM10-mediated cleavage events. By utilizing *lck-cre* transgenic mice, Tian *et al.* reported impaired development and suppressed *Notch1* signaling in ADAM10-deficient thymocytes(14). ADAM10-deficient thymocyte development resembled that of *dnKuz* and *lck*-directed *Notch1*-deficient mice. The authors further demonstrated that production of the NICD was not detectable in ADAM10-deficient thymocytes.

B2 lymphocytes, which comprise the majority of circulating B cells, develop from CLPs and differentiate into pro-, then pre-, and ultimately immature B cells prior to

exiting the BM. Given that the Notch1 cleavage site recognized by ADAMs is not present in murine Notch2, and that B cells express Notch2 rather than Notch1, ADAM10 has not been attributed to B2 B cell development(5,13,28-31). Just as Notch signaling promotes CLP commitment to the T cell lineage, it prevents B lineage fate. Thus, multiple studies have demonstrated that enforced expression of active NICD in BM progenitors completely abrogates B2 cell development(22,31). Following exit from the BM, B2 cells enter a transitional stage and undergo further maturation in the spleen, where the majority differentiate into follicular B cells, while a subset develop into cells of the marginal zone B (MZB) cell lineage, including pre-MZBs and MZB cells. By generating B-cell specific Notch2 knockout mice, Saito *et al.* demonstrated that Notch2 signaling is required for development of the MZB cell lineage, which initiates immune responses to blood-borne infections and transports antigen into the spleen follicles(13,32). Moreover, by deleting ADAM10 in a mature B cell-specific manner with CD19-cre knockin mice, Gibb *et al.* also demonstrated an absolute requirement for ADAM10 in MZB development(33). Analysis of Notch target gene expression revealed a dramatic defect in Notch2 signaling. Furthermore, in contrast to WT B cells, ADAM10-null cells were completely unresponsive to DLL-1-induced Notch stimulation. These findings not only revealed the importance of ADAM10 in B cell development, but also demonstrated that ADAM10 is responsible for activating RIP-mediated signaling through Notch2. In contrast to reports of ADAM10 deletion in thymocytes, deletion in mature B cells did not result in any compensatory Notch2 cleavage by other proteases, including

ADAM17. Thus, ADAM10 may be the only protease that can recognize the Notch2 cleavage site, which is distinct from the Notch1 site(5).

C. ADAM10 in myeloid cell development.

In the classical model of hematopoiesis, hematopoietic stem cells (HSCs) differentiate to yield common myeloid progenitors (CMPs) and CLPs(34). CMPs undergo further differentiation into mature myeloid cells, including the recently identified myeloid-derived suppressor cells (MDSCs). Due to the deleterious role of these suppressor cells in anti-tumor responses, myeloid differentiation has been the subject of many recent investigations(35). As discussed further below, numerous hematopoietic pathways, including Notch signaling, have been implicated in MDSC development but remain controversial. Several investigators have reported that alterations in Notch signaling have minimal effects on the myeloid compartment(22,36,37). Yet, Kawamata *et al.* reported that constitutive Notch signaling promotes myeloid differentiation in a non-cell autonomous manner(31). This is supported by a report of abrogated B cell and myeloid cell development in mice deficient in downstream targets of Notch(38). However, other studies have indicated that Notch signaling inhibits myeloid differentiation(39,40). Qyang *et al.* demonstrated that blockade of Notch signaling at the γ -secretase cleavage site induces myeloid accumulation(40).

Many of these alterations were observed in mice with altered lymphocyte development. This suggests that ADAM10 directed Notch signaling may modulate the differentiation pathway of CLPs and CMPs from a common upstream progenitor. Two

groups have recently described common myelo-lymphoid progenitors (CMLPs) that are indistinguishable from HSCs(34,41). Although these multipotent cells often commit to the B or T cell lineage, they retain the potential for myeloid development. Therefore, alterations in Notch signaling during lymphocyte development could also affect myelopoiesis. Collectively, these findings indicate that myeloid differentiation may be regulated by the signal strength and temporal stage of Notch signaling. Therefore, examination of other Notch regulators, including ADAM10, could clarify the role of Notch signaling in myeloid differentiation.

II. Development of myeloid derived suppressor cells (MDSCs).

As mentioned earlier, hematopoiesis is the hierarchal differentiation of multipotent progenitors into mature blood cells of various lineages and functions. The BM microenvironment provides a complex network of cytokines, transcription factors, and intercellular signaling pathways, to tightly regulate the progressive lineage commitment of hematopoietic stem cells(42). HSCs themselves are characterized by a lack of lineage markers and by expression of the receptor tyrosine kinase (RTK) c-kit and the surface protein Sca-1, together termed Lin⁻Sca-1⁺c-kit⁺ (LSK)(43). The expression of Flt3 RTK and CD34 further subdivide the LSK compartment into self-renewing long and short-term HSC populations as well as a multipotent progenitor (MPP) population. This hierarchy descends deeper into lymphoid and myeloid compartments where the progenitors are again subdivided into three distinct populations based upon CD34 and low affinity IgG Fc receptors (FcγRII/RIII). These include CD34⁺FcγRII/III^{lo} Common

Myeloid Progenitors (CMP), CD34⁺FcgRII/III^{hi} Granulocyte-Macrophage Progenitors (GMP), and CD34⁻FcgRII/III^{lo} Megakaryocyte-Erythroid Progenitors (MEP)(44).

In the classical dichotomous model of differentiation, MPPs give rise to either lineage restricted common myelo-erythroid progenitors (CMEP) or common lymphoid progenitors (CLPs)(45). This process is dependent upon the expression of three proteins: c-kit, the IL-7 receptor and recombination activation gene 1 (RAG1)(46). The relative expression of these species act as a 'switch' that determines the ultimate hematopoietic endpoint. While RAG1 and IL-7R expressing CLPs give rise to T and B lymphocytes, c-Kit⁺ CMEPs are observed to generate myeloid and erythroid cells(47,48). This classical model has been recently challenged by the idea that lineage restriction is not necessarily permanent. Several independent observations indicate that early thymocyte and B cell progenitors retain myeloid potential, leading to an alternative, 'myeloid-based' mechanism of hematopoiesis(34,41). CMLPs that are phenotypically indistinguishable from LSKs have been observed, and these cells give rise to either B cells, T cells, or myeloid cells. The myeloid-based mechanism excludes the existence of CLPs, stating that differentiation occurs from these CMLP and CMEP progenitors(49). This suggests that, although committed toward T or B lineage, progenitor cells retain the potential towards myeloid lineage. The existence of progenitor myeloid, B and T (p-MBT) cells which are committed B or T cells, yet retain the ability to revert back to myeloid cells further support this possibility(50). Additionally, the observation of several leukemic disease states containing cells of both myeloid and lymphoid origin indicate that a close relationship exists between p-MTB progenitors(34). This process is heavily influenced

by the microvasculature in terms of cytokines and signaling pathways it can provide to a developing HSC. Therefore, the unique cytokine profiles in any given pathologic state can also heavily impact hematopoiesis and thus the subsequent immune response.

In response to physiological insult, such as that generated by a pathogenic organism, the host enters a state of ‘emergency’ hematopoiesis characterized by increased recruitment of myeloid lineage cells such as neutrophils and macrophages(51). However, during chronic inflammatory stimuli such as in cancer progression, severe hematopoietic dysregulation can occur at the CMLP or GMP/CMP stages of differentiation(52). This results in the premature BM recruitment of a heterogeneous population of mononuclear ($CD11b^+Gr-1^{int}Ly6G^-Ly6C^+$) and polymorphonuclear ($CD11b^+Gr-1^{high}Ly6G^+Ly6C^-$) MDSCs(35). As discussed below, MDSCs accumulate in response to a variety of cytokine and transcription factor alterations unique to a given disease-state such as cancer, the natural aging process, solid organ transplantation, parasitic infections, sepsis, autoimmune disease, trauma, and burns(53-56). The phenotypic and functional characteristics of MDSCs strongly underscore their myeloid origin. Common cytokines involved in myeloid development have been implicated in the induction of MDSCs. These factors include macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin-6 (IL-6), vascular endothelia growth factor (VEGF), and granulocyte-colony stimulating factor (G-CSF). Several transcription factors involved in myelopoiesis such as interferon-regulatory factor 8 (IRF-8), CAAT/enhancer binding protein-beta (C/EBP- β), and PU.1 have also been demonstrated to regulate MDSC development(57). In addition to myeloid

differentiation antigen Gr-1 and CD11b, MDSCs also express various other markers that are traditionally used to define myeloid cells such as CX₃CR1, CCR2, CXCL10, CD206, and IL-1B. In fact, CD11b⁺Gr-1⁺CCR2⁺CX₃CR1^{low} MDSCs can be considered as inflammatory monocytes and can differentiate into DC and macrophages under appropriate stimuli. The expression of IL-4Ra further supports the differentiation of MDSCs into monocytic (IL-4Ra⁺) and polymorphonuclear cells (IL-4Ra⁻) with the monocytic fraction expressing a more substantial immunosuppressive character due to its ability to secrete IL-13 and IFN- γ (58,59). Recent investigations demonstrate that, at the tumor site, MDSCs bear several classically activated (M1) and alternatively activated (M2) macrophage phenotypic and functional characteristics(59). MDSCs express IL-1B, TNF- α , CXCL10, and *inducible* nitric oxide synthase (iNOS) that is characteristic to M1 macrophages as well as CD206, and arginase I (Arg I), which are M2 specific. Since MDSC development and proliferation occur in an asynchronous manner along the differentiation of CMPs and GMPs toward mature myeloid cells, it is not surprising that they retain a ‘fluid’ spectrum of myeloid cell characteristics.

Despite the ambiguity surrounding their origin, MDSCs are most prominently recognized for their role in the anti-tumor immune response. MDSCs exert their pro-neoplastic effects through the release of small soluble oxidizers, the impairment of T-cell-antigen recognition, and the depletion of essential amino acids from the local extracellular environment, all ultimately leading to suppression of T cell responses(60-64). Additionally, through overproduction of cytokines and angiogenic factors, MDSCs shift immune regulation to a state favoring both tumor escape and proliferation(64).

Although the biological and functional properties of myeloid derived suppressor cells have been well characterized and described elsewhere, the mechanism(s) of their development remains controversial(35,65,66). This review outlines the role of commonly implicated cytokines and cytokine-mediated transcription factors in MDSC expansion. Furthermore, several controversial mechanisms of MDSC mobilization are discussed, including the recent observation of MDSC expansion induced by Notch-mediated hematopoietic dysregulation.

A. Cytokine and cytokine-induced transcriptional regulation of MDSC expansion.

A1. Interleukin-6. As mentioned earlier, emergency granulopoiesis is the natural immune response to an infection, which results in the overproduction of myeloid cells. Through differentiation, these myeloid cells afford increased populations of monocytes, granulocytes, neutrophils, and more recently, MDSCs in the circulatory system to serve as regulators of the immune response(67). Inflammatory cytokines play a critical role in this process, particularly interleukin 6 (IL-6). IL-6 is a pleiotropic cytokine secreted by numerous immune and non-immune cells. In a state of emergency granulopoiesis, IL-6 induces the production of acute phase proteins and antibodies by B lymphocytes, while simultaneously mediating hematopoietic progenitor differentiation(68). One of the multiple biological activities of IL-6 comes from its interaction with the CAAT/enhancer binding protein-beta (C/EBP- β) protein, a transcription factor of the leucine zipper family(69). C/EBP transcription factors regulate granulopoiesis; C/EBP- α is required in steady-state granulopoiesis, while C/EBP- β is exploited as a mediator for emergency

granulopoiesis. C/EBP- α is itself an activator for several requisite genes in myeloid development, in particular the granulocyte-colony stimulating factor receptor (G-CSFR)(70). In the absence of C/EBP- α , abnormal myeloid differentiation from CMP to GMP has been reported(71). Although C/EBP- β has been observed during myeloid differentiation, its abrogation does not result in abnormal granulopoiesis. Nonetheless, C/EBP- β appears to specifically affect MDSC production, as myeloid precursors in C/EBP- $\beta^{-/-}$ mice are unable to differentiate into MDSCs(72,73). Additional observations suggest that the role of C/EBP- β extends beyond being requisite for MDSC development; C/EBP- β itself may exert a pro-MDSC effect. *In vitro* experiments have indicated that C/EBP- β promotes the generation of functionally active MDSCs in the presence of IL-6 and the granulocyte-monocyte colony stimulating factor (GM-CSF)(67). Furthermore, using an *in vivo* murine model, upregulation of C/EBP- β has been shown to correlate with an increase in the degree of immunosuppression induced by a growing tumor(67,74). While not directly attributable to MDSC production, this observation is consistent with the immune suppression expected from enhanced MDSC expansion.

In direct support of the myeloid-based model of differentiation, IL-6 acts on lymphoid cells possessing myeloid potential, favoring myelopoiesis. The importance of IL-6 is clearly underscored in transgenic mice containing a deletion of the 5' inositol phosphatase (SHIP) gene. This modification mimics a state of emergency granulopoiesis with concomitant overproduction of IL-6(75). As a consequence, these mice phenotypically exhibit severe splenomegaly due to the accumulation of functionally active CD11b⁺Gr-1⁺ cells. Like tumor-derived MDSCs, these cells significantly impair

priming of allogeneic T cell responses and render the animals less susceptible to Graft versus Host Disease (GVHD)(76,77).

A2. GM-CSF. The granulocyte-macrophage colony stimulating factor (GM-CSF) is recognized as a critical hematopoietic cytokine, mediating the differentiation of myeloid progenitors into granulocytes, eosinophils, monocytes, megakaryocytes, and erythrocytes(1). Remarkably, depending on the concentration of GM-CSF, this factor can exert either a stimulatory or an inhibitory effect on the immune system. In low concentrations, GM-CSF enhances antigen presentation by dendritic cells (DC) as well as cytotoxic CD8⁺ and T helper CD4⁺ T cell responses(78). However, as the concentration of GM-CSF is increased, downregulation of DC differentiation and induction of CD11b⁺Gr-1⁺ MDSC accumulation can occur(79). Ultimately, this process results in a state of severe immunosuppression, as observed in most cancers. The accumulation of MDSCs is believed to be a specific consequence of GM-CSF's ability to skew hematopoiesis towards myeloid development. Several studies have reported GM-CSF induced myelopoiesis that is consistent with the myeloid-based model of hematopoiesis(80-82). Transgenic expression of GM-CSFR in hematopoietic tissue results in a preferential shift towards myeloid progenitors at the CMP, GMP and MEP stages(83). Accordingly, these subpopulations favor myeloid development at the expense of lymphoid committed progenitors, hence preventing subsequent lymphopoiesis(84). This is further supported by the observation of myeloid development from committed CLPs when the IL-2RB receptor is inserted into CLPs to upregulate GM-CSFR(85). Accordingly, it is not unreasonable to believe that excessive GM-CSF production, as

seen in conditions leading to MDSC accumulation, can skew CMLP and CMEP lineage commitment towards dysregulated myelopoiesis.

A3. G-CSF. The granulocyte-colony stimulating factor (G-CSF) and its receptor are critical regulators of steady state and emergency granulopoiesis. G-CSF promotes the survival, mobilization, and proliferation of myeloid progenitor cells alongside neutrophil differentiation. G-CSF likewise enhances myriad neutrophil functions including phagocytosis, cytokine production, antibody-dependent cellular cytotoxicity, and bactericidal activity(86,87). Mice deficient in G-CSF or G-CSFR suffer from neutropenia and succumb to bacterial infections due to the decreased activity of resident neutrophils(87). *In vivo* and *in vitro* administration of G-CSF reflects its immunomodulatory effects. In the clinical setting, G-CSF is used to treat neutropenia resulting from congenital pathologies or immunosuppressive therapy. However, a fraction of patients receiving G-CSF have developed myeloproliferative disorders such as acute myeloid leukemia (AML)(88). In addition, G-CSF administration can reduce DC and cytotoxic T cell activity, and promote the induction of IL-10 producing myeloid cells(79).

G-CSF mediated MDSC accumulation has been reported in both tumor models and *in vitro* studies(55,64). This process is attributed to the interplay between G-CSF and several cytokines and transcription factors known to induce MDSCs. In this case, the relationship with other mediators is immediately apparent: the transcriptional initiation site of G-CSF itself contains binding sites for both IL-6 and NFkB. While the role of IL-6 was clearly outlined earlier, NFkB provides a second pathway by which MDSC

accumulation may occur. In fact, MDSC accumulation has been observed in pathologies during which NF κ B is activated through toll-like receptor (TLR) mediated myeloid differentiation primary response gene 88 (MyD88) signaling, including sepsis, trauma, and particular microbial and viral infections(73,89). Moreover, other inflammatory mediators implicated in MDSC expansion such as IL-1 and IL-17 also activate G-CSF(90-94). Additionally, the G-CSF responsive genes, PU.1 and C/EBP- β are positively correlated with MDSC upregulation(38,94).

Nonetheless, the primary consequences of G-CSF mediated myelopoiesis emerge from activation of the G-CSF receptor (G-CSFR) in the BM. Following activation, G-CSFR initiates signaling cascade through the Janus Kinase (Jak) / signal transducer and activator of transcription (STAT) pathway(95). STAT proteins are latent cytoplasmic transcription factors activated by a variety of hematopoietic and immune-mediated cytokine receptors. The critical role STATs in hematopoiesis is illustrated by the close association between lymphoproliferative and myeloproliferative disorders with dysregulated STAT signaling(96). In hematopoietic tissue, G-CSFR is the main activator of STAT3 and, to a lesser degree, STAT1 and 5(95). Phosphorylation of STAT3 is markedly increased in tumor-derived MDSCs, and inhibition of STAT3 phosphorylation in hematopoietic progenitors markedly diminishes MDSC expansion(97). Likewise, the activity of STAT3 in myeloid progenitors prevents differentiation and induces immature myeloid cell expansion(94). The downstream signaling partners of STAT3, in particular S100A8 and S100A9 also modulate behavior in the myeloid compartment. Overexpression of S100A9 results in an enhanced c-kit⁺ myeloid compartment in the

BM(98). S100A9 acts in concert with S100A8 to inhibit DC differentiation and promote MDSC expansion. Further MDSC-mediated immune suppression and survival is mediated through STAT1 and STAT5 pathways(73). Given the wide range of G-CSF activities in the BM, it is not surprising that G-CSF secretion by tumor cells and its *in vitro* administration can skew hematopoiesis in favor of MDSCs.

A4. M-CSF. The macrophage-colony stimulating factor (M-CSF), also known as CD115, is a critical regulator of monocytic development. M-CSF signals through its corresponding tyrosine kinase receptor, which is widely expressed on the CMP and GMP myeloid compartments(57). M-CSF serves a physiological role in the proliferation and differentiation of monocytes, macrophages, and DCs. In genetically altered murine models, the absence of M-CSF and / or its receptor induces a dramatic reduction of peripheral monocyte populations that is only restored upon transgene expression of M-CSF(99).

The M-CSFR promoter contains binding sites for several transcription factors implicated in myeloid development, including PU.1, C/EBP- α , and C/EBP- β (100,101). Furthermore, dimerization of the M-CSF receptor activates numerous downstream pathways, including Stat1, the Ras/Raf/MEK/ Erk pathway, and the phospholipase C γ (PLC γ) pathway(102). These pathways exert manifold regulatory roles in cell-fate determination. In particular, activation of protein kinase c (PKC) by PLC γ promotes monocyte differentiation, while blockade of ERK signaling *in vitro* induces subsequent abrogation of monocytic differentiation from FDC-P1 cells(103). Signaling through the ERK pathway is necessary to modulate commitment of lymphoid-myeloid progenitor

cells toward CMPs rather than CLPs. Accordingly, hematopoietic cells with myeloid and lymphoid potential exhibit a high sensitivity to M-CSF and undergo differentiation towards myeloid cells at high M-CSF concentrations(104). In fact, under high M-CSF concentrations inhibit proper myeloid differentiation and promote the differentiation of DCs into monocytes-like cells with macrophage like characteristics(105).

Several markers indicate that M-CSF exerts a prominent role in MDSCs-related disease states. In particular, M-CSFR mRNA is induced in several pathological conditions in which MDSCs have been implicated. Once recruited to the site of an immune insult, macrophages initiate an autocrine loop of M-CSF production. This, in turn, alters hematopoiesis and induces the recruitment MDSCs to the site of insult(106). Furthermore, once recruited to the site, MDSCs perpetuate the loop by producing M-CSF on-site(107). As a consequence, it is not surprising the MDSCs have been observed in many infections and pathological conditions in which macrophage response is desirable.

In a second pathway, M-CSF is regulated by the transcription factor MafB to restrict lineage commitment toward myelopoiesis(108). The relationship between MafB and M-CSF is evident in murine models, in which the overexpression of MafB limits myeloid differentiation. Conversely, a MafB deficiency in these models has been shown to render myeloid progenitors more sensitive to M-CSF. As a consequence, increased PU.1 activity is observed, shifting hematopoiesis toward myeloid development(109). In addition, MafB is highly expressed in LT-HSCs and becomes downregulated during intermediate stages of HSC differentiation with subsequent upregulation occurring upon lineage commitment(57). Although unclear at this time, one can speculate that in line

with myeloid-based models of HSC differentiation, it is plausible that MafB regulates the balance of lineage potential and commitment early in CMLPs and later in p-MBTs.

A5. IFN- γ . The Th1 cytokine Interferon-gamma (IFN γ) plays a paradoxical role in pathologic states and MDSC expansion. IFN γ 's proapoptotic and antiangiogenic effect in innate and adaptive immune responses have made this cytokine an attractive therapy for numerous hematologic malignancies. IFN γ production has been demonstrated to prevent the development of both primary and transplanted tumors. However, it is increasingly recognized that IFN γ may have negative oncological outcomes, specifically with regard to tumor development(110). In particular, IFN γ production by antigen-activated T cells synergizes with MDSC-produced IFN γ to further enhance the suppressive function of MDSCs in a STAT1 dependent and independent manner(111). Conversely, blockade of IFN γ production diminishes MDSC-mediated T cell suppression(58). Perhaps this controversial role of IFN γ can be attributed to its production unique to a given pathological condition and regulation of interferon regulatory factor-8 (IRF-8). Known as the interferon consensus sequence binding protein (ICSBP), IRF-8 is a member of the IRF family of IFN- α and IFN- β dependent transcription factors(112). However, IRF-8 is sensitive to IFN γ and is expressed in hematopoietic myeloid and lymphoid progenitors. Within these cells, IRF-8 serves as a positive modulator of the EBF/E2A transcription system, which regulates B cell development (Figure 2)(113). IRF-8 is also a negative modulator of PU.1, master regulator of myelopoiesis. Inhibition of PU.1 alleviates PAX-5 suppression, which is required for normal B cell development(114). The interplay between PU.1 and B cell

development is further supported by the observation in which ectopic expression of PU.1 and C/EBP α induces macrophage cell fate in developing B cells(115). In the absence of IRF-8, PU.1 activity is not inhibited and myeloid differentiation is promoted(113,116). This is further supported in IRF-8 deficient mice which develop chronic myelogenous-like syndrome (CML) subsequent to robust MDSC accumulation(117). Thus, tumor-induced IFN γ dysregulation and IRF-8 reduction would be expected to skew hematopoiesis away from lymphoid development towards myelopoiesis.

A6. VEGF. Vascular endothelial growth factor (VEGF) is a prototypic growth factor consisting of five family members with tissue specific physiological effects that signal through either of the two high affinity tyrosine kinase receptors: VEGFR1, c fms-like tyrosine kinase (Flt1) and VEGFR2, fetal liver kinase-1 (Flk-1)(118,119). The upregulation and contribution of VEGF to pathogenesis and hematopoietic dysregulation has been well documented in many cancer patients and animal models of neoplasia(120,121). Within the BM microenvironment, VEGF is secreted by HSCs to ensure cellular survival. In particular, VEGF-deficient HSCs are unable to repopulate in lethally irradiated mice, even with co-administration of WT HSCs. Conversely, stimulation of VEGFR1 rescued their survival of these cells *in vivo* and *in vitro* administration of VEGF promoted colony formation. These results indicate that VEGF may regulate hematopoiesis in a cell-autonomous manner by establishing an autocrine loop(120). This assumption is further supported by the autocrine production of VEGF by malignant myeloid precursors in leukemic patients, leading to mobilization of immature myeloid progenitors upon stimulation of both VEGFR1 and VEGFR2(121). VEGF itself

binds CD34⁺ hematopoietic progenitors via VEGFR1, enhancing CD34⁺ MPP and MEP early progenitor cell production while skewing hematopoiesis in favor of myeloid development. This observation supports the myeloid-based model of hematopoiesis, since an increase in VEGF stimulation can induce lineage redistribution of p-MBT cells. In fact, VEGF increased CD34⁺ progenitor cell production with myeloid and lymphoid potential from mouse embryonic stem cells. Furthermore, these cells have been demonstrated to form myeloid colony forming units as well as B220⁺, CD19⁺ B lymphocytes when cultured on stromal cells in the presence of IL-2 and IL-7(122).

VEGF differentially regulates hematopoietic alterations contingent upon stimulation of a given VEGFR subpopulation. Signaling through VEGFR1 boosts populations of immature Gr-1⁺ myeloid and B lymphocytes. Furthermore, ablation of VEGFR1 is associated with decreased HSC survival and differentiation(119,123). VEGFR1 activation likewise populates the immature myeloid cell compartment by inhibiting the transcription factor NFκB in hematopoietic progenitors. Continuous infusion of VEGF abrogates NFκB and downregulates stimulatory FLT3L, thereby reducing DC differentiation and maturation. As a consequence, the immature myeloid compartment is further populated(124). This observation is consistent with observations that alterations in NF-κB subunits induce enhanced myelopoiesis and reduced lymphopoiesis(125). The behavior of VEGFR1 is in direct contrast to that mediated by VEGFR2 activation, which promotes CD11b⁺Gr-1⁺ MDSCs at the expense of B cells at the pro-B cell state(126). In particular, continuous infusion of VEGF results in decreased lymphoid compartment concomitant with increased myeloid populations, thereby

inducing splenomegaly(124). The consequence of increased VEGF signaling in dysregulated hematopoiesis perhaps occurs through VEGF/VEGFR modulation of p-MBT cells, redirecting their commitment towards lymphoid or myeloid cells.

III. Accumulation and differential activity of MDSC subsets in disease.

As mentioned earlier and demonstrated by Figure 3, MDSC induction occurs in a myriad of disease states, impairing both the innate and adaptive arms of the immune response. MDSCs exert most deleterious effects on T cell activation via the release of small soluble oxidizers, the impairment of T-cell-antigen recognition, and the depletion of essential amino acids from the local extracellular environment. This MDSC mediated immune suppression and impairment of cancer immunotherapy is well established(66,127). However, these studies are challenged by observations in which MDSCs are not always immunosuppressive. Reports suggest that the immunostimulatory or immunoinhibitory potential of MDSCs depends upon the dual role of nitric oxide, the cytokine milieu, the interaction between MDSCs, T cells, other myeloid cells, and the tumor microenvironment. In fact, infections with microorganisms can skew MDSCs towards antitumor properties and for this reason, *Salmonella*-based cancer immunotherapies have been pursued with promising preclinical results(128,129). Furthermore, by limiting immune activation, MDSCs serve a beneficial role in transplantation, autoimmunity, and sepsis(130,131). These paradoxical observations are due to the differential accumulation and activity of granulocytic Ly6G⁺ (G-MDSC) monocytic Ly6C⁺ (G-MDSC) subtypes in disease progression. Although much attention

has been dedicated to elucidate MDSCs as a group, a thorough characterization of each subset is required to demonstrate that despite being categorized as a heterogeneous group of cells, G-MDSCs and M-MDSCs are functionally and phenotypically distinct.

A. Cytokine consortium of M-MDSC and G-MDSC development.

The phenotypic and functional characteristics of MDSCs underscore their myeloid origin. Common cytokines involved in myeloid commitment that are dysregulated in cancer and other disease states have been implicated in the induction of MDSCs. These factors include M-CSF, GM-CSF, IL-6, VEGF, and G-CSF. Thus the unique immune response elicited by a given pathological condition can lead to the accumulation of MDSCs. Moreover, as demonstrated in Figure 4, the subsequent cytokine consortium dictates the accumulation of monocytic or granulocytic MDSCs. GM-CSF and G-CSF are two cytokines predominately associated with *in vivo* MDSC accumulation and *in vitro* MDSC generation(35,132). GM-CSF is recognized as a critical hematopoietic cytokine, mediating the differentiation of myeloid progenitors into granulocytes, eosinophils, monocytes, megakaryocytes, and erythrocytes. G-CSF and its receptor are critical regulators of steady state and emergency granulopoiesis. G-CSF promotes the survival, mobilization, and proliferation of myeloid progenitor cells alongside neutrophil differentiation. Several studies indicate that each cytokine preferentially induces granulocytic or monocytic MDSCs. Exposure to GM-CSF alone induces immunosuppressive M-MDSCs while G-CSF induces G-MDSCs(133,134). In fact, glioblastoma patients exhibit high levels of G-CSF concomitant with G-MDSC accumulation(135). Under steady state conditions, G-CSF acts in concert with CXCR2

ligands, CXCL1 and CXCL8 to induce mobilization and recruitment of neutrophils, respectively. Therefore, it has been proposed that G-CSF may promote expansion of G-MDSCs via CXCL1 and mobilization via CXCL8(136). In terms of GM-CSF, Dolcetti *et al.* demonstrated that a 4T1 mammary cancer cell line deficient in GM-CSF but not G-CSF, is less immunosuppressive due to the differential expansion of G-MDSCs over M-MDSCs(134). The reduced tumor growth is accompanied by an increase in antigen specific T cells in lymph nodes of mice challenged with GM-CSF deficient 4T1. These mice also exhibited an accumulation of G-MDSCs that did not affect the generation of tumor specific T cells(137). Moreover, Greten and colleagues indicate that human CD14⁺ monocytes purified from healthy donors can be differentiated into suppressive M-MDSCs upon co-culture with IL-4 and GM-CSF(132). Interestingly, the combination of GM-CSF and G-CSF induces M-MDSCs from BM cells after 4-day co-culture. Additionally, IL-1 β has been indicated to inhibit NK cell differentiation in favor of G-MDSCs in 4T1 mammary carcinoma(138,139). Lastly, Simpson and colleagues demonstrate that the inflammatory cytokine, macrophage inhibitory factor (MIF), induces M-MDSC differentiation rather than G-MDSCs(140).

B. Immunophenotyping.

Although MDSCs collectively represent a heterogeneous cell population with different patterns of surface proteins, they are unified by expression of Gr-1 and CD11b in the murine system. The Gr-1 epitope consists of Ly6C and Ly6G molecules that are members of the Ly-6 family of low molecular weight phosphatidylinositol-anchored cell surface glycoproteins(135,141,142). Gr-1^{high} Ly6G⁺ G-MDSCs morphologically

resemble polymorphonuclear granulocytes while Gr-1^{low} Ly6C⁺ M-MDSCs resemble mononuclear monocytes. Studies indicate that engagement of Gr-1 epitope through Ly6G or Ly6C can differentially regulate myeloid cell activity and the subsequent immune response(136,141,143). For example, ligation through Ly6G can induce cell death while Ly6C ligation can lead to cellular expansion and differentiation. In addition to Gr-1 and CD11b in the murine system, both populations are also identified by functional markers such as Dectin-1, a non-toll like pattern recognition receptor, death receptor FAS, and ADAM17 (144-146). In humans, MDSC subset accumulation is dependent on the type of tumor but overall defined as Lin⁻HLA-DR⁻CD33⁺ and further categorized into monocytic CD14⁺ and granulocytic CD15⁺ cells(127).

As summarized in Figure 5, many groups have reported the utilization of other markers to further delineate G-MDSCs and M-MDSCs. In humans, G-MDSCs are defined as CD11b⁺CD33⁺CD14^{lo}CD15^{hi} and further characterized by expression of CD66b, a member of the carcinoembryonic antigen family that is commonly expressed on human neutrophils. G-MDSCs have also been indicated to express VEGFR1 and low levels of CD62L(147-149). Although these surface proteins are often associated with neutrophils, the main differentiating factor between G-MDSCs and neutrophils is the immunosuppressive capabilities of G-MDSCs. In contrast to neutrophils, G-MDSCs are purified from the mononuclear cell fraction in a density gradient of peripheral blood(147). Furthermore, other groups report IL-4R α (CD124) in patients with non-small cell lung cancer(150) and higher levels of S100 proteins, S100A8/A9 complex as compared to S100A9 on M-MDSCs, in patients with head and neck squamous cell

carcinoma (HNSCC)(151). Human G-MDSCs express IL-13R under suppressive conditions and lower levels of CD33 as compared to M-MDSCs(127,152,153). In the murine system, Youn and colleagues demonstrated the expression of M-CSFR and utilization of CD244 as a marker of G-MDSC cells with T cell suppressing activity. The authors indicate that CD244⁺ G-MDSCs are capable of inhibiting antigen specific T cell responses while CD244⁻ G-MDSCs are unable to do so(154). Additionally, Toh *et al.* report that G-MDSCs express CXCR2 (IL-8R β), which binds CXCL1, CXCL2, and CXCL5. G-MDSCs also express CXCL1 and CXCL2 while tumors express CXCL5. The ligation of these particular molecules leads to the accumulation of G-MDSCs and not M-MDSCs in the tumor microenvironment. In fact, inhibition of CXCR2 inhibited G-MDSC trafficking to primary melanoma tumor(7). This is supported by another finding in a murine model of traumatic spinal cord injury, indicating the expression of CXCR1 and CXCR2 on G-MDSCs(155).

M-MDSCs are mainly defined as CD11b⁺CD33⁺CD14^{hi}CD15^{lo} in humans and as CD11b⁺Gr-1^{int}Ly6G⁻ Ly6C⁺ in mice. In addition to S100A9 and CD124 as mentioned above, M-MDSCs are also characterized by expression of CCR2, which is mainly expressed by monocytes and not granulocytes(111). In tumor conditions, CCR2 induces monocyte trafficking from the BM, limits entry of tumor specific T cells into the tumor microenvironment, and leads to T cell inhibition by MDSCs in a contact-dependent manner. Murine M-MDSCs are further defined by CD49d, CCR7, and CX3CR1(132,155,156). Another group reported the presence of F4/80 and CD93 on M-MDSCs in the context of Experimental Autoimmune Encephalomyelitis (EAE)(157).

C. Mechanism of action.

While the immunosuppressive functions of MDSCs are highly pleiotropic, the mechanism of action differs according to each subset. Compared to M-MDSCs, G-MDSCs are not very suppressive unless present in high numbers(111,134,137). G-MDSCs are thought to induce antigen specific T cell suppression while M-MDSCs promote both antigen specific and non-specific T cell suppression. This is a consequence of a differential enzymatic, signaling, and cytokine profile of each subset.

C1. Enzymatic activity. While both populations of MDSCs express arginase 1 (Arg1), reactive oxygen species (ROS) and inducible nitric oxide (*i*NOS) are specific to G-MDSCs and M-MDSCs, respectively(35). Arg1 is widely expressed in murine myeloid cells but limited to neutrophils in humans. The expression of Arg1 allows G-MDSCs to promote cancer progression via T cell suppression but also limit graft versus host disease (GVHD)(150,154). Arg1 acts by consuming the essential amino acid _L-arginine (L-Arg) and metabolizes it to _L-ornithine and urea, thus depleting critical components of cellular proliferation and detoxification(158). *In vitro* L-Arg depletion by MDSCs inhibits antigen-specific T cell proliferation in OT-1 and OT-2 transgenic mice. Furthermore, in the absence of L-Arg, T cells exhibit decreased CD3 ζ expression, Jak-3, NF κ B-p65 translocation and IFN- γ production(148,159). CD3 ζ is an essential component of the T cell receptor (TCR), imperative for the intracellular signaling cascade and subsequent T cell activation(160). Loss of L-Arg decreases cyclin D mRNA transcriptional rate as well as translation, thus arresting T cells in G₀-G₁ phase(161). However the depletion of L-Arg does not result in apoptosis, and both CD3 ζ expression

and proliferative potential can be restored upon L-Arg administration(162). In addition to Arg1, G-MDSCs induce T cell suppression via the production of ROS. Although ROS can be produced by several mechanisms, NADPH oxidase (NOX2) is primarily responsible for ROS production in leukocytes(163). The main consequence of MDSC-mediated ROS production is inhibition of T cells in an antigen-specific manner by inducing MHC class-I restricted T cell tolerance(164). Nagaraj *et al.* report that ROS nitrates tyrosine residues on CD8 and TCR to alter peptide recognition but does not induce T cell deletion(165). Finally, G-MDSCs limit NK-cell responses via H₂O₂ production(166). However, additional experiments are required to further elucidate the mechanism of this interaction.

M-MDSCs are more potent suppressors of the immune response(111). Perhaps one of the main contributions of M-MDSC in pathologic conditions is the upregulation of *i*NOS, which generates NO. Mazzoni *et al.* report that NO production by MDSCs blocks peptide-specific T cell proliferation. Further analysis revealed this results from prevention of T cells entering the cell cycle. Interestingly, this was reversible; after T cells were stimulated with Con A in the presence of MDSCs, they regained their proliferative potential upon removal of MDSCs. Thus, it is plausible that although M-MDSCs prevent T cell proliferation, they do not induce apoptosis but transiently induce T cell suppression(167). The contribution of NO is further elucidated by the observation that MDSCs generated from *i*NOS-deficient mice are not suppressive(168). Another deleterious consequence of *i*NOS is the consumption of L-arginine; under conditions of

limited L-Arg concentration, NOS produces peroxynitrites (ONOO_2), which are strong oxidizing agents that nitrate proteins and cause T cell apoptosis(164,169).

C2. Cytokine production. Although cytokine profile is disease dependent, emerging literature is beginning to identify each population of MDSCs with certain cytokines. For example, G-MDSCs secrete $\text{TNF-}\alpha$ upon exposure to LPS, $\text{INF}\gamma$, and $\text{TGF-}\beta$ while M-MDSCs mainly produce IL-6, IL-10, IL-23, $\text{TGF-}\beta$, and $\text{VEGF-}\alpha$ (132,156). The cytokine consortium subsequently affords the observed differential role of each subset. In fact, the concomitant secretion of IL-10 and $\text{TGF-}\beta$ also allows M-MDSCs to be potent inducers of $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ T-regulatory cells(132,136,156,170).

C3. STAT signaling. Both M-MDSCs and G-MDSCs signal through Janus Kinase (Jak) / signal transducer and activator of transcription (STAT) pathways(95,97). STAT proteins are latent cytoplasmic transcription factors activated by a variety of hematopoietic and immune-mediated cytokine receptors. M-MDSCs utilize STAT1 to induce downstream T cell suppression(111). Of the STAT signaling pathways, STAT3 has been strongly linked with MDSC proliferation and survival(56,111). STAT3 also promotes the activity of MDSCs, particularly Arg1 in CD14^+ M-MDSCs(171). Using the receptor tyrosine kinase inhibitor, sunitinib, Ko and colleagues demonstrated differential STAT3 and STAT5 signaling in M-MDSCs and G-MDSCs. The authors indicate that sunitinib downregulates STAT3, subsequently abrogating M-MDSC proliferation and impairing G-MDSC survival. However, the addition of GM-CSF provided protection via STAT5 signaling(172).

C4. Interaction with other cells. Recent findings are beginning to suggest that mast cells (MCs) contribute to the recruitment and activity of MDSCs(10,173,174). While MCs have well been documented to mediate allergic inflammation, their demonstrated involvement with neoplastic disease is more recent. Mast cells accumulate in the tumor microenvironment and correlate with poor prognosis(175). This is partly a consequence of the secretion of various pro-inflammatory cytokines such as TNF- α , IL-6, IL-10, IL-13, and IL-17(176). MCs facilitate MDSC migration to the tumor microenvironment via the utilization of 5-lipoxygenase and IL-17(173,174).

Perhaps another intriguing component of MDSC/MC interaction is the biogenic amine, histamine. Although MCs have previously been known to be the major contributors to the histamine pool, recent studies indicate that other myeloid cells can produce histamine, albeit at lower levels(177). Endogenous histamine is generated via the conversion of L-histidine to histamine by histidine decarboxylase (HDC). Histamine acts on a spectrum of cell types through G-protein coupled receptors, Histamine Receptor (HR) 1 – HR4. Studies demonstrate that histamine alters the Th1/Th2-attracting chemokine distribution, leading to a more pronounced Th2 response. In fact, co-culture of monocytes with histamine increases IL-10 but inhibits IL-12 cytokine production by the monocytes, further skewing towards Th2 immunity(178,179). It was recently reported the HR antagonists can lead to MDSC apoptosis and subsequent tumor regression(180). Intriguingly, while both G-MDSCs and M-MDSCs express HR1 and HR2, the net affect of histamine appears to be subset specific(181). Indeed, HR2 blockade is more effective in limiting M-MDSC activity(180). Exogenous administration

of histamine promoted the expansion and proliferation of M-MDSCs rather than G-MDSCs.

Macrophages are capable of fighting both intracellular and extracellular pathogens and are thus classified into two distinct subsets according to the cytokines required for their activation and the pathogens they target(182). Classically activated M1 Macrophages are normally activated by IFN- γ or LPS to release NO and TNF- α in targeting intracellular pathogens as well as IL-12 and IL-23(183). M1 macrophages are better able to partake in antigen presentation and T cell activation as compared to alternatively activated M2 macrophages. Upon stimulation by IL-4 or IL-13, M2 macrophages secrete IL-10, TGF- β , and upregulate Arg-1 in response to extracellular pathogens(184). MDSCs have not only been shown to interact with macrophages but also resemble M2 macrophages. MDSCs decrease IL-12 production by macrophages and skew their differentiation towards an M2 phenotype and macrophages in turn promote MDSC IL-10 production(185). Although the authors of this study did not differentiate the subset of MDSCs, it can be deduced based on morphology and cytokine production that this interaction is mainly within the monocytic fraction.

Another cell known to interaction with MDSCs are dendritic cells (DCs). Dendritic cells are professional antigen presenting cells that identify, process, and present antigens to naïve, resting T cells(186). This is critical for the generation of an appropriate immune response. MDSCs secrete IL-10 that abrogates toll like receptor (TLR) ligation and subsequent IL-12 production by DCs. This in turn reduces DC mediated T cell activation(187). While MDSCs as a group have been shown to limit DC maturation, a

recent study indicated that CD14⁺ M-MDSCs impair the quality of a DC vaccine. The authors indicate that M-MDSCs not only prevent DC maturation but also antigen presentation and migration(188). Interestingly, MDSCs can also be converted to DCs. Two studies have been done to this effect, one indicating the requirement of NKT Cells and α -galactosylceramide; and another suggesting interaction of iNKT with CD1d on MDSCs(132,139,156,189).

D. Expansion and activation.

D1. Survival and Proliferation. G-MDSCs have a reduced life-span, similar to the 3.8 days reported for neutrophils and are more sensitive to cryopreservation as compared to M-MDSCs(150,190,191). However, G-MDSCs are quickly repopulated from the BM and exhibit up to 80% survival when exposed to tumor explant supernatant and GM-CSF(136,147,154,190). This has been attributed to delayed apoptosis by increased CXCL8(192). Despite the prolonged life-span, these conditions do not induce G-MDSC proliferation. This has led to the current notion that M-MDSCs are the main subset capable of proliferation. In fact, in the same study, Youn *et al.* demonstrated that splenic M-MDSCs incorporated more than twofold more BrdU as compared to G-MDSCs in tumor bearing mice(170).

D2. Polarization and differentiation. In addition to a shorter life span, G-MDSCs are also incapable of differentiation, while M-MDSCs can give rise to mature myeloid cells(154). In fact, CD11b⁺Gr-1⁺CCR2⁺CX₃CR1^{low} M-MDSCs can mature into DC and macrophages under appropriate stimuli(156). A recent study indicates that M-MDSCs can also differentiate into G-MDSCs via epigenetic silencing of the

retinoblastoma gene. Interestingly, the same study also suggests that G-MDSCs can lose their immunosuppressive characteristics and become phagocytic upon *ex vivo* exposure to tumor explant supernatants and GM-CSF(170).

Moreover, MDSCs can be polarized towards immunostimulatory type1 or immunoinhibitory type2 cells. Murine Paired immunoglobulin-like receptors A and B (PIR-A and PIR-B) along with human PIR homologues belong to the immunoglobulin super family. These molecules have recently been demonstrated to differentially regulate MDSC polarization. PIRs were first identified as homologues to the human Fc receptor for immunoglobulin A (IgA). It has recently been reported that PIR signaling can alter polarization of M-MDSCs between classically activated proinflammatory phenotype (M1) and alternatively activated anti-inflammatory (M2) phenotype. PIR-A delivers activation signals in complex with a homodimeric Fc common γ chain, which harbors an immunoreceptor tyrosine-based activation motif (ITAM), resulting in cytokine production in myeloid cells(193-195). In contrast, PIR-B engagement results in negative signal transduction upon phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and recruitment of SH₂-homology-containing protein-tyrosine phosphatase-1 (SHP-1) and SHP-2. In fact, SHP1 inhibition can shift M2 M-MDSCs to M1-MDSCs. Furthermore, Fridlender et al. demonstrated that G-MDSCs can also be polarized between G1 and G2 phenotypes, but this is dependent upon TGF- β signaling. MDSCs and tumor cells secrete large amounts of TGF- β that induces pro-tumor G2 G-MDSCs. However, blockade of TGF- β signaling via TGF- β R antagonization reprogrammed G2 G-MDSCs to tumoricidal G1 G-MDSCs(196).

E. Subset accumulation in pathological conditions.

E1. Neoplasia. The expansion of MDSC phenotypes is dependent upon the immunoregulatory factors altered by a given tumor model (Fig. 6). In humans, M-MDSCs are mostly associated with melanoma, prostate, lung, gastrointestinal, and hepatocellular carcinoma while G-MDSCs are associated with breast, colon, pancreatic, bladder, HNSC carcinoma(132). A detailed list is summarized elsewhere, but the overall trend is the dominant accumulation of G-MDSCs(132,136,156,170).

In naïve mice, MDSCs comprise 2-4% of total nucleated splenocytes with a 3:1 ratio of G-MDSC to M-MDSCs(132). However, MDSCs can increase to 50% of total splenocytes with a 5:1 G-MDSC to M-MDSC ratio in tumor bearing mice(170). The population of G-MDSCs becomes consistently increased in all tumor models, whereas the frequency of monocytic MDSCs is only significantly increased in a few models such as EL-4 and ANV. Only in mice bearing Lewis Lung Carcinoma, G-MDSCs and M-MDSCs are equally elevated(197). In humans, G-MDSCs are found to be the most abundant population in solid tumors while M-MDSCs are more frequent in leukemia(198). Moreover, in a given host, G-MDSCs tend to locate at the primary tumor site (24.5%) vs spleen (4.2%), while M-MDSCs are found in cutaneous tumors (3.9%) vs. spleen (2.8%) (7). This is further supported by the observation that MDSC depletion results in reduction of primary but not cutaneous tumors(7). In a mouse model of Multiple Myeloma, M-MDSCs begin to accumulate within one week of tumor induction while G-MDSCs accumulate weeks later. Despite the delay in kinetics, G-MDSCs continue to be the dominant population both in the BM and peripheral blood(199). In

tumor bearing mice, G-MDSCs are recruited out of the BM in response to stromal cell derived factor-1 α (SDF-1 α), ligand for CXCR4. In humans with renal cell carcinoma (RCC) and HNSCC, G-MDSCs accumulation is associated with an increase in intratumoral and serum expression of CXCL8 (IL-8), a potent inducer of neutrophil motility to sites of inflammation(192). This is corroborated by another report indicating the importance of CXCR2 ligand in the recruitment of G-MDSCs to primary tumor sites. In fact, G-MDSCs were 5 times more abundant as M-MDSCs, and CXCR2 inhibition significantly abrogated their migration(7).

While most attention has been given to the pathogenic mechanisms of M-MDSCs, G-MDSCs are now beginning to be appreciated for their contribution to tumor progression. Using a model of spontaneous melanoma, Toh and colleagues found that G-MDSCs are needed for the early stages of cancer dissemination, as late depletion did not alter metastasis to skin, lymph nodes, and lungs. Additionally, upon co-culture with G-MDSCs, NBT-II bladder carcinoma cells acquired a mesenchymal morphology, consisting of actin microfilament redistribution and downregulation of E-Cadherin. This was attributed to the production of Hepatocyte Growth Factor (HGF) and TGF- β , known inducers of epithelial-mesenchymal transition (EMT). Moreover, the same study reported that G-MDSCs promoted tumor cell proliferation in a contact-independent manner(7).

E2. Infection. MDSCs, predominantly G-MDSCs, have been shown to accumulate during infections with microorganisms. In this context, they play a beneficial role in limiting unwanted inflammation. In fact, mice unable to expand MDSCs exhibit markedly higher mortalities to sepsis-associated inflammation(56,200,201). As

mentioned earlier, bacterial infections can skew MDSCs towards antitumor properties. The administration of attenuated *Salmonella enterica* serovar Typhimurium vaccine (RASV) results in an accumulation of MDSCs, particularly G-MDSCs. These MDSCs were tumoricidal, sensitive to LPS stimulation, and released large amounts of TNF- α . RASV treatment reduced Her-2/neu expressing tumor growth, shifted MDSC differentiation towards G-MDSCs, and enhanced anti-tumor CTL responses. It is for this reason that *Salmonella*-based cancer immunotherapies have been pursued with promising preclinical results(202,203). Furthermore, by limiting immune activation, MDSC play a protective role in sepsis. Delano *et al.* demonstrated that polymicrobial sepsis induced significant MDSC accumulation. Additional reports are surfacing and lending support to the protective role of MDSCs in microbial infections, especially *Pseudomonas aeruginosa* colonization in burn victims(56). More recently, it was reported that murine MDSCs express dectin-1, a non-Toll-like pattern recognition receptor for β -Glucans, which are the main components of numerous bacteria, fungi, and yeast cell walls. The authors report that whole β -Glucans particle (WPG) administration preferentially enhanced the survival of G-MDSCs but did not induce their differentiation. In contrast, WPG treatment reduced M-MDSC survival but promoted differentiation towards CD11c⁺F4/80⁺ non-immunosuppressive cells. Moreover, M-MDSCs were no longer able to suppress T cell proliferation in the presence of WPG(145). This could be attributed to the fact that WPG reduced NO and Arg1 expression in M-MDSCs while it did not alter ROS expression in G-MDSCs.

While the accumulation of MDSCs in parasitic infection has been reported, the immunomodulatory potential of these cells remains largely unknown(204). However, MDSCs have recently been characterized in *Trypanosoma cruzi* infection. The protozoan parasite is the causative agent of myocarditis that manifests in Chagas disease. MDSCs comprise the majority of the inflammatory cell infiltrates in the heart. The most abundant subset in this case is M-MDSCs (70%) as compared to G-MDSCs (20%)(205). The authors of this finding indicate that this is mainly mediated via CCL2 chemokine. Interestingly, these infiltrating M-MDSCs in the heart do not traffic from the BM but rather become replenished from the spleen. Like those isolated from the tumor microenvironment, the infiltrating M-MDSCs also express high levels of Arg and *i*NOS, and inhibit T cell proliferation. Given another report indicating the accumulation of G-MDSCs in the peripheral organs of *T. cruzi* infected mice, it is possible that G-MDSCs and M-MDSCs preferentially migrate to specific anatomical sites in a given disease state(205,206).

E3. Autoimmunity. Experimental autoimmune encephalomyelitis (EAE) is a well characterized and utilized murine model of human multiple sclerosis (MS). While MDSC expansion has been reported, conflicting results exist surrounding subset accumulation and contribution to MS. Zhu *et al.* found M-MDSCs to be the dominant population shortly after EAE induction, comprising 30% of infiltrating inflammatory cells in the central nervous system (CNS). They suggest that M-MDSCs can have both protective and harmful roles in the CNS in that they can suppress excessive T cell activation but damage neuronal tissue via NO over-production(157). In a separate

investigation, Yi *et al.*, while also reporting M-MDSCs accumulation in EAE, suggest that M-MDSCs promote the pathogenesis of EAE. In this model, MDSCs induced Th17 differentiation from naïve CD4⁺T helper cells via IL-17A production and upregulation of orphan nuclear receptor RORA and RORC. The severity of EAE was reduced upon MDSC depletion(207). Although contradictory to our understanding of MDSCs, this observation is supported by another model of MS, which utilizes Theiler's murine encephalomyelitis (TMEV), a natural mouse pathogen known to promote demyelination in susceptible strains. Parallel to Zhu *et al.*'s observations, M-MDSCs comprise the majority of infiltrating immune cells in TMEV infected mice and contribute to myelin attack and inflammation in the CNS. M-MDSC depletion resulted in diminished viral load, reduced T cell accumulation, and increased IFN- γ and IL-12 production with simultaneous IL-10 reduction(157,208). The disease promoting capability of M-MDSCs in this context is a salient contradiction to the MDSC paradigm, since given the overall immunosuppressive nature of MDSCs; one would anticipate that M-MDSCs would limit EAE progression. This negative role of MDSCs and dominant accumulation of M-MDSCs is challenged by Ioannou and colleagues. The authors demonstrated that G-MDSCs are the major population that expand in EAE and serve a protective role. They showed that AT of G-MDSCs decreases the severity of EAE via immunosuppression induced by PD-L1-PD-1 ligation. The authors argue that the disparity in these findings results from the inflammatory milieu induced in each model as well as emphasis on the effects of MDSCs on Th17 expansion or the immunosuppressive nature of MDSCs(209). Perhaps these controversial findings can be remedied by the observation that the

damaging activity of MDSCs in MS lies within the M-MDSC population and beneficial within G-MDSCs.

E4. Chronic inflammation/ metabolic dysregulation. Although both subsets of MDSCs are associated with inflammation, M-MDSCs are the main pro-inflammatory subset. In a report by Zigmond *et al.*, M-MDSCs were shown to promote inflammatory bowel disease (IBD) via the release of IL-6, IL-23, VEGF- α , and induction of iNOS(156). IBD is defined as a heterogeneous group of chronic inflammatory disorders of the gastrointestinal tract with two major forms-Crohn's disease and ulcerative colitis(210,211). While the therapeutic potential of MDSCs in inflammatory disease has been proposed, a recent study by Su *et al.* demonstrates that G-MDSCs could be the subset most effective. The authors report that G-MDSCs can be purified from BM of naïve mice and subsequently utilized for AT in a model of murine colitis. G-MDSCs, albeit not entirely curative, relieved inflammation, increased survival rate, and decreased disease score(211).

Traumatic spinal cord injury (SCI) results in significant acute inflammation, characterized by mobilization of immune cells, including MDSCs and production of proinflammatory cytokines. Using a murine model of SCI, Saiwai and colleagues demonstrated that M-MDSCs are the most abundant population. The authors attribute this preferential accumulation to the expression of CCR2 by M-MDSCs. They indicate that M-MDSCs exhibit higher expression of anti-inflammatory mediators such as IL-10, TGF- β , and VEGF while G-MDSCs express IL-1 β and TNF- α . The infiltrating M-MDSCs accelerated the removal of hematomas and enhanced the coagulation process,

promoting tissue repair. They also demonstrated that IL-10 released by M-MDSCs induces heme-oxygenase 1 expression in macrophages, which prevents heme toxicity(155,212). Thus the authors argue that AT of M-MDSCs can have therapeutic anti-inflammatory benefits not only in SCI but other disease states involving severe inflammation.

Obesity is known to induce a low-grade chronic inflammatory state and it has recently been associated with MDSC accumulation. Taking advantage of mice genetically predisposed to obesity; Xia *et al.* demonstrated that both G-MDSCs and M-MDSCs expand with increased weight and higher fasting glucose. They report that MDSC depletion leads to impaired insulin sensitivity and glucose tolerance that can be remedied upon AT of MDSCs. This increase in MDSCs is restricted mainly to the liver and other peripheral organs while BM levels remain comparable to lean mice. The authors argue that in this case, MDSCs inhibit proinflammatory cytokines such as IL-6 and TNF- α and skew macrophages towards insulin sensitizing, alternatively activated M2 macrophages. This suggests that the AT of MDSCs promotes a Th2 skewed immune response(213).

IV. Dissertation Objective.

To address the role of ADAM10 in hematopoietic cell differentiation, we generated a strain of mice overexpressing ADAM10 at early stages of HSC development. These mice (A10Tg) not only allowed for the elucidation of ADAM10 in cell differentiation but also gave rise to myeloid derived suppressor cells. After confirming that A10Tg MDSCs were similar to tumor-derived MDSCs, we utilized A10Tg MDSCs

to address the differential activity of granulocytic and monocytic MDSC subsets in neoplasia and helminth infections in an environment devoid of confounding tumors and tumor-derived factors. Furthermore, A10Tg MDSCs were exploited to further dissect the interaction of mast cells and mast cell released histamine with MDSCs.

MATERIALS AND METHODS

I. Mice.

A. ADAM10 transgenic mice.

ADAM10 transgenic (A10Tg) mice were generated with the ADAM10-pHSE3' transgene construct. ADAM10-pHSE3' was produced by subcloning the murine ADAM10-HA cDNA from mADAM10-pcDNA3.1/Zeo into the previously described pHSE3' vector, containing the murine H-2Kb promoter and IgH enhancer regulatory elements(214-216). Briefly, the ADAM10-HA cDNA was excised using BamHI/SalI and ligated into BamHI/XhoI cut pHSE3'. ADAM10-pHSE3' was amplified and analyzed by restriction endonuclease digestion and sequence analysis. A 7.5-kb XhoI fragment containing both cDNA and regulatory elements was excised from ADAM10-pHSE3' and injected into C57BL/6 (A10Tg line 240) or C57BL/6 x Balb/c (A10Tg line 258) embryos by the Virginia Commonwealth University Transgenic/Knockout Mouse Core. The resulting offspring were screened for the presence of the ADAM10-HA cDNA by PCR analysis of genomic tail DNA using ADAM10 cDNA sense (5'-CCGACAGTGTTAATTCTGCTCC-3') and anti-sense (5'TTCTTTCAGCCAGAGTTGTGCG-3') primers. Amplification of DNA from A10Tg founders generated a 652-bp PCR product. Transgene integrity was verified and transgene copy number determined for both A10Tg lines by Southern blot analysis. Briefly, genomic tail DNA from both founders and their F2 progeny was digested with AccI and electrophoresed on a 0.9% agarose gel, and the injection fragment from

ADAM10-pHSE3' was used as both probe and copy number control. A10Tg line 258 was backcrossed with C57BL/6 mice for at least five generations.

B. Mast cell deficient mice.

Genetically mast cell-deficient *c-kit* mutant mice were purchased from Jackson laboratory. These mice carry spontaneous loss-of-function mutations at both alleles of the dominant *white spotting (W)* locus (i.e., *c-kit*). They exhibit a marked reduction in *c-kit* tyrosine kinase-dependent signaling, resulting in disrupted normal mast cell development and survival(217). The Cpa3-Cre; Mcl-1^{fl/fl} mice (C57BL6 background) are a generous gift from Dr. Stephen Galli and express Cre recombinase under the control of a segment of the carboxypeptidase A3 (Cpa3) promoter. C57BL/6-Cpa3-Cre; Mcl-1^{fl/fl} mice are severely deficient in MCs and basophils with no other apparent hematologic changes(218).

C. Other mice.

C57BL/6 and congenic CD45.1⁺ (B6-Ly5.2) mice were purchased from Jackson Laboratories and the National Cancer Institute, respectively. Pmel-1 mice were progeny of breeding pairs purchased from Jackson Laboratories.

II. Cell Lines.

OP9-GFP and OP9-DL1 cells were kindly provided by J.C. Zuniga-Pflucker (University of Toronto) and maintained in OP-9 medium as previously described(6). Lewis Lung Carcinoma (LLC) cell line was obtained from the American Type Culture Collection (ATCC) and maintained in DMEM as per ATCC'. B16-melanoma and B16-GM-CSF cells were provided by H. Bear and maintained as previously described(219).

III. Adoptive immunotherapy (AIT) of B16-melanoma.

AIT subsequent to B16-melanoma challenge was performed as previously described(219). Briefly, donor WT or pmel-1 mice were sensitized in the left footpad with 1×10^6 B16-GM-CSF melanoma cells. Ten days later, popliteal draining lymph nodes were harvested, dispersed into a single cell suspensions in complete RPMI 1640 (cRPMI) at 1×10^6 cells/mL, and activated with bryostatin 1 (5 nM, provided by the National Cancer Institute), ionomycin (10nM, Calbiochem) (B/I), and rIL-2 (80U/mL, Chiron) at 37°C for 18 h. Cells were washed 3x with cRPMI 1640, resuspended at $1-2 \times 10^6$ cells/mL in the presence of IL-7 and IL-15 (10 ng/mL each, Peprotech), cultured for five days and maintained at $1-2 \times 10^6$ cells/mL in the presence of fresh cytokines. Recipient mice were injected *i.v.* with 0.25×10^6 B16-melanoma cells. Three days subsequent to B16 inoculation and one day prior to AIT treatment, mice were pre-treated *i.p.* with 2 mg cyclophosphamide (CYP, Mead Johnson). For gemcitabine treatment, mice were injected *i.p.* with 1.2 mg three days prior to B16 challenge and every five days thereafter. For AIT, 2×10^7 expanded donor cells were washed (2x) in PBS, filtered through a 70- μ m nylon mesh strainer (Invitrogen), and injected *i.v.* in recipient B16-challenged mice. No systemic cytokines or vaccinations were administered to these tumor-bearing mice. After 14 days, mice were euthanized by CO₂ inhalation and lungs were harvested, fixed in 10% formaldehyde, and black lung nodules were counted under a dissecting scope. When nodules were too many to count they were given a value of 250.

IV. ARG and NO detection.

A. Arginase.

MDSCs were purified from A10Tg and LLC bearing mouse spleens, cytoplasmic extracts were prepared and tested for arginase activity by the production of urea as described in manufacturers instructions (DARG-200, *BioAssay Systems*). Briefly, 1×10^6 splenic MDSCs were purified and washed with PBS and centrifuged at 1000Xg at 4°C for 10 min. Pellets were then lysed for 10 min in 100 μ L of 10mM Tris-HCL (PH 7.4) containing 1 mM pepstain A, 1 mM leupeptin, and 0.4% Triton X-100. Subsequently, cells were centrifuged at 10,000Xg at 4°C for 10 min and transferred to 96-well plates, incubated for 30 mins at 37°C with arginine (4X) and Mn (1X) buffer. Arginase reaction was then stopped using 200 μ L of urea reagent and incubated at RT for 60 mins. The urea concentration was then measured by light absorption at 520 nm wavelength using a microplate reader.

B. Nitric Oxide.

Nitric Oxide was measured using Greiss Reagent per manufacturers instructions (*Molecular Probes*, G7921). Briefly, 150 μ L of cell supernatants were mixed with 20 μ L of Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride), and 130 μ L of deionized water. After 30 min incubation at RT, the absorbance was measured by light absorption at 548 nm wavelength using a microplate reader.

C. Arginase 1 and iNOS Quantitative PCR.

MDSCs were isolated as previously described and cultured with MCs or 100 μ M histamine (Invitrogen) for 24 hours. RNA was extracted and cDNA was generated as previously described(220). Primers for running an iQ SYBR Green Supermix (Biorad) quantitative PCR (qPCR) assay were purchased from IDT. Primers are as listed for Arg1 forward 5'-GAC CAC AGT CTG GCA GTT GG-3', Arg1 reverse 5'-TGG TTG TCA GGG GAG TGT TG-3', *i*NOS forward 5'-CAC CCC AAG TTC GAC TGG TT-3' and *i*NOS reverse 5'-CTA AAG GGA CAG GCG CTG AA-3'. Results were analyzed with Biorad iQ5 real-time PCR software (version 2.0)

V. B16 Challenge and MDSC adoptive transfer (AT).

C57/BL6 WT and mast cell deficient Kit^{Wsh/Wsh} mice were given 5-10 X 10⁶ A10Tg MDSCs via tail vein injection one day prior to challenge with 0.25x10⁶ B16-melanoma cells. In the subpopulation studies, WT mice were injected with the same amount of either granulocytic or monocytic fraction of MDSCs. Mice were then injected again with 5-10 X 10⁶ A10Tg MDSCs every three days for three weeks. Mice were then euthanized by CO₂ inhalation and lungs were harvested, fixed in 10% formaldehyde, and black lung nodules were counted under a dissecting scope as described above.

VI. BMDC culture.

Mouse bone marrow-derived mast cells (BMDC's) were derived from femurs of WT mice and cultured in cRPMI 1640 containing 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 1mM HEPES (Quality Biological, Inc.), and 1mM sodium pyruvate (Cellgro). Cultures were supplemented with IL-3-containing supernatant from WEHI-3 cells and stem cell factor (SCF)-containing supernatant from

BHK-MKL cells. The final concentrations of IL-3 and SCF were adjusted to 1ng/mL and 10ng/mL, respectively. Mature BMMC were used after 28 of days of culture.

VII. BMMC co-culture with A10Tg MDSCs.

BMMCs were washed and resuspended at 0.5×10^6 cells/mL; half were loaded with 0.5ug/mL of mouse IgE (Purified mouse IgE kappa isotype control, BD Pharmingen) in 10ng/mL of recombinant IL-3 24hrs prior to the co-culture. Following MDSC isolation, BMMCs were washed twice and cultured at a 1:1 ratio with MDSC in 10ng/mL IL-3 and 10ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) overnight. Dinitrophenylated human serum albumin (DNP-HSA) was added to all wells at a concentration determined to be optimal for MC activation (20-100ng/mL). Supernatants were collected 6 or 18 hours after cross-linking and kept at -80°C until analysis by ELISA.

VIII. Bone marrow chimera studies.

Recipient CD45.2^+ A10Tg (F240) and CD45.1^+ WT mice were irradiated with 950 rads using a ^{137}Cs source (Mark I , Model 68-0146; JL Shepherd & Associates). Donor Lineage $^{-}$ Sca-1 $^{+}$ c-Kit $^{+}$ (LSK) cells were isolated from mouse tibia, femur, and humerus by magnetic cell sorting with a lineage cell depletion kit. (MACS; Miltenyi Biotec). 24 hours after irradiation, recipient CD45.2^+ A10Tg and CD45.1^+ WT mice were injected *i.v.* with 2.5×10^6 CD45.1^+ WT and 5×10^6 A10Tg LSK cells, respectively. For generation of mixed BM chimeras, a mixture of LSK cells from CD45.2^+ A10Tg (2.5×10^6) and CD45.1^+ WT (1.25×10^6) mice were injected *i.v.* in to irradiated CD45.1^+ WT mice. Cell populations were analyzed 42 and 63 days after reconstitution.

IX. Determination of supernatants, serum, or cellular cytokine levels by ELISA and Multiplex Assays.

A. MC/MDSC co-culture supernatants.

All ELISAs for MDSC/MC co-cultures were obtained from Peprotech and used as per manufacturer's instructions. Supernatants collected after 6hrs were analyzed for TNF- α and supernatants collected after 18hrs were analyzed for IL-6, IL-13, and MIP-1 α .

B. Serum cytokine levels.

Serum from Nb infected mice were collected and analyzed for cytokines using the Millipore Milliplex Mouse Cytokine Panel and Bio-Rad Bioplex according to the manufacturer's guidelines.

C. Hepatic MDSCs.

Livers were harvested from Nb infected mice on Day 7 post infection and AT with MDSCs from A10Tg spleens. Livers were isolated as described(221). Recovered liver MDSCs as well as MDSCs isolated from A10Tg spleens were cultured for 48hrs in cRPMI. Supernatants were harvested and cytokines were analyzed with a Bioplex Pro Multiplex Cytokine Assay (Biorad).

X. Flow cytometry and cellular imaging.

Cell isolation and labeling were conducted as previously described(6). Additionally, peritoneal fluid cells were obtained by flushing the peritoneal cavity with PBS (5mL). BM cells were isolated by flushing excised tibias and femurs with cRPMI. Single cell suspensions of PLN cells, thymocytes, and splenocytes were created by disrupting inguinal, brachial, axillary lymph nodes, thymus, and spleens, respectively, with glass

slides. Cells were labeled following red blood cell lysis and filtration through 40 μ M cell strainers. Antibodies included anti-mouse unlabeled 2.4G2, biotinylated CD135 (A2F10), FITC-conjugated IL7R (A7R34), anti-CD62L (MEL-14), CD44 (IM7), B220 (RA3-6B2), and Gr-1 (RB6-8C5); PE-conjugated Ly6G (A18), B220, CD8 (53-6.7), Gr-1, Ter-119, Thy1.2, (30-H12) CD11b (M1/70), CD3 ϵ (2C11); APC-labeled B220, Ly6C (HK1.4)CD4 (RM4-5), CD5 (53-7.3), CD45.2 (104) and c-kit (2B8); PE/Cy7-conjugated CD11b and sca-1 (D7), PE/Cy5 CD34 (A2F10), APC/Cy7-conjugated CD19 (6D5) and CD45.1 (A20), PerCP/Cy5.5-conjugated IL-7R, IgM (RMM-1); and anti-human PERCP/Cy5.5 CD15 (W6D3); and CD33 (WM-53) all from Biolegend. CD34-FITC (RAM34), Ly6G-FITC (1A8), c-kit-PE (2B8), Sca-1-PE/Cy7, and APC-conjugated CD3 (145-2C11), B220 (RA3-6B2), Ly6C/Ly6G (RB6-8C5), CD11b (M1/70), and TER-119 (TER-119) from BD Biosciences, and ADAM10-FITC (FAB946) from R&D Systems. PE/Cy5-conjugated anti-mouse HR1 (polyclonal) from Bioss; FITC anti-human HLA-DR (LN3), APC anti-human CD11b (ICRF44), and PE anti-human CD14 (61D3) from eBiosciences Anti-mouse Fc γ R2/3 (in-house) and anti-mouse Ly6D (49-H4) (BD Biosciences) were biotinylated with EZ-Link Sulfo-NHS-biotin (Pierce), followed by dialysis to remove free NHS-biotin. Streptavidin-ECD (Beckman Coulter) was used for secondary labeling of biotinylated-Ly6D and Fc γ R2/3 labeled cells. Flow cytometric analysis was performed using a Canto or AriaII (BD Biosciences), and data analysis was conducted with FCS Express V3 software. Histogram overlays were generated in SigmaPlot 10.0. as line plots and smoothed using the SMOOTH transform. For fluorescence activated cell sorting (FACS) of LSKs, lineage positive cells were depleted

with a lineage cell depletion kit (MACS; Miltenyi Biotec/ Stemcell Technologies). Remaining LSKs (Lin⁻IL7R⁻ckit^{hi}sca-1^{hi}) were sorted with an Aria II. Lineage positive cocktail included anti-CD3 ϵ , Gr-1, CD11b, B220, and Ter119 antibodies. Splenic MDSCs (CD11b⁺Gr-1⁺) were sorted for photomicrographs. Purity exceeded 95% and were subsequently cytopun on glass slides and stained with the HEMA 3 stain set (Fisher Scientific). Photographs were taken with a BIOQUANT NOVA camera attached to an Olympus BH-2 microscope.

XI. Human MDSCs.

A. Allergic patients.

Human studies were conducted under Institutional Review Board (IRB) protocol #MCC-13740 for cancer patients and 00870 for allergic patients. All patients had the capacity to give informed consent to participate in this research. Twenty mLs of blood was collected in EDTA coated vacuum tubes from allergic patients identified as symptomatic or non-asymptomatic controls and peripheral blood leukocytes were isolated using Ficoll-Paque separation medium (GE Healthcare) according to manufacturer's protocol.

B. Cancer patients.

For the isolation of MDSCs from the peripheral blood of a stage I primary breast cancer patient, erythrocytes were lysed from whole blood treated with ACK lysing buffer. Total leukocytes were then stained and sorted into a CD33⁺ CD11b⁺ HLA-DR^{lo/-} population, representative of MDSCs, into 100% FBS using a Becton Dickinson FACSAria III. Flow cytometry was performed on pretreatment MDSCs and 48hr post addition of

histamine or RPMI alone on a BDCanto II. Data analysis was conducted using FlowJo software v10.0.5.

XII. Isolation of MDSCs, AT, and labeling.

Spleens were harvested from A10Tg or LLC bearing WT mice, depending upon experimental protocol. They were then dispersed into single cell suspensions and filtered through 70- μ m nylon mesh strainers (Invitrogen). Erythrocytes were lysed using an ammonium chloride potassium (ACK) lysing buffer (Quality Biological). Gr-1⁺, Ly6G⁺, or Ly6C⁺ cells were purified using the Easy Sep PE-selection kit (Stem Cell Technologies) according to the manufacturer's protocol. For AT studies, 5-10 x 10⁶ naïve MDSCs were injected into the tail vein of each experimental group every three days. For AT of dye labeled MDSCs, PKH26GL dye linker kit was purchased from Sigma and MDSCs were stained according to the manufacturer's protocol.

XIII. LSK cultures.

BM-derived LSK cells were isolated via magnetic cell sorting and FACS, were cultured in the presence of IL-7 (1 ng/mL, Peprotech) and Flt3L (5ng/mL, R&D Systems) on OP9-GFP or OP9-DL1 as previously described(26). LSK differentiation was examined via flow cytometric analysis, and differentiated cells were passed onto freshly plated OP9 cells with additional cytokines every 4-5 days. Compound E (100nM, Alexis Biochemicals) and GI254023X (5 μ M, Glaxo Smith Kline) were used for blockade of γ -secretase and ADAM10 activity, respectively.

XIV. MDSC/Histamine Cultures and Migration Assays.

Five million MDSCs were cultured with 100 μ M histamine (Invitrogen) and counts were made with trypan blue dead cell exclusion daily for three days. For dose response assays, MDSCs were cultured with 5 μ M, 10 μ M or 100 μ M histamine (Invitrogen). All cell concentrations started with 50,000 cells/well. After 48 hours of growth, a 24hr pulse of [H^3]-thymidine, 1mCi/mL (Perkin Elmer) was used. Plates were then harvested using a Filtermate cell harvester onto GFC plates. Assays were read using a Topcount Plate Counter (Perkin Elmer, Waltham, MA). For migration assays, 2×10^5 B16 melanoma cells, 2×10^5 MCs derived from BMMC or media alone were loaded into the lower well of 8 micron transwell plates (Costar). 2×10^5 MDSCs were loaded into the upper well. Plates were incubated for four hours at 37 °C and the lower well was harvested for flow cytometry to determine migration through the membrane.

XV. *N. brasiliensis* infection, gemcitabine treatment, and HR antagonization.

A. Nb infection.

N. brasiliensis (Nb) larvae were generously provided by Joe Urban (Agriculture Research Station, Beltsville, MD) and were maintained as previously described(222). A10Tg or WT animals were infected with approximately 650 L3 Nb in groups of four or more. Alternatively, Kit^{wsh/wsh} mice and WT controls were infected with approximately 500 L3 Nb. Additional groups of animals were sacrificed on Day 7 post infection and the proximal halves of the small intestines were harvested. Adult worms were then purified and enumerated. Fecal egg burdens were assessed on days 5-13 and enumerated on McMaster counting chamber slides. Serum was collected by tail vein nick on days 7, 14, 21 and 35.

B. Gemcitabine treatment.

For mice injected with GEM, 1.2 mg was injected *i.p.* starting on Day 0 and repeated every five days throughout the experiment. For AT studies, WT mice were injected with 5×10^6 A10Tg MDSCs via tail vein injection starting at day four of infection and repeated every three days thereafter. MDSCs were incubated with FITC labeled anti-Gr-1 (Biolegend, RB6.C85) and selected using anti-FITC magnetic beads (MACS, Miltenyi Biotec).

C. HR antagonization.

Mice were injected with cetirizine (CT), 0.5mg/kg in 100 μ l saline *i.p.* starting on day -1 and repeated daily. For cimetidine (CIM), 20mg/kg was injected in 100 μ L saline *i.p.* starting on day -1 and repeated every two days thereafter.

XVI. Proliferation Assays.

MDSCs were cultured for 15 mins with 10 μ M CT, CIM, or alone prior to the addition of 5 μ M, 10 μ M or 100 μ M histamine (Invitrogen). All cell concentrations started with 50,000 cells/well. After 48 hours of growth, a 24hr pulse of [H^3]-thymidine, 1mCi/mL (Perkin Elmer) was used. Plates were then harvested using a Filtermate cell harvester onto GFC plates. Assays were read using a Topcount Plate Counter (Perkin Elmer, Waltham, MA).

XVII. T cell suppression assays.

For polyclonal T cell activation, CD90.2⁺ T cells (6.67×10^4) were sorted from spleen via FACS and activated with immobilized anti-CD3 (2C11, 10 μ g/mL) and soluble anti-CD28 (37.51, 1 μ g/mL) in 96-well plates. Sorted CD11b⁺ MDSCs (Ly6G⁺, Gr-1⁺, and Ly6G⁻) from A10Tg or LLC tumor bearing mice were then added at increasing T

cell:MDSC ratios. To achieve a total cell number of 2×10^5 /well, CD90.2⁺ WT splenocytes were added. For antigen-specific T cell suppression assays, soluble gp100 (1 μ g/mL) was added to defined ratios of pmel-1 transgenic splenocytes (6.67×10^4) and A10Tg MDSCs. In both situations, after 54hrs of culture, 1 μ Ci of [³H]-thymidine was added to each well for an additional 18 hrs and thymidine incorporation was measured.

XVIII. Western Blot.

A. ADAM10.

Whole cell lysates (30ug) of BM cells generated with lysis buffer containing 0.5% NP40 and protease inhibitors (Roche) were applied to SDS-PAGE and blotted on nitrocellulose membranes. Blots were probed with HRP-conjugated anti-mouse β -actin (Sigma-Aldrich) or unlabeled rabbit anti-ADAM10 (AnaSpec Inc.) followed by HRP-conjugated anti-rabbit IgG (Southern Biotech). Chemiluminescence was visualized with the Pico chemiluminescent kit (Pierce).

B. HR expression.

For HR1 and HR2, five million naïve MDSCs were lysed and prepared as described above. Blots were stained with an H1 Receptor (P-20) and H2-I/Ad β (5K43) both from Santa Cruz, for HR1 and HR2 and detected with Rabbit F(ab')₂ Anti-Goat IgG and Goat Anti-Mouse IgG1-HRP respectively both from Southern Biotechnology Associates, Inc. Equal loading was confirmed by staining with Monoclonal Anti- β -Actin-Peroxidase, antibody produced in mouse from Sigma.

C. STAT signaling.

Five million Ly6G⁺ or Ly6C⁺ cells were cultured for 24 hours with 10 μ M histamine, harvested and lysed in Cell Lysis Buffer (Cell Signaling) on ice for 30 minutes following the manufacturer's protocol. Proteins were run on a NuPage 10% Bis-Tris gel (Invitrogen) and then transferred to nitrocellulose. Blots were stained with pSTAT5 (Y694) from Cell Signaling and pSTAT3 (Tyr 705)-R from Santa Cruz. Detection was performed with Goat anti-Rabbit IgG (H+L)-HRP from Southern Biotechnology Associates, Inc. and detected with SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific).

XIX. Statistical Analysis

P-values were calculated using unpaired two-tailed Student's t-tests or unpaired Mann-Whitney analysis in Graphpad Prism v5. Error bars represent the SEM between samples. $p < 0.05$ is considered statistically significant.

RESULTS

I. ADAM10 overexpression alters hematopoietic cell differentiation by dysregulating S2/S3 cleavage of Notch.

A. Generation of ADAM10 transgenic mice.

To examine the role of ADAM10 in hematopoiesis, we generated A10Tg mice that overexpress murine ADAM10 cDNA under control of the H-2Kb promoter and the IgH enhancer region (Fig. 7A). These transcriptional regulatory units were previously utilized to generate multiple transgenic mouse lines, including TCR, CD23, and bkl transgenics. Thus this vector allows expression in early lymphocyte progenitors(215,223,224). Inclusion of the IgH enhancer results in preferential expression on B lineage cells. Two founder lines, F240 and F258, were generated, and Southern blot analysis of genomic DNA from F2 progeny demonstrated that both lines contain similar copy numbers of the transgene (Fig. 7B). Because progeny of both lines have nearly identical phenotypes, the following data are presented from line F240, unless otherwise stated.

B. ADAM10 overexpression prevents B2 but not B1 lymphocyte development.

Western blot and flow cytometric analysis demonstrated that transgene expression resulted in elevated levels of ADAM10 in BM cells, including pro/pre B cells (B220⁺IgM⁻) (Fig. 8A-C). Unexpectedly, overexpression markedly reduced the levels of pro/pre B cells and immature B cells (B220^{hi}IgM⁺) in BM (Fig. 8A). This led to a near complete loss of peripheral B cells in peripheral organs including blood, lymph nodes and spleen (Fig. 9A). Analysis of B cells from peritoneal fluid revealed that levels of B1a

(B220^{int}CD11b⁺CD5⁺) and B1b cells (B220^{int}CD11b⁺CD5⁻) in A10Tg mice were not significantly altered compared to littermate (LM) controls, whereas B2 cells (B220^{hi}CD11b⁻CD5⁻) were nearly absent (Fig. 9B). Thus, the block in B cell development was specific to BM-derived B2 cells.

C. Reduced thymocyte progenitors in A10Tg mice.

Transgene expression suppressed development of thymocyte progenitors. A ten-fold reduction was observed in Lin⁻CD44⁺c-kit⁺ early thymocyte progenitors (ETPs) in A10Tg BM compared to LM (Fig. 9C). Accordingly, A10Tg mice exhibited reduced levels of total, DN and DP thymocytes concomitant with a small thymus. However, numbers of single positive thymocytes and peripheral T cells in PLN and the spleen were not altered in A10Tg mice (Fig. 9D).

D. MDSC expansion in A10Tg mice.

Concomitant to the reduction in lymphocyte levels, A10Tg animals exhibited significant myeloid accumulation. In fact, the spleens of A10Tg mice were noticeably enlarged, weighing an average of 2.5 fold more than LM spleens and contained twice as many nucleated cells (Fig. 10A). The forward and side scatter pattern of A10Tg splenocytes indicated the presence of large granular myeloid cells (Fig. 10B). While the majority of WT CD11b⁺Gr-1⁺ cells differentiate into mature myeloid cells prior to exiting the BM, A10Tg CD11b⁺Gr-1⁺ cells expanded in BM, constituting 93.5% of BM cells, and entered the spleen and PBL at dramatically high levels (Fig. 10C). CD11b⁺Gr-1⁺ cells outside the bone marrow are classified as MDSCs, consisting of monocytic (CD11b⁺Gr-1^{int}Ly6G⁻) and granulocytic (CD11b⁺Gr-1^{hi}Ly6G⁺) subsets(111). Light

micrographs of sorted A10Tg CD11b⁺Gr-1⁺ splenocytes and flow cytometry confirmed that A10Tg mice contain both monocytic and granulocytic MDSCs in PBL and spleen (Fig. 10D-F).

E. ADAM10 prevents commitment of CLPs to the B cell lineage.

The expansion of myeloid cells in conjunction with blockade of B2 cell development in A10Tg mice indicates that ADAM10 may regulate the commitment of BM progenitors to myeloid or lymphoid lineages. Thus, we attempted to determine the developmental stage at which this occurs. According to the classical model of hematopoiesis, HSCs in the BM develop into CMPs that differentiate into myeloid cells or CLPs that give rise to early thymocyte precursors or pro-B cells(34). Although a small level of B220⁺ cells was present in A10Tg, further analysis revealed that they express CD11b and Gr-1 (Fig. 11A,B), indicating that alterations in hematopoiesis occur prior to the pro-B cell stage. This prompted us to examine the levels of HSCs, CLPs, and CMPs in A10Tg mice. Analysis of BM lineage positive cells (Ter119, CD3ε, CD11b, Gr-1) and CD19⁺ cells demonstrated the near absence of Lin⁻CD19⁺ B cells in A10Tg mice. However, the percentage of Lin⁻CD19⁻ cells was similar to LM levels (Fig. 12A). Lin⁻CD19⁻ LM cells contain B220⁺ early B cell precursors, which were absent from A10Tg BM (Fig. 12B). Lin⁻CD19⁻B220⁻ cells can be further divided based upon IL-7 receptor (IL-7R) expression. Gating on IL-7R⁻ cells demonstrated that the percentage of HSCs (Lin⁻CD19⁻B220⁻IL-7R⁻c-kit^{hi}sca^{hi}) was slightly lower in A10Tg BM, while the percentage of CMPs (Lin⁻CD19⁻B220⁻IL-7R⁻c-kit^{hi}sca⁺) was 2-fold higher than the level of LM CMPs (Fig. 12C). In contrast, analysis of IL7R⁺ cells revealed a 2-fold decrease in

CLP (Lin⁻CD19⁻B220⁻IL-7R⁺c-kit^{int}sca^{int}) levels in A10Tg BM (Fig. 12D). Additionally, Inlay *et al.* recently demonstrated that Ly6D⁺ CLPs are committed to the B cell lineage, whereas Ly6D⁻ CLPs are uncommitted lymphoid progenitors(225). In accordance with their report, 89% of LM B220⁺ cells and 30% of LM CLPs expressed high levels of Ly6D (Fig. 12E,F). However, only 0.16% of A10Tg CLPs were Ly6D⁺(Fig. 12E). Finally, A10Tg HSCs and CMPs do not overexpress ADAM10 on the cell surface. However, 55.3% of A10Tg CLPs express elevated levels of ADAM10 compared to 28.4% of LM CLPs (Fig. 11C). These findings indicate that hematopoietic alterations in A10Tg mice occur prior to the commitment of CLPs to the B cells.

F. ADAM10 alters myeloid but not LSK development.

To further determine the stage at which A10 overexpression altered cell differentiation, we analyzed numerous subsets of LSK cells and myeloid precursors. As mentioned above, multiple studies have shown that BM LSK cells can be subdivided into various distinct populations based upon CD34 and fms-related tyrosine kinase 3 (Flt3) expression: CD34⁻Flt3⁻ long-term HSCs (LT-HSCs), CD34⁺Flt3⁻ short-term HSCs (ST-HSCs), and finally CD34⁺Flt3⁺ multipotent progenitor populations (MPPs)(44,226,227). We therefore analyzed these distinct populations within the LSK compartment in A10Tg mice. Although the percentage of LSK cells was slightly lower in A10Tg BM (Fig. 13A), we did not observe any differences in the levels of LT-HSC, ST-HSCs nor MPPs between LM and A10Tg mice (Fig. 13C). However, the percentage of myeloid progenitors (Lin⁻c-kit^{hi}sca⁻) in A10Tg mice was approximately 2-fold greater than the level in LM mice (Fig. 13B). It was previously shown that myeloid progenitors can also

be subdivided into three distinct populations based upon CD34 and low affinity IgG Fc receptors (FcγRII/RIII). These include CD34⁺FcγRII/III^{lo} Common Myeloid Progenitors (CMP), CD34⁺FcγRII/III^{hi} Granulocyte-Macrophage Progenitors (GMP), and CD34⁺FcγRII/III^{lo} Megakaryocyte-Erythroid Progenitors (MEP)(44). As indicated above, there is a striking difference in the myeloid compartment of A10Tg compared to LM BM. GMP and MEP populations are nearly absent from A10Tg mice. Additionally, approximately 80% of the A10Tg myeloid progenitors are in the CMP stage compared to 31.6% in the LM. This finding may account for the systemic expansion of CD11b⁺Gr-1⁺ MDSCs in A10Tg mice but not other mature myeloid cells such as neutrophils or macrophages.

G. ADAM10-mediated hematopoietic dysregulation is cell-intrinsic.

To confirm that the observed phenotype was the result of transgene expression by hematopoietic cells and not a dysregulation in the BM stromal environment, mixed BM assays were conducted. Irradiated CD45.2⁺ A10Tg hosts were reconstituted with LSK cells from WT CD45.1⁺ congenic mice, and irradiated WT CD45.1⁺ congenic hosts were reconstituted with CD45.2⁺ A10Tg LSK BM cells. Despite being in a WT host, A10Tg BM recapitulated the observed alteration in hematopoiesis of A10Tg mice: lack of B cells and increased MDSCs(Fig. 14A). Despite being in an A10Tg host, WT BM cells demonstrated normal cell differentiation: higher levels of B cells and minimal circulating MDSCs (Fig. 14B). This indicated that altered cell differentiation in A10Tg mice was due to alterations in signaling pathway(s) within hematopoietic cells rather than the microenvironment. Mixed BM chimera studies further confirmed this, as irradiated

CD45.1⁺ WT hosts were reconstituted with a mixture of LSK BM cells from CD45.2⁺ A10Tg and CD45.1⁺ WT donors. This resulted in similar reconstitution of host BM by A10Tg and WT donor cells. Hematopoietic development of CD45.1⁺ WT cells was similar to development in LM control mice, and development of CD45.2⁺ A10Tg cells closely mimicked development in A10Tg mice. MDSCs only expanded from A10Tg BM cells, and B lineage cells predominantly differentiated from WT BM (Fig. 15A,B). These results demonstrate that ADAM10-mediated MDSC expansion is not the indirect result of abrogated B cell development, trans-cleavage of BM stromal cell ligands, nor cytokine secretion, which would cause WT MDSC expansion. They also illustrate that ADAM10 overexpression on hematopoietic cells causes MDSC expansion via an intrinsic cell autonomous mechanism.

H. ADAM10 overexpression alters hematopoiesis by dysregulating Notch signaling.

Recent studies have demonstrated a critical role for ADAM10 and perturbed Notch signaling in embryonic, thymocyte, and MZB development(12,14,228). Therefore, to further elucidate this interaction and determine the effect of ADAM10 overexpression on Notch signaling in HSC development, we cultured purified HSCs from LM and A10Tg mice on OP9 stromal cells that lack Notch ligand (OP9-GFP) or express Notch ligand, Delta-1, (OP9-DL1). The addition of IL-7 and Flt3L promote WT HSC differentiation into T cells on OP9-DL1 cells and differentiation of B cells on OP9-GFP cells(229). Examination of OP9-GFP cultures showed that while LM HSCs developed into B cells, A10Tg HSCs only generated CD11b⁺Gr-1⁺ myeloid cells (Fig. 16A). Analysis of OP9-DL1 cultures demonstrated that A10Tg HSCs are capable of T cell

differentiation in the presence of Notch ligand (Fig. 16B). However, their development was delayed compared to LM HSCs. This further supports the adverse effect of ADAM10 overexpression on early thymocyte development.

I. Differential effects of S2 and S3 cleavage of Notch.

In order to further test the hypothesis that ADAM10 overexpression perturbs Notch signaling, we examined the effects of dysregulating Notch signaling in LM and A10Tg HSCs. Inhibition of Notch signaling with the addition of a γ -secretase inhibitor (GSI) or the ADAM10 inhibitor (GI254023X) to OP9-DL1 cultures prevented LM and A10Tg T cell development (Fig. 16C). In accordance with reports of ADAM10's critical role in Notch S2 cleavage in other cell types, this result demonstrates that ADAM10 also mediates S2 cleavage in developing hematopoietic precursors(12,16,20). Most interestingly, although both inhibitors equally blocked Notch-dependent T cell differentiation, their effects on the development of myeloid and B lineage cells were distinct. ADAM10 inhibition in OP9-DL1 cultures resulted in B cell development of LM and A10Tg HSCs, whereas GSI inhibition caused myeloid expansion (79.4% of LM and 76.7% of A10Tg cells) that was strikingly similar to the myeloid differentiation of A10Tg cells in OP9-GFP cultures (88.5%) (Fig. 17A). Additionally, ADAM10 inhibition in OP9-GFP cultures not only resulted in enhanced LM B cell development, but it also rescued B cell development of A10Tg cells and prevented myeloid differentiation (Fig. 17B). This further supports our finding that ADAM10 activity inhibits B cell development. Finally, GSI treatment of LM OP9-GFP cultures had no effect on HSCs, demonstrating that GSI treatment directs myeloid development only upon S2 product

accumulation. Accordingly, A10Tg HSCs exclusively developed into Gr-1⁺ myeloid cells in the absence of ligand or following GSI treatment. However, ligand engagement and GSI activity prevented S2 accumulation and myeloid development in LM and A10Tg cultures. Thus, these results indicate that ADAM10 overexpression may also direct myeloid development by generating excessive S2 product that is not processed in the absence of ligand-induced γ -secretase activity.

II. A10Tg MDSCs are analogous to tumor-derived MDSCs and require MCs for their activity.

A. A10Tg MDSCs are analogous to tumor-induced MDSCs.

Although MDSC accumulation is a byproduct of ADAM10 overexpression in early hematopoietic progenitors, ADAM10 expression is not altered in these cells. Therefore, we examined whether A10Tg mice could be exploited to study MDSC-mediated immune regulation in an environment devoid of confounding tumor derived factors. To this end, we conducted T cell proliferation assays with A10Tg MDSCs in order to ensure that A10Tg derived MDSCs are functional. Accordingly, *ex vivo* T cell suppression assays were performed. A10Tg MDSCs inhibited T cell proliferation of both A10Tg and WT purified T cells with anti-CD3/anti-CD28 polyclonal T cell stimulation (Fig. 18A, B). We also tested antigen specific T cell stimulation using T cells from pmel-1 mice, which have T cell receptor transgenic that specifically recognize the melanocyte differentiating antigen gp100 peptide(230). Indeed, MDSCs purified from A10Tg mice also suppressed antigen specific T cell proliferation (Fig. 18C). Like tumor-derived MDSCs, A10Tg MDSCs also consists of two subsets: granulocytic (CD11b⁺Ly6G^{high})

and more immunosuppressive monocytic (CD11b⁺ Ly6G⁻Ly6C^{high}) subsets(231). As demonstrated in Figure 18A-C, the more immunosuppressive Ly6G⁻ population of MDSCs suppressed T cell proliferation to a larger degree as compared to Ly6G⁺ or Gr-1⁺ MDSCs. Both A10Tg and tumor-induced MDSCs demonstrated similar differential T cell suppression for these two subsets (Fig. 18D). Furthermore, A10Tg T cells also exhibited reduced levels of L-selectin (CD-62L), a receptor required for homing of naïve T cells to the lymph nodes (Fig. 18E,F), similar to what has been previously reported for tumor-induced MDSCs(144). Additionally, MDSCs impair the T cell response through the activity of arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS)(232). Accordingly, A10Tg MDSCs had comparable levels of these enzymes to tumor derived MDSCs (Fig. 18G,H). These *ex vivo* observations indicate that MDSCs present in A10Tg animals behave similarly to tumor-derived MDSCs.

B. M-MDSCs promote tumor progression and limit AIT.

The B16 melanoma model was employed to redundantly validate the functionality of A10Tg MDSCs and to assess their *in vivo* contribution to tumor growth and therapeutic intervention. This model is well established for characterization of metastatic behavior and response to adoptive immunotherapy (AIT)(219). After two weeks of challenge with B16, A10Tg animals were more susceptible to metastasis than LM controls (Fig. 19A). In fact, A10Tg animals exhibited tumor nodules that were too numerous to count and subsequently assigned a count of 250. AIT exerted a minimal effect in A10Tg mice, suggesting that the presence of MDSCs diminished the activity of adoptively transferred T cells. Conversely, while AIT treatment did not completely

abolish metastasis in LM lungs, it nonetheless induced a significant decrease in tumor load. This may be attributed to the use of non-antigen specific T cells and the lack of lymphodepleting chemotherapy, highlighting the importance of lymphodepletion for optimal activated T cell infusion in immunotherapy. Accordingly, our AIT protocol was modified to incorporate the chemotherapeutic agents cyclophosphamide (CYP) and gemcitabine (GEM). CYP lymphodepletes recipient mice, permitting homeostatic proliferation of transferred T cells and GEM preferentially decreases MDSC levels in tumor-bearing mice(233,234). A10Tg mice treated with GEM alone exhibited a response comparable to their LM counterparts (Fig. 19B,C), demonstrating that T cell functionality is retained upon MDSC depletion. Incorporation of GEM concomitant with CYP and activated T cell transfer resulted in complete regression of metastasis in both groups. Inspection of lungs from A10Tg mice illustrates the significant abrogation of metastasis arising from tritherapy (Fig. 19D). It is important to note that in A10Tg mice, GEM administration selectively diminished MDSCs levels but did not alter the lymphocyte population (Fig. 20A).

The preceding experiments were performed using LM T cells. Given the profound ability of MDSCs to suppress antigen-specific T cell stimulation, the ideal AIT would utilize tumor-specific T cells alongside chemotherapy. Accordingly, our protocol was adjusted to incorporate pmel-1 mice that are T cell receptor transgenic for gp100 melanoma peptide(219). As predicted, A10Tg MDSCs exerted parallel effects regardless of antigen specificity. A10Tg mice had much higher metastasis levels than LM without treatment and only responded favorably to AIT upon MDSC depletion (Fig. 20B). These

studies indicate that A10Tg MDSCs promote tumor metastasis and compromise the efficacy of cancer immunotherapy. Lastly, given the published literature and our observation that M-MDSCs are more immunosuppressive than G-MDSCs, we conducted AT of both MDSC subsets with B16 Melanoma. As shown in Figure 21, M-MDSCs were more potent promoters of B16 colonization to the lungs compared to G-MDSCs. Taken together, our data indicate that A10Tg MDSCs are functionally and phenotypically parallel to tumor-derived MDSCs.

C. G-MDSCs enhance parasite clearance.

Next, we examined the effect of MDSCs on the immune response in helminth infections. While MDSC accumulation with *Nippostrongylus brasiliensis* (Nb) has been reported, the direct immunomodulatory role of MDSCs in anti-Nb responses has not been examined(204). A WT response to Nb is characterized by production of Th2 associated cytokines that ultimately mediate adult worm (L5) clearance 10-12 days post-inoculation(235). Following Nb challenge, A10Tg animals had significantly reduced fecal egg counts compared to WT controls (Fig. 22A). This is highly correlated with 50 percent reduction in L5 burden in A10Tgs (Fig 22B). This indicated that MDSCs may facilitate parasite clearance. To determine if the increased worm clearance was correlated with an increase in Th2 cytokines, we examined a panel of Th2 cytokines. Analysis of peak infection (day 7) serum levels of IL-4, IL-5 and IL-13 demonstrated significantly enhanced levels in Nb infected A10Tg animals as compared to infected LM (Fig. 22C). Additionally IL-17 and IL-33, which have been reported to recruit MDSCs, were also

elevated in serum of A10Tg mice(173,236). These serum cytokines then decreased as infection began to decline (data not shown).

To determine whether enhanced Nb clearance is dependent on MDSC-activity, MDSCs were depleted from A10Tg mice. GEM treatment resulted in elevated egg counts in A10Tg mice similar to WT levels (Fig. 22A). However, GEM-mediated MDSC depletion in A10Tg mice significantly exacerbated the peak level of Nb infection. Taken together, the data indicate that the enhanced immune response in A10Tgs is a consequence of elevated MDSC levels. Next, to eliminate off target effects of GEM, A10Tg MDSCs were purified and adoptively transferred to WT mice over the course of Nb infection. AT of MDSCs resulted in significantly reduced egg counts, comparable to A10Tgs (Fig. 22A). To determine which MDSC subset was responsible for this observation, MDSCs were purified based upon Ly6G and Ly6C expression and AT into WT Nb infected mice. The Ly6G^{high} (G-MDSCs) population had the same effect as total Gr-1⁺ purified MDSCs while the AT Ly6C^{high} (M-MDSCs) had no effect. This indicates that the granulocytic population of MDSCs mediates the anti-Nb immunity (Fig. 22E). The direct role of MDSCs was further supported in WT mice that were T cell depleted. These mice exhibited the same rapid rate of clearance as WT mice infused with MDSCs (Fig 22A). As expected, Nb clearance was significantly slower in T depleted WT mice without AT of MDSCs (Fig. 22D).

D. Interaction with MCs is critical for MDSC-mediated Nb clearance.

Mucosal MC hyperplasia is a hallmark of gastrointestinal helminth infection which enhances the immune response, leading to parasite clearance(237). In fact, mice

deficient in MCs exhibit delayed clearance kinetics(238). Given that MCs mainly produce Th2 polarizing and pro-inflammatory cytokines and that MCs chemoattract MDSCs, we examined whether the interaction between the two cell types could be contributing to the enhanced Nb expulsion. Surprisingly, AT of MDSCs into MC deficient mice did not enhance Nb expulsion, indicating that a MDSC/MC interaction is critical for MDSC-mediated anti-Nb responses (Fig. 22F). To assess this interaction *ex vivo*, MCs were co-cultured with MDSCs. This resulted in synergistic increases in IL-6, IL-13, TNF- α and MIP-1 α (Fig. 23).

E. In the absence of MCs, MDSCs fail to promote B16 colonization to the lungs.

Given that MDSCs require MCs to enhance anti-Nb immunity, the B16 metastasis study was re-evaluated in MC deficient mice. Several groups have demonstrated that MCs recruit MDSCs, which accumulate in the tumor microenvironment and correlate with poor prognosis(173,239,240). In addition, MC-deficient mice have decreased rates of tumor growth that increase to WT levels subsequent to MC reconstitution(241). Therefore, we anticipated a lower degree of B16 metastasis in *Kit^{Wsh/Wsh}* compared to WT controls, which would be enhanced in the presence of MDSCs. To this end, *Kit^{Wsh/Wsh}* and WT mice were injected with B16 with and without AT of MDSCs. WT mice were more susceptible to B16 metastasis compared to *Kit^{Wsh/Wsh}*. Upon AT of MDSCs, WT mice exhibited similar levels of tumor nodules as B16 A10Tgs, indicating the direct contribution of MDSCs to suppression of the immune response. However, much to our surprise, AT of MDSCs into *Kit^{Wsh/Wsh}* mice failed to increase B16 metastasis (Fig. 24A,B). Thus, these results demonstrate that MDSCs

promote B16 melanoma metastasis in a MC-dependent manner and indicate that MC/MDSC interactions significantly enhance the MDSC-mediated suppression of antitumor immunity but accelerate parasite clearance.

III. Histamine contributes to MC-mediated MDSC regulation.

A. MCs single-handedly promote MDSC-mediated parasite clearance.

To confirm that enhanced parasite clearance and necessity of MCs was not unique to A10Tg MDSCs or our *Kit^{Wsh/Wsh}* mice, similar studies were repeated with tumor derived MDSCs as well as another strain of MC deficient mice. Mice were injected *s.c.* in the flanks with Lewis Lung Carcinoma (LLC) cells and MDSCs were isolated from tumor bearing mice and adoptively transferred into WT mice. As shown in 25, MDSCs derived from LLC-bearing mice also accelerated Nb expulsion. This demonstrated that our observations were not unique to A10Tg MDSCs. Additionally, to confirm that the reduced parasite clearance in *Kit^{Wsh/Wsh}* MC deficient mice was due to lack of MCs and not the mouse model, *Kit^{Wsh/Wsh}* were reconstituted with MCs. Upon reconstitution, AT of MDSCs promoted parasite clearance similar to our observations in WT mice, suggesting the requirement of MCs (Fig. 26). We further confirmed this observation using another strain of MC deficient mice, *Cpa3^{cre}*; *Mcl-1^{fl/fl}* mice (C57BL/6 background). These mice express Cre recombinase under the control of a segment of the carboxypeptidase A3 (Cpa3) promoter. C57BL/6-*Cpa3^{cre}*; *Mcl-1^{fl/fl}* mice are severely deficient in MCs and basophils with no other apparent hematologic changes(218). Parallel to *Kit^{Wsh/Wsh}* mice, AT of MDSCs also failed to accelerate the clearance of Nb in the *Cpa3^{cre}*; *Mcl-1^{fl/fl}* mice

(Fig. 27). Overall, these studies confirm that MCs are indeed required for MDSC-mediated parasite clearance.

B. MCs induce MDSC migration and activation in the liver.

Given the literature highlighting MCs as attractants of MDSCs and the importance of MDSC recruitment in an immune response, the trafficking behavior of MDSCs was analyzed after AT in the presence or absence of MCs. To monitor their migration, MDSCs were labeled with the PKH26GL lipid dye and AT *i.v.* into naïve mice. In agreement with the published literature, we observed a preferential migration of MDSCs 18hrs post AT to the liver (Fig. 28A)(221). To determine if this trafficking pattern is utilized in helminth infection, MDSCs were labeled and infused *i.v.* concomitant with Nb challenge in WT and Kit^{Wsh/Wsh} mice. Indeed, MDSCs trafficked to the liver in infected WT mice. However, the accumulation of MDSCs in the liver was significantly reduced in Kit^{Wsh/Wsh} mice, indicating the role of MCs in MDSC recruitment (Fig. 28B,C). These results were confirmed by *in vitro* migration assays, in which MDSCs exhibited a high degree of migration toward MCs (Fig. 28D). This is consistent with published literature indicating that MDSCs traffic to the liver in tumor models and that MCs secrete mediators in the liver through the bile(242).

Based on the literature and our previous observations, we hypothesized that upon migration to the liver, MDSC interaction with MCs culminates into increased cytokine production that mediates Nb expulsion. Accordingly, MDSCs were compared pre and post AT to the liver. Intriguingly, we saw an increase in IL-4 and eotaxin, both important

for Nb clearance from post AT MDSCs (Fig. 29). This suggests that perhaps cross-talk with MCs in the liver is necessary to enhance MDSC activity.

C. MDSCs express HR1 and HR2.

Given the contribution of histamine to myeloid activity, cell proliferation, and Th2 skewed immune responses, we examined whether histamine could serve as a potential mediator in the MC/MDSC cross communication(178,181). Given the recent reporting of HR1 and HR2 expression on tumor-derived MDSCs, we wanted to determine whether A10Tg MDSCs also expressed these receptors. Indeed, both G-MDSCs and M-MDSCs purified from A10Tg mice expressed HR1 (Fig. 30A). Western blot not only confirmed HR1 expression but also demonstrated HR2 on MDSCs (Fig. 30B), thus making the contribution of histamine a very likely mediator in the MC/MDSC interaction.

D. Histamine promotes MDSC survival and proliferation.

Given the expression of HR on MDSCs, we next analyzed the effect of histamine on MDSCs directly. Accordingly, MDSCs were purified from spleens of A10Tg mice and cultured in the presence of histamine. Indeed, exposure to histamine prolonged MDSC survival and induced proliferation in a dose dependent manner, compared to cRPMI(Fig. 31A,B). The contribution of histamine was confirmed using cetirizine (CT), an HR1 antagonist. As shown in Fig. 32, CT inhibited histamine-mediated cell proliferation and cimetidine (CIM), an HR2 antagonist exhibited similar results. The next logical step in our assays was to determine which fraction of MDSCs was most affected by histamine. Using proliferation assays, we demonstrate that both M-MDSCs and G-

MDSCs are responsible to the proliferative effects of histamine (Fig. 33). Although both subsets exhibited increased proliferation in the presence of histamine, M-MDSCs appeared to be more proliferative.

E. Histamine enhances MDSC enzyme expression.

Given the role of histamine in the immune system, and its ability to induce MDSC proliferation *in vitro*, a logical next step was to examine its role in modulating MDSC enzymatic activity. Accordingly, MDSCs were cultured with histamine or MCs and subsequently analyzed for expression of Arg 1 and iNOS via quantitative PCR (qPCR). Indeed, histamine enhanced the expression of both Arg 1 and iNOS (Fig. 34A,B). Intriguingly, in the presence of MCs, only Arg1 was upregulated. We next wanted to determine if the effects of histamine was subset specific parallel to proliferation. We compared iNOS and Arg 1 levels in G-MDSC and M-MDSC subsets. In line with their proliferative response, M-MDSCS exhibited increased Arg1 and iNOS over the G-MDSCs (Fig. 34C,D). Its important to note that these studies need to be confirmed but nonetheless provide the platform for a very novel role of histamine for MDSC activity.

F. MDSC-mediated parasite clearance is abrogated by HR antagonism.

To confirm the effects of histamine on MDSC activity *in vivo*, we conducted Nb infection studies with HR1 and HR2 blockade. Accordingly, mice were challenged with 650 L3 Nb and received MDSC *i.v.* in conjunction with CT. Administration of CT significantly abrogated the ability of MDSCs to accelerate Nb expulsion (Fig. 35A). This was correlated with reduced infiltrating MDSCs in the liver (Fig. 35B) and diminished

phenotypic conversion towards G-MDSCs (Fig. 35C). Given the spectrum of histamine receptors and our observation of HR1 and HR2 expression on MDSCs, we examined whether histamine was acting through HR1 independently or congruent with HR2. Accordingly, we incorporated CIM into our experiments. As shown in Figure 32, CIM also blocked histamine mediated MDSC proliferation *ex vivo*. This was confirmed with *in vivo* administration of CIM to Nb infected mice also receiving AT of MDSCs. Parallel to CT, CIM also inhibited the ability of MDSCs to accelerate Nb expulsion, albeit to a lower degree (Fig. 35D). Administration of CIM also reduced MDSC migration and phenotype conversion in the liver (Fig. 35E,F). This suggests distinct yet overlapping roles of histamine mediated via HR1 and HR2 on MDSCs in generating and anti-parasitic immune response.

G. HR antagonization inhibits the immunosuppressive potential of MDSCs.

As mentioned earlier, several studies have indicated the contribution of MCs and histamine to tumor progression(243,244). Accordingly, we were interested in determining if MDSCs potentially served as bridge linking histamine release and tumor progression. To do this, we challenged mice with B16 melanoma concomitant with MDSC administration in the presence or absence of histamine. Intriguingly, mice given B16, AT of MDSCs, and treated with CIM had significantly reduced colonization to the lungs compared to mice given B16 and AT of MDSCs without CIM treatment, as determined by lung weight (Fig. 36). It should be noted that CIM alone had no significant effect on tumor burden in the absence of AT of MDSCs. This is consistent

with data showing similar results utilizing a model of murine LLC lung tumor where CIM treatment resulted in reduced tumor size(180).

H. Histamine induces differential STAT signaling.

Histamine has been shown to induce Stat3 signaling, and the importance of STAT3 in MDSCs is well documented(35,42,56,73). Furthermore, many groups have shown that STAT3 and STAT5 are differentially regulated in myeloid cells(245). Therefore, we wanted to examine which signaling mechanism is triggered by histamine in MDSCs. To do this, monocytic and granulocytic MDSCs were cultured in the presence of histamine and proteins isolated for STAT3 and STAT5 proteins. Although rather preliminary, our data thus far indicate that histamine promotes STAT3 signaling in M-MDSCs and STAT5 in G-MDSCs (Fig. 37). This is consistent with our observation that proliferation of M-MDSCs is stimulated by histamine and numerous studies indicate STAT3 as the main signal transducer of MDSC differentiation and proliferation(94,171).

I. Symptomatic allergic patients have higher circulating MDSCs.

Allergy is a MC-mediated, Th2-dependent immune response. Allergic patients experiencing symptoms have increased levels of circulating MC derived histamine(246). To elucidate the translational relevance of our findings and our prediction that allergic patients will have higher circulating MDSCs, we isolated PBL from symptomatic allergic patients and non-allergic controls. Human MDSCs are defined as HLA-DR⁻ CD33⁺CD11b⁺ cells that can also be differentiated into monocytic (CD14⁺) and granulocytic (CD15⁺) subsets(132). In accordance with our prediction, allergic patients exhibited a larger percentage of circulating MDSCs compared to non-allergic control

patients (Fig. 38A,B). These findings require analysis of additional patient samples but nonetheless indicate a role of histamine in MDSC subset accumulation.

J. Histamine promotes the ex vivo survival of MDSCs generated in stage1 primary breast cancer.

Given the ability of histamine to prolong life-span of murine MDSCs, we wanted to examine if the same would hold true for human MDSCs. To do this, we utilized previously purified and cryopreserved samples of MDSCs from a stage1 primary breast cancer patient. In line with published observations, these human MDSCs did not survive in cultured medium in the 48hr window. However, we observed a steady population and enhanced life span of MDSCs even at 48hrs in the presence of histamine (Fig. 39). While quite preliminary, this observation has important implications for the utilization of histamine to prolong MDSC survival in *ex vivo* studies.

DISCUSSION

I. ADAM10 is required for appropriate hematopoietic cell development and differentiation.

Because ADAM10-mediated S2 cleavage is required for the initiation of the canonical Notch signaling pathway, we hypothesized that ADAM10 serves as a critical regulator in the differentiation of early hematopoietic progenitors. Our experimental observations demonstrate that ADAM10 overexpression attenuates the development of thymocytes, abrogates B2 cell development, and promotes expansion of functional MDSCs via a cell-intrinsic mechanism. Furthermore, our studies indicate that S2 and S3 cleavage products of Notch differentially regulate hematopoietic cell fate determination.

Although effects of Notch signaling in B cell and T cell lineage commitment has been extensively described, the effect of ADAM10 activity in cell differentiation and early B lineage commitment was previously uncharacterized(228). Here, we demonstrate that overexpression of ADAM10 in early lymphoid precursors prevented B2 cell commitment from CLPs. Interestingly, this was specific to BM-derived B2 cells, while development of B1 cells which reside in peritoneal and pleural cavities was not affected, presumably due to the unique developmental pathways of B1 and B2 cells. B1 cells largely originate from fetal liver and the molecular pathways that direct B1 cell development are quite distinct from those critical to B2 cell commitment(247). The sparing of B1 cell development in A10Tg mice is consistent with the finding that B cell-specific deletion of ADAM10 and other regulators of Notch signaling do not affect B1 cell development(13,28,33). Given the lack of B2 cells in A10Tg mice and that we only

observed B2 cell development from A10Tg HSCs upon ADAM10 inhibition in OP9 cultures, we are confident that ADAM10 is heavily involved in B2 lineage commitment. Although ADAM10 may influence B cell development by cleaving multiple substrates, its prominent role in hematopoiesis is the regulation of Notch signaling. In fact, ADAM10 inhibition in OP9-DL1 cultures prevented Notch-dependent T cell development while simultaneously promoting B cell development. This finding is consistent with the report demonstrating that deletion of Notch1 from CLPs results in the development of B cells in the thymus(21). However, we cannot rule out the possibility that other ADAM10 substrates could potentially contribute to the altered hematopoiesis observed in A10Tg mice.

The development of thymocytes from BM precursors in A10Tg mice was delayed in our studies. This was demonstrated by diminished thymus size, a relative inability of A10Tg thymocyte precursors to develop in mixed BM chimeras, and delayed development of A10Tg T cells in OP9-DL1 cultures. The data are consistent with ligand independent cleavage of Notch S2 as a consequence of increased ADAM10 activity. It is possible that, in the absence of ligand, γ -secretase complex is not recruited to the cell membrane and/or becomes saturated, leading to less efficient S3 cleavage and less T cell development. Given the high ligand expression levels in OP9-DL1 cells, we anticipated proper T cell differentiation in A10Tg HSCs as the S2 product would not be in excess. Indeed, T cell development in A10Tg HSCs was comparable to LM HSCs. It should be noted that because A10Tg HSCs are not different from LM, this cannot be attributed to

seeding of different progenitor populations on OP9s but rather excessive ADAM10 causing ligand-independent S2 cleavage.

While the effects of Notch signaling in B and T cell lineage commitment have been thoroughly described, its role in myeloid development is controversial(248). Kawamata *et al.* reported that enforced expression of NICD or Notch target genes, Hes 1 or Hes5, caused non-cell autonomous expansion of Mac-1/CD11b⁺Gr-1⁺ myeloid cells from BM precursor cells(31,249). This is consistent with our model system of increased CD11b⁺Gr-1⁺ myeloid cells in A10Tg MDSCs. Moreover, the elevated levels of CMPs in A10Tg BM and the lack of ADAM10 overexpression by CMPs, CD11b⁺Gr-1⁺ BM cells, and splenic MDSCs indicate that HSC development is altered at an early stage prior to mature cell lineage commitment. Additionally, mixed BM chimeras revealed that myeloid expansion in A10Tg mice occurs by a cell autonomous mechanism. Furthermore, complete γ -secretase inhibition of LM and A10Tg HSCs on OP9-DL1 cultures resulted in myeloid expansion. This is in agreement with other studies that have shown γ -secretase blockade to result in accumulation of the S2 product, that could direct myeloid development(16,20). Indeed, diminished presenilin (PS) dependent γ -secretase activity in PS1^{+/-}PS2^{-/-} mice results in myeloproliferative disease, characterized by accumulation of Mac-1/CD11b⁺Gr-1⁺ cells, causing splenomegaly(40). Thus, ligand independent S2 Notch cleavage and ADAM10 overexpression may direct myeloid development by independent mechanisms. Nevertheless, these studies indicate that perturbation of Notch signaling in HSC development can induce myeloid expansion.

The classical model of hematopoiesis describes the initial dichotomous differentiation of HSCs into CLPs or CMPs. However, this has been challenged by observations in which lymphoid progenitors retain myeloid potential(250-254). For this reason, a myeloid-based model of HSC development is beginning to emerge(34,41). In A10Tg mice, the moderate effects on thymocyte development in combination with the more pronounced effects on B lineage commitment and myeloid expansion indicate that B2 cells and expanded myeloid cells develop from common progenitor(s), whereas thymocytes may develop from a unique precursor. It should be noted that upon lineage depletion, comparable BM cell numbers were recovered from both A10Tg and LM mice. Therefore, the percentage of ETPs obtained is reflective of absolute numbers of thymocyte precursors in the BM. Although peripheral T cell levels are comparable, the observed reduction in ETPs is nonetheless very intriguing and will require further study.

Clearly the differential effects of ADAM10 overexpression on lymphocyte and myeloid development are in congruence with the myeloid-based model of hematopoiesis. The shift in favor of CMPs could explain the robust accumulation of MDSCs in the periphery. Further analysis is required to ascertain whether the lower levels of MEPs and GMPs are a result of a halt in development at the CMP stage, or simply due to the high level of CMP formation. Additionally, because CMPs have been shown to retain erythromyeloid potential, it is not surprising that despite the decreased MEP and GMP compartments, erythropoiesis remains unaffected.

Based on our data, we have formulated a model through which Notch cleavage mediates cell fate determination (Figure 40). In the presence of ligand, the Notch

receptor undergoes cleavage at both S2 and S3 sites, resulting in two products and ultimately promoting T cell development. In the absence of Notch ligand or in the case of blocked S2 cleavage, the Notch receptor remains intact and B cell development results. However, S2 cleavage without S3 cleavage, as observed during ADAM10 overexpression, induces myelopoiesis and delayed T cell development. All of the aforementioned scenarios are predicted to occur under basal physiologic conditions in WT mice and thus, cell differentiation is regulated by the S2/S3 product ratio and the abundance of cleaved Notch products. This concept is further supported by our S2/S3 inhibitor assays in the OP9 culture system. Although further studies are needed to identify the mechanism at a transcriptional level, it appears that three branch points exist in the Notch signaling pathway. The first pathway occurs when an intact Notch receptor is present, resulting in B cell differentiation. This case occurs when either the S2 cleavage site is blocked or in the absence of ligand in WT animals. The second pathway is determined by ligand-independent S2 cleavage, which ultimately results in myeloid expansion, whereas the third case is determined by cleavage at both S2 and S3 sites, affording T cells. The A10Tg animal is representative of the second case, in which ADAM10 overexpression results in excessive ligand-independent Notch cleavage leading to extensive myeloid development. Furthermore, the delayed T cell maturation in both the A10Tg animals and in OP9-DL1 cell cultures can be attributed to limited Notch ligand engagement and cleavage.

The differential effect of S2 and S3 blockade on WT Notch signaling has significant implications for the treatment of Notch-related diseases. Many reports have

proposed the use of GSIs for the treatment of T-ALL and B cell lymphoma, however; our findings indicate that GSI treatment could cause MDSC expansion that would ultimately induce immunosuppression and enhance tumor growth(23,255). Thus, studies of GSI treatment in mice and clinical trials should include careful monitoring of the myeloid compartment. Our studies suggest that the addition of ADAM10 inhibitors to GSI treatment may be a more advantageous strategy.

II. A10Tg MDSCs are functionally analogous to tumor-derived MDSCs and require MCs for their activity.

Despite the profound increase in myeloid precursors, A10Tg mice do not develop any confounding pathologies such as chronic myelogenous leukemia (CML) or accelerated tumor development. Therefore, A10Tg mice afford a unique tool to characterize the immunomodulatory potential of MDSCs in an environment devoid of established tumor and tumor-derived factors. While this property makes our model ideal for the study of neoplasia, we, by extension, demonstrate that A10Tg mice are likewise a viable system to elucidate disease states in which MDSCs are not detrimental to the host. Here we report that 1) MDSCs generated in a tumor free environment, at least with this A10Tg model, are functionally and phenotypically analogous to tumor derived MDSCs. 2) MDSC-mediated augmentation of tumor growth is enhanced by mast cells (MCs). 3) MDSCs exert an immune enhancing potential in promoting a robust anti-helminth immunity that is also largely dependent upon interaction with MCs. 4) MC/MDSC interaction augments cytokine production by both cells, promoting a Th2 skewed immune response that is harmful in neoplastic progression but beneficial in parasitic

infections. Our studies demonstrate that MDSCs possess a duality in terms of their function, contingent upon the pathophysiological context to which they are subjected.

MDSCs derived from A10Tg mice suppress T cell proliferation under both polyclonal and antigen specific stimulation to a degree comparable to tumor-derived MDSCs. A10Tg MDSCs also exhibit comparable Arg1 and iNOS levels to tumor-associated MDSCs. Furthermore, the dramatic increase in metastatic progression and resistance to AIT in A10Tg mice compared to LM controls demonstrates that A10Tg MDSCs contribute to tumor-associated immune suppression. In fact, the increased sensitivity of T cells to MDSCs in A10Tgs illustrates that the *in vivo* presence of MDSCs is sufficient to diminish T cell responses, even in a tumor-free environment. This effect can be rescued upon MDSC depletion, which is evidenced through the ability of A10Tg T cells to halt B16 progression to a similar degree as in LM with GEM treatment. This observation also indicates that MDSC-mediated T cell suppression is not permanent. Even more convincing is the complete regression of the B16 melanoma observed following tri-therapy (GEM + CYP + AIT). These collective observations clearly delineate that the increased metastatic progression in A10Tg mice is a consequence of ADAM10-mediated expansion of functional MDSCs rather than impaired T cell response. It is important to note that we did not observe significant MDSC accumulation in WT mice upon B16 challenge. This makes the B16 model ideal to state the consequence of MDSC administration on B16 progression.

MDSCs are widely recognized for their negative role in the context of neoplasia and antineoplastic therapy. Nonetheless, eradication of these cells may have detrimental

consequences for the host. In fact, studies suggest a beneficial role of MDSCs in graft-versus-host disease (GVHD), autoimmunity, bacterial infections, and more recently, MDSC driven cancer vaccines(55,130,189). In the case of parasitic infections, MDSCs have been reported to accumulate but their effect on the immune response remained unclear(256). Therefore, we utilized A10Tg mice and a model of helminth infection, *N. brasiliensis* to answer the following questions: 1) Whether MDSCs could differentially regulate the immune response depending on the Th1 or Th2 inducing disease state? 2) Whether MCs only function in recruiting MDSCs or if they could actually affect the immunoregulatory activity of MDSCs?

Our results were quite exciting and provide novel insight into the role of MDSCs in regulating the immune response in the context of interaction with MCs and disease progression. The results indicate that MDSCs promote robust anti-helminth immunity. A10Tg mice had a dramatically increased rate of worm expulsion as compared to LM controls. This resistance occurs quite early, as lungworm counts at day 2-post infection were significantly lower in A10Tgs. The restoration to LM susceptibility upon GEM treatment in A10Tg mice coupled with increased resistance in LM animals with AT of MDSCs strongly indicates the involvement of MDSCs. This finding was further solidified by AT of MDSC subsets, which demonstrated that the enhanced anti-helminth immunity was indeed MDSC driven, and that the granulocytic Ly6G⁺ and not the monocytic Ly6C⁺ population of MDSCs enhanced anti-Nb immunity.

Physiologically, the observed anti-helminth immune response in A10Tg mice was accompanied by an upregulation of multiple Th2 cytokines that are critical mediators of

anti-Nb immunity. These observations highlight a mechanistic foundation for MDSC-mediated anti-helminth immunity, namely increased production of Th2 associated cytokines. It is possible that MDSCs may not only secrete Th2 polarizing cytokines, but also stimulate their production by other immune cell populations. The most logical candidate for this relationship comprises MDSCs and MCs. Within the context of helminth infections, IgE mediated MC degranulation initiates cytokine production, ultimately accelerating worm expulsion(237). Given that MCs recruit MDSCs and they mediate parasite clearance, we tested whether this interaction was also critical in the immune response. The challenge of MC deficient Kit^{Wsh/Wsh} mice with Nb concomitant with MDSC AT provided novel insight. The data showed that while MDSCs can accelerate parasite clearance, they require MCs for their activity. This is likely to be cytokine mediated. In fact, *in vitro* analysis of MC/MDSC co-cultures demonstrate a synergistic increase in measured cytokines; especially IL-13 which is critical for helminth expulsion(257).

Given our observation of MDSC's dependency on MCs in anti-Nb immunity, increased cytokine production in MC/MDSC co-culture, and recent data of tumor development indicating that MCs are capable of mobilizing MDSC populations, we tested the contribution of MC/MDSC crosstalk to metastasis. Prior to our investigations, it was unclear if once recruited by MCs, MDSCs would then act independently or require further interaction with MCs. Therefore MC deficient Kit^{Wsh/Wsh} WT controls were utilized to examine MDSC-mediated B16 progression in the presence or absence of MCs. While we agreed with published literature that B16 colonization to the lungs would be

reduced in MC deficient mice, we nonetheless expected increased colonization upon MDSC AT (241). However, much to our surprise, the presence of MDSCs had little effect on tumor growth in these MC deficient hosts, suggesting the requirement of MCs for MDSC's immunosuppressive tumor-promoting activity. This finding was quite exciting and prompted us to further confirm and examine the crosstalk between MCs and MDSCs.

III. Histamine is a key player in the MC/MDSC interaction.

While our results were quite intriguing, we needed to first ensure that our observations were not unique to MDSCs isolated from A10Tg mice and the *Kit^{Wsh/Wsh}* MC deficient model. To this end, we employed an additional model of MC deficiency as well as conducted a parallel MDSC AT into Nb infected mice with MDSCs generated in WT LLC bearing mice. Our assays have demonstrated that regardless of source, MDSCs exert an immunoregulatory role depending on disease state and absolutely require MCs. By extension, we have also determined histamine to be an important mediator in the observed MC/MDSC interaction.

The ability of MDSCs derived from LLC bearing mice to clear Nb, clearly demonstrates that enhanced parasite clearance is not unique to A10Tg animals. The observation that MDSCs also failed to promote Nb clearance in *Cpa3-Cre; Mcl-1^{fl/fl}* MC deficient mice but regained activity in MC reconstituted *Kit^{Wsh/Wsh}*, clearly emphasize that MDSCs depend on MCs to exert their effects. Furthermore, AT assays of labeled MDSCs in these model systems allowed us to examine the trafficking pattern of MDSCs in relation to MCs. Previous literature indicates that during tumor progression, MDSCs

traffic and accumulate in the liver, where they inhibit Kupffer cells and T cells to dampen anti-tumor immune responses(221,258,259). Moreover, hepatic MDSCs have been shown to produce a wide array of proinflammatory and regulatory cytokines and chemokines in response to tumor challenge(221). Indeed, in our Nb infection model, MDSCs preferentially migrate to the liver. Thus, it is possible that in the liver, MDSC interact with hepatic MCs and become further activated. In fact, it was reported that hepatic MCs release histamine and other mediators through the portal bile ducts and into the bile(242), which can have significant implications for parasite load in the gut. Interestingly, we observed a significant increase in c-kit⁺ cells in the liver after Nb infection. This highlights the fact that the liver may be an important site for MC accumulation and activity in Nb infection. The interaction between MCs and MDSCs in the liver would presumably lead to increased cytokine production and soluble mediator release such as histamine that would enhance MDSC activity and subsequent Nb expulsion (Fig. 41). This could provide a potential explanation for our observations that in the absence of MCs, MDSC migration to the liver and parasite expulsion are significantly reduced.

MCs and histamine have been shown to play an important role in parasitic clearance. Livestock animals with increased numbers of MCs and concentrations of histamine are positively correlated with natural helminth resistance or tolerance(260). Acting in conjunction with IL-4 and IL-13, histamine has been associated with increased smooth muscle cell contractions to enhance the ‘sweep’ portion of the ‘weep and sweep’

method of parasitic clearance(261,262). Our studies also demonstrate that histamine further promotes parasite expulsion through MDSC activation and proliferation.

We show that MDSCs express both HR1 and HR2, which are classically linked to the immune system. The addition of histamine to MDSCs protected against cell death and increased cellular proliferation. Interestingly, this was more prominent in M-MDSCs than in G-MDSCs. This could be attributed to the fact that granulocytes have a shortened half-life as compared to monocytes in culture(150,172). Furthermore, histamine enhanced Arg1 and *i*NOS expression by MDSCs. Interestingly, uncrosslinked MCs co-cultured with MDSCs upregulated Arg1 but not *i*NOS expression. This could indicate that uncrosslinked MCs do not release histamine sufficiently to induce *i*NOS activation of MDSCs, demonstrating that MC degranulation is needed for optimal MDSC activation. These studies are supported by a recent publication in which histamine blockade with CIM increased MDSC apoptosis, reduced Arg1 and *i*NOS enzyme expression, and decreased tumor burden(180). More recently, it was shown that MDSCs are also capable of synthesizing low levels of histamine, which directed their differentiation and survival (181).

The interaction between histamine and MDSCs became more apparent upon histamine receptor blockade in our *in vivo* model systems of disease progression and *in vitro* mechanistic assays. Administration of HR1 and HR2 antagonists in mice challenged with Nb and given MDSC AT recapitulated our findings in MC deficient mice. This suggests that histamine signaling is the bridge between MCs and MDSCs. CT has been previously shown to affect migration of cells of myeloid lineage(263,264). This may be

the reason that MDSC trafficking to the liver is reduced but still does not explain why G-MDSCs and not M-MDSCs were reduced. HR2 antagonists have been shown to alter T cell production of IL-14, IL-5 and IL-13, all of which are involved in appropriate anti-helminth immunity(257,265,266). Although preliminary, our findings also indicate that hepatic MDSCs produce IL-4 after Nb infection, indicating that perhaps CIM works by reducing MDSC cytokine production involved in parasite clearance rather than their migration.

One of the most important signaling pathways for MDSC accumulation has been STAT3(245,267). Interestingly, we found that histamine induced differential STAT signaling; predominantly STAT3 in monocytic MDSCs and STAT5 in granulocytic MDSCs. This observation is in line with previously published data indicating that the upregulation of STAT3 is associated with increased cellular proliferation and enzymatic activity of MDSCs(42,94,171). Because neutrophils have a short half-life, it was not surprising that the neutrophil-like G-MDSCs exhibited a lower degree of proliferation and STAT3 signaling as compared to M-MDSCs(150). While the MC/MDSC interaction is multifarious, we propose that MC-derived histamine drives the distinct MDSC subpopulations to differential STAT signaling, inducing distinct phenotypic activation that further directs MC activity.

Upregulation of MDSCs in patient peripheral blood has been indicated in many cancers and suggested as a marker of poor prognosis(268-270). The link between inflammation and cancer has been heavily studied in the last decade but remains elusive (271-273). We suggest that MC/MDSC interactions can potentially contribute to this

phenomenon. To our knowledge, we are the first to report MDSC expansion in symptomatic allergic patients; especially G-MDSCs. The link between histamine and human MDSCs is further supported by increased survival of human MDSCs cultured with histamine.

Collectively, our results demonstrate that MCs are required for MDSC motility and activity. Given our findings and current literature on MDSCs, we have generated a model of this interaction (Fig. 41). We hypothesize that in the liver, as well as other sites of immune response, resident MCs attract MDSCs and activate them via histamine release. Upon activation, MDSCs secrete Th2 cytokines, most notably IL-4 and IL-13, which promote Th2 differentiation, along with IL-6 and TGF- β that chemoattract MCs. In this context, T cells not only contribute to the pool of IL-4 and IL-13 but also increase IgE synthesis by B cells. IgE in turn further activates MCs. These events afford a self-sustaining and synergistic cycle of MC/MDSC activation that results in increased survival, proliferation, and activation. This ultimately leads to a Th2 skewed immune response that is detrimental in neoplasia but beneficial for helminth expulsion. This interaction has important implications for regulation of MDSC activity via antihistamines and MC stabilizing agents for disease states in which the accumulation of MDSCs is detrimental to the host.

IV. Conclusions and Significance.

The goal of this dissertation project was to determine the physiologic significance of ADAM10-mediated Notch cleavage in hematopoiesis. To this end, we generated mice with increased ADAM10 expression in early progenitor cells. Consequently, A10Tg

mice exhibit abrogated B cell development, delayed T cell maturation, and increased immature myeloid cells. As a result, these mice have allowed our laboratory to make significant contributions to the literature in the context of hematopoietic cell development, neoplastic progression, and anti-parasite immunity.

Our data demonstrate that the proteolytic activity of ADAM10 regulates the lineage commitment of B2 cells and the expansion of functional MDSCs in a cell-intrinsic manner. Moreover, A10Tg mice provide a model system to further examine MDSC expansion and MDSC-mediated immune suppression in the absence of confounding tumor-derived factors. Consequently, we have shown that increased MDSCs render A10Tgs more susceptible to tumor metastasis but resistant to helminth infection. MCs are required for both the observed immunosuppressive and immunoenhancing properties of MDSCs. MDSCs traffic to MCs, affording a synergistic Th2 skewed immune response. While undesirable in the context of neoplasia, the observed response is ideal in parasitic (at least with respect to helminth) infections. This augmentation of MDSC activity in the presence of MCs was further evaluated using the mast cell derived factor, histamine. We demonstrate that MDSCs express HR1 and HR2 with increased expression on M-MDSCs. Histamine promotes MDSC activity and proliferation, particularly in M-MDSCs. Furthermore, HR 1 and HR2 antagonization abrogate MDSC activity in our murine model of parasitic infection as well as tumor progression.

Unresolved inflammation increases myelopoiesis, resulting in premature bone marrow release of a heterogeneous group of mononuclear (CD11b⁺Ly6C^{high}) and

polymorphonuclear (CD11b⁺Ly6G^{high}) cells, known as myeloid derived suppressor cells (MDSCs). MDSCs have been associated with increased risk of metastatic progression and poor therapeutic response in cancer patients. We have recently begun to demonstrate an important role for Mast Cells (MCs) and MC-derived histamine in this MDSC-mediated immune suppression. Furthermore, the data presented here suggest that MDSCs may serve as the bridge between controversial correlation of allergic inflammation and tumor progression. Our data underscore a previously unappreciated functional duality for MDSCs, ranging from immunosuppressive to immunosupportive contingent upon physiological context. These studies have important implications for enhancing tumor rejection by the modulation of MDSC activity and application of pharmacologic inhibitors of MC function in new therapeutic strategies for cancer treatment.

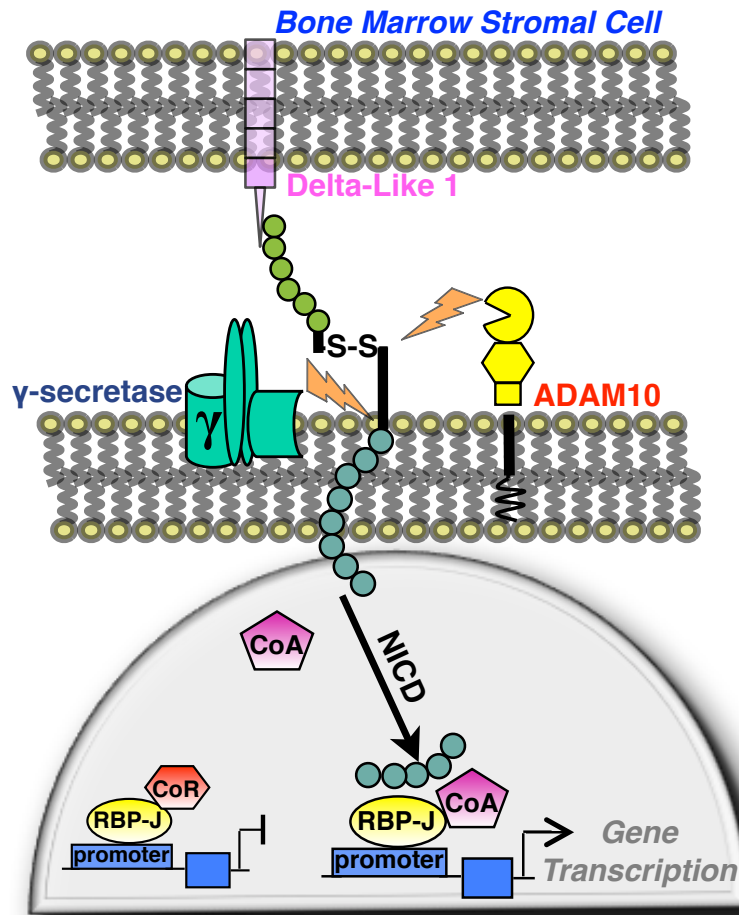


Figure 1. ADAM10-mediated Notch signaling. At the surface, Notch is expressed as an integral membrane protein, consisting of both extracellular (NEXT) and intracellular domains (NICD). Once engaged with its ligand (Delta-Like 1), the extracellular domain undergoes an ADAM10-mediated cleavage at site 2 (S2). This event generates a substrate for γ -secretase complex to perform a final cleavage of Notch at site 3 (S3), releasing the transcriptionally active NICD that subsequently translocates to the nucleus to induce downstream signaling pathways.

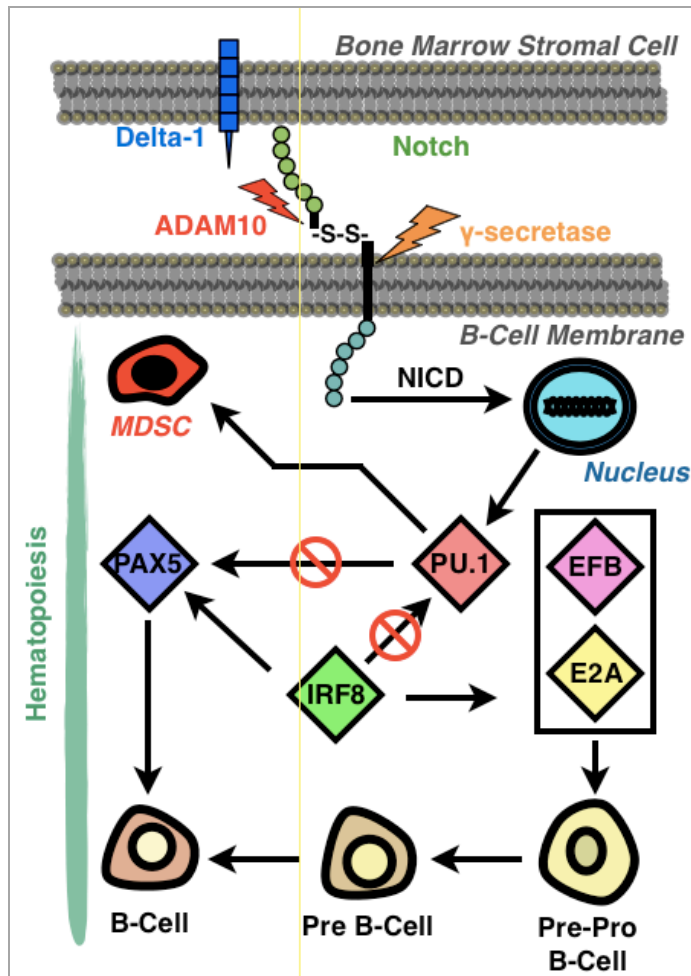


Figure 2. Putative role of ADAM10 in hematopoietic cell differentiation. Subsequent to ligand engagement and appropriate cleavage by ADAM10 and γ -secretase, Notch intracellular domain (NICD) translocates to the nucleus and facilitates transcriptional upregulation of PU.1, mediating differentiation between MDSCs and B cells. IRF-8 serves as a positive EBF/E2A transcription system, which regulates B cell development. IRF-8 also acts as negative modulator of PU.1, alleviating suppression of the PAX5 transcription factor required for B-cell development. Therefore, the expression of IRF-8 and PU.1 transcription factors as well as Notch signaling regulates MDSC development.

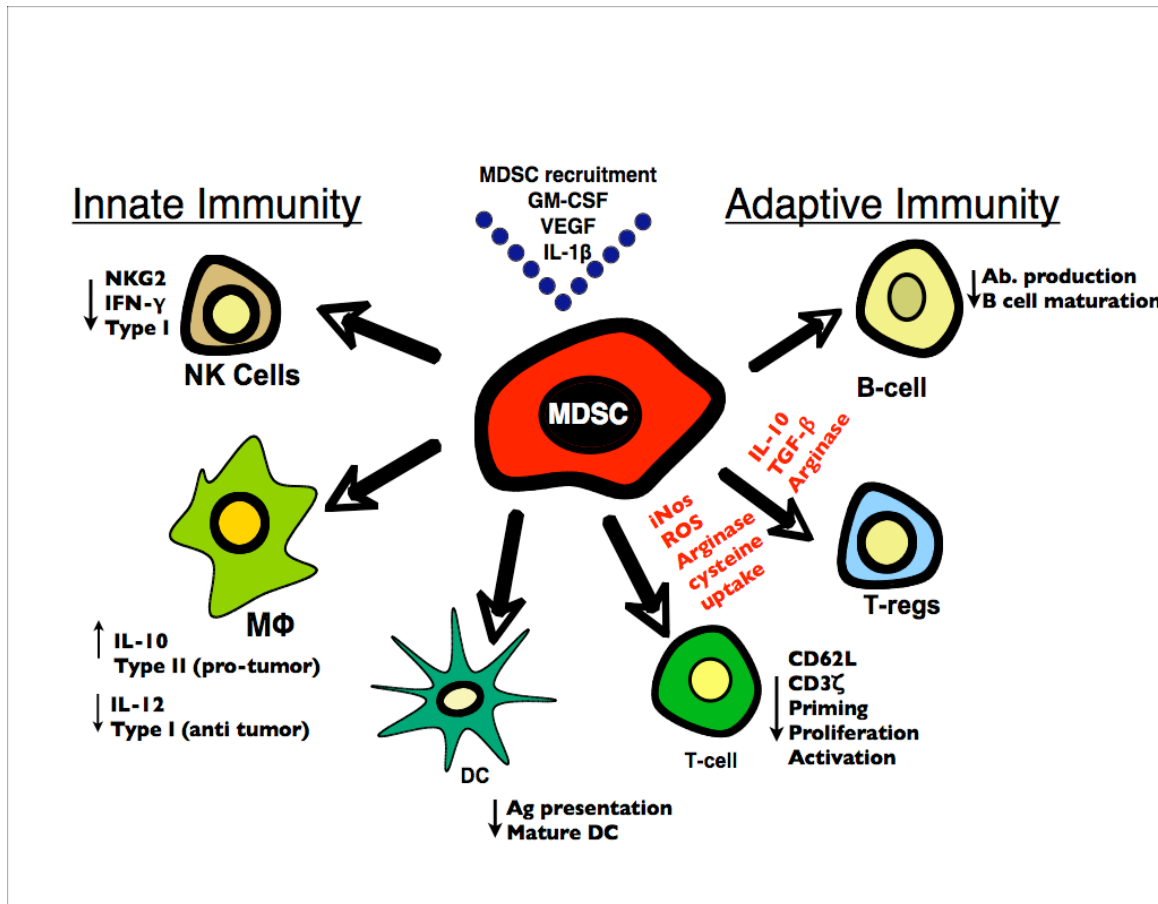


Figure 3. MDSC induction and function. Various hematopoietic cytokines such as GM-CSF and VEGF promote the premature release of MDSCs from the BM. Once recruited to the site of immune response, MDSCs impair both arms of the immune response. In particular, they inhibit T cell activation, induce T-regulatory cells, limit B cell antibody production, restrict DC maturation, promote the activation of immunosuppressive Type 2 macrophages, and impair appropriate NK activity.

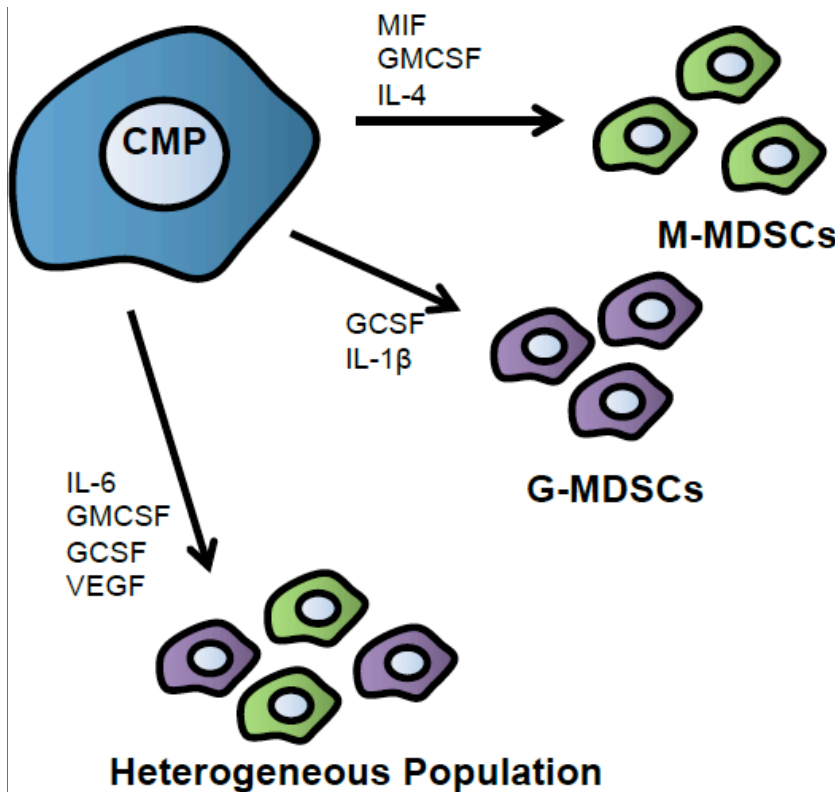


Figure 4. Cytokine mediated MDSC generation. Heterogeneous MDSC generation is a consequence of numerous cytokines. However, the development of granulocytic or monocytic MDSCs is unique to certain cytokines; GM-CSF and IL-4 promote M-MDSCs while G-CSF and IL-1 β promote G-MDSCs.

	G-MDSC	M-MDSC
T cell suppression	Antigen specific	Antigen specific or Antigen independent
Enzymatic Activity	ROS, Arg1	iNOS, Arg1
Cytokines	TNF- α , IFN γ , TGF- β	IL-6, IL-10, IL-23, TGF- β , VEGF- α
Survival	Short life span Non-proliferative	Long life span Highly proliferative
Phenotypic Markers Human	CD15, CD66b, VEGFR1, CD62L, IL-4R(CD124), IL-13R	CD14, CCR2, CD124, S100A9
Mouse	Ly6G, CD244, CXCR2(IL-8R β), CXCL1, CXCL2, CXCR1, CXCR2, CSF-R(CD115), SLAMF4(CD244)	CCR2, CD49d, S100A9, CD124, CD115, CCR7, CX3CR1, F4/80, CD93, Dectin-1, FAS, ADAM17

Figure 5. MDSC subset identification based upon protein expression and function.

MDSCs can further be delineated into granulocytic or monocytic subpopulations based upon surface marker expression, cytokine production, and function.

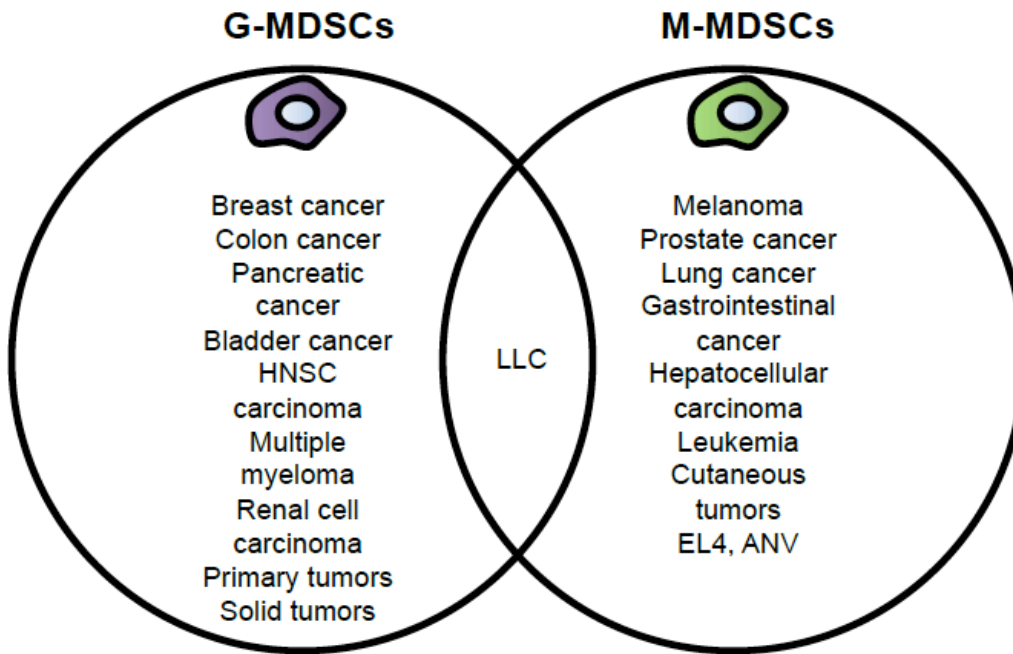


Figure 6. MDSC accumulation in neoplasia is type specific. Granulocytic or monocytic MDSC accumulation is unique to anatomical location of neoplastic progression. Overall, G-MDSCs tend to comprise most cancers, particularly solid tumors.

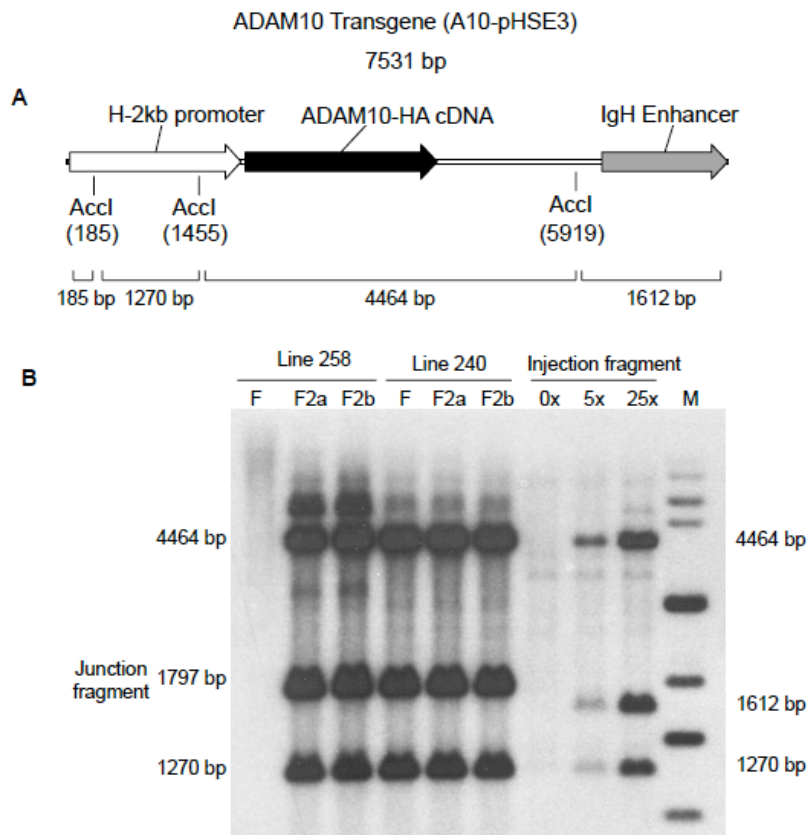


Figure 7. Generation of ADAM10 transgenic mice. (A) Schematic of the 7.5-kb XhoI injection fragment containing the murine ADAM10-HA cDNA and the murine H-2Kb promoter and IgH enhancer regulatory elements. (B) Southern blot analysis of genomic tail DNA from both founders (F) and their F2 progeny (F2a and F2b) digested with AccI and electrophoresed on a 0.9% agarose gel along with 1 kb DNA Ladder markers (M). The injection fragment shown in (A) was used as both probe and copy number control (5x and 25x). A10Tg lines 240 and 258 possess greater than 25 copies of the transgene, generating 1797 bp junction fragments (1612 bp + 185 bp) indicative of head-to-tail arrays, as well as 1270 bp and 4464 bp internal fragments. The founder of line 258 appears to be mosaic, identifiable as a transgenic by PCR only.

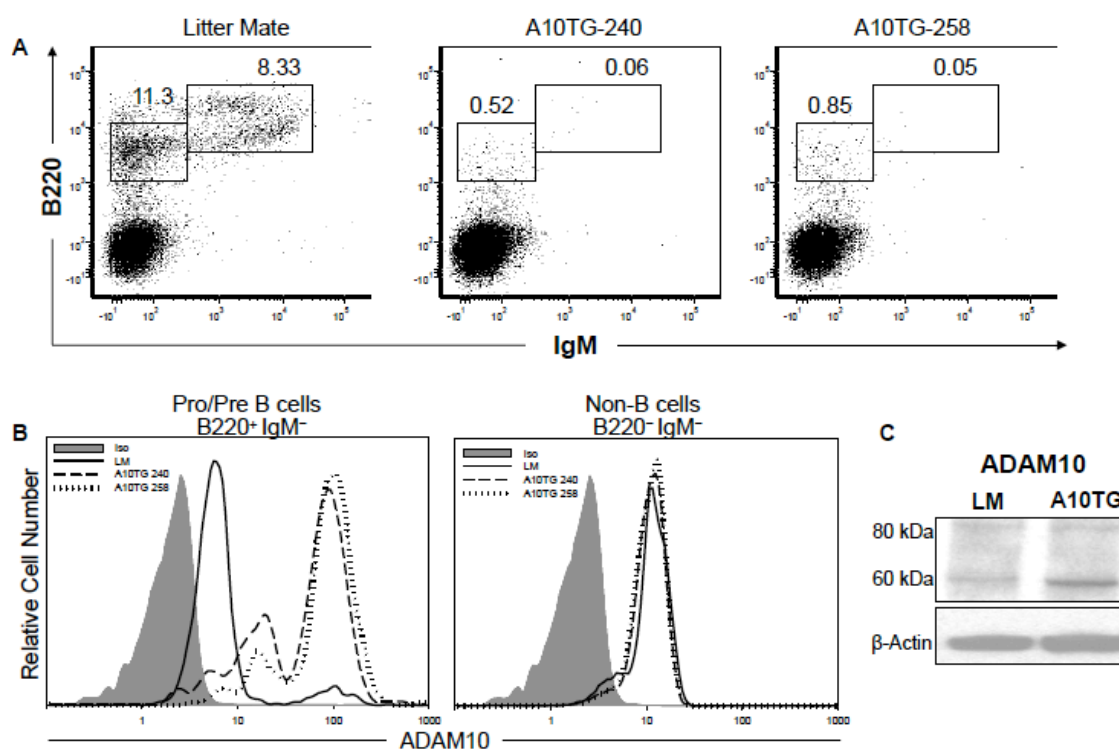


Figure 8. ADAM10 Overexpression blocks B2 B cell development. (A) Flow cytometric analysis of pro/pre B cells (B220⁺IgM⁻) and immature B cells (B220^{hi}IgM⁺) in BM of littermate (LM) and A10Tg progeny. (B) ADAM10 surface expression by B220⁺IgM⁻ cells and B220^{hi}IgM⁺ cells. Dot plots and histograms in (A,B) are representative of 6 independent experiments. (C) Western blot analysis of ADAM10 and β-actin protein levels in whole cell lysates of BM cells from indicated mice; ADAM10 pro-form (80 kDa) and ADAM10 mature form (60 kDa), representative of 3 independent experiments.

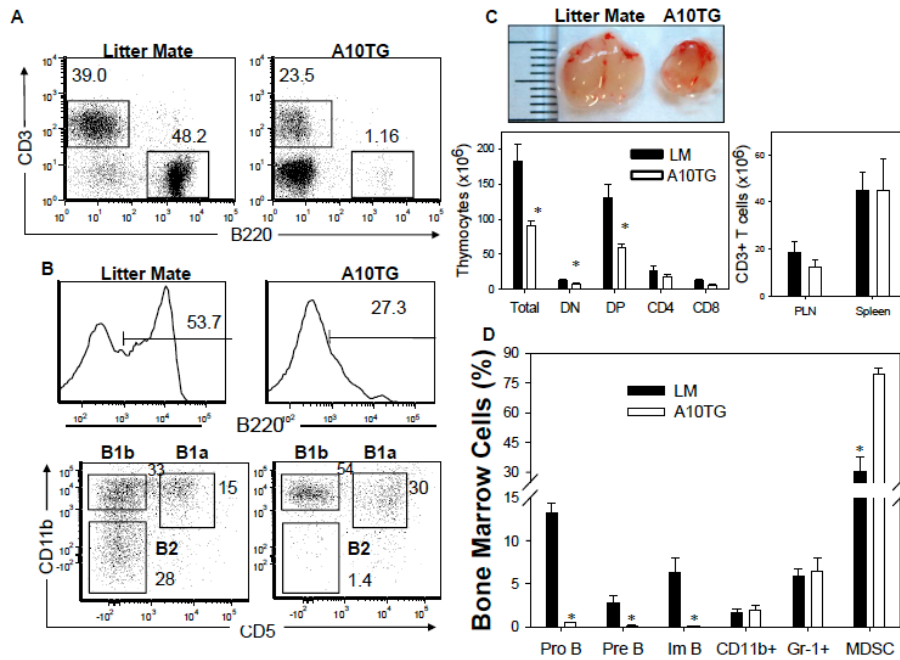


Figure 9. ADAM10 overexpression does not affect B1 B cell development but suppresses thymocyte progenitors. (A,B) Flow cytometric analysis of (A) T cells (CD3⁺) and B cells (B220⁺) in spleen (SPL); and (B) B cell subsets in peritoneal fluid. Lower two panels are gated on B220⁺ cells in upper histograms. B2 cells: B220^{hi}CD11b⁻CD5⁻, B1a cells: B220^{int}CD11b⁺CD5⁺, B1b cells: B220^{int}CD11b⁺CD5⁻. (C) Representative thymi from indicated mice. Bone Marrow cells were analyzed for Lin⁻CD25⁻CD44⁺c-kit⁺ early thymocyte progenitors (ETPs). Lineage cocktail includes B220, Ter-119, CD11b, Gr-1, CD3, CD4, CD8. (D) Amount of thymocyte subsets and CD3⁺ T cells in the spleen and PLN; n=4, mean ± SEM, DN: CD4⁻CD8⁻, DP: CD4⁺CD8⁺, CD4: CD4⁺CD8⁻, CD8: CD4⁻CD8⁺. Dot plots and histograms are representative of 6 (A), 4(B), 3(C) independent experiments. Numbers on plots indicated percent of gated cells within box. * p<0.05

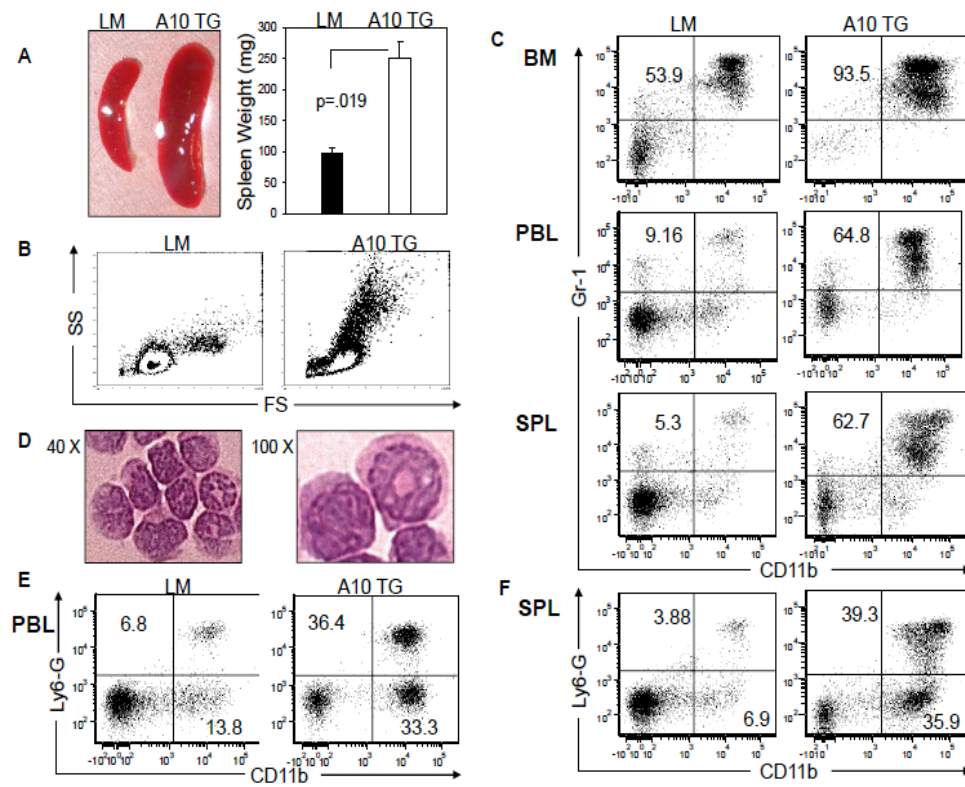


Figure 10. ADAM10 overexpression causes the expansion of myeloid-derived suppressor cells. (A) Representative spleens and average spleen weight of indicated mice, $n=4$, mean \pm SEM. Flow cytometric analysis of (B) forward scatter (FS) vs. side scatter (SS) of splenocytes, the percentage of (C) $CD11b^{+}Gr-1^{+}$ MDSCs present in the bone marrow (BM), PBL, and spleen (SPL), and the percentage of (E,F) $CD11b^{+}Ly6-G^{+}$ and $CD11b^{+}Ly6-G^{-}$ MDSCs in (E) PBL and (F) spleen of indicated mice. (D) 40X and 100X photomicrographs of sorted $CD11b^{+}Gr-1^{+}$ splenocytes from A10Tg mice. Flow cytometry plots and photomicrographs are representative of 4 independent experiments. Numbers on plots indicate the percentage of cells in the UR (C) and UR,LR quadrants (E) and (F).

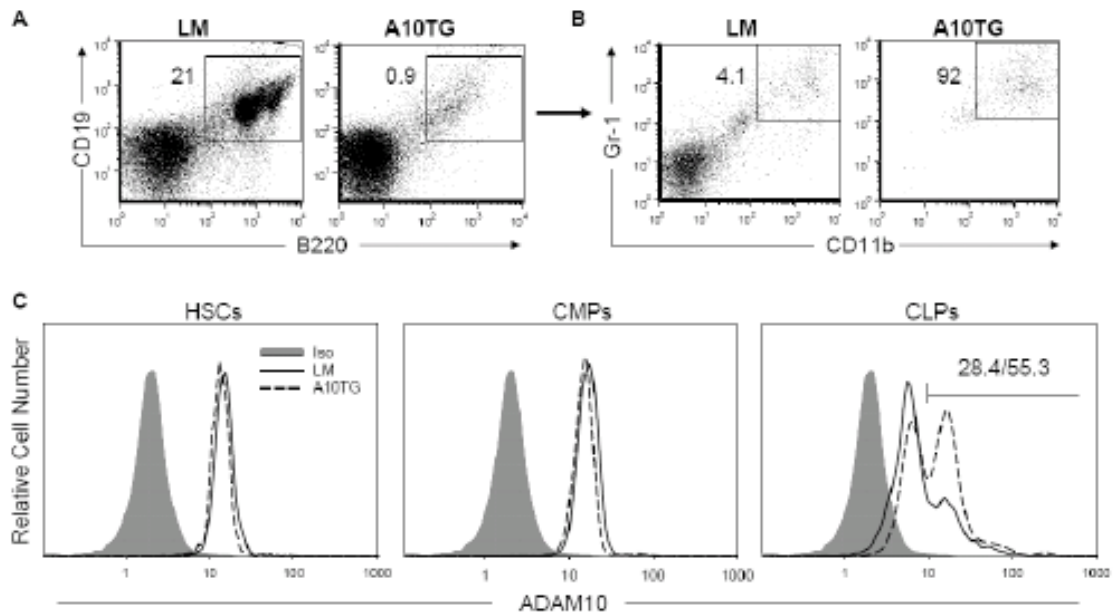


Figure 11. A10Tg B220⁺CD19⁺ bone marrow cells express myeloid markers. (A,B) Flow cytometric analysis of (A) CD19 and B220 labeled cells and (B) Gr-1 and CD11b labeled cells, gated on B220⁺CD19⁺ cells shown in (A). (C) ADAM10 surface expression by hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and common lymphoid progenitors (CLPs) from BM. Numbers on dot plots and histograms indicate percent of gated cells shown in box or linear markers, respectively; representative of 3 independent experiments.

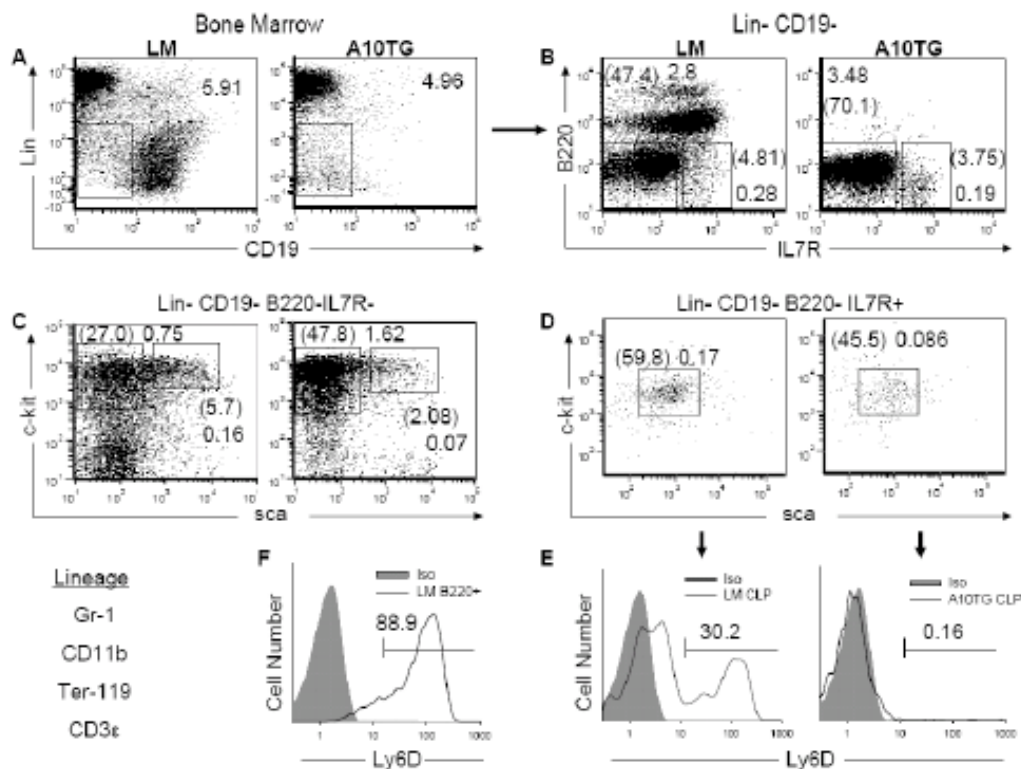


Figure 12. Overexpression of ADAM10 alters hematopoiesis prior to CLP commitment to the B cell lineage. Flow cytometric analysis of (A) Lineage negative (Lin⁻) and CD19 labeled BM cells, (B) B220 and IL-7 receptor (IL7R) expression by Lin⁻ CD19⁻ gated cells, (C,D) c-kit and sca-1 expression of (C) Lin⁻CD19⁻B220⁻IL7R⁻ cells and (D) Lin⁻CD19⁻B220⁻IL7R⁺ cells. (E,F) Expression of Ly6D by (E) CLPs of LM and A10Tg mice, and (F) Lin⁻CD19⁻B220⁺ BM cells from LM mice. Numbers on dot plots and histograms indicate the percentage of BM cells (percentage of gated cells) in box or linear marker, respectively. The cell lineage includes Ter-119, CD11b, Gr-1, and CD3ε positive cells. Plots are representative of 3 independent experiments. Lin⁻ populations are defined as follows, HSCs: Lin⁻CD19⁻B220⁻IL7R⁻c-kit⁺sca-1^{hi}; CMPs: Lin⁻CD19⁻B220⁻IL7R⁻c-kit⁺sca-1^{int}; CLPs: Lin⁻CD19⁻B220⁻IL7R⁺c-kit^{int}sca-1^{int}.

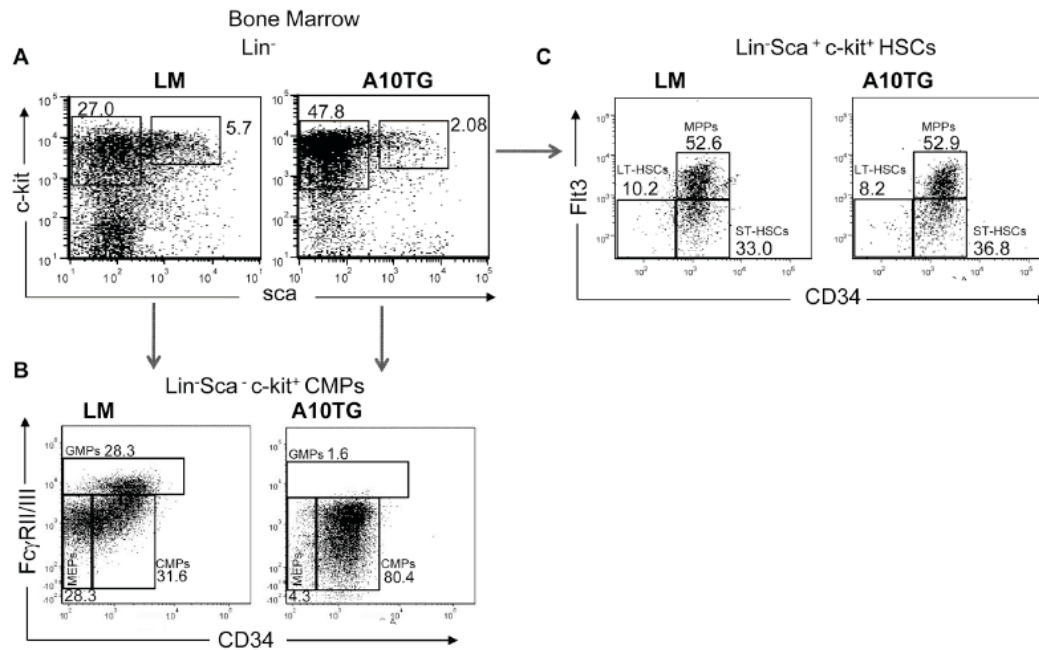


Figure 13. ADAM10 overexpression dysregulates development of myeloid and T cell progenitors, but not LSK subsets. Flow cytometric analysis of (A) Lin⁻ bone marrow cells. (B) CD34 and FcγRII/III expression on CMP gate from (A). (C) CD34 and Flt3 expression in the Lineage⁻Sca-1⁺c-kit⁺ (LSK) gated cells from (A). (D) Bone marrow cells were also analyzed for early thymocyte progenitors (ETPs). For D, CD25 was added to the lineage negative cocktail. Numbers on dot plots indicate the percentage of gated BM cells. Cell lineage cocktail includes B220, Ter-119, CD11b, Gr-1, and CD3 positive cells. Plots are representative of 3 independent experiments. LSKs were gated as Lin⁻sca-1^{hi}c-kit⁺ and subdivided to Long-term Hematopoietic Stem Cells (LT-HSCs): CD34⁻Flt3⁻, Short-term Hematopoietic Stem Cells ST-HSCs: CD34⁺Flt3⁻, and Multipotent Progenitors (MPPs): CD34⁺Flt3⁺. The c-kit⁺ myeloid cells were gated and

defined as Common Myeloid Progenitors (CMPs): $CD34^{+}Fc\gamma RII/III^{lo}$, Granulocyte-Macrophage Progenitors (GMPs): $CD34^{+}Fc\gamma RII/III^{hi}$, and Megakaryocyte-Erythroid Progenitors (MEPs): $CD34^{+}Fc\gamma RII/III^{lo}$.

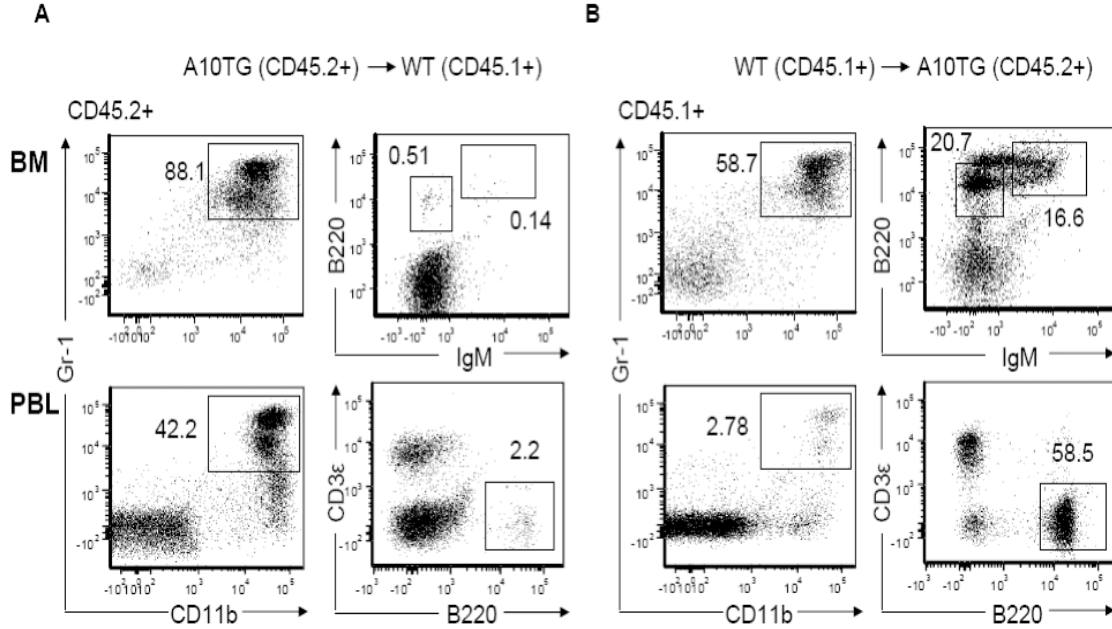


Figure 14. Altered hematopoiesis is unique to A10Tg HSCs. Flow cytometric analysis of BM and PBL cells gated on (A) CD45.2⁺ cells developing from adoptively transferred A10Tg Lin⁻ BM cells in CD45.1⁺ congenic WT mice, and cells gated on (B) CD45.1⁺ cells developing from adoptively transferred WT Lin⁻ BM cells in A10Tg mice, 6 weeks after transfer. BM and PBL plots are representative of 2 and 4 independent experiments, respectively. Numbers on plots indicate the percentage of gated cells within box.

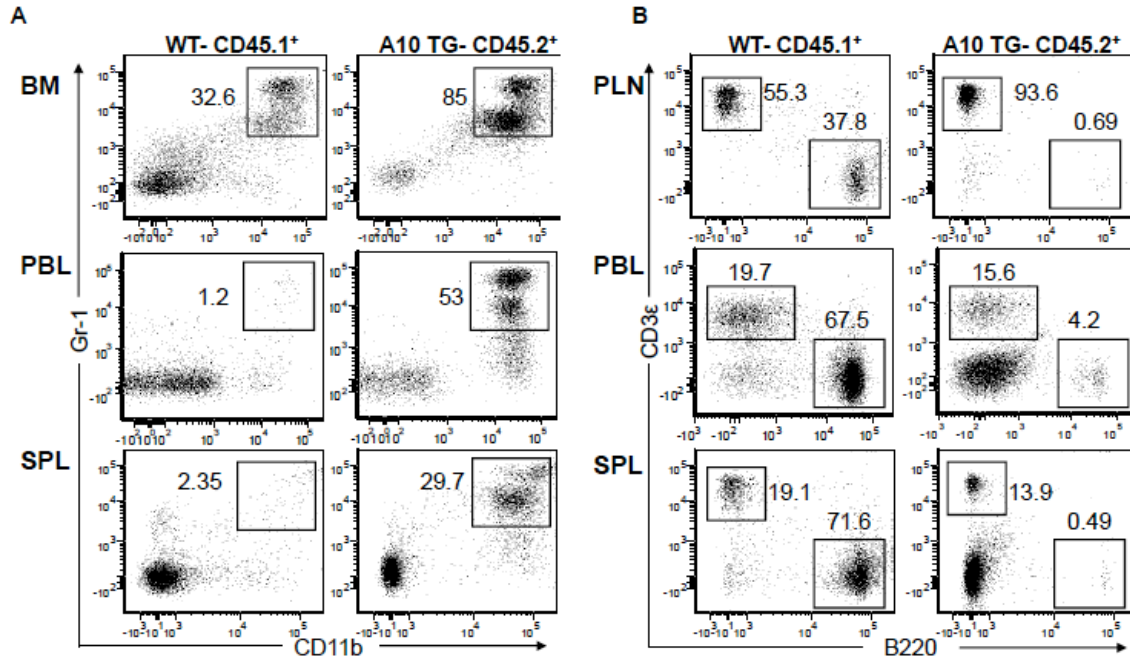
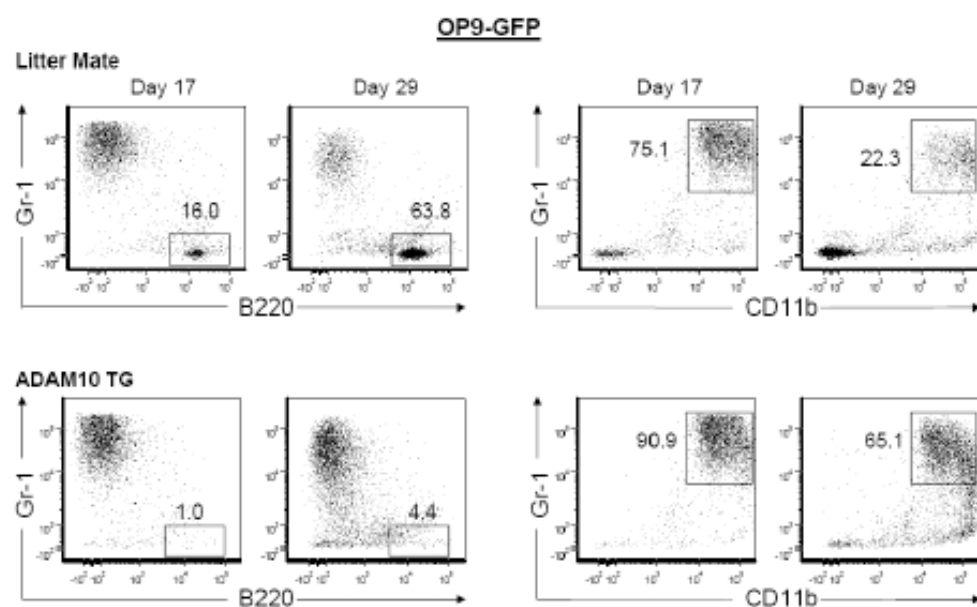
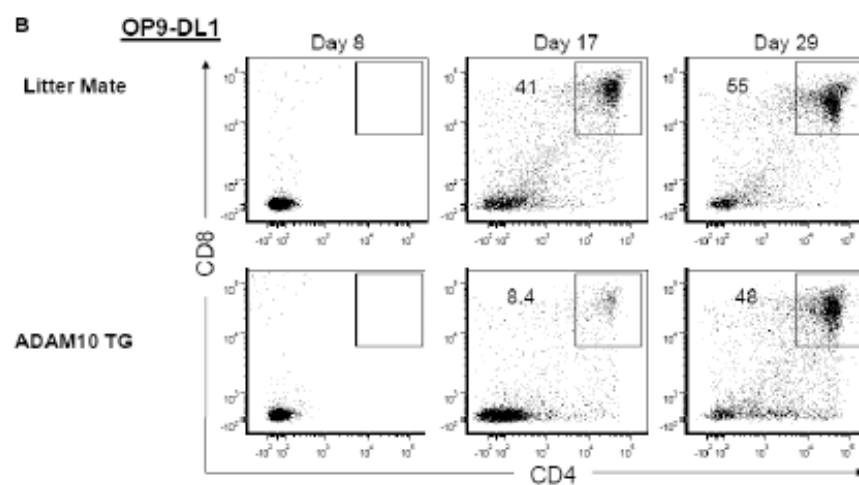


Figure 15. ADAM10 alters hematopoiesis by a cell-autonomous intrinsic mechanism. Flow cytometric analysis of (A) myeloid and (B) lymphocyte differentiation in mixed BM chimeras generated as described in the *Methods* 42 days after cell transfer. CD45.1⁺ and CD45.2⁺ gated cells differentiated from WT and A10Tg LSK BM, respectively. Data are representative of 3 independent experiments, except PBL data is representative of 6 independent experiments; numbers on dot plots indicate the percent of CD45.1 or CD45.2 gated cells within boxes.

A



B



C

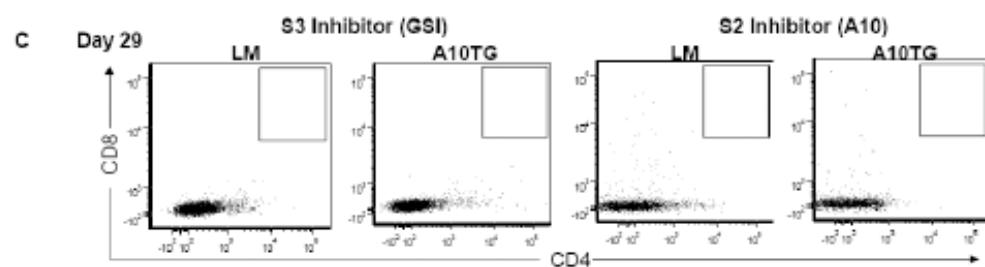


Figure 16. Inhibition of γ -secretase or ADAM10 activity prevents Notch-dependent T cell development. (A,B) Flow cytometric analysis of differentiated HSCs co-cultured with (A) OP9-GFP or (B) OP9-DL1 stromal cells for 8, 17, and 29 days; representative of 4 independent experiments. (C) T cell development of HSCs co-cultured with OP9-DL1 cells in the presence of a γ -secretase inhibitor, Compound E, or an ADAM10 inhibitor, GI254023X; representative of 2 independent experiments.

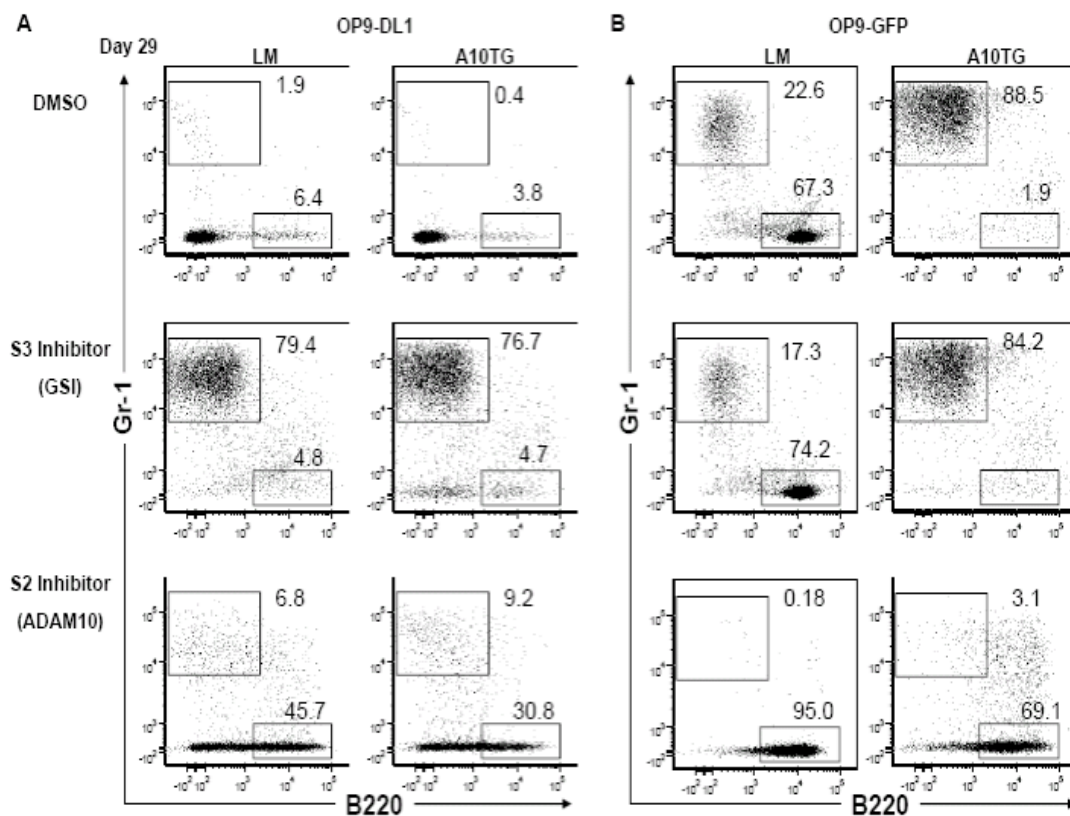


Figure 17. γ -secretase and ADAM10 inhibition have differential effects on Notch-mediated HSC differentiation. Flow cytometric analysis of HSC differentiation after 29 days of HSC co-culture with (A) OP9-DL1 or (B) OP9-GFP stromal cells as described in the Methods. Compound E, GI254023X, or control DMSO was added to media to inhibit γ -secretase or ADAM10 activity, respectively. DMSO-treated plots are representative of 4 independent experiments; plots of inhibitor-treated cultures are representative of 2 independent experiments. Numbers on plots indicate the percentage of gated cells within boxes.

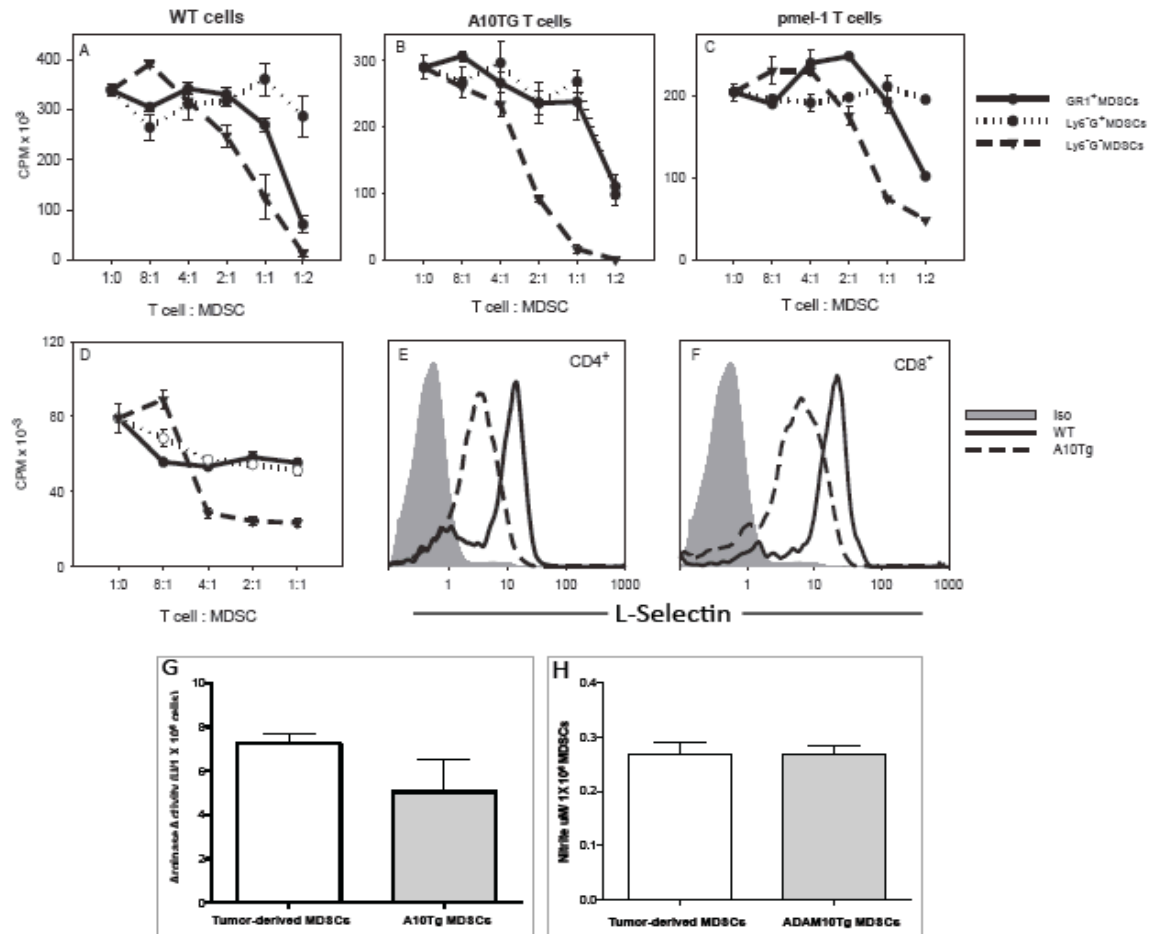


Figure 18. MDSCs from A10Tg mice are phenotypically and functionally analogous to tumor-derived MDSCs. (A) Proliferation of purified WT and A10Tg splenic T cells in the presence of increasing amounts of A10Tg CD11b⁺ MDSCs (Ly6G⁺, Ly6G⁻, or Gr-1⁺); stimulated with immobilized anti-CD3 and soluble anti-CD28. (C) Purified pmel-1 TCR transgenic splenocytes were cultured in the presence of increasing A10Tg MDSC subsets and stimulated with gp100 peptide. (D) Tumor derived MDSCs were purified from LLC bearing mice and used in suppression assays with Pmel1 splenocytes at increasing ratios (T cells or splenocytes: MDSCs, *p<0.05), stimulated with gp100 peptide. (E) Cell surface expression of L-selectin

(CD62L) by CD4⁺ and CD8⁺ (F) gated T cells from peripheral lymph nodes (PLNs). Lysates of MDSCs derived from Lewis Lung Carcinoma bearing WT hosts and A10Tg MDSCs were analyzed for the activity of (G) Arginase by urea production and (H) Nitric oxide by Greiss Reagent. The data is representative of at least three independent experiments with splenocytes from three or more mice.

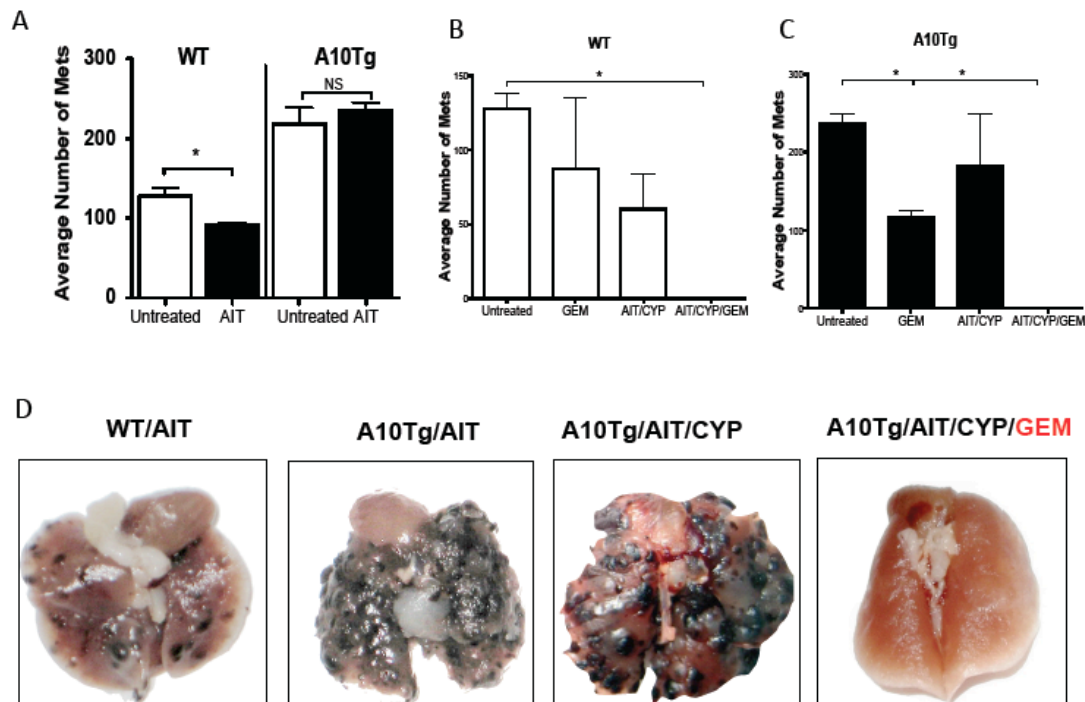


Figure 19. MDSC depletion restores the anti-tumor response and prevents metastatic progression of B16 melanoma in A10Tg mice. (A) Number of lung metastases in LM and A10Tg animals two weeks after B16 melanoma challenge *i.v.* with or without adoptive immunotherapy (AIT) as described in *Methods*. Number of lung metastases of LM (B) or A10Tg (C) mice challenged with B16 and treated as in A with the addition of cyclophosphamide (CYP) and gemcitabine (GEM). (D) Representative lungs of LM and A10Tg mice with AIT with and without CYP and GEM. * $p < 0.05$ in comparison to respective untreated controls.

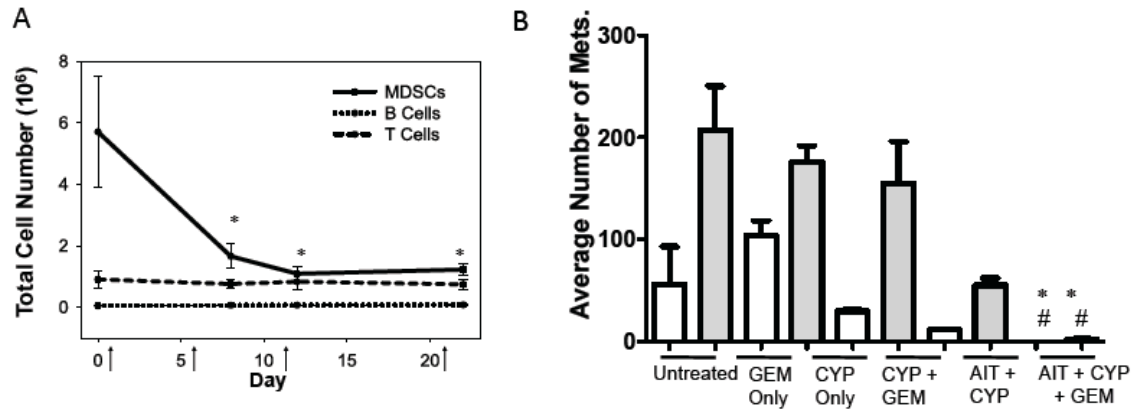


Figure 20. Gemcitabine selectively depletes MDSCs, which allows for effective AIT with tumor specific T cells. (A) FACS analysis of peripheral blood leukocyte levels in A10Tg mice following *i.p.* injections with gemcitabine (upward arrow) every five days for three weeks, * $p < 0.05$. (B) Number of B16 lung metastases in WT (white bar) and A10Tg mice (gray bar) treated with AIT comprised of pmel-1 transgenic T cells and chemotherapeutics as described in *Methods*. * $p < 0.05$ in comparison to respective untreated controls and # $p < 0.05$ in comparison to respective AIT+ CYP treatment.

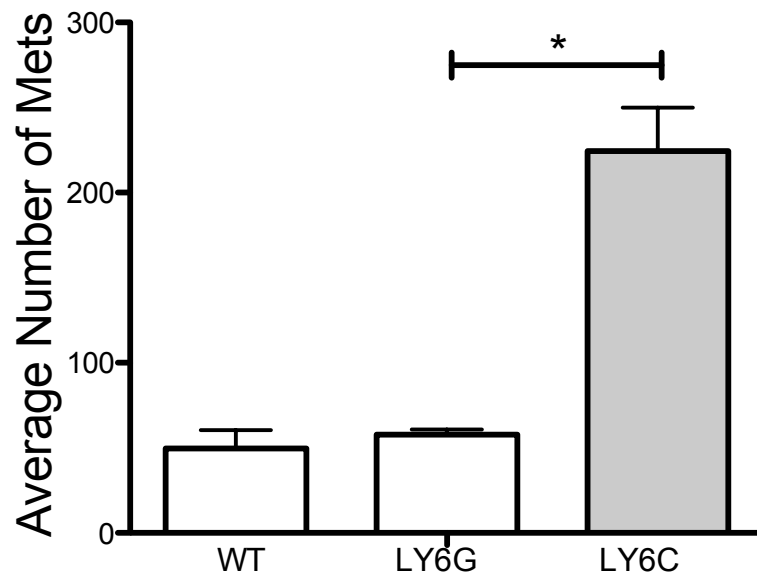


Figure 21. M-MDSCs promote B16 melanoma colonization to the lungs.

Quantification of B16 lung metastasis in WT C57/BL6 mice with AT of either G-MDSCs (CD11b⁺Ly6G⁺) or M-MDSCs (CD11b⁺Ly6C⁺) purified based upon surface marker expression, as described in *Methods*. More than five mice were used per group in three independent experiments. *p<0.05 in comparison to respective G-MDSC group.

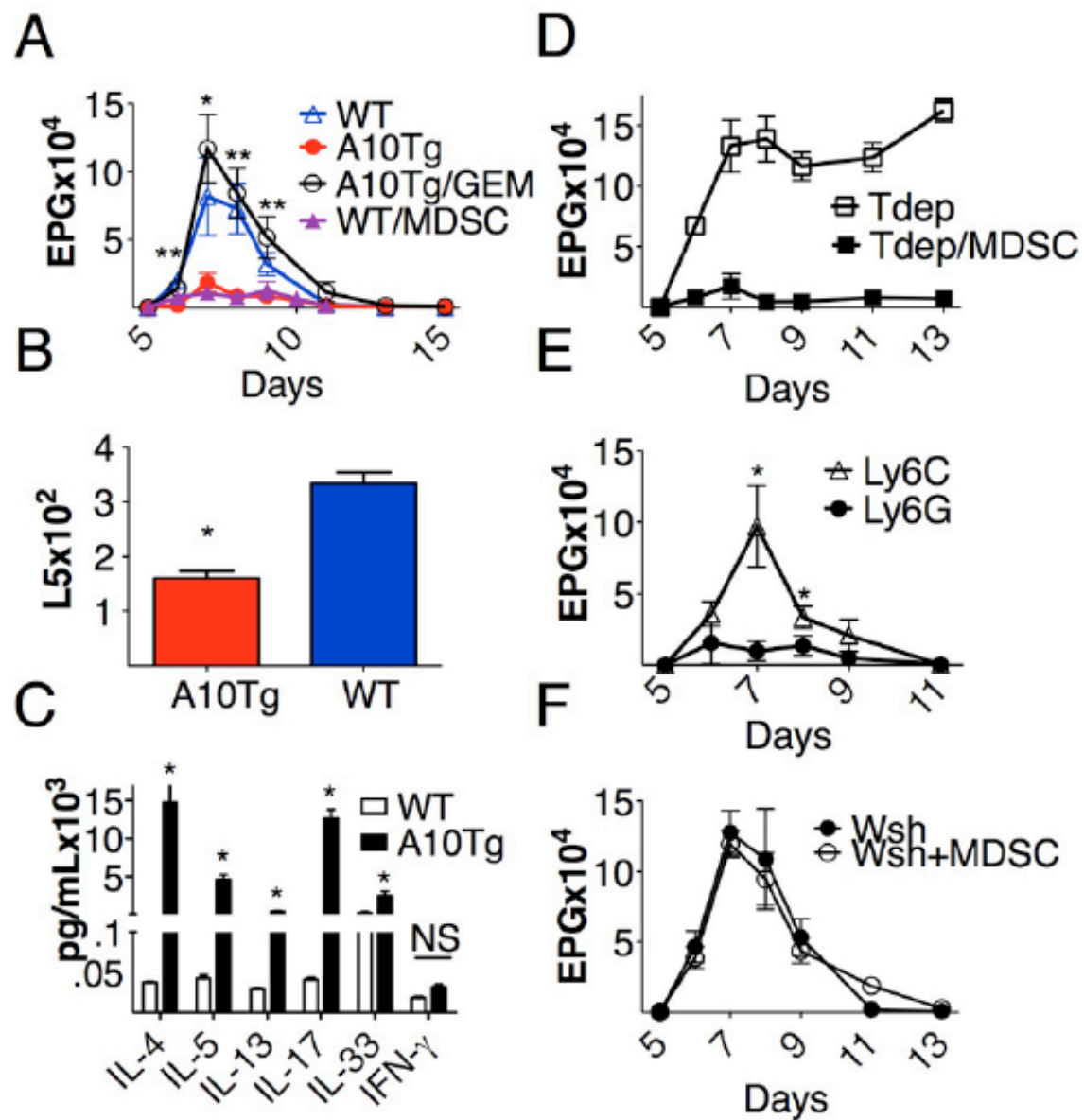


Figure 22. A10Tg mice are resistant to Nb infection. (A) Eggs/gram feces were determined at indicated times in WT and A10Tg mice as well as A10Tgs treated with GEM or WT with adoptive transfer (AT) of MDSCs upon infection with 650 Nb L3 worms. (B) WT and A10Tg mice were examined on day seven for adult worm recovery as described in *Methods*. (C) Serum was collected at day seven and analyzed for the

above cytokines using a Milliplex Mouse Cytokine Kit. * $p < 0.05$ in comparison to infected WT controls. (D) Eggs/gram feces of WT mice adoptively transferred with MDSCs or WT controls, both depleted of T cells (T dep) as described in *Methods*. (E) Enumerated eggs/gram of feces of infected WT with AT of either $CD11b^+Ly6^{high}Ly6C^{low}$ or $CD11b^+Ly6^{low}Ly6C^{high}$ population of MDSCs. (F) Enumerated eggs/gram feces of mast cell deficient $Kit^{Wsh/Wsh}$ mice with and without MDSCs upon infection with 650 Nb L3 worms. The data represents five mice per group.

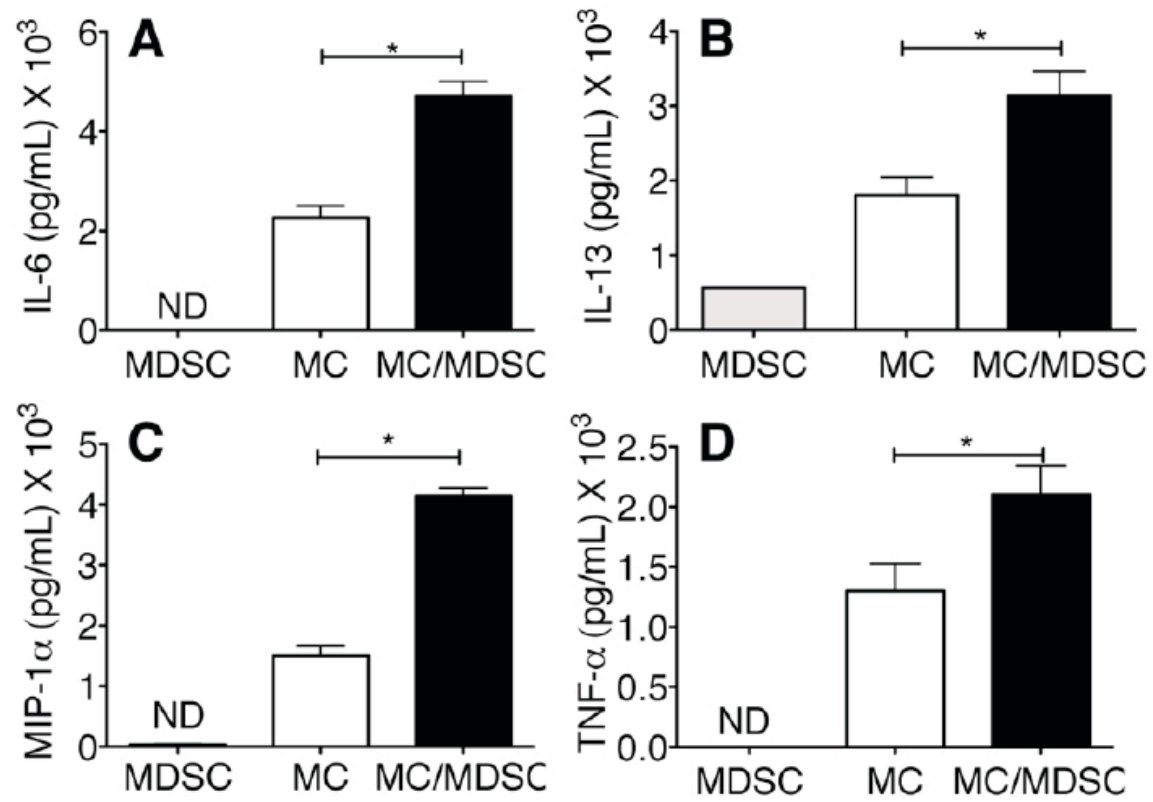


Figure 23. Mast cell/MDSC interaction augments cytokine production. BMMCs were co-cultured with A10Tg MDSCs as described in *Methods*. Supernatants were collected and analyzed for the production of (A) IL-6, (B) IL-13, (C) MIP-1 α , and (D) TNF- α . The data represent pooled spleens of at least three mice per group. * $p < 0.05$ in comparison to mast cells (MCs) alone.

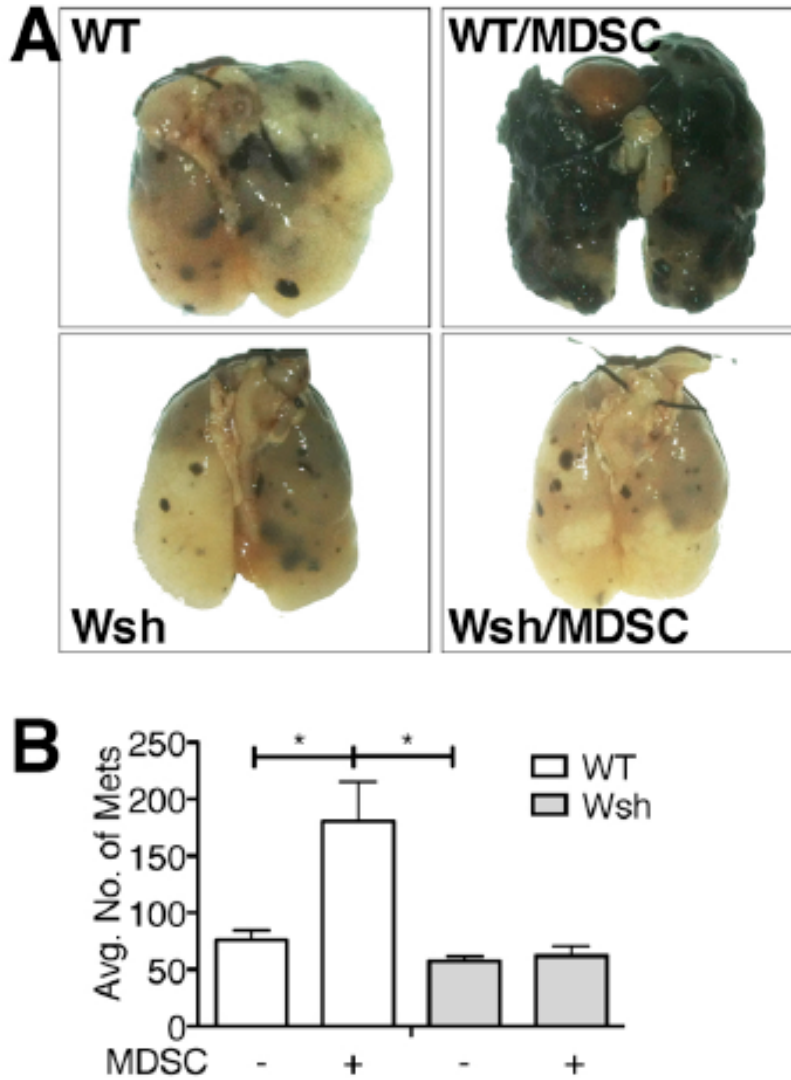


Figure 24. MC/MDSC interaction is required for MDSC-mediated immune suppression. Photographic representation (A) and quantification (B) of B16 lung metastasis in control C57 and $\text{Kit}^{Wsh/Wsh}$ mice with and without the AT of MDSCs. The data is representative of with at least five mice per group. * $p < 0.05$ in comparison to WT alone or $\text{Kit}^{Wsh/Wsh}$ with MDSCs.

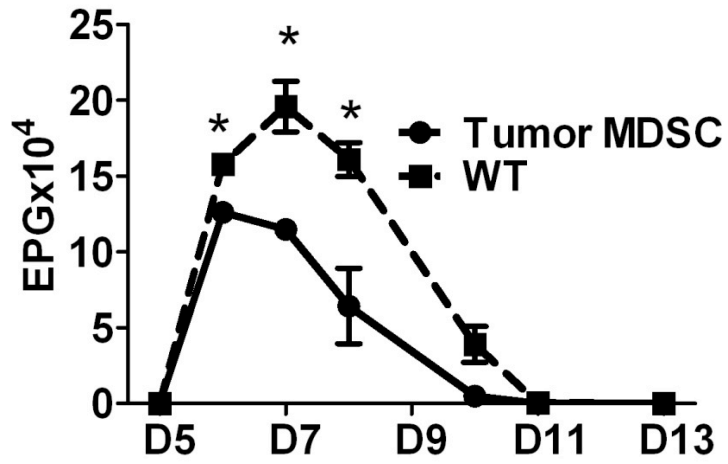


Figure 25. Tumor derived MDSCs promote Nb clearance. MDSCs isolated from LLC injected mice and i.v. AT into wild type mice on days (-1,2,5,8) or controls, were infected with 650L3Nb *P<0.05.

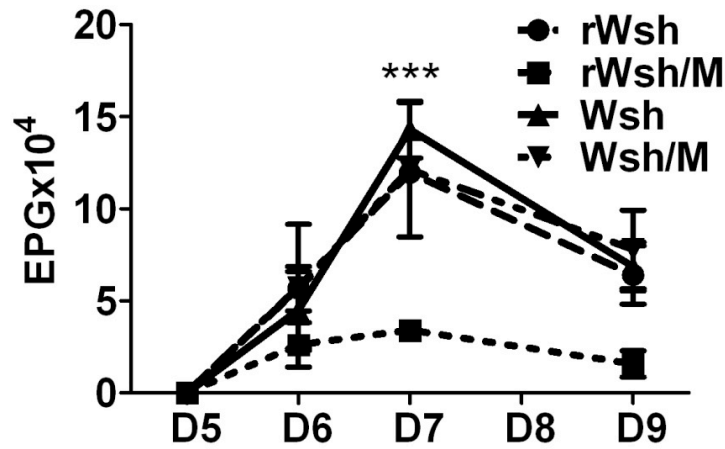


Figure 26. MDSCs regain their effect in $\text{Kit}^{\text{Wsh/Wsh}}$ mice upon reconstitution with MCs. $\text{Kit}^{\text{Wsh/Wsh}}$ mice were reconstituted with BMMC 20 weeks prior to experiment (rWsh). Age matched unreconstituted $\text{Kit}^{\text{Wsh/Wsh}}$ mice (Wsh) mice were used as controls. Mice were challenged with Nb in the presence or absence of MDSCs. Eggs per gram of feces were determined on indicated days. *** $P < 0.0005$ between rWsh and rWsh/M.

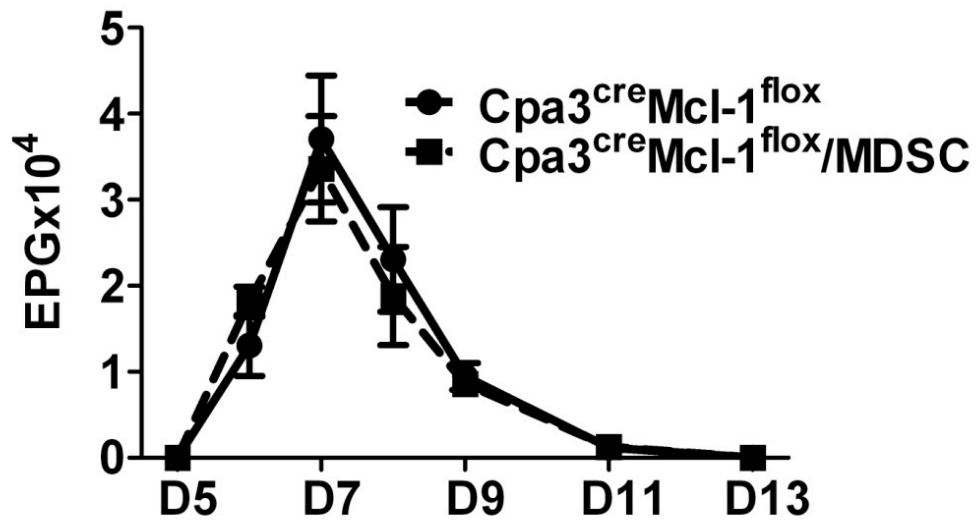


Figure 27. MDSC-mediated parasite expulsion is abrogated in MC deficient Cpa3^{cre} Mcl-1^{fl/fl} mice. WT and Cpa3^{cre} Mcl-1^{fl/fl} mice were infected with Nb with and without the AT of MDSCs as described in Methods. Eggs per gram of feces were determined on indicated days.

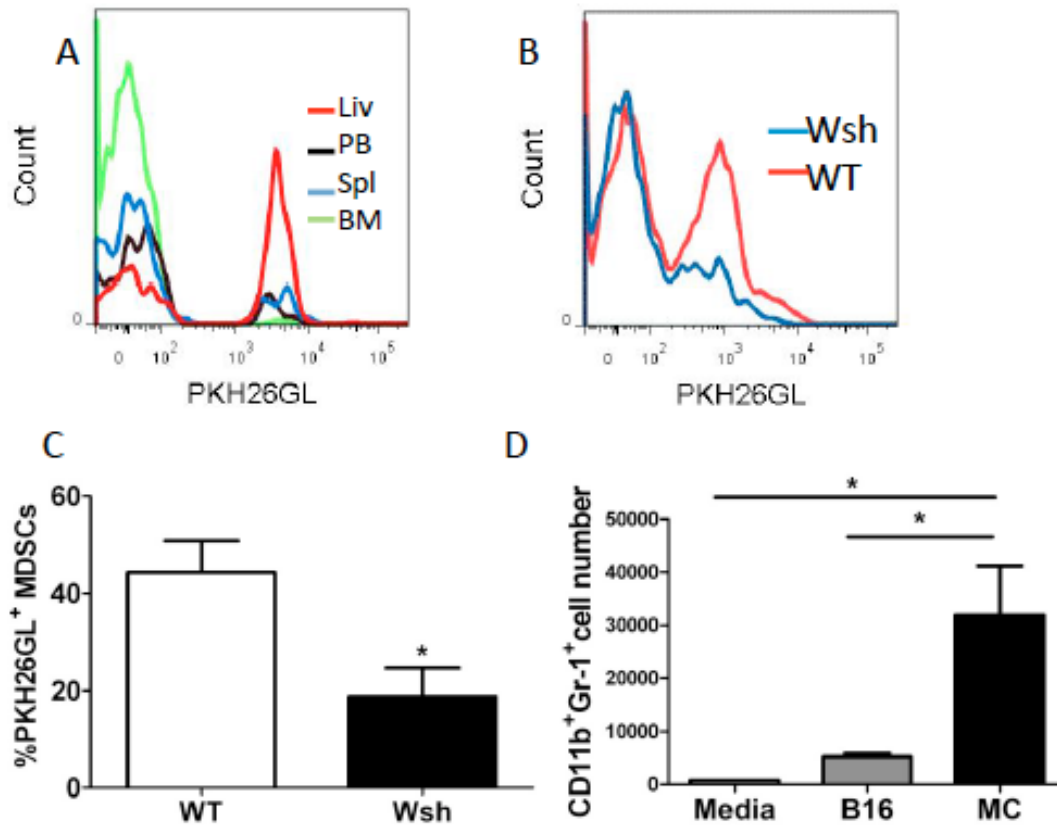


Figure 28. MDSCs preferentially migrate to the liver in a MC-dependent manner.

(A) Wild type mice were adoptively transferred with PKH26GL labeled MDSCs 18hrs post injection. PKH26GL staining was assessed on CD11b⁺Gr1⁺ cells in liver (Liv), peripheral blood (PB), spleen (Spl) and bone marrow (BM). (B) Kit^{Wsh/Wsh} mice (Wsh) or wildtype (WT) were *i.v.* AT with PKH26GL labeled MDSCs on days (-1,2,5) and infected (day 0) with 650L3 Nb. (C) Day 7 post Nb infection PKH26GL staining was assessed on CD11b⁺Gr-1⁺ cells in the liver. (D) MDSCs were cultured in the top well of 8-micron transwell plates. B16 melanoma cells, BMMC cells or media alone were placed in the bottom well. After 4hr incubation, cells were harvested from bottom wells and CD11b⁺Gr-1⁺ MDSC migration was determined by flow cytometry.

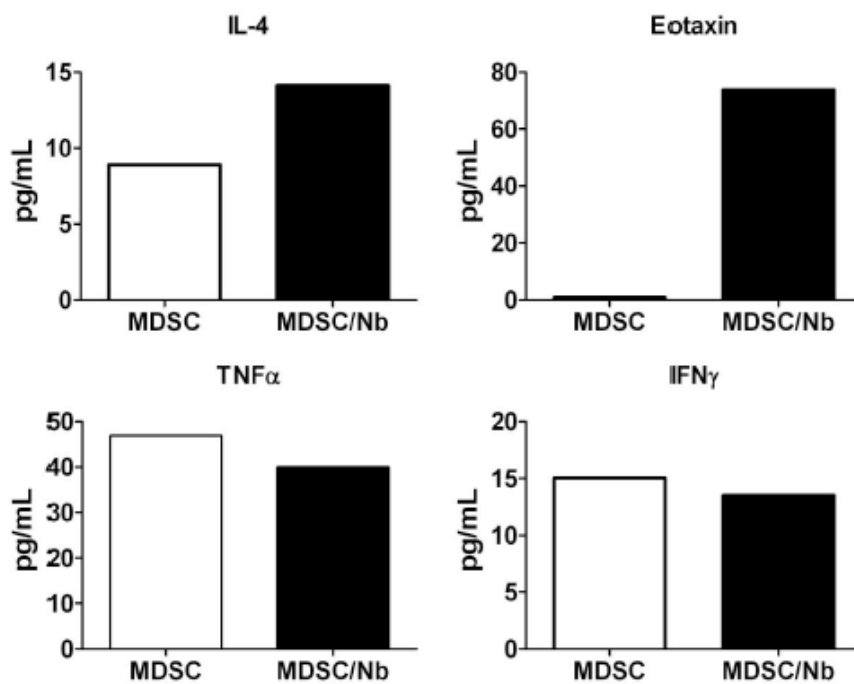


Figure 29. Hepatic MCs activate MDSCs and enhance cytokine production.

Cytokine analysis on supernatants of CD11b⁺Gr1⁺ MDSCs recovered from pooled livers of WT Nb infected mice after adoptive transfer with MDSCs and cultured for 48hrs in cRPMI. Pre-adoptive transfer MDSCs were used as control.

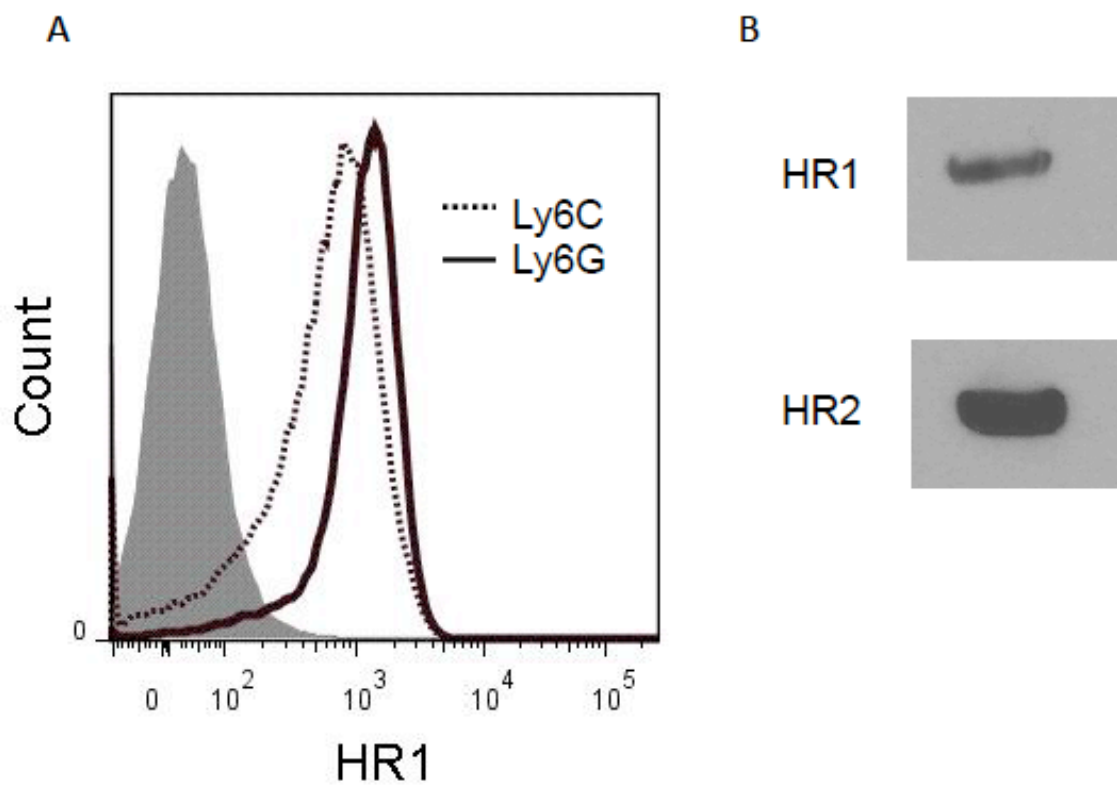


Figure 30. MDSCs express HR1 and HR2. A. HR1 staining was determined on CD11b⁺Ly6G⁺ (solid line), CD11b⁺Ly6C⁺ (dotted line) or CD11b⁻ (grey) cells by FACS. B. HR1 and HR2 presence was confirmed by western blotting.

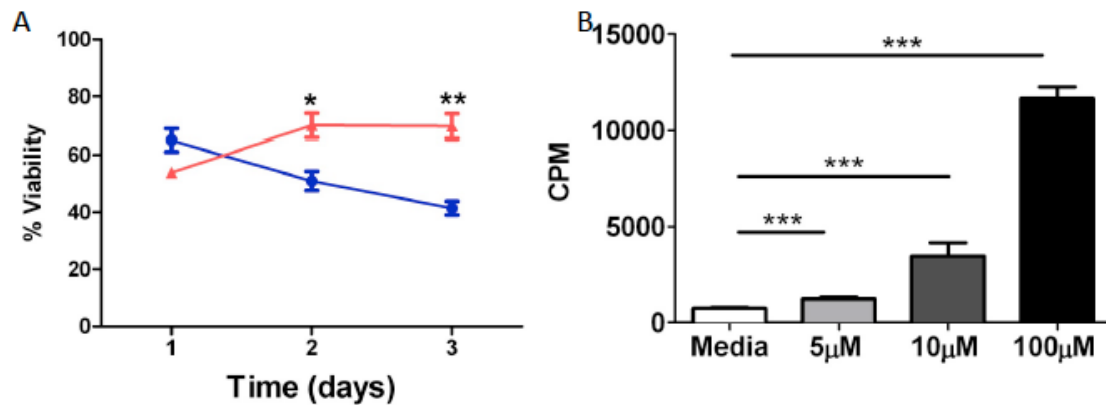


Figure 31. Histamine increases MDSC survival and proliferation. A. MDSCs were purified and cultured in the presence of 100 μ M histamine. Cellular viability was quantified using trypan blue exclusion over a 72 hr period. B. MDSCs were cultured with 5 μ M, 10 μ M or 100 μ M histamine (Invitrogen). All cell concentrations started with 50,000 cells/well in 96 well plates. To determine proliferation, cells were then labeled with [H^3]-thymidine. Plates were then harvested 24hrs later, using a Filtermate cell harvester onto GFC plates, and read with a Topcount Plate Counter (Perkin Elmer, Waltham, MA).

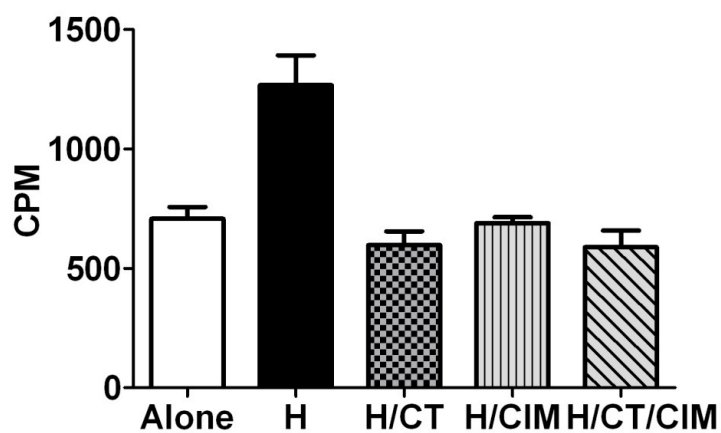


Figure 32. Histamine receptor blockade abrogates MDSC proliferation. MDSCs were pre-treated with CIM, CT or both and subsequently cultured with 100 μ M histamine for 48 hours, and proliferation was determined using [H]³-thymidine incorporation.

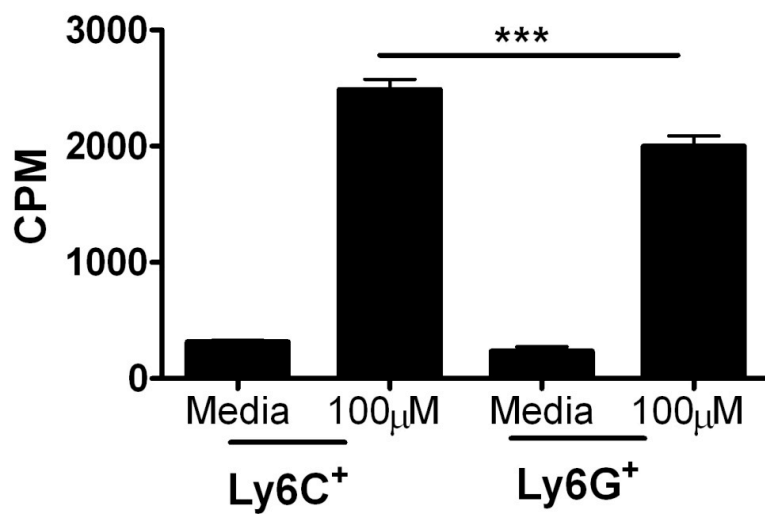


Figure 33. Both M-MDSCs and G-MDSCs are sensitive to the proliferative effects of histamine. Ly6C⁺ or Ly6G⁺ MDSCs were cultured with 100µM histamine and proliferation was determined using [H]³-thymidine incorporation.***P<0.0005. Data represents Mean ± SEM.

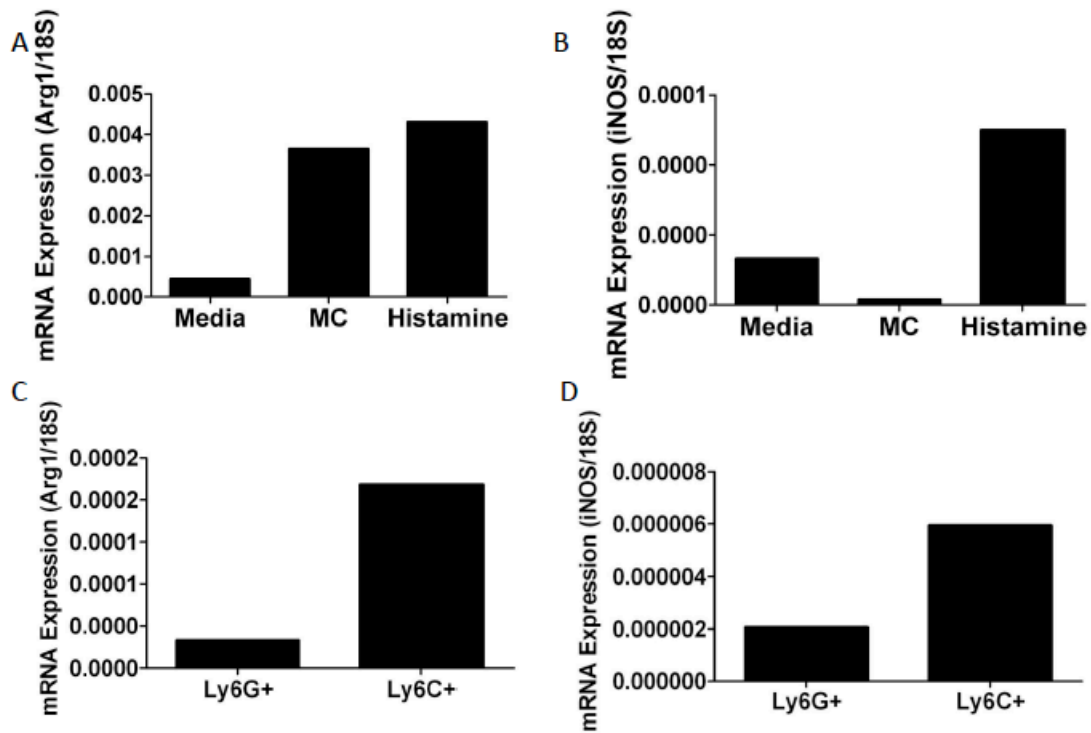


Figure 34. Histamine increases enzymatic activity of MDSCs. MDSCs were purified as a heterogeneous group based on CD11b+Gr-1+ expression or as subsets based upon Ly6G and Ly6C expression. They were subsequently cultured in the presence of 100 μ M histamine or in 04 μ m transwells with MCs. 24 hrs post co-culture, mRNA was isolated and analyzed for expression of Arg1/18S (A,C) or iNOS/18S (B,D) by qPCR.

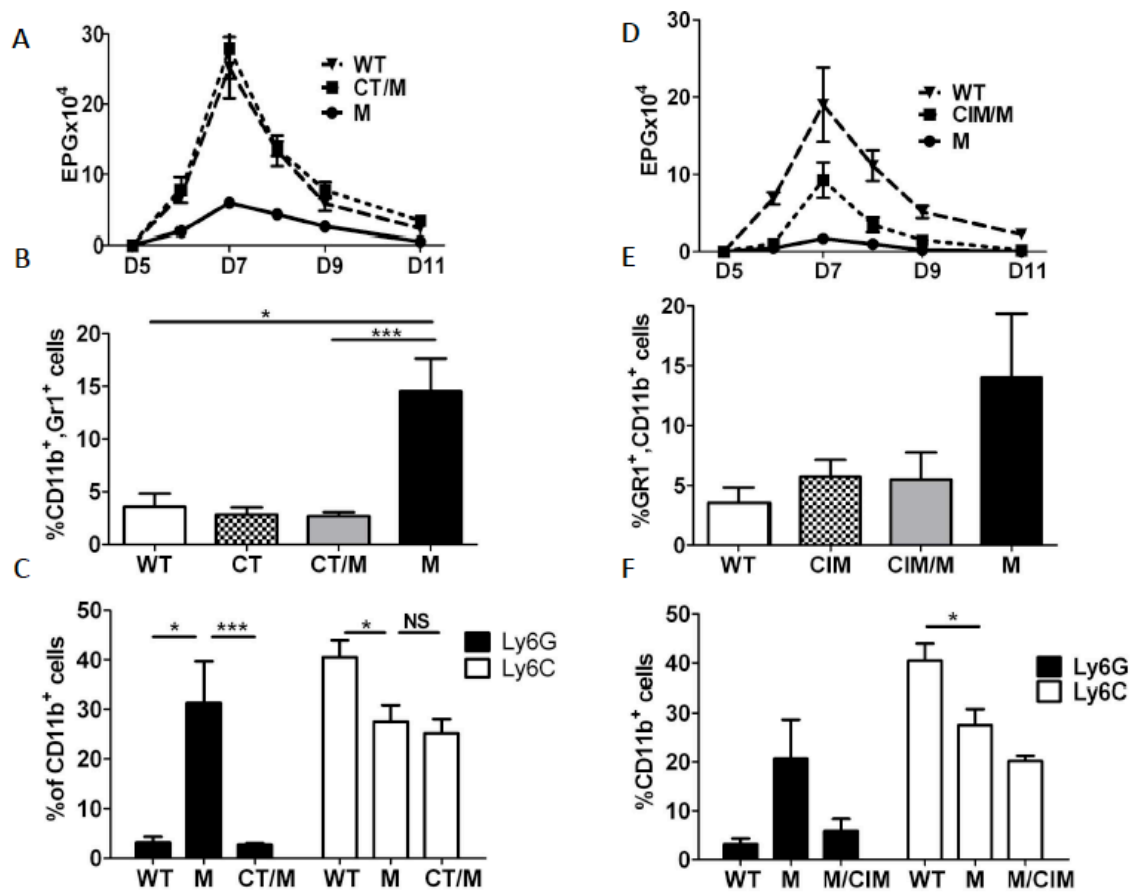


Figure 35. HR antagonist blocks MDSC mediated Nb clearance. (A) Eggs per gram of feces was determined over a time course in mice infected with 650L3 Nb, with or without AT of MDSCs on days (-1,2,5,8,11) in the presence or absence of *i.p.* CT treatment or CIM (D) treatment every other day. B, E. Day 14 analysis of total CD11b⁺Gr1⁺ MDSCs and (C,F) CD11b⁺Ly6C⁺ or CD11b⁺Ly6G⁺ populations in the liver determined by flow cytometry from CT (B,C) or CIM (E,F) treated mice. *P<0.05, ***P<0.0005, NS=not significant. Data represents Mean ± SEM.

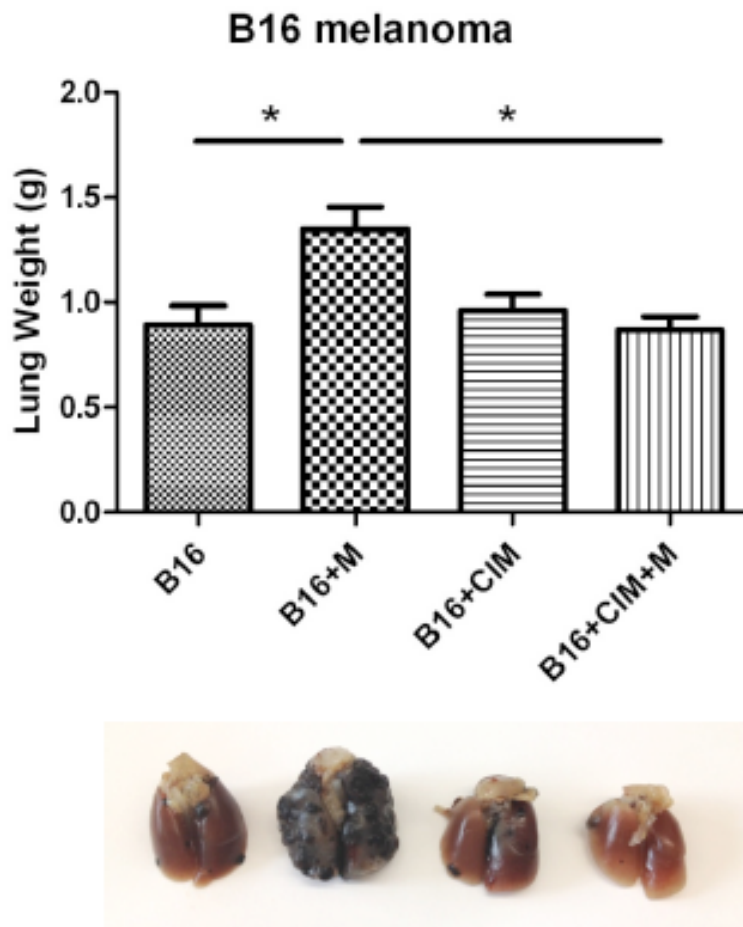


Figure 36. HR antagonization inhibits MDSC mediated tumor progression. Mice were challenged with B16 concomitant with MDSCs in the presence or absence of cimetidine (CIM), an HR2 antagonist. Mice were subsequently sacrificed and their lungs weighed for B16 colonization. * $P < 0.05$

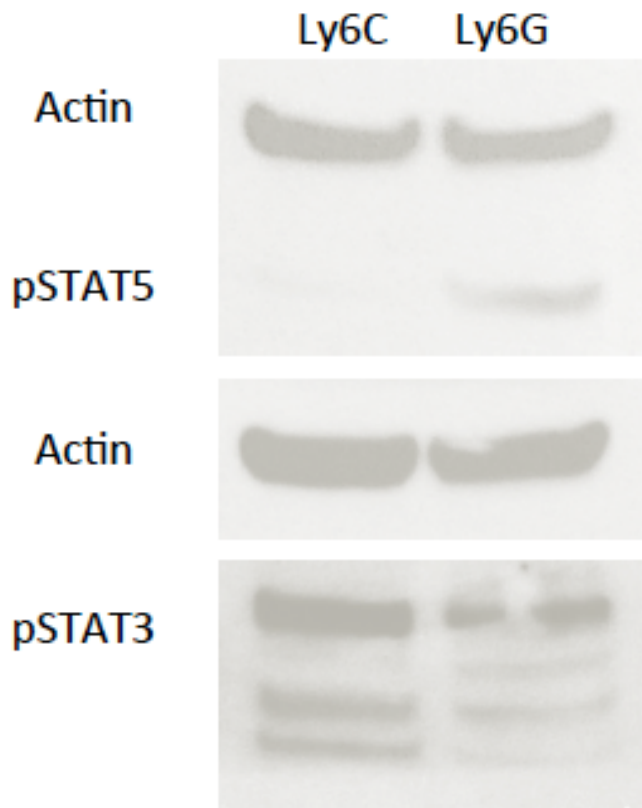


Figure 37. Histamine differentially regulates STAT signaling in MDSC subpopulations. The levels of pSTAT5 and pSTAT3, on Ly6C⁺ or Ly6G⁺ MDSC populations, after 24 hours of culture with 100μM histamine was determined by western blotting. Equal loading was confirmed with β-actin.

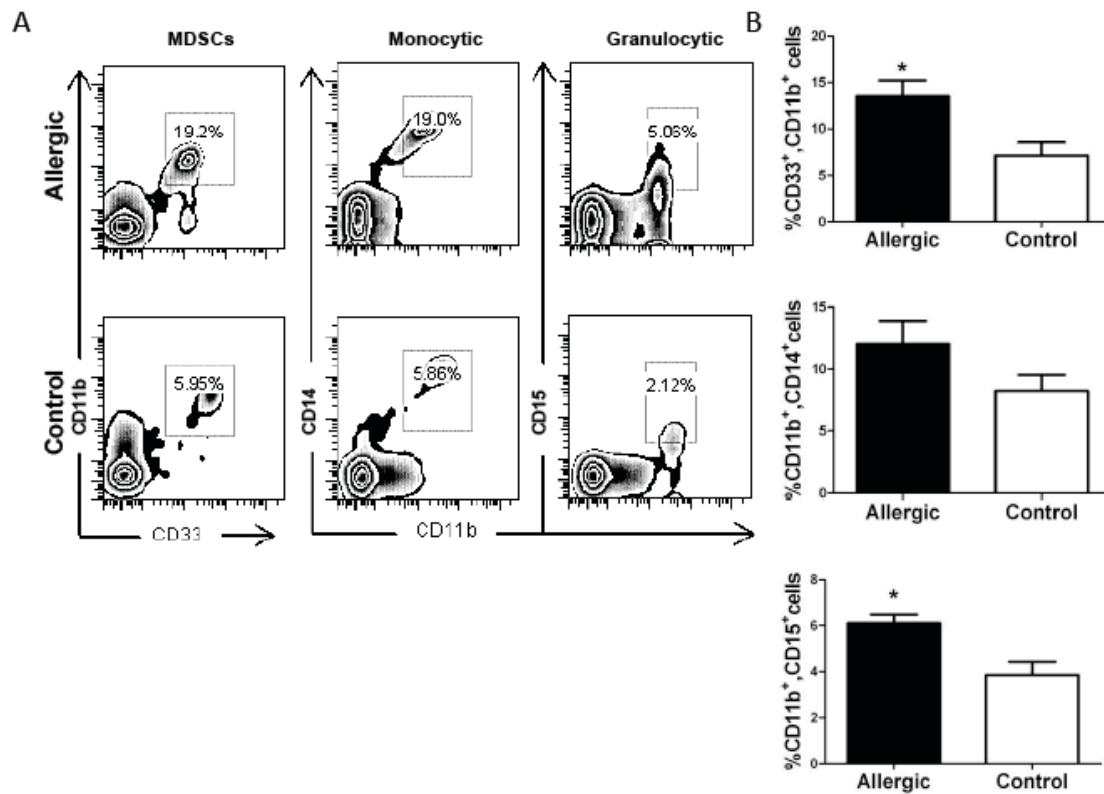


Figure 38. Allergic Patients have increased circulating MDSCs. Allergic Patients were defined as patients in active allergy, experiencing symptomology and compared to non-allergic controls. Cells were isolated from peripheral blood and percent MDSCs and subpopulations were determined by flow cytometry. A. Representative allergic patient vs. control and (B) compiled.* $P < 0.05$. Data represents Mean of 9 patients \pm SEM.

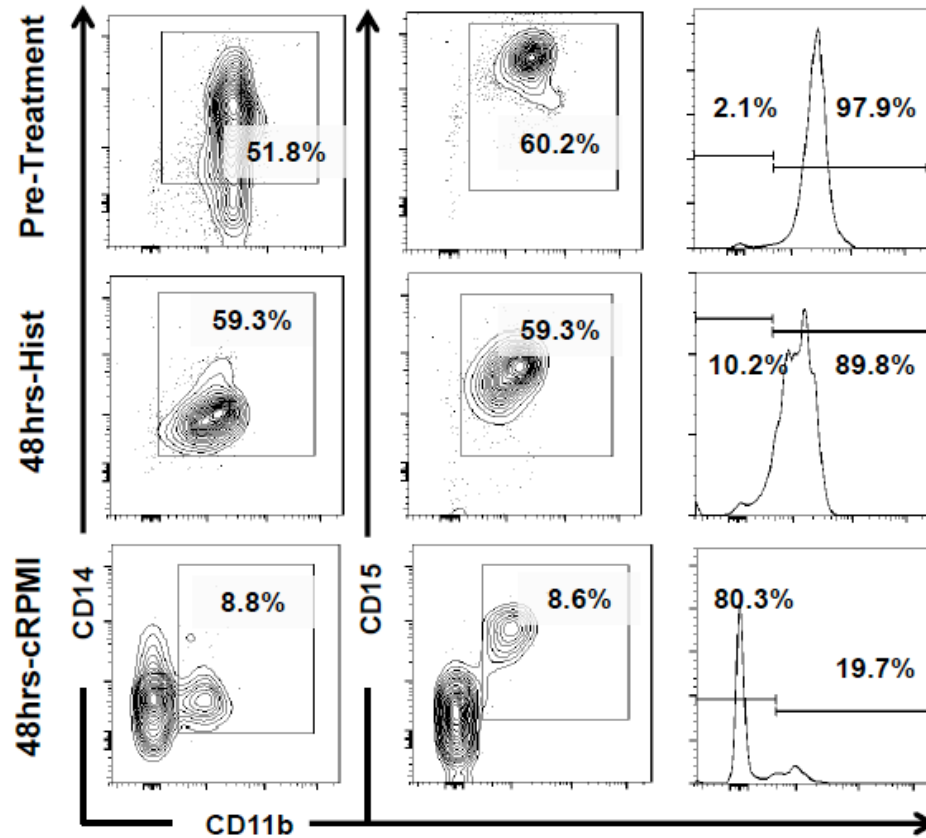


Figure 39. Human MDSCs have increased survival in the presence of histamine.

MDSCs sorted as $\text{HLA-DR}^{\text{lo/-}}\text{CD11b}^+\text{CD33}^+$ and from peripheral blood of a stage I breast cancer patient. Cells were stained for flow cytometry prior to culture and after 48 hours of culture with 100 μM histamine or in media alone.

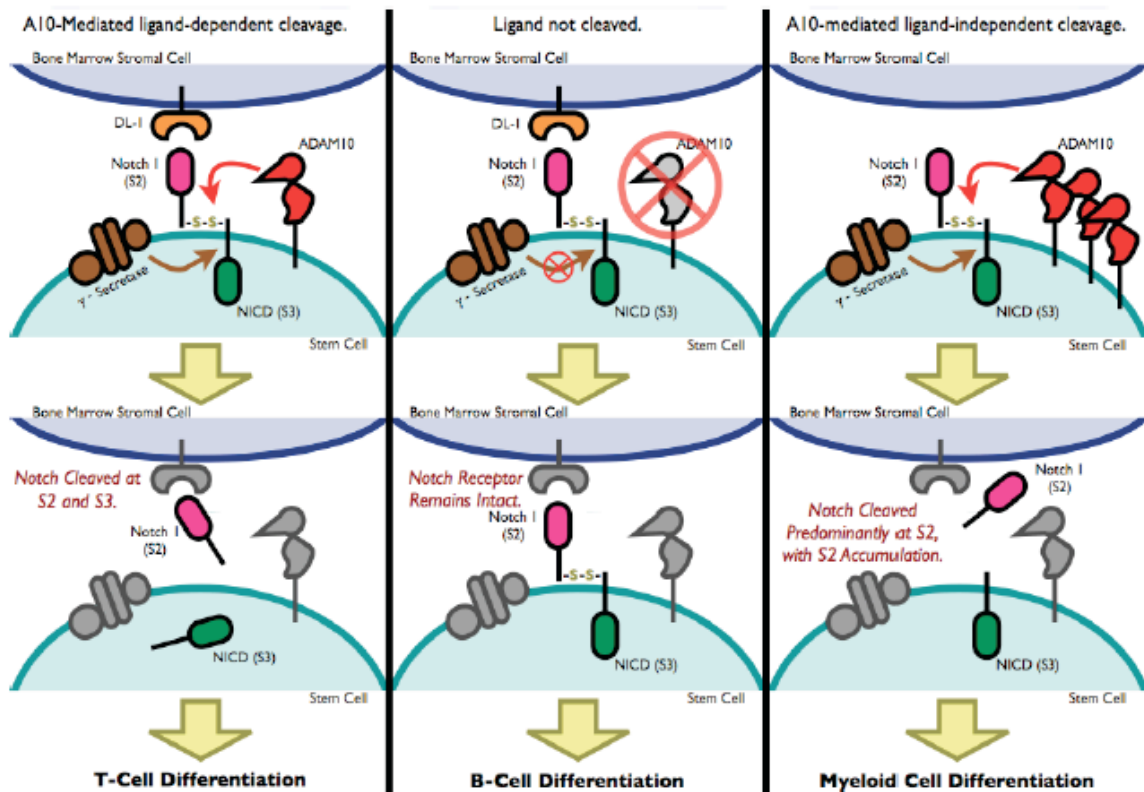


Figure 40. An illustration of differential effects of Notch S2/S3 cleavage in hematopoietic differentiation. In the presence of ligand, the Notch receptor undergoes both S2 and S3 cleavage, resulting in two cleaved products and ultimately promoting T cell development. In the absence of ligand or in the case of blocked S2 cleavage, the Notch receptor remains intact and B cell development results. However, S2 cleavage without S3 due to increased ADAM10 activity, results in myelopoiesis.

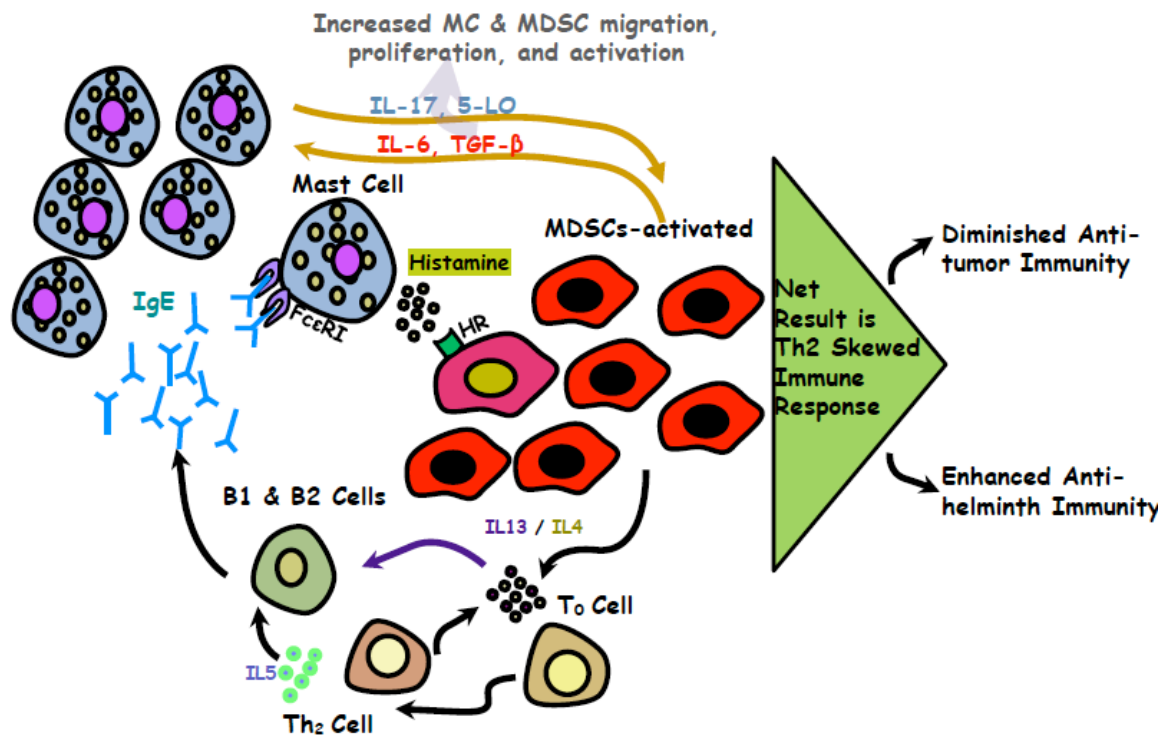


Figure 41. Model of MDSC/MC interaction. MCs are required for MDSC activity. MCs release mediators such as histamine that induce MDSC activation, proliferation and Th2 cytokine production. This enhanced cytokine production culminates in Th2 skewed immune responses that promotes allergy and parasitic clearance and diminish anti-tumor responses.

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