STAT1-Regulated Lung MDSC-like Cells Aid Resolution of Inflammation After Bacterial Pneumonia

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We have recently identified a $CD11b^{+}Gr1^{int}F4/80^{+}$ ($Gr1^{int}$) regulatory, non-migratory, cell type in the lung that is able to suppress a Th2 effector response via secretion of Interleukin-10 (IL-10), arginase 1 (Arg1) and nitric oxide (NO). These regulatory lung $Gr1^{int}$ cells also secrete Interleukin-6 (IL-6) and Granulocyte macrophage-colony stimulating factor (GM-CSF), but low levels of Interleukin-12 (IL-12). Our studies show that although $Gr1^{int}$ cells are present in the lung of naïve mice, their frequency increases in the interstitium following endotoxin/lipopolysaccharide (LPS) exposure in a TLR4/MyD88-dependent fashion. The suppressive function of these $Gr1^{int}$ cells is significant in the context of allergic inflammation, and because this suppressive cell type accumulates in the lung in response to a bacterial cell wall component, they may be a mechanism underlying the hygiene hypothesis, which was postulated in 1989 to describe the observation that despite increased sanitation in westernized countries, the prevalence of allergies is steadily increasing. Because of the potent suppressive capacity of lung $Gr1^{int}$, they are now recognized as lung myeloid derived suppressor cells (lung-MDSCs)[1]. We sought to gain a better understanding of their development to allow for improved therapeutic targets during allergic inflammation.

Secondly, the rapid appearance of these cells in response to a TLR ligand, combined with their non-migratory nature and their anatomical location in the lung interstitium, suggests a unique function during host defense against bacterial infections. Much is already known about the role of alveolar macrophages and neutrophils in host defense against *Klebsiella pneumoniae*,

but little is known about host defense mechanisms in the lung tissue/interstitium. Therefore, we have also examined the role of MDSC-like cells in defense against *K. pneumoniae*.

Briefly, although an inflammatory response in the lung is required to fight the causative agent, persistent tissue-resident neutrophils can induce collateral tissue damage and precipitate acute lung injury during non-resolving pneumonia. Little is known about mechanisms orchestrated in the lung tissue that remove apoptotic neutrophils to restore tissue homeostasis. We show that these MDSC-like cells increase following exposure to *K. pneumoniae*, are able to efferocytose apoptotic neutrophils and are a major source of IL-10 in the lung late after infection. Furthermore, using IL-$10^{-/-}$ mice, we show that IL-10 is critical for the control of neutrophilia and restoration of homeostasis. Finally, because IFNγ-STAT1 signaling is known to inhibit IL-10, we examined the lungs of $STAT1^{-/-}$ mice. The lung neutrophil burden was attenuated in infected $STAT1^{-/-}$ mice with concomitant increase in the frequency of the MDSC-like cells and lung IL-10 levels. Thus, inhibiting STAT1 in combination with antibiotics may be a novel therapeutic strategy to address inefficient resolution of bacterial pneumonia. The inhibition of STAT1 may also be relevant in the context of allergic inflammation when a greater immunosuppressive force, or increased numbers of lung MDSCs, would be advantageous to temper lung inflammation.

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To all of you, I pray that health and true joy may be yours always.

LIST OF ABBREVIATIONS

A

AF: autofluorescence

AHR: airway hyperresponsiveness

AMs: alveolar macrophages

B

BALF: bronchoalveolar lavage fluid

BM: bone marrow

BMDC: bone marrow-derived dendritic cell

C

C/EBP: CCAAT Enhancer Binding Protein

CLP: common lymphoid precursor

CMP: common myeloid precursor

CTL: cytotoxic T lymphocyte

D

d: day(s)

DC: dendritic cells

dLN: draining lymph node

DSS: dextran sulfate sodium

E

E. coli: Escherichia coli

Eos: eosinophils

F

FSC: forward scatter

G

GM-CSF: Granulocyte-macrophage colony-stimulating-factor

GMP: granulocyte macrophage progenitor

GPI: glycoslphosphatidylinositol

GSK3: glycogen synthase kinase 3

H

HDM: house dust mite

h: hour(s)

HSC: hematopoietic stem cell

HSPC: hematopoietic stem and progenitor cell

Hsp: heat shock protein

I

IL: interleukin

IM: interstitial macrophage

iNOS: inducible nitric oxide synthase

i.t.: intratracheally

IRAK: interleukin-1 receptor-associated kinase

ITAM: intracellular tyrosine kinase activation motif

J

JAK: Janus kinase

K

KC/CXCL1: Chemokine (C-X-C motif) ligand 1

KO: knockout (mice)

L

L-NAME: L-NG-Nitroarginine methyl ester

LPS: lipopolysaccharide

M

MAP: Mitogen-activated protein

MDP: macrophage/dendritic cell progenitors

MDSC: myeloid-derived suppressor cell

MFI: mean fluorescence intensity

MIP: macrophage inflammatory protein

miR: microRNA

MPO: myeloperoxidase

N

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

Neu: neutrophils

P

PAMP: pathogen-associated molecular pattern

pDC: plasmacytoid DC

PI3K: Phosphoinositide 3-kinase

PMN: polymorphonuclear

PRR: pattern recognition receptor

PTX3: pentraxin 3

Q

qRT-PCR: quantitative reverse-transcription PCR

R

RANTES/CCL5: Regulated upon Activation, Normal T Cell Expressed, and Secreted

S

SCF: stem cell factor

SSC: side scatter

STAT: signal transducers and activators of transcription

T

TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TRAM: TRIF related adaptor molecule

TRIF: TIR-domain-containing adaptor-inducing interferon-β

TRAF6: TNF receptor associated factor 6

TAK1: transforming growth factor β-activated kinase 1

TCR: T cell receptor

Tg: transgenic

TLR: toll-like receptor

V

VEGF: vascular endothelial growth factor

W

WT: wild type

1.0 INTRODUCTION

1.1 THE IDENTIFICATION OF A LUNG-MDSC LIKE CELL

The orchestration of an appropriate immune response is dependent upon the ability of the immune system to discriminate between self and non-self and, the timely resolution of inflammation to prevent tissue injury. Both inappropriate and appropriate immune responses must be counteracted by regulatory mechanisms to maintain body-wide homeostasis. Over the years many investigators have questioned the nature of these interactions, experimentally detailing the derivation of inflammatory vs. regulatory responses, cellular differentiation, and the importance of cytokines and intracellular signaling molecules for the determination of immune outcomes. Much of this work is organ specific and it has become increasingly clear that it is not only the particular cell types involved, but the nature of the infection and extracellular milieu that contributes to their function[2], the culmination of which defines the result.

The so-called "hygiene hypothesis" was proposed to explain the ongoing increase in allergy and autoimmunity in westernized cultures. Essentially, it postulates that early exposure of the immune system to otherwise innocuous antigens is necessary to induce immunological tolerance, and thus prevent aberrant immune responses leading towards allergic and autoimmune conditions later in life. Studies, initiated in our laboratory in 2007 (described in detail in Section 1.1.3), introduced a connection between environmental exposure to endotoxin and the

establishment of immunological tolerance, thereby providing a potential mechanism underlying the hygiene hypothesis. Briefly, in response to intratracheal administration of LPS, an interleukin-10 (IL-10) producing myeloid-lineage cell population expanded in the lung tissue, which was phenotypically similar to the myeloid-derived suppressor cells (MDSCs) commonly associated with anti-tumor immunity[3]. These regulatory cells expressed CD11b, intermediate levels of Gr1, and F4/80, and were expanded in a TLR4/MyD88-dependent manner. As such, they were phenotypically and morphologically similar to cells described as being present in tumor microenvironments[4]. The MDSC-like cells we described expanded in the lung tissue in a dose dependent manner in response to intratracheal administration of bacterial lipopolysaccharide (LPS). In contrast to lung DC, these MDSC-like cells did not traffic to the lung draining lymph nodes, allowing them to act in a dominant fashion suppressing DC-mediated regulation of Th2 responses in situ. The MDSC-like cells were found to blunt the ability of the lung DCs to induce GATA-3 upregulation or STAT5 activation in primed Th2 cells, both transcription factors having critical roles in Th2 effector function. The findings, published in Mucosal Immunology in 2010, characterized these cells for the first time as lung MDSCs, with the ability to suppress HDM-driven eosinophilic inflammation via IL-10, nitric oxide (NO), and arginase-1 (Arg1) expression[5]. Overall, these studies identified a cellular mechanism that may underlie the hygiene hypothesis, thus providing the field with a tangible explanation for why the use of antibiotics and improved sanitation in the Western world correlates with an increase in atopic disease. Essentially, decreased exposure to bacterial products including LPS particularly early in life may reduce MDSC number and function thereby leading to crippling of immunological tolerance and potentially deleterious immune responses.

Although this work identified a lung MDSC, greatly advanced the field of lung biology, and expanded our understanding of cellular-mediated immunosuppression, we had not yet addressed the origin of these lung MDSCs, or the mechanism(s) that controlled their expansion in the lung tissue. Further elucidation of these processes holds the potential for therapeutic development of biologically-based approaches to re-establish immunological tolerance in allergy and autoimmunity.

This thesis work is two-fold. It first focuses on the LPS/TLR4-mediated generation of lung MDSCs, and identifies a novel mechanism by which their expansion is regulated. Secondly, given the expansion of this cell type in response to a bacterial component, it characterizes the importance of lung MDSCs and their IL-10 production in the control of neutrophils following bacterial pneumonia. Taken together, the data generated herein contribute to our current understanding of MDSC biology and make advances towards harnessing them for therapeutic intervention.

1.1.1 The relationship between endotoxin exposure and immunosuppression; the hygiene hypothesis

The chronic inflammatory state found in asthmatics is maintained due to repeated interactions between an allergen(s) and the immune system of susceptible individuals. Once initiated, disease is perpetuated upon epithelial cell involvement and leukocyte influx, with chronic inflammation finally resulting in excessive collagen deposition and the development of fibrosis in the lung tissue. To explain the recent increase in the prevalence of atopic diseases in developed countries, the hygiene hypothesis was postulated in 1989, contending that due to improved sanitation and cleaner lifestyles, microbial exposures in childhood are reduced[6].

Several investigators reported findings supporting this concept, most notably, von Mutius and colleagues, who documented an inverse correlation between endotoxin levels measured in children's mattresses and the development of hay fever and atopic asthma[7]. Additionally, allergic disease was lower in children who were enrolled in day care at an early age, which presumably was mediated by community-associated exposure to a wide range of environmental antigens[8].

From an immunological perspective, the observed inverse relationship between allergic disease, hay fever, and family size was initially attributed to the ability of early pathogenic exposures to tip the balance away from the reported default Th2 bias in neonates[9]. However, it is now accepted that the mechanism cannot simply be Th1/Th2 cross-regulation since parasitic exposure resulting in high IL-10 levels also protected from allergic disease[10]. Additionally, Th1/Th17-driven pathology in autoimmune diseases such as multiple sclerosis and diabetes mellitus have also been similarly reported with increased frequency in western societies[11]. Therefore, it appears that the breach of tolerance observed in allergic disease and other disorders may be due to a failure in fundamental tolerogenic mechanisms, and that lack of homeostatic maintenance leads to aberrant immune responses. Indeed, the publication from our laboratory described above suggests a connection between environmental exposure to endotoxin and the promotion of tolerance via the induction of lung-MDSCs, thus representing a mechanism of regulation potentially applicable to a wide range of conditions characterized by heightened immune activation[5].

Although bacterial lipopolysaccharide (LPS) has been described as an adjuvant in the development of allergic inflammation, it also can have differential effects depending on the dose used. A low dose (<1 ng) has been shown to promote Th2 eosinophilic inflammation while a

higher dose (~100 ng) was shown to favor neutrophilia suggestive of a Th1 or Th17 response[12]. Higher doses (in the μg range) actually inhibit airway inflammation[12,13]. The increased neutrophilia in response to an intermediate dose of LPS is interesting given that the traditional method of quantifying neutrophilia is based upon morphology and phenotypic markers. It is plausible that, although neutrophilia is indeed a response to high-dose LPS, a subset of those "neutrophils" were actually MDSC-like cells, similar on the basis of CD11b and Gr1 expression, two phenotypic markers that are shared between these myeloid subsets, but that are functionally distinct.

A mechanism underlying this low to high dose-response continuum of LPS in the promotion of allergic immunological responses was uncovered in 2009 with the discovery that Der p 2, the main allergen in house-dust mite (HDM) has structural homology with MD-2, the LPS binding component of the TLR4 signaling complex[14]. Airway epithelial cells do not express MD-2, but are known to express TLR4[15]. HDM-Der p 2, functioning as a molecular mimic of MD-2, may explain why a very low dose of LPS in the presence of HDM, can enhance a Th2 response in the lung. Furthermore, the presence of TLR4 on epithelial cells is critical for the initiation of HDM-driven allergic airway inflammation[16]. The development of immunosuppressive MDSC-like cells in response to endotoxin suggests a mechanism by which nature may limit atopic disease in cases where environmental exposure to LPS is increased or even extreme.

1.1.2 MDSCs and allergic inflammation

Prior to our publication demonstrating in vivo generation of lung MDSCs following exposure to LPS[5], little had been established regarding the role of MDSCs in the down-regulation of allergic

inflammation. In fact, the majority of publications involving MDSCs/MDSC-like cells or MDSC expansion described a cell type which actually favors a Th2 response[17-20]. Discrepancies between these studies and ours can likely be explained by differing microenvironments contributing to the generation and function of these MDSC like cell subsets in particular situations.

1.1.3 Lung MDSC suppression of Th2 immunity

MDSCs are historically known to inhibit both T-cell proliferation and Th1-immune responses in the context of anti-tumor immunity[3,21,22], but in 2010 we demonstrated the ability of lung MDSC-like cells to blunt the Th2 effector response in the lung[5]. Using both an HDM-driven model of allergic inflammation as well as *in vitro* co-culture of lung MDSCs with primed Th2 cells, we showed that LPS-generated lung MDSCs could dampen Th2 effector mechanisms. In the presence of lung MDSCs, lung DCs could not promote Th2 cytokine production, upregulate GATA-3 expression, or induce STAT5 activation in primed Th2 cells[23]. GATA3 and STAT5 are critical transcription factors for Th2 effector function, with GATA3 being the factor which drives Th2 development, and activated STAT5 being required for the solidification of a potent Th2 cell in the lung once it traffics from the lymph node[24,25]. Activated STAT5 is required for T1ST2 expression, a molecule required for Th2 effector function[26]. The LPS-mediated lung MDSCs produce nitric oxide, Arg1, and IL-10, but also relatively large amounts of IL-6, which was noted in culture supernatants. IL-10 and Arg1 cooperate to suppress GATA3 and nitric oxide was responsible for the suppression of pSTAT5. Nitric oxide is known to block Jak3-mediated STAT5 activation[27,28].

Preliminary studies revealed that one role for MDSC-derived IL-6 production was the suppression of DC-induced IFNγ production from T-cells in co-culture. Previous work from our laboratory also recognized IL-6 as a potent inhibitor of Th1[29]. Importantly, those particular experiments were designed as co-cultures of lung DC and MDSC (in varying ratios) together with naïve CD4 T-cells. It was later determined that lung MDSC were not present in lung draining lymph nodes (LNs), strongly suggesting that they are primarily tissue resident myeloid cells and therefore exert their immunosuppressive force in the periphery. That being the case, it was obvious that co-culture of lung MDSC with naïve T-cells did not accurately recapitulate the in vivo cell-cell dynamics. Therefore, it was deemed necessary to co-culture lung DC and lung MDSC with T-cells already primed towards the Th2 lineage, as this would best represent the physiological situation found in the lung. Studies are currently underway to determine the influence of MDSCs on Th1 and Th17 effector cell function

1.2 REGULATION OF MDSC ACCUMULATION AND FUNCTION

The accumulation of MDSC-type cells in the tumor microenvironment is thought to be dependent upon a number of factors such as VEGF, IL-6, GM-CSF, M-CSF, SCF, cycloxygenase-2, prostaglandins, TLR ligands and members of the S100 protein family[30-35]. Tumor-generated MDSCs have been characterized with the basic phenotype of $CD11b^{+}Gr1^{+}$, although additional surface molecules such as IL-4Rα, CD115, CD124, PD-L1, and CD80 are variable based upon particular microenvironments and tumor types[36,37]. Our study demonstrated the expansion of $CD11b^{+}Gr1^{int}F4/80^{+}$ cells in a dose dependent manner following LPS administration[5]. Their expansion was greatly blunted in MyD88-deficient mice suggesting

dependence upon this adaptor protein for their accumulation. It is important to note that a number of other studies have also documented the expansion of $CD11b^{+}Gr1^{+}$ cells in response to repeated LPS exposure[17,38,39]. $CD11b^{+}Gr1^{+}$ cells were also identified in the context of microbial sepsis and were shown to promote immune suppression via Th2 polarization[17]. Although both this study and ours demonstrated MyD88 dependence in the accumulation of these myeloid cells, their function in terms of Th2 regulation are very different, likely due to differences in microenvironment and organ specificity.

$CD11b^{+}Gr1^{+}$ MDSC-like cells are present in the bone marrow under homeostatic conditions. Furthermore, suppressive MDSC-like cells have been shown to be generated in culture under conditions of low or high concentrations of GM-CSF[40]. We demonstrated the in vitro generation of $CD11b^{+}Gr1^{int}$ from bone marrow cells under conditions of LPS and GM-CSF[5]. While the presence of GM-CSF alone favors myeloid DC differentiation, the addition of LPS blocks DC generation in favor of MDSC-like cells. In a similar manner, the combination of GM-CSF with inflammatory cytokines such as IL-6 or IL-1beta favors the generation of MDSCs with T cell suppressive capacity[30]. Suppressors of cytokine signaling (SOCS) proteins have additionally been shown to play a role in blocking DC differentiation presumably for the purpose of favoring macrophage differentiation under conditions where innate host defense is needed for protection[41].

Because of the initial association of MDSCs with tumor progression described in the literature, a better understanding of their generation and function is necessary for the development of novel immunotherapies, not only in the cancer field but also in organ transplantation, autoimmune diseases, and allergy[42-44]. Importantly, although many investigators have sought the mechanisms by which MDSCs are generated via exposure to tumor-secreted

factors as well as recruitment into the tumor microenvironment, these processes are still not well understood[1,21].

1.2.1 Development of murine myeloid cell lineages.

The discovery of the hematopoietic stem cell (HSC) provided the basis for the recent exploration of lineage differentiation of various types of cells[45]. Common-lymphoid, -myeloid, or -granulocyte progenitors (CLP, CMP, and GMP respectively) can be derived from a single HSC from the adult bone marrow. The process is dependent upon the surrounding growth factors and various transcription factors, which allow these progenitors to commit to a particular cell lymphoid or myeloid cell type, many of which have been characterized on the basis of surface phenotype and function[45-47].

The CMP lineage can give rise to myeloid cells encompassing granulocyte-macrophage, macrophage, and granulocyte populations differentiated via GM-CSF, M-CSF, and G-CSF, respectively[48]. Much of this work has been verified *in vitro*[49]. The addition of GM-CSF to bone marrow cells in culture is one method used for the generation of myeloid/conventional dendritic cells (cDCs) and M-CSF is added to similar cultures in order to promote bone marrow macrophage differentiation.

PU.1, and C/EBPα/β are two of the critical transcription factors shown to play a role in lineage commitment[50]. PU.1 plays a fundamental role and is required for HSC to proceed to CMP stage, since inhibition of PU.1 results in loss of CMPs, GMPs, and CLPs[46]. In contrast, CCAAT Enhancer Binding Protein alpha deficient (C/EBPα$^{-/-}$) mice lack neutrophils and eosinophils, and C/EBPβ was recently recognized as a critical transcription factor for neutrophil development during stress-induced hematopoiesis[51]. C/EBPβ is also critical for bone marrow

MDSC generation[52]. Furthermore, signal transducers and activators of transcription (STAT) molecules as well as interferon regulatory factors (IRFs) are highly involved in regulating cellular differentiation[53,54]. Myeloid dendritic cell generation requires signaling through STAT5 downstream of GM-CSF and it was recently shown that plasmacytoid dendritic cell (pDC) development involves a blockade of IRF8 by STAT5 during differentiation[55].

1.2.2 TLR signaling and regulation of inflammation

The toll-like receptors (TLRs) are a family of 13 (in mice) pattern recognition receptors, which represent the first line of defense against a variety of microbial pathogens. Expressed primarily on cells involved in innate immunity (epithelial cells, macrophages and dendritic cells (DCs)), these TLRs are type 1 membrane proteins with a Toll/interleukin-1 receptor (TIR) domain responsible for signal transduction via the TIR domain-containing adaptor molecules including MyD88, MyD88-like adaptor (Mal/TIRAP), TRAM, and TRIF. TLRs form homo or heterodimers for pattern recognition and TLR ligation induces DC maturation and triggers inflammatory cytokine production (TNFα, IL-6, IL-1β, IFN's) for host defense[56]. TLRs 1, 2, 4 and 6 are expressed on the cell surface whereas TLRs 3, 7, 8 and 9 are located intracellularly for recognition of pathogen-associated nucleic acids. All TLRs, except for TLR3, associate with MyD88 to initiate a common pathway of signal transduction involving activation of IRAK4 and IRAK1. This in turn induces the recruitment and activation of TRAF6, subsequent activation of the TAK1 complex, and culminates in IκB phosphorylation and NF-κB activation. In contrast, TLR3 utilizes TRIF and induces activation of interferon regulatory factor 3 (IRF-3), the MAPK-AP-1 pathway, and/or NF-κB[57]. TLR4 is unique given its ability to signal through two adaptor proteins, MyD88 and TRIF. Due to the bipartite nature of TLR4 signaling, there have been

many questions regarding how these pathways are controlled. LPS internalization is independent of TLR4 but both LPS and TLR4 are known to traffic between the Golgi apparatus and the plasma membrane in a manner independent of signaling[58,59]. Furthermore, it has been shown that endocytosis of TLR4 is necessary for signaling via the MyD88-independent pathway. Upon TLR4 ligation, MyD88 is recruited to TLR4 at the plasma membrane via TIRAP. Following signaling via MyD88, TRAM is able to bind TLR4 and facilitate interactions between TLR4 and TRIF in an endosomal location. Presumably, in the absence of TLR4 endocytosis, signaling through TRIF cannot occur[60]. Interestingly, while MyD88 has been linked to an early phase activation of NF-κB and MAP kinases upon LPS stimulation, TRIF-dependent signaling has been linked to later phase NF-κB and MAP kinase activation[61]. Downstream of TLRs, there are five NF-κB family members, all having the ability, with the exception of RelB, to form hetero- or homodimers for their function, with each member implicated in distinct roles.

TLR4 signaling has been characterized in a variety of cell types and has a primary function of coupling innate and adaptive immunity, but TLR4 has also been shown to promote immune homeostasis in the intestine[62]. This work, published in 2004, started to uncover the delicate balance between TLR stimulation as a way to alert the immune system of foreign invaders, while simultaneously maintaining homeostasis in the presence of commensal microflora. Ligation of TLRs is known to provoke an inflammatory response but using a toxic model of intestinal inflammation (oral administration of dextran sulfate sodium (DSS), Rakoff-Nahoum and colleagues demonstrated that TLR ligation was actually required for protection from inflammation. This protective response required MyD88 signaling downstream of TLR since MyD88-/- mice exhibited severe mortality following DSS administration. Although it was initially thought that the recognition of commensal bacteria by TLRs would be important to limit

infiltrating leukocytes and control bacterial growth; however, it was instead demonstrated that TLR ligation was critical for the expression of both heat shock proteins and cytokines, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).

TLRs expressed on precursors of the lymphoid and myeloid derived cell subsets in the bone marrow are thought to be important for replenishment of the innate immune system during infection. These progenitors express various TLRs, as well as MD2 and CD14, and have been shown to respond to TLR stimulation[63]. In 2008 a study showed that migratory hematopoietic stem and progenitor cells (HSPCs) traffic continually through extramedullary tissues such as the lung and kidneys and can proliferate within these tissues in response to LPS. TLR4 ligation halted the circulation of these progenitor cells via down regulation of sphingosine phosphate receptor (SP1), and induced rapid proliferation in situ giving rise to myeloid cells that express CD11c and a subset that expressed Gr1 at an intermediate level. This was one of the first studies suggesting the ability of HPCs to differentiate in response to TLR stimulation outside of the bone-marrow niche. The proposed function of these cells was immunosurveillance but the authors did not go on to show if or how this cell type contributed to host defense via rapid expansion in the tissue[64]. To address this issue, we performed experiments utilizing intravenous adoptive transfer of a GFP^+ lineage negative bone marrow population followed by subsequent LPS administration and demonstrated their expansion in the lung parenchyma[5].

1.2.3 GM-CSF and its role in augmenting LPS-TLR4 signaling

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a growth factor produced by many cell types including T cells, macrophages, and tumor cells. Fibroblasts, endothelial and epithelial tissue types can also produce GM-CSF. This small glycomeric protein is responsible

for the recruitment and production and function of macrophage and neutrophils, as well as eosinophils[65]. GM-CSF signals via the JAK2/STAT5 pathway, although the cell-specificity of GM-CSF signaling is intriguingly complex[66]. STAT5 is encoded by two separate genes resulting in two isoforms, STAT5A and STAT5B. $STAT5A^{-/-}$ mice have a defect in bone marrow GM-CSF-induced proliferation while STAT5B is known to play a role in growth hormone regulation[50]. However, partially distinctive functions for the two isoforms during monocyte/macrophage differentiation have been suggested, again highlighting the cell-specificity of STAT5 signaling[66,67]. A recent study performed in a human leukemic cell line demonstrated chronic STAT5A signaling as a promoter of M1 macrophage differentiation. High IL-6 production along with enhanced activity of NF-κB was critical for this STAT5A dependent differentiation[68]. Furthermore, GM-CSF is known to promote the accumulation of MDSCs in the tumor microenvironment[69], validating our interest in the role of GM-CSF in the lung.

In the lung GM-CSF is critical for homeostasis[70,71], as its absence results in alveolar proteinosis. It is also critical for alveolar macrophage development and function[72]. A basal level of GM-CSF is present at all times in the lung, a major source being epithelial cells[65,71], and it has been shown to augment signaling pathways downstream of TLR4 via augmentation of LPS-induced Akt and Erk1/2 signaling[73]. GM-CSF was found to be important for the second wave of LPS-TLR4 induced signaling pathways and nuclear localization of NF-κB (p50/65) augmenting neutrophil influx into the lung. Furthermore, neutralization of GM-CSF resulted in lower physiological expression of TLR4 and decreased TNF-α levels following LPS administration, also resulting in decreased neutrophil recruitment[74]. Taken together, these studies reveal the importance of GM-CSF signaling in the lung, regulating the ability of LPS to trigger a robust inflammatory response.

1.2.4 IL-6 and STAT3 signaling in MDSC differentiation, function, and lung homeostasis.

Interleukin-6 (IL-6) was initially cloned as an interferon species [75,76], and called by a number of names including B-cell stimulatory factor 2 (BSF-2) [77], interferon-β_2 (IFN-β_2)[75], and 26-kDa protein[78]. First recognized as a B-cell proliferative factor[79], it is now has an appreciated, yet complex, role in affecting numerous cell types under many disease conditions. Although initially thought of primarily as an inflammatory cytokine, it has been reported that levels of pro-inflammatory cytokines, TNF-α and MIP-2, are higher in the lungs of IL-$6^{-/-}$ following LPS exposure suggestive of a regulatory function at least in certain circumstances[80]. IL-6 is also an important regulator of Th2 inflammation[81]. Furthermore, evidence of IL-6 as a regulatory cytokine is demonstrated by the fact that IL-$6^{-/-}$ mice succumb during hyperoxic conditions within 96 hours[82]. The emerging role of IL-6 as a regulatory cytokine, along with its role in B-cell proliferation, points towards it being a major regulator of cytokine networks and thus a significant player in cellular differentiation.

Jak2/STAT3 signaling is involved in tumor-associated MDSC generation[83], and it has been shown that the presence of IL-6 with concomitant activation of STAT3 inhibits DC maturation resulting in the formation of an immature myeloid cell population[4]. *In vitro* inhibition of STAT3 abolishes MDSC-suppressive activity, and blocking STAT3 in vivo abrogates MDSC expansion in tumor-bearing mice[1].

Because STAT3 is now recognized as the major regulator of tumor MDSC, it can be appreciated that cytokines and molecules that themselves induce STAT3 activation could also modulate MDSC accumulation and function and there are several examples of this concept in the literature. For example, tumor derived Hsp72 regulates immunosuppressive functions of MDSCs via STAT3[84]. S100A9, which is regulated via STAT3, has also been shown to favor

MDSC differentiation[33]. Modulation of STAT3 via miR-17-5p and miR-20a also alleviates the suppressive potency of MDSC[85]. Furthermore, G-CSF, M-CSF, GM-CSF, and VEGF can all activate STAT3 and these have been linked to MDSC expansion[1], and STAT3 promotion of CXCL1 by hepatocytes is an indirect way by which STAT3 directs MDSC accumulation[86].

Lastly, continual signaling through STAT3 by IL-6 induces up-regulation of the SOCS3 protein, which has been shown to be a regulator of IL-6 signaling, and SOCS3 is a known modulator of cellular differentiation[41,87].

1.2.5 STAT1/STAT3 Crosstalk

Although the role of STAT3 is well appreciated in MDSC biology, STAT1 is another transcription factor known to regulate the immunosuppressive function of MDSCs via promotion of nitric oxide. Responsible for the regulation of nitric oxide synthase (iNOS), the ability of MDSC to suppress T cell activation is compromised in the absence of STAT1[3]. STAT1 is the main transcription factor transducing signals via IFNγ, a major cytokine involved in Th1-associated host defense[88]. Cross-regulation between Th1 and Th2 cytokines is well appreciated in the literature and recent evidence suggests that downstream interaction between STAT molecules, primarily STAT1 and STAT3, is partially responsible[89,90].

STAT1 and STAT3 compete for binding to the interferon gamma receptor 1 (IFNGR1) and STAT3 phosphorylation is stronger and more prolonged in STAT1-null cells[90]. Additionally, overexpression of a constitutively active STAT1 mutant protein can enhance IFNγ-induced anti-proliferative activity[91,92]. In contrast, IFNγ can induce the proliferation and survival of primary bone marrow cells from STAT1-null mice but not WT, revealing the presence of an alternative signaling pathway downstream of IFNγ which takes over in the absence of STAT1

and which renders IFNγ proliferative rather than anti-proliferative. Also, SOCS1 is a regulator of IFNγ induced responses by virtue of its activity in inhibiting STAT1[93]. SOCS1-/- mice are hypersensitive to LPS-induced endotoxic shock suggesting the need to regulate STAT1 signaling in the presence of LPS[94,95].

STAT1 signaling has been shown to inhibit IL-10 in a variety of models[96-98], demonstrating the suppressive nature of STAT1 on production of this regulatory cytokine. Most interesting to our studies is the role of STAT1 in the regulation of LPS-induced inflammation. Aryl hydrocarbon receptor (AhR) was shown to interact with STAT1 to inhibit production of IL-6, a known inducer of MDSC differentiation and function via STAT3[4,99].

1.3 THE COORDINATION OF EARLY AND LATE PULMONARY HOST DEFENSE

The development of regulatory myeloid cells in the lung, now recognized as lung MDSCs, in response to bacterial endotoxin instinctively prompted the investigation of their possible role in host defense against Gram negative pathogens. We chose to use *Klebsiella pneumoniae* as our model, given that *K. pneumoniae* is a common isolate found in nosocomial infections and its potency as an opportunistic pathogen in the lung results in a high rate of mortality and morbidity[100,101].

1.3.1 The four phases of host defense

To quote Rodger P. McEver: "There is a delicate balance between initiating inflammation to control microbial invasion and dampening inflammation to prevent untoward tissue injury." [102]. This quote describes Mantovani's work on long-pentraxin 3 (PTX3)[103], a molecule which acts as a molecular switch to either enhance or diminish neutrophil influx into the lung[104]. In the context of host defense against *K. pneumoniae*, PTX3 has been described as having seemingly contradictory functions whereby, depending on the degree of infection, it may increase or oppose the recruitment of neutrophils into the tissue. Although just one example, these observations highlight the complexity of a robust innate immune response because although necessary for protection against microbial pathogens, the resolution of inflammation and restoration of tissue homeostasis is equally important. Control of inflammation must be carefully coordinated, the complexity of which is only starting to be understood.

The innate immune system response can be broken down into four phases, all of which must occur for successful host defense[105]. Phase I is triggered by the initial recognition of pathogen by patrolling antigen-presenting cells (APCs). These APCs provide chemotactic signals, which trigger neutrophil egress from the bone marrow and into the tissue. Phase II occurs as the neutrophils reach the site of infection, degranulate, and trigger further monocyte extravasation. Phase III is a delicate balance of signal recognition between the APCs and neutrophils. Neutrophils have a short life span and rapidly begin to undergo apoptosis at the site of inflammation, somewhat aided by signals provided by macrophages. These apoptotic neutrophils in turn trigger a regulatory program in the macrophages, including the production of interleukin-10, which prevents further neutrophil infiltration. Phase IV is the result of a properly functioning Phase III, where the interaction with and clearance of apoptotic neutrophils by

macrophages further accentuates anti-inflammatory activity of macrophages in order to restore homeostasis.

The sub-populations of macrophages, as defined by location within particular tissues, which play a roll in neutrophil recruitment and/or elimination are not yet well understood and this topic remains largely unexplored in the literature[106].

1.3.2 Innate Host Defense Against *Klebsiella pneumoniae*

Bacterial pneumonia is a leading cause of morbidity and mortality worldwide causing 40,000 to 70,000 deaths annually. *K. pneumoniae*, a Gram negative bacterium with lipopolysaccharide as a major component of its cell wall, is a commonly isolated causative agent of pneumonia in hospital-acquired infections[101].

Innate host defense against *K. pneumoniae* has been shown to involve both alveolar macrophages (AMs), the resident phagocytes in the alveolar space, and neutrophils (PMNs). AMs, due to their anatomical location serve as the first line of cellular host-defense against *K. pneumoniae*. Both experiments where neutrophil influx was decreased, as in the case of TIRAP-/- mice in comparison to WT, or experiments where neutrophil influx was increased, as induced by over-expression of KC, demonstrate the crucial role of neutrophils in bacterial killing[107 108]. Selective depletion of AMs has been attempted through the use of intratracheal administration of liposomes containing dichloromethyene diphosphonate, or DMDP[109]. Braoug-Holub and colleagues depleted AMs using DMDP-liposomes and revealed that AMs are a critical component of host defense against *K. pneumoniae*[110]. In AM-depleted animals, enhanced neutrophil recruitment and MPO activity, which corresponded with increased levels of TNF-α was observed. Strikingly, in spite of increased neutrophilia, enhanced bacterial burden was

evident in both the lungs and plasma of AM-depleted animals suggesting that neutrophils alone are not sufficient for bacterial clearance. AM depletion in the case of a sub-lethal dose of 100 CFU *K. pneumoniae* suggests that not only do AMs participate in initial host defense, but that signals provided by the AMs enhance neutrophil killing capacity. In our studies we also noted that in the case of 100 CFU *K. pneumoniae*, neutrophil recruitment is minimal, presumably due to rapid clearance of bacteria by the AMs.

Recognition of *K. pneumoniae* occurs via the TLR4/MyD88 signaling pathway and TLR4, involving both the MyD88 and TRIF dependent pathways[111-113]. Functional TLR2 and TLR4 expression is required for adequate host defense suggesting cooperation between these two signaling pathways[114].

Downstream of TLR ligation and signaling, NF-κB activation serves to promote the release of TNF-α, MIP-2, and KC, three critical cytokines for protection against *K. pneumoniae*. All three play a role in neutrophil recruitment, and while MIP-2 is a C-X-C chemokine that aids in neutrophil recruitment, TNF-α is also linked directly to macrophage activation[115]. Nitric oxide is also critical for host defense against *K. pneumoniae* as demonstrated by increased bacterial survival in the presence of L-NAME, an inhibitor of iNOS[116], and interleukin-17 (IL-17) also plays a roll in enhanced neutrophil recruitment and TNF-α induction[117].

There is evidence that neutrophils aid in killing of *K. pneumoniae*[118]. However, the neutrophil influx induced by *K. pneumoniae* can be followed by extensive lung destruction if left unchecked[119]. Furthermore, although the role of AMs is well appreciated, the cells involved in host defense in the lung interstitium are not well defined.

1.3.3 Alveolar Macrophages vs Tissue Macrophage Subsets

Interactions between subsets of antigen-presenting cells (APCs) within the respiratory tract are an important determinant of the immune response in the lung[120]. Macrophage and DC subsets must cooperate to properly modulate an immune response to invading pathogens, innocuous substances, and inhaled allergens[2].

With meticulous dissection, von Garnier and colleagues utilized flow cytometry of cells within the parenchymal tissue, the conducting airways, and the bronchial alveolar lavage fluid (BALF), to reveal differing subsets of APCs within the respiratory tract based on anatomical location[120]. Dual staining with antibodies against CD11c and MHC II was used to discern the presence of four subsets, which differed in their frequency between tissue, airway, and BALF. The four subsets consisted of cells that were $CD11c^{neg}I\text{-}A^{d\ high}$(R1), $CD11c^{high}I\text{-}A^{d\ high}$(R2), $CD11c^{int\text{-}high}I\text{-}A^{d\ low}$(R3), $CD11c^{int}I\text{-}A^{d\ neg}$(R4). R1 and R2 were found in both the parenchyma and the conducting airways, while R3 and R4 were largely located in the parenchymal lung tissue. Strikingly, one of these subsets (R3) located predominantly within the lung parenchyma, characterized by intermediated expression of CD11c and low expression of MHC II bore close resemblance to lung MDSCs although no function was attributed to them at that time.

AMs present within the alveolar space are known to regulate immune responses, and in 2009, a role for interstitial macrophages (IMs)[121] in the suppression of Th2 inflammation has been documented[122]. However, although there were similarities between the lung MDSCs we have described[5] and the IMs in this report, the latter were not found to be $Gr1^{int}$, nor did they express CD11c. It is worth noting that the characterization of IMs was performed on lungs from naïve animals inviting speculation as to what the phenotype of IMs would be in LPS treated animals. It is likely that due to the heterogeneity of lung MDSC-like cells[36], IMs may be one

cellular component. However, their generation in situ in response to LPS was not examined. Therefore, it is not known whether or not IMs develop from trafficking lineage negative precursors as previously demonstrated[5].

Finally, control of neutrophil numbers maintained in part by alveolar macrophages under steady-state conditions is a critical component of lung homeostasis[123]. Alveolar macrophages, through a process known as efferocytosis, or the phagocytosis of apoptotic cells, participate in the removal of dying neutrophils from the alveolar space[124]. Although efferocytosis (described in greater detail in Section 1.3.7) by macrophages has also been investigated *in vitro*[125,126], macrophage populations within the interstitium have not yet been explicitly recognized as efferocytic cells.

1.3.4 STAT1 in host defense

The use of embryonic stem cells deficient in STAT1, as well as STAT1-/- mice, revealed that this molecule is essential for anti-viral immunity and responsiveness to IFNα and IFNγ as evidenced by the fact that 100% of animals succumbed by 5 days post infection with *L. monocytogenes* or vesicular stomatitis virus[88,127]. STAT1 is the main transcription factor induced downstream of IFNγ[96], is responsible for eliciting macrophage activation[128], and is a major player in the induction of inflammatory responses[129]. Interestingly, IFNγ induced STAT1 is augmented when cells are simultaneously or pre-exposed to LPS, highlighting the role of STAT1 as a central effector molecule for macrophage function during host defense against Gram negative pathogens. Of relevance, the role of STAT1 in host defense against *K. pneumoniae* has not been explored, although STAT1 is a critical molecule for the regulation of iNOS induction

and nitric oxide production[88], which are known to be important for protection against *K. pneumoniae*[130].

STAT1 also plays a role in expression of chemokines in the lung, regulating not only macrophage activation via IFNγ, but also cellular recruitment[131]. Of note, freshly isolated monocytes stimulated with IFNγ are not available to migrate in response to CCL2, therefore IFNγ-STAT1 signaling has a negative regulatory effect on monocyte chemotaxis[132].

1.3.5 Neutrophils

Neutrophils are the most abundant white blood cell in the circulation and are rapidly recruited to sites of infection. Highly phagocytic, they are crucial for host defense based upon their strong anti-microbial properties via the production of antimicrobial peptides, serine proteases, and large amounts of reactive-oxygen species (ROS)[133,134]. The number of neutrophils present in the circulation at any one time has been described as being the result of a combination of generation, recruitment, survival, and removal[133].

Neutrophils are consistently generated in the BM, and in the steady state, about 1-2% are released from the BM. Because their half-life is less than 6 hours in the circulation, this is adequate in order to maintain peripheral homeostasis[135]. It is critical that during homeostatic conditions, neutrophil removal matches their production[133] since many of the anti-microbial effectors produced by neutrophils can inflict tissue damage[119].

G-CSF is the principal cytokine for neutrophil development, and both G-CSF-/- and G-CSFR-/- mice have compromised basal levels of granulopoiesis[136]. Apart from G-CSF, other cytokines, such as GM-CSF[137] and IL-6[138] can stimulate granulopoiesis in vivo. Furthermore,

IL-6 and TNF-α regulate neutrophil function[139] inducing their migration and activation at the site of infection[80].

Neutrophils can be identified microscopically in the lungs by their distinctive multi-lobed nuclei (based on histological examination following Wright's stain), and phenotypically by expression of characteristic surface molecules such as Gr1 (both $Ly6G^{hi}$ and Ly6C expressing), CD11b, CD62L, and CXCR2[135].

1.3.6 Neutrophil Recruitment in Response to *K. pneumoniae*.

Deficiencies in neutrophil recruitment are linked to increased host susceptibility during *K. pneumoniae* infection[140]. Conversely, increasing neutrophil recruitment has proven to be beneficial[108]. During host defense, neutrophils are recruited from the bone marrow to the site of inflammation. Transendothelial migration of leukocytes from the blood to sites of inflammation includes several key steps including, rolling, adhesion strengthening, intraluminal crawling, paracellular or transcellular migration, and migration through the basement membrane. Integrins and selectins are the major adhesion molecules regulating each of these aspects of neutrophil emigration[141].

The most crucial neutrophil chemoattractants are CXC chemokines including IL-8 (CXCL8) (present in humans but not in mice), MIP-2 (CXCL2) and KC (CXCL1) (present in both humans and mice)[142]. Both MIP-2 and KC signal through the receptor CXCR2, and also play a role in neutrophil activation after they arrive at the site of infection.

CXC chemokines act on neutrophils to affect their release from the BM as well as at multiple steps of neutrophil migration. CXC chemokines such as KC can desensitize the responsiveness of neutrophils to SDF-1, therefore disrupting the BM SDF-1/CXCR4 retention

signaling and thus facilitating neutrophil egress[143]. MIP-2 and KC are each capable of inducing a dose and time-dependent increase in neutrophil rolling on endothelial cells, which was inhibited by anti-P-selectin antibody, suggesting that these chemokines induce a P-selectin-dependent rolling[144]. In vivo studies further confirmed that CXCR2, the receptor for KC and MIP-2, was required for neutrophil tight adhesion to inflamed venules[145]. Recent work in our laboratory has also demonstrated that TNF-α and IL-17 contribute to neutrophil recruitment and survival in the lung[146].

A previous report from our laboratory implicated RANTES in neutrophil accumulation in the lungs[147]. Interestingly, we observed decreased RANTES expression in STAT1-/- lung tissue following infection with *K. pneumoniae* and STAT1 regulation of RANTES-induced neutrophilia may be one reason we observe decreased neutrophils in STAT1-/- mice (See 3.0). Finally, it is interesting to note that we detected substantial levels of these neutrophil recruiting chemokines in culture supernatants of purified lung-MDSCs (data not shown). Therefore, it may be that lung MDSCs participate in neutrophil/macrophage recruitment for necessary host defense, while simultaneously being a source of tissue IL-10 for the tempering of inflammation.

1.3.7 Neutrophil Apoptosis and Clearance (Efferocytosis)

Neutrophils release oxidants, necessary for bacterial killing, but if left unchecked, these oxidants and enzymes can cause tissue damage[119,148]. In the absence of inflammation, circulating neutrophils are quickly removed with a half-life of 6-8 hours[149], and this normal turnover is accomplished through constitutive apoptosis, followed by clearance by macrophages[150] by a process is referred to as efferocytosis meaning 'to carry the grave'[133]. Neutrophil apoptosis is

critical for the resolution of inflammation, but host-derived factors (such as GM-CSF and G-CSF) and microbial products can delay neutrophil apoptosis[105].

During apoptosis, neutrophils shed receptors and are functionally compromised, no longer secreting pro-inflammatory cytokines, and therefore becoming anti-inflammatory instead of pro-inflammatory upon recognition by a macrophage. Indeed, the production of IL-10, PGE_2 and TGF-β has been linked to macrophage recognition of apoptotic neutrophils[105,124]. In order for a neutrophil to be recognized, it must express appropriate molecules on its surface, such as bridge molecules and signaling receptors including phosphatidylserine (PS), cardiolipin, CD14, PTX3, and CD43[133]. These molecules are recognized by scavenger receptors on macrophages such as CD36 (recognizes PS), ICAM3 (recognizes CD14), nucleolin (recognizes CD43) and CD91 (recognizes PTX3).

CD14 is a GPI anchored surface molecule, and is part of the LPS/LBP complex, found on the surface of monocytes and macrophages. The soluble form of CD14 aids LPS recognition by CD14 negative cells. Importantly, bacterial phagocytosis via CD14 is independent from FcγR-mediated phagocytosis. Recently IL-10 was shown to enhance CD14-mediated uptake of apoptotic cells[151], and this recognition of apoptotic cells via CD14 can protect against LPS-induced shock[152]. Of note, IFNγ was found to have the opposite effect[153].

CD36 has been shown to bind multiple ligands including phospholipids, long-chain fatty acids, and sickle cell erythrocytes[154]. Expressed on a variety of cells types it is defined as a class B scavenger receptor, and importantly, $CD36^{-/-}$ mice exhibit up to 80% reduction in OxLDL binding and uptake[155]. Furthermore, DCs employ CD36 during cross-presentation of apoptotic cell antigens to induce CTL activation[156].

Several other scavenger receptors, some newly identified have also been reported. The urokinase receptor (uPAR/CD87) negatively regulates efferocytosis and uPAR-/- macrophages have increased efferocytic ability[157] as well as an enhanced capacity to engulf viable neutrophils. Park et al. demonstrated that uPAR/uPA binding enhanced phagocytosis by either macrophages or neutrophils and that uPAR expression on one of the two cell types was necessary. uPAR expression seems to inhibit integrin binding to uPAR on nearby cells[157]. Previously, IL-10 was shown to inhibit uPAR expression, a plausible mechanism contributing to the ability of IL-10 to promote efferocytosis[158]. Urokinase and its receptor are implicated in migration of monocytes and adherence to both fibrinogen and vitronectin, which is critical for immunity/inflammation.

Rac1 and Cdc42 signaling (both small GPTases) are regulated by IFNγ and IL-10. IFNγ activates Rac1, decreasing the amount available for F-actin remodeling, a necessary mechanism during phagocytosis of FcγR-mediated internalization of IgG opsonized particles. IL-10 stimulation increases FcγR expression [159]. Interestingly signaling via the macrophage G-protein-coupled receptor G2A regulates enhanced efferocytosis via augmentation Rac1 signaling[160]. The opposition of these two studies could point to the critical control of Rac1 signaling during phagocytic events, dependent upon the type of cargo and state of the surrounding microenvironment. Additionally, the binding of phosphatidylserine (PS) on dying cells and subsequent efferocytosis, likely employs different mechanisms than FcγR-mediated internalization.

Taken together, the interplay between neutrophils and macrophages is a complex process. Enhanced understanding of these processes will lead to therapeutic development to prevent lung injury following bacterial pneumonia.

1.4 INTERLEUKIN-10

Interleukin-10 (IL-10) is a potent regulatory cytokine. Discovered in 1990 by Mossman and colleagues[161], it was found to suppress not only Th1 responses, but also curb inflammation by limiting activation of a variety of cell types[89]. Of importance for our studies, IL-10-/- mice develop inflammatory bowel disease[162], suggesting that IL-10 functions, at least in part, in a non-redundant manner as a regulator of inflammation.

1.4.1 Early vs. Late IL-10 production in host defense

Numerous studies have demonstrated the negative impact of IL-10 on host defense[124,163-165]. In all of these cases, IL-10 was shown to inhibit the initial host response to infection. Given the potency of IL-10 as an anti-inflammatory cytokine, these results are not surprising. In the case of infection with *K. pneumoniae*, neutralization of IL-10 prior to infection proved to be beneficial and resulted in increased bacterial clearance[165]. Similar results were shown with *Pseudomonas aeruginosa*[166]. In the case of both of these infections, high IL-10 was detrimental to the host due to impairment of neutrophil recruitment via suppression of neutrophil attracting chemokines. However, in order to prevent tissue injury to maintain organ function, inflammation must not be permitted to continue in an uncontrolled manner[102]. As demonstrated in the case of infection by *Pneumocystis carinii*, treatment with viral IL-10 was beneficial and decreased tissue inflammation without altering pathogen clearance[167]. In a sepsis model it was demonstrated that a balance of pro and anti-inflammatory cytokines is necessary[168]. The authors showed that severe sepsis, which results in greater mortality than moderate sepsis, was due to unopposed inflammatory responses.

These studies suggest a role for IL-10 in appropriate resolution of inflammation. Although there is a recognized need for such regulation, an interstitial cellular source of IL-10, which may aid in homeostatic resolution, has not been identified. A review article published in the Journal of Immunology in 2008 put it well by stating that although many cell types can produce IL-10, "the priming of these various populations during infections is not well understood and it is not clear whether the cellular source of IL-10 dictates its cellular target and thus its outcome"[169].

1.4.2 IL-10 production by myeloid cells

Because of the potent anti-inflammatory properties of IL-10, its production by innate cells is regulated at multiple levels including changes in chromatin structure, regulation of transcription factors and their activation, and post-transcriptional modifications[170]. In addition, positive or negative regulation of IL-10 can be influenced by multiple soluble factors. The complexity of IL-10 regulation makes comprehensive assessment of all of its functions difficult[171].

Microbial products induce IL-10 secretion from DCs and macrophages, and although these cells as well as MDSCs are all known to produce IL-10, neutrophils have also recently been reported to express IL-10 *in vivo*[172]. Upon TLR ligation, both MyD88-dependent and independent (TRIF) pathways are involved in promoting the production of IL-10[171]. Downstream of MyD88, both the MAPK/ERK cascade and NF-κB family members induce IL-10 transcription. The amount of ERK activation, a MAPK family member, dose dependently regulates production of IL-10 by DCs and macrophages[173]. Additionally, activation of p38 kinases can trigger IL-10 production, and both ERK and p38 dependent IL-10 expression can be dampened by IFNγ[174]. In brief, the release of GSK3 is typically held in check by PI3K, but

because IFNγ negatively regulates PI3K, GSK3 is released to subsequently act by inhibiting binding of activator protein 1 (AP1) to the IL-10 promoter significant since the activity of AP1 on the IL-10 promoter induces IL-10 transcription downstream of TLR[174]. Interestingly, IL-10 also regulates its own production by promoting dual-specificity protein-phosphatase 1 (DUSP1), a p38 regulator[175], and a second wave of IL-10 production from myeloid cells has been attributed to type I IFNs via TRAF3 signaling[176]. Various studies have revealed that C/EBPβ, STAT3, and IRF1 can all bind to the IL-10 promoter to induce IL-10 transcription[171]. With regard to NF-κB family members, NF-κB p50 deficient mice display reduced IL-10 expression[177].

The production of IL-10 is also regulated by a number of soluble factors. LPS has been shown to induce IL-10 expression by triggering release of IL-27 and type I IFNs, a process dependent upon IL-27 activation of both STAT1 and STAT3[178]. IFNγ can cross-regulate IL-10 the effects of IL-10, not only its production. IFNγ was shown to switch the balance of STAT activation by IL-10 from STAT3 to STAT1 in the presence of IFNγ[98], and in fact, IL-10 only triggered STAT1 when IFNγ was present. Due to the centrality of STAT3 in MDSC biology, STAT3 regulated IL-10 production is of special interest.

IL-27 and IL-6 trigger the production of IL-10[179], and given that GM-CSF can promote IL-6 production[180], GM-CSF activation of STAT proteins likely regulates both IL-6 and IL-10 production to direct immune outcome. Of great interest, GM-CSF blocks STAT1 activation downstream of IFNs (IFNα and IFNγ)[181], suggesting that some of the observed effects of GM-CSF in our model (Further discussed in Section 2.0) may be a result of STAT1 inhibition.

1.4.3 IL-10 contributes to Neutrophil Clearance and Resolution of Inflammation

The resolution of inflammation (Phase III-IV of host defense as described in Section 1.3.1) is characterized by removal of apoptotic neutrophils and a shift in pro-inflammatory mediators to anti-inflammatory signals, some of which are lipoxins, protectins, and resolvins. How exactly this switch occurs is an area of ongoing investigation, but is thought to involve the production of protectins by macrophages that have engulfed apoptotic neutrophils, and lipoxins appear to play a role in discontinuing neutrophil influx to the site of infection[106,182,183].

Ample evidence regarding the immunobiology of IL-10 points to it being a central regulator of inflammation, not only for the prevention of autoimmune disease, development of regulatory T-cells and DCs, and dampening of Th2-allergic inflammation, but now for the resolution of inflammation following bacterial pneumonia. IL-10 plays a role in dampening neutrophil influx and function by counter-regulating TNF-α, KC, RANTES, and MIP-2 production[165,184-187], and recent studies report IL-10 release during efferocytosis[124].

The role of IL-10 in regulation of efferocytosis is of considerable interest. Michlewska and colleagues demonstrated that ingestion of apoptotic cells by IL-10-/- bone marrow macrophages is markedly reduced as compared to WT, suggesting a role for IL-10 in the promotion of efferocytosis[126]. LPS and TNF-α are known inhibit neutrophil apoptosis[188,189], but this inhibition can be counteracted in the presence of IL-10 suggesting that the presence of IL-10, even in low levels, dampens the inflammatory effects of both LPS and TNF-α[126,185].

IL-10 has also been shown to maintain FcγR expression for the function of FcγR mediated phagocytosis, suggesting that IL-10 can play a positive role in the internalization of IgG coated particles with implications for host defense[190]. Due to its regulatory properties, IL-10 may promote phagocytosis to prevent exacerbated inflammatory cytokine release but how

exactly this process occurs is not known. As indicated above, IL-10 plays a role in the regulation of several scavenger receptors but details of their engagement and role during efferocytosis is an area of ongoing investigation.

There is much interest in further understanding the mechanisms that may regulate MDSC accumulation and function in different disease settings. Due to our discovery of a lung MDSC-like cell in response to LPS[5], the following data explore mechanisms by which LPS/TLR4 signaling contributes to their generation, including the role of GM-CSF, a central growth factor in lung biology. Furthermore, how neutrophil influx and clearance is regulated is of increasing interest since the deleterious effects of neutrophils in the induction of lung injury are being increasingly recognized. The following data uncover the role of GM-CSF/STAT5A signaling in lung MDSC development, and reveal a yet unrecognized role of STAT1 as a negative regulator of MDSC accumulation. Furthermore, we investigated a cellular mechanism that regulates neutrophil clearance in the lung interstitium, an area unexplored in the literature to date.

2.0 THE DEVELOPMENT OF A LUNG MDSC-LIKE CELL

2.1 ABSTRACT

Our laboratory has studied the effects of lipopolysaccharide (LPS) exposure on the murine lung as well as on bone marrow progenitor cells *in vitro*. These studies initially led to the discovery that intratracheal administration of LPS, a toll-like receptor 4 (TLR4) ligand, induces the appearance of suppressive $CD11b^{+}Gr1^{int}F4/80^{+}$ MDSC-like cells in the lung, which secrete nitric oxide (NO), Arginase-1, interleukin-6 (IL-6), interleukin-10 (IL-10) and granulocyte macrophage colony-stimulating factor (GM-CSF). Furthermore, the secretion of IL-10 and Arg-1 in combination with NO, serves to suppress Th2-driven inflammation. Therefore, pulmonary MDSC-like cells likely help to control inappropriate inflammatory responses in the airways[5], and thus may be a potential mechanism underlying the hygiene hypothesis.

This present work expands upon these initial findings to illustrate that GM-CSF and LPS act in concert to induce MDSCs in vitro from lineage-negative progenitor cells. GM-CSF is known to strengthen signaling downstream of TLR4, and is also linked to IL-6 production in a variety of cell types. These studies were carried out *in vitro*, but importantly, it was also found by *in vivo* neutralization of GM-CSF that it is similarly required for the development of functional MDSC-like cells in the lung. Since GM-CSF is known to signal via STAT5, we observed a reduction in the $CD11b^{+}Gr1^{int}F4/80^{+}$ population in STAT5A-/- mice upon exposure

to LPS, suggesting at least a partial dependence on GM-CSF-STAT5A signaling for their expansion. These data strongly suggest that cooperation between GM-CSF and TLR signaling induces the development of lung MDSCs.

2.2 INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) have historically been investigated largely in the context of cancer development and tumor growth[1]. In contrast to a negative connotation of MDSCs in cancer where immune suppression has deleterious effects, pulmonary MDSCs may help to control inappropriate inflammatory responses to inhaled allergens and represent an important mechanism for the suppression of the initiation and perpetuation of asthma[5]. These findings, published in 2010, are summarized below and in **Figure 1.**

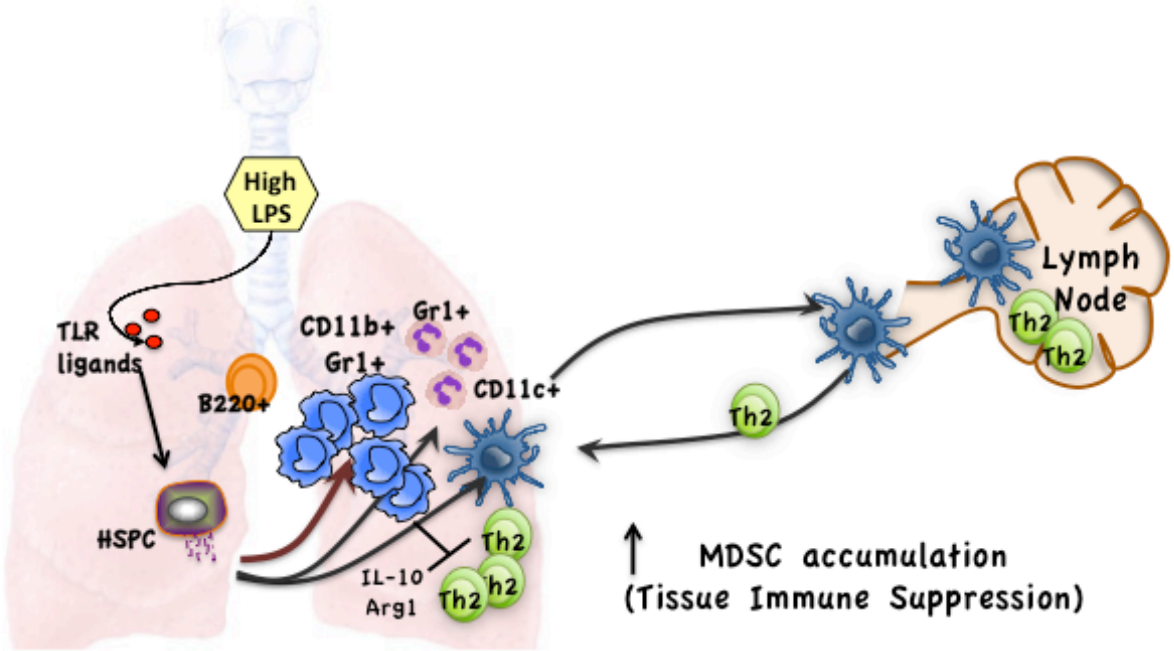

Figure 1. Schematic depicting regulation of lung-MDSC accumulation and function.

A relatively high dose of LPS inhalation induces the differentiation and expansion of lung trafficking HSPCs into a variety of innate cell types, including $CD11b^{+}Gr1^{int}F4/80^{+}$ MDSC-like cells in the lung tissue. In contrast to activated lung DC, these cells do not traffic to the draining lymph nodes which causes a 5–10-fold enrichment of these cells relative to DCs in the tissue. Molecules expressed by the $CD11b^{+}Gr1^{int}F4/80^{+}$ cells such as arginase 1 and IL-10 suppress reactivation of Th2 cells by the resident DCs[23].

Allergic asthma is a chronic inflammatory condition of the lower airways, and is promoted by repeated interactions between allergen(s) and the immune system. Although the presence of allergen is necessary for disease development and exacerbation, the prevalence of allergic disease has been paradoxically increasing in developed countries where exposure to allergens is reduced. In light of this observation, the hygiene hypothesis was postulated in 1989, stating that early childhood exposure to pathogen-associated products, like endotoxin, may actually be protective against future allergic responses via the induction of immunological tolerance[6]. It is established that bacterial endotoxin (lipopolysaccharide-LPS) has adjuvant-like properties and can promote an inflammatory response in the lung[12,14,16]. However, pulmonary LPS exposure has also been linked to immune suppression[13], and importantly, findings by von Mutius *et al*. showed that endotoxin exposure was inversely correlated with development of asthma in children[7]. In an attempt to recapitulate the idea behind the hygiene hypothesis, our laboratory has studied the effects of LPS exposure on the murine lung as well as on bone marrow progenitor cells *in vitro*. These ongoing studies have led to the discovery that intratracheal administration of LPS, a toll-like receptor 4 (TLR4) ligand, induces the appearance of MDSC-like cells in the lung, which secrete nitric oxide (NO), arginase 1, interleukin-6 (IL-6), interleukin-10 (IL-10), and granulocyte macrophage colony-stimulating factor (GM-CSF). We have shown that signaling through the TLR4-MyD88 pathway results in this suppressive phenotype[5]. Furthermore, MDSC secretion of NO, IL-10, and Arg-1, serves to suppress Th2

responses. In vitro, GM-CSF induces conventional, myeloid-like DC (cDC) development, but the addition of LPS results in the generation of cells with suppressive function that are phenotypically similar to MDSCs found in vivo[5]. This link between LPS exposure and MDSCs is intriguing in light of the hygiene hypothesis, prompting many questions regarding the role of both LPS and GM-CSF in their development. We investigated the role of GM-CSF in the expansion of lung MDSCs *in vivo*, as well as the contribution of STAT5 signaling downstream of GM-CSF. Our data strongly suggest a cooperative role for LPS and GM-CSF in MDSC development both *in vivo* and *in vitro*. We now show that signaling mechanisms downstream of GM-CSF and TLR4 signaling are required for the full development of MDSC-like cells (**Figure 2**).

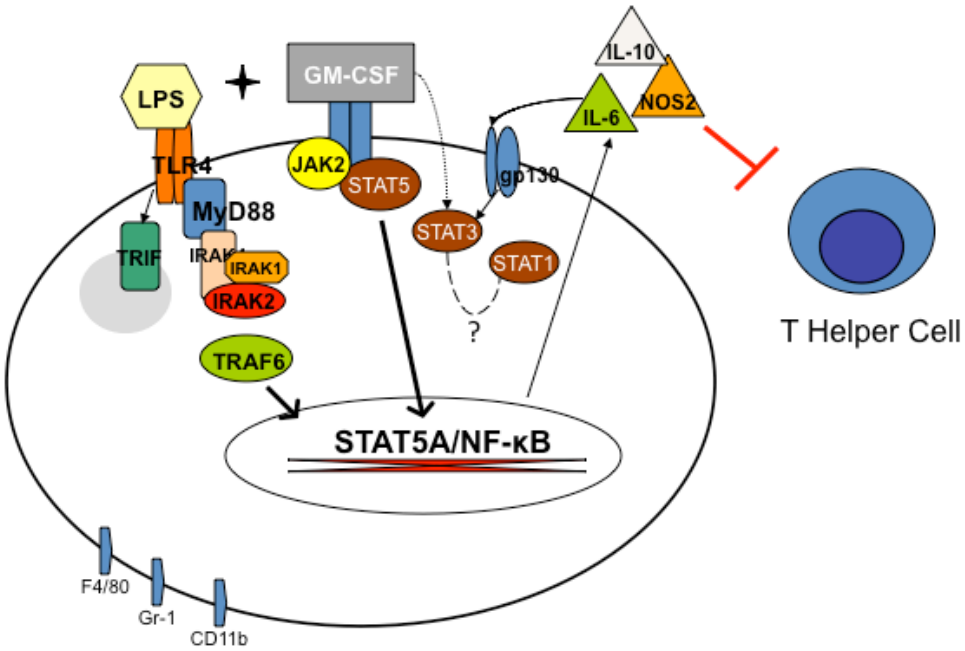

Figure 2: Generation of lung MDSC requires GM-CSF and LPS, signaling via STAT5 for the production of IL-6.

2.3 MATERIALS AND METHODS

2.3.1 Animals and reagents

Male 6- to 8-week old BALB/c ByJ or C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). DO11.10 transgenic mice were originally provided by Dr. Kenneth Murphy at Washington University (St Louis, MO) and were bred at the animal facility at the University of Pittsburgh. STAT5A$^{-/-}$ and IL-6$^{-/-}$ were purchased from the Jackson Laboratory (Bar Harbor, ME). All studies with mice were approved by the animal care and use committee at the University of Pittsburgh. Recombinant GM-CSF was purchased from PeproTech (Rocky Hill, NJ). LPS from *Escherichia coli*, strain O26:B6, was obtained from Sigma (St. Louis, MO). HDM (*Dermatophagoides Pteronyssinus*) was purchased from Greer Laboratories (Lenoir, NC) and was stripped of LPS using Endo-Trap columns. A number of directly labeled monoclonal antibodies were used in cell phenotyping experiments as follows: phycoerythrin (PE)-labeled anti-mouse CD40 (clone 3/23), PE-labeled anti-mouse CD86 (clone GL1), APC-labeled anti-mouse CD11c (clone HL3), APC-labeled anti-mouse Gr1 (clone RB6-8C5), fluoresceine isothiocyanate-labeled anti-mouse Ly6C (clone AL-21), PE-labeled anti-mouse Ly6G (clone 1A8), and neutralizing anti-mouse IL-6, all purchased from BD Biosciences (San Jose, CA). PE-labeled rat anti-mouse CD80 was purchased from Serotec (Raleigh, NC). PE-labeled anti-mouse major histocompatibility complex II (clone NIMR-4) was purchased from Southern Biotech (Birmingham, AL). PE- and APC-labeled anti-mouse F4/80 were purchased from Caltag Laboratories (Carlsbad, CA). Neutralizing GM-CSF antibody (clone MP122E9) and isotype control Rat IgG_{2A} were purchased from R&D Systems (Minneapolis, MN).

2.3.2 Induction of allergic airway inflammation by HDM

BALB/c mice were exposed to HDM (100 μg per mouse per treatment) intratracheally on days 0, 7, 13, and 21 with or without concomitant treatment with LPS (0.05-10 μg per mouse per treatment). At 72h after the final HDM exposure, BAL fluid and lung tissue were obtained. Lung inflammation was gauged based upon BAL fluid differential cell counts by hematoxylin and eosin (H&E) staining. Production of cytokines was assessed by preparation of whole lung homogenates and analysis by multiplex assay (Bioplex; Bio-Rad, Hercules, CA).

2.3.3 Generation of LPS-induced cells *in vivo*

LPS (up to 10 ug per mouse per dose) was instilled intratracheally in 100 ul into C67BL/6J or Balb/c daily for 3 or 4 consecutive days, respectively. At 24h after the final exposure, mice were anesthetized with ketamine (100 $mgkg^{-1}$)/xylazine (10 $mgkg^{-1}$) mixture. Brochial alveolar lavage fluid was obtained by inflating the lungs with 1mL phosphate-buffered saline (PBS), and then removing. After exsanguinations through cardiac puncture, the aorta was severed and the pulmonary vasculature was perfused with sterile PBS to remove peripheral blood cells. The perfused lungs were placed into C tubes associated with the AutoMacs Dissociator (Miltenyi Biotec), chopped twice using Program Lung_01, and incubated in an enzyme solution containing 0.7 mg ml^{-1} collagenase A (Boehringer Mannheim (Roche), Indianapolis, IN) and 30 ug ml^{-1} type IV bovine pancreatic DNase I (Sigma-Aldrich) for 45 min at 37'C. Digested lungs were then removed from 37'C and subjected to program Lung_02. Cells were then passed through a 70 um cell strainer and the filtered cells were further enriched by positive selection using magnetic bead separation with anti-CD11b microbeads and an AutoMACs automated cell

separation instrument (Miltenyi Biotec, Auburn, CA). $CD11b^+$ cells were then stained with ApC-labeled anti-Gr1 and PE-labeled anti F4/80 antibodies (BD Biosciences). Cells expressing F4/80 and an intermediate level of Gr1 ($Gr1^{int}F4/80^+$) were isolated by fluorescence-activated cell sorting using a BD Biosciences FACSAria flow cytometer running FACSDiva software.

Conventional Dendritic Cells (cDCs) were isolated from mouse lungs. Single cell lung suspensions were prepared as above except that magnetic bead selection was performed using anti-CD11c microbeads. Then, $CD11c^+$ cells, with low autofluorescence detected by the E PMT of the FACSAria cytometer, were sorted from highly autofluorescent cells (largely $CD11c^+$ alveolar macrophages) and were used as cDCs.

Cytokine production from $CD11b^+Gr1^{int}$ cells was assessed by culturing the cells overnight at 37'C and then examining culture supernatants by multiplex cytokine assay (Bio-Rad).

2.3.4 Generation of DCs and $CD11b^+Gr1^+$ cells from bone marrow cells *in vitro*

Femurs of mice were removed aseptically and cleaned of surrounding muscle tissue. Bone marrow cells flushed from the femurs using PBS were grown in RPMI medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini), 100 U ml^{-1} of penicillin and 100 ug ml^{-1} of streptomycin sulfate (Gibco), 1 mM sodium-pyruvate (Gibco), and 50 uM 2-mercaptoethanol (Sigma, Carlsbad, CA). The cultures were supplemented with 10 ng ml^{-1} of GM-CSF in the absence or presence of LPS (*Escherichia coli*, strain O26:B6) and were incubated for 8-9 days at 37'C. At the end of the culture period, non-adherent cells were collected and purified by magnetic bead positive selection (Miltenyi Biotec) for CD11c-expressing cells in the case of cultures with GM-CSF alone, or for Thy1.2-expressing cells with

LPS plus GM-CSF-treated cultures. Similar cultures were also started from lineage^{neg} cells enriched using a lineage depletion kit (Miltenyi Biotec). Cell purity, assessed by flow cytometry, was >95% in both the cases. CD11c^{+} cells were considered to be conventional myeloid bone marrow DCs (BMDCs).

2.3.5 Phenotypic evaluation of CD11b^{+}Gr1^{int} cells and cDCs

The surface phenotype of cells was assessed by flow cytometry using standard methods. In brief, cells were first incubated with unlabeled CD16/CD32 monoclonal antibody (Fc block) for 5 minutes on ice, then with specifically conjugated monoclonal antibodies for an additional 15 minutes. In all cases, control staining with appropriate, matched isotype control antibodies was similarly performed. All incubations were conducted at 4°C in PBS supplemented with 2% fetal bovine serum. Analysis was carried out on a BD Biosciences FACSCalibur flow cytometer using CellQuest Pro software. At least 10,000 events within the live cell gate were acquired for each sample. Analysis was performed using FlowJo.

2.3.6 Colorimetric assay for Nitric Oxide (NO)

NO production by DCs and CD11b^{+}Gr1^{int} cells was quantified by measuring nitrite in culture supernatants using a commercially available colorimetric assay for nitrite determination (Griess Reagent Kit for Nitrite Determination, Molecular Probes - Invitrogen).

2.3.7 Neutralization of GM-CSF *in vivo*

Neutralizing GM-CSF antibody (R&D Systems) was administered i.t. on days 0, 2, and 4 of lung-MDSC generation by 1 μg LPS i.t. days 1-4. Lung MDSC-like cells were isolated from isotype or anti-GM-CSF treated mice and cultured ex vivo for 24 hours. IL-6 was measured by ELISA.

2.3.8 Statistical analyses

Comparisons of means± s.d. were performed using one-way analysis of variance or Student's t-test using GraphPad Prism software (La Jolla, CA). Differences between the groups were considered significant if $p < 0.05$.

2.4 RESULTS

2.4.1 Lung MDSC-like cells expand in response to LPS in contrast to lung DC.

We demonstrated the expansion of a suppressive $CD11b^{+}Gr1^{int}F4/80^{+}$ cell in the lung in response to intratracheal LPS administration. Although the presence of an MDSC-like cell could be detected 24 hours after LPS administration, the kinetics of this accumulation in combination with potency of cytokine production was not addressed. Additionally, we observed a suppressive dominance of lung MDSCs over DCs *ex vivo* in co-culture experiments with T cells, but had not

quantified the numbers of both cell types side-by-side post LPS administration to determine if the ratios used ex vivo represented the physiological situation in the lung. In order to accurately quantify lung MDSCs in vivo, we developed a 3-color staining scheme, depicted in **Figure 3.** Ongoing studies in our lab developed a method by which to quantify lung cDCs based on low autofluorescence, and dual staining with CD11c and major histocompatibility complex Class II (MHC-II) (**Figure 4a-b). Figure 5** reveals the actual frequency of these two cell types in the lung post-LPS administration. Of note, alveolar macrophages (AMs) can be identified by high autofluorescence and high CD11c expression. In **Figure 4b** a gate was drawn around the AM population to demonstrate how the percentage of AMs fluctuates in the lung following LPS treatment. After 1 instillation of LPS, there was an observed increase, which decreases at 72 h post-LPS.

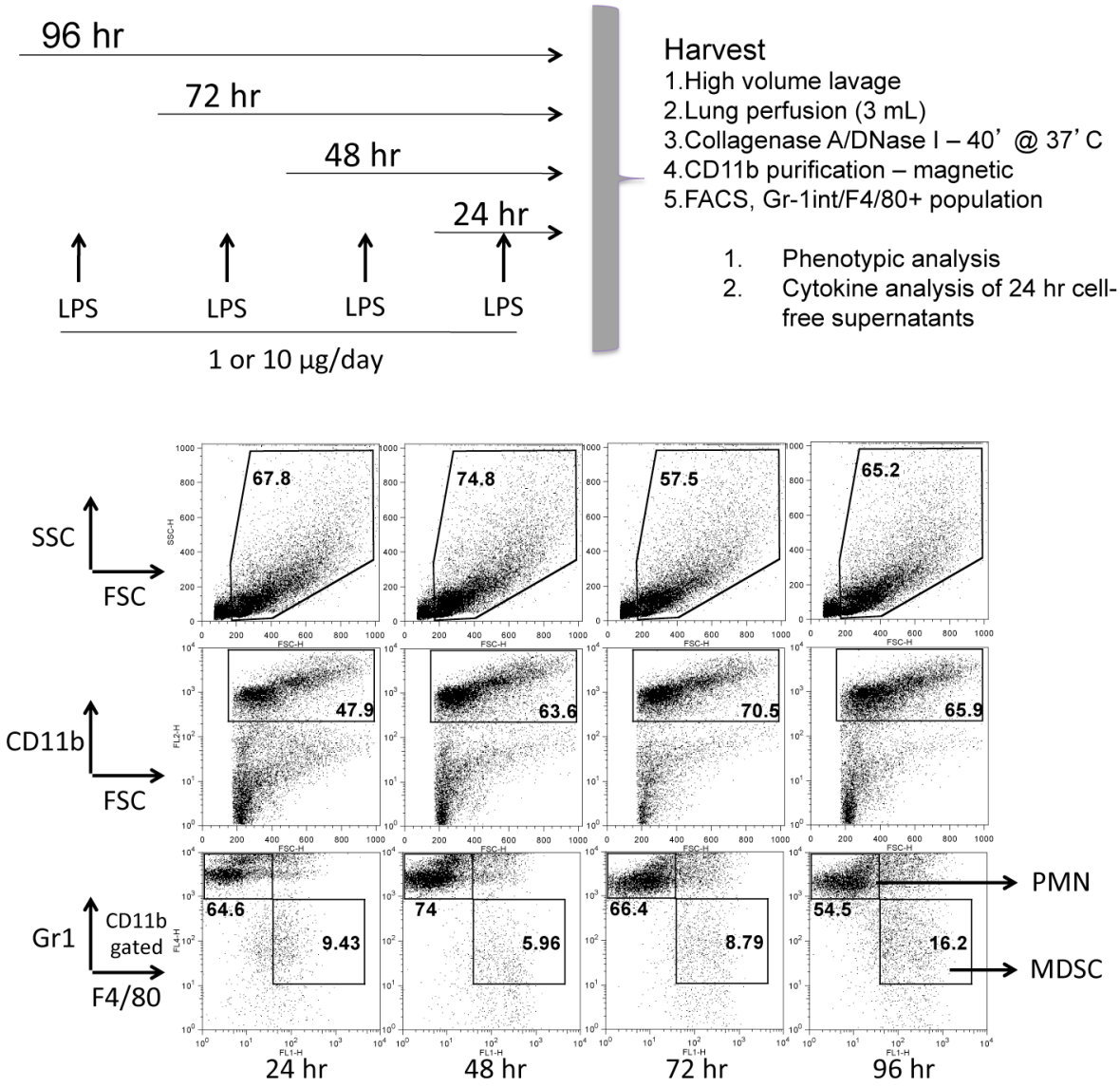

Figure 3. MDSC-like cells ($Gr1^{int}$) expand in response to LPS.

Mice were treated for 4 consecutive days with 1 µg LPS (schematic show). Lung MDSC-like cells were identified by expression of CD11b, intermediate expression of Gr1, and expression of F4/80. Data shown are representative of 3 independent experiments.

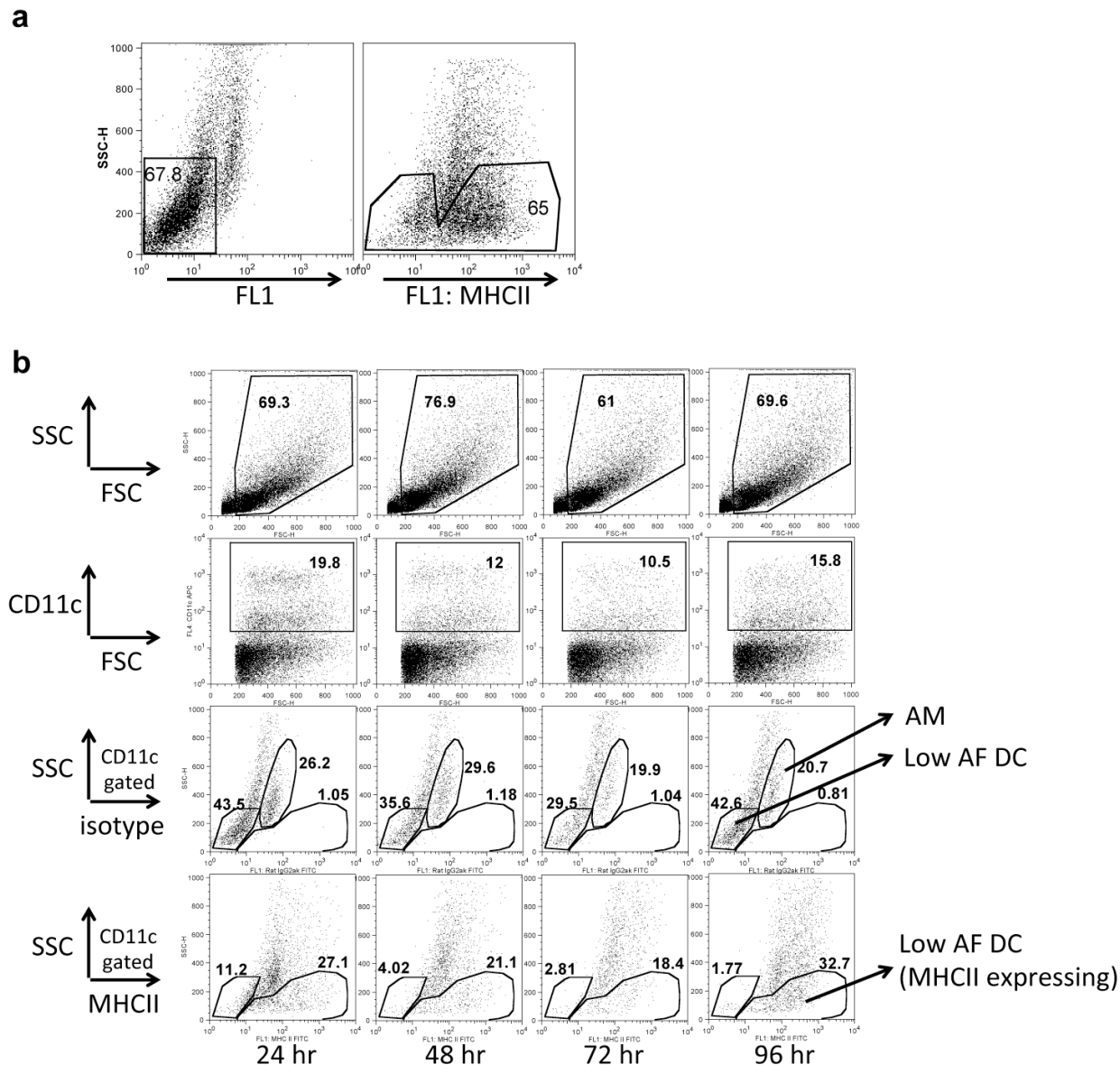

Figure 4. Lung dendritic cells are identified by low autofluorescence and MHCII expression, and their numbers decrease in the lung after LPS administration.

Mice were treated for 4 consecutive days with 1 μg LPS. (a) Lung DCs are identified by low autofluorescence and MHCII expression. (b) Lung DCs can be quantified by flow cytometric staining and are identified by low autofluorescence, CD11c expression and MHCII expression. Data shown are representative of 3 independent experiments.

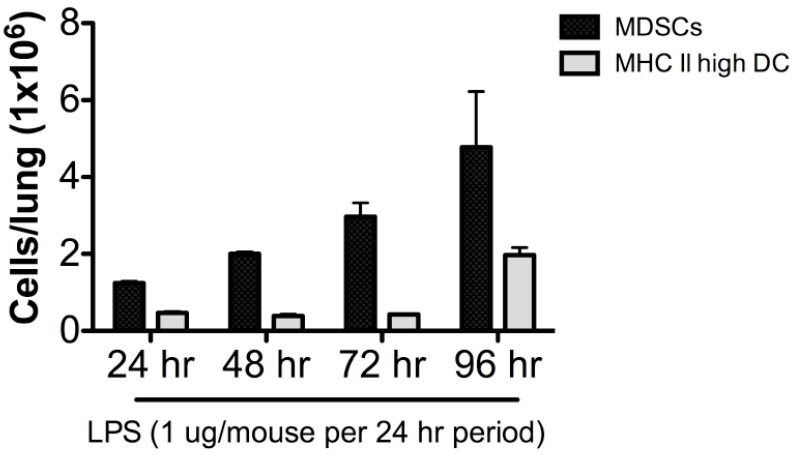

Figure 5. Ratio of MDSC:DC increases over time in the lung post LPS-treatment.

Mice were treated for 4 consecutive days with 1 μg LPS. Lung MDSC-like cells were identified using flow cytometry and expression of CD11b, intermediate expression of Gr1, and expression of F4/80. Quantification was determined by back calculating to the number of total lung cells. Lung DCs were identified by low autofluorescence, CD11c expression and MHCII expression and back calculated to the number of total lung cells. Data shown are representative of 3 independent experiments.

2.4.2 Lung MDSC produce IL-10 and NO in a dose dependent manner.

Because it is known that lung MDSC-like cells utilize both IL-10 and NO to suppress T-cell responses[5], we investigated the production of these mediators from MDSCs isolated at various time points following LPS administration. A high concentration of nitric oxide was noted in bone marrow cultures treated with GM-CSF + LPS, together which induced bone marrow MDSC development, in contrast to cultures with GM-CSF alone, which is known to promote DC development[191]. Nitric oxide is known to be an inhibitor of cell proliferation[192], which likely explains the lag in culture growth rate observed in the bone marrow cultures containing GM-CSF + LPS (not shown). Although there was an initial lag in proliferation, the GM-CSF and LPS promoted a robust expansion of bone marrow MDSCs by day 8-9 of bone marrow culture (see **Figure 8**). IL-10 and NO could be detected from bone marrow MDSCs, in higher amounts than

from BMDCs (not shown). When soluble factor production by lung MDSCs was examined *ex vivo*, we observed that both IL-10 and NO production increased dose-dependently in response to LPS (**Figure 6**). Lung-MDSCs isolated following 4 administrations of LPS produced more IL-10 and NO on a per cell basis, than lung MDSCs isolated after just one LPS administration. NO plays a role in endotoxin tolerance[193], suggesting a role for lung MDSCs in the protection of lung integrity during high exposure to endotoxin. The lungs of mice exhibited greater evidence of hemorrhagic damage after 1 day of LPS administration compared to 4 days, suggesting the development of some sort of tolerance mechanism. It is interesting to consider that although the lungs of mice show less injury following 4 days of LPS treatment, higher amounts of NO were observed when 10 μg of LPS was used repeatedly in comparison to 1 μg LPS. 10 μg administration also correlated with increased weight loss (20% of body weight) suggesting that a large induction of NO may lead to the development of peroxynitrites[194], known to be cytotoxic via induction of mitochondrial injury. This is an area of future interest.

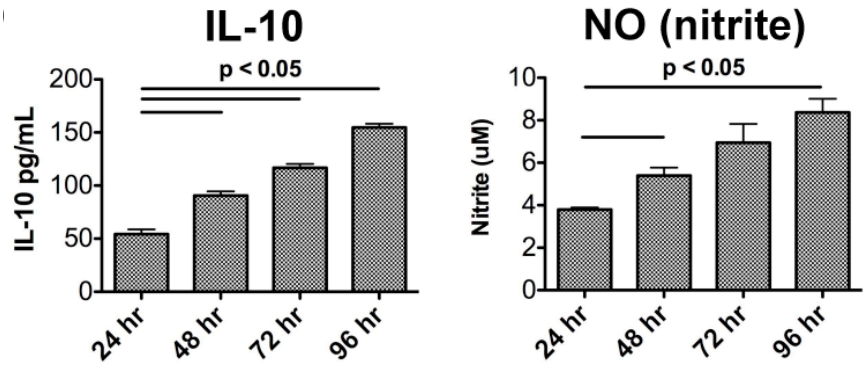

Figure 6. MDSC-like cells ($Gr1^{int}$) expand in response to LPS and produce IL-10 and Nitric Oxide.

Mice were treated for 4 consecutive days with 1 μg LPS. $Gr1^{int}$ were purified and cultured overnight at 37°C in RPMI+10% serum. IL-10 and nitrite were measured in cell free supernatants by ELISA and Greiss assay, respectively. These results are representative of 3 independent experiments.

2.4.3 High dose LPS is required to suppress HDM-driven eosinophilia.

The robust expression of IL-10 in lung MDSCs isolated after 4 daily administrations of LPS is suggestive of a requirement for a high dose of LPS in the production of IL-10. We previously showed that IL-10 was critical for the suppression of Th2 cytokines by MDSC[5]. Others have shown that low levels of LPS exposure in combination with ovalbumin (OVA) antigen are correlated with the induction of Th2 driven eosinophilic inflammation, whereas a high dose of LPS is associated with a reduced Th2 response[12]. Our data suggested that a threshold level of LPS exposure required for protection from Th2 was associated with MDSC development which was confirmed *in vivo* by adoptive transfer of lung or bone marrow MDSC into an OVA/CT model of allergic inflammation resulting in suppressed Th2-driven inflammation and pathology[5]. Using an HDM-driven model of allergic inflammation thought to more closely resemble naturally-occurring asthma, the threshold level of LPS required for induction of MDSCs and suppression of allergen-induced Th2 inflammation in the lung was investigated(**Figure 7**). A high dose of LPS (10 ug) was required for the suppression of HDM-induced eosinophilic infiltration, which was quantified by examining cellular infiltrates in the bronchoalveolar lavage fluid.

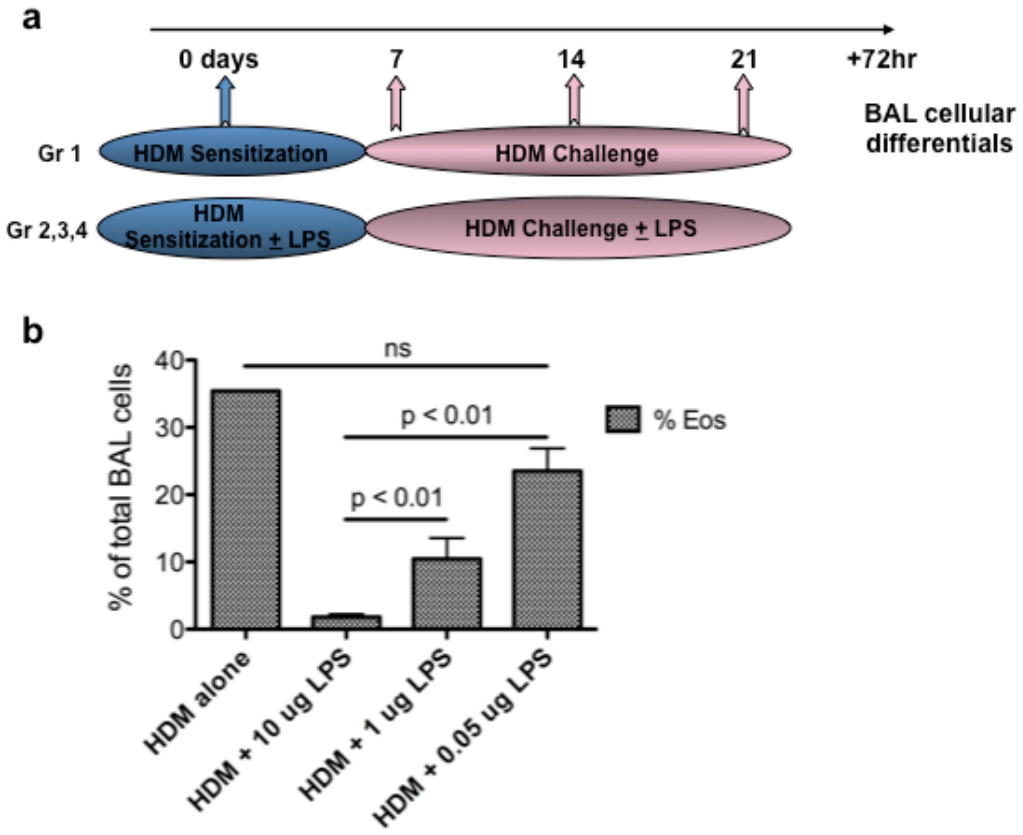

Figure 7. Suppression of house dust mite (HDM)-driven eosinophilic inflammation in the airways by LPS.

(a) Diagram of the treatment protocol. HDM (100 µg per mouse) ±LPS (0.05-10 µg per mouse) was administered intratracheally (i.t.) at the indicated time points. After the final instillation, bronchoalveolar lavage (BAL) was performed with 1 mL PBS. (b) Cellular differentials were determined following cytospin. Values are mean ± s.d. These results are representative of 2 independent experiments (n = 4 mice per group).

2.4.4 Lung MDSC-like cells can be generated from lineage negative progenitors *in vitro.*

Since an MDSC-like cell phenotype could be generated from bulk bone marrow culture by the addition of GM-CSF + LPS, in contrast to bone marrow DC generation (GM-CSF alone) (**Figure 8 and Figure 9**), the origin of these lung MDSC-like cells was investigated. We sought to determine whether lineage negative progenitors could differentiate into MDSC-like cells, a

phenomenon that could have application for treatment of human conditions characterized by an aberrant Th2-biased immune response.

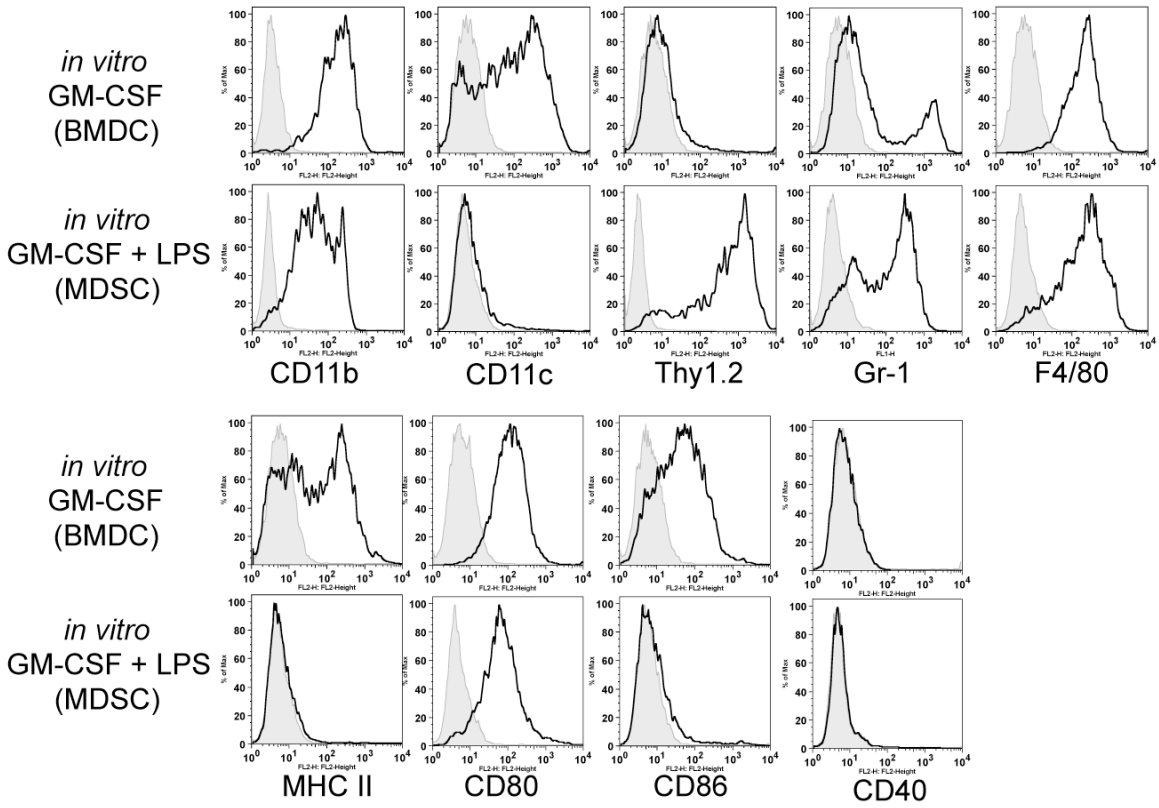

Figure 8. MDSC-like cells can be generated in vitro under conditions of GM-CSF and LPS.

Bone marrow cells were flushed aseptically from the femurs of WT mice. The cells were cultured at a concentration of $1x10^6$ cells/ml and were supplemented with 10 ng ml^{-1} of GM-CSF in the absence or presence of LPS (*Escherichia coli*, strain O26:B6). On day 7, GM-CSF alone cultures were harvested and examined for phenotypic markers (BMDC). On day 9, GM-CSF+LPS cultures (MDSC) were harvested and cells were examined for phenotypic markers. Results are representative of at least 4 independent experiments.

Lineage negative progenitors were purified from the bone marrow of naïve mice and cultured under conditions of GM-CSF (10 ng/ml) or GM-CSF + LPS (1 μg/ml). This experiment revealed that purified lineage negative progenitors could differentiate into MDSC-like cells essentially identical to those that developed from cultures of total bone marrow cells under

similar conditions (**Figure 10a-b**). Furthermore, adoptive transfer GFP+ lineage negative cells intravenously, followed by i.t. LPS administration resulted in GFP+ cells in the lung with a $CD11b^{+}Gr1^{int}$ phenotype, indicating that the *in vivo* precursor for MDSC may be lineage negative bone marrow progenitors that traffic to the lung via the peripheral circulation[5]. Collectively, these data suggest that generation of MDSCs is one critical aspect of how GM-CSF and LPS contribute to immune regulation of the fine balance between inflammation and tolerance in the lung.

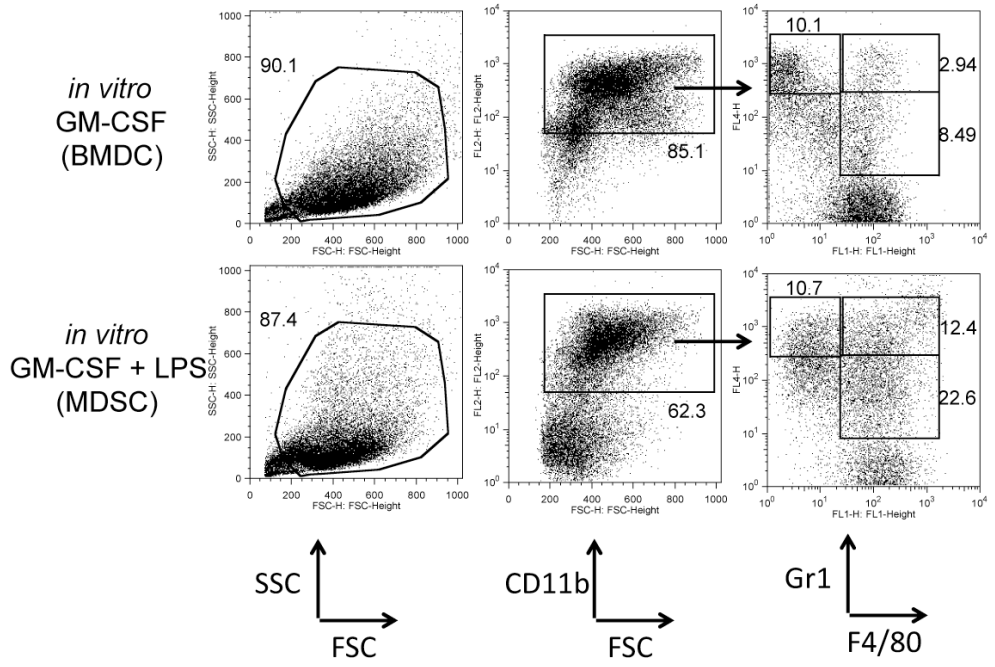

Figure 9. A triple positive ($CD11b^{+}Gr1^{int}F4/80^{+}$) cell population is found with increased frequency in GM-CSF + LPS culture conditions.

Bone marrow cells were flushed aseptically from the femurs of WT mice. The cells were cultured at a concentration of $1x10^{6}$ cells/ml and were supplemented with 10 ng ml^{-1} of GM-CSF in the absence or presence of LPS (*Escherichia coli*, strain O26:B6). On day 7, GM-CSF alone cultures were harvested and examined for phenotypic markers (BMDC). On day 9, GM-CSF+LPS cultures (MDSC) were harvested and cells were examined for phenotypic markers. Results are representative of at least 2 independent experiments.

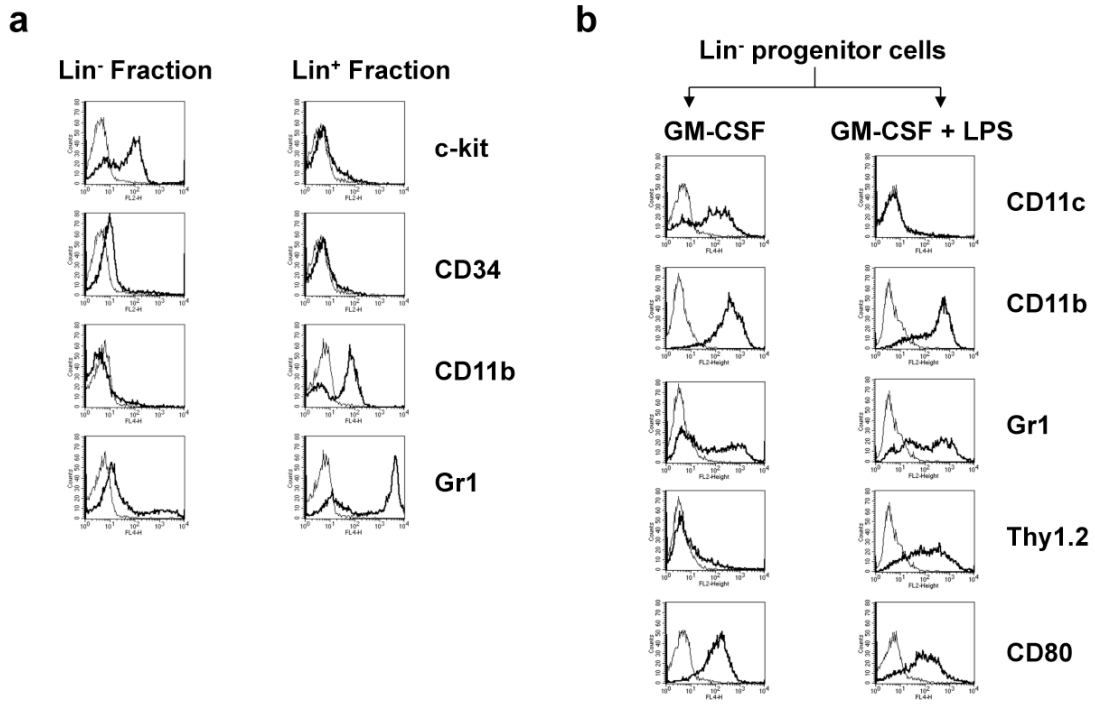

Figure 10. MDSC like cells can be generated from lineage negative progenitors *in vitro*.

LPS treatment generates $CD11b^{+}Gr1^{int}$ cells from lineage negative progenitor cells. (**a**) The lineage negative population of bone marrow progenitor cells was enriched using a lineage depletion kit and phenotypic analysis was performed by flow cytometry. (**b**) The lineage negative population of bone marrow progenitor cells was cultured with GM-CSF (10 ng/ml) in the absence or presence of LPS (1 μg/ml) for 9 days and phenotypic analysis was performed. These results are representative of at least 3 independent experiments. Cells were pooled from individual mice prior to flow cytometric analysis.

2.4.5 Neutralization of GM-CSF alters lung-MDSC cytokine profile.

GM-CSF is critical for homeostasis in the lung microenvironment as its absence results in alveolar proteinosis[48]. It is also critical for alveolar macrophage development and function[72], and promotes differentiation of cDCs[191]. A basal level of GM-CSF is present at all times in the lung, a major source being lung epithelial cells[65,71]. GM-CSF being a critical growth factor, as well as an adjuvant[48,195], was necessary for the development of a healthy and functional MDSC

like cell *in vitro*, which could be discerned from bone marrow progenitors based upon expression of Thy1.2, among other markers (**Figures 8 & 10**). When LPS alone was added to cultures of bone marrow cells in the absence of GM-CSF, MDSCs did not develop based upon Thy1.2 expression (**Figure 11**), and in fact, there were very few living cells left at the end of 6 days (data not shown). These studies allowed us to observe that in the absence of GM-CSF, we did not achieve a cell type phenotypically similar to a MDSC.

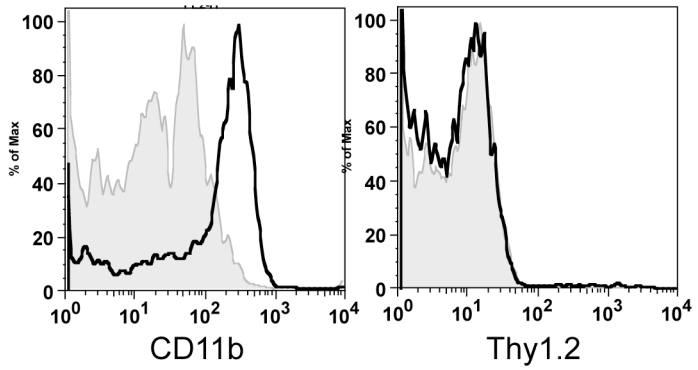

Figure 11. LPS alone does not generate a Thy1.2^{+} bone marrow MDSC-like cell.

Bone marrow cells were flushed aseptically from the femurs of WT mice. The cells were cultured at a concentration of 1x10^{6} cells/ml and were supplemented with 1 μg/ml LPS (*Escherichia coli*, strain O26:B6). On day 7, the cultures were harvested and cells were examined for phenotypic markers. Results are representative of at least 2 independent experiments. Cells were pooled from mice prior to culture and flow cytometric analysis.

Given the lack of bone marrow MDSC development in the absence of GM-CSF, we sought to determine whether neutralization of GM-CSF *in vivo* would result in a defect in lung MDSC development and/or function. Based upon the work of Anderson and colleagues utilizing a model of GM-CSF neutralization during LPS administration[73], we administered neutralizing GM-CSF antibody intratracheally 2 hours prior to LPS administration. The basic model is shown in **Figure 12a** and antibody administration was found to be effective in neutralization of GM-CSF in the lung tissue (**Figure 12b**). Strikingly, lung MDSCs produced reduced IL-6 and

increased NO (as quantified by measurement of nitrite) *ex vivo* when purified from animals treated with neutralizing GM-CSF antibody (**Figure 12c**). Neutralization of GM-CSF also reduced dual expression of Gr1 and F4/80 in the lung tissue (**Figure 13**) further indicating that GM-CSF is an important cytokine for lung-MDSC generation.

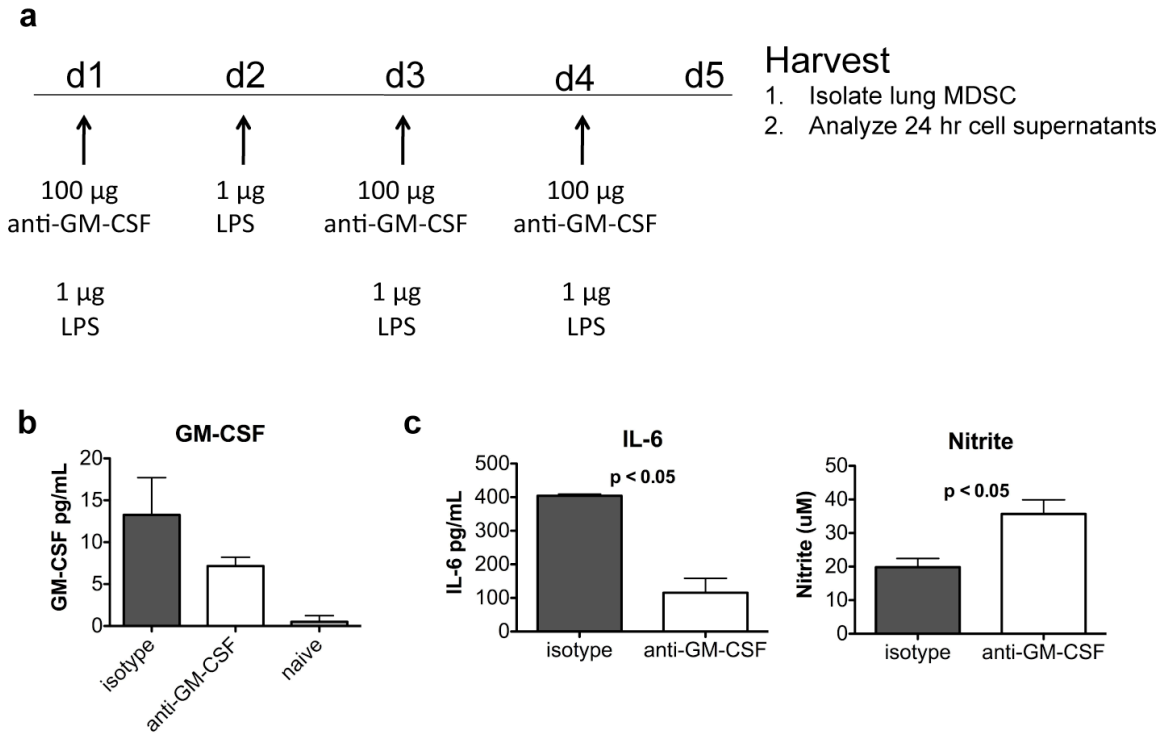

Figure 12. Neutralization of GM-CSF during LPS administration alters cytokine profile of lung MDSC-like cells.

A neutralizing GM-CSF antibody (R&D Systems) was administered on day 1, 3 and 4 of LPS administration (1 µg/mouse/day). (**a**) Schematic of protocol. On day 5, mice were harvested. (**b**) GM-CSF was measured by ELISA in total lung homogenate. (**c**) $Gr1^{int}$ were purified and put in culture overnight at 37°C. IL-6 and nitrite were measured in cell free supernatants by ELISA and Greiss assay, respectively. Results are representative of 3 independent experiments. Cells were pooled from mice prior to cell culture.

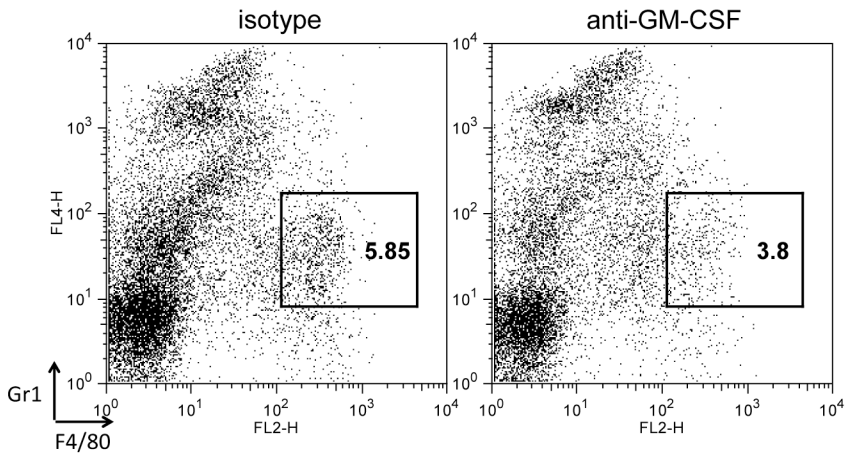

Figure 13. Reduced F4/80^{+}Gr1^{int} expression in the presence of neutralizing GM-CSF antibody.

A neutralizing GM-CSF antibody (R&D Systems) was administered on day 1, 3 and 4 of LPS administration (1 μg/mouse/day). On day 5, following lung tissue digestion, total lung cells were examined by flow cytometry. Results are representative of 2 independent experiments (n = 3-4 per group).

2.4.6 Generation of MDSC is reduced in the absence of STAT5A signaling *in vitro* and *in vivo.*

With the understanding that GM-CSF and LPS are both critical for MDSC development *in vitro*, we investigated the role of STAT5, an intracellular mediator downstream of both LPS and GM-CSF. GM-CSF is known to signal via STAT5, and LPS triggers STAT5 activation via the induction of GM-CSF[196]. Myeloid-lineage cells deficient in STAT5A show reduced proliferation in response to GM-CSF[197]. Therefore we hypothesized that MDSC development involves cooperative effects of the GM-CSF/STAT5 and TLR/MyD88/NF-κB pathways.

To determine the importance of STAT5 for MDSC development, we utilized STAT5A$^{-/-}$ mice to investigate the development of both bone marrow-derived MDSCs *in vitro* and lung MDSCs *in vivo*. We observed reductions in both BMDC and bone marrow MDSC generation in the STAT5A$^{-/-}$ cells (**Figure 14a**). Gr1 expression was also increased in STAT5A$^{-/-}$ BMDCs

revealing a role for GM-CSF-STAT5 signaling in the balance between granulocyte and macrophage differentiation in agreement with previously published reports[67,198]. Furthermore, CD11b-positive cells purified from STAT5A$^{-/-}$ bone marrow MDSC cultures produced less IL-6 in the presence of either GM-CSF alone or GM-CSF + LPS over the course of 24 hours (**Figure 14b**), highlighting the importance of STAT5A signaling in IL-6 production.

Lung MDSC accumulation was reduced to ~50% of WT in the absence of STAT5A (**Figure 15a**), which was also accompanied by a reduction in IL-6 production. Interestingly, a statistically significant increase in NO production from STAT5A$^{-/-}$ bone marrow or lung MDSCs was not observed(not shown), indicating that the regulation of NO by GM-CSF (**Figure 12c**) is STAT5A independent. Utilization of STAT5A$^{-/-}$ mice thus uncovered a mechanism by which accumulation of LPS-generated lung MDSCs is regulated *in vivo*.

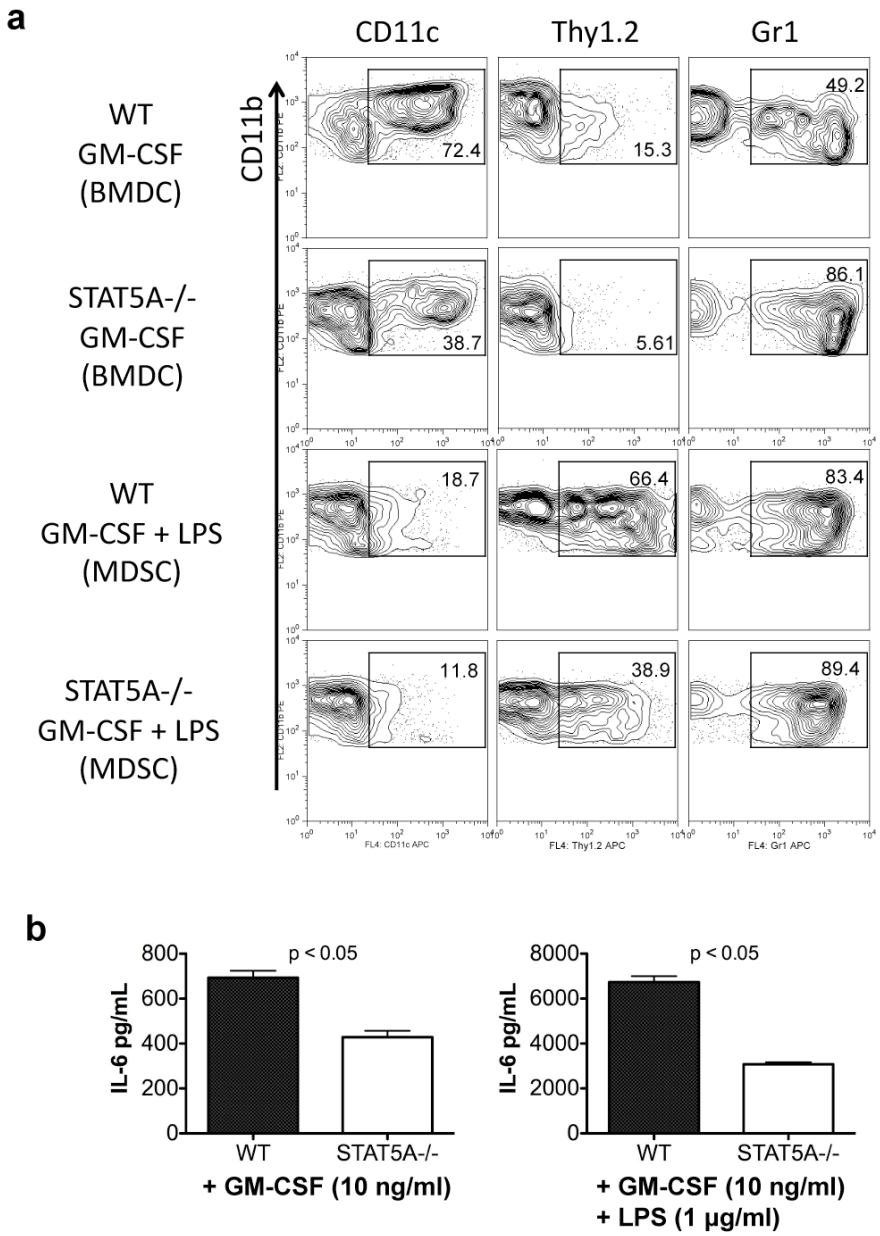

Figure 14. STAT5A deficiency alters BMDC and MDSC generation in vitro with reduced IL-6 production.

Bone marrow cells were flushed aseptically from the femurs of WT and STAT5A$^{-/-}$ mice. The cells were cultured at a concentration of 1x10^{6} cells/ml and were supplemented with 10 ng ml^{-1} of GM-CSF in the absence or presence of LPS (*Escherichia coli*, strain O26:B6). (**a**) On day 7, GM-CSF alone cultures were harvested and examined for phenotypic markers (BMDC). On day 9, GM-CSF+LPS cultures (MDSC) were harvested and cells were examined for phenotypic markers. (**b**) CD11b purified cells from 9 day GM-CSF + LPS cultures were washed twice and

seeded at a concentration of $1x10^6$ cells/mL overnight in the presence of GM-CSF or GM-CSF + LPS. Cell free supernatants were analyzed for IL-6 by ELISA. Results are representative of at least 2 independent experiments.

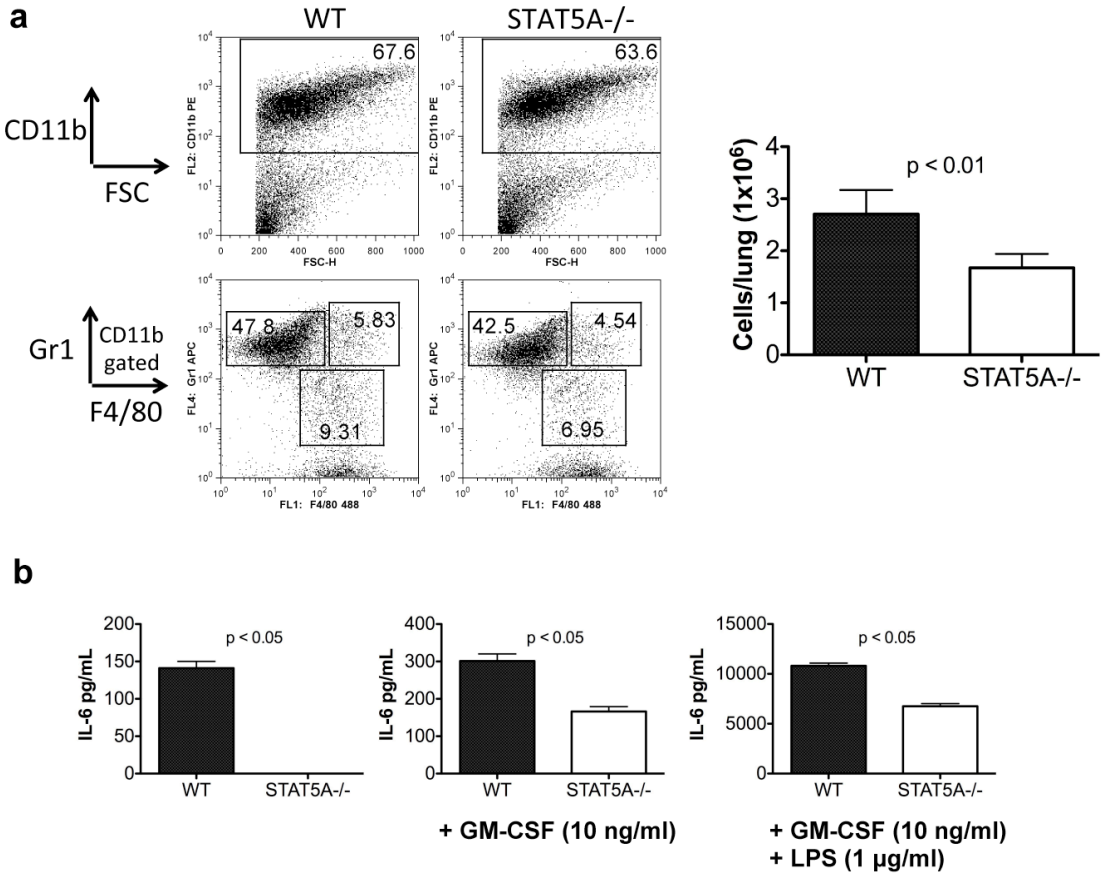

Figure 15. $Gr1^{int}$ accumulation is reduced in $STAT5A^{-/-}$ mice, and $Gr1^{int}$ cells produce less IL-6 as compared to WT MDSC-like cells in the lung.

1 µg LPS was administered to WT and $STAT5A^{-/-}$ mice for 4 consecutive days. (**a**) $Gr1^{int}$ accumulation was less in the $STAT5A^{-/-}$ mice. (**b**) $STAT5A^{-/-}$ $Gr1^{int}$ cells were purified and put in culture overnight at 37°C in the presence or absence of GM-CSF (10 ng/ml) and LPS (1 µg/ml). IL-6 was measured in cell free supernatants by ELISA. Data representative of 2 independent experiments (n = 3 mice per group). Cells from individual mice were pooled for cell culture cytokine analysis.

2.4.7 IL-6 is an MDSC-survival factor *ex vivo.*

As shown in Figures 14 and 15, GM-CSF signaling plays a role in the production of IL-6, and is critical for the development of lung MDSCs (**Figures 11-15**). LPS-generated lung-MDSCs produce IL-6 (**Figure 16a**). Furthermore, IL-6 signals via STAT3, a critical mediator of tumor MDSC generation[1,83]. Therefore, we examined lung MDSC survival *ex vivo* in the presence of neutralizing IL-6 antibody and found that IL-6 is critical for MDSC survival ex vivo (**Figure 16b-c**).

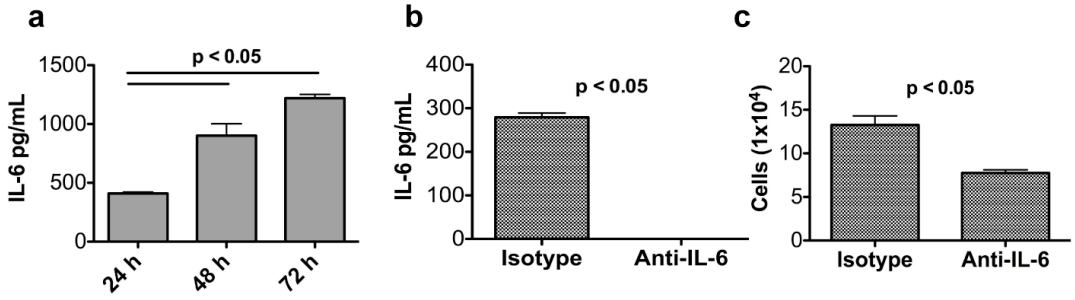

Figure 16. Gr1^{int} cells produce IL-6 and neutralization of IL-6 reduces the number of Gr1^{int} cells cultured *ex vivo*.

(**a**) 1 mg of LPS was intratracheally administered every 24 h. Mice were sacrificed at the indicated time points. Gr1^{int} cells were isolated, cultured overnight and cell free supernatants were analyzed by ELISA for IL-6. (**b**) Antibody mediated neutralization of IL-6 in culture resulted in (**c**) fewer live Gr1^{int} cells after 24 h culture as assessed by trypan blue exclusion. Results representative of 2 independent experiments.

2.4.8 Interleukin-6 is critical for survival following intratracheal LPS administration.

These studies revealed that lung-MDSC development is dependent upon LPS, GM-CSF, and STAT5A signaling, as well as IL-6 production for their survival. We went on to utilize IL-6$^{-/-}$ animals in an attempt to further understand the importance of IL-6 in the development of lung-

MDSCs. Strikingly, IL-6$^{-/-}$ were unable to survive following 2 administrations of LPS (**Figure 17**) highlighting the importance of IL-6 in the regulation of a response to lung endotoxin exposure.

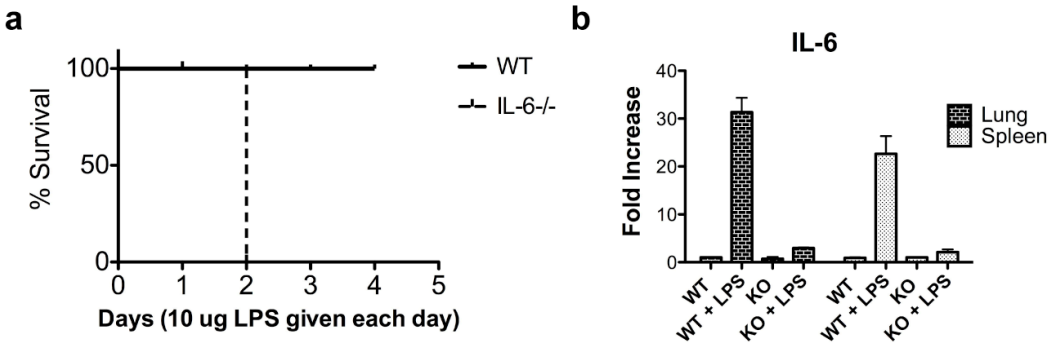

Figure 17. IL-6 is critical for survival following intratracheal LPS administration.

WT and IL-6$^{-/-}$ mice were treated with 10 μg LPS/day. (**a**) After 2 LPS administrations, all the IL-6$^{-/-}$ succumbed, while the WT had 100% survival. (**b**) Lungs and spleens were harvested from naïve WT and IL-6$^{-/-}$ mice. Total lung or spleen cells were purified and cultured (1x10^6 cells/ml) for 24 hours at 37°C ± LPS (1 μg/ml). Cells were pelleted and resuspended in TRIZOL Reagent. mRNA was purified and IL-6 expression was quantified by QRT-PCR. Data combined from 2 independent experiments.

2.4.9 STAT1 is a negative regulator of MDSC-like cell accumulation in the lung.

Given the relationship between STAT3 and STAT1, we explored STAT1 as a negative regulator of lung MDSC development and function. The findings regarding STAT1 as a negative regulator are discussed in greater detail in the context of bacterial pneumonia (Section 3.4.6), but in brief, it was found that numbers of lung MDSCs double in STAT1$^{-/-}$ mice, which correlated with increased IL-6 and IL-10 in lung homogenates. These results illustrate the potential for therapeutic treatment modalities targeting STAT1 in the context of bacteria pneumonia.

2.5 DISCUSSION

These data highlight the therapeutic potential of MDSCs in the control of atopic disease. In light of the novel finding that addition of LPS to bone marrow cultures induces the generation of a suppressive cell type, the majority of our studies focused on LPS as the major player in MDSC generation. However, GM-CSF is recognized as an equally contributing factor, given that MDSCs cannot be generated in vitro in its absence. Additionally, our data indicate that in vivo neutralization of GM-CSF during MDSC development results in a deficiency in MDSC-IL-6 production in comparison to WT MDSC. Reduced IL-6 production also correlated with reduced lung MDSC in the STAT5A$^{-/-}$ as well as reduced survival *ex vivo* in the presence of neutralizing IL-6 antibody.

Unique to the biology of the lung is the continual inhalation of microbial products and exposure to pathogens. Endotoxin exposure in the lung induces rapid neutrophil influx and GM-CSF production, facilitating recruitment of myeloid cell populations and hematopoietic progenitors from the bone marrow. Although there is not a profound defect in myeloid-cell development in GM-CSF-deficient mice, GM-CSF signaling in the lung is crucial for lung homeostasis as evidenced by the development of alveolar proteinosis in its absence[48]. Of great relevance to our studies, GM-CSF has recently been shown to promote bone metastasis of breast cancers[199] and high doses of GM-CSF alone have been shown to convert human monocytes into a regulatory DC phenotype[200]. Additionally, a murine trial using a GM-CSF vaccine strategy demonstrated rapid tumor progression rather than the intended boost in anti-tumor immunity[69]. GM-CSF and TLR4 signaling are integrally related for their function. GM-CSF has been shown to prevent endotoxin-mediated tolerance by promoting interaction between MyD88 and IRAK1[201]. It has also been shown to be important for macrophage-derived IL-6 production in

response to high concentrations of LPS[72]. In addition, GM-CSF is vital for pulmonary immunity to LPS, since the amplification of the LPS-induced response requires Akt/Erk activation of NFkB and AP-1 by GM-CSF[73]. Taken together, we hypothesize that GM-CSF plays an integral role in the development of LPS-induced lung MDSCs.

We have demonstrated that lung MDSC-like cells can be expanded *in vitro* from lineage negative progenitor cells in a manner dependent on both LPS and GM-CSF. Furthermore, LPS administration following adoptive transfer of lineage negative progenitors demonstrates the potential for a lineage negative progenitor to be the precursor of *in vivo* MDSC-like cells in the lung, complementing studies done by von Andrian and colleagues[64].

Functioning downstream of GM-CSF, STAT5A is an important signaling molecule for IL-6 production and both *in vitro* and *in vivo* expansion of MDSC-like cells. Although STAT5 is a key transcription factor involved in GM-CSF function, $STAT5A^{-/-}$ mice do not show defects in myeloid development. On the contrary, the transcription factor PU.1 is known to regulate lineage differentiation during the earliest stages of macrophage and granulocyte differentiation. It belongs to the Ets family of transcription factors, is activated by both TLR and GM-CSF signaling, and is essential for myeloid differentiation[45,202]. As a determinant of cellular differentiation, PU.1 may play a fundamental role in MDSC development from a lineage negative progenitor cell in vitro, as well as in vivo. More relevant, PU.1 over-expression can bypass the need for endogenous GM-CSF in lung alveolar macrophages [72]. Indeed, the effects that have been observed by blocking GM-CSF in vivo may be mediated through PU.1 in addition to STAT5A. We chose to focus on STAT5 due to its potential role in driving IL-6 production, and investigated the contribution of STAT5A in MDSC development and function due to its specific association with IL-6 production. However, STAT5B is also activated downstream of

GM-CSF and may be equally important for lung-MDSC generation. PU.1 gene disruption results in severe defects in myeloid differentiation and a conditional knockout would be necessary to investigate its role in MDSC development[203,204].

Taken together, these studies reveal the contribution of STAT5 and GM-CSF, in combination with LPS, as critical mediators of bone marrow and lung-MDSC generation. Administration of GM-CSF in the lung may have application for reduced Th2 inflammation, and blockade of STAT5 is a yet unappreciated mechanism by which to control MDSC generation.

3.0 A ROLE FOR IL-10 IN EFFEROCYTOSIS OF APOPTOTIC NEUTROPHILS AND RESOLUTION OF INFLAMMATION PRODUCED BY STAT1-REGULATED LUNG MDSC-LIKE CELLS

3.1 ABSTRACT

Bacterial pneumonia remains a significant health care burden worldwide. Although an inflammatory response in the lung is required to fight the causative agent, persistent tissue-resident neutrophils in non-resolving pneumonia can induce collateral tissue damage and precipitate acute lung injury. However, little is known about mechanisms orchestrated in the lung tissue that remove apoptotic neutrophils to restore tissue homeostasis under normal circumstances. In mice infected with *Klebsiella pneumoniae*, a bacterium commonly associated with hospital-acquired pneumonia, we show that interleukin-10 is essential for resolution of lung inflammation and recovery of mice after infection. Although IL-$10^{-/-}$ mice cleared bacteria, they displayed increased morbidity with progressive weight loss and persistent lung inflammation in the later phase of the immune response to infection. A source of tissue IL-10 was found to be resident $CD11b^{+}Gr1^{int}F4/80^{+}$ cells resembling myeloid-derived suppressor cells that appeared with a delayed kinetics after infection. These cells efficiently efferocytosed apoptotic neutrophils, a process which was aided by IL-10. The lung neutrophil burden was attenuated in infected STAT$1^{-/-}$ mice with concomitant increase in the frequency of the MDSC-like cells and

lung IL-10 levels. Thus, inhibiting STAT1 in combination with antibiotics may be a novel therapeutic strategy to address inefficient resolution of bacterial pneumonia.

3.2 INTRODUCTION

Bacterial infection leading to pneumonia is a leading cause of morbidity and mortality worldwide with more than 3 million cases occurring annually in the United States alone and resulting in 40,000 to 70,000 deaths each year [205]. Additionally, the majority of deaths during the 1918-19 influenza pandemic were actually due to secondary bacterial infections following viral clearance [206]. Characteristically, defense against pathogenic bacteria involves a set of finely tuned orchestrated events whose goal is to rapidly mount an innate immune response to clear the pathogen [105,106]. The cells of the innate immune response that are able to phagocytose and kill the bacteria once internalized are the alveolar macrophages and neutrophils [105,106]. Typically, alveolar macrophages (AMs) participate initially and their numbers dwindle as neutrophils are rapidly recruited to the site of infection [207]. Neutrophils produce a range of products including reactive oxygen species and proteases that are not only harmful for the pathogen but also for the host's own cells [135,205,208-210]. Therefore, once the pathogen is cleared, it is crucial for the host to mount an appropriate anti-inflammatory response to limit further neutrophil recruitment and to clear the dead cells. However, bystander tissue injury caused by unmitigated inflammation after infection can initiate and lead to the progression of acute lung injury (ALI) that can be fatal. Indeed, severe pneumonia is a common underlying cause of acute lung injury and its more formidable form, acute respiratory distress syndrome (ARDS) [205,211]. It is, therefore, important to understand the molecular and cellular mechanisms that mediate innate immune system

dysfunction so that tissue homeostasis after infection can be rapidly restored with therapeutic intervention, if necessary.

Excess tissue IL-10 levels early after infection inhibits bacterial clearance thereby acting as a negative regulator of host defense [105,124,165]. However, IL-10 is also known to be important for maintenance of homeostasis as well as regulation of neutrophil clearance (decrease survival/efferocytosis) [105,124,126,190,212]. These two observations, while seemingly contradictory, point to the need for early absence of IL-10 to facilitate bacterial clearance but with its presence being necessary during the resolution phase of the immune response. A central unanswered question in the context of bacterial pneumonia is which cell type(s), with the potential to produce IL-10 in the lung tissue, like AMs in the alveolar lumen, aid in the removal of apoptotic neutrophils (efferocytosis) effecting resolution of inflammation. This question is important because neutrophils in the alveolar space represent only a fraction of the extravasated neutrophils in the inflamed lung and do not necessarily track with parenchymal neutrophil burden [213,214]. In contrast, interstitial neutrophils have been found to be more intimately associated with clinically relevant measures of ALI, such as increased microvascular permeability and decreased lung compliance [135,208,209]. Thus, there appears to be an indispensible role for additional cellular players in the alveolar interstitium to limit lung injury by inhibiting the continuous tissue influx of neutrophils after infection and to efferocytose apoptotic neutrophils.

We recently described a $CD11b^{+}Gr1^{int}F4/80^{+}$ regulatory cell population in the lung that expands in response to LPS exposure in a TLR4/MyD88-dependent manner [5]. We showed that these cells have the ability to suppress Th2 effector responses via secretion of IL-10. These cells also secrete IL-6 and GM-CSF, but low levels of IL-12. Due to their close resemblance to myeloid-derived suppressor cells (MDSCs), these $Gr1^{int}$ cells are now recognized as lung

MDSCs [1]. It is known that mice lacking functional MyD88 signaling have increased susceptibility to a number of infectious pathogens, including the Gram negative bacterium *Klebsiella pneumoniae* [112]. *K. pneumoniae* is a common bacterial species acquired by nosocomial infections that can cause pneumonia in severely ill patients with a high rate of morbidity and mortality. In fact, *K. pneumoniae* was found to be the third most commonly isolated organism from intensive care units in the US [101]. In our previous study, we observed IL-10 production by tissue-resident MDSC-like cells in response to LPS [5]. In the present study we addressed whether these cells represent a source of IL-10 after infection with *K. pneumoniae* and if so, whether this is beneficial for the host. This question arose because presence of IL-10 early after infection with *K. pneumoniae* was deleterious and increased bacterial load in the lung. However, IL-10 was crucial for resolution of inflammation and eventual recovery of mice late after infection. The MDSC-like cells were found to expand in the lungs with delayed kinetics in response to bacterial infection and therefore produced IL-10 only in the later phase of infection. Functionally, the cells efferocytosed apoptotic neutrophils, which was partially dependent on IL-10. In our efforts to identify mechanisms that would increase the MDSC:neutrophil ratio which presumably would help in the resolution process of neutrophilia, we found that deletion of STAT1 caused a doubling of MDSC-like cells with concomitant reduction of tissue neutrophils. In the absence of STAT1 signaling, IL-6 and IL-10 levels in the lung increased, both of which signal through STAT3, a known mediator of proliferation and survival of MDSC-like cells [1,83].

3.3 MATERIALS AND METHODS

3.3.1 Animals and reagents

Male 6-8-week old C57BL/6 mice, and IL10$^{-/-}$ mice on C57BL/6 background (stock number 002251), were purchased from the Jackson Laboratory (Bar Harbor, ME). STAT1$^{-/-}$ mice previously generated [127] were bred at the animal facility at the University of Pittsburgh. All mice were housed under pathogen-free conditions and were used between 6 and 10 weeks of age. Within experiments, the mice were age and sex matched. All studies with mice were approved by the Animal Care and Use Committee at the University of Pittsburgh. LPS from *Escherichia coli*, strain O26:B6, was obtained from Sigma. A number of fluorochrome-confugated monoclonal antibodies were used in cell phenotyping experiments as follows: PE-labeled anti-mouse CD11b, APC-labeled anti-mouse CD11c (clone HL3), APC-labeled anti-mouse Gr1 (clone RB6-8C5), PE-labeled anti-mouse Ly6G (clone 1A8), Alexa 647-conjugated mouse anti-STAT3 (pY705, clone 4/P-STAT3), neutralizing anti-mouse IL-6, and neutralizing anti-mouse IL-10, all of which were purchased from BD Biosciences. Alexa 488-, PE- and APC-labeled anti-mouse F4/80 were purchased from Caltag Laboratories.

3.3.2 Infection

All infections were done with *K. pneumoniae* ATCC 43816 as described previously [117]. Bacterial growth medium was seeded with a 1:100 dilution of an overnight culture of *K. pneumoniae* and the culture was incubated for 2 h to attain mid logarithmic phase. The OD_{600} of the culture was determined (1 OD_{600} = $5x10^8$ CFU) and the inoculum was diluted accordingly.

For infection, the mice were anesthetized with isoflurane and $1x10^2$ or $1x10^3$ CFU were delivered by intratracheal instillation[215]. The exact CFU was routinely estimated by plating dilutions of the inoculum on tryptic soy agar plates. Lung and systemic burden of *K. pneumoniae* were determined by plating dilutions of homogenized lung tissue or blood in PBS on tryptic soy agar plates. CFU were enumerated following overnight incubation at 37°C.

3.3.3 Generation and isolation of MDSC-like $Gr1^{int}$ cells from the lung

MDSC-like cells were induced in the lung and isolated as previously described in **2.3.3**. The $Gr1^{int}$ were purified in an identical manner 24 or 72 h post infection with *K. pneumoniae*. The staining procedures to identify $CD11b^+Gr1^{int}F4/80^+$ cells were described previously in **2.3.3**.

3.3.4 Histology and quantitation using Metamorph

Tif images of Hematoxylin & Eosin (H&E) stained lung sections (5 μm) were scanned using a Mirax MIDI (Zeiss, Budapest, Hungary) microscope. Digital images were captured using Panoramic Viewer (Version 1.14; 3D Histech) and analyzed using Metamorph. Following color separation, the blue layer was converted to monochrome. The entire lung was selected as a region and the threshold was set to 195 for all images. Region statistics were calculated. % Threshold area was determined by dividing the threshold area by the total area of the lung (region). Threshold Area/Total Area x 100 = % Threshold Area.

3.3.5 Efferocytosis assay

Neutrophils (PMNs) were purified from the lungs of LPS-treated mice by FACS of the $Ly6G^{hi}F4/80^{-}$ population following CD11b purification (Miltenyi Biotec Automacs technology). PMNs were cultured overnight at 37°C under serum free condition to induce apoptosis [125]. Apoptotic PMNs were detected by Annexin V/propidium iodide (PI) staining. The PMNs were labeled with DDAO-SE (Molecular Probes). $Gr1^{int}$ cells were purified by FACS from $CD11b^{+}$ lung cells labeled with Alexa 488-conjugated anti-mouse F4/80 and PE-conjugated anti-mouse Gr1. Apoptotic PMNs and Alexa 488 labeled $Gr1^{int}$ cells were incubated together (10:1 ratio) for 30 minutes at 37°C. % uptake was quantified by flow cytometry. Confocal images were captured on an Olympus Fluoview FV1000 confocal microscope using a 60x Objective (NA 1.35). Following the efferocytic assay, $Gr1^{int}$ MDSC-like cells labeled with Alexa 488-conjugated anti-mouse F4/80 and DDAO-SE (Molecular Probes)-labeled PMNs were detected using the excitation wavelengths 488 nm and 635 nm, respectively. Z-stack projections were created from 0.75-μm optical increments spanning the thickness of the cell.

3.3.6 Intracellular cytokine staining

Freshly isolated PMNs, $Gr1^{int}$ and $F4/80^{+}$ cells were incubated with LPS (1μg/ml) and Brefeldin A (10 μg/ml) (Sigma) for 4 h. Cells were harvested, washed twice with PBS-2% fetal bovine serum (FBS) and then fixed in 4% paraformaldehyde solution for 15 min at room temperature. After washing the cells twice, cells were incubated in permeabilization buffer for 30 min at room temperature. Cells were then centrifuged, washed in permeabilization buffer and resuspended in APC-labeled anti-mouse IL-10 (BD Biosciences) or APC-labeled Rat IgG2b control antibody for

30 min at room temperature. Cells were finally sequentially washed in permeabilization buffer and PBS-2% FBS and analyzed by flow cytometry.

3.3.7 pSTAT3 analysis

Lung cells were isolated as previously described [5]. Intracellular staining for phsophoSTAT3 (pY705) was performed according to BD Phosflow protocol for intracellular staining. Briefly, cells were left unstimulated or stimulated with recombinant mouse IL-6 at 100 ng/mL for 15 minutes at 37°C. Cells were then fixed using Fix Buffer 1 (Cat#: 557870) for 10 minutes at 37°C, washed, and stained for CD11b and Gr1 to phenotypically identify the $CD11b^{+}Gr1^{int}$ population. Cells were permeabilized with Perm Buffer III (Cat#: 558050) on ice for 30 minutes, washed twice, and stained with pYSTAT3 (Cat#: 557815) or isotype control (Mouse IgG2ak Cat#: 558052). Intracellular staining was performed at room temperature for 30 min.

3.3.8 qRT-PCR

Quantitative RT-PCR was performed as previously described [146] as follows. Tissues or cells were treated with TRIzol (Invitrogen). RNA was isolated with the RNeasy kit (Qiagen) and treated with RNase-free DNase (Qiagen). cDNA was synthesized and used for quantitative PCR with TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The level of mRNA was normalized to *HPRT1* expression, and the results were analyzed by the $2^{-\Delta\Delta Ct}$ method. Fold expression of IL-10 in $STAT1^{-/-}$ $Gr1^{int}$ cells was calculated relative to expression of IL-10 in WT $Gr1^{int}$ cells both harvested 72 h post infection with 1000

CFU *K. pneumoniae.* The level of expression was normalized to that of the housekeeping gene HPRT and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

3.3.9 Cytokine analysis by ELISA

Cytokine production from $CD11b^{+}Gr1^{int}F4/80^{+}$ cells and $CD11c^{+}$ alveolar macrophages was assessed by culturing the cells ($1x10^{6}$ cells/ml) overnight at 37°C in RPMI 1640 (Gibco) medium supplemented with 10% heat-inactivated FBS (Gemini), 100 U/ml of penicillin, 100 mg/ml of streptomycin sulfate (Gibco), 1 mM sodium pyruvate (Gibco) and 50 μM 2-mercaptoethanol (Sigma). All reagents used had <0.6 U/ml of LPS . Secreted cytokines were measured by multiplex cytokine assay (Bio-Rad Laboratories) or ELISA (murine Duo Set; R&D Systems), according to the manufacturers' instructions.

3.3.10 Statistical analyses

Comparisons of means± s.d. were performed using one-way analysis of variance or Student's t-test using GraphPad Prism software (La Jolla, CA). Differences between the groups were considered significant if $p < 0.05$.

3.4 RESULTS

3.4.1 Establishment of an LD_{50} dose of *K. pneumoniae*

IL-10 has been negatively associated with the deployment of rapid defense mechanisms against bacterial infection [105,124,165]. However, its role in the resolution of tissue inflammation induced to clear the pathogen has not been adequately addressed. Our goal was to determine whether wild-type (WT) and IL-$10^{-/-}$ mice differentially respond to acute bacterial infection over time. To address this goal, we wished to use a dose of the bacterium that would allow ~50% of the wild-type (WT) mice to recover from infection and observe whether lack of IL-10 would make a difference in this recovery. Towards this end, we infected WT mice with two doses of the bacterium, 100 and 1000 colony forming units (CFU), to compare the outcome. Mice infected with 100 CFU experienced less weight loss (**Figure 18a**) and systemic dissemination on day 3 after infection (**Figure 18b**) compared with those infected with 1000 CFU. Systemic cytokine levels were also lower in mice infected with the lower dose (**Figure 18c**) and the lungs appeared less inflamed (**Figure 18d**). Histological examination of lung sections of mice infected with 1000 CFU showed significantly more inflammation at 72 h compared to that observed at earlier time points (**Figure 18e**). At 72 h after infection, all of the mice infected with 100 CFU had recovered.

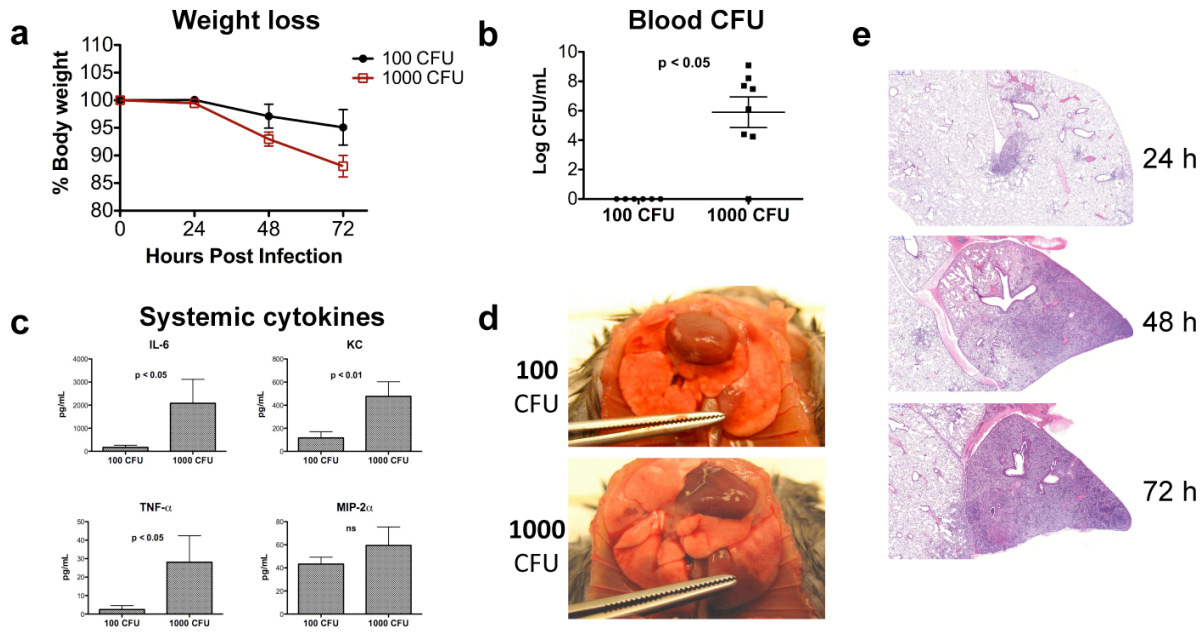

Figure 18. Establishment of an LD_{50} dose of *K. pneumoniae* infection

WT C57BL/6 mice were infected intratracheally with either 100 or 1000 CFU *K. pneumoniae* (ATCC: 43816, serotype 2) and various parameters were recorded. (**a**) % Body weight loss from baseline was recorded at indicated time points. (**b**) Blood colony forming units (CFU) 72 h post infection. (**c**) Serum cytokine and chemokine levels 24 h post infection. Data shown are means ± s.d. (**d**) Representative photographs of lungs showing more injury at the higher bacterial dose. (**e**) Increased cellular infiltration over time is visible via hematoxylin and eosin (H & E) staining of lung sections of mice infected with 1000 CFU of *K. pneumoniae*.

With the higher dose of 1000 CFU, however, at 72 h after infection, ~ 50% of the WT mice displayed continued loss of weight and body temperature, greater systemic dissemination of bacteria, (**Figure 19a-c**) and appeared huddled and withdrawn from food. These moribund mice were placed in one group while the remaining 50% that received the same high bacterial dose, but did not appear sick and were active, were labeled survivors and placed in the other group. We compared various parameters between these two groups of mice all sacrificed on day 3 post-infection. The level of IL-10 was significantly higher in the lungs of the mice with more sever disease, and correspondingly, the percentage of polymorphonuclear neutrophils (PMNs),

required for rapid bacterial clearance, was lower in the lungs of this group (**Figure 19, d-e**). Thus a dose of 1000 CFU was chosen to examine whether presence or absence of IL-10 made any difference to the recovery of mice after bacterial infection. All of the subsequent experiments were performed using the LD_{50} dose of 1000 CFU to address the importance of IL-10 in resolution of lung inflammation and recovery after bacterial infection.

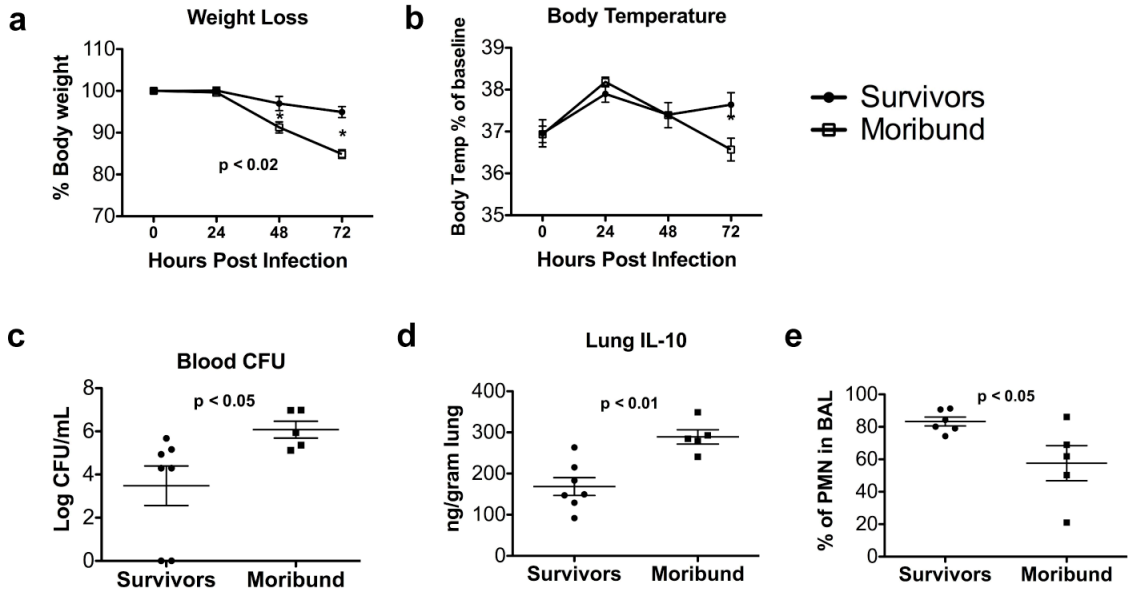

Figure 19. Survivors display better parameters of health compared to moribund mice, both infected with 1000 CFU of bacteria.

At 72 h post infection with 1000 CFU *K. pneumonia*, 50% of the sick/moribund mice showed (a) increased weight loss and (b) decreased body temperature. (c) Increased bacterial dissemination, as measured by estimation of log CFU/mL in blood, was also observed in mice displaying increased morbidity, which correlated with (d) increased lung IL-10 and (e) decreased PMN in BAL. Data shown are representative of 3 independent experiments with n=10-12 mice/group.

3.4.2 Early Versus Late Interleukin-10 during infection By *Klebsiella pneumoniae*

To assess the role of IL-10 in defense against *K. pneumoniae*, WT and IL-$10^{-/-}$ mice were infected with 1000 CFU of the bacterium. We compared the outcome in the two groups with respect to inflammation, bacterial burden in the lung, systemic bacterial load and weight loss. Early after infection at 48 h, the IL-$10^{-/-}$ mice showed limited lung pathology and carried lower bacterial burden in the lung compared to the WT mice (**Figure 20a**). These results were in line with previous observations of delayed mortality and reduced bacterial burden in the lungs of mice in which IL-10 was neutralized prior to infection with a dose of *K. pneumoniae* that was lethal for the strain of mouse used (CD-1)[165]. The difference between the prior study and ours is that we used a LD_{50} dose, where 50% of mice repeatedly succumbed post infection, to study effects of complete IL-10 deficiency on lung health and bacterial dissemination late after infection. The rationale for our experimental design was that while lack of IL-10 initially might help in bacterial clearance, it is unknown how its absence would impact resolution of lung inflammation and recovery after infection.

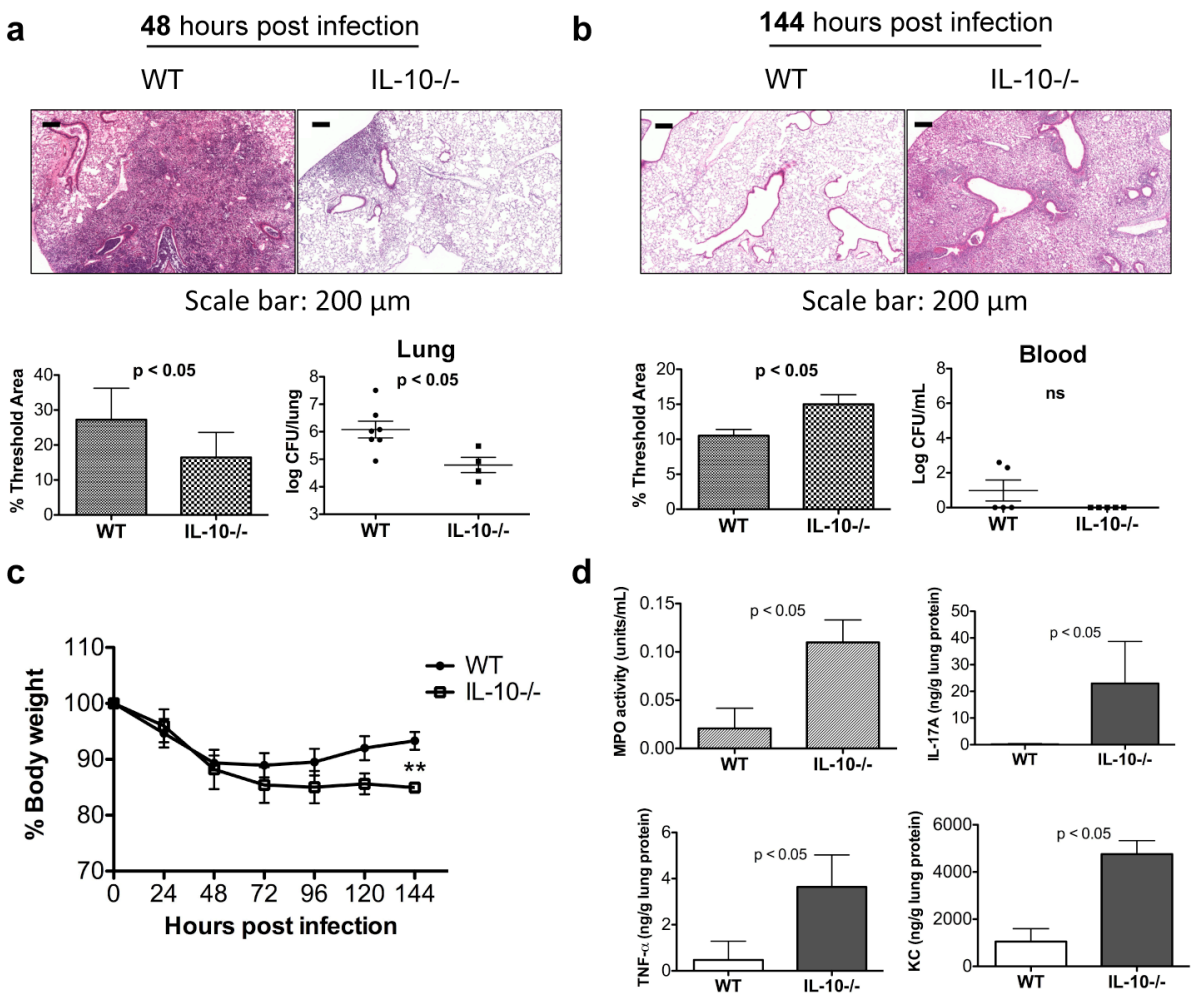

Figure 20. IL-10 deficiency worsens outcome late after infection.

To determine the role of IL-10 early versus late after infection, WT and IL-$10^{-/-}$ were infected with 1000 CFU of *K. pneumoniae*. (**a**) 48 h post infection mice were sacrificed. Cellular infiltration was determined by H&E staining of lung sections and inflammation in whole lung was quantified using Metamorph, as indicated by % Threshold Area. The bacterial burden in the lung was estimated by log CFU/lung. (**b**) To examine the role of IL-10 late after infection, WT and IL-$10^{-/-}$ mice were infected with 1000 CFU. 50% of WT mice and 20% of IL-$10^{-/-}$ mice were sacrificed prior to 144 h on the basis of continued weight loss, drop in body temperature and appearance (data not shown). The mice that did not show signs of morbidity were monitored until day 6 (144 h). (**b**) At 144 h post-infection, WT and IL-$10^{-/-}$ were examined for cellular infiltration by H&E staining and quantified by % Threshold Area. Bacterial dissemination was measured as log CFU/mL blood. (**c**) Surviving WT mice recovered from the initial weight loss unlike IL-$10^{-/-}$ mice. Data shown are means ± s.d. **p< 0.01. (**d**) MPO activity was measured in the BAL fluid 144 h post infection and total lung homogenates were analyzed for IL-17A, TNF-α, and KC levels (n=5-6 mice per group). Data shown are representative of 2 independent experiments.

The 50% of WT mice that appeared close to death on day 3 after infection by virtue of huddled appearance, withdrawal from food and loss of body weight and temperature were euthanized. In contrast, fewer (~20%) of the IL-$10^{-/-}$ mice looked moribund at this time point. By 144 h, while the remaining 50% WT mice looked active and on the road to recovery, all of the IL-10-/- mice met criteria for euthanasia, which included continued loss of body weight, withdrawal from food, loss of body weight and temperature and needed to be sacrificed along with the WT survivors (which were active and appeared to have recovered) to assess various outcome measures. Analysis of lung histology using Metamorph software showed that compared to the lungs of WT mice, those of IL-$10^{-/-}$ mice at 144 h post-infection harbored significantly greater inflammation (**Figure 20b**). However, we did not observe increased bacterial dissemination in the IL-$10^{-/-}$ mice at 144 h (**Figure 20b**). Strikingly, using weight loss as a global measure of health decline, we observed that the IL-$10^{-/-}$ mice continued to lose weight after infection whereas the WT survivors began to regain the weight lost during the initial phase of infection with the two groups beginning to diverge at 72 h after infection (**Figure 20c**). Myeloperoxidase [123] activity (a measure of PMN infiltration) was also increased in the IL-$10^{-/-}$ mice 144 h post infection (**Figure 20d**). Upon further investigation of what criteria distinguished the WT from their IL-$10^{-/-}$ counterparts at 72 h, a time point at which the surviving WT and the IL-$10^{-/-}$ mice began to diverge with respect to weight loss, we found that the MPO activity and the PMN:Mac ratio in the BAL fluid was greater in the IL-$10^{-/-}$ mice as compared to that in the WT mice (**Figure 21, a-b**). However, the majority of the IL-$10^{-/-}$ mice had 2 logs lower CFU in their blood at this time point compared to the WT mice (**Figure 21c**). Thus, in our analysis, the key difference at 72 h that was disadvantageous to the IL-$10^{-/-}$ mice was more

inflammation in the lungs as compared to that in the WT counterparts. No systemic bacteria were detected in the surviving WT mice after 8 days of infection (**Figure 21d).**

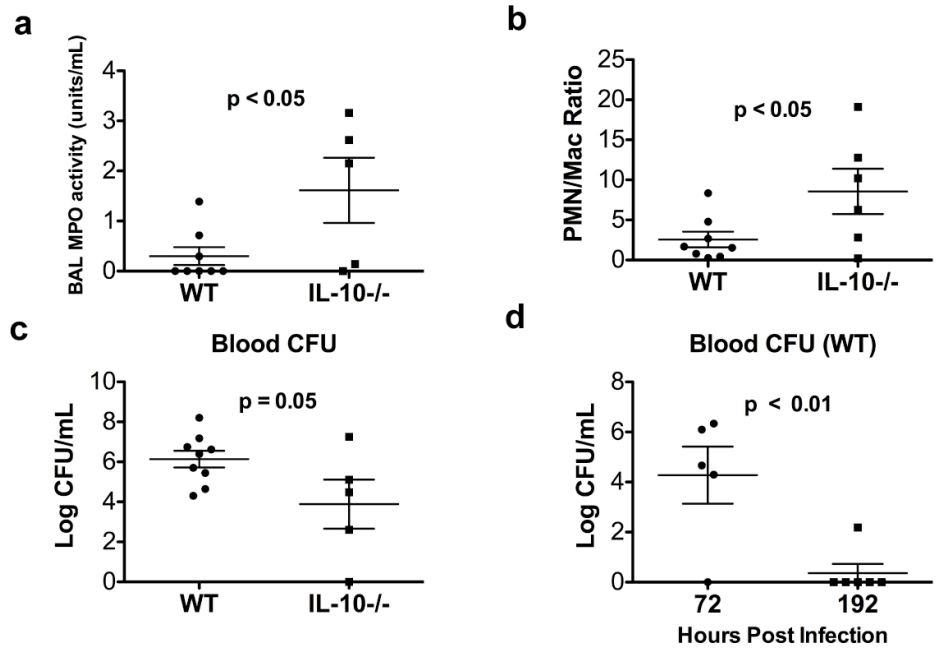

Figure 21. Improved bacterial clearance and increased PMN recruitment in IL-$10^{-/-}$ mice.

WT and IL-$10^{-/-}$ mice were infected with 1000 CFU of *K. pneumoniae*. At 72 h post-infection, (**a**) MPO activity was measured in cell free BAL fluid, and (**b**) PMN and Macrophage (Mac) numbers in BAL cells were assessed (expressed as a ratio). (**c**) Bacterial dissemination was lower in IL-$10^{-/-}$. (**d**) Clearance of systemic bacteria is achieved in surviving WT mice by day 8 after infection with 1000 CFU. Data shown are representative of 2 independent experiments (n=6-8 mice per group).

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We examined the levels of two cytokines, IL-17A and TNF-α, and the neutrophil chemoattractant KC in the lungs of the mice 144 h after infection, the combination of which was found to promote lung neutrophilia in response to fungal infection in our recent study[146]. As shown in **Figure 20d**, the levels of all of these molecules were significantly higher in the lungs of the IL-10-deficient mice at 144 h after infection, which might explain the increased PMN burden in these mice late after infection (**Figure 20 and Figure 21**). qRT-PCR analysis of steady-state RNA levels at 144 h for the most part also matched the protein data (**Figure 22**).

The lack of correspondence between RNA and protein for TNF-α may be due to posttranscriptional regulation of protein expression [216]. Taken together, these results suggested that the increased morbidity observed in the IL-10$^{-/-}$ mice over time was not due to failure to clear bacteria but that the mice continued to harbor an overwhelming inflammatory response in the lungs in the absence of IL-10, which was already evident at 72 h after infection.

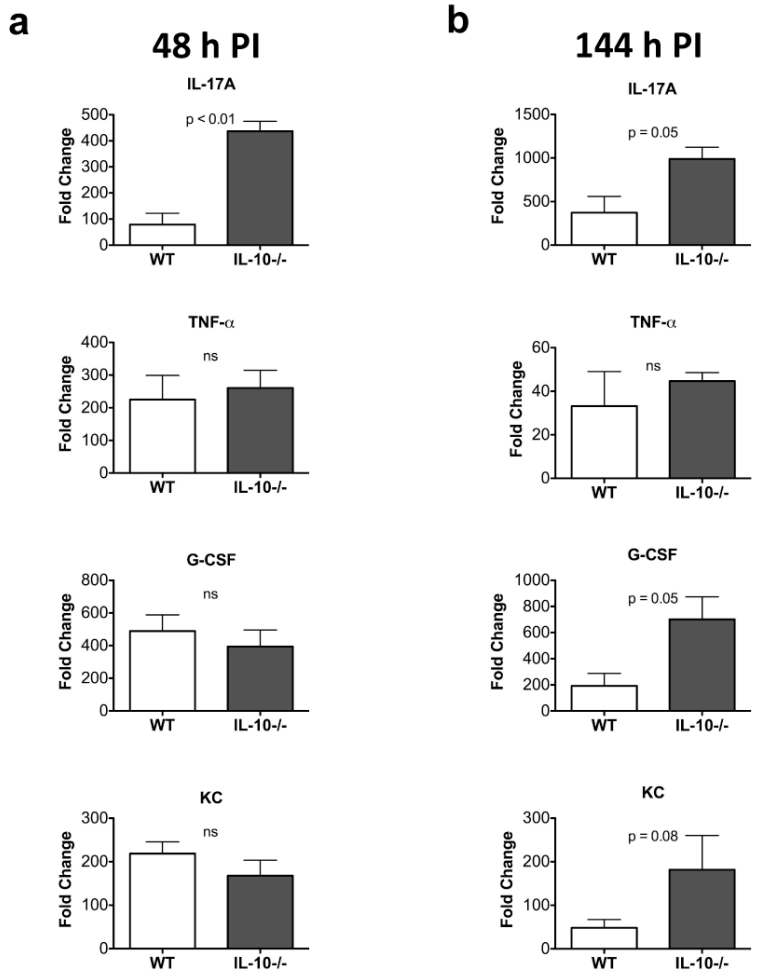

Figure 22. Increased expression of cytokines and chemokines associated with neutrophil recruitment in IL-10$^{-/-}$ mice.

WT and IL-10$^{-/-}$ mice were infected with 1000 CFU of *K. pneumoniae*. Lung mRNA was analyzed for expression of cytokines and chemokines by qRT-PCR techniques 48 and 144 h post infection. Data shown are representative of 2 independent experiments (n=5-6 mice per group).

3.4.3 Gr1^{int} MDSC-like cells expand in response to *K. pneumoniae* and produce IL-10.

Alveolar macrophages (AMs) are known to participate in the removal of cellular debris following infection. However, because they are confined to the alveolar lumen, there is a requirement for additional cellular players to remove apoptotic neutrophils in the lung interstitium in order to restore tissue homeostasis. Our previous work identified a myeloid cell with the phenotype CD11b^{+}Gr1^{int}F4/80^{+} resembling myeloid-derived suppressor cells (MDSCs) whose numbers increase in the lung tissue in response to LPS in a dose-dependent fashion and which produce IL-10[5]. As described previously[5], the cells are largely Ly6G^{int}/Ly6C$^{lo/-}$ and resemble granulocytic MDSCs. These cells constitute >60% of F4/80^{+} cells in the lung at 72 h after LPS instillation or bacterial infection. Given the anatomical location of these lung MDSC-like cells as well as their ability to proliferate in response to LPS, we examined the kinetics of their expansion and IL-10-producing ability in response to *K. pneumoniae*. As shown in **Figure 23**, the number of the Gr1^{int} MDSC-like cells (labeled as Gr1^{int} cells) did not change at 24 h after infection but increased significantly at 72 h after infection. Since AMs are also known to produce IL-10, we next simultaneously investigated the expansion of both AMs and the Gr1^{int} cells after infection with 1000 CFU of *K. pneumoniae*. More AMs than Gr1^{int} cells were recovered from the lungs of naïve mice. At 72 h after bacterial infection, however, the profile was reversed with fewer AMs than MDSC-like cells present in the lungs of the infected mice (**Figure 23a**). Typically, AMs participate very early after infection and their numbers dwindle as

neutrophils are rapidly recruited to the site of infection [207], which was observed by us as well (**Figure 24**). However, although the AMs reappear over time to be able to clear dying neutrophils in the alveolar lumen, at 72 h post-infection, the MDSC-like cells were clearly more abundant compared to AMs (**Figure 23a**). These data suggest a carefully orchestrated mechanism the host has evolved to simultaneously allow for an appropriate inflammatory response to bacterial challenge with subsequent expansion of MDSC-like $Gr1^{int}$ cells 72 h post infection, to temper inflammation and prevent tissue damage. Importantly, although both AMs and lung $Gr1^{int}$ cells were able to secrete IL-10, the total contribution of IL-10 from the interstitial $Gr1^{int}$ cells outweighed the amount of IL-10 from the AMs in the lumen late after infection (**Figure 23b**). We examined IL-10 production from tissue PMNs, $Gr1^{int}$ and total F4/80+ cells isolated from the lungs of mice at 72 h after infection with 100 versus 1000 CFU of bacteria by intracellular staining techniques. As shown in **Figure 23c**, the frequency of IL-10-secreting cells was highest in the $Gr1^{int}$ population with 100 CFU of infection. The frequency of IL-10-secreting $Gr1^{int}$ cells appeared to diminish with the higher bacterial dose. The results of these experiments showed that with the passage of time after infection when bacteria and PMNs infiltrate the tissue, the $Gr1^{int}$ cells expand as IL-10-producing cells in the lung. They appear with a delayed kinetics so as not to produce IL-10 too early to impede PMN recruitment for bacterial clearance.

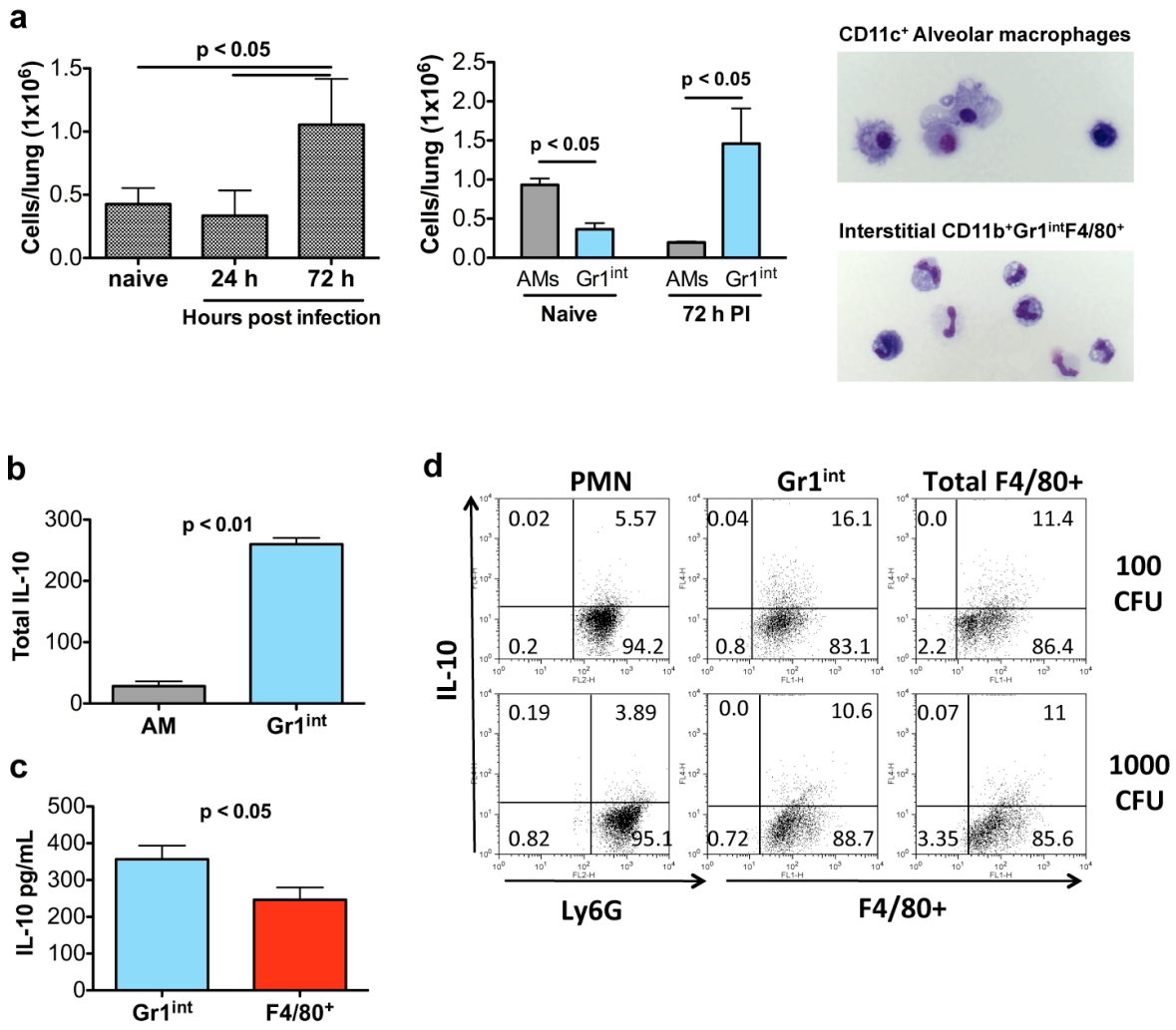

Figure 23. Gr1^{int} MDSC-like cells are the IL-10 producers in the lung interstitium 72 h post infection.

(**a**) Gr1^{int} cell numbers were assessed at indicated times after infection of mice with 1000 CFU of *K. pneumoniae*. AMs and Gr1^{int} cells were quantified by flow cytometric analysis in naïve mice as well as in mice at 72 h post infection (PI). AMs were identified by flow cytometry based on CD11c expression and high autofluorescence while Gr1^{int} cells were CD11b^{+}Gr1^{int}F4/80^{+}. Morphology of each cell type is also shown. (**b**) At 72 h post infection, Gr1^{int} were purified as previously described [5]. AMs were purified by high volume lavage and CD11c magnetic purification. Cells were seeded in equal numbers and after 24 h, cell-free supernatants were analyzed by ELISA for IL-10 levels. Total IL-10 was determined by multiplying the amount of IL-10 produced per cell by the number of cells present in the lung 72 h post-infection. (**c**) IL-10 secretion was greater from purified Gr1int on a per cell basis than total F4/80+ cells isolated 72 h post infection with 1000 CFU *K. pneumoniae*. (**d**) Intracellular cytokine staining for IL-10 was performed on neutrophils (PMNs), Gr1^{int}, and total F4/80+ cells isolated 72 h post infection with 100 or 1000 CFU *K. pneumoniae*. CD11b^{+} cells were purified by AutoMACS technology. Within the CD11b+

cells, PMNs were CD11b^{+}Ly6G^{hi}F4/80^{-}, Gr1^{int} cells were CD11b^{+}Gr1^{int}F4/80^{+}, and total F4/80+ were CD11b^{+}F4/80^{+}. Data shown are means ± s.d. and are representative of 2 independent experiments. For cellular quantification mice were kept individually (n = 4-5 mice per group). For cell purification and sorting to determine cytokine production (n = 3-4 mice pooled per group).

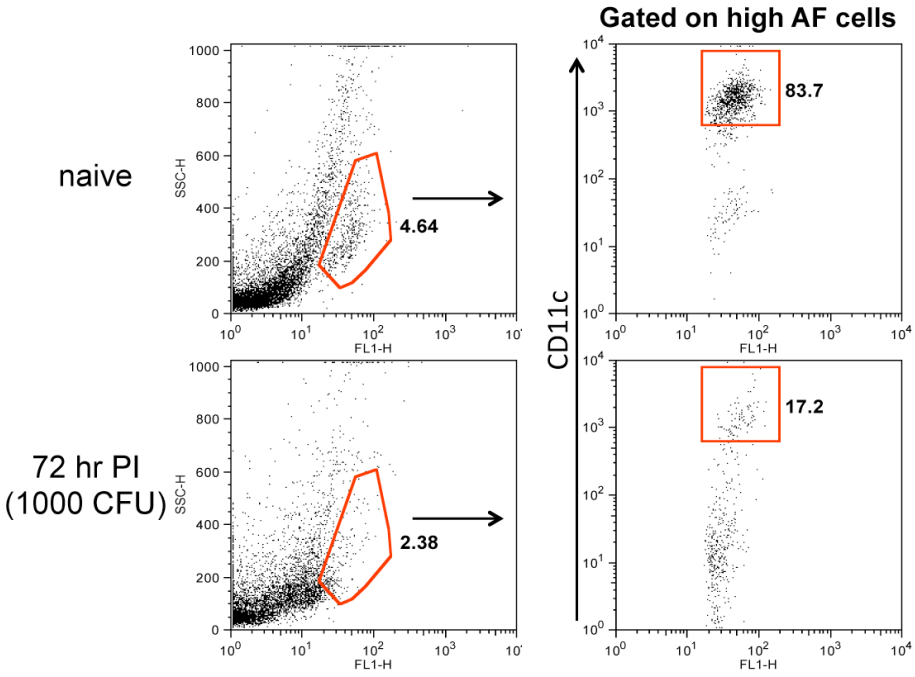

Figure 24. AMs decrease over time post infection.

High autofluorescent cells were gated and CD11c expression was examined. AMs were identified based on high autofluorescence and high CD11c expression. Data shown are representative of 2 independent experiments.

3.4.4 Gr1^{int} MDSC-like cells are outnumbered by neutrophils at high bacterial dose.

While our studies and previous literature collectively show that the host deploys its armamentarium of cells in a highly regulated fashion to combat infection, the delicate balancing act of mounting inflammation to eliminate the pathogen and then later curbing it to restore homeostasis is not always successful. In patients who fail to recover from bacterial pneumonia, overwhelming tissue inflammation precipitates ALI [135,205,208-210]. Since a dose of 100 CFU of

bacteria led to complete recovery of all mice while 1000 CFU caused 50% mortality in our study, we examined the PMN:Gr1^{int} ratio in the lungs of the mice in response to these doses 24 and 72 h after infection. Our analysis showed that the numbers of Gr1^{int} cells do not keep up with those of infiltrating neutrophils at a higher bacterial burden (**Figure 25a,c**). In particular, in moribund mice infected with 1000 CFU of bacteria, the ratio of PMN:Gr1^{int} cells was ~20, compared to a ratio of ~7 in mice with less weight loss (survivors) (**Figure 25b**) and 2-4 in mice infected with 100 CFU (**Figure 25c**).

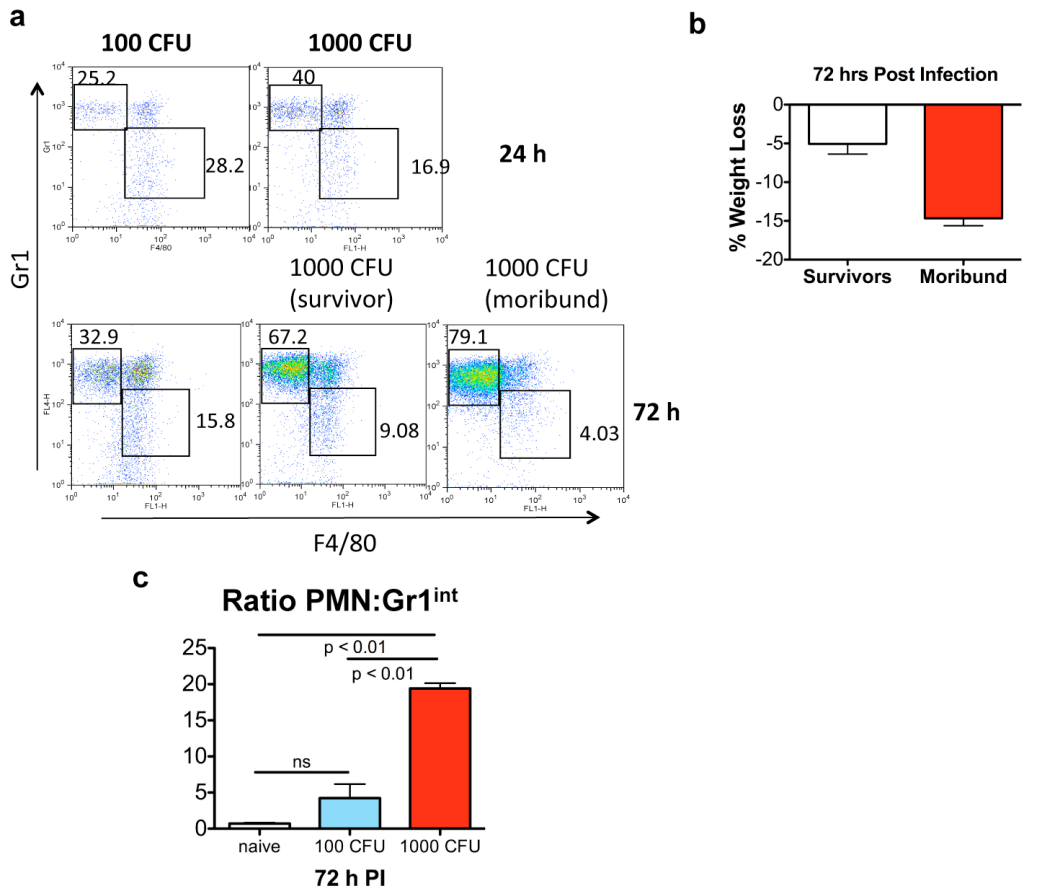

Figure 25. Increased PMN infiltration in the lung at higher bacterial burden resulting in an increased PMN:Gr1^{int} ratio.

(**a**) The CD11b^{+} population in the lung was analyzed for Ly6G or Gr1 and F4/80 expression. Ly6G^{hi}F4/80^{-} cells represent PMNs whereas Gr1^{int}F4/80^{+} cells are the Gr1^{int} lung-MDSC like cells. 1000 CFU of bacteria induced more PMN infiltration than 100 CFU at both 24 and 72 h post infection. (**b**) Mice infected with 1000 CFU can be divided

into 2 groups on the basis of weight loss 72 h post infection. Therefore, panel (**a**) also depicts representative flow plot of a moribund versus a surviving mouse. (**c**) A significantly higher PMN to $Gr1^{int}$ ratio in the lung tissue with 1000 CFU as compared to 100 CFU of *K. pneumoniae*. Total cell numbers (in millions) 72 h post infection were PMN: 5.03 +/-1.54; $Gr1^{int}$: 1.03 +/-0.37 (with 100 CFU) and PMN:15.3 +/-4.3; $Gr1^{int}$: 0.94 +/-0.36 (with 1000 CFU). Data shown are means ± s.d. and are representative of 2 independent experiments. For cellular quantification and determination of ratios, mice were kept individually (n = 4-5 mice per group).

3.4.5 Lung MDSC-like cells have the ability to efferocytose apoptotic neutrophils.

Our next question in the study was whether IL-10-secreting $Gr1^{int}$ cells- cells, appearing with a delayed kinetics in the tissue, have the functional ability to remove dying PMNs. The term "efferocytosis" describes the phagocytosis of apoptotic cells, a process shown to promote as well as require IL-10 [124-126]. Although AMs are known to participate in PMN clearance in the lumen, which cell types mediate a similar process in the interstitium is poorly understood. In order to investigate whether the IL-10-producing lung $Gr1^{int}$ cells have efferocytic potential, we labeled both apoptotic PMNs and lung $Gr1^{int}$ cells. The labeled cells were incubated together in culture medium and uptake of the fluorescently labeled PMNs by the $Gr1^{int}$ cells was examined. Flow cytometric analysis suggested that the $Gr1^{int}$ cells had the ability to efferocytose apoptotic PMNs (**Figure 26a**), which was further substantiated by confocal imaging methods (**Figure 26c**). Z-stack projections confirmed that the neutrophils were contained within the $Gr1^{int}$ cells (not shown). Prior to the efferocytosis assay, PMNs were confirmed to be apoptotic by Annexin V/PI flow cytometric analysis and were hypersegmented in morphology (characteristic of apoptotic neutrophils) as compared to freshly isolated PMNs (**Figure 26b**). Furthermore, neutralization of IL-10 by neutralizing antibody in the efferocytosis assay dose-dependently reduced the efferocytic efficiency of the $Gr1^{int}$ cells (**Figure 26d**). Thus, IL-10-producing lung $Gr1^{int}$ cells

show the capacity to ingest apoptotic neutrophils and IL-10 enhances the efferocytic potential of these Gr1^{int} cells.

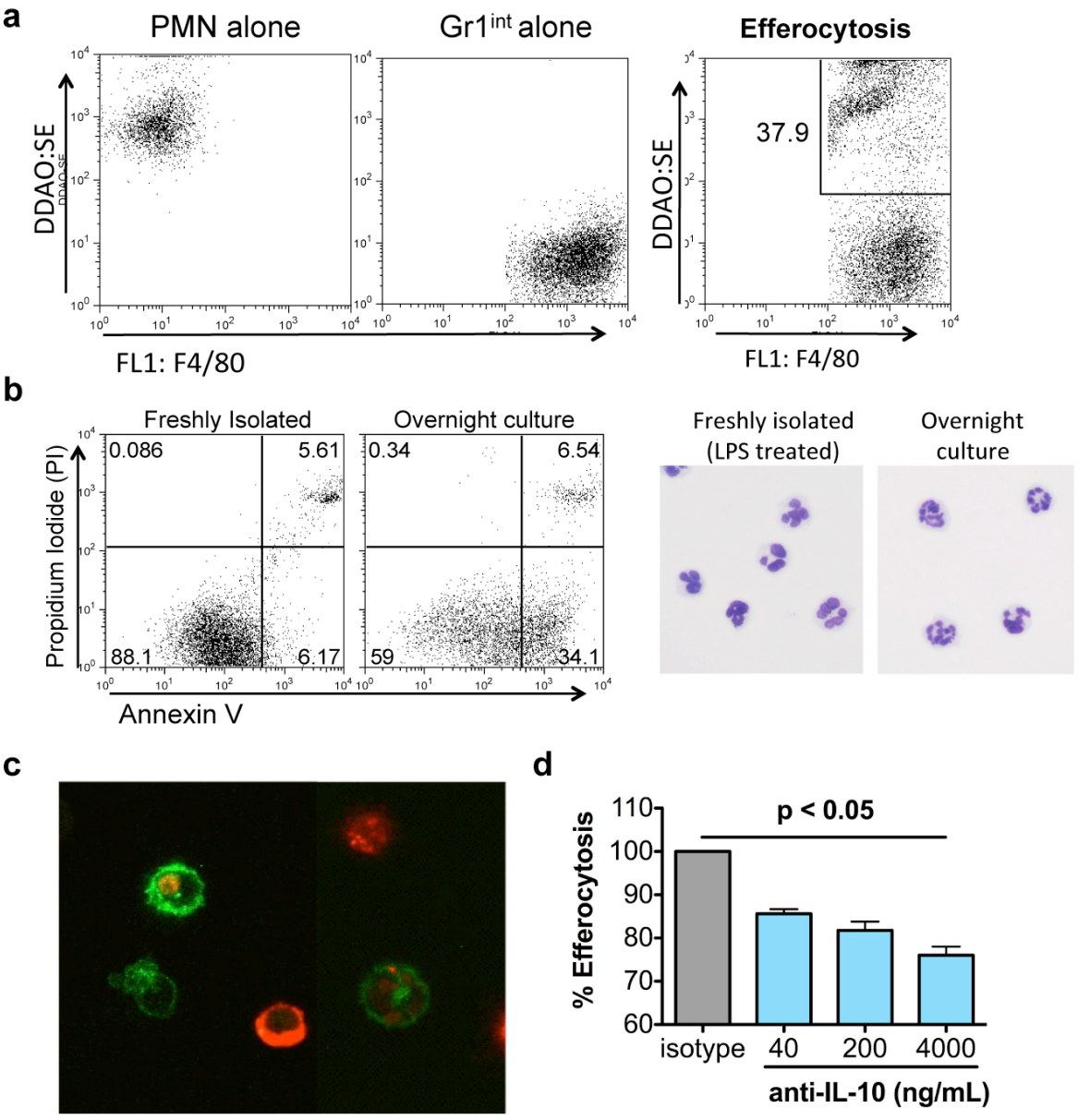

Figure 26. Gr1^{int} cells efferocytose apoptotic PMNs.

(**a**) PMNs were purified from the lungs of LPS treated mice (10 μg/day for 3 days) and plated overnight under serum free conditions (2x10^6 cells/mL) to induce apoptosis. Gr1^{int} cells were purified from the lungs of LPS-treated mice as F4/80$^+$Gr1^{int} cells by FACS of Alexa-488- and PE-labeled CD11b+ cells. PMNs were labeled with DDAO-SE (Molecular Probes). Gr1^{int} and PMNs were cultured together in a 1:10 ratio for 30 min at 37^{0}C. The cells were

washed twice and percent uptake was determined by flow cytometry. (**b**) 35% of the live PMNs (based on forward and side scatter) were annnexin V^+/PI^-. Morphology is also shown. (**c**) Confocal images of apoptotic PMNs (red) inside of $Gr1^{int}$ (green) cells. Z-stack projections were taken to confirm intracellular location (not shown). (**d**) Antibody-mediated neutralization of IL-10 in culture during efferocytosis assay reduced uptake of PMNs by $Gr1^{int}$ cells. Data shown are representative of 2 independent experiments. For cell purification and sorting of lung-MDSCs and PMNs, 3-4 mice were treated and total lung cells were pooled for subsequent purification.

3.4.6 Regulation of $Gr1^{int}$:PMN ratio by STAT1

Our data showing efferocytic potential of the $Gr1^{int}$ cells (**Figure 26**) and blunting of their numbers compared to PMNs with high bacterial burden (**Figure 25**) led us to then ask what mechanisms limit $Gr1^{int}$ cellular expansion in the interstitium since if their numbers could be expanded, their regulatory properties could be better harnessed to limit immune-mediated pathology.

Tumor-associated MDSCs have been shown to be dependent on STAT3 for both expansion and soluble mediator production [1]. In fact, a recent publication highlights the importance of STAT3 signaling downstream of Hsp72/TLR2 via autocrine IL-6 production to illicit MDSC suppressive function [84]. In our experiments, neutralization of IL-6 blunted $Gr1^{int}$ numbers in *ex vivo* cultures of the cells (**See Figure 14**).

STAT1 and STAT3 are known to counterbalance each other with effects on both cytokine production and cellular plasticity [1,83,90,91,96]. Given our interest in expanding the $Gr1^{int}$ MDSC-like cell type in the lung thereby theoretically enhancing clearance of apoptotic PMNs, we asked whether deletion of STAT1 signaling would help promote $Gr1^{int}$ cells and lower PMNs in defense against *K. pneumoniae*. Towards this end, WT and $STAT1^{-/-}$ mice were infected with 1000 CFU of bacteria. At 72 h post infection, the STAT1-/- mice survived and histological examination revealed less inflammatory cell infiltration into the lungs compared to the WT mice

(**Figure 27a**). Not surprisingly, since STAT1 is downstream of type I and II IFNs and controls activation of phagocytic cells for pathogen killing[217], bacterial dissemination was higher in the STAT1-deficient mice (**Figure 28a**). However, it is possible that the mice did not immediately succumb to infection because STAT1 deficiency was somewhat compensated by adequate levels of TNF-α (**Figure 28b**), which is also important for phagocyte activation[218]. Using weight loss as an overall measure of health, there was no significantly difference between WT and $STAT1^{-/-}$ (**Figure 28c**) suggesting that, most importantly, the reduced lung histopathology may have also sustained the mice. With regard to effects on the myeloid cells, the $STAT1^{-/-}$ mice showed almost a doubling in the numbers of $Gr1^{int}$ cells in the lung with significant reduction in PMN numbers as compared to the WT mice (**Figure 27b**). Representative flow cytometry data along with cell quantifications are shown in **Figure 29a-b.** As a side note, we also observed a doubling of $Gr1^{int}$ cells in LPS treated $STAT1^{-/-}$ mice (**Figure 29c)**. The lower numbers of PMNs in the $STAT1^{-/-}$ mice also correlated with reduced MPO activity in the BAL fluid (**Figure 27c**). The tissue IL-10 level as well as IL-10 gene expression in the $Gr1^{int}$ cells was greater in the $STAT1^{-/-}$ mice (**Figure 27d-e**). The efferocytic potential of the STAT1-deficient $Gr1^{int}$ cells was found to be largely intact (**Figure 27f**). This is important since STAT1 controls the expression of selected molecules involved in phagocytosis[219]. Since the IL-6/STAT3 axis promotes proliferation of MDSCs, we were curious to determine whether the IL-6 level in the lungs of the $STAT1^{-/-}$ mice was increased as compared to that in WT mice which was found to be true (**Figure 27g**). Because the level of IL-6 was higher in the lungs of $STAT1^{-/-}$ mice, we asked whether STAT3 signaling was enhanced in $Gr1^{int}$ cells isolated from these mice. In these experiments, LPS was used as a surrogate for *K. pneumoniae* since treatment of $STAT1^{-/-}$ mice with LPS also resulted in increased frequency of the MDSC-like cells (not shown). As shown in

Figure 27h, IL-6 efficiently induced STAT3 phosphorylation in MDSC-like cells harvested from naïve WT (shown) or STAT1$^{-/-}$ mice. When cells were isolated from LPS-treated WT and STAT1$^{-/-}$ mice, higher pSTAT3 levels were detected in response to IL-6 in the STAT1-deficient Gr1^{int} cells (**Figure 30a**). Representative flow cytometry is shown in **Figure 30b**. Thus, the increased IL-6 levels in the lungs of STAT1$^{-/-}$ mice (**Figure 27g**) combined with the enhanced ability of STAT1-deficient Gr1^{int} cells to respond to IL-6 in the context of inflammation (**Figure 30**) may contribute to the increased frequency of the Gr1^{int} cells under STAT1-deficient conditions (**Figures 27b and 29**).

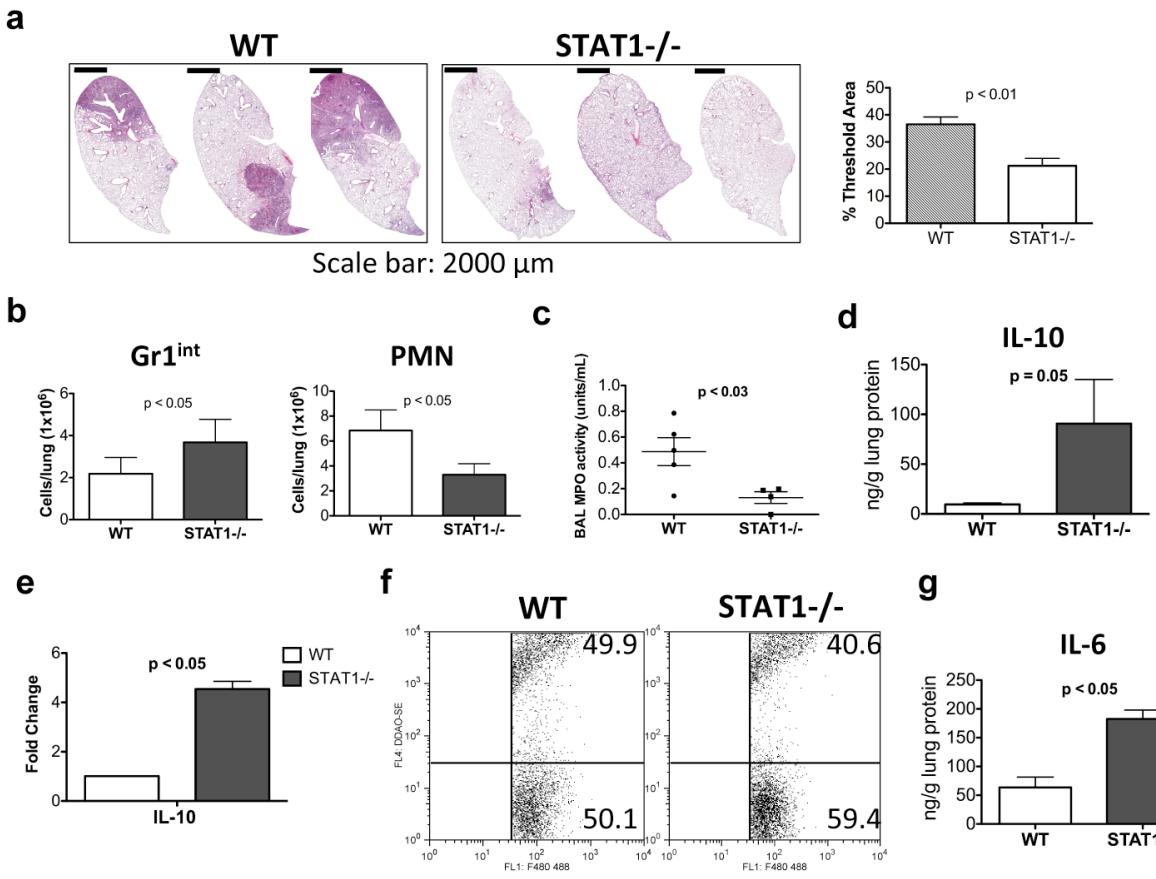

Figure 27. STAT1$^{-/-}$ mice show reduced cellular infiltration upon gross examination with decreased frequency of PMNs and increased frequency of Gr1^{int} cells in the lungs following 1000 CFU *K. pneunoniae* infection.

(**a**) WT and STAT1$^{-/-}$ were infected with 1000 CFU. 72 h post infection lungs were examined for histopathology by H&E staining and the stained lung sections were quantified using Metamorph. (**b**) The numbers of Gr1^{int} cells increases and PMNs decrease in STAT1$^{-/-}$ as compared to WT 72 h post infection with 1000 CFU of *K. pneumoniae*. (**c**) STAT1$^{-/-}$ mice showed reduced MPO activity in the BAL fluid compared to that observed in WT mice. (**d**) Lung IL-10 levels were increased in STAT1$^{-/-}$ mice as compared to that in WT mice. (**e**) Increased IL-10 mRNA levels in STAT1-deficient Gr1^{int} cells as determined by qRT-PCR analysis (n=3-4 mice/group). (**f**) STAT1$^{-/-}$ Gr1$^{int\ cells}$ can efferocytose apoptotic PMNs similar to WT Gr1^{int} cells. (**g**) Lung IL-6 levels were increased in STAT1$^{-/-}$ mice as compared to that in WT mice. Data shown are means ± s.d. (n=3 mice/group) and are representative of two independent experiments.

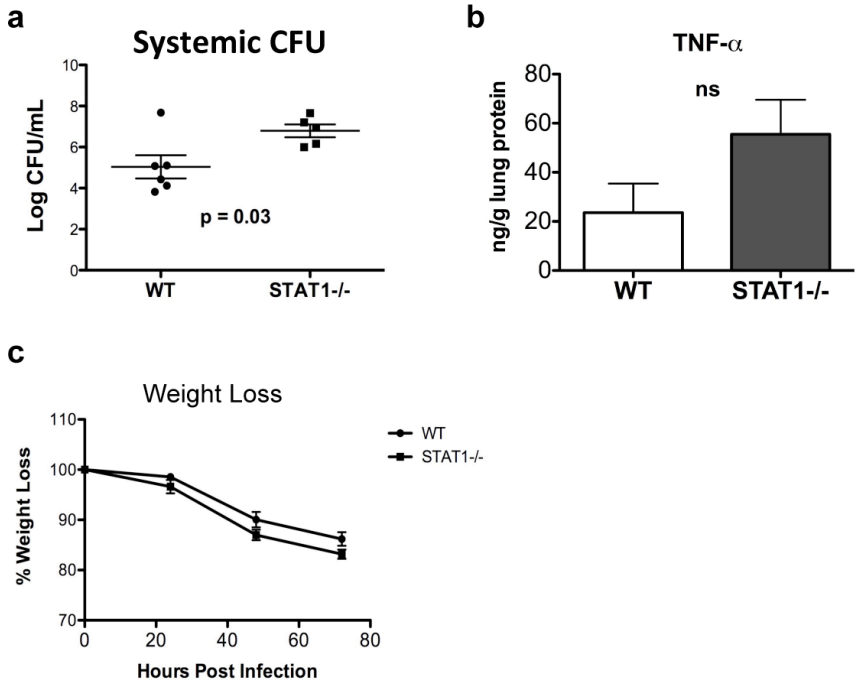

Figure 28. STAT1$^{-/-}$ mice show increased systemic CFU 72 hours post infection.

(a) WT and STAT1$^{-/-}$ mice were infected with 1000 CFU of *K. pneumoniae*. Blood CFU was determined 72 hours post infection and (b) TNF-α was measured in lung homogenates by multiplex. (c) Weight loss was not statistically different between WT and STAT1$^{-/-}$ mice. Data shown are means ± s.d. and are representative of 2 independent experiments. For cellular quantification and determination of ratios, mice were kept individually (n = 4-5 mice per group).

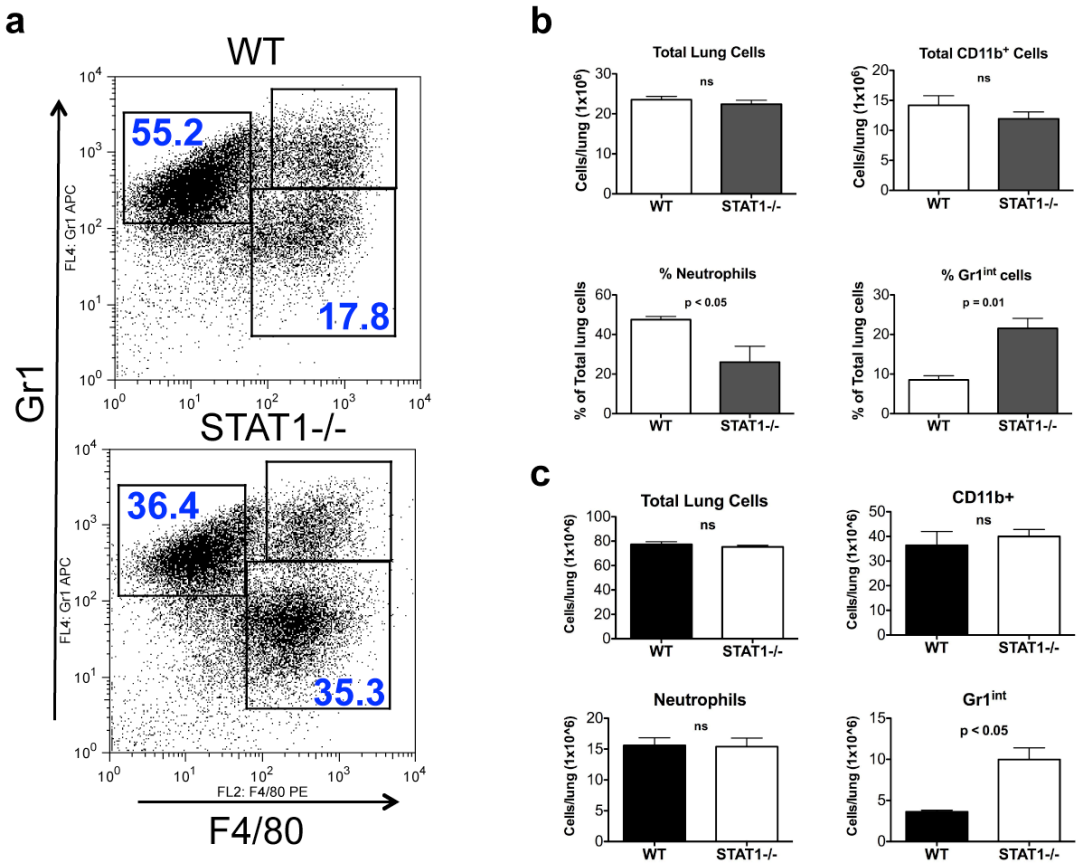

Figure 29. STAT1$^{-/-}$ have increased Gr1^{int} following infection with 1000 CFU *K. pneumoniae* or LPS administration.

WT and STAT1$^{-/-}$ were infected with 1000 CFU *K. pneumoniae*. (**a**) CD11b purified cells were stained with anti-Gr1 and F4/80 and analyzed by flow cytometry. (**b**) The numbers of Gr1^{int} cells increases and PMNs decrease in STAT1$^{-/-}$ as compared to WT 72 h post infection with 1000 CFU of *K. pneumoniae*. (**c**) Increased Gr1^{int} is also observed in mice treated with LPS (10ug/day for 3 consecutive days). Data shown are means ± s.d. and are representative of 2 independent experiments. For cellular quantification and determination of ratios, mice were kept individually (n = 3-4 mice per group).

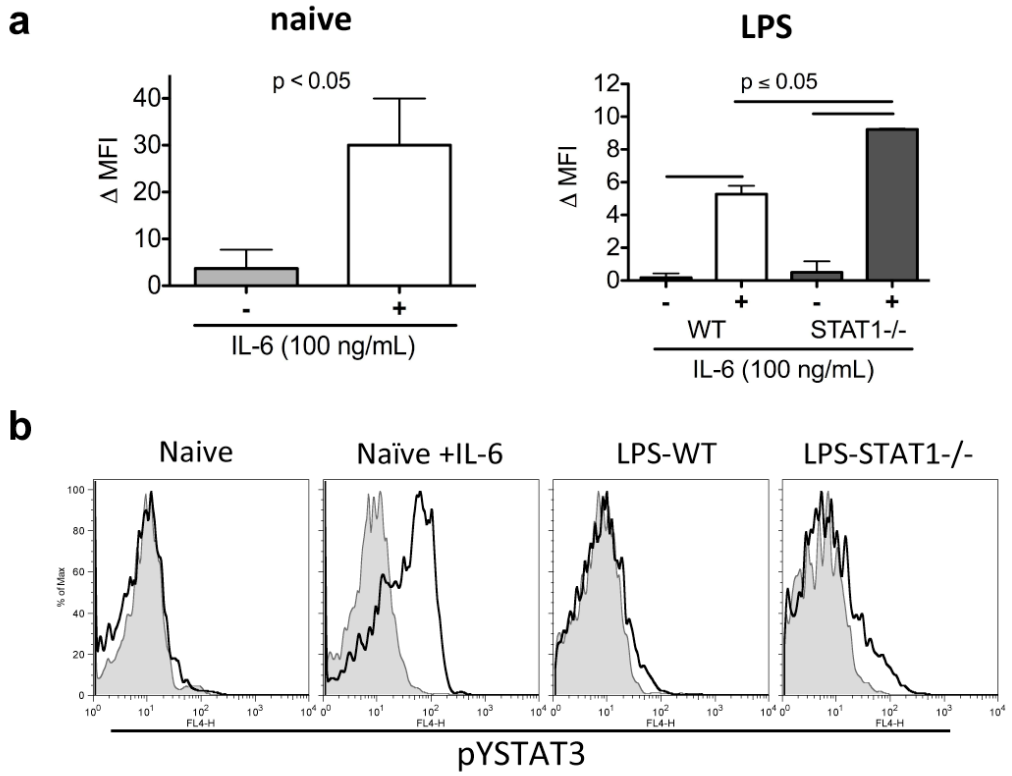

Figure 30. STAT1$^{-/-}$ Gr1int have increased pSTAT3 in response to IL-6 treatment.

(a) The level of Gr1^{int} pSTAT3 expression was analyzed in naïve mice or mice treated with LPS (10 μg/day for 3 days). Analysis of pSTAT3 levels in WT and STAT-1-deficient MDSC-like cells ± IL-6 (100 ng/ml) reveals that pSTAT3 level is increased in STAT1$^{-/-}$ Gr1^{int} compared to WT. (b) Representative flow cytometry plots are shown. The level of pSTAT3 expression expressed as ΔMFI represents the difference in mean channel fluorescence intensity of positive staining cells and of those stained with isotype controls. Data shown are means ± s.d. (n=3 mice/group) and are representative of two independent experiments.

3.5 DISCUSSION

Previously shown to be a modulator of allergic inflammation [5], our data now demonstrate a role for an MDSC-like cell population in the lung as an innate regulator of inflammation in a model of acute bacterial pneumonia. The delicate balance required between appropriate inflammation to clear pathogens in infected tissue and the mitigation of associated immune-mediated

pathology to minimize collateral tissue damage is well appreciated in the literature but not adequately studied [105]. We show that although early IL-10 production hampers bacterial clearance from the lung, its complete absence prevents resolution of neutrophilic inflammation and recovery of mice. In the lung, accumulation of interstitial neutrophils during non-resolving pneumonia has been associated with the hallmarks of acute lung injury [135,208,209]. However, no study to date has described which cells participate in the normal resolution of inflammation after bacterial infection so that the process can be improved when the potential for complete recovery is in jeopardy. We show that the host expands $Gr1^{int}$ MDSC-like cell numbers in the tissue in response to bacterial infection with a delayed kinetics. These MDSC-like $Gr1^{int}$ cells produce IL-10 and can efferocytose apoptotic neutrophils. Interestingly, efferocytosis by MDSC-like cells is aided by IL-10, inviting speculation regarding autocrine/paracrine enhancement of efferocytosis during the resolution phase of inflammation. We show that deletion of STAT1 lowers the neutrophilic burden in the lung with concomitant increase in the proportion of MDSC-like cells without causing undue morbidity or mortality of the mice. This is important since a combination of a STAT1 inhibitor and appropriate antibiotic therapy may help address non-resolving pneumonia and thereby decrease associated mortality.

An important initial goal in this study was to determine a dose of *K. pneumoniae* that would cause ~50% lethality early in WT mice but would be less lethal to $IL\text{-}10^{-/-}$ mice. This is because from the existing literature, it was clear that early production of IL-10 inhibits neutrophil recruitment [105,124,165], neutrophils being important in defense against this bacterium. We therefore expected that we would be able to arrive at a dose that at an early time point would impart a survival benefit to the $IL\text{-}10^{-/-}$ mice as compared to the WT mice. We reproducibly observed a 100% survival rate in WT mice when a 100 CFU of this bacterium was used but only

50% survival when a log higher dose was used. 100 CFU induced minor cellular infiltration and very minimal lung tissue injury in all WT or IL-10$^{-/-}$ animals (data not shown). Thus a low dose would not have allowed us to address our main question, which was the role of IL-10 in host defense against *K. pneumoniae* late after infection, this question being pertinent in resolving versus non-resolving pneumonia. Using the 1000 CFU dose, although the IL-10$^{-/-}$ mice fared better initially, they were near death by 144h (6 days) and had to be sacrificed. Along with the dying IL-10$^{-/-}$ mice we also sacrificed the surviving WT mice in order to identify parameters that were different between the WT and the IL-10$^{-/-}$ mice 144 h post infection (Fig. 1, Supp Figs S1-S3). With a lower dose of 100 CFU, there would not have been enough early immune activation to trigger continued neutrophil influx, which contributed to mortality in the IL-10$^{-/-}$ mice at the 144h time point. Collectively, while inclusion of data from mice that needed to be sacrificed early could have influenced the results presented, we feel that our analysis of mice that survived up to 144h has not biased our data addressing differences between IL-10-sufficient and IL-10-deprived state late after infection. Since *K. pneumoniae* is an extremely virulent bacterium for many strains of mice, the LD_{50} is quite low. For reproducibility, we infected mice with 1000 CFU each time, which ensured a difference in the outcome between WT and IL-10$^{-/-}$ mice.

IL-10 can have opposite effects depending on context and timing. For example, during murine pneumonia induced by a lethal dose of *K. pneumoniae*, instillation of anti-IL-10 neutralizing antibody prior to infection improved bacterial clearance [165]. A similar observation was made when early production of anti-inflammatory mediators including IL-10 was shown to be deleterious to recruitment of neutrophils for defense against *Streptococcus pneumoniae* [220]. In each of these studies, anti-IL-10 neutralizing antibody was administered immediately before infection and lethal doses of bacteria were used. In contrast, complete absence of IL-10 has been

shown to increase tissue injury in multiple infection models involving protozoa, bacteria, and nematodes [169] a phenomenon that was similarly evident in our model of bacterial pneumonia in which an LD_{50} dose of *K. pneumoniae* was used. In our previous study, we showed that the expansion of the $Gr1^{int}$ cells in response to LPS is greatly blunted in MyD88-deficient mice suggesting dependence on this adaptor protein for their accumulation [5]. Additionally, using GFP to track cells *in vivo*, we showed that a lineage negative bone marrow progenitor population when introduced intravenously into mice has the ability to differentiate into MDSC-like cells in the lung after intratracheal delivery of LPS [5]. This finding was in agreement with a report showing the ability of hematopoietic stem and progenitor cells (HSPCs) in extramedullary tissues including the lung to differentiate into $CD11c^{+}Gr1^{+}$ cells in the presence of LPS [64]. It was speculated that the purpose of LPS-induced differentiation of HSPCs into myeloid cells was to boost immune surveillance although this idea was not experimentally interrogated. Taken together, it seems likely that resident HSPCs differentiate into IL-10-producing $Gr1^{int}$ MDSC-like cells with a delayed kinetics in response to pathogen-derived signals.

The molecular regulation of IL-10 in the lung MDSC-like cells has yet to be determined. However, data generated through the use of $STAT1^{-/-}$ mice are suggestive of mechanisms underlying IL-10 production by these cells. Given the increased levels of IL-6-induced pSTAT3 in the STAT1-deficient $Gr1^{int}$ cells, and with IL-6 being a key cytokine produced by these cells, a likely positive regulator of IL-10 in the $Gr1^{int}$ cells is STAT3[171,179]. Reciprocally, as previously observed[97,174], STAT1 may negatively regulate IL-10 in the $Gr1^{int}$ cells since a significantly higher level of IL-10 was detected in the lungs of STAT1-deficient mice along with higher levels of IL-10 mRNA in STAT1-deficient MDSC-like cells which may be mediated by increased levels of pSTAT3[171,179].

We observed significantly higher levels of KC expression late after infection in the lungs of IL-10$^{-/-}$ mice as compared to that in WT mice. KC is a known neutrophil chemoattractant during acute pulmonary inflammation[221]. Furthermore, IL-10 is known to inhibit LPS induced KC-mRNA stability [187] suggesting that lack of IL-10 is a primary mechanism contributing to increased KC in the IL-10$^{-/-}$ mice which is not advantageous to the host during the resolution phase. Although our primary focus was on regulation of MDSC-like cellular expansion, we also observed a decrease in RANTES production in the absence of STAT1 signaling during *K. pneumonia* infection (not shown). Indeed, RANTES has been linked to neutrophil accumulation in the lungs and is also known to be negatively regulated by IL-10, further substantiating our finding of a lower neutrophilic burden in the STAT1$^{-/-}$ mice[186,222]. Together with expansion of MDSCs in the STAT1$^{-/-}$, the observed reduction in RANTES expression and implicated reduction in RANTES-induced neutrophil accumulation further warrants the use of STAT1 inhibition in the lungs post bacteria infection.

STAT1 is important for antibacterial host defense as was shown for clearance of *Listeria monocytogenes* from livers and spleens of infected mice[129]. STAT1 expression in MDSCs was shown to promote Arg1 and NOS2 expression and to be important for the suppressive effects of MDSCs on T cells[223]. Our previous study also demonstrated the importance of Arg1 and IL-10 expression in the lung MDSC-like cells for inhibition of Th2 effector function [5]. However, since to date Arg1 has not been shown to promote anti-bacterial defense and on the contrary increased Arg1 in macrophages compromises bacterial clearance [224], enhancement of IL-10 and decrease in Arg1 expression via inhibition of STAT1 in combination with antibiotics may be an ideal therapeutic modality for non-resolving pneumonia.

In summary, in a model of bacterial pneumonia, we have elucidated a requirement for IL-10 in resolution of lung inflammation. We show that $Gr1^{int}$ MDSC-like cells within the tissue compartment are a cellular source of IL-10 and that this cell type expands late after infection and aids in efferocytosis of apoptotic neutrophils. MDSCs, including the lung $Gr1^{int}$ cells, harbor characteristics of both macrophage/monocytes and neutrophils, and therefore it has not yet been possible to selectively deplete these cells by molecular targeting, which would be beneficial in the case of tumor-associated MDSCs. However, given that a desirable goal in non-resolving pneumonia is to augment rather than delete this cell type, we postulate that STAT1 may be a suitable target to elicit that effect with a concomitant decrease in lung neutrophil burden. Our study further emphasizes the continued need for improved understanding of organ and cell-specific regulation of IL-10 for the purposes of immune regulation and therapeutic intervention.

3.6 ACKNOWLEDGEMENTS

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4.0 CONCLUSIONS

Following up on the initial recognition of the presence of lung $CD11b^{+}Gr1^{int}F4/80^{+}$ MDSC-like lung cells in response to LPS, the current studies now reveal a role for GM-CSF/STAT5A signaling in combination with LPS for the generation of MDSCs. Furthermore, this work has identified lung MDSCs as interstitial cells, which produce IL-10 in response to *K. pneumoniae*, and participate in resolution of inflammation following bacterial pneumonia.

In our attempts to elucidate mechanisms by which the lung returns to homeostasis following bacterial infection we have detailed the coordinated expansion of these cells in the lung tissue. Lung MDSCs exist in very small numbers during the initial phase of host defense followed by expansion over the course of 72 hours, well beyond the time when PMNs were initially recruited to participate in bacterial clearance. Although alveolar macrophages are known to participate in efferocytosis within the alveolar space, the dynamics between the cells involved in host defense in the lung tissue/interstitium are not well defined and no cell has yet been found to participate in clearance of apoptotic neutrophils in the intersititum. This is a critical question given that "interstitial neutrophils are more intimately associated with clinically relevant measures of acute lung injury" (Fessler, 2005 #380).

IL-10 has been shown to impair neutrophil recruitment thereby dampening host defense[166]. This suggests that high IL-10 concentration during the initiation phase is detrimental to the host because neutrophil recruitment is impaired via suppression of relevant

chemokines[165,166]. However, IL-10 is also known to be important for maintenance of homeostasis as well as regulation of neutrophil clearance, specifically promoting phagocytosis of apoptotic neutrophils (efferocytosis)[126]. The role of IL-10 as both a negative regulator of bacterial clearance but a positive regulator of homeostasis pointed to a seemingly contradictory need for a source of IL-10 which is absent during initial host defense but present following infection. Until now, a cellular source of IL-10, which aids in homeostatic resolution in the lung interstitium, had not been identified. Furthermore, the requirement for late IL-10 production following infection had not been rigorously tested. Our studies reveal the role of lung MDSCs as tissue dwelling cells with efferocytic and IL-10 producing capability. They expand over the course of 72 hours to exert a regulatory force via IL-10 production after initial host defense and adequate neutrophil recruitment has occurred.

Finally, we wanted to better understand the mechanism(s) the host may use to control MDSC expansion at earlier time points but allow for proliferation when resolution is necessary. Since we demonstrated that lung MDSC participate in clearance of apoptotic neutrophils, more detailed understanding of the mechanisms that control their development would theoretically result in novel clues towards potential future therapeutic development. To address this issue we explored a number of possibilities including signaling through both the GM-CSF-STAT5A, and IL-6 pathways in an attempt to better understand MDSC development in the lung tissue. Furthermore, we investigated the role of STAT1 in lung MDSC development and function given the role of STAT1 as a STAT3 antagonist and the published centrality of STAT3 signaling in tumor MDSC development and function[1]. STAT1/STAT3 antagonism has been discussed extensively in the literature. For example, IFNγ is able to signal via STAT3 in the absence of STAT1 suggesting some redundancy in their role but also the opportunity for counter-regulatory

control[90]. We now show that ablation of STAT1 results in increased frequency of lung MDSCs as well as increased production of IL-10. We propose the use of STAT1 inhibition in combination with systemic antibiotic administration as a potential novel therapy for the treatment of non-resolving pneumonia.

5.0 FUTURE DIRECTIONS

The current work has contributed to the field of MDSC biology and the understanding of regulation of inflammation in the lung following bacterial pneumonia. However, many questions remain unanswered and many of these are extremely relevant for the development of potential future therapies.

This discovery of MDSC accumulation in the lung, presumably resulting from trafficking of progenitor cells into the tissue, highlights a way the immune system has evolved to minimize global immunosuppression. In the case of immunosuppression in the lung after bacterial clearance, it is necessary for the MDSCs to remain compartmentalized, lest their immunosuppressive effects result in sepsis. Futher work as to the origin and accumulation of lung MDSCs is of great interest and relevant for harnessing their therapeutic potential. To this end, we have already initiated studies regarding the role of CX3CR1 vs CCR2 in the accumulation of lung MDSCs.

We have also observed that MDSC IL-10 production seems to be inhibited during overwhelming infection, suggesting that although the immune system may dampen IL-10 in the hopes of augmenting host defense and facilitating neutrophil influx, this dampening of IL-10 could inevitably result in increased mortality due to destructive lung inflammation. Identification of the soluble factors and signaling molecules related to the decrease in IL-10 production is an area of interest for our laboratory. As one example, STAT3 has two isoforms

STAT3α and STAT3β. STAT3α has recently been linked to an IL-6 response and production of IL-10 in macrophages indicating that further investigation into IL-10 production by lung MDSCs is warranted[225].

Another area of interest revolves around the role of MDSCs in actual bacterial clearance. We observed lung MDSCs to be highly phagocytic both *in vitro* even to the extent of having phagocytosed *K. pneumoniae in vivo* (**Figure 31**). Although MDSCs were able to also phagocytose *E. coli in vitro*, the observation of *K. pneumoniae* inside isolated MDSCs ex vivo may not be necessarily due to direct phagocytosis. We have not attempted to address whether the *K. pneumoniae* is directly phagocytosed by MDSCs, or if what we observed is simply the result of MDSCs efferocytosing neutrophils that have previously phagocytosed *K. pneumoniae in vivo*. The possibility exists that it may be a combination of the two, but the engulfment of bacteria-containing neutrophils may be one way that host defense is perpetuated towards resolution of inflammation. Referred to as nonphlogistic clearance of bacteria, MDSCs may be a major player in the lung due to the simultaneous engagement of apoptotic surface molecules on PMNs. Blander and colleagues have elegantly discussed this concept in their work regarding promotion of IL-17 responses downstream of simultaneous engagement of TLRs in the presence of apoptotic cells[226,227]. Indeed, the regulation of a Th17 response by lung MDSCs is an area of future interest for our laboratory.

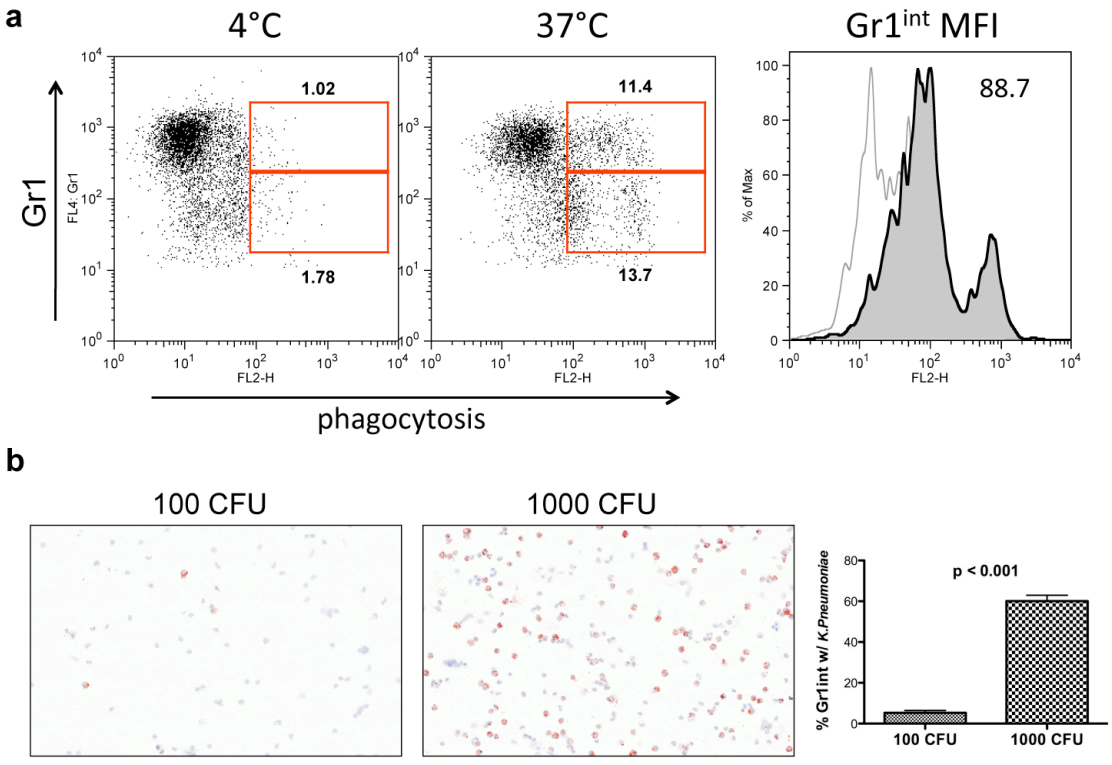

Figure 31. Gr1^{int} cells can phagocytose Gram-negative bacteria *in vitro* and *in vivo*.

(a) CD11b cells were purified from the lungs following LPS treatment by magnetic separation and incubated with pHrodo dye-conjugated E.coli (invitrogen). Using flow cytometry Gr1^{int} and Gr1^{hi} cells were distinguished and examined for labeled E.coli. Incubation at 4°C was used as a negative control for physiological internalization of bacteria. (b) Gr1^{int} cells isolated from *K. pneumoniae* infected mice contain *K. pneumoniae* with increased frequency in 1000 CFU infected animals as compared to 100 CFU infection, 72 hours post infection. Cytospins of purified Gr1^{int} cells were immunostained for *K. pneumoniae* and counterstained with hematoxylin. Percent positive for *K. pneumoniae* was determined.

Speculation regarding the role of lung MDSCs in nonphlogistic clearance of bacteria would also involve future studies determining which receptors are involved in MDSC recognition and efferocytosis of apoptotic neutrophils in the lung. Although we investigated the transcription levels of many receptors via qPCR, none stood out as being differentially expressed, suggesting that regulation of function and downstream signaling may be more

relevant than simple expression levels. If relevant cellular receptors were to be identified, then their associated signaling pathways would represent ideal targets for modulation, with the goal of enhancing neutrophil clearance *in vivo*.

Finally, and most relevant for therapeutic development in the context of the current state of knowledge, is the potential for inhibition of STAT1 as a therapy for non-resolving pneumonia. We feel that simultaneous administration of systemic antibiotics with local delivery of a STAT1 inhibitor around 48 hours post infection will yield the best result towards the timely resolution of inflammation in the lung. We are currently conducting these studies in the laboratory.

6.0 PUBLICATIONS

Poe, S.L., M. Arora, T.B. Oriss, M. Yarlagadda, K. Isse, A. Khare, D.E. Levy, J.S. Lee, R. Mallampalli, A. Ray and P. Ray. 2012. STAT1-Regulated Lung MDSC-like Cells Produce IL-10 and Efferocytose Apoptotic Neutrophils With Relevance In Resolution of Bacterial Pneumonia. Mucosal Immunol. (In press).

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Arora M, **S.L. Poe**, A. Ray and P. Ray. 2011. LPS-induced CD11b+Gr1(int)F4/80+ regulatory myeloid cells suppress allergen-induced airway inflammation. Int Immunopharmacol. Jul;11(7):827-32. PMID: 21320637

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APPENDIX A

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3. Arora M, **S.L. Poe**, A. Ray and P. Ray. 2011. LPS-induced CD11b+Gr1(int)F4/80+ regulatory myeloid cells suppress allergen-induced airway inflammation. Int Immunopharmacol. Jul;11(7):827-32. PMID: 21320637

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