IMPACT OF MYELOID DERIVED SUPPRESSOR CELLS DURING POLYMICROBIAL SEPSIS

By

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Sepsis is defined as bacterial infection accompanied by an overwhelming systemic inflammatory response that results in both innate and adaptive immune system dysfunction. Specifically, polymicrobial sepsis alters the adaptive immune response, and induces T-cell suppression and T_H2 immune polarization. In this dissertation, we identified a population of GR-1^+CD11b$^+$ cells whose numbers dramatically increase and remain elevated in the spleen, lymph nodes and bone marrow during polymicrobial sepsis. Phenotypically, these cells are heterogeneous, immature, predominantly myeloid progenitors that express IL-10, TNFα, MCP-1 and a number of other cytokines and chemokines. Splenic GR-1^+ cells effectively suppress antigen-specific CD8$^+$ T-cell interferon-γ production, but only modestly suppress antigen-specific and nonspecific CD4$^+$ T-cell proliferation. GR-1^+ cell depletion *in vivo* prevents both the sepsis-induced augmentation of T_H2-dependent and depression of T_H1-dependent antibody production. Signaling through MyD88 and the SDF-1 pathways, but not TLR4, TRIF, the IFNα/β receptor, CCR2 receptor, IL-10, IL-4 or the M-CSF pathways are required for complete GR-1^+CD11b$^+$ expansion. GR-1^+CD11b$^+$ cells contribute to sepsis-induced T-cell suppression and preferential T_H2 polarization.

CHAPTER 1
INTRODUCTION

Sepsis Significance

Sepsis occurs when an overwhelming microbial infection leads to a systemic inflammatory response, manifesting clinically as fever, leukocytosis, increased cardiac output, and reduced peripheral vascular resistance, leading to multi-system organ failure and death. Despite progress in antibiotic administration, ventilator management, and fluid resuscitation over the past 20 years, sepsis remains the leading cause of death in the intensive care with over 750,000 cases and 210,000 deaths annually in the United States (1, 2). Recently, significant advancements in understanding sepsis pathophysiology have occurred with a better appreciation of inflammation science and the innate immune system (3, 4). Unfortunately, our scientific understanding has had little impact on the mortality rate from severe sepsis (1, 2), with the sepsis incidence compounded by the ever increasing elderly U.S. population. A multitude of proposed approaches, including anti-tumor necrosis factor-α (TNF) therapies, corticosteroids, antibodies against endotoxin, inhibitors of prostaglandins, bradykinins, PAF, and interleukin (IL)-1 receptor antagonist, have all failed during clinical trials (5). There are only partially efficacious sepsis-related therapies currently available, including activated protein C (6), replacement steroids for sepsis-associated adrenal insufficiency (7), and insulin therapy for blood glucose maintenance (8). However, as monotherapy or in combination, these approaches still only modestly improve sepsis survival (5). Furthermore most laboratory work investigating these early anti-inflammatory therapies were based on intravenously or intraperitoneally administered bacteria or endotoxin, or pretreatment prophylactic approaches which did not replicate the septic patients' pathophysiology or the disease process adequately (9, 10) . For the aforementioned reasons, many investigators have focused recent attention on experimental models of sepsis that modulate

cellular apoptosis and effector cell populations of both the innate and adaptive immune systems during sepsis to produce in animal models what is observed in human sepsis.

Sepsis, the Cecal Ligation and Puncture (CLP) Model, and Immune Dysfunction

For years, investigators have observed that severe sepsis produces a state immune suppression illustrated by a loss of delayed type hypersensitivity (11), an inability to eradicate primary infections (12), a predisposition to develop secondary nosocomial infections(12, 13), and a failure to respond to skin testing with specific antigens from microbes to which they were already exposed and had tested positive (11, 14). Furthermore, animal models of sepsis indicate that immune dysfunction is crucial to the pathogenesis of sepsis with a plethora of immune responses intertwining both the innate and adaptive immune systems (15-17). Loss of MHC II expression (18), defects in antigen presentation (19), apoptosis induced depletion of $CD4^+$ T cells (20), dendritic cell apoptosis (21), dendritic cell exhaustion of paralysis (22, 23), suppression of T-cell proliferative responses (24-27), reduced inflammatory and T_H1 cytokine production by monocytes and tissue macrophages (28), all contribute to immunologic compromise during sepsis and culminate in a shift from the proinflammatory T_H1 to the anti-inflammatory T_H2 immune profile (29-32). Moreover, recent attention has focused on the T_H1 to T_H2 immune profile shift as an explanation for post-sepsis immune suppression (33-35); however, the underlying mechanisms that orchestrate the shift in immune polarization during sepsis are still unknown.

With the failure of therapies in human sepsis, further research has resulted in a better understanding of the immune dysfunction during sepsis and the development of more appropriate murine models to better mimic human sepsis. Many of these findings can be recapitulated using the murine cecal ligation and puncture model (CLP) of sepsis (21, 36-39). Although the CLP model is valuable and replicates most of the manifestations of human sepsis, several limitations

to this approach remain. First, it is difficult to hemodynamically monitor mice. Second, although acute fluid resuscitation is provided, actual fluid requirements initially and chronically are not easily measurable or administratable. Furthermore, the pathophysiologic changes that occur in human sepsis occur in a more abbreviated time frame compared to human sepsis. Lastly, antibiotic administration varies among investigators and is not tailored to target the specific infective microbe, but is usually a broad spectrum antibiotic administered one time (39).

Although there are some inherent differences in human sepsis and experimental murine sepsis from a CLP model, the similarities are profound and generally characterized by two distinct immune phases that occur either concomitantly or sequentially (40). The early phase to a CLP is characterized by a hyperdynamic state with elevated cardiac output, tissue perfusion, and decreased vascular resistance. The hallmark of the early phase is inflammation mediated by neutrophils, macrophages, dendritic cells and monocytes, stimulated by microbes and/or their toxins. The second phase is characterized by a hypodynamic response beginning between 12-24 hrs after CLP and includes decreased macrovascular and microvascular blood flow, decreased cardiac function and output, and increased organ injury and dysfunction. Along with the physiologic and inflammatory alterations observed during the first 24 hours of sepsis,there is concomitant adaptive immune system dysfunction,where the adaptive immune system exhibits defective antigen presentation, decreased major histocompatibility complex type II (MHC II) expression, loss of phagocytic function, decreased T_H1 $CD4^+$ lymphocyte cytokine production and decreased T_H1 proliferative response to exogenous mitogens (41). However, in the face of this T_H1 immune dampening, there is a concomitant expansion of T_H2 $CD4^+$ lymphocyte cytokine production, expansion in regulatory T cell populations (42, 43), immature myeloid populations, and an increased susceptibility to secondary infections (3, 4). Insights into the

specific immune modulatory cell populations have in promoting sepsis induced immune suppression will help us understand sepsis syndromes.

Myeloid Derived Suppressor Cells (MDSCs) and Disease

Currently little is known about the origins and function of the MDSC population. The cancer literature describes them as a heterogenous, immature population of the myeloid lineage derived from stem cells and bone marrow progenitors. This population has been referred to as: "natural suppressor cells"(44), "myeloid derived suppressor cells"(45), "early myeloid cells"(46), and "inhibitory macrophages"(47) in the past decades and now the population goes by the name of myeloid derived suppressor cells. Phenotypically, these cells exhibit a high expression of cell surface markers CD11b and GR-1. However, other cells of myeloid lineage can express low levels of these receptors such as macrophages and neutrophils. Other cell surface markers including CD115, CD31, CD34, c-Kit, and F4/80 may also be displayed.

Recent studies suggest that the accumulation of MDSCs in bone marrow, spleen and lymph nodes is a conserved response to an array of disparate insults (48-50) which may explain the altered immune reactivity associated with these insults. Bronte and colleagues have postulated that MDSCs play an important role inhibiting T cell activation during the resolution phase of an inflammatory insult or an immune response (47, 48, 51). Several cytokines, such as GM-CSF, CSF-1, IL-6, IL-10 and VEGF have been shown to regulate the expansion of MDSC populations (48, 52).

Because of their relative immature and undifferentiated phenotype, there is considerable functional variability among these cells with a suppressive phenotype elicited upon exposure to T_H2 cytokines (IL-4, IL-10 and TGFβ) often increased in sepsis (47, 53). On the other hand exposure of MDSCs to T_H1 cytokines (TNFα) stimulates differentiation along macrophage pathways, and enhances T cell cytotoxic responses. For example when MDSCs obtained from

tumor bearing mice were administered into healthy naïve animals, they differentiated into normal dendritic cells and macrophages, but when administered into other tumor bearing animals, they maintained their suppressor cell phenotype (54).

Most of the information on the MDSC populations stems from the oncology literature, using human and murine tumors that result in MDSC expansion (49, 55-57). In mouse tumor models, immune suppression to growing tumors could be ameliorated by GR-1^{+}CD$11b^{+}$ cell depletion with *all trans* retinoic acid restoring T-cell responses (58, 59). MDSCs can inhibit T cell activation through cell-cell contact or immediate juxtaposition. The mechanisms of MDSC T-lymphocyte inhibition are not yet fully understood; however, they appear to depend on L-arginine metabolism to decrease T lymphocyte responsiveness to subsequent antigen stimulation (48, 49). Furthermore induction of iNOS with NO release and peroxynitrites formation only accounts for some of the T cell unresponsiveness in tumor models (60-62). Over production of arginase I by MDSCs can result in "local arginine starvation" that can inhibit T-lymphocyte proliferation (63, 64). In addition GR-1^{+} cells also secrete IL-4 and IL-10 (65), reactive oxygen species, and TGFβ, all of which can have immunosuppressive properties.

Myeloid Derived Suppressor Cell's Function During Inflammation

The role that MDSCs play in acute inflammation has undergone little investigation. Many of the proinflammatory cytokines, factors and mediators that are produced during acute inflammation could play a role on the development of MDSCs and their immune modulatory phenotype. G-CSF, GM-CSF, CSF-1, IL-4, IL-6, and IL-10 are just a few of the mediators; however, few investigators have studied the MDSC populations during sepsis. Holda and colleagues demonstrated that small injections of lipopolysaccharide markedly enhanced what he termed natural suppressor cell activity (66). Murphey, Sherwood and colleagues reported a two-

fold increase in "natural suppressor cells" in mouse spleens five days after CLP (67). Ochoa and colleagues reported a seven fold increase in the GR-1^{+}CD11b^{+} cell population in the spleens of mice 12 hours following operative trauma (68), and demonstrated that the MDSCs suppress T-cell proliferation to anti-CD3, anti-CD28 stimulation through depletion of the amino acid, L-arginine. These recent studies explain some of the earlier findings that arginase activity is increased in myeloid cells after trauma (69-72), and its increase is modulated by T_H2 cytokines often increased in trauma (73).

Hypothesis

Although the role of MDSCs in tumor-induced immune suppression is evolving, the contribution of GR-1^{+}CD11b^{+} MDSCs to the development of immune suppression in sepsis is currently unknown. We propose that expansion of the MDSC populations in the secondary lymphoid organs during sepsis contributes to the adaptive immune system defects observed in sepsis, and also serves as a target for therapeutic intervention. Preliminary research shows that MDSCs serve disparate immune modulatory roles in acute infection and tumor-associated inflammation. MDSCs simultaneously promote antigen specific T effector cell tolerance, while concomitantly modulating B cell antibody production culminating in a shift from a T_H1 to T_H2 type immune profile. Therefore, our overarching hypothesis is that MDSCs play simultaneous and competing roles in sepsis where the host utilizes their suppressive potential to dampen the magnitude of the inflammatory response facilitating an anti-inflammatory T_H2 immune polarization; however, accumulation or expansion of these populations may also lead to a compensatory anti-inflammatory immune suppression and increase the host susceptibility to secondary infections.

Specific Aim 1

To characterize the biological impact of MDSCs during a prolonged model of polymicrobial sepsis and whether the expansion of MDSC populations in the secondary lymphoid organs is dependent upon a sustained microbial infection as seen in sepsis. The goal was to determine whether a polymicrobial infection and an accompanying systemic inflammatory response provide the proper milieu to facilitate the expansion of the MDSC population in the spleen, lymph nodes, and bone marrow. Mice underwent a prolonged infectious sepsis stimulus (sublethal CLP, LD_{20}) and the MDSC numbers and activity were determined in a time course fashion at various intervals up to 16 weeks. MDSC numbers ($GR\text{-}1^{+}CD11b^{+}$) were determined and phenotypically characterized by co-staining with various myeloid markers and analyzed using flow cytometry and histologic sectioning.

Specific Aim 2

The goal of this aim was to determine the effect of MDSC expansion on the adaptive immune system in a prolonged model of polymicrobial sepsis. The suppressor cell function was evaluated by coculturing the MDSCs *in vivo* with T-cell receptor specific $CD8^{+}$ lymphocytes and measuring the T lymphocyte specific responses to antigen specific proliferative signals. Since the MDSCs must be in physical proximity with other cells to impart their immunosuppressive properties (56, 74, 75), and are known to secrete IL-4, IL-10 (65) and nitric oxide (NO) (49), we tested whether these mediators contribute directly to the MDSC suppression in sepsis by generating MDSCs from IL-4, IL-10 and iNOS (NOS2) null animals.

Specific Aim 3

The goal in this aim was to confirm the signaling pathways that are required for the expansion of the MDSC populations in polymicrobial sepsis. Our preliminary data suggests that MyD88 null animals fail to undergo peripheral expansion of their MDSC populations in sepsis;

however, the role that MyD88 signaling is playing is unclear. We demonstrate that MyD88 null mice experience the same fluctuations in bone marrow stem cells and myeloid progenitors as wild-type mice at both 1 and 7 days after sepsis distinguishing the MyD88 induced signaling defects in the bone marrow from any effects on peripheral MDSC expansion. Since the absence of MyD88 signaling only delayed, and not prohibited, the ultimate expansion of the splenic MDSC population, we attempted to investigate other signaling pathways that are involved in myeloid cells proliferation and differentiation during chronic sepsis. We found that signaling through MyD88 and the SDF-1 pathways, but not TLR4, TRIF, IFNα/β receptor, CCR2 receptor, IL-10, IL-4 or the M-CSF pathways are required for complete GR-$1^{+}$$CD11b^{+}$ expansion.

CHAPTER 2
EXPANSION OF AN IMMATURE GR-1^{+}CD11B^{+} POPULATION INDUCES T-CELL SUPPRESSION AND T_H2 POLARIZATION IN SEPSIS

Specific Aim 1

As discussed earlier, we set out in Specific Aim 1 to characterize the biological impact of MDSCs during a model of prolonged polymicrobial sepsis and whether the expansion of MDSC populations in the secondary lymphoid organs is dependent upon a sustained microbial infection as seen in sepsis. The goal was to determine whether a polymicrobial infection and an accompanying systemic inflammatory response provide the proper milieu to facilitate the expansion of the MDSC population in the spleen, lymph nodes, and bone marrow. The proceeding experimental design was developed to achieve the goals set forth in Specific Aim 1.

Introduction

Sepsis is the systemic inflammatory response to severe microbial infection. It is well recognized that patients with sepsis are often immune suppressed, as illustrated by failure to eradicate their primary infections, a predisposition to develop secondary nosocomial infections, and an attenuated delayed type hypersensitivity response (4, 76). Animals with polymicrobial sepsis also exhibit widespread dysfunction in both antigen-presenting cells and T-lymphocytes. Reduced CD4^{+} T-cell numbers due to apoptosis-induced depletion (20, 77) and a suppression of T-cell proliferative responses (43) have been shown to contribute to sepsis-associated morbidity. Moreover, interest has focused on the shift from a T_H1 to a T_H2 profile as contributing to sepsis-associated immune dysfunction (78, 79).

The role that suppressor cell populations play in polymicrobial sepsis is unknown. The present report examined the role of myeloid derived suppressor cells in sepsis, and their contribution to the sepsis-induced defects in acquired immunity. Myeloid derived suppressor cells with suppressor functions have been previously observed in the spleens and tumors of mice

with transplantable tumors (48, 80) and in models of chronic inflammation (50). In tumor-bearing mice, these cells contribute to the tumor-associated antigen specific T-cell dysfunction and tolerance (54, 59, 74, 80-82). Splenic GR-1$^+$CD11b$^+$ cells may also play an instrumental role in priming of B cell antibody production (65).

However, there has been only modest exploration of these cell populations in sepsis or other acute inflammatory processes. Here, we observed that an ongoing septic process induces a dramatic expansion of the GR-1$^+$CD11b$^+$ population in bone marrow, spleen and lymph nodes. The splenic infiltration with these cells was associated with splenic enlargement and lymphoid follicle disruption. The GR-1$^+$CD11b$^+$ cells contained immature progenitors and expressed IL-10, TNFα and other cytokines and chemokines. Furthermore, using a depleting antibody, we demonstrated that expansion of GR-1$^+$ cells in vivo contributed to the induced T_H2 polarization of antibody responses following sepsis. These cells were also capable of causing CD8$^+$ T-cell tolerance, as demonstrated by the suppression of antigen specific interferon-γ (IFN-γ) production by CD8$^+$ T-lymphocytes in non-septic immunized mice. Finally, we observed that signaling through MyD88, but not TLR4, TRIF, or the IFNα/β receptor, was required for the early and full expansion of this cell population, highlighting the importance of inflammation and TLR signaling other than that induced by microbial endotoxin in the regulation of myeloid derived suppressor cells in sepsis.

Results

GR-1$^+$CD11b$^+$ Cells Accumulate in the Spleen after Sepsis

To examine the long-term effects of polymicrobial sepsis on the expansion of the GR-1$^+$CD11b$^+$ populations, the studies were conducted in a murine model of polymicrobial sepsis (generalized peritonitis) induced by ligation of the cecum and a double enterotomy created with a 27 gauge needle. Mortality in this model was approximately 10-20%, and occurred

predominantly in the first 96 hours; thereafter, surviving mice developed abscesses surrounding the devitalized cecum. As shown in Figure 2-1, the presence of sepsis was confirmed for at least ten days by a transient bacteremia (lasting 24 hours) and prolonged bacterial contamination of the peritoneal cavity. The animals exhibited a significant early leukopenia, followed by a profound granulocytosis, Figure 2-2. Plasma cytokine concentrations over the first ten days were consistent with an early exaggerated systemic inflammatory response, and by sustained elevations in the plasma IL-6, KC and MIP-1α concentrations, Figure 2-3.

Interestingly, surviving septic mice developed a dramatic splenomegaly with the spleen mass increasing by 300% 10 days after initiation of polymicrobial sepsis, Figure 2-4, panel A. The dramatic increase in spleen mass suggested an expansion of one or more cell populations within the spleen. Periodic histological analysis of the spleens of mice over 10 days of sepsis, Figure 2-4 panel B, demonstrated that the apparent splenomegaly was associated with extramedullary hematopoeisis and marked expansion of immature myelomonocytic cells, including forms with ringed nuclei in the periarteriolar sheaths and subcapsular space, with the focal involution of lymphoid follicles. Analysis of lymphoid and non-lymphoid cell populations did not reveal an increase in the number of cells expressing CD3, B220, or CD11c. However, a dramatic accumulation of cells expressing GR-1 and CD11b occurred. As shown in Figure 2-5, splenocytes harvested from septic mice at various intervals after cecal ligation and puncture, or sham procedures, exhibited a striking increase in the percentage and absolute number of GR-1^{+}CD11b^{+} cells (Panels A, and B). The dramatic increases in the percentage and absolute numbers of GR-1^{+}CD11b^{+} cells did not occur until at least three days after the induction of sepsis, and the percentages continued to increase to a plateau, until about 7-10 days. The numbers and proportion of these cells remained elevated even out to 12 weeks in surviving mice.

By ten days, the absolute numbers of these GR-1$^+$CD11b$^+$ cells in the spleen had increased 50-fold.

GR-1$^+$CD11b$^+$ Cells are Phenotypically, Heterogenous Cells

GR-1$^+$CD11b$^+$ cells represent a heterogenous population of cells encompassing mature and immature myeloid forms. To further characterize these GR-1$^+$CD11b$^+$ cells, splenocytes were also stained for CD31, a marker of immature myeloid development that is lost with more terminal cell differentiation (83), and Ter119, a marker of erythroid lineage, as well as F4/80, a marker for myeloid lineage development. As shown in Figure 2-5, panel C, approximately 40% of the GR-1$^+$CD11b$^+$ cells were also CD31 positive, and the numbers of GR-1$^+$CD11b$^+$CD31$^+$ cells were increased nearly 70-fold during sepsis. Similar results were obtained examining the GR-1$^+$CD11b$^+$F4/80$^+$ triple positive cells in the spleen and bone marrow (*data not shown*), suggesting that the GR-1$^+$ cells contained a subpopulation of developing myeloid cells. In contrast, only 6% of the GR-1$^+$CD11b$^+$ positive cells were Ter119$^+$, suggesting that only a small proportion of these cells may still possess the ability to differentiate into cells of erythroid lineage, Figure 2-6. Interestingly, however, less than 3% of these GR-1$^+$CD11b$^+$ cells were also MHC II$^+$, Figure 2-7.

As shown in Figure 2-8, panel D, the enriched GR-1$^+$ cell population from the spleens of septic mice contained large numbers of phenotypically heterogeneous cells. Many of these cells had characteristic circular or 'ringed-shaped' nuclei. Gauging on their nuclear size, complexity, and cytoplasmic granularity as described previously in the literature, most of these cells were determined to be immature myeloid forms (18). These cells were identified on the cell-sorted cytospin preparations.

To examine whether the GR-1$^+$CD11b$^+$ population were an immature proliferating precursor population sensitive to myeloid growth factors, the GR-1$^+$ enriched splenocytes from

septic mice were cultured ex vivo on 24 well-plates with GM-CSF. In the absence of GM-CSF, these cells rapidly died. In contrast, seven days of culture with GM-CSF led to approximately 17% of these cells differentiating into conventional $CD11c^{high}MHCII^{high}$ dendritic cells and approximately 22% differentiating into $F4/80^{high}$ macrophages (Figure 2-8 Panels A, B and C) and all of these cells demonstrated increased MHCII expression over freshly isolated $Gr\text{-}1^{+}CD11b^{+}$ cells. In addition, culturing these $GR\text{-}1^{+}$ cells from septic mice in soft methylcellulose with either GM-CSF or G-CSF, but not erythropoietin, for seven days led to a significant increase in the numbers of colonies formed (Figure 2-8 Panel E). Interestingly, the $GR\text{-}1^{+}$ cells from sham-treated mice did not contain any significant number of progenitors capable of forming colonies in response to these growth factors. The failure of $GR\text{-}1^{+}$ cells from septic mice to generate a significant number of colonies in response to erythropoietin, and the low $Ter119^{+}$ staining, suggest that during sepsis, these expanded numbers of $GR\text{-}1^{+}CD11b^{+}$ cells represent a mixed population of immature, proliferating, progenitors committed predominantly to a myeloid, and not an erythroid, pathway.

Myeloid derived suppressor cells Accumulate in Secondary Lymphoid Organs after Sepsis.

We also looked for these $GR\text{-}1^{+}CD11b^{+}$ cells in other secondary lymphoid and reticuloendothelial organs. For these cross-sectional analyses, we selected intervals after cecal ligation and puncture or a sham procedure when the changes in the spleen were maximal. No significant increases in $GR\text{-}1^{+}CD11b^{+}$ cells were seen in either the liver or lung (*data not shown*). In peripheral lymph nodes, however, marked increases in the percentage and numbers of these $GR\text{-}1^{+}CD11b^{+}$ cells were evident at 10-14 days after sepsis, and were still increased at 12 weeks (Figure 2-9, Panel B and C). Numbers remained significantly elevated in mesenteric lymph nodes in direct proximity to the cecal ligation and puncture at 12 weeks, whereas numbers declined somewhat, but still remained significantly elevated, in more distal inguinal and axillary

lymph nodes at 12 weeks. Furthermore, in the bone marrow (Figure 2-9, Panel A), the numbers of these GR-1^{+}CD11b^{+} cells doubled within three days, and by seven days after sepsis, accounted for nearly 90% of the cells in the bone marrow.

Histological confirmation of these cells in the spleens of mice with severe sepsis is shown in Figure 2-10 panels A, B, and C. Over the course of 10 days, there was progressive expansion of the red pulp by extramedullary hematopoiesis and marked expansion of the periarteriolar sheaths (Figure 2-10, Panel B) and subcapsular space (Figure 2-10, Panel C) by immature mononuclear cells and myelomonocytic cells with ringed nuclei. These cells were also found in small clusters within the interfollicular areas. The myeloid derived suppressor cells with ringed nuclei in all of these locations were uniformly CD11b^{+} in the spleens of mice that underwent cecal ligation and puncture (Figure 2-10 Panel D, E, and G). In contrast, CD11b showed only scattered reactivity in the sham treated mice, mainly highlighting mature granulocytes within the interfollicular areas (Figure 2-10, Panel E). The increasing numbers and overall percentage of immature myelomonocytic cells with ringed nuclei correlated with the time progression after sepsis and was associated with cuffing in the perivascular/periarteriolar sheaths and subcapsular spaces. Finally, there was focal involution of the lymphoid follicles that paralleled the expansion of the red pulp. No immature myelomonocytic cells with ringed nuclei were identified within the follicular areas.

Myeloid derived suppressor cells are Capable of Inflammatory Mediator Production

Since myeloid derived suppressor cells obtained from tumor bearing hosts are known to be immunomodulatory (48, 80), we next examined whether cells obtained from septic mice could produce immunosuppressive and inflammatory mediators, including IL-10, a cytokine generally regarded as a requisite for T-cell suppression and T_H2 polarization during sepsis. When GR-1^{+} enriched splenocytes from septic mice were cultured *ex vivo*, they produced low levels of several

inflammatory mediators, including IL-10, TNF-α, RANTES (CCL5) and MIP-1β (CCL4). When stimulated ex vivo with bacterial lipopolysaccharide, the GR-1^+ cells from septic mice produced significantly greater amounts (>5 fold) of IL-10 than similar GR-1^+ cells from sham-treated animals (Figure 2-11). They also produced increased quantities of TNF-α, RANTES and MIP-1β but did not produce increased quantities of other T_H2 cytokines IL-4 or IL-13. Unstimulated or lipopolysaccharide stimulated GR-1^+ splenocytes also did not produce measurable quantities of GM-CSF, IL-12p40, IL-12p70, IL-2, IL-3, IL-5, IL-9, IL-17, VEGF or IFN-γ (*data not shown*). In addition, GR-1^+ splenocytes obtained from septic mice also produced TNFα (218 ± 67 vs 4 ± 1 pgs/ml) and IL-10 (165 ± 43 pgs/ml) in response to stimulation with 5 μg/ml flagellin (TLR5 agonist), albeit in lesser quantities than with LPS stimulation.

Myeloid Derived Suppressor Cells Affect Adaptive Immune Responses In Sepsis

To examine whether these cells could affect adaptive immune function, sham-treated and septic mice were immunized with NP-KLH using the adjuvant alum when GR-1^+ cells reached a maximal proportion in the spleen (ten days following cecal ligation and puncture), and the NP specific serum immunoglobulin response determined ten days later. The serum immunoglobulin response to NP-KLH with alum is a T-cell dependent, B-cell response, and the immunoglobulin isotypes can be used to assess polarization of the T-cell response (19). Polymicrobial sepsis was not associated with any significant changes in total IgM or IgG responses (Figure 2-12, Panel A and B); however, when the total IgG response was dissected into its isotypic components, the serum IgG_{2a} response was significantly decreased while the IgG_1 response increased in the septic mice, consistent with a shift from a T_H1 to a T_H2 T-cell response (Figure 2-12 Panel C and D). When the septic mice were treated with a GR-1 depleting antibody, producing a greater than 80% reduction in total GR-1^+CD11b^+ splenocytes (Figure 2-13), the sepsis-induced increase in IgG_1 and the decrease in IgG_{2a} responses were abolished, demonstrating the involvement of the

GR-1$^+$ cells in this polarization. As expected by the T-cell dependent nature of this antibody response, depletion of CD4$^+$ cells significantly attenuated the IgM responses and completely prevented the IgG class switching in the septic animals (Figure 2-14, Panels A, B, C, and D).

To further confirm the in vivo role that these cells play in suppressing an antigenic T-cell response, the effect of GR-1$^+$ cells on the CD8$^+$ T-cell IFN-γ response by splenocytes from OT-I TCR-transgenic mice (C57BL/6-Tg(TCRαTCRβ)1100mjb) immunized with OVA-derived peptide (H-2Kb restricted, aa 257–264, SIINFEKL) was examined (Figure 2-15 Panel A). GR-1$^+$ cells were obtained from either ten day septic or sham-treated mice, and were infused into C57BL/6 mice that had previously received CD8$^+$ T-cells from OT-1 TCR-transgenic mice, and simultaneously immunized with OVA-derived specific peptide. Ten days later, the spleens from these animals were removed, and IFN-γ responses to ex vivo stimulation with OVA-derived specific peptide were examined. IFN-γ production was markedly reduced when the animals were administered GR-1$^+$ splenocytes from septic animals when compared to sham-treated mice, confirming that GR-1$^+$ splenocytes from these septic mice could suppress a CD8$^+$ T-cell IFN-γ response.

To determine whether GR-1$^+$ cells could directly suppress an antigen specific or nonspecific CD4$^+$ T cell proliferative response, D011.10 OVA-TCR transgenic mice were made septic and at 10 days, CD4$^+$ splenocytes from septic mice were cultured with irradiated GR-1$^+$ containing antigen presenting cells from the spleens of 10 day septic, sham-treated, and control mice, and were incubated with either OVA peptide, bovine serum albumin or on CD3/CD28 coated plates. As shown in Figure 2-15, Panel B, culturing CD4$^+$ cells with irradiated GR-1$^+$ cells from septic mice only modestly, but still significantly, reduced both the antigen-specific (OVA) and non-specific (CD3/CD28) proliferative responses.

MyD88 Signaling Pathways Are Required for GR-1$^+$CD11b$^+$ Cells to Accumulate in the Spleen after Polymicrobial Sepsis.

Polymicrobial sepsis produced by cecal ligation and puncture releases a large number of microbial products that are recognized by the innate immune system, in large part through toll-like receptor signaling pathways. To examine the cell signaling pathways required to elicit the expansion of these immunomodulatory GR-1$^+$CD11b$^+$ cells, wild-type C57BL/6 mice were first injected with a sublethal dose (5 mg/kg BW) of the TLR4 agonist, bacterial lipopolysaccharide. The numbers of these GR-1$^+$CD11b$^+$ splenocytes were examined at daily intervals after lipopolysaccharide injection. As shown in Figure 2-16, panel A, the administration of bacterial lipopolysaccharide produced a more rapid, but transient increase in the percentage and absolute numbers of GR-1$^+$CD11b$^+$ splenocytes suggesting the likely pathway involves TLR4 signaling. The expansion of GR-1$^+$CD11b$^+$ splenocytes by lipopolysaccharide occurred by 24 hours, reached a peak by five days, and declined thereafter until it reached a near-baseline level by day 10. Interestingly, the increased numbers of splenic GR-1$^+$CD11b$^+$ cells were very modest compared to mice undergoing a cecal ligation and puncture, and even the sham procedure produced some modest increase in GR-1$^+$CD11b$^+$ cell numbers.

To determine whether TLR4 signaling is a requirement for the expansion of these cell populations in our model of polymicrobial sepsis, cecal ligation and puncture was performed in C3H/HeJ mice that have a spontaneous mutation in TLR4, and the results were compared to C3H/OuJ mice. As shown in Figure 2-16, Panel B, TLR4 mutant C3H/HeJ mice had a comparable increase in splenic GR-1$^+$CD11b$^+$ cell numbers as in the C3H/OuJ controls at seven days, suggesting that intact TLR4 signaling is not required for the early expansion of these GR-1$^+$CD11b$^+$ cells in sepsis. Thus, although lipopolysaccharide signaling via TLR4 can induce an

expansion of this GR-1^{+}CD11b^{+} cell population, TLR4 signaling is not required during polymicrobial sepsis, and there are redundant signaling pathways.

Such findings are not completely unexpected since polymicrobial sepsis is generally associated with the release of large numbers of different microbial products that can signal simultaneously through a number of different TLR receptors. Since TLR signaling occurs through MyD88- and TRIF-dependent pathways, and may involve the secretion of type I interferons, cecal ligation and puncture was also performed in MyD88$^{-/-}$, TRIF$^{-/-}$ and IFNα/βR$^{-/-}$ mice. The increased expansion of GR-1^{+}CD11b^{+} cells after cecal ligation and puncture at seven days was markedly attenuated in only the MyD88$^{-/-}$ (B6x129) mice and not in either the TRIF$^{-/-}$ or IFNα/βR$^{-/-}$ mice, highlighting the requirement of MyD88 signaling for the early expansion of this cell population during sepsis (Figure 2-16, Panel C, D and E).

To confirm that the requirement for MyD88 signaling was not dependent upon the background of the animals, and was sustained during prolonged sepsis, the studies were repeated in MyD88$^{-/-}$ animals backcrossed onto a C57BL/6 background. As shown in Figure 2-17, after seven days of sepsis, there was again no expansion of the GR-1^{+}CD11b^{+} population in the MyD88$^{-/-}$ (B6) mice. By 14 days, there was some expansion of the GR-1^{+}CD11b^{+} cell populations, but it was still markedly attenuated when compared to the wild-type B6 controls.

Discussion

T-cell dysfunction is a common response to polymicrobial sepsis (4), ultimately leading to increased susceptibility to ongoing and opportunistic infections, and poor outcome. Recent attention has focused on the shift from a more proinflammatory T_H1 to a more anti-inflammatory T_H2 immune profile as an explanation for post-sepsis immune suppression; however, the underlying mechanisms that orchestrate this T-cell suppression and T_H2 polarization during sepsis are still unknown.

Although there has been some speculation that endogenous and inducible T-regulatory cells may contribute to the T-cell suppression and T_H2 polarization in sepsis (42, 84), more recent studies have refuted some of those claims (85, 86). Several years ago, Murphey and colleagues observed an increased number of macrophage-like cells in the spleens of mice surviving a cecal ligation and puncture (67), although they did not explore their suppressor cell function. More recently, Makarenkova and colleagues observed increased numbers of myeloid derived suppressor cells in the spleens of mice within 12 hours of a traumatic injury, and identified them as a source of arginase I activity (87).

Using a model of polymicrobial sepsis that produces only limited early mortality but sustained inflammation as determined by elevated plasma cytokine concentrations and neutrophilia, we observed a profound splenomegaly that persisted for weeks in surviving animals. On closer examination, it became evident that associated with this ongoing septic process, there was marked disintegration of the follicular regions, increased extramedullary hematopoiesis, and replacement of the splenic cellularity with large numbers of myeloid derived suppressor cells (Figure 2-5). Hotchkiss and others, including ourselves, had previously shown that in similar models of polymicrobial sepsis, there is a rapid apoptotic loss of $CD4^+$ T-cells and dendritic cells in the first 24 hours of sepsis, and these cellular losses contribute to the adverse outcome (17, 21, 88). Although there has been considerable exploration into the role of extramedullary hematopoiesis in chronic infectious and inflammatory processes, none have explored or even described this massive expansion of an immature myeloid cell population ($GR\text{-}1^+CD11b^+CD31^+$) in the bone marrow, spleen and lymph nodes of mice with ongoing septic processes. We have clearly shown that by ten days after cecal ligation and puncture, almost 40% of the spleen and 90% of the bone marrow cellularity represent immature $GR\text{-}1^+CD11b^+$ cells.

Interestingly, the administration of near lethal doses of bacterial lipopolysaccharide could also produce some expansion of this immature myeloid cell population. However, the increases were transient and modest compared to that seen in the septic animals, suggesting that an ongoing inflammatory process may be required for complete manifestation of the response (50).

The phenotype of these cells, the kinetics of their expansion, and their anatomical location in the spleen argue against these cells being functionally or phenotypically similar to those reported previously by Makarenkova and colleagues (68) immediately after trauma, or as merely components of extramedullary hematopoiesis. Makarenkova et al. observed a rapid influx of $GR1^{+}CD11b^{+}$ cells into the spleens of mice 12-24 hours after traumatic injury. However these cells, which produced large quantities of arginase I, were located in the mantle surrounding the lymphocyte rich follicles, and very few ring cells were detected. In contrast, we saw a transient decline in $GR1^{+}CD11b^{+}$ splenocytes in the first 24 hours of sepsis, and only saw expansion of our splenocyte population after three to five days of sepsis. Furthermore, although both populations are clearly heterogenous, our cells contained higher proportions of immature precursors that were concentrated in perivascular/periarteriolar and subcapsular regions of the spleen, exhibited less MHC class II expression, and made copious amounts of IL-10 when stimulated ex vivo. These cells obtained from the spleens of septic animals contained predominantly precursors committed to a myeloid and not erythroid lineage. Less than 6% of the $GR\text{-}1^{+}CD11b^{+}$ cells recovered from the spleens of septic mice were $Ter119^{+}$ positive, and when cultured in soft methylcellulose with erythropoietin, had only a minimal capacity for colony or burst formation (Figure 2-6). In contrast, almost 40% of the cells differentiated into macrophages or dendritic cells when cultured with GM-CSF (Figure 2-8 panel C), and large

numbers were capable of forming colonies when incubated in soft methylcellulose with either G-CSF or GM-CSF (Figure 2-8 panel E).

This dramatic expansion of the GR-1^+CD$11b^+$ response to sepsis in the spleen is very similar to the response previously observed in mice with actively growing tumors. Similar immature myeloid cell populations have been shown to accumulate in the spleens, infiltrating into tumors in several animal tumor models (54, 55, 74, 81, 82, 89), and also in the blood of some patients with cancer (90). In mice with transplantable tumors, these myeloid-derived suppressor cells, as they are now being termed (91), have also been shown to inhibit antigen-specific and nonspecific T-cell functions via several different mechanisms, including arginase I, nitric oxide, reactive oxygen species, and TGFβ.

Given the abilities of myeloid derived suppressor cells to facilitate immune suppression in murine cancer models, as well as to suppress antigen-specific T cell responses and to influence B cell antibody production, the expansion of these immature myeloid populations in sepsis may similarly orchestrate the T_H1 to T_H2 immune polarization that is known to occur in sepsis. Challenging mice with T-cell dependent antigens, such as NP-KLH, offers the opportunity to explore in vivo the shift in antibody class switching from IgG_{2a} to IgG_1 production, which is dependent upon cytokines including IFNγ and IL-4, and reflects this predilection towards a T_H2 versus a TH_1 $CD4^+$ T-cell response (65, 92). Ten days after sepsis, immunization with NP-KLH led to an increase in the IgG_1 production at the expense of IgG_{2a}, consistent with a preferential T_H2 response. In contrast, partial depletion of the GR-1^+ cells in vivo blocked the characteristic increase in the IgG_1 secretion while also preventing the fall in the IgG_{2a} responses, demonstrating a contributory role for these GR-1^+ cells in this shift from a T_H1 to T_H2 response.

Consistent with these findings was the observation that GR-1^+ cells from septic mice significantly attenuated the IFN-γ response by $CD8^+$ T-cells to specific antigenic stimulation. These results are very similar to the suppression of cytotoxic T-cells to antigen-specific stimulation by GR-1^+ cells obtained from tumor-bearing hosts (81). Surprising, however, were the only modest reductions in antigen-specific $CD4^+$ T-cell proliferation that we saw when GR-1^+ cells were administered to DO11.10 $CD4^+$ OVA transgenic mice immunized with OVA peptide. The answer may simply be that these GR-1^+ cells express very little MHC class II, although they retain relatively high levels of MHC class I expression (81). Therefore, in the DO11.10 $CD4^+$ OVA transgenic mouse model, these GR-1^+ cells were probably unable to present antigen via MHC class II, and therefore, could not directly affect $CD4^+$ T-cell proliferative responses. In contrast, they could present antigen in the context of class I expression and suppress $CD8^+$ T-cell IFN-γ responses.

Interestingly, the numbers of myeloid derived suppressor cells decreased in mice in which the abscess had spontaneously resolved 12 weeks after sepsis. Furthermore, when these same animals with abscess resolution were immunized with NP-KLH and alum, the antibody response more closely approximated baseline isotype levels Figure 2-18. Similar decreases in the numbers of myeloid derived suppressor cells have been reported in tumor bearing animals upon resection of their primary tumor (93).

The similarities in the appearance of these myeloid-derived suppressor cells in sepsis, and in animals with tumors or other chronic inflammatory processes, suggest that there are common signals involved in the expansion of these cell populations. Polymicrobial sepsis produced by a ligation of the cecum and expression of fecal contents releases large quantities of microbial products into the peritoneum and systemic circulation. It is not at all surprising that

polymicrobial sepsis produced a comparable expansion of the $GR1^{+}CD11b^{+}$ population in C3H/HeJ mice lacking a functional TLR4 receptor, suggesting that although lipopolysaccharide contributes to the septic response in wild-type mice, signaling through TLR4 is not required during polymicrobial sepsis as signaling through other TLRs by pathogen associated molecular patterns likely also contributes to the expansion of these cell populations. Considering the fact that mice with defective TLR4 signaling also exhibit comparable mortality to wild-type mice after sublethal polymicrobial sepsis (94), the findings suggest that neither murine expansion of myeloid-derived suppressor cells nor survival after sepsis are entirely TLR4 dependent.

Two major pathways activated by TLR signaling include the induction of both inflammatory mediators through MyD88 and type I interferon production. Since both MyD88 and TRIF pathways can lead to type I interferon production, mice deficient in the type I IFN receptor were used to ascertain the role of type I IFN in sepsis-induced myeloid cell accumulation. The increases in the $GR1^{+}CD11b^{+}$ population were unaffected in mice lacking TRIF or IFNα/βR signaling, indicating that TLR signaling to Type I interferons is not important in the induction of these cells. However, their expansion was completely prevented for the first seven days in septic mice lacking MyD88 signaling, and was attenuated after 14 days. The studies were confirmed in $MyD88^{-/-}$ mice on two backgrounds B6 and B6x129(F1)) to assure that these findings were not strain dependent. This observation in particular suggests that the expansion of this cell population represents a fundamental component of inflammatory signaling in the host innate immune response to TLR ligation by pathogens. The observation that even $MyD88^{-/-}$ mice could expand their cell population to some extent after a cecal ligation and puncture reveals the fundamental nature of this response and the redundant signaling pathways involved in its invocation. In response to microbial products released during cecal ligation and

puncture, activation of inflammatory signaling through MyD88, presumably through ligation of multiple TLR receptors, plays some still indeterminant role in the expansion of these cell populations.

In summary, the results demonstrate for the first time that the numbers of GR-1^+ $CD11b^+$ cells increase dramatically in the spleen, lymph nodes and bone marrow during polymicrobial sepsis, and remain elevated for up to 12 weeks. These cells are heterogenous, metabolically active, can secrete a number of cytokines, and are immature, but are predominantly committed to development along myeloid pathways. Signaling through MyD88 is required for the full expansion of these cell populations, with an incomplete increase in the numbers of these cells in the spleen or bone marrow in the absence of MyD88 signaling. In mice, these cells contribute to the T-cell suppression seen after sepsis by suppressing $CD8^+$ T cell IFNγ production, and the repolarization from a T_H1 to a T_H2 type immune response exhibited by augmenting B cell antibody production towards IgG_1 (T_H2) and away from IgG_{2a} (T_H1). What remains unresolved is whether a comparable expansion of these immature myeloid populations also occurs in human sepsis, and contributes to the immune suppression and polarization that occurs. Further studies will be required to determine whether these findings translate to a better understanding of the human innate and adaptive immune responses to severe sepsis.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committees at the University of Florida College of Medicine, Rhode Island Hospital and Brown University or Schering-Plough Biopharma. Specific pathogen-free C57BL/6 mice, C3H/HeJ mice (TLR4 receptor mutation) and their control mice (C3H/HeOuJ), OT-1 TCR transgenic mice C57BL/6-Tg(TcrαTcrβ)1100Mjb/J, D011.10 OVA TCR transgenic mice (BALB/c-TgN(DO11.10)10Loh)

and B6.129PF1/J mice were all purchased from The Jackson Laboratory (Bar Harbor, ME). IFN-αβR/A129 mice on the 129S6/SvEv background (H-2b) and wildtype Sv129 mice were purchased from B & K Universal (Hull, East Yorkshire, UK). All mice were maintained at the University of Florida College of Medicine. MyD88$^{-/-}$ mice on a B6x129(F1) background and TRIF$^{-/-}$ mice were a kind gift of Dr. Shizuo Akira to Schering-Plough Biopharma, and were maintained at Schering-Plough Biopharma, Palo Alto, CA. MyD88$^{-/-}$ mice on a B6 background were obtained from Dr. Shizuo Akira, and maintained at Rhode Island Hospital and Brown University.

Cecal Ligation and Puncture

For induction of polymicrobial sepsis, mice underwent a cecal ligation and puncture or sham procedure as previously described (4, 32) to obtain a mortality of 10-20% by 10 days. At various intervals, bacterial counts from the blood and peritoneal wash (3 mls of phosphate-buffered saline) were determined by culturing aliquots on sheep RBC-agar plates. Total and differential white blood cell counts were also determined using an automated cell counter. Plasma cytokines were determined by Luminex™ technology using reagents obtained from Upstate Cell Signaling Solutions (Beadlyte™ Mouse Multi-Cytokine Detection System) (Temecula, CA).

Flow Cytometry

Spleens, lymph nodes and bone marrow cells were analyzed by flow cytometry as previously described (95). Antibodies included anti-GR-1 (Ly6G and Ly6C (RB6-8C5)) conjugated to APC, anti-CD11b (Integrin aM, chain Mac-1a chain (M1/70)) conjugated to FITC, anti-MHC II (I-A/I-E (2G9)) conjugated to FITC, anti-F4/80 Antigen (Pan Macrophage Marker (BM8)) conjugated to PE, Ter119 conjugated to FITC (clone ter119) and anti-CD11c (N418) conjugated to APC, Fc-Block (CD16/CD32 Fc g III/II Receptor (2.4G2), and 7 amino actinomycin D

(7AAD) F4/80 and CD11c specific antibodies were purchased from eBioscience all other antibodies were purchased from BD Pharmingen.

Cell Purification

All magnetic bead kits were obtained from Miltenyi Biotec. Erythrocyte-depleted splenocytes and lymphocytes were isolated using either anti-GR-1 (Ly6G and Ly6C (RB6-8C5)) conjugated to APC followed by anti-APC MicroBeads for GR-1^+ splenocytes. Anti-CD8a (Ly-2) MicroBeads were used alone for OT-1 $CD8^+$ splenocyte isolation.

Ex vivo Stimulation and Cytokine Production

Enriched GR-1^+ cells were plated at 1 x 10^6 cells/well with RPMI 1640 supplemented with 10% fetal calf sera, 2 mM L-glutamine, 200 units/ml penicillin and 50 µg/ml streptomycin, and stimulated with 10 µg/ml of bacterial lipopolysaccharide (E. coli 0111:B4). The culture supernatant was analyzed for cytokines.

Ex vivo Differentiation and Colony Formation

1 x 10^6 GR-1^+ splenocytes positively enriched and cultured with RPMI 1640 medium supplemented with 10% fetal calf sera, 2 mM L-glutamine 200 units/ml penicillin and 50 µg/ml streptomycin. Cells were stimulated for 7 days with 10 ng/ml of GM-CSF (R&D Systems). The cells were phenotyped by flow cytometric analyses.

For the colony forming assays, 1 x 10^5 GR-1^+ splenocytes from sham and septic mice were cultured in Methocult™ methylcellulose media (Stem Cell Vancouver, Canada) containing recombinant mouse G-CSF, GM-CSF, or erythropoeitin (R&D Systems; all at 10 ng/ml) for ten days. Colonies containing >30 cells were enumerated.

Antigen Specific $CD8^+$ T-Cell IFNγ Production

3–5 x 10^6 purified T-cells from OT-1 TCR transgenic mice were injected i.v. into naive C57BL/6 recipient mice. Two days later, mice were injected i.v. with 5 x 10^6 GR-1^+ cells

obtained ten days after sepsis or sham procedure. Mice were immunized subcutaneously with the specific peptide (100 μg of OVA-derived peptide SIINFEKL) mixed with Incomplete Freund 's Adjuvant. Ten days later, lymph node and spleen cells were isolated, and restimulated in vitro with specific (OVA-derived peptide SIINFEKL) or control peptide (RAHYNIVTF), and analyzed via IFN-γ ELISpot.

Enzyme-Linked Immunospot Assay (ELISpot)

Millipore MultiScreen HA plates were coated with and blocked with phosphate-buffered-saline with 1% bovine serum albumin prior to plating. Spleen and lymph node cells were plated at 2.5 x 10^5 cells/well with HL-1 (Cambrex) supplemented with 2 mM L-glutamine, 200 units/ml penicillin and 50 μg/ml streptomycin. Cells were stimulated with either 10 μM OVA257-264 or bovine serum albumin for 48 hours. The cells were treated with 1 μg/ml biotinylated anti-mouse-IFN-γ ((XMG1.2) BD Bioscience), then a 1:1000 dilution of strepavidin-alkaline phosphatase conjugate and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Pierce). The spots per well were manually counted.

Antigen Specific and Nonspecific $CD4^+$ T-cell proliferation

The MHC class II-restricted OVA T-cell transgenic mouse strain DO11.10 was utilized to determine whether $GR\text{-}1^+$ cells suppress antigen specific or nonspecific $CD4^+$ T-cell proliferation. DO11.10 transgenic mice were made septic and immunized with 100 μg OVA323–339 peptide (Genscript) in alum. At day 7, erythrocyte-depleted splenocytes and lymph node cells underwent $CD4^+$ T-cell purification by positive selection (>98% purity). The cells were irradiated with 3000 rads. 2 x 10^5 cells from sham-treated mice (containing <1x10^4 $GR\text{-}1^+$ cells) or 2 x 10^5 cells from septic mice (containing 7.5 x 10^4 $GR\text{-}1^+$ cells) were mixed with 2.5 x 10^4 $CD4^+$ T cells. Cells were restimulated with 10 μg/ml OVA323–339 peptide or anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) or 10 μg/ml of bovine serum albumin for 48 hours. In the final

16 hours of culture, 1 μCi ^{3}H-thymidine (Amersham Biosciences,) was added. Proliferation was determined by the incorporation of 3H-thymidine in the cell coculture.

Immunization with NP-KLH and Humoral Immune Responses

An NP-KLH immunization model was used as described by Hurov and colleagues (96). When indicated, animals were depleted of either GR-1^{+} cells or CD4^{+} T-cells as described above. At day 10 post surgery, mice were immunized subcutaneously with 100 μg of the T-cell-dependent antigen 4-hydroxy-3-nitrophenyl acetyl–keyhole limpet hemocyanin (NP-KLH) (Biosearch Tech) and alum. Serum titers of NP specific antibodies were determined by ELISA. Immulon 4HBX 96 well plates (Dynex Tech) were coated with 1 mg of NP-bovine serum albumin (Biosearch Tech) per ml. NP-specific antibodies were bound to biotin-conjugated goat anti-mouse Ig isotype antibodies, anti-IgM, anti-IgG1, anti-IgG_{2a}, and anti-IgG_3 (CalTag). Streptavidin-conjugated horseradish peroxidase was incorporated to detect the biotin-Ig with 2,2'-azino-di(3-ethylbenzthiazolinesulfonate) (ABTS) substrate.

Morphologic and Histologic Analysis

For cytosipns 1 x 10^{5} enriched GR-1^{+} splenocytes were stained using One Step II Wright-Giemsa Stain Solution® (Criterion Sciences). Hematoxylin and eosin staining tissues were fixed in 10% neutral buffered formalin with 0.03% Eosin (Sigma Aldrich), paraffin embedded, sectioned (5 μm) and mounted for staining. For immunohistochemical staining, spleens were mounted in Tissue Tek O.C.T. compound (Sakura Finetek), and flash-frozen. 4 μm cryosections were stained with a rat anti-mouse CD11b at a 1:50 dilution (BD Pharmingen), followed by a biotinylated rabbit anti-rat (Vector Laboratories) secondary antibody, and visualized using Vulcan Fast Red (VFR) (Biocare Medical). Slides were counterstained with hematoxylin (Vector Laboratories) followed by bluing with Tris-buffered saline.

Statistics

Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric analyses were evaluated by analysis of variance for multiple groups and Student's t-test for two groups. Significance was designated at the 95% confidence level.

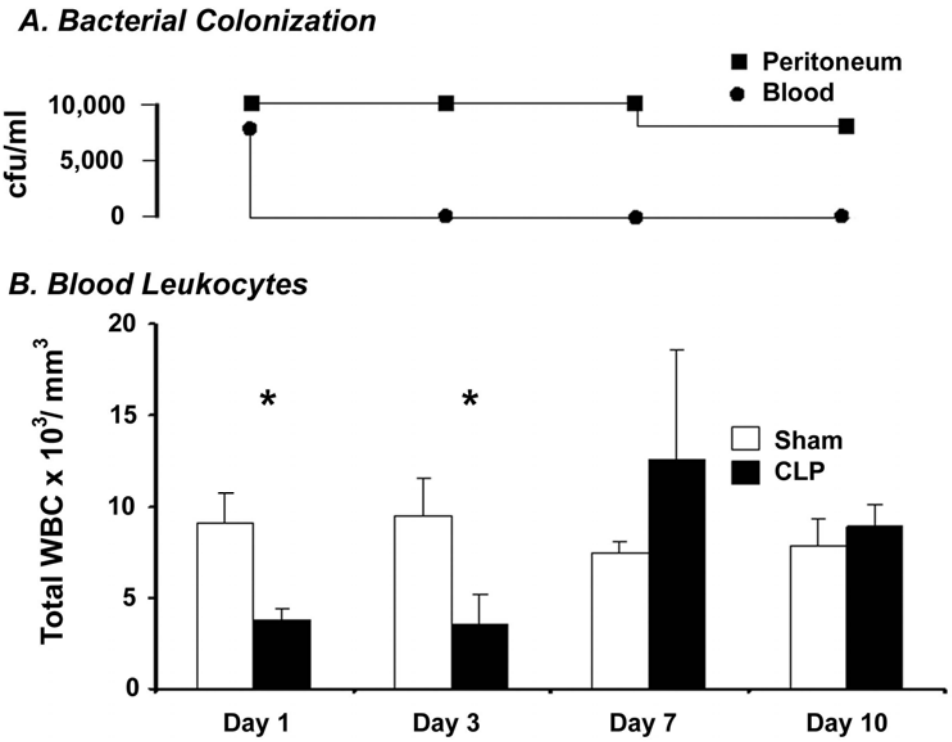

Figure 2-1. Bacteremia and blood leukocyte responses to sepsis produced by a cecal ligation and puncture. Mice underwent either a cecal ligation and puncture, or a sham procedure, as described in the Materials and Methods. At selected intervals, surviving animals were killed and blood and peritoneal bacteremia were determined. Blood bacteremia was only detected on the first day after cecal ligation and puncture, whereas bacteria could be recovered from the peritoneal cavity for up to ten days. Blood and peritoneal washes were sterile from sham-treated animals (data not shown). Sepsis was associated with a profound leukopenia that lasted three days. Values represent the mean of three (sham) to five (septic) animals per group (± S.D.). Differences in the response were determined by Student's t-test.

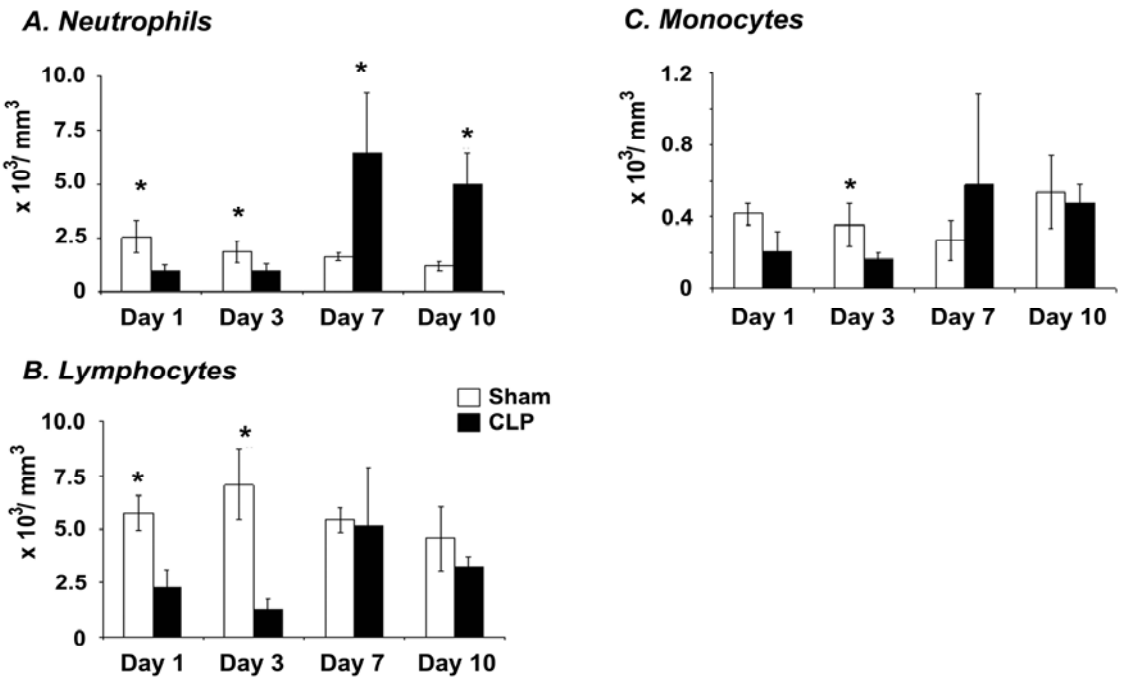

Figure 2- 2. Differential white blood cell counts in response to sepsis. Total differential leukocyte counts were performed at intervals in septic and sham-treated mice. Sepsis was associated with a transient pan cytopenia lasting approximately three days. Afterwards, there was a sustained neutrophilia that lasted approximately 7-10 days. Values represent the mean of three (sham) to five (septic) animals per group (± S.D.). Differences in the response were determined by Student's t-test.

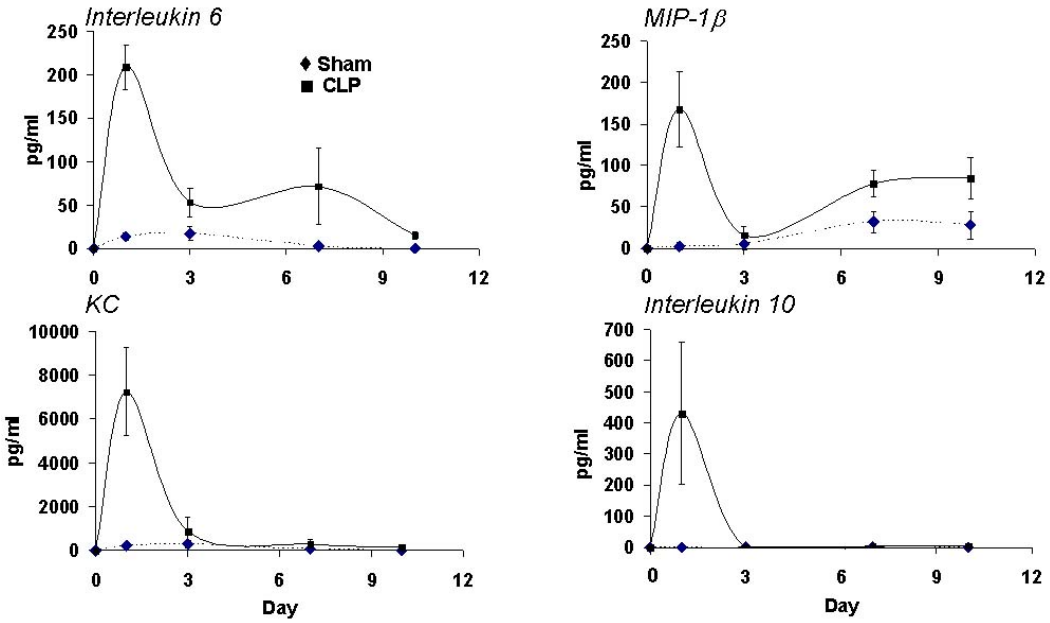

Figure 2-3. Selected plasma cytokine concentrations in septic and sham-treated mice. The plasma concentrations of 22 cytokines were determined after sepsis. The concentrations of 17 cytokines significantly changed in response to the cecal ligation and puncture (CLP), although only four are graphically presented. Concentrations peaked one-three days after sepsis, but remained significantly elevated for at least seven to ten days. Values represent the mean of three (sham) to five (septic) animals per group (± S.D.). Differences in the response were determined by Student's t-test.

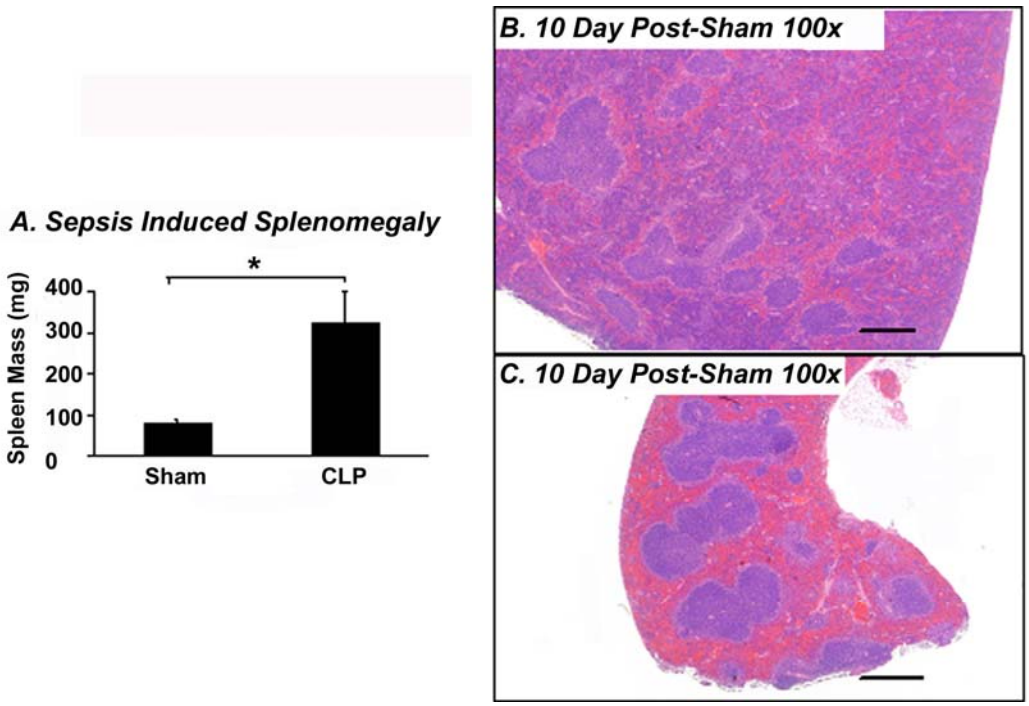

Figure 2- 4. Sepsis induced splenomegaly. Spleens were harvested from mice ten days after an LD10 cecal ligation and puncture (CLP). Panel A. Spleen mass increased nearly fivefold in ten days. Panel B. Hemotoxylin and eosin stained spleen from sham mouse taken ten days after surgical procedure (magnification 100 x). Panel C. Hematoxylin and eosin stained spleen from 10 day septic mouse (magnification 100 x). Splenomegaly and loss of follicular architecture is evident in spleens from septic animals 10 days after CLP. Values represent the mean and standard error of 5-10 animals per group. * $p<0.001$ by Student's t-test. (Bar, 1000 μm.)

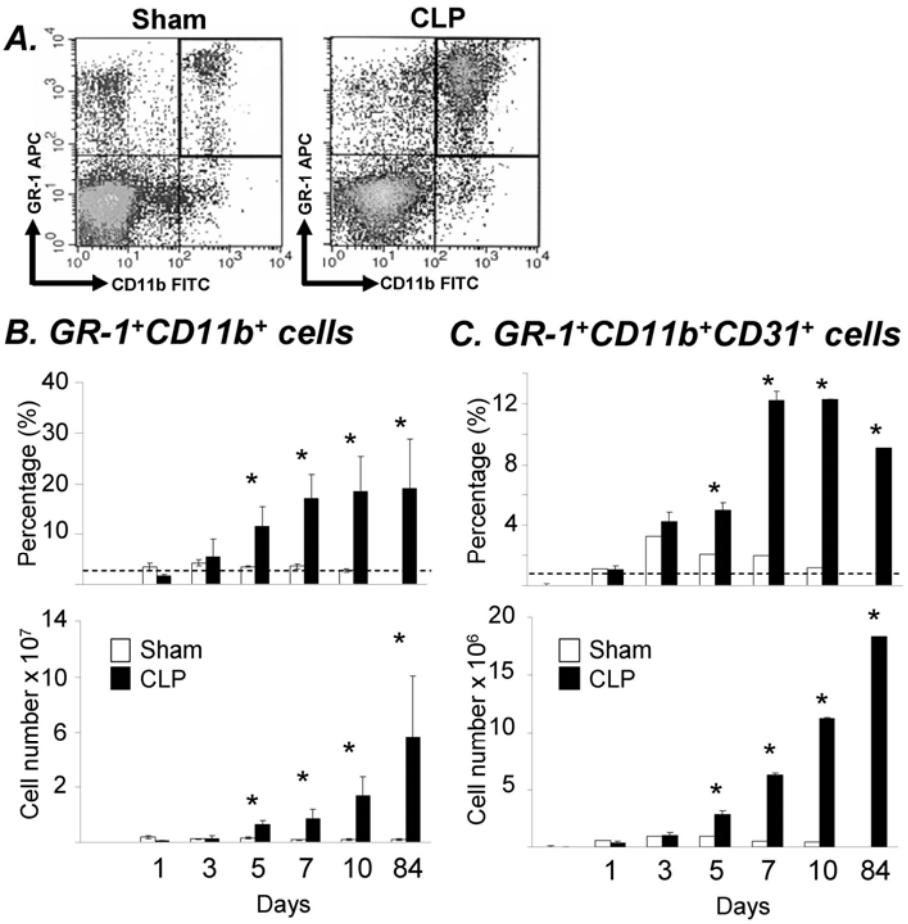

Figure 2-5. Appearance of GR-1$^+$CD11b$^+$ cells in spleens from septic and sham mice. Panel A. Flow cytometry histogram of viable splenocytes gated on GR-1$^+$ and CD11b$^+$ staining. Panel B. Percentage and total numbers of GR-1$^+$CD11b$^+$ splenocytes recovered from mice at intervals after CLP and sham-treatment. Panel C. Percentage and total numbers of GR-1$^+$CD11b$^+$CD31$^+$ splenocytes recovered from mice at intervals after CLP and sham-treatment. Sepsis induced by CLP produced 50-fold increases in numbers of GR-1$^+$CD11b$^+$ splenocytes. Values represent the mean and standard error of 5-10 animals per group. * $p<0.01$ by analysis of variance and Student-Newman Keuls multiple range test.

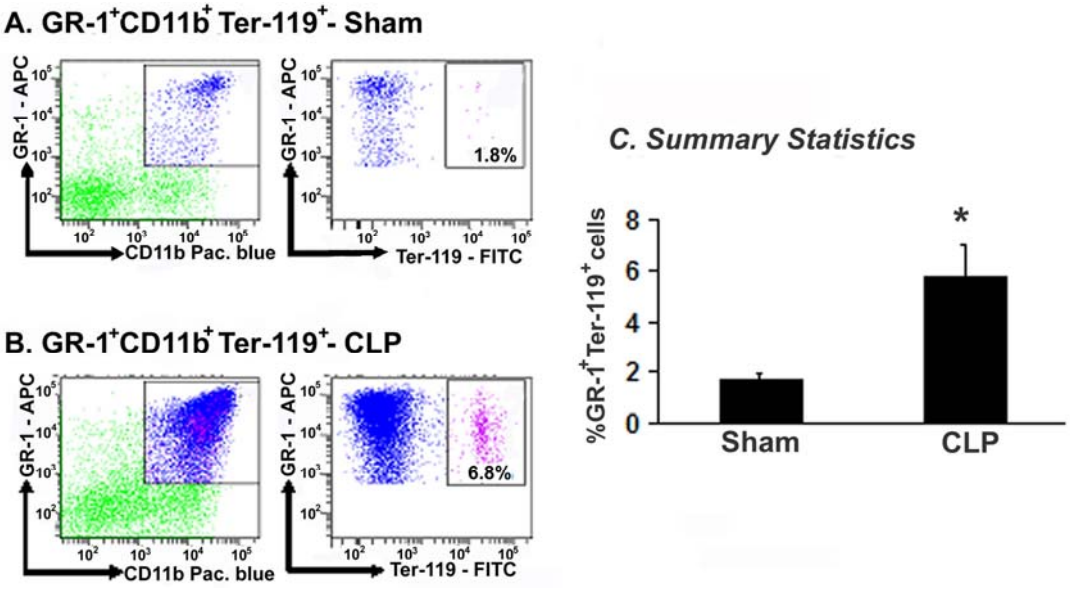

Figure 2-6. Ter119 Staining of GR-1$^+$CD11b$^+$ splenocytes harvested from sham and septic mice. Spleens were harvested from mice ten days after induction of sepsis by cecal ligation and puncture (CLP) and splenocytes were stained for GR-1, CD11b and Ter119. The percentage of Ter119$^+$ cells increased in sepsis, but still remained less than 6% of the GR-1$^+$CD11b$^+$ population. Values represent the mean of three to five animals per group (± S.D.). * Differences in the response were determined by Student's t-test ($p<0.05$).

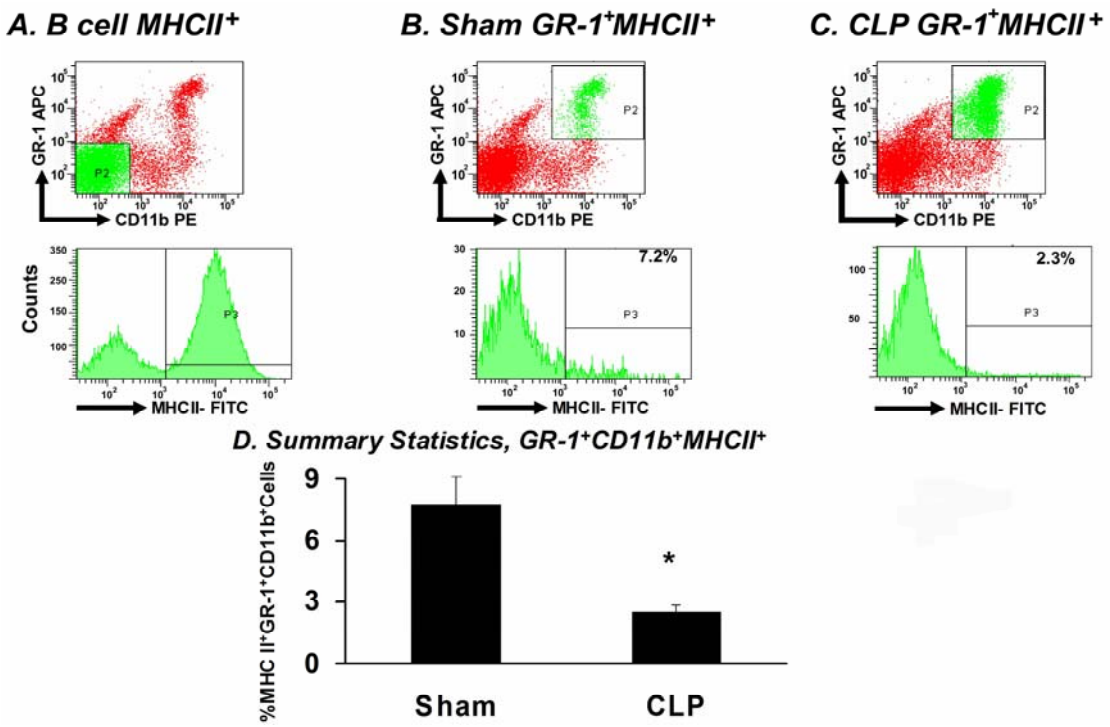

Figure 2-7. MHC Class II expression in GR-1$^+$CD11b$^+$ splenocytes harvested from sham and septic mice. Spleens were harvested from mice ten days after the induction of sepsis by cecal ligation and puncture (CLP) and splenocytes were stained for GR-1, CD11b and MHC II. A GR-1-CD11b- splenocyte population (presumably B cells) were used to gate the MHCII$^+$ population, and those gates were then applied to the GR-1$^+$CD11b$^+$ populations from the sham and septic mice. Values represent the mean of three to five animals per group (± S.D.). * Differences in the response were determined by Student's t-test ($p<0.05$).

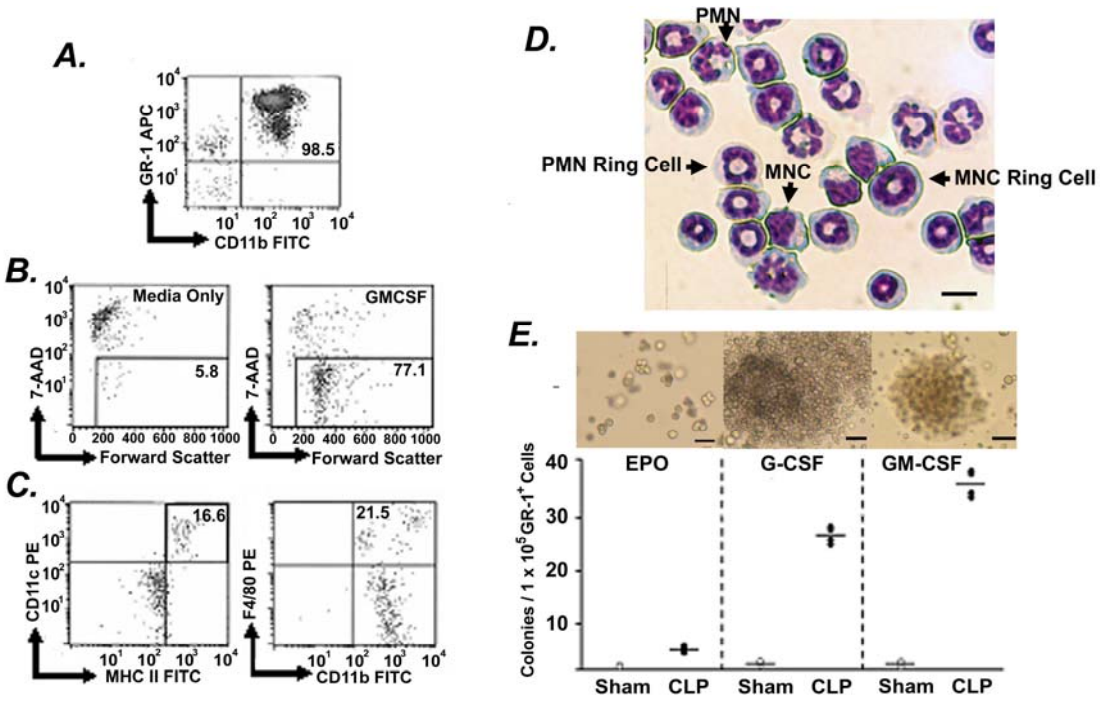

Figure 2-8. Flow cytometric analysis of GR-1$^+$ splenocytes cultured with GM-CSF, G-CSF or erythropoietin (EPO) ex vivo. Panel A. Flow cytometric analysis of enriched GR-1$^+$ splenocytes obtained from septic mice 10 days after CLP. Panel B. Cells were cultured for 7 days with either nothing or GM-CSF and cell viability was determined by 7-AAD staining. Cells cultured without GM-CSF rapidly died. Panel C. GR-1$^+$ enriched splenocytes cultured with GM-CSF for seven days yielded a phenotypically diverse cell population staining positive for CD11c and F4/80. Panel D. Cytospin preparation of enriched GR-1$^+$ cells 10 days after cecal ligation and puncture demonstrated immature heterogeneous myeloid phenotypes with characteristic ring shaped nuclei. (Bar, 5μm.) Panel E. Colony forming units of GR-1$^+$ enriched splenocytes cultured with G-CSF, GM-CSF or erythropoietin (EPO) for ten days in soft methylcellulose. The photographs distinguish the nature of the colonies, reflecting primarily neutrophil and monocyte like colonies in the G-CSF and GM-CSF-treated groups, respectively. (Bar, 15μm.) Values for panels A-C represent the mean and standard error of 5-10 animals per group. * $p<0.01$ by between CLP and sham treated animals, by Student's t-test. Hatched line indicates mean percentage from healthy control animals not subjected to CLP or sham procedures.

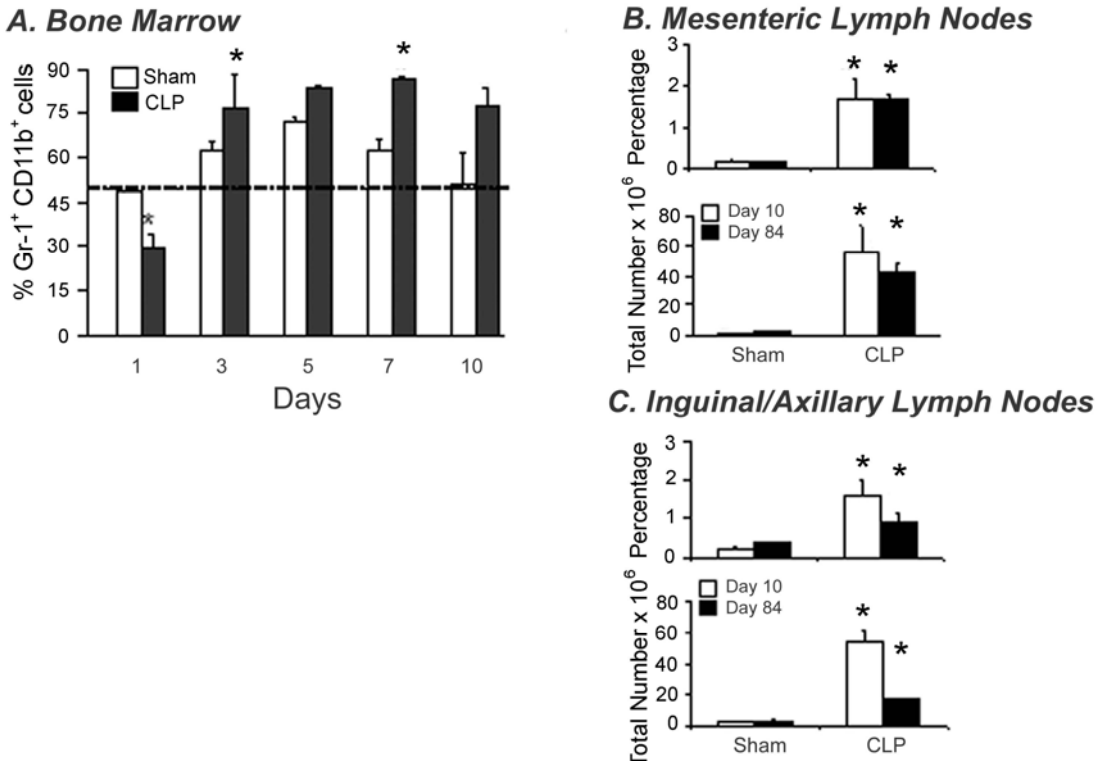

Figure 2-9. Bone marrow and lymph node GR-1$^+$CD11b$^+$ cells from septic and sham mice. CLP produced a rapid increase in the numbers of GR-1$^+$CD11b$^+$ cells in bone marrow (Panel A) and lymph nodes (Panels B and C). A sham procedure produced a more transient modest increase. Values represent the mean and standard error of 5-10 animals per group. * $p<0.01$ by between CLP and sham treated animals, by Student's t-test. Hatched line indicates mean percentage from healthy control animals not subjected to CLP or sham procedures.

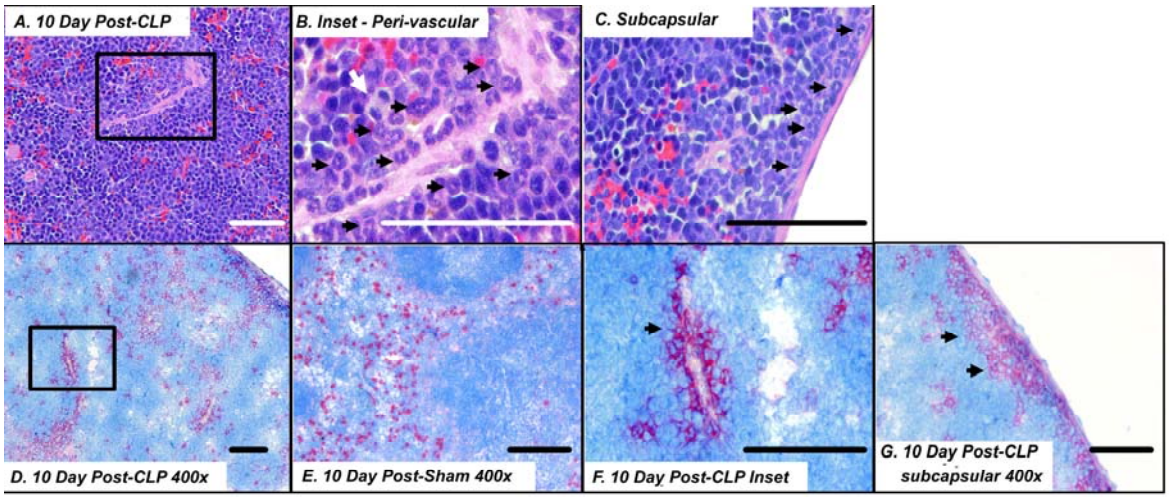

Figure 2-10. Hematoxylin and eosin stained spleens from septic mice ten days after CLP. Panel A. 400 x magnification with perivascular region identified as an inset. Panel B. High powered view of perivascular inset showing cuffing and infiltration with myeloid cells showing characteristic signet ring features. Note mitotically active cell (white arrow). Panel C. High powered view of subcapsular region also showing infiltration with myeloid cells exhibiting characteristic signet ring features. Panel D. $CD11b^{+}$ staining of spleen from septic animal 10 days after CLP. Panel E. $CD11b^{+}$ staining of spleen from sham-treated animal 10 days after surgical procedure. In the sham animal, $CD11b^{+}$ staining is distributed in the mantel region surrounding T-cell rich follicles. After 10 days of sepsis, additional $CD11b^{+}$ staining appears in the perivascular and subcapsular regions. Panel F. Higher magnification staining of perivascular region showing $CD11b^{+}$ staining from a 10 day septic animal. Panel G. Higher magnification showing resolution of subcapsular region showing $CD11b^{+}$ staining from septic animal. (Bar, 100µm.)

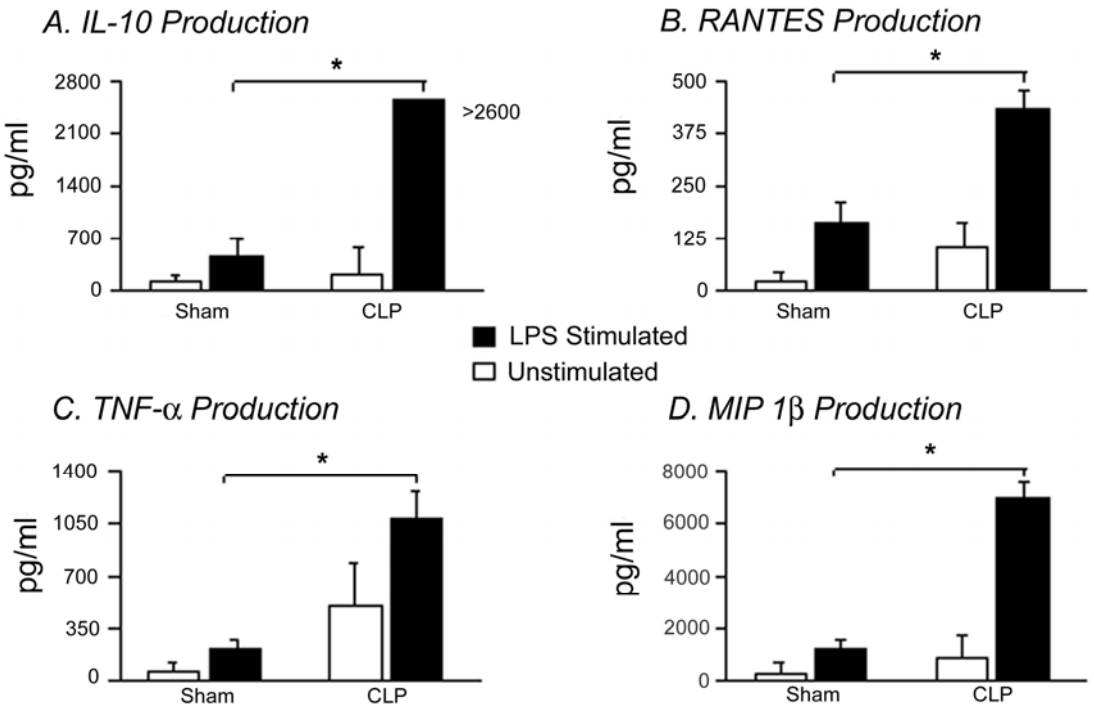

Figure 2-11. Effect of ex vivo lipopolysaccharide stimulation on cytokine expression in GR-1^+ splenocytes obtained from septic mice. When GR-1^+ splenocytes were harvested from seven day sham-treated or septic mice, and stimulated with 10 μg/ml of bacterial lipopolysaccharide, IL-1α, IL-1β, IL-6, IL-10, TNFα, RANTES, MIP-1β and KC production were significantly increased in all groups stimulated with lipopolysaccharide. Importantly, GR-1^+ splenocytes from septic mice secreted more IL-10, TNFα, RANTES and MIP-1β production after lipopolysaccharide administration than GR-1^+ splenocytes from sham-treated animals. Values represent the mean (± S.E.M.) of between four and six samples. * $p<0.05$ by Student's t-test.

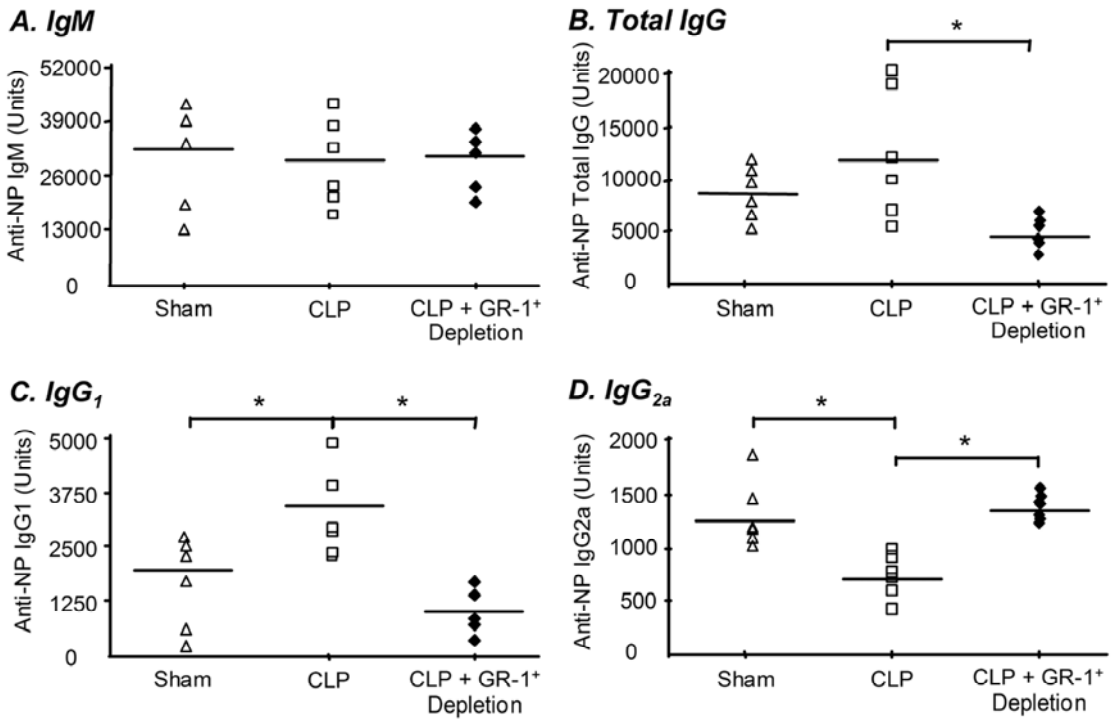

Figure 2-12. Immunoglobulin production following NP-KLH immunization in sham, septic and septic mice depleted of GR-1^+ cells. Nine and ten days after induction of sepsis by CLP, mice were depleted of GR-1^+ cells by the intraperitoneal administration of RB6-8C5 anti-GR-1 antibody, as described in the Methods. Mice were then immunized with NP-KLH. Seven days later, mice were bled and serum IgM (Panel A), total IgG (Panel B), IgG_1 (Panel C) and IgG_{2a} (Panel D) responses to NP-KLH immunization were determined. Sepsis produced no difference in the IgM response while concomitantly producing an increase in the total serum IgG and IgG_1 and a decrease in the serum IgG_{2a} response consistent with a T_H2 type polarization. The total IgG, IgG_1, and IgG_{2a} responses after sepsis were prevented by depletion of the GR-1^+ cells. * $p<0.01$ by ANOVA and Student-Newman Keuls multiple range test.

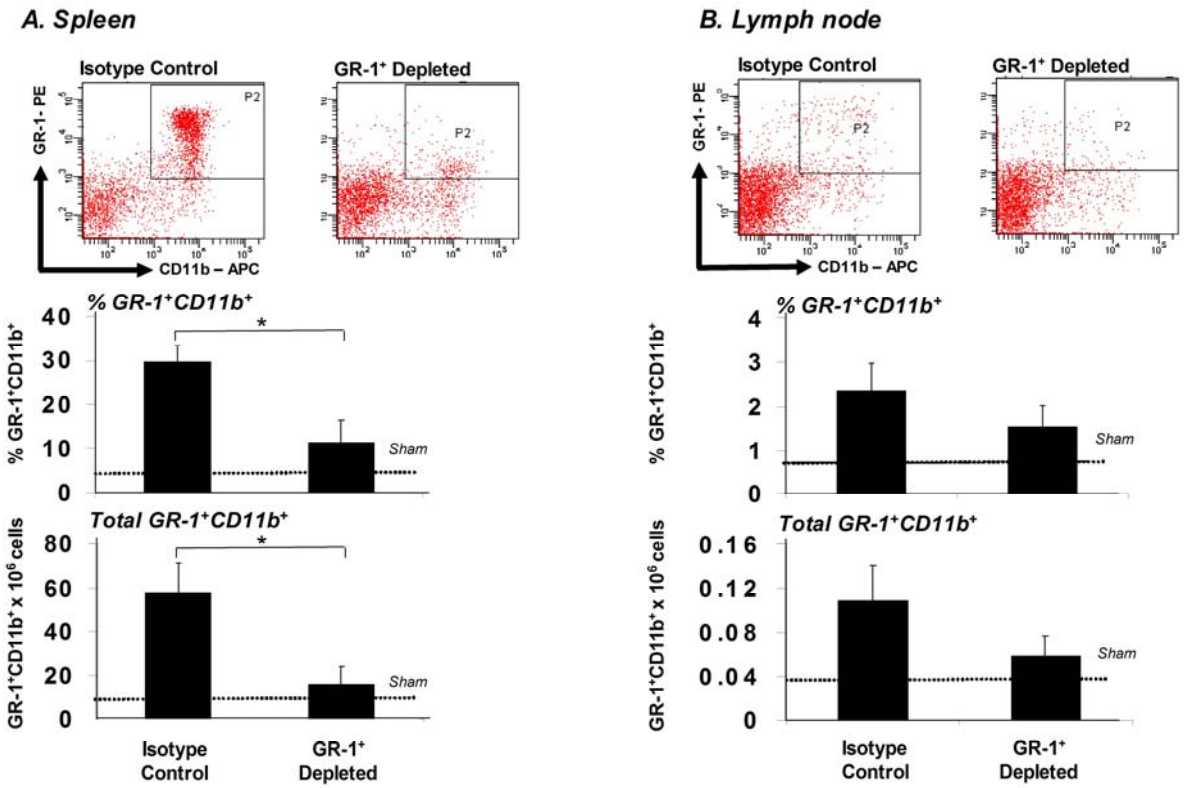

Figure 2-13. Effect of GR-1 antibody depletion on $CD11b^+GR\text{-}1^+$ populations in the spleen and lymph nodes. Nine days after a cecal ligation and puncture, mice were treated with the intraperitoneal injection of 500 µg of purified rat, antimouse GR-1 IgG, followed by a second intraperitoneal injection of 250 µg the next day, or equivalent quantities of an isotype control (IgG_3). Twenty-four hours later, the mice were killed and $GR\text{-}1^+CD11b^+$ cells in the spleen and lymph nodes were determined. Results were also compared to the numbers of $GR\text{-}1^+CD11b^+$ cells obtained in the same organs from a sham animal. $GR\text{-}1^+$ produced a significant decrease in the relative and absolute numbers of $GR\text{-}1^+CD11b^+$ cells in the spleens of the septic animals. Although depletion was not complete, the number of remaining $GR\text{-}1^+CD11b^+$ cells were only modestly increased, albeit not significantly, from the number of cells in the sham animals. Values represent the mean of three to five samples, and differences were determined by the Student's t-test (*$p<0.05$).

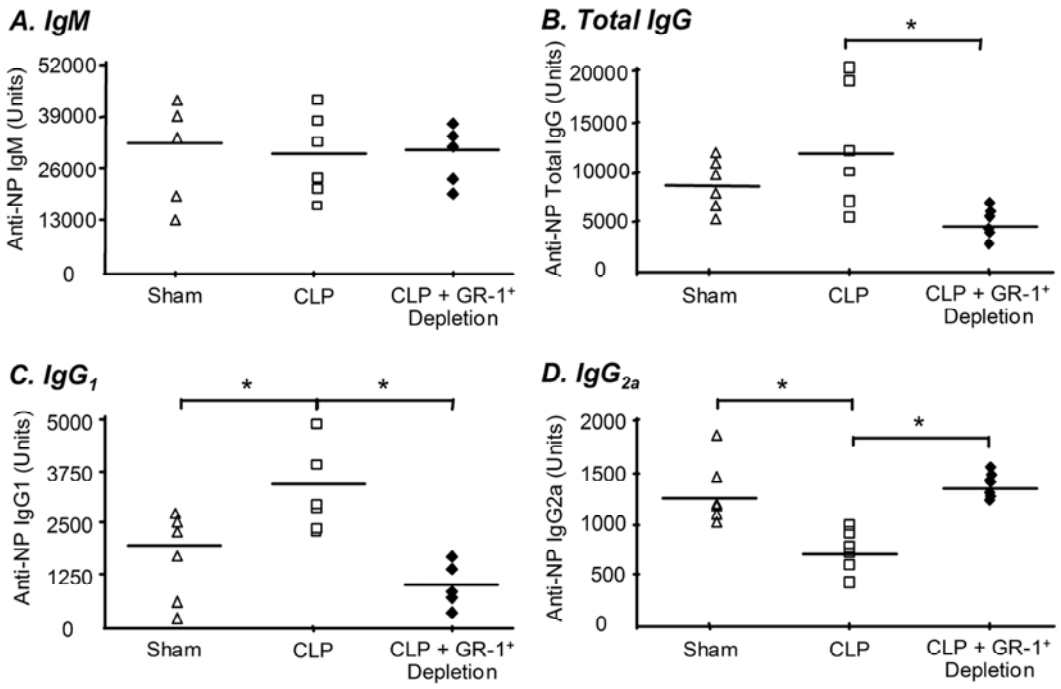

Figure 2-14. Immunoglobulin production following NP-KLH immunization in sham, and septic mice. Nine and ten days after induction of sepsis by CLP, mice were depleted of $CD4^+$ cells by the intraperitoneal administration of an anti-CD4 antibody (GK1.5 hybridoma). Mice were then immunized with NP-KLH. Seven days later, mice were bled and serum IgM (Panel A), total IgG (Panel B), IgG_1 (Panel C) and IgG_{2a} (Panel D) responses to NP-KLH immunization were determined. Sepsis produced no difference in the IgM response while concomitantly producing an increase in the total serum IgG and IgG_1 and a decrease in the serum IgG_{2a} response consistent with a T_H2 type polarization. However depletion of $CD4^+$ cells significantly attenuated the IgM responses and completely prevented the IgG class switching in the septic animals (Panels A, B, C, and D). * $p<0.01$ by ANOVA and Student-Newman Keuls multiple range test.

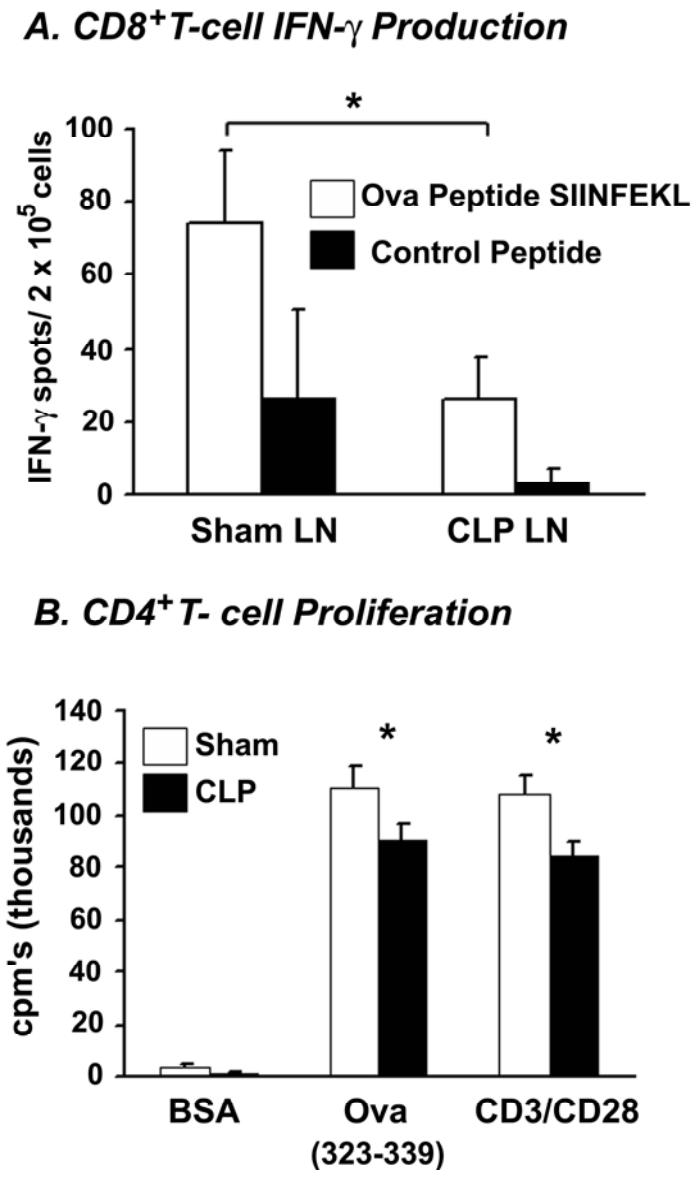

Figure 2-15. Effect of GR-1^+ cells from septic mice on antigen specific $CD4^+$ T-cell proliferation and $CD8^+$ T-cell IFNγ responses, Mice were treated as described in the Materials and Methods. GR-1^+ cells from septic animals markedly attenuated the IFNγ production (determined by ELISpot™) by OT-1 splenocytes stimulated with either control peptide or OVA-derived peptide SIINFEKL ex vivo following administration and immunization in C57BL/6 mice. In contrast, injection of GR-1^+ splenocytes from ten day septic mice had only a minimal but significant suppressive effect on antigen specific and nonspecific $CD4^+$ T-cell proliferative response in OVA antigen specific, D011.10 mice (Panel B). Values represent the mean and standard error of 5 animals per group. The experiment was repeated twice and values presented are from one of the representative experiments. * $p<0.05$ by ANOVA and Student's-Newman-Keuls multiple range test.

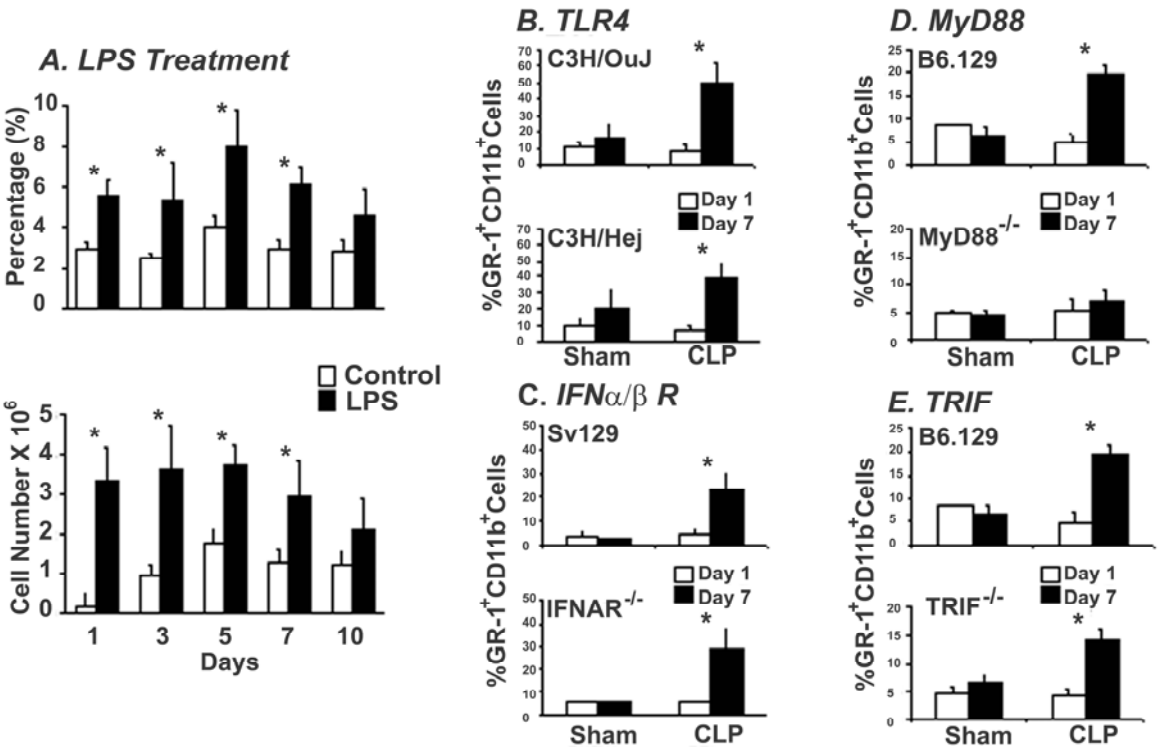

Figure 2-16. Effects of lipopolysaccharide (LPS) and transgenic mice on expansion of the splenic GR-1$^+$CD11b$^+$ population ten days after CLP. (Panel A) Mice received either nothing or the intraperitoneal injection of 5 mg/kg BW of bacterial lipopolysaccharide, and were sacrificed at intervals thereafter. Lipopolysaccharide injection increased the percentage of GR-1$^+$CD11b$^+$ cells in the spleen within one day, and expansion of this cell population remained for about seven days. CLP was induced in C3H/HeJ (TLR4 mutant) (Panel B.), IFNα/βR$^{-/-}$ (Panel C), MyD88$^{-/-}$ (B6x129)(Panel D) and TRIF$^{-/-}$ (Panel E) as described in the Methods. At one and seven days later, splenic GR-1$^+$CD11B$^+$ populations were examined in the spleens of knockout mice and their appropriate background controls. Normal expansion of the GR-1$^+$CD11b$^+$ splenocytes was seen in all mice at seven days with the exception of the MyD88$^{-/-}$ mice that failed to demonstrate an increase in their GR-1$^+$CD11b$^+$ population. Values represent the mean and standard error of 5 animals per group. *$p<0.01$ versus control at the same time point, as determined by Student's t-test.

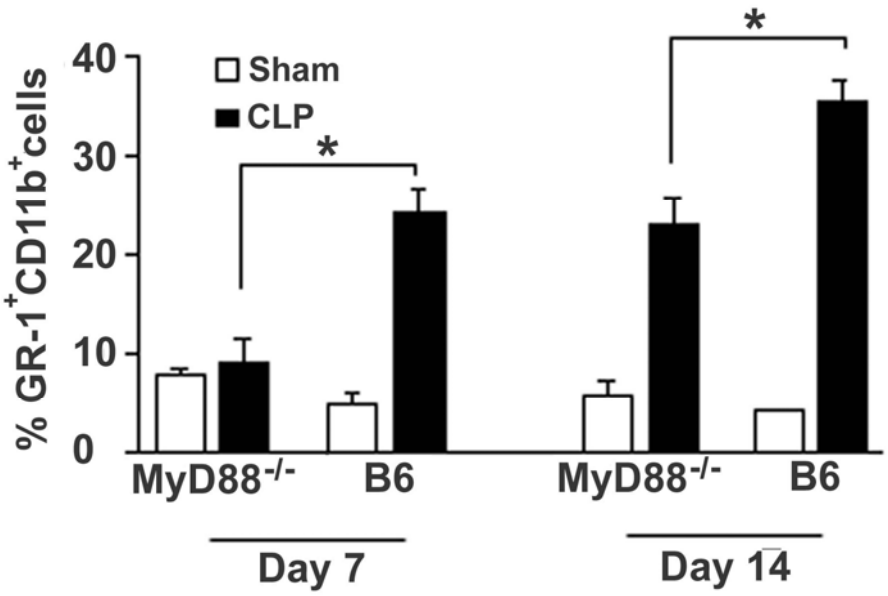

Figure 2-17. Requirement for MyD88$^{-/-}$ signaling in the expansion of GR-1^{+}CD11b^{+} splenocytes in response to prolonged sepsis. MyD88$^{-/-}$ mice on a C57Bl/6 background and wild-type controls underwent a cecal ligation and puncture, and representative animals were sacrificed at seven and 14 days after induction of sepsis. The relative and absolute numbers of GR-1^{+}CD11b^{+} splenocytes were determined, as previously described. As seen in the MyD88$^{-/-}$ B6x129 animals, there was no increase in the GR-1^{+}CD11b^{+} splenocyte population seven days after sepsis. However, at 14 days, there was an increase in the GR-1^{+}CD11b^{+} population, although it was still less compared to the wild-type animals. *$p<0.01$ versus control at the same time point, as determined by Student's t-test.

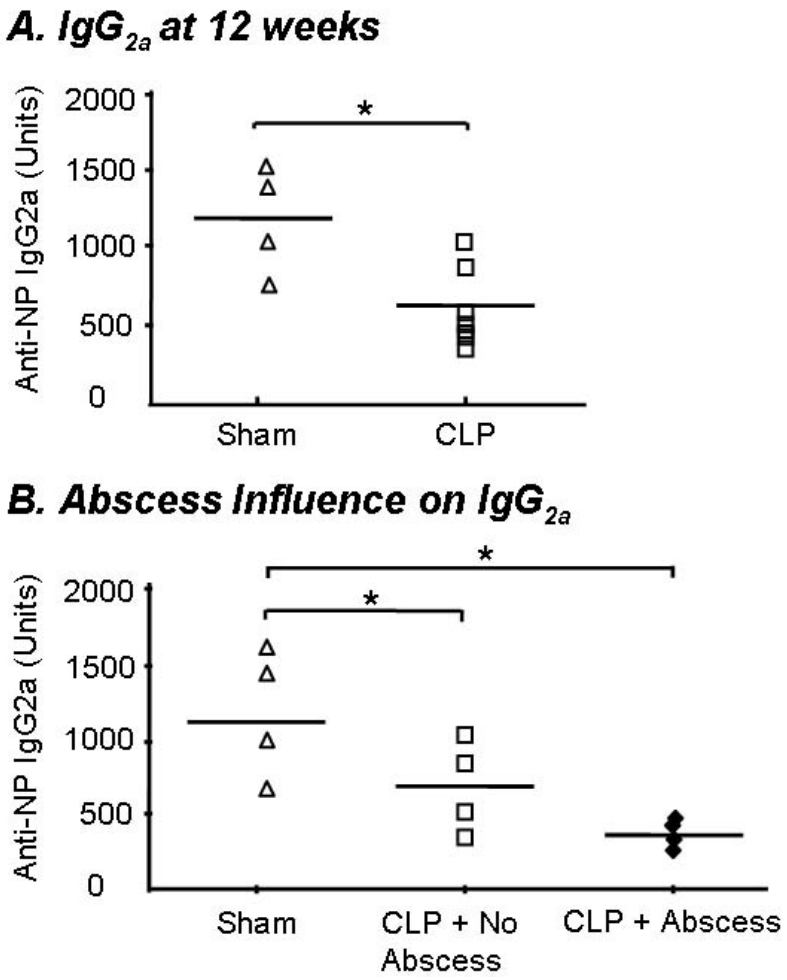

Figure 2-18. Immunoglobulin production following NP-KLH immunization in sham, and septic mice 12 weeks after cecal ligation and puncture. Mice were immunized with NP-KLH with alum 12 weeks after cecal ligation and puncture. Seven days later, mice were bled and serum IgG_{2a} (Panel A) responses to NP-KLH immunization were determined. Sepsis produced a decrease in the serum IgG_{2a} response consistent with a T_H2 type polarization. However, in mice that exhibited resolved abscesses the IgG_{2a} responses were more elevated compared with mice with ongoing abscesses (Panel B). * $p<0.01$ by ANOVA and Student-Newman Keuls multiple range test.

CHAPTER 3
IL-4, IL-10 AND NITRIC OXIDE SIGNALING DO NOT CONTRIBUTE TO THE MYELOID DERIVED SUPPRESSOR CELL EXPANSION OR SUPPRESSOR FUNCTION DURING POLYMICROBIAL SEPSIS

Specific Aim 2

The goal of Specific Aim 2 aim was to determine the effect of MDSC expansion on the adaptive immune system using a prolonged model of polymicrobial sepsis. The suppressor cell function was evaluated by incorporating the MDSCs *in vivo* with T-cell receptor specific $CD8^+$ lymphocytes and measuring the T lymphocyte specific responses to antigen specific proliferative signals. Since the MDSCs must be in physical proximity with other cells to impart their immunosuppressive properties (56, 74, 75), and are known to secrete IL-4, IL-10 (65) and nitric oxide (NO) (49), we examined whether these mediators contribute directly to the MDSC suppression in sepsis by generating MDSCs from IL-4, IL-10 and iNOS (NOS2) null animals.

Introduction

Sepsis is the result of a severe microbial infection that leads to both an exaggerated systemic inflammatory response and immune suppression (4, 40, 76), as evidenced by a failure to eradicate primary infections (12), a predisposition to develop secondary nosocomial infections (12, 13), and an attenuated delayed type hypersensitivity response (11). The etiology of sepsis associated immune suppression is thought to be multifactoral, with defects in antigen-presentation (19), apoptosis of B cells, T cells, and dendritic cells (20, 77) development of an anti-inflammatory response (97), and the increased presence of regulatory cell populations (85, 98).

Expansion of the myeloid derived suppressor cell (MDSC) population (91) has also been observed in the spleens and tumors of mice with transplantable tumors (48, 80), and in models of chronic inflammation (50). In tumor-bearing mice, these cells contribute to tumor-associated

antigen specific T cell dysfunction and tolerance (74, 80, 81, 99). We have recently described a similar heterogenous population of GR-1$^+$CD11b$^+$ immature myeloid cells whose numbers dramatically increase in the spleen, lymph nodes and bone marrow during polymicrobial sepsis. These MDSCs are capable of inhibiting antigen-specific CD8$^+$ T cell interferon-γ production and antigen nonspecific CD4$^+$ T cell proliferation during sepsis, and the polarization of the T helper cell response from a T_H1 to a T_H2 profile. MDSCs are known to secrete a number of biologically active mediators, including IL-10, TGFβ, chemokines, oxygen free radicals, nitric oxide and peroxynitrites (48). However, the specific mediators responsible for either the sepsis-induced myeloid cell expansion or the associated T cell suppressor activity of these immature myeloid cells are yet unknown.

Previously, we established that the sepsis-induced MDSC population is an immature, heterogenous group of cells capable of polarizing T and B cell responses during sepsis (99). Bronte and colleagues reported that MDSCs play an important role in inhibiting T cell activation during inflammatory responses (80). Suppressor activity requires the MDSCs to be in immediate proximity to the effector cells (54, 87), suggesting that the MDSC suppressor activity is mediated through a paracrine or juxtacrine mechanism. A variety of candidate molecules have been proposed, including nitric oxide, reactive oxygen species, arginine depletion, TGFβ, IL-4 or IL-10 (49). During sepsis, increased IL-4 and IL-10 production have been associated with an anti-inflammatory response and a T-helper cell T_H2 polarization (4). Since the MDSC population is capable of producing increased levels of IL-10, and to a lesser extent IL-4 during sepsis, MDSCs may utilize these cytokines to mediate their suppressive effects.

Here, we observed that an ongoing septic process produces a dramatic expansion of GR-1$^+$CD11b$^+$ cells in the spleen that is not dependent on IL-10, IL-4 or nitric oxide signaling.

Although MDSCs express large quantities of IL-10, and lesser quantities of IL-4, the suppression of antigen-specific $CD8^+$ T-cell interferon-γ production is also not dependent on MDSC expression of either mediator *in vivo*.

Results

MDSCs are Capable of Immune Modulatory Cytokine Production

Since myeloid derived suppressor cells obtained from tumor bearing hosts are known to be immune modulatory (48, 80), we sought to examine whether myeloid derived suppressor cells obtained from septic mice could produce immune modulatory mediators responsible for T-cell suppression and polarization during sepsis. When $GR\text{-}1^+$ enriched splenocytes from septic mice were cultured *ex vivo* with bacterial lipopolysaccharide, the $GR\text{-}1^+$ cells from septic mice produced significantly greater amounts (>5 fold) of IL-10 than similar $GR\text{-}1^+$ cells from sham-treated animals (Figure 3-1 Panel B). They also produced increased quantities of IL-4 and MIP-1β (Figure 3-1 Panel A and C).

In order to confirm the ability of the splenic myeloid cells to produce increased levels of immune modulatory cytokines, we used anti-IL-4 and anti-IL-10 staining antibodies and flow cytometry analysis to confirm the increased production of both cytokines. Intracellular staining revealed that the total percentage and absolute numbers of splenic IL-10 or IL-4 expressing $GR\text{-}1^+CD11b^+$ cells increased seven days after sepsis (Figure 3-2 Panels A-D), although the proportion of IL-10 or IL-4 expressing cells remained relatively small.

MDSCs Inhibit Antigen Specific $CD8^+$ T-Cell IFN-γ Production Independent of Interleukin-4, Interleukin-10, and iNOS Production

By determining the effect of $GR\text{-}1^+$ cells on the $CD8^+$ T-cell IFN-γ response by splenocytes from OT-I TCR-transgenic mice (C57BL/6-Tg(TCRαTCRβ)1100mjb) immunized with OVA-derived peptide (H-2Kb restricted, aa 257–264, SIINFEKL), we were able to

demonstrate that GR-1$^+$ splenocytes inhibit CD8$^+$ T-cell IFN-γ production ex vivo(99) . However, the specific mechanism of GR-1$^+$ splenocyte suppression of CD8$^+$ T- cell IFN-γ production is yet unknown. In light of the myeloid derived suppressor cells' ability to produce immune modulatory cytokines, we postulated that IL-4 and IL-10 may be responsible for the observed myeloid cell induced CD8$^+$ T-cell tolerance. GR-1$^+$ splenocytes from IL-4 and IL-10 null mice were obtained either 10 days after sepsis or a sham procedure. As shown in Figure 3-3 Panels A-C, greater than 20 million GR-1$^+$ cells/spleen were obtained from the IL-4 null (23 million GR-1$^+$ cells/spleen), IL-10 null (24 million GR-1$^+$ cells/spleen), iNOS (*NOS2*) null (*data not shown*) and wild type (22 million GR-1$^+$ cells/spleen) mice indicating that the deficit in IL-10, IL-4, or iNOS production did not hinder the expansion of the GR-1$^+$ population. Next, the GR-1$^+$ cells were adoptively transferred into C57BL/6 mice that had previously received CD8$^+$ T-cells from OT-1 TCR-transgenic mice, and were simultaneously immunized with OVA-derived specific peptide. Ten days later, the spleens from these animals were harvested, and IFN-γ responses to ex vivo stimulation with OVA-derived specific peptide were examined. IFN-γ production was markedly reduced when the animals were administered GR-1$^+$ splenocytes from septic IL-10 null, IL-4 null, iNOS null, and wild type C57BL/6 animals, when compared to sham-treated mice (Figure 3-4 Panels A-C), indicating that the MDSC-induced CD8$^+$ T-cell suppression is not IL-4, IL-10 or iNOS dependent.

MDSCs Inhibit Antigen Specific CD8$^+$ T-cell Cytotoxicity

Although the signaling pathways impacting the antigen specific CD8$^+$ T cell IFNγ secretion reduction induced by MDSCs are unclear and do not involve IL-4, IL-10 or iNOS, the other uncertainty that exists is the direct effect of the MDSCs on T cell cytotoxic function. Since antigen specific cytokine secretion is only an indirect method or ascertaining T cell function, we sought to find a more definitive assessment of the impact of MDSCs on the function of CD8$^+$ T

cells. We incorporated an antigen specific, non radioactive cytotoxicixty assay (see **Materials and Methods**) to directly determine the cytotoxic function of $CD8^+$ T cells that have been cultured with GR-1^+ cells from either sham or CLP treated mice. As Figure 3-5 demonstrates, there was an appreciable reduction in $CD8^+$ T cell cytotoxic ability when $CD8^+$ T cells were cultured with GR-1^+ cells taken from septic animals when compared to sham controls. This *in vitro* cytotoxic reduction indicates that the MDSC effect on $CD8^+$ cells may also occur *in vivo* and predispose the host to invasion from organisms that require $CD8^+$ T cells to mount an immune response.

Discussion

Although the etiology of sepsis-induced immune dysfunction is unknown, recent interest has focused on the effects of regulatory cell populations responsible for the shift from a T_H1 to a T_H2 immune profile that results in a state of T cell anergy and immune suppression (42, 85, 98). We have previously described a substantial expansion of a GR-1^+CD11b^+ myeloid population with an immature phenotype capable of antigen specific $CD8^+$ T-cell suppression and T_H1 to T_H2 immune polarization. However, the mechanisms driving this immature myeloid cell expansion and antigen specific T-cell suppression remain elusive. In the current study, we observed that an ongoing septic process produces a dramatic expansion of GR-1^+CD11b^+ cells in the spleen that is independent of IL-4, IL-10, and iNOS. Although MDSCs produce IL-10 and IL-4, their suppression of antigen-specific $CD8^+$ T-cell IFN-γ production is also not dependent on either cytokine.

T cell apoptosis and loss during sepsis significantly contribute to immune dysfunction and mortality (4, 17, 88). Recently, Monneret suggested that T regulatory cell inhibition of T effector cells may account for the $CD4^+$ T cell dysfunction during sepsis (42), although our more recent studies, and those of Ayala, have recently questioned the role of T-regulatory cells in

outcome to polymicrobial sepsis (95, 98). Other investigators (54, 74, 80, 82, 89) have demonstrated that immature myeloid cells are able to induce T cell tolerance through a variety of mediators including nitric oxide, reactive oxygen species, arginine depletion, TGFβ, IL-4 and IL-10 (49). Due to the fact that sepsis induced myeloid cells are capable of producing increased levels of IL-10, and IL-4 upon ex vivo stimulation with endotoxin (Figure 3-1 Panels A and B), we examined whether these cytokines are responsible for the myeloid cell induced $CD8^+$ T cell suppression. Despite the fact that Serafini and colleagues demonstrated in vitro that both IL-10 and IL-4 are essential to the suppressor function of MDSCs in cancer (49), our data suggest that neither IL-4 nor IL-10 production by MDSCs is necessary for the suppression of antigen specific $CD8^+$ T-cell IFN-γ production commonly exhibited by MDSCs *in vivo*. IL-10-mediated T-cell tolerance in sepsis has been repeatedly observed and is one of the most accepted mechanisms of T cell suppression during sepsis (12). We can only speculate whether the increased IL-10 and IL-4 production by MDSCs may contribute to other components of the immune tolerance seen in sepsis, but we can conclude that IL-10 and IL-4 do not contribute to the suppression of $CD8^+$ T-cell IFN-γ production by sepsis derived MDSCs.

An alternative explanation is that MDSC induced T cell tolerance is due to the increased production of oxygen free radicals and peroxynitrites. Recently, Nagar and colleagues found that MDSC inhibition of $CD8^+$ T-cells results in part from the nitrosylation of the MHCII complex which inhibits the binding of the $CD8^+$ T-cell receptor with the specific peptide MHC II complex (75).

In conclusion, we observed that an ongoing septic process produces a dramatic expansion of GR-1^+CD11b^+ cells in the spleen that is likely independent of G-CSF and neutrophil elastase activity, and is also independent of IL-10, and IL-4 signaling. Although MDSCs express large

quantities of IL-10, and lesser quantities of IL-4, the suppression of antigen-specific $CD8^+$ T-cell interferon-γ production is also not dependent on MDSC expression of either mediator in vivo.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine. Specific pathogen-free C57BL/6 mice, OT-1 TCR transgenic mice [C57BL/6-Tg(TcrαTcrβ)1100Mjb/J], IL-4 (C57BL/6-Il4tm1Nnt/J) and IL-10 (B6.129P2-Il10tm1Cgn/J) null mice (on a C57BL/6 background), mice were all purchased from The Jackson Laboratory (Bar Harbor, ME). iNOS null mice ($NOS2^{-/-}$) were a gift from Dr Edward Scott at the University of Florida. All mice were maintained at the University of Florida College of Medicine and were studied between 6-12 weeks of age.

Cecal Ligation and Puncture

For induction of polymicrobial sepsis, mice underwent sham laparotomy or cecal ligation and puncture induced by ligation of the cecum and a double enterotomy created with a 27 gauge needle. Mortality in this model was approximately 10-15%, and occurred predominantly in the first 3 days; thereafter, surviving mice developed abscesses surrounding the devitalized cecum as previously described (77, 95, 99).

Flow Cytometry

Spleens and bone marrow cells were analyzed by flow cytometry as previously described (95, 99). Antibodies included anti-GR-1 (Ly6G and Ly6C (RB6-8C5)) conjugated to APC, anti-CD11b (Integrin aM, chain Mac-1a chain (M1/70)) conjugated to Pacific Blue, Fc-Block (CD16/CD32 Fc g III/II Receptor (2.4G2), and Sytox. All antibodies were purchased from BD Pharmingen.

Cell Purification

All magnetic bead kits were obtained from Miltenyi Biotec. Erythrocyte-depleted splenocytes and lymphocytes were isolated using either anti-GR-1 (Ly6G and Ly6C (RB6-8C5)) conjugated to APC followed by anti-APC MicroBeads for GR-1^+ splenocytes. Anti-CD8a (Ly-2) MicroBeads were used alone for OT-1 $CD8^+$ splenocyte isolation. Purities for either population after magnetic separation were >95% (data not shown).

***Ex vivo* Stimulation and Cytokine Production**

Enriched GR-1^+ cells were plated at 1 x 10^6 cells/well with RPMI 1640 supplemented with 10% fetal calf sera, 2 mM L-glutamine, 200 units/ml penicillin and 50 μg/ml streptomycin, and stimulated with 10 μg/ml of bacterial lipopolysaccharide (E. coli 0111:B4). The culture supernatant was analyzed for cytokines using Luminex™ technology using reagents obtained from Upstate Cell Signaling Solutions (Beadlyte™ Mouse Multi-Cytokine Detection System) (Temecula, CA).

Antigen Specific $CD8^+$ T-Cell IFN-γ Production

3–5 x 10^6 purified T-cells from OT-1 TCR transgenic mice were injected i.v. into naive C57BL/6 recipient mice. Two days later, mice were injected i.v. with 5 x 10^6 GR-1^+ cells obtained ten days after sepsis or sham procedure. Mice were immunized subcutaneously with the specific peptide (100 μg of OVA-derived peptide SIINFEKL) mixed with Incomplete Freund's Adjuvant. Ten days later, lymph node and spleen cells were isolated, and restimulated in vitro with specific (OVA-derived peptide SIINFEKL) or control peptide (RAHYNIVTF), and analyzed via IFN-γ ELISpot.

Enzyme-Linked Immunospot Assay (ELISpot)

Millipore MultiScreen HA plates were coated with and blocked with phosphate-buffered-saline with 1% bovine serum albumin prior to plating. Spleen and lymph node cells were plated

at 2.5 x 10^5 cells/well with HL-1 (Cambrex) supplemented with 2 mM L-glutamine, 200 units/ml penicillin and 50 μg/ml streptomycin. Cells were stimulated with either 10 μM OVA257-264 or bovine serum albumin for 48 hours. The cells were treated with 1 μg/ml biotinylated anti-mouse-IFNγ ((XMG1.2) BD Bioscience), then a 1:1000 dilution of strepavidin-alkaline phosphatase conjugate and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Pierce Fine Chemicals, Rockford IL). The spots per well were manually counted.

$CD8^+$ T Cell-Cytotoxicity

A total of 3–5 x 10^6 purified T-cells from OT-1 TCR transgenic mice were injected i.v. into naive C57BL/6 mice. Two to three days later, these mice are injected intravenously with 5 x 10^6 GR-1^+ cells obtained from C57Bl/6 mice obtained ten days after CLP or sham procedure. Within 1 hour after cell transfer, mice were immunized subcutaneously with the specific peptide (100 μg of OVA-derived peptide SIINFEKL for OT-1 mice) mixed with Incomplete Freund's Adjuvant. Ten days later, OT-1 cells from lymph nodes were isolated, washed and cultured with varying ratios of (1:1, 5:1, 10:1, and 20:1) E.G7-OVA (ATCC# CRL-2133TM) cells that constitutively express H-2 Kb restricted OVA 258-276 peptide as target cells. Different ratios of MDSCs:T cell responders (4:1, 2:1, 1:1, 1:2, 1:4, and 1:8) were added to each well. Cells were cultured for 24 hours, the supernatants then harvested, and the lactate dehydrogenase (LDH) release measured using a Cyto Tox 96TM Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's directions.

Statistics

Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric analyses were evaluated by analysis of variance for multiple groups and Student's t-test for two groups. Significance was designated at the 95% confidence level.

Figure 3-1. Effect of *ex vivo* lipopolysaccharide stimulation on cytokine expression in GR-1^+ splenocytes obtained from septic mice. When GR-1^+ splenocytes were harvested from seven day sham-treated or septic mice, and stimulated ex vivo with 10 μg/ml of bacterial lipopolysaccharide, IL-1α, IL-1β, IL-6, IL-10, TNF-α, RANTES, MIP-1β, KC, and MCP-1 production were significantly increased in all groups. Notably, GR-1^+ splenocytes from septic mice secreted more IL-4 (Panel A), IL-10 (Panel B), TNF-α, RANTES, MIP-1β (Panel C), and MCP-1 production after lipopolysaccharide administration than GR-1^+ splenocytes from sham-treated animals. Values represent the mean (± S.E.M.) of between four and seven samples. * $p<0.05$ by Student's t-test.

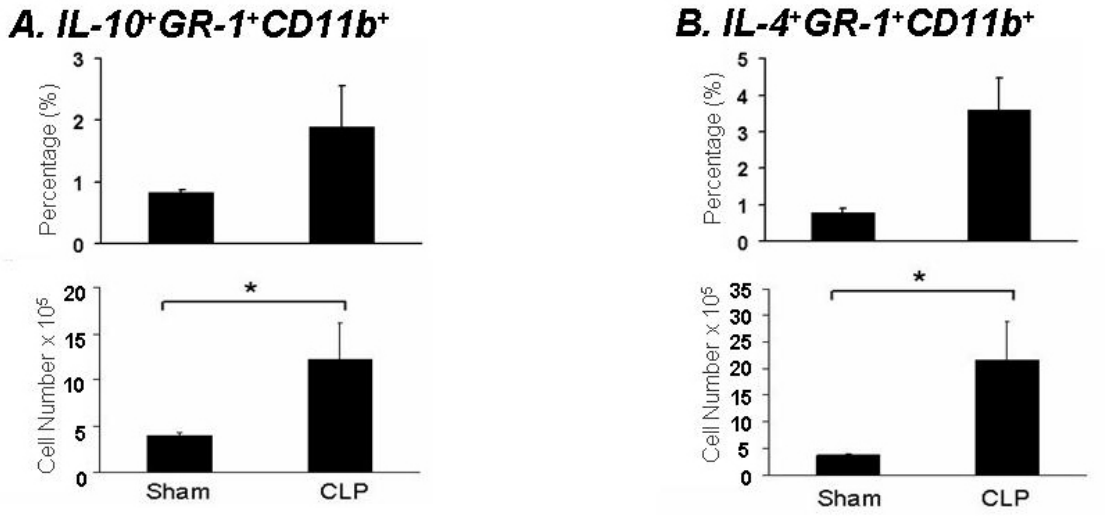

Figure 3-2. Flow cytometry analysis of IL-10 and IL-4 expression in response to ex vivo lipopolysaccharide stimulation. Total erythrocyte-depleted splenocytes from seven

day septic and sham-treated mice were enriched for GR-1^+ cells and then stimulated ex vivo with lipopolysaccharide for 48 hours. The cells were then harvested and fixed in buffer containing 1% formaldehyde for 30 min, permeabilized by washing in flow buffer containing 0.5% saponin, and stained with either anti-IL-10 (JES5-16E3) conjugated to FITC or anti-IL-4 (11B11) conjugated to PE. The absolute numbers of IL-10 (Panel A) and IL-4 (Panel B) expressing $GR1^+CD11b^+$ cells increased significantly in the septic mice. Values represent the mean (± S.E.M.) of five samples. *$p<0.05$ by Student's t-test.

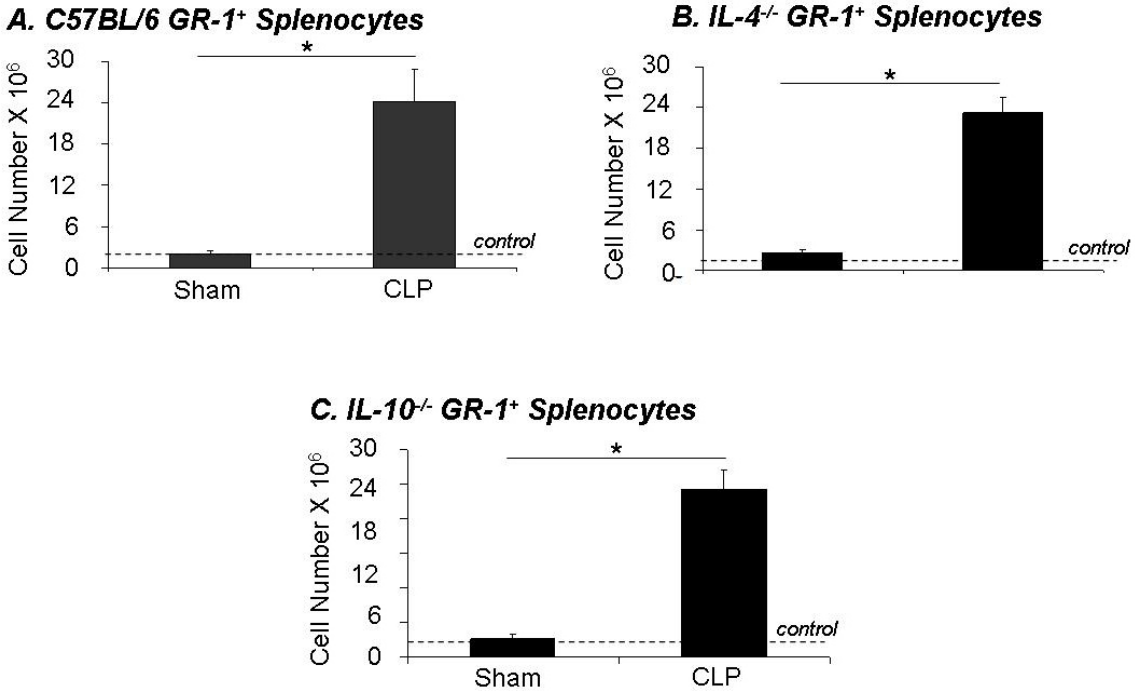

Figure 3-3. GR-1^{+}CD11b^{+} myeloid cell expansion is not dependent on the presence of IL-4 or IL-10 production. Wild type, IL-10 or IL-4 null animals underwent CLP or sham procedure and at 7 days after sepsis the spleens harvested and analyzed for the total numbers of GR-1^{+} cells per spleen. Splenic expansion of GR-1^{+} cells in wild type C57BL/6 mice (Panel A) is similar to that of the IL-4 (Panel B) and IL-10 null mice (Panel C). Values represent the mean (± S.E.M.) of three to five samples. *$p<0.05$ by Student's t-test.

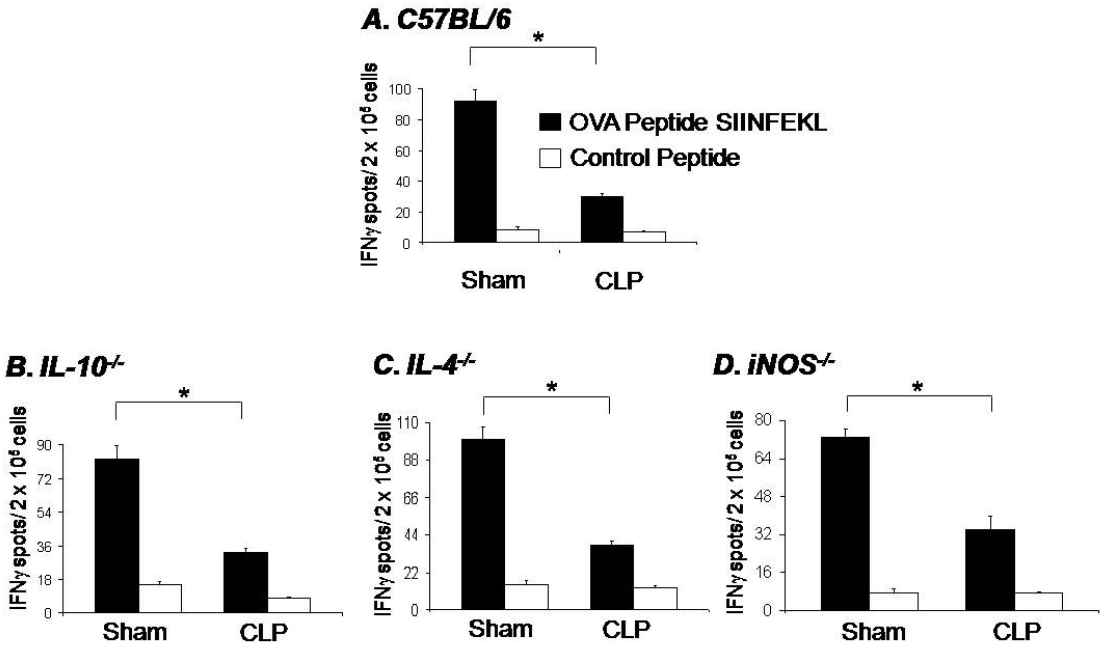

Figure 3-4. Effect of GR-1$^+$ cells from septic mice on antigen specific CD8$^+$ T-cell IFN-γ responses. Mice were treated as described in the Materials and Methods. GR-1$^+$ cells from septic wild type control C57BL/6 mice (Panel A), IL-10$^{-/-}$ (Panel B), IL-4$^{-/-}$ (Panel C), and iNOS$^{-/-}$ (Panel D),markedly attenuated the IFN-γ production (determined by ELISpot™) by OT-1 splenocytes stimulated with either control peptide or OVA-derived peptide SIINFEKL *ex vivo* following administration and immunization in C57BL/6 mice. Antigen specific CD8$^+$ T-cell IFN-γ production suppression is unaltered by GR-1$^+$ cells lacking the ability to secret IL-4, IL-10, or iNOS implying that IL-4, IL-10 or iNOS production by GR-1$^+$ splenocytes is not integral to immature myeloid cell CD8$^+$ T-cell IFN-γ production. Values represent the mean and standard error of five animals per group. The experiments were each repeated twice and values presented are from one of the representative experiments. * $p<0.05$ by ANOVA and Student's-Newman-Keuls multiple range test.

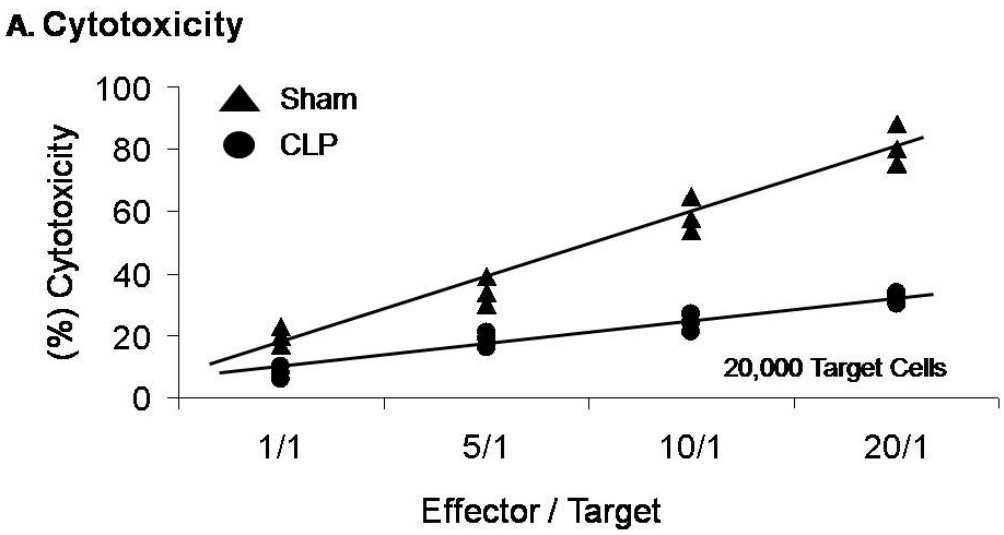

Figure 3-5. Effect of GR-1^+ cells from septic mice on antigen specific $CD8^+$ T-cell cytotoxic function. Mice were treated as described in the Materials and Methods. GR-1^+ cells from septic animals attenuated the ability of $CD8^+$ T cells (Panel A) to execute their cytotoxic functions on OVA expressing target cells (E.G7-OVA cells, ATCC# CRL-2133TM)

CHAPTER 4
MYELOID DERIVED SUPPRESSOR CELL EXPANSION IS DEPENDENT ON CXCL12 MEDIATED COMMON MYELOID PROGENITOR EXPANSION DURING SEPSIS

Specific Aim 3

The goal in Specific Aim 3 was to confirm the signaling pathways that are required for the expansion of the MDSC populations in polymicrobial sepsis. Our preliminary data suggests that MyD88$^{-/-}$ animals display a delay in the peripheral expansion of their MDSC populations in sepsis; however, the role that MyD88 signaling occupies in MDSC expansion is still not fully known. The following experimental approaches were devised to answer the questions set forth in Specific Aim 3.

Introduction

Expansion of a myeloid derived suppressor cell (MDSC) population (91) has been observed in the spleens and tumors of mice with transplantable tumors (48, 80), and in models of chronic inflammation (50). In tumor-bearing mice, these cells contribute to tumor-associated antigen specific T cell dysfunction and tolerance (74, 80, 81, 99). We have recently described a similar heterogeneous population of GR-1^{+}CD11b^{+} immature myeloid cells whose numbers dramatically increase in the spleen, lymph nodes and bone marrow during polymicrobial sepsis. These MDSCs are capable of inhibiting antigen-specific CD8^{+} T cell interferon-γ production and antigen nonspecific CD4^{+} T cell proliferation during sepsis, and the polarization of the T helper cell response from a T_H1 to a T_H2 profile. However, the specific signaling mediators responsible for this sepsis-induced immature myeloid cell expansion in the spleen are yet unknown.

Recent reports suggest that the expansion of immature myeloid cells in the bone marrow, spleen and lymph nodes is a highly conserved response that occurs in a multitude of insults and may be dependent on Toll-like receptor (TLR) signaling (48, 100). Kinkade and colleagues observed this TLR dependent myeloid cell expansion first hand by demonstrating that

hematopoietic stem cells (HSCs) in culture with TLR2 and TLR4 agonist (Pam3CSK4 and LPS respectively), undergo proliferation and preferential differentiation into myeloid lineage cells (101). We too, have observed a partial dependence of splenic immature myeloid cell expansion on the MyD88 pathway in vivo, although not as stunning as Kincade and associates saw with lipopolysaccharide, an exclusive TLR4 agonist. However after further investigation we found that MyD88$^{-/-}$ animals exhibit only a delayed expansion of GR-1^{+}CD11b^{+} cells in the spleen that approached wild type control levels with the progression of sepsis (99). This evidence leads us to believe that there must be other signals that regulate myeloid expansion during sepsis.

In the oncology literature, multiple studies have shown that cytokines and prostaglandins, including GM-CSF, G-CSF-1, IL-6, IL-10 and PGE2 may regulate the expansion of immature myeloid populations (49, 63). In states of inflammatory stress, hematopoietic stem cells (HSCs) continually circulate between the bone marrow and peripheral organs to maintain or expand lymphoid and myeloid populations.(102) Although the exact mechanisms of stem cell egression and myeloid expansion are still unknown, Petit and colleagues demonstrated that granulocyte-colony stimulating factor (G-CSF) facilitates bone marrow egression through the activity of bone marrow derived neutrophil elastase on CXCL12 (stromal cell derived factor-1;SDF-1). The authors further demonstrated that this egression can be inhibited by using specific elastase inhibitors and anti-CXCR4 neutralizing antibodies, which disrupt the CXCL12-CXCR4 signaling axis, reducing the number of progenitor cells found in the spleen (103) In addition, recent reports have shown that the C-C chemokine receptor-2 (CCR2) is essential for monocyte recruitment from the bone marrow in times of bacterial infection; myeloid expansion in the spleen is reduced in infected mice devoid of CCR2 (100, 104). Interestingly, CCR2-CCL2 (MCP-1) interactions were shown to facilitate the expansion and migration of myeloid derived

suppressor cells in cancer patients and mice with growing tumors (105). However, as the MDSC population is heterogeneous, so are the signaling pathways involved in myeloid expansion. The c-*fms* receptor (CD115, M-CSFR, CSF-1R), is a myeloid lineage cell marker that progressively increases from stem cell precursors to monocytes and macrophages (106). C-*fms* receptor ligation with its ligand M-CSF stimulates the differentiation, proliferation and survival of HSCs as they undergo myeloid development (107). Several studies have demonstrated that administration of the c-*fms* receptor antibody AFS98 reduces the relative percentages and absolute numbers of macrophages and other myeloid derived cells after various injuries (108, 109). Taken together, these data provide potential targets for the amelioration of MDSC expansion during sepsis.

In this study, we have demonstrated that the overall sepsis induced immature myeloid cell population expansion is only modestly dependent on CSF-1 and CCR2 signaling with immature monocyte accumulation mainly dependent on these two pathways. In contrast, CXCL12 signaling during sepsis proved necessary to the complete expansion of the immature myeloid population in sepsis with CXCL12 blockade inhibiting nearly 70% of the overall myeloid expansion 7 days after the initiation of sepsis. Further analysis of cells along the myeloid differentiation pathway demonstrated that CXCL12 inhibition effectively reduced the splenic accumulation of the common myeloid progenitor cell population-the earliest and most immature cell in the myeloid differentiation pathway. Therefore, we conclude that CXCL12 signaling is imperative for the complete immature myeloid expansion cell expansion that occurs during polymicrobial sepsis.

Results

Hematopoietic Stem Cell Proliferation and Differentiation do not Dependent on MyD88 or TRIF Signaling *in vivo*.

We have previously reported that complete splenic expansion of the MDSC population may have some dependence on MyD88 signaling which resolves with sepsis progression beyond 7 days (99). Several reports also indicate that HSC proliferation and preferential differentiation into myeloid lineage cells are dependent on the MyD88 signaling pathway in vitro (101, 110-112). Since MDSCs are an immature heterogeneous contingent of cells at various stages of development in the myeloid differentiation pathway, we speculated, based on the aforementioned data, that HSC proliferation and myeloid differentiation should be impaired using an in vivo system devoid of MyD88 signaling, and may account for the delay that we have observed in MDSC expansion in MyD88$^{-/-}$ mice during sepsis. Using a low mortality (LD_{20}) cecal ligation and puncture (CLP) model of polymicrobial sepsis and incorporating wild type, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice at 1 and 7 day periods, were able to determine the fluctuations that occur in the HSC, progenitor cell, and immature myeloid cell populations in the bone marrow after the induction of sepsis. In the wild type B6.129 mice, we found that sepsis produced a 2 fold increase in the relative percentages (Figure 4-1 Panel A) and absolute numbers (*data not shown*) of HSCs (Lineage^{neg}c-Kit^{high}Sca-1^{high}) in the bone marrow within 24 hours after sepsis that persisted through 7 days in the CLP group compared to sham controls. Interestingly, and in contrast to Kincade and associates (101), we found that MyD88$^{-/-}$ and TRIF$^{-/-}$ animals exhibited the same increase in HSC numbers at 24 hours and 7 days post-sepsis as did sham and wild type control animals (Figure 4-1 Panels B and C). This finding suggests that MyD88 and TRIF signaling are not imperative for early or prolonged HSC proliferation in vivo during sepsis.

When HSCs proliferate they give rise to multipotent progenitors (MPPs) that lose their ability to differentiate as they mature into cells in the lymphoid and myeloid lineages. MMPs differentiate into common lymphoid progenitors (CLPs) in the lymphoid lineage and common myeloid progenitors (CMPs) which further mature into megakaryocyte erythrocyte progenitors (MEPs) and granulocyte macrophage progenitors (GMPs)-all of which are bone marrow progenitors in the myeloid lineage. After finding a substantial elevation in the HSC numbers in MyD88$^{-/-}$ mice within 24 hours after sepsis, we next sought to determine if this HSC increase actually translated into increased numbers of bone marrow progenitor cells and whether the absence of MyD88 would hinder HSC differentiation along the myeloid lineage in vivo. As shown in Figure 4-2 Panel A, we observed little change in the percentages of total bone marrow progenitors (Lineage^{neg}c-Kit^{high}sca-1^{neg}) at 24 hours after sepsis; however, by 7 days we found a significant increase in the quantity of progenitor cells in the sepsis mice compared to sham controls. When we evaluated the bone marrow progenitor population in the MyD88 and TRIF mice after sepsis we found little change in progenitor numbers at 24 hours; surprisingly, we found the same increase in progenitor cell numbers at seven days post sepsis compared to wild type controls (Figure 4-2 Panels B and C). This finding implies that although HSC differentiation in vitro may be dependent on MyD88 mediated TLR signaling, that in vivo, HSC differentiation into progenitor cells can still occur in the absence of MyD88 signaling.

Although we did not observe a difference in the relative fluctuations in HSC and progenitor populations between wild type, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice, we postulated that a MyD88 dependent differentiation between the progenitor population and more terminally differentiated myeloid cells may exist, and halt progenitor maturation accounting for the increases in progenitor cell observed in the MyD88$^{-/-}$ and TRIF$^{-/-}$ mice at 7 days post sepsis. In

contrast, as Figure 4-3 Panels A, B and C suggest, the same immature myeloid cell (GR-1^{+}CD11b^{+}) fluctuations occur in all three mouse types at 24 hours and 7 days after sepsis initiation. The reduction in GR-1^{+}CD11b^{+} cells at 24 hours is similar in all the mouse types tested and represents immature granulocyte egression from the bone marrow in response to sepsis. By 7 days after CLP sepsis, there was an equivalent and significant increase in the immature myeloid population across all three mouse types indicating that differentiation from the HSC population through the progenitor population and on to the immature myeloid population can occur in the absence of MyD88 and TRIF signaling in vivo.

MDSC Expansion During Sepsis Occurs Independently of the CCR2 Signaling Pathway.

Given our finding that immature myeloid cells can expand even in the absence of MyD88 signaling in the bone marrow and have only partial dependence on MyD88 signaling to expand in the spleen, we began to investigate other potential mediators known to effect myeloid cells in other various models of inflammation. Previously, we reported a substantial increase in the GR-1^{+}CD11b^{+} myeloid population in the spleen and bone marrow beginning three days after sepsis (99). Phenotype analysis revealed that over 40% of the GR-1^{+}CD11b^{+} cells were also CD31^{+}, a marker of immaturity, and could form colonies ex vivo when cultured with G-CSF or GM-CSF, but not erythropoietin.

Given the increased number of immature myeloid cells in secondary lymphoid organs following sepsis, we sought to investigate the underlying mechanism(s) responsible for this myeloid cell expansion. Several reports indicate that the C-C chemokine receptor 2, (CCR2) signaling pathway is required for GR-1^{high} monocyte egression from the bone marrow into the peripheral circulation during *listeriosis*.(104) We found that *ex vivo* stimulation (10 μg/mL bacterial lipopolysaccharide) of enriched GR-1^{+} splenocytes obtained 7 days after CLP but not sham treatment demonstrated increased production of MCP-1, the ligand for CCR2 (Figure 4-4

Panel A). Since the splenic myeloid population is capable of MCP-1 production, we sought to determine whether CCR2 is involved in the myeloid bone marrow egression and lymphoid expansion during polymicrobial sepsis. Seven days after sepsis, we examined the spleen and bone marrow myeloid cell numbers in wild type control C57BL/6 mice and in $CCR2^{-/-}$ mice. As demonstrated in Figure 4-4 panels B and C, the $GR\text{-}1^{+}CD11b^{+}$ population in the spleen increased comparably in wild type and $CCR2^{-/-}$ mice. Similar increases in the myeloid population were obtained in the bone marrow of wild type and $CCR2^{-/-}$ mice *(data not shown)*. We did not observe any significant differences in the absolute numbers and relative percentages of $GR\text{-}1^{+}CD11b^{+}F4/80^{+}$ population or the total $F4/80^{+}$ population between CCR2 null and control mice *(data not shown)*. Furthermore, no differences were observed in the absolute numbers and relative percentages of $Ly6C^{high}$ cells between the CCR2 null and the wild type mice, suggesting that CCR2 signaling is not essential for immature myeloid cell expansion in the spleen during polymicrobial sepsis.

Although the overall myeloid expansion in the spleen of both wild type and CCR2 null mice was similar at 7 days post sepsis, there were some substantial differences in the expansion of the $GR\text{-}1^{intermediate}CD11b^{+}$ populations. These $GR\text{-}1^{intermediate}$ cell populations represent more immature monocytic cell populations. In Figure 4-5 panel A, there was a 50% reduction in the numbers of the $GR\text{-}1^{intermediate}$ population in the sham $CCR2^{-/-}$ mice, as compared to the wild type sham mice. After induction of sepsis, there was a 7-fold increase (Figure 4-5 panel B) in the $GR\text{-}1^{intermediate}$ splenocytes in the wild type group, compared to only a 3-fold increase in the $CCR2^{-/-}$ mice, indicating that CCR2 may participate in the expansion of certain subpopulations of splenic MSDCs. Based on CD11b, F4/80, and MHC II cell surface analysis, the four fold

reduction consisted of mainly immature monocytes. Analysis of this same subpopulation in the bone marrow revealed no significant differences between the two groups (*data not shown*).

In order to explain the overall expansion of the myeloid population in the absence of CCR2, we examined whether the frequency of myeloid precursor cells in the bone marrow changes in response to sepsis. Broxmeyer and colleagues demonstrated that there are increased numbers of early myeloid progenitors in $CCR2^{-/-}$ mice compared to wild type littermate controls (113). Thus, we postulated that in $CCR2^{-/-}$ mice, the absence of MCP-1 signaling on the myeloid progenitor population may increase the numbers of bone marrow myeloid progenitors whose proliferation is dependent on the CCR2-MCP-1 axis. The relative percentages of $lineage^{-}c\text{-}kit^{+}sca\text{-}1^{-}$ bone marrow and splenic progenitor cells in $CCR2^{-/-}$ and wild type litter mates were determined seven days after induction of sepsis (Figure 4-6, panels A and C). As depicted in Figure 4-6 panel B and D, there was no significant difference in the percentages of myeloid progenitor cells in the bone marrow and spleen, also suggesting that MCP-1-CCR2 does not participate in myeloid progenitor expansion during sepsis.

M-CSF Receptor Signaling Modestly Inhibits Immature Myeloid Cell Expansion

Due to the fact that CCR2-MCP-1 inhibition provided little impairment of the immature myeloid cell expansion that occurs during sepsis, we sought to investigate other signals that have been shown to govern myeloid cell development and expansion in other model systems. The c-*fms* receptor and it's ligand M-CSF have been shown to play a pivotal role in the differentiation and proliferation of monocytes and macrophages (107). AFS98, an anti-murine c-*fms* antibody which inhibits M-CSF–dependent growth and development by binding to the c-*fms* receptor, has been shown to have a profound effect on monocyte/macrophage peripheral expansion (114). Therefore, we hypothesized that c-*fms* inhibition by AFS98 may ameliorate secondary lymphoid organ myeloid cell expansion. AFS98 was administered 12 hours prior to sepsis and once daily

thereafter (see **Materials and Methods**) for a total of six consecutive days after CLP treatment. We evaluated the bone marrow, lymph node, and splenic myeloid cell populations and determined the amount of myeloid cell expansion compared to isotype control treated septic mice. As shown in Figure 4-7 Panel A and B, AFS98 treatment produced only a modest reduction (25%) in the overall splenic GR-1^{+}CD$11b^{+}$ population with no differences seen in the bone marrow or lymph node GR-1^{+}CD$11b^{+}$ populations (*data not shown*) compared to CLP plus isotype control treatment. More detailed analysis reveal a more substantial reduction in the GR-$1^{intermediate}$CD$11b^{+}$ population analogous to the reduction observed in the CCR$2^{-/-}$ septic animals; however, again there was little absolute change in the cell numbers in the overall myeloid population. Further investigation using CD11b, MHC II, F4/80, markers revealed that this GR-$1^{intermediate}$CD$11b^{+}$ population consists almost entirely of immature monocytes and macrophages (*data not shown*). To evaluate the efficacy of the AFS98 anti-c-fms receptor inhibitor in our model of polymicrobial sepsis, we evaluated the levels of remaining c-fms receptor positive (CD115^{+}) cells after AFS98 administration compared to isotype control treated animals. Interestingly, we found only minimal reduction in the triple positive (GR-1^{+}CD$11b^{+}$CD115^{+}) cells as shown in Figure 4-7 Panel C. This finding leads to us believe that first, AFS98 inhibition is ineffectual in the setting of murine polymicrobial sepsis based on the plethora of CD115^{+} cells acquired after AFS98 treatment and secondly, based on the CCR2 and AFS98 findings, the myeloid expansion observed during sepsis is largely due to other expanding populations in the myeloid differentiation pathway and not monocytes and macrophages.

MDSC Expansion During Sepsis Occurs Independently of Neutrophil Elastase Activity.

Since the expansion of myeloid cells occurs largely independent of CCR2- MCP-1 and c-fms-CSF-1 signaling pathways, we next tested whether the MDSC expansion in the spleen may be the result of G-CSF mediated progenitor egression from the bone marrow to the secondary

lymphoid organs. Petit and colleagues previously demonstrated that G-CSF is responsible for bone marrow stem cell mobilization through CXCL12-CXCR4 signaling, via the increased activity of neutrophil elastase (103). Neutrophil elastase inhibition also decreased stem cell mobilization from the bone marrow. Therefore, we hypothesized that neutrophil elastase inhibition may ameliorate the bone marrow egression of myeloid progenitor cells and inhibit the expansion of immature myeloid cells in the spleen. Neutrophil elastase inhibitor (MeOSuc-Ala-Ala-Pro-Val-CMK 1.5 mg/day I.P) was administered on days 3, 4, 5 and 6 following sepsis. On day 7 during sepsis, we evaluated the bone marrow and splenic myeloid and myeloid progenitor cells. As demonstrated in Figure 4-8 Panels A and B, there was no appreciable difference in the percentages of splenic GR-1^{+}CD$11b^{+}$ cells or splenic progenitor cells in mice treated with neutrophil elastase inhibitor compared to mice undergoing sepsis alone. Similarly, as shown in Figure 4-8 Panels C and D, there was also no appreciable difference between the numbers of bone marrow myeloid cells or bone marrow progenitors between neutrophil elastase treated mice compared to mice undergoing sepsis alone. Although neutrophil elastase activity may be necessary for stem cell migration from the bone marrow, our data would indicate that the same does not hold true for the egress of the bone marrow myeloid population.

CXCL12 is Required for Complete MDSC Expansion during Sepsis.

Since the expansion of the overall myeloid cell population is not entirely dependent on TLR-MyD88, CCR2- MCP-1 or c-*fms*-MCSF-1 signaling pathways, we next investigated a pathway more central to immature cell trafficking. Recent reports have shown that the CXCL12-CXCR4 signaling axis is paramount to hematopoietic progenitor cell bone marrow egression and splenic homing along with other cell populations in the myeloid lineage. Kelsoe and colleagues have identified CXCL12 signaling as a major determinant of immature myeloid and lymphoid cell expansion in the spleen and bone marrow using immunization models (115, 116). Moreover

immature myeloid cell expansion in the spleen may be the result of G-CSF mediated CXCL12 dependent progenitor egression from the bone marrow to the secondary lymphoid organs. Petit and colleagues previously demonstrated that G-CSF is responsible for HSC and progenitor cell mobilization from the bone marrow through CXCL12-CXCR4 signaling.(103). Considering these reports we next hypothesized that the CXCL12 signaling pathway may mediate the splenic myeloid expansion during sepsis. To test this hypothesis we obtained a preparation of anti-CXCL12 antibodies (117) (see **Materials and Methods**) that have been shown to block CXCL12 in murine models of pulmonary fibrosis (118, 119). We administered 200 mg of anti-CXCR12 i.p. daily beginning 12 hours prior to CLP sepsis through to 7 days and administered heat inactivated goat serum (200 mL) i.p. as an isotype control. As shown in Figure 4-9 Panel A anti-CXCL12 treatment during sepsis substantially reduced the relative percentage of GR-1^+CD11b^+ splenocytes over 60% compared with isotype treatment and sepsis. Not only were the relative percentages reduced but also the total number of GR-1^+CD11b^+ splenocytes were reduced to almost the level of sham treatment (Figure 4-9 Panel B). In addition, the total numbers of splenic CD3^+CD4^+, CD3^+CD8^+, and B220^+ cells were unaltered with anti-SDF-1 treatment (*data not shown*).

Over the last decade, it has been demonstrated that CXCL12 signaling is an integral component of not only HSC and progenitor mobilization, but also for progenitor cell proliferation and survival (120-122). When HSCs proliferate they give rise to MPPs that differentiate into CLPs in the lymphoid lineage and CMPs that further mature into MEPs and GMPs all of which are bone marrow progenitors in the myeloid lineage (123). We evaluated the bone marrow and spleen for each of the progenitor cell types in the myeloid lineage to determine if CXCL12 inhibition had an impact on immature myeloid cell precursors. At 7 days after

treatment with daily anti-CXCL12 therapy in septic mice, we harvested the bone marrow and spleen and evaluated the relative percentages and absolute numbers of HSC's (Lineage^{neg}c-Kit^{high}sca-1^{high}), CMP (Lineage^{neg}c-Kit^{high}sca-1^{neg}CD34^{high}FcγR^{low}), GMP(Lineage^{neg}c-Kit^{high}sca-1^{neg}CD34^{high}FcγR^{high}), and MEPs (Lineage^{neg}c-Kit^{high}sca-1^{neg}CD34^{neg}FcγR^{low})(123). The gating strategy employed consisted of first eliminating debris and dead cells based on forward and side scatter and Sytox staining (Figure 4-10 Panel A). Next the lineage^{neg} live non-debris cells were gated based on their c-Kit and sca-1 expression and the c-Kit^{+} cells were further evaluated based on their FcγR and CD34 expression. As shown in Figure 4-10 Panels B, C and D, there was little difference in the splenic HSC percentages; however, there was a significant decrease in the percentage of total progenitors as well as the specific CMPs in the mice receiving anti-CXCL12 treatment during sepsis (Figure 4-10 Panels C and D). There were no differences in the splenic MEP or GMP populations (*data not shown*).

Inhibiting the CXCR4-CXCL12 signaling axis has been shown to reduce bone marrow egression of progenitor cells into the periphery (103). We hypothesized that if CXCL12 was inhibiting bone marrow egression of progenitor cells that there could possibly be a buildup of progenitor cells in the bone marrow that are unnable to exit to the periphery during CXCL12 inhibition. We evaluated the bone marrow HSC, CMP, GMP and MEP populations, and found no increase in the anti-CXCL12 treated animal's HSC or CMP populations compared with control treatment and sepsis (Figure 4-11 Panel A and B). Furthermore, there was also no difference in the bone marrow MEP or GMP populations (*data not shown*) between the control and the anti-CXCL12 treatment group.

Discussion

Although the etiology of sepsis-induced immune dysfunction is unknown, recent interest has focused on the effects of regulatory cell populations responsible for the shift from a T_H1 to a

T_H2 immune profile that results in a state of T cell anergy and immune suppression (42, 85, 98). We have previously described a substantial expansion of a GR-1^+CD$11b^+$ myeloid population with an immature phenotype capable of antigen specific $CD8^+$ T-cell suppression and T_H1 to T_H2 immune polarization. However, the mechanisms driving this immature myeloid cell expansion remain elusive. In the current study, we observed that an ongoing septic process produces a dramatic expansion of GR-1^+CD$11b^+$ cells in the spleen that is independent of CCR2, c-*fms* receptor, neutrophil elastase effects, and independent of MyD88 and TRIF signaling events in the bone marrow. However, what we did demonstrate is that myeloid expansion during sepsis requires the presence of an intact CXCL12-CXCR4 signaling axis to achieve full MDSC expansion.

We have previously demonstrated that MDSC expansion in the spleen is delayed in the absence of MyD88 signaling; however, as sepsis progresses MDSC expansion begins to approach wild type control levels implying that MyD88 signaling may be involved in the early events mediating MDSC expansion yet MyD88 may not be mandatory for ultimate splenic MDSC expansion (99). Based on the work of Kincade and colleagues (101) demonstrating that HSC proliferation and preferential differentiation toward the myeloid lineage is dependent on MyD88 signaling *in vitro*, we believe that the absence of MyD88 signaling *in vivo* may account for the delay in MDSC splenic accumulation we observed during long term sepsis. Contrary to this supposition in this study, we found little evidence that HSC or progenitor cell proliferation early in sepsis requires MyD88 signaling. The absence of MyD88 signaling produced no deficiency in bone marrow HSCs, hematopoietic progenitors, or immature myeloid cells (Figures 1, 2, and 3) suggesting to us that MyD88 signaling may have little effect on bone marrow myelopoesis in an *in vivo* model of polymicrobial sepsis. Although these findings are in

opposition to those of Kincade and associates, any more meaningful conclusions are hard to formulate since the model systems used in the respective studies were very different. While we used an *in vivo* model of polymicrobial sepsis to evaluate the impact of MyD88 signaling on hematopoietic progenitors, Kincade *et. al.* employed an *in vitro* model where hematopoietic progenitors were placed in culture with various TLR agonists.

Given the innate complexity of overlapping signaling pathways and redundant nature of the host response to injury, it is not surprising that hematopoietic cell expansion during sepsis is not dependent on one signaling mediator or pathway. Moreover the fact the MyD88$^{-/-}$ mice displayed no reduction in numbers of HSCs, CMPs, and immature myeloid bone marrow cells suggests that there are indeed other signaling mediators involved in HSC proliferation. Two such mediators may be prostaglandin E2 (124) and 5-lipooxygenase (125) both of which have been shown to directly impact HSC proliferation and survival.

Several reports have demonstrated that the CCR2-MCP-1 interaction is necessary for myeloid cell recruitment during times of bacterial infection (126, 127). Since MDSCs produced increased levels of MCP-1 (Figure 4-1 Panel A) in response to endotoxin stimulation, we postulated that the CCR2 pathway may play a role in their expansion during polymicrobial sepsis. However we found few differences in the relative percentages of GR-1^{+}CD11b^{+} bone marrow and spleen in the CCR2 null mice compared to the wild type controls (Figures 4-1 and 3). The only appreciable difference was observed in the GR-1^{intermediate} CD11b^{+} population which expanded ~60% less in the CCR2 null mice (Figure 4-2, Panels A and B). Although it was originally proposed that this GR-1^{intermediate} sub-population may represent a more monocytic than granulocytic phenotype, when the GR-1^{intermediate} sub-population was further analyzed, no difference in cell surface expression of F4/80 was found *(data not shown)*. Peters and colleagues

created $CCR2^{-/-}$ chimeras and found a reduction in the lung recruitment of $F4/80^{dim}$ macrophages in response to *M. tuberculosis*, infection (100). During *L. monocytogenes* infection, Sebrina *et al*. demonstrated an accumulation of $L6C^{high}$ monocytes in the bone marrow that failed to egress into the circulation in CCR2 null mice (104). The differences in CCR2 dependency are likely due to the complexity of the polymicrobial sepsis model, when compared to a single microbial insult used in the other reports. Unlike a single microbial infection, polymicrobial sepsis exposes the host to a multitude of intestinal pathogens and microbial products (PAMPS) that signal through multiple overlapping signaling pathways (TLR dependent and independent) that appear not to be dependent upon a functional CCR2.

Realizing that myeloid expansion during sepsis is CCR2 independent, we evaluated an alternative pathway that could account for the myeloid proliferation. Petit demonstrated that G-CSF mediated neutrophil elastase degradation of bone marrow CXCL12 was responsible for bone marrow progenitor egression into the blood (103). Postulating that egression of bone marrow progenitor cells could be responsible for the splenic myeloid expansion, and since we have observed an increase in splenic progenitor cells in C57BL/6 mice (Figure 4-6 Panel C and D), we administered a neutrophil elastase inhibitor in an attempt to block bone marrow progenitor egression at doses that were effective in earlier studies (103). The results of the experiment were equivocal implying that neutrophil elastase plays no significant role in polymicrobial sepsis MDSC expansion.

CXCL12 has been demonstrated by Rafii *et. al*. to play an essential role in hematopoietic progenitor cell mobilization and progenitor cell homing. More specifically, the authors demonstrate that in response to physiologic stress, plasma CXCL12 levels rise and mediate hematopoietic cell mobilization and repopulation of peripheral organs. Others have found using

models of inflammatory stress such as cardiac surgery (128), tuberculosis infection (129) and breast cancer tumor models (130) that CXCL12 is required for hematopoietic progenitor cell peripheral recruitment. In a model of malarial infection, de Andrade demonstrated that splenic CXCL12 levels continued to increase until the infection was under control in C57BL/6 mice and facilitated $CD11c^+$ dendritic cell recruitment to the spleen (131). Our data support this notion of CXCL12 mediated recruitment given that fact that CXCL12 inhibition substantially reduced splenic MDSC accumulation (Figure 4-9). More importantly, anti-CXCL12 therapy produced an even greater reduction in the numbers of splenic CMPs-the precursor that gives rise to the other myeloid lineage cells (Figure 4-10). This suggests, although with further investigation necessary, that MDSCs arise from CMPs that have migrated from the bone marrow to the spleen and undergo myelopoesis generating the dramatic numbers of myeloid cell observed in the spleen during long term sepsis.

In conclusion, we observed that an ongoing septic process produces a dramatic expansion of $GR\text{-}1^+CD11b^+$ cells in the spleen that is independent of CCR2 and c-fms receptor signaling, bone marrow MyD88 signaling, and neutrophil elastase activity. In contrast we found that the splenic MDSC population is partially dependent on CXCL12 signaling. CXCL12 depletion substantially reduced the accumulation of the MDSC population in the spleen during sepsis. Furthermore, CXCL12 inhibition also reduced the CMP-the earliest myeloid precursor cell type suggesting that MDSC expansion depends on CMP splenic expansion.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine or Schering-Plough Biopharma. Specific pathogen-free C57BL/6 mice and CCR2 null (B6.129S4-Ccr2tm1Ifc/J) mice were purchased from The

Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the University of Florida College of Medicine and were studied between 6-12 weeks of age. MyD88$^{-/-}$ mice on a B6x129(F_1) background and TRIF$^{-/-}$ mice were a kind gift of Dr. Shizuo Akira to Schering-Plough Biopharma, and were maintained at Schering-Plough Biopharma, Palo Alto, CA.

Inhibitors

When indicated, mice were injected with 500 μL/day i.p polyclonal goat anti-CXCL12 antibodies beginning 12 hours prior to the initiation of sepsis. The anti-CXCL12 antibody preparation was a gift from Dr. Robert Strieter at the University of Virginia, Charlottesville, VA (117). As a control heat inactivated polyclonal goat serum (Sigma) was used (500 μL/day i.p). AFS98, a rat monoclonal anti-murine c-*fms* antibody (IgG2a), which inhibits M-CSF–dependent growth and development by binding of M-CSF to its receptor (114) was also used when indicated. The AFS98 hybridoma cell line was a gift from Dr E. Richard Stanley at Albert Einstein College of Medicine, Bronx, NY. The AFS98 hybridoma was cultured and the AFS98 antibody harvested and purified by Klaus Lubbe (Bio Express, Inc. West Lebanon, NH). Two milligrams of AFS98 in 200 μL of PBS were administered i.p. per mouse daily, PBS or 2 mg (200 μL/i.p. daily) of an irrelevant isotype-matched rat IgG used as described (108, 109) as a control. Mice were also treated with the neutrophil elastase inhibitor, MeOSuc-Ala-Ala-Pro-Val-CMK (1.5 mg/day) by intraperitoneal injection (Calbiochem, La Jolla, CA) on days 3, 4, 5 and 6 following the induction of sepsis (103). All inhibitors were injected 12 hrs before the induction of sepsis and continued daily as described for 6 consecutive days.

Cecal Ligation and Puncture

For induction of polymicrobial sepsis, mice underwent sham laparotomy or cecal ligation and puncture induced by ligation of the cecum and a double enterotomy created with a 27 gauge needle. Mortality in this model was approximately 10-15%, and occurred predominantly in the

first 3 days; thereafter, surviving mice developed abscesses surrounding the devitalized cecum as previously described (77, 95, 99).

Flow Cytometry

Spleens and bone marrow cells were analyzed by flow cytometry as previously described (95, 99). Antibodies included anti-GR-1 (Ly6G and Ly6C (RB6-8C5)) conjugated to APC, anti-CD11b (Integrin aM, chain Mac-1a chain (M1/70)) conjugated to Pacific Blue, anti-MHC II (I-A/I-E (2G9)) conjugated to FITC, anti-F4/80 Antigen (Pan Macrophage Marker (BM8)) conjugated to PE, Fc-Block (CD16/CD32 Fc g III/II Receptor (2.4G2), Lineage Cocktail conjugated to biotin [CD3e(145-2C11), CD11b(M1/70), CD45R/B220(RA3-6B2), Ly6G and Ly6C(RB6-8C5), TER-119(TER-119)], Sca-1 conjugated to either PE (D7), c-Kit conjugated to either FITC or APC (2B8). CD34 conjugated to Alexa Fluor 647 (RAM34), FcγR conjugated to Pac Blue (CD16/32 clone 93), and Sytox Blue. F4/80, CD11c, CD34, and FcγR specific antibodies were purchased from eBioscience and all other antibodies were purchased from BD Pharmingen. Spleens, peripheral blood, and bone marrow were harvested after either CLP or Sham surgery and single cell suspensions were created by passing the cells through 70 μm pore sized cell strainers (Falcon). Erythrocytes were then lysed using ammonium chloride lysis buffer and washed two times using PBS without calcium, phenol red, or magnesium. Samples were acquired and analyzed using a LSRII flow cytometer (BD Biosciences). A minimum of 5×10^4 live non debris cells ($Sytox^{negative}$) were collected and analyzed.

Ex vivo Stimulation and Cytokine Production

Enriched GR-1^+ cells were plated at 1×10^6 cells/well with RPMI 1640 supplemented with 10% fetal calf sera, 2 mM L-glutamine, 200 units/ml penicillin and 50 μg/ml streptomycin, and stimulated with 10 μg/ml of bacterial lipopolysaccharide (*E. coli 0111:B4)*. The culture supernatant was analyzed for cytokines using Luminex™ technology using reagents obtained

from Upstate Cell Signaling Solutions (Beadlyte™ Mouse Multi-Cytokine Detection System) (Temecula, CA).

Statistics

Continuous variables were first tested for normality and equality of variances. Differences among groups in flow cytometric analyses were evaluated by analysis of variance for multiple groups and Student's t-test for two groups. Significance was designated at the 95% confidence level.

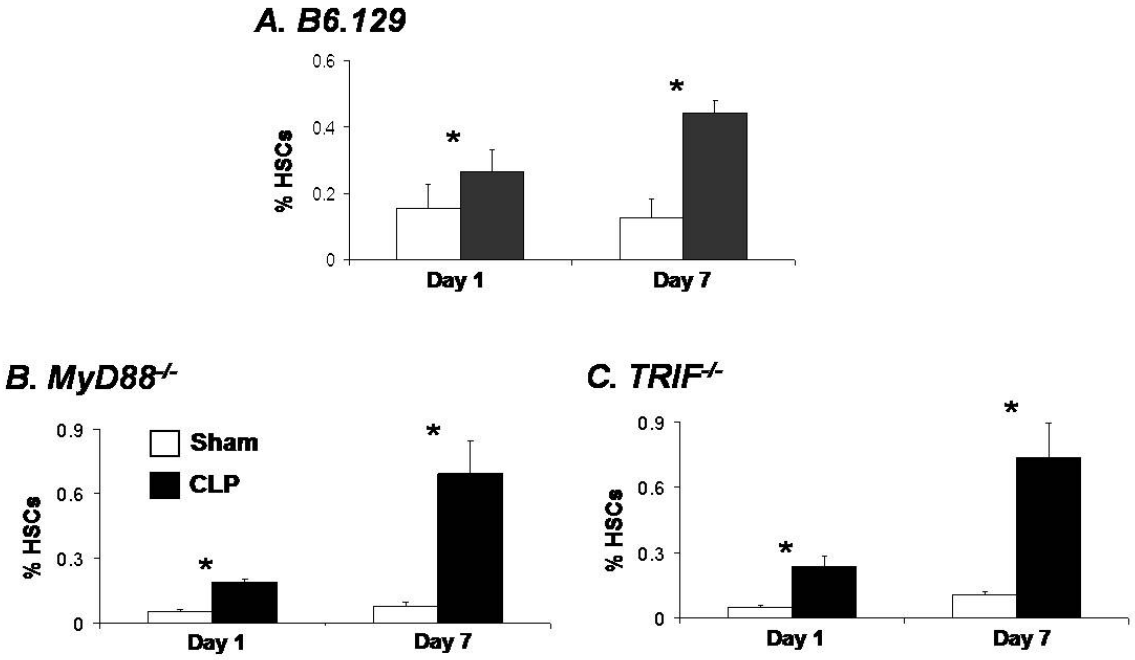

Figure 4-1. The absence of MyD88 and TRIF signaling pathways does not impact HSC expansion during sepsis. MyD88$^{-/-}$, TRIF$^{-/-}$, and B6.129 wild type mice underwent either sham or CLP induced sepsis. At days 1 and 7 after sepsis, induction total bone marrow cells were harvested and analyzed for HSCs via flow cytometry. Panel A. The relative percentage of HSCs (Lineage^{neg}c-kit^{high}sca-1^{high}) is elevated at 24 hours and remains elevated through 7 days after sepsis initiation in wild type B6.129 mice. Panels B and C. MyD88$^{-/-}$ and TRIF$^{-/-}$ mice demonstrated the same increase in HSCs as did the wild type mice in Panel A at both 1 and 7 days after sepsis induction. This implies that HSC proliferation occurs independently of both MyD88 and TRIF signaling during sepsis. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

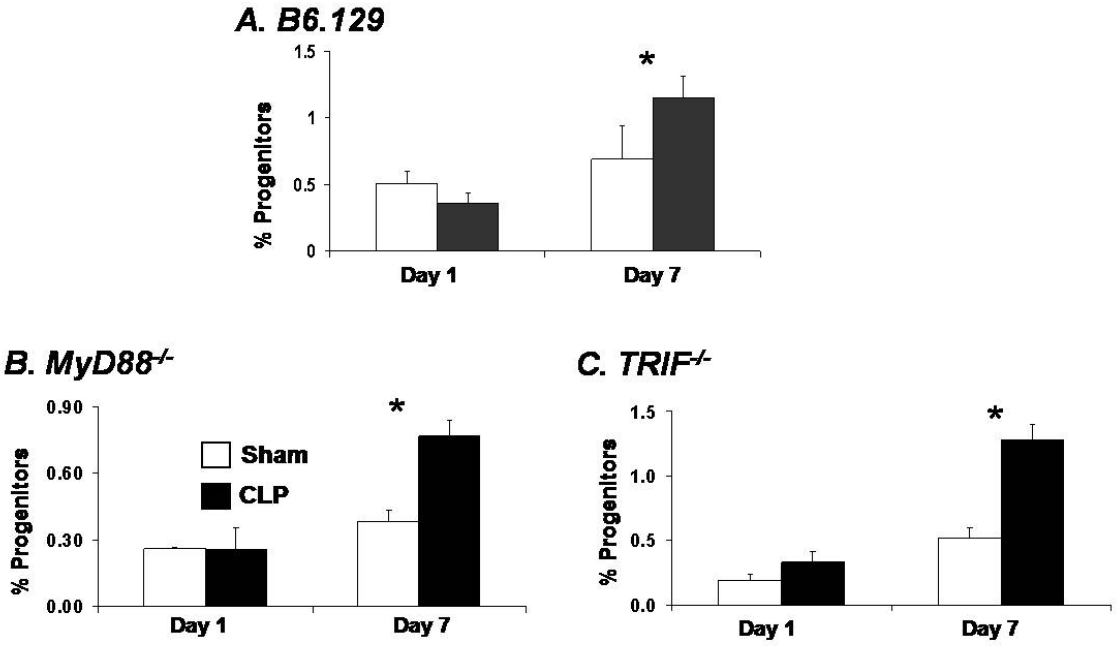

Figure 4-2. MyD88 and TRIF signaling do not impact bone marrow progenitor expansion during sepsis. MyD88$^{-/-}$, TRIF$^{-/-}$, and B6.129 wild type mice underwent either sham or CLP induced sepsis. At days 1 and 7 after sepsis induction total bone marrow cells were harvested and analyzed for bone marrow progenitor cells via flow cytometry. Panel A. The relative percentage of bone marrow progenitors (Lineage^{neg}c-kit^{high}sca-1^{neg}) is unaltered at 24 hours, however increases 2 fold over sham levels at 7 days after sepsis initiation in wild type B6.129 mice. Panels B and C. MyD88$^{-/-}$ and TRIF$^{-/-}$ mice demonstrate the same unaltered levels of bone marrow progenitors as do the wild type mice at 24 hours after sepsis induction. Both the MyD88$^{-/-}$ and TRIF$^{-/-}$ animals exhibit 2 fold increases in bone marrow progenitor levels 7 days after sepsis induction implying that bone marrow progenitor expansion occurs independently of both MyD88 and TRIF signaling during sepsis. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

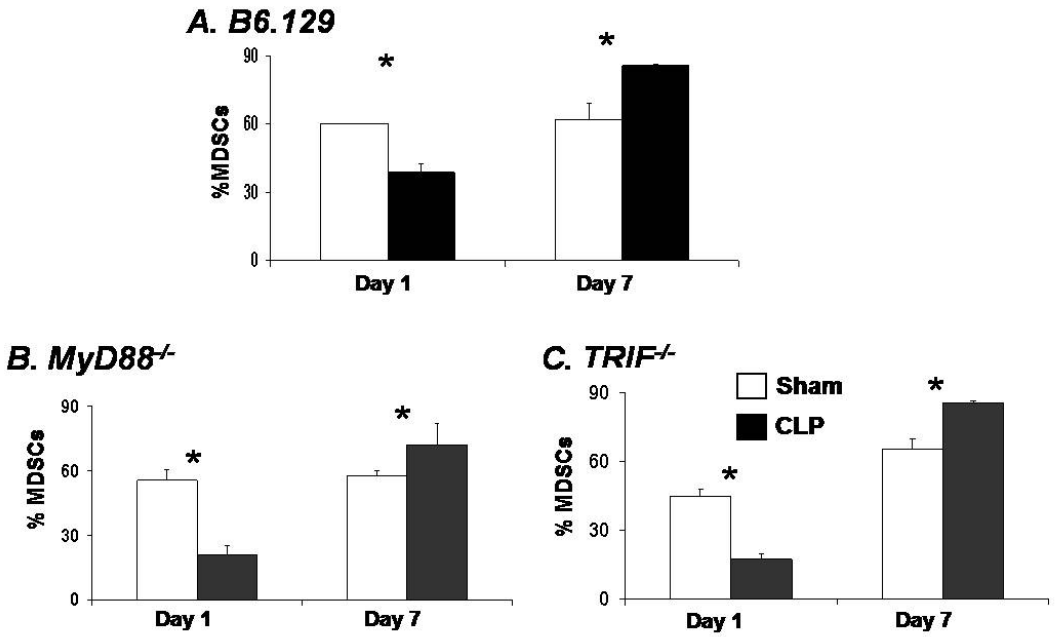

Figure 4-3. MyD88 and TRIF signaling do not effect immature myeloid cell expansion in the bone marrow during sepsis. MyD88$^{-/-}$, TRIF$^{-/-}$, and B6.129 wild type mice underwent either sham or CLP induced sepsis. At days 1 and 7 after sepsis, induction total bone marrow cells were harvested and analyzed for immature myeloid cells via flow cytometry. Panel A. The relative percentage of immature myeloid cells (GR-1^{+}CD11b^{+}) is reduced at 24 hours as a result of bone marrow degranulation into the circulation, however increases by 30% over sham levels at 7 days after sepsis initiation in wild type B6.129 mice. Panels B and C. MyD88$^{-/-}$ and TRIF$^{-/-}$ mice demonstrate the same reductions in immature myeloid cells as do the wild type mice 24 hours after sepsis. Both the MyD88$^{-/-}$ and TRIF$^{-/-}$ animals exhibit significant increases in bone marrow immature myeloid cell levels 7 days after sepsis induction. This data implies that bone marrow immature myeloid cell expansion occurs independently of both MyD88 and TRIF signaling during sepsis. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

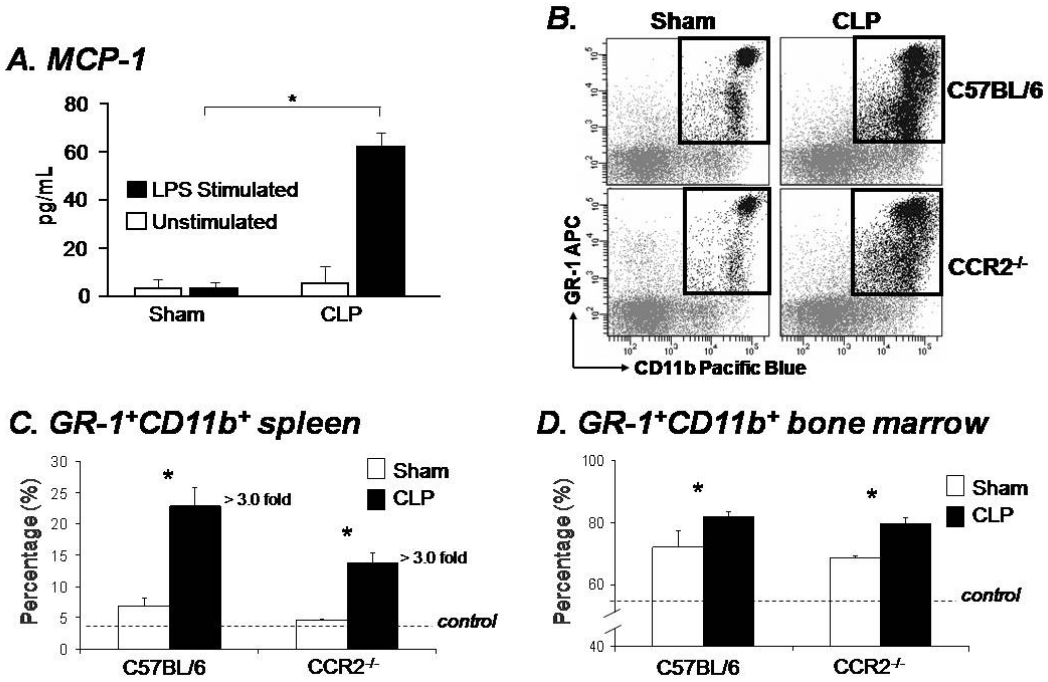

Figure 4-4. Splenic and bone marrow MDSC expansion is not dependent on CCR2 receptor signaling pathway. Panel A. GR-1^+ splenocytes harvested seven days after CLP or sham treatment produce MCP-1 upon ex vivo stimulation with 10 µg/ml of bacterial lipopolysaccharide. Panel B. Flow cytometry dot plot of viable, GR-1^+CD11b^+ total splenocytes from wild type C57BL/6 or CCR2 null mice 7 days after sham or CLP treatment. Panel C and Panel D. Percentage of GR-1^+CD11b^+ total splenocytes and bone marrow cells recovered fromC57BL/6 wild type or CCR2 null mice at 1 and 7 days after CLP or sham treatment. The absence of a functional CCR2 receptor has no significant effect on the expansion of the GR-1^+CD11b^+ population of immature myeloid cells in the spleen or bone marrow. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

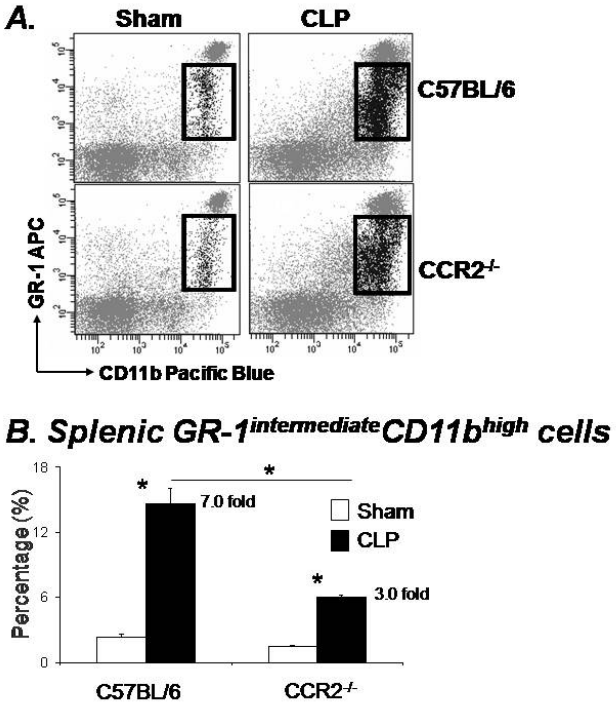

Figure 4-5. CCR2$^{-/-}$ mice exhibit fewer GR-1^{intermediate} cells. Panel A. Although the same number of total events was collected in each plot, the CCR2 null animals displayed fewer GR-1^{intermediate} cells at base line and at 7 days after sepsis treatment. Panel B. While there was a 7 fold increase in the GR-1^{intermediate} population in the wild type animals there was only a 3 fold increase in the CCR2 null mice. Values represent the mean (± S.E.M.) of between 3 and five samples. * $p<0.05$ by Student's t-test.

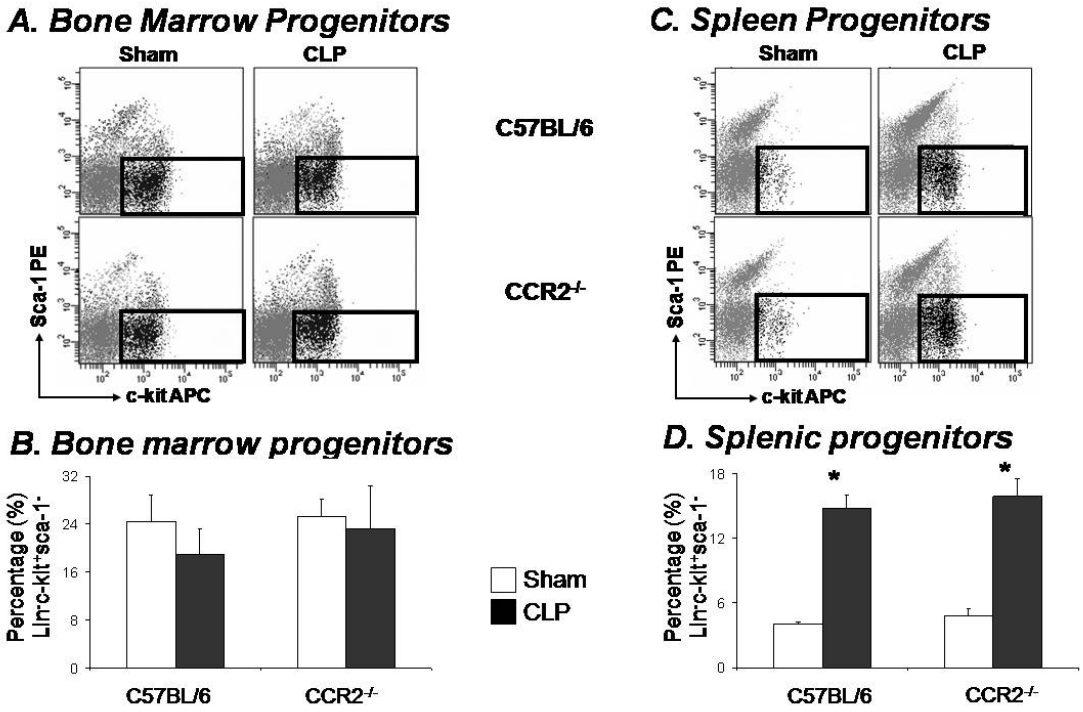

Figure 4-6. Splenic and bone marrow expansion of progenitor cells is not dependent on CCR2 receptor signaling pathway. Total bone marrow and spleen cells were harvested at 1 and 7 days after CLP or sham treatment and the percentage of bone marrow and splenic progenitor cells were determined by immune phenotyping the Lineage$^-$c-kit$^+$sca-1$^-$ fraction of viable cells using flow cytometry analysis. Panel A. Flow cytometry dot plot of the c-kit$^+$sca-1$^-$ bone marrow progenitor cells (gated off of the Lineage$^-$ cells) from wildtype (C57/BL6) and CCR2 null mice 7 days after CLP and sham treatment. Panel B and C. The absence of a functional CCR2 receptor (CCR2 null mice) has no effect on the percentage of bone marrow progenitors seven days after sham and CLP treatment. Panel C. Although significance between sham and CLP treated animals was achieved, there was no significant difference in the expansion of the splenic progenitor population 7 days after sepsis treatment between the CCR2 null animals and the wild type control (C57BL/6) animals. Both the CCR2 null and the C57BL/6 wild type control mice exhibited an equivalent 3 fold increase in the percentage of Lin-c$^-$kit$^+$Sca-1$^-$ cell fraction. * Values represent the mean (± S.E.M.) of between 3 and five samples. *$p<0.05$ by Student's t-test.

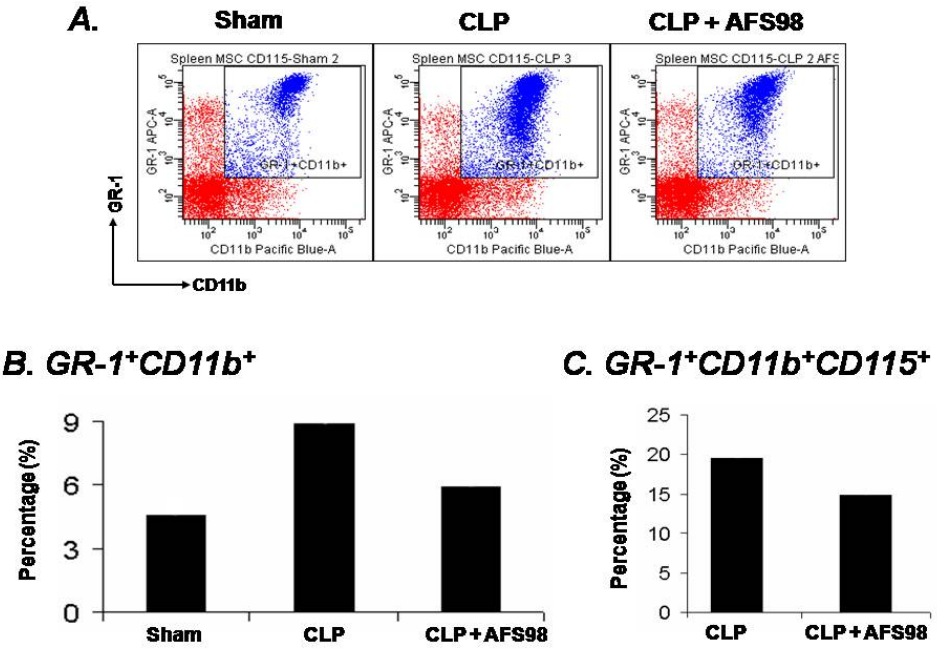

Figure 4-7. Splenic MDSC expansion is not dependent on c-*fms* receptor signaling. Panel A. Flow cytometry dot plot of viable, GR-1^{+}CD11b^{+} total splenocytes from mice that underwent sham, CLP alone or CLP with AFS98 (ant-c-*fms* receptor) treatment daliy for 7 days. Panel B. Graphic representation of the modest reduction in the relative percentages of immature myeloid cells observed after CLP plus daily AFS98 treatment compared to sham and CLP alone. Panel C. Although AFS98 is an anti-c-*fms* receptor inhibitor, AFS98 treatment only minimally reduced the percentage of c-*fms* (CD115) positive (GR-1^{+}CD11b^{+}CD115^{+}) cells in the spleen 7 days after the initiation of sepsis. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

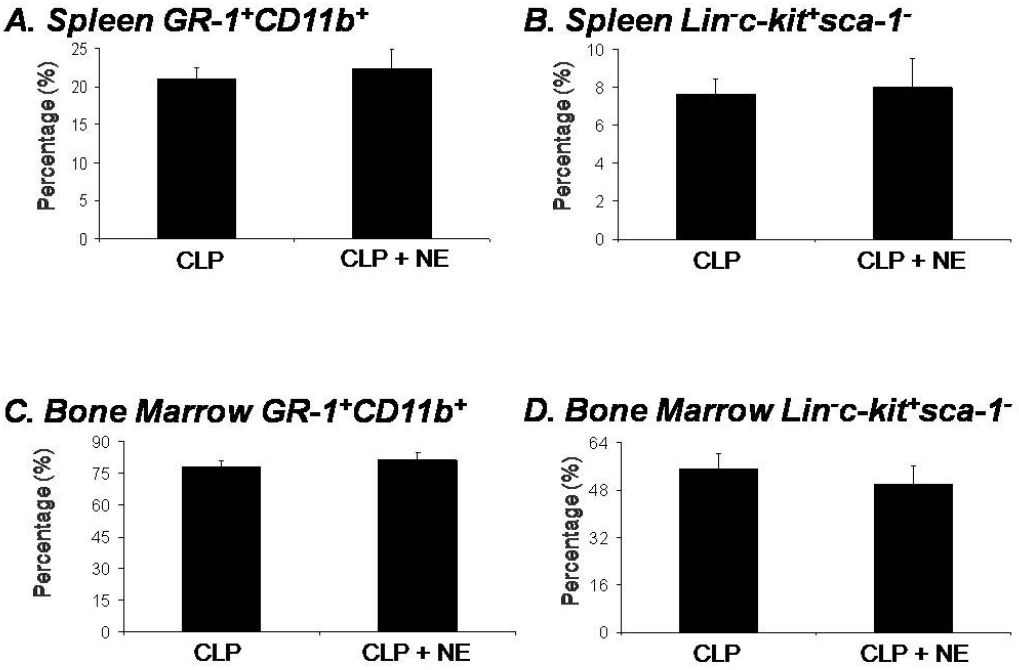

Figure 4-8. Splenic and bone marrow expansion of immature myeloid and bone marrow progenitor cells is not dependent on neutrophil elastase activity. Total bone marrow and spleen cells were harvested 7 days after CLP or sham treatment and the percentages of bone marrow and splenic immature myeloid cells and progenitor cells were determined by phenotyping the viable cell population using flow cytometry analysis. Bone marrow and splenic progenitors were defined as c-kit^{+}sca-1^{-} cells gated off of the lineage^{-} bone marrow and spleen populations. Panel A and Panel B represent the immature myeloid and progenitor populations from the spleens of mice 7 days after either CLP treatment alone or CLP treatment with neutrophil elastase inhibitor administration. Neutrophil elastase inhibition had no significant effect on the expansion of splenic progenitor or immature myeloid cell populations. Panel C and Panel D represent the immature myeloid and progenitor populations from the spleens of mice 7 days after either CLP treatment or CLP treatment with neutrophil elastase inhibitor administration. Neutrophil elastase inhibition had no significant effect on the expansion of splenic progenitor or immature myeloid cell populations. Panel C and Panel D represent the immature myeloid and progenitor populations from the bone marrow of mice 7 days after CLP treatment or CLP treatment with neutrophil elastase inhibitor administration. Neutrophil elastase inhibition had no significant effect on the expansion of bone marrow progenitor or immature myeloid cell populations. Values represent the mean (± S.E.M.) of between 3 and 5 samples. *$p<0.05$ by Student's t-test.

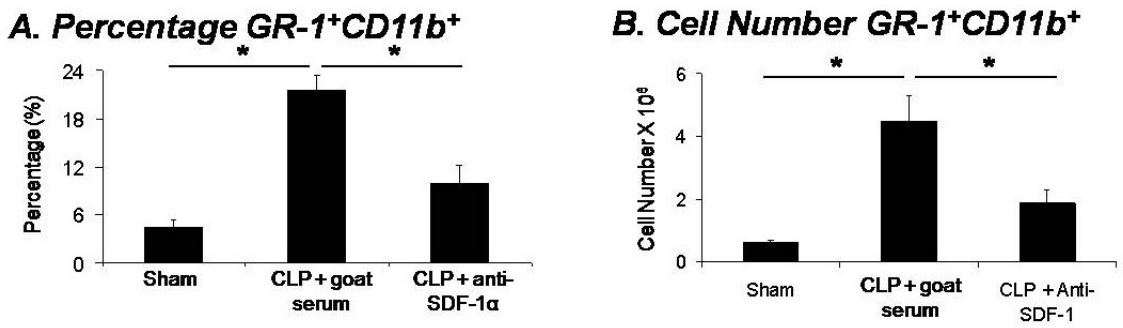

Figure 4-9. Splenic expansion of myeloid derived suppressor cells is dependent on CXCL12 signaling. Total spleen cells were harvested 7 days after CLP or sham treatment and the percentages of splenic MDSCs determined by phenotyping the viable cell population using flow cytometry analysis. Splenic MDSCs were defined as GR-1^{+}CD11b^{+} cells gated off spleen populations. Panel A and Panel B represent the immature myeloid cells from the spleens of mice 7 days after either CLP treatment alone or CLP treatment with anti-CXCL12 treatment. CXCL12 depletion reduced the splenic GR-1^{+}CD11b^{+} immature myeloid cell population by over 60% compared with CLP alone. Values represent the mean (± S.E.M.) of between 3 and 5 samples. *$p<0.05$ by Student's t-test.

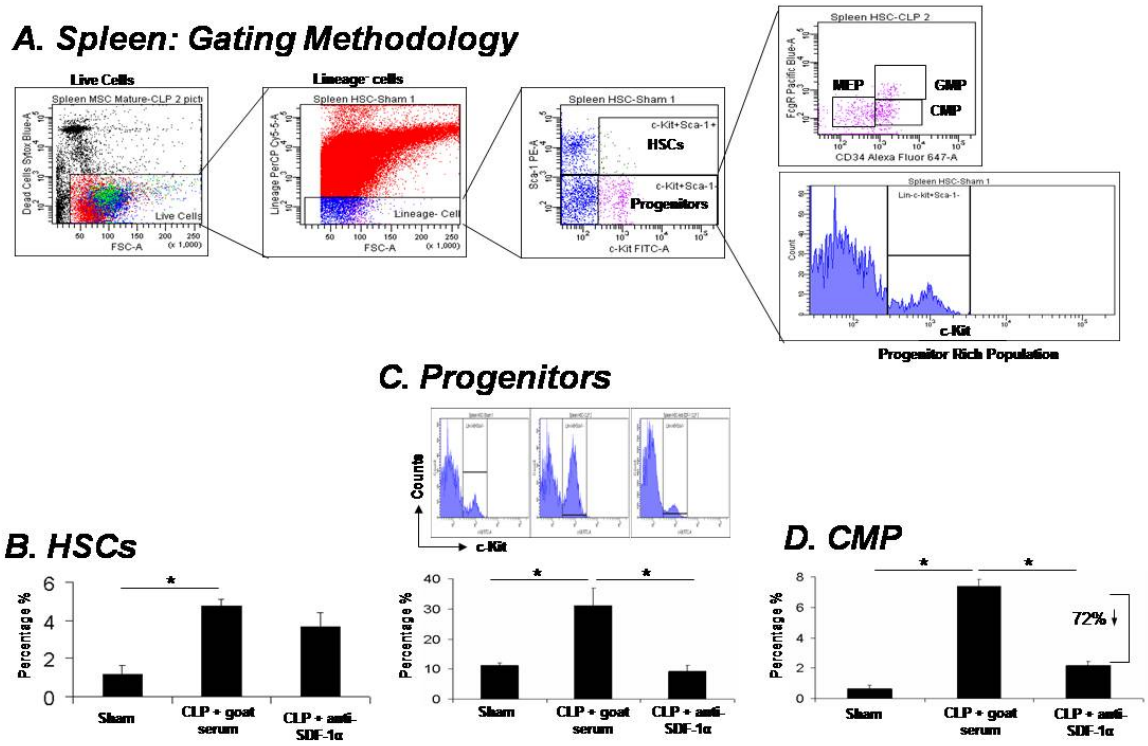

Figure 4- 10. CXCL12 inhibited the splenic accumulation of common myeloid progenitors during sepsis. Total spleen cells were harvested at 7 days after CLP or CLP with anti-CXCL12 treatment and the percentage of splenic HSCs ($Lineage^{neg}c\text{-}kit^{high}sca\text{-}1^{high}$), total progenitor cells ($Lineage^{neg}c\text{-}kit^{high}sca\text{-}1^{neg}$), and CMPs ($Lineage^{neg}c\text{-}kit^{high}sca\text{-}1^{neg}CD34^{high}Fc\gamma R^{low}$) were determined by immune phenotyping the fraction of viable cells using flow cytometry analysis. Panel A. Flow cytometry gating strategy. The live non debris cells were gated based on forward side scatter and $Sytox^{neg}$. The Lineage negative cells were then gated based on c-Kit and sca-1 expression and the $Lineage^{neg}c\text{-}Kit^{high}sca\text{-}1^{neg}$ cells were further analyzed based on their CD34 and FcγR expression. Panel B depicts the increase in splenic HSCs that occurs during sepsis and is unaltered by anti-CXCL12 treatment. Panel C illustrates the overall reduction in the splenic progenitor population after 7 days of CXCL12 inhibition. Panel D shows the reduction in the specific CMP population in the spleen at 7 days after CXCL12 depletion. * Values represent the mean (± S.E.M.) of between 3 and five samples. *$p<0.05$ by Student's t-test.

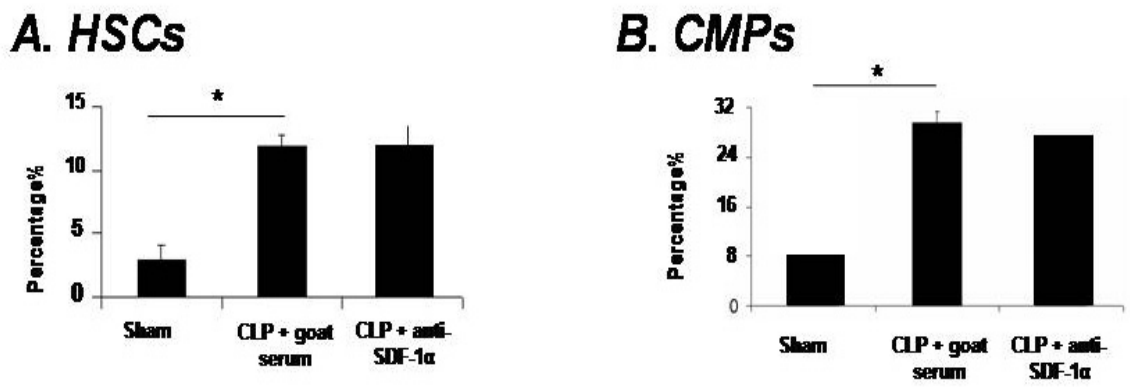

Figure 4-11. Bone marrow HSC and CMP expansion does not depend on CXCL12 during sepsis. Seven days after sham, CLP or CLP with anti-CXCL12 treatment, total bone marrow cells were harvested and analyzed via flow cytomery for the quantity of HSCs (Lineage^{neg}c-kit^{high}sca-1^{high}) and CMPs (Lineage^{neg}c-kit^{high}sca-1^{neg}CD34^{high}FcγR^{low}). Panel A demonstrates that there is no impact of the percentages of HSCs in the bone marrow with anti-CXCL12 treatment, however there is a significant increase in the percentage of HSCs during sepsis compared to sham treatment. Panel B. Although there was a substantial increase in the percentage of CMPs after sepsis initiation, no effect on the CMP population was observed after anti-CXCL12 treatment compared to sepsis alone. Values represent the mean (± S.E.M.) of between three and five samples. * $p<0.05$ by Student's t-test.

CHAPTER 5
MYELOID-DERIVED SUPPRESSOR CELLS AND THEIR CONTRIBUTION TO POST-INJURY AND SEPSIS IMMUNE SUPPRESSION

Introduction

Sepsis occurs when an overwhelming microbial infection leads to a systemic inflammatory response, manifesting clinically as fever, leukocytosis, and reduced vascular resistance, often leading to multi-system organ failure and death. Despite progress over the past 2 decades sepsis remains the leading cause of death in the intensive care unit with over 750,000 cases and 210,000 deaths annually in the United States (1, 2). Many significant advancements in sepsis pathophysiology have occurred (3, 4), but unfortunately, this progress has had only a minimal impact on the mortality rate (1, 2). A number of approaches, including anti-tumor necrosis factor-α (TNF) therapies, corticosteroids, antibodies against endotoxin, inhibitors of prostaglandins, bradykinins, and interleukin (IL)-1 receptor antagonist, have all failed in clinical trials (5). The only efficacious sepsis-related therapies currently available are activated protein C administration (Xigris^{TM}) (6), replacement steroids for sepsis-associated adrenal insufficiency (7), and insulin therapy for blood glucose maintenance (8). However, as a monotherapy or in combination, these approaches still only modestly improve outcome (5).

Sepsis and Immune Dysfunction

Much of the early work on sepsis-induced immune dysfunction focused on inflammation and the 'cytokine storm' that characterized the early response to microbial invasion. It has also been known for several decades that both severe injury and sepsis simultaneously produce a state of immune suppression illustrated by a loss of delayed type hypersensitivity (11), an inability to eradicate primary infections (12), a predisposition to develop secondary nosocomial infections (12, 13), and a failure to respond to skin testing with specific antigens (11, 132). Furthermore, animal models of sepsis indicate that acquired immune dysfunction is an intrinsic property of

sepsis, and results from defects in both the innate and acquired immune responses. Monocyte deactivation (133, 134), apoptosis induced depletion of $CD4^+$ T cells (20) and dendritic cells (21, 135), dendritic cell exhaustion or paralysis (22), suppression of T-cell proliferative responses (24, 26, 136), and reduced inflammatory and T_H1 cytokine production by monocytes and tissue macrophages (28), all contribute to immunologic compromise during sepsis, and culminate in a shift from a more proinflammatory T_H1 to a more anti-inflammatory T_H2 immune profile (4). Moreover, attention has focused on the T_H1 to T_H2 immune profile shift as an explanation for post-sepsis immune suppression (34, 137); however, the underlying mechanisms that orchestrate the shift in immune polarization during sepsis are still unknown.

Myeloid Derived Suppressor Cells Play a Role in Injury, Sepsis and Trauma

In recent years, there has been increasing interest in the role that regulatory cell populations play in the immune suppression that accompanies sepsis. There are a number of different regulatory cell populations that could potentially be important for the development of sepsis-induced immune suppression, including regulatory T cells (natural Tregs, T_H3 and TR1 cells), regulatory dendritic cells (DC3 cells), and myeloid-derived suppressor cell (MDSC) populations.

Natural regulatory T cells have been recently hypothesized to contribute to the acquired immune deficits that occur in human sepsis (42). Recent studies have demonstrated that after a burn injury in rodents (43, 138), or after trauma in human subjects (139), there is an increased number of natural regulatory T cells in the draining lymph nodes and blood, respectively. Oppenheim has recently reported that depletion of regulatory T cells with an anti-CD25 antibody reduced mortality to polymicrobial sepsis (140). Those findings, however, have not been confirmed by other investigators. We and the Ayala lab have independently demonstrated that natural regulatory T cells are only transiently increased in murine polymicrobial sepsis, but both

of us were unable to demonstrate that this expansion of endogenous regulatory T cells contributes significantly to the immune suppression or outcome in this model (86, 141).

Due to the inability of T regulatory and regulatory dendritic cell populations to fully explain the observed post-injury and post sepsis immune suppression, investigators have begun to search for other immunologically active cell types that may be responsible for mediating the observed post-injury induced immune dysfunction. One of the more recent candidate cell types focused on by investigators is a heterogeneous group of myeloid derived suppressor cells (MDSCs)(91). Currently very little is known about the origins and suppressor function of MDSCs. Much of what we know about these cell populations comes from the cancer literature, although in the past two years, there has been a dramatic increase in the interest in these cell populations in surgical trauma (68), sepsis (99), and burn injury (142-144).

We do know that MDSCs are a heterogeneous subpopulation of immature cells of the myeloid lineage that are probably both derived from the bone marrow and/or develop independently in secondary lymphoid organs-mainly the spleen but also the lymphnodes to a lesser degree. Hematopoiesis occurs normally in the bone marrow, but after acute inflammatory conditions, such as in burns, trauma and sepsis, extramedullary hematopoiesis and more specifically, myelopoiesis, increases dramatically in the spleen.(45, 145, 146) In mice, for example, it is well known that burn injury induces increased numbers of myeloid progenitors in the spleen, with maximal levels occurring after one week (143). We have also seen increased numbers of myeloid progenitors in the bone marrow of mice early after a septic event (99). The expansion of the MDSC population appears to be secondary to the normal reprioritization of the bone marrow and spleen in the injured or septic host to promote myelopoiesis at the expense of both lymphopoiesis and erythropoiesis (101). When HSCs proliferate they give rise to

multipotent progenitors (MPPs) that lose their ability to differentiate as they mature into cells in the lymphoid and myeloid lineages. MMPs differentiate into common lymphoid progenitors (CLPs) in the lymphoid lineage and common myeloid progenitors (CMPs) which further mature into megakaryocyte erythrocyte progenitors (MEPs) and granulocyte macrophage progenitors (GMPs)-all of which are bone marrow progenitors in the myeloid lineage (123). Under times of trauma, burns and immune stress hematopoietic progenitors from the bone marrow egress into the circulation and home to peripheral organs to initiate repair and regenerative processes (102). We see this process first hand in the spleen 7 days after sepsis with a substantial increase in HSCs, CMP, GMPs, MEPs, and GR-1^{+}CD$11b^{+}$immature myeloid cells illustrating the shift toward the myeloid lineage and the simultaneous contribution that both the bone marrow and spleen make to this post-injury myelopoesis.

In the past, these cells in the spleen and lymph nodes have been referred to as: "natural suppressor cells" (44), "myeloid derived suppressor cells" (45), "early myeloid cells" (46), and "inhibitory macrophages" (47). Phenotypically, these cells exhibit a high expression of cell surface markers CD11b and GR-1. However, other cells of myeloid lineage can also express low levels of these receptors, such as more mature macrophages and neutrophils. Figure 5-1 shows a cytospin preparation of GR-1^{+} cells obtained from the spleen of a mouse ten days post-induction of a cecal ligation and puncture. As is evident from a simple Wright stain, the cells are phenotypically diverse with groups of both immature monocyte-like and neutrophil like cell forms.

Other cell surface markers that these MDSCs express include ER-MP54, ER-MP58, CD115, CD31, F4/80, c-Kit, and FcγR (80, 83, 99, 116, 123, 147). Table 5-1 represents several recent attempts by various authors to define the cell surface phenotype of the MDSC population

in three different injury models. It is evident that the population is heterogenous and immature; however, there are many similarities in the cell surface markers between models especially the markers GR-1 and CD11b.

We do know, however, that a large proportion of these cells are immature, proliferating, committed to a myeloid lineage, and responsive to growth factor stimulation. As shown in Table 5-2, when GR-1^+ cells were harvested from the spleen of a sham mice and cultured with either GM-CSF, G-CSF or erythropoietin (99), there was no evidence of either proliferation or the generation of myeloid colonies. Conversely, when GR-1^+ cells were harvested from the spleen of a mouse 10 days after polymicrobial sepsis, a large number of colonies were generated in response to culture with GM-CSF and G-CSF, but not to erythropoietin (99, 144). Approximately 22% of the enriched GR-1^+ placed in culture with GM-CSF became $CD11c^+$ conventional dendritic cells, and 18% differentiated into $F4/80^+$ macrophages. Similar results were reported by Ogle and colleagues in which splenocytes from eight day burned mice (but not sham controls) were incubated with either M-CSF (CSF-1), GM-CSF or G-CSF, and dramatic increases in the numbers of colonies were observed (142). Under *in vivo* conditions, we have also observed that treatment of septic mice with the nucleoside analogue, gemcitibine (Figure 5-2), completely prevented the expansion of this $CD11b^+$GR-1^+ population in the spleen and lymph nodes, confirming that under in vivo conditions, these cells are rapidly dividing.

In addition, we are beginning to resolve the signaling pathways which drive the expansion of MDSCs in sepsis. In one of the earliest studies looking at these myeloid derived suppressor cells, Holda demonstrated that very modest injections of lipopolysaccharide markedly enhanced what he termed natural suppressor cell activity (66). We also showed that administration of lipopolysaccharide and the TLR5 agonist, flagellin, significantly increased the number of

$CD11b^{+}GR\text{-}1^{+}$ cells in the spleen and lymph nodes ((99) and *unpublished findings*). Almost four years ago, Murphey, Sherwood and colleagues reported that five days following polymicrobial sepsis, there was a near two-fold increase in the number of what they termed "natural suppressor cells" in the spleens of mice (67), although they did not look at suppressor activity directly. Around the same time Cora Ogle's group in Cincinnati reported that "macrophage progenitors" increased in the spleen 10-20 fold eight days after a scald burn (143). In 2006, Dr. Juan Ochoa and colleagues at the University of Pittsburgh reported a seven fold increase in the $CD11b^{+}GR\text{-}1^{+}$cell population in the spleens of mice 12 hours following an experimental laparotomy (68). All of these studies suggest that expansion of an immature myeloid suppressor cell population is a common finding in a number of acute inflammatory states, including surgical injury, trauma, burns and sepsis.

Over the past couple of years, we too have been exploring under what inflammatory conditions does this expansion of this immature myeloid cell population occurs, and whether the expansion of this cell population is an integral component of the injury response. One of the most striking findings is that rather modest noninfectious inflammatory states produce rather immediate and significant increases in the numbers of these cell populations. As previously noted, Ochoa and colleagues showed that a surgical injury dramatically increased the numbers of these cell populations in the spleen within 12 hours (68). In our own studies where we performed a sham surgical procedure to mobilize the bowel, and then closed the peritoneal cavity, we observed a 50% increase in the $CD11b^{+}GR\text{-}1^{+}$ population after 3 days. (Table 5-3) We also saw a similar expansion of the population in mice that underwent a surgical procedure and 45 minutes of warm ischemia to the liver (*Unpublished observations of O'Malley and Moldawer)*. Similar to the findings of Ogle and colleagues, a scald burn in the mouse produced a

significant expansion of the $CD11b^{+}GR\text{-}1^{+}$ cells in both the spleen and the draining lymph nodes that increased with the size of the burns (*Unpublished data of Moreno and Moldawer)*. Such data suggest that surgical inflammation and noninfectious stimuli can produce expansion of these immature cell populations.

With that said, however, the most dramatic changes in the expansion of these cell populations occur in sepsis or in microbial infections. Using either a cecal ligation and puncture or a fecal peritonitis model of polymicrobial sepsis (148), the increases in the expansion of these cell populations were dramatic and often resulted in over 40% of the spleen cellularity being MDSCs. In addition, when *Pseudomonas* was injected subcutaneously in healthy and scald burned mice, increased numbers of $CD11b^{+}GR\text{-}1^{+}$ cells were observed in both spleen and lymph nodes, and when *Pseudomonas* was superimposed on the burn injury, the numbers of $CD11b^{+}$ $GR\text{-}1^{+}$ cells increased dramatically (*Unpublished data of Moreno and Moldawer)*. According to the data presented in Table 3 from injury models in our own laboratory and the laboratories of other investigators, it is apparent that although non-infectious insults such as organ ischemia or acute trauma produce significant increases in the MDSC population, the most dramatic expansion in the MDSC population is produced from infectious stimuli. Furthermore, the longer the infection continues, the more substantial the myeloid cell accumulation in the primary and secondary lymphoid organs.

These findings suggest that although both noninfectious and infectious challenges can increase the expansion of these cell populations in the spleen and lymph node, infectious challenges, and those that are ongoing and sustained, generally produce the greatest and most profound expansion. Along the same lines, polymicrobial sepsis appears to produce a delayed expansion of these cell populations, as after surgical injury, these $CD11b^{+}GR\text{-}1^{+}$ cells peaked in

the spleen 12-48 hours after the insult, whereas in the cecal ligation and puncture models of sepsis, total $CD11b^{+}GR\text{-}1^{+}$ cells decreased in the first 24 hours, and peaked at 7-10 days. There is, however, an important caveat, and that is that we are dealing with heterogenous cell populations that may not be identical with the different injury models. For example, we have reported that the $CD11b^{+}GR\text{-}1^{+}$ cells recovered from the spleens of septic mice express extremely low levels of MHC class II (<5%), and are localized in periarterial and subcapsular regions of the spleen (99), whereas the $CD11b^{+}GR\text{-}1^{+}$ cells identified by Ochoa and colleagues have a much higher proportion of MHC class II expression and are located in the mantle surrounding the lymphoid follicles (68). Such findings suggest that the heterogenous populations may be similar, but still phenotypically and functionally distinct.

Since the expansion of the MDSC population appears to be a highly conserved phenomenon that occurs in response to both infectious and non-infectious stimuli, we also examined the signaling pathways responsible for their invocation. Because both bacterial lipopolysaccharide and flagellin could increase the expansion of this cell population, a natural first focus was on signaling pathways derived from the family of TLR receptors and mediated through MyD88 and TRIF signaling pathways. There is growing appreciation that the ligands for TLR signaling involve not only microbial products, but also endogenous products, such as heat-shock proteins, mammalian nucleic acids, and HMGB1, that can serve as endogenous danger signals (149). Simultaneously, since the production of type I interferon is a consequence of TLR activation, we examined whether these signaling pathways were required for the expansion of this cell population.

Surprisingly, we found that mice defective in either TLR4 signaling, TRIF or type I interferon had no attenuation in their ability to increase the numbers of MDSCs in the bone

marrow, or in secondary lymphoid organs, in response to a cecal ligation and puncture (99). Similarly, mice deficient in IL-10, IL-4, and IL-6 *(data not shown*) also have a normal expansion of their MDSC population. Despite the fact that a TLR4 agonist could induce the expansion of this cell population, the absence of TLR4 did not prevent any increase in response to sepsis, suggesting that TLR4 signaling could induce expansion of this population, but wasn't required in polymicrobial sepsis. In contrast, the absence of MyD88 signaling appeared to delay the expansion of these cells over the first seven days of a cecal ligation and puncture, which began to approach wild type levels at 14 days. Since MyD88 signaling is linked to both the recognition of microbial products and endogenous danger signals, the requirement for MyD88 would suggest that the expansion of the MDSC population is a fundamental component of the host response.

So the question remains: do MDSCs themselves require MyD88 signaling necessary to either emigrate from the bone marrow to the secondary lymph organs, or to promote extramedulllary hematopoiesis or myelopoesis, or is MyD88 signaling required to facilitate the mediators that do control myelopoesis? Bronte administered GM-CSF to healthy mice and showed a modest expansion of the MDSC population, suggesting that GM-CSF may contribute, but cannot recapitulate in its entirety, the expansion seen in inflammation (150). Ramphal and associates demonstrated that MyD88 null mice lack the ability to produce G-CSF after *Pseudomonas* infection which directly reduced neutrophil recruitment to the lung and increased mortality(151). The expansion of these MDSCs may also simply be a component of the larger extramedullary hematopoietic response that is conserved in many animal models of acute injury. We definitely witness evidence of extramedullary hematopoiesis beginning at three days after the initiation of sepsis, and as seen in Figure 5-3; by 10 days there is a fourfold increase in the number of megakaryocytes in the spleens of septic mice. Whether the myeloid expansion is a

required component of extramedullary hematopoiesis or occurs independently remains to be seen.

We have previously reported that complete splenic expansion of the MDSC population has some dependence on MyD88 signaling which diminishes with sepsis progression beyond 7 days (99). Several reports also indicate that HSC proliferation and preferential differentiation into myeloid lineage cells is dependent on the MyD88 signaling pathway in vitro (101, 110-112). Since MDSCs are an immature heterogeneous contingent of cells at various stages of development in the myeloid differentiation pathway we speculated, that HSC proliferation and myeloid differentiation should be impaired using an *in vivo* system devoid of MyD88 signaling, and may account for the modest delay that we have observed in MDSC expansion in MyD88$^{-/-}$ mice during sepsis. Much to our surprise, we discovered that there was no MyD88 dependency on HSCs (Lineage^{neg}c-Kit^{high}Sca-1^{high}) proliferation in the bone marrow after sepsis. More importantly and in contrast to Kincade and associates (101), we found that MyD88$^{-/-}$ and TRIF$^{-/-}$ animals exhibited the same increase in HSC numbers at 24 hours and 7 days post-sepsis as did sham and wild type control animals. This finding suggests that MyD88 and TRIF signaling are not essential for early or prolonged HSC proliferation in vivo during sepsis.

When HSCs proliferate they give rise to multipotent progenitors (MPPs) that lose their ability to differentiate as they mature into cells in the lymphoid and myeloid lineages. MMPs differentiate into common lymphoid progenitors (CLPs) in the lymphoid lineage and common myeloid progenitors (CMPs) which further mature into megakaryocyte erythrocyte progenitors (MEPs) and granulocyte macrophage progenitors (GMPs)-all of which are bone marrow progenitors in the myeloid lineage. After finding a substantial elevation in the HSC numbers in MyD88$^{-/-}$ mice within 24 hours after sepsis, we next sought to determine if this HSC increase

actually translated into increased numbers of bone marrow progenitor cells and whether the absence of MyD88 would hinder HSC differentiation along the myeloid lineage in vivo. When we evaluated the bone marrow progenitor population ($Lineage^{neg}c\text{-}Kit^{high}sca\text{-}1^{neg}$) in the MyD88 and TRIF mice after sepsis, we found little change in progenitor numbers at 7 days post sepsis compared to wild type controls. This finding implies that although HSC differentiation *in vitro* may be dependent on MyD88 mediated TLR signaling, that *in vivo*, HSC differentiation into progenitor cells can occur in the absence of MyD88 signaling.

Although we did not observe a difference in the relative fluctuations in HSC and progenitor populations between wild type, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice, we postulated that a MyD88 dependent differentiation between the progenitor population and more terminally differentiated myeloid cells may exits and halt progenitor maturation accounting for the increases in progenitor cell observed in the $MyD88^{-/-}$ and $TRIF^{-/-}$ mice at 7 days post sepsis. In contrast, the same immature myeloid cell ($GR\text{-}1^{+}CD11b^{+}$) fluctuations occur in all three mouse types at 24 hours and 7 days after sepsis initiation. By 7 days after CLP sepsis, there was an equivalent and significant increase in the immature myeloid population in wild type, MyD88 and TRIF null mice indicating that differentiation from the HSC population through the progenitor population and on to the immature myeloid population can occur in the absence of MyD88 and TRIF signaling in vivo.

We have demonstrated that myeloid expansion during sepsis is modestly CCR2 independent, and evaluated some alternative pathways that could account for the splenic myeloid expansion. Petit demonstrated that G-CSF mediated neutrophil elastase degradation of bone marrow CXCL12 was responsible for bone marrow progenitor egression into the blood (103). Postulating that egression of bone marrow progenitor cells could be responsible for the splenic

myeloid expansion, and since we have observed an increase in splenic progenitor cells in C57BL/6 mice, we administered a neutrophil elastase inhibitor in an attempt to block bone marrow progenitor egression at doses that were effective in earlier studies (103). However, the results of the experiment were equivocal implying that neutrophil elastase plays no role in polymicrobial sepsis MDSC expansion.

Still other signals that have been shown to govern myeloid cell development and expansion in other model systems. The c-*fms* receptor and it's ligand M-CSF (CSF-1) have been shown to play a pivotal role in the differentiation and proliferation of monocytes and macrophages (107). AFS98, an anti-murine c-*fms* antibody which inhibits M-CSF–dependent growth and development by binding to the c-*fms* receptor, has been shown to have a profound effect on monocyte/macrophage peripheral expansion (114). We hypothesized that c-*fms* inhibition by AFS98 may ameliorate secondary lymphoid organ myeloid cell expansion. AFS98 treatment produced only a modest reduction (25%) in the overall splenic GR-$1^{+}$$CD11b^{+}$ population with no differences seen in the bone marrow of lymph nodes GR-$1^{+}$$CD11b^{+}$ populations (*data not shown*) compared to CLP plus isotype control treatment. More detailed analysis reveals a more substantial reduction in the GR-$1^{intermediate}$$CD11b^{+}$ population analogous to the reduction observed in the $CCR2^{-/-}$ septic animals; however, again there was little absolute change in the cell numbers in the overall myeloid population. Further investigation using CD11b, MHC II, F4/80, markers revealed that this GR-$1^{intermediate}$$CD11b^{+}$ population consists almost entirely of immature monocytes and macrophages (*data not shown*). To evaluate the efficacy of the AFS98 anti-c-fms receptor inhibitor in our model of polymicrobial sepsis we evaluated the levels of remaining c-fms receptor positive ($CD115^{+}$) cells after AFS98 administration compared to isotype control treated animals. Interestingly, we found only minimal reduction in the triple

positive (GR-1^{+}CD11b^{+}CD115^{+}) cells. This finding leads to us believe that first, AFS98 inhibition is ineffectual in the setting of murine polymicrobial sepsis based on the plethora of CD115^{+} cells acquired after AFS98 treatment, and secondly, based on the CCR2 and AFS98 findings, the myeloid expansion observed during sepsis is largely due to other expanding populations in the myeloid differentiation pathway and not monocytes and macrophages. In two other independent studies Jose (108) and Murayama (109) found that AFS98 inhibition of c-fms signaling inhibited mainly monocytes and macrophages using chronic models of inflammation and not acute infection. This finding is not entirely surprising given the fact that c-*fms* expression progressively increases from stem cell precursors to monocytes and macrophages (106). Since our GR-1^{+}CD11b^{+} cells are more immature than most monocytes and macrophages they probably have a lower cell surface density of the c-*fms* receptor, making c-*fms* inhibition a minimal factor in immature myeloid cell survival.

The chemokine CXCL12 (stromal cell derived factor-1) has been demonstrated by Rafii *et. al*. to play an essential role in hematopoietic progenitor cell mobilization and progenitor cell homing to peripheral organs during times of stress. Others have found using models of inflammatory stress such as cardiac surgery (128), tuberculosis infection (129) and breast cancer tumor models (130) that CXCL12 is imperative for hematopoietic progenitor cell peripheral recruitment. In a model of malarial infection, de Andrade demonstrated that splenic CXCL12 levels continued to increase until the infection was under control in C57BL/6 mice and facilitated CD11c^{+} dendritic cell recruitment to the spleen (131). Applying the knowledge of the MDSCs that we have available, namely that they are immature, rapidly proliferating, precursors to more mature phenotypes in the myeloid differentiation pathway and are ultimately derived from common myeloid progenitors, it is not hard for us to see the inextricable link between peripheral

expansion of these cells with CXCL12 and hematopoietic progenitor cell homing. Our data support this notion of CXCL12 mediated recruitment given that fact that CXCL12 inhibition substantially reduced splenic MDSC accumulation. More importantly, anti-CXCL12 therapy produced an even greater reduction in the numbers of splenic CMPs-the precursor that gives rise to the other myeloid lineage cells. This suggest, although with further investigation necessary, that MDSCs arise from CMPs that have migrated from the bone marrow to the spleen and undergo myelopoesis generating the dramatic numbers of myeloid cell observed in the spleen during long term sepsis.

The is data suggest that an ongoing septic process produces a dramatic expansion of GR-1^{+}CD11b^{+} cells in the spleen that is independent of CCR2 and c-*fms* receptor signaling, bone marrow MyD88 and TRIF signaling, and neutrophil elastase activity. In contrast, we have demonstrated that the splenic MDSC population is dependent on CXCL12 signaling. CXCL12 depletion substantially reduced the accumulation of the MDSC population in the spleen during sepsis. Furthermore, CXCL12 inhibition also reduced the CMP-the earliest myeloid precursor cell type suggesting that MDSC expansion depends on CMP splenic expansion.

MDSCs and Sepsis-induced Immune Suppression

The inevitable question is: what are the immunological consequences of this cell population in terms of the host response to severe injury or sepsis? Do these cells contribute to the immune suppression and T_H2 polarization that are seen in sepsis? The rather late appearance (days 3-8) of these cells in burns and sepsis (99, 142, 143) suggest that they probably play little role in the early inflammatory response, and more likely contribute to the later responses seen in animals that survive the early "cytokine storm". It is easy to speculate that any cell population which contributes as much as 40% of the cells in the spleen, 10% in the lymph nodes and 90% in the bone marrow is very likely to have some impact on the immunological response, although

this has not been definitively shown. Studies from our own laboratory and those of Cora Ogle reveal that these cells obtained from septic and burned mice, respectively, produce a large number of inflammatory mediators, including TNF-α, MCP1, and MIP1β (99, 142). These cells also produce a large number of mediators and cytokines that are immune suppressive, including reactive oxygen species, nitric oxide, peroxynitrites, TGFβ, IL-4 and IL-10. There is also a significant amount of indirect, associative data suggesting that the expansion of these cell populations is temporally related to the development of immune suppression and increased susceptibility to secondary infections. Murphey and colleagues, for example, showed in septic mice that the increased number of what they termed suppressive macrophages occurred simultaneously with the reduced bacterial clearance to a *Pseudomonas* infection (67). Similarly, the increased appearance of these $CD11b^{+}GR\text{-}1^{+}$ cells after a burn injury coincides with increased lethality to a secondary *Pseudomonas* pneumonia.

Of course, association is not cause and effect, and studies to date have not convincingly demonstrated that these defects in acquired immunity are due to the presence of these suppressor cells in sepsis. Much of the difficulty has to do with the heterogeneity of the cell population and the lack of experimental approaches to selectively deplete the host of these $CD11b^{+}$ $GR\text{-}1^{+}$ cells. For example, we have used a $GR\text{-}1^{+}$ depleting antibody to prevent the expansion of these immature cell populations, and can successfully prevent their increase in sepsis (99). However, mature neutrophils also express $GR\text{-}1^{+}$, and a depleting antibody indiscriminately removes both immature MDSCs and terminally differentiated neutrophils. Thus, the effects on survival to a bacterial infection in the presence of $GR\text{-}1^{+}$ depletion cannot easily distinguish between MDSCs and neutrophils. Similar concerns exist with gemcitibine, a nucleoside analog that inhibits rapidly dividing cells, which not only kills replicating MDSCs, but also other proliferating cells

required for a successful response to microbial infection (Figure 5-2). There are, however, other approaches under consideration. Gabrilovich and colleagues have used the subcutaneous implantation of pellets containing *all trans* retinoic acid (ATRA) to force the differentiation of MDSCs in tumor-bearing mice (59). Recent studies suggest, however, that this approach may also lack some specificity, since ATRA also will stimulate the expansion of some regulatory DC populations (152). Other approaches under consideration involve the use of specific tyrosine kinase inhibitors involved in growth factor signaling (153). These prevent the expansion of myeloid derived suppressor cells by blocking growth factor signaling, and can be used short term to block the expansion of these cell populations.

With this said, however, there is actually considerable information known about the immune suppressive phenotype of MDSCs. However, very little of that information comes from the sepsis and injury field, while most comes from the oncology literature. Because of the heterogeneity of the myeloid populations and the probability that chronic tumor growth may stimulate the expansion of a similar but distinct MDSC population as seen in sepsis, caution should be employed when directly comparing the populations. What we do know from both sets of literature, however, is that these cells are quite immunologically active. Because of their relative undifferentiated phenotype, there is considerable functional plasticity of these cells with a suppressive phenotype elicited upon continued exposure to T_H2 cytokines (IL-4, IL-10 and TGFβ) often increased in sepsis (47, 53). On the other hand exposure of MDSCs to T_H1 cytokines (TNFα) stimulates differentiation along macrophage pathways, and enhances T cell cytotoxic responses. For example, when MDSCs obtained from tumor bearing mice were administered to healthy naïve animals, they differentiated into normal dendritic cells and

macrophages, but when administered into other tumor bearing animals, they maintained their suppressor cell phenotype (54).

In mouse tumor models, the immune suppression to growing tumors could be ameliorated by $CD11b^{+}GR\text{-}1^{+}$ cell depletion with *all trans* retinoic acid, thereby restoring T-cell responses (58, 59). MDSCs can inhibit T cell activation through cell-cell contact or immediate juxtaposition. The mechanisms of MDSC T-lymphocyte inhibition are not yet fully understood; however, they appear to depend in part on L-arginine metabolism to decrease T lymphocyte responsiveness to subsequent antigen stimulation (48, 49). Furthermore induction of iNOS with NO release and peroxynitrite formation can account for some of the T cell unresponsiveness in tumor models (60-62). Over production of arginase I by MDSCs can result in "local arginine starvation" that can inhibit T-lymphocyte proliferation (63, 64). In addition, $GR\text{-}1^{+}$ cells also secrete IL-4 and IL-10 (65), reactive oxygen species, and TGFβ, all of which can have immunosuppressive properties. Recent studies by Gabrilovich have proposed a novel mechanism by which MDSCs can suppress $CD8^{+}$ T-cell function. They had previously shown that much of the tumor evasion of host immunity was mediated by MDSC abrogation of $CD8^{+}$ T cell function, including cytolytic killing of tumor cells, proliferation and IFNγ production (55). They reported that increased peroxynitrite production by MDSCs could directly nitrosylate the TCR complex and prevent MHC complex dimers-T cell receptor interactions (154).

In contrast, little is known about the potential mechanisms by which MDSCs can produce immune suppression and T_H2 polarization in severe injury and sepsis. Ochoa examined the functionality of these $CD11b^{+}GR\text{-}1^{+}$ cells recovered from the spleen of mice after traumatic injury, and showed that these cells express large quantities of arginase (68). These $CD11b^{+}GR\text{-}1^{+}$ cells obtained from the spleen of traumatized mice significantly inhibited CD3/CD28-

mediated T cell proliferation, TCR zeta-chain expression, and IL-2 production. The suppressive effects could be overcome by blocking arginase activity or by supplementation of medium with L-arginine.

To examine whether these cells can affect adaptive immune responses in sepsis, sham-treated and septic mice were immunized with NP-KLH using alum as an adjuvant and the serum immunoglobulin response determined ten days later (99). The serum immunoglobulin response to NP-KLH with alum can be used to determine the predilection of the $CD4^+$ T-cell response (92). Polymicrobial sepsis was not associated with any disturbances in the total serum IgG and IgM responses in the mice immunized with NP-KLH with alum, but the serum IgG_{2a} response was significantly decreased while the IgG_1 response increased in the septic mice, consistent with a shift from a T_H1 to a T_H2 T-cell response. When the septic mice were depleted of their total GR-1^+ cells by antibody treatment, the sepsis-induced increase in IgG_1 and the decrease in IgG_{2a} response were abolished, suggesting that GR-1^+ cells were contributing to this $T_H2$polarizing response (99).

To further confirm the in vivo role that these cells play in suppressing an antigenic T-cell response, the effect of GR-1^+ cells on the cytotoxic T-cell IFNγ response by splenocytes from OT-I TCR-transgenic mice immunized with OVA-derived peptide was examined. GR-1^+ cells were obtained from either ten day septic or sham-treated mice, and were infused into C57BL/6 mice that had previously received $CD8^+$ T-cells from OT-1 TCR-transgenic mice, and simultaneously immunized with OVA-derived specific peptide. Ten days later, the spleens from these animals were removed, and IFN-γ responses to *ex vivo* stimulation with OVA-derived specific peptide were examined. The IFN-γ (99) production and cytotoxicity were markedly reduced when the animals were administered GR-1^+ splenocytes from septic animals when

compared to GR-1^+ splenocytes from sham-treated mice, confirming that GR-1^+ splenocytes from these septic mice could suppress antigen specific T-cell IFNγ and cell cytotoxicity responses.

To determine whether GR-1^+ cells could directly suppress an antigen specific or nonspecific $CD4^+$ T-cell proliferative response, D011.10 OVA-TCR transgenic mice were made septic and at 10 days, $CD4^+$ splenocytes from septic mice were cultured with irradiated GR-1^+ containing antigen presenting cells from the spleens of 10 day septic or sham-treated mice, and with either OVA peptide, bovine serum albumin or on CD3/CD28 coated plates. Culturing $CD4^+$ cells with irradiated GR-1^+ cells from septic mice only modestly, but still significantly, reduced both the antigen-specific (OVA) and non-specific (CD3/CD28) proliferative responses (99).

Since these MDSCs secrete increased quantities of IL-10 and IL-4, we asked whether MDSCs obtained from IL-10 and IL-4 null mice had the same phenotype as MDSCs obtained from wild-type mice. Sepsis was induced in C57BL/6 IL-10 and IL-4 null mice, and these animals showed a similar expansion of their MDSC population in the spleens and lymph nodes. GR-1^+ splenocytes from 10 day septic IL-10 or IL-4 null mice were injected into naïve B6 mice along with OT-1 OVA-specific $CD4^+$ T cells and then immunized with OVA peptide, their ability to suppress a $CD8^+$ T cell IFN-γ response was comparable to that seen from GR-1^+ cells obtained from a septic, wild-type mouse. Such findings suggest that the suppressive phenotype is seen in MDSCs from these null animals and that their suppressive effects maybe mediated through other signaling pathways.

As summarized in Figure 5-4, there are a number of mechanisms by which these MDSCs could directly impact both innate and acquired immune responses. The increased production of a

number of inflammatory mediators, such as TNFα, suggests that in response to a secondary infectious challenge and microbial products, the increased numbers of these MDSCs could contribute to a secondary cytokine storm. Simultaneously, increased production of IL-4, IL-10 and TGFβ by MDSCs could promote the further development of other regulatory/inhibitory cell populations, such as T_H3, TR1 and regulatory dendritic cells, as well as directly suppress T_H1 effector cell populations. Interestingly, the increased production of reactive oxygen species, peroxynitrites and the depletion of arginine in the T cell microenvironment could contribute to both suppressed $CD4^+$ and $CD8^+$ proliferative and effector responses by multiple mechanisms, including inactivation of the T cell receptor complex by nitrosylation and amino acid starvation during proliferation. All in all, these metabolically active cells are well positioned in bone marrow, spleen and secondary lymph nodes to wreak havoc on normal acquired and innate immune responses. The sepsis state promotes the continued expansion and suppressive phenotype of these cells.

Conclusions

Studies to date have focused on a novel MDSC population in bone marrow, spleen and secondary lymph nodes that has previously attracted only a limited amount of research attention by the shock, trauma and sepsis communities. Although there is now convincing evidence to suggest that this cellular population is dramatically increased in mice after a number of acute inflammatory challenges, including surgical trauma, burns, ischemia/reperfusion injury and polymicrobial sepsis, little is known about either the mediators that drive this expansion, or their functional consequences. Furthermore, there is at present no information whether these MDSC populations increase in septic patients or individuals after severe trauma or burn injury. Data from other literature suggests, however, that increases in the MDSC population are likely to have

dramatic consequences on acquired immunity, similar or greater than the effects attributed to regulatory T cell and dendritic cells populations. Thus, future studies are required to further resolve the role that this novel suppressor cell population plays as a potential mechanism for immune suppression and a target for therapeutic intervention during sepsis.

Myeloid Derived Suppresor Cells and Future Directions

Although we have described an immature heterogenous myeloid cell population that is capable of dramatic expansion after a variety of inflammatory insults ranging from organ ischemia to bacterial infections, we still know very little regarding the true phenotypes of these cells and what purpose they serve in hematopoiesis. Kelsoe and colleagues have demonstrated that in the bone marrow the GR-1^{+}CD11b^{+} population can actually be divided into 3 different subpopulations based on their cell surface expression of c-Kit and FcγR (115). One avenue that would benefit our understanding is to phenotype the MDSC stage of development in the myeloid differentiation pathway. In addition to cell surface expression of GR-1, CD11b, and F4/80, we would also incorporate c-Kit and FcγR to further compare the cells we observe in the spleen and bone marrow after sepsis to the published reports. By separating out the subpopulations that comprise the GR-1^{+}CD11b^{+} cohort of cells we will then be able to investigate the subpopulation's myeloid transcription factors (155) and in conjunction with the immune phenotyping, determine exactly what stage of myeloid development these cells persist. Knowing this information would provide us the necessary understanding to better tailor therapeutic interventions to arrest MDSC expansion without negatively affecting the whole myeloid lineage.

Another avenue for consideration is the mechanism of expansion of the cells during sepsis. Our CXCL12 data shows that MDSC expansion is dependent on an uninterrupted CXCL12 signaling cascade; however, the events surrounding the CXCL12 signaling remain a mystery. Heissig demonstrated that after immune stress with 5-FU, bone marrow egression and splenic

homing of hematopoietic progenitors is dependent on MMP-9 mediated SDF-1 induced release of the Kit ligand (122). If we consider sepsis an immune stress and consider the loss of lymphocytes that Hotchkiss (88) and others have shown as providing a spleen niche, then it is not so large of a stretch to imagine that all of these events happen in concert to allow the expansion of the myeloid lineage and extramedullary hematopoiesis in the spleen. Although there are some assumptions being made on our part, based on data in other model systems, the events that are happening in sepsis are the same namely myeloid expansion after immune stress. Consider that 90% of the bone marrow consists of immature myeloid cells by five days after sepsis and then that level begins to recede back to baseline at seven days. In the meantime, the splenic levels of immature myeloid cells are just beginning to rise and by ten days are completely expanded. The question is: what governs this initial bone marrow myeloid expansion and once the bone marrow levels return towards baseline, what is mediating the continued splenic expansion of the myeloid lineage? The involvement of CXCL12 only explains the bone marrow egression and possibly the splenic homing; however, it does not explain the initial events that signal stem cells and bone marrow progenitors to proliferate and differentiate. These are the questions that need to be resolved in order to truly understand the myeloid cell expansion during sepsis.

Lastly, and probably most important is the establishment of a link between what we understand to be true in our mouse model of polymicrobial sepsis and what actually happens in humans during sepsis. Are we beneficially impacting sepsis understanding and human myeloid biology or are we just studying various differences in immune system function between various animals? Currently, aside from a few scant publications in the oncology literature pertaining to proposed phenotypes of MDSCs in humans (156), there have been no definitive reports.

Probably the most important understanding that we could have would be whether this myeloid expansion occurs in human sepsis, and to determine the cell phenotype involved. Currently we have obtained several histological sections of human spleen tissue from septic patients. H&E staining revealed that there is indeed some initial promise to demonstrate myeloid expansion in the periarterioler areas in the spleen. However to say these findings are analogous to what we have demonstrated in mouse sepsis are very premature. First off, there are no human correlates of the GR-1 marker that we use to define the population in mice. Secondly, our mouse model is a model of long term sepsis unabated by the administration of any medical care such as antibiotics or nutrition support. In human sepsis, rarely is there a patient that has a chronic inflammatory process that progresses without medical attention, antibiotic treatment and nutritional support. The caveat is that the myeloid expansion we see in mouse sepsis could just be the product of uncontrolled infection that does not occur in human sepsis. It is this very question that needs to be investigated and answered.

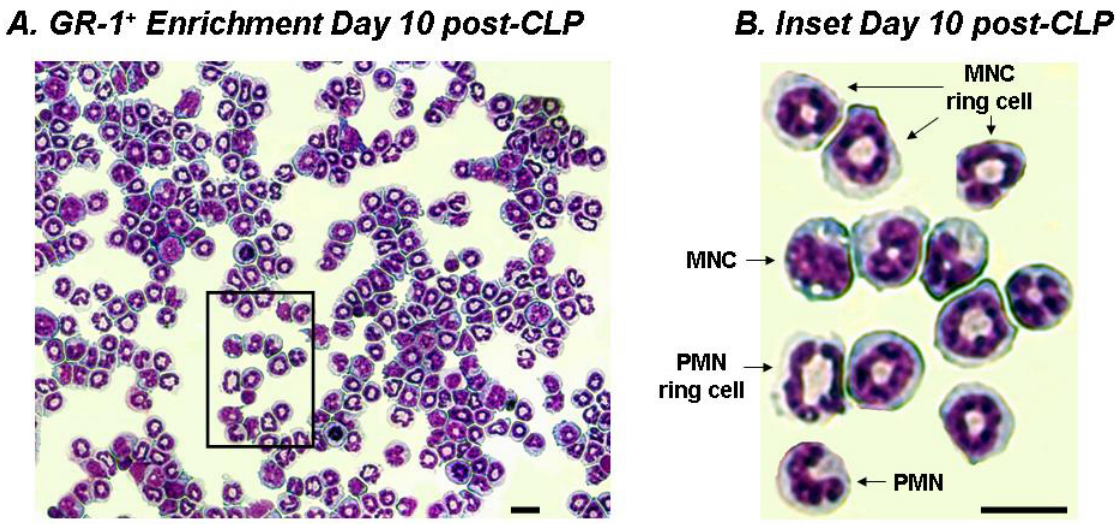

Figure 5-1. Panels A and B represent a cytospin preparation of GR-1^+ cells isolated from the spleen of a 10 day septic mice. Mice underwent a cecal ligation and puncture. After ten days, the mouse was euthanized , and the GR-1^+ splenocytes were enriched using Miltenyi columns (33). The GR-1^+ cells were stained with a Wright stain and visualized at low and high magnifications. Most evident is the heterogeneity of the cell population (Panels A), with cells phenotypically varying from mature neutrophils and monocytes to immature ring-shaped cells that appear to be neutrophil and monocyte precursors (Panel B).

Table 5-1. MDSC Phenotypes in Various Inflammation Models.

	GR-1	CD11b	MHC I	MHC II	CD80	CD86	CD40	CD11c	CD34	F4/80	CD16/32	CD31	Ter 119	c-kit	CD115	
Operative Trauma	H	H	H	L	L	L	L	L	L	L	L	L				Makarenkova *et al.* (32)
Polymicrobial Sepsis	H	H		L				L	L	I	L	L	L	L	I	Delano *et al.* (33)
Burn Sepsis	H	H								L						Noel *et al.* (34)

Cell surface expression of various receptors and markers on the MDSCs as reported from our laboratory and from other investigators using sepsis and injury models. It is obvious that the GR-1 and CD11b markers broadly define the population however the heterogeneity of the cells is reflected in the various expression of a wide array of cell surface markers. Relative cell surface expression of each marker was interpreted from the authors publishes work and classified as H for High, I for Intermediate, and L for Low surface levels of the respective markers. Empty cells mean that the authors presented no data on the marker.

Table 5-2. MDSC's Proliferate When Cultured With Growth Factors.

	Sham (cfu)	CLP (cfu)	Reference
Erythropoietin	1	4	(33)
GM-CSF	2	38	(33)
G-CSF	2	26	(33)
CSF-1/M-CSF	5	30	(34)

Effect of growth factors on the proliferation and differentiation of immature MDSCs obtained from burned and septic mice. *Data was adapted from Neol et al.(142) based on the number of cells in their initial cultures.

Table 5-3. MDSC Fold Change Increases in Various Models of Inflammation.

	Time (days)	Fold Change: Increase	Reference
Burn	8	4	Noel *et al.* (34)
Liver Ischemia	7	1.5	O'Malley, K. (unpublished data)
Kidney Ischemia	7	2	Delano, M.J. (unpublished data)
Acute Trauma	3	6	Makarenkova *et al.* (32)
Polymicrobial Sepsis	10	12	Delano *et al.* (33)
Pseudomonas Infection	10	9	Moreno, C. (unpublished data)
Cecal Slurry Peritonitis	2	4	Wynn, J.L. (unpublished data)

Relative fold change increases between injured and control animals observed in various injury models. The infectious injuries such as polymicrobial sepsis or *Psuedomonas* infection seem to more dramatically induce MDSC population expansion compared to the non-infectious models such as organ ischemia.

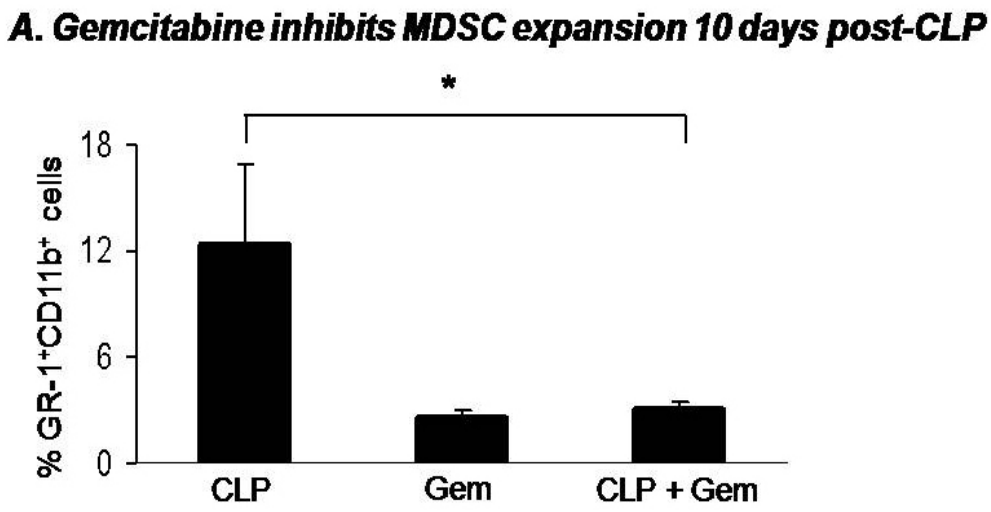

Figure 5-2. Gemcitibine, a nucleoside analog that inhibits rapidly proliferating cells, was administered i.p. at 120 mg/kg/mouse and was able to inhibit the expansion of the myeloid population after 10 days in the spleens of septic mice (n=5) as compared to septic mice without gemcitibine treatment (n=5). * $P<0.05$ (Student's t test) between mice that underwent CLP alone and mice that underwent CLP and gemcitibine treatment (CLP + Gem). CLP, Cecal ligation and puncture, Gem, Gemcitibine.

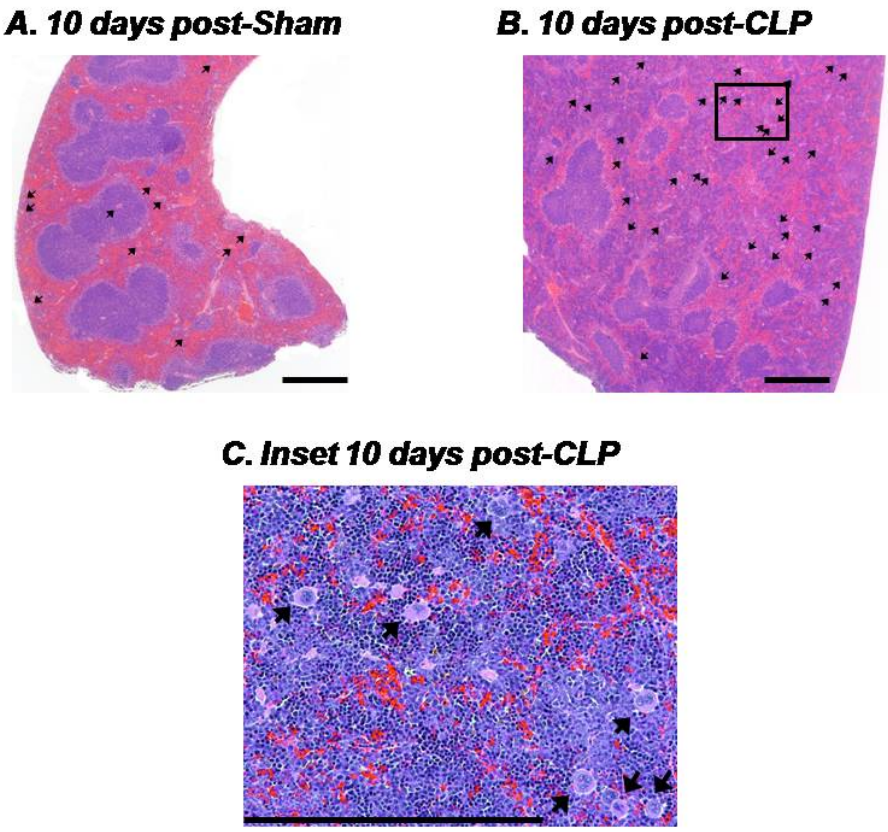

Figure 5-3. H&E preparations of mouse spleens 10 days after either sham or sepsis treatment. Panels A and B are low power views of sham and sepsis treated mouse spleens. The arrows indicate the presence of megakaryocytes. There is a 4 fold increase per low power field in the number of megakaryocytes in the septic spleen compared to the sham spleen. Panel C is a high power view of the megakaryocytes in the septic mouse spleens.

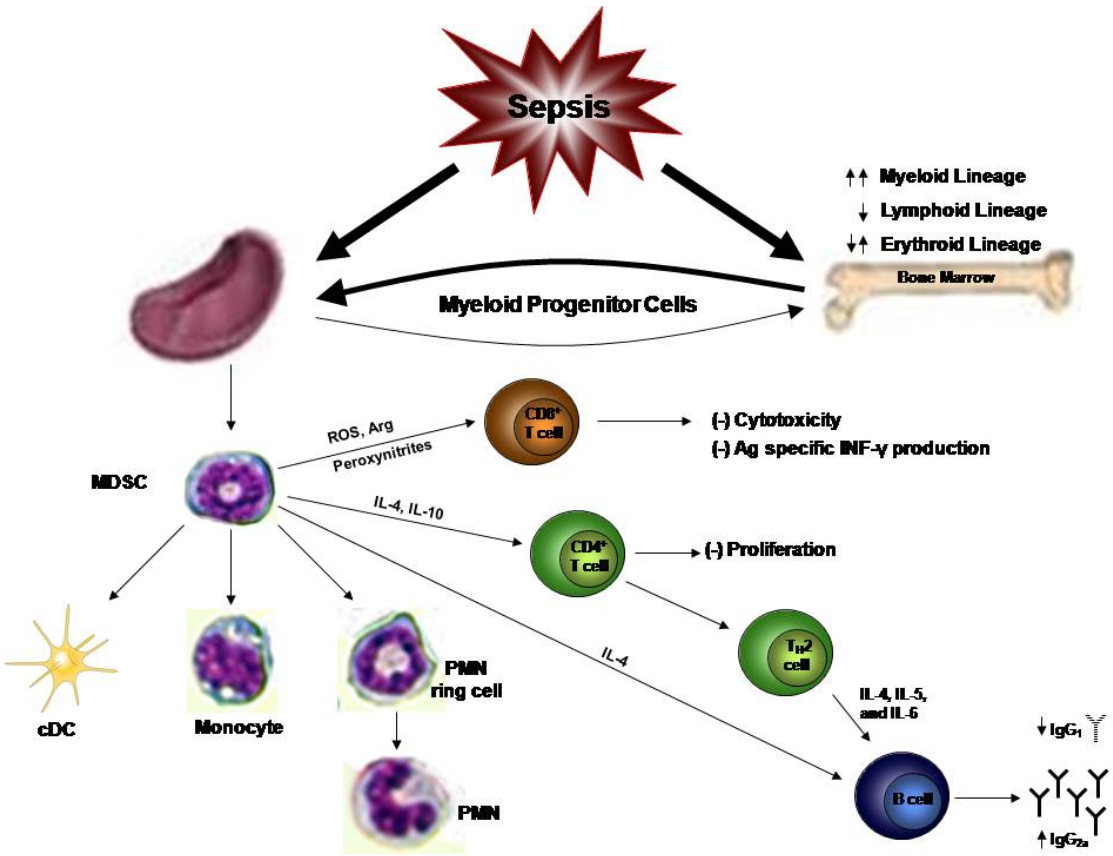

Figure 5-4. Proposed model by which MDSCs can impact both components of acquired and innate immunity. Expansion of the MDSC population leads to increased numbers of myeloid derived suppressor cells in direct proximity to both $CD4^+$ and $CD8^+$ T cells with the ability to secrete large numbers of immunoregulatory peptides. It is presumed that the immunosuppressive phenotype is achieved through both direct contact and immediate proximity with CD4/CD8 T cells as well as the release of both paracrine and endocrine-like mediators of inflammation. cDC, conventional dendritic cell.

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BIOGRAPHICAL SKETCH

Matthew J. Delano was born in Butler, PA in 1974. He attended East Brady High School in East Brady, PA and then graduated from Armstrong Central High School in Kittanning, PA in 1992. He then enrolled in Saint Vincent College where he graduated with Honors with a Bachelor of Science degree in Chemistry. Matt then worked for Costello Pharmaceuticals for two years before entering Temple Medical School in Philadelphia, PA in the fall of 1998. From 2001-2002 Matt was awarded a stipend to study pancreatic cancer under the direction of Dr. Howard Reber at the David Geffen School of Medicine at the University of California at Los Angeles. In 2002 Matt resumed his medical school curricula and graduated from Temple Medical School with a Medical Doctor degree in the spring of 2003 with a categorical residency position in general surgery in the University of Florida, College of Medicine, Department of Surgery, beginning in July of 2003. After completing two years of a 5 year residency in general surgery, Matt entered the laboratory of Dr. Lyle Moldawer and gained acceptance to the Immunology Microbiology Advanced Concentration in the IDP program at the University of Florida. Matt's plans are to finish his general surgery residency and to pursue a career structured around inflammation biology and its' impact on post surgical immune suppression.