Dysfunctional Expansion of Hematopoietic Stem Cells and Block of Myeloid Differentiation in Lethal Sepsis

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Running Title: Block of myeloid differentiation in lethal sepsis

Key words: Pseudomonas aeruginosa; neutropenia; hematopoietic stem cells; myeloid progenitors; LPS, Toll-like Receptor 4.

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ABSTRACT

Severe sepsis is one of the leading causes of death worldwide. High mortality rates in sepsis are frequently associated with neutropenia. Despite the central role of neutrophils in innate immunity, the mechanisms causing neutropenia during sepsis remain elusive. Here, we show that neutropenia is caused in part by apoptosis and is sustained by a block of hematopoietic stem cell (HSC) differentiation. Using a sepsis murine model, we found that the human opportunistic bacterial pathogen *Pseudomonas aeruginosa* caused neutrophil depletion and expansion of the HSC pool in the bone marrow. “Septic” HSCs were significantly impaired in competitive repopulation assays and defective in generating common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs), resulting in lower rates of myeloid differentiation in vitro and in vivo. Delayed myeloid-neutrophil differentiation was further mapped by using a lysozyme-GFP reporter mouse. *Pseudomonas* lipopolysaccharide (LPS) were necessary and sufficient to induce myelosuppression and required intact TLR4 signaling. Our results establish a previously unrecognized link between HSC regulation and host response in severe sepsis and demonstrate a novel role for TLR4.
INTRODUCTION

Sepsis is a complex clinical syndrome, a devastating consequence of bacterial infection that frequently causes severe organ dysfunction, and is the leading cause of death in non-coronary intensive care units. The severe complications present during sepsis are largely due to the excessive release of cytokines that lead progressively to endothelial dysfunction, coagulation cascade activation, microvascular injury and in many cases, multiple organ failure. The systemic inflammation underlying sepsis results initially from the “failure” of the host innate immune system to control invasive pathogens. A key component of the host innate response to bacterial pathogens is the neutrophil. During infection, neutrophils rapidly migrate to the site of inflammation where they initiate their anti-microbial activity. Due to their short life-span, neutrophils have to be supplied continuously during infection by expansion of myeloid progenitors in the bone marrow (BM). Thus, the ability of the BM to respond to infections by expanding the progenitors and producing differentiated cells capable of destroying the microbial pathogens, while preserving an intact stem cell pool, is a critical feature of host defense that translates into the difference between resolving an infection or succumbing to it. Despite the central role of neutrophils in innate immunity, the mechanisms of neutropenia and myelosuppression during sepsis remain elusive.

The use of steady-state hematopoiesis mouse models and bone marrow transplantation-induced stress have permitted great progress in identifying key molecules that coordinate HSC self-renewal, proliferation and differentiation during normal adult hematopoiesis. However, the in vivo mechanisms by which the HSC compartment responds to sepsis is poorly understood. Severe sepsis is one of the most dramatic examples of inadequate host response to inflammation, where an initial hyper-reactive response is often followed by profound neutropenia and “immune paralysis.” However, the precise dynamic of HSC response and the cause of neutropenia during sepsis have not been investigated.

Previous studies had shown that P. aeruginosa can cause profound neutropenia in burns. Using a burn mouse model that closely recapitulates the lethal sepsis occurring in patients with overwhelming gram negative bacterial infection, we have mapped the cellular dynamics that are altered in the BM compartment during sepsis. We observed that P. aeruginosa induced a dramatic expansion of the stem cell compartment associated with failure to regenerate neutrophils. These effects were not induced by a non-lethal isogenic P. aeruginosa mutant associated with high survival and defective in LPS production, but were recapitulated by injection of purified lipopolysaccharide (LPS). In addition, we show that TLR4 signaling, triggered by LPS, directs a dysfunctional expansion of HSCs in response to bacterial infection, resulting in low engraftment potential, both at long- and short-term, and in defective ability to generate myeloid progenitors.
MATERIALS AND METHODS

Mice. Bacteremia and endotoxemia models were performed in several mouse strains: CD1 and C57BL/6J mice (Jackson Labs); FVB/N-TgN Lys-GFP reporter mice (Dr. Graf laboratory); C3H/HeJ and C3H/OuJ mice (Jackson Labs). Gender-matched mice of both sexes between the ages of 8 and 12 weeks were used. B6.SJL-PtrcaPep3b/BoyJ (BoyJ; CD45.1) mice (In vivo Therapeutics Core, Wells Center, IU) were used as recipient for transplants. All animal studies were reviewed and approved by the Institutional Animal Care of the Massachusetts General Hospital and by the Indiana University LARC Committee on Animal Research.

Bacteremia and Endotoxemia models. A mouse full-thickness skin burn model was used. Briefly, mice were subjected to a 7% scalding injury on the abdominal surface and the bacterial inoculum was delivered s.c. under the scald eschar. Independent sets of mice were infected with bacterial inoculums of PA14 or 33C7 strains (~2-3 × 10^5 CFU/ml). Mice that received scalding injury only and healthy mice were used as controls. Blood samples from challenged animals were collected and dilutions were plated to assess the number of bacteria present at different time points. Mice were subjected to i.p. inoculation of 0.8 mg/Kg (approx 20 ug/mouse) of *P. aeruginosa* LPS purchased from Sigma (L 8643).

Bone Marrow Transplantation. Competitive repopulation assay (CRA) was used to evaluate repopulation ability of Lin^- or LSK cells sorted from controls, septic or LPS challenged animals. Recipients (C57BL/6-CD45.1, female; Jackson Laboratories) were irradiated with a split dose of 10 Gy 16 hours before transplantation. Lin^- or LSK donor cells were obtained from 8-12 week-old female C57BL/6-CD45.2 mice (Jackson Laboratories). Competitive BM cells were prepared as single-cell suspension from Boy/J CD45.1 mice and admixed with CD45.2 test cells. Engraftment was evaluated at 4 week intervals by collecting the peripheral blood (PB) for analysis of CD45.2 and lineage markers.

Reagents. Recombinant mouse stem cell factor (SCF), interleukin 3 (IL-3) and granulocyte –colony stimulating factor (G-CSF) were purchased from R&D systems (Minneapolis, MN).

Morphology and histologic analysis. BM smears from tibias and cytospins (10^5 cells) were stained with Wright-Giemsa using the Hema-Tek system. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 μm and mounted for staining with hematoxylin and eosin.

Flow Cytometry and Identification of Hematopoietic Stem and Progenitor Cells. BM cells were incubated with a lineage antibody cocktail (anti-Mac-1, anti-Gr1, anti-B220, anti-Ter119, anti-CD3, anti-
CD8 and anti-CD4) followed by negative selection by MACS separation system (Miltenyi Biotec) or sorting by FACS. Lineage-depleted cells were stained with anti-c-Kit, anti-Sca1 and anti-IL7R and sorted. Identification of defined subsets was conducted as following: HSC (Lin⁻ IL-7Rα⁻ CD34⁺ c-Kit⁺, Sca1⁺), CMP (Lin⁻ IL-7Rα⁻ c-Kit⁺, Sca1⁻ CD34⁺ FcγRII/IIIlo), GMP (Lin⁻ IL-7Rα⁻ c-Kit⁺, Sca1⁻ CD34⁺ FcγRII/IIIhi), MEP (Lin⁻ IL-7Rα⁻ c-Kit⁺, Sca1⁻ CD34⁻ FcγRII/IIIhi), CLP (Lin⁻ IL-7Rα⁻ c-Kitlo, Sca1lo). Staining includes the following antibodies: FITC-conjugated CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), Ter119, Gr1 (RB6-8C5); PECy7-IL7Rα (A7R34), PECy5.5-Sca1, Alexa750-c-Kit (2B8), APC-FcγRII/III (93), Pacific blue-CD34 (RAM34), PE-conjugated anti-TLR4-MD2 Complex (MTS510). PB was labeled with anti-CD45.1-FITC and anti-CD45.2-APC plus CD3-PE, CD4-PE, B220-PE, CD8-PE Gr-1-PE, Mac1-PE or F4/80-PE. Sca1-FITC (E13-161.7), Gr1/Mac1/F4-80-PE and c-Kit-APC (2B8) were used in in vitro differentiation. LSRII, FACSCalibur and FACSaria (BD biosciences) were used for analysis and sorting. Data were analyzed with FlowJo and Cellquest software. For analysis of rare populations 5x10⁵ to 1x10⁶ events were collected.

**Serum-Free Cell Cultures.** BM cells from control, PA14 or LPS challenged mice were harvested from femurs by flushing with PBS/0.2mM EDTA. Cells were lineage depleted using the lineage cell depletion kit from Miltenyi Biotec or sorted for the LSK subset. Cells were grown in X-VIVO-15 media (BioWHITTAKER, Lonza) supplemented with SCF (50 ng/ml; R&D), IL-3 (20 ng/ml; R&D) and G-CSF (10 ng/ml), seeded at 5x10⁵ cells/ml in 48 or 96-well plates and cultured at 37°C, 5% CO₂ for up to 7 days.

**Cell Cycle and Apoptosis analysis.** BM fraction enriched in Lin⁻ cells by immunomagnetic separation was labeled with antibodies detecting the LSK population and by Hoechst and propidium iodide (PI) as described and analyzed by FACS. In independent experiments, Lin⁻ cells, immediately after harvest and purification were cultured in X-VIVO-15 media supplemented with BrdU (10 μM), and no growth factors, for a 2h pulse. Cells were collected and cell cycle analysis was performed using the BrdU-APC Flow kit (BD Pharmingen). Cell death was measured by labeling BM cells with Annexin V and PI following manufacturer’s instructions, in combination with markers for LSK, common progenitors, Gr1 and Mac1. Samples were analyzed by FACS.

**Long-term culture with limiting dilutions and colony assays.** Cobblestone area forming cell (CAFC) assay was used to quantify primitive cells within the BM. Lin⁻ cells from PA14 or LPS challenged mice and controls were seeded in limiting dilution over feeder layers of murine BM stroma cultured for 5 weeks and measured as described. BM cells from control and LPS challenged mice were seeded in methylcellulose at 10,000 /ml and evaluated for colony-forming ability at days 7-10.
Quantitative qRT-PCR analysis of Gene Expression. Total RNA was isolated using RNAeasy kit (Quiagen). DNase (Quiagen)-treated RNA was used to generate cDNAs by reverse transcription according to the manufacturer’s instructions (SuperScript II kit; Invitrogen). PCR reactions were performed in an MX3000 detection system using SYBR green PCR reagents following the manufacturer’s instructions (Stratagene). For each gene analyzed a calibration curve was performed and all the oligonucleotides were tested to ensure specificity and sensitivity. Primers for C/EBPα and PU.1 were generated as described 12. These primers were used for the following sequences: SKP2-F (TGGGATCTTTTCTGTCTGTG), SKP2-R (TACCCGGAAAGAGCTGAAGC); p21-F (GAGCCACAGGCACCATGTCC), p21-R (AGACCTTGGGCGAGCCCTAGG); LRG47-F (TGAGCCTCAGCCTCCCCTTT), LRG47-R (TGGGACAATGTGCGCCACAGT).

Statistical analysis. Equality of distributions for matched pairs of observations was tested using the T-Test. Non-Gaussian distributed data were analyzed by the Wilcoxon Signed-Rank Test using the StatPlus™ 2008 Professional software package. Error bars in panels represent STD; SEM when sample number is equal or larger than 10.

RESULTS

P. aeruginosa infection decreases the number of neutrophils in the bone marrow

To study the changes occurring in the BM compartment during sepsis, we used an established murine model that recapitulates lethal sepsis in humans 7,8. In this model, a limited thermal injury in healthy animals followed by inoculation of the virulent strain of P. aeruginosa UCBPP-PA14 (PA14; 2-3 x10^5 CFU/ml) induces sepsis and ~ 90% mortality within 48-72 hours. Burn injury alone (B) is associated with modest local inflammation and 100% survival 7. We performed multiple trials of burn and inoculation with PA14 and collected BM, PB and parenchymal tissue specimens at specific time points. Occurrence of sepsis during time was confirmed by the presence of PA14 in the blood (data not shown). PA14 infection induced a significant decrease of neutrophils in the circulation, as previously documented 13, coupled with the absence of inflammatory leukocyte infiltrates at the site of inoculation and in the parenchymal organs, which had extensive areas of necrosis due to bacteria presence (Supplementary Fig. 1A-B).

Total BM cellularity was progressively reduced in PA14 infected mice and decreased to 40% in normal controls by 24 hours (Supplementary Fig. 2). Immunophenotypic analysis indicated that the cellular loss was within the BM Gr1^-Mac1+ population, which includes maturing myeloid cells, monocytes and neutrophils. The decrease in mature neutrophils was confirmed by analysis of Gr1^-Mac1+ cells with low forward scatter (FSC) and high side scatter (SSC), and by morphologic analysis of sorted populations (Fig. 1A). The reduction of neutrophils in PA14 infected mice was progressive and greater...
than 70% at 24 hours, whereas in B mice neutrophils levels in the BM decreased less than 10% (Fig. 1B-C), likely due to trafficking to the site of thermal injury (Suppl. Fig. 1A). Next, we examine whether neutrophil loss was due to increased apoptosis during sepsis. Evaluation of apoptosis within the Gr1+Mac1+ population showed a higher percentage of Annexin V positive cells in PA14 infected mice at 12 and 24 hours (Fig. 1D). However, the level of apoptosis (15-20%) in PA14 infected mice suggests that apoptosis accounted partially for the neutrophils shortage observed during sepsis (Fig. 1E). Evaluation of all mature subsets during sepsis confirmed that the cell loss was specific for Gr1+Mac1+ cells whereas no substantial changes were observed in mature T, NK cells and erythroid progenitors (Ter119+); B cells showed an increase (data not shown). These findings clearly show that *P. aeruginosa* infection induces a profound depletion of neutrophils in the BM resulting in a general state of neutropenia and lack of inflammatory infiltrates in the parenchymal organs.

**P. aeruginosa infection induces expansion of the LSK cell pool in the bone marrow.**

Neutrophils have a short half life and their destruction needs to be continuously compensated by rapid regeneration by the progenitor and stem cell compartment. To test whether the exhaustion of the BM neutrophil pool during sepsis was due to a defect in regeneration, we examined the BM progenitor/stem cell compartment by immunophenotypic analysis. This analysis demonstrated that LSK cells (cells negative for lineage markers, Lin−, and positive for Sca1 and c-Kit), a cell subset greatly enriched for hematopoietic stem and progenitor cells (HSPC), were significantly increased in PA14 infected mice at 24 hours from bacterial challenge, both as percentage and in absolute number (5 to 8 fold increase; Fig. 2A-C and Suppl. Fig.2B). Interestingly, analysis of the cell cycle showed that LSK cells of septic animals were in a more quiescent state (Fig. 2D). Enrichment of the more quiescent and primitive pool was also substantiated by increase in the frequency of cobblestone-area forming cells (CAFC) in the BM of septic mice (Fig. 2E). Despite the significant expansion of the LSK pool, the pool of progenitor Lin−/Sca1−/Kit+ cells (Kit+; enriched in myeloid and erythroid progenitors) was reduced by 40% in PA14 infected mice (Fig. 2F), and the ratio progenitors/HSPC (Kit+/LSK) was significantly lower in the BM of PA14 challenged mice compared to controls (Fig. 2G), suggesting a block of differentiation.

To determine the physiological relevance of these observations, parallel trials were conducted with an isogenic non-lethal PA14 mutant, 33C7. In these trials, survival was associated with presence of leukocyte infiltrates in the peripheral organs and lack of the dramatic HSPC expansion observed during infection with the PA14 strain. Analysis of the BM of mice infected with the 33C7 non-lethal mutant strain showed only a modest increase of LSK cells (two-fold at 24 hours), similar to that observed in mice subjected to burn injury only (Fig. 2C and suppl. Fig. 2B). This correlated with a modest decrease of the number of neutrophil levels in the BM (Fig. 1C), likely due to migration and abundant infiltration of leukocytes in the peripheral organs until resolution of the infection (Supp. Fig.1B).
Overall, these findings demonstrate that *P. aeruginosa* infection induces an abnormal expansion of LSK cells coupled with a profound depletion of BM neutrophils and suggest that their regeneration is impaired by a block of differentiation.

**P. aeruginosa LPS is sufficient to cause BM neutrophils depletion and LSK pool expansion.**

Next, we investigated the mechanisms triggering the BM effects during infection. Despite the large number of studies on the role of bacterial LPS in the host immune response, its *in vivo* effects on HSPCs and progenitors have not been investigated. To test whether LPS from *P. aeruginosa* was critical and sufficient to induce the BM phenotype observed during sepsis, we injected increasing concentrations of purified *P. aeruginosa* LPS into mice. Analysis of BM showed that injection of 0.8 mg/Kg (comparable to the amount of *P. aeruginosa*’s LPS present in the blood at 24-30 hours from inoculation with live bacteria) was sufficient to induce the same effects observed during bacterial sepsis. Similar to the observations during PA14 infection, *P. aeruginosa*’s LPS induced a decrease in neutrophils and an increase in LSK cells (Fig. 3A, 2C and 4A). Histological analysis of the BM confirmed hypocellularity with visible decrease in neutrophils and increased frequency of immature forms (Fig. 3B). As observed during sepsis in PA14 infected mice, LSK cells from LPS challenged mice showed reduced cell cycle (Fig. 3C), further confirmed by evidence of transcriptional downregulation of SKP2, a key regulator promoting cell cycle entry (Fig. 3D). Similar results were found on Lin− cells (Suppl. Fig. 3A-D). In agreement with the increased number of immature cells, BM from LPS challenged mice showed a higher frequency of primitive cells capable of generating CAFCs (Fig. 2E), and a lower ability to generate colonies derived from more committed progenitors (CFU-C; Fig. 3E).

In conclusion, our data show that LPS from *P. aeruginosa* is sufficient to induce the effects observed in the BM during *P. aeruginosa* sepsis. Given the causal role of LPS in determining the BM phenotype and the challenges of manipulating bacterial infected BM cells, further analysis of the BM compartment was performed by using *P. aeruginosa*’s LPS inoculation as surrogate for the sepsis model.

**P. aeruginosa’s LPS induces reduction of BM multipotential progenitors, CMPs and GMPs, and defective myeloid differentiation.**

To address the mechanisms involved in the differentiation block we characterized the stem and progenitor subsets and measured the egress from the LSK pool into the more mature pool of progenitors CMP and GMP, which give rise to the myeloid progeny and ultimately to neutrophils. Analysis of distinct Lin− subsets by multiparameter immunophenotypic analysis demonstrated that LPS caused a dramatic reduction of CMPs (Lin−IL7R−Sca−Kit−CD34−FcγRII/IIIlow) and GMPs (Lin−IL7R−Sca−Kit−CD34−FcγRII/IIIhi) (Fig. 4A-B). Apoptosis evaluation revealed negligible differences in these subsets in the two conditions (data not shown). Next, we compared the rates of progenitor differentiation by analysis of lysozyme’s expression, a marker of myeloid differentiation increasingly expressed from CMPs to neutrophils. To
this end, we used Lys-EGFP reporter mice in which GFP expression is driven by the lysozyme promoter. Lys-EGFP mice challenged with *P. aeruginosa’s* LPS, showed that the CMP, GMP and neutrophils had a much lower expression of lysozyme than controls, with prevalence of immature forms (Lys-EGFP negative or dim) in each subset (Fig. 4B), confirming lower rates of myeloid differentiation. Evaluation of the Lys-EGFP expression in LSK cells confirmed a similar expression in PBS and LPS challenged mice, thus excluding the possibility that the expansion of LSK pool is due to CMP and GMP progenitors’ failure to downregulate Sca1 (Suppl. Fig. 4A).

Global analysis of Lin− subpopulations revealed also a decrease in megakaryocytic-erythroid precursors (MEPs, Lin-IL7R-Sca-Kit+CD34-FcγRII/IIIlo; Fig. 4A and C), which, however, was not associated with decreased erythroid progenitors, and an increase in common lymphoid precursors (CLPs; Fig. 4C). Further distinction of the LSK subsets in LSK IL7R−CD34+FLT3+ (multipotential progenitors, MPP; short-term, ST-HSC) and IL7R−CD34+FLT3− (long-term, LT-HSC), indicated that LPS induced a greater increase in cells with LT-HSC phenotype (average 4.75 % vs 0.43% control) than in cells with ST-HSC (average 1.0 % vs 0.47% control). qRT-PCR analysis of PU.1 and C/EBPα, transcription factors involved in myeloid differentiation, showed that they were expressed at significant lower levels in LSK cells sorted from LPS challenged mice than in controls (Fig. 4D).

To confirm that the altered representation of LSK, MPP, CMP and GMP had impact on differentiation dynamics, Lin− cells purified from LPS mice or PBS controls were tested for their ability to differentiate under myelodifferentiative conditions *in vitro*. Lin− cells from controls rapidly generated differentiated myeloid cells expressing Mac1, Gr1 and F4/80 (Fig. 5A and B). In contrast Lin− cells from LPS challenged mice maintained high levels of the primitive markers c-Kit and Sca1 during time and required more than 5 days to generate equivalent proportion of mature myeloid cells (Fig. 5C). In conclusion, these data suggest that during sepsis, *P. aeruginosa* LPS skews primitive hematopoietic cells toward a more immature state, characterized by increased numbers of LSK cells and decreased output into the CMP and GMP pools, resulting in a lower ability to progress toward myeloid differentiation. As neutrophils have a very short life span, low efficiency in myeloid differentiation has a greater impact on their levels, as clearly visible *in vivo* during sepsis.

**Septic LSK cells show decreased short-term engraftment and reduced long-term repopulating ability**

We examined the functional properties of the “septic” HSPCs by competitive BM transplantation assays. Equivalent numbers of CD45.2 Lin− cells sorted from BM of PBS or LPS challenged mice were transplanted into lethally irradiated CD45.1 recipients together with competitor cells (CD45.1). Engraftment analysis by PB chimerism (CD45.2 vs CD45.1) was performed at successive 4-week time points from transplant. As the Lin− cells derived from LPS challenged mice contained higher percentages of LSK cells than controls, we expected to observe a significant increase in the long-term engraftment...
ability of this pool in transplanted animals. However, Lin− cells from LPS challenged mice showed a long-term repopulating ability not significantly different from the Lin− cells of controls (Fig. 6A), indicating no improvement in overall stem cell activity. Multilineage analysis performed in the PB in conjunction with CD45.1 and CD45.2 markers at 6, 9 and 12 months, showed that all blood lineages were reconstituted in both groups (data not shown).

Next, we evaluated the specific competitive repopulating activity and differentiation ability of LSK cells. LSK cells sorted from BM of LPS challenged mice showed a significant lower level of engraftment at long-term (3 to 12 months) and at short-term (4 to 12 weeks) and this difference was further increased when lower cell doses were used for transplantation (Fig. 6B and C). Multilineage analysis of the peripheral blood of transplanted animals showed a dramatic decrease of donor Gr1 positive cells compared to competitor (Fig. 6D), accompanied by absence of CMPs and GMPs in the BM (data not shown), confirming a defective ability of the “septic” LSK to generate CMPs and GMPs and mature myeloid cells. Taken together, these results show that during sepsis, the dramatic immunophenotypic expansion of both ST-HSC and LT-HSC is associated with defective stem cell activity and decreased ability to generate CMPs and GMPs.

**Loss of TLR4 rescue the myeloid differentiation block observed during sepsis.**

Toll-like receptor 4 (TLR4), is a critical component in cellular recognition and activation by LPS 17. TLR4 is expressed by several cell types in the bone marrow, including LSK cells 21. Interestingly, we observed that TLR4 expression was significantly upregulated in LSK cells during LPS challenge in vivo (Fig. 7A). Furthermore, LSK cells from LPS treated mice showed transcriptional downregulation of IKBα, indicating their ability to participate to the TLR4/NF-κB signaling circuitry (Suppl. Fig. 4B).

To determine whether TLR4 signaling was required for the BM alterations observed in our model, we injected LPS from *P. aeruginosa* in C3H/HeJ mice, which have a spontaneous mutation in the TLR4 gene resulting in its loss of function (TLR4−), and the results were compared with C3H/OuJ mice, which have functional TLR4 receptors (TLR4wt). As anticipated, TLR4wt mice responded to LPS challenge with a significant decrease in neutrophil levels and a great expansion of LSK cells. In contrast, in TLR4− mice LSK cells were not significantly increased and neutrophils levels remained unchanged (Fig. 7B and C). The modest expansion of LSK cells and the partial normalization of CMP and GMP in TLR4− mice challenged with LPS is likely due to TLR2 activation by residual peptidoglycan-associated lipoprotein (PAL) contaminating the LPS (data not shown) 21,22. Further analysis of the CMP and GMP subsets showed that these progenitor pools were greatly protected by loss of TLR4 signaling in TLR4− mice challenged by LPS (Fig. 7D and E). In conclusion, TLR4 loss greatly reduces the myeloid suppression induced by LPS.
DISCUSSION

The results described here provide the first evidence of the physiological mechanism causing neutropenia during lethal sepsis. By using an in vivo model of P. aeruginosa infection, we show that the neutropenia occurring during sepsis is caused in part by apoptosis and is sustained by a block of hematopoietic stem progenitor cell (HSPC) differentiation. Specifically, we demonstrated that in response to the high consumption of mature cell production during sepsis, the HSPCs expand at the expense of myeloid differentiation resulting in the dramatic reduction of CMPs and GMPs, the common myeloid progenitors that produce neutrophils (Fig. 4E). Moreover, we found that these effects are TLR4 dependent. More broadly, our data establish a link between HSC regulation and host response in severe sepsis, a previously unrecognized connection.

One of the major challenges in this area of study is represented by the variability and limitations of the existing experimental models of sepsis in recapitulating the human pathophysiologic conditions. Thus, it is very important that the results are viewed in the context of the in vivo model of infection used. There is evidence that the mechanisms regulating neutrophil production and migration may change considerably with the severity of sepsis within the same experimental model. The burn P. aeruginosa inoculation model used in this study closely resembles the late stages of sepsis in patients with overwhelming gram negative bacteremia. This model has a very brief early phase of hyperresponsive inflammatory response and is characterized by rapid onset of neutropenia, immunoparalysis and death. In contrast, models of sublethal multimicrobial sepsis, such as cecal ligation and puncture (CLP), or chronic infections, are characterized by a longer hyperactive immunoresponse and by a significant myelostimulation with increase of myeloid progenitors and neutrophils.

Previous studies conducted on the burn P. aeruginosa model revealed the existence of myelosuppression in addition to neutrophils consumption and apoptosis during sepsis. Interestingly, a recent study from Zhu et al. showed that Listeria monocytogenes, a gram positive bacteria with a TLR4 agonist component, can induce myelosuppression and neutropenia correlating with lethality. Although these studies clearly indicated the presence of a myelosuppressive activity during lethal sepsis, they were limited to the analysis of progenitor colonies and terminal differentiation of BM myeloid cells, and the mechanisms mediating this process were not elucidated. To address this question, we investigated how the stem and progenitor dynamics were altered during P. aeruginosa induced sepsis. A central finding of our work is the discovery that the common myeloid progenitors, CMPs and GMPs, which give rise to granulocytes and monocytes, are dramatically decreased during sepsis. Their decrease does not seem to be caused by cell death, as apoptosis evaluation in these subsets did not detect substantial differences between control and septic animals. Furthermore, CMPs and GMPs of septic animals are not only altered in numbers but also in their progress toward differentiation, as indicated by analysis of the lysozyme’s expression. By using a transgenic Lys-EGFP reporter mouse model we show that “septic” myeloid progenitors are characterized by a much lower expression of Lys-
EGFP at all stages, from CMP to neutrophils, indicating delayed kinetic of differentiation. Overall, our data indicate that the decline in BM neutrophils observed in our model can largely be attributed to the decrease of common progenitors, although we do not exclude the possibility that other factors may contribute to the inhibition of myeloid differentiation at later stages.

Interestingly, we found that during sepsis the MEP subset, which contains erythroid multipotential progenitors, was significantly decreased without resulting in decreased Ter119 erythroid progenitors, supporting the notion that these cells can be also generated directly by the LSK fraction in . In contrast, common lymphoid progenitors (CLP) were significantly increased and correlated with augmented levels of B220 cells in the BM (data not shown).

What causes the reduction of common myeloid progenitors during sepsis? Alteration of the common progenitor subsets during sepsis was associated with abnormal expansion of the LSK pool. LSK increased 5 to 8 fold in absolute numbers per femur within 24 hours. Surprisingly, this expansion was not associated with increased cell cycle, but with a block of differentiation. LSK cells showed a more quiescent status with evidence of decreased expression of SKP2, a positive cell cycle regulator in . This observation was extended also to the progenitor pool of Lin- cells, characterized by decreased BrDU incorporation, decreased SKP2 and increased in the cell cycle inhibitor p21cip1. Although this finding was unexpected, it is supported by the recent observation that expansion of HSCs following 5FU is associated with upregulation of LRG47, a negative regulator of cell proliferation . Interestingly, the GTPase LRG-47 is involved in host defense and has shown to mediate hematopoietic recovery following bacterial infection . In our study, we have also confirmed the increased expression of LRG47 in Lin- cells from LPS challenged mice. Indeed, cell cycle arrest is a protective mechanisms and a common cellular response to stress and injury .

In steady-state hematopoiesis a delicate balance exists between the stem cell pool and the compartments of progressively more committed and differentiated cells of all lineages. Egress from a compartment to a more mature one is regulated by the combinatorial effects of microenvironmental cues, transcription factors and cell cycle regulation. Failure to orderly progress from one stage to another characterizes hematopoietic disorders such as myelodysplastic syndromes and leukemias, in which mature cell production (i.e neutrophils and monocytes) is impaired by both increased apoptosis and block of differentiation at the stem cell/progenitor level . Our results show a decrease of mature myeloid cells and myeloid common progenitors associated with LSK expansion, strongly suggesting a block of differentiation. Our hypothesis is further supported by the in vitro and in vivo functional characterization of “septic” LSK cells. Consistent with the finding of decreased CMPs and GMPs in septic animals, LSK from septic mice had significant lower expression of C/EBPα and PU.1, transcription factors critical for myeloid differentiation , and were defective in generating CMPs and GMPs progenitors in vivo, resulting in very low levels of donor myeloid cells at short-term in competitive transplantation assays. Furthermore, stem cells from septic mice acquired myeloid differentiation markers significantly later than controls when
cultured \textit{in vitro} in pro-differentiative LPS-free conditions. Despite the increase of immunophenotypically defined long-term HSC (Lin\textsuperscript-IL7R\textsuperscript-Sca\textsuperscript+Kit\textsuperscript+CD34\textsuperscript-FLT3\textsuperscript-)\textsuperscript{35}, functional analysis by competitive repopulation assay demonstrated that “septic” LSK cells have a significant defect in sustaining long-term engraftment. Engraftment of Lin\textsuperscript- cells from LPS- or PBS-treated mice showed similar long-term reconstitution despite the > 10-fold increase in LSK content in the LPS-treated population. This is more clearly demonstrated in the subsequent experiments using equivalent numbers of LSK from LPS- and PBS-treated mice, in which LSK from septic mice displayed markedly lower repopulating ability. In conclusion, the majority of the LSK cells that expanded during sepsis are dysfunctional, as they do not display long-term repopulating ability and do not generate an adequate output of multipotential myeloid progenitors.

The cellular and molecular mechanisms inducing myeloid-suppression may be potential target for therapy aimed at preserving key cellular components of the host innate immune system during sepsis. This concept is supported by the study of BitMansour and colleagues, in which transplantation of HSC in combination with myeloid progenitors showed protection against \textit{P. aeruginosa} sepsis\textsuperscript{39}. The effects of the isogenic \textit{P. aeruginosa} mutant provided us with critical insights. The non-lethal strains had in common LPS defects and resulted in infections characterized by the absence of LSK alterations and preservation of myeloid progenitors and neutrophils regeneration (unpublished observations). Despite the large number of studies on the role of bacterial LPS in the host immune response\textsuperscript{17}, its \textit{in vivo} effects on HSPCs and progenitors have not been investigated. In this report, we show that injection of LPS from \textit{P. aeruginosa} is sufficient to induce \textit{in vivo} the expansion of HSPC and the decrease of myeloid progenitors and neutrophils observed during \textit{P. aeruginosa} infection. This model, characterized by the absence of active bacterial infection and neutrophil consumption, has enabled us to dissect the host response to sepsis and to further prove a direct link between neutropenia and HSC function.

Finally, the critical importance of LPS-TLR4 signaling is validated by experiments in C3H/HeJ mice lacking TLR4 function. Previous \textit{in vitro} experiments have suggested a role of TLR4 in HSC regulation\textsuperscript{21}. In our study, we demonstrated the critical role of TLR4 in regulating BM dynamics \textit{in vivo} during sepsis by showing that functional TLR4 signaling is necessary to mediate HSPC expansion and reduction of myeloid progenitors. This observation is particularly relevant, as it demonstrates that the alterations of HSC compartment and the resulting myelosuppression are part of the host innate response to TLR4 activation by pathogens. Future studies will explore the contribution of direct versus indirect effects of TLR4 signaling on stem and progenitor cells, as several factors triggered by TLR4 signaling may converge on the hematopoietic stem cell and contribute to alter its normal activity.

In conclusion, our results establish a link between HSC regulation and host response in severe sepsis and demonstrate a novel role for TLR4 signaling in acute infections. These findings also suggest that prevention of the myeloid block at HSC level could be a potential therapeutic target to complement the existing therapeutic strategies for the treatment of severe sepsis.
ACKNOWLEDGMENTS

We would like to thank Dr. S. Spinola (IUSM), Dr. W. Clapp and Dr. M. Yoder for helpful discussion. We thank Dr. J. Hellman (MGH) for quality control test on LPS and Regina Baldini (MGH) for bacterial cell culture preparation.

This work was supported by NIH (grant R01-HL068256-05A2 to N.C), the Shawalter grant (N.C) and Shriners grant 8710 (to L.G.R.)

AUTHOR CONTRIBUTIONS

S.R. designed experiments, performed animal studies, flow cytometry analysis and sorting, cell culture experiments and qRT-PCR and wrote part of the manuscript.
A.C. performed bacteremia experiments and flow cytometry analysis.
B.G. designed experiments, performed animal studies, and performed and interpreted histo-pathological analysis.
C.M. performed bone marrow transplants and endotoxemia studies.
S.G. contributed to bone marrow transplant experiments.
L.F. performed endotoxemia study and flow cytometry analysis.
H.B. performed animal studies
H. H. analyzed histological sections
D.D. performed cell sorting and flow cytometry analysis
C.A.K. performed statistical analysis
S.R. performed cell sorting and 7-multicolor analysis
L.G.R. designed experiments, interpreted and supervised the bacteremia experiments.
N.C. Designed the study and the experiments, analyzed the data, supervised the study and wrote the manuscript.

The authors have no conflict of interest.
REFERENCES


LEGENDS

Figure 1. Loss of BM neutrophil during sepsis. CD1 mice challenged with burn and inoculation of the PA14 (PA14) or 33C7 (33C7) strain, burn only (B) and normal controls (N) were sacrificed at the indicated time points. BM cells were harvested and stained with antibodies directed to Gr1 and Mac1 markers and analyzed by flow cytometry. (A) Dot blots in the left panel show FSC (indicative of size) and SSC (indicative of granularity) of total BM samples. The FCS\textsuperscript{low}/SSC\textsuperscript{high} population was gated (R1) and analyzed for Gr1/Mac1 expression (middle panel), and sorted for morphological analysis (right panel). (B) Dot blots show Gr1 and Mac1 expression in a representative experiment. Numbers indicate percentage of cells within the gate. (C) Line graph summarizes 3 independent experiments. Values are the average of 6 to 10 mice and indicate percentages of BM Gr1\textsuperscript{+}/Mac1\textsuperscript{+} cells. The value at time 0 is the average of 15 normal controls. Error bars indicate standard deviation. (D) Bar graph shows percentages of Annexin V expression on Gr1\textsuperscript{+}/Mac1\textsuperscript{+} population in N, B and PA14 mice. Values are averages of 3-4 mice. Error bars indicate standard deviation. *p = 0.04. (E) Bar graph shows the average percentage of total BM Gr1\textsuperscript{+}/Mac1\textsuperscript{+} cells in PA14 challenged mice; percentage are normalized to Gr1\textsuperscript{+}/Mac1\textsuperscript{+} cells in normal controls = 100\%. The light portion of the column represents the average percentage of alive cells, the dark portion of the column represents average percentage of apoptotic cells Annexin V positive, (n = 4). Error bars indicate standard deviation.

Figure 2. Sepsis induces increase of primitive cells with LSK phenotype. CD1 mice challenged with burn and inoculation of the PA14 (PA14) or 33C7 (33C7) strain, burn only (B) and normal controls (N) were sacrificed at the indicated time points. BM cells were stained with antibodies directed to lineage positive markers and to Sca1 and c-Kit markers. Samples were analyzed by multicolor flow cytometry analysis. (A) Dot blots show Sca1 and c-Kit expression on gated Lin negative (Lin\textsuperscript{-}) cells in N, B and PA14 mice at 24 hours after challenge in a representative experiment. Numbers indicate percentage of cells within the Lin\textsuperscript{-} gate. (B) Line graph indicate percentage of LSK cells on overall BM with exclusion of the R1 region in Fig. 1A (to avoid relative increase of percentage due to loss of the FCS\textsuperscript{low}/SSC\textsuperscript{high} population), in a representative experiment. Values are averages of 4-8 mice. Time 0 is the average of 8 normal animals. (C) Bar graph shows average fold increase in LSK absolute number at 24 hours from challenge. In each column values are the average of 10 mice compared to normal controls in 3 independent experiments. Error bars indicate standard error. LPS: *p = 0.03; PA14: *p = 0.01. (D) Bar graph indicates percentages of LSK cells in the S+G2M phase of the cell cycle, as determined by Hoechst staining. Values indicate average of 3 mice at 24 hours from challenge in a representative experiment. Error bars indicate standard deviation. *p< 0.01. (E) Long term culture with limiting dilution was performed on BM cells to quantify the frequencies of hematopoietic progenitors and stem cells. Data
indicate frequency of CAFCs and are represented as mean ± SD. (F) Bar graph indicates percentage of Lin positive (Lin+) c-Kit+ cells in N, B and PA14 mice at 24 hours from challenge. Values indicate average of 6 mice in a representative experiment at 24 hours from challenge. Error bars indicate standard deviation. *p=0.0018. (G) Measure of LSK cell output. Bar graph shows ratio of percentages of Lin+Kit+ (Kit+) over LSK cells (r = Kit+/LSK). Percentages of each population were measured on identical gate = total BM excluded R1, as in Fig.1A. Values indicate average of 6 mice in two independent experiments at 24 hours from challenge. Error bars indicate standard deviation. *p= 0.016.

**Figure 3.** *P. aeruginosa*’s LPS is sufficient and necessary to induce the BM alterations observed during sepsis. Coherent of CD1 and of C57BL/6J mice were injected i.p. with *P. aeruginosa*’s LPS (0.8 mg/kg) or PBS and compared with mice challenged with PA14. Mice were sacrificed at the indicated time points and their BM cells analyzed. (A) BM cells harvested at 24 hours from LPS or PBS injection, or PA14 challenge, were labeled with antibodies anti-Gr1 and anti-Mac1. Bar graph shows percentage of Gr1+/Mac1+ cells in total BM. Gr1+/Mac1+ . Values are average of 6 mice in 3 independent experiments. Error bars indicate standard deviation. *p <0.0001. (B) BM smears from PBS or LPS challenged mice. Samples were stained by hematoxilin/eosin and morphology was evaluated by light microscopy (100 X magnification). Circles indicate the presence of neutrophils in PBS condition and of immature cells in LPS (arrows). (C) Bar graph indicates percentages of LSK cells in the S+G2M phase of the cell cycle, as determined by Hoechst staining. Values indicate average of 2 mice at 24 hours from challenge in a representative experiment. Error bars indicate standard deviation. (D) Each sample of sorted LSK was derived from a pool of BM of 4-6 mice. RNA was extracted from LSK (IL7R') cells from PBS and LPS challenged mice at 24 hours. Samples were analyzed for expression of SKP2 by qRT-PCR. Bar graphs represent averages of fold changes in expression in three independent samples from two independent experiments. Error bars indicate standard deviation; LPS vs PBS: p=0.04. (E) BM cells were analyzed for colony-forming ability by methylcellulose colony assay. Bar graph shows average number of myeloid colonies (CFU-GM + CFU-G + CFU-M) per 10,000 cells in 3 independent samples, each of them in quadruplicate. *p<0.001.

**Figure 4.** *P. aeruginosa*’s LPS causes reduction of BM CMPs and GMPs. Coherent of CD1 or C57BL/6J mice were injected i.p. with *P. aeruginosa*’s LPS (0.8 mg/kg) or PBS. (A) BM was harvested 24 hours after injection, stained with specific antibodies and analyzed by multicolor flow cytometry. Dot blot on the left insight shows expression of c-Kit and Sca-1 in Lin’/IL7R’ cells. Dot blot on the right insight shows FcγRII/III expression on gated Lin’/IL7R'/Sca'/Kit+ cells to determine CMP, GMP and MEP subsets. Bar graphs indicate percentages of CMPs and GMPs on the Lin’ population. Values are average of 10 to 12 mice and summarize 3 independent experiments. Error bars indicate standard error. CMP: LPS vs. PBS *p<0.001; GMP: LPS vs PBS *p<0.0001. (B) Lys-GFP reporter mice were challenged with
P. aeruginosa's LPS or PBS and the BM was harvested at 24 hours from injection. Dot plots show GFP expression on CMPs, GMPs and granulocytes in a representative experiment. Values in the bar graph indicate percentage of cells that express no or low levels of Lys-GFP (gate A) within each specific subset and are average of 4 mice. CMP: LPS vs. PBS *p=0.05; GMP: LPS vs PBS *p= 0.0015; granulocytes: LPS vs PBS *p= 0.035  (C) BM of C57/B6 mice challenged with LPS or PBS was analyzed for the presence of MEP (left panel), CLP (middle panel) and MPP (right panel) at 24 hours from challenge. Bar graphs indicate percentages of MEP and CLP on the Lin− population. Values are average of 10 to 12 mice and summarize 3 independent experiments. Error bars indicate standard error. MEP: LPS vs. PBS *p<0.001; CLP: LPS vs PBS *p<0.0001. ST-HSC (or MPP) were defined as Lin−/IL7R−/Sca+/Kit+/CD34+/Flt3+ cells. Bar graph shows percentage of ST-HSC (lower, light columns) and LT-HSC (upper, dark columns) on total Lin− population. Values are average of 12 mice and summarize 3 independent experiments. Error bars indicates standard error. *p<0.001. (D) In each experiment, a sample of sorted LSK was derived from a pool of BM of 4-6 mice. RNA was extracted from LSK (IL7R−) cells from PBS and LPS challenged mice. Samples were analyzed for expression of C/EBPα and PU.1 by qRT-PCR. Bar graphs represent averages of fold changes in expression in three independent samples. Error bars indicate standard deviation; LPS vs PBS: C/EBPα, *p=0.033; PU.1 *p=0.029.  

(E) **Sepsis causes alterations in HSC differentiation (working model).** During severe sepsis, bacterial LPS induces TLR4-dependent expansion of dysfunctional LSK cells displaying defective ability to progress into the pool of myeloid progenitors: CMPs and GMPs. Reduction in CMPs and GMPs results in neutropenia.

**Figure 5.** P. aeruginosa's LPS causes defective myeloid differentiation. C57BL/6J mice were injected i.p. with P. aeruginosa's LPS (0.8 mg/kg) or PBS. BM was harvested at 24 hours from injection and Lin− cells were sorted by FACS and grown *in vitro* in pro-differentiative conditions in the presence of SCF, IL-3 and G-CSF. Cells were harvested at the indicated time points and analyzed for expression of differentiation markers by flow cytometry. (A) Dot blots shows expression of c-Kit and of a combination of myeloid differentiation markers (Gr1 + Mac1 + F4/80) at day 1 and day 3 of culture in a representative experiment. (B) Line graph represents acquisition of the myeloid markers (Gr1, Mac1, F4/80) over time. Values represent average percentages of 3 samples from a representative experiment out of 3 independent experiments. Error bars indicate standard deviation. Day 3 *p= 0.00012. (C) Line graph shows kinetic of LSK cells in culture in 3 independent experiments. Values represent average percentage of LSK cells (n = 7).

**Figure 6.** Septic HSPCs show functional defects in bone marrow transplantation assays. C57BL/6J CD45.2 mice were injected i.p. with LPS from P. aeruginosa (0.8mg/kg) or PBS. BM was harvested at 24
hours from injection and Lin− or LSK cells, IL7 receptor negative (to exclude contamination from common lymphoid progenitors), were sorted by FACS. Sorted populations were transplanted into irradiated CD45.1 donors and engraftment was evaluated at each indicated time point by analysis of CD45.2 positive cells in the peripheral blood. (A) 2x10⁵ Lin−CD45.2+ sorted cells were injected into a lethally irradiated CD45.1+ Boy/J recipient together with 3x10⁵ Lin−CD45.1+ competitor cells. Line graph shows engraftment as percentage of CD45.2 cells during time. Values are average of 10 to 12 mice from 3 independent experiments. Bars indicate standard error. p=NS. (B) 2500 LSK/CD45.2+ sorted cells were injected into a lethally irradiated CD45.1 Boy/J recipient together with 1x10⁵ CD45.1+ competitor total BM cells. Line graph shows engraftment as percentage of CD45.2 cells during time. Values are average of 10 to 12 mice from 3 independent experiments. Bars indicate standard error. *p<0.001. (C) Bar graph shows level of donor engraftment in the PB at 4 weeks of transplantation in mice receiving decreasing doses (2500, 1000, 500) of LSK cells derived from PBS controls or LPS challenged mice. Values are average of 5 to 8 mice. *p<0.001. (D) PB cells were collected and stained with antibodies directed to the myeloid lineage. Bar graph shows percentages of donors Gr1+/CD45.2+ cells on total GR1+ cells (100%) at 2, 4 and 8 weeks from transplant. Values are average of 8 to 12 mice. Error bars indicate standard error. *p<0.001

Figure 7. The effects induced by LPS on HSC and neutrophils are abrogated in a TLR4 null phenotype. (A) C57BL/6J CD45.2 mice were injected i.p. with LPS from P. aeruginosa (0.8 mg/kg) or PBS. BM was harvested at 24 hours from injection and Lin− cells were purified and stained with c-Kit, Sca1 and TLR4/MD2 antibodies. Histograms show TLR4 expression on LSK cells (black line) superimposed to TLR4 expression on LK cells (Lin−Kit+ Sca−; gray filled curve) in PBS control (left panel) and in LPS challenged mice (right panel). TLR4 expression on LK cells was at limit of detection and superimposed with IgGs control. (B) C3H/OuJ(TLR4 wt) and C3H/HeJ (TLR4−) mice were injected i.p. with LPS from P. aeruginosa (0.8 mg/kg) or PBS. BM was harvested at 24 hours, stained with monoclonal antibodies necessary to identify the indicated subsets and analyzed by multicolor flow cytometry analysis. Bar graphs show: (B) average percentage of LSK on the Lin− cells. TLR4⁵WT: PBS vs LPS *p=0.045; (C) average percentage of Gr1+/Mac1+ neutrophils in the total BM population. TLR4⁵WT: PBS vs LPS *p=0.0001; (D) average percentage of CMPs in the Lin− population. TLR4⁵WT: PBS vs LPS *p=0.0001; (E) average percentage of GMPs in the Lin− population. TLR4⁵WT: PBS vs LPS *p=0.0001. In all graphs, values are average of 6 mice from two independent experiments. Error bars show standard deviation.
Figure 6
Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in lethal sepsis

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