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MyD88-dependent expansion of an immature GR-1⁺CD11b⁺ population induces T cell suppression and Th2 polarization in sepsis

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Polymicrobial sepsis alters the adaptive immune response and induces T cell suppression and Th2 immune polarization. We identify a GR-1⁺CD11b⁺ population 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 interleukin 10 and several other cytokines and chemokines. Splenic GR-1⁺ cells effectively suppress antigen-specific CD8⁺ T cell interferon (IFN) 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 Th2 cell-dependent and depression of Th1 cell-dependent antibody production. Signaling through MyD88, but not Toll-like receptor 4, TIR domain-containing adaptor-inducing IFN- β , or the IFN- α/β receptor, is required for complete GR-1⁺CD11b⁺ expansion. GR-1⁺CD11b⁺ cells contribute to sepsis-induced T cell suppression and preferential Th2 polarization.

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Abbreviations used: APC, allophycocyanin; MIP, macrophage inflammatory protein; NP-KLH, 4-hydroxy-3-nitrophenyl acetyl-KLH; RANTES, regulated on activation, normal T cell expressed and secreted; TLR, Toll-like receptor; TRIF, TIR domain-containing adaptor-inducing IFN- β .

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 (1, 2). Animals with polymicrobial sepsis also exhibit widespread dysfunction in both antigen-presenting cells and T lymphocytes. Reduced CD4⁺ T cell numbers caused by apoptosis-induced depletion (3, 4) and a suppression of T cell proliferative responses (5) have been shown to contribute to sepsis-associated

morbidity. Moreover, interest has focused on the shift from a Th1 to a Th2 cell profile as contributing to sepsis-associated immune dysfunction (6, 7).

The role that suppressor cell populations play in polymicrobial sepsis is unknown. The present study examined the role of immature myeloid cells in sepsis, as well as their contribution to the sepsis-induced defects in acquired immunity. Immature myeloid cells with suppressor functions have been previously observed in the spleens and tumors of mice with transplantable tumors (8, 9) and in models of chronic inflammation (10). In tumor-bearing mice, these cells contribute to tumor-associated antigen-specific T cell dysfunction and tolerance (8, 11–15).

The online version of this article contains supplemental material.

Splenic GR-1⁺CD11b⁺ cells may also play an instrumental role in the priming of B cell antibody production (16).

There has been only modest exploration of these cell populations in sepsis or other acute inflammatory processes. In this paper, we observed that an ongoing septic process induces a dramatic expansion of the GR-1⁺CD11b⁺ population in the bone marrow, spleen, and lymph nodes. Expansion of these cells was associated with splenic enlargement and lymphoid follicle disruption. The GR-1⁺CD11b⁺ cells contained immature progenitors and expressed IL-10, TNF- α , and other cytokines and chemokines. Furthermore, using a depleting antibody, we demonstrated that expansion of GR-1⁺ cells *in vivo* contributed to the induced Th2 polarization of antibody responses after sepsis. These cells were also capable of causing CD8⁺ T cell tolerance, as demonstrated by the suppression of antigen-specific IFN- γ production by CD8⁺ T lymphocytes in nonseptic immunized mice. Finally, we observed that signaling through MyD88, but not Toll-like receptor (TLR4), TIR domain-containing adaptor-inducing IFN- β (TRIF), or the IFN- α/β receptor, was required for the early and complete expansion of this cell population, highlighting the importance of inflammation and TLR signaling other than that induced by microbial endotoxin in the regulation of immature myeloid 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 experiments were conducted in a mouse 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 ~10–20% and predominantly occurred during the first 96 h; thereafter, surviving mice developed abscesses surrounding the devitalized cecum. As shown in Fig. S1 (available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>), the presence of sepsis was confirmed for at least 10 d by a transient bacteremia (lasting 24 h) and prolonged bacterial contamination of the peritoneal cavity. The animals exhibited a significant early leukopenia, followed by a profound granulocytosis (Fig. S2). Plasma cytokine concentrations over the first 10 d were consistent with an early exaggerated systemic inflammatory response and with sustained elevations in the plasma IL-6, KC/CXCL1, and macrophage inflammatory protein (MIP) 1 β concentrations (Fig. S3).

Interestingly, surviving septic mice developed a dramatic splenomegaly, with the spleen mass increasing by 300% by 10 d after initiation of polymicrobial sepsis (Fig. S4 A, available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>). 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 d of sepsis (Fig. S4 B) demonstrated that the apparent splenomegaly was associated with extramedullary hematopoiesis and marked expansion of immature myelomonocytic cells, including forms with ringed nuclei in the periarteriolar sheaths and subcapsular

space, including focal involution of lymphoid follicles. Analysis of lymphoid and nonlymphoid cell populations did not reveal an increase in cells expressing CD3, B220, or CD11c. However, a dramatic accumulation of cells expressing GR-1 and CD11b occurred. As shown in Fig. 1 (A and B), splenocytes harvested from septic mice at various intervals after cecal ligation and puncture, or sham procedures, exhibited a striking increase in the percentages and absolute numbers of GR-1⁺CD11b⁺ cells. The dramatic increases in the percentages and absolute numbers of GR-1⁺CD11b⁺ cells did not occur until at least 3 d after the induction of sepsis, and the percentages continued to increase to a plateau (~7–10 d). The numbers and proportion of these cells remained elevated even out to 12 wk in surviving mice. By 10 d, the absolute numbers of these GR-1⁺CD11b⁺ cells in the spleen had increased 50-fold.

GR-1⁺CD11b⁺ cells are phenotypically heterogeneous cells

GR-1⁺CD11b⁺ cells represent a heterogeneous population of cells encompassing mature and immature myeloid forms.

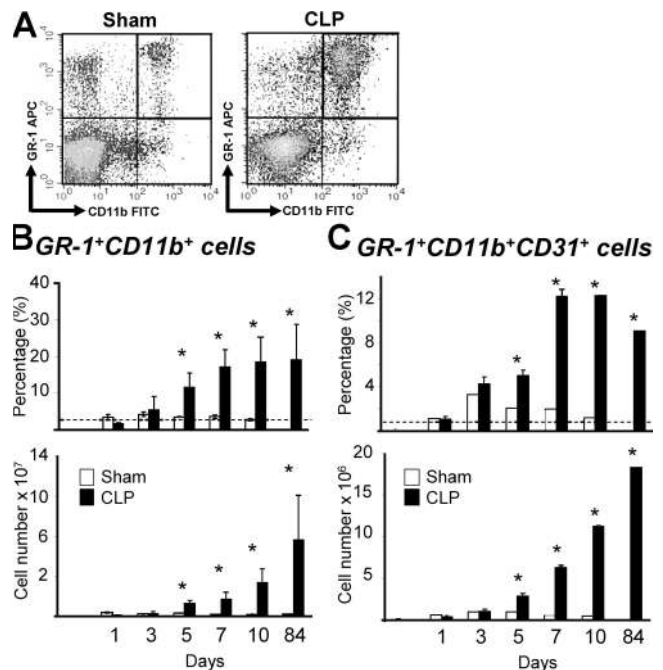


Figure 1. Appearance of GR-1⁺CD11b⁺ cells in spleens from septic and sham mice. (A) Flow cytometry dot plot of viable splenocytes gated on GR-1⁺ and CD11b⁺ staining. (B) Percentages and total numbers of GR-1⁺CD11b⁺ splenocytes recovered from mice at intervals after CLP and sham treatment. (C) Percentages 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. The dashed lines represent the percentages of GR-1⁺CD11b⁺ cells (B) and the percentages of GR-1⁺CD11b⁺CD31⁺ cells (C) in control animals that are neither sham nor CLP treated. Values represent the mean and standard error of 5–10 animals per group. *, $P < 0.01$ by analysis of variance and the Student-Newman-Keuls multiple range test. CLP, cecal ligation and puncture.

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 (17), and Ter119, a marker of erythroid lineage, as well as F4/80, a marker for myeloid lineage development. As shown in Fig. 1 C, ~40% of the GR-1⁺CD11b⁺ cells were also CD31⁺, 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 (unpublished data), suggesting that the GR-1⁺ cells contained a subpopulation of developing myeloid cells. In contrast, only 6% of the GR-1⁺CD11b⁺ cells were Ter119⁺, suggesting that only a small proportion of these cells may still possess the ability to differentiate into cells of erythroid lineage (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>). Interestingly, however, <3% of these GR-1⁺CD11b⁺ cells were also MHC class II⁺ (Fig. S6).

As shown in Fig. 2 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, 7 d of culture with GM-CSF led to ~17% of these cells differentiating into conventional CD11c^{high}MHC class II^{high} dendritic cells and ~22% differentiating into F4/80^{high} macrophages (Fig. 2, A–C), and all of these cells demonstrated increased MHC class II expression over freshly isolated Gr-1⁺CD11b⁺ cells. In addition, culturing these GR-1⁺ cells from septic mice in soft methylcellulose with either GM-CSF or G-CSF, but not erythropoietin, for 7 d led to an increase in the number of colonies formed (Fig. 2 E). Interestingly, the GR-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-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

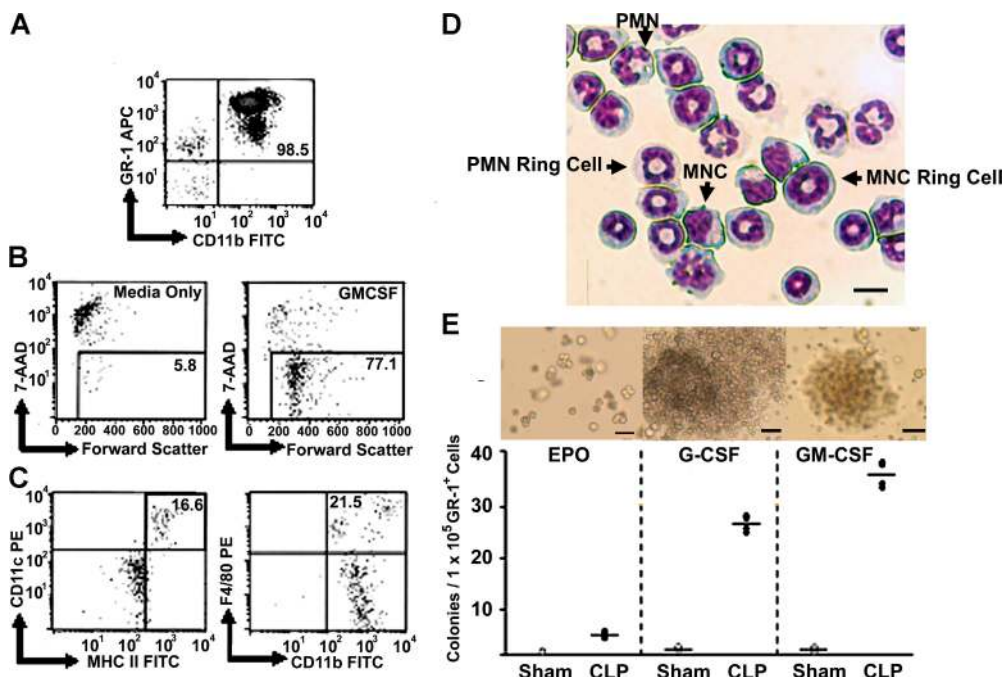


Figure 2. Flow cytometric analysis of GR-1⁺ splenocytes cultured with GM-CSF, G-CSF, or erythropoietin *ex vivo*. (A) Flow cytometric analysis of enriched GR-1⁺ splenocytes obtained from septic mice 10 d after CLP. (B) Cells were cultured for 7 d with media alone or media and GM-CSF, and cell viability was determined by 7-amino-actinomycin D staining. Cells cultured without GM-CSF rapidly died. (C) GR-1⁺-enriched splenocytes cultured with GM-CSF for 7 d yielded a phenotypically diverse cell population staining positive for CD11c and F4/80. (D) Cytospin preparation of enriched GR-1⁺ cells 10 d after CLP demonstrated immature heterogeneous myeloid phenotypes with characteristic ring shaped nuclei.

Bar, 5 μ m. (E) Colony-forming units of GR-1⁺-enriched splenocytes cultured with G-CSF, GM-CSF, or EPO for 10 d in soft methylcellulose. The images distinguish the nature of the colonies, reflecting primarily neutrophil- and monocyte-like colonies in the G-CSF- and GM-CSF-treated groups, respectively. Bars, 15 μ m. Values in A–C represent the mean and standard error of 5–10 animals per group, and the numbers in the quadrants represent the percentage of cells in that quadrant based on the total number of cells that were gated. Horizontal lines indicate the mean number of colonies per group. CLP, cecal ligation and puncture; EPO, erythropoietin; MNC, mononuclear cell; PMN, polymorphonuclear.

expanded numbers of GR-1⁺CD11b⁺ cells represent a mixed population of immature, proliferating, progenitors committed predominantly to a myeloid, and not an erythroid, pathway.

Immature myeloid cells accumulate in secondary lymphoid organs after sepsis

We also looked for these GR-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-1⁺CD11b⁺ cells were seen in either the liver or lung (unpublished data). In peripheral lymph nodes, however, marked increases in the percentages and numbers of these GR-1⁺CD11b⁺ cells were evident at 10–14 d after sepsis and were still increased at 12 wk (Fig. 3, B and C). Numbers remained significantly elevated in mesenteric lymph nodes in direct proximity to the cecal ligation and puncture at 12 wk, whereas numbers declined somewhat, but still remained significantly elevated, in more distal inguinal and axillary lymph nodes at 12 wk. In the bone marrow (Fig. 3 A), the numbers of these GR-1⁺CD11b⁺ cells doubled within 3 d and accounted for nearly 90% of the cells by 7 d after sepsis.

Histological confirmation of these cells in the spleens of mice with severe sepsis is shown in Fig. 4 (A–C). Over the course of 10 d, there was progressive expansion of the red pulp by extramedullary hematopoiesis and marked expansion of the periarteriolar sheaths (Fig. 4 B) and subcapsular space (Fig. 4 C) by immature mononuclear cells and myelomonocytic cells with ringed nuclei. These cells were also found in small clusters within the interfollicular areas. The immature myeloid cells with ringed nuclei in all of these locations were uniformly CD11b⁺ in the spleens of mice that underwent cecal ligation and puncture (Fig. 4, D, F, and G). In contrast, CD11b showed only scattered reactivity in the sham-treated mice, mainly highlighting mature granulocytes within the interfollicular areas (Fig. 4 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.

Immature myeloid cells are capable of inflammatory mediator production

Because immature myeloid cells obtained from tumor-bearing hosts are known to be immunomodulatory (8, 9), we next examined whether cells obtained from septic mice could produce inflammatory mediators, including IL-10, a cytokine generally regarded as a requisite for T cell suppression and Th2 cell polarization during sepsis. When GR-1⁺-enriched splenocytes from septic mice were cultured *ex vivo*, they produced low levels of several inflammatory mediators, including

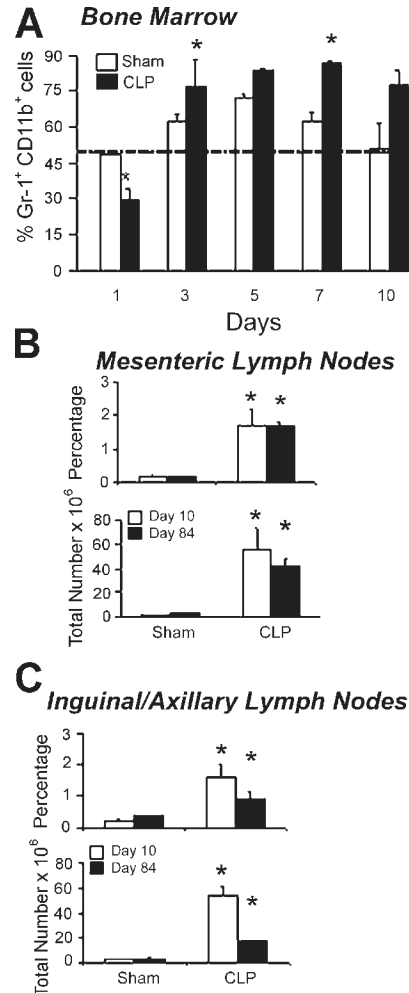


Figure 3. Bone marrow and lymph node GR-1⁺CD11b⁺ cells from septic and sham mice. CLP produced a rapid increase in the numbers of GR-1⁺CD11b⁺ cells in the bone marrow (A) and lymph nodes (B and C). A sham procedure produced a more transient modest increase. Values represent the mean and standard error of 5–10 animals per group. Dashed line indicates the mean percentage from healthy control animals not subjected to CLP or sham procedures. *, $P < 0.01$ between CLP and sham-treated animals by the Student's *t* test. CLP, cecal ligation and puncture.

IL-10, TNF- α , regulated on activation, normal T cell expressed and secreted (RANTES; CC chemokine ligand 5), and MIP-1 β (CC chemokine ligand 4). When stimulated *ex vivo* with bacterial LPS, the GR-1⁺ cells from septic mice produced significantly greater amounts (more than fivefold) of IL-10 than similar GR-1⁺ cells from sham-treated animals (Fig. 5). They also produced increased quantities of TNF- α , RANTES, and MIP-1 β but did not produce the Th2 cytokines IL-4 or IL-13. Unstimulated or LPS-stimulated GR-1⁺ splenocytes did not produce measurable quantities of GM-CSF, IL-12p40, IL-12p70, IL-2, IL-3, IL-5, IL-9, IL-17, vascular endothelial growth factor, or IFN- γ (unpublished data). In addition, GR-1⁺ splenocytes obtained from septic mice also produced TNF- α (218 ± 67 vs. 4 ± 1 pg/ml) and IL-10

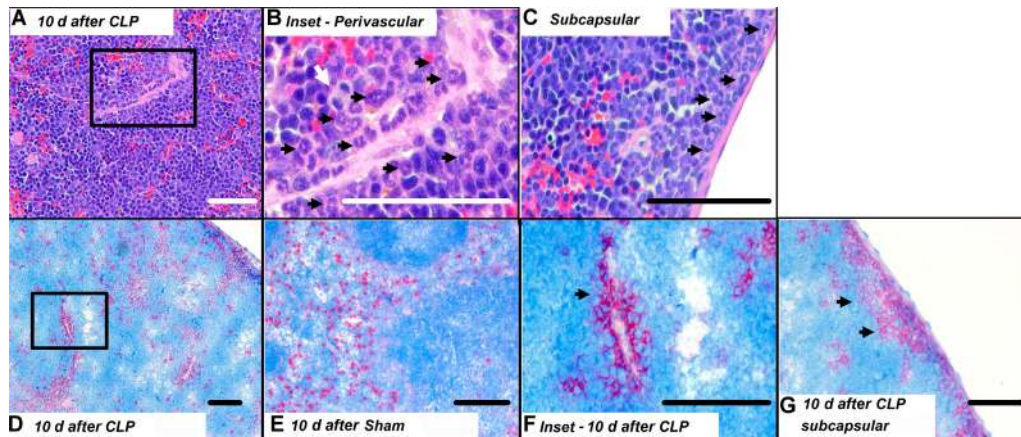


Figure 4. Hematoxylin and eosin–stained spleens from septic mice 10 d after CLP. (A) Low power view, with the perivascular region identified (inset). (B) High power view of the perivascular region showing cuffing and infiltration, with myeloid cells showing characteristic ring features (black arrows). Note the mitotically active cell (white arrow). (C) High power view of the subcapsular region also showing infiltration, with myeloid cells exhibiting characteristic ring features (arrows). (D) CD11b⁺ staining of the spleen from a septic animal 10 d after CLP. (E) CD11b⁺

staining of the spleen from a sham-treated animal 10 d after surgical procedure. In the sham animal, CD11b⁺ staining is distributed in the mantle region surrounding T cell-rich follicles. After 10 d of sepsis, additional CD11b⁺ staining appears in the perivascular and subcapsular regions. (F) High power view of staining of the perivascular region showing CD11b⁺ staining from a 10-d septic animal. (G) High power view showing resolution of the subcapsular region showing CD11b⁺ staining from a septic animal. Bars, 100 μm. CLP, cecal ligation and puncture.

(165 ± 43 pg/ml) in response to stimulation with 5 μg/ml flagellin (TLR5 agonist), albeit in lesser quantities than with LPS stimulation.

Immature myeloid 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 4-hydroxy-3-nitrophenyl acetyl–KLH (NP-KLH) using the adjuvant alum when GR-1⁺ cells reached a maxi-

mal proportion in the spleen (10 d after cecal ligation and puncture), and the NP-specific serum immunoglobulin response was determined 10 d 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 (Fig. 6, A and B); however, when the total IgG response was dissected into its isotypic components, the serum IgG_{2a} response was significantly decreased

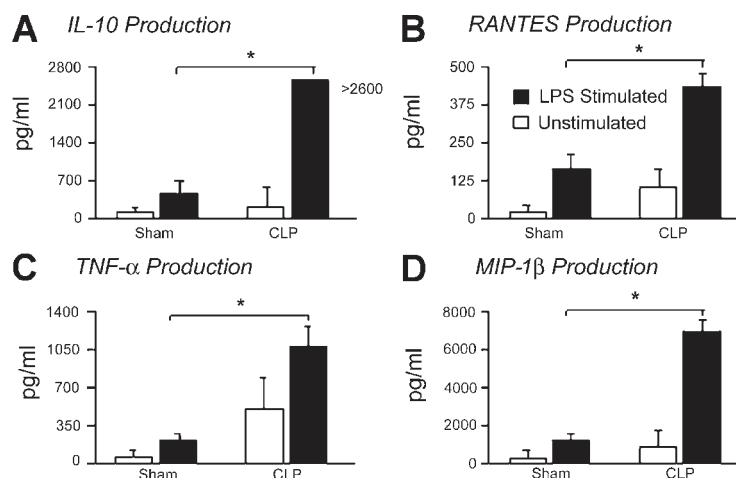


Figure 5. Effect of ex vivo LPS stimulation on cytokine expression in GR-1⁺ splenocytes obtained from septic mice. When GR-1⁺ splenocytes were harvested from 7-d sham-treated or septic mice and stimulated with 10 μg/ml of bacterial LPS, IL-1α, IL-1β, IL-6, IL-10, TNF-α, RANTES, MIP-1β, and KC/CXCL1 production was significantly increased in

all groups stimulated with LPS. Importantly, GR-1⁺ splenocytes from septic mice secreted more IL-10, TNF-α, RANTES, and MIP-1β production after LPS administration than GR-1⁺ splenocytes from sham-treated animals. Values represent the mean ± SEM of between four and six samples. *, P < 0.05 by the Student's *t* test.

while the IgG₁ response increased in the septic mice, consistent with a shift from a Th1 to a Th2 response (Fig. 6, C and D). When the septic mice were treated with a GR-1–depleting antibody, producing >80% reduction in total GR-1⁺CD11b⁺ splenocytes (Fig. S7, available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>), the sepsis-induced increase in IgG₁ 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 (Fig. S8, A–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(TCR α TCR β)1100mjb/J) immunized with OVA-derived peptide (H-2K^b restricted, aa 257–264; SIINFEKL) was examined (Fig. 7 A). GR-1⁺ cells obtained from either 10-d septic or sham-treated mice were infused into C57BL/6 mice that had previously received CD8⁺ T cells from OT-1 TCR transgenic mice, and mice were simultaneously immunized with OVA-derived specific peptide. 10 d 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 with 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, DO11.10 OVA-TCR transgenic mice were made septic, and CD4⁺ splenocytes were cultured with irradiated GR-1⁺–containing antigen-presenting cells from the spleens of 10-d septic or sham-treated mice and were incubated with either OVA peptide, bovine serum albumin, or on CD3/CD28-coated plates. As shown in Fig. 7 B, culturing CD4⁺ cells with irradiated GR-1⁺ cells from septic mice only modestly, but still significantly, reduced both the antigen-specific (OVA) and nonspecific (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 TLR 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 injected with a sublethal dose (5 mg/kg body weight) of the TLR4 agonist bacterial LPS. The numbers of these GR-1⁺CD11b⁺ splenocytes were examined at daily intervals after LPS injection. As shown in Fig. 8 A, the administration of bacterial LPS produced a more rapid but transient increase in the percentages and absolute numbers of GR-1⁺CD11b⁺ splenocytes, suggesting that the likely pathway involves TLR4

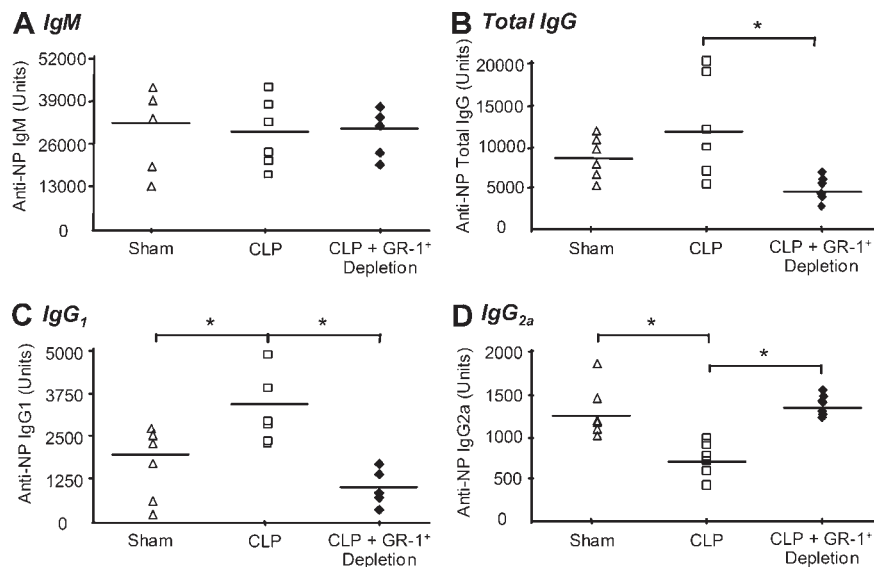


Figure 6. Ig production after NP-KLH immunization in sham mice, septic mice, and septic mice depleted of GR-1⁺ cells. 9 and 10 d after induction of sepsis by CLP, mice were depleted of GR-1⁺ cells by the *i.p.* administration of RB6-8C5 anti-GR-1 antibody, as described in Materials and methods. Mice were then immunized with NP-KLH. 7 d later, mice were bled, and serum IgM (A), total IgG (B), IgG₁ (C), and IgG_{2a} (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 IgG₁ and a decrease in the serum IgG_{2a} response consistent with Th2 polarization. The total IgG, IgG₁, and IgG_{2a} responses after sepsis were prevented by depletion of the GR-1⁺ cells. Horizontal lines represent the means for each group of samples. *, $P < 0.01$ by analysis of variance and the Student-Newman-Keuls multiple range test. CLP, cecal ligation and puncture.

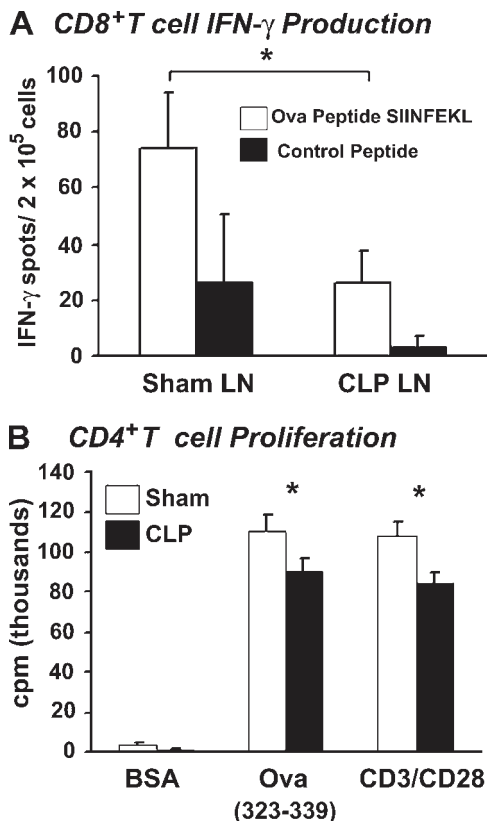


Figure 7. Effect of GR-1⁺ cells from septic mice on antigen-specific CD4⁺ T cell proliferation and CD8⁺ T cell IFN- γ responses. Mice were treated as described in Materials and methods. GR-1⁺ cells from septic animals markedly attenuated the IFN- γ production (determined by ELISPOT) by OT-1 splenocytes stimulated with either control peptide or OVA-derived peptide SIINFEKL ex vivo after administration and immunization in C57BL/6 mice (A). In contrast, GR-1⁺ splenocytes from 10-d septic mice had only a minimal but significant suppressive effect on antigen-specific and nonspecific CD4⁺ T cell proliferative responses in OVA antigen-specific, DO11.10 mice (B). Values represent the mean and standard error of five animals per group. The experiment was repeated twice, and the values presented are from one representative experiment. *, $P < 0.05$ by analysis of variance and the Student-Newman-Keuls multiple range test.

signaling. The expansion of GR-1⁺CD11b⁺ splenocytes by LPS occurred by 24 h, reached a peak by 5 d, and declined thereafter until it reached a near-baseline level by day 10. Interestingly, the increased number of splenic GR-1⁺CD11b⁺ cells was very modest compared with mice undergoing 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 with C3H/OuJ mice. As shown in Fig. 8 B, TLR4 mutant C3H/HeJ mice had a comparable increase in splenic GR-1⁺CD11b⁺ cell

number as the C3H/OuJ controls at 7 d, suggesting that intact TLR4 signaling is not required for the expansion of these GR-1⁺CD11b⁺ cells in sepsis. Thus, although LPS 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, because polymicrobial sepsis is generally associated with the release of large numbers of different microbial products that can simultaneously signal through several different TLR receptors. Because TLR signaling occurs through MyD88- and TRIF-dependent pathways and may involve the secretion of type I IFNs, cecal ligation and puncture were also performed in MyD88^{-/-}, TRIF^{-/-}, and IFN- α/β R^{-/-} mice. The increased expansion of GR-1⁺CD11b⁺ cells after cecal ligation and puncture at 7 d was markedly attenuated in only the MyD88^{-/-} (B6 \times 129) mice and not in either the TRIF^{-/-} or IFN- α/β R^{-/-} mice, highlighting the requirement of MyD88 signaling for the early expansion of this cell population during sepsis (Fig. 8, C-E).

To confirm that the requirement for MyD88 signaling was not dependent on 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 Fig. 9, after 7 d of sepsis, there was again no expansion of the GR-1⁺CD11b⁺ population in the MyD88^{-/-} (B6) mice. By 14 d, there was some expansion of the GR-1⁺CD11b⁺ cell populations, but it was still markedly attenuated when compared with the wild-type B6 controls.

DISCUSSION

T cell dysfunction is a common response to polymicrobial sepsis (1), ultimately leading to increased susceptibility to ongoing and opportunistic infections and poor outcome. Recent attention has focused on the shift from a more proinflammatory Th1 to a more antiinflammatory Th2 cell immune profile as an explanation for postsepsis immune suppression; however, the underlying mechanisms that orchestrate this T cell suppression and Th2 cell 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 Th2 polarization in sepsis (20, 21), more recent studies have refuted some of those claims (22, 23). Several years ago, Murphey et al. observed an increased number of macrophage-like cells in the spleens of mice surviving cecal ligation and puncture (5), although they did not explore their suppressor cell function. More recently, Makarenkova et al. observed increased numbers of immature myeloid cells in the spleens of mice within 12 h of a traumatic injury and identified them as a source of arginase I activity (24).

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

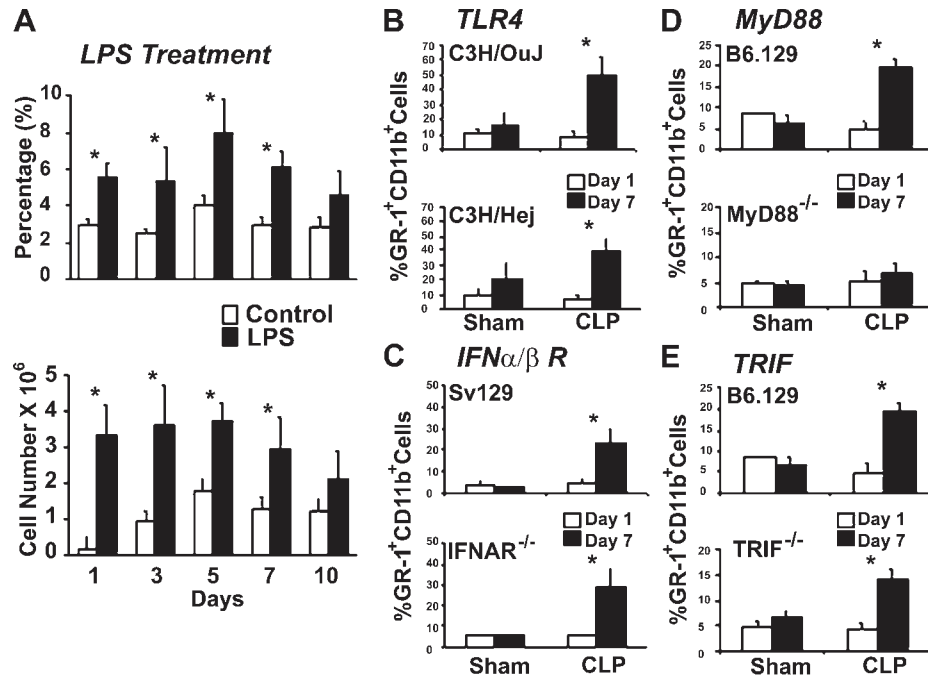


Figure 8. Effects of LPS and transgenic mice on the expansion of the splenic GR-1⁺CD11b⁺ population 7 d after CLP. (A) Mice received either nothing or the i.p. injection of 5 mg/kg body weight of bacterial LPS and were killed at intervals thereafter. LPS injection increased the percentage of GR-1⁺CD11b⁺ cells in the spleen within 1 d, and expansion of this cell population remained for about 7 d. CLP was induced in C3H/HeJ (TLR4 mutant; B), IFN- α/β R^{-/-} (C), MyD88^{-/-} (B6 \times 129; D), and TRIF^{-/-} (E) mice, as described in Materials and methods. 1 and 7 d 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 7 d, 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 five animals per group. *, P < 0.01 versus control at the same time point, as determined by the Student's *t* test. CLP, cecal ligation and puncture.

became evident that, in association 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 immature myeloid cells (Fig. 1). Hotchkiss and co-workers, as well as us, 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 h of sepsis and that these cellular losses contribute to the adverse outcome (25–27). 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-1⁺CD11b⁺CD31⁺) in the bone marrow, spleen, and lymph nodes of mice with ongoing septic processes. We have clearly shown that by 10 d after cecal ligation and puncture, almost 40% of the spleen and 90% of the bone marrow cellularity represent immature GR-1⁺CD11b⁺ cells. Interestingly, the administration of near lethal doses of bacterial LPS could also produce some expansion of this immature myeloid cell population. However, the increases were transient and modest compared with those seen in the septic animals, suggesting that an ongoing inflammatory process may be required for complete manifestation of the response (10).

The phenotype of these cells, the kinetics of their expansion, and their anatomical location in the spleen argue against them being functionally or phenotypically similar to those reported previously by Makarenkova et al. (24) immediately after trauma, or as mere components of extramedullary hematopoiesis. Makarenkova et al. observed a rapid influx of GR1⁺CD11b⁺ cells into the spleens of mice 12–24 h 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 during the first 24 h of sepsis and only saw expansion of our splenocyte population after 3–5 d of sepsis. Furthermore, although both populations are clearly heterogeneous, our cells contained higher proportions of immature precursors that were concentrated in perivascular/periarterial 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-1⁺CD11b⁺ cells recovered from the spleens of septic mice were Ter119⁺ and, when cultured in soft methylcellulose with erythropoietin, had only a minimal

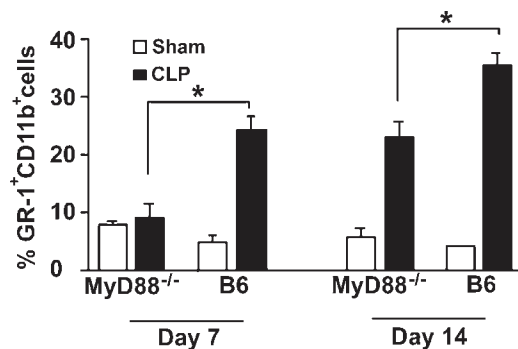


Figure 9. Requirement for MyD88^{-/-} signaling in the expansion of GR-1⁺CD11b⁺ splenocytes in response to prolonged sepsis.

MyD88^{-/-} mice on a C57BL/6 background and wild-type controls underwent CLP, and the representative animals were killed at 7 and 14 d after induction of sepsis. The relative and absolute numbers of GR-1⁺CD11b⁺ splenocytes were determined, as previously described in Fig. 1. As seen in the MyD88^{-/-} B6 × 129 animals, there was no increase in the GR-1⁺CD11b⁺ splenocyte population 7 d after sepsis. However, there was an increase in the GR-1⁺CD11b⁺ population at 14 d, although it was modest compared with the wild-type animals. *, *P* < 0.01 versus control at the same time point, as determined by the Student's *t* test. CLP, cecal ligation and puncture.

capacity for colony or burst formation (Fig. S5). In contrast, almost 40% of the cells differentiated into macrophages or dendritic cells when cultured with GM-CSF (Fig. 2 C), and large numbers were capable of forming colonies when incubated in soft methylcellulose with either G-CSF or GM-CSF (Fig. 2 E).

This dramatic expansion of the GR-1⁺CD11b⁺ 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 (11–15), and also in the blood of some patients with cancer (28). In mice with transplantable tumors, these myeloid-derived suppressor cells, as they are now being termed (29), 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 TGF-β.

Given the abilities of immature myeloid cells to facilitate immune suppression in mouse 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 Th1 to Th2 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 to IgG_{2a} or IgG₁ production, which is dependent on cytokines, including IFN-γ and IL-4, and reflects this predilection toward a Th2 versus a Th1 CD4⁺ T cell response (16, 19). 10 d after sepsis, immunization with NP-KLH led to an increase in the IgG₁ production at the expense of IgG_{2a}, consistent with a preferential Th2

response. In contrast, partial depletion of the GR-1⁺ cells in vivo blocked the characteristic increase in the IgG₁ 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 Th1 to Th2 response.

Consistent with these findings was the observation that GR-1⁺ cells from septic mice substantially 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 (11). Surprising, however, were the modest reductions in antigen-specific CD4⁺ T cell proliferation that we observed when GR-1⁺ cells were cultured with DO11.10 CD4⁺ OVA transgenic T cells immunized with OVA peptide. The answer may simply be that these GR-1⁺ cells express very little MHC class II, whereas they retain relatively high levels of MHC class I expression (11). 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 immature myeloid cells decreased in mice in which the abscess had spontaneously resolved 12 wk after sepsis (unpublished data). Furthermore, when these same animals with abscess resolution were immunized with NP-KLH and alum, the antibody response more closely approximated baseline isotype levels (Fig. S9, available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>). Similar decreases in the numbers of immature myeloid cells have been reported in tumor-bearing animals upon resection of their primary tumor (30).

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 and puncture of the cecum 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 GR-1⁺CD11b⁺ population in C3H/HeJ mice lacking a functional TLR4 receptor, suggesting that although LPS 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 mortality comparable with wild-type mice after sublethal polymicrobial sepsis (31), the findings suggest that neither mouse 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 IFN production. Because both MyD88

and TRIF pathways can lead to type I IFN 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 IFN- α / β R signaling, indicating that TLR signaling to type I IFNs is not important in the induction of these cells. However their expansion was completely prevented for the first 7 d in septic mice lacking MyD88 signaling and was still considerably attenuated after 14 d. The studies were confirmed in MyD88^{-/-} mice on two backgrounds (B6 and B6 \times 129(F₁)) to ensure 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. 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 a key 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 wk. These cells are heterogenous, metabolically active, can secrete several cytokines, and are immature, but they are predominantly committed to development along myeloid pathways. Signaling through MyD88 is required for the complete 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 IFN- γ production, and to the polarization from a Th1 to a Th2 immune response exhibited by augmenting B cell antibody production toward IgG₁ (Th2) and away from IgG_{2a} (Th1). 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/HeOJ), OT-1 TCR transgenic mice (C57BL/6-Tg(TCR α TCR β)1100Mjb/J), DO11.10 OVA TCR transgenic mice (BALB/c-TgN(DO11.10)10Loh), and B6.129PF1/J mice were all purchased from the Jackson Laboratory. IFN- α R/A129 mice on the 129S6/SvEv background (H-2b) and wild-type Sv129 mice were purchased from B&K Universal. All mice were maintained at the University of Florida College of Medicine. MyD88^{-/-} mice on a B6 \times 129(F₁) background and TRIF^{-/-} mice were a gift of S. Akira (Hyogo College of Medicine, Hyogo, Japan) to Schering-Plough Biopharma and were maintained at Schering-Plough Biopharma. MyD88^{-/-} mice on a B6 background were

obtained from S. Akira and were maintained at Rhode Island Hospital and Brown University.

Cecal ligation and puncture. For induction of polymicrobial sepsis, mice underwent cecal ligation and puncture or a sham procedure, as previously described (4, 32), to obtain a mortality of 10–20% by 10 d. At various intervals, bacterial counts from the blood and peritoneal wash (3 ml 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 (Hemavet 1700 FS; Drew Scientific Inc.). Plasma cytokines were determined by Luminex technology (Beadlyte Mouse Multi-Cytokine Detection System; Upstate Cell Signaling Solutions).

Flow cytometry. Spleens, lymph nodes, and bone marrow cells were analyzed by flow cytometry, as previously described (32). Antibodies included anti-GR-1 (Ly-6G and Ly-6C; clone RB6-8C5) conjugated to allophycocyanin (APC), anti-CD11b (integrin α M chain, Mac-1 α chain; clone M1/70) conjugated to FITC, anti-MHC class II (I-A/I-E; 2G9) conjugated to FITC, anti-F4/80 antigen (panmacrophage marker; clone BM8) conjugated to PE, Ter119 conjugated to FITC (clone ter119), anti-CD11c (clone N418) conjugated to APC, Fc-Block (CD16/CD32 Fc γ III/II receptor; 2.4G2), and 7-amino-actinomycin D F4/80- and CD11c-specific antibodies purchased from eBioscience; all other antibodies were purchased from BD Biosciences.

Cell purification. All magnetic bead kits were obtained from Miltenyi Biotec. Erythrocyte-depleted splenocytes and lymphocytes were isolated using either anti-GR-1 (Ly-6G and Ly-6C; clone 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 10⁶ cells/well with RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 200 U/ml penicillin, and 50 μ g/ml streptomycin, and stimulated with 10 μ g/ml of bacterial LPS (*Escherichia coli* 0111: B4). The culture supernatant was analyzed for cytokines.

Ex vivo differentiation and colony formation. 10⁶ GR-1⁺ splenocytes were positively enriched and cultured with RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 200 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were stimulated for 7 d with 10 ng/ml GM-CSF (R&D Systems). The cells were phenotyped by flow cytometric analyses.

For the colony-forming assays, 10⁵ GR-1⁺ splenocytes from sham and septic mice were cultured in Methocult methylcellulose media (StemCell Technologies Inc.) containing recombinant mouse G-CSF, GM-CSF, or erythropoietin (all at 10 ng/ml; R&D Systems) for 10 d. Colonies containing >30 cells were enumerated.

Antigen-specific CD8⁺ T cell IFN- γ production. 3–5 \times 10⁶ purified T cells from OT-1 TCR transgenic mice were injected i.v. into naive C57BL/6 recipient mice. 2 d later, mice were injected i.v. with 5 \times 10⁶ GR-1⁺ cells obtained 10 d after sepsis or sham procedure. Mice were immunized s.c. with the specific peptide (100 μ g of the OVA-derived peptide SIINFEKL) mixed with incomplete Freund's adjuvant. 10 d later, lymph node and spleen cells were isolated and restimulated in vitro with the specific (SIINFEKL) or control (RAHYNIVTF) peptide and analyzed via IFN- γ ELISPOT.

ELISPOT assay. MultiScreen HA plates (Millipore) were coated with and blocked with phosphate-buffered saline with 1% bovine serum albumin before plating. Spleen and lymph node cells were plated at 2.5 \times 10⁵ cells/well with HL-1 (Cambrex) supplemented with 2 mM L-glutamine, 200 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were stimulated with either 10 μ M OVA_{257–264} or bovine serum albumin for 48 h. The cells were treated with 1 μ g/ml biotinylated anti-mouse-IFN- γ (XMG1.2; BD Bioscience) and a 1:1,000 dilution of streptavidin-alkaline phosphatase

conjugate and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Pierce Chemical Co.). 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 used to determine whether GR-1⁺ cells suppress antigen-specific or nonspecific CD4⁺ T cell proliferation. DO11.10 transgenic mice were made septic and immunized with 100 μ g 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 negative fraction was irradiated with 3,000 rads. 2×10^5 antigen-presenting cells from sham-treated mice (containing $<10^4$ GR-1⁺ cells) or 2×10^5 antigen-presenting cells from septic mice (containing 7.5×10^4 GR-1⁺ cells) were mixed with 2.5×10^4 CD4⁺ T cells. Cells were restimulated with 10 μ g/ml OVA₃₂₃₋₃₃₉ peptide or 1 μ g/ml anti-CD3, and 1 μ g/ml anti-CD28 or 10 μ g/ml of bovine serum albumin for 48 h. In the final 16 h of culture, 1 μ Ci [³H]thymidine (GE Healthcare) was added. Proliferation was determined by the incorporation of [³H]thymidine in the cell co-culture.

Immunization with NP-KLH and humoral immune responses. An NP-KLH immunization model was used, as described by Hurov et al. (33). When indicated in the figures, animals were depleted either of GR-1⁺ cells or CD4⁺ T cells by i.p. injection of purified rat anti-mouse GR-1 hybridoma IgG₁ (RP865) or rat anti-mouse CD4 hybridoma IgG₁ (GK1.5). At day 10 after surgery, mice were immunized s.c. with 100 μ g of the T cell-dependent antigen NP-KLH (Biosearch Tech) and alum. Serum titers of NP-specific antibodies were determined by ELISA. 96-well plates (Immulon 4HBX; Dynex Tech) were coated with 1 mg of NP-bovine serum albumin (Biosearch Tech) per milliliter. NP-specific antibodies were bound to biotin-conjugated goat anti-mouse Ig isotype antibodies, anti-IgM, anti-IgG1, anti-IgG2a, and anti-IgG3 (CalTag). Streptavidin-conjugated horseradish peroxidase was incorporated to detect the biotin-Ig with 2,2'-azino-di(3-ethylbenzthiazolinesulfonate) substrate.

Morphologic and histologic analysis. For cytopsins, 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- μ m sections), and mounted for staining. For immunohistochemical staining, spleens were mounted in Tissue Tek optimum cutting temperature compound (Sakura Finetek) and flash frozen. 4- μ m cryosections were stained with a rat anti-mouse CD11b at a 1:50 dilution (BD Biosciences), followed by a biotinylated rabbit anti-rat (Vector Laboratories) secondary antibody, and visualized using Vulcan Fast Red (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 the Student's *t* test for two groups. Significance was designated at the 95% confidence level.

Online supplemental material. Fig. S1 relates the bacteremia and blood leukocyte responses to sepsis produced by cecal ligation and puncture. Fig. S2 displays the mouse differential white blood cell counts in response to sepsis. Fig. S3 exhibits the selected plasma cytokine concentrations in septic and sham-treated mice. Fig. S4 shows the splenomegaly that results from sepsis. Fig. S5 depicts the Ter119 surface staining of GR-1⁺CD11b⁺ splenocytes harvested from sham and septic mice. Fig. S6 relates the MHC class II expression in GR-1⁺CD11b⁺ splenocytes harvested from sham and septic mice. Fig. S7 details the effect of GR-1 antibody depletion on CD11b⁺GR-1⁺ populations in the spleen and lymph nodes. Fig. S8 depicts the immunoglobulin production after NP-KLH immunization in sham and septic mice. Fig. S9 exhibits the immunoglobulin production after NP-KLH immunization in sham and septic mice 12 wk after cecal ligation and

puncture. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20062602/DC1>.

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MyD88^{-/-} mice on a B6 \times 129(F₁) background and TRIF^{-/-} mice were a gift of Dr. Shizuo Akira to Schering-Plough Biopharma and were maintained at Schering-Plough Biopharma. MyD88^{-/-} mice on a B6 background were obtained from Dr. Shizuo Akira and maintained at Rhode Island Hospital and Brown University.

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