

Toll-like Receptors on Hematopoietic Progenitor Cells Stimulate Innate Immune System Replenishment

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cytes (Iwasaki and Medzhitov, 2004). Cells responsible for both innate and adaptive immunity have finite life spans and must be constantly replenished from hematopoietic stem cells (HSCs) and progenitors in bone marrow (Kondo et al., 2003). Although TLRs on mature immune cells have been well studied, little is known about when maturing cells in bone marrow acquire functional TLRs and whether those receptors influence hematopoietic development.

HSCs give rise to a series of progenitors that gradually lose differentiation options and produce cells of a given type. For example, multipotent progenitors (MPPs) spawn common myeloid progenitors (CMPs) that give rise to either megakaryocyte and erythrocyte progenitors (MEPs) or granulocyte and macrophage progenitors (GMPs) (Akashi et al., 2000). Early lymphoid progenitors (ELPs) capable of producing T, B, and NK cells give rise to polymphocytes or common lymphoid progenitors (CLPs) that can then become pre-B cells (Igarashi et al., 2002; Kouro et al., 2002; Kondo et al., 1997). All information available to date indicates that commitment to, and progression within, these lineages requires well-studied growth and differentiation factors. These and other extracellular cues control expression of key transcription factors such as EBF, C/EBP α , and PU.1 (Henderson and Calame, 1998; Rosmarin et al., 2005).

There is considerable controversy concerning the plasticity of stem cells and progenitors, and there are numerous examples where lymphoid lineage cells were experimentally converted to macrophages (Kondo et al., 2000; Iwasaki-Arai et al., 2003; Xie et al., 2004). Although there has been no physiological basis for this phenomenon, it is interesting that plasmacytoid dendritic cells (DCs) can be converted to myeloid DCs during viral infection (Zuniga et al., 2004).

The TLR family recognizes well-conserved microbial and viral components. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS) from Gram-negative bacteria (Hoshino et al., 1999), whereas TLR2 recognizes peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Takeuchi et al., 1999). Effective stimulation of cells via some TLRs requires cooperation with other molecules. MD-2 is associated with TLR4 and is essential for LPS recognition (Nagai et al., 2002). On B lymphocytes, the RP105-MD-1 complex cooperates with TLR2 and TLR4-MD-2 to cause antibody production to microbial membranes (Nagai et al., 2005). However, unlike B cells, RP105 can be a negative regulator of TLR4 signaling on macrophages (Divanovic et al., 2005). CD14 is known to cooperate with TLR2 and the TLR4-MD-2 complex in responses to lipoproteins and LPS, respectively (Yoshimura et al., 1999; Means et al., 1999). In addition, TLRs require intracellular adaptor proteins for effective signaling. All TLRs except for TLR3 use Myd88 for the production of inflammatory cytokines (Takeda and Akira, 2005). In addition, TLR3 and TLR4 use a Myd88-independent pathway, which is triggered by the TRIF (also known as TICAM) protein critical for induction of interferon-inducible genes (Yamamoto et al., 2003; Oshiumi et al., 2003).

Summary

Toll-like receptors (TLRs) are best known for their ability to recognize microbial or viral components and initiate innate immune responses. We showed here that TLRs and their coreceptors were expressed by multipotent hematopoietic stem cells, whose cell cycle entry was triggered by TLR ligation. TLR expression also extended to some of the early hematopoietic progenitors, although not the progenitor cells dedicated to megakaryocyte and erythroid differentiation. TLR signaling via the Myd88 adaptor protein drove differentiation of myeloid progenitors, bypassing some normal growth and differentiation requirements, and also drove lymphoid progenitors to become dendritic cells. CD14 contributed to the efficiency of lipopolysaccharide (LPS) recognition by stem and progenitor cells, and LPS interacted directly with the TLR4/MD-2 complex on these cells in bone marrow. Thus, the preferential pathogen-mediated stimulation of myeloid differentiation pathways may provide a means for rapid replenishment of the innate immune system during infection.

Introduction

The survival of an organism depends on an innate immune system that can quickly recognize and respond to microbial and viral products. Toll-like receptors (TLRs) are responsible for much of that recognition and consequently have vital roles in survival (Takeda et al., 2003). Activation via TLRs couples innate immunity with the adaptive immunity provided by lympho-

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A variety of defense mechanisms are triggered when microbial and viral products engage TLRs on innate immune cells. For example, TLR2 and TLR4 are linked to macrophage phagocytosis of bacteria (Blander and Medzhitov, 2004). TLR signaling in DCs induces the expression of histocompatibility complex (MHC) and costimulatory molecules as well as the production of IL-12, a key cytokine for the induction of Th1 immune responses (Iwasaki and Medzhitov, 2004). In addition to these well-studied examples involving mature cells, there have been some hints that TLR might influence hematopoiesis. For example, chronic inflammation such as that elicited with endotoxin alters myeloid/lymphoid ratios in marrow (Ueda et al., 2004, 2005), and maturation of osteoclasts is altered by TLR ligands (Sato et al., 2004; Hayashi et al., 2003). Furthermore, although the Toll pathway in *Drosophila* has a developmental role in determining dorsoventral polarity (Ferrandon et al., 2004), it could regulate hemocyte division and differentiation in the steady state (Qiu et al., 1998).

We now report that early hematopoietic progenitor cells expressed functional TLR2 and TLR4-MD-2. When stimulated with TLR ligands in culture, myeloid progenitors gave rise to monocytes and/or macrophages in the absence of exogenous growth and differentiation factors, and lymphoid progenitors became DCs. These results indicate that undifferentiated progenitor cells can distinguish “non-self” from “self” via TLRs and that microbial components could be cues for hematopoietic cell development. Normally delivered within the context of cytokines and chemokines, such signals may help replace and/or increase cells that constitute a first line of defense against pathogens.

Results

Toll-like Receptors and Associated Molecules Are Expressed on Stem Cells and Early Hematopoietic Progenitors

We wanted to determine when TLRs are acquired by maturing hematopoietic progenitor cells, and whether they have roles in hematopoiesis. Therefore, we traced the expression of TLRs and their coreceptors on bone marrow cells by flow cytometry. An initial finding was that HSCs and early progenitors enriched in the lineage marker-negative (Lin^-) c-Kit^+ subset, as well as mature immune cells in the periphery, expressed TLRs and associated molecules. These included TLR2 and TLR4, as well as MD-2 and CD14 (Figure S1A; see the Supplemental Data available with this article online).

Next, we divided Lin^- c-Kit^+ cells into five major subsets and evaluated them by flow cytometry (Figure 1A). Gating and flow cytometry analysis of the progenitor cells were performed as described by Kondo et al. (2003) (see Method 1 in the Supplemental Data). The HSC-enriched Lin^- $\text{IL-7R}\alpha^-$ c-Kit^{hi} Sca-1^+ fraction (hereafter referred to as LKS^+) had uniformly high expression of TLR2, and the same was true for Lin^- $\text{IL-7R}\alpha^-$ c-Kit^{lo} Sca-1^{lo} , prolymphocytes, or CLPs. Although this was also the case for the Lin^- $\text{IL-7R}\alpha^-$ c-Kit^{hi} Sca-1^- (LKS^-) fraction, CD34^- $\text{Fc}\gamma\text{R}^{\text{lo}}$ MEPs within the LKS^- subset had very little TLR2 (Figure 1A). The Lin^- $\text{IL-7R}\alpha^-$ c-Kit^{hi} Sca-1^- CD34^+ $\text{Fc}\gamma\text{R}^{\text{hi}}$ GMPs subset was uniformly TLR2⁺, and Lin^- $\text{IL-7R}\alpha^-$ c-Kit^{hi} Sca-1^-

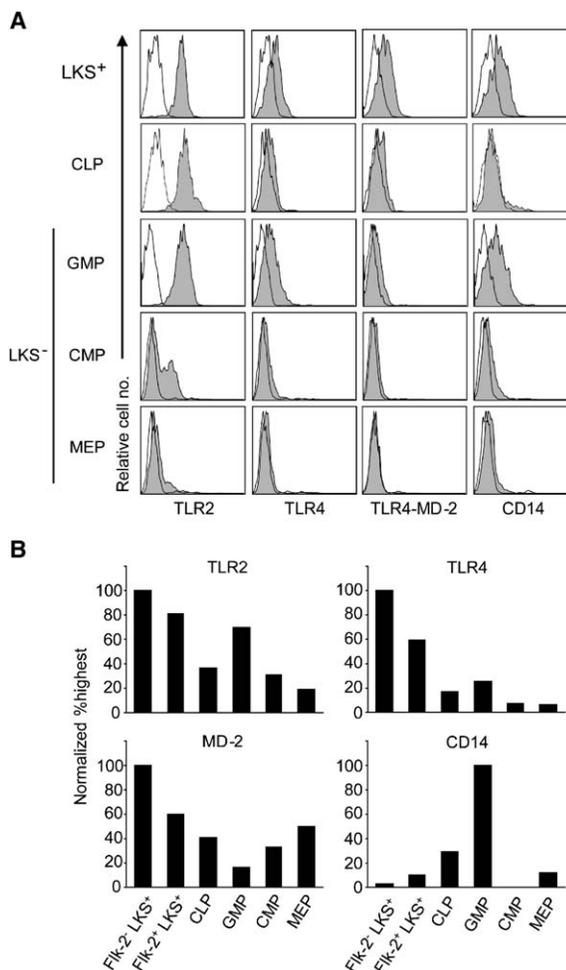


Figure 1. TLRs and Related Molecules Are Expressed by Hematopoietic Stem Cells and Progenitor Cells

(A) Lineage marker-negative cells were enriched from bone marrow suspensions before being stained with antibodies to hematopoietic subsets and TLR2, TLR4, TLR4-MD-2, or CD14. Open histograms depict staining with the appropriate isotype-matched Abs. The results shown are representative of three independent experiments.

(B) Total RNA was extracted from each progenitor subset, and semi-quantitative RT-PCR was conducted to detect mRNA encoding TLR4, MD-2, TLR2, and CD14. The results are shown as values normalized to peak expression for each of the transcripts and are representative of two independent experiments.

CD34^+ $\text{Fc}\gamma\text{R}^{\text{lo}}$ CMPs were heterogeneous with respect to this receptor (Figure 1A). A new TLR4-specific antibody or one directed to the TLR4-MD-2 complex stained the HSC-enriched fraction, and CD14 was also conspicuous. In contrast to HSCs, CLPs were very similar to mature spleen B cells that have a high density of TLR2 and trace amounts of TLR4-MD-2 (Nagai et al., 2005). TLR4 and CD14 were more easily seen on GMPs than the two LKS^- companion subsets (Figure 1A).

Some TLRs are not displayed on the cell surface, so each of the above-described fractions was analyzed by RT-PCR (Figure 1B). In general, there was good correlation between transcript expression and flow cytometry results. Long-term repopulating HSCs are known to be enriched in the Fik-2^- subset of the LKS^+ fraction (Christensen and Weissman, 2001). Interestingly, this

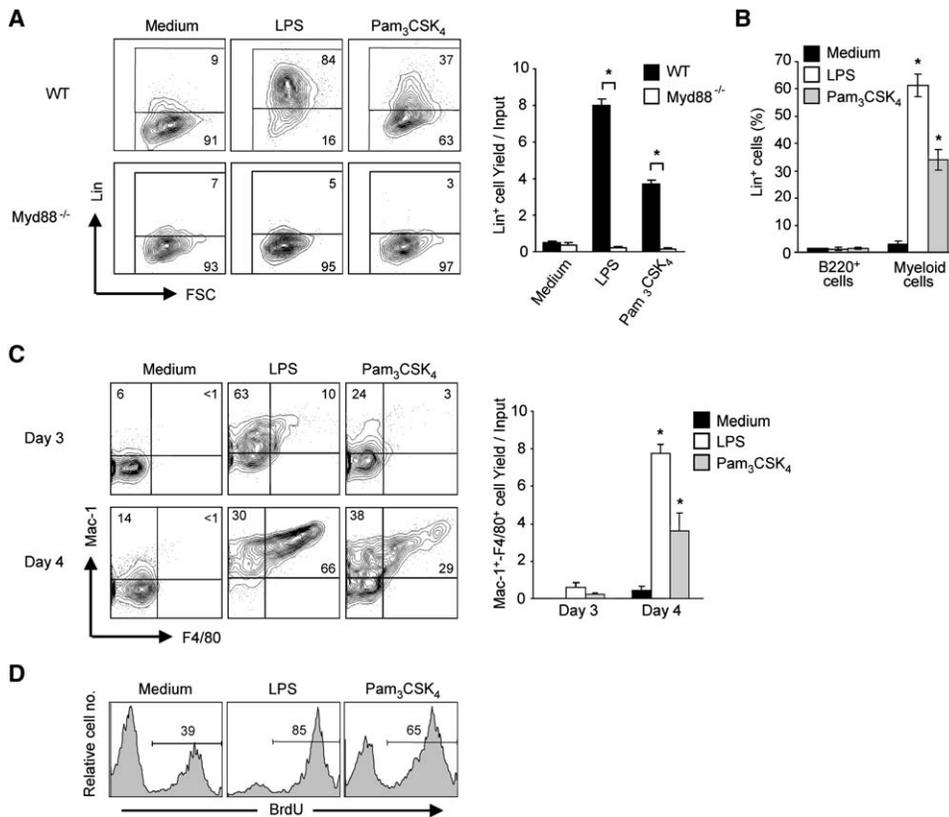


Figure 2. Activation of TLRs through Myd88 on LKS⁺ Cells Leads to Myeloid Cell Differentiation, and Stem Cell-Rich Flk-2⁻ Cells Enter the Cell Cycle with TLR Ligation

(A) (Left) Sorted LKS⁺ cells (10,000 cells/well) from C57BL/6 or Myd88^{-/-} mice were cultured in the presence of FL and SCF with medium alone, LPS (10 μg/ml), or Pam₃CSK₄ (1 μg/ml). After 72 hr in culture, cells were analyzed by flow cytometry for expression of lineage markers. Percentages indicate the frequencies of Lin⁺ or Lin⁻ cells. (Right) The bar graph depicts yields, i.e., numbers of Lin⁺ cells recovered per input progenitor, and the data represent mean values with standard deviations from triplicate cultures (*p < 0.01). The results are representative of five independent experiments. Similar results were obtained when 1600, 5000, or 7500 LKS⁺ cells were plated per well (data not shown).

(B) Expression of CD45R/B220 or myeloid cell markers (Mac-1 and/or Gr-1) on cultured cells. The bar graph depicts percentages of recovered cells bearing B220, CD11b/Mac-1, and/or Gr-1 lineage markers. Data represent mean values with standard deviations from triplicate cultures (*p < 0.001). Similar results were obtained in three independent experiments.

(C) (Left) Sorted LKS⁺ cells (10,000 cells/well) from C57BL/6 mice were cultured in the presence of FL and SCF with medium alone, LPS (10 μg/ml), or Pam₃CSK₄ (1 μg/ml). After 72 or 96 hr in culture, cells were analyzed by flow cytometry for Mac-1 and F4/80. (Right) The bar graph depicts cell yields. Data represent mean values with standard deviations from triplicate cultures and are representative of three independent experiments (*p < 0.002).

(D) Sorted Flk-2⁻ LKS⁺ (10,000 cells/well) were cultured in the presence of SCF with medium alone, LPS (10 μg/ml), or Pam₃CSK₄ (1 μg/ml) for 50 hr and were pulsed with 10 μM BrdU for the final 18 hr. Cells were then stained with anti-BrdU. Percentages indicate the frequencies of BrdU⁺ cells, and the results are representative of three independent experiments.

population had the highest expression of TLR2, TLR4, and MD-2 mRNA, but little CD14. By flow cytometry analysis, the Flk-2⁻ and Flk-2⁺ subsets expressed similar densities of cell surface TLR2, TLR4-MD-2, and CD14 (Figure S1B).

The analysis was extended by staining progenitors with antibodies to RP105 and MD-1 (Figures S2A and S2B). Lin⁻ c-Kit⁺ progenitors in bone marrow expressed the RP105-MD-1 complex, but the densities were less than those found on mature cells. Among the progenitors, RP105 was highest on CLPs detectable on GMPs and was near background on the other subsets. The density of MD-1 on most of these progenitors corresponded with RP105 expression.

We conclude that some of the most primitive of hematopoietic progenitors in bone marrow, and especially a rare stem cell-enriched fraction, express TLRs and associated molecules.

TLR Signaling Drives Myd88-Dependent Myeloid Differentiation of Primitive Hematopoietic Cells

Our experiments then focused on potential functions for TLRs on hematopoietic progenitors. We isolated LKS⁺ cells and stimulated them in serum-free, stromal cell-free cultures with either LPS, a TLR4 ligand, or Pam₃CSK₄, a ligand for TLR2. We determined in preliminary experiments that the cytokines Flt-3 ligand (FL) and Stem cell factor (SCF) promoted cell viability, but alone caused little differentiation under these conditions. The frequency of Lin⁺ cells increased as a result of TLR stimulation in as little as 24 hr and progressed with time (Figure 2A; Figure S3A). Absolute numbers of Lin⁺ cells increased ~8-fold within 72 hr, and this response did not occur with cells from Myd88-deficient mice (Figure 2A). To exclude the possibility that contaminated mature cells might respond to TLR ligands, the number of LKS⁺ cells plated per well was reduced.

Similar results were obtained when only 1,600 LKS⁺ cells were plated per well (data not shown). Expanding cells expressed the Mac-1 and/or Gr-1 myeloid markers, but not B220 (Figure 2B). The mature monocyte and macrophage marker F4/80 was acquired by some cells in as little as 72 hr, and levels increased dramatically with time (Figure 2C). In parallel with these changes, substantial numbers of LKS⁺ bone marrow cells acquired Fc γ R in response to LPS or Pam₃CSK₄ exposure within 24 hr (Figure S3B).

The dose of LPS required to stimulate hematopoietic cells under serum-free conditions was high, and we found minimal responses at concentrations below 10 μ g/ml (Figure S3C). Serum is known to contain soluble CD14, which enhances LPS and TLR2 ligand recognition by mature cells (Yoshimura et al., 1999; Means et al., 1999). Therefore, we added mouse CD14-Fc protein to this culture system and found that it augmented the sensitivity of LKS⁺ cells to LPS after 72 hr (Figures S3C and S3D). That is, we recorded responses to as little as 0.1 μ g/ml LPS and found coexpression of Mac-1 and F4/80 within 72 hr (Figure S3D). A recombinant human CD14 was similar to mouse CD14-Fc in augmenting LPS responses (data not shown). Exogenous CD14 had less influence on responses to Pam₃CSK₄ (Figure S3C).

Similar studies were performed with the LKS⁺ fraction subdivided on the basis of Flk-2. The long-term repopulating, stem cell-rich Flk-2⁻ subset responded to TLR stimulation, but it did so less dramatically than multipotent Flk-2⁺ progenitors (Figure S3E). BrdU labeling experiments showed that increased proportions of cells stimulated with TLR ligands entered the cell cycle (Figure 2D). Thus, the quiescent long-term repopulating HSC subset could be driven into the cycle by TLR ligation.

A preliminary RT-PCR analysis was performed with the same experimental design to verify TLR ligand-induced differentiation (Figure S4). Transcripts for the M-CSF receptor (*c-fms*) increased upon stimulation with TLR ligands, consistent with monocyte and/or macrophage differentiation, but the GM-CSF receptor was highly expressed and unchanged. Another objective was to determine if key transcription factors associated with lineage fate decisions were altered. Depression of *SCL* and *GATA2* transcripts and a slight increase in *PU.1* were seen, as expected for myeloid progenitors (Akashi et al., 2000). However, levels of the *C/EBP α* transcription factor that can drive macrophage differentiation (Rosmarin et al., 2005; Xie et al., 2004) declined with TLR stimulation (Figure S4).

It is clear from these findings that two TLR ligands delivered Myd88-dependent signals, promoting myeloid lineage progression of primitive hematopoietic cells. Moreover, highly enriched populations of HSCs displayed functional receptors for these substances.

Signaling via TLRs on Granulocyte and Macrophage Progenitors Obviates the Need for Growth and Differentiation Factors

The above-described findings show that highly enriched HSCs can be stimulated via TLRs to produce myeloid lineage cells. We next asked if LKS⁻ committed myeloid and erythroid progenitors would be similarly responsive. Serum-free, stromal cell-free culture conditions were

used to determine whether the normal cues for differentiation might be overcome. LKS⁻ cells responded to TLR2 and TLR4 ligands and acquired F4/80 dramatically (Figure 3A). Responses at 72 hr were indistinguishable from those obtained when the same population was stimulated with M-CSF. In contrast to the homogeneous differentiation seen with those three stimuli, only a subset of LKS⁻ myeloid progenitors responded to GM-CSF. Signaling via TLR, but not the CSFs, was dependent on Myd88 (Figures 3A and 3B). In some experiments, yields of F4/80⁺ cells obtained with LPS were nearly equal to those derived with CSF stimulation, whereas Pam₃CSK₄ was consistently a less potent stimulus (Figure 3B). When shorter culture intervals (24 or 48 hr) were used to compare TLR and CSF receptor signaling (Figure S5), TLR2 and TLR4 ligands caused much more rapid acquisition of F4/80 than CSFs did.

It seemed possible that the hematopoietic cells were induced to produce their own growth and differentiation factors. Therefore, neutralizing monoclonal antibodies to either an M-CSF receptor or GM-CSF were added to the culture system (Figure 3C). Although the amounts used were sufficient to completely block responses to M-CSF, and greatly diminish those to GM-CSF, the antibodies had no significant effect on TLR stimulation.

Three subsets of LKS⁻ cells were then sorted and stimulated with TLR ligands (Figure 3D) to precisely identify cellular targets for TLR stimulation. MEPs died in culture, regardless of stimulus, and cells expressing the erythrocyte-associated TER119 marker were never recovered (data not shown). In contrast, GMPs produced F4/80⁺ cells within 24 hr (Figure 3D). Although CMPs also produced F4/80⁺ cells, more time was required, and the calculated yield of F4/80⁺ cells at 72 hr was less than one per input progenitor. These results would be consistent with the lower expression of TLR2 and TLR4 on CMPs, and it seemed possible that some of the CMPs can differentiate into even more TLR-responsive GMPs in culture. Addition of exogenous CD14 to this culture augmented stimulation via TLR2 and TLR4 (data not shown), but less than that described above for stem cells.

We conclude that myeloid progenitors representing a range of differentiation stages react to TLR ligands via a Myd88-dependent pathway, and that GMPs represented the most TLR-responsive of myeloid progenitors in bone marrow. There was no absolute requirement for typical growth and differentiation factors under these defined conditions of culture, and the responses were distinct from those observed with CSFs both in terms of time required and dependence on Myd88.

Monocyte and/or Macrophage Subsets Are Rapidly Produced from Committed Myeloid Progenitors after TLR Ligation

We wondered if a normal range of myeloid cell types would be produced in response to TLR ligation. Accordingly, GMPs were placed in defined culture conditions for 72 hr, and the recovered cells were evaluated (Figure 4). In contrast to cultures stimulated with M-CSF or GM-CSF, in which typical foamy macrophages or neutrophils predominated, macrophage-like cells in LPS-containing cultures had more basophilic cytoplasm with small granules. Cells recovered from Pam₃CSK₄-stimulated

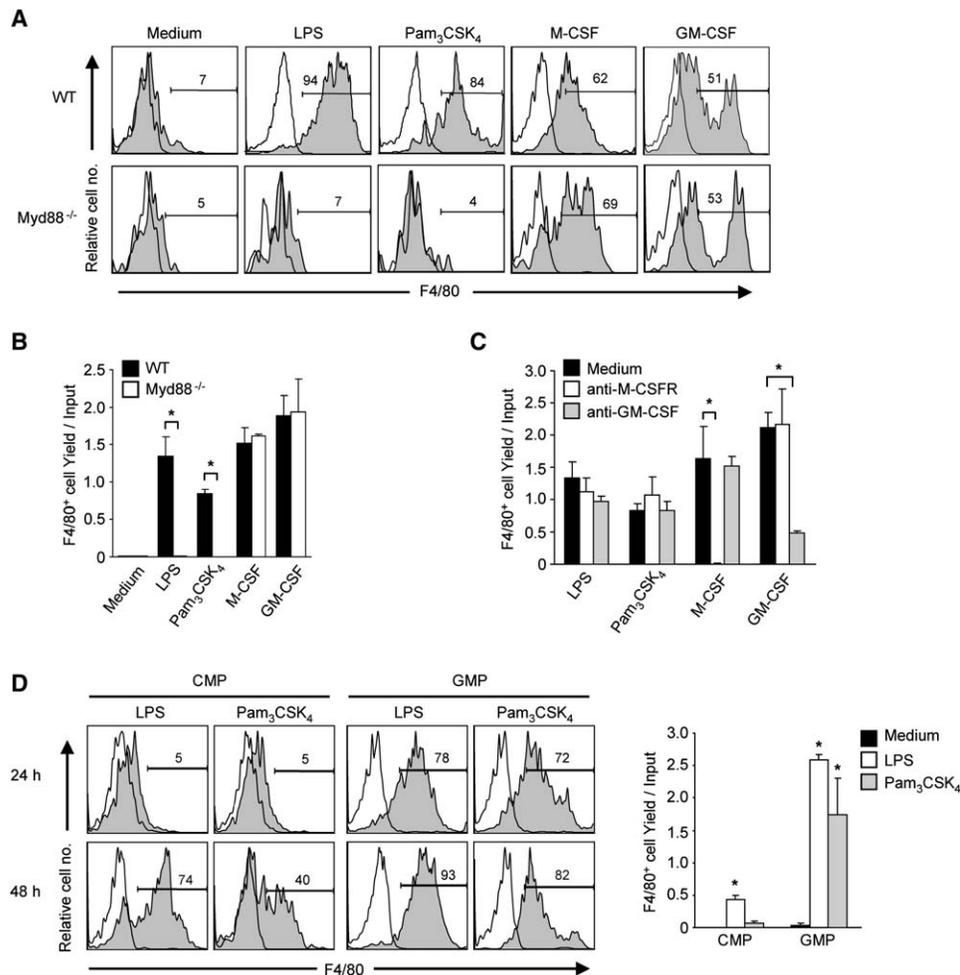


Figure 3. Activation of TLRs through Myd88 Bypasses Normal Differentiation Cues and Drives Monocyte and/or Macrophage Differentiation of Myeloid Progenitors

(A) Sorted LKS⁻ cells from C57BL/6 or Myd88^{-/-} mice were stimulated with medium alone, LPS (10 μg/ml), Pam₃CSK₄ (1 μg/ml), M-CSF, or GM-CSF. After 72 hr in culture, cells were analyzed by flow cytometry for expression of F4/80. Open histograms depict staining with the isotype-matched Ab for F4/80. Percentages given in each histogram indicate the frequencies of F4/80⁺ cells, and the results are representative of three independent experiments.

(B) The bar graph depicts yields, i.e., numbers of F4/80⁺ cells recovered per input progenitor. Data represent mean values with standard deviations from triplicate cultures, and the results are representative of three independent experiments (*p < 0.005).

(C) Sorted LKS⁻ cells from C57BL/6 mice were stimulated with LPS (10 μg/ml), Pam₃CSK₄ (1 μg/ml), M-CSF, or GM-CSF in the presence of anti-M-CSFR (10 μg/ml) or anti-GM-CSF (10 μg/ml). After 72 hr in culture, cells were analyzed by flow cytometry for expression of F4/80. Data represent mean values with standard deviations from triplicate cultures, and the results are representative of three independent experiments (*p < 0.01).

(D) (Left) Sorted CMPs or GMPs from C57BL/6 mice were stimulated with LPS (10 μg/ml) or Pam₃CSK₄ (1 μg/ml). After 24 or 48 hr in culture, cells were analyzed by flow cytometry for expression of F4/80. Open histograms depict staining with the isotype-matched Abs for F4/80. Frequencies of F4/80⁺ cells are given in each histogram, and the bar graphs on the right depict cell yields. The data represent mean values with standard deviations from triplicate cultures and are representative of three independent experiments (*p < 0.02).

cultures were less homogeneous in appearance (Figure 4A). Virtually all cells produced in response to LPS or Pam₃CSK₄ expressed the Mac-1 marker, and the staining intensities tended to correlate with densities of F4/80 (Figure 4B). A conspicuous subset of Mac-1⁺ F4/80⁻ cells seen in response to GM-CSF was not present in TLR ligand-stimulated cultures. LPS was particularly efficient in driving production of Mac-1^{hi} F4/80^{hi} cells (Figure 4C). Previous studies showed that inflammatory Mac-1⁺ F4/80⁺ monocytes preferentially expressed Gr-1 and CD62L (Geissmann et al., 2003). This inflammatory phenotype was observed for the Mac-1^{hi}

F4/80^{hi} cells derived by TLR ligand or GM-CSF stimulation, but not for the M-CSF-stimulated cells (Figure 4B). The costimulatory CD86 molecule was present on TLR-generated cells, but at lower expression than on cells induced with M-CSF. Additionally, only 9% of the cells recovered from LPS-stimulated cultures after 72 hr, and none from any of the other cultures, coexpressed the Mac-1 and CD11c markers typical of DCs (data not shown). These results indicate that TLR stimulation induces committed myeloid progenitors to differentiate into Mac-1^{hi} F4/80^{hi} monocytes and/or macrophages with inflammatory characteristics. The phenomenon

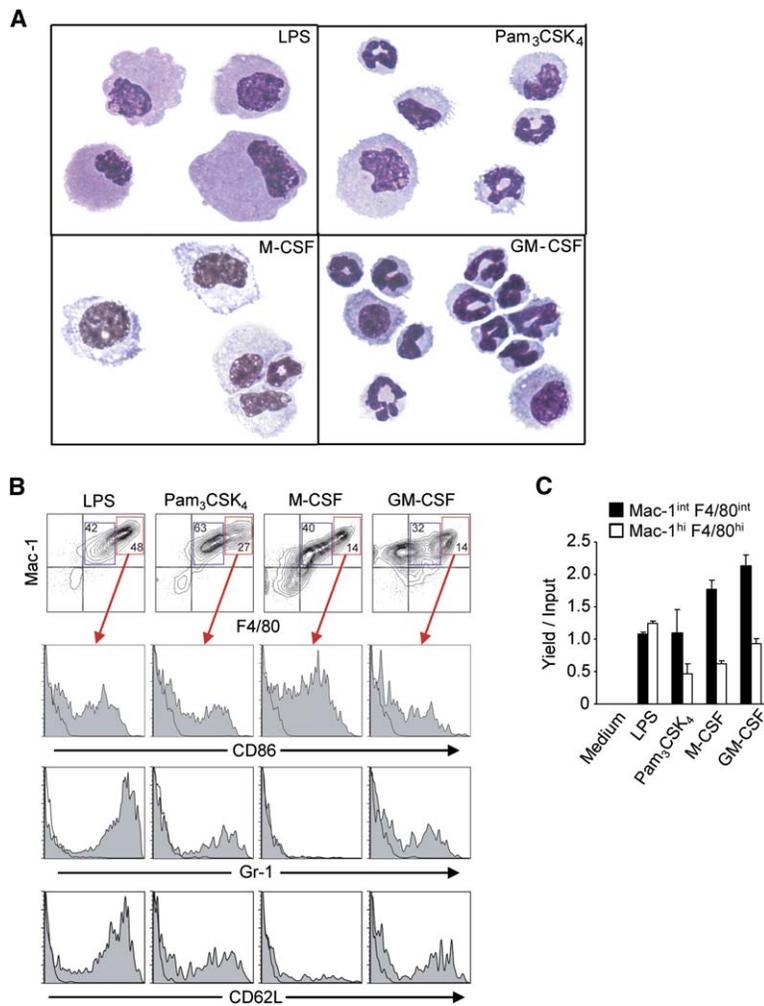


Figure 4. TLR Stimulation Drives Differentiation of GMPs into F4/80^{hi} Monocytes and/or Macrophages

(A) These photomicrographs were prepared with Giemsa-May-Grünwald-stained cyto-centrifuged slides.

(B and C) Sorted GMPs were stimulated with LPS (1 μ g/ml), Pam₃CSK₄ (100 ng/ml), M-CSF, or GM-CSF. After 72 hr in culture, cells were analyzed by flow cytometry for expression of F4/80 and Mac-1. Percentages indicate the frequencies of Mac-1^{lo} F4/80^{lo} or Mac-1^{hi} F4/80^{hi} cells. CD86, Gr-1, or CD62L were analyzed by flow cytometry on Mac-1^{hi} F4/80^{hi} cells. Open histograms depict staining with the appropriate isotype-matched Abs. The results are representative of those obtained in two independent experiments. (C) The bar graphs depict cell yields of Mac-1^{lo} F4/80^{lo} or Mac-1^{hi} F4/80^{hi} cells. The data represent mean values with standard deviations from triplicate cultures and are representative of three independent experiments.

may even be observed in the absence of exogenous growth factors.

Preferential Dendritic Cell Differentiation of Lymphoid Progenitors in Response to TLR Ligands

Under experimental conditions, CLPs can give rise to T, B, NK, and DCs (Kouro et al., 2002; Kondo et al., 1997; Karsunky et al., 2003; Shigematsu et al., 2004). Although essentially pure populations of CD19⁺ B lineage cells were produced from CLPs in serum-free, stromal cell-free culture with cytokines (Figure 5A), only Mac-1⁺ cells emerged when LPS was added. A mixture of the two cell types was found in Pam₃CSK₄-containing cultures. This dramatic change was dependent on Myd88, as only CD19⁺ lymphocytes were produced when CLPs from Myd88-deficient mice were stimulated. Most Mac-1⁺ cells had DC morphology on Giemsa-May-Grünwald-stained slides (Figure 5B). Further analysis of these cultures revealed that almost all of the Mac-1⁺ cells produced from CLPs in response to LPS were CD11c⁺ Gr-1⁻ (Figure 5C). Cells recovered from Pam₃CSK₄-containing cultures were Gr-1⁻, but were less homogenous.

Because CLPs express very little TLR4-MD-2 or CD14 (Figure 1), and the experiments described in Figure 5 were conducted with serum-free medium, exogenous CD14 was added to the cultures to determine if this

would influence efficiency of TLR4-MD-2 signals. The addition of CD14 dramatically augmented DC production by low doses of LPS in serum-free cultures, while it had little effect on Pam₃CSK₄ responses (Figure 5D and data not shown).

It seemed possible that TLR ligation induced production of cytokines that could influence the differentiation patterns we observed. GM-CSF and TNF α were of particular interest because they can promote the production of DCs from bone marrow in vitro (Gilliet et al., 2002), and TNF α can suppress B lymphopoiesis (Sedger et al., 2002). However, we found that neutralizing antibodies to either GM-CSF or TNF α did not inhibit the production of DCs from CLPs induced by TLR ligands (data not shown).

Although CLPs are largely B lineage restricted when held in defined conditions, these progenitors produce DCs in stromal cell cocultures and transplantation assays (Karsunky et al., 2003; Shigematsu et al., 2004). It seemed possible that TLR ligation quickly substitutes for that permissive signal and selects for clones that are not fully B lineage committed. Therefore, CLPs were sorted to high purity and incubated for 24 hr with LPS, Pam₃CSK₄, or medium alone. Each group was then harvested, and single cells were plated on OP9 stromal cells with cytokines to assess differentiation

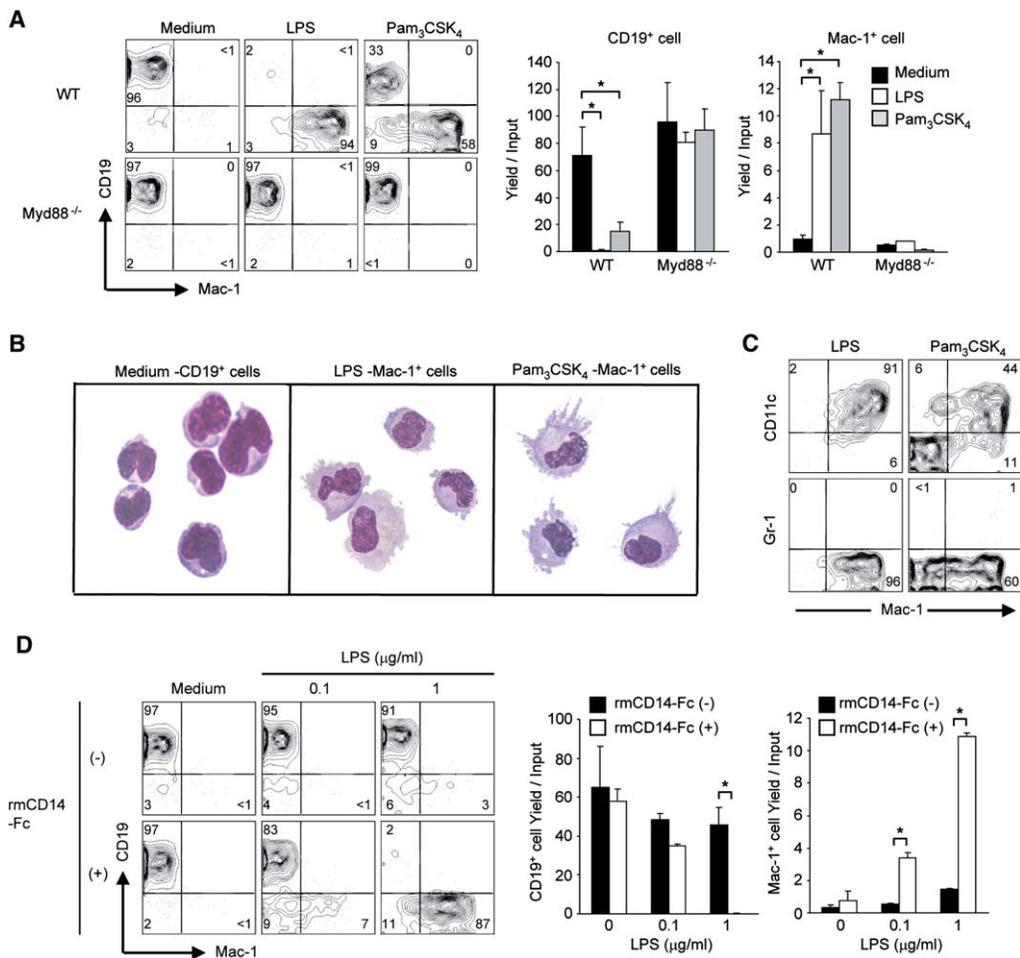


Figure 5. TLR Stimulation Allows Lymphoid-Biased Progenitors to Produce Dendritic Cells at the Expense of B Lymphopoiesis (A) Sorted CLPs (5000/well) from C57BL/6 or Myd88^{-/-} mice were stimulated in X-VIVO15 medium alone, LPS (10 μg/ml), or Pam₃CSK₄ (100 ng/ml) plus SCF, FL, and IL-7. After 7 days in culture, cells were analyzed by flow cytometry for expression of CD19 and Mac-1 (Left). The bar graphs depict cell yields for CD19⁺ cells or Mac-1⁺ cells (right), and the data represent mean values with standard deviations from triplicate cultures (*p < 0.02). The results are representative of those obtained in five independent experiments. (B) Subsets of the recovered cells described in panel (A) were sorted and used to prepare Giemsa-May-Grünwal-stained slides. (C) Cultured cells from C57BL/6 mice were also analyzed by flow cytometry for expression of Gr-1 and CD11c. (D) Sorted CLPs from C57BL/6 mice were cultured in the presence of SCF, FL, and IL-7 with a range of concentrations of LPS or combinations of recombinant mouse CD14-Fc protein (1 μg/ml) plus LPS. After 7 days in culture, cells were analyzed by flow cytometry for expression of CD19 and Mac-1 (left). The bar graph depicts cell yields for CD19⁺ cells or Mac-1⁺ cells (right). Data represent mean values with standard deviations from triplicate cultures (*p < 0.003). The results are representative of three independent experiments.

potential (Figure 6). Four types of clones were detected 10 days later (pure CD19⁺ B lineage, pure CD11c⁺ single positive, CD19⁺ plus CD11c⁺ mixed, or CD11c⁻ CD19⁻ clones). Pure B lineage colonies were the largest in size (data not shown). Most of the CD11c⁺ cells were also Mac-1⁺, and CD11c⁺ single positive DC or mixed colonies were very small (data not shown). Numbers of pure B cell clones consistently declined when LPS was present, while the same treatment increased numbers of pure DC colonies. Pam₃CSK₄ also enhanced production of colonies with pure DC, but did not alter the production of CD19⁺ B cell clones. Thus, the results of these clonal assays concur with those obtained with serum-free, stromal-cell free bulk cultures.

RT-PCR was then used to evaluate CLPs 24 hr after stimulation with TLR ligands (Figure S6). As might be expected with maturing DCs, transcripts corresponding to GM-CSF receptors increased with stimulation. Reduc-

tions were recorded in the EBF, E12, and RAG-1 transcription factors required for lymphopoiesis, in parallel with increases in M-CSF receptor, but changes in three others, PU.1, E47, and Pax-5, were not remarkable. As noted above for stem cells, levels of the C/EBP α transcription factor actually declined after TLR ligation.

These results demonstrate that lymphoid-biased progenitors can be driven to a DC fate by Myd88-dependent TLR signals. Even though CLPs express little TLR4-MD-2, LPS alters their differentiation with sufficient amounts of CD14. TLR signals suppress B lymphopoiesis and stimulate the dendritic potential of lymphoid-biased progenitors.

Exposure to LPS Modulates the TLR4-MD-2 Complex on Hematopoietic Progenitor Cells In Vivo

The above-described results showed that highly purified progenitors can respond to TLR ligands in defined

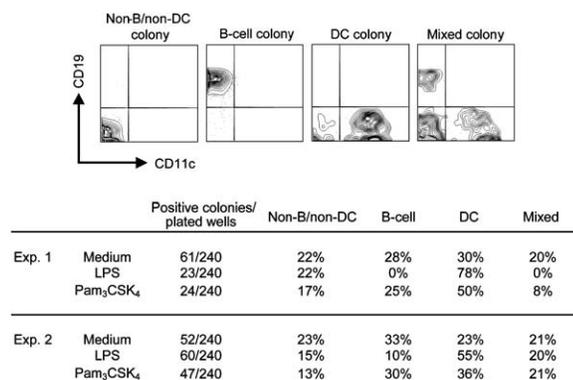


Figure 6. Altered Differentiation Patterns of Single Lymphoid Progenitors Activated via TLRs

Sorted CLPs were cultured in the presence of SCF, FL, and IL-7 with medium alone, LPS (10 μ g/ml), or Pam₃CSK₄ (1 μ g/ml). After 24 hr, cultured cells were harvested and washed three times with medium. Single cultured cells were then sorted and recultured on OP9 stromal cells in 96-well plates for 10 days in the presence of SCF, FL, and IL-7. Positive colonies were examined by flow cytometry (representative examples shown in the top row). The frequencies of wells with each of these differentiation patterns are shown along with the total numbers of clones observed.

culture conditions. It was important to know if these phenomena pertain to progenitors in the bone marrow. LPS is known to cause a very rapid change in the TLR4-MD-2 complex, and this LPS-specific conformational change results in diminished staining with a unique TLR4-MD-2-specific mAb (Akashi et al., 2003). We exploited this phenomenon to ask if LPS can interact directly with hematopoietic progenitors in a physiological setting. First, whole bone marrow cells were stimulated with LPS *in vitro* and then stained with the mAb 1 hr later (Figure 7, left). While untreated bone marrow cells were clearly recognized by this mAb, staining for TLR4-MD-2 was essentially negative after just 1 hr of LPS exposure. We then harvested bone marrow cells from wild-type mice 1 hr after treatment with LPS or PBS (Figure 7, right). Brief *in vivo* exposure to LPS completely abolished TLR4-MD-2 staining on these progenitors.

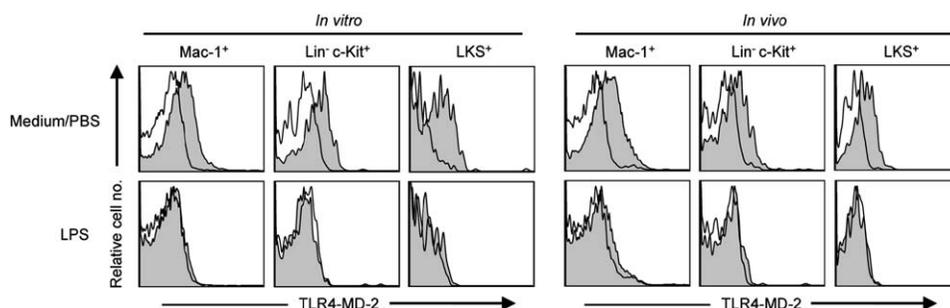


Figure 7. LPS Rapidly Changes the TLR4-MD-2 Complex on Hematopoietic Stem/Progenitors

(Left) Whole bone marrow cells from C57BL/6 mice were cultured with medium alone or LPS (1 μ g/ml) for 1 hr. The cells were then harvested and stained with mAbs to TLR4-MD-2, Mac-1, lineage markers as described in Experimental Procedures, Sca-1, and c-Kit. The MTS510 reagent is unique in detecting a conformation-dependent epitope on TLR4-MD-2 (Akashi et al., 2003). Open histograms depict staining with the isotype-matched Ab for TLR4-MD-2. The results are representative of three independent experiments. (Right) C57BL/6 mice were intravenously or intraperitoneally injected with PBS or 100 μ g LPS from *E. coli*. After 1 hr, mice were sacrificed, and whole bone marrow cells were harvested and stained with mAbs to TLR4-MD-2, Mac-1, lineage markers, Sca-1, and c-Kit. Open histograms depict staining with the isotype-matched Ab for TLR4-MD-2. The results are representative of four independent experiments.

Bone marrow and spleens were then evaluated after LPS injection to determine if this treatment caused perturbations in hematopoietic cells. As has been noted in a previous study (Ueda et al., 2004), LPS depressed numbers of newly formed AA4.1⁺ B220⁺ B lineage lymphocytes in the bone marrow (Figure S7A). Reciprocally, numbers of Mac-1⁺ F4/80⁺ monocytes and/or macrophages increased in the bone marrow and spleen (Figure S7B). Increases were also recorded in the numbers of Mac-1⁺ CD11c⁺ DCs in spleen (Figure S7C).

We conclude that hematopoietic progenitor cells within bone marrow are directly and quickly affected by exposure to this TLR ligand. Changes in hematopoietic cells *in vivo* resemble the patterns of differentiation seen under highly defined conditions of culture.

Discussion

A principal finding is that HSCs and some of their derivative-committed progenitors display functional TLR2 and TLR4-MD-2. The TLR4-MD-2 complex cooperates with CD14 at the cell surface, and optimal responses to LPS or a synthetic lipopeptide require Myd88. TLR ligation obviated some of the normal differentiation cues and stimulated progenitors to become monocytes and/or macrophages with inflammatory phenotypes. Additionally, lymphoid-biased progenitors were directed to a DC fate in response to TLR signals. Members of the TLR family may allow hematopoietic stem/progenitors to directly sense microbial and viral products and alter normal differentiation patterns. When integrated with signals from cytokines and chemokines, this mechanism could provide a means for boosting the innate immune system during life-threatening infection.

A key question is how TLR signals stimulate hematopoietic cells to adopt a particular fate and diminish the need for some cytokines. Progenitors from Myd88-deficient mice were unresponsive to two TLR ligands, so at least some of the previously described intracellular TLR signaling pathways are used. The Myd88-independent TRIF pathway, which contributes to production of type I IFN (Yamamoto et al., 2003; Oshiumi et al., 2003), might not be important to the responses we observed. TLR

stimulation of stem cells depressed SCL and GATA2 transcripts while only slightly changing PU.1, but we were surprised to find declines in the C/EBP α transcription factor that can drive macrophage differentiation (Rosmarin et al., 2005; Xie et al., 2004). Many of the other transcription factor changes were consistent with increased myeloid differentiation. We considered the possibility that TLR ligands might stimulate hematopoietic cells to make their own factors. However, addition of neutralizing antibodies to M-CSF, GM-CSF, or TNF α provided no evidence for autocrine stimulation. Additional experiments are required to exclude the potential contribution of other cytokines.

The results of stromal cell cocultures initiated with a single CLP extend those obtained in bulk, stromal cell-free cultures and would be consistent with either of two possible interpretations. TLR ligands could suppress individual progenitors that are inherently biased for lymphopoiesis while simultaneously stimulating those destined to produce DCs. Alternatively, single progenitors could be “reprogrammed” to adopt different fates as a result of TLR stimulation (Xie et al., 2004).

HSCs divide infrequently under normal circumstances, and retention of their unique potential for self-renewal may require residence in specialized niches near the endosteal surface (Zhang et al., 2003). Such an environment could protect the majority of stem cells from systemic events that would have potentially deleterious effects. An additional expectation is that stem cells express only those receptors needed to maintain their quiescence or allow them to actively differentiate as needed to replenish blood cell populations. Therefore, finding that functional TLRs and associated molecules are present at the stem cell stage was unexpected and raises the possibility that TLR ligands influence numbers and characteristics of stem cells in treated animals. Repeated infections could theoretically exhaust the self-renewal potential or have other long-term consequences for the stem cell pool. Consequently, protective mechanisms may have evolved to block this response. Several negative regulators for TLR signaling have been reported that might work in a cell type-dependent manner (Liew et al., 2005). Furthermore, the RP105-MD-1 complex can be a negative regulator of TLR4 signaling on macrophages (Divanovic et al., 2005). It will therefore be important to learn if signals delivered via inhibitory molecules influence stem cell behavior. On the other hand, recognition that functional TLRs are expressed on progenitors may suggest new ways to manipulate their activity for therapeutic purposes.

Curiously, HSCs are present in the circulation as well as tissues such as skeletal muscle (Kawada and Ogawa, 2001). Since almost all blood cell formation occurs in bone marrow, the biological significance of this distribution is unclear. It is attractive to consider that dispersed stem cells sense foreign materials and become locally converted to innate effector cells.

It is difficult to use surface TLR expression to predict the efficiency of responsiveness to particular TLR ligands because many TLRs are intracellular, and coreceptor components could influence the avidity of receptor-ligand interactions (Matsumoto et al., 2003; Latz et al., 2004; Takeuchi et al., 2001, 2002). Despite high-level display of TLR2, responses of hematopoietic pro-

genitors to the TLR2 ligand lipopeptide were often weaker than those to LPS. Responses of LKS and lymphoid progenitors to LPS in serum-free cultures were significantly augmented by provision of soluble CD14, but there was much less influence on lipopeptide responses. It will be important to study additional TLR ligands in the same way and learn if coreceptors can modulate the nature or magnitude of differentiation changes.

There is a huge body of literature concerning physiological responses to bacterial components (Beutler and Rietschel, 2003; Taylor, 2001). Multiple cell types respond, various cell types are redistributed in the body, cytokines are released, and the coagulation system is activated. Dissecting discrete stimulatory pathways and mechanisms is extremely difficult in those circumstances. However, mobilization and redistribution of hematopoietic cells in response to inflammatory processes has been known for some time (Ueda et al., 2004, 2005). In our experiments, LPS injections depleted B lineage lymphocytes from bone marrow, as previously described by others (Ueda et al., 2004), and there were corresponding increases in macrophages and DCs. Multiple mechanisms may account for these changes, but they are consistent with the responses of highly purified progenitors in defined culture conditions. The TLR4-MD-2 complex undergoes a rapid change upon interaction with LPS such that staining with the MTS510 mAb is abolished (Akashi et al., 2003). We exploited this response to learn that injected LPS rapidly diffuses into the bone marrow cavity and engages the receptors of stem cells and progenitors. Thus, our findings suggest that rapid interactions between foreign substances and hematopoietic progenitors could occur in bone marrow. Signals delivered via that mechanism would be in addition to, and in context with, those associated with cytokines and chemokines.

Our findings suggest that stem cells and progenitors need to be investigated from the perspective of disease-related processes. As just one example, full maturation of osteoclast progenitors is known to be altered by ligation of TLRs on hematopoietic cells (Sato et al., 2004; Hayashi et al., 2003). An imbalance of osteoclast versus osteoblast activity and abnormal bone density might result from chronic microbial and viral infections. A report has just appeared that shows that human CD34⁺ cord blood cells express functional TLR9 (Kim et al., 2005). It is therefore important to learn if TLR-expressing hematopoietic cells are involved in pathogenic mechanisms.

In addition to the importance of TLRs in sensing pathogen-associated molecular patterns, a number of endogenous TLR ligands have been described (Tsan and Gao, 2004). Experimental TLR-dependent responses to these substances might have been due to trace contamination with bacterial products. However, the innate immune system could use this mechanism to sense danger represented by inflamed or remodeled tissues (Seong and Matzinger, 2004). There are no published descriptions of hematopoietic maturational defects in TLR gene targeted mice. This could reflect functional redundancy among TLRs or indicate that hematopoietic cells use these receptors only in response to infections.

The Toll receptor was first discovered because of its importance to the establishment of dorsoventral polarity

in *Drosophila* embryos (Ferrandon et al., 2004). While the best-known functions of TLRs in higher species involve mature cells, our results indicate that self/non-self discrimination may begin at the level of HSCs and progenitors. TLR signals could help to activate quiescent stem cells and influence the types of cells they produce. This may augment the capability for rapidly replenishing the innate immune system during infection.

Experimental Procedures

Mice

C57BL/6 and Myd88^{-/-} mice were used at 8–10 weeks of age. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Myd88^{-/-} mice were maintained in our laboratory animal resource facility (LARC) at the Oklahoma Medical Research Foundation.

Reagents

Recombinant mouse IL-7, stem cell factor (SCF), Flt-3 ligand (FL), M-CSF, GM-CSF, recombinant mouse CD14/Fc chimera protein, and recombinant human CD14 were purchased from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO). A synthetic lipopeptide, Pam₃CSK₄, was purchased from InvivoGen (San Diego, CA).

Antibodies and Flow Cytometry

The following antibodies for flow cytometry were purchased from eBioscience (San Diego, CA): biotinylated anti-TLR2 (clone GC2), biotinylated anti-TLR4-MD-2 (clone MTS510), biotinylated anti-RP105 (clone RP14), biotinylated anti-MD-1 (clone MD113), biotinylated anti-CD14 (clone Sa2-8), PE-conjugated anti-CD62L (clone MEL-14), allophycocyanin (APC)-conjugated anti-F4/80 (clone BM8), anti-c-Kit (clone ACK2), phycoerythrin (PE)-Cy5-conjugated anti-Sca-1 (clone D7), biotinylated anti-Flk-2 (clone A2F10), purified anti-mouse CD115 (CSF-1R, clone AFS98), purified anti-mouse GM-CSF (clone MP1-22E9), and purified anti-mouse TNF α (clone MP6-XT22).

The following antibodies for flow cytometry were purchased from BD Pharmingen (San Diego, CA): FITC-conjugated anti-CD2 (clone RM2-5), anti-CD3 ϵ (clone 145-2C11), anti-CD8 α (clone 53-6.7), anti-CD45R (B220; clone RA3-6B2), anti-Ly6G (clone RB6-8C5), anti-CD11b/Mac-1 (clone M1/70), anti-TER119, anti-CD34 (clone RAM34), anti-CD11c (clone HL3), PE-conjugated IL-7R α (clone SB/119), anti-CD19 (clone 1D3), anti-Ly6G (clone RB6-8C5), anti-CD11c (clone HL3), anti-Fc γ R2/3 (clone 2.4G2), anti-AA4.1, anti-CD86 (clone GL1), APC-conjugated anti-CD45R (clone RA3-6B2), anti-CD11b/Mac-1 (clone M1/70), biotin-conjugated anti-VCAM-1 (clone 429, MVCAM.A), PE-conjugated streptavidin, and PE Texas-Red-conjugated streptavidin. For analyzing cultured cells by flow cytometry, 7-AAD (BD Pharmingen) was always used to exclude dead cells.

Flow cytometry analyses were conducted on a FACSCan, FACS-Calibur, or FACSAria (Becton Dickinson & Co., Mountain View, CA), and the data were analyzed with FlowJo software (Treestar, San Carlos, CA).

Establishment of the Anti-Mouse TLR4 Monoclonal Antibody, Clone UT49

A full description of the preparation and validation of this reagent will be published elsewhere. Briefly, TLR4-deficient mice were intraperitoneally injected four times at 1 week intervals with 1×10^7 Ba/F3 cells expressing mouse TLR4 and mouse MD-2. Three days after the last injection, mice were euthanized and spleens were removed. Spleen cells were dispersed and fused with SP2/O cells by using a standard fusion protocol with polyethylene glycol 1500 (Roche, Basel, Switzerland). Hybridoma cells were selected in hypoxanthine/aminopterin/thymidine medium and screened by flow cytometry with Ba/F3 cells expressing mouse TLR4-MD-2 complex and parent Ba/F3 cells. Biotin-conjugated antibody was used for flow cytometry.

Isolation of Hematopoietic Stem and Progenitor Cells

Bone marrow cells were harvested and enriched for lineage-negative cells by incubation with antibody to CD11b/Mac-1 (clone M1/70), anti-Ly6G (clone RB6-8C5), anti-CD45R (B220; clone RA3-6B2), anti-CD19 (clone 1D3), and anti-TER119, followed by negative selection with the MACS cell separation system (Miltenyi Biotec, Auburn, CA). For sorting of Flk-2⁻ or Flk-2⁺ LKS⁺ (Lin⁻ IL-7R α ⁻ c-Kit^{hi} Sca-1⁺), LKS⁻ (Lin⁻ IL-7R α ⁻ c-Kit^{hi} Sca-1⁻), and CLP (Lin⁻ IL-7R α ⁺ c-Kit^{lo} Sca-1^{lo}), these partially lineage-depleted cells were further stained with FITC-conjugated lineage markers: anti-CD2, anti-CD3 ϵ , anti-CD8 α , anti-CD45R, anti-Ly6G, anti-CD11b/Mac-1, anti-TER119, PE-conjugated IL-7R α , APC-conjugated anti-c-Kit, PE-Cy5-conjugated anti-Sca-1, and biotinylated anti-Flk-2 combined with PE Texas-Red-conjugated streptavidin. For sorting of CMP (Lin⁻ IL-7R α ⁻ c-Kit^{hi} Sca-1⁻ CD34⁺ Fc γ R2/3^{lo}), GMP (Lin⁻ IL-7R α ⁻ c-Kit^{hi} Sca-1⁻ CD34⁺ Fc γ R2/3^{hi}), and MEP (Lin⁻ IL-7R α ⁻ c-Kit^{hi} Sca-1⁻ CD34⁻ Fc γ R2/3^{lo}), the sorted LKS⁻ cells were further stained with FITC-conjugated anti-CD34 and PE-conjugated anti-Fc γ R2/3. Cells were sorted on a FACSAria (Becton Dickinson & Co.) The sort gates and the post-sort analyses are illustrated in Method 1 in the Supplemental Data.

Serum-free, Stromal Cell-free Cell Cultures

Round-bottomed, 96-well plates or flat-bottomed, 24-well plates (Corning, Inc.) were used for these cultures. Sorted cells were cultured with X-VIVO15 medium (Biowhittaker, Walkersville, MD) or StemPro-34 SFM medium (Invitrogen, Carlsbad, CA). X-VIVO15 medium, which seemed to be optimal for lymphoid cultures, contained 1% detoxified bovine serum albumin (Stem Cell Technologies, Vancouver, Canada), 5×10^{-5} M 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. StemPro-34 SFM medium containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin was usually used for myeloid cultures. The concentrations of cytokines were IL-7, 1 ng/ml; FL, 100 ng/ml; SCF, 20 ng/ml, M-CSF, 20 ng/ml; GM-CSF, 20 ng/ml. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Single Cell Culture on OP9 Stromal Cells

Sorted CLPs were cultured with X-VIVO15 in the presence of SCF (20 ng/ml), FL (100 ng/ml), and IL-7 (1 ng/ml) with or without LPS (10 μ g/ml) or Pam₃CSK₄ (1 μ g/ml). After 24 hr, cultured cells were harvested and washed with medium three times. A single cell sorting for the cultured cells was then conducted on a FACSAria, and a single cell was plated on OP9 stromal cells in a 96-well plate and cultured for 10 days in the presence of SCF (20 ng/ml), FL (100 ng/ml), and IL-7 (1 ng/ml). The conditioning medium for OP9 was MEM α medium (Invitrogen), containing 20% FCS, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Positive wells (more than 30 cells) were determined by microscopic observation. Cells were harvested and then stained with mAbs to CD19, CD11c, and Mac-1 and were analyzed by flow cytometry. Cells were also stained with a mAb to VCAM-1 to exclude stromal cells. The OP9 stromal cell line was kindly provided by Dr. Shin-ichi Hayashi (Tottori University, Japan).

In Vitro BrdU Incorporation

Sorted Flk-2⁻ LKS⁺ cells (10,000 cells/well) were cultured with or without LPS (10 μ g/ml) or Pam₃CSK₄ (1 μ g/ml) in the presence of SCF (20 ng/ml) for 50 hr and were pulsed with 10 μ M 5-bromo-2'-deoxyuridine (BrdU) for the final 18 hr. The cells were then stained with anti-BrdU. A BrdU flow kit was purchased from BD Pharmingen. Analyses by flow cytometry were conducted on a FACSCan.

Interaction of LPS with the TLR4-MD-2 Complex

Whole bone marrow cells from C57BL/6 mice were cultured in RPMI1640 medium (Mediatech, Inc., Herndon, VA) containing 10% FCS, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin and were stimulated with or without 1 μ g/ml LPS for 1 hr. Then, cells were harvested and stained with mAbs to TLR4-MD-2, Mac-1, lineage markers (CD2, CD3 ϵ , CD8 α , CD45R/B220, CD11b/Mac-1, Ly6G, TER119), Sca-1, and c-Kit. Alternatively, C57BL/6 mice were intravenously or intraperitoneally injected with PBS or 100 μ g LPS. After 1 hr, whole bone marrow cells were harvested from femurs and tibiae and stained with mAbs to

TLR4-MD-2, Mac-1, lineage markers, Sca-1, and c-Kit. The MTS 10 reagent is unique in detecting a conformation-dependent epitope on TLR4-MD-2 (Akashi et al., 2003).

Cell Morphology

Cytospins of cultured progenitor cells were stained with Giemsa-May-Grünwald (Sigma Diagnostics, St. Louis, MO), mounted in Immersol (Zeiss, Thornwood, NY), and analyzed in the OMRFs Biological Imaging Facility on a Zeiss Axioplan 2i microscope with a 100×/1.4 NA Plan Achromat objective. Photomicrographs were made with an AxioCam MRc color camera (Zeiss) and AxioVision 3.1 software (Zeiss).

Semiquantitative RT-PCR Analysis of Gene Expression

The mRNAs were isolated from sorted cells by using MicroPoly(A) Pure (Ambion, Austin, TX). The cDNAs were then prepared from DNase I-treated mRNA by using oligo-dT and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR reactions were conducted in buffer containing 200 μM dATP, dGTP, dTTP; 100 μM dCTP; and 0.5 μCi [α -³²P]dCTP. Aliquots were removed at cycles 25, 28, and 31 for β -actin and cycles 32, 35, and 38 for all others to insure that PCR remained within the exponential range of amplifications. Aliquots (5 μl) were denatured in a formamide-loading buffer and applied to a 6% polyacrylamide gel containing 7 M urea. Incorporation of [α -³²P] dCTP into PCR product bands was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The primer sequences for each gene are given in Method 2 in the Supplemental Data.

Statistical Analysis

The statistical significance of differences between group means was determined with the Student's t test. p values less than 0.05 were considered significant.

Supplemental Data

Supplemental Data include seven additional figures, Method 1, and Method 2 and are available at <http://www.immunity.com/cgi/content/full/24/6/801/DC1/>.

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