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

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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 co-receptors were expressed by multipotential hematopoietic stem cells, whose cell cycle entry was triggered by TLR ligation. TLR expression extended also 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 lymphocytes (Iwasaki and Medzhitov, 2004). Cells responsible for both innate and adaptive immunity have finite lifespans 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)

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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 pro-lymphocytes or common lymphoid progenitors (CLPs) that can then become pre-B cells (Igarashi et al., 2002, Kouro et al., 2002 and 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/EBPα and PU.1 (Henderson and Calame, 1998 and Rosmarin et al., 2005).

There is considerable controversy concerning the plasticity of stem 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, and Xie et al., 2004). Although there has been no physiological basis for this phenomenon, it is interesting that plasmacytoid DCs can convert 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 and Means et al., 1999). In addition, TLRs require intracellular adaptor proteins for effective signaling. All TLRs except for TLR3 use the 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 and Oshiumi et al., 2003).

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 co-stimulatory 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 and Ueda et al., 2005) and maturation of osteoclasts is altered by TLR ligands (Sato et al., 2004 and Hayashi et al., 2003). Furthermore, although the Toll pathway in *Drosophila* has a developmental role in determining dorso-ventral 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 dendritic cells. 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 co-receptors on bone marrow cells by flow cytometry. An initial finding was that HSCs and early progenitor 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 (Supplementary Figure 1A).

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 as described by Kondo and colleagues (Kondo et al., 2003, Supplementary Method 1). The HSC enriched Lin^- IL-7Rα^- c-Kit^{hi} Sca-1^+ fraction (hereafter referred to as LKS^+) had uniformly high expression of TLR2, and the same was true for Lin^- IL-7Rα^+ c-Kit^{lo} Sca-1^{lo}, pro-lymphocytes or common lymphoid progenitors (hereafter referred to as CLPs). Although this was also the case for the Lin^- IL-7Rα^- c-Kit^{hi} Sca-1^- (LKS^-) fraction, $CD34^-$ FcγR^{lo} megakaryocyte and erythrocyte progenitors (MEPs) within the LKS^- subset had very little TLR2 (Figure 1A). The Lin^- IL-7Rα^- c-Kit^{hi} Sca-1^- $CD34^+$ FcγR^{hi} granulocyte and macrophage progenitors (GMPs) subset was uniformly $TLR2^+$ and Lin^- IL-7Rα^- c-Kit^{hi} Sca-1^- $CD34^+$ FcγR^{lo} common myeloid progenitors (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 HSCs 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 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 Flk-2^- subset of the LKS^+ fraction (Christensen and Weissman, 2002). Interestingly, this population had the highest expression of TLR2, TLR4, and MD-2 mRNA, but little CD14. By flow cytometry analysis, Flk-2^- and Flk- 2^+ subsets expressed similar densities of cell surface TLR2, TLR4-MD-2, and CD14 (Supplementary Figure 1B).

The analysis was extended by staining progenitors with antibodies to RP105 and MD-1 (Supplemental Figure 2A, B). 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 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 Pam_3CSK_4, 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 and Supplementary Figure 3A). Absolute numbers of Lin^+ cells increased approximately eight 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 increased dramatically with time (Figure 2C). In parallel with these changes, substantial numbers of LKS^+ bone marrow cells acquired the FcγR in response to LPS or Pam_3CSK_4 exposure within 24 hr (Supplementary Figure 3B).

The dose of LPS required to stimulate hematopoietic cells under serum-free conditions was high, and we found minimal responses at concentrations below 10 μg/ml (Supplementary Figure 3C). Serum is known to contain soluble CD14 that enhances LPS and TLR2 ligand recognition by mature cells (Yoshimura et al., 1999 and 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 (Supplementary Figure 3C and D). That is, we recorded responses to as little as 0.1 μg/ml LPS and found co-expression of Mac-1 and F4/80 within 72 hr (Supplementary Figure 3D). 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 Pam_3CSK_4 (Supplementary Figure 3C).

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 less dramatically than multipotent Flk-2^+ progenitors (Supplementary Figure 3E). BrdU labeling experiments showed that increased proportions of cells stimulated with TLR ligands entered cell cycle (Figure 2E). Thus, the quiescent long-term repopulating HSC subset could be driven into cycle by TLR ligation.

A preliminary RT-PCR analysis was performed with the same experimental design to verify TLR ligand induced differentiation (Supplementary Figure 4). Transcripts for the M-CSF receptor (c-fms) increased on 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, the C/EBPα transcription factor that can drive macrophage differentiation (Rosmarin et al., 2005 and Xie et al., 2004) declined with TLR stimulation (Supplementary Figure 4).

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 findings show that highly enriched hematopoietic stem cells 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 (Figure 3A, B). In some experiments, yields of $F4/80^+$ cells obtained with LPS were nearly equal to those derived with CSF stimulation, whereas Pam_3CSK_4 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 (Supplementary Figure 5), 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 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 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 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 following 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, where typical foamy macrophages or neutrophils predominated, macrophage-like cells in LPS containing cultures had more basophilic cytoplasm with small granules. Cells recovered from Pam_3CSK_4 stimulated cultures were less homogeneous in appearance (Figure 4A). Virtually all cells produced in response to LPS or Pam_3CSK_4 expressed the Mac-1 marker, and the staining intensities tended to correlate with densities of F4/80 (Figure 4B). A conspicuous subset of $Mac\text{-}1^+$ $F4/80^-$ cells seen in response to GM-CSF was not present in TLR ligand stimulated cultures. LPS was particularly efficient in driving production of $Mac\text{-}1^{hi}$ $F4/80^{hi}$ cells (Figure 4C). Previous studies showed that inflammatory $Mac\text{-}1^+$ $F4/80^+$ monocytes preferentially expressed Gr-1 and CD62L (Geissmann et al., 2003). This inflammatory phenotype was observed for the $Mac\text{-}1^{hi}$ $F4/80^{hi}$ cells derived by TLR ligand or GM-CSF stimulation, but not for the M-CSF stimulated cells (Figure 4B). The co-stimulatory 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 hours and none from any of the other cultures co-expressed the Mac-1 and CD11c markers typical of dendritic cells (data not shown). These results indicate that TLR stimulation induces committed myeloid progenitors to differentiate into $Mac\text{-}1^{hi}$ $F4/80^{hi}$

monocytes and/or macrophages with inflammatory characteristics. The phenomenon 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 and 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 $Mac\text{-}1^+$ cells emerged when LPS was added. A mixture of the two cell types was found in Pam_3CSK_4 containing cultures. This dramatic change was dependent on Myd88 as only $CD19^+$ lymphocytes were produced when CLPs from Myd88-deficient mice were stimulated. Most $Mac\text{-}1^+$ cells had DC morphology on Giemsa-May-Grünwald stained slides (Figure 5B). Further analysis of these cultures revealed that almost all of the $Mac\text{-}1^+$ cells produced from CLPs in response to LPS were $CD11c^+$ $Gr\text{-}1^-$ (Figure 5C). Cells recovered from Pam_3CSK_4 containing cultures were $Gr\text{-}1^-$ but 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 having little effect on Pam_3CSK_4 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 TNFα were of particular interest because they can promote the production of DCs from bone marrow *in vitro* (Gilliet et al., 2002) and TNFα can suppress B-lymphopoiesis (Sedger et al., 2002). However, we found that neutralizing antibodies to either GM-CSF or TNFα 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 co-cultures and transplantation assays (Karsunky et al., 2003 and 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, Pam_3CSK_4 or medium alone. Each group was then harvested, and single cells were plated on OP9 stromal cells with cytokines to assess differentiation potential (Figure 6). Four types of clones were detected ten 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 $Mac\text{-}1^+$ 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. Pam_3CSK_4 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 (Supplementary Figure 6). As might be expected with maturing DCs, transcripts corresponding to GM-CSF receptors increased with stimulation. Reductions 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, the C/EBPα 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 results showed that highly purified progenitors can respond to TLR ligands in defined 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.

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 (Supplemental Figure 7A). Reciprocally, numbers of $Mac\text{-}1^+$ $F4/80^+$ monocytes and/or macrophages increased in the bone marrow and spleen (Supplemental Figure 7B). Increases were also recorded in numbers of $Mac\text{-}1^+$ $CD11c^+$ DCs in spleen (Supplemental Figure 7C).

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 hematopoietic stem cells 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 new 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 and 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/EBPα transcription factor that can drive macrophage differentiation (Rosmarin et al.,

2005 and 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 TNFα provided no evidence for autocrine stimulation. Further experiments are required to exclude the potential contribution of other cytokines.

The results of stromal cell co-cultures initiated with 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).

Hematopoietic stem cells 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 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, hematopoietic stem cells 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 TLR are intracellular and coreceptor components could influence avidity of receptor-ligand interactions (Matsumoto et al., 2003, Latz et al., 2004, Takeuchi et al., 2001, and Takeuchi et al., 2002). Despite high level display of TLR2, responses of hematopoietic progenitors 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 co-receptors 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 and Taylor, 2001). Multiple cell types respond, various cell types are re-distributed 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 re-distribution of hematopoietic cells in response to inflammatory processes has been known for some time (Ueda et al., 2004 and Ueda et al.,

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 on 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 new 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 and 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 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 pathogenetic 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 establishment of dorso-ventral 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 hematopoietic stem cells and progenitors. TLR signals could help to activate quiescent stem cells and influence the types of cells they produce. This may augment 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 Pam_3CSK_4 was purchased from InvivoGen (San Diego, CA).

Antibodies and flow cytometry

The following Abs for flow cytometry were purchased from eBioscience (San Diego, CA): biotinylated anti-TLR4-MD-2 (clone MTS510), biotinylated anti-RP105 (clone RP/14), 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 TNFα (clone MP6-XT22).

The following Abs for flow cytometry were purchased from BD Pharmingen (San Diego, CA): FITC-conjugated anti-CD2 (clone RM2-5), anti-CD3ε (clone 145-2C11), anti-CD8α (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-7Rα (clone SB/119), anti-CD19 (clone 1D3), anti-Ly6G (clone RB6-8C5), anti-CD11c (clone HL3), anti-FcγR2/3 (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 was conducted on a FACSCan™, FACSCalibur™ 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 a week intervals with $1x10^7$ 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 using a standard fusion protocol with polyethylene glycol 1500 (Roche, Basel, Switzerland). Hybridoma cells were selected in hypoxanthine/aminopterine/thymidine medium and screened by flow cytometry with Ba/F3 cells expressing mouse TLR4-MD-2 complex and parent Ba/F3 cells. Biotin-conjugated this 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 using the MACS cell separation system (Miltenyi Biotec, Auburn, CA). For sorting of Flk-2$^-$ or Flk-2$^+$ LKS$^+$ (Lin$^-$ IL-7Rα$^-$ c-Kit^{hi} Sca-1$^+$), LKS$^-$(Lin$^-$ IL-7Rα$^-$ c-Kit^{hi} Sca-1$^-$) and CLP (Lin$^-$ IL-7Rα$^+$ c-Kit^{lo} Sca-1^{lo}), these partially lineage-depleted cells were further stained with FITC-conjugated lineage markers; anti-CD2, anti-CD3ε, anti-CD8α, anti-CD45R, anti-Ly6G, anti-CD11b/Mac-1, anti-TER119, PE-conjugated IL-7Rα, 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-7Rα$^-$ c-Kit^{hi} Sca-1$^-$ CD34$^+$ FcγR2/3^{lo}), GMP (Lin$^-$ IL-7Rα$^-$ c-Kit^{hi} Sca-1$^-$ CD34$^+$ FcγR2/3^{hi}) and MEP (Lin$^-$ IL-7Rα$^-$ c-Kit^{hi} Sca-1$^-$ CD34$^-$ FcγR2/3^{lo}), the sorted LKS$^-$ cells were further stained with FITC-conjugated anti-CD34 and PE-conjugated anti-FcγR2/3. Cells were sorted on a FACSAria™ (Becton Dickinson & Co.) The sort gates and the post sort analyses are illustrated in Supplementary Method 1.

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, that 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% CO_2 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 μg/ml), or Pam_3CSK_4 (1 μg/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 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 MEMα 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 analyzed by flow cytometry. Cells were also stained with 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 μg/ml) or Pam_3CSK_4 (1 μg/ml) in the presence of SCF (20 ng/ml) for 50 hr, pulsing with 10 μM 5-bromo-2'-deoxyuridine (BrdU) for the final 18 hr. The cells were then stained with anti-BrdU. 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 stimulated with or without 1 μg/ml LPS for 1 hr. Then cells were harvested and stained with mAbs to TLR4-MD-2, Mac-1, lineage markers (CD2, CD3e, CD8a, 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 μg 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 OMRFs Biological Imaging Facility on a Zeiss Axioplan 2i microscope with a 100x/1.4 NA Plan Achromat objective. Photomicrographs were made using an AxioCam MRc color camera (Zeiss), and AxioVision 3.1 software (Zeiss).

Semi-quantitative RT-PCR analysis of gene expression

The mRNAs were isolated from sorted cells using MicroPoly(A) Pure (Ambion, Austin, TX). The cDNAs were then prepared from DNase I-treated mRNA 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 ^{32}P] dCTP. Aliquots were removed at cycle 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. Five micro liter aliquots were denatured in a formamide-loading buffer and applied to a 6 % polyacrylamide gel containing 7 M urea. Incorporation of [α ^{32}P] dCTP into PCR product bands was quantified by PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The primer sequences for each gene are given in Supplementary Method 2.

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.

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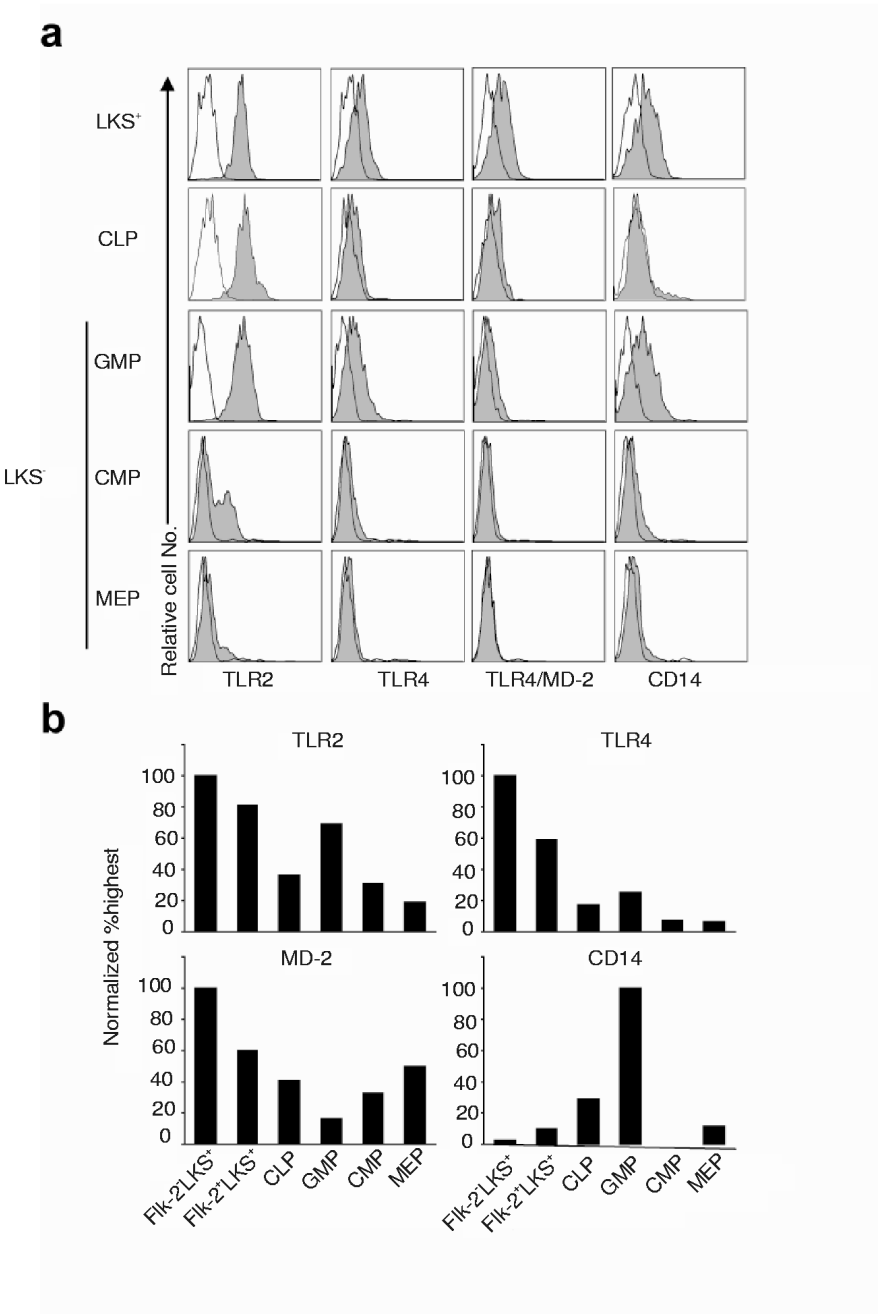


Figure 1.

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 staining 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 representative of two independent experiments.

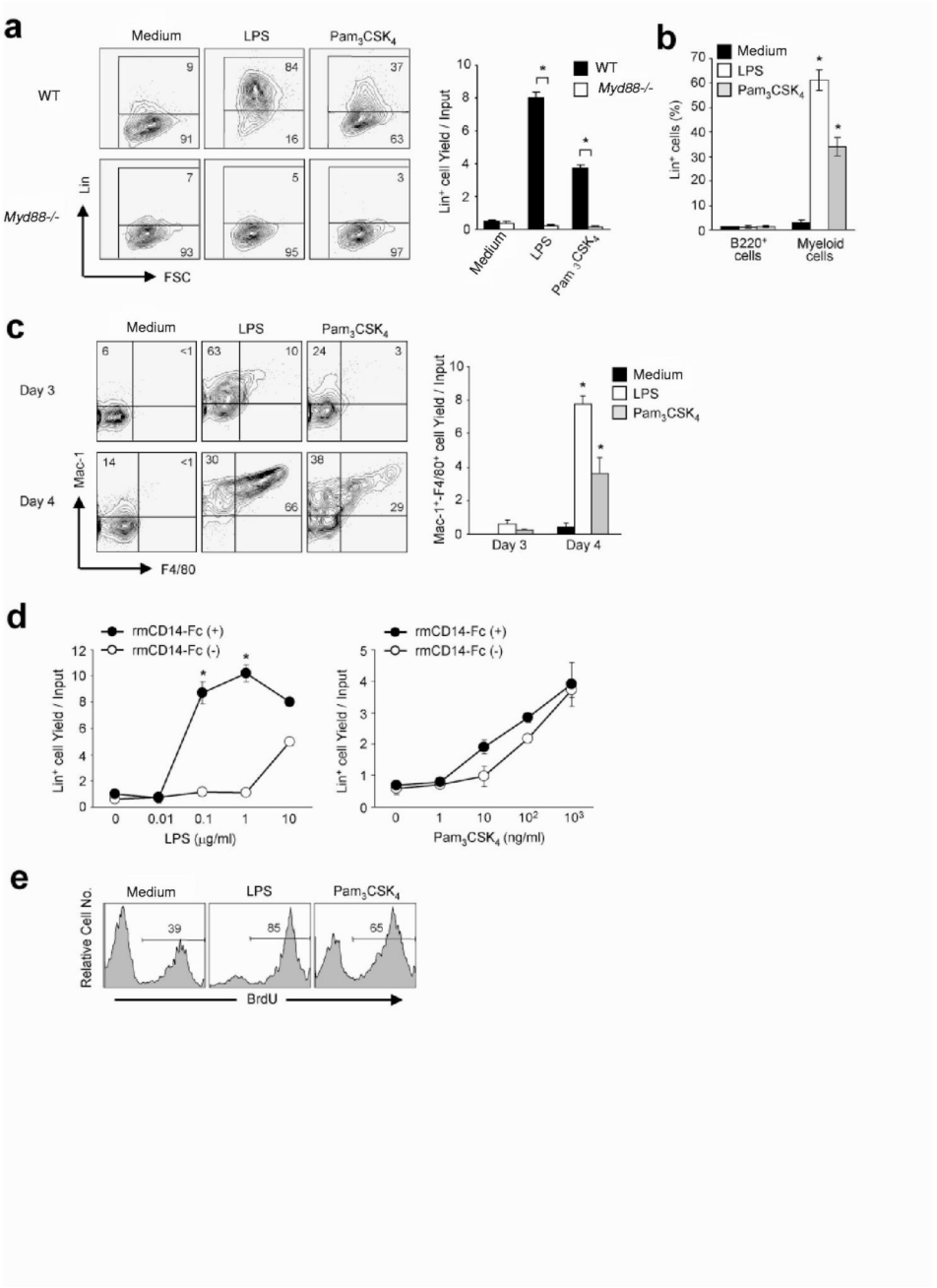


Figure 2.

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 Pam_3CSK_4 (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$^+$ cell 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 1,600, 5,000, or 7,500 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 Pam_3CSK_4 (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 $Flk\text{-}2^{-}$ LKS^{+} (10,000 cells/well) were cultured in the presence of SCF with medium alone, LPS (10 μg/ml) or Pam_3CSK_4 (1 μg/ml) for 50 hr, pulsing 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.

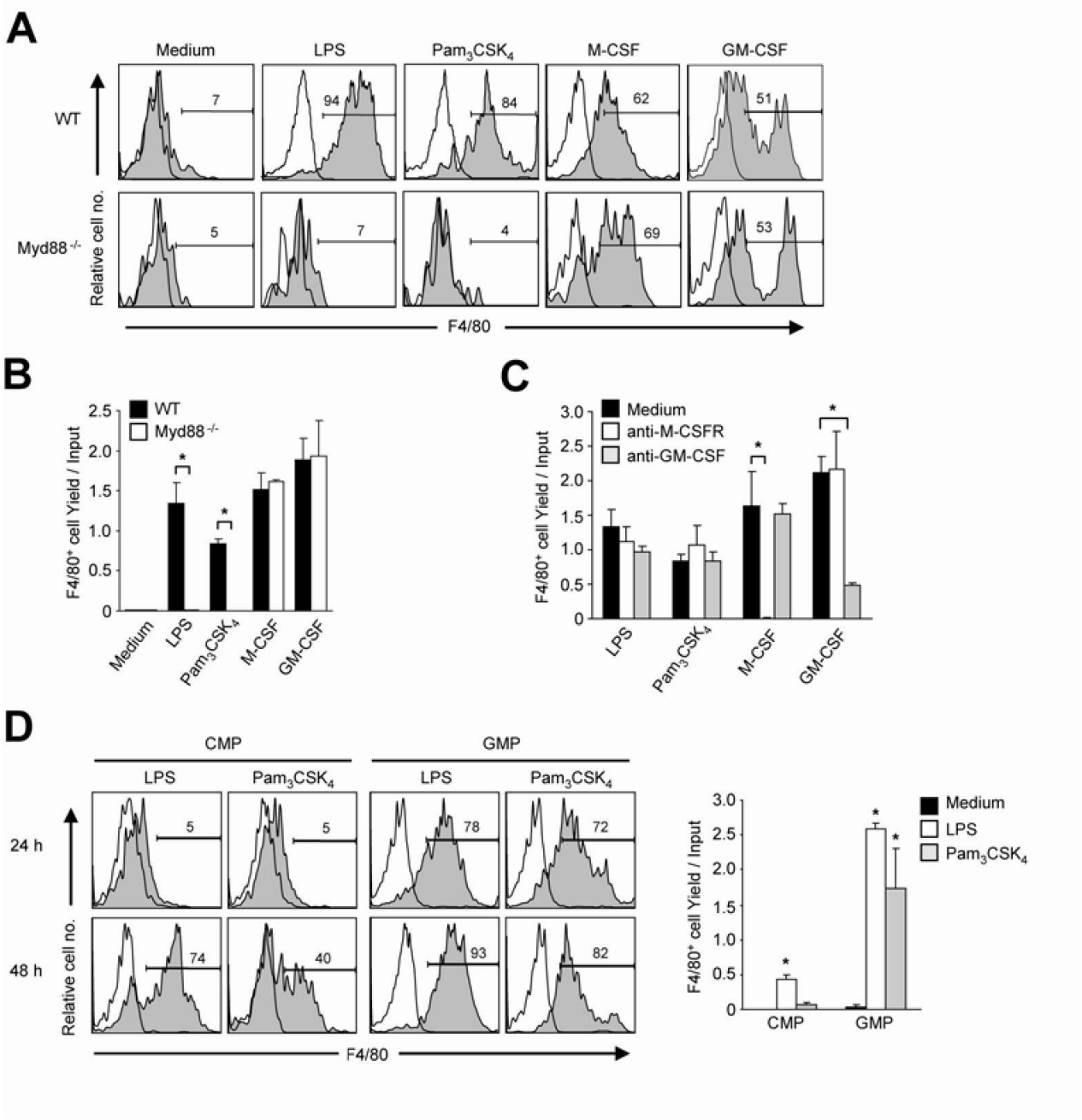


Figure 3.

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), Pam_3CSK_4 (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$^+$ cell 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), Pam_3CSK_4 (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 Pam_3CSK_4 (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 ($^{*}P<0.02$).

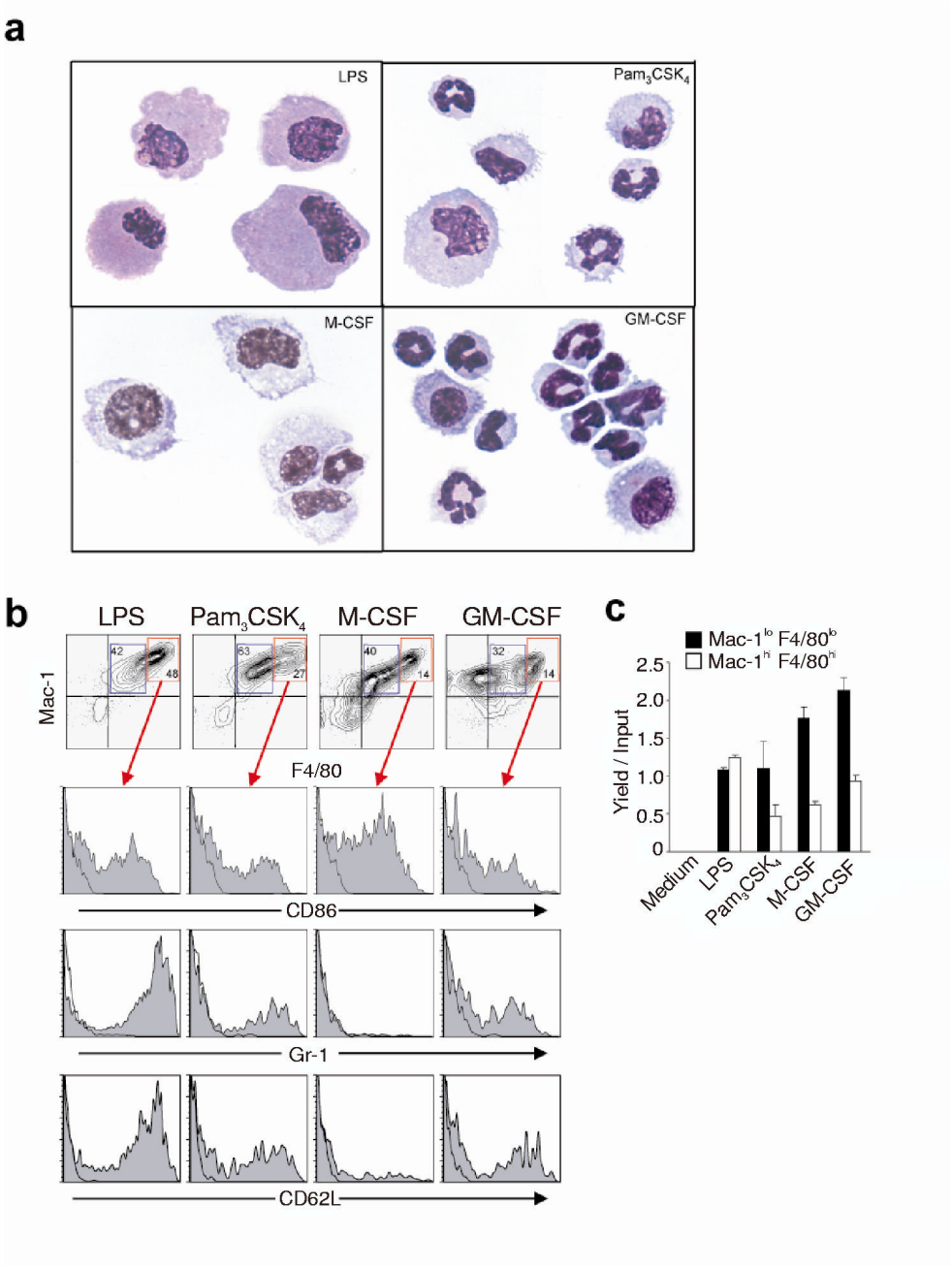


Figure 4.

TLR stimulation drives differentiation of GMPs into F4/80^{hi} monocytes and/or macrophages.(A) These photomicrographs were prepared with Giemsa-May-Grünwald stained cytocentrifuged slides. (B, C) Sorted GMPs were stimulated with LPS (1 μg/ml), Pam_3CSK_4 (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.

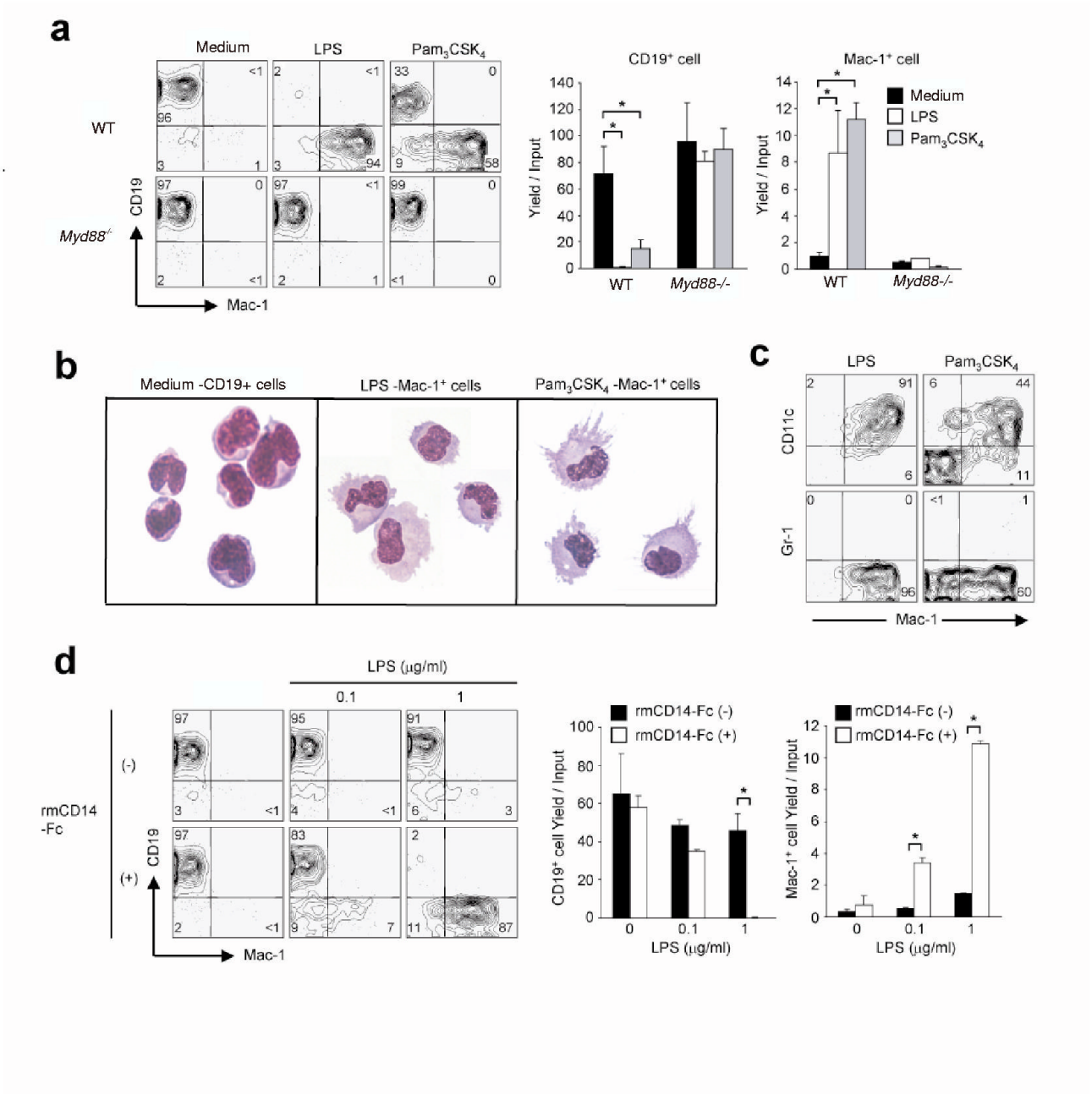
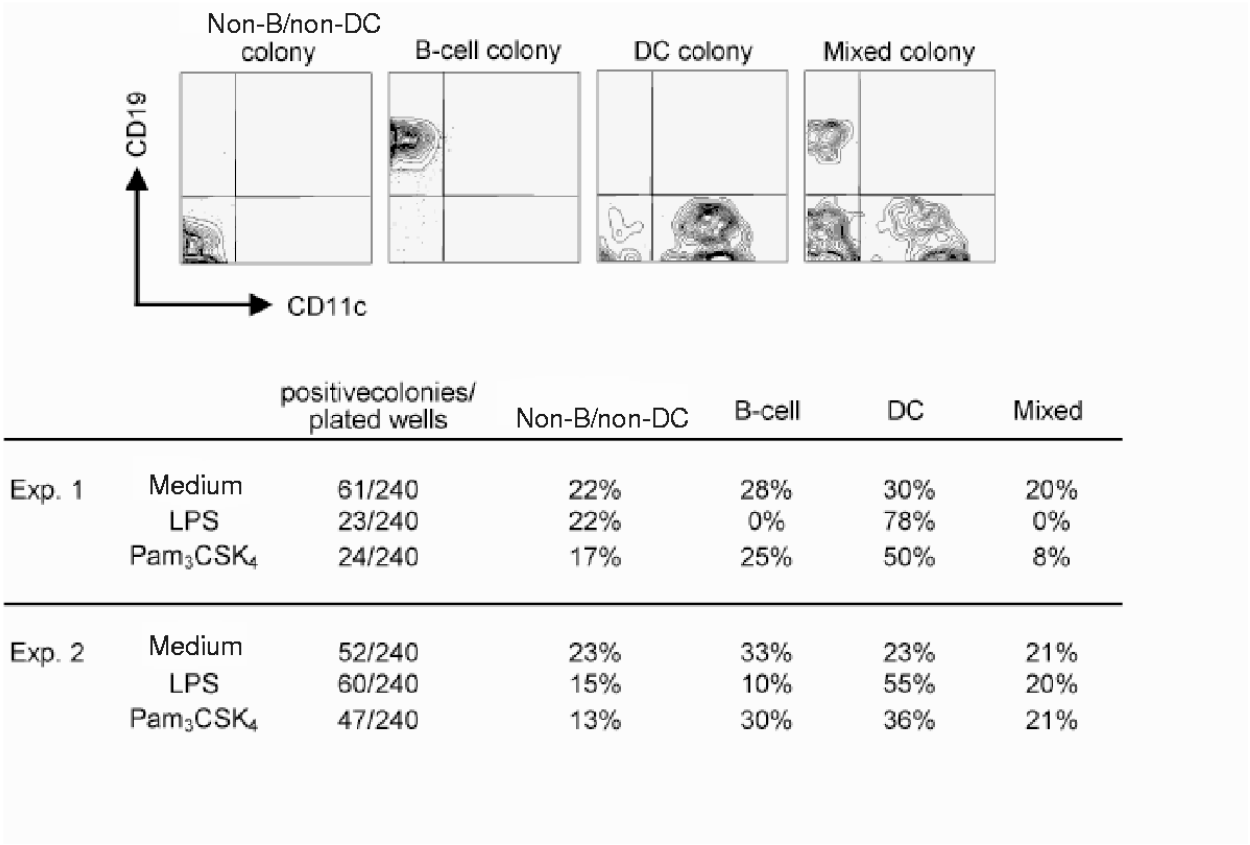


Figure 5.

TLR stimulation allows lymphoid biased progenitors to produce dendritic cells at the expense of B lymphopoiesis.(A) Sorted CLPs (5,000/well) from C57BL/6 or Myd88$^{-/-}$ mice were stimulated in X-VIVO15 medium alone, LPS (10 μg/ml) or Pam_3CSK_4 (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 $Mac\text{-}1^+$ cells (Right). Data represent mean values with standard deviations from triplicate cultures ($^*P<0.003$). The results are representative of three independent experiments.



		positivecolonies/ plated wells	Non-B/non-DC	B-cell	DC	Mixed
Exp. 1	Medium	61/240	22%	28%	30%	20%
	LPS	23/240	22%	0%	78%	0%
	Pam_3CSK_4	24/240	17%	25%	50%	8%
Exp. 2	Medium	52/240	23%	33%	23%	21%
	LPS	60/240	15%	10%	55%	20%
	Pam_3CSK_4	47/240	13%	30%	36%	21%

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 μg/ml) or Pam_3CSK_4 (1 μg/ml). After 24 hr, cultured cells were harvested and washed three times with medium. Single cultured cells were then sorted and re-cultured 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 on top row). The frequencies of wells with each of these differentiation patterns are shown along with total numbers of clones observed.

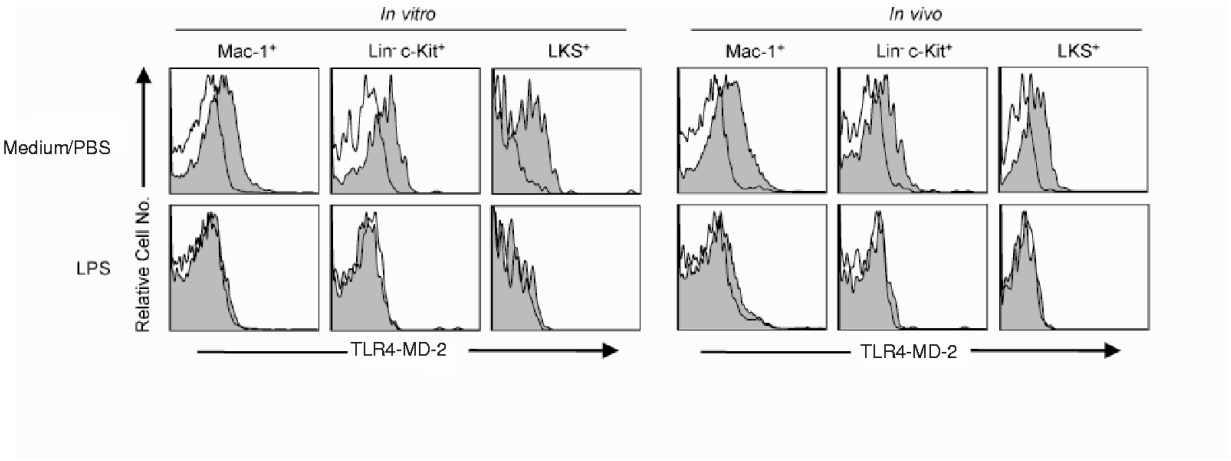
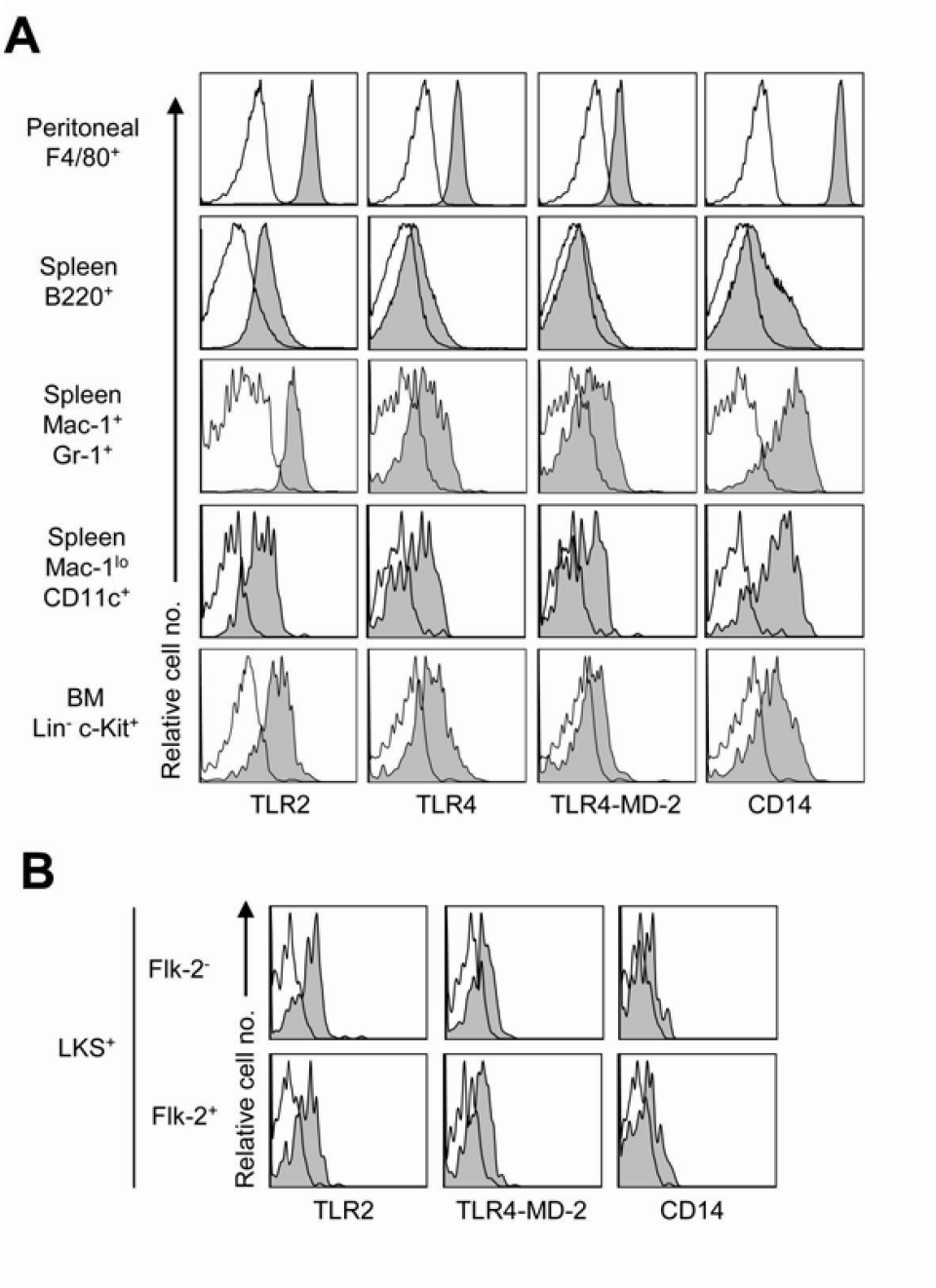


Figure 7.

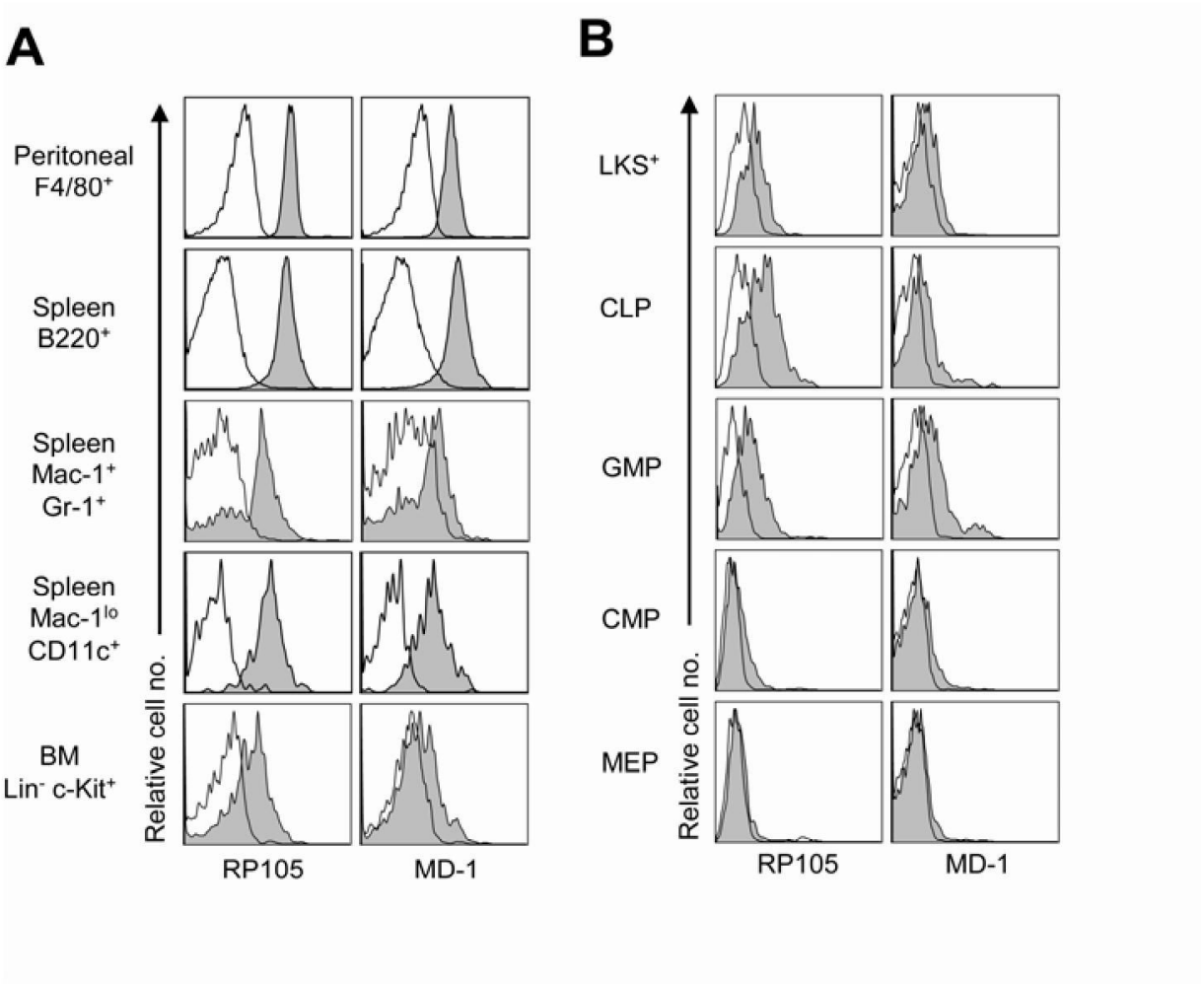
LPS rapidly changes the TLR4-MD-2 complex on hematopoietic stem/progenitors. Left, whole bone marrow cells from C57BL/6 mice were cultured with medium alone or LPS (1 μg/ml) for 1 hr. The cells were then harvested and stained with mAbs to TLR4-MD-2, Mac-1, lineage markers as described in Methods, Sca-1, and c-Kit. The MTS 10 reagent is unique in detecting a conformation dependent epitope on TLR4-MD-2 (Akashi et al., 2003). 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 μg 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.



Supplementary Figure 1.

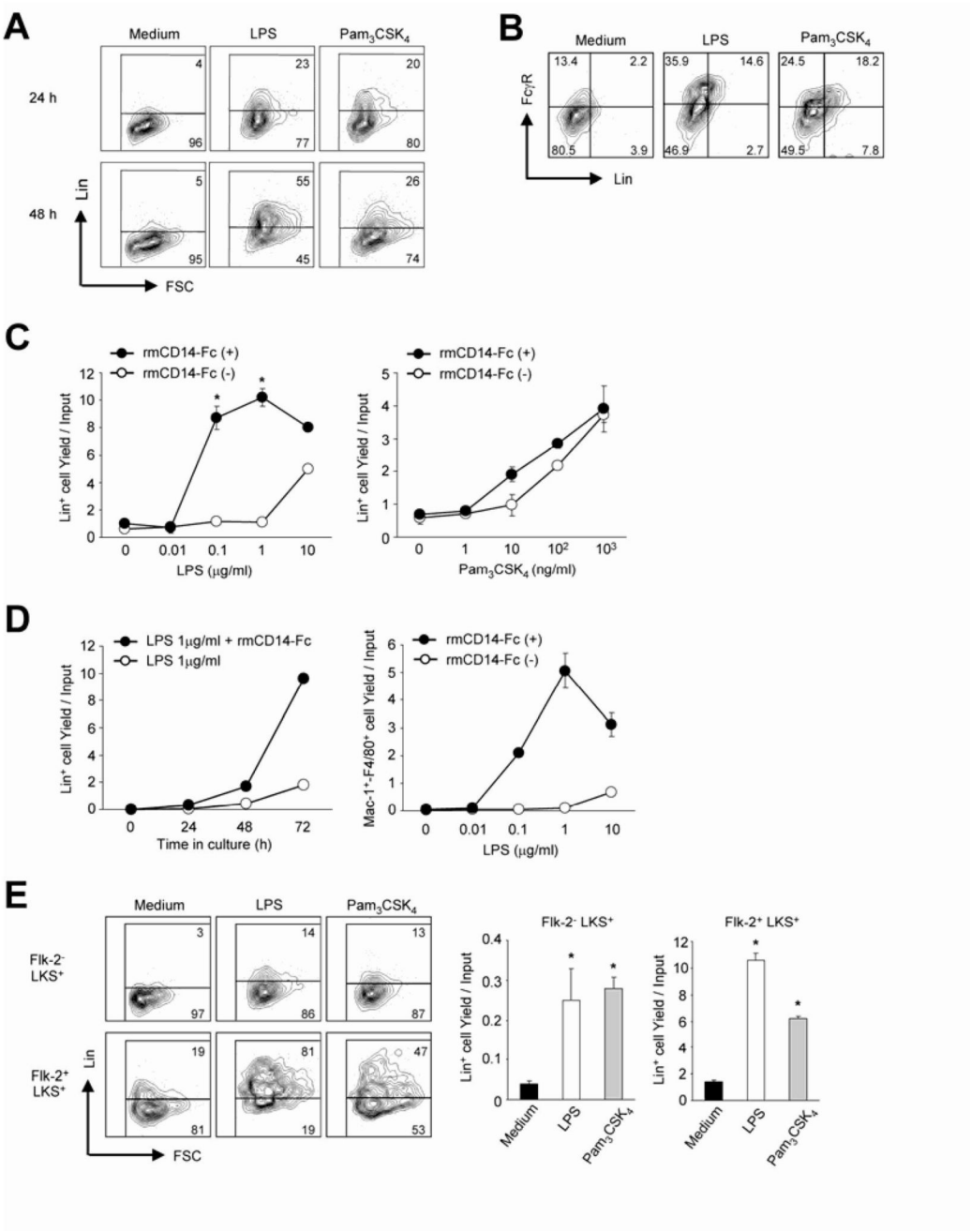
Lin$^-$ c-Kit$^+$ progenitors in bone marrow express TLRs and their co-receptors.(A) TLR2, TLR4, TLR4-MD-2, or CD14 were analyzed by flow cytometry on mature peripheral cells or Lin$^-$ c-Kit$^+$ bone marrow cells. Peritoneal lavage cells from C57BL/6 mice were stained with mAbs to F4/80 together with TLR2, TLR4, TLR4-MD-2, or CD14. Whole spleen cells from C57BL/6 mice were stained with mAbs to B220, Mac-1, Gr-1, and CD11c together with TLR2, TLR4, TLR4-MD-2, or CD14. Whole bone marrow cells from C57BL/6 mice were stained with mAbs to lineage markers as described in Methods and c-Kit together with TLR2, TLR4, TLR4-MD-2, or CD14. Open histograms depict staining with the isotype matched Abs. The results shown are representative of three independent experiments. (B) Whole bone marrow

cells from C57BL/6 mice were stained with mAbs to lineage markers, Sca-1, c-Kit, and Flk-2 together with TLR2, TLR4-MD-2, or CD14. Open histograms depict staining with the isotype matched Abs. The results shown are representative of two independent experiments.



Supplementary Figure 2.

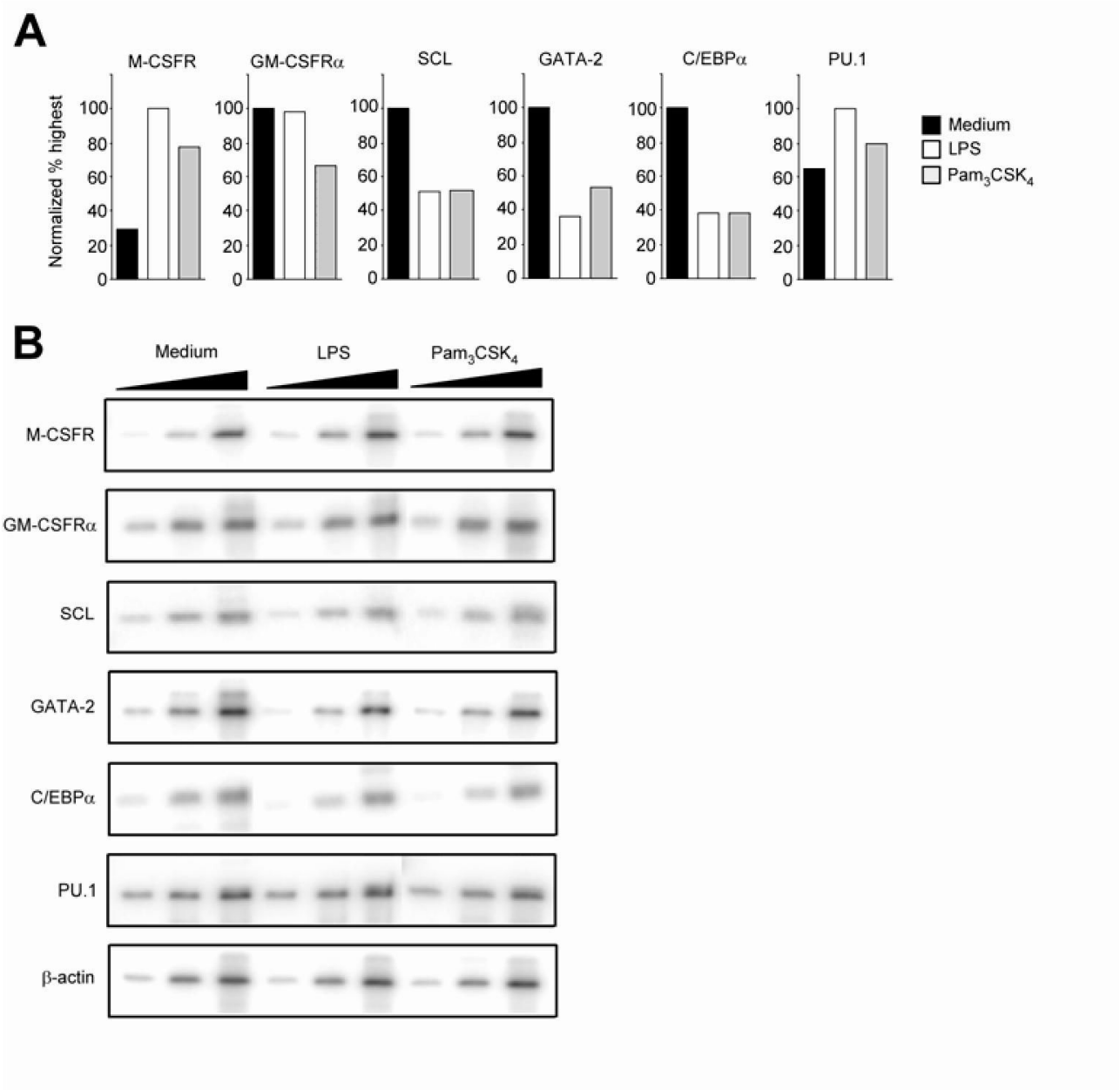
Lin$^-$ stem/progenitor cells express the RP105-MD-1 complex.(A) RP105 or MD-1 were analyzed by flow cytometry on mature peripheral cells or Lin$^-$ c-Kit$^+$ bone marrow cells. Peritoneal lavage cells from C57BL/6 mice were stained with mAbs to F4/80 together with RP105 or MD-1. Whole spleen cells from C57BL/6 mice were stained with mAbs to B220, Mac-1, Gr-1, and CD11c together with RP105 or MD-1. Whole bone marrow cells from C57BL/6 mice were stained with mAbs to lineage markers as described in Methods, and c-Kit was used together with RP105 or MD-1. Open histograms depict staining with the isotype matched Abs. The results shown are representative of three independent experiments. (B) The RP105-MD-1 complex is expressed by hematopoietic progenitor cells. Open histograms depict staining with the isotype matched Abs and the results are representative of two independent experiments.



Supplementary Figure 3.

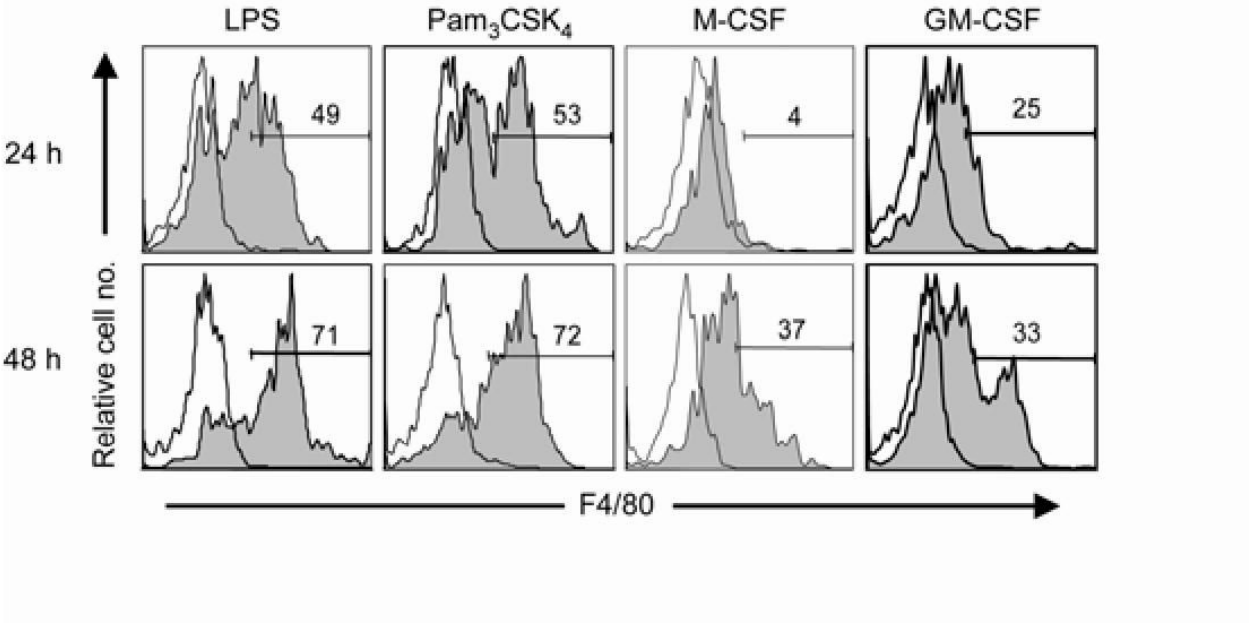
TLR stimulation causes a progression of Lin$^+$ cells from LKS$^+$ cells and soluble CD14 augments the acquisition of Mac-1 and F4/80.(A) Sorted LKS$^+$ cells (10,000 cells/well) from C57BL/6 mice were cultured in the presence of FL and SCF with medium alone, LPS (10 μg/ml) or Pam_3CSK_4 (1 μg/ml). After 24 or 48 hr in culture, cells were analyzed by flow cytometry for expression of lineage markers and percentages of Lin$^+$ or Lin$^-$ cells are indicated. (B) 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 Pam_3CSK_4 (1 μg/ml). After 24 hr in culture, cells were analyzed by flow cytometry for expression of lineage markers and FcγR. (C) Sorted LKS$^+$ cells from C57BL/6 mice were cultured in the presence of FL and SCF with a range of

concentrations of LPS (Left, open circles), Pam_3CSK_4 (Right, open circles) or a combination of mouse CD14-Fc protein (1 μg/ml) plus LPS (Left, filled circles) or Pam_3CSK_4 (Right, filled circles). After 72 hr in culture, cells were analyzed by flow cytometry for expression of lineage markers. Data represent mean values with standard deviations from triplicate cultures ($^*P<0.001$). The results are representative of three independent experiments. (D) Left, sorted LKS^+ cells from C57BL/6 mice were cultured in the presence of FL and SCF with 1 μg/ml LPS (Left, open circles) or a combination of recombinant mouse CD14-Fc protein (1 μg/ml) plus LPS (Left, filled circles). After 24, 48, or 72 hr in culture, cells were analyzed by flow cytometry for expression of lineage markers. The graph depicts cell yields and the results are representative of two independent experiments. Right, sorted LKS^+ cells from C57BL/6 mice were cultured in the presence of FL and SCF with a range of concentrations of LPS (Right, open circles) or a combination of recombinant mouse CD14-Fc protein (1 μg/ml) plus LPS (Right, filled circles). After 72 hr in culture, cells were analyzed by flow cytometry for expression of Mac-1 and F4/80. The graph depicts cell yields and the data are representative of two independent experiments. (E) Sorted Flk-2^- or Flk-2^+ LKS^+ cells (10,000 cells/well) were cultured with medium alone, LPS (10 μg/ml) or Pam_3CSK (1 μg/ml). Flk-2^- LKS^+ cells were cultured with SCF. Flk-2 LKS^+ cells were cultured with SCF and FL. After 72 h in culture, cells were analyzed by flow cytometry for expression of lineage markers (left panels). Percentages given in quadrants indicate the frequencies of Lin^+ or Lin^- cells and the bar graphs on the right depict cell yields. The data represent mean values with standard deviations from triplicate cultures and the results are representative of three independent experiments. ($^*P<0.03$: Flk-2^- LKS^+, $^*P<0.002$: Flk-2^+ LKS^+)



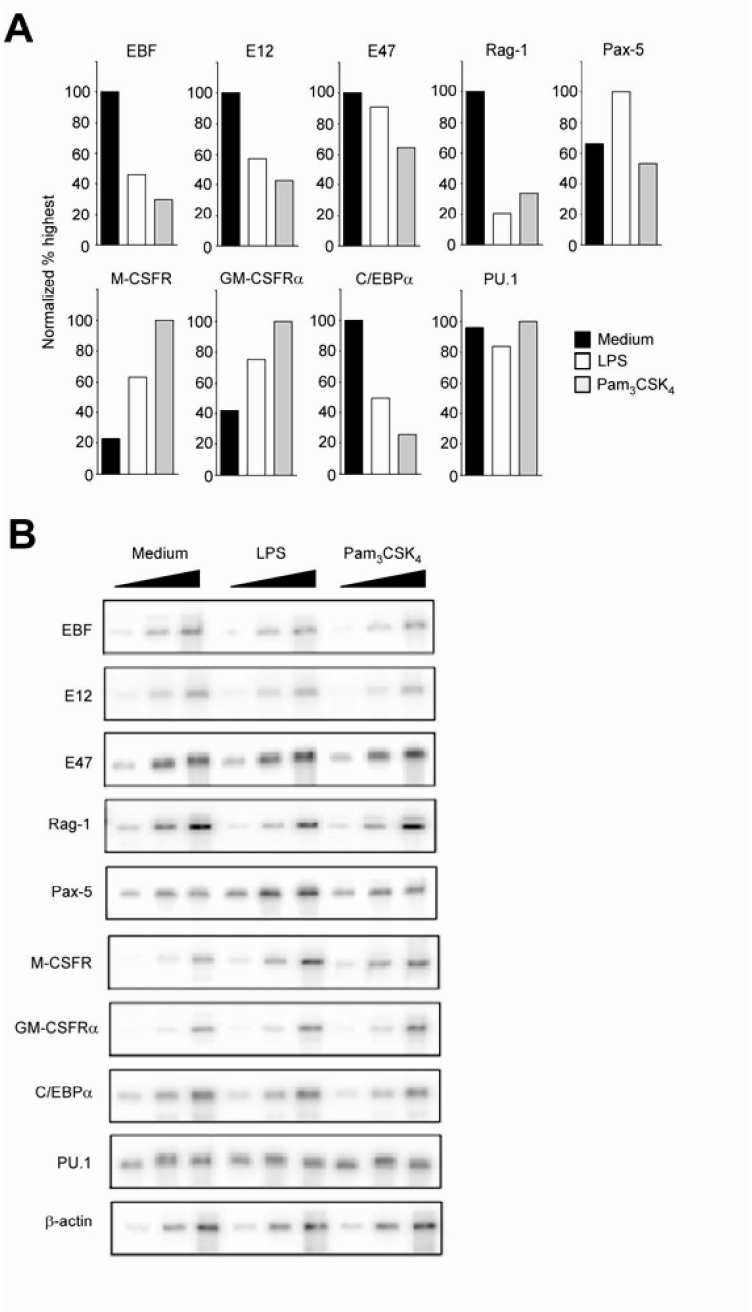
Supplementary Figure 4.

TLR stimulation alters some lineage-associated gene patterns in Flk-2$^-$ HSCs. Sorted Flk-2$^-$ LKS$^+$ cells from C57BL/6 mice were stimulated with medium alone, LPS (10 μg/ml) or Pam_3CSK_4 (1 μg/ml) in the presence of SCF. After 24 hr in culture, cells were harvested and mRNAs were isolated from cultured cells. Semi-quantitative RT-PCR was carried out to amplify transcripts for the indicated genes in each population. (A) The results are shown as values normalized to peak expression for each of the transcripts and actual bands are shown in (B).



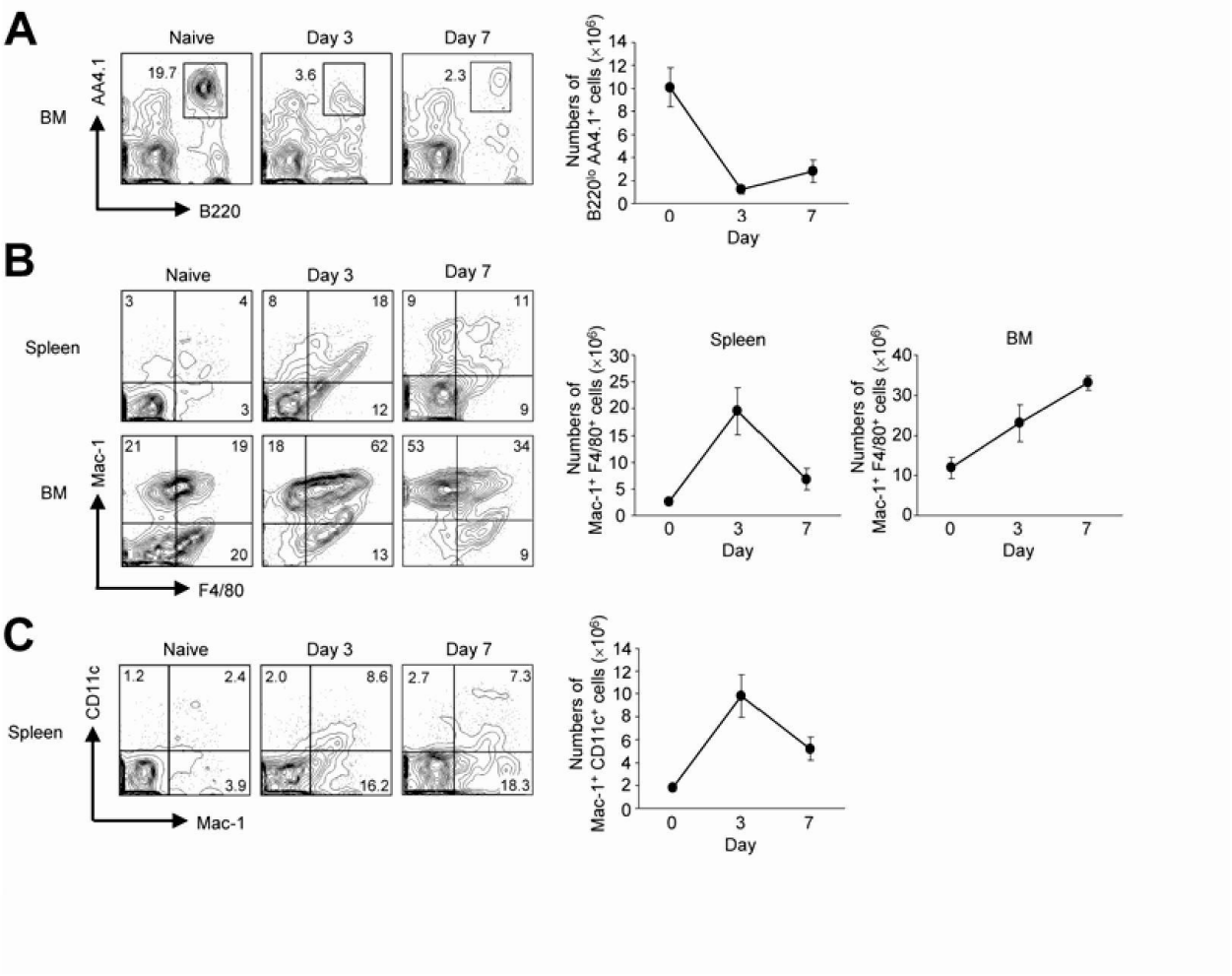
Supplementary Figure 5.

TLR stimulation causes rapid production of F4/80$^+$ cells from LKS$^-$ cells. Sorted LKS$^-$ cells (10,000 cells/well) from C57BL/6 mice were cultured with LPS (10 μg/ml), Pam_3CSK_4 (1 μg/ml), M-CSF, or GM-CSF. Virtually no viable cells were recovered from wells with no stimulus and were not studied further. Stimulated wells were analyzed after 24 or 48 hr of culture by flow cytometry for expression of F4/80. Open histograms depict staining with the isotype matched Ab for F4/80. Percentages of F4/80$^+$ cells are representative of three independent experiments.



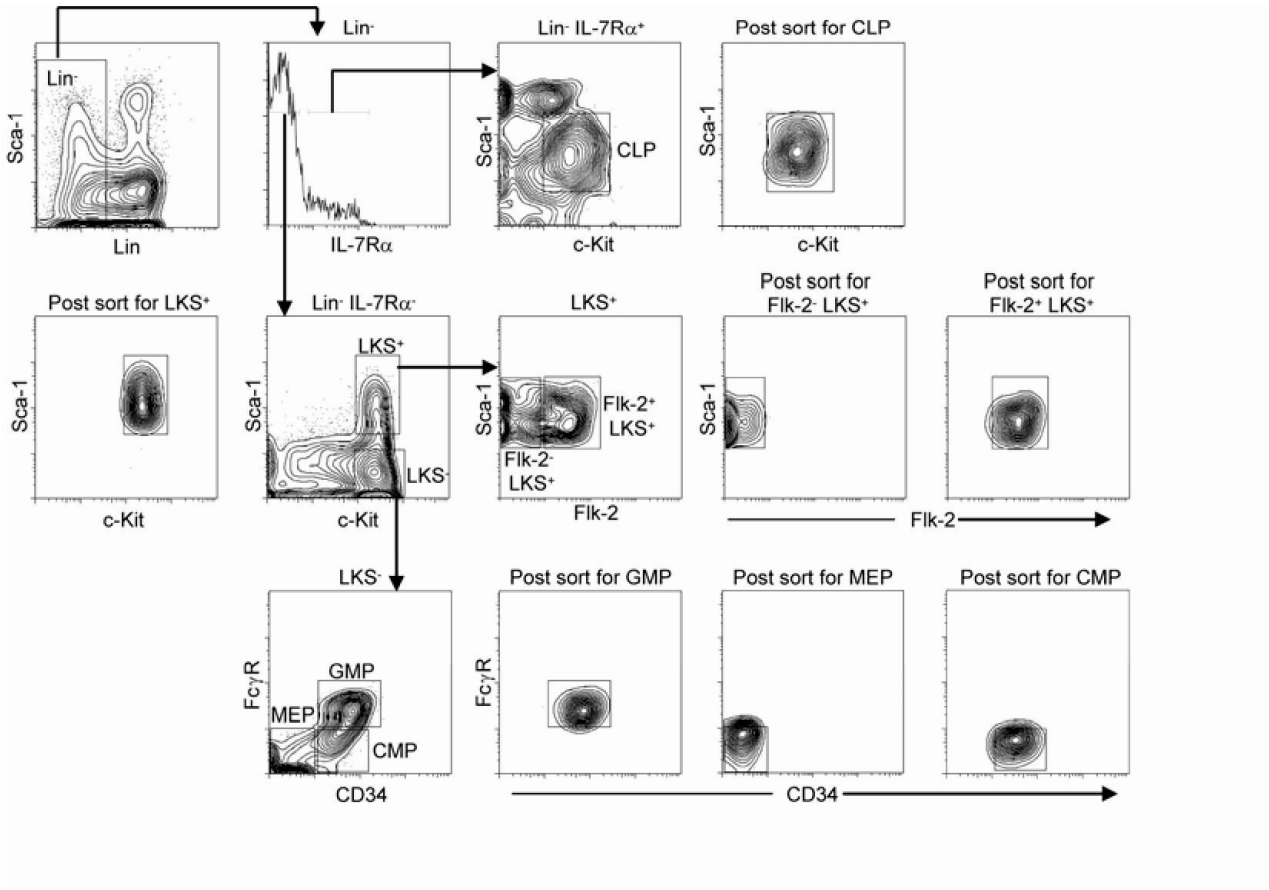
Supplementary Figure 6.

Alteration of some lineage-associated gene patterns in TLR ligated CLPs. Sorted CLPs from C57BL/6 mice were stimulated with medium alone, LPS (10 μg/ml) or Pam_3CSK_4 (1 μg/ml) in the presence of IL-7, FL, and SCF. After 24 hr in culture, cells were harvested and mRNAs were isolated from cultured cells. Semi-quantitative RT-PCR was carried out to amplify transcripts for the indicated genes in each population. (A) The results are shown as values normalized to peak expression for each of the transcripts and actual bands are shown in (B).



Supplementary Figure 7.

Dramatic alterations in B lineage cells, monocytes and/or macrophages and dendritic cells in LPS treated mice. C57BL/6 mice were injected intraperitoneally with 100 μg LPS from *E. coli*. After 3 or 7 days, bone marrow cells from femurs and tibiae or spleen cells were stained with mAbs to the indicated markers and analyzed by flow cytometry. Percentages of $B220^{lo}$ $AA4.1^{+}$ (A), $Mac\text{-}1^{+}$ $F4/80^{+}$ (B), or $Mac\text{-}1^{+}$ $CD11c^{+}$ (C) cells are indicated. The graphs depict cell numbers of $B220^{lo}$ $AA4.1^{+}$ (A), $Mac\text{-}1^{+}$ $F4/80^{+}$ (B), or $Mac\text{-}1^{+}$ $CD11c^{+}$ (C) cells. Data represent mean values with standard deviations from four mice and are representative of two independent experiments.



Supplementary Method 1.

	Primers (5' to 3')	
Gene	sense	anti-sense
TLR4	AGTGGGTCAAGGAACAGAAGCA	CTTTACCAGCTCATTTCTCACC
TLR2	TCTAAAGTCGATCCGCGACAT	TACCCAGCTCGCTCACTACGT
MD-2	ATGTTGCCATTTATTCTCTTTTCGACG	ATTGACATCACGGCGGTGAATGATG
CD14	ACATCTTGAACCTCCGCAAC	AGGGTTCCTATCCAGCCTGT
M-CSFR	TCATTCAGAGCCAGCTGCCCAT	ACAGGCTCCCAAGAGGTTGACT
GM-CSFRα	GCACTGGAGGCTGAGCTTCGTC	CACCTTGACCTTGTGACCTCCG
EBF	GAGATTTTTCCACAAGAAAAGGTTG	GGAAGAACCTGTCAATTATCACTGG
E12	GACGCCGAAGAGGACAAGAA	TGGTGCAGGATGAGCAGTTT
E47	CGCACTGACCACGAGCTTCAC	TCCAGGGACAGCACCTCATCTG
Pax-5	CTACAGGCTCCGTGACGCAG	GTCTCGGCCTGTGACAATAGG
Rag-1	TGCAGACATTCTAGCACTCTGG	ACATCTGCCTTCACGTCGAT
SCL	TCCCCATATGAGATGGAGATTT	ATTGATGTACTTCATGGCAAGG
GATA2	CGGAATTCGACACACCACCCGATACC	CGGAATTCGCCTACGCCATGGCAGT
C/EBPα	GAACAGCAACGAGTACCGGGT	GCCATGGCCTTGACCAAGGAG
PU.1	CGGATGACTTGGTTACTTACG	GTAGGAAACCTGGTGACTGAG
β-actin	CCTAAGGCCAACCGTGAAAAG	TCTTCATGGTGCTAGGAGCCA

Supplementary Method 2.