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Chronic Exposure to a TLR Ligand Injures Hematopoietic Stem Cells

Brandt L. Esplin,*†,1 Tomoyuki Shimazu,*† Robert S. Welner,* Karla P. Garrett,* Lei Nie,* Qingzhao Zhang,* Mary Beth Humphrey,‡,§ Qi Yang,¶ Lisa A. Borghesi,¶ and Paul W. Kincade*

Hematopoietic stem cells (HSC) can be harmed by disease, chemotherapy, radiation, and normal aging. We show in this study that damage also occurs in mice repeatedly treated with very low doses of LPS. Overall health of the animals was good, and there were relatively minor changes in marrow hematopoietic progenitors. However, HSC were unable to maintain quiescence, and transplantation revealed them to be myeloid skewed. Moreover, HSC from treated mice were not sustained in serial transplants and produced lymphoid progenitors with low levels of the E47 transcription factor. This phenomenon was previously seen in normal aging. Screening identified mAbs that resolve HSC subsets, and relative proportions of these HSC changed with age and/or chronic LPS treatment. For example, minor CD150loCD48− populations lacking CD86 or CD18 expanded. Simultaneous loss of CD150lo−CD48− HSC and gain of the normally rare subsets, in parallel with diminished transplantation potential, would be consistent with age- or TLR-related injury. In contrast, HSC in old mice differed from those in LPS-treated animals with respect to VCAM-1 or CD41 expression and lacked proliferation abnormalities. HSC can be exposed to endogenous and pathogen-derived TLR ligands during persistent low-grade infections. This stimulation might contribute in part to HSC senescence and ultimately compromise immunity. The Journal of Immunology, 2011, 186: 5367–5375.

The bone marrow (BM) is a target for some pathogens, including CMV, parvovirus, dengue virus, hepatitis, and HIV (1–5). Animal studies indicate that hematopoiesis can also be affected by Cryptosporidium parvum, pertussis, malaria, influenza, vaccinia virus, and immunization (6–11). Depending on the agent, hematopoietic stem cells (HSC), hematopoietic progenitor, and marrow stromal cells can be infected. The same cells can also respond to inflammatory cytokines (12–14).

We discovered another mechanism through which stem and progenitor cells are influenced by pathogen products and endogenous danger signals (15). These cells express TLR, coreceptors, and associated signaling molecules. Moreover, several responses were recorded when highly purified cells were exposed to TLR ligands such as LPS under defined culture conditions. HSC were driven into cycle and stimulated to acquire lineage markers, whereas committed myeloid progenitors differentiated even when no exogenous growth and differentiation factors were added. In addition, we observed dramatic changes in lymphoid progenitors; B lymphopoiesis was arrested, and CLP were directed to become dendritic cells (DC). Following i.p. injection, LPS travels quickly to the marrow and engages the TLR4 receptor (15). HSC are then mobilized to the periphery where their differentiation can again be affected by this ligand (16, 17). This ability of stem and progenitor cells to sense pathogen products may be protective, allowing rapid mobilization and generation of cells in the innate immune system. However, it is also possible that this mechanism has pathological consequences in some situations, such as during prolonged systemic exposure to TLR ligands. This would be the case in such conditions as Gram-negative periodontitis, subacute bacterial endocarditis, and other chronic infectious circumstances (18). Furthermore, TLR4 can be engaged by endogenous ligands and fatty acids, suggesting that HSC might be altered by inflammation associated with tissue damage and obesity (19, 20). This could account for the fact that HSC from some TLR knockout mice have an engraftment advantage over wild-type HSC during transplantation (21).

HSC have sufficient potential to replenish all blood cell types for several lifetimes, and numbers of transplantable HSC do not decline (22–24). However, many studies suggest they undergo important age-related changes, including the selective loss of lymphopoietic potential (25–28). This intrinsic tendency for lineage skewing has not been reported in other situations in which HSC are injured. For example, a shortening of telomeres causes myeloid skewing, but largely because of systemic factors, and HSC remain relatively normal (29, 30). The same is true of animals with naturally occurring DNA damage or defects in DNA repair enzymes (31). That is, the potential to replenish both lymphoid and myeloid lineage cells is compromised. Accelerated HSC senescence has been reported in other stressful conditions that include ionizing radiation and chemotherapy (32, 33). Finally,

*Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104; †Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; ‡Veterans Affairs, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; †Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; and ‡Department of Immunology, University of Pittsburgh, Pittsburgh, PA 15261

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Address correspondence and reprint requests to Dr. Paul W. Kincade, Oklahoma Medical Research Foundation, 825 Northeast 13th Street, Oklahoma City, OK 73104. E-mail address: kincade@omrf.org

The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CLP, common lymphoid progenitor; DC, dendritic cell; HSC, hematopoietic stem cell; Lin−, lineage marker negative; LSK, lineage marker Sca-1+c-Kithi; MFI, mean fluorescence intensity; MPP, multipotent progenitor; PC, peritoneal cavity; RU, repopulating unit.
the marrow can be influenced by cytokines released during systemic infections (13, 14). Again, these seem to be examples in which hematopoietic functions are uniformly compromised. We report in these studies that repeated exposure to small amounts of LPS is harmful to long-term repopulating stem cells. HSC cycling was elevated and remained so during 8 mo of serial transplantation within untreated recipients. Initially able to reconstitute multiple hematopoietic lineages, stem cells from LPS-treated mice preferentially lost the ability to generate lymphocytes. This lymphoid versus myeloid bias became more obvious with time, and there was evidence of HSC exhaustion. Some, but not all, changes resemble those occurring during aging.

Materials and Methods

Mice

C57BL/6 (CD45.2 alloantigen; The Jackson Laboratory, Bar Harbor, ME), aged C57BL/6 (National Institute on Aging), B6-JIL/J (CD45.1 alloantigen; The Jackson Laboratory), and C57BL/6 × SJL/JL5.1 F1 (CD45.1 and CD45.2 alloantigens) were bred and maintained in the Laboratory Animal Resource Center at the Oklahoma Medical Research Foundation (Oklahoma City, OK). All mice were 8–15 wk old, and male and female mice were used without gender discrimination. Experiments were performed in accordance with approved Institutional Animal Care and Use Committee protocols.

Intraperitoneal LPS injections

Daily injections containing 6 μg LPS (O55:B5; Sigma-Aldrich, St. Louis, MO) in 200 μl PBS or PBS alone were administered during the 4–6 wk treatment period. C57BL/6 mice received LPS, and B6SJL/J mice received PBS. Analysis of hematopoietic tissues was performed after 4–6 wk of injections.

Isolation of cell populations and flow cytometry

Flow cytometry analyses and sorting were performed as previously described (15, 34). Tissue and cell manipulations were performed in HBSS plus 5% FCS. Marrow cells were isolated from the long bones of donor mice, and erythrocytes were lysed in NH₄Cl–hypotonic solution. Peritoneal cavity (PC) cells were acquired by lavage with 10 ml HBSS plus 5% FCS. To isolate progenitor populations for culture and transplantation, BM cells were enriched by negative selection by labeling marrow with Ly6G/CGr1 (R6C-8C5), CD11b/Mac-1 (M1/70), TER-119, CD11c (17A2), CD8 (53-6.7), CD19 (1D3), and B220 (14.8) and then immunomagnetically depleted using goat anti-rat IgG (Qiagen, Valencia, CA). All cells were treated with Fc-receptor block (2.4G2) prior to fluorescence staining and sorting. After staining marrow with biotin–antilineage markers (Gr-1, CD11b, CD11c, CD45R/B220, TER-119, CD3, CD8, and NK1.1), anti-c–Kit (2B8), anti–Sca-1 (D7; eBioscience, San Diego, CA), and anti–IL-7Rα (ATR3; eBioscience); anti–Flt3 (A2F10; Biologend); and secondary streptavidin PE-Texas Red (Caltag Laboratories, Burlingame, CA), and lineage marker-negative (Lin−) populations were sorted using either a MoFlo (DakoCytomation, Ft. Collins, CO) or FACSAria cytometer (BD Biosciences, San Diego, CA) into specific populations. Dead cells were excluded by propidium iodide staining (Molecular Probes, Eugene, OR). Purification of each subset was achieved by double sorting and confirmed by postsort analysis. Cells harvested from sacrificed recipients were analyzed by flow cytometry to determine the phenotypes of resident or donor cells. Abs included CD3 (145-2C11), B220 (RA3-6B2), CD8 (53-6.7), CD11b (M1/70), TER-119, Gr-1 (R6C-8C5), IgM (R6-60.2), NK1.1 (PK136), CD19 (1D3), CD48 (H48-M14; Biologend), CD135/Flt3 (A2F10; Biologend), CD11c (HL3), CD34 (HM34; Biologend), FcyRII/III (93), CD150 (TC12-1F2; Biologend), CD48 (GL1), CD18 (C7/11L), Vcam-1 (429 [MVCAM.A]), CD41 (WMReg30), CD39 (3F2; Biologend), CD25 (PC61), CD44 (IM7), CD45.1 (A20), and CD45.2 (104). Intracellular staining with FITC–anti-E47 was accomplished by fixation in 100% ethanol, followed by permeabilization in 0.2% Tween 20. After E47 staining, cells were washed three times prior to flow cytometry analysis in HBSS plus 5% FCS. Flow cytometry was performed on a BD LSRII (BD Biosciences, San Jose, CA), and Flowjo software (Tree Star, San Carlos, CA) was used for data analysis. All Abs came from BD Pharmingen, unless otherwise stated.

Intravenous serial competitive transplantations

A chimerism-based transplantation method was developed for these studies. Recipients were 8–12-wk-old F1 (CD45.1+CD45.2−) mice lethally irradiated (2 × 650 rad) in a 137Cs source (Mark 1 irradiator; J. L. Shepard and Associates, Glendale, CA). Mice were anesthetized with isoflurane (IsoSol; Vedco, St. Joseph, MO), and cells were infused i.v. by retro-orbital injection. F1 recipients allowed precise demarcation of both competing donor marrow grafts (CD45.1+ control and CD45.2+ LPS-treated) from residual host cells (CD45.1+CD45.2− F1). Transplant grafts consisted of equal-part chimeric mixtures containing 2 × 10⁶ nucleated whole marrow cells each from CD45.1+ control- and CD45.2+ LPS-treated mice. Pretransplant chimeric ratios were confirmed by FACS analysis. Competitive repopulation was assessed at 4-wk intervals by peripheral blood analysis, as described below. Primary F1 recipients were sacrificed at 16 wk, and total, multilineage, hematopoietic contributions of the competing grafts was assessed in the BM, spleen, thymus, and PC of the F1 recipient mice. Equal numbers of whole marrow-nucleated cells collected from one femur of each primary recipient were pooled, and 2 × 10⁶ of these mixed cells were retransplanted into secondary F1 recipients in a similar fashion.

Peripheral blood preparations

Mice were briefly anesthetized with isoflurane, and ~0.05 ml peripheral blood was obtained from each mouse by retro-orbital bleeding with heparin-coated 10-inch elongated glass pipettes. Heparinized blood from the pipettes was emptied directly into glass centrifuge tubes containing 0.4 ml 2% Dextran solution and mixed thoroughly. The mixture was incubated for 20 min at 37°C to allow sedimentation of RBCs. The supernatant, BrdU-enriched phase was retained, and cells were washed before brief resuspension in hypotonic NH₄Cl solution as an additional means of removing erythrocytes. The cells were washed in HBSS and stained with immunofluorescent Abs for analysis by flow cytometry. Complete blood counts were performed using heparin-coated capillary tubes and a cell counter (Serono 9000, Serono-Parker Diagnostics, Allentown, PA).

Cell-cycle kinetics analyses

Ki-67. Single-cell suspensions of C57BL/6 marrow were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with FITC anti–Ki-67 (B56) or MOPC-21 isotype control (BD Biosciences), with continual administration of BrdU-enriched drinking water (0.8 mg/ml; Sigma-Aldrich) thereafter during the final 4 d of the injection period. Mice from a single femur were collected and analyzed by intracellular staining with FITC anti-BrdU (FITC BrdU flow kit; BD Biosciences) and compared with isotype control stains.

Serum-free, stromal cell-free cultures

Sorted cells were cultured in round-bottom 96-well plates (Corning) with X-VIVO15 medium (BioWhittaker, Walkersville, MD) containing 1% de- toxified BSA (StemCell Technologies, Vancouver, BC, Canada), 5 × 10⁻³ M 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Culture medium was enriched with 100 ng/ml Flt3 ligand, 20 ng/ml stem cell factor, 1 ng/ml IL-7, and 20 ng/ml M-CSF in various combinations as indicated in the Results section. DC-lineage–promoting conditions included stem cell factor and Flt3 ligand without IL-7. Incubation was maintained at 37°C in a 5% CO₂-humidified atmosphere. Cells were fed by replacing half culture volume with fresh media and cytokines every 3 to 4 d. Cells were harvested at designated times and stained with mAbs to CD19, B220, CD11c, Ly6c, CD11b/Mac-1, and NK1.1.

Methylcellulose cultures

Sorted cells were added to 2 ml Methocult 3434 (StemCell Technologies) medium and plated at 100 cells/plate in 35-mm culture dishes. Incubation was carried out as described above, and colonies were counted with a dissecting microscope after 10 d.

Western blot analysis of E2A in pro-B cells

Equal numbers (1.5 × 10⁵ to 5.0 × 10⁵) of sorted pro-B cells (CD19⁺ CD220⁺ CD19⁺Im7) were lysed in RIPA and separated on 9% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The immunoreactive proteins on the membrane were detected using ECL reagents (Amersham Biosciences, Piscataway, NJ). The Abs used for probing E2A and α-tubulin were purchased from Santa Cruz Biototechnology (Santa Cruz, CA).

Statistics

Prism V3.02 software (GraphPad, San Diego, CA) was used for statistical analysis. Unpaired, two-tailed t test analyses were employed for intergroup comparisons, and p values were considered significant if <0.05.
Serum cytokine analyses

Nonheparinized peripheral blood samples were obtained from mice 2 h after the daily i.p. LPS injection. Blood samples were allowed to clot for 1 h at room temperature, and then briefly centrifuged to optimize serum separation. A total of 100 μl aliquots of serum were assayed for TNF-α abundance by ELISA (R&D Systems, Minneapolis, MN). The PE-based Cytometric Bead Array (BD Biosciences) was also employed for simultaneous analysis of multiple serum cytokines (TNF-α, IFN-γ, IL-6, GM-CSF, IL-10, and IL-12p70), according to manufacturer instructions.

Bone density analyses

The right proximal tibiae were scanned by microcomputed tomography (mCT-20; Scanco Medical, Basserdorf, Switzerland), as described previously (35). Briefly, the proximal tibiae were fixed in PBS plus 4% paraformaldehyde overnight at 4°C and then stored in 70% ethanol. The bones were placed in a 17-mm holder, and an image consisting of 200 slices at 9-mm voxel size through a region of 1.08 mm in all three axes was generated. Evaluation was done on 120 slices initiating 0.1 mm from the lowest point of the growth plate. A three-dimensional cubical voxel model of bone was built, and the following calculations were made: relative bone volume, trabecular number, thickness, spacing, connectivity density, and cortical thickness.

Results

A model for chronic TLR stimulation

There is no evidence that TLRs are needed to build the hematopoietic system under normal steady-state conditions (36). However, we hypothesized that chronic exposure might have longer-term consequences and sought conditions in which mice could be safely exposed to a defined TLR ligand for extended periods of time. Following a published protocol, animals were implanted with time-release pellets calibrated to deliver ∼3.5 μg LPS/d (37). Although this treatment was well tolerated, the method gave variable mouse-to-mouse hematopoietic responses. We subsequently found that daily LPS injections of 6 μg/d worked well when delivered for 4–6 wk, as treatment caused no noticeable wasting or morbidity. Complete blood counts and thymus cellularity were normal at that time (Table I and data not shown).

Although serum ELISA analyses during the first week of injection revealed slight increases in levels of TNF-α, no abnormalities were found after 4 wk of treatment in TNF-α, IFN-γ, IL-6, GM-CSF, IL-10, or IL-12p70 (not shown).

Although bone densities were significantly reduced (Supplemental Fig. 1A), total numbers of marrow-nucleated cells were unchanged in LPS-treated mice after 4–6 wk of daily injection (Table I). However, percentages of CD11b+Gr-1+ myeloid cells elevated during the final weeks of treatment, Sca-1 densities and Consistent with our findings that inflammatory cytokines were not elevated during the final weeks of treatment, Sca-1 densities and numbers of cells in the primitive LSK fraction were normal in LPS-treated mice (Fig. 1D, 1E). Long-term repopulating HSC represent only a small fraction of LSK, so we employed more rigorous analyses using Flt3+ LSK (39, 40) and Lin−Sca-1+c-Ki67+CD48+CD150+ (41) definitions for HSC (Supplemental Fig. 1B). Absolute numbers of such long-term repopulating stem cells in chronically stimulated marrow were significantly elevated in three independent experiments (Fig. 1E). It has been reported that TLR stimulation increases numbers of HSC in the periphery (16). We found that animals sacrificed after the last treatment had enlarged spleens, and absolute numbers of CD150+CD48−LSK defined HSC in that site were elevated an average of 28-fold (Fig. 1F).

Therefore, mice can tolerate repeated exposure to very low doses (∼1% of the LD50) of LPS. The treatment caused modest increases in HSC numbers as well as depressions in B and T lymphoid progenitors. Expansion of HSC in the spleen could reflect mobilization from the marrow or stimulation in the periphery.

Chronic TLR ligation alters cell division and differentiation potential of primitive hematopoietic cells

Assessments were then made of cycle status in HSC. Fig. 2A shows small but statistically significant elevations in percentages of CD150+CD48−LSK for the intracellular Ki-67 Ag, and representative staining is given in Supplemental Fig. 2A. Similar results were obtained when HSC were more generously gated as Flt3+LSK (data not shown). Furthermore, increased numbers of Flt3−LSK incorporated BrdU during the last 4 d of LPS treatment, indicating a persistently elevated cycling rate for HSC-enriched cells chronically exposed to LPS (Supplemental Fig. 2B). The differentiation potential of primitive cells in the LSK subset was then assessed with two types of culture conditions. LSK sorted from marrow of LPS-treated mice and placed into myeloid-differentiation from the marrow or stimulation in the periphery.

Purified hematopoietic progenitors respond to TLR ligands in defined liquid cultures (15, 34), and we found similar changes in chronically LPS-treated mice. That is, CLP lost the potential to generate CD11b−Gr-1−cDC, conventional DC.

Table I. Relatively minor changes in hematopoietic cells in low-dose LPS-treated mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total BM (one femur)</td>
<td>23.6 ± 3.7 × 10^6</td>
<td>24.7 ± 4.0 × 10^6</td>
</tr>
<tr>
<td>Total spleen</td>
<td>1.61 ± 0.3 × 10^8</td>
<td>2.8 ± 0.9 × 10^8</td>
</tr>
<tr>
<td>Total thymus</td>
<td>1.33 ± 0.3 × 10^9</td>
<td>1.01 ± 0.2 × 10^9</td>
</tr>
<tr>
<td>No. of myeloid/femur</td>
<td>9.76 ± 0.03 × 10^5</td>
<td>13.5 ± 0.08 × 10^5</td>
</tr>
<tr>
<td>No. of pre-B/femur</td>
<td>6.58 ± 0.18 × 10^5</td>
<td>2.5 ± 0.9 × 10^5</td>
</tr>
<tr>
<td>No. of cDC/femur</td>
<td>3.5 ± 0.2 × 10^5</td>
<td>5.9 ± 1.1 × 10^5</td>
</tr>
<tr>
<td>No. of B cells/spleen</td>
<td>6.0 ± 2.1 × 10^5</td>
<td>11.7 ± 0.8 × 10^5</td>
</tr>
<tr>
<td>No. of cDC/spleen</td>
<td>3.0 ± 0.9 × 10^5</td>
<td>5.6 ± 1.1 × 10^5</td>
</tr>
<tr>
<td>No. of myeloid/spleen</td>
<td>25.0 ± 6.8 × 10^6</td>
<td>34.5 ± 4.8 × 10^6</td>
</tr>
</tbody>
</table>

cDC were gated as CD19−B220−CD11b−CD11c+. Myeloid cells were gated as CD11b−Gr-1−. Pre-B were gated as B220+CD43−IgM−. Representative of four experiments with at least four mice per group per experiment. *p < 0.05 as determined by t test.

cDC, conventional DC.
low-grade TLR stimulation increased numbers of phenotypically defined HSC and caused some of them to enter cell cycle and have reduced lymphopoietic potential.

**Chronic TLR stimulation causes durable changes in long-term repopulating HSC**

Experiments involving transplantation of TLR knockout BM indicated that these receptors have important functions on long-term repopulating HSC (21). Furthermore, the above data show that repeated exposure to LPS interfered with quiescence and skewed differentiation of the HSC-rich fractions away from the adaptive immune system. It was unclear if these abnormalities were persistent or would resolve with time, so formal assessments of HSC function were made in competitive transplantation experiments.

Equal numbers of control (CD45.1+) and LPS-treated (CD45.2+) BM cells were transplanted together into lethally irradiated (CD45.2 × CD45.1) F1 mice (Fig. 3). This should have given a slight (1.3-fold) advantage to the treated HSC (Fig. 1E, top). However, HSC derived from the control marrow dominated with respect to peripheral blood chimerism and particularly with respect to CD11b+Gr-1+CD19+CD3+ lymphocytes (Fig. 3A). In contrast, the two donors equally contributed to production of large CD11b+ peripheral blood myeloid cells, and this myeloid bias increased when marrow cells were retransplanted for an additional 16 wk (Fig. 3B). Lymphoid/myeloid ratios reflected enduring, heritable myeloid bias and lymphoid deficiency in HSC originating from marrow chronically exposed to LPS (Fig. 3C).

A thorough analysis of hematopoietic chimerism was performed with tissues harvested from the recipients 16 wk after each transplant (Fig. 3D). Marrow cells originating in chronically treated mice became progressively defective in repopulating lymphocytes in the thymus, PC, spleen, and marrow.

As noted above, low-grade TLR ligation did not immediately change the myeloid potential of LSK (Fig. 2B), and the same was found in Methocel culture experiments using LSK harvested from primary transplant recipients (Supplemental Fig. 3A). However, CD45.2+ LSK recovered from secondary transplants had increased ability to form colonies in this assay compared with CD45.1+ controls (Supplemental Fig. 3A). As was the case for the initial marrow donors, we found statistically significant increases in...
statistical significance was determined by independent experiments with at least four culture wells per group. SEM per input progenitor are shown. Similar results were obtained in four independent experiments with five to eight mice per group. All error values depict SEM.

Six d later by flow cytometry. Absolute yields of myeloid cells were determined and placed in defined, lymphoid-supporting liquid cultures for 12 d before flow cytometry. Boxes show percentages of B220+CD19+ lymphocytes that were generated. p = 0.02. These data are representative of that found in four independent experiments with five to eight mice per group. All error values depict SEM. D, CLP defined as in Supplemental Fig. 1 were recovered from the two groups of mice after 4–6 wk of chronic exposure and placed to high purity. These were placed in defined lymphoid-supporting cultures (see Materials and Methods) for 8 d before harvest and flow cytometry. Yields of CD19+B220+B-lineage lymphocytes and CD11c+ DC ± SEM per input progenitor were calculated. E, CMP sorted as shown in Supplemental Fig. 1B were placed in myeloid-supporting cultures, and their potential for generation of myeloid marker bearing cells was determined 8 d later by flow cytometry. Absolute yields of myeloid cells ± SEM per input progenitor are shown. Similar results were obtained in four independent experiments with at least four culture wells per group. Statistical significance was determined by t test analysis.

cycling Ki-67+Flt3− LSK cells recovered from primary and secondary transplant recipients (Supplemental Fig. 3B).

Analysis of the primitive LSK fraction of marrow in recipients also suggested that stem cells harvested from LPS-treated donors were compromised in long-term self-renewal potential (Fig. 3E). This was apparent even in the primary transplants, and cells originating from LPS-treated animals (CD45.2+) competed poorly with control donor (CD45.1+) stem cells. Secondary transplants revealed that LPS-treated stem cells were defective even relative to radio-resistant host F1 (CD45.1+CD45.2+) stem cells (Fig. 3E). This was also apparent in terms of repopulating units (RU) (42). Although these values were unchanged when control marrow was transplanted, there was an 88% reduction on secondary transplantation of LPS-treated HSC (Fig. 3F). All of these results indicate that chronic LPS exposure permanently harms self-renewal capacity and functional integrity of HSC. A similar experimental design was used to assess the differentiation potentials of HSC highly enriched as CD150+CD48− LSK before transplantation and marrow analysis (Supplemental Fig. 4). Although the sorting procedure might have normalized some of the LPS-induced changes in HSC subsets (see below), HSC derived from treated mice displayed strong myeloid bias in three of five recipient mice.

Thus, residence in chronically LPS-treated mice diminished the ability of long-term HSC to sustain their numbers through serial transplantation, and lymphopoietic potential was selectively lost, whereas myeloid output was maintained. These characteristics were durable, with no signs of recovery through two rounds of transplantation in untreated recipient mice over a period of 8 mo.

Some, but not all, hematopoietic changes resemble those described for normal aging

The myeloid skewing we observed in LPS-treated mice has been shown to be a characteristic of old mice (25–28), so we investigated other possible similarities. Members of the E protein family of transcription factors have critical roles at early lymphoid progenitor, MPP, and stem cell stages (43–47). Importantly, it has been demonstrated that levels of the E47 transcription factor markedly decline in CD19+B220+CD43+IgM− pro-B cells during aging (48). Therefore, we sorted pro-B cells according to origin from primary or secondary transplant recipients and then evaluated them by Western blot analyses (Fig. 4A, 4B). E47 protein levels were significantly reduced in pro-B cells generated from marrow originating in LPS-treated mice. A similar assessment was made of pro-B cells, as well as the HSC-containing Flt3− LSK subset of secondary transplants using flow cytometry (Fig. 4C, 4D). Again, there was significant depression of E47 levels. Mean fluorescence intensity (MFI) values ± SEM for pro-B cells generated from control and LPS-treated marrow in secondary transplants were 425 ± 81 and 175 ± 19 (p = 0.02), respectively. Values for Flt3− LSK generated from control and LPS-treated marrow were 2610 ± 358 and 1780 ± 180 (p = 0.04), respectively. Thus, pro-B cells generated after two successive, 4-mo transplants of treated BM had undergone a change previously described in aged mice. Furthermore, the depression of E47 was apparent even in the Flt3− LSK subset.

Recent papers revealed that functionally distinct HSC can be partially resolved by immunostaining and that many HSC in aged mice have high levels of CD150 (49–51). We found that total numbers of CD150−CD48− LSK were elevated by chronic LPS treatment (Fig. 1E, top); this was most apparent for the brightest CD150+ cells, and MFI for CD150 were always increased (Figs. 5A, 5B, 6, bottom panel). Additional informative Abs were identified by microarray analysis and screening (not shown). Whereas CD86 (B7.2) stained most HSC in normal, young mice, a negative population expanded with LPS treatment and aging (48). Therefore, we sorted pro-B cells according to origin from primary or secondary transplant recipients and then evaluated them by Western blot analyses (Fig. 6A, 6B). E47 protein levels were significantly reduced in pro-B cells generated from marrow originating in LPS-treated mice. A similar assessment was made of pro-B cells, as well as the HSC-containing Flt3− LSK subset of secondary transplants using flow cytometry (Fig. 4C, 4D). Again, there was significant depression of E47 levels. Mean fluorescence intensity (MFI) values ± SEM for pro-B cells generated from control and LPS-treated marrow in secondary transplants were 425 ± 81 and 175 ± 19 (p = 0.02), respectively. Values for Flt3− LSK generated from control and LPS-treated marrow were 2610 ± 358 and 1780 ± 180 (p = 0.04), respectively. Thus, pro-B cells generated after two successive, 4-mo transplants of treated BM had undergone a change previously described in aged mice. Furthermore, the depression of E47 was apparent even in the Flt3− LSK subset.

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significantly reduced in LPS-treated but not aged mice (not shown). Although the CD41 integrin chain has typically been used to study HSC in early embryos (53), we observed significant staining of adult HSC with a CD41 Ab labeled with two different fluorochromes (Fig. 6 and data not shown). Although there was no change with LPS treatment, HSC staining for CD41 became much more homogeneous with aging.

Thus, persistent TLR stimulation causes HSC in young mice to acquire some, but not all, properties of HSC in mice maintained for 2 y under pathogen-free conditions. Expansion of rare CD150 Hi HSC subsets in parallel with functional defects is consistent with some type of irreversible injury.

Discussion
The expression of pathogen receptors on hematopoietic cells may be life sparing in some circumstances, such as when production of innate effector cells is boosted during infections. However, these new findings show that HSC are injured by persistent exposure of mice to small amounts of a single TLR ligand. Thus, low-grade host–pathogen interactions may be detrimental to stem cells over time.

Administering very low doses of LPS for an extended period was done to avoid major inflammation and generalized stress. This protocol might reflect conditions in chronic infections such as Gram-negative periodontitis and subacute bacterial endocarditis (18). The fact that thymus cellularity and blood counts were normal indicates that the LPS treatment was well tolerated. However, bone densities were reduced, and other subtle changes were occurring after 4–6 wk of LPS treatment. Previous culture experiments showed that differentiation of myeloid progenitors is promoted, and lymphoid progenitors are directed to become DC by exposure to TLR ligands (15, 34). Similar changes were seen in the marrows of low-dose LPS-treated mice. That is, we found elevated percentages of myeloid lineage and DC. In addition, progenitors isolated from those animals were clearly deficient with respect to lymphopoietic potential.

Although phenotypically defined HSC increased in LPS-treated mice, their repopulating and lymphopoietic potentials dramatically declined. It is clear that this reflects stem cell injury, but we do not know if they were direct and/or indirect targets of TLR ligands.

Subendosteal osteoblasts are thought to be essential components of HSC niches, and experimental manipulations affecting these cells...
influence HSC (54, 55). Osteoblast precursors could be affected by cytokines released during inflammation or express TLR that would allow them to be direct LPS targets (56). In addition to osteoblastic niches, HSC reside in perivascular areas of the BM and recirculate throughout the body (17, 41). LPS-induced chronic inflammation leads to bone remodeling and mobilizes additional hematopoietic cells to the periphery (16, 57). Extended mobilization would have the effect of reducing their residence time in supporting niches.

Selective loss of lymphopoietic potential in HSC and progenitors has also been seen in studies of normal aging and could conceivably contribute to immunosenescence (25–27, 58). The incidence of myeloid versus lymphoid leukemias is also age-related (59). The cause of lineage skewing is unknown, but could involve stable epigenetic changes (60, 61). We report in this study that long-term competence of HSC to produce B and T lymphocytes was severely compromised in low-dose LPS-treated mice. Given that 8 mo had elapsed since LPS treatment, these changes may result from epigenetic regulation.

Chromatin modifications could result in altered patterns of transcription factor abundance, and helix-loop-helix family proteins are attractive candidates. E2A degradation occurs through an MAPK-dependent ubiquitination process (62), and the same mechanism may explain the reduced protein levels in lymphocytes of aged mice (63). Recent studies suggest that members of the E2A family have pivotal importance in the earliest hematopoietic stages (43–47). That is, control of net helix-loop-helix activity permits appropriate lymphoid/myeloid fate decisions, maintenance of stem cell quiescence, and ultimately long-term repopulation capability of HSC. Subnormal levels of E2A transcription factor proteins in pro-B cells correlate with aging (48). We found that the same was true for stem and pro-B cells made from LPS-treated marrow in long-term transplant recipients.

Although rare, HSC are surprisingly heterogeneous, and subsets have been described to be deeply quiescent, latent, intermediate term repopulating, myeloid biased, balanced, or lymphoid biased (49, 51, 64–67). The degree of overlap between subpopulations is far from clear, but a series of recent reports suggest that flow cytometry analyses can be highly informative (49–51). For example, high CD150 density in young mice correlates with high self-renewal potential and myeloid skewing. CD150<sup>hi</sup>/CD48<sup>−</sup> HSC expand during normal aging and are myeloid biased. This could mean that proportions of specialized subsets change over time (68). However, all HSC in old animals differ from those in young mice with respect to homing and self-renewal (24, 50).

We found that TLR stimulation elicited some, but not all, of these changes in HSC subsets. The density of Slamf1<sup>+</sup>/CD150<sup>−</sup> HSC in BM. Marrow was harvested from mice given daily injections of saline or LPS for 4–6 wk. Subsets of HSC in the LSK gate illustrated in Supplemental Fig. 1B were resolved on the basis of CD48 and/or Flt3 expression. A, Densities of CD150 are reflected in MFI. B, HSC identified as CD150<sup>−</sup>/CD48<sup>−</sup> LSK were subdivided into CD150<sup>low</sup> and CD150<sup>hi</sup> fractions (see Supplemental Fig. 1B) that were enumerated in control and LPS-treated mice.
β2) expanded. It is interesting that marrow stromal cells express the CD28 counterreceptor for CD86 (69), and it will be important to learn if either of these molecules are associated with HSC functions. Their absence correlated with poor self-renewal and lymphopoietic potential in parallel with depletion of CD150hi CD48– HSC. Transplantation experiments suggest that CD150hi CD48– HSC can give rise to CD150lo and CD150– subsets with lymphopoietic potential (50, 51, 70). It seems possible that these primordial HSC become injured and unable to make the transition to lymphopoietic cells as a result of age or TLR stimulation.

Some HSC recovered from persistently LPS-treated animals were in cell cycle, but this is not a characteristic of HSC in aged mice (31, 60, 71 and data not shown). We found that two additional parameters could be used to discriminate HSCs in these situations. The VCAM-1 cell adhesion molecule was downregulated by LPS treatment, but not aging. Reciprocally, the CD41 integrin was uniformly upregulated on old, but not TLR-stimulated by LPS treatment, but not aging. Therefore, HSC can give rise to CD150 Lo and CD150hi HSC. Transplantation experiments suggest that CD150 hi HSC can give rise to CD150lo and CD150– subsets with lymphopoietic potential in parallel with depletion of CD150hi CD48– HSC. Transplantation experiments suggest that CD150hi CD48– HSC can give rise to CD150lo and CD150– subsets with lymphopoietic potential (50, 51, 70). It seems possible that these primordial HSC become injured and unable to make the transition to lymphopoietic cells as a result of age or TLR stimulation.

We conclude that HSC in chronically LPS-treated animals are functionally compromised and have some, but not all, age-related characteristics. This and other recent studies suggest that mAb staining as well as transcription patterns can be informative about functionally specialized HSC subsets and serve as biomarkers of normal aging. It should now be possible to determine what population changes correlate with loss of HSC integrity and develop new strategies to protect them. More aggressive management of chronic infections could ensure the competency of HSC to replenish the immune system.

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Disclosures

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