The Journal of Immunology

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J Immunol 2021; 207:1078-1086; Prepublished online 2 August 2021;
doi: 10.4049/jimmunol.2100168
http://www.jimmunol.org/content/207/4/1078

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-6 Generated from Human Hematopoietic Stem and Progenitor Cells through TLR4 Signaling Promotes Emergency Granulopoiesis by Regulating Transcription Factor Expression

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Emergency granulopoiesis, also known as demand-adapted granulopoiesis, is defined as the response of an organism to systemic bacterial infections, and it results in neutrophil mobilization from reservoir pools and increased myelopoiesis in the bone marrow. Indirect and direct initiating mechanisms of emergency granulopoiesis have been hypothesized. However, the detailed mechanism of hyperactive myelopoiesis in the bone marrow, which leads to granulocyte left shift, remains unknown. In this study, we report that TLR4 is expressed on granulo-monocytic progenitors, as well as mobilized human peripheral blood CD34$^+$ cells, which account for 0.2% of monocytes in peripheral blood, and ~ 10% in bone marrow. LPS, a component of Gram-negative bacteria that results in a systemic bacterial infection, induces the differentiation of peripheral blood CD34$^+$ cells into myelocytes and monocytes in vitro via the TLR4 signaling pathway. Moreover, CD34$^+$ cells directly responded to LPS stimulation by activating the MAPK and NF-κB signaling pathways, and they produced IL-6 that promotes emergency granulopoiesis by phosphorylating C/EBPα and C/EBPβ, and this effect was suppressed by the action of an IL-6 receptor inhibitor. This work supports the finding that TLR is expressed on human hematopoietic stem and progenitor cells, and it provides evidence that human hematopoietic stem and progenitor cells can directly sense pathogens and produce cytokines exerting autocrine and/or paracrine effects, thereby promoting differentiation. *The Journal of Immunology*, **2021, 207: 1078–1086.**

Neutrophils are cells of the innate immune system and function as the body's first line of defense against pathogens. Mature neutrophils differentiate from hematopoietic stem and progenitor cells (HSPCs) and are first distributed in the postmitotic pool of the bone marrow, and they are then distributed in the circulating and marginated pools of peripheral blood vessels (1). In addition to the mobilization of neutrophils from these storage pools, granulocyte hematopoiesis in the bone marrow is enhanced during severe systemic infection with classical Gram-positive or Gram-negative bacteria, a process termed emergency granulopoiesis. This enhancement may result in the appearance of immature neutrophil precursor cells in the peripheral blood, a mechanism referred to as the left shift. The mechanisms of emergency granulopoiesis and left shift, considered as a part of the clinical general knowledge, remain elusive.

The detection of infection is mediated by innate pattern recognition receptors (PRRs), which include TLRs, retinoic acid–inducible gene I (RIG-I)-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors (2–4). Each PRR activates a specific signaling cascade to induce the expression of factors such as proinflammatory cytokines through microbe-specific pathogen-associated molecular patterns (5). TLRs play a role in hematopoiesis (6–8) and the regulation of myeloid lineage commitment (9).

Two principal mechanisms of initiation of emergency granulopoiesis, namely indirect and direct mechanisms, mediated by the action of nonhematopoietic and hematopoietic cells have been reported (10). Several nonhematopoietic cells, including endothelial cells, mesenchymal stromal cells, reticular cells, and hematopoietic cells, interact intricately to elicit an appropriate hematopoietic response to an immunological emergency (11–13). Pathogenic microorganisms are recognized via PRRs such as TLRs, regardless of the indirect or direct pathway involved. Boettcher et al. (14) reported that LPS, a TLR4 ligand, induced emergency granulopoiesis by activating the MyD88 signal in endothelial cells, which constitutes an indirect pathway. Ziegler et al. (15) reported that LPS-stimulated human bone marrow mesenchymal stromal cells support myeloid cell development by increasing the secretion of cytokines in an indirect pathway. Wang et al. (16) also reported similar observations using human cord blood CD34$^+$ cells. A recent study reported by Kwak et al. (17) revealed that mature myeloid cell–derived reactive

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Received for publication February 19, 2021. Accepted for publication June 16, 2021.

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This work was supported by the Japan Society for the Promotion of Science (KAKENHI Grants 17K16177 to Y.-M.G. and 18K08315 to K.U.) and by the Rechallenge Fund from Akita University to Y.-M.G.

Y.-M.G. designed the study, designed and performed experiments, analyzed data, and wrote the manuscript; Y.S. designed and performed experiments, analyzed data, and wrote the manuscript; T.G., K.U., K.A., and I.K. performed experiments and contributed to data analysis; and K.S., H.W., and N.T. interpreted the data and contributed to writing the manuscript. All authors read and approved the final manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: EPO, erythropoietin; GPA, glycophorin A; HSPC, hematopoietic stem and progenitor cell; LAP*, liver-enriched activating protein*; LIP, liver-enriched inhibitory protein; MD-2, myeloid differentiation protein-2; MRA, tocilizumab; PI, propidium iodide; PRR, pattern recognition receptor; SCF, stem cell factor; TAK1, TGF-β-activated kinase 1; TPO, thrombopoietin; 5*z*-7, (5*Z*)-7-oxozeaenol.

oxygen species externally regulate the proliferation of myeloid progenitors during emergency granulopoiesis. In contrast, Zhao et al. (18) reported that HSPCs respond to TLR agonist stimulation directly and secrete considerable amounts of inflammatory cytokines and growth factors, which promote the development of demand-adapted granulopoiesis in mice. De Luca et al. (19) have reported that TLR1/2 agonists promote the differentiation of hematopoietic stem cells into early myeloid progenitors by upregulating the expression of transcription factors in cord blood cells. The detailed mechanism of emergency granulopoiesis in humans remains to be elucidated.

Myeloid specification in early HSPCs and subsequent differentiation of granulo-monocytic progenitors during steady-state conditions require the coordinated temporal activation of a considerable number of myeloid transcription factors, including the Ets family of transcription factors, C/EBP, and core-binding factor families, among others (20, 21). The Ets family transcription factor PU.1 and the representative members of the C/EBP family of transcription factors C/EBPα and C/EBPβ are essential for myeloid lineage differentiation. C/EBPα is the key transcription factor of neutrophil differentiation at a steady state, whereas C/EBPβ is known to play an important role in emergency granulopoiesis (22). The three isoforms of C/EBPβ, liver-enriched activating protein* (LAP*), LAP, and liver-enriched inhibitory protein (LIP), are transcription factors that exert a major cell proliferation-expanding effect (23). The signaling pathways responsible for the switch from C/EBPα-dependent steady-state granulopoiesis to C/EBPβ-dependent emergency granulopoiesis are poorly understood.

$CD34^+$ cells are found in 0.2% of PBMCs, which is ∼10% of the frequency in bone marrow. Peripheral blood $CD34^+$ cells may be part of the stem cell population that responds to emergency granulopoiesis. In the current study, we investigated the mechanism of emergency granulopoiesis by inducing in vitro differentiation of mobilized human $CD34^+$ cells, obtained from peripheral blood, into granulocytes using LPS to mimic a severe systemic Gram-negative bacterial infection. Furthermore, we attempted to elucidate the mechanism of switching from C/EBPα-dependent steady-state granulopoiesis to C/EBPβ-dependent emergency granulopoiesis.

Materials and Methods

Reagents

BSA, IMDM, propidium iodide (PI), and LPS were purchased from Sigma (St. Louis, MO). FCS was purchased from Flow Laboratories (McLean, VA) and HyClone Laboratories (Logan, UT). Penicillin and streptomycin were purchased from Invitrogen (Tokyo, Japan). Insulin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Vitamin B12 was purchased from Eisai (Tokyo, Japan), and folic acid was purchased from Takeda Pharmaceutical (Osaka, Japan). IL-3, stem cell factor (SCF), and thrombopoietin (TPO) were generously gifted by the Kirin Brewery (Tokyo, Japan), and erythropoietin (EPO) and G-CSF were purchased from Chugai Pharmaceutical (Tokyo, Japan). C34 (an amino monosaccharide that inhibits TLR4 signaling by blocking the TLR4 coreceptor, myeloid differentiation protein-2 [MD-2]) and (5*Z*)-7-oxozeaenol (5z-7, a TGF-β-activated kinase 1 [TAK1] inhibitor) were purchased from Tocris (Bristol, UK). U0126 (an ERK inhibitor) was purchased from Enzo (Farmingdale, NY). SP600125 (a JNK inhibitor), BAY117082 (an NF-κB inhibitor), and TAK242 (a specific inhibitor of TLR4 signaling that inhibits myeloid differentiation primary response [MyD88] and Toll/IL-1R domain-containing adapter inducing IFN-β [TRIF]–dependent pathways by binding to Cys747 in the intracellular domain of TLR4) were purchased from InvivoGen (San Diego, CA). SB203580 (a p38 MAPK inhibitor) was purchased from EMD Millipore (Burlington, MA). ST2825 (a MyD88 dimerization inhibitor) was purchased from Chemscene (NJ, USA). MRA (tocilizumab; anti–IL-6 receptor Ab) was purchased from Chugai Pharmaceutical (Tokyo, Japan). FITC-labeled mAb specific for CD15 (HI98), FITC-labeled CD45RA, and PE-labeled Abs for CD13 (WM15) were purchased from Becton Dickinson (Mountain View, CA). PE-labeled CD16 was purchased from BioLegend (San Diego, CA). PI was purchased from Sigma-Aldrich (Tokyo, Japan). FITC-labeled glycophorin A (GPA; JC159) and PE-labeled CD34 (BIRMA-k3) were purchased from Dako Japan (Kyoto, Japan). FITC-labeled CD61 and CD14 were purchased from BD Biosciences (Franklin Lakes, NJ). CFSE was purchased from Invitrogen (Tokyo, Japan). TLR4, phospho-NF-κB p65, NF-κB p65, and C/EBPβ Abs were purchased from Santa Cruz; phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, PU.1, phospho-C/EBPα, C/EBPα, and phospho-C/EBPβ Abs were purchased from Cell Signaling Technology; and GAPDH Ab was purchased from MBL International. HRP-conjugated secondary anti-mouse (from KPL) or anti-rabbit Abs were purchased from Cell Signaling Technology.

Cell preparation

G-CSF–mobilized $CD34^+$ cells were purified from peripheral blood samples obtained from healthy volunteers and were stored in liquid nitrogen until further use as per methods described previously (24). Informed consent was obtained from each subject before participation in the study, which was pre-approved by the Akita University School of Medicine Committee for the Protection of Human Subjects.

To generate granulocyte progenitor cells, $CD34^+$ cells were thawed and prepared for culture (24). Cells were cultured in granulocytic medium (IMDM containing 20% FCS, 10% heat-inactivated pooled human AB serum, 1% BSA, 10 μg/ml insulin, 0.5 μg/ml vitamin B_{12}, 15 μg/ml folic acid, 50 nM 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin) in the presence of 50 ng/ml G-CSF at a cell density of 1×10^5 cells/ml. Cells were maintained in an incubator at 37°C in a 5% CO_2 atmosphere (25). The cells were harvested on respective days and resuspended in 2 ml of IMDM containing 0.1% BSA. In this culture system, ∼10% of the monocytic cells were also generated simultaneously.

To simultaneously generate erythroid, neutrophilic, and megakaryocytic progenitor cells, $CD34^+$ cells were suspended at a density of 2×10^4 to 3×10^4 cells/ml in the presence of 50 ng/ml IL-3, 50 ng/ml SCF, 2 IU/ml EPO, 50 ng/ml G-CSF, and 100 ng/ml TPO (24, 26). The yield and viability were assessed using dye exclusion with 0.2% trypan blue dye and a hemocytometer.

Flow cytometry

The cells were washed twice with IMDM containing 0.3% BSA solution. The cells were then incubated with FITC and PE-labeled mAbs and washed twice with a staining medium containing 10 mM PBS (pH 7.4), 0.5% BSA, and 2 mM EDTA, and analyzed using a FACSCanto II (BD Biosciences, Franklin Lakes, NJ) (24). Each isotype Ab was used as a negative control for gating strategies. Dead cells were excluded using PI.

CFSE dilution assay

Mobilized human peripheral blood $CD34^+$ cells were thawed and washed three times with IMDM containing 20% FCS, IMDM containing 10% FCS, and IMDM containing 0.3% BSA, respectively. The cells were resuspended in 1 ml of PBS containing 1% FCS, and 0.25 μl of 10 mM CFSE was added and vortexed quickly. The cells were promptly incubated at 37°C in a water bath for 7 min and washed with 10 ml of PBS.

Immunoblotting

$CD34^+$ cells were treated with C34 or MRA and left untreated, followed by exposure to 10 μg/ml LPS for the indicated time. Cells were lysed in sample buffer (0.25 mM Tris-HCl [pH 6.8], 5% glycerol, 1% SDS, 17.5 mM DTT, and 0.01% bromophenol blue). Depending on the condition, lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100: TBS) and protease and phosphatase inhibitor cocktail (Abcam, Cambridge, UK) were added, and the mixture was boiled for 15 min. Equal amounts of lysate were analyzed by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride or nitrocellulose membranes and the membranes were blocked using TBST containing 3–5% skim milk for 1 h before incubation with primary Abs (TLR4, phospho-NF-κB p65, NF-κB p65, C/EBPα, phospho-C/EBPα, C/EBPβ, phospho-C/EBPβ, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, PU.1, and GAPDH Ab) overnight in 5% BSA and TBST at room temperature. The membranes were incubated with HRP-conjugated secondary anti-mouse or anti-rabbit Abs for 1 h in 5% BSA and TBST or 5% skim milk and TBST. The ECL Prime Western blotting detection reagent or ECL Select Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) was used for color development. The intensities of immunoreactive protein bands were quantified using the ChemiDoc XRS imaging system (Bio-Rad, Tokyo, Japan). The relative expression levels of the analyzed proteins were normalized with GAPDH or total protein expression levels.

ELISA

$CD34^+$ cells were pretreated with 5z-7 for 1 h and the procedure was followed by exposure to 10 μg/ml LPS for 48 h. The supernatants after centrifugation were collected and stored at −20°C until further use. The IL-6 expression levels in the culture supernatants were analyzed using the human IL-6 ELISA MAX standard set (BioLegend, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the Student *t* test for parametric data and the Mann–Whitney *U* test for nonparametric data. The *p* values <0.05 were considered to be significant in all analyses.

Results

LPS promotes the differentiation of isolated human $CD34^+$ cells to granulocytes and monocytes

To examine the effects of LPS on the growth of hematopoietic progenitors, human $CD34^+$ cells were cultured for 7 d in a liquid medium in the presence of multiple growth factors, including G-CSF, EPO, and TPO. These conditions led to the simultaneous differentiation of myeloid ($CD13^+$), erythroid (GPA^+), and megakaryocytic ($CD61^+$) progenitors. We observed that LPS promoted the growth of both myeloid (42.3 ± 3.4% $CD13^+$ cells, 24.6 ± 4.6% $CD15^+$ cells, and 7.8 ± 1.3% $CD14^+$ cells) and erythroid (48.2 ± 5.3% GPA^+ cells) progenitor lineages, but it did not affect the growth of megakaryocytic (0.6 ± 0.2% $CD61^+$ cells) progenitors (Fig. 1A, 1B). When only G-CSF was used to differentiate $CD34^+$ cells into myeloid cells, $CD13^+$ cells were 98.6 ± 0.6%, and the effects of LPS on the growth of granulocytes and monocytes were dose-dependent (Fig. 1C). The percentages of granulocytes (53.5 ± 4.6% $CD15^+$ cells) and monocytes (25.6 ± 5.4% $CD14^+$ cells) were almost the same in the presence and absence of LPS, but the number of granulocytes and monocytes increased by 1.5- to 2-fold in the presence of LPS (Fig. 1D, 1E). The cells on day 7 were weakly positive for CD16 (0.8 ± 0.5%) and negative for CD45RA (Fig. 1E). Based on the cell morphology (Fig. 1F), the $CD15^+$ cells on day 7 were considered to be myelocytes-metamyelocytes. When $CD34^+$ cells were stained with CFSE and cultured in G-CSF medium, with or without LPS, the cells cultured with LPS exhibited a higher CFSE intensity on days 3, 4, and 5 (Supplemental Fig. 1, top panel). These findings suggest that LPS promotes myeloid progenitor cell proliferation, and the addition of LPS to the culture medium may simulate emergency granulopoiesis observed during bacterial infection in vitro by promoting granulo-monocytic progenitor differentiation.

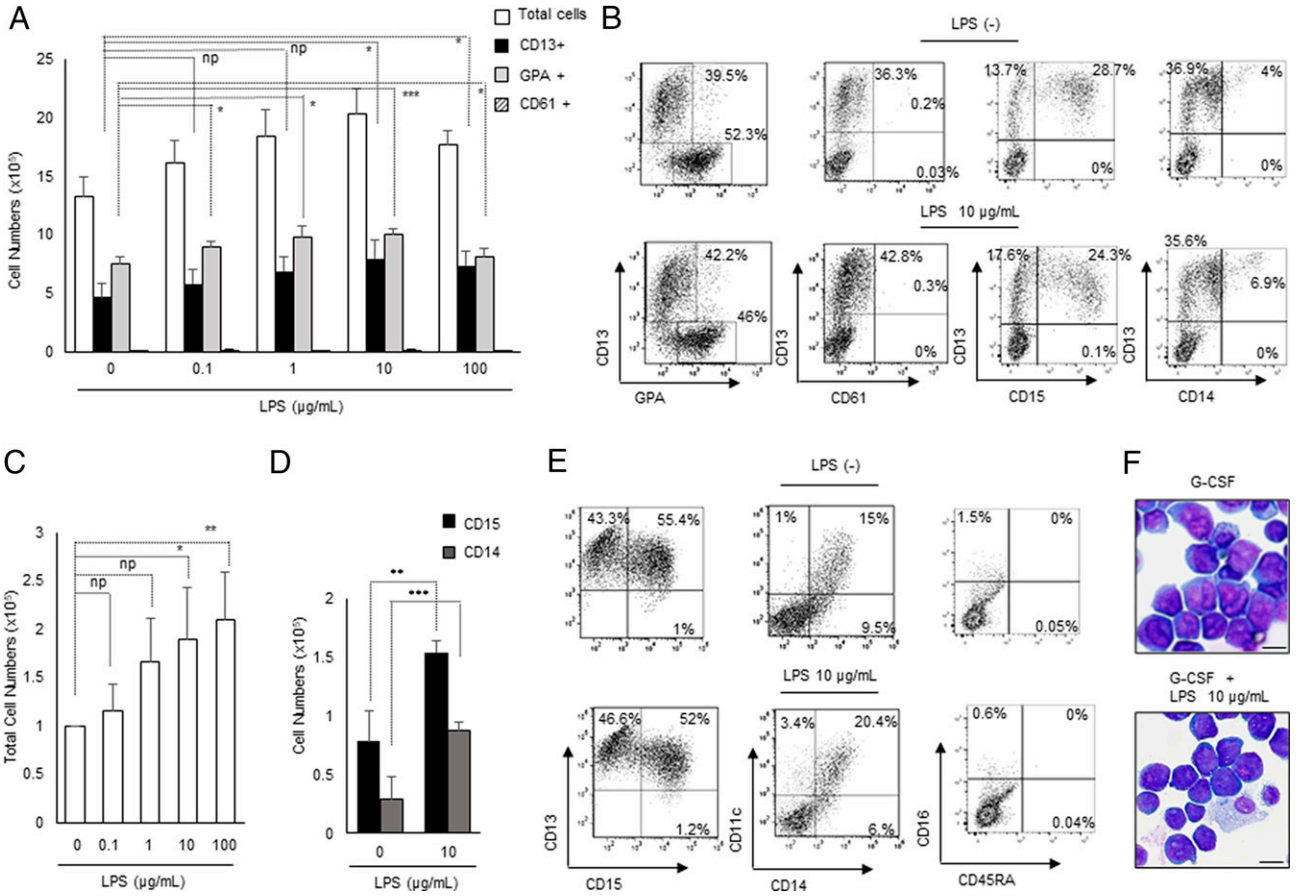

FIGURE 1. Effects of LPS on the growth of hematopoietic progenitors derived from $CD34^+$ cells. (**A**) Purified human $CD34^+$ cells at a cell density of 2×10^4 cells/ml were cultured with multilineage medium containing IL-3, SCF, EPO, G-CSF, TPO, and various concentrations of LPS ranging from 0 to 100 μg/ml. On day 7, the total cell yield was estimated, and CD13, GPA, and CD61 expression levels were assessed using flow cytometry. Data represent mean ± SD of four independent experiments. (**B**) Representative flow cytometry profile as described in (A). (**C**) $CD34^+$ cells, at a cell density of 1×10^5 cells/ml, were cultured using a single-lineage medium containing G-CSF, as well as various concentrations of LPS, ranging from 0 to 100 μg/ml. On day 7, the total cell yield was estimated and all values were normalized to the value of control (LPS-negative). (**D**) $CD34^+$ cells were cultured with G-CSF at a cell density of 1×10^5 cells/ml, with or without 10 μg/ml LPS. On day 7, the total cell yield was estimated and CD15, CD14, CD16, and CD45RA expression levels were determined using flow cytometry. Data represent mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. np indicates no significance. (**E**) Representative flow cytometry profile as described in (D). (**F**) Representative May–Grünwald–Giemsa staining of cytospin preparations of (C). Original magnification, ×400. Scale bars, 10 μm.

LPS promotes the early stages of granulo-monocytic progenitor differentiation and promyelocyte proliferation

To further understand the kinetics of effects of LPS on myeloid progenitor cell growth, purified CD34$^+$ cells were cultured in the G-CSF medium in the presence and absence of LPS. Total cells, CD15$^+$ cells, and CD14$^+$ cells were counted after 7 d of culture (Fig. 2A). The cell yield substantially increased in cultures that contained LPS, after 4 d in culture, but the number of cells did not increase substantially in culture containing only G-CSF. Dead cells were found for several days after the start of culture, and cell viability with LPS was better than without LPS (Fig. 2B). When CD34$^+$ cells were cultured for 7 d in G-CSF medium, the addition of LPS to the medium even after 3 d of culture resulted in a significant increase in cell numbers (Fig. 2C). To eliminate the effect of cytokine circulation generated due to long-term exposure of LPS, the medium was changed every 6 h on days 2 and 3. Although the effect of cytokine circulation cannot be completely eliminated, short-term exposures of LPS resulted in similar LPS-stimulated cell growth–promoting effects with these long-term exposures (Fig. 2D). Surface markers of the cells on day 2 were similar, with or without LPS, but on day 4, there were more CD34$^-$ cells among the LPS-treated cells (Fig. 2E). CD34 was present on myeloblasts, monoblasts, and granulo-monocytic progenitors, but absent on promyelocytes and promonocytes. The expression of CD13 and CD15 was similar on day 4, but the expression of CD34 lowered on the cells treated with LPS, suggesting that the cells differentiated more under LPS stimulation. CD34$^+$ cells were labeled with CFSE and cultured in G-CSF medium, with or without LPS. CD34$^-$ cells showed lower CFSE intensity in the presence of LPS on days 3, 4, and 5 of culture (Supplemental Fig. 1, second panel), but the expression of CFSE in CD34$^+$ cells was similar with and without LPS (Supplemental Fig. 1, third panel). Moreover, CFSE intensity in

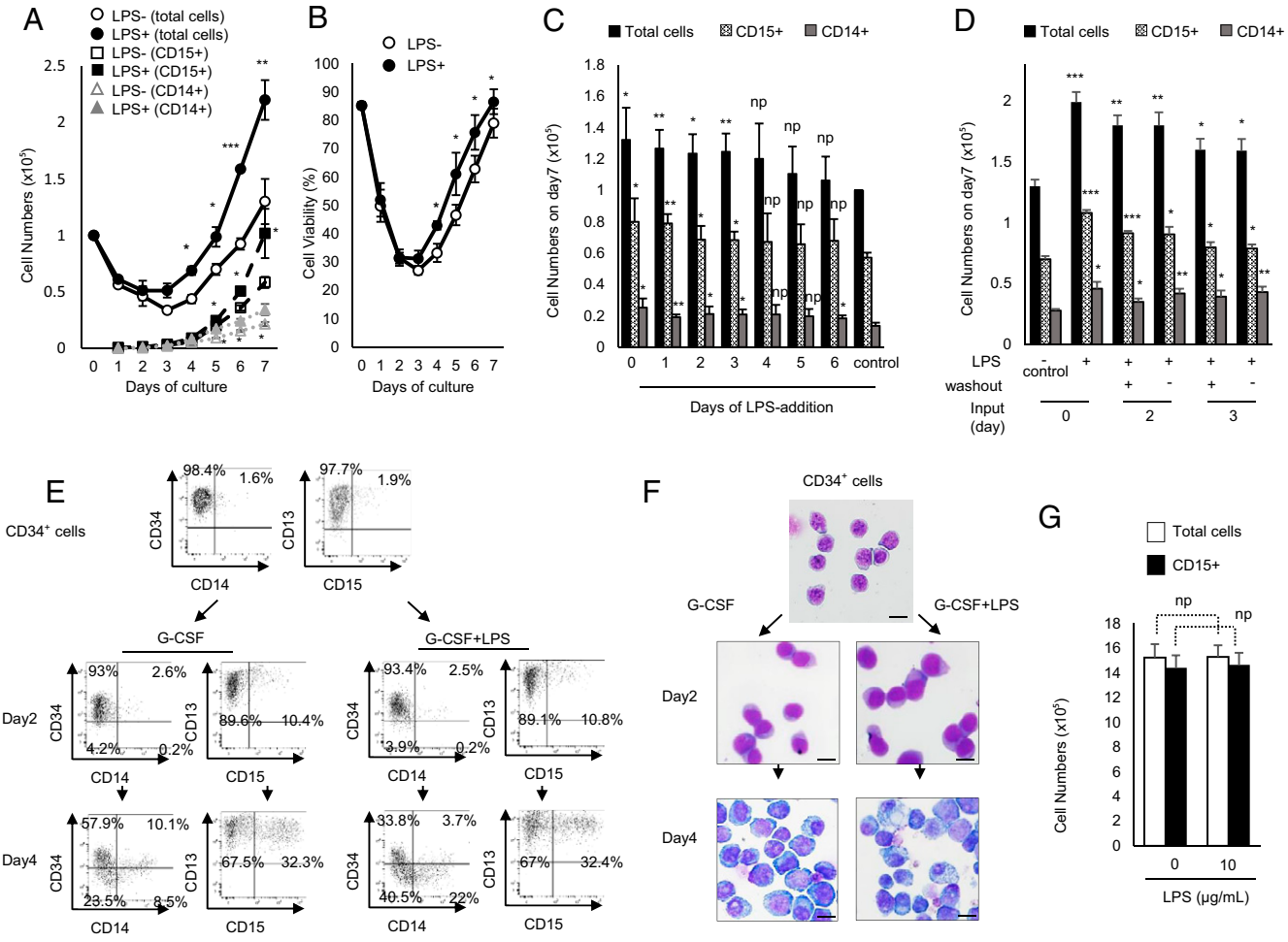

FIGURE 2. LPS promotes the early stages of granulo-monocytic progenitor differentiation. (**A**) Purified human CD34$^+$ cells were cultured in a G-CSF medium, with or without 10 μg/ml LPS. On the indicated days, the total cells were collected and counted. CD15 and CD14 expression levels were determined using flow cytometry. Data represent mean ± SD of three independent experiments. (**B**) CD34$^+$ cells were cultured in a G-CSF medium, with or without 10 μg/ml LPS. On the indicated days, the cells were harvested and viability was analyzed with PI using flow cytometry. Data represent mean ± SD of three independent experiments. (**C**) CD34$^+$ cells were cultured for 7 d in a G-CSF medium. On the indicated days, 10 μg/ml LPS was added to the medium and the total cell yield was estimated on day 7, and CD15 and CD14 expression levels were determined using flow cytometry. Data represent mean ± SD of four independent experiments. (**D**) Input 0: CD34$^+$ cells were cultured in a G-CSF medium and 10 μg/ml LPS was added from day 0 onward. Input 2 or input 3: LPS was removed by washing with 0.3% BSA/IMDM after preincubation with LPS for 6 h and culture was continued without LPS addition until day 7. The total cell yield was estimated on day 7, and CD15 and CD14 expression levels were determined using flow cytometry. Data represent mean ± SD of three independent experiments. (**E**) CD34$^+$ cells were cultured in a G-CSF medium with or without 10 μg/ml LPS. CD34$^+$ cells and the cells on the indicated days were harvested and surface markers were analyzed using flow cytometry. Results are representative of two independent experiments. (**F**) Morphology of the generated cells. CD34$^+$ cells were cultured in a G-CSF medium, with or without 10 μg/ml LPS, for 2 or 4 d and subjected to May–Grünwald–Giemsa staining. Results are representative of three independent experiments. Original magnification, ×400. Scale bars, 10 μm. (**G**) CD34$^+$ cells were cultured in G-CSF medium. After 7 d, the cells were collected and washed using 0.3% BSA/IMDM and then cultured for an additional 5 d (until day 12) in a G-CSF medium, with or without 10 μg/ml LPS. Data represent mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. np indicates no significance.

$CD15^+$ cells was lower in the presence of LPS (Supplemental Fig. 1, bottom panel). These findings suggested that LPS promoted the proliferation of the cells that differentiated to promyelocytes.

On day 2, the cells had uniform cell morphology and were considered to be granulo-monocytic progenitors (Fig. 2F). On day 4, the size of the cells increased and azurophilic granules also appeared, suggesting a promyelocytic stage (Fig. 2F). Although the proportion of monocytes continued to increase, there was no significant difference in granulocyte morphology, which was considered to be in the myelocytic-metamyelocytic stage of differentiation, after 7 d of culture using LPS-mediated stimulation (Fig. 1F). In contrast, LPS did not affect the proliferation of more mature granulocytes, which were obtained after the late myelocytic stage (Fig. 2G). These findings indicate that LPS promotes HSPC differentiation to granulo-monocytic progenitors and promotes proliferation of promyelocytes in the early stage of development, but it may not significantly affect myelocyte-metamyelocyte proliferation in later stages.

LPS promotes $CD34^+$ cell differentiation to granulo-monocytic progenitors via TLR4 signaling

Purified $CD34^+$ cells were cultured in a G-CSF medium in the presence or absence of LPS, and their TLR4 protein expression was determined at the indicated time points by immunoblotting. THP-1 cells, monocyte-like cells, were used as a positive control (data not shown). As illustrated in Fig. 3A, $CD34^+$ cells and the cultured cells, from days 1 to 3, expressed TLR4. Although there is no significant difference, TLR4 expression seemed to increase in the presence of LPS on days 1–3.

To verify that LPS promoted differentiation of granulo-monocytic progenitors via the TLR4 pathway, $CD34^+$ cells were incubated with C34 (an inhibitor of the TLR4 coreceptor MD-2) (Fig. 3B) or TAK242 (a specific inhibitor of TLR4 signaling) (Fig. 3C) or ST2825 (a MyD88 inhibitor) (Fig. 3D) in a G-CSF medium in the presence or absence of LPS. The addition of any of the inhibitors suppressed the activated myeloid expansion by LPS. However, TLR4 expression was not attenuated by the addition of any of these inhibitors (Supplemental Fig. 2). C34 is an amino monosaccharide that inhibits TLR4 signaling by docking with the hydrophobic pocket of the TLR4 coreceptor MD-2. TAK-242 is a specific inhibitor of TLR4 signaling that inhibits MyD88 and TRIF-dependent pathways by binding to Cys747 in the intracellular domain of TLR4. ST2825 is a specific MyD88 dimerization inhibitor. These inhibitors block the TLR4 signaling pathway, but they may not affect TLR4 expression itself. The TAK1 inhibitor, 5z-7, inhibited the downstream signal of MyD88 and the upstream signal of both the NF-κB and MAPK pathways, thus inhibiting the promotion of activated myeloid expansion by LPS (Fig. 3E). These results suggest

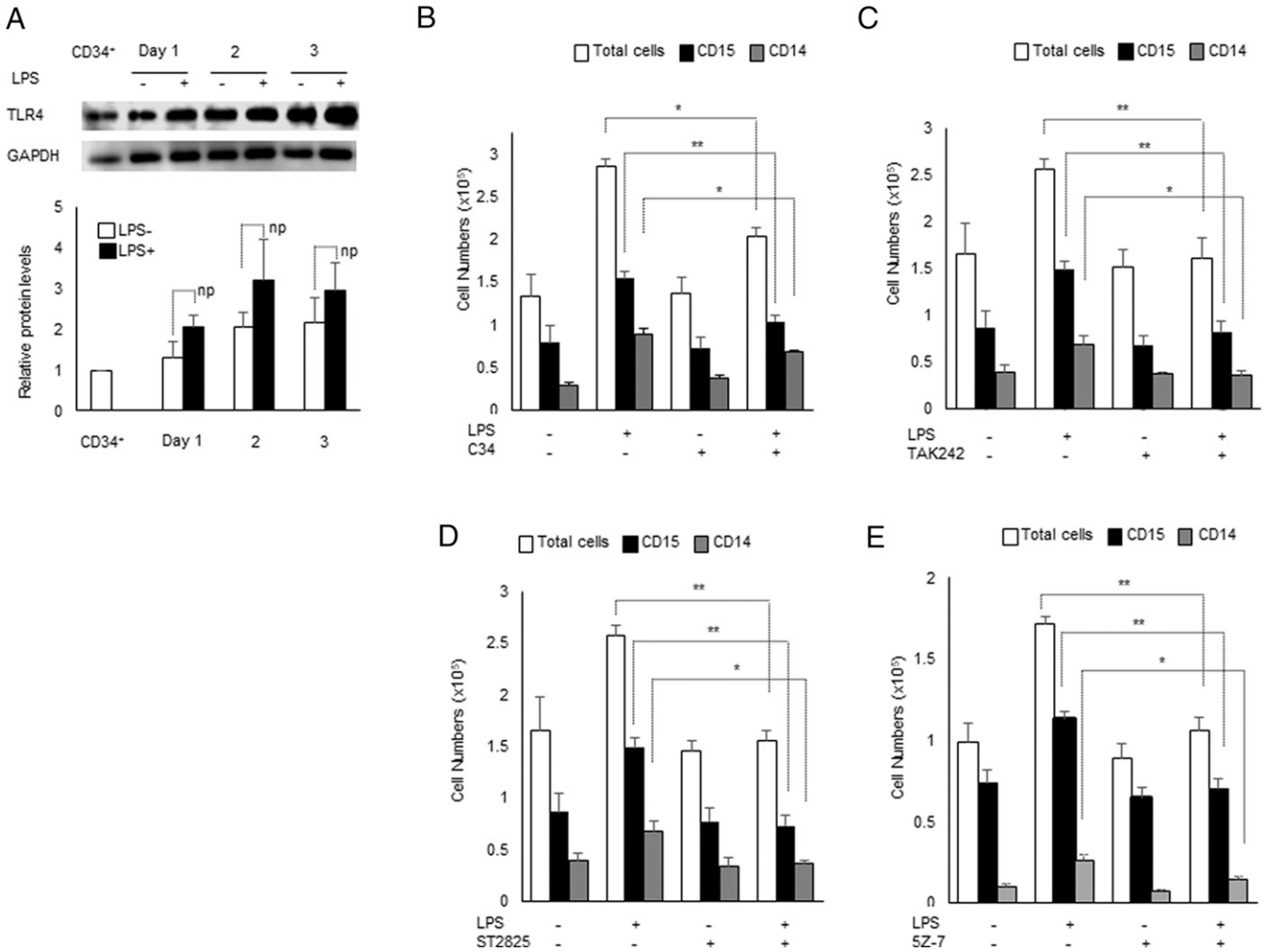

FIGURE 3. LPS promotes $CD34^+$ cell differentiation to granulo-monocytic progenitors via TLR4 signaling. (**A**) TLR4 expression on hematopoietic stem cells and hematopoietic progenitor cells. $CD34^+$ cells were cultured in G-CSF medium with or without the addition of 10 μg/ml LPS until the indicated days. Then, cells were extracted for immunoblotting analysis. GAPDH expression was considered as a loading control. (**B–E**) Effects of the inhibition of TLR4 signaling on $CD34^+$ cell differentiation. $CD34^+$ cells (1×10^5) were pretreated with (B) 10 μM C34 (TLR4 inhibitor), (C) 10 μM TAK242 (TLR4 inhibitor), (D) 2.5 μM ST2825 (MyD88 inhibitor), or (E) 0.5 μM 5z-7 (a TAK1 inhibitor) for 6 h, then cultured with or without addition of 10 μg/ml LPS. After 7 d, the cells were harvested and analyzed using flow cytometry. Data represent mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$. np indicates no significance.

that LPS promotes CD34$^+$ cell differentiation to granulo-monocytic progenitors via TLR4 signaling.

LPS-stimulated CD34$^+$ cells activate the NF-κB and MAPK signaling pathways

Because the NF-κB and MAPK signaling pathways are the classical downstream pathways of TLR4 signaling, we investigated whether these pathways were activated by LPS stimulation of CD34$^+$ cells. As shown in Fig. 4A, levels of phosphorylated p65, a subunit of the NF-κB, ERK, JNK, and p38 MAPK signaling pathways, were enhanced by LPS stimulation and eliminated by C34, the TLR4 inhibitor, except for p38. Next, we assessed whether these pathways were involved in the promotion of activated myeloid expansion by LPS using BAY117082 (an NF-κB inhibitor), u0126 (an ERK MAPK inhibitor), SP600125 (a JNK MAPK inhibitor), and SB203580 (a p38 MAPK inhibitor). Except for the p38 MAPK inhibitor, all inhibitors significantly suppressed the promotion of activated myeloid expansion by LPS (Fig. 4B). Consistent with previous reports, the LPS-activated TLR4 signaling pathway in human peripheral blood CD34$^+$ cells also uses NF-κB and MAPK signaling pathways.

LPS promotes differentiation of CD34$^+$ cells to granulo-monocytic progenitors via IL-6

IL-1β, IL-6, TNF-α, and GM-CSF are known to be the major cytokines produced by cells of the myeloid lineage and are important for the sustenance of the myeloid lineage. Thus, we analyzed the changes in the levels of these cytokines after the addition of LPS. IL-6 secretion was significantly increased after the addition of LPS, from day 1 of culture, and the apparent increase in cell number coincided with the rapid increase in IL-6 secretion on day 4 (Figs. 2A, 5A). However, IL-1β, TNF-α, and GM-CSF levels did not change (data not shown). The 5z-7, which significantly suppressed the activated myeloid expansion effect of LPS, also suppressed the increased secretion of IL-6 (Fig. 5B). To confirm whether IL-6 was directly associated with increased granulocyte and monocyte differentiation, we added MRA, an anti–IL-6 receptor Ab to the medium. MRA markedly inhibited the activated myeloid expansion effect of LPS (Fig. 5C), suggesting that LPS promoted CD34$^+$ cell differentiation to granulo-monocytic progenitors, primarily via IL-6.

IL-6 promotes emergency granulopoiesis by enhancing the phosphorylation of transcription factor C/EBPβ

Because PU.1, C/EBPα, and C/EBPβ are essential components for myeloid lineage differentiation, and as C/EBPβ is known to play an important role in emergency granulopoiesis, we analyzed the changes in the expression of these transcription factors, after addition of LPS, using immunoblotting. Furthermore, to confirm whether IL-6 was directly associated with the changes in the expression of these transcription factors, PU.1, C/EBPα, and C/EBPβ protein expression levels and the phosphorylation levels of C/EBPα and C/EBPβ were determined by immunoblotting after the addition of MRA (anti–IL-6 receptor Ab). The expression of PU.1 was increased by LPS addition, but IL-6 inhibition did not suppress the increase in PU.1 level (Fig. 6A), suggesting that IL-6 was not involved. The total

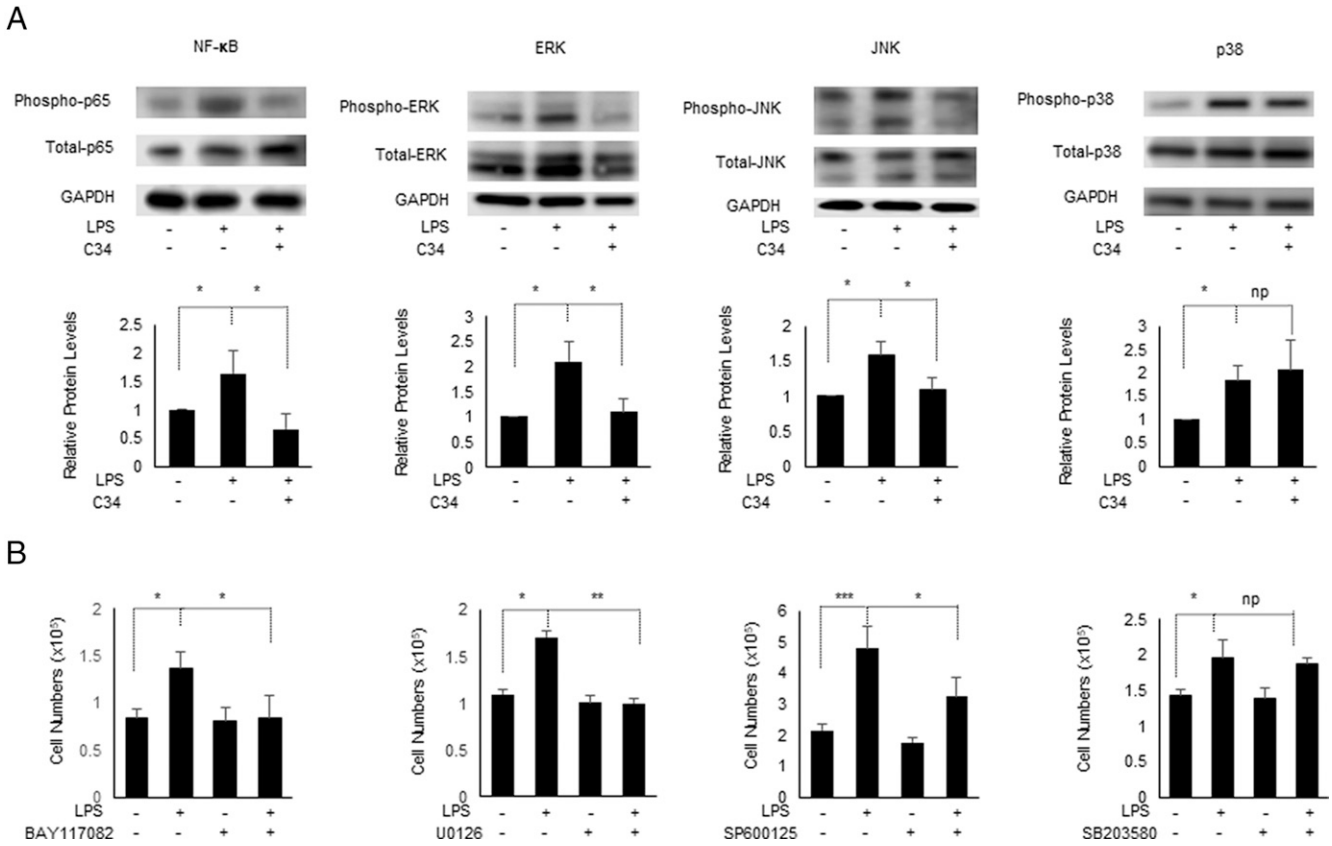

FIGURE 4. LPS stimulates CD34$^+$ cells and activates the NF-κB and MAPK signaling pathways. (**A**) After 2 days of incubation in a G-CSF medium without added LPS, CD34$^+$ cells were pretreated with or without 10 μM C34, and then stimulated for 4 h using 10 μg/ml LPS on day 2. Equal amounts of total proteins were electrophoresed and proteins were blotted for the detection of phosphorylated NF-κB p65 and total p65, phosphorylated ERK and total ERK, phosphorylated JNK, and total JNK, and phosphorylated p38 and total p38, respectively. The relative expression levels of the analyzed proteins were normalized with total protein expression levels. (**B**) CD34$^+$ cells were pretreated in the presence of 1 μM BAY117082 (an NF-κB inhibitor) for 2 h, in the presence of 5 μM U0126 (an ERK inhibitor) for 3 h, in the presence of 10 μM SP600125 (a JNK inhibitor) for 1 h, and in the presence of 10 μM SB203580 (a p38 inhibitor) for 45 min, and cells were then cultured with or without the addition of 10 μg/ml LPS. Seven days later, the cells were harvested and the total cell number was determined. Data represent mean ± SD of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. np indicates no significance.

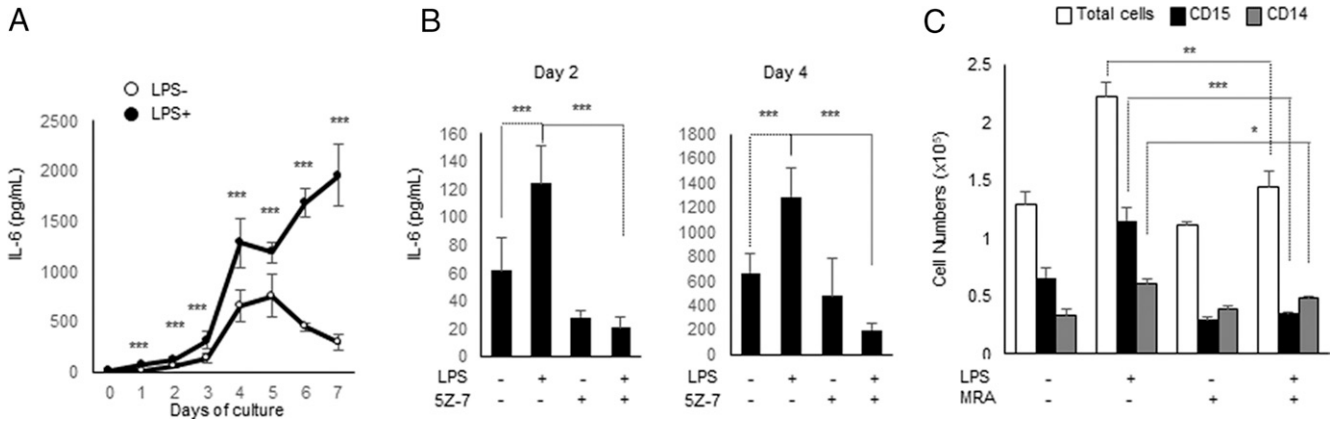

FIGURE 5. LPS promotes $CD34^+$ cell differentiation to granulo-monocytic progenitors via IL-6. (**A**) $CD34^+$ cells were cultured in G-CSF medium with or without the addition of 10 μg/ml LPS, and the supernatant was harvested on the indicated days. IL-6 levels in the supernatant were analyzed using ELISA. (**B**) $CD34^+$ cells (1×10^5) pretreated for 1 h with 0.5 μM 5z-7 were incubated with or without the addition of 10 μg/ml LPS, and the supernatant was harvested after 2 or 4 days. IL-6 levels in the supernatant were analyzed using ELISA. (**C**) $CD34^+$ cells (1×10^5) pretreated for 45 min with 10 μg/ml MRA (anti–IL-6 receptor inhibitor) were incubated with 10 μg/ml LPS. Seven days later, the cells were harvested and analyzed using flow cytometry. For (A)–(C), data represent mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. np indicates no significance.

protein expression of C/EBPα did not increase after treatment with LPS, but the phosphorylation of C/EBPα increased. However, MRA inhibited this increase in phosphorylation (Fig. 6B). Phosphorylation of LAP*, LAP, and LIP was enhanced by LPS addition, and it was diminished by MRA addition (Fig. 6C). These results suggest that IL-6 promoted emergency granulopoiesis and the differentiation of monocyte-macrophage lineage by enhancing C/EBPβ and C/EBPα phosphorylation, respectively, following LPS stimulation.

Discussion

In this study, we confirmed the expression of LPS on mobilized human peripheral blood $CD34^+$ cells and created a model of emergency granulopoiesis in human peripheral blood $CD34^+$ cells during bacterial infection, using LPS, under defined experimental conditions in vitro. LPS promoted the differentiation of human peripheral blood $CD34^+$ cells to granulo-monocytic progenitors and proliferation of promyelocytes via the TLR4 signaling pathway. $CD34^+$ cells directly responded to LPS stimulation by activating the MAPK and NF-κB signaling pathways, as in other reports. IL-6 was generated in HSPCs and promoted emergency granulopoiesis by phosphorylating C/EBPα and C/EBPβ. This mainly affects the early differentiation stage from HSPCs to granulo-monocytic progenitors, thus promoting differentiation of monocytes as well as granulocytes.

TLRs are one of the four PRRs to sense pathogenic agents and play an important role in regulating innate immunity. Several previous studies have demonstrated a direct or indirect association

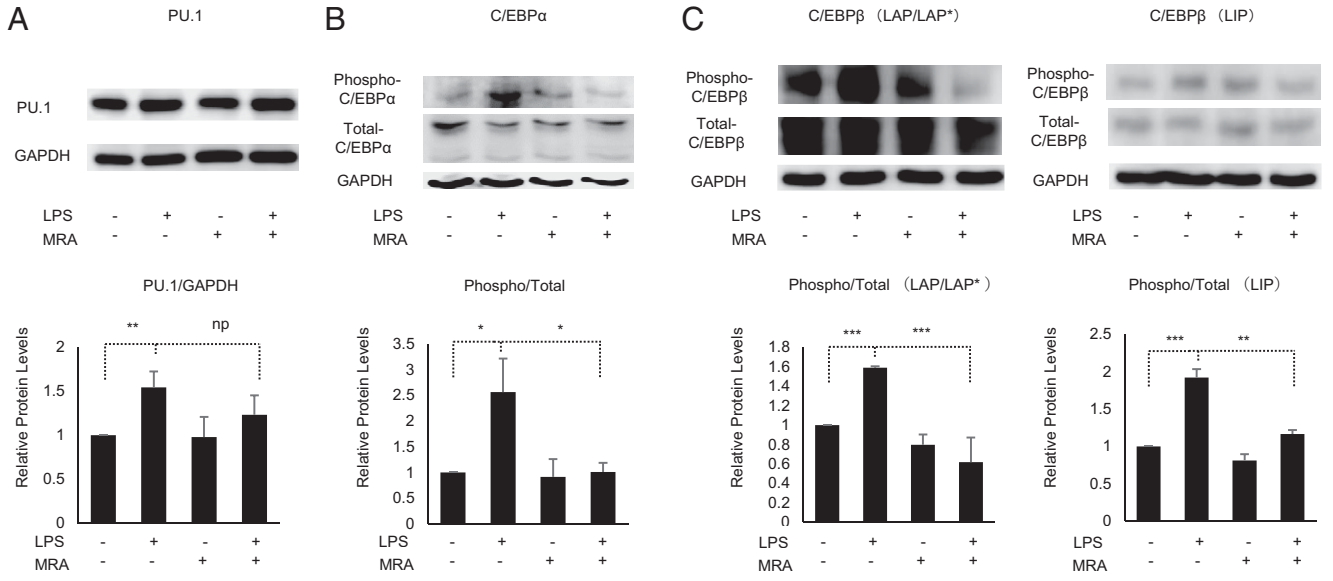

FIGURE 6. IL-6 promotes emergency granulopoiesis by enhancing the phosphorylation of transcription factor C/EBPβ. (**A**) $CD34^+$ cells were pretreated with or without 10 μg/ml MRA for 24 h and then stimulated for 48 h with 10 μg/ml LPS in G-CSF medium. Equal amounts of total proteins were electrophoresed and proteins were blotted for detection of PU.1. The relative expression levels of the analyzed proteins were normalized using GAPDH expression levels. (**B** and **C**) After 2 days of incubation in G-CSF medium without LPS, cells were pretreated with or without 10 μg/ml MRA for 1 h and then stimulated for 4 h with 10 μg/ml LPS addition on day 2. Equal amounts of total proteins were electrophoresed and proteins were blotted for detection of phosphorylated C/EBPα and total C/EBPα and phosphorylated C/EBPβ and total C/EBPβ, respectively. The relative expression levels of the analyzed proteins were normalized with total protein expression levels. For (A)–(C), data represent mean ± SD of four independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. np indicates no significance.

between TLRs and hematopoiesis (6–8). Nagai et al. (7) found that TLRs and their coreceptors were expressed on multipotential hematopoietic stem cells, and TLRs activated the cell cycle by TLR ligation. They also showed that TLR signaling, via the MyD88 adaptor protein, drove differentiation of myeloid progenitors and lymphoid progenitors to dendritic cells. Sioud et al. (8) showed that human bone marrow $CD34^+$ progenitor cells constitutively expressed functional TLR7/8, a receptor that recognizes ssRNAs from RNA viruses, and the ligation of which can induce differentiation along the myeloid lineage without the addition of any exogenous cytokines. In our previous reports, we demonstrated selective inhibition of erythroid growth and downregulation of the expression of erythropoietin receptor mRNA in human $CD34^+$ cells by CpG (cytosine linked to guanine by a phosphate bond) oligodeoxynucleotide-2006 (CpG-ODN2006), a TLR9 ligand that shares a consensus sequence with the parvovirus B19 genome (6), and rapid induction of apoptosis in $CD34^+$ cells by synthetic dsRNA and poly(I:C), a TLR3 ligand (27). Thus, nucleic acids derived from viruses and bacteria directly or indirectly affect hematopoiesis. LPS, which mimics severe systemic Gram-negative bacterial infection, initiates TLR4 signaling by binding to the TLR4/MD-2 receptor complex on cellular membranes and leads to the activation of multiple signaling pathways including MAPK and NF-κB and results in inflammatory cytokine gene transcription. LPS, both in vitro and in vivo, modulates cord blood progenitor cells and bone marrow progenitor cell differentiation (7, 28, 29). Stimulation by TLRs activates the MAPK signaling pathway in human monocytes, macrophages, eosinophils, and cord blood progenitor cells (12, 16, 28). In the current study, we confirmed the expression of LPS on mobilized human peripheral blood $CD34^+$ cells, as in other reports (8, 19), using immunoblotting, and we demonstrated that $CD34^+$ cells stimulated with LPS activated the NF-κB signaling pathway and all MAPK signaling pathways, except for the p38 pathway, as previously reported (7, 28, 29). This study shows, to our knowledge for the first time, that LPS promoted the differentiation of mobilized human peripheral blood $CD34^+$ cells to granulo-monocytic progenitors, and LPS mainly promoted the early differentiation stage from HSPCs to granulo-monocytic progenitors, but it did not significantly affect the late myelocyte stage.

IL-6 is mainly responsible for the regulation of steady-state and emergency granulopoiesis (30, 31). Schürch et al. (32) showed that bone marrow stromal cells responded to $CD8^+$ T cell–derived IFN-γ by releasing IL-6, which promoted myelopoiesis and monocyte differentiation in mice. Zhao et al. (18) showed that emergency granulopoiesis was severely diminished in mice transplanted with IL-$6^{-/-}$ cells compared with mice transplanted with wild-type cells. They also suggested that HSPCs might not only act as the targets for cytokine signals but might also directly sense pathogen signals and increased proinflammatory cytokine levels in an autocrine and/or paracrine manner through single-cell cytokine chip analysis. Our study shows, to our knowledge for the first time, that human peripheral blood $CD34^+$ cells directly respond to LPS stimulation and LPS-induced IL-6 secretion from mobilized human peripheral blood $CD34^+$ cells, which confirms the autocrine and/or paracrine function of human HSPCs in vitro. Although gene transfer to hematopoietic stem cells is difficult because 70% of the cells die after the start of culture, the purified human peripheral blood $CD34^+$ cells showed a very high purity of 97–98% $CD34^+$ cells. IL-6 has been reported to play an important role in the proliferation of granulocytes, but the detailed mechanism remains unclear. Recently, the exact mechanism by which disseminated bacterial infection is sensed and translated into increased G-CSF levels, which stimulates the switch from the steady state to emergency granulopoiesis, has been elucidated (33). Because the medium used in our study already contained a high concentration of G-CSF, it was difficult to verify whether these results were due to G-CSF, thus motivating us to explore the relationship between IL-6 and transcription factors of granulocytes.

PU.1, an Ets family transcription factor, and C/EBPα and C/EBPβ, members of the C/EBP family, are essential for myeloid lineage differentiation. PU.1 competes with C/EBPα at the bifurcation stage between monocytes-macrophages and granulocytes, and C/EBPα predominance promotes differentiation into granulocytes, whereas PU.1 predominance promotes differentiation into monocyte-macrophage lineage (34). C/EBPα is the key transcription factor of neutrophil differentiation at a steady-state condition, but phosphorylation of C/EBPα favors monocyte differentiation by blocking granulopoiesis (35, 36). In contrast, C/EBPβ, which belongs to the same family as C/EBPα, is known to play an important role in emergency granulopoiesis (22). C/EBP$\beta^{-/-}$ mice failed to elicit emergency granulopoiesis, although steady-state granulopoiesis was unaffected (22, 37–39). Furthermore, C/EBPβ promotes monocyte-to-macrophage differentiation by regulating constitutive gene expression (40). LAP*, LAP, and LIP, the three isoforms of C/EBPβ, are translated from their unique mRNA and exhibit distinct functions (41, 42). Sato et al. (23) observed that LIP was the first to be upregulated in long-term HSCs under stress, and early upregulation of LIP promoted differentiation of quiescent HSCs into progenitors. Thereafter, LAP/LAP* were upregulated, and this amplified the myeloid differentiation of HSPCs. Phosphorylation of C/EBPβ on Thr235 increased the promoter activation potential (43). In our study, PU.1 expression was enhanced by LPS, but the addition of MRA, an anti–IL-6 receptor inhibitor, did not inhibit the LPS-induced increase of PU.1 expression, suggesting that PU.1 promoted monocyte-macrophage lineage differentiation, but it was not associated with enhanced IL-6 secretion. LPS increased levels of phosphorylated C/EBPα and C/EBPβ, which were inhibited by the addition of MRA. These data suggest that LPS activates MAPK as well as NF-κB signaling pathways via TLR4 to stimulate increased IL-6 production from HSPCs, which in turn partially controls the switching from C/EBPα-dependent steady-state granulopoiesis to C/EBPβ-dependent emergency granulopoiesis. Briefly, enhanced expression of PU.1 and phosphorylation of C/EBPα contribute to the differentiation of the monocyte-macrophage lineage, and the enhanced phosphorylation of C/EBPβ by IL-6 contributes to the development of emergency granulopoiesis.

Skirecki et al. (44) reported that LPS promoted proliferation of $CD34^+$ cells in a humanized mouse model. Ziegler et al. (15) reported that LPS-stimulated human bone marrow mesenchymal stromal cells support myeloid cell development by indirectly increasing the secretion of cytokines. This study showed that human peripheral blood $CD34^+$ cells were stimulated by LPS to produce IL-6 and further promote their own differentiation, without the influence of other cells. Although this study is limited to in vitro experiments, it suggests that human HSPCs can sense pathogens and produce cytokines exerting autocrine effects, thereby directly promoting differentiation. To the best of our knowledge, the relationships between left-shifted granulopoiesis in infection, IL-6 levels, and expression of granulocyte transcription factors in human $CD34^+$ cells remain to be examined. We propose a mechanism for left shift resulting from changes in the bone marrow. Understanding the biology of emergency granulopoiesis induced by TLR-mediated IL-6 may aid in devising new therapeutic strategies to treat human infectious and hematologic diseases. Further studies are needed to elaborate the mechanism of cytokine production by human HSPCs directly.

Acknowledgments

We thank Hiromi Kataho, Yukiko Abe, and Yuko Chiba of Akita University for valuable technical assistance with cell staining.

Disclosures

The authors have no financial conflicts of interest.

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