C/EBPβ Is Involved in the Amplification of Early Granulocyte Precursors during Candidemia-Induced "Emergency" Granulopoiesis

Sakiko Satake, Hideyo Hirai, Yoshihiro Hayashi, Nobuaki Shime, Akihiro Tamura, Hisayuki Yao, Satoshi Yoshioka, Yasuo Miura, Tohru Inaba, Naohisa Fujita, Eishi Ashihara, Jiro Imanishi, Teiji Sawa and Taira Maekawa

*J Immunol* 2012; 189:4546-4555; Prepublished online 28 September 2012; doi: 10.4049/jimmunol.1103007

http://www.jimmunol.org/content/189/9/4546

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/09/28/jimmunol.1103007.DC1

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article *cites 37 articles*, 18 of which you can access for free at: http://www.jimmunol.org/content/189/9/4546.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
C/EBPβ Is Involved in the Amplification of Early Granulocyte Precursors during Candidemia-Induced “Emergency” Granulopoiesis

Sakiko Satake,*†,1 Hideyo Hirai,*†,4 Yoshihiro Hayashi,*†,1 Nobuaki Shime,*‡
Akihiro Tamura,* Hisayuki Yao,* Satoshi Yoshioka,* Yasuo Miura,* Tohru Inaba,*
Naohisa Fujita,* Eishi Ashihara,*‡,4 Jiro Imanishi,‖ Teiji Sawa,† and Taira Maekawa*†

Granulopoiesis is tightly regulated to meet host demands during both “steady-state” and “emergency” situations, such as infections. The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) plays critical roles in emergency granulopoiesis, but the precise developmental stages in which C/EBPβ are required are unknown. In this study, a novel flow cytometric method was developed that successfully dissected mouse bone marrow cells undergoing granulopoiesis into five distinct subpopulations (#1–5) according to their levels of c-Kit and Ly-6G expression. After the induction of candidemia, rapid mobilization of mature granulocytes and an increase in early granulocyte precursors accompanied by cell cycle acceleration was followed by a gradual increase in granulocytes originating from the immature populations. Upon infection, C/EBPβ were upregulated at the protein level in all the granulopoietic subpopulations. The rapid increase in immature subpopulations #1 and #2 observed in C/EBPβ knockout mice at 1 d postinfection was attenuated. Candidemia-induced cell cycle acceleration and proliferation of hematopoietic stem/progenitors were also impaired. Taken together, these data suggest that C/EBPβ is involved in the efficient amplification of early granulocyte precursors during candidemia-induced emergency granulopoiesis. The Journal of Immunology, 2012, 189: 4546–4555.

Neutrophilic granulocytes are the major cell type at the front line of host defense (1, 2). Granulocytes are continuously produced in the bone marrow (BM) and supplied to the peripheral blood or tissues, where they fight microorganisms via their bactericidal activities (1, 2). A shortage of granulocytes rapidly causes fatal infections, whereas an excess of granulocytes triggers conditions such as acute respiratory distress syndrome and sepsis-related other acute organ dysfunctions (1, 3). Therefore, the number of granulocytes must be tightly regulated to meet host demands. Granulopoiesis, the process of granulocyte production in the BM, is under the control of both intrinsic and extrinsic cellular factors. In mice deficient in the transcription factor CCAAT/enhancer binding protein (C/EBPα), granulocytes are completely absent, suggesting a central role for C/EBPα in “steady-state” granulopoiesis (4, 5). During “emergencies” such as infection, the supply of granulocytes from the marginal and BM pool increases, and the cells are mobilized to the site of infection. In addition, BM granulopoiesis is increased in response to various cytokines including IL-3, GM-CSF, and G-CSF (6–9). We and others have previously shown that, in contrast to steady-state granulopoiesis, which is dependent on C/EBPα, emergency granulopoiesis is dependent on the transcription factor C/EBPβ (8, 10). Although C/EBPβ plays a critical role in the transition from common myeloid progenitors (CMPs) to granulocyte-macrophage progenitors (GMPs) (5), the precise developmental stage at which C/EBPβ plays important roles during emergency granulopoiesis is unknown.

All hematopoietic cells, including granulocytes, originate from a small number of hematopoietic stem cells (HSCs) residing within the BM (11, 12). During the process of differentiation, HSCs lose their multipotency and their ability for self-renewal and gain lineage-specific functions. Recent advances in flow cytometry have enabled us to prospectively identify the intermediate stages between HSCs and mature cells. Long-term HSCs gradually lose the self-renewability as they differentiate and mature into granulocytes, first becoming short-term HSCs (13) and then multipotent progenitors (14). Multipotent progenitors then begin to lose their multipotency and give rise to specialized progenitors, including CMPs and GMPs (15–17). After passing through the GMP stage, granulocytic lineage cells gradually acquire specific features. This maturation process is nicely characterized by the development of distinct morphological features and the expression of granule proteins. Giemsa staining clearly shows distinct differentiation steps in the progression from myeloblasts, promyelocytes, myelocytes,
metamyelocytes (MM), and band cells (BC) to segmented granulocytes. Three types of granule proteins are expressed during specific developmental stages: azurophil granule proteins (primary granules), specific granule proteins (secondary granules), and gelatinase granule proteins (tertiary granules) (18, 19). However, despite the relatively well-known characteristics found among cells intermediate between HSCs and specialized progenitors, flow cytometric techniques that can dissect the process of granulocyte maturation have not been fully established. In this study, we developed a novel flow cytometric method for analyzing the process of mouse granulopoiesis and applied it to the investigation of candidemia-induced emergency granulopoiesis to determine the developmental stages that require C/EBPβ.

Materials and Methods

Mice

C57BL/6 mice (8–12 wk old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) orCLEA Japan (Tokyo, Japan). Mice deficient in C/EBPβ (20) were bred and maintained under specific pathogen-free conditions in Kyoto University. Littermates were always used as controls for the experiments involving C/EBPβ knockout (KO) mice. Approval for the animal protocols was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Flow cytometric analysis and cell sorting

Peripheral blood samples were obtained from the tail vein, and blood cell count and differential were analyzed with a SE-9000 instrument (Sysmex, Kobe, Japan). For flow cytometric analysis of peripheral blood, RBCs were lysed by treatment with Pharm Lyse reagent (BD Biosciences). BM cells were stained with fluorescence-conjugated Abs and analyzed either using a FACSCalibur or a FACSaria instrument (BD Biosciences). The Abs used were FITC-conjugated anti-CD34 (RAM34); PE-conjugated anti–Ly-6G (1A8); allophycocyanin-conjugated anti–c-KiThigh (2B8); and PerCP-Cy5.5–conjugated lineage markers, including CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), B220 (RA3-6B2), and Ter119. Allophycocyanin-conjugated anti–c-KiThigh PE-Cy7–conjugated anti–Sca-1, PE-conjugated anti-CD16/32, and FITC-conjugated anti-CD34 Abs were used for staining HSCs and myeloid progenitor cells. All Abs were purchased from BD Biosciences, eBioscience, BioLegend, or Caltag Laboratories. To exclude dead cells, BM cells were stained with propidium iodide. Data were analyzed using the FlowJo software (Tree Star).

Infection with Candida albicans

Experiments involving infection with C. albicans were carried out as previously described, with minor modulations (21). Briefly, C. albicans (18804; American Type Culture Collection) was plated onto Luria–Bertani broth agar plates, and the plates were stored at 4˚C for a maximum of 4 wk. Before each experiment, several colonies were picked from the plate and grown in 3 ml Sabouraud dextrose broth (Sigma-Aldrich, St. Louis, MO) at 37˚C for 24 h. The fungi were washed twice with pyrogen-free PBS and resuspended in PBS, and 4 × 10⁶ CFU/20 g body weight/mouse were i.v. injected via the tail vein to induce disseminated candidiasis.

Quantitative RT-PCR

Total RNA from sorted cells was extracted using an RNeasy Micro Kit (Qiagen), according to the manufacturer’s instructions. RNA was reverse transcribed and subsequently amplified using a LightCycler System (Roche Diagnostics) programmed with the following parameters: 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 s and 60˚C for 30 s. The PCR mixture contained TaqMan Master Mix (Roche Diagnostics), cDNA, the relevant primer pairs, and a TaqMan probe (Universal Probe Library, Roche Diagnostics). The sequences of the primers and probes are listed in Table 1. The expression level of each gene was normalized against that of GAPDH.

In vivo BrdU incorporation analysis

Mice were injected i.p. with 1 mg BrdU 1 h before euthanasia. BM cells were harvested and stained with fluorescent-conjugated Abs. Labeled BM cells were fixed, permeabilized, and treated with DNase to expose the incorporated BrdU using a BrdU Flow Kit (BD Biosciences). Cells were then stained with an FITC-labeled anti-BrdU and analyzed using a FACSCalibur instrument.

Western blotting

Sorted cells were diluted in Laemmli sample buffer and boiled for 10 min. Samples were then separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. A “Can Get” Signal immunoreaction enhancer kit (Toyobo, Osaka, Japan) was used to dilute the primary and secondary Abs. Abs specific for phospho-C/EBPβ (Thr235 or Thr188 in mouse) (3084; Cell Signaling Technology), C/EBPβ (sc-150; Santa Cruz Biotechnology), and GAPDH (sc-25778; Santa Cruz Technology) were used as primary Abs. Immunoreactive proteins were detected using HRP-conjugated anti-rabbit IgG (NA934V; GE Healthcare) and visualized using ECL (GE Healthcare).

Statistical analysis

Statistical differences were determined using the Student t test. A p value < 0.05 was considered statistically significant.

Results

Flow cytometric dissection of mouse granulopoiesis

To elucidate the molecular mechanisms involved in granulopoiesis, we devised a novel flow cytometry method. The strategy is shown in Fig. 1A. First, cells that have lost the potential to give rise to granulocytes are removed from the target population (Fig. 1B–D). Cells expressing lineage markers for T lymphocytes (CD4 and CD8), B lymphocytes (CD19 or B220), and erythroid cells (Ter119) are excluded, together with propidium iodide-stained dead cells (R1 in Fig. 1B). Because a discrete population of SSc-high/FSClow cells (R2 in Fig. 1C) was enriched in CD11b+Ly-6G+7/4(SSc-high)/Ter119 eosinophilic granulocytes (eosinophils) (9; Supplemental Fig. 1A–C), these cells were also removed. When the cells within R3 (Fig. 1C) were analyzed for the expression of CD34 and c-KiThigh, the c-KiThigh CD34low cells (R4 in Fig. 1D) were megakaryocyte–erythroid progenitors (MEP) that have been previously defined as c-KiThigh CD34+FCrIII/IIlow/lin− cells (see Supplemental Fig. 1D) (15). Thus, R4 cells were also eliminated from the analysis. The remaining cells (R5) were then analyzed for the expression of c-KiThigh and lymphocyte Ag 6 complex, locus G (Ly-6G; a member of the Ly-6 family of GPI-anchored proteins), which is specifically expressed by granulocytes (Fig. 1E) (22). During the process of granulocytic differentiation, cells are expected to acquire Ly-6G expression and gradually lose the expression of c-KiThigh. The cells within R5 were then divided into five cell subpopulations (#1–#5), as shown in Fig. 1E. Subpopulation #1 was c-KiThigh/Ly-6G−, #2 was c-KiThigh/Ly-6G−, and #5 was c-KiThigh/Ly-6Ghigh discrete population. The remaining cells between #2 and #5 were divided equally into subpopulations #3 and #4, according to the expression levels of Ly-6G (Fig. 1C). The c-KiThigh/Ly-6G− population comprised cells that stained negative for all other markers and was enriched in monocytes expressing M-CSF receptor (Supplemental Fig. 1E, 1F). During granulopoiesis, the cells are hypothesized to progress from stage 1 to 5 (in that order).

Five subpopulations dissected by flow cytometry precisely reflect the stepwise progression of granulopoiesis

To validate the flow cytometric dissection, the morphological characteristics of these five subpopulations were analyzed. After sorting, the cells were stained with Wright–Giemsa solution (Fig. 2A). Subpopulation #1 comprised mainly myeloblasts with large, round nuclei and a narrow, basophilic cytoplasm (71.5 ± 3.5%). Subpopulation #2 contained an abundance of PMs with oval-shaped nuclei and a wider, less basophilic cytoplasm (78.5 ± 2.1%). Segmentation of the nuclei became gradually more evident between stages 3 and 4 (myelocytes, 71.5 ± 5.0% in subpopulation #3; MM, 64.0 ± 7.1% in subpopulation #4); subpopulation #5 mainly comprised cells with donut-shaped nuclei (BC and segmented cells; 60.5 ± 2.1 and 29.5 ± 3.5%, respectively). Cyto-

The Journal of Immunology 4547

Downloaded from http://www.jimmunol.org/ by guest on October 30, 2017
plasmic staining became weaker as the cells moved toward stage 5. These morphological changes within the defined subpopulations correlate well with granulocytic maturation.

Next, the expression of granule proteins by each population was examined (Fig. 2B–D, Table I), because granule proteins are known to be expressed only at specific stages in differentiation (19). First, the expression of azurophil granule proteins, including cathepsin G, proteinase 3, and neutrophil elastase 2, which are synthesized at the promyelocytic stage, was examined (Fig. 2B). The level of cathepsin G transcripts was high in subpopulations #1 and #2, much lower in #3, and almost undetectable in #4 and #5. This expression pattern was similar for all the azurophil granule proteins tested. Lactoferrin, a granule protein expressed after the myelocyte stage, was highly expressed within subpopulation #4 (Fig. 2C). The gelatinase matrix metallopeptidase 9, a granule protein expressed at the MM and BC stages (18, 23), was expressed in subpopulation #4 and, at much higher levels, in #5 (Fig. 2D). The expression patterns of the different granule proteins within the defined subpopulations, together with the observed morphological characteristics, confirmed that the process of granulopoiesis progresses from subpopulations #1 to #5 in this order. These results validate the flow cytometric method for the analysis of granulopoiesis.

Flow cytometric analysis of emergency granulopoiesis

To assess changes in granulopoiesis during the early phase of an infection, the flow cytometry method was applied to a mouse candidemia model. *C. albicans* (4 × 10^6 CFU/20 g body weight) was i.v. injected into mice and peripheral WBC counts were taken on days 0–4 (Fig. 3A, 3B). The number of WBCs and granulocytes, as well as the frequency of CD11b+Ly-6G+ granulocytes, increased during the observation period, suggesting that granulocyte production and mobilization were significantly enhanced, as described previously (8, 21). The BM cells from infected mice were then analyzed on days 0–4 postinfection (Fig. 4A). BM cells from mice in the steady state were always analyzed before flow cytometric analysis of BM cells from infected mice. The gates were then set as described in Fig. 1 and applied to the analysis of BM cells from infected mice. The gates were then set as described in Fig. 1 and applied to the analysis of BM cells from infected mice. Downregulation of c-Kit was observed in subpopulations #2–#5 during the early phase of infection (days 1 and 2); therefore, the gates were adjusted on with the y-axis (corresponding to c-Kit expression). To verify whether the five subpopulations defined during the steady state were still present during this emergency situation, the expression levels of various granule proteins were measured within each subpopulation on days 0, 2, and 4 postinfection. As shown in Fig. 3C, the expression profile of each of the granule proteins was identical to that observed during the steady state (Fig. 2B–D), suggesting that the flow cytometric method was also valid for the analysis of emergency granulopoiesis.

**Candidemia-induced dynamic changes in granulopoiesis**

The flow cytometric profiles of BM cells during candidemia from days 0 to 4 are shown in Fig. 4A. The cell numbers per 5 × 10^5 BM cells within each subpopulation are shown in Fig. 4B. The number of total BM cells did not significantly change throughout the observation period (data not shown); the cell numbers shown in Fig. 4B reflect the absolute numbers in the BM. On day 1, the cell number (per 5 × 10^5 BM cells) in subpopulation #1 (which included c-Kit+ hematopoietic stem/progenitor cells) significantly increased from 8,076 ± 868 (mean ± SD) to 12,776 ± 831 (p < 0.01; n = 3), and the cells in subpopulation #5 decreased from 125,675 ± 15,920 (p < 0.01; n = 3). The cell number in subpopulation #5 started to increase on day 2, whereas the increase in cell number for subpopulations #2, #3, and #4

---

**Table I. Primers and probes used for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Universal Probe (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin G</td>
<td>5′-CAACGTTCTGCTGGAAAGATGC-3′</td>
<td>5′-CTTCCTGGCCTCAGAATGAT-3′</td>
<td>15</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>5′-GGAGGAGAGCCTAGAGTTTTG-3′</td>
<td>5′-TAGACAGAGAACGCCGTAATG-3′</td>
<td>7</td>
</tr>
<tr>
<td>Elastase 2</td>
<td>5′-ACCTGGCTCCACGATGCTGCT-3′</td>
<td>5′-GCCCAAACAACTCTTGAGG-3′</td>
<td>107</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>5′-AGTCACCTACCCACCAAGG-3′</td>
<td>5′-CTGCTGCACACTCACAATT-3′</td>
<td>4</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>5′-GGCAGCTGCGCTGTTAGTT-3′</td>
<td>5′-CTGCTCTGGTGTTCTGAAAT-3′</td>
<td>53</td>
</tr>
<tr>
<td>MMP9</td>
<td>5′-ACGACATAGAGCAGCCTCCA-3′</td>
<td>5′-TGACCTCTTCCTCCCTGCT-3′</td>
<td>77</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>5′-CCTTCAGACGACGTTGCTGg-3′</td>
<td>5′-TGGCCTTCTCCTCGTGC-3′</td>
<td>11</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>5′-ATGCTGCGAGCAGCTCACT-3′</td>
<td>5′-TATGTCGAGGAAGAGG-3′</td>
<td>55</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TGTCCTGGCTTGGAGAATGAC-3′</td>
<td>5′-CGTGATTAGGACCTG-3′</td>
<td>80</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Flow cytometric analysis of murine granulopoiesis. (A) Strategy used for flow cytometric analysis of mouse granulopoiesis. (B–E) Staining and gating of mouse BM cells. First, cells that had lost the potential to give rise to granulocytes were removed from the target population (B–D). The remaining cells (R5) were then analyzed for expression of c-Kit and Ly-6G (E). Subpopulation #1 was c-Kit^hiLy-6G^, #2 was c-Kit^loLy-6G^, and #5 was a discrete c-Kit^hiLy-6G^ population. The remaining cells between #2 and #5 were divided into subpopulations #3 and #4 according to their expression levels of Ly-6G. (E) Ery, Erythrocyte; PI, propidium iodide.
began after day 3. Within the granulopoietic compartment (Fig. 4C), the frequency of subpopulation #5 cells decreased, all the other subpopulation cell levels increased on day 1, and the timing of the next peak level (arrowhead in Fig. 4C) gradually progressed from #1 to #5. These data suggest that, upon infection, mature granulocytes (#5) were immediately mobilized from the BM and that the enhancement of granulopoiesis was initiated in subpopulation #1 on day 1, and this population differentiated to stage 5 over time.

The cell cycle was accelerated in subpopulations #1 and #2 in the early phase of emergency granulopoiesis

To further investigate the dynamic changes in the cell populations during the candidemia-induced emergency response, the cell cycle status of the five subpopulations was analyzed using in vivo BrdU labeling experiments. On day 1 postinfection, the numbers of early granulopoietic precursors (subpopulation #1) had already begun to increase, and therefore, cell cycle changes at these early times were examined. BM cells were harvested 1 h after i.p. injection of BrdU on day 1 postinfection (Fig. 5A). The cells in subpopulations #1–#3 had already incorporated BrdU. In contrast, very few numbers of the cells in subpopulation #5 showed BrdU uptake during the steady state (day 0 in Fig. 5B, 5C). By 24 h after the induction of candidemia, the frequency of BrdU-positive cells within subpopulations #1 and #2 significantly increased (from 28.3 ± 4.5 to 41.8 ± 4.1%, p < 0.05; and 40.6 ± 2.0 to 53.5 ± 3.1%, p < 0.01, respectively), whereas BrdU incorporation by subpopulations #3–#5 was not affected, suggesting that the enhancement of granulopoiesis in response to infection was regulated at the level of the earliest granulocytic precursors, the cells in subpopulations #1 and #2.
Candidemia upregulates C/EBPb protein in all granulopoietic subpopulations

To determine the precise developmental stage at which C/EBPb plays a critical role, the mRNA and protein levels of C/EBPb were measured in each of the subpopulations on days 0 and 1 postinfection (Fig. 6A, 6B). During the steady state, the mRNA levels of C/EBPb were upregulated in line with the progression of granulocytic maturation (from subpopulations #1 to #5; Fig. 6A). The mRNA levels on day 1 postinfection were unchanged in all the granulopoietic subpopulations.

In addition to transcriptional regulation, C/EBPb is also regulated at the translational and posttranslational levels, including the phosphorylation of a threonine residue (Thr188 in mice) (24). Western blotting analysis revealed that both phospho-C/EBPb and total C/EBPb increased in all the granulopoietic subpopulations by day 1 postinfection. These data suggested that C/EBPb is upregulated at the translational level across all stages of granulopoiesis during the early phase of an infection.

C/EBPb is required for candidemia-induced increases in the early granulopoietic subpopulation

Our previous findings showed that emergency granulopoiesis induced by candidemia is impaired in C/EBPb KO mice (8). To further characterize the defect in granulopoiesis of C/EBPb KO mice, the novel flow cytometric method was used to examine candidemia in C/EBPb KO mice (Fig. 7). On day 1 postinfection, an increase in the levels of subpopulations #1 and #2 cells (from 3.6 ± 1.6 to 10.2 ± 2.4% and 4.7 ± 0.8 to 11.6 ± 4.1%, respectively) and a decrease in subpopulation #5 cells (from 66.3 ± 2.9 to 41.2 ± 11.5%) were observed in wild-type (WT) mice (Fig. 7A, left panels, Fig. 7B). In C/EBPb KO mice, the distribution of cells at steady state was identical to that of WT mice (Fig. 7A, upper panels, 7B). In contrast, the increases in subpopulations #1 and #2 and the decreases in subpopulation #5 cells were significantly impaired in KO mice (from 3.1 ± 1.3 to 5.9 ± 1.8, 4.5 ± 1.0 to 6.4 ± 1.5, and 67.5 ± 4.7 to 58.6 ± 6.5%, respectively; Fig. 7A, right panels, 7B). The levels of mRNA expression for granule proteins within each subpopulation from WT and KO mice were identical at both steady state and during infection (Supplemental Fig. 2). When the cell cycle status of these models was evaluated using in vivo BrdU labeling experiments, incorporation of BrdU in subpopulations #1 and #2 in C/EBPb KO mice was always slightly lower than in WT mice, but the differences were not statistically significant. These findings suggest that C/EBPb is required for efficient proliferation of early granulocytic precursors.

C/EBPb is involved in the amplification of early granulocyte precursors

To elucidate the roles of C/EBPb in the proliferation of the early granulopoietic subpopulations, the HSC and myeloid progenitor
compartments were analyzed in WT and KO mice (Figs. 8, 9). The frequency and number of c-Kit^Sca-1^ lineage markers HSC were identical between WT mice and C/EBP\(\beta\) KO mice during the steady state and were not significantly affected on day 1 postinfection.

**FIGURE 4.** Flow cytometric analysis of candidemia-induced granulopoiesis. (A) Flow cytometric analysis of candidemia-induced emergency granulopoiesis. BM cells were harvested on days 0–4 after i.v. injection of *C. albicans* and analyzed by flow cytometry. (B) Chronological changes in the cell numbers of each population per \(5 \times 10^5\) BM cells after the onset of infection. (C) Chronological changes in the frequency of the indicated granulopoietic subpopulations within the granulopoietic compartment, including subpopulations #1–#5. Arrowheads indicate the peak levels after day 2 within the observation period. Data are representative of three independent experiments (\(n=3; *p<0.05\)).

**FIGURE 5.** Cell cycle changes during emergency granulopoiesis. (A) In vivo BrdU incorporation assay. Flow cytometric analyses of the BrdU-positive cells in each population are shown in (B) and (C). Data are representative of two independent experiments (\(n=3; *p<0.05\)).
Infection (Fig. 8A–C). Myeloid progenitors, including CMP, macrophage-granulocyte progenitors (GMP), and MEP, were detected at the same frequency in WT mice and C/EBPb KO mice before infection with C. albicans (Fig. 8D–F). Induction of candidemia significantly increased the frequency and number of GMP in WT mice, and these increases were significantly attenuated in C/EBPb KO mice. At steady state, the cell cycle status of HSC and myeloid progenitors (as assessed by in vivo BrdU labeling experiments) was identical in both WT and C/EBPb KO mice (Fig. 9A, 9B). Upon induction of candidemia, the frequency of BrdU-positive cells in the HSC and CMP populations from WT mice increased significantly; however, an increase of BrdU-positive cells was observed only within the HSC compartment in C/EBPb KO mice and at a lower level than that in WT mice (Fig. 9A, 9B). These results suggest that C/EBPb is involved in the efficient proliferation of HSCs and myeloid progenitors.

Discussion

This paper describes a novel flow cytometric method for the analysis of mouse granulopoiesis and its use in elucidating the molecular mechanisms underlying emergency granulopoiesis.

The prospective identification and isolation of distinct cellular populations is of fundamental importance, especially in the fields of hematology and immunology, and flow cytometry is a powerful tool for this purpose. For successful analysis of a specific hematopoietic lineage, the choice of lineage-specific surface Ag is a central issue in identifying the cell population. In this study, Ly-6G was used as a marker for granulocytic differentiation. An anti-granulocyte receptor 1 Ab (RB6-8C5), which has been widely used for the detection of mouse granulocytes (21, 25, 26), recognizes both Ly-6G and Ly-6C (22, 27). However, the expression of Ly-6C is not restricted to granulocytes, and so, the Ab cross-reacts with nongranulocytic lineages such as dendritic cells (28), monocytes (29, 30), and a subpopulation of lymphocytes (31). In contrast, Ly-6G, which was cloned as a member of the Ly-6 allogeneic family (32), is expressed exclusively on granulocytes (22, 33), and treatment with an anti–Ly-6G Ab specifically depletes granulocytes in vivo (34, 35). Although the physiological ligand and biological function of Ly-6G are unclear, the granulocyte-specific expression of Ly-6G makes it an ideal marker of granulocytic differentiation. To our knowledge, this study shows for the first time that Ly-6G expression correlates with the degree of granulocytic differentiation and maturation. The five subpopulations identified by our flow cytometry method precisely reflect the stepwise differentiation of granulocytes, confirming that this method is useful for analyzing stage-specific gene expression and function during both steady state and emergency granulopoiesis.

Granulopoiesis is a continuous process for producing sufficient numbers of granulocytes for the demands of the host during both
steady state and emergency conditions. In this study, upon infection, mice exhibited an immediate (day 1) decrease in subpopulation #5 and an increase in subpopulation #1. Subsequently, increases in the intermediate subpopulations were observed (Fig. 4A, 4B). These results suggested that the immediate mobilization of mature granulocytes from the BM was followed by a more gradual enhancement of granulopoiesis originating from changes in the earliest granulopoietic precursors. The cell cycle status of each subpopulation was also evaluated. A cell cycle acceleration was observed in subpopulation #1 on day 1 and was accompanied by an increase in the number of cells in subpopulation #1. A cell cycle acceleration was also observed in subpopulation #2 on day 2 but did not result in an increase in cell numbers within subpopulation #2 (Figs. 4B, 5C). However, it did lead to an increase in cell numbers within subpopulation #5 on day 2. Acceleration of the cell cycle within a subpopulation did not necessarily correlate well with an increase in the cell number of that subpopulation. When each subpopulation was sorted and subjected to propidium iodide staining after fixation, sub-G1 cells were rarely observed, either in the presence or absence of infection; this suggests that apoptosis was not involved in the process (Supplemental Fig. 3). Taken together, these results suggest that cell cycle acceleration during granulopoiesis is tightly coupled to extremely rapid differentiation and maturation (from subpopulations #2 to #5). Such fine-tuning of the proliferation and differentiation of granulocytic precursors is requisite for emergency granulopoiesis, and our flow cytometric method provides an excellent platform for investigating the underlying regulatory mechanisms.

The expression of C/EBPβ is regulated at the transcriptional, translational, and posttranslational levels; posttranslational modifications include phosphorylation and sumoylation (36). We have previously shown that C/EBPβ transcripts were upregulated after cytokine stimulation or infection (8). However, in this study, C/EBPβ mRNA levels were unchanged in all the subpopulations, although the protein levels were markedly upregulated on day 1 postinfection. In fact, in the current series of experiments, C/EBPβ mRNA levels were substantially upregulated by day 4 postinfection (data not shown), which suggests that C/EBPβ is upregulated at the translational level as an immediate response and, subsequently, at the transcription level as a prolonged response. Our previous work identified distinct roles for C/EBPβ and C/EBPα during granulopoiesis (8). C/EBPα is prerequisite during steady-state granulopoiesis; naive mice deficient for C/EBPα lack granulocytes in healthy steady-state conditions (4, 5). In vivo production of granulocytes can be induced in C/EBPβ KO mice only in the presence of cytokine stimulation (8). In contrast, granulopoietic responses to cytokine stimulation or infection are impaired in C/EBPβ KO mice, whereas steady-state granulopoiesis is normal, suggesting a specific requirement for C/EBPβ during emergency granulopoiesis (8, 10). In agreement with these previous findings, we showed in this study that C/EBPβ is dispensable in steady-state granulopoiesis but is clearly required for efficient amplification of early granulopoietic subpopulation #1 and #2 cells, on day 1 postinfection. Subpopulation #1 comprised more immature hematopoietic cells, including HSCs and myeloid progenitors. Further analysis of HSC and myeloid progenitors clearly showed...
that C/EBPβ is necessary for cell cycle acceleration in both HSC and CMP and for the increase in GMP numbers during the early phase of candidemia-induced emergency granulopoiesis (Figs. 8, 9). Previous findings showed that C/EBPβ and C/EBPα share the ability to induce granulocyte differentiation but that the strong inhibitory effect on the cell cycle is specific to C/EBPα (8, 37), which is downregulated during emergency granulopoiesis (data not shown) (8). The defects in emergency granulopoiesis observed in C/EBPβ KO mice may be attributable to an imbalance between proliferation and differentiation of granulocytic precursors caused by the downregulation of C/EBPα in addition to the loss of C/EBPβ. Further investigation of the target molecules driven by C/EBPβ in the proliferation of granulocytic precursors is necessary to understand the molecular mechanism underlying emergency granulopoiesis.

Granulopoiesis involves the loss of self-renewal by HSCs, proliferation, and lineage specification, followed by the maturation and acquisition of specific functions. As increased levels of C/EBPβ protein were observed in all the granulopoietic subpopulations, C/EBPβ may play a role in many of these processes. In this study, the expression levels of granule proteins by each subpopulation were not affected in the absence of C/EBPβ, suggesting that up-regulation of C/EBPβ is not directly involved in the differentiation or maturation of granulocytes in response to infection. Therefore,

**FIGURE 9.** C/EBPβ is involved in cell cycle acceleration in HSC and progenitors during emergency granulopoiesis induced by candidemia. (A) In vivo BrdU incorporation assay. Flow cytometric analyses of the BrdU-positive cells in each population are shown in (A) and (B). HSC, c-Kit+Sca-1+Lin− HSC. Data are representative of two independent experiments (n = 4; *p < 0.05).
we have not yet fully elucidated the stage-specific significance of C/EBPβ during emergency situations. We are currently investigating the role of C/EBPβ in HSC regulation and granulocyte function during emergencies using C/EBPβ KO mice.

In summary, our novel flow cytometric analysis method revealed that C/EBPβ is involved in the efficient amplification of early granulocytic precursors. The method developed in this study will provide an excellent platform for studies investigating granulopoiesis, will further elucidate the molecular mechanisms involved in the shift from C/EBPα- to C/EBPβ-dependency during emergency granulopoiesis, and will facilitate a greater understanding of the hematopoietic system as a part of host defense.

Acknowledgments
We thank Y. Nakagawa, M. Katakami, and Y. Manabe for excellent technical assistance and all the laboratory members in the Department of Transfusion Medicine and Cell Therapy for valuable discussions.

Disclosures
The authors have no financial conflicts of interest.

References