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Alcohol Impairs the Myeloid Proliferative Response to Bacteremia in Mice by Inhibiting the Stem Cell Antigen-1/ERK Pathway

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Enhancement of stem cell Ag-1 (Sca-1) expression by myeloid precursors promotes the granulopoietic response to bacterial infection. However, the underlying mechanisms remain unclear. ERK pathway activation strongly enhances proliferation of hematopoietic progenitor cells. In this study, we investigated the role of Sca-1 in promoting ERK-dependent myeloid lineage proliferation and the effects of alcohol on this process. Thirty minutes after i.p. injection of alcohol, mice received i.v. challenge with $5 \times 10^7$ Escherichia coli for 8 or 24 h. A subset of mice received i.v. BrdU injection 20 h after challenge. Bacteremia increased Sca-1 expression, ERK activation, and proliferation of myeloid and granulopoietic precursors. Alcohol administration suppressed this response and impaired granulocyte production. Sca-1 expression positively correlated with ERK activation and cell cycling, but negatively correlated with myeloperoxidase content in granulopoietic precursors. Alcohol intoxication suppressed ERK activation in granulopoietic precursors and proliferation of these cells during bacteremia. Granulopoietic precursors in Sca-1$^{-/}$ mice failed to activate ERK signaling and could not increase granulomacrophagic CFU activity following bacteremia. These data indicate that Sca-1 expression promotes ERK-dependent myeloid cell proliferation during bacteremia. Suppression of this response could represent an underlying mechanism for developing myelosuppression in alcohol-abusing hosts with severe bacterial infection. The Journal of Immunology, 2012, 188: 1961–1969.

Polymeronuclear leukocytes (referring specifically to neutrophilic granulocytes or neutrophils) represent the largest population of phagocytes in the circulation. They constantly patrol the bloodstream and can quickly migrate to tissue sites of infection to exert microbicidal activity. Through the course of bacterial infection, mediators generated from inflammatory tissues and substances derived from invading microbes stimulate bone marrow granulopoietic activity by enhancing granulopoietic precursor cell proliferation, accelerating granulocyte maturation, and increasing the release of these phagocytes into the bloodstream (1, 2). Multiple humoral factors including G-CSF, GM-CSF, IL-1, IL-6, and androgens have been reported to play different roles in the regulation of granulopoiesis and the granulopoietic response (3–7). Hematopoietic stem cells and progenitors at different stages of differentiation express TLRs and their coreceptors. TLR4, as well as coreceptors MD-2 and CD14, are required for the recognition of LPS from Gram-negative bacteria (8). Recent studies from our group have revealed that in response to Escherichia coli infection or LPS stimulation, primitive hematopoietic precursors, including hematopoietic stem cells, up-regulate their surface expression of stem cell Ag-1 (Sca-1, an 18-kDa glycophosphatidylinositol-anchored cell surface protein associated with cell cycle activation) (9–11). This enhancement of Sca-1 expression is associated with increases in hematopoietic precursor cell proliferation and their commitment toward granulocyte lineage development during bacterial infection (10–13).

It has long been recognized that excessive alcohol consumption injures bone marrow granulopoietic function (14–17). Such compromised innate immunity predisposes alcohol abusers to the development of severe bacterial infections (14, 18). Alcohol abusers with severe infections, particularly sepsis, frequently present with granulocytopenia, which is an indicator of increased mortality (19). Examination of the bone marrow from alcoholic patients has shown vacuolated granulopoietic progenitors with a significantly reduced number of mature granulocytes (16, 20, 21). However, mechanisms underlying alcohol-induced impairment of marrow granulopoietic activity have remained unclear.

Activation of the ERK pathway following ligand engagement of TLR4 has been known to provide a strong signal mediating the proliferative response of hematopoietic precursors (8, 22). Our current investigation focused on the role of enhanced Sca-1 expression in facilitating ERK signaling, as well as the associated myeloid and granulopoietic precursor cell proliferation in response to bacteremia. The results indicate that upregulation of Sca-1 expression promotes ERK-dependent myeloid cell proliferation following Escherichia coli bacteremia. Alcohol intoxica-
tion suppresses this response, which may represent an underlying mechanism for developing granulocytopenia in alcohol-abusing hosts with severe bacterial infection.

Materials and Methods

Animals

Male BALB/c mice (7–10 wk old; Charles River Laboratories, Wilmington, MA) with a body weight of 21.8 ± 2.10 g were housed in a specific pathogen-free facility with a 12-h light/dark cycle. Acute alcohol binge was administered by i.p. injection of 20% ethanol (EtOH; 5 g/kg) in saline. Blood alcohol levels achieved with this model are in the ranges of 106.3–132.8, 87.7–122.4, and 48.4–61.4 mM, respectively, at 90 min, 3 h, and 6 h after alcohol administration, as previously reported (11, 23). The alcohol dose used in our experiments was selected to model the kinetic changes in blood alcohol levels frequently observed in clinical patients with acute alcohol intoxication. Mice commonly awakened from the intoxication within 6 h after alcohol administration. Thirty minutes after i.p. injection with EtOH or saline, mice received i.v. challenge with 5 × 105 E. coli (E11775 from the American Type Culture Collection, Manassas, VA; in 100 μl saline/mouse) by penile vein injection under isoflurane anesthesia. Control mice received an equal volume of saline. Animals were sacrificed San Jose, CA) 4 h prior to sacrifice.

Preliminary results showed that fixation and permeabilization of murine bone marrow cells produced differential effects on cell surface staining (Supplemental Fig. 1). Flow cytometry

Phospho-specific flow cytometry enables the study of intracellular signaling events at the single cell level. To investigate intracellular signaling mechanisms, we developed a phospho-specific flow cytometry protocol for analyzing intracellular ERK signaling in distinct stages of myeloid and granulocyte lineage development. After 8 h E. coli challenge, bone marrow was collected and RBCs in the bone marrow sample were lysed with Purescript RBC lysis solution (Qiagen, Valencia, CA) as previously described (10). Thereafter, nucleated bone marrow cells were fixed and permeabilized according to the previously reported protocol, with some modifications (26). Nucleated cells were washed in 1× PBS after RBC lysis. Cells were fixed immediately with 1% paraformaldehyde for 10 min. Fixed cells were washed twice with staining buffer (PBS plus 0.5% BSA plus 0.1% NaN3). A mixed panel of biotinylated anti-mouse lineage markers (CD3e [clone 145-2C11], CD45/B220 [clone RA3-6B2], CD11b/CD18 [Mac-1; clone M1/70], Gr1 [Ly6G/Ly6C; clone RB6-8C5], or TER119) or isotype control Abs (clones A19-3, B35-95, A95-1; BD Biosciences) were then incubated for 20 min at room temperature. PE-conjugated streptavidin (BD Biosciences; 10 μg/ml) and anti-Gr1 (Ly6a/E, clone D7; BD Biosciences; 10 μg/ml) were then added to the incubation. The cell mixture was incubated another 20 min. Cells were washed once with staining buffer. Fixed cells were then permeabilized with 100% ice-cold acetone for 10 min at 4°C. Acetone was chosen as a permeabilizing agent to preserve Sca-1 antigenicity (26). Following permeabilization, cells were washed twice with staining buffer. Fixed and permeabilized samples were incubated with fluorochrome-conjugated anti-c-Kit (CD117, clone 2B8; BD Biosciences; 10 μg/ml), anti-Gr1 (granulocyte differentiation Ag-1 or Ly6G/Ly6C, clone RB6-8C5; BD Biosciences; 10 μg/ml), and anti–phospho-p44/42 (E10, Thr202/Tyr204; Cell Signaling Technology, Danvers, MA; 10 μg/ml) 20 min at room temperature. Cells were then washed and resuspended in staining buffer for FACS analysis. Phospho-ERK expression was quantified by integrated mean fluorescence intensity (representing the percentage positive cells multiplied by their mean channel fluorescence) (27). Cell cycle activity was determined by measuring the DNA content of granulopoietic precursors following 24 h E. coli challenge using the DNA QC Particles kit (BD Biosciences). Following surface staining, Gr1+/Sca-1+ (Sca-1+/granulopoietic precursors) and Gr1+2/Sca-1+ (Sca-1+/granulopoietic precursors) were sorted directly into DNA QC buffer. DNA content of sorted cells was then assessed by measuring the propidium iodide content of each cell type on a BD FACSaria. Cell cycle analysis was performed using ModFit LT software (Verity Software House, Topsham, ME).

Myeloid progenitors were identified by the lineage (lin)− c-Kit+Sca-1+ (LKS+) surface phenotype (28). Granulocyte lineage cells were studied using the expression of Gr1. Immature granulopoietic precursors express low levels of Gr1 Ag (Gr1+ cell; granulopoietic precursor). As these precursors terminally differentiate, Gr1 surface expression increases, with mature granulocytes expressing the highest level of Gr1 (Gr1hi cell; mature granulocyte) (29, 30).

Cell cycle activity was determined by measuring the DNA content of granulopoietic precursors following 24 h E. coli challenge using the DNA QC Particles kit (BD Biosciences). Following surface staining, Gr1+Sca-1+ (Sca-1+ granulopoietic precursors) and Gr1+Sca-1− (Sca-1− granulopoietic precursors) were sorted directly into DNA QC buffer. DNA content of sorted cells was then assessed by measuring the propidium iodide content of each cell type on a BD FACSaria. Cell cycle analysis was performed using ModFit LT software (Verity Software House, Topsham, ME).

To determine myeloperoxidase (MPO) expression, bone marrow samples were prepared as described above. After RBC lysis, nucleated marrow cells were suspended in 1× PBS. Abs against surface Ags Sca-1 and Gr1 were added into the cell suspension. After 20 min incubation at room temperature, cells were washed with 1× PBS and permeabilized with 1× FACS detergent solution containing 0.1% saponin, 1% paraformaldehyde, 1% Triton X-100, and 50 mM sodium chloride. Permeabilized samples were washed with staining buffer, and anti-MPO (Abcam, Cambridge, MA; 10 μg/ml) was added and incubated for 30 min. Cells were then washed with staining buffer, and PE-conjugated anti-mouse IgG secondary Ab (BD Biosciences; 10 μg/ml) was added and incubated for 30 min. After incubation, samples were washed with staining buffer and fixed with 1% paraformaldehyde in 1× PBS in readiness for FACS. MPO content was quantified by integrated mean fluorescence intensity.

Granulopoietic response to bacteremia

Bone marrow was collected following an 8-h challenge with 5 × 105 E. coli in the presence and absence of acute alcohol administration. Protein was extracted with a lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSE, 50 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin [pH 7.6]). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Twenty micrograms protein sample was used with 8% SDS-PAGE ready gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Phosphorylation of ERK1/2 was detected by incubating the polyvinylidene difluoride membrane with phospho-p44/42 (Thr202/Tyr204; MAPK, Thr202/Tyr204; Cell Signaling Technology) and then HRP-conjugated goat anti-rabbit IgG (1:1000 with blocking buffer) secondary Ab. Determination of bound Ab was conducted using the ECL Western blotting detection system (GE Healthcare, Piscataway, NJ) and imaged using the Kodak Gel Logic 2200 imaging system (Carestream Molecular Imaging, Rochester, NY) with Kodak Molecular Imaging Software.

Statistical analyses

Data are presented as mean ± SEM. The sample size is indicated in each figure legend. Statistical analysis was conducted using an unpaired Student t test (for comparison between two groups) and one-way ANOVA, followed by Student–Newman–Keuls test. Asterisk and bars with different letters in each panel are statistically different (p < 0.05). Regression analysis was performed with the Microsoft Excel Analysis ToolPak (Microsoft, Redmond, WA).

Results

Granulopoietic response to bacteremia

We first sought to determine how the bone marrow supported increased granulocyte turnover during bacteremia. Twenty-four
hours after *E. coli* challenge, the number of mature granulocytes was significantly reduced in the bone marrow with a concomitant increase in the immature granulopoietic precursor population (Fig. 1A). These alterations in the marrow granulocyte population likely represent mobilization of the bone marrow reserve of mature granulocytes into the circulation and subsequent expansion of marrow granulopoietic precursors in response to bacterial infection (31, 32). Alcohol administration further decreased the number of mature granulocytes but did not alter the increase in marrow granulopoietic precursor fraction during bacteremia (Fig. 1A). *E. coli* challenge significantly increased the proliferation and expansion of Sca-1⁺ granulopoietic precursors (Fig. 1B, 1C). Although the number of Gr1<sup>hi</sup>Sca-1<sup>+</sup>BrdU<sup>+</sup> cells (BrdU<sup>+</sup>Sca-1⁺ mature granulocytes) similarly increased, the total number of Gr1<sup>hi</sup>Sca-1<sup>+</sup> cells (Sca-1⁺ mature granulocytes) was reduced during bacteremia (Fig. 1B, 1C). Alcohol treatment suppressed the expansion and proliferation of marrow Sca-1⁺ granulopoietic precursors. The total number and proliferation rate of marrow Sca-1⁺ mature granulocytes were also reduced by alcohol during bacteremia.

In saline-treated control mice, proliferative granulocyte lineage committed cells (BrdU⁺Gr1⁺; Table I) accounted for ~18% of all cellular proliferation in the bone marrow. The proliferative activity of marrow B cells (CD19<sup>+</sup>), monocytes (F4-80<sup>+</sup>), or erythroid precursors (TER119<sup>+</sup>) was similar to that of marrow granulocytes (Gr1<sup>+</sup>). Multipotent precursors (lin<sup>−</sup>) and T cells (CD3<sup>+</sup>) contributed a lesser percentage (Table I). Alcohol administration alone decreased the ratio of proliferating mature granulocyte to granulopoietic precursor cells in the bone marrow. Both granulopoietic precursors and mature granulocytes had incorporated BrdU during the last 4 h *E. coli* challenge. Because mature granulocytes are postmitotic and arise from granulopoietic precursor maturation, Gr1<sup>hi</sup>BrdU<sup>+</sup> cells (BrdU⁺ mature granulocytes) may represent newly produced neutrophils from granulopoietic precursors since the time of BrdU injection (29, 30, 33). Therefore, within this BrdU⁺ population, the immature granulopoietic precursor population was greater in cells expressing Sca-1<sup>+</sup> that ERK signaling was upregulated in myeloid progenitors and granulopoietic precursors during bacteremia. Mature granulocytes failed to increase ERK signaling, which may correspond to their postmitotic, differentiated state. Alcohol suppressed ERK activation in marrow myeloid progenitors and granulopoietic precursors during bacteremia (Fig. 1D). Alcohol administration suppressed marrow granulocyte production and reduced the BrdU⁺ mature granulocyte/granulopoietic precursor ratio in the bone marrow during bacteremia (Fig. 1D).

**Enhanced marrow Sca-1 expression and ERK activation during bacteremia**

To study the correlation between upregulation of Sca-1 expression and activation of the ERK pathway during bacteremia, we customized a phospho-specific flow cytometry protocol for studying specific myeloid and granulopoietic cell subpopulations. ERK activation is important for G<sub>1</sub>/S phase transition during proliferation of hematopoietic precursors (22). Western blot analysis demonstrated that ERK signaling was strongly activated in the bone marrow 8 h after *E. coli* challenge (Fig. 2A). Alcohol administration inhibited this response. Phospho-specific flow cytometry showed that enhanced Sca-1 expression by myeloid and granulopoietic precursors corresponded with increased ERK activation in marrow myeloid progenitors and granulopoietic precursors during bacteremia. Mature granulocytes were also reduced by alcohol during bacteremia.

In saline-treated control mice, proliferative granulocyte lineage committed cells (BrdU⁺Gr1⁺; Table I) accounted for ~18% of all cellular proliferation in the bone marrow. The proliferative activity of marrow B cells (CD19<sup>+</sup>), monocytes (F4-80<sup>+</sup>), or erythroid precursors (TER119<sup>+</sup>) was similar to that of marrow granulocytes (Gr1<sup>+</sup>). Multipotent precursors (lin<sup>−</sup>) and T cells (CD3<sup>+</sup>) contributed a lesser percentage (Table I). Alcohol administration alone decreased the ratio of proliferating mature granulocyte to granulopoietic precursor cells in the bone marrow. Both granulopoietic precursors and mature granulocytes had incorporated BrdU during the last 4 h *E. coli* challenge. Because mature granulocytes are postmitotic and arise from granulopoietic precursor maturation, Gr1<sup>hi</sup>BrdU<sup>+</sup> cells (BrdU⁺ mature granulocytes) may represent newly produced neutrophils from granulopoietic precursors since the time of BrdU injection (29, 30, 33). Therefore, within this BrdU⁺ population, the mature granulocyte/granulopoietic precursor may serve as an indicator for the activity of granulocyte maturation in the bone marrow. After 24 h *E. coli* challenge, enhanced marrow Sca-1 expression corresponded with an increase in marrow granulocyte production (Table I). Greater granulocyte production resulted from an increase in the proliferation of marrow hematopoietic (lin<sup>−</sup>) and granulopoietic precursors during bacteremia. Expanded production of granulocytes during bacteremia also corresponded with decreased B cell and monocyte proliferation (Table I). Production of marrow T cells and erythroid precursors remained relatively unchanged. Alcohol administration suppressed marrow granulocyte production and reduced the BrdU⁺ mature granulocyte/granulopoietic precursor ratio in the bone marrow during bacteremia (Fig. 1D).

**FIGURE 1.** Granulopoietic response to bacteremia. *A*, Changes in the marrow granulopoietic precursor and mature granulocyte cell populations during 24 h *E. coli* challenge. *B*, Number of marrow Sca-1⁺ granulopoietic precursor cells and Sca-1⁺mature granulocytes during bacteremia. **Proliferative response of marrow Sca-1⁺ granulopoietic precursor cells and Sca-1⁺mature granulocytes during bacteremia.** *C*, Total granulocyte compartment (Gr1<sup>+</sup> + Gr1<sup>hi</sup>) and the mature granulocyte/granulopoietic precursor represent the percentage of mature granulocytes to granulopoietic precursors contributing to granulocyte lineage proliferation. This ratio can be an indicator of the rate of granulopoietic precursor differentiation into a mature granulocyte (*n = 5*). Bars with different letters are statistically different (*p* ≤ 0.05). BMCs, bone marrow cells.
by Sca-1+ granulopoietic precursor cells was associated with a further increase in ERK activation (Fig. 3C). Alcohol suppressed the increased ERK signaling in Sca-1+ granulopoietic precursor cells during bacteremia (Fig. 3C). Enhanced Sca-1 expression by granulopoietic precursors also correlated ($r = 0.6037$) with ERK pathway activation in these cells during *E. coli* challenge (Fig. 3D). Bacteremia did not induce an increase in ERK signaling in Sca-1− granulopoietic precursor cells. Suppression of Sca-1 expression by alcohol correlated with reduced ERK activation during *E. coli* challenge (Fig. 3D).

**Table I.** Constitution of Sca-1+BrdU+ cells in the bone marrow following 24 h *E. coli* challenge

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Saline</th>
<th>EtOH</th>
<th><em>E. coli</em></th>
<th>EtOH + <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Sca-1+ (SEM) of each population</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multipotent precursors</td>
<td>&lt;0.1 (0.0)</td>
<td>&lt;0.1 (0.0)</td>
<td>56.8 (1.9)</td>
<td>11.3 (2.2)</td>
</tr>
<tr>
<td>Granulocyte precursors</td>
<td>2.6 (0.5)</td>
<td>1.6 (0.4)</td>
<td>19.1 (1.1)</td>
<td>3.6 (0.8)</td>
</tr>
<tr>
<td>Mature granulocytes</td>
<td>11.4 (1.1)</td>
<td>7.3 (1.9)</td>
<td>31.1 (2.0)</td>
<td>8.1 (0.6)</td>
</tr>
<tr>
<td>Erythroid precursors</td>
<td>0.6 (0.1)</td>
<td>0.6 (0.2)</td>
<td>1.0 (0.1)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.8 (0.2)</td>
<td>1.1 (0.1)</td>
<td>16.8 (1.6)</td>
<td>3.9 (0.6)</td>
</tr>
<tr>
<td>T cells</td>
<td>3.4 (0.4)</td>
<td>2.8 (0.6)</td>
<td>53.7 (3.5)</td>
<td>15.9 (1.5)</td>
</tr>
<tr>
<td>B cells</td>
<td>0.8 (0.1)</td>
<td>1.3 (0.2)</td>
<td>18.1 (1.7)</td>
<td>4.6 (0.6)</td>
</tr>
<tr>
<td>% total BMCs (SEM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multipotent precursors</td>
<td>2.7 (0.2)</td>
<td>4.5 (0.7)</td>
<td>5.3 (0.4)</td>
<td>3.6 (0.3)</td>
</tr>
<tr>
<td>Granulocyte precursors</td>
<td>6.5 (0.5)</td>
<td>12.3 (2.5)</td>
<td>20.2 (0.8)</td>
<td>22.0 (1.3)</td>
</tr>
<tr>
<td>Mature granulocytes</td>
<td>11.6 (1.0)</td>
<td>10.9 (1.6)</td>
<td>10.8 (0.4)</td>
<td>4.0 (0.3)</td>
</tr>
<tr>
<td>Erythroid precursors</td>
<td>27.5 (2.1)</td>
<td>38.5 (4.9)</td>
<td>27.1 (2.2)</td>
<td>43.2 (1.9)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>26.2 (1.8)</td>
<td>19.8 (2.0)</td>
<td>19.9 (1.2)</td>
<td>16.7 (0.3)</td>
</tr>
<tr>
<td>T cells</td>
<td>0.5 (0.1)</td>
<td>0.6 (0.1)</td>
<td>0.8 (0.0)</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>B cells</td>
<td>24.9 (2.0)</td>
<td>13.4 (4.1)</td>
<td>15.9 (1.2)</td>
<td>9.9 (0.6)</td>
</tr>
</tbody>
</table>

**BMCs**, bone marrow cells.

**Enhanced Sca-1 expression and granulocyte cell cycle activity**

Because Sca-1 expression correlated with ERK activation in myeloid and granulopoietic precursors, we then examined whether enhanced Sca-1 expression was also associated with an increase in cell cycle activity. In control animals, marrow Sca-1+ granulopoietic precursor cells had greater cell cycle activity than did Sca-1− granulopoietic precursor cells (Fig. 4A, 4B). Following 24 h *E. coli* challenge, the percentage of cycling Sca-1+ granulopoietic precursor cells slightly decreased (Fig. 4B). However, Sca-1− granulopoietic precursor cell cycle activity declined

**FIGURE 2.** Enhanced marrow Sca-1 expression and ERK activation during bacteremia. *A*, Representative Western blot of phospho-ERK expression during 8 h *E. coli* challenge (quantification of $n = 4$). *B*, Representative dot plots of enhanced Sca-1 and pERK expression in the bone marrow during 8 h bacteremia. *C*, Increase in the number of marrow Sca-1+pERK+ cells and percentage of Sca-1+ in pERK+ cells during challenge. Data are combined from two independent experiments ($n = 5–8$). Bars with asterisks or different letters are statistically significant ($p < 0.05$). **BMCs**, bone marrow cells.
markedly, and fewer proliferating Sca-1− granulopoietic precursor cells were resident in the bone marrow during bacteremia (Fig. 4B, 4C). The number of proliferating marrow Sca-1+ granulopoietic precursor cells was significantly fewer than Sca-1− granulopoietic precursor cells in the absence of infection (Fig. 4C). This is reflective of the far greater marrow Sca-1− granulopoietic precursor cell population in controls. With an increase in the marrow Sca-1+ granulopoietic precursor cell population during bacteremia (Fig. 1B), there were significantly more proliferating Sca-1+ granulopoietic precursor cells than Sca-1− granulopoietic precursor cells in the bone marrow (Fig. 4C). Alcohol suppressed the proliferation of Sca-1+ granulopoietic precursors during bacteremia (Fig. 4B). Suppression of Sca-1+ granulopoietic precursor cell cycle activity by alcohol was associated with a decrease in the number of proliferating Sca-1+ granulopoietic precursors in the bone marrow during bacteremia as determined by BrdU incorporation.

Alcohol-induced suppression of Sca-1+ granulopoietic precursor cell proliferation also coincided with a slight increase in the number of proliferative marrow Sca-1− granulopoietic precursor cells during bacteremia (Fig. 4C). However, the cell cycle activity of this population did not change with infection. These data may partially explain the reduced ratio of mature granulocyte/granulopoietic precursor of proliferative cells in the bone marrow with alcohol administration during bacteremia (Fig. 1A). Our results suggest that the Sca-1− granulopoietic precursor population became the predominant cell population contributing to granulocyte production in the bone marrow during the granulopoietic response to bacteremia, and the expansion of this population was inhibited by alcohol.

**Sca-1 expression and maturation of granulopoietic precursors**

We next measured MPO content as a marker of granulocyte maturation to determine whether Sca-1 expression also correlated with increased differentiation of granulopoietic precursors. Proliferation of granulopoietic precursors terminates at the myelocyte stage, as these cells begin to produce the antimicrobial components characteristic of mature granulocytes (34). Production of MPO, the principal enzyme of cytoplasmic, peroxidase-positive granules, increases from the myeloblast to myelocyte stages (35, 36). As expected in control animals, immature granulopoietic precursor cells represented a lesser percentage than did mature granulocytes of the total MPO+ population in the bone marrow. Sca-1− cells composed relatively little of either granulocyte population (Fig. 5A). Following 24 h *E. coli* challenge, the number of MPO+Sca-1− cells was reduced and coincided with a slight increase in the number of marrow MPO+Sca-1+ cells. This increase, however, did not achieve statistical significance (Fig. 5B). Focusing on granulopoietic precursor development, we found that Sca-1− granulopoietic precursor cells had greater MPO content than did Sca-1+ granulopoietic precursor cells in control animals (data not shown). During bacteremia, the MPO content of Sca-1− granulopoietic precursors was reduced, whereas the MPO content of Sca-1− granulopoietic precursor cells remained unchanged. Alcohol exacerbated the decrease in MPO content of Sca-1+ granulopoietic precursor cells but did not affect the MPO content of Sca-1− granulopoietic precursor cells during bacteremia (Fig. 5C). Additionally, we found that the reduction in MPO content of Sca-1+ granulopoietic precursors inversely correlated ($r = 0.6178$) with the increase in number of marrow Sca-1+ granulopoietic precursor cells during *E. coli* challenge (Fig. 5D). With alcohol administration prior to challenge, MPO content and Sca-1+ granulopoietic precursor cell number remained inversely correlated ($r = 0.6987$) but were further reduced during bacteremia (Fig. 5D). The relationship between Sca-1 expression and MPO content was specific to granulopoietic precursors (Supplemental Fig. 2). There was no correlation between Sca-1 and MPO in mature granulocytes.

**Granulomacrophagic CFU production by Sca-1−/− myeloid and granulopoietic precursors during bacteremia**

To further determine the importance of Sca-1–associated proliferation in increasing granulocyte production during bacteremia granulomacrophagic CFU (CFU-GM) assays were performed using myeloid and granulopoietic precursors from wild-type C57BL/6 and Sca-1−/− mice. As expected in the absence of infection, CFU-GM production by myeloid precursors (30%; 30/100 lin−c-Kit+ cells) was higher than downstream granulopoietic precursor cells (0.5%; 10/2000 Gr1− cells) in wild-type controls. The CFU-GM activity of marrow Sca-1−/− myeloid precursors was significantly
reduced compared with that of wild-type cells. We observed that Sca-1^{-} granulopoietic precursor cells partially compensated for this deficiency with greater CFU-GM production than wild-type granulopoietic precursor cells in the absence of infection (Fig. 6A, 6B). These results agree with earlier findings by Ito et al. (25).

During the granulopoietic response to bacteremia, the CFU-GM activity of wild-type myeloid precursors and granulopoietic precursor cells increased 1.5- and 3-fold, respectively (Fig. 6A, 6B). During this response, ERK signaling was also activated in total bone marrow and granulopoietic precursor cells (Fig. 6C, 6D). In Sca-1^{-} mice, bacteremia did not stimulate a statistical increase in CFU-GM production by marrow myeloid precursor or granulopoietic precursor cells (Fig. 6A, 6B). Impaired CFU-GM production by Sca-1^{-} granulopoietic precursors during bacteremia was also associated with a failure to activate ERK signaling in total bone marrow and marrow granulopoietic precursor cells (Fig. 6C, 6D). These results suggest that granulopoietic precursors maintain a large reserve capacity to enhance granulocyte production during bacterial infection that is facilitated by upregulation of Sca-1 expression.

**Discussion**

Hematopoietic stem and progenitor cells respond to systemic infection by expansion, differentiation, and mobilization (37). Our previous studies have shown that enhanced surface expression of Sca-1 by marrow primitive hematopoietic precursors, including hematopoietic stem cells, plays an important role in the granulopoietic response to bacteremia. In response to Gram-negative or Gram-positive bacterial infection, enhanced Sca-1 expression by marrow hematopoietic precursors corresponds with enhanced proliferation and preferential commitment toward myeloid lineage development (10, 11, 38). Sca-1 expression can also be upregulated in granulopoietic precursors, leading to increased mitosis and subsequent granulocyte production (Supplemental Fig. 3) (23). Results presented in this study suggest that upregulated Sca-1 expression may promote granulocyte lineage expansion through enhancing ERK pathway activation.

Excessive alcohol consumption dysregulates multiple structural, cellular, and humoral aspects of the immune system, including granulopoiesis. We have previously reported that alcohol suppresses enhanced Sca-1 expression by primitive hematopoietic precursors and marrow granulocytic lineage expansion during bacterial infection (11, 23). Our current study extends this line of investigation by demonstrating that alcohol suppresses the proliferation of granulopoietic precursors by impairing their upregulation of Sca-1 expression and subsequent activation of the ERK signaling pathway during the granulopoietic response.

Granulocyte production is dynamically regulated in the bone marrow during bacterial infection. Our current data show that the marrow granulopoietic precursor population expanded following E. coli challenge, coinciding with reduction of the marrow mature granulocyte reserve. Following 24 h bacteremia, activated marrow granulopoiesis was associated with an increase in proliferation of myeloid and granulopoietic precursors and development into mature granulocytes. A prominent feature of this process was the significant upregulation of Sca-1 expression by cells in both myeloid and granulopoietic precursor populations, which correlated with their robust increase in proliferation and granulocyte production.

ERK activation plays an important role in hematopoietic precursor proliferation and myeloid lineage commitment (22, 39).
Therefore, we studied the role of Sca-1 in promoting ERK signaling during the granulopoietic response to bacteremia. Our data demonstrate that enhanced Sca-1 expression closely correlated with ERK activation in the bone marrow during E. coli challenge. Bacteremia induced a nearly 3-fold increase in the contribution of Sca-1+ granulopoietic precursor cells to total marrow cells with ERK activation. Increased ERK signaling occurred in myeloid and granulopoietic precursors but not mature granulocytes during bacteremia. Moreover, enhanced Sca-1 expression by granulopoietic precursors directly correlated with increased ERK pathway activation. These data support previous reports that ERK signaling is important for granulopoietic precursor expansion rather than differentiation (22).

We further assessed whether greater ERK activation by Sca-1+ granulopoietic precursors also corresponded with greater cell cycle activity. Our data demonstrate that Sca-1+ granulopoietic precursor cells were more actively involved in cell cycling than were Sca-1− granulopoietic precursor cells in the absence of infection. Moreover, during the granulopoietic response to bacteremia, the Sca-1+ granulopoietic precursor cell population became the predominant cell type contributing to marrow granulocyte production.

Multiple host- and pathogen-derived factors are involved in stimulating granulopoiesis during a systemic bacterial infection. Although inflammatory cytokines, including G-CSF, GM-CSF, IL-1, and IL-6, are well-recognized regulators of granulopoiesis, increasing evidence suggests that pathogens can directly modify marrow hematopoiesis through TLR stimulation of hematopoietic precursors (5, 6, 8, 40, 41). The ERK pathway is a signaling cascade activated by ligand engagement of both TLR and cytokine receptors, which are critically involved in the granulopoietic response (2). Activation of ERK signaling enhances marrow granulopoiesis by promoting the proliferation over differentiation of granulopoietic precursors during infection. Activated ERK negatively regulates the master myeloid lineage transcription factor C/EBP-α. Upon stimulation, C/EBP-α suppresses further granulopoietic precursor proliferation in favor of terminal granulocyte differentiation (42, 43). Because enhanced Sca-1 expression promoted ERK activation in granulopoietic precursors, we then studied whether Sca-1 expression was also related to their terminal differentiation during bacteremia. As predicted, expansion of the marrow Sca-1+ granulopoietic precursor cell pool inversely correlated with the MPO content of this population. As granulopoietic precursors develop into more mature myelocytes, their proliferation ceases and MPO content increases. Enhanced Sca-1 expression by granulopoietic precursors may therefore promote proliferation over subsequent differentiation of granulopoietic precursors by supporting increased ERK activation during bacteremia. Alcohol intoxication impaired the upregulation of Sca-1 expression and ERK activation by granulopoietic precursors in response to bacteremia. Concurrently, alcohol suppressed Sca-1+ granulopoietic precursor cell cycle activity, as well as BrdU incorporation into Sca-1+ granulopoietic precursors during bacteremia. Alcohol administration also exacerbated the decrease in MPO content of Sca-1+ granulopoietic precursor cells following systemic infection with E. coli. These data indicate that marrow granulopoietic precursors are one of the major cell types targeted by alcohol in alcohol-induced impairment of the granulopoietic response to bacteremia.

To confirm the role of Sca-1 in achieving maximal granulopoietic precursor proliferation during bacterial infection, we challenged Sca-1−/− mice with i.v. E. coli. Previous studies with Sca-1−/− mice have identified deficiencies in hematopoietic and mesenchymal stem cell self-renewal and myeloid progenitor cell production (25, 44). Our studies have also found Sca-1−/− mice to have a suppressed marrow granulopoietic response and reduced production of circulating granulocytes during bacteremia (23). Because maximal granulocyte production is dependent on lineage-committed precursors, we determined the approximate stage of granulocyte development that Sca-1 was most important for enhancing granulocyte production during bacteremia (45). Our observations indicate that Sca-1 expression is necessary for granulocyte production by myeloid precursors in the absence of infection. Sca-1−/− granulopoietic precursors partially compensate for this deficiency with greater proliferation than do wild-type cells, in
Support of earlier observations (25). Following 24 h E. coli bacteremia, granulocyte production increased in wild-type, but not Sca-1–/–, myeloid precursor cells. In wild-type animals, bacteremia induced a greater increase in granulocyte production by downstream granulopoietic precursor cells. In response to 24 h E. coli challenge, granulocyte production by wild-type granulopoietic precursors increased 3-fold, doubling the increase observed in wild-type myeloid precursor cells. In contrast, Sca-1–/– granulopoietic precursor cells were unable to further enhance granulocyte production during bacteremia. This impaired capacity for Sca-1–/– granulopoietic precursors to contribute to granulocyte lineage expansion during bacteremia was associated with failure to activate ERK signaling. After 8 h E. coli challenge, the ERK pathway was activated in granulopoietic precursor cells from wild-type mice but was absent in Sca-1–/– mice.

Growing evidence suggests that Sca-1 expression is regulated by both proinflammatory and anti-inflammatory stimuli. Components of a proinflammatory milieu including IFN-α/γ, IL-6, TNF-α, and LPS can upregulate Sca-1, whereas anti-inflammatory factors including TGF-β have been shown to downregulate Sca-1 expression (10, 46). Therefore, Sca-1 expression and its role in hematopoietic precursor proliferation are dynamically regulated during the generation and resolution of the host immune response to bacteremia. It is likely that LPS/TLR4 signaling plays a direct role in upregulating Sca-1 expression at the initial stage of E. coli infection in our model system. As the host response to infection proceeds, other tissue-generated inflammatory mediators may subsequently join in the regulation of Sca-1 expression by hematopoietic cells. Further investigation on the dynamic regulation of the Sca-1 response will provide additional information for understanding this important host defense process.

Suppression of Sca-1 induction and ERK signaling in myeloid precursors by alcohol may result from impaired pathogen recognition (11, 47). Szabo et al. (48) have shown that alcohol can impair LPS-mediated activation of phagocyes by inducing the redistribution of TLR4 complex components within membrane lipid rafts, impeding receptor clustering, and suppressing the transduction of intracellular signaling cascades. Because Sca-1 is a glycoposphatidylinositol-anchored protein that localizes to and may facilitate interactions within lipid rafts, the inhibition of Sca-1 expression by alcohol might be an underlying mechanism for the suppression of proproliferative signaling during the granulopoietic response to bacteremia (9, 49).

In our present investigation, we incorporated phospho-specific flow cytometry to understand the cell signaling events related to enhanced Sca-1 expression in myeloid and granulopoietic precursors. Phospho-specific flow cytometry is a significantly more powerful tool for studying cellular signaling events than conventional Western blot, because simultaneous analysis of cell surface marker expression and signaling can be resolved at a single cell level. This technique however, involves several technical hurdles, including the negative effects of fixation and permeablization on cell surface staining (26, 50). In this study, cell surface staining was not performed prior to fixation to avoid the possibility that mAbs would stimulate intracellular signaling events. Our protocol optimized fixation/permeablization procedures, specifically for studying intracellular signaling at different stages of myeloid lineage development during the host immune response to infection.

In summary, our present study reveals that enhanced Sca-1 expression facilitates activation of the ERK pathway and supports the proliferation of myeloid and granulopoietic precursors during bacteremia. Enhanced Sca-1 expression favors further proliferation over terminal differentiation of these precursors. Whereas both myeloid and granulopoietic precursors contribute to granulocyte lineage expansion, granulopoietic precursors have a larger reserve capacity to support increased granulocyte production during bacteremia that may be facilitated by increased Sca-1 expression. Suppression of enhanced Sca-1 expression by alcohol correlates with impaired ERK pathway activation and proliferation of granulopoietic precursors, resulting in impaired marrow granulocyte lineage expansion. These results highlight a critical role of Sca-1 in the granulopoietic response and a target for alcohol-induced myelosuppression in the alcohol-abusing host.
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Disclosures

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References


