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# Alcohol Suppresses the Granulopoietic Response to Pulmonary *Streptococcus pneumoniae* Infection with Enhancement of STAT3 Signaling

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Enhanced granulopoietic activity is crucial for host defense against bacterial pneumonia. Alcohol impairs this response. The underlying mechanisms remain obscure. G-CSF produced by infected lung tissue plays a key role in stimulating bone marrow granulopoiesis. This study investigated the effects of alcohol on G-CSF signaling in the regulation of marrow myeloid progenitor cell proliferation in mice with *Streptococcus pneumoniae* pneumonia. Chronic alcohol consumption plus acute alcohol intoxication suppressed the increase in blood granulocyte counts following intrapulmonary challenge with *S. pneumoniae*. This suppression was associated with a significant decrease in bone marrow granulopoietic progenitor cell proliferation. Alcohol treatment significantly enhanced STAT3 phosphorylation in bone marrow cells of animals challenged with *S. pneumoniae*. In vitro experiments showed that G-CSF-induced activation of STAT3-p27<sup>Kip1</sup> pathway in murine myeloid progenitor cell line 32D-G-CSFR cells was markedly enhanced by alcohol exposure. Alcohol dose dependently inhibited G-CSF-stimulated 32D-G-CSFR cell proliferation. This impairment of myeloid progenitor cell proliferation was not attenuated by inhibition of alcohol metabolism through either the alcohol dehydrogenase pathway or the cytochrome P450 system. These data suggest that alcohol enhances G-CSF-associated STAT3-p27<sup>Kip1</sup> signaling, which impairs granulopoietic progenitor cell proliferation by inducing cell cycling arrest and facilitating their terminal differentiation during the granulopoietic response to pulmonary infection. *The Journal of Immunology*, 2011, 186: 4306–4313.

Representing the largest population of WBCs in the circulation, polymorphonuclear leukocytes (PMNs or granulocytes) constitute the first line of phagocytic defense. These cells patrol in the circulatory system and quickly migrate to infected tissue sites to eliminate pathogenic microbes by phagocytosis and intracellular killing. Due to the relatively short life span of PMNs, the bone marrow continuously produces these phagocytes from hematopoietic precursors to maintain homeostatic levels of circulating PMNs (1). During bacterial infection, bone marrow production of PMNs is significantly enhanced to reinforce host defense against invading pathogens. Impairment of granulopoiesis and the granulopoietic response by inherited or acquired abnormalities results in ineffective control of infection with increased morbidity and mortality (2).

Alcohol abuse predisposes the host to severe bacterial infections, particularly pneumonia (3, 4). A prominent feature of alcohol-abusing patients with severe pulmonary infection is the frequent occurrence of granulocytopenia, which predicts poor outcome (3, 5–7). Analysis of bone marrow from these individuals shows a reduction in the number of mature granulocytes with vacuolization of myeloid progenitor cells (8–10). Incubation of bone marrow cells with alcohol at concentrations commonly observed in intoxicated patients has been reported to suppress granulocyte colony formation (8, 11). Currently, the underlying mechanisms for these effects remain unclear.

G-CSF is a lineage-specific growth factor that stimulates granulopoiesis in the bone marrow. During bacterial infection, cells of infected tissues produce large quantities of inflammatory mediators, including G-CSF (12–15). Studies on clinical patients and experimental animals have repeatedly shown that the level of G-CSF in the systemic circulation is significantly increased during bacterial infection (16–19). This elevated G-CSF concentration stimulates marrow granulocyte lineage development and enhances PMN mobilization from the bone marrow into the circulation (12, 14, 15, 20). Previous investigations have shown that acute alcohol intoxication causes a temporary inhibition of the G-CSF response to bacterial infection (21). However, the inhibition of the G-CSF response is brief, which suggests that the prolonged impairment of the granulopoietic response during bacterial infection in alcohol abusers may involve other factors yet to be defined (21).

Engagement of G-CSF with its receptor triggers the p44/42-cyclin D pathway, which mediates myeloid progenitor cell proliferation under both normal and emergency conditions (22). G-CSF also activates STAT3-cyclin-dependent kinase (CDK) inhibitor p27<sup>Kip1</sup> pathway, which serves as a negative signal causing cell cycle arrest at the G<sub>1</sub> checkpoint (23–25). These two pathways are complementary in their roles of promoting progeni-

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The online version of this article contains supplemental material.

Abbreviations used in this article: ADH, alcohol dehydrogenase; BAL, bronchoalveolar lavage; CDK, cyclin-dependent kinase; CYP450, cytochrome P450; GM, granulocyte macrophage; i.t., intratracheal; MCF, mean channel fluorescence; 4-MP, 4-methylpyrazole; PMN, polymorphonuclear leukocyte; SOCS, suppressor of cytokine signaling; TYK2, tyrosine kinase 2.

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tor cell pool expansion and terminal granulocyte differentiation. In this study, we determined the impact of alcohol on cell signaling regulation of myeloid progenitor cell development. Our observations indicate that alcohol treatment causes a profound enhancement of STAT3-p27<sup>Kip1</sup> negative signaling in myeloid precursor cells, which is associated with impairment of the granulopoietic response in mice with *Streptococcus pneumoniae* lung infection.

## Materials and Methods

### Animals

Male BALB/c mice (7 wk old; Charles River Laboratories) with a body weight of  $22.7 \pm 0.1$  g were housed in a specific pathogen-free facility with a 12-h light/dark cycle. Mice were maintained on the Lieber-DeCarli low-fat liquid alcohol diet (supplies 36% of calories as ethanol; Dyets 710261, Bethlehem, PA) for 5 d and standard laboratory diet plus 20% alcohol in drinking water for 2 d/wk for a total of 5 wk (26). Control animals were fed the isocaloric Lieber-DeCarli low-fat liquid control diet (Dyets 710028) and standard laboratory diet with drinking water utilizing the same schedule. This alcohol-feeding protocol is well tolerated by mice (26). The median alcohol concentration in the blood (from random morning samples) is 8.5 mM (39 mg/dl) (26). Acute alcohol intoxication (i.p. injection with 20% alcohol in saline at a dose of 5 g alcohol/kg) was induced after mice had been on chronic alcohol diets for 5 wk. The blood alcohol levels were  $119.7 \pm 1.3$ ,  $106.3 \pm 1.5$ ,  $87.7 \pm 3.6$ , and  $48.4 \pm 3.5$  mM, respectively, at 45 min, 90 min, 3 h, and 6 h postalcohol injection in naive mice, as reported by our group previously (27). Control mice were i.p. injected with an equal volume of saline.

Thirty minutes after i.p. ethanol injection, *S. pneumoniae* (type 3, 6303; American Type Culture Collection, Manassas, VA) ( $1 \times 10^5$  CFU in 50  $\mu$ l pyrogen-free isotonic saline) or saline was administered intratracheally (i.t.) to mice under isoflurane anesthesia. The animals were sacrificed at scheduled time points after the i.t. challenge, as indicated in each figure legend. In a subgroup of mice, BrdU (1 mg in 100  $\mu$ l PBS/mouse; BD Biosciences, San Jose, CA) was administered i.v. at the same time of the i.t. challenge. Upon sacrifice, a heparinized blood sample was obtained by cardiac puncture. WBCs were quantified under a light microscope with a hemocytometer, and differential WBC counts were performed on Wright-Giemsa smears. Plasma was separated and stored at  $-80^\circ\text{C}$ . PBMCs were isolated using Lympholyte-Mammal density separation medium (Cedarlane Laboratories, Burlington, NC) with the procedure provided by the manufacturer. Bronchoalveolar lavage (BAL) was performed to obtain BAL cells, as described previously (28). BAL cells were enumerated and differential cell counts were performed. Femurs and tibias were collected. Bone marrow cells were collected, as described previously (27). The above described experiments were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. All experimental protocols were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

### Culture of bacteria

For each experiment, frozen stock cultures of *S. pneumoniae* were added to 100 ml Todd Hewitt broth and incubated for 14 h in a  $\text{CO}_2$  incubator. Bacteria were collected and washed twice with PBS. A suspension of bacteria in PBS at a concentration of  $1 \times 10^8$  CFUs/ml was prepared based on its OD at 600 nm. Actual numbers of viable bacteria were verified by standard plate counts of the bacterial suspensions on Columbia agar with sheep blood plates (BBL 221165; BD Biosciences) following 24-h culture at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator.

Bacterial load was determined in the lungs of control and alcohol-treated animals. Lung samples were collected, weighed, and homogenized with 9 vol PBS (mg/ $\mu$ l) using sterilized glass homogenizers equipped with a NSI-12 Fractional Horsepower Motor (Bodine Electric, Chicago, IL). Serial dilutions (1:10) of tissue homogenates were plated on Columbia agar with sheep blood plates and cultured for 24 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator. Bacterial colonies were enumerated after culture.

### CFU assay

CFU assay of bone marrow cells was performed by culturing the cells in Methocult GF M3534 medium (StemCell Technologies, Vancouver, BC, Canada). One milliliter of Methocult GF M3534 medium containing 20,000 total bone marrow cells was plated in a 35-mm Nunclon dish (Nunc, Rochester, NY). Each sample was cultured in triplicate for 7 d at  $37^\circ\text{C}$  in

an atmosphere of 5%  $\text{CO}_2$ . Colonies containing 50 or more cells were then enumerated.

### Development of 32D-G-CSFR cell line

The 32Dcl3 cell line (American Type Culture Collection CRL-11346, Manassas, VA) is an IL-3-dependent murine myeloid progenitor cell line. Switching 32Dcl3 cell line to G-CSF dependence was achieved by electroporation of cells with a pCR3.1 vector (Invitrogen, Carlsbad, CA) encoding wild-type murine G-CSFR gene, followed by G418 selection. Expression of G-CSFR mRNA and protein was confirmed by real-time RT-PCR and Western blot analysis. These selected 32D-G-CSFR cells were then cultured with 1 ng/ml murine G-CSF for maintenance.

### Flow cytometric analysis

Nucleated bone marrow cells suspended in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 2% FCS ( $2 \times 10^6$  cells in 100  $\mu$ l medium) were incubated with 10  $\mu$ g/ml fluorochrome-conjugated anti-mouse Gr1 (Ly6G, RB6-8C5; BD Biosciences) or isotype control Ab in the dark for 15 min at  $4^\circ\text{C}$ . For measuring BrdU incorporation, the cells were further processed using a BD BrdU flow kit (BD Biosciences). At the end of the staining procedure, cells were suspended in 0.5 ml PBS containing 1% paraformaldehyde. Analysis of cell phenotype and BrdU incorporation was performed on a FACSAria or a LSR-II flow cytometer with FACSDiva software (BD Biosciences). For each sample, 300,000 cells were acquired for analysis.

Additionally, 32D-G-CSFR cells were cultured in RPMI 1640 containing 10% FCS, 10 ng/ml G-CSF, and various concentrations of alcohol. On day 6 of the culture, BrdU (1 mM) was added to cells. The cells were harvested 90 min later to determine incorporation of BrdU into cells by flow cytometry using a BD BrdU kit and manufacturer's protocols. BrdU incorporation was enumerated on a FACSCalibur flow cytometer (BD Biosciences). Viability was assessed by trypan blue exclusion for all in vitro and ex vivo studies (Supplemental Table I).

### Analysis of cell proliferation with CFSE staining

The 32D-G-CSFR cells were incubated in HBSS containing 10% FCS and 5  $\mu$ M CFSE (Molecular Probes/Invitrogen, Eugene, OR) for 5 min. After washing twice with HBSS, the stained cells were suspended in RPMI 1640 containing 10% FCS and murine rG-CSF (10 ng/ml). The cells were cultured with various concentrations of alcohol. In a subset of cell cultures, 4-methylpyrazole (4-MP, which blocks alcohol dehydrogenase [ADH]; Sigma-Aldrich, St. Louis, MO) or metyrapone (which blocks cytochrome P450 [CYP450] enzymes; Sigma-Aldrich) was added to the culture media. The cells were cultured with G-CSF (10 ng/ml) in the absence and presence of 50 mM alcohol. Analysis of cell proliferation based on the reduction of cell fluorescence was performed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

### Sample preparation for Western blot analysis

Nucleated bone marrow cells isolated from naive mice were plated in a 12-well plate with  $5 \times 10^6$  cells/well. Cells were incubated in RPMI 1640 containing 10% FCS with different concentrations of alcohol (0, 50, and 100 mM) 30 min prior to the addition of G-CSF (10 ng/ml). The cells were harvested 10 min after addition of G-CSF. In a separate set of experiments, 32D-G-CSFR cells were cultured in RPMI 1640 containing 10% FCS with various concentrations of alcohol (0, 50, and 100 mM) for 4 h. G-CSF (10 ng/ml) was then added to the cultures. Following 30 min of stimulation, cells were harvested. Freshly isolated bone marrow cells, cultured bone marrow cells, and cultured 32D-G-CSFR cells were lysed with lysis buffer to prepare cell lysates for Western blot analysis (29).

### Western blot analysis of phospho-JAKs and phospho-STAT3

Western blot procedures were performed, as previously reported, with minor modifications (29). After gel transfer onto a polyvinylidene difluoride membrane, the membrane was blocked with 5% milk in TBST buffer and hybridized sequentially with primary Ab against phospho-STAT3 (Tyr<sup>705</sup>), phospho-JAK1 (Tyr<sup>1022/1023</sup>), phospho-JAK2 (Tyr<sup>1007/1008</sup>), phospho-tyrosine kinase 2 (TYK2) (Tyr<sup>1054/1055</sup>; Cell Signaling Technology, Danvers, MA), and HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology). The membrane was stripped and then reprobed with rabbit anti- $\beta$ -actin Ab (Cell Signaling Technology) and HRP-conjugated goat anti-rabbit IgG to determine  $\beta$ -actin content in each lane of the gel. Semiquantification was performed using a Kodak Gel Logic 2,200 Imaging System. Data are presented as the normalized mean intensity ratio of either the

phospho-STAT3 or phospho-JAK2 protein band versus the corresponding  $\beta$ -actin band.

#### Preparation of p27<sup>Kip1</sup> RNA standard and real-time RT-PCR determination of p27<sup>Kip1</sup> mRNA

Total RNA was isolated from 10<sup>7</sup> 32Dcl3 cells, and p27<sup>Kip1</sup> cRNA standards were produced, as previously reported (30). Forward and reverse cloning primers for p27<sup>Kip1</sup> were 5'-ATGTC AACGTGAGAGTGTC-TAACGG-3' and 5'-TTACGTCTGGCGTCAAGGC-3', respectively. For each subsequent real-time quantitative RT-PCR assay, standard curves of p27<sup>Kip1</sup> and 18S rRNA (Taqman; Applied Biosystems, Carlsbad, CA) (ranging from 10<sup>2</sup> to 10<sup>9</sup> copies of p27<sup>Kip1</sup> RNA per reaction and 10<sup>-5</sup> to 10<sup>-1</sup> ng 18S rRNA per  $\mu$ l reaction volume) were generated by serial dilution of stock standard RNA aliquots. Real-time RT-PCR determination of p27<sup>Kip1</sup> mRNA expression by 32D-G-CSFR cells was performed, as described previously (29). The amplification primers and probes used for determination of p27<sup>Kip1</sup> expression were as follows: forward primer, 5'-GACTCGTCAGACAATCAGGCT-3'; reverse primer, 5'-CCCTTTT-GTTTTGCCGAAGAAGATC-3'; and probe, 5'-AGGTCGCTTCCTCAT-CCCTGGACTG-3'. This set of primers and probe was designed using Primer Express software (Applied Biosystems). The primers and probe for detection of 18S rRNA were purchased from Applied Biosystems. The p27<sup>Kip1</sup> mRNA quantity in each sample was determined by comparing its cycle threshold number with those of the p27<sup>Kip1</sup> RNA standard curve and then normalized to the content of 18S rRNA in each sample. The results are expressed as copies mRNA/ng rRNA.

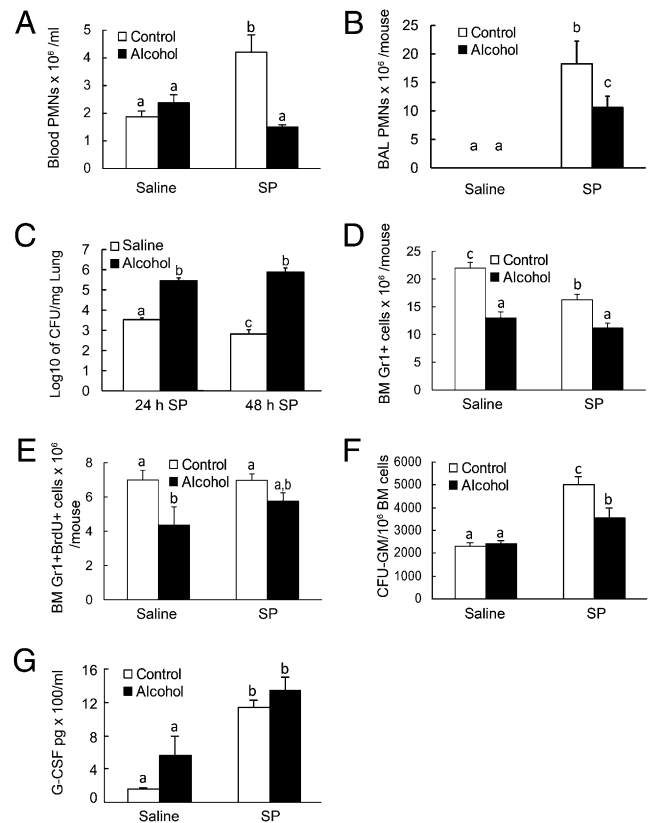
#### Statistical analysis

Data are presented as mean  $\pm$  SEM. The sample size is indicated in the legend of each figure. Statistical analyses of data were conducted using unpaired Student *t* test (for comparison between two groups) and one-way ANOVA, followed by Student-Newman-Keuls test or Proc Mixed (SAS Institute; 2004) two-way ANOVA (for comparisons among multiple groups). Differences were considered statistically significant at  $p < 0.05$ .

## Results

### Alterations of bone marrow granulocyte production

To examine the effects of alcohol treatment on the circulating levels of granulocytes, blood was harvested 24 h after i.t. challenge of saline or *S. pneumoniae*. Alcohol had no effect on blood PMNs from mice receiving saline; however, alcohol suppressed the increase in the number of circulating PMNs in response to pneumococcal infection (Fig. 1A). Alcohol treatment also impaired PMN recruitment into the alveolar space following pulmonary infection with *S. pneumoniae* (Fig. 1B). This impaired recruitment of PMNs was associated with a significantly higher bacterial burden in the lung at both 24 and 48 h post-*S. pneumoniae* inoculation (Fig. 1C). Bacterial clearance was evident in the lungs from mice without alcohol treatment at 48 h. To assess the role of the bone marrow in this PMN response, we analyzed total nucleated bone marrow cells for Gr1 expression at 24 h post-challenge. Gr1 is a surface marker of the Ly6 family that is found on cells of the granulocyte lineage. Alcohol treatment diminished the bone marrow pool of Gr1<sup>+</sup> cells in both saline- and pneumococcus-inoculated animals (Fig. 1D). To determine whether this decreased bone marrow granulocyte pool was due to inhibited cell proliferation, we analyzed BrdU incorporation into the Gr1<sup>+</sup> cells in the bone marrow (Fig. 1E). Alcohol treatment decreased the number of Gr1<sup>+</sup>BrdU<sup>+</sup> cells in saline-challenged mice, suggesting an impaired proliferation capacity of the myeloid progenitors. We next performed CFU-granulocyte macrophage (GM) assays on bone marrow cells from mice sacrificed 48 h postchallenge with pneumococcus to examine granulopoietic potential. Pneumococcal infection enhanced the CFU-GM activity of the bone marrow progenitors, and alcohol inhibited this enhancement (Fig. 1F). Because G-CSF is a major cytokine stimulating granulocyte lineage development, we tested whether the effects of alcohol on progenitor proliferation were due to inhibited G-CSF

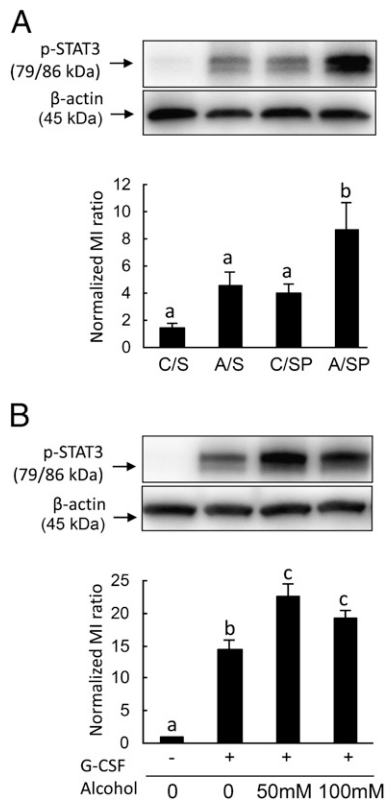


**FIGURE 1.** Granulopoietic response to pneumonia. *A*, Changes in blood PMN counts 24 h post-i.t. challenge. *B*, BAL PMN counts 24 h post-i.t. challenge. *C*, Bacterial load in the lung 24 and 48 h post-i.t. inoculation of *S. pneumoniae*. *D*, Changes in the number of bone marrow Gr1<sup>+</sup> cells 24 h post-i.t. challenge. *E*, Changes in the number of bone marrow Gr1<sup>+</sup>BrdU<sup>+</sup> cells 24 h post-i.t. challenge. *F*, Changes in bone marrow cells 48 h post-i.t. challenge. *G*, Plasma G-CSF concentration 10 h after i.t. challenge. Saline: intratracheal challenge with saline; SP: intratracheal challenge with *S. pneumoniae*.  $n = 4-8$ . Bars with different letters in each panel are statistically different.  $p < 0.05$ .

secretion by the infected tissues. The circulating level of G-CSF 10 h postinoculation of *S. pneumoniae* was significantly increased as compared with the control values (Fig. 1G). Alcohol treatment did not affect pneumococcal infection-induced increase in plasma G-CSF concentration at this time point. This observation would suggest that the granulopoietic progenitor cell response to G-CSF may play an important role in alcohol-elicited adverse effects on granulocyte production in the bone marrow following pulmonary infection with *S. pneumoniae*.

### Alcohol enhances STAT3 phosphorylation

In an effort to elucidate the mechanism by which alcohol impairs neutrophil number and function during infection, we assessed the activation of STAT3 in nucleated bone marrow cells. Under control conditions, mice displayed a low level of Tyr<sup>705</sup> phosphorylation on STAT3. Alcohol treatment enhanced STAT3 activation in bone marrow cells 10 h postpulmonary infection with *S. pneumoniae* (Fig. 2A). This pattern of alcohol-enhanced STAT3 activation was evident as early as 6 h post-*S. pneumoniae* challenge (data not shown). STAT3 phosphorylation is a key effector molecule in the signal transduction cascades for many cytokine receptors, including G-CSFR. To assess the role of G-CSFR in alcohol-enhanced p-STAT3 levels, nucleated bone marrow cells isolated from naive mice were cultured in vitro with G-CSF and various concentra-



**FIGURE 2.** STAT3 phosphorylation in bone marrow cells. *A*, In vivo effects of alcohol treatment on STAT3 activation (Tyr<sup>705</sup> phosphorylation) in bone marrow cells 10 h post-i.t. challenge with *S. pneumoniae*. Western blot images are representative of three to four experiments. Each lane was loaded with 20  $\mu$ g protein. C/S, control mice with i.t. saline; A/S, alcohol-treated mice with i.t. saline; C/SP, control mice with i.t. *S. pneumoniae*; A/SP, alcohol-treated mice with i.t. *S. pneumoniae*. *B*, In vitro effects of alcohol on STAT3 activation in bone marrow cells 10 min following G-CSF (10 ng/ml) stimulation. The Western blot images are representatives of three experiments. Each lane was loaded with 20  $\mu$ g protein. Bars with different letters in each panel are statistically different.  $p < 0.05$ .

tions of alcohol. As shown in Fig. 2*B*, G-CSF increased STAT3 phosphorylation in cultured bone marrow cells. Exposure to 50 and 100 mM alcohol enhanced G-CSF-induced STAT3 phosphorylation in these cells. These ex vivo results mirror those seen from pneumococcus-challenged animals, indicating G-CSF signaling through STAT3 is enhanced by alcohol treatment.

#### Alcohol inhibits myeloid progenitor cell proliferation

To further test whether alcohol inhibits myeloid progenitor cell proliferation, we employed a cell culture model using 32D-G-CSFR cells prepared in our laboratory. The IL-3-dependent parent cell line, 32Dcl3, was derived from mouse bone marrow and maintains an immature, proliferative myeloid phenotype. Cells were labeled with CFSE and grown in the presence of G-CSF and various concentrations of alcohol for 3 and 6 d. Fluorescence of CFSE-labeled cells decreases proportionally with cell divisions. Gating strategy and initial CFSE-loading intensity are shown in Fig. 3*A* and 3*B*. Alcohol dose dependently inhibited 32D-G-CSFR proliferation at day 3 of culture (Fig. 3*C*, 3*D*). The antiproliferative effects of the lowest alcohol dose (25 mM) were abrogated by day 6 (Fig. 3*E*, 3*F*). However, cells treated with 50 and 100 mM alcohol still showed a dose-dependent impairment of cell proliferation by day 6, as reflected by the higher mean channel fluorescence (MCF; Fig. 3*E*, 3*F*).

To supplement the CFSE studies, we also treated the 32D-G-CSFR cells with various concentrations of alcohol for 6 d and performed BrdU incorporation assays. BrdU is a thymidine analog that incorporates into DNA during the S phase of the cell cycle. Consistent with our CFSE findings, alcohol dose dependently prevented BrdU incorporation into the 32D-G-CSFR cells at 50 and 100 mM (Fig. 4).

To determine whether the effects that we observed were caused by either the direct action of alcohol or alcohol metabolism, we cultured CFSE-labeled 32D-G-CSFR cells in 50 mM alcohol with either 4-MP or metyrapone for 3 d. Inhibition of ADH with 4-MP or CYP450 with metyrapone further enhanced the alcohol-induced inhibition of 32D-G-CSFR cell proliferation in response to G-CSF (Fig. 5). These data indicate that alcohol is acting directly on the cells to inhibit their proliferative response to G-CSF.

#### Alcohol selectively enhances the JAK2–STAT3–p27<sup>Kip1</sup> pathway

G-CSF signaling through the STAT3–CDK inhibitor p27<sup>Kip1</sup> pathway causes cell cycling arrest (23). JAK (JAK1, JAK2, and TYK2) associated with the G-CSFR are activated after receptor ligation. To further test how alcohol affects this pathway, 32D-G-CSFR cells precultured with various concentrations of alcohol were stimulated with G-CSF, and, as expected, G-CSF treatment led to enhancement of JAK2 phosphorylation (Fig. 6). However, alcohol treatment had no effect on phospho-JAK2 levels (Fig. 6). We did not detect JAK1 and TYK2 in this culture system (data not shown). STAT3 is immediately downstream of JAK2 in the G-CSFR signal transduction cascade. Consistent with our in vivo and primary cell culture results, phospho-STAT3 levels were elevated by G-CSF treatment. Alcohol exposure enhanced G-CSF-induced STAT3 phosphorylation.

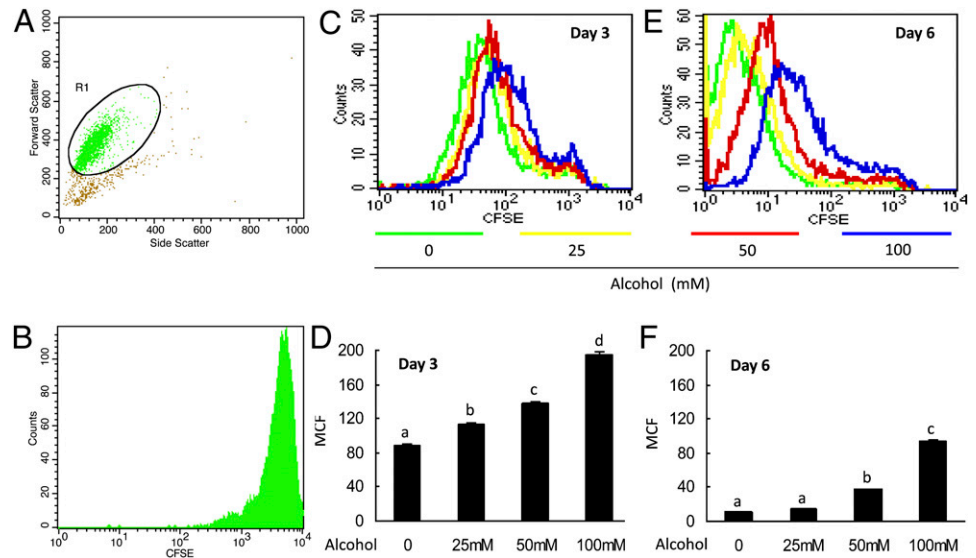
Activated STAT3 directly increases CDK inhibitor p27<sup>Kip1</sup> mRNA transcription, which is largely responsible for G<sub>1</sub> cell cycle arrest and terminal differentiation in hematopoietic progenitor cells (23, 31, 32). To determine whether the alcohol-induced increase in phospho-STAT3 was associated with changes in p27<sup>Kip1</sup> expression, we harvested 32D-G-CSFR cells after 24 h of culture in various concentrations of alcohol in the presence of 10 ng/ml G-CSF. Indeed, exposure to 25 and 50 mM alcohol caused a dose-dependent increase in p27<sup>Kip1</sup> mRNA expression (Fig. 7). However, no further enhancement of p27<sup>Kip1</sup> expression was observed when alcohol concentration exceeded 50 mM treatment.

## Discussion

During bacterial infection, hematopoietic activity in the bone marrow is shifted toward granulocyte production, which is critical for enhancing host defense against invading pathogens. G-CSF is the predominant cytokine stimulating granulopoiesis. G-CSF binding to its receptor can activate multiple signal transduction cascades. The activation of p44/42–cyclin D pathway through ligand engagement of G-CSFR mediates myeloid progenitor cell proliferation (33, 34). In contrast, activation of the STAT3–CDK inhibitor p27<sup>Kip1</sup> pathway following G-CSFR stimulation can cause cell cycle arrest and terminal differentiation (23, 32). These opposite signaling events differentially function to expand the granulopoietic progenitor cell pool (p44/42 pathway) and to promote terminal differentiation of PMNs (STAT3 pathway) (32).

The function of STAT3 signaling in the regulation of cell proliferation has drawn wide attention in recent years. The impact of STAT3 activation is largely context dependent. Multiple tyrosine and serine residues can be phosphorylated in the native STAT3 protein, often eliciting different effects (35, 36). Phosphorylation of Tyr<sup>705</sup> is required for STAT3 dimerization, nuclear translo-

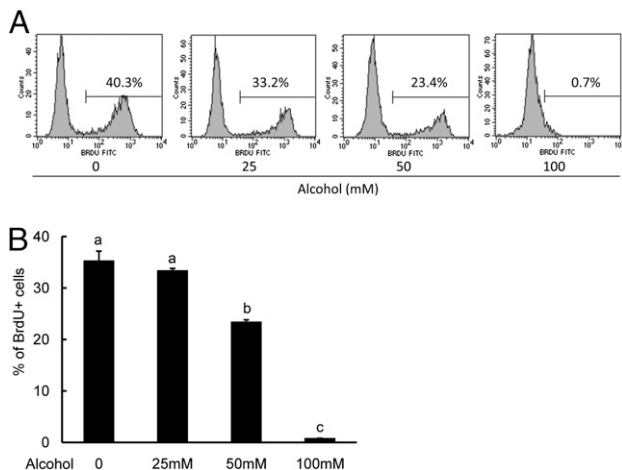
**FIGURE 3.** Effects of alcohol on G-CSF-stimulated 32D-G-CSFR cell proliferation determined by CFSE reduction. *A*, Gating strategy for CFSE experiments. Potential dead cells and cell debris were not gated for analysis. *B*, Representative histogram of initial CFSE-loading intensity. *C* and *E*, Representative histograms for CFSE fluorescence on days 3 and 6, respectively. *D* and *F*, Quantified MCF for days 3 and 6, respectively.  $n = 5$ . Bars with different letters in *B* and *D* are statistically different.  $p < 0.05$ .



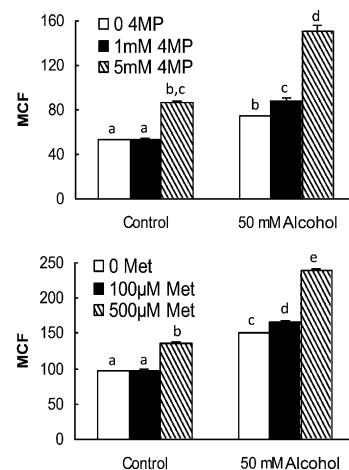
cation, and transcriptional activation (23). The CDK inhibitor p27<sup>Kip1</sup> is a direct target of STAT3-mediated transcription (37). STAT3-induced p27<sup>Kip1</sup> expression promotes cell cycle arrest and causes terminal differentiation in myeloid precursor cells (23, 32, 38). Thus, alcohol may be causing premature terminal differentiation in the myeloid progenitor cells.

Alcohol has been reported to suppress mitogenesis in a variety of tissues and cell lines (39–44). However, knowledge regarding mechanisms underlying the effects of alcohol on myeloid progenitor cell proliferation remains limited. A few in vitro studies have shown that alcohol inhibits CFU-GM formation in cultured bone marrow cells (45). Recently, our group showed that alcohol intoxication impairs proliferation of bone marrow lineage<sup>-</sup>c-Kit<sup>+</sup>Sca-1<sup>+</sup> cells, which include the short- and long-term repopulating hematopoietic stem cells in mice (27, 46). These studies demonstrated that alcohol impairs hematopoietic precursor cell responses to inflammatory stimulants, including cytokines and microbe-derived cell wall components. In light of these observations, we extended our investigations to determine whether alcohol impairs granulocyte production via disrupting cell signaling at the myeloid

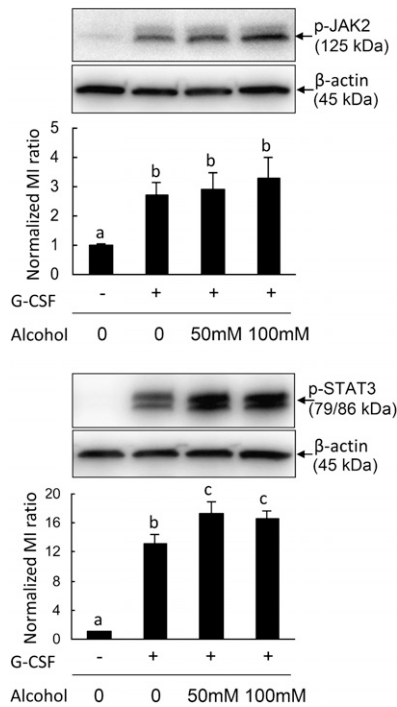
progenitor cell stage during bacterial pneumonia. The results of our current study showed that alcohol treatment suppressed the increase in the number of circulating granulocytes following *S. pneumoniae* infection in the lung, which was associated with impairment of PMN recruitment into the alveolar space and clearance of bacteria from the lung. Alcohol-treated mice exhibited a reduced storage pool of PMNs in the bone marrow in the absence and presence of pulmonary infection with *S. pneumoniae*. The inability to increase the number of circulating granulocytes during infection in alcohol-treated animals can partially be explained by attenuated progenitor cell amplification. Chronic alcohol consumption plus acute alcohol intoxication significantly inhibited proliferation of Gr1<sup>+</sup> bone marrow cells in uninfected mice. Furthermore, alcohol treatment attenuated *S. pneumoniae*-stimulated increases in CFU-GM activity in the bone marrow. Because the CFU-GM assay primarily tests progenitor cell amplification potential, these results suggest that in vivo alcohol exposure limits the number of myeloid progenitor cell divisions before terminal differentiation. Because alcohol did not significantly affect plasma G-CSF concentration in our model, we pre-



**FIGURE 4.** Effects of alcohol on BrdU incorporation into 32D-G-CSFR cells. Cells were cultured with the indicated alcohol concentrations for 6 d in G-CSF (1 ng/ml). BrdU incorporation was assessed 90 min after the addition of BrdU to the cultures. *A*, Representative histograms are shown. *B*, MCF was quantified as shown in the bar graph.  $n = 4$ . Bars with different letters are statistically different.  $p < 0.05$ .



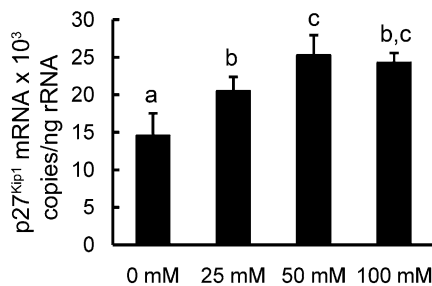
**FIGURE 5.** Effects of 4-MP and metyrapone (MET) on alcohol-induced inhibition of 32D-G-CSFR cell proliferation. Cells were loaded with CFSE prior to the initiation of culture with indicated alcohol doses, G-CSF (10 ng/ml), and the respective inhibitors. MCF was measured following 3 d of cell culture.  $n = 5$ –6. Bars with different letters in each panel are statistically different.  $p < 0.05$ .



**FIGURE 6.** Effects of alcohol on phospho-JAK2 (Tyr<sup>1007/1008</sup>) and phospho-STAT3 (Tyr<sup>705</sup>) levels in 32D-G-CSFR cells. The 32D-G-CSFR cells were cultured in alcohol 4 h prior to the addition of G-CSF (10 ng/ml). Cells were harvested 30 min post-G-CSF exposure. Each lane was loaded with 20 μg protein. The image is a representative of four to five experiments. Bars with different letters in each panel are statistically significant. *p* < 0.05.

dicted that the inhibited granulopoietic response was most likely occurring at the level of the bone marrow myeloid progenitor cell.

Western blot analysis in our current study showed that chronic alcohol consumption plus acute alcohol intoxication markedly enhanced STAT3 activation in nucleated bone marrow cells following pulmonary infection with *S. pneumoniae*. Furthermore, in vitro exposure to alcohol markedly enhanced G-CSF-induced STAT3 phosphorylation in cultured bone marrow cells. Previous investigations have shown that alcohol exposure may exert contrasting effects on STAT3 signaling in different cell types (47–49). Our results showed that alcohol dose dependently inhibited G-CSF-induced proliferation of these myeloid progenitor cells. These data strongly support our in vivo observations in alcohol-treated animals and further confirm that alcohol impairs granulopoietic precursor cell proliferation in response to G-CSF stimulation.



**FIGURE 7.** Effects of alcohol on p27<sup>Kip1</sup> mRNA expression by 32D-G-CSFR cells. Cells were cultured for 24 h in the indicated alcohol doses with G-CSF (10 ng/ml). *n* = 5. Bars with different letters are statistically different. *p* < 0.05.

Previous studies have shown that leukocytes and bone marrow cells metabolize alcohol (50). Alcohol metabolism through both the ADH pathway and the CYP450 system generates reactive oxygen species (51). Reactive oxygen metabolites have been reported to injure various cell functional activities and proliferation (52). Therefore, disruption of G-CSF-stimulated proliferation of myeloid progenitors may also be caused indirectly by alcohol metabolites. To delineate whether the observed negative effect of alcohol on myeloid progenitor cell proliferation is directly caused by alcohol or indirectly resulted from alcohol metabolites, myeloid progenitor cells were cultured with 4-MP (ADH inhibitor) or metyrapone (CYP450 inhibitor) to block alcohol metabolism. Our results showed that blockade of alcohol metabolism enhanced the alcohol-induced inhibition of myeloid progenitor proliferation, which may be a result of increased intracellular alcohol concentration. These data indicate that the impairment of myeloid progenitor cell proliferation is directly caused by alcohol.

To identify the level at which alcohol modified G-CSF signal transduction, we examined JAK1, JAK2, and TYK2 activation. JAK are effector molecules immediately proximal to G-CSFR (2, 53–59). JAK1 and TYK2 were not detected in our model system. G-CSF did stimulate JAK2 phosphorylation, but this signal was not altered by alcohol administration. This finding suggested that the target of alcohol was downstream of JAK2. Because activated JAK2 promotes STAT3 phosphorylation, we measured phospho-STAT3 levels after G-CSF addition to the culture system of myeloid progenitor cells in the absence and presence of alcohol. Consistent with our in vivo findings and observations from primary bone marrow cell cultures, alcohol enhanced the level of STAT3 phosphorylation in G-CSF-stimulated myeloid progenitor cells. These data demonstrate that alcohol enhances STAT3 signaling at the level of STAT3 activation. Molecular mechanisms underlying alcohol-enhanced STAT3 phosphorylation remain to be further explored. Because activated STAT3 induces expression of suppressor of cytokine signaling (SOCS) proteins, including SOCS1 and SOCS3, and these SOCS proteins in turn inhibit activation of STAT3, one potential mechanism might be that alcohol impairs SOCS expression or function, resulting in loss of this negative feedback (60–62). Previous investigations in a rat model have shown that chronic alcohol consumption completely suppresses growth hormone-induced SOCS3 expression in the liver (63).

An important component in the STAT3 signal pathway is that activated STAT3 promotes expression of the CDK inhibitor p27<sup>Kip1</sup>, which causes G<sub>1</sub> cell cycle arrest and promotes terminal neutrophil differentiation (23–25, 32). To determine whether alcohol-induced enhancement of STAT3 activation has a downstream consequence, we analyzed p27<sup>Kip1</sup> mRNA expression by myeloid progenitors. The results showed that in the presence of G-CSF stimulation, p27<sup>Kip1</sup> transcription in myeloid progenitor cells was dose dependently increased by alcohol exposure. It has been known that during the process of maturation, hematopoietic progenitors traverse a stage termed transit amplification in which progenitors undergo maximal mitotic expansion (64). Our data support that alcohol enhances STAT3–p27<sup>Kip1</sup> negative signaling leading to cell cycle arrest and enhanced terminal differentiation (32). Therefore, the ultimate outcome of chronic alcohol consumption plus acute alcohol administration is restricted transit amplification potential of myeloid progenitor cells during the granulopoietic response to pulmonary bacterial infection.

In summary, the results of our current investigation demonstrate that alcohol enhances STAT3 negative signaling in myeloid progenitor cells in response to pneumococcal infection and G-CSF

stimulation. This alcohol-induced STAT3 activation is associated with inhibition of myeloid progenitor cell proliferation. Impaired expansion of the myeloid progenitor cell population restricts the marrow storage pool of granulocytes and consequently limits the increase in the number of circulating granulocytes and recruitment of these phagocytes into the infected alveolar space during the host response to pulmonary infection. These findings highlight an important mechanism underlying alcohol-induced myelosuppression in alcohol-abusing hosts.

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## Disclosures

The authors have no financial conflicts of interest.

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Table 1: Percentage of Viable Cells (mean  $\pm$  SE) by Figure Number

			Treatment					
Figure	Cell Cultures		Control	GSCF	0 mM Alcohol	25 mM Alcohol	50 mM Alcohol	100 mM Alcohol
Figure 2	BM Cells		91.30 $\pm$ 1.65	93.58 $\pm$ 1.28			89.74 $\pm$ 1.43	91.18 $\pm$ 1.28
Figure 3	32D Cells	Day 0			93.00			
		Day 3			95.33 $\pm$ 1.42	94.77 $\pm$ 1.26	91.61 $\pm$ 1.23	89.12 $\pm$ 1.19
		Day 6			82.90 $\pm$ 1.82	73.46 $\pm$ 0.78	75.88 $\pm$ 1.98	73.98 $\pm$ 3.11
Figure 4	32D Cells	Day 0			91.00			
		Day 3			87.29 $\pm$ 1.25	89.34 $\pm$ 1.42	79.48 $\pm$ 2.65	70.49 $\pm$ 0.87
		Day 6			91.84 $\pm$ 0.79	91.58 $\pm$ 0.58	73.63 $\pm$ 1.85	62.79 $\pm$ 5.14
Figure 5A	32D Cells	0mM 4MP			96.98 $\pm$ 0.46		92.60 $\pm$ 1.69	
		1mM 4MP			96.20 $\pm$ 0.62		94.46 $\pm$ 0.46	
		5mM 4MP			88.93 $\pm$ 1.82		73.50 $\pm$ 2.67	
Figure 5B	32D Cells	0 mM MET			94.14 $\pm$ 0.83		90.86 $\pm$ 1.38	
		100mM MET			93.64 $\pm$ 1.26		93.08 $\pm$ 1.25	
		500mM MET			85.83 $\pm$ 1.17		75.08 $\pm$ 2.51	
Figure 6	32D Cells		90.93 $\pm$ 0.53	92.13 $\pm$ 1.17			91.66 $\pm$ 1.19	90.02 $\pm$ 1.35
Figure 7	32D Cells				89.22 $\pm$ 0.46	90.03 $\pm$ 0.44	91.99 $\pm$ 0.73	90.28 $\pm$ 1.21