Bcl3 prevents acute inflammatory lung injury in mice by restraining emergency granulopoiesis

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Granulocytes are pivotal regulators of tissue injury. However, the transcriptional mechanisms that regulate granulopoiesis under inflammatory conditions are poorly understood. Here we show that the transcriptional coregulator B cell leukemia/lymphoma 3 (Bcl3) limits granulopoiesis under emergency (i.e., inflammatory) conditions, but not homeostatic conditions. Treatment of mouse myeloid progenitors with G-CSF — serum concentrations of which rise under inflammatory conditions — rapidly increased Bcl3 transcript accumulation in a STAT3-dependent manner. Bcl3-deficient myeloid progenitors demonstrated an enhanced capacity to proliferate and differentiate into granulocytes following G-CSF stimulation, whereas the accumulation of Bcl3 protein attenuated granulopoiesis in an NF-κB p50–dependent manner. In a clinically relevant model of transplant-mediated lung ischemia reperfusion injury, expression of Bcl3 in recipients inhibited emergency granulopoiesis and limited acute graft damage. These data demonstrate a critical role for Bcl3 in regulating emergency granulopoiesis and suggest that targeting the differentiation of myeloid progenitors may be a therapeutic strategy for preventing inflammatory lung injury.

Introduction
Granulocytes are important orchestrators of host defense responses. Their accumulation in target tissues must be strictly controlled, as too few granulocytes may not allow for adequate immunity and tissue repair, while too many granulocytes can promote tissue injury (1, 2). Under steady-state or homeostatic conditions, the number of circulating granulocytes is strictly maintained by low serum levels of G-CSF (3). Under inflammatory conditions, serum G-CSF concentrations rise, which results in rapid increases in granulocyte production in the bone marrow and a large expansion of granulocyte numbers in the periphery (4). This demand-driven or “emergency” granulopoiesis can also be regulated by other granulopoietic cytokines such as GM-CSF and IL-3 (5, 6).

Granulocytes arise from a small number of myeloid lineage-restricted progenitors within the bone marrow (7). HSCs eventually give rise to common myeloid progenitors (CMPs), which can differentiate into granulocyte-macrophage progenitors (GMPs) (8). Proliferation and differentiation of myeloid progenitors is required to support both homeostatic and emergency granulopoiesis. In response to saturating amounts of granulopoietic cytokines, myeloid progenitors divide more frequently to sustain the granulocyte output necessary to promote emergency granulopoiesis. However, the molecular mechanisms that differentially regulate these 2 states of granulopoiesis remain elusive. One hypothesis is that the expression patterns of transcription factors that control the proliferation of myeloid progenitors are differentially regulated to allow for accelerated granulocyte production. This has been observed for CAAT enhancer binding protein (C/EBP) family members (9, 10). For example, C/EBPα inhibits the proliferation of myeloid progenitors to a greater extent than does C/EBPβ. In response to high concentrations of granulopoietic cytokines, C/EBPα expression is attenuated, while C/EBPβ expression, which has been previously demonstrated to be required for emergency granulopoiesis, is upregulated. However, whether there are other transcription factors that specifically control emergency granulopoiesis remains largely unclear. For instance, despite their dominant role in regulating inflammatory responses, the expression patterns of the NF-κB family of transcription factors during emergency granulopoiesis is not well understood.

The IκB family member B cell leukemia/lymphoma 3 (Bcl3) was originally identified as a proto-oncogene (11). However, unlike other classical IκB family members, Bcl3 does not sequester NF-κB transcription complexes to the cytoplasm, but instead mainly resides in the nucleus. Here it is thought to play a critical role in counter-regulating inflammatory responses through limiting the transcription of NF-κB–dependent genes (12). For example, Bcl3 promotes IL-10–mediated inhibition of LPS-induced TNF-α expression in macrophages (13, 14). We have recently shown that Bcl3 can negatively regulate TLR4-mediated inflammatory gene expression by promoting the stability of NF-κB p50 homodimers, which can compete for κB elements otherwise occupied by pro-inflammatory RelA p65 and c-Rel–containing NF-κB transcription factor heterodimers such as NF-κB p50/p65 (15). In the absence of Bcl3 expression, mice become hyperresponsive to TLR signals and are incapable of achieving tolerance to repeated LPS challenge.

The fundamental mechanisms of human acute lung injury have been difficult to investigate, as clinical data are descriptive regarding the evolution of inflammatory changes inside and outside pulmonary tissue. As it concerns granulocyte-mediated lung injury there has been a lack of a model that allows the genetic dissection between lung-resident and peripheral responses in regulating emergency granulopoiesis. We have recently developed a vascularized and aerated orthotopic lung transplant model in

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Bcl3 prevents acute inflammatory lung injury in mice by restraining emergency granulopoiesis

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The fundamental mechanisms of human acute lung injury have been difficult to investigate, as clinical data are descriptive regarding the evolution of inflammatory changes inside and outside pulmonary tissue. As it concerns granulocyte-mediated lung injury there has been a lack of a model that allows the genetic dissection between lung-resident and peripheral responses in regulating emergency granulopoiesis. We have recently developed a vascularized and aerated orthotopic lung transplant model in
the mouse, which recapitulates the ischemia-reperfusion induced acute graft injury observed in human lung recipients (16, 17). Utilizing this transplant model we selectively analyzed the role of Bcl3 in granulocyte production by studying the effects of wild-type lung engraftment into recipients that are Bcl3 deficient in the hematopoietic cell compartment, thereby eliminating the potential inflammatory contributions of Bcl3 in alveolar macrophages. Here, we report a new and unexpected role for Bcl3 in regulating emergency granulopoiesis. While Bcl3 expression is not required to maintain homeostatic granulopoiesis, we show that a lack of Bcl3 expression in lung recipient hematopoietic cells results in exacerbation of pulmonary tissue injury. Bcl3 transcripts accumulate in myeloid progenitors in response to G-CSF stimulation and act to limit their capacity to promote emergency granulopoiesis in an NF-κB p50-dependent manner.

**Results**

**Pulmonary injury is exacerbated in Bcl3−/− lung graft recipients.** To examine the role of Bcl3 in emergency granulopoiesis, we reconstituted wild-type B6 mice with Bcl3−/− bone marrow (B6 [Bcl3−/−]), since Bcl3−/− mice have a primary defect in their stromal cells that prevents the full development of secondary lymphoid organs, while B6 (Bcl3+/+) mice have normal secondary lymphoid tissue (15, 18–20). B6 lungs were transplanted into syngeneic B6 (Bcl3−/+) or control B6 lung recipients reconstituted with wild-type B6 bone marrow (B6 [B6]) and evaluated for graft function and injury (Figure 1, A and B). Both B6 (B6) and B6 (Bcl3−/+) lung recipients had comparable graft function (PaO2) and pulmonary edema as measured by Evans Blue Dye (EBD) exclusion at 6 hours following transplantation. However, at 24 hours following transplantation, B6 → B6 (Bcl3−/+) lung recipients had significantly worse graft function and pulmonary edema as well as marked alveolar thickening and congestion when compared to B6 → B6 (Bcl3+/+) lung recipients. We also observed a significantly higher degree of myeloperoxidase (MPO) activity in graft tissue (Figure 1C) and a greater abundance of granulocytes in both lung grafts and airways after transplantation into B6 (Bcl3−/+) recipients at this time point (Figure 1D). Resting B6 (Bcl3−/+) mice had equivalent hematological parameters and no evidence of pulmonary neutrophilia when compared to B6 (B6) mice (Table 1 and Supplemental Figure 1; supplemental material available online).
able online with this article; doi:10.1172/JCI42596DS1). Importantly, these patterns of acute pulmonary injury were not unique to lung transplantation, as intratracheal LPS instillation resulted in worse lung injury and function and pulmonary neutrophilia in B6 (Bcl3−/−) when compared with B6 (B6) mice (Supplemental Figure 2). As granulocytes have been shown to be critical regulators of solid organ injury, we asked whether reduction of granulocytes in B6 (Bcl3−/−) lung recipients. Thus, these data indicated that elevated granulocyte numbers in the blood of B6 → B6 (Bcl3−/−) lung recipients is due to augmented bone marrow granulopoiesis.

The cytokines G-CSF, GM-CSF, and IL-3 are well characterized stimulators of granulocyte production. We next assessed concentrations of these cytokines in the peripheral blood of B6 (B6) and B6 (Bcl3−/−) mice, which received B6 lungs (Figure 3C). B6 (B6) and B6 (Bcl3−/−) lung recipients produced nearly equivalent amounts of granulopoietic cytokines. Notably, early after engraftment the serum concentration of G-CSF transiently increased almost 10-fold relative to pre-transplant levels. By contrast, GM-CSF and IL-3 serum concentrations showed only modest elevations following lung transplantation. To assess the functional significance of these observations, we administered recombinant G-CSF, GM-CSF, or IL-3 to B6 (B6) or B6 (Bcl3−/−) mice and measured granulocyte accumulation in the peripheral blood (Figure 3D). G-CSF–treated B6 (Bcl3−/−) mice accumulated approximately twice as many peripheral blood granulocytes as did B6 (B6) mice. By contrast, IL-3 and GM-CSF administration produced comparable increases of granulocytes in the peripheral blood of B6 (Bcl3−/−) mice relative to B6 (B6) mice. As granulocyte numbers in resting B6 (B6) and B6 (Bcl3−/−) mice are equivalent, our data collectively show that Bcl3 is a negative regulator of G-CSF–mediated emergency granulopoiesis.

**Bcl3 controls myeloid progenitor mobilization.** The finding that B6 (Bcl3−/−) mice have the capacity to produce more granulocytes could be due to greater numbers of myeloid progenitors within the bone marrow. To assess this possibility, we performed methylcellulose colony assays with bone marrow from B6 (B6) and B6 (Bcl3−/−) mice in the presence of G-CSF, GM-CSF, or IL-3 (Figure 4A). Bone marrow from both of these mice generated similar numbers of colonies irrespective of cytokine treatment, demonstrating that B6 (Bcl3−/−) and B6 (B6) mice have equivalent numbers of myeloid progenitors. However, unlike the case for GM-CSF or IL-3, G-CSF–stimulated Bcl3−/− bone marrow generated greater numbers of cells per colony progenitor relative to G-CSF–stimulated B6 bone marrow (Figure 4A). These data suggested that Bcl3−/− myeloid progenitors had an augmented capacity to produce granulocytes after stimulation with G-CSF. To confirm these findings, we used flow cytometry to sort CMPs from Bcl3−/− or B6 bone marrow, cultured them with G-CSF, GM-CSF, or IL-3, and assessed their differentiation into granulocytes. At 18 hours G-CSF–stimulated Bcl3−/− CMP cultures produced a markedly higher percentage of MPO+ cells relative to measured the survival B6 and Bcl3−/− granulocytes ex vivo (Figure 2F). While spontaneous death was nearly equivalent for both types of granulocytes, stimulation of B6 granulocytes with G-CSF was more effective at increasing survival when compared with G-CSF stimulation of Bcl3−/− granulocytes.

These findings prompted us to examine whether differences in granulocyte production played a role in the lung graft injury after transplantation into B6 (Bcl3−/−) recipients. We therefore assessed granulocyte accumulation in the peripheral blood following B6 → B6 (B6) and B6 → B6 (Bcl3−/−) lung transplantation (Figure 3A). Between 1 and 3 days after engraftment, B6 (Bcl3−/−) recipients had significantly more blood granulocytes than B6 (B6) recipients. We next examined the bone marrow of B6 → B6 (Bcl3−/−) lung recipients for evidence of augmented production of granulocytes (Figure 3B). One day after engraftment, there was an overall greater abundance of Gr1+ MPOlohi cells, along with a greater fraction of the mature Gr1+ MPOhi subset in the bone marrow of B6 → B6 (Bcl3−/−) lung recipients as compared with the bone marrow of B6 → B6 (B6) recipients. Thus, these data indicated that elevated granulocyte numbers in the blood of B6 → B6 (Bcl3−/−) lung recipients is due to augmented bone marrow granulopoiesis.

### Table 1

**Hematological parameters**

<table>
<thead>
<tr>
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<th>B6 (B6)</th>
<th>B6 (Bcl3−/−)</th>
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</thead>
<tbody>
<tr>
<td>Neutrophils (10⁶/µl)</td>
<td>1.15 ± 0.18</td>
<td>1.32 ± 0.44</td>
</tr>
<tr>
<td>Lymphocytes (10⁶/µl)</td>
<td>5.93 ± 1.27</td>
<td>6.79 ± 0.82</td>
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<tr>
<td>Monocytes (10⁶/µl)</td>
<td>0.19 ± 0.10</td>
<td>0.27 ± 0.14</td>
</tr>
<tr>
<td>Eosinophils (10⁶/µl)</td>
<td>0.15 ± 0.01</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Basophils (10⁶/µl)</td>
<td>0.27 ± 0.02</td>
<td>0.34 ± 0.09</td>
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Differential counts. Peripheral blood analysis of B6 (B6) and B6 (Bcl3−/−) mice. n = 7/group. All values are P = 0.17 for B6 (B6) versus B6 (Bcl3−/−).
G-CSF–stimulated B6 CMP cultures (Figure 4B), while GM-CSF– or IL-3–stimulated Bcl3−/− and B6 CMP cultures generated similar percentages of cells that expressed MPO. By 3 days of culture there were significantly higher numbers of granulocytes in G-CSF–stimulated Bcl3−/− CMP cultures as compared with G-CSF–stimulated B6 CMP cultures (Figure 4C). By contrast, Bcl3−/− and B6 CMP cultures stimulated with either GM-CSF or IL-3 produced equivalent numbers of granulocytes. We also assessed granulocyte-associated gene expression in peripheral blood granulocytes isolated from G-CSF–treated B6 (B6) and B6 (Bcl3−/−) mice (Figure 4D). Compared with peripheral blood granulocytes from G-CSF–treated B6 (B6) mice, we observed significantly higher levels of MPO and neutrophil elastase transcript levels but comparable accumulation of lysozyme transcripts. In addition to elevations in transcript lev-
els of MPO and neutrophil elastase, we also observed significantly increased accumulation of lysozyme transcripts in CMPs from G-CSF–treated B6 (Bcl3−/−) mice (Figure 4E). Of note, we detected relatively higher transcript accumulation of MPO and neutrophil elastase in CMPs isolated from G-CSF–treated B6 (Bcl3−/−) as compared with G-CSF–mobilized peripheral blood granulocytes from the same mice (Figure 4, E and D), indicating that Bcl3 has a more prominent role in gene expression in myeloid progenitors than in circulating granulocytes.

These observations raised the possibility that Bcl3 could also regulate the proliferation of myeloid progenitors. To address this question, we BrdU-pulsed CMP cultures that were stimulated with graded amounts of G-CSF, GM-CSF, or IL-3 (Figure 4F). Compared with B6 CMP cultures, Bcl3−/− CMP cultures had significantly higher percentages of BrdU+ cells following treatment with saturating concentrations of G-CSF. GM-CSF– and IL-3–treated B6 and Bcl3−/− cultures had comparable percentages of BrdU+ cells irrespective of cytokine concentration. Additionally, proliferative responses in G-CSF–stimulated Bcl3−/− CMPs were associated with elevated transcript levels of the cell cycle regulator cyclin D3 (Figure 4F). We then investigated myeloid progenitor proliferation in vivo by BrdU-pulsing B6 → B6 (B6) or B6 → B6 (Bcl3−/−) lung recipients and assessing BrdU incorporation in bone marrow–resident CMPs and GMPs 24 hours after engraftment (Figure 4G). Although B6 (B6) and B6 (Bcl3−/−) lung recipients had comparable bone marrow fractions of myeloid progenitors, we observed a markedly higher percentage of BrdU+ CMPs and GMPs in B6 (Bcl3−/−) as compared with B6 (B6) recipients. Thus, Bcl3 regulates both the differentiation and proliferation of myeloid progenitors during emergency granulopoiesis.

Regulation of Bcl3 expression. As Bcl3 is a co-transcriptional regulator, we considered the possibility that it differentially regulates the expression of genes that have been reported to have a role in G-CSF–mediated granulopoiesis (Supplemental Figure 3). In particular, the transcription factors C/EBPα and -β both have been shown to regulate granulopoiesis through promoting the differentiation of myeloid progenitors into granulocytes (10). However, we were not able to detect differences in the accumulation of C/EBPα and -β transcripts in B6 and Bcl3−/− myeloid progenitors. Additionally, transcript levels of critical components of the G-CSF...
Figure 4
Proliferation and differentiation of Bcl3-deficient myeloid progenitors. (A) Left: Methylcellulose colony count from B6 or Bcl3−/− bone marrow cells cultured with indicated cytokines. Right: Number of cells per colony. Data are representative of 2 independent experiments. (B) Representative FACS analysis (n = 4) of MPO expression in liquid CMP cultures following 18 hours of stimulation with indicated cytokines. Numbers within histograms indicate percent abundance of MPO+ cells. (C) Granulocyte output from CMP liquid cultures following 72 hours of stimulation with indicated cytokines. (D) Indicated transcript accumulation in peripheral blood granulocytes isolated from B6 (B6) or B6 (Bcl3−/−) mice 24 hours following a 5-μg injection of G-CSF. (E) Indicated transcript accumulation in CMPs isolated from B6 (B6) or B6 (Bcl3−/−) mice 24 hours following injection of 5 μg of indicated cytokines. (F) Bottom: CMP BrdU incorporation in response to indicated concentrations of G-CSF, GM-CSF, or IL-3 following 18 hours of liquid culture. Data are representative of at least 3 independent experiments. Top: Cyclin D3 transcript accumulation in CMPs isolated from B6 (B6) or B6 (Bcl3−/−) mice 24 hours following injection of 5 μg of indicated cytokines. (G) Representative (n = 3) BrdU incorporation of bone marrow Lin−Sca-1−c-Kit+ cells (GMPs; CD34+CD16/32+) and (CMPs; CD34+CD16/32−) in B6 →B6 (B6) or B6 (Bcl3−/−) lung recipients 24 hours following transplantation. Dot plot numbers indicate percent abundance. Data represent mean ± SD. *P < 0.05; **P < 0.01.
signaling pathway, G-CSFR, SOCS3, and STAT3, were also similar in B6 and Bcl3−/− myeloid progenitors with or without G-CSF stimulation. We then considered whether Bcl3 expression in myeloid progenitors is modulated by cytokine stimulation. Purified B6 myeloid progenitors were stimulated with G-CSF, GM-CSF, or IL-3 and analyzed for Bcl3 transcript levels (Figure 5A). Notably, Bcl3 transcript accumulation increased approximately 4-fold in G-CSF–treated CMP cultures and was also significantly elevated in HSC and GMP cultures. By contrast, no significant changes in Bcl3 transcript levels were observed in myeloid progenitors following stimulation with GM-CSF or IL-3. Additionally, in mature granulocytes, Bcl3 transcript levels were also elevated after G-CSF but not GM-CSF or IL-3 treatment. To analyze changes in Bcl3 expression in vivo, we isolated RNA from myeloid progenitors and granulocytes in the airways of B6 (B6) recipients of B6 lungs that had been treated with G-CSF–neutralizing antibodies or control
As these data suggested that G-CSF signaling enhances Bcl3 expression, we next analyzed the role of STAT3, a principal transcription factor that regulates granulopoiesis following G-CSF engagement but is less critical for IL-3 receptor or GM-CSF receptor function (22–24). To accomplish this we isolated myeloid progenitors from mice with a cytoplasmic truncation in their G-CSFR, which has been previously shown to activate STAT3 in G-CSFR–/– bone marrow cells (26, 27) (Figure 5D). We observed STAT3 binding to this enhancer region following stimulation with G-CSF, GM-CSF, or IL-3 (Figure 5F). Irrespective of cytokine treatment, ectopic expression of Bcl3 in NF-κB–/– bone marrow cells greatly reduced granulocyte production, indicating that the accumulation of either Bcl3 or NF-κB p50 following stimulation with G-CSF, GM-CSF, or IL-3 (Figure 5F). Irrespective of cytokine treatment, ectopic expression of Bcl3 in NF-κB–/– bone marrow cells greatly reduced granulocyte production, indicating that the accumulation of either transcription factor is sufficient to inhibit granulopoiesis. However, ectopic expression of Bcl3 in NF-κB p50–deficient bone marrow cells did not markedly inhibit cytokine-mediated granulopoiesis, demonstrating a requirement for NF-κB p50 expression for Bcl3 to negatively regulate granulopoiesis (Supplemental Figure 4). Thus, the accumulation of Bcl3 protein attenuates the production of granulocytes in a NF-κB p50–dependent manner.

G-CSF blockade prevents lung graft injury in B6 (Bcl3−/−) recipients. The observation that G-CSF is highly prevalent in the serum of lung transplant recipients led us to hypothesize that blocking G-CSF activity in B6 → B6 (Bcl3−/−) lung recipients can attenuate graft injury. Treatment of B6 (Bcl3−/−) recipients of B6 lungs with G-CSF–neutralizing antibodies significantly reduced granulocyte numbers in peripheral blood, airways, and lung tissue (Figure 6A) and was associated with marked improvement in lung function and graft histology as well as reduction in pulmonary edema (Figure 6B). These results demonstrate that blockade of G-CSF–mediated emergency granulopoiesis attenuates graft injury in B6 (Bcl3−/−) lung recipients.

**Discussion**

Our results reveal a previously unknown function for Bcl3. In addition to formerly described roles in secondary lymphoid organ development, cell survival, and inflammatory cytokine gene expression, we now demonstrate that Bcl3 expression is critical to controlling G-CSF–mediated emergency granulopoiesis (15, 28, 29). Excessive granulocyte accumulation has been shown to pro-
mote lung graft injury both in animal models and in the clinical setting (30, 31). Also, granulocytes play a key role in exacerbating LPS-mediated acute lung injury (32, 33). Interestingly, in humans, intra-bronchial LPS instillation results in G-CSF accumulation in the blood and bronchoalveolar lavage (BAL), which drives granulocyte accumulation in the airway (34). We observed a large accumulation of granulocytes in the peripheral blood and lung tissue of both B6 (Bcl3−/−) mice treated with LPS and B6 (Bcl3−/−) lung recipients, underscoring the role that Bcl3 plays in controlling granulopoiesis to prevent acute lung injury.

Our use of a transplant model to study the role of Bcl3 in acute lung injury has several advantages. First, lung ischemia reperfusion injury is associated with robust recruitment of granulocytes (7). STAT3 promotes CXCR2-mediated chemotactic responses (40). SOCS3, a negative regulator of G-CSF–mediated emergency granulopoiesis, inhibits G-CSF–mediated survival and may be required to temper intrinsic granulocyte activity, as mice with a SOCS3-specific deletion in their hematopoietic cells develop solid organ damage from an inflammatory neutrophilia (41). However, consistent with previous reports of Bcl3 as a pro-survival factor (42), we observed that Bcl3 deficiency in activated granulocytes was associated with a decrease in their survival. Interestingly, a coupling of cell survival to proliferation has been previously observed for other pro-survival molecules such as Bcl2 (43). Moreover, Bcl3 expression in T lymphocytes promotes their survival while inhibiting proliferation and differentiation (29, 44).

To better understand how Bcl3 controls G-CSF–mediated emergency granulopoiesis, we analyzed differential and proliferative responses of myeloid progenitors. After only 18 hours, G-CSF–treated Bcl3−/− CMP cultures accumulated more MPO-expressing cells than equivalently stimulated wild-type B6 CMPs. In vivo, G-CSF–stimulated Bcl3−/− CMPs accumulated higher levels of transcripts associated with granulocyte function as compared with comparably stimulated B6 CMPs. We also observed the augmented transcript accumulation of the cell cycle regulator cyclin D3 in Bcl3−/− CMPs following G-CSF, but not GM-CSF or IL-3 stimulation. Consistent with this finding is a previous report of the cyclin D3 expression in myeloid progenitors during G-CSF–mediated emergency granulopoiesis (45). Moreover, we noted that enhancement of proliferation in Bcl3−/− CMP cultures was prominent only in response to high G-CSF concentrations, indicating a selective role for Bcl3 in attenuating granulocyte production under inflammatory conditions. However, as compared with CMPs, granulocyte function–associated transcripts accumulate to a larger extent in peripheral blood granulocytes isolated from G-CSF–treated B6 (Bcl3−/−) mice. Collectively, these observations support a model in which Bcl3 expression plays a predominant role in myeloid progenitors by tempering gene expression that is required for emergency granulopoiesis.

Depending on the inflammatory stimulus, Bcl3 gene expression may be regulated through different transcription factors. For example, it has been noted that rapidly after TLR engagement Bcl3 gene transcription is dependent on NF-κB activation, raising the possibility that Bcl3 expression can be regulated independently of STAT3 (46, 47). However, TLR stimulation has been also shown to lead to Bcl3 transcription in a STAT3-dependent manner through the autocrine effects of IL-6 and IL-10 production (13, 14). Likewise, in STAT3-hyporesponsive mutant G-CSFRΔ715F myeloid progenitors, we failed to observe Bcl3 transcript accumulation after stimulation with G-CSF. Moreover, we did not observe accumulation of Bcl3 transcripts following GM-CSF stimulation of myeloid progenitors. As the GM-CSF receptor has been reported to be a strong activator of NF-κB but not STAT3 (48, 49), our data collectively support a model in which STAT3 activation is required to drive Bcl3 expression in myeloid progenitors. Interestingly, the ability of IL-6 to stimulate Bcl3 transcript accumulation in myeloid progenitors raises the possibility that this cytokine could play a role in regulating emergency granulopoiesis following lung ischemia reperfusion injury (50). However, unlike G-CSF, which is the primary regulator of granulocyte production, IL-6 also controls the production of monocytes from hematopoietic progenitors (51, 52). Moreover, IL-6 has also been shown to protect lung grafts from injury, as it reduces endothelial cell disruption, which inhibits granulocyte tissue sequestration (53). Therefore, a selec-
tive role for IL-6 in emergency granulopoiesis in the context of solid organ injury would be difficult to discern.

To confirm the functional significance of Bcl3 transcript accumulation in myeloid progenitors we ectopically expressed Bcl3 protein in bone marrow cells. Notably, we observed an attenuation of granulocyte formation in Bcl3 transgene–positive bone marrow cells that is not G-CSF specific and requires NF-κB p50 expression. Thus, these data suggest that when Bcl3 protein accumulates at sufficiently high levels it acts through transcriptional mechanisms that globally regulate granulopoiesis. In this context C/EBPs are likely targets of Bcl3-mediated effects on granulopoiesis, as their transcriptional activity is controlled by association with NF-κB p50 (12, 54). Also, C/EBPs are regulators of GM-CSF– and IL-3–dependent granulopoiesis (10). However, Bcl3 is unlikely to control granulopoiesis by directly regulating C/EBP expression. In the absence of Bcl3 we observed comparable C/EBPα or β transcript levels irrespective of cytokine stimulation. In particular, the maintenance of C/EBPα expression in Bcl3+/− cells is notable; in a recent report NF-κB p50+/− mice were shown to have reduced levels of C/EBPα expression (55).

Importantly, our data show that Bcl3 deficiency does not result in complete elimination of NF-κB p50 protein, indicating that there is still sufficient NF-κB p50 expression to maintain C/EBPα gene transcription. As we demonstrated that NF-κB p50 expression is required for Bcl3-mediated control of granulopoiesis, these data collectively suggest a mechanism by which altering NF-κB p50 levels in the nucleus is critical to control granulocyte production. Consistent with such a role is the observation that mutating the bZIP domain in C/EBPα to reduce the formation of C/EBPα:NF-κB p50 transcriptional complexes promotes granulopoiesis in myeloid progenitor cell lines (56, 57). In addition, C/EBPβ has an identical bZIP domain to C/EBPα, and coimmunoprecipitation studies have demonstrated an interaction between NF-κB p50 and C/EBPβ (58). Thus, it is conceivable that Bcl3 may act to stimulate the formation of NF-κB p50:C/EBPα transcriptional complexes, which in turn promote gene expression patterns that limit granulocyte production. A potential therapeutic role of inhibiting emergency granulopoiesis is substantiated by our demonstration that G-CSF blockade in B6 (Bcl3+/−) lung recipients significantly reduces graft injury and improves function. Moreover, G-CSF blockade effects on lung injury may also be favorably affected by the inhibition of granulopoiesis-independent functions of G-CSF such as the blunting of bone marrow mobilization (59), survival (60), or tissue migration of granulocytes (61). In summary, we have identified what we believe is a novel role for Bcl3 in negatively regulating G-CSF–mediated emergency granulopoiesis. Recent reports linking increased G-CSF serum levels or G-CSF treatment to acute lung injury in the clinical setting highlight the importance of understanding mechanisms that calibrate granulocyte production under emergency conditions (62, 63).

Methods

Mice. C57BL/6j (B6) and NF-κB p50−/− (B6 background) mice were purchased from The Jackson Laboratory. Bcl3+/− mice (B6 background) were a gift from Y. Chen (University of Pennsylvania, Philadelphia, Pennsylvania, USA); G-CSFR551S mice (B6 background) were a gift from D.C. Link (Washington University in St. Louis). All mice were maintained in a pathogen-free facility at Washington University School of Medicine. All experiments were reviewed and approved by the Animal Studies Committee of Washington University in St. Louis.

Lung transplantation, LPS administration, antibodies, and BrdU assays. Orthotopic left vascularized lung transplants were performed as previously described (17). LPS (300 μg) from strain O111:B4 (Sigma-Aldrich) was administered down the airway in 100 μl of PBS. Granulocytes were partially depleted with a 250-μg dose of Ly6G-specific antibodies (1A8; Bio-X-Cell) administered intravenously 4 hours prior to surgery. G-CSF was neutralized with 200 μg of G-CSF–specific antibodies (Peprotech) administered intravenously 1 hour prior to surgery. In vivo BrdU was administered intraperitoneally 4 hours before sacrifice for analysis. In vitro BrdU was added to myeloid progenitor cultures at a final concentration of 10 μM, and cells were analyzed 30 minutes later for BrdU incorporation. BrdU assay was conducted by flow cytometric (FACS) analysis using BrdU-specific antibodies (Invitrogen) and permeabilization/fixation reagents from a BrdU Flow Kit (BD Biosciences) in accordance with the manufacturer’s recommendations.

Granulocyte purification, survival, and graft infiltration assay. Granulocytes were purified from the bone marrow using a previously described 3-layer Percoll (GE Healthcare Life Sciences) gradient method optimized to collect mature neutrophils (64). Granulocyte preparations were routinely greater than 90% Gr1+MPO+ cells with high granularity as determined by side scatter. To measure granulocyte infiltration in lung grafts, freshly isolated bone marrow granulocytes were stimulated for 1 hour in 30 ng/ml of G-CSF, stained with 5 μM CFSE (Invitrogen), quenched in 50% PBS (Stem Cell Technologies)/PBS, and washed twice in PBS before adoptive transfer into B6 → B6 lung recipients. To measure their survival, freshly isolated bone marrow or BAL granulocytes were stained with Annexin V–FITC (BD Biosciences – Pharmingen) as per the manufacturer’s recommendations.

Bone marrow transplantation. Bone marrow chimeras were created as previously described (65).

Granulocyte analysis. BAL and lung tissue digest were prepared as previously described (16). Lung cell isolates were analyzed by FACS analysis by staining with Gr1 (RB6-8C5), CD11b (M1/70), and CD62L (MEL-14)–specific antibodies (ebioscience). Intracellular staining for MPO (8F4; Cell Sciences) was conducted after cell surface staining using a fixation/permeabilization reagent (ebioscience) in accordance with the manufacturers’ recommendations. Granulocytes were counted in the BAL and peripheral blood with a HEMAVET analyzer (Drew Scientific).

Myeloblastic colony assay. Bone marrow suspensions were mixed with MethoCult M3234 (Stemcell Technologies) and treated with mouse recombinant cytokines (Peprotech) G-CSF (10 ng/ml), GM-CSF (10 ng/ml) or IL-3 (10 ng/ml). Colonies were counted on day 7 with an inverted microscope, and cells per colony were calculated by counting the total number of cells and dividing by the number of colonies.

Cell sorting. Lin− bone marrow cell preparation, and culture. HSCs, CMPs, and GMPs were purified as previously described (8). Bone marrow cells were stained with the following biotin-conjugated antibody cocktail for the identification of lineage-specific markers: CD3e (145-2C11), CD4 (L3T4), CD8a (Ly-2), CD19 (eBio1D3), B220 (RA3-6B2), Gr-1 (RB6-8C5), Ter-119, CD49b (DX5), CD11c (N418), and CD11b (M1/70). Preparations were then stained with PerCP-Cy5.5–conjugated streptavidin, FITC–conjugated anti-CD34 (RAM34), APC–conjugated c-Ki67 (2B8), APC–750–conjugated Sca-1 (D7) and PE–conjugated CD16/32 (clone 93). All antibodies were obtained from ebioscience. Cells were then sorted on a custom configured Reflection Cell Sorter through a Lin− c-kit−Sca-1− gate for HSCs, a Lin− c-kit−Sca-1 CD34+CD16/32+ gate for CMPs and a Lin− c-kit−Sca-1 CD34+CD16/32+ gate for GMPs. Lin− bone marrow cells were prepared by 2 rounds of negative selection using the above described lineage-specific antibody cocktail and anti-biotin microbeads. Myeloid progenitors were cultured in round-bottom 96-well plates in medium containing StemPro 34 SFM (Gibco) supplemented with 20% FBS (Stem Cell Technologies) and indicated cytokines at 10 ng/ml.

Immunoblot. Nuclear protein was prepared from 105 Lin− bone marrow cells using a Nuclear Extract Kit (Active Motif) in accordance with the man-
manufacturer’s recommendations. For whole cell lysates, protein was prepared from an equivalent number of Lin– bone marrow cells. Protein extracts were then heat-denatured in SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 50 mM EDTA, 0.01% bromophenol blue), resolved on a denaturating 10% SDS-PAGE gel, transferred to nitrocellulose membranes via XCell II blotter (Invitrogen), and probed with either FLAG [M2, Sigma-Aldrich], NF-κB p105/50 (Cell Signaling), Oct-1 (Abcam), or β-actin–specific (AC-15; Santa Cruz Biotechnology Inc.) antibodies. Immuneoreactivity was detected with horseradish peroxidase–conjugated antibodies specific for either rabbit (Cell Signaling) or mouse (Cell Signaling) IgG and visualized on X-Omat film (Kodak) by chemiluminescence (ECL kit; Amersham Biosciences).

Retroviral vector transduction and culture. N-FLAG Bcl3 cDNA or N-FLAG NF-κB p50 cDNA, provided by Y. Chen, were subcloned into the EcoRI site located within a bicistronic expression cassette of pMSCV-NFκB (MSCV), a gift from W. Pear (University of Pennsylvania, Philadelphia, Pennsylvania, USA), which contains an internal ribosomal entry site that drives the simultaneous expression of a truncated form of the human nerve growth factor receptor (NGFR) to constructs pMSCV-Bcl3 or pMSCV-NF-κB p50. Retrovirus was packaged in Plat-E cells (Cell Biolabs) in a manner previously described (66), and high-titer retroviral supernatant was spin transfected with 5 μg/ml of polybrene into Lin– bone marrow cells stimulated in DMEM (Invitrogen), 20% FBS (Stem Cell Technologies), 10 ng/ml IL-3, 50 ng/ml GM-CSF (10 ng/ml), or IL-3 (10 ng/ml) for 3 days and then assessed for granulocyte accumulation with Gr1–, CD11b–, and MPO-specific antibodies.

Chromosomal immunoprecipitation assay. Lin– bone marrow cells (10⁶) were stimulated with 30 ng/ml of G-CSF, IL-6, or IL-3 for 30 minutes, resuspended in cell lysis buffer (5 mM PIPES, pH 8.1; 10 mM KCl; 0.5% Igepal; 10 mM PMSF, Sigma-Aldrich; 10 μg/ml aprotenin, Sigma-Aldrich; and 10 μg/ml leupeptin, Sigma-Aldrich), and nuclei were released with a Dounce Homogenizer. To prepare chromatin, nuclei were resuspended in nuclear lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors), incubated on ice for 20 minutes, and freeze-thawed twice in liquid nitrogen. Ruptured nuclei were sonicated at 50% power in a Branson 250 Sonicator for five 30-second pulses spaced by 1 minute. Chromatin DNA was cleared by 14,000-g centrifugation for 10 minutes and dialyzed into immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, 167 mM NaCl, and protease inhibitors). Following an overnight incubation at 4°C with either STAT3 antisense or sense (5′-TCCCCGGATCTGGC-CAGGCTTC-3′; antisense 5′-AAGCGTCACCCGCTTGGCTC-3′) specific for the distal enhancer region within intron 2 of the Bcl3 gene, chromatin was immunoprecipitated with Protein A/G agarose (Santa Cruz Biotechnology Inc.), eluted off with 1% SDS, 0.1 M NaHCO₃, and 0.01 mg/ml Herring sperm DNA, and re-equilibrated in 10 mM Tris EDTA buffer, pH 8.0. DNA was amplified with primers (sense 5′-TCCCCGGATCTGGCCAGGCTTC-3′; antisense 5′-AAGCGTCACCCGCTTGGCTC-3′) specific for the distal enhancer region within intron 2 of the Bcl3 gene.

Results. Generation of ROS was assessed by FACS analysis by detection of Rhodamine 123 accumulation in cultured granulocytes following treatment with DHR123 (Invitrogen) and either 0.2 μg/ml PMA (Sigma-Aldrich), 0.5 μM fMLP (Sigma-Aldrich), or vehicle for 10 minutes at 37°C. MPO lung tissue level activity was determined as previously described (67). Optical density was measured at 460 nm with a spectrophotometer (model PMQ II; Carl Zeiss). One unit of enzyme activity was defined as 1.0 optical density (AU) min−1 mg protein−1 at room temperature.

ELISA. Serum G-CSF, GM-CSF, and IL-3 concentrations were measured with Quantikine ELISA kits (R&D Systems).

EBD exclusion and lung function. EBD was administered intravenously 4 hours prior to sacrifice, at which point lung grafts were excised and flushed with 20 ml of PBS. To extract EBD, lung tissue was homogenized in 5 ml of formamide. The homogenate was incubated at 37°C for 24 hours and centrifuged at 3,500 g for 30 minutes. The optical density of the supernatant was measured at 620 nm and expressed as milligrams of EBD per gram of wet lung weight. To assess graft function, arterial blood gases were measured using an iSTAT Portable Clinical Analyzer (iSTAT) at a FiO2 of 1.0 after the right pulmonary hilum was clamped for 5 minutes.

Real-time PCR. FACs-sorted myeloid progenitors or Percoll-purified granulocytes were placed into TRIzol (Invitrogen), and RNA extraction was performed in accordance with the manufacturer’s instructions. Quantitative real-time RT-PCR was conducted on an ABI 7900 using TaqMan Gene Expression Assay system (Applied Biosystems) in accordance with manufacturer’s recommendations. Amplification of target sequences was conducted as follows: 50°C for 20 minutes and 95°C for 10 minutes, followed by 38–45 cycles of 95°C for 15 seconds and 60°C for 1 minute. All primers and MGB probes were purchased as kits from Applied Biosystems and can be identified in the following manner: C/EBPα (Mm01265914_s1), G-CSFR (Mm00432735_m1), ST3β (Mm01219775_m1), SOCS3 (Mm01249143_g1), Bcl3 (Mm00504306_m1), cyclin D3 (Mm01273583_m1), MPO (Mm00447886_m1), lysozyme (Mm01289278_m1), neutrophil elastase (Mm00463910_m1), and 18S rRNA (Hs0392931_g1).

Statistics. Data were analyzed using GraphPad Prism, version 5.0, and results are presented as mean ± SD. An unpaired 2-tailed Student’s t test was used to evaluate pairs of means for significance. Values of P < 0.05 were considered significant.

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DC. Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor (G-CSF) concentration.


