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*J Immunol* 2011; 186:5956-5967; Prepublished online 6 April 2011; doi: 10.4049/jimmunol.1003558

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Prevention of Bone Marrow Cell Apoptosis and Regulation of Hematopoiesis by Type I IFNs during Systemic Responses to Pneumocystis Lung Infection

David Taylor,1 Michelle Wilkison,1 Jovanka Voyich, and Nicole Meissner

We recently demonstrated that lack of type I IFN signaling (IFNAR knockout) in lymphocyte-deficient mice (IFrag−/−) results in bone marrow (BM) failure after Pneumocystis lung infection, whereas lymphocyte-deficient mice with intact IFNAR (RAG−/−) had normal hematopoiesis. In the current work, we performed studies to define further the mechanisms involved in the induction of BM failure in this system. BM chimera experiments revealed that IFNAR expression was required on BM-derived but not stroma-derived cells to prevent BM failure. Signals elicited after day 7 postinfection appeared critical in determining BM cell fate. We observed caspase-8– and caspase-9–mediated apoptotic cell death, beginning with neutrophils. Death of myeloid precursors was associated with secondary oxidative stress, and decreasing colony-forming activity in BM cell cultures. Treatment with acetylcysteine could slow the progression of, but not prevent, BM failure. Type I IFN signaling has previously been shown to expand the neutrophil life span and regulate the expression of some antiapoptotic factors. Quantitative RT-PCR demonstrated reduced mRNA abundance for the antiapoptotic factors BCL-2, IAP2, MCL-1, and others in BM cells from IFrag−/− compared with that in BM cells from RAG−/− mice at day 7. mRNA and protein for the proapoptotic cytokine TNF-α was increased, whereas mRNA for the growth factors G-CSF and GM-CSF was reduced. In vivo anti–TNF-α treatment improved precursor cell survival and activity in culture. Thus, we propose that lack of type I IFN signaling results in decreased resistance to inflammation-induced proapoptotic stressors and impaired replenishment by precursors after systemic responses to Pneumocystis lung infection. Our finding may have implications in understanding mechanisms underlying regenerative BM depression/failure during complex immune deficiencies such as AIDS. The Journal of Immunology, 2011, 186: 5956–5967.

Subj ecting the host to infectious agents commonly results in rapid recruitment and accumulation of leukocytes at the site of insult to attain clearance and tissue homeostasis. In response to most bacterial as well as fungal infections, neutrophils constitute the first line of defense and are subsequently followed by cells of the acquired immune response. All leukocytes are derived from the bone marrow (BM), and circulating neutrophils in particular have, under steady-state conditions, a very short life span of ∼12 h (1). This can be extended after activation and recruitment to specific tissue sites to up to 2 d. However, neutrophils will subsequently undergo apoptosis, and the rate is controlled by the activation state and various antiapoptotic factors such as MCL-1 (2).

Hematopoiesis and inflammation are tightly linked (3). Upon infection at local sites, resident phagocytes release inflammatory mediators, such as TNF-α, IL-1, and IL-6 and thus elicit a systemic response (acute-phase response), resulting in increased production and release of neutrophils from the BM to sites of infection (4, 5). This process of supply and demand is tightly regulated. With regard to granulopoiesis, it is known that distinct transcription factors regulate basal (C/EBPα) and emergency (C/EBPβ) hematopoiesis (6). However, continuous increased demand of inflammatory cells during chronic inflammatory conditions can also be associated with abnormal hematopoiesis. Patients with chronic inflammation, such as rheumatic diseases, (7–9) or chronic infections often have additional blood abnormalities, including RBC anemia (10, 11), thrombocytopenia (12), or neutropenia either as lineage-specific presentations or pancytopenia (13). Neutropenia is generally associated with pancytopenia. In this regard, in virtually all patients with advanced-stage AIDS (stage IV), pancytopenia is the rule due to regenerative BM failure, and it appears that primarily on-demand hematopoiesis is suppressed (14). Mechanisms involved are certainly multifactorial; however, whether the underlying immune defect directly contributes to these BM dysfunctions has not been elucidated.

Clearly, immune-compromised patients with defects of lymphocyte functions and/or a defect of the type I IFN system, such as in AIDS (15–17), are at risk to develop opportunistic infections. In this regard, Pneumocystis infection of the lung is the most common opportunistic infection in AIDS causing severe pneumonia (Pneumocystis pneumonia); however, it also affects patients undergoing chemotherapy for cancer, anti–TNF-α therapy for some rheumatic diseases (18–20), or patients with inherited CD4 T cell defects (21). During Pneumocystis pneumonia, significant numbers of neutrophils are recruited from the BM to the lung, although they are unable to clear the pathogen (22, 23).

Type I IFNs are important regulators of the innate and acquired immune response and have also been therapeutically used for their...
antiviral and antiproliferative properties. However, when administered in high therapeutic dosages for hepatitis C or cancer treatment, type I IFNs can induce BM suppression (24). In contrast, we recently demonstrated that specifically in response to pulmonary infection with the fungal pathogen pneumonia, the lack of type I IFN signaling in lymphocyte-deficient mice (IFrag-/- mice) results in rapidly progressing BM failure (25). Lymphocyte-competent but IFNAR-deficient mice (IFNAR-/- mice) developed mild BM depression with induction of extramedullary hematopoiesis, whereas lymphocyte-deficient mice with intact IFNAR (RAG-/- mice) as well as wild-type mice demonstrate normal hematopoiesis after pneumonia lung infection. This occurred although the infection remained in the lung without evidence of dissemination. BM failure in IFRag-/- mice could be prevented via immune reconstitution with wild-type or IFNAR-/- splenocytes and specifically B cells (25).

In the current study, we further examined mechanisms involved in the induction of BM failure to discern how type I IFN signaling acted in protecting hematopoiesis in the absence of lymphocytes and under the stress of increased demand after pneumonia lung infection. By comparing responses in IFRag-/- versus RAG-/- mice, we demonstrate that IFNAR signaling is required on BM-derived but not stromal-derived cells to prevent BM failure after pneumonia lung infection. Failure is initiated by accelerated apoptosis of the neutrophil lineage, mainly via the extrinsic pathway of apoptosis, followed by the additional loss of myeloid precursors. BM failure is preceded by reduction of transcription or rapid degradation of mRNA for some key antiapoptotic and survival factors and increased mRNA abundance for proapoptotic TNF-α when assessed in total BM cells. This is also followed by evidence for oxidative stress in all myeloid and other precursors associated with caspase-9 activation.

These data show that a state of immunodeficiency involving the type I IFN system may negatively affect the regenerative capabilities of the BM in response to increased demand due to opportunistic lung infections with pneumonia by regulating the balance of proapoptotic and antiapoptotic mechanisms. This could have implications in understanding the causes underlying regenerative BM failure as it can occur in AIDS and possibly other BM-failure diseases associated with immune defects (14, 26).

Materials and Methods
Mice, treatment procedures, and BM chimera generation
C.B17 SCID mice, as a source for pneumonia murina organisms, were bred and maintained at the Montana State University Animal Resource Center. RAG1-/- mice (C57/BL6 background) were initially purchased from Jackson Laboratories (stock no. 002096) and then bred at Montana State University. IFRag-/- mice were generated by crossing IFNAR knock-out mice (C57/BL6 background and maintained at Montana State University) with RAG1-/- mice (C57/BL6 background), as previously described (25), and have since been backcrossed two more times on a C57/BL6 background. Animals were kept in ventilator cages with sterilized food and water. Some mice received N-acetylcysteine (NAC) as an anti-oxidant treatment at 7.6 mM (1.25 g/l) in acidic drinking water starting 1 d prior to and continuing through the course of the experiment. BM chimeric mice between IFRag-/- and RAG-/- were generated as previously described (19). Briefly, two groups of recipient IFRag-/- and Rag-1-/- mice were lethally irradiated with 9.5 Gy from a 60Co source in a split dose, and 10^7 BM cells from IFRag-/- donor animals was i.v. injected into one group of RAG-/- recipient mice (IFrag-/- BM into RAG-/- mice) and into one group of IFrag-/- recipient mice (IFrag BM into IFRag-/- mice), and 10^7 BM cells from RAG-/- mice was injected into IFRag-/- recipient mice (RAG-/- BM into IFRag-/-) and into RAG-/- recipient mice (RAG-/- BM into RAG-/- mice). Eight weeks after BM engraftment, the chimeric mice were infected with pneumonia species as described later.

All mouse studies conformed to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at Montana State University.

Pneumocystis infection and enumeration
Experimental animals were intratracheally infected with 10^7 Pneumocystis nuclei in 100 μl lung homogenate from infected source mice diluted in PBS buffer, and Pneumocystis burden of infected animals was assessed microscopically by enumeration of trophozoite nuclei count in lung homogenates in 10–50 oil immersion fields as previously described. The limit of detection for this technique is log_{10} 4.43 (27).

Collection and differentiation of BM cells
BM cells from femur and tibia was collected as previously described by flushing 2 ml PBS through the BM canal using a 26 1/2 g needle and brought into a single-cell suspension (28). BM cells were diluted 1:10 in PBS. Cells numbers were enumerated, spun onto glass slides, and stained with Diff-Quick solution (Dade/Behring). Cell differentiation was performed based on morphology and staining pattern to distinguish myeloid (including myeloblast–myelocyte and metamyelocyte stage), erythroid cells, neutrophils, and eosinophils (28).

FACS analysis was also applied for BM cell subset differentiation using Abs for the cell surface molecules CD11b, Ly6G/6C (Gr-1), and Ter119, as described later, in combination with assessment for caspase-3, -8, and -9 activities at the single-cell level. Cell-sorting analysis confirmed that CD11bGr-1BM cells consisted of cells in the myeloblast–meta-myelocyte stage of differentiation, whereas CD11bGr-1 B cells consisted of both neutrophil and eosinophil granulocytes. Staining against eosinophil marker CCR3 gave inconsistent results, and thus we were unable to distinguish between eosinophils and neutrophils by FACS analysis. The Ter119 marker identified cells of the erythroid lineage. Hematopoietic stem cells were identified by combining a lineage marker mix with Abs for Sca1, c-Kit, and CD150 (SLAM) followed by gating on cells negative for lineage marker expression but that were Sca1, c-Kit, and CD150 (29). FACS staining, RBC lysis of BM cell samples was performed using ACK lysis buffer. Cells were then suspended at 1 x 10^6/ml in FACS buffer (PBS/10% calf serum) containing Fc-block (mouse clone 24G2; Pharmingen) at a 1:800 dilution. Sets of 5 x 10^5 cells were stained with specific Abs: TER-119 (PerCP-Cy5.5, clone TER-119; BioLegend), anti-CD11b (Alexa Fluor 700, clone M1/70, BioLegend), anti-Ly-6G/6C (allophycocyanin-Cy7, clone RB6-8C5; Pharmingen). After cell surface staining, cells were assessed for the presence of total activated caspases in live cells using the CaspGLOW Red Caspase staining kit (Bio Vision), the presence of activated caspase-8 using CaspaseGLOW Red Caspase-8 staining kit (Bio Vision), the presence of activated caspase-9 using CaspGLOW Red Caspase-9 staining kit (Bio Vision), or the presence of activated caspase-3 using CaspGLOW Red Caspase-3 (Bio Vision). The manufacturer’s protocols were followed for these CaspGLOW products. Hematopoietic stem cells were identified as described above using a FITC-labeled lineage marker mix (Invitrogen), anti-c-Kit (allophycocyanin-Cy7, clone 2B8; BioLegend), anti-CD150 (PerCP, clone TC15-12F12.2; BioLegend), and anti-Sca1 (allophycocyanin, clone D7; eBioscience). BCL-2 expression in BM cells was assessed using a PE-labeled mouse BCL-2–specific Ab and an isotype-specific irrelevant control Ab (PE Hamster anti-mouse Bcl-2; Set: BD Pharmingen), and an intracellular staining was performed according to the manufacturer’s protocol. For cell fixation and permeabilization, Cytofix–Cytoperm reagents from BD Pharmingen were used. Cell acquisition was performed using an LSRII flow cytometer (BD) and analyzed by FlowJo software.

Detection of oxidative stress in BM cells using dichlorodihydrofluorescein diacetate
Baseline reactive oxygen species (ROS) production in BM cells from IFRag-/- mice compared with that in RAG-/- mice was quantified during the course of pneumonia lung infection as an indicator of cell stress (30). Briefly, BM cell suspensions were diluted to 2 x 10^5/ml in MEM, and 5 x 10^5 cells were placed in flat-bottom, black, 96-well plates (Costar) in the presence of 8 μM cell-permeant dichlorodihydrofluorescein diacetate (H2DCF-DA=DCF), a fluorometric detector of ROS. Each cell sample was measured in quadruplicate in a Fluoroskan Ascent FL fluorometer (Thermo Electron) read at 2-min intervals over the course of 120 min (excitation 485 nm and emission 530 nm). Oxidation of DCF (H2DCF-DA) resulted in increased fluorescence of the product relative to the amount of ROS production in the cells, which was displayed in arbitrary fluorescent units. Accumulated ROS production was compared between the experimental groups at 120 min.
Quantitative gene expression analysis by RT-PCR and protein analysis

Total RNA was harvested from total BM cells from three individual Ifrag−/− and RAG−/− mice at days 0, 7, and 10 after Pneumocystis lung infection using an RNasey Plus Mini Extraction Kit (Qiagen). RNA quality (RNA integrity number) was assessed with an Agilent BioAnalyzer following the Nano Kit Lab-On-A-Chip procedure (Agilent Technologies). RNA with an RNA integrity number of ≥7.6 was used to be analyzed using pathway-focused RT-PCR array systems for apoptosis (RT2 Profiler PCR Array Mouse Apoptosis PAMM-012C; SABiosciences) using an Applied Biosystems 7500 Fast Real-Time PCR machine. Ct values were gathered using www.SABiosciences.com/pcrarraydataanalysis.php to determine relative expression differences between the comparison groups. Changes of mRNA abundances by 2-fold and higher with a p value <0.05 were considered significantly different between the comparison groups as shown. Confirmatory studies and relative quantification of additional genes was performed using QuantíFast SYBR Green RT-PCR Kit (no. 204154; Qiagen) with 100 ng RNA/reaction and following the manufacturer’s protocol. Specific primers were ordered from Qiagen: mouse β-Actin (no. QT01136772), G-CSF (no. QT00105140), GM-CSF (no. QT00251286), TNF-α (no. QT00104006). Data were analyzed with 7500 Fast System SDS Software. All assays were done in triplicate. TNF-α protein was detected in cell lysates of Ifrag−/− and RAG−/− BM cells at day 9 post-infection. Cell lysates were generated in RIPA buffer, and 60 μg total protein was assayed in duplicate using a commercially available mouse TNF-α ELISA kit from R&D Systems according to the manufacturer’s protocol.

Bone histology

For detection of apoptosis of BM cells, in situ TUNEL staining was performed on decalcified and paraffin-embedded bone sections. Cleaned femur and tibia were placed in 10% buffered formalin solutions for 24 h. Bones were decalcified in 14% EDTA in DPBS, pH 7.4–7.6, at 4°C for approximately 1 wk, rinsed in tap water for 30 min, and placed into 70% ethanol until further processing. Paraffin-embedded bones were cut in 5-μm sections and H&E stained (Richard Allen Scientific). TUNEL staining was performed on adjacent slides using the Roche Apoptosis Kit (TUNEL assay) following the manufacturer’s protocol.

Colonies-forming cell assay for mouse BM cells

Hematopoietic precursor cell activity in BM from Ifrag−/− and RAG−/− mice was assessed by performing colony-forming cell (CFC) assays in methylcellulose media. For this, 105 BM cells per animal and group of each time point was plated in MethoCult GF M3534 media (StemCell Technologies), which has been formulated to support the optimal growth of granulocyte and macrophage precursor cells. Cells from each sample were plated in duplicate using a commercially available mouse TNF-α ELISA kit from R&D Systems according to the manufacturer’s protocol.

Anti-TNF-α treatment of Ifrag−/− mice

Anti-TNF-α treatment of Ifrag−/− mice was performed via biweekly i.p. injection of 250 μg hamster anti-mouse mAb anti–TNF-α Ab (clone TN3-19.12, Leinco Technology) as previously described (31).

Microscopy and statistical analysis

Microscopy was performed using a Zeiss Axiostar microscope (Zeiss, Jena, Germany).

Statistical analysis was performed using either a one-way ANOVA or two-way ANOVA followed by either a Tukey or a Bonferroni post test.

Results

Hematopoietic cells of Ifrag−/− mice undergo apoptosis in response to Pneumocystis lung infection

As previously reported, Ifrag−/− but not RAG−/− mice develop rapidly progressing bone BM failure in response to Pneumocystis lung infection. Furthermore, BM failure in Ifrag−/− mice can be prevented by immune reconstitution with splenocytes from wild-type or IFNAR−/− mice, and the rescuing activity is specifically provided by B cells (25). To determine whether apoptosis plays an essential role in Pneumocystis-induced BM failure, we assessed morphological features of BM cells from Pneumocystis-infected Ifrag−/− mice with BM failure compared with those of BM cells from immune-reconstituted and infected Ifrag−/− mice without BM failure. Morphological analysis revealed that BM cells from infected and unreconstituted Ifrag−/− mice without BM failure. Morphological analysis revealed that BM cells from infected and unreconstituted Ifrag−/− mice demonstrated nuclei fragmentation and cell membrane blebbing compared with BM cells from infected but immune-reconstituted Ifrag−/− mice without BM failure (Fig. 1A). Such changes are consistent with apoptosis. To confirm increased apoptosis in the BM of Pneumocystis-infected Ifrag−/− mice, bone sections from the two comparison groups were H&E stained to assess BM cellularity in situ (Fig. 1B), and adjacent sections were TUNEL stained to detect DNA fragmentation (Fig. 1C). BM cellularity was clearly reduced in Pneumocystis-infected Ifrag−/− mice compared with that in immune-reconstituted and infected littersmates (Fig. 1B). Moreover, despite reduced BM cellularity, there was an ~5-fold greater signal for TUNEL-positive cells (green dots/field of view in Fig. 1C) in unreconstituted and Pneumocystis-infected Ifrag−/− mice compared with that in immune-reconstituted and infected Ifrag−/− mice (Fig. 1C).

Type I IFN signaling is relevant on BM-derived cells and not on radio-resistant stromal cells to prevent BM failure in lymphocyte-deficient mice.
To elucidate further the mechanism involved in the induction of BM failure in our model, and how type I IFN signaling acts to prevent it, we focused our analysis on the comparison of the BM responses between Ifrag-/- and RAG-/- mice during the course of Pneumocystis lung infection. To determine whether IFNAR signaling is essential on BM-derived cells or on radio-resistant stromal cells, BM chimeric mice were generated by lethally irradiating two sets of Ifrag-/- mice as well as RAG-/- mice and then reconstituting one group of Ifrag-/- mice with BM from RAG-/- donor mice (RAG-/- BM into Ifrag-/-) and one group as a control with BM from Ifrag-/- littermates (Ifrag-/- BM into Ifrag-/-). Conversely, each group of irradiated RAG-/- mice was reconstituted either with BM from Ifrag-/- donor mice (Ifrag-/- BM into RAG-/-) or with BM from RAG-/- littermates (RAG-/- BM into RAG-/-). After BM engraftment, all groups were Pneumocystis-infected, and the BM response was assessed at day 16 postinfection and compared with the response of Ifrag-/- and RAG-/- mice with previously unmanipulated BM and with that of uninfected control groups. RAG-/- mice engrafted with Ifrag-/- BM experienced BM failure in response to Pneumocystis lung infection, whereas Ifrag-/- mice that were engrafted with RAG-/- BM demonstrated normal hematopoiesis in response to the infection (Fig. 2A). All control groups behaved as expected: irradiated Ifrag-/- mice engrafted with Ifrag-/- BM and nonchimeric Ifrag-/- mice developed BM failure, and RAG-/- mice engrafted with RAG-/- BM as well as nonchimeric RAG-/- mice maintained hematopoiesis after Pneumocystis lung infection. Thus, type I IFN signaling is required on BM-derived cells to maintain hematopoiesis during the systemic stress response to Pneumocystis lung infection. Notably, in BM chimeric RAG-/- mice that had received Ifrag-/- BM (Ifrag-/- into RAG-/- mice), BM failure appeared to have progressed more rapidly compared with that in control groups in which both stromal and BM cells lacked IFNAR. This more pronounced loss of BM cells did not appear to be triggered by systemic signals released due to higher Pneumocystis lung burdens, as no significant difference between the infected groups could be detected (Fig. 2B). Nevertheless, BM differentiations demonstrated that regardless of whether IFNAR was lacking on only BM cells or on both BM and stromal cells, loss of band neutrophils was equally severe when assessed by relative and absolute numbers (Fig. 2C, 2D). However, in mice in which both stromal and BM was lacking

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**FIGURE 2.** Type I IFN signaling on BM-derived cells prevents BM failure after Pneumocystis lung infection. BM chimeric mice were generated between Ifrag-/- and RAG-/- mice. After complete engraftment at 6 wk posttransplantation, mice were Pneumocystis-infected and the BM response evaluated at day 16 postinfection. A, BM cell counts flushed from one femur and tibia per mouse with six mice per group. Data demonstrate that RAG-/- mice that received Ifrag-/- BM developed BM failure after Pneumocystis lung infection whereas Ifrag-/- mice receiving RAG-/- BM did not (white bars). All other comparison groups served as controls to the experiment. B, Pneumocystis lung burden of all experimental groups was evaluated microscopically in lung homogenates. The detection limit of the method is 4.43 log_{10}. C-F, BM cell differentiation was performed microscopically using Diff-Quick–stained BM cytopsins. C and D, Relative and absolute neutrophil numbers in BM of all comparison groups. E and F, Relative and absolute eosinophil numbers. Differences were compared with uninfected control groups. *p < 0.05, **p < 0.01, ***p < 0.001.
IFNAR, BM eosinophils and their precursors appeared to be more resistant to induction of cell death and lingered longer in the marrow (Fig. 2E, 2F).

BM failure in Ifrag−/− mice is associated with increased oxidative cell stress and global caspase activity.

Previous analysis had determined that BM failure in Ifrag−/− mice becomes obvious between days 7 and 10 and is commonly severe by day 16 postinfection. Neutrophil counts in the BM are sensitive indicators of this progression. Figure 3A shows comparative cytospins from BM samples of Ifrag−/− and RAG−/− mice at days 0, 7, 10, and 16 postinfection. The data demonstrate a progressive loss of neutrophils during the course of infection and morphological changes consistent with apoptosis in younger myelocytes and metamyelocytes from Ifrag−/− but not from RAG−/− mice. Significant differences between the comparison groups were evident by day 10 postinfection (Fig. 3B). FACS analysis also verified the complete loss of neutrophils by day 16 postinfection (Fig. 3C). The percentage of neutrophils was determined using a two-way ANOVA for B. Values shown are means (±SEM), n = 5, **p < 0.01, ***p < 0.001.

Activated caspases initiate and facilitate apoptosis. The percentage of cells positive for global caspase activity was determined with FACS analysis in all comparison groups throughout the course of infection. Comparative histogram plots show staining for global caspase activity (mainly executioner caspases 1, 3, 4, 7, 10) at the single-cell level for BM cells from Ifrag−/− and RAG−/− mice over the course of infection (Fig. 4A). Accumulation of ROS in BM cells from untreated Ifrag−/− mice over the course of infection was associated with a progressive decrease in cell numbers. However, despite significant reduction of ROS levels, NAC treatment could only postpone but not prevent the induction of BM failure in Ifrag−/− mice. In this group, BM cell numbers remained normal until day 10 postinfection but then also rapidly decreased by day 16 postinfection (Fig. 4B).

ROS production has been shown to induce apoptotic cell death in neutrophils (32, 33) and is causally linked to the development of progressive BM failure in models for Fanconi anemia (34) and ataxia telangiectasia (35). Thus, baseline ROS production in BM cells from Ifrag−/− compared with RAG−/− mice was assessed in response to Pneumocystis lung infection. We established that ROS accumulation in H2DCF-DA–loaded BM cells from infected Ifrag−/− mice at day 10 after Pneumocystis infection, whereas ROS production remained low in BM cells from RAG−/− mice. Furthermore, NAC treatment reduced ROS production significantly in BM cells of infected Ifrag−/− mice (Fig. 4A). Accumulation of ROS in BM cells from untreated Ifrag−/− mice over the course of infection was associated with a progressive decrease in cell numbers. However, despite significant reduction of ROS levels, NAC treatment could only postpone but not prevent the induction of BM failure in Ifrag−/− mice. In this group, BM cell numbers remained normal until day 10 postinfection but then also rapidly decreased by day 16 postinfection (Fig. 4B).

FIGURE 3. Day 7 postinfection marks a turning point, and neutrophil loss is a sensitive indicator of BM failure-progression in Ifrag−/− mice. Comparative cytospin analysis of BM flushes from Ifrag−/− versus RAG−/− mice is depicted at days 0, 7, 10, and 16 postinfection demonstrating progressive loss of first band neutrophils, and then younger myeloid precursors in Ifrag−/− but not RAG−/− mice. Loss of neutrophils was assessed microscopically on Diff-Quik–stained BM cytospins. Pictures were taken using an Axio Imager with a digital imaging system (original magnification ×100) (A) and the percentage of BM-neutrophil determined by differential cell count using Diff-Quik–stained cytospin slides (B). FACS analysis allowed for the identification of granulocytes (mainly neutrophils) via staining for Gr-1 versus CD11b. Additional BM cell FACS analysis confirms loss of CD11b+Gr-1hi–expressing granulocytes in BM from Ifrag−/− but not RAG−/− mice at day 16 postinfection (C). Statistical analysis was performed using a two-way ANOVA for B. Values shown are means (±SEM), n = 5, **p < 0.01, ***p < 0.001.
Multicolor FACS analysis demonstrated that particularly CD11b
Gr-1<sup>hi</sup>-expressing cells (identifying granulocytes) were the first to
become caspase<sup>+</sup> (data not shown) consistent with accelerated
apoptosis in this cell subset.

Mechanisms initiating both the intrinsic and extrinsic pathways
of apoptosis are involved in the induction of BM failure in IFrag<sup>−/−</sup>
mice.

Executioner caspase activation is induced via either the intrinsic
or extrinsic pathway of apoptosis (36, 37). Understanding the
pathway responsible for apoptosis induction in this model of
*Pneumocystis* -infected BM failure could provide key insight into
how type I IFNs act to prevent BM failure. Thus, a kinetic study
days 0, 7, 10, and 16 postinfection was performed to determine
whether either the initiator caspase-8 (extrinsic pathway) or
caspase-9 (intrinsic pathway) was predominately activated.
Furthermore, using FACS analysis we assessed whether this also led
to activation of the primary executioner caspase-3 in BM cells
from IFrag<sup>−/−</sup> compared with those from RAG<sup>−/−</sup> mice. Repre-
sentative FACS plots of BM cells from IFrag<sup>−/−</sup> versus RAG<sup>−/−</sup>
mice over the course of infection showing the percentage of cells positive for activated
caspases (C), and data from all treatment
groups are summarized (D). The percen-
tage of neutrophils in BM differentiation of the comparison groups over the course of infection is demonstrated (E).

To gain further insight into the mechanisms underlying the
initiation of both pathways of apoptosis in BM cells from IFrag<sup>−/−</sup>
mice, pathway-focused PCR arrays related to apoptotic and anti-
apoptotic mechanisms were performed (results of the complete
study can be found in the Gene Expression Omnibus database
under accession number GSE27835 under the following link:
For this experiment, three experimental groups were compared: IFrag\(^{−/−}\), RAG\(^{−/−}\), and IFrag\(^{+/−}\) mice receiving NAC treatment. Three independent BM cell samples per group at days 0 and 7 postinfection were analyzed. In this experiment, gene expression of all assessed genes was not significantly different at day 0 between the comparison groups (data not shown). However, at day 7 postinfection, mRNA abundance of important antiapoptotic genes known to interfere with caspase activation, such as Bcl-2, Birc2
FIGURE 6. Reduced mRNA for key antiapoptotic genes and myeloid growth factors in IFrag−/− BM cells precedes BM failure. Relative quantification of mRNA abundance of IFrag−/− versus RAG−/− BM cells at day 7 postinfection for some antiapoptotic genes, growth factors, and cytokines was performed. A, Fold difference of mRNA abundance for some key antiapoptotic regulators such as Bcl-2, IAP-2, Mcl-1, and Pim-2. For this, mRNA from three animals per group was analyzed in triplicate. B, Fold difference of mRNA abundance for the growth factors GM-CSF and G-CSF as well as the apoptosis-inducing cytokine TNF-α. For this, mRNA of six mice per group was analyzed in triplicate. C and D, The corresponding data for mRNA abundance differences comparing NAC-treated IFrag−/− mice with RAG−/− mice at day 7 postinfection. E, TNF-α protein concentrations in BM cell lysates from IFrag−/− compared with RAG−/− mice at day 10 postinfection (n = 4). F, The percentage of myeloid progenitor cells (gated on CD11b+/Gr-1− cells and set as 100%) expressing high levels of BCL-2 protein in IFrag−/− versus RAG−/− BM at day 10 after Pneumocystis infection as analyzed by intracellular FACS staining (n = 4). Values shown are means (±SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

(IFrag−/−, IAP), Pim-2, and Mcl-1, were significantly reduced in infected IFrag−/− mice compared with that in infected RAG−/− mice and preceded the induction of BM failure in these mice (Fig. 6A). Furthermore, quantitative PCR analysis for some (apoptosis-inducing) cytokines and growth factors not only confirmed data of the PCR array but also demonstrated reduced mRNA abundance for some key antiapoptotic genes and myeloid growth factors, and cytokines not only confirmed data of the PCR array but also demonstrated reduced mRNA abundance for some key antiapoptotic genes and myeloid growth factors. GM-CSF and G-CSF as well as the apoptosis-inducing cytokine TNF-α. For this, mRNA from three animals per group was analyzed in triplicate. C and D. The corresponding data for mRNA abundance differences comparing NAC-treated IFrag−/− mice with RAG−/− mice at day 7 postinfection. E, TNF-α protein concentrations in BM cell lysates from IFrag−/− compared with RAG−/− mice at day 10 postinfection (n = 4). F, The percentage of myeloid progenitor cells (gated on CD11b+/Gr-1− cells and set as 100%) expressing high levels of BCL-2 protein in IFrag−/− versus RAG−/− BM at day 10 after Pneumocystis infection as analyzed by intracellular FACS staining (n = 4). Values shown are means (±SEM), *p < 0.05, **p < 0.01, ***p < 0.001.

Hematopoietic precursor cell activity decreases over the course of Pneumocystis lung infection in BM of IFrag−/− mice, and TNF-α activity is a contributing factor.

Caspase activity was also increased in myeloid precursor cells of IFrag−/− mice. Thus, the question arose whether loss of neutrophils in our system was predominately due to lack of replenishment rather than accelerated apoptosis of band neutrophils themselves. Therefore, CFC assays from BM cells of both IFrag−/− and RAG−/− mice were performed in methylcellulose-based media formulated to support optimal growth of granulocyte and macrophage progenitors. BM cells from both comparison groups were harvested and equal cell numbers seeded into culture at days 0, 7, 10, and 16 postinfection. Colony formation was assessed microscopically 7 d postseeding. Fig. 7A shows the total BM cell numbers in the comparison groups during the course of infection, which recapitulates the previous findings. Colony numbers established with BM cells from the comparison groups at each time point are shown in Fig. 7B. The data demonstrate a significant reduction in colony numbers from seeded BM cells of IFrag−/− mice after day 7 postinfection compared with that of seeded BM cells from RAG−/− mice, indicating a reduction in viable BM precursor cells from IFrag−/− mice. Microscopic differentiation of established colonies revealed that the relative distribution of GM-, G-, and M-forming colonies was not different in the comparison group.
groups up to day 7 (Fig. 7C–E). However, after day 7, particularly the relative (and absolute) number of G-forming colonies significantly increased in BM cultures from RAG<sup>2/2</sup> mice but decreased in those from I<sup>Frag</sup>2/2 mice (Fig. 7D). To assess whether increased TNF-α activity found in BM lysates from I<sup>Frag</sup>2/2 mice negatively affected myeloid precursor viability, two groups of I<sup>Frag</sup>2/2 mice were Pneumocystis-infected. One of the groups received anti–TNF-α treatment via i.p. injection (250 μg twice weekly) whereas the other infected group did not. BM cells were harvested at day 16 postinfection for counts, differentiation, as well as assessment of precursor cell activity in CFC assays. Fig. 8A demonstrates that total BM cell numbers were low and not significantly different between the infected I<sup>Frag</sup>2/2 groups. However, cell numbers were consistently higher in the TNF-α–treated group compared with those in the untreated but infected I<sup>Frag</sup>2/2 group. Microscopic BM differentiation revealed that neutrophils were also rapidly lost in the anti–TNF-α–treated group (data not shown). However, myeloid precursor cells remained significantly higher in anti–TNF-α–treated I<sup>Frag</sup>2/2 mice (Fig. 8B) and also appeared microscopically more viable (data not shown). Colony assays performed with BM cells from the comparison groups confirmed this observation. Fig. 8C shows significantly higher CFCs in BM cell cultures from anti–TNF-α–treated I<sup>Frag</sup>2/2 mice compared with those in BM cultures from untreated but infected I<sup>Frag</sup>2/2 mice indicating a negative effect of TNF-α on BM precursor cell viability in our system.

**FIGURE 7.** Myeloid precursor cell activity decreases in BM from I<sup>Frag</sup>2/2 mice in response to Pneumocystis lung infection. Hematopoietic precursor cell activity in BM from I<sup>Frag</sup>2/2 and RAG<sup>2/2</sup> mice was assessed by performing CFC assays in methylcellulose media at days 0, 7, 10, and 16 postinfection. BM cells (10<sup>5</sup>) per animal and group of each time point were plated in MethoCult<sup>®</sup> GF M3534 media (StemCell Technologies), which supports the optimal growth of granulocyte and macrophage precursor cells. A, BM cell numbers flushed from two hind legs of each mouse per group over the course of infection. B, Colony numbers counted in each group and time point. C–E, The percentage of respective colonies identified in each group and time point. Colonies were identified as GM (C), G (D), and M (E). To show representative distributions, colony differentiation data were not plotted if total colony counts were below 10. Values shown are means (±SEM), n = 4 per group and time point. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 8.** Neutralization of TNF-α in I<sup>Frag</sup>2/2 mice during Pneumocystis lung infection positively affects precursor cell viability. Anti–TNF-α treatment of I<sup>Frag</sup>2/2 mice was performed via biweekly i.p. injection of 250 μg hamster anti-mouse mAb anti–TNF-α Ab (clone TN3-19.12; Leinco Technology) and the effects on BM cell numbers, differentiation, and precursor cell activity assessed at day 16 postinfection in comparison with untreated but infected I<sup>Frag</sup>2/2 mice as well as uninfected I<sup>Frag</sup>2/2 mice. A, Total BM cell numbers from two hind legs. B, Total number of myeloid precursor cells in BM. C, Total colony numbers counted from equal number of BM cells plated in CFC assay. Values shown are means (±SEM), n = 4 per group. Statistical analyses between the three comparison groups were generated using a one-way ANOVA and a Tukey post hoc test. **p < 0.01, ***p < 0.001 (compared with infected but untreated I<sup>Frag</sup>2/2 mice).
Discussion

Systemic responses to local infections are necessary to allow recruitment of inflammatory cells from the BM and signal increased demand to the system (5). We recently demonstrated that type I IFN signaling is instrumental for the maintenance of on-demand hematopoiesis after pulmonary Pneumocystis infection (25). In this study, we further examined mechanisms underlying the development of BM failure in the absence of type I IFN signaling after Pneumocystis lung infection.

In the presented studies, type I IFN signaling was found to be relevant on BM-derived cells and not on stromal-derived cells to maintain hematopoiesis after the systemic response to Pneumocystis lung infection by modulating neutrophil and precursor cell apoptosis. Neutrophils are high-turnover cells that are continuously replenished from BM precursors and form the first line of defense after acute-phase response signals during inflammation. Neutrophils are also recruited to the lung in response to Pneumocystis infection (22, 23). Their short life span can be extended via suppression of their intrinsic apoptotic program with growth factor stimulation (32). Type I IFNs are proinflammatory cytokines that, when administered in therapeutic dosages, can be proapoptotic and result in BM suppression (38, 39). In contrast, recent research also demonstrated that type I IFNs extend neutrophil life span similarly to G-CSF in culture (40) and during certain inflammatory responses in vivo, which can also contribute to prolonged inflammation (41). The regulation of antiapoptotic factors has been shown to be critically involved in this process (42).

Mechanisms underlying BM failure in response to Pneumocystis infection in our model are certainly multifactorial. We demonstrated that lack of type I IFN signaling in lymphocyte-deficient mice (Ifrag−/−) resulted in increased apoptosis of hematopoietic cells. BM-residing band neutrophils were the first cells affected in Ifrag−/− mice. In addition to possibly accelerated apoptotic turnover, CFC assays of precursor cells indicated that band neutrophils may also not be sufficiently replenished by their progenitor cells as they also undergo apoptosis in vivo and successively lost their ability to form colonies under optimal culture conditions in vitro.

Events near day 7 postinfection appeared vital in determining the fate of BM cells. Transcriptional analysis using quantitative RT-PCR revealed that apoptosis in the BM of Ifrag−/− mice was preceded by significantly reduced mRNA abundance for relevant antiapoptotic regulators such as IAP2 (Birc2), MCL-1, BCL-2, and others at day 7 postinfection. Indeed protein expression levels for BCL-2 were significantly lower in myeloid precursor cells from infected Ifrag−/− compared with those in myeloid precursor cells from RAG−/− mice in response to Pneumocystis lung infection and were associated with increasing percentage of caspase+ cells in this subset. BCL-2 is only expressed in hematopoietic cells up to the promyelocyte stage but not in band neutrophils (43, 44). Decreasing expression of BCL-2 in these subsets is consistent with increasing global caspase activity in these precursors and their declining viability as evident by diminishing activity in CFC assays. However, various other antiapoptotic molecules have been identified to be important in directly prolonging neutrophil survival. One of these factors is IAP2 (Birc2), which is downregulated in Ifrag−/− mice after Pneumocystis lung infection. IAP2 expression is upregulated in response to type I and type II IFNs via STAT3-mediated pathways, resulting in prolonged human neutrophil survival in vitro (42). This is consistent with our unpublished finding that RAG/STAT1−/− mice do not develop BM failure in response to Pneumocystis lung infection (N. Meissner, unpublished observation). STAT1 is an important signaling molecule for IFNAR, and we had anticipated BM failure to develop in these mice.

Although downregulation of IAP2 in BM of Ifrag−/− mice could be a direct link to the apoptosis of band neutrophils in our system, MCL-1 is also known to play a major role in controlling the rate at which neutrophils undergo apoptosis (2, 45). Cellular levels of MCL-1 are regulated by the rate of transcription as well as the rate of protein turnover, which is accelerated by TNF-α (46). Both TNF-α mRNA abundance as well as protein levels were found increased in BM from Ifrag−/− but not RAG−/− in response to Pneumocystis lung infection. Yet, treatment of infected Ifrag−/− mice with neutralizing anti–TNF-α Ab could not significantly prevent loss of neutrophils from the BM. However, it accomplished prolonged survival of BM myeloid precursor cells, which also maintained their ability to form colonies in CFC assays compared with BM cells from untreated but infected Ifrag−/− mice. These findings suggest that increased TNF-α levels in Ifrag−/− BM do not directly affect the life span of band neutrophils but that of their precursor cells. However, the continued loss of neutrophils in anti–TNF-α-treated Ifrag−/− mice must have resulted either from additional lack of sufficient growth factors to allow for rapid replenishment from viable precursor cells or from the presence of other apoptosis-inducing factors specifically targeting band neutrophils, or a combined effect.

Although loss of neutrophils was a sensitive indicator of BM failure in our model, precursors of the myeloid and erythroid lineage also showed morphological changes consistent with apoptosis and subsequently disappeared from the BM of infected Ifrag−/− mice (25). Single-cell analysis revealed that neutrophil death in Ifrag−/− mice was associated with a slightly more pronounced activation of initiator caspase-8 over caspase-9 followed by increased executioner caspase-3 activity. Apoptosis of all other lineages was associated with an equal activation of both initiator caspases. Indeed, differences of caspase activity in the myeloid precursor subset of each comparison group were initially due to a decrease in baseline caspase activity for initiator caspases 8 and 9 as well as the executioner caspase-3 in cells from RAG−/− mice beginning at day 7 and were significant at day 10 after Pneumocystis infection. Only after day 10 was there an actual increase in the baseline percentage of precursor cells positive for caspases from Ifrag−/− mice compared with RAG−/− mice. These findings could point to a potential difference in the induction of myeloid growth factor activity between the two comparison groups to meet increased hematopoietic demand in response to Pneumocystis lung infection. Caspase-9 is activated by internal stressors such as growth factor deficiencies (37), and its activity was increased during the progression of BM failure particularly in early precursors of Ifrag−/− mice. This was associated with reduced mRNA abundance for key myeloid growth factors such as GM-CSF and G-CSF was well as reduced mRNA abundance of the antiapoptotic factor PIM-2 in BM cells from Ifrag−/− mice at day 7 postinfection. PIM-2 expression has been demonstrated to confer long-term resistance to a variety of apoptotic signals in hematopoietic cells. However, growth factor withdrawal results in rapid decline of PIM-2 transcripts and cell apoptosis (47).

Activation of the intrinsic pathway of apoptosis is associated with generation of ROS due to cell stress (48). Baseline ROS production indeed increased in BM cells from Ifrag−/− but not RAG−/− mice by day 10 postinfection and continued to increase over time as BM failure progressed. At day 10, many band neutrophils had already disappeared from Ifrag−/− BM. Thus, the source for ROS was likely the remaining precursor cells, and their
loss and dysfunction resulted in lack of neutrophil replenishment.

Models for inherited BM-failure diseases, such as Fanconi anemia, have demonstrated an increased sensitivity to inflammation-induced (TNF-α mediated) ROS production in hematopoietic precursor cells (34, 49) and protection from BM failure via antioxidant treatment. Antioxidant treatment of IFRag−/− mice during Pneumocystis lung infection also significantly suppressed ROS production in BM cells. However, BM failure could only be delayed but not prevented. These data indicate that increased ROS production was likely an additional stressor or a result of other underlying mechanisms of cell stress but not the primary cause of BM failure in our model.

Signals activating both the intrinsic and extrinsic pathways of apoptosis appear involved in the induction of BM failure in our model. Caspase-8 activation is elicited via death receptor-mediated apoptosis (51, 52), and the absence of IFNAR signaling (112: 935–945). Our previous results had demonstrated that TNF-α mRNA abundance and protein concentrations in BM cells from IFRag−/− mice were elevated, other mediators, including TRAIL, were not increased. However, we found reduced mRNA abundance for the TRAIL-decay receptor osteoprotegerin (data not shown), which, if also reduced on protein level, could allow for increased TRAIL activity in IFRag−/− BM due to reduced decay activity. Based on these data, it appears that complex mechanisms of cell regulation are disrupted during the systemic response to Pneumocystis lung infection in the absence of type I IFN signaling. TNF-α and TRAIL are important apoptosis-inducing cytokines relevant to the regulation of hematopoiesis (51, 52). We hypothesize that their potential increased activity, in combination with reduced expression of key growth factors for the myeloid lineage (GM-CSF and G-CSF), may have important implications in the pathogenesis of BM failure in our system by affecting cellular turnover. What signals in the lung elicited in response to Pneumocystis lung infection due to the absence of IFNAR signaling result in such a profound deregulation of BM homeostasis is currently not clear and under investigation.

Overall, our system demonstrates a key regulatory role of type I IFN signaling in balancing proapoptotic and antiapoptotic signals in the BM after the systemic inflammatory response initiated during pulmonary infection with the opportunistic fungal pathogen Pneumocystis. Our previous results had demonstrated that Pneumocystis lung burden is only very low at the time BM failure is progressing (25), and a pulmonary affliction may thus not be a primary presenting symptom. Collectively, our findings could have implications for understanding how regenerative BM failure might be triggered in the context of immune-deficiency syndromes that also involve the type I IFN system such as AIDS.

Acknowledgments
We thank Dr. David Pascual and Dr. Mark Quinn for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


