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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 N-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.

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Address correspondence and reprint requests to Dr. Nicole Meissner, Department of Immunology and Infectious Diseases, Montana State University, 960 Technology Boulevard, Bozeman, MT 59718. E-mail address: nicolem@montana.edu

Abbreviations used in this article: BM, bone marrow; CFC, colony-forming cell; G, granulocyte; GM, granulocyte-macrophage; H,DCF-DA, dichlorodihydrofluorescein diacetate; M, macrophage; ROS, reactive oxygen species.

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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 \textit{Pneumocystis}, 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 \textit{Pneumocystis} 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 \textit{Pneumocystis} 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 \textit{Pneumocystis} 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 \textit{Pneumocystis} 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

\textbf{Mice, treatment procedures, and BM chimera generation}

C.B17 SCID mice, as a source for \textit{Pneumocystis 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 \textit{anti-CD2} (clone R73; BioLegend), anti–CD45.2 (clone 104.17; BioLegend), anti–CD44 (clone IM7; BioLegend), and anti–CD4 (clone 14-2-2; BioLegend) and were maintained at the \textit{Cardinal Rex Laboratory} (Excelsior, MN) with \textit{fodder} for the duration of all experiments. All mice were housed under a 12L:12D light schedule and were approved by the Institutional Animal Care and Use Committee at Montana State University.

\textbf{Pneumocystis infection and enumeration}

Experimental animals were intratracheally infected with 10^7 \textit{Pneumocystis} nuclei in 100 µl lung homogenate from infected source mice diluted in PBS buffer, and \textit{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).

\textbf{Collection and differentiation of BM cells}

BM cells from femur and tibia were 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. Cell 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 CD11b^+Gr-1^- BM cells consisted of cells in the myeloblast–meta-myelocyte stage of differentiation, whereas CD11b^+Gr-1^- cells consisted of immature myeloid and eosinophilic granulocytes. Staining with an 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 and 10^5/ml in FACS buffer (PBS/10% calf serum) containing Fc-block (mouse clone 24G2; Pharmingen) at a 1:800 dilution. Sets of 5 × 10^3 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–Cy-7, 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 (Inovitrogen), anti–c-Kit (allophycocyanin–Cy-7, clone 28B; 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 \textit{Pneumocystis} lung infection as an indicator of cell stress (30). Baseline ROS activities were diluted to 2 × 10^5 cells/plating and 5 × 10^3 cells were placed in flat-bottom, black, 96-well plates (Costar) in the presence of 8 µM cell-permeant dichlorodihydrofluorescein diacetate (H_2DCF-DA= DCF), a fluorimetric detector of ROS. Each cell sample was measured in quadruplicate using 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 (H_2DCF-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 RNeasy 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 by 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 7500 Fast System SDS Software. Comparative data analysis was performed via the ΔΔCt method using the PCR Array Data Analysis Web Portal (http://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. Confirmation studies and relative quantification of additional genes was performed using QuantiFast 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. Cleared 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 approx. 1 wk, rinsed in tap water for 30 min, and placed in 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.

Colony-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 according to the manufacturer’s protocol in 35-mm dishes in the presence of one 35-mm dish containing sterile water. Cultures were incubated for 7 d in a water-jacketed incubator maintained with 5% CO2. Colony recognition (granulocyte–macrophage [GM-], granulocyte [G]-, and macrophage [M]-forming colonies) and enumeration was performed according to StemCell Technologies guidelines.

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 Axioskop 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 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 littermates (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...
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. Fig. 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 disappearance of the CD11bGr-1hi-expressing granulocyte population at day 16 postinfection (Fig. 3C, compare 1.58% in Ifrag−/− versus 61.5% in RAG−/− mice).

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 uninfected Ifrag−/− and RAG−/− mice was essentially equivalent. However, at day 10 after Pneumocystis infection, ROS levels were significantly higher in cells from Ifrag−/− mice compared with those in cells from RAG−/− mice (data not shown). To test further whether BM failure was indeed causally linked to increased ROS production, a kinetic study was performed to investigate the effects of neutralizing ROS production via antioxidant treatment in Ifrag−/− mice. In this experiment (Fig. 4A), we compared BM responses of infected Ifrag−/− mice either untreated (open circle) or orally treated with NAC (open triangle) and those of infected RAG−/− mice (open square). We found that ROS production in BM cells from Ifrag−/− mice progressively increased over the course of 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).

Activated caspases initiate and facilitate apoptosis. The percentage of cells positive for global caspase activity was determined by FACS analysis in all comparison groups throughout the course of infection. Comparative histogram plots show staining for Gr-1 versus CD11b in mice treated with NAC (open triangle) or untreated (open circle) or NAC-treated infected Ifrag−/− mice (open square). We found that ROS production in BM cells from Ifrag−/− mice progressively increased over the course of 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).

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Multicolor FACS analysis demonstrated that particularly CD11b+ Gr-1hi-expressing cells (identifying granulocytes) were the first to become caspase+ (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 I FRag−/− 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-induced 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 I FRag−/− compared with those from RAG−/− mice. Representative FACS plots of BM cells from I FRag−/− versus RAG−/− mice over the course of infection display all cells stained for caspase-8 and caspase-9 (intrinsic pathway) was predominately activated. Further, by day 10 postinfection, the percentage of cells positive for both initiators caspase-8 and caspase-9 and executioner caspase-3 was significantly higher in I FRag−/− mice than that in RAG−/− mice and progressed this way throughout day 16 postinfection (Fig. 5B). This was consistent with cell loss (data not shown; see Fig. 4). When caspase activity was assessed on a cell-subset level, we found that under steady-state conditions, caspase activities were negligible in the CD11b+ Gr-1hi subset (granulocytes) between the groups. However, at day 10 postinfection, there was a significant difference in the percentage of cells positive for caspase-8 between I FRag−/− and RAG−/− mice (Fig. 5D).

In response to the infection, this activity persisted unchanged in I FRag−/− mice out to day 10 and then increased for both initiator caspase-8 to ~25%. In contrast, in CD11b+ Gr-1−/low myeloid precursors in both comparison groups already demonstrated significant caspase activity under steady-state conditions (day 0, ~10% of cells are positive for initiator and executioner caspases).

To gain further insight into the mechanisms underlying the initiation of both pathways of apoptosis in BM cells from I FRag−/− 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: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27835).
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 anti-apoptotic genes and myeloid growth factors in IFRag<sup>−/−</sup> BM cells precedes BM failure. Relative quantification of mRNA abundance of IFRag<sup>−/−</sup> versus RAG<sup>−/−</sup> 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<sup>−/−</sup>, IAP-2, Mcl-1, and Pim-2. For this, mRNA from three animals per group was analyzed in triplicate. E, Fold difference of mRNA abundance for the anti-apoptotic genes compared with RAG<sup>−/−</sup> mice at day 7 postinfection. F, The percentage of myeloid progenitor cells (gated on CD11b+ Gr-1<sup>+</sup>/low cells and set as 100%) expressing high levels of BCL-2 protein in IFRag<sup>−/−</sup> versus RAG<sup>−/−</sup> BM cells 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<sup>−/−</sup>) mice compared with that in infected RAG<sup>−/−</sup> 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 the two important myeloid growth factors GM-CSF and G-CSF as well as the apoptosis-inducing cytokine TNF-α when assessed by ELISA (Fig. 6E). In addition, intracellular staining for BCL-2 expression in BM cells demonstrated a reduced percentage of BCL-2-expressing myeloid progenitor cells in IFRag<sup>−/−</sup> mice compared with that in RAG<sup>−/−</sup> mice and a particular decrease of cells with high expression for BCL-2 (Fig. 6F). This is consistent with increasing caspase-9 activity in these subsets over time and with decreased mRNA abundance for the latter as assessed in total BM cells.

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

Caspase activity was also increased in myeloid precursor cells of IFRag<sup>−/−</sup> 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<sup>−/−</sup>- and RAG<sup>−/−</sup>- 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 post seeding. 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<sup>−/−</sup> mice after day 7 postinfection compared with that of seeded BM cells from RAG<sup>−/−</sup> mice, indicating a reduction in viable BM precursor cells from IFRag<sup>−/−</sup> mice. Microscopic differentiation of established colonies revealed that the relative distribution of GM-, G-, and M-forming colonies was not different in the comparison...
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 RAG2/2 mice was assessed by performing CFC assays in methylcellulose media at days 0, 7, 10, and 16 postinfection. BM cells (10⁵) 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 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.
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 caspase8 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, which also maintained their ability to form colonies 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.

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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 signals provided by cytokines such as FASL, TNF-α, TRAIL, TWEAK, and others via their specific receptors (50–52). Analysis currently not clear and under investigation.

Pneumocystis lung infection due to the absence of IFNAR signaling might be triggered in the context of immune-deficiency syndromes (GM-CSF and G-CSF), may have important implications in the size that their potential increased activity, in combination with cytokines such as FASL, TNF-α, TRAIL, and TRAIL are important apoptosis-inducing cytokines that also involve the type I IFN system such as AIDS.

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Disclosures

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