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Type I Interferon Signaling and B Cells Maintain Hemopoiesis during Pneumocystis Infection of the Lung1

Nicole Meisssner,2 Melanie Rutkowski, Ann L. Harmsen, Soo Han, and Allen G. Harmsen

Loss of CD4 T cells is the hallmark of HIV infection. However, type I IFN-producing plasmacytoid dendritic cells may also be lost. This results in susceptibility to an opportunistic infection such as Pneumocystis pneumonia. In addition, regenerative bone marrow failure resulting in pancytopenia is another common problem in advanced stage AIDS. This may be linked to both the failing immune system and recurrent opportunistic infections. We generated lymphocyte-deficient type I IFN receptor-deficient mice (IFrag−/−) to study the effects on Pneumocystis infection of the lung. When IFrags−/− mice were infected with Pneumocystis they died between days 16 and 21 postinfection with minimal pneumonia but severe anemia due to complete bone marrow failure. This included the loss of uncommitted hemopoietic precursor cells. Bone marrow failure was prevented by the reconstitution of IFrags−/− mice with wild-type lymphocytes, especially B cells. T and B cells lacking type I IFN receptor signaling could only partially prevent bone marrow failure in response to Pneumocystis infection. However, the presence of T and B cells lacking type I IFN signaling resulted in compensatory extramedullary hemopoiesis in the liver and spleen. Lymphocyte support of the regenerative capacity of the bone marrow was provided by both type I IFN-dependent and -independent mechanisms that acted synergistically. Our findings point to the requirement of both type I IFNs and lymphocytes in the regenerative capabilities of the hemopoietic system under the pressure of Pneumocystis infection, but not during steady-state hemopoiesis. This may have implications in the management of pancytopenia in AIDS.  


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Loss of CD4 T cells is the hallmark of HIV infection. However, the concomitant loss of type I IFN-producing plasmacytoid dendritic cells contributes independently to the susceptibility of AIDS-defining illnesses and the recovery of immune cell functions in response to highly active antiretroviral therapy (HAART)3 (1–3). This adds to the complexity of the immunodeficiency in AIDS.

In addition to AIDS-defining illnesses like Pneumocystis pneumonia (4), another frequently observed problem in AIDS is a wide variety of blood abnormalities including anemia, thrombocytopenia, and neutropenia. These abnormalities occur as either lineage-specific deficiencies or as pancytopenia (5, 6). These alone can have major impacts on the patient’s overall well being and also be life threatening (6, 7). However, the mechanisms that cause these blood abnormalities remain poorly understood.

Inflammation and hemopoiesis are closely linked, as virtually all immune cells are bone marrow-derived cells and are released upon demand (8). However, at the same time chronic inflammatory stimuli may negatively affect hemopoiesis and decrease the cell numbers of either isolated or all lineages. In AIDS, RBC anemia can occur due to alimentary iron-deficiency, iron reutilization disturbances during chronic inflammation, or RBC precursor infection with parvovirus B19 (9, 10). Isolated thrombocytopenia may be due to immune-mediated destruction of platelets (idiopathic thrombocytopenic purpura) and is a common finding in AIDS patients (11). Neutropenia is generally associated with pancytopenia. In virtually all patients in advanced stage AIDS (stage IV) pancytopenia is the rule and is commonly due to regenerative bone marrow suppression (12). Bone marrow suppression may, in some cases, be attributed to infectious diseases like disseminated mycobacterium infections or be a result of the toxic side effects of antiretroviral, antifungal, or antibacterial drug treatments (13–15). Apart from these explanations, there is evidence that HIV infection itself may influence the regenerative capacity of the bone marrow (12, 16). This suggests that pancytopenia in AIDS is either due to a dysfunction of the hemopoietic stem cell (HSC) or the stromal support system where HSC reside. Sound evidence that HIV infects the HSC is lacking, although there is evidence that it does infect supporting stromal cells, particularly CD14+ bone marrow-derived monocyctic cells, which function as nurse cells for some lineages (12, 17). It has not been established that HIV infection of CD14+ monocyctic bone marrow stromal cells is solely responsible for pancytopenia during HIV infection. Thus, as pancytopenia increases with AIDS progression, it is possible that the loss of acquired and innate immune functions not only directly affects susceptibility to opportunistic infections but directly affects the regenerative capacity of the bone marrow itself.

Hemopoietic stem cells colocalize with spindle-shaped osteoblasts (18, 19). This colocalization is instrumental in the maintenance of the HSC niche. Genetic defects resulting in a lack of osteoblast development lead to compromised hemopoiesis with bone marrow aplasia (20). These findings intimately link bone metabolism and hemopoiesis. Immune cell functions influence bone metabolism and are controlled by a tight balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Recently,
RANKL (receptor activator of NF-κB ligand), an activated T cell-derived factor, has been shown to induce osteoclast activation, resulting in bone loss (21, 22). Both type I and type II IFNα (IFN-α or -β and IFN-γ) counteract this activation of osteoclasts. Furthermore, type I IFN signaling is involved in the induction of osteoblast activities and differentiation (23). These data suggest a close connection of the immune system with osteogenesis and hemopoiesis.

We created T cell- and B cell-deficient type I IFN receptor-deficient mice (IFNAR−/−) by crossing RAG−/− and IFNAR−/− mice (I-Frag−/−) to examine the role of both a functional type I IFN system and a functional acquired immune system in Pneumocystis infection of the lung. When these highly immune-compromised animals were infected with Pneumocystis, they died within 16–21 days postinfection with signs of severe anemia, despite only minimal pathogen growth and inflammation of the lung. Bone marrow analysis of these animals revealed complete aplasia of all hemopoietic lineages. Immune reconstitution of these animals with either wild-type or IFNAR-deficient splenocytes restored an avid inflammatory response in the lung and rescued the animals from bone marrow failure in response to Pneumocystis infection. IFNAR−/− mice and IFNAR−/− splenocyte-immune-reconstituted I-Frag−/− mice showed some reduction in bone marrow cellularity in response to the infection. This led to compensatory extramedullary hemopoiesis in the liver and spleens of IFNAR−/− mice. These data indicate that either an intact type I IFN signaling system or an intact acquired immune system is critical for the maintenance of the regenerative capabilities of bone marrow when under the increased demand of opportunistic infections. This may, therefore, have implications in the management of pancytopenia in AIDS.

Materials and Methods

Mice

C.B17 SCID mice, as a source for Pneumocystis organisms, were bred and maintained at the Montana State University Animal Resource Center (Bozeman, MT). A breeder pair of IFNAR knockout (KO) mice on a 129/ SvEv background (24) that share the same major histocompatibility Ags with C.B17/SCID mice was provided by Prof. D.E. Levy (New York University School of Medicine, New York, NY) and was maintained at the Montana State University Animal Resource Center. RAG1−/− mice (C57BL6 background), B cell-deficient μMT mice (C57BL/6 background) and either C.B17/SCID mixed background wild-type mice (B6129PF2/J; stock no. 100903) as well as C57/BL6 wild-type mice were purchased from The Jackson Laboratory. To generate T cell- and B cell-deficient IFNAR KO mice (I-Frag−/− mice), IFNAR−/− mice were crossed with RAG1−/− mice. The F2 generation was screened by FACS analysis for the absence of T and B cells. Those animals that were T cell- and B cell-deficient in the F2 generation were screened by PCR for the disruption of the IFNAR gene using primers flanking the neomycin insertion site into exon III of the IFNAR gene. The primer used pair was 5'-CAGCCCAAGGATATACTTCTAACCC-3' (mouse IFNAR-a) and 5'-CAGAGAAGCGCCCTTGAATGC-3' (mouse IFNAR-b). The fragment generated due to IFNAR gene disruption by neomycin gene insertion was ~1500-bp long, and the wild-type gene product was 371-bp long. Animals that were phenotypically T cell- and B cell-deficient and genotypically IFNAR−/− were continued to be bred and in addition screened by PCR for the disruption of the RAG1 gene. For this the following primer triplet was provided by Dr. E. Schmidt (Montana State University, Bozeman, MT): 5'-GCTTATCTGGATACGCTG-3' (ES 2); 5'-GCTCTATCTAAATTCCTGACTGTG-3' (ES 339); and 5'-CAGAGTAGGCGCCGACCAGCCCGAG-3' (ES 340).

Homozygous litters disrupted in both IFNAR−/− and Rag1−/− genes (I-Frag−/−) were on a 129/C57BL6 mixed background, normal in size, and displayed no obvious abnormalities upon birth or in their postnatal development. Animals were kept in ventilator cages with sterilized food and water.

All animal experiments were approved by the Institutional Animal Care and Use Committee (Montana State University, Bozeman, MT) and followed all requirements of the animal welfare act.

Pneumocystis infection and enumeration

Lung homogenates from Pneumocystis-infected SCID mice were used as a source of Pneumocystis and animals under deep isoflurane anesthesia were infected intratracheally with 107 Pneumocystis nuclei in 100 μl of lung homogenate diluted in PBS buffer. The Pneumocystis burden of infected animals was assessed microscopically by the enumeration of trophozoite nuclei count in lung homogenates in 10–50 oil immersion fields as previously described (25). The limit of detection for this technique is log5, 4.43.

Collection of bone marrow cells

To obtain bone marrow, mice were euthanized via pentobarbital injection (90 mg/kg). Hind legs were removed from the hip joint and fur, muscles, and fat were trimmed. The femur and tibia were separated and the epicondyles were cut off. A 26.5-gauge needle was inserted into the bone marrow canal and the bone marrow was flushed out with 2 ml of PBS solution from both the femur and the tibia. Healthy bone marrow was generally flushed out of the bone marrow canal in a solid plug and cells were brought into a single cell suspension by repeatedly and gently aspirating the cells and flushing out again through the same needle (26).

Bone marrow cell enumeration and differentiation

Single cell suspensions of bone marrow cells diluted 1/10 were spun onto glass slides and stained with Diff-Quick solution (Dade/Behring) for cell differentiation. Cell numbers were determined using a hemocytometer. Bone marrow differentiation was performed based on morphology and the staining pattern. Cells were distinguished as myeloid, erythroid, neutrophils, eosinophils, and lymphoid cells (26).

Histological analysis of bone structure and bone marrow

Cleared femur and tibia were placed in 10% buffered formalin solutions for at least 24 h. Bone decalcification was performed using 14% EDTA in Dulbecco’s PBS (pH 7.4–7.6) at 4°C for ~1 wk. EDTA was removed by rinsing bones in tap water for at least 30 min. Bones were placed into 70% ethanol until further processed. After processing and paraffin embedding, bones were cut in 4-μm thick sections on a Leica RM2155 microtome and an H&E stain was performed using Richard Allen Scientific reagents and protocols.

Immune reconstitution and T cell depletion studies

Immune reconstitution studies of I-Frag−/− mice was performed using splenocytes derived from either C57/BL6 or 129/BL6 mixed background wild-type animals, T cell- and B cell-competent IFNAR−/− mice, or B cell-deficient μMT mice. Donor animals were euthanized via CO2, narcosis, and mice on spleens and on as previously described or both Abs together (27, 28).

Enumeration of lineage-negative and Sca-1-positive hemopoietic precursor cells (HPC) vs lineage negative/Sca-1-negative stromal cells by FACS analysis

To determine the number of lineage-uncommitted HPC, including HSC, vs lineage-committed hemopoietic and lineage-negative stromal cells in the bone marrow, FACS analysis was performed. Bone marrow cells obtained by the flush technique were stained with an Alexa Fluor 488-labeled mouse lineage Ab mixture (Caltag Laboratories) according to the manufacturer’s protocol in combination with a biotinylated anti-Sca-1 Ab in a 1/200 dilution (BD Pharmingen) and subsequent detection with PE-labeled Streptavidin (BD Pharmingen) in a 1/1000 dilution. Alexa Fluor 488-positive cells were lineage-committed hemopoietic cells, whereas lineage marker-negative cells could be divided into Sca-1−/− uncommitted HPC and Sca-1− mesenchymal derived stromal cells. Absolute cell numbers of the individual subsets were determined based on total cell numbers in the bone marrow flush and their percentages were determined by FACS analysis.

Statistical analysis

For statistical analysis a one-way ANOVA followed by a Tukey test as a post hoc test was performed. p ≤ 0.05 was considered statistically significant. Shown in graphs are the means ± SEM, and each group consisted of
4–5 animals. Each experiment was repeated at least three times, \( p < 0.05 \) is marked as *, \( p < 0.01 \) is marked as **, and \( p < 0.001 \) is marked as ***.

**Results**

*Lack of both type I IFNs as well as T and B cell leads to aplastic anemia in response to Pneumocystis infection of the lung in IFrag\(^{−/−}\) mice*

We generated T cell- and B cell-deficient IFNAR\(^{−/−}\) mice by crossing RAG\(^{−/−}\) mice with IFNAR\(^{−/−}\) mice. Mice homozygous for defects in both genes are henceforth referred to as IFrag\(^{−/−}\) mice (Table I). When IFrag\(^{−/−}\) animals were infected with *Pneumocystis* they died between day 16 and day 21 postinfection despite minimal signs of lung inflammation. The *Pneumocystis* lung burden was low at this early time point in the infection with an average *Pneumocystis*-trophozoite count on day 16 postinfection as follows: 7.1 log\(_{10}\) \pm 0.07 SEM for IFNAR\(^{−/−}\) mice; 6.5 log\(_{10}\) \pm 0.09 SEM for IFrag\(^{−/−}\) mice; 7.0 \pm 0.15 SEM for RAG\(^{−/−}\) mice; and 5.2 log\(_{10}\) \pm 0.28 SEM for wild-type mice. (The presented

![FIGURE 1. IFrag\(^{−/−}\) mice respond with bone marrow failure to *Pneumocystis* infection of the lung while steady-state bone marrow production is not affected. A, total bone marrow cell counts flushed from one femur and tibia of IFrag\(^{−/−}\), RAG\(^{−/−}\), wild-type, and IFNAR\(^{−/−}\) mice at day 16 (d 16) post *Pneumocystis* infection of the lung. Each experimental group consisted of four animals. Shown is one representative experiment of at least three. Values are means ± SEM. Asterisks mark groups with significant differences in bone marrow cell counts compared with those of the 16-day infected wild-type group. Furthermore, the cell numbers of uninfected animals of each mouse strain are compared with those of its 16-day *Pneumocystis*-infected littermate, indicating that steady-state bone marrow cell production is not affected in IFrag\(^{−/−}\) mice compared with wild-type, RAG\(^{−/−}\), and IFNAR\(^{−/−}\) mice. The numbers of asterisks in the graph represent the following \( p \) values: *, \( p < 0.05 \); and **, \( p < 0.01 \). B, cytospin preparations of bone marrow cells derived from the above groups in a 1/10 dilution followed by a Diff-Quick stain for differentiation.*
*Pneumocystis* burden is given for a representative experiment, the bone marrow data from which are shown in Fig. 1A). However, the animals appeared severely anemic upon necropsy based on a pale-colored liver and spleen when compared with uninfected *IFrag*−/− mice or wild-type animals and only minimal blood flow when the abdominal aorta was clipped. Although full blood cell counts were not taken, peripheral blood smears appeared low in nucleated cell numbers, suggesting that red and white cell lineages were affected (data not shown). To determine whether the suspected anemia was due to direct effects on hemopoiesis, bone marrow analysis was performed and total cell numbers were determined. Fig. 1A shows total bone marrow cell numbers of *IFrag*−/− mice, RAG−/− mice, IFNAR−/− mice, and wild-type mice on day 16 after *Pneumocystis* infection as well as numbers from uninfected animals of the same strains. Bone marrow cell counts were significantly reduced in *Pneumocystis*-infected *IFrag*−/− compared with all other infected animal groups. Although bone marrow cell counts were not different in wild-type and RAG−/− mice in response to the infection, the cell counts in IFNAR−/− single KO mice were slightly reduced. However, bone marrow cell counts of all uninfected groups were not different. These data suggested that type I IFN signaling is involved in the maintenance of bone marrow functions in response to *Pneumocystis* infection whereas steady-state production is not affected. Additional type I IFN-independent factors provided by lymphocytes could at least partially compensate for the lack of type I IFN-signaling and support hemopoiesis during *Pneumocystis* infection of the lung. Cytoospin preparations of recovered bone marrow cells of infected *IFrag*−/− mice showed severely pyknotic cell nuclei, and some bone marrow was completely “empty” of cells (Fig. 1B). This suggested increased apoptosis in the hemopoietic compartment in *IFrag*−/− mice. Furthermore and consistent with the above finding, the bone marrow of wild-type, RAG−/−, and IFNAR−/− mice always flushed out of the bone marrow canal in a solid plug. The bone marrow of *IFrag*−/− mice was always liquid and very red due to an accumulation of anucleated RBC and seemed to lack a connecting matrix.

To assure that differences in cell numbers in response to *Pneumocystis* infection were not due to an inefficient bone marrow flushing technique, histological analysis of the bones at day 16 postinfection was performed. H&E staining of femur and tibia sections confirmed the dramatic loss of nucleated cells and the accumulation of anucleated RBC only in the bone marrow of *IFrag*−/− mice (Fig. 2A) compared with RAG−/− mice (Fig. 2B), IFNAR−/− mice (Fig. 2C), and wild-type mice (Fig. 2D).

To determine whether the loss of bone marrow cellularity in response to *Pneumocystis* infection of the lung could be due to a transient aplastic crisis, we determined bone marrow cell numbers in *IFrag*−/− mice over an extended period of time throughout the infection. Fig. 3 demonstrates the successive loss of bone marrow cells in *IFrag*−/− mice over the course of infection, eventually resulting in complete aplasia. There was no evidence of recovery and only rarely were animals able to survive to day 28 postinfection. Bone marrow cell differential counts revealed that neutrophils (Fig. 4C) and myelocytic precursors (Fig. 4A) disappeared first from the bone marrow, whereas later-stage erythroid precursors (normoblasts) persisted the longest (Fig. 4B). One consistent finding in response to *Pneumocystis* infection in RAG−/− mice was a transient and relative (%) increase of bone marrow eosinophil cell numbers. Bone marrow eosinophilia preceded complete bone marrow failure at ~16 days postinfection (Fig. 4D). This indicated either prolonged retention of these cells in the bone marrow or prolonged survival compared with other cell types. Megakaryocyte counts were not determined in numbers but were noted in our experiments as present vs absent. In this regard, megakaryocytes were completely absent in all bone marrow counts performed at day 16 postinfection.

Immune reconstitution with splenocytes from wild-type or IFNAR−/− mice prevented aplastic crisis in *IFrag*−/− mice in response to *Pneumocystis* infection of the lung

Uninfected *IFrag*−/− mice showed normal bone marrow cell numbers under steady-state conditions and neither RAG−/− nor IFNAR−/− single KO mice developed complete bone marrow failure in response to *Pneumocystis* infection of the lung. We therefore...
hypothesized that type I IFN signaling is important in bone marrow maintenance during increased demand and that acquired immune signals are not required as long as type I IFN signaling is intact. However, other factors and signals provided by lymphocytes act synergistically and can compensate for a lack of type I IFN signaling.

To further determine which immune cells are involved in this process, we performed complete and partial immune reconstitution studies. In an initial experiment, we reconstituted Ifrag⁻/⁻ mice with wild-type splenocytes either derived from C57/BL6 mice or 129/BL6 mixed background mice. For this, Ifrag⁻/⁻ mice were injected with 3–5 × 10⁷ splenocytes via the tail vein. Animals were infected with Pneumocystis 5–7 days after immune reconstitution and bone marrow cell numbers as well as the inflammatory response in the lung was determined on days 16, 21, and 28 postinfection.

Pneumocystis burden as well as inflammatory cell numbers in the lung were significantly higher on both day 16 and day 21 in immune-reconstituted Ifrag⁻/⁻ mice compared with unreconstituted Ifrag⁻/⁻ mice, although the animals appeared clinically well (data not shown). However, all unreconstituted animals were moribund between 16 and 21 days postinfection. Consistent with this, bone marrow cell counts were not significantly different between uninfected and immune-reconstituted and infected animals through day 28 postinfection (Fig. 5A). In contrast and in accordance with previous experiments, unreconstituted and infected Ifrag⁻/⁻ mice showed severe bone marrow depression on day 16 postinfection, resulting in almost complete aplasia at day 21. The relative increase of bone marrow eosinophils in unreconstituted animals at day 16 postinfection served as a predictor of bone marrow failure in these animals (Fig. 5B).

**FIGURE 4.** Dynamic changes in the bone marrow cell composition of Ifrag⁻/⁻ mice in response to Pneumocystis infection of the lung. Microscopic bone marrow differentiation was performed in Ifrag⁻/⁻ mice in response to Pneumocystis infection of the lung. Cells were differentiated into myeloid precursors (A), erythroid precursors (all nucleated red cells) (B), neutrophils (Neutro) (C), and eosinophils (Eos) (D).

**FIGURE 5.** Immune reconstitution (reconst.) of Ifrag⁻/⁻ mice with wild-type splenocytes (WT spl.) prevents bone marrow failure in response to Pneumocystis infection of the lung. A. Bone marrow cell numbers of wild-type splenocyte-reconstituted Ifrag⁻/⁻ mice were followed in response to Pneumocystis infection of the lung throughout day (d) 28 postinfection and compared with those of unreconstituted (unreconst.) and infected (infect.) Ifrag⁻/⁻ mice. Each experimental group consisted of four animals; values are means ± SEM. B. Bone marrow cell differentiation of the same experimental groups revealed a transient relative bone marrow eosinophilia only noticed in unreconstituted and infected animals with a peak at day 16 postinfection. Bone marrow eosinophil counts are given as percentages, and values are means ± SEM. Numbers of asterisks in the graphs represent the following p values using a one-way ANOVA: *, p < 0.05; and ***, p < 0.001.
In previous experiments we found some reduction of bone marrow cell numbers in IFNAR−/− single-KO mice in response to Pneumocystis infection of the lung (see Fig. 1). This raised the question of whether the bone marrow protective and supportive activity derived from lymphocytes is truly type I IFN independent.

We therefore reconstituted IFRag−/− double-KO mice, as well as RAG−/− single-KO mice with 3 × 10^7 splenocytes from either IFNAR−/− mice or wild-type animals and compared the bone marrow responsiveness at day 16 after Pneumocystis infection to that of unreconstituted animals of both strains as well as to that of infected wild-type and IFNAR−/− single-KO mice. Results are shown in Fig. 6. Again, unreconstituted IFRag−/− mice showed severe bone marrow failure at day 16 postinfection, whereas unreconstituted RAG−/− mice had normal cell numbers and actually showed a relative increase in band neutrophils as an indicator of increased production upon demand (data not shown). Reconstitution with wild-type splenocytes resulted in a slight increase of total bone marrow cell numbers in RAG−/− mice in response to the infection and completely rescued IFRag−/− mice from bone marrow failure. Cell numbers were comparable to the total bone marrow cell counts of infected wild-type animals that could clear the pathogen within 21 days. Reconstitution of IFRag−/− mice with the splenocytes of IFNAR−/− mice protected the mice from complete bone marrow failure; however, the bone marrow cell numbers were significantly lower compared with those of animals reconstituted with wild-type splenocytes. The numbers were similarly low as seen in IFNAR−/− single-KO mice in response to the infection. Bone marrow cell numbers of RAG−/− mice reconstituted with IFNAR−/− splenocytes remained unchanged compared with the cell numbers of unreconstituted but infected RAG−/− animals. These data indicate that the presence of type I IFN signaling on nonlymphocytoid cells (e.g., other bone marrow-derived cells) plays a dominant and independent role in the maintenance of the
Materials and Methods. Another group of animals was reconstituted with wild-type spleen minus CD4, one group was CD8 T cell depleted (and wild-type spleen minus CD4), and one group was CD4 and CD8 T cell depleted (and WT spleen minus CD4 and CD8) using specific mAbs as described in Materials and Methods. Another group of animals was reconstituted with splenocytes derived from B cell-deficient μMT mice, IFrag<sup>−/−</sup> mice (and μMT spleen), and one IFrag<sup>−/−</sup> group was left unreconstituted. As control, groups served uninfected IFrag<sup>−/−</sup> as well as infected wild-type mice. In A bone marrow cell counts of all immune cell-reconstituted groups were compared with bone marrow cell numbers of infected IFrag<sup>−/−</sup> mice reconstituted with complete wild-type splenocytes. Because B cells appeared critical but not solely responsible for the maintenance of hemopoiesis in IFrag<sup>−/−</sup> mice in response to Pneumocystis infection, more detailed immune reconstitution studies were performed. B shows an experiment in which one group of IFrag<sup>−/−</sup> mice were reconstituted with complete wild-type splenocytes (and wild-type WT spleen) and three groups were reconstituted with B cell-deficient μMT splenocytes (and μMT spleen). In the μMT splenocyte-reconstituted groups one group was in addition CD4 T cell-depleted (and μMT minus CD4) and one group was in addition CD8 T cell-depleted (and μMT minus CD8). In addition, one IFrag<sup>−/−</sup> group was left unreconstituted (IFrag<sup>−/−</sup> unreconst.). All animals were Pneumocystis-infected and bone marrow cell numbers were assessed at day 16 postinfection. Bone marrow cellularity of all groups was compared with those IFrag<sup>−/−</sup> mice that had received complete wild-type splenocytes (B). Each group consisted of four animals and values are presented ± SEM. The numbers of asterisks in the graphs represent the following p values: *, p < 0.05; ***, p < 0.001.

Indeed, Pneumocystis infection of the lung in IFNAR<sup>−/−</sup> single-KO mice resulted in a stressed hemopoietic system, as they reacted to the infection with a significant splenomegaly and hepatectomy. Histological analysis of both organs showed extended extramedullary hemopoiesis. This was not visible in IFrag<sup>−/−</sup> mice, RAG<sup>−/−</sup>, or wild-type mice (Fig. 7). These data indicated that the type I IFN-independent signals provided by lymphocytes are required for the induction of extramedullary hemopoiesis in response to bone marrow stress.

B cells play an important role in bone marrow maintenance in response to Pneumocystis infection of the lung in the absence of type I IFN signaling

Because immune reconstitution with wild-type and less efficiently with IFNAR<sup>−/−</sup> splenocytes rescued IFrag<sup>−/−</sup> mice from complete bone marrow failure in response to Pneumocystis infection of the lung, we wanted to determine which cell subsets were involved.

Therefore, IFrag<sup>−/−</sup> mice were reconstituted with 3–5 × 10<sup>7</sup> wild-type splenocytes 2 days before infection with Pneumocystis; the animals were then depleted of either CD4 T cells or CD8 T cells or both subsets and the bone marrow cell numbers were again assessed at day 16 postinfection. These groups were compared with wild-type mice, splenocyte-reconstituted IFrag<sup>−/−</sup> mice, B cell-deficient μMT splenocyte-reconstituted IFrag<sup>−/−</sup> mice, and unreconstituted IFrag<sup>−/−</sup> mice as well as with wild-type and IFNAR<sup>−/−</sup> single-KO mice. As demonstrated in Fig. 8A, the depletion of CD8 T cells, CD4 T cells, or both did not lead to a drop in bone marrow cellularity in response to Pneumocystis infection of the lung. The reconstitution of animals with splenocytes from B cell-deficient μMT mice lead to a reduction in bone marrow cell numbers within 16 days after Pneumocystis infection; however, it was not as dramatic as that observed in unreconstituted and Pneumocystis-infected IFrag<sup>−/−</sup> mice. These data indicated that B cells function to maintain hemopoiesis during stress responses of the bone marrow; however, they are to a lesser extent supported by T cells. To determine which of the T cells is of greater support to B cells in maintaining bone marrow integrity, we again reconstituted one group of IFrag<sup>−/−</sup> mice with wild-type splenocytes and three groups of IFrag<sup>−/−</sup> mice with splenocytes from B cell-deficient μM mice. B cell-deficient splenocyte-reconstituted animals were then either depleted of CD4 T cells or CD8 T cells or left undepleted. All animals were infected with Pneumocystis and bone marrow cellularity was assessed on day 16 postinfection. As demonstrated in Fig. 8B, all μM splenocyte-reconstituted animals showed a significant drop in bone marrow cellularity in response to Pneumocystis infection when compared with wild-type splenocyte-reconstituted animals. However, only those animals that were
Pneumocystis infection of the lung leads to a loss of both lineage marker-positive and lineage marker-negative early bone marrow precursor cells, indicating an effect on both hemopoietic precursor cells and mesenchymal cells.

To assess whether the induction of bone marrow aplasia in Ifrag<sup>−/−</sup> mice in response to Pneumocystis infection of the lung was due to the loss of uncommitted HPC early during the course of infection, these cells were enumerated. FACS analysis for lineage-negative and lineage-positive bone marrow cells was determined in uninfected Ifrag<sup>−/−</sup> mice as well as Pneumocystis-infected Ifrag<sup>−/−</sup> mice on days 7 and 10 postinfection. A mouse lineage marker antibody mixture in combination with a Sca-1 antibody was used. Uncommitted HPC, including HSC, are negative for any lineage markers but positive for Sca-1 expression in increasing intensity (Sca-1<sup>low</sup> vs Sca-1<sup>high</sup>). Mesenchymal cells are negative for both lineage markers as well as Sca-1. The percentage of total lineage marker-negative cells as well as the percentage of lineage marker-negative and Sca-1-positive (lin<sup>−/−</sup>Sca<sup>+</sup>) HPC numbers was determined. Absolute numbers were calculated based on total bone marrow cell numbers obtained from bone marrow flushes. As shown in Fig. 9, absolute numbers of lineage-negative cells in bone marrow flushes decreased dramatically during the course of infection (Fig. 9A) and affected both lin<sup>−</sup>Sca<sup>−/−</sup> (Fig. 9B) and lin<sup>−</sup>Sca<sup>−</sup> cell population (Fig. 9C). This experiment indicated that the loss of type I IFN signaling in T cell- and B cell-deficient mice resulted in the reduction or loss of all HPC, possibly including HSC. Furthermore, it led to a loss of lin<sup>−</sup>Sca<sup>−</sup> cells that are possibly part of the mesenchymal support system. As this preceded the loss of lin<sup>−</sup>Sca<sup>−</sup> cells by several days, it is possible that the lack of type I IFN signaling affects the integrity of the HSC niche in response to an acute stimulus and in this way results in bone marrow failure in response to Pneumocystis infection of the lung.

Bone marrow failure syndrome in Ifrag<sup>−/−</sup> mice does not occur by infection with any pathogen.

The bone marrow failure syndrome in Ifrag<sup>−/−</sup> mice in response to Pneumocystis infection of the lung raised the question whether this was a general response to any acute inflammatory stimulus, specific to the site of insult, or specific to the kind of offending pathogen.

We therefore infected Ifrag<sup>−/−</sup> mice with several different pathogens via different routes of infection and subsequently assessed the bone marrow responsiveness as a result of these insults. Toxoplasma gondii, an opportunistic pathogen typically affecting AIDS patients (29), and the Herpes simplex virus can lead to severe ascending encephalitis in immunosuppressed hosts (30).

We infected Ifrag<sup>−/−</sup> mice either with 10<sup>6</sup> tachyzoites of the Toxoplasma VEG-strain i.p. (provided by Dr. J. Radke, Montana State University) or the Herpes simplex virus via intraocular infection (performed by Dr. W. Halford, Montana State University). Toxoplasma-infected Ifrag<sup>−/−</sup> animals became moribund between 10 and 16 days postinfection, and intraocular infection with the Herpes simplex virus led to severe ascending encephalitis within 10 days. However, as shown in Fig. 10 bone marrow cell numbers remained unaffected in response to both infections.

Aspergillus fumigatus is another fungal infection of the lung affecting immune compromised patients. It is commonly controlled by neutrophils and elicits strong neutrophil recruitment to the lung in immune-competent animals (31, 32). Ifrag<sup>−/−</sup> mice were infected with 5 × 10<sup>6</sup> Aspergillus conidia and bone marrow cellularity was assessed at 24 h, 48 h, and 14 days postinfection with no

FIGURE 9. Pneumocystis infection of the lung results in loss of lin<sup>−</sup>stromal cells and HPC cells in Ifrag<sup>−/−</sup> mice. Numbers of lin<sup>−</sup>Sca-1<sup>−/−</sup> hemopoietic stem/precursor cell numbers (HPC) and lin<sup>−</sup>Sca-1<sup>−/−</sup> mesenchymal stromal cells were assessed in the bone marrow in Ifrag<sup>−/−</sup> mice at days (d) 0, 7 and 10 after Pneumocystis infection using FACS analysis. A, total lin<sup>−</sup> cell numbers (counting HPC and stromal cells), B, lin<sup>−</sup>Sca-1<sup>−/−</sup> cell number, representing total numbers of HPC. C, total number of stromal cells determined by subtracting number of Sca-1<sup>−/−</sup> lin<sup>−</sup> cell numbers from total lineage-negative cell numbers. Values are means ± SEM. Numbers of asterisks in the graphs represent the following p values using a one-way ANOVA: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
high-dose IFN- in some tumors (35, 36). A common side effect of particularly agents in the treatment of both hepatitis B and C infections and antitumor properties and in this regard are used as therapeutic However, these results are in contrast to those of previous stud-

hemopoietic system in response to induction and protection of the regenerative capabilities of the bone marrow, which is therefore the paradigm of hemopoietic failure syndromes. This may result in a damaging reaction to the bone marrow in IFrag mice as is assumed to happen in some patients suffering from acquired aplastic anemia. Aplastic anemia is a relatively rare, severe condition characterized by an "empty" bone marrow and is therefore the paradigm of hemopoietic failure syndromes. Aplastic anemia is generally divided into acquired and inherited conditions (45). Although exposure to chemicals and some drugs has been shown to induce acute bone marrow failure syndromes, most cases of acquired aplastic anemia are now thought to have an immunological origin and be the result of a T cell-mediated, organ-specific destruction of bone marrow hemopoietic cells via FAS-mediated destruction of CD34+ hemopoietic precursor cells. In addition, excessive secretion of IFN-γ and TNF-α by activated T cells are thought to be inhibitory to hemopoietic precursor cells. Therefore, most acquired aplastic anemias are categorized as autoimmune-like illnesses (46, 47). In our IFrag−/− mice autoimmune phenomena are unlikely, as the animals are deficient of T and B cells and are indeed protected from bone marrow loss in the presence of these cells. Furthermore, although autoimmune phenomena can be observed in patients suffering from AIDS, general immune suppression in the advanced stages of AIDS is so severe and the remaining T cells in these patients are generally hyporesponsive (48, 49). Furthermore, HAART and the improvement of immune functions can result in the improvement of the pancytopenia in AIDS patients. In this regard a recent published report described the case of a child that presented with aplastic anemia and was subsequently diagnosed with HIV infection. The bone marrow failure syndrome in IFrag−/− mice is caused by a lack of type I IFNs. Type I IFNs are known to be important regulators of both innate as well as acquired immune responses. They are secreted in large amounts during infections of the lung Pneumocystis infection of the lung is critical to sustain on-demand hemopoiesis but is not needed during steady-state production. In response to Pneumocystis infection, the absence of type I IFN signaling resulted in the loss of uncommitted HPC and subsequently led to complete bone marrow failure. Our findings point to an important role of type I IFNs in the induction and protection of the regenerative capabilities of the hemopoietic system in response to Pneumocystis infection. However, these results are in contrast to those of previous studies suggesting that type I IFNs are negative regulators of hemopoiesis (34). Type I IFNs have direct antiviral as well as antitumor properties and in this regard are used as therapeutic agents in the treatment of both hepatitis B and C infections and in some tumors (35, 36). A common side effect of particularly high-dose IFN-α treatment is myelosuppression resulting in pancytopenia in patients (37). Although this side effect is not wanted in otherwise healthy individuals, it is used, therapeutically, to control ineffective hemopoiesis in myelodysplastic syndrome and malignant proliferation in some types of leukemia (36). This suppressive effect has been shown in vitro to be mediated via phosphorylation of the p38 MAPK pathway in CD34+ hemopoietic progenitor cells and affects all lineages (38). However, therapeutic dosages of type I IFNs, as used during treatment of chronic hepatitis, can be as high as multiple mega units and therefore exceed physiological concentrations (37). Thus, it is likely that type I IFNs have dosage-dependent opposing effects on bone marrow regeneration. This may explain the differences between previously published findings and our own. Under physiological conditions type I IFNs may actually support hemopoietic precursors during stress responses. Infectious agents commonly induce a strong inflammatory stimulus resulting in a systemic acute phase response. Released cytokines such as IL-1, IL-6, and TNF-α will regulate the release of acute phase proteins, the temperature set point, and the release of inflammatory cells from the bone marrow (39, 40). Particularly the IL-6 cytokine family has been shown to be involved in various steps of hemopoiesis and has been used to expand hemopoietic cells in vitro (41). Type I IFNs are known to be important regulators of both innate as well as acquired immune responses. They are secreted in large amounts during early responses to an invading pathogen and can be considered a danger signal to the immune system (42, 43). It is therefore possible that type I IFNs influence the acute phase response. The absence of the latter may result in the reduced secretion of cytokines such as IL-6 that, in turn, act on the mobilization of hemopoietic cells in response to inflammation. We recently showed that, in CD4 T cell-depleted mice suffering from Pneumocystis pneumonia, the lack of type I IFN signaling reduces the inflammatory response and recruitment of CD8 T cells to the site of infection. This is associated with reduced secretion of proinflammatory cytokines such as IL-6, TNF-α, and IFN-γ (44). However, because steady-state hemopoiesis in uninfected IFrag−/− mice appears to be normal and comparable to that in uninfected wild-type, IFNAR−/−, and RAG−/− mice, one would think that the lack of acute phase response cytokines should only result in a lack of responsiveness of the bone marrow to infection with Pneumocystis. Instead, we found bone marrow aplasia in IFrag−/− mice in response to the infection. We therefore postulated that Pneumocystis infection of the lungs resulted in a damaging reaction to the bone marrow in IFrag−/− mice as is assumed to happen in some patients suffering from acquired aplastic anemia. Aplastic anemia is a relatively rare, severe condition characterized by an "empty" bone marrow and is therefore the paradigm of hemopoietic failure syndromes. Aplastic anemia is generally divided into acquired and inherited conditions (45). Although exposure to chemicals and some drugs has been shown to induce acute bone marrow failure syndromes, most cases of acquired aplastic anemia are now thought to have an immunological origin and be the result of a T cell-mediated, organ-specific destruction of bone marrow hemopoietic cells via FAS-mediated destruction of CD34+ hemopoietic precursor cells. In addition, excessive secretion of IFN-γ and TNF-α by activated T cells are thought to be inhibitory to hemopoietic precursor cells. Therefore, most acquired aplastic anemias are categorized as autoimmune-like illnesses (46, 47). In our IFrag−/− mice autoimmune phenomena are unlikely, as the animals are deficient of T and B cells and are indeed protected from bone marrow loss in the presence of these cells. Furthermore, although autoimmune phenomena can be observed in patients suffering from AIDS, general immune suppression in the advanced stages of AIDS is so severe and the remaining T cells in these patients are generally hyporesponsive (48, 49). Furthermore, HAART and the improvement of immune functions can result in the improvement of the pancytopenia in AIDS patients. In this regard a recent published report described the case of a child that presented with aplastic anemia and was subsequently diagnosed with HIV infection. The bone marrow aplasia in IFrag−/− mice was i.p. infected with T. gondii or intracutaneously infected with the Herpes simplex virus and total bone marrow cell numbers were assessed at various time points. Each animal group consisted of five animals, values are means ± SEM.

FIGURE 10. Bone marrow failure in IFrag−/− mice does not occur in response to any infection. IFrag−/− mice were i.p. infected with T. gondii toxoplasites or intracutaneously infected with the Herpes simplex virus and total bone marrow cell numbers were assessed at various time points. Each animal group consisted of five animals, values are means ± SEM.
Bone marrow failure syndrome in this patient responded well under HAART with the normalization of bone marrow cellularity and peripheral cell numbers (50). This case report supports our hypothesis that a direct link between immune failure and failure in hemopoiesis exists.

Most inherited forms of aplastic anemias, however, commonly occur in the context of more complex syndromes and are associated with bone and subtle immune defects (45). Fanconi anemia, e.g., is an autosomal recessive disorder and a defect in the Fancc gene is thought to interfere with the STAT-1 signaling-pathway and affect both the type I and type II IFN pathways (51). Therefore, the pathways used by the type I IFN system appear to be affected in these inherited bone marrow failure syndromes that may influence immune functions as well as bone metabolism and, therefore, hemopoiesis.

Recent progress in hemopoietic stem cell research has demonstrated that HSC reside in specialized niches in the bone marrow in close proximity to spindle-shaped osteoblasts. Interaction between these two cells appears to be of great importance in the maintenance of the HSC (19). Lack of osteoblasts leads to reduced numbers of HSC, resulting in bone marrow aplasia, whereas increased numbers of osteoblasts results in increased numbers of HSC. Therefore, bone marrow stromal cells and particularly osteoblasts are of critical importance in both bone metabolism and hemopoiesis (20, 52).

Type I IFNs influence the balance of osteoblast vs osteoclast activities in favor of increased osteoblast function. IFNAR−/− mice reportedly have decreased bone density and their bones fracture more easily compared with those of wild-type animals (23, 53, 54). Indeed, we observed in our IFrag−/− mice increased bone brittleness in response to Pneumocystis infection of the lung compared with wild-type and infected IFrag−/− animals when the femur is removed from the hip joint (results not shown). However, these data were not quantified and bone density measurements should be performed in subsequent research.

FACS analysis of bone marrow from IFrag−/− mice during the early course of infection demonstrated a loss of lineage marker-negative bone marrow cells. This included SCA-1-positive HPC as well as SCA-1-negative mesenchymal cells such as osteoblasts. Furthermore, it appeared that loss of SCA-1-negative and lineage marker-negative cell numbers preceded the loss of HPC numbers. This implies that type I IFNs may indeed influence the HSC niche by regulating the stromal support system. In this regard, it is interesting that the rescue of IFrag−/− mice from bone marrow failure in response to Pneumocystis infection of the lung was critically mediated by B cells, while T cell subsets provided support to a lesser extent. Recently, there has been accumulating evidence of a complex relationship between bone cell and B cell development. Some of the research suggests that B cells and osteoclasts share a common precursor and that factors like RANKL and osteoprotegerin regulate both osteoclast and B cell numbers and activities similarly (55). Although the links provided pertain to osteoclast and not osteoblast activities, it is possible that B cells support the HSC niche, providing either cell contact-dependent or -independent factors that support the hemopoietic system and can substitute for direct type I IFN-dependent mechanisms. However, optimal support requires the help of T cells, in particular the help of CD8 T cells. Mechanisms of this interaction are however unclear.

The proliferation of HSC and HPC is maintained by an array of growth factors. Recently, a receptor tyrosine kinase (fetal liver tyrosine kinase 3 or FLT3) has been described as being expressed in hemopoietic stem/progenitor cells. Through interactions with its ligand (FLT3L) it is an important regulator of normal lymphohemopoietic stem cell functions. Activating mutations of FLT3 or overexpression of its ligand have been implicated in the pathogenesis of acute myeloid leukemia, whereas the lack of either due to targeted disruptions in mouse models results in reduced hemopoietic precursors and particularly affects myeloid cell numbers, namely those of dendritic cells and lymphocyte cell numbers in particular, resulting in a lack of B cells and NK cells (56, 57). Conversely, the overexpression of FLT3 or the treatment of bone marrow cultures with FLT3L induces the expansion of the above cells, promotes dendritic cell differentiation, and enhances type I IFN-producing plasmacytoid dendritic cell development (58, 59). However, whether a feedback mechanism exists between type I IFN-production and FLT3/FLT3L expression and function and whether this plays a role in the observed bone marrow failure syndrome in our system remains to be elucidated.

B cell numbers in AIDS patients are not necessarily reduced, but functional defects are implicated by increased autoantibodies titers, increased IgE titers, and poor response to recall Ags (60, 61). Therefore, IFrag−/− mice could model the complex immune deficiency as it occurs in patients with advanced stages of AIDS. Immunodeficiency and inflammatory stress responses due to opportunistic infection of the lung may result in severe impact on the regenerative ability of the hemopoietic system in these patients.

Thus far, only infection with Pneumocystis of the lung resulted in acute aplastic anemia. Other infections such as herpes simplex virus encephalitis or systemic Toxoplasma infection resulted in the death of IFrag−/− mice with no evidence of bone marrow failure. Pneumocystis infection usually does not disseminate (62, 63) and microscopic bone marrow analysis did not indicate any evidence for Pneumocystis spread (data not shown). It is possible that the systemic acute phase response to the infection of the lung or the pathogen itself induced a strong signal to the bone marrow that negatively affected the microenvironment of the HPC and resulted in their death. What signals are involved in this is currently not known, and global cytokine screening of both bone marrow cells and lung tissue homogenates may give insight into the mechanisms involved.

Therefore, our research leaves several major questions unanswered. Is the impact on the bone marrow a result of any slowly progressing infection of the lung? Is it the result of a specific damaging molecule released during the slow growth of Pneumocystis, or is it the specific kind of immune signal that Pneumocystis elicits that leads to such a deleterious systemic response resulting in complete bone marrow failure?

Pulmonary infections and the resulting complications are important aspects in disease progression in AIDS (64). It will be interesting to determine whether the integrity of the lung plays an important role in the sustainability of the myeloid lineage in particular during on-demand hemopoiesis. Conversely, Pneumocystis infection of the lung, even if clinically not apparent, may have a great systemic impact on the sustainability of the hemopoietic system in patients with complex immune deficiencies as it occurs in AIDS.

We therefore propose an important role for type I IFNs in the regenerative capability of the bone marrow in response to Pneumocystis infection of the lung, in particularly during the absence of an intact acquired immune system. Furthermore our results demonstrate an intricate interplay between the innate and acquired immune system not only in the prevention of infectious diseases but in the maintenance of the hemopoietic system that provides both
the immune cells as well as the cells involved in oxygen transport and hemostasis. In AIDS patients the absence of both a functional type I IFN system as well as a functional acquired immune system may result in an impairment of the regenerative capabilities of the bone marrow under chronic stimulation due to opportunistic and other infections.

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