Neutralization of Interferon-γ Exacerbates Pneumocystis-driven Interstitial Pneumonitis After Bone Marrow Transplantation in Mice

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Abstract

The role of IFNγ in the development of infection-driven interstitial pneumonitis in a model of murine graft-versus-host disease was investigated. Mice were given either syngeneic or allogeneic bone marrow transplants along with lung *Pneumocystis carinii* infections and were treated with either control mAb or anti-IFNγ mAb. At day 21 after transplant, lung weights were elevated nearly twofold in all groups. By day 41, mice in all groups had cleared the *P. carinii* but only the mice given allogeneic transplants and anti-IFNγ had increased lung weights. Increased lung weights in the anti-IFNγ–treated mice corresponded to alveolar infiltration of eosinophils, neutrophils, and multinucleated giant cells and exacerbated interstitial pneumonitis compared with mice treated with control antibody. Intracellular staining indicated that there were 3- to 10-fold more $CD4^+$ cells producing IFNγ than those producing IL-4 in the lung lavages of mice given either syngeneic or allogeneic transplant. Treatment of transplanted mice with anti-IFNγ resulted in a significant decrease in IFNγ-producing $CD4^+$ and $CD8^+$ cells in the lung lavages but no change in the number of IL-4–producing $CD4^+$ cells. These data indicate that IFNγ is critical for controlling the development of *P. carinii*–driven interstitial pneumonia after either syngeneic or allogeneic bone marrow transplant in mice. (*J. Clin. Invest.* 1997. 99: 1637–1644.) Key words: T cells • immunity • pneumonitis • opportunistic infection • inflammation

Introduction

Graft-versus-host disease (GVHD)[1] and interstitial pneumonitis are the most serious complications of clinical bone marrow transplantation (BMT) and together account for the majority of deaths after BMT (1). Interstitial pneumonitis is most often associated with an infectious etiology, particularly viral or fungal (2, 3). In this regard, we have recently reported that *Pneumocystis carinii* is capable of driving the development of interstitial pneumonitis after whole body irradiation and bone marrow transplantation in mice (4). Furthermore, the pneumonitis was $CD4^+$ T cell dependent and exacerbated by GVHD, which suggested that severity of interstitial pneumonitis after BMT may be mediated by cytokines produced by alloreactive T cells.

It has recently been reported that Th1-like cytokine responses (IL-2, IFNγ) are predominant in acute forms of GVHD whereas Th2-like cytokine profiles (IL-4, IL-10) are predominant in chronic forms of GVHD (5–10). Furthermore, IFNγ, a cytokine secreted by Th1 cells (10), has been shown to be secreted by alloreactive T cells in a minor Mls antigen model of murine acute GVHD (11). IFNγ has been implicated in immunosuppression characterized by the ability of cells from mice with GVHD to suppress both in vitro activation of normal T cells (12–14) and bone marrow B cell development (15). Neutralization of IFNγ either in vivo or in vitro has been shown to remove immunosuppression associated with alloreactive cells (14, 15). However, it is unclear if neutralization of IFNγ is capable of altering the course of GVHD since it has been reported to either reverse (14) or exacerbate (12) the severity of the disease.

It is currently thought that the "cytokine storm" released during the onset of acute GVHD is responsible for the organ injury that makes this a life-threatening disease (reviewed in reference 7). Similarly, it has been found that the pulmonary fibrosis associated with exposure to high doses of irradiation is associated with a persistent cascade of cytokine release (16). Furthermore, Wilkes et al. reported that instillation of allogeneic bronchoalveolar lavage cells into the lungs of mice resulted in the generation of local Th1 type cytokine responses (IFNγ and IL-2 production) and histological changes consistent with lung allograft rejection (17). It is clear that cytokines are an integral part of the pathological changes in the lung associated with irradiation and transplantation. The experiments presented in this paper were designed to determine the role of IFNγ in *P. carinii*–driven development of interstitial pneumonitis using a minor Mls antigen disparate model of GVHD. Neutralization of IFNγ in vivo after BMT resulted in exacerbated inflammatory responses in the lungs and severe interstitial pneumonitis that persisted after clearance of *P. carinii* infection.

Methods

Mice. Donor B10.D2oSnJ (H-2^d, Mls $1^b2^b3^b$) female mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred at the Trudeau Institute Animal Breeding Facility. Recipient BALB/cBaileyJ (H-2^d, Mls $1^b2^a3^a$) female mice were obtained from the Trudeau Institute Animal Breeding Facility. C.B-17 severe combined immunodeficiency (SCID) mice infected with *P. carinii* were obtained from a colony maintained at the Trudeau Institute (18). Mice were routinely used at about 8 wk of age and were maintained on

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1. *Abbreviations used in this paper:* BMT, bone marrow transplant; GVHD, graft-versus-host disease; MNGC, multinucleated giant cell; PE, phycoerythrin; SCID, severe combined immunodeficiency.

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acidified water or, in some experiments, water containing tetracycline after transplantation. Animal housing facilities at the Trudeau Institute are routinely screened for common mouse pathogens by serology of sentinels. The mice used in these experiments were housed in rooms determined to be free of common pathogens.

Bone marrow transplants and IFNγ neutralization. Bone marrow was flushed from femurs and tibias of donor B10.D2 mice and spleens pushed through mesh to form single cell suspensions in HBSS. To induce GVHD, whole body-irradiated (7.5 Gy) recipient BALB/c mice were given 10^7 bone marrow cells along with 10^7 splenocytes by intravenous injection. As controls, some irradiated BALB/c mice received syngeneic transplants (BALB/c bone marrow and splenocytes). Rat anti–mouse IFNγ mAb (IgG1) from clone R4-6A2 (19) was partially purified from ascites and tested for neutralizing activity in an in vitro assay in which the neutralizing titer was expressed as the reciprocal of the highest mAb dilution, which neutralized 50% or more of the antiviral activity of IFNγ (20 U/ml) on vesicular stomatitis virus-infected L929B cells. IFNγ was neutralized in vivo by intraperitoneal injection of 2×10^4 neutralizing units (200 μg) three times per week (20). As a control, some mice were injected with equal quantities of an isotype-matched mAb specific for horseradish peroxidase (a gift from Dr. C.J. Dean, ICR, Sutton, Surrey, United Kingdom).

Infection with Pneumocystis carinii. C.B-17 SCID mice were used as a source of *P. carinii.* Lungs were removed from infected mice and pushed through screens into 5 ml PBS. *P. carinii*, in these homogenates were enumerated, adjusted to 10^8 *P. carinii* nuclei per milliliter in PBS containing gentamicin and recipient BALB/c mice were lightly anaesthetized with halothane gas and inoculated intratracheally (21) with 10^7 *P. carinii* nuclei.

Lung lavage and FACS analysis of cells. Lungs were lavaged as previously described (21) with 5 ml PBS containing 0.3 mM EDTA. Cells were placed into tubes for subsequent surface staining and analysis using FACS®. Splenocytes were obtained by pushing spleens through mesh screens in PBS to obtain single cell suspensions. Cells were incubated with anti-CD4-FITC (clone GK1.5), anti-CD8-phycoerythrin (clone 53-6.7; PharMingen, San Diego, CA), and anti-CD45RB-biotin (clone 16A; PharMingen) or anti-Ig-FITC (H&L) (Southern Biotech, Birmingham, AL) and anti-B220-phycoerythrin (clone RA3.6B2; PharMingen). Streptavidin-Cy-Chrome (PharMingen) was used to label biotinylated antibodies. Fluorescence-labeled cells were analyzed using a FACScan® (Becton Dickinson and Co., Mountain View, CA) and 4,000–5,000 events were routinely acquired.

Intracellular cytokine staining for FACS® analysis. Staining for intracellular cytokines was performed using a modification of the protocol of Openshaw et al. (22). Lung lavage cells were cultured in Iscove's modified Dulbecco's media (GIBCO BRL, Grand Island, NY) with 10% FCS, antibiotics, and 5×10^{-5} M 2-mercaptoethanol and stimulated for 4 h with 50 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) and 500 ng/ml ionomycin (Sigma Chemical Co.) in the presence of 10 μg/ml brefeldin A (Sigma Chemical Co.) for the final 2 h. After washing, cells were stained for surface markers as described above but in the presence of 10 μg/ml brefeldin A. Cells were washed by centrifugation and fixed for 20 min in 2% formaldehyde. After extensive washing, cells were suspended in PBS and refrigerated overnight. Cells were pelleted and resuspended in permeabilization buffer (0.05% saponin in HBSS) for 10 min at room temperature, pelleted, and resuspended in 1 μg rat anti–mouse Fcγ receptor (clone 2.4G2) for 10 min. The appropriate dilution of phycoerythrin (PE)-conjugated anti–IL-4 (clone 11B11; PharMingen), PE-conjugated anti-IFNγ (clone XMG1.2; PharMingen), or an isotype-matched PE-conjugated rat IgG1 (PharMingen) was added to the cells and incubated for 20 min. Cells were washed with permeabilization buffer followed by PBS containing 0.1% BSA and 0.02% NaN_3. Cells were resuspended in PBS for FACS® analysis. Splenocytes were used to set a lymphocyte gate on the forward versus side scatter plot and the percentage of fluorescence-positive lymphocytes was determined. Nonspecific binding of PE-conjugated rat IgG (always $< 0.5\%$) for each individual sample was subtracted from the IL-4 or IFNγ-positive cells and data expressed as the number of fluorescence positive cells for each lung lavage sample.

Enumeration of P. carinii in the lungs. Mice were anaesthetized deeply and the right bronchi were tied off and the right lobes of the lungs were excised, blotted and weighed, and pushed through stainless steel screens in 3 ml PBS. Aliquots of the homogenates were diluted and spun onto glass slides using a cytocentrifuge. The smear was stained with Diff-Quik and the number of *P. carinii* nuclei per 25–50 oil emersion fields determined (18). Data are reported as *P. carinii* nuclei per right lungs and the limit of detectability was $10^{3.84}$ nuclei.

Lung histopathology. Left lobes of the lungs were inflated with 10% neutral buffered formalin through a tracheal catheter as previously described (23). Left lobes were removed en bloc and embedded in paraffin using standard techniques. Sections were cut 4 μm thick and stained with hematoxylin and eosin. All sections were examined microscopically for the presence of cellular infiltrates.

Statistics. Statistical analysis was performed using Whitney rank sum test or Kruskall-Wallis analysis of variance on ranks followed by Dunn's post hoc tests where appropriate. Differences were considered significant at $P \leq 0.05$.

Results

P. carinii infection is cleared from the lungs by day 41 after transplant. BALB/c mice were irradiated and given either syngeneic or allogeneic BMT along with lung *P. carinii* infections. Subsequently, they were given triweekly injections of either anti-IFNγ mAb or a control rat IgG1 mAb specific for horseradish peroxidase. At day 21 after BMT, mice that had received either BMT and anti-IFNγ or allogeneic BMT and con-

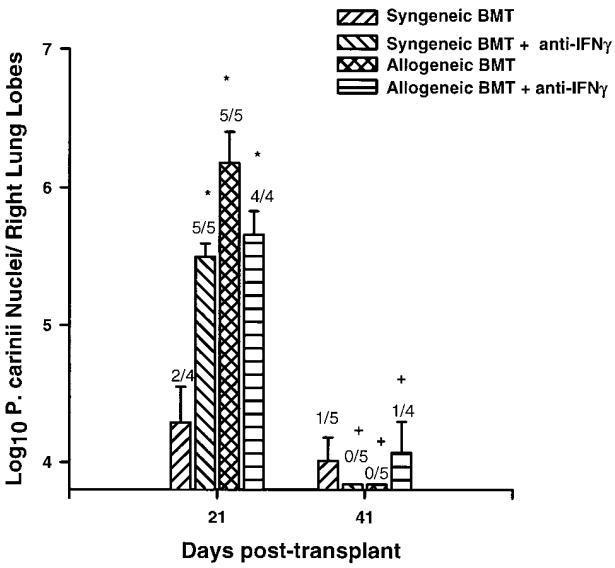

Figure 1. Resolution of *P. carinii* lung infection is complete by 41 d after BMT. Aliquots of right lung lobe homogenates from mice given either syngeneic or allogeneic BMT and treated with either anti-IFNγ or control mAb were spun onto glass slides, stained with Diff-Quik, and *P. carinii* nuclei enumerated microscopically. The limit of detection of *P. carinii* nuclei was $\log_{10}$ 3.84. The number of mice in each group with detectible *P. carinii* nuclei per total in the group is indicated above each bar. Data are expressed as the mean±SEM and are representative of two separate experiments. *Significantly different than similarly treated BMT mice given control mAb at the same time point, $P < 0.05$. +Significantly different than similarly treated mice at day 21 after BMT, $P < 0.05$.

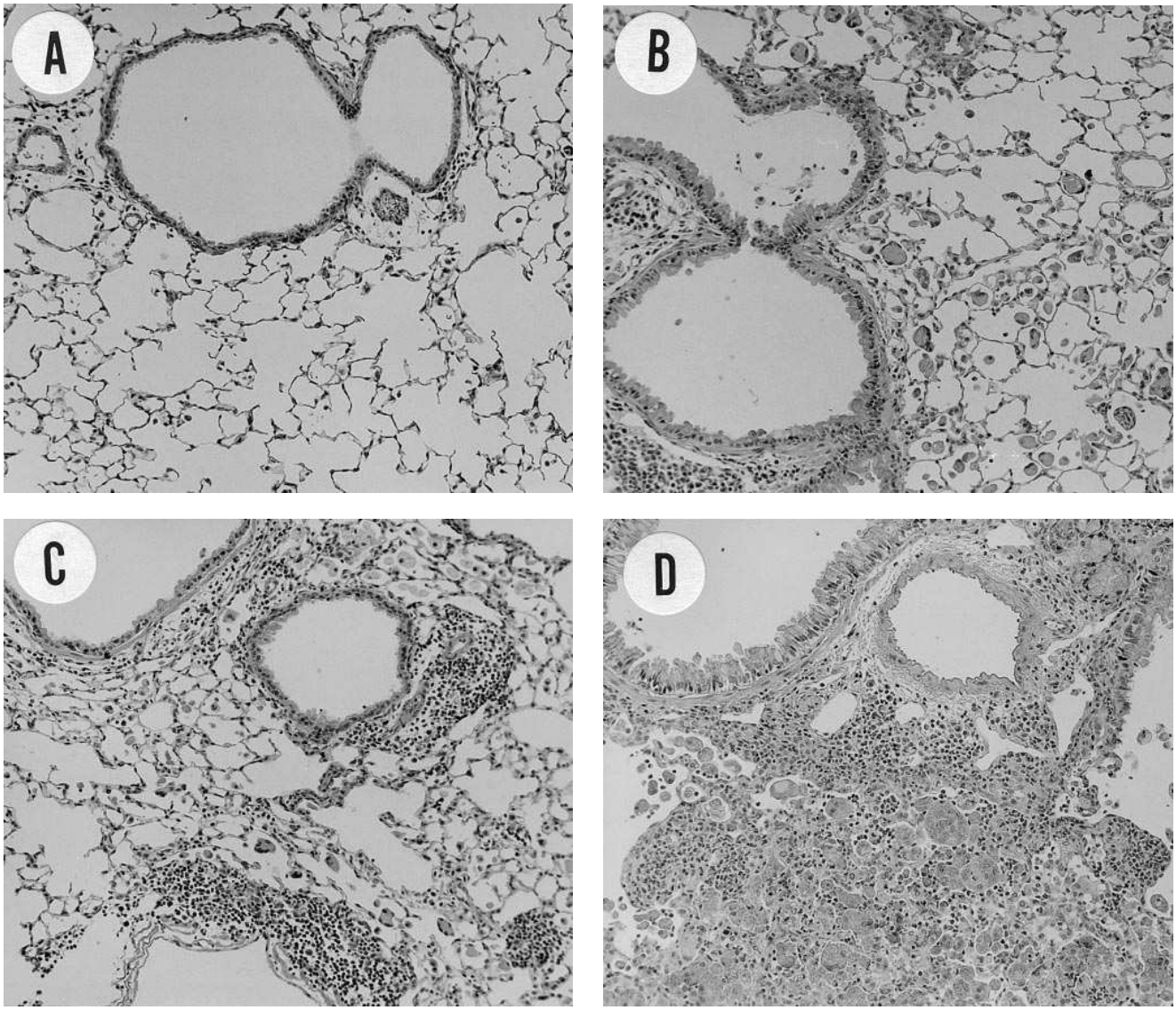

Figure 2. Lung histopathology 41 days after BMT is more severe in mice treated with anti-IFNγ than in similarly treated BMT mice given control mAb. Sections from paraffin-embedded left lung lobes were stained with hematoxylin and eosin. Sections shown are from *P. carinii*–infected mice given (*A*) syngeneic BMT and control mAb, (*B*) syngeneic BMT and anti-IFNγ, (*C*) allogeneic BMT and control mAb, (*D*) allogeneic BMT and anti-IFNγ. Sections are representative of four to five mice per group and two separate experiments. ×100.

trol mAb had $> 10^5$ *P. carinii* nuclei in their lungs (Fig. 1). However, two of four mice given syngeneic BMT and control mAb had no detectable *P. carinii* in the lungs at day 21. Significantly, by day 41 after BMT *P. carinii* had been almost completely cleared from the lungs of mice in all of the experimental groups (Fig. 1).

Interstitial pneumonitis in mice given anti-IFNγ was more severe than those given control mAb. Wet weights of the right lobes of the lungs were determined at death as an indication of lung pathology. Lung histopathologic changes were determined from hematoxylin and eosin–stained 4-μm sections of the lung left lobes. At day 21 after BMT, mice of all groups that had received *P. carinii* infection had elevated lung weights compared to a group of mice that were given allogeneic BMT and anti-IFNγ but were not infected with *P. carinii* (Table I). Increased lung weights in syngeneic BMT-mice corresponded to perivascular and peribronchiolar cuffing and focal areas of macrophage, lymphocyte, and PMN infiltration in the alveoli (not shown). In mice given syngeneic BMT and anti-IFNγ, the cellular infiltration occurred in the alveolar spaces and within alveolar septa and was more intense and widespread compared with that in mice given syngeneic BMT and control mAb (not shown). There were also large numbers of multinucleated giant cells (MNGC) and eosinophils in the alveoli of mice given syngeneic BMT and anti-IFNγ. At day 21 after BMT, cellular infiltration in the alveolar spaces and within alveolar septa of mice given allogeneic BMT and control mAb was similar in intensity to that of mice given syngeneic BMT and anti-IFNγ (not shown). However, the presence of MNGC and eosinophils was not as widespread in the lungs of mice given control mAb compared with those given anti-IFNγ. In this regard, there was widespread cellular infiltration consisting of lympho-

Table I. Wet Lung Weights after BMT

Treatment¶	Right lung weights (g)	
	Day 21*	Day 41
Syngeneic BMT	0.33±0.05	0.21±0.01‡
Syngeneic BMT + anti-IFNγ	0.44±0.01§	0.29±0.02‡
Allogeneic BMT	0.35±0.03	0.25±0.03‡
Allogeneic BMT + anti-IFNγ	0.38±0.03	0.41±0.08
Allogeneic BMT + anti-IFNγ (no *P. carinii*)	0.24±0.01	0.19±0.03

*Days after transplant. Data are expressed as the mean±SEM, $n = 5$ mice. ‡Significantly different than similarly treated group at day 21, $P < 0.05$. §Significantly different than similarly treated group given control mAb, $P < 0.05$. ¶All groups were infected with *P. carinii* but the last group in the column, which received allogeneic BMT and anti-IFNγ, was not infected.

cytes, macrophages, and PMN in the alveolar spaces and within alveolar septa of mice given allogeneic BMT and anti-IFNγ as well as large numbers of MNGC in the alveolar spaces. It should be noted that, as has been previously published (4), mice given allogeneic BMT but not *P. carinii* infections did not develop interstitial pneumonia. Indeed, the lungs of mice given anti-IFNγ and allogeneic BMT but not *P. carinii* infections did not have any abnormal histopathology (not shown) indicating that the interstitial pneumonia observed after BMT was driven by *P. carinii* infection.

At day 41 after BMT, the lung weights of mice given syngeneic BMT and anti-IFNγ as well as those of both groups of mice given control mAb had decreased significantly. This decrease in lung weights corresponded to clearance of *P. carinii* (Table I and Fig. 1). However, even though mice that had been given allogeneic BMT and anti-IFNγ had also nearly cleared their *P. carinii* infection, three of four of these mice still had very high lung weights (though not statistically different than the mice given syngeneic BMT and control mAb) at day 41 after BMT indicating the presence of a pathological state. Consistent with decreased lung weight, the lungs of mice given syngeneic BMT and control mAb were cleared of inflammatory cell infiltrates and histologically resembled those of uninfected mice given allogeneic BMT (Fig. 2). Although lung weights had decreased significantly by day 41 in mice given syngeneic BMT and anti-IFNγ as well as in those given allogeneic BMT and control mAb, lung weights for these mice were still higher than those in mice given syngeneic BMT and control mAb (Table I). In this regard, focal areas of cellular infiltrates in the alveolar septa as well as light perivascular and peribronchiolar cuffing was still visible in the lungs of mice given either syngeneic BMT and anti-IFNγ or allogeneic BMT and control mAb (Fig. 2). Furthermore, modest numbers of MNGC were still found in the alveoli of these two groups of mice. In contrast, elevated lung weights in the mice given allogeneic BMT and anti-IFNγ corresponded to intense and widespread cellular infiltration, consisting of lymphocytes, macrophages, neutrophils, and eosinophils in both the alveolar spaces and within alveolar septa (Fig. 2). Large numbers of MNGC were also found in the alveoli giving the sections the appearance of being consolidated (Fig. 2). Additionally, close examination of the lung sections from these mice revealed evidence of epithelial cell damage in the form of fibrous material sloughed off into the alveolar spaces.

Inflammatory response detected in lung lavages was more intense in mice given BMT and treated with anti-IFNγ than in those treated with control mAb. Lung lavage cell types were identified using differential counting of Diff-Quik–stained cells spun onto glass slides or by FACS® analysis. At day 21 after BMT, $> 10^6$ neutrophils were found in the alveoli of all of the groups of mice that had been infected with *P. carinii* (Fig. 3). There were no significant differences between the experimental groups at day 21. It should be noted that in uninfected mice, macrophages make up > 98% and neutrophils < 2% of the cells in lung lavages (data not shown). At day 41 after BMT, neutrophil numbers in the lung lavage fluids had decreased to near background levels in all of the groups except the mice given allogeneic BMT and anti-IFNγ. These mice had $> 9 \times 10^6$ neutrophils in their alveoli at day 41 compared with $< 5 \times 10^5$ in each of the other experimental groups (Fig. 3).

At day 21 after BMT, lung lavage eosinophils were significantly higher in the mice given anti-IFNγ than in the mice given control mAb (Fig. 3). However, by day 41, the number of eosinophils in the alveoli of mice given allogeneic or synge-

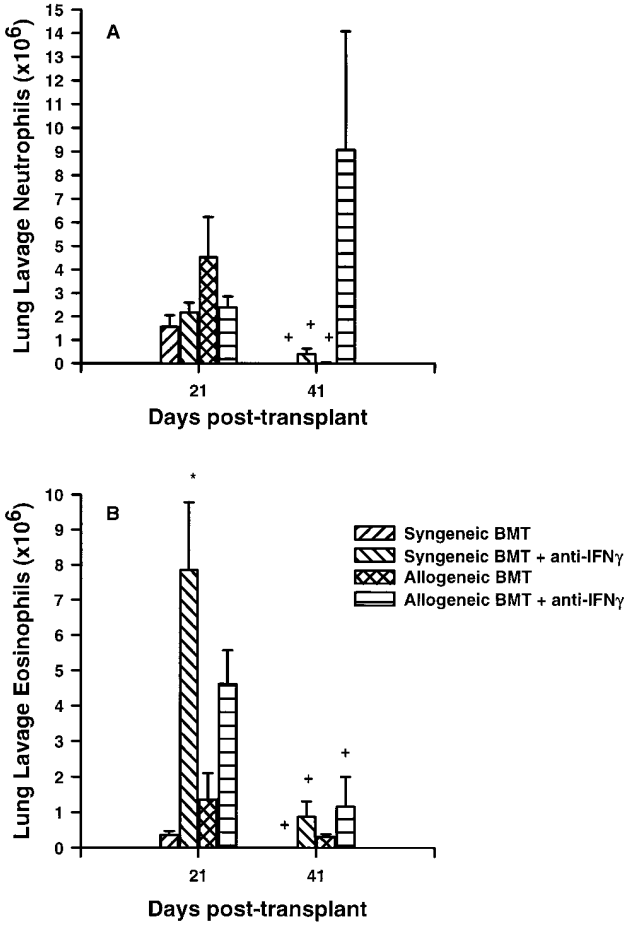

Figure 3. Infiltration of PMN into the alveolar spaces is exacerbated with treatment with anti-IFNγ. Aliquots of lung lavage fluids were spun onto glass slides, stained with Diff-Quik, and neutrophils (*A*) and eosinophils (*B*) enumerated microscopically. Data are expressed as the mean±SEM of four to five mice per group and are representative of two separate experiments. *Significantly different than similarly treated BMT mice given control mAb at the same time point, $P < 0.05$. +Significantly different than similarly treated mice at day 21 after BMT, $P < 0.05$.

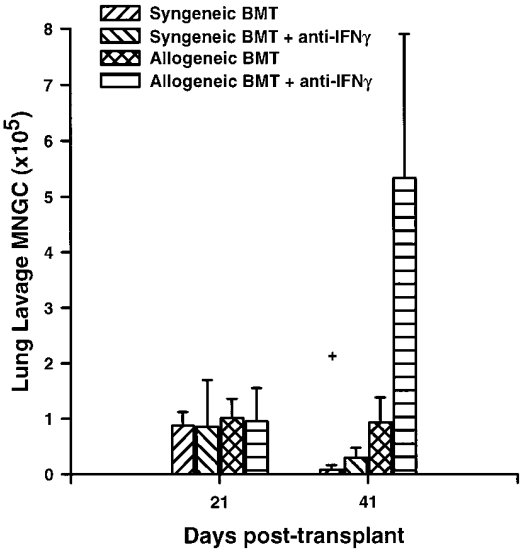

Figure 4. Formation of multinucleated giant cells in the alveolar spaces is exacerbated by treatment with anti-IFNγ in mice given allogeneic BMT. Aliquots of lung lavage fluids were spun onto glass slides, stained with Diff-Quik, and MNGC (two or more nuclei) enumerated microscopically. Data are expressed as the mean±SEM of four to five mice per group and are representative of two separate experiments. *Significantly different than similarly treated BMT mice given control mAb at the same time point, $P < 0.05$. $^+$Significantly different than similarly treated mice at day 21 after BMT, $P < 0.05$.

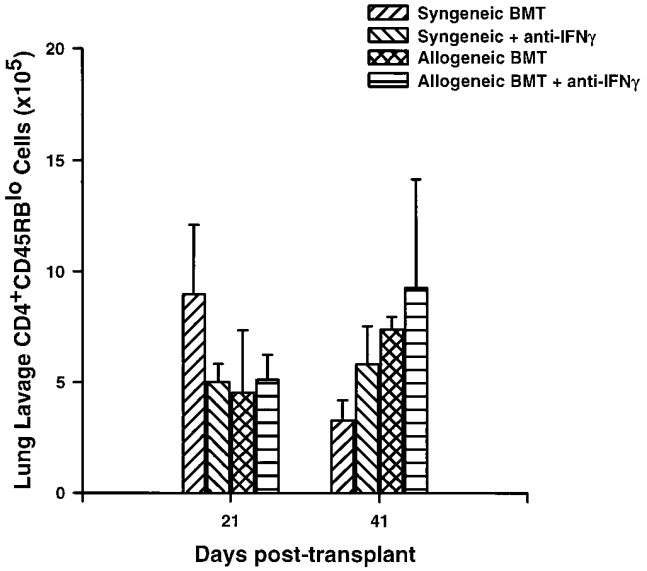

Figure 5. The number of activated CD4$^+$CD45RB^{lo} cells in the alveolar spaces was not affected by treatment with anti-IFNγ. FACS® analysis was used to enumerate the CD4$^+$CD45RB^{lo} cells in the lung lavages fluids at 21 and 41 days after BMT. Data are expressed as the mean±SEM of four to five mice per group and are representative of two separate experiments. There were no statistically significant differences detected between groups at the same time point or between time points.

neic BMT and anti-IFNγ had decreased three to eightfold, respectively. Eosinophil numbers did not change from day 21–41 after BMT in the groups of mice given control mAb.

At day 21 after BMT, the number of MNGC (defined as having two or more nuclei) in the lung lavage fluids of all the experiment groups was $\sim 10^5$ (Fig. 4). By day 41, the number of MNGC had decreased significantly in the alveoli of mice given syngeneic BMT and control mAb and remained unchanged in the mice given either syngeneic BMT and anti-IFNγ or allogeneic BMT and control mAb (Fig. 4). However, there was an approximately fivefold increase in the number of MNGC in the lung lavages of mice given allogeneic BMT and anti-IFNγ (Fig. 4) consistent with the presence of chronic lung inflammation (24).

The number of CD4$^+$ T cells in the lung lavage fluids having a phenotype consistent with activation (CD45RB^{lo}) (25) was determined using FACS®. There was a significant infiltration of CD4$^+$CD45RB^{lo} cells in the alveoli of mice from all experimental groups at day 21 after BMT, which persisted through day 41 (Fig. 5). There was a twofold decrease in the number of CD4$^+$CD45RB^{lo} cells in the mice given syngeneic BMT and control mAb from day 21–41 after BMT, however this was not a statistically significant difference. There was also an upward trend in the number of CD4$^+$CD45RB$^+$ cells in the lung lavages of mice given allogeneic BMT, though these increases were not statistically significant. Notably, lymphocytes are normally undetectable in the lung lavage fluids of uninfected mice (data not shown).

The number of IL-4 or IFNγ-producing CD4$^+$ cells in lung lavage is not different in mice given syngeneic as compared with allogeneic BMT. To determine if there was a relationship between the severity of lung disease and the type of T helper cytokine responses after BMT, FACS® analysis was used to determine the presence of IL-4 or IFNγ in lymphocytes after BMT. Lung lavage cells were stimulated nonspecifically with ionomycin and PMA for 4 h in the presence of brefeldin A to prevent protein secretion and labeled with fluorescent antibodies specific to IL-4 or IFNγ. Data are expressed as the number of CD4$^+$ (Fig. 6 *A*) or CD8$^+$ (Fig. 6 *B*) cells with intracellular IFNγ or IL-4–specific fluorescence. At day 32 after BMT, there was no significant difference between experimental groups in the numbers of CD4$^+$ cells producing IL-4. There was also no difference in the numbers of CD4$^+$ cells producing IFNγ between the mice given syngeneic or allogeneic BMT and control mAb (Fig. 6). However, there was a significant reduction in the number of IFNγ-producing CD4$^+$ cells in the mice given syngeneic or allogeneic BMT and anti-IFNγ resulting in a decreased IFNγ/IL-4 ratio. This decreased number of CD4$^+$ cells producing IFNγ was associated with elevated lung weights and a significant interstitial inflammatory response in the lungs. Mice given syngeneic or allogeneic BMT and control mAb had significantly more lung lavage CD8$^+$ cells producing IFNγ compared with those given anti-IFNγ. Notably, in mice given allogeneic BMT and anti-IFNγ but no *P. carinii* infection (inoculated with uninfected C.B-17 mouse lung homogenate), the number of CD4$^+$ and CD8$^+$ cells in the lung lavages was $< 0.3 \times 10^5$ which is near the limit of detection (not shown).

Discussion

In previous studies, we reported that *P. carinii* infection after allogeneic BMT (B10.D2 into BALB/c) led to persistent interstitial pneumonitis (4). Furthermore, development of this pneu-

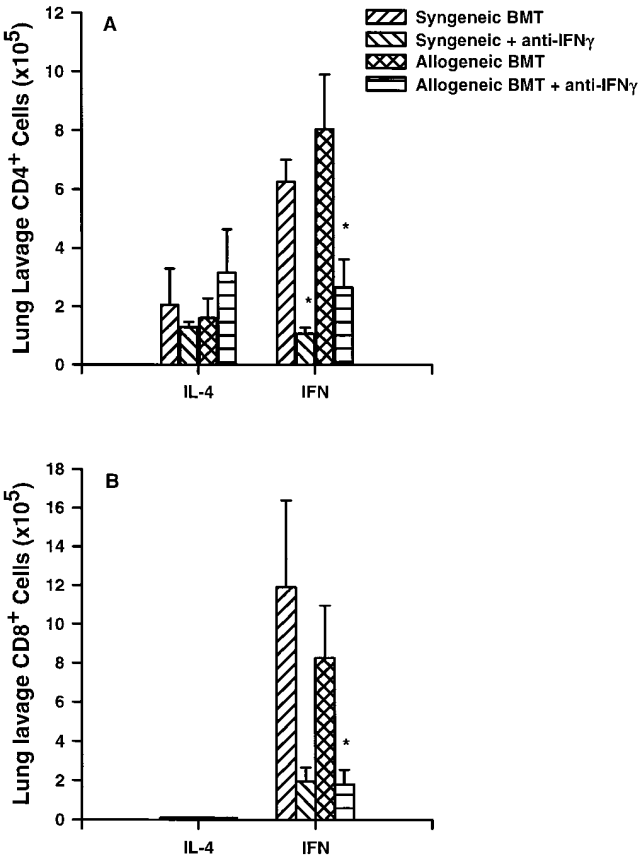

Figure 6. The number of $CD4^+$ and $CD8^+$ cells in the alveolar spaces of *P. carinii*–infected mice given BMT which produce IL-4 or IFNγ ex vivo. Lung lavage cells were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin and stained for production of intracellular cytokines analyzed by FACS®. Data represent the number of $CD4^+$ (*A*) or $CD8^+$ (*B*) cells which exhibited positive fluorescence when stained with PE-conjugated antibodies specific for IL-4 or IFNγ. Lung lavage samples were also stained with a PE-conjugated rat IgG as a nonspecific control. Fluorescence from the nonspecific staining was $< 0.5\%$ and was subtracted from the specific fluorescence. Data are representative of three separate experiments and are expressed as the mean±SEM of four to five mice per group. *Significantly different than mice given similar BMT and control mAb, $P < 0.05$.

monitis was infection and $CD4^+$ T cell dependent (4). IFNγ has been shown to be secreted by alloreactive $CD4^+$ T cells expressing Vβ3 of the T cell receptor in this same Mls-mismatched model of murine GVHD (11). In an effort to discover the mechanism involved in development of this infection-driven pneumonitis, in the present investigation, IFNγ was neutralized in mice after BMT. Neutralization of IFNγ led to severe and prolonged interstitial pneumonitis in mice undergoing GVHD. Furthermore, neutralization of IFNγ in mice given syngeneic BMT also led to exacerbation of the lung inflammatory response compared to that of mice given syngeneic BMT and control mAb. Therefore, IFNγ appears to be important in the control of the inflammatory responses that lead to interstitial pneumonitis after BMT. In this regard, it has been recently reported that stimulation of inflammation in IFNγ receptor knockout mice by exogenous IL-12 led to the development of pulmonary pathology similar to that reported here (26). Apparently, endogenous IFNγ is critical for controlling severe pulmonary pathology caused by inflammation induced by a number of stimuli (26).

Interestingly, neutralization of IFNγ in mice given allogeneic BMT did not abrogate the development of GVHD. These mice had signs of GVHD that were similar to mice given allogeneic BMT and control mAb. These signs included cellular infiltrates in the skin, scaly tails and ears, and loss of body weight. In contrast, Klimpel et al. found that neutralization of IFNγ in this same B10.D2 into BALB/c model of BMT led to amelioration of the clinical signs of GVHD (14). However, in that model recipient mice were given a sublethal dose of irradiation (6 Gy) and were reconstituted with splenocytes and not bone marrow (14).

As we have previously reported, mice did not develop pneumonitis after BMT unless they were infected with *P. carinii* (4). Interestingly, although infection was necessary to drive the initiation of pneumonitis after BMT, clearance of *P. carinii* did not necessarily lead to resolution of interstitial pneumonitis. Mice from all experimental groups had cleared the infection by day 41 after BMT. However, all groups of mice, except the mice given syngeneic BMT and control mAb still had cellular infiltrates in the alveolar septa. Indeed, mice given allogeneic BMT and anti-IFNγ had severe interstitial pneumonitis at 41 days after BMT although only one of four of these mice had detectable *P. carinii* in their lungs. This indicated that in this group of mice, once initiated, interstitial pneumonitis developed uncontrollably in the absence of further stimulus. The limit of detection in the lung homogenates was $10^{3.84}$ *P. carinii* nuclei and, therefore, it is possible that there were some *P. carinii* left in the lungs which were undetected. However, the exacerbated interstitial pneumonia was prolonged even while the *P. carinii* was being actively cleared from the lungs.

The presence of $CD4^+$ T cells has been shown previously to be required for the development of *P. carinii*–driven interstitial pneumonitis after BMT in this model (4). It is evident that alloreactive T cells exacerbate the pneumonitis compared to syngeneic T cells because by day 41 after BMT, mice given syngeneic BMT had largely resolved the *P. carinii*–induced pneumonitis whereas mice given allogeneic BMT had an intense interstitial pneumonitis. Neutralization of IFNγ, that was likely secreted by engrafted T cells, appeared to result in dysregulation of the inflammatory response and exacerbated interstitial pneumonitis in both the syngeneic and allogeneic BMT groups compared with similarly treated mice given control mAb. However, mice given allogeneic BMT and anti-IFNγ had more severe lung histopathology at day 41 after BMT than did mice given syngeneic BMT and anti-IFNγ. This suggests that there was a significant difference in the response of allogeneic T cells as opposed to syngeneic T cells. This was not the result of a difference in the number of T cells that accumulated in the lungs of the two groups nor in their activation state ($CD45RB^{hi}/CD45RB^{lo}$) or CD4/CD8 ratio of the cells. It is, therefore, possible that the syngeneic and alloreactive T cells differed in either the types of signals or the cytokines they produced. In this regard, it has been reported that the cytokine profiles of T cells from syngeneic versus allogeneic transplants differ significantly (8, 11).

Intracellular cytokine staining of lung lavage $CD4^+$ and $CD8^+$ cells indicated that at days 32 and 41 after BMT mice given either syngeneic or allogeneic BMT had 3 to 10 times the number of cells producing IFNγ than IL-4, indicative of a predominant Th1 response in both groups. Neutralization of IFNγ

in vivo resulted in a decrease in the number of IFNγ-producing $CD4^+$ and $CD8^+$ cells in the lung lavages although the absolute number of IL-4–producing $CD4^+$ cells was not different than in the lung lavages of mice receiving control mAb. There was not a compensatory increase in IL-4–producing cells when the number of IFNγ-producing cells was greatly decreased. Thus, whether treatment of BMT mice with anti-IFNγ drove a more effective Th2 response is not clear. In other studies, depletion of IFNγ in vivo in mice infected with *Listeria monocytogenes* resulted in a compensatory increase in serum IL-4 compared with mice given control antibody (27). Similarly, mice with disrupted IFNγ receptor genes and challenged with *Schistosoma mansoni* cercariae had increased levels of IL-4, IL-5, and IL-10 in their lungs compared to wild-type mice (28). Although, in the experiment presented herein, the ratio of IFNγ/IL-4 producing cells was significantly decreased by neutralization of IFNγ, it is not clear that this resulted in a Th2-type response since the amount of IL-4 acting locally was not measured.

Clearly, the presence of IFNγ is critical in controlling the inflammatory response to *P. carinii* and so the development of interstitial pneumonia after BMT. In this regard, it was recently found in this laboratory that mice with disrupted IFNγ genes resolve *P. carinii* lung infections but have a protracted inflammatory response in the lungs (29). It is not clear, however, whether IL-4 or other Th2 cytokines (IL-5, IL-10) are responsible for exacerbated interstitial pneumonia in the absence of IFNγ. It is possible that the number of cells secreting IL-5 and/or IL-10 was elevated in the mice treated with anti-IFNγ or in mice given allogeneic BMT compared with those given syngeneic BMT. Although there was not a consistent difference in the number of IL-4–producing $CD4^+$ cells between the mice given syngeneic BMT and allogeneic BMT, in some experiments the number of IL-4 producing cells in the lung lavages was significantly elevated in the mice given allogeneic BMT. It is possible that at earlier time points there may be more Th2-type cells responding to *P. carinii* in the lungs of mice given allogeneic BMT compared with syngeneic BMT. However, it is evident that the mechanism responsible for exacerbated *P. carinii*–driven interstitial pneumonia in mice given allogeneic BMT compared with syngeneic BMT is more complex than a simple difference between Th1 and Th2 responses.

The development of abnormal lung pathology in the mice treated with anti-IFNγ was characterized by a large influx of PMN and MNGC. Notably, large numbers of eosinophils were present at day 21 after BMT in the mice given syngeneic or allogeneic BMT and anti-IFNγ, which then decreased by day 41. Eosinophil accumulation generally occurs in the lungs as a result of Th2-type responses characteristic of airway hyperreactivity (30). Furthermore, IFNγ receptor knockout mice have been shown to develop generalized alveolar and interstitial pneumonitis with predominant eosinophilia in response to *S. mansoni* infection that normally produces a Th1 response (28). Since IL-5 is associated with eosinophilia in the airways, it is possible that the number of IL-5–secreting cells was elevated in the lungs of mice given either allogeneic BMT or BMT and anti-IFNγ as compared with mice given syngeneic BMT and control mAb that do not develop eosinophilia (30).

The studies presented here clearly indicate that IFNγ is critical in controlling the *P. carinii*–driven lung inflammatory response that leads to interstitial pneumonitis after BMT. However, the presence of IFNγ-producing cells was not sufficient to control the development of pneumonitis since mice given allogeneic BMT had numerous IFNγ-producing cells as well as interstitial pneumonitis at day 41 after BMT. However, neutralization of IFNγ resulted in dysregulation of the inflammatory response and severe interstitial pneumonitis in mice undergoing GVHD. Although IFNγ is associated with immunosuppression after BMT (12–15), it is clear from the studies presented here that the presence of IFNγ after allogeneic BMT is beneficial in controlling *P. carinii*–driven inflammation in the lungs.

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