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Blockade of CXCR3 Receptor:Ligand Interactions Reduces Leukocyte Recruitment to the Lung and the Severity of Experimental Idiopathic Pneumonia Syndrome

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Idiopathic pneumonia syndrome (IPS) is a frequently fatal complication after allogeneic stem cell transplantation (allo-SCT) that responds poorly to standard immunosuppressive therapy. The pathophysiology of IPS involves the secretion of inflammatory cytokines including IFN-γ and TNF-α along with the recruitment of donor T cells to the lung. CXCR3 is a chemokine receptor that is expressed on activated Th1/Tc1 T cell subsets and the expression of its ligands CXCL9 (monokine induced by IFN-γ (Mig)) and CXCL10 (IFN-γ-inducible protein 10 (IP-10)) can be induced in a variety of cell types by IFN-γ alone or in combination with TNF-α. We used a lethally irradiated murine SCT model (B6 → bm1) to evaluate the role of CXCR3 receptor:ligand interactions in the development of IPS. We found that Mig and IP-10 protein levels were significantly elevated in the bronchoalveolar lavage fluid of allo-SCT recipients compared with syngeneic controls and correlated with the infiltration of IFN-γ-secreting CXCR3+ donor T cells into the lung. The in vivo neutralization of either Mig or IP-10 significantly reduced the severity of IPS compared with control-treated animals, and an additive effect was observed when both ligands were blocked simultaneously. Complementary experiments using CXCR3+/− mice as SCT donors also resulted in a significant decrease in IPS. These data demonstrate that interactions involving CXCR3 and its primary ligands Mig and IP-10 significantly contribute to donor T cell recruitment to the lung after allo-SCT. Therefore, approaches focusing on the abrogation of these interactions may prove successful in preventing or treating lung injury that occurs in this setting. The Journal of Immunology, 2004, 173: 2050–2059.

A llogeneic stem cell transplantation (allo-SCT) is an important therapy for a number of malignant and nonmalignant diseases. However, the utility of allo-SCT is limited by serious complications including the development of pulmonary toxicity and acute graft-vs-host disease (GVHD) (1–6). Diffuse lung injury has been reported in 25–55% of allo-SCT recipients and significantly contributes to nonrelapse mortality (1, 6–8). Noninfectious diffuse lung injury, defined as idiopathic pneumonia syndrome (IPS), contributes to 50% of cases and is associated with mortality rates of >70% (1, 4). The pathophysiology of both IPS and GVHD involves toxic damage due to irradiation and chemotherapy (9, 10), an allospecific donor T cell response (11–14), and the production of inflammatory cytokines (15–19). Although the lung has not been traditionally considered an acute GVHD target organ, data from murine studies strongly suggest that pulmonary injury after allo-SCT occurs in the context of an acute GVH reaction.

Although the contribution of cellular effectors to the development of experimental IPS has been clearly established (11–13), the mechanisms by which these cells are recruited to the lung are not fully understood. Cell-mediated injury by CTLs is thought to contribute to the destruction of GVHD target tissues particularly when the GVH response is mediated by CD8+ donor T cells (20–22). CTLs leave the systemic circulation and migrate into sites of inflammation through a series of events involving inflammatory chemokines and their receptors (23). In particular, the chemokine receptor CXCR3 is strongly expressed on activated Th1 and Tc1 cells (24–26). CXCR3 mediates chemotaxis in response to its ligands, CXCL9/monokine induced by IFN-γ (Mig), CXCL10/IFN-γ-inducible protein 10 (IP-10), and CXCL11/IFN-γ-inducible T cell α chemoattractant (I-TAC) (27). Mig, IP-10, and I-TAC can be produced by various cell types including endothelium, macrophages, and neutrophils, and the expression of each is enhanced by IFN-γ (26, 28–31).

Murine models of IPS after allo-SCT are well established (13, 17, 32), and mechanistic insights provided by these experimental models are being translated back to the clinic in the form of novel treatment strategies for this complication (33, 34). Histopathologic

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4 Abbreviations used in this paper: allo-SCT, allogeneic stem cell transplantation; BAL, bronchoalveolar lavage; BALF, BAL fluid; BMT, bone marrow transplantation; GVHD, graft-vs-host disease; IP-10, IFN-γ-inducible protein 10; IPS, idiopathic pneumonia syndrome; I-TAC, IFN-γ-inducible T cell α chemoattractant; Mig, monokine induced by IFN-γ.
changes in these models include a diffuse pneumonitis involving both the alveolar and interstitial space, as well as periluminal mononuclear cell infiltrates around vascular and bronchial structures (13, 32, 35), and directly correlate with finding observed during human disease (1, 36–38). Pathologic changes are further associated with decreased pulmonary function in these mice, which strongly supports the physiological relevance of these models (11, 13).

Using a murine SCT model wherein the allospecific graft-vs-host response is mediated by donor CD8⁺ T lymphocytes to an isolated MHC class I mismatch between donor and host, we tested the hypothesis that CXCR3 receptor:ligand interactions significantly contribute to donor effector T cell recruitment to the lung during IPS. Increases in pulmonary Mig and IP-10 expression were associated with donor CD8⁺ CXCR3⁺ T cell recruitment into the lung. In vivo neutralization of these chemokines individually and in combination, as well as the transplantation with CXCR3-deficient donor cells, resulted in the reduction of IPS severity. However, a reduction in T cell recruitment was not found when CCR5-deficient donor cells were used, demonstrating that CXCR3, rather than CCR5, is critical for donor CD8⁺ T cell recruitment to the lung after allo-SCT.

Materials and Methods

Mice and SCT

Female C57BL/6 (H-2b, CD45.2⁺), B6 Ly5.2 (H-2b, CD45.1⁺), and B6.C-H2/bm1/BjJ (H-2.b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or from the Frederick Cancer Research and Development Center (National Cancer Institute, Frederick, MD). CXCR3⁻/⁻ mice were generated (39) and kindly provided by Dr. B. Lu. CXCR3⁻/⁻ mice used in this study were backcrossed at least six generations onto C57BL/6 strain. CCR5⁻/⁻ mice were backcrossed onto a B6 background for eight generations and were kindly provided by Dr. W. A. Kaziel (40). Recipient animals used for SCT and animals used for in vitro experiments were between 10 and 14 wk old. Donor animals were between 10 and 30 wk old and appropriate age-matched controls were used. All experiments were approved by the University of Michigan Committee on the Use and Care of Animals.

Mice were transplanted according to a standard protocol as described previously (41). Briefly, bm1 or B6D2F1 recipients were transplanted with cell mixtures of 5 × 10⁶ bone marrow cells supplemented with 1.8–2.0 × 10⁶ splenic T cells from either syngeneic (bm1 or B6D2F1) or allogeneic C57BL/6, B6 Ly5.2, CXCR3⁻/⁻, or CCR5⁻/⁻ donors. T cell purification was performed by magnetic bead separation using CD4 and CD8 MicroBeads and the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol, with >85% of cells obtained being positive for CD4 or CD8 surface Abs (data not shown). Percentages of purified CD4⁺ and CD8⁺ T cells did not significantly differ between donors. Before transplant, host mice received 11 Gy of total body irradiation (¹³¹I Cs source) delivered in two fractions separated by 3 h to reduce gastrointestinal toxicity. Mice were subsequently housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated water for the first 3 wk after SCT and filtered water thereafter.

Bronchoalveolar lavage (BAL)

At the time of analysis, mice were killed by exsanguination and BAL was performed as previously described (32). Supernatant from the first lavage was frozen for subsequent analysis of cytokine/chemokine content. Cell pellets were combined, washed, and counted. In some experiments, aliquots of cell suspensions were stained with fluorescent Abs to cell surface Ags and analyzed by FACS analysis (see below).

Cell surface phenotype and intracellular cytokine analysis

To analyze cell surface phenotype, cells were stained with FITC-conjugated mAbs to CD4, CD8, CD45.1, and CD8, PE-conjugated mAbs to CD4, CD8, CD11c, and IFN-γ, or allophycocyanin-conjugated mAbs to CD4 and CD8 for flow cytometric analysis as previously described (16). All mAbs were purchased from BD Pharmingen (San Diego, CA). For intracellular IFN-γ staining, BAL cells were stimulated with the Leukocyte Activation Cocktail in the presence of Golgi Plug (BD Pharmingen) for 4 h, and then stained using reagents from the Intracellular Cytokine Staining Kit (BD Pharmingen) according to the manufacturer’s protocol. CXCR3 staining was performed using either unlabelled rabbit anti-CXCR3 (mouse Abs (Zymed Laboratories, San Francisco, CA) or rabbit isotype IgG control Abs as primary Abs, and for subsequent detection, was performed using FITC-conjugated goat anti-rabbit IgG secondary Ab (Zymed Laboratories). Three-color flow analysis was performed using a FACSCalibur (BD Biosciences, San Jose, CA). The FACScan was calibrated using FITC-, PE-, and allophycocyanin-conjugated nonspecific IgG Abs.

Semiquantitative evaluation of lung histopathology

Pulmonary toxicity after SCT was determined by examination of lung histopathology in transplanted animals 28 days (bm1 recipients) after transplant (42) as previously described (32). H&E-stained lung sections from individual mice were coded without reference to mouse type or prior treatment regimen and independently examined by C.L. to establish an index of injury. Lung tissue was evaluated for the presence of periluminal infiltrates (around airways and vessels) or parenchymal pneumonitis (involving the alveoli or interstitium) using a semiquantitative scoring system as previously described that incorporates both the severity (periluminal infiltrates: 0, no infiltrates; 1, 1–3 diameter cells thick; 2, 4–10 diameter cells thick; 3, >10 diameter cells thick; pneumonitis: 0, no infiltrates; 1, increased cells, only visible at high magnification (×400); 2, easily seen cellular infiltrate or interstitial thickening; 3, consolidation by inflammatory cells and interstitial thickness) and extent (percentage of lung tissue involved: 1, <5%; 2, >5–25%; 3, >25–50%; 4, >50%) of histopathology (32).

Immune neutralization of Mig and IP-10

Polyclonal Abs against Mig and IP-10 were generated in multiple-site immunized New Zealand white rabbits using the peptides NH₂-CH₂-DDGDGVRMRRAIGK-COOH for IP-10 and NH₂-CTISRSTGHYKLKDQKL FAPS-COOH for Mig (Bio Synthesis, Lewisville, TX). The specificity and the neutralizing capacity of rabbit antiserum developed to these peptides has been previously demonstrated in other experimental systems (42).

Allo-SCT recipients were injected i.p. with 250 μl of anti-Mig or anti-IP-10 antiserum plus 250 μl of control rabbit serum (total volume, 500 μl), 250 μl each of both anti-Mig and anti-IP-10 antiserum (total volume, 500 μl), or with 500 μl of control rabbit serum on day 0, 2, 5, 11, 14, 17, 23, and 26 after transplant. Syngeneic SCT recipients received control serum only.

Splenocyte T cell expansion and serum cytokine analysis after SCT

Spleens were harvested 7 days after transplant and single cell suspensions were generated from individual animals. Splenocytes were subsequently counted and stained for CD45.1, CD4, and CD8. For cytokine analysis, animals were exsanguinated 7 days after SCT, and blood samples were collected in 1.5-ml Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 5000 rpm for 5 min. Serum supernatants were harvested for subsequent analysis for TNF-α and IFN-γ by ELISA as described below.

Cell culture, analysis of proliferative T cell response, and CTL function

All culture medium reagents were purchased from Invitrogen Life Technologies (Gaithersburg, MD). Cell cultures were performed at 37°C in a humidified incubator supplemented with 7.5% CO₂. T cell proliferation in response to alloantigen was measured by coculturing 2 × 10⁵ splenic T cells from B6 wild-type, B6 CXCR3⁻/⁻, and bm1 mice in flat-bottom 96-well, Falcon plates (BD Biosciences, Franklin Lakes, NJ) for 120 h in the presence of 5 × 10⁵ irradiated (20 Gy) naive bm1 CD11c⁺ cells. CD11c⁺ cells were isolated by magnetic bead separation using CD11c MicroBeads and the autoMACS system (Miltenyi Biotec) according to the manufacturer’s protocol, and normalized for numbers of CD11c⁺ cells by flow cytometry. After 96 h, supernatants were obtained and analyzed for IFN-γ as described below. Proliferative response was measured by using a 1205 Betaplate reader (PerkinElmer Wallac, Turku, Finland) after 120 h by incorporation of [³H]thymidine (1 μCi) for the last 12 h of incubation. In some experiments, B6 wild-type T cells and B6 CXCR3⁻/⁻ T cells were cocultured with/without splenic-derived dendritic cells from B6D2F1 mice and IFN-γ secretion and proliferative response were determined as above after 72 and 96 h, respectively.

CTL function of donor lymphocytes was assessed by using a previously published ⁵¹Cr release assay (43). Cytotoxic effectors were generated in vivo following the transplant protocols described above and splenic CTLs were isolated on day 7 after bone marrow transplantation (BMT). To assess alloantigen-specific CTL function, allogeneic B6 wild-type or B6 CXCR3⁻/⁻ donor cells were transplanted into lethally irradiated allogeneic
B6D2F1 recipients and the P815 (H-2b) and EL-4 cells (H-2d) were labeled with chromium and used as allogeneic and syngeneic targets respectively. Nonallospecific CTL activity was also assessed using a lectin-redirected \(^{51}\)Cr release assay as previously described (44). Briefly, effector cells were generated 7 days after transplantation of B6 wild-type and B6 CXCR3\(^+\) cells into allogeneic bm1 recipients, and both EL-4 or P815 were again used as target cells. In these CTL assays, Con A (5 \(\mu\)g/ml) was added to the donor lymphocyte/target cell culture and CTL activity against lecithin-bond target cells was measured (45).

**Measurement of cytokine and chemokine protein levels by ELISA**

Concentrations of specific cytokines and chemokines were measured in BAL fluid (BALF) and serum using ELISA kits for IFN-\(\gamma\) (BD Pharmingen), TNF-\(\alpha\) (R&D Systems, Minneapolis, MN for serum; BioSource International, Camarillo, CA for BALF samples), Mig (R&D Systems), IP-10 (R&D Systems), and I-TAC (R&D Systems). Assays were performed according to the manufacturer’s protocol. ELISA plates were read by microplate reader (Bio-Rad, Hercules, CA). Total protein in the BALF was determined by using the Bio-Rad Protein Assay (Bio-Rad).

**Statistical considerations**

All values are expressed as the mean \(\pm\) SEM. Statistical comparisons between groups were completed using the parametric independent sample \(t\) test if \(n > 5\) animals per group and using the Mann-Whitney \(U\) test if \(n < 5\) animals per group.

**Results**

**IFN-\(\gamma\)-producing CD8\(^+\) donor T cells are increased in the bronchoalveolar space of mice with lung injury after allo-SCT**

Lethally irradiated bm1 mice received SCT from either syngeneic (bm1) or allogeneic (B6 CD45.1\(^+\)) donors as described in Materials and Methods. In this model, donor and host differ at an isolated MHC class I mismatch, and allospecific target organ injury is mediated by CD8\(^+\) T cells. By 28 days after transplant, 10–15% of allo-SCT recipients died of acute GVHD, and all surviving mice showed significant signs of systemic GVHD. By contrast, all recipients of syngeneic SCT survived and were indistinguishable from naive controls in appearance (data not shown). At this time point, mice with GVHD developed significant lung histopathology, whereas the lungs of syngeneic recipients remained normal (Fig. 1a). Lung pathology was associated with significant increases in the number of total cells and of CD8\(^+\) but not CD4\(^+\) T cells in the BALF (Fig. 1, a and b). Similarly, when lymphocytes were obtained from whole lung digests, ~95% of T cells were CD8\(^+\) after allo-SCT, whereas only 40% of T cells obtained from syngeneic or naive animals stained positive for CD8 (data not shown).

Costaining of lymphocyte subsets for allelic differences in the common leukocyte Ag (CD45) revealed that >95% of T cells were of donor (CD45.1) and not of host (CD45.2) origin by day 7 after SCT. Next, we examined intracellular IFN-\(\gamma\)-expression of donor CD8\(^+\) cells in the BALF of transplanted animals. At day 7 and 28, approximately one-third of CD8\(^+\) T cells were positive for IFN-\(\gamma\), resulting in a significant increase in the absolute number of CD8\(^+\)IFN-\(\gamma\)-T cells at each time point after allo-SCT compared with syngeneic controls (Fig. 1, c and d).

**Increased BALF levels of the IFN-\(\gamma\)-inducible chemokines Mig and IP-10 are associated with the recruitment of CXCR3 expressing donor CD8\(^+\) T cells to the lung after allo-SCT**

As the term implies, the expression of the “IFN-\(\gamma\)-inducible” chemokines Mig and IP-10, and I-TAC is increased by IFN-\(\gamma\)-inducible cytokines. To evaluate whether the presence of IFN-\(\gamma\)-secreting CD8\(^+\) effectors cells in the lungs of mice with IPS was associated with enhanced chemokine production in the lung after allo-SCT, we measured BALF levels of Mig, IP-10, and I-TAC at 1 and 4 wk after SCT. At each time point, levels of both Mig and IP-10 were significantly elevated in allo-SCT recipients compared with syngeneic controls (Fig. 2, a and b), and levels of IP-10 directly correlated with increasing numbers of CD8\(^+\) effector cells (Fig. 1d). By contrast, I-TAC levels did not differ between syngeneic and allogeneic recipients 7 days after transplant (Fig. 2c) and were comparable to levels measured in naive animals (data not shown). Surprisingly, I-TAC levels in allogeneic recipients decreased at day 28, whereas levels in syngeneic SCT recipients remained unchanged (Fig. 2c). Interactions between chemokines and their corresponding receptors are involved in leukocyte trafficking to sites of inflammation and CXCR3 has been identified as the primary

![FIGURE 1.](http://www.jimmunol.org/) Allo-SCT results in recruitment of IFN-\(\gamma\)-CD8\(^+\) T cells into the bronchoalveolar space. Lethally irradiated bm1 mice received SCT from either syngeneic (bm1, □) or allogeneic (B6, ■) donors as described in Materials and Methods. Animals were analyzed by day 28 for lung histology and BALF cellularity (a), as well as BALF T cell subsets (b). c and d. Next, numbers of IFN-\(\gamma\)-CD8\(^+\) T cells were determined 7 and 28 days after transplant by flow cytometry. Data are presented as mean \(\pm\) SEM and are from one of two comparable experiments; \(n = 3\)–6 per group; *, \(p < 0.05\), ■ vs □.
receptor for both Mig and IP-10 (46). Therefore, we used flow cytometry to determine whether CXCR3 was detectable on the CD8⁺ donor T cells recruited to the lungs of mice with IPS. As shown in Fig. 2, d and e, CXCR3 was strongly expressed on nearly all donor CD8⁺ T cells present in the BALF as early as day 7 after allo-SCT.

In vivo neutralization of the CXCR3-ligands Mig and IP-10 reduces IPS severity after allo-SCT but has no effect on systemic T cell expansion or activation

We hypothesized that elevated pulmonary levels of Mig and IP-10 were responsible for the migration of CXCR3⁺ donor effector T cells to the lung after allo-SCT. To test this hypothesis, we first determined the effects of in vivo neutralization of Mig and IP-10 on the development of IPS. bm1 mice received syngeneic or allo-SCT as described above. Allo-SCT recipients were injected i.p. from day 0 to 26 with polyclonal Abs to Mig and IP-10 (500 μl total) or control serum as described in Materials and Methods. We chose this schedule to: 1) neutralize Mig and IP-10 before both the increased expression of each chemokine and the influx of donor T cells to the lung after SCT, and 2) maintain this blockade through the development of fulminant IPS. Syngeneic SCT recipients were treated with control serum only at the same volume and schedule. All groups were analyzed by day 28 for lung histopathology and BALF cellularity. Administration of control serum had no effect on lung histopathology in syngeneic animals, nor were clinical symptoms of a xenogenic, rabbit anti-mouse response (serum sickness) observed. As expected, control-treated allo-SCT recipients developed significant lung injury, whereas immunoneutralization of Mig and IP-10 resulted in a 60% reduction in lung histopathology (Fig. 3, a and b), along with significant decreases in total BALF cellularity (Fig. 3c), the number of BALF CD8⁺ T cells (Fig. 3d) and BALF total protein levels (Fig. 3e), a hallmark for lung injury and increased pulmonary vascular permeability in several models of lung inflammation (47–51). Previous work has shown that allo-reactive donor T cells, monocytes, and macrophages are significant producers of TNF-α during the development of IPS (35). Therefore, we measured TNF-α levels in the BALF and found that the decrease in lung injury was also associated with a significant reduction in TNF-α levels (Fig. 3f).

We hypothesized that the reduction of pulmonary injury seen after the administration of Mig and IP-10 Abs was secondary to impaired donor T cell migration to this target tissue rather than to alterations in allospecific T cell proliferation and activation or to the depletion of these alloreactive cells. To test this hypothesis, we measured splenic donor T cell expansion and serum levels of IFN-γ early after SCT. Systemic neutralization of Mig and IP-10 had no effect on the expansion of donor CD8⁺ T cells in the spleen by day 7 after SCT, which was significantly increased in both allogeneic groups compared with syngeneic controls (Fig. 4a). As observed in the lung, significant expansion of CD4⁺ T cells was not observed after allo-SCT (Fig. 4a), again demonstrating that the allo-immune response in this murine model is driven by CD8⁺ T cells. Once activated, donor T cells secrete IFN-γ and TNF-α, which can contribute to target tissue injury after allo-SCT (35, 52, 53). As shown in Fig. 4b, no differences in serum cytokine concentrations were observed after allo-SCT, and levels in both allogeneic groups were significantly increased compared with syngeneic controls. Taken together, these findings demonstrate that neutralization of the CXCR3-ligands Mig and IP-10 had no effect on allospecific T cell expansion in vivo, nor on the systemic inflammation engendered by T cell activation.

The contribution of Mig and IP-10 to T cell recruitment has been shown to vary depending upon the disease and experimental model studied (42, 54–56). Therefore, we next determined whether neutralizing Mig or IP-10 individually would significantly reduce lung injury after allo-SCT and whether a predominant role could be ascribed to either chemokine in this context. Allo-SCT recipients were then treated with polyclonal Abs to Mig or IP-10 alone in the dose and schedule described above. As before, groups of syngeneic and allogeneic mice treated with control serum were also included. As shown in Table I, the neutralization of either Mig or IP-10 alone reduced the severity of IPS. Moreover, the reduction of lung injury after either strategy was equivalent but less
CXCR3:Mig/IP-10 INTERACTIONS IN IDIOPATHIC PNEUMONIA SYNDROME

FIGURE 3. In vivo neutralization of Mig and IP-10 after allo-SCT results in significantly reduced IPS severity. Lethally irradiated bm1 mice received BMT from either syngeneic (bm1) or allogeneic wild-type B6 donors as described in Fig. 1. Syngeneic recipients and a subset of allogeneic recipients were treated with preimmune control serum, whereas a second group of allogeneic recipients was treated with polyclonal Abs against murine Mig and IP-10 as described in Materials and Methods. Animals were analyzed on day 28 for lung histopathology (a and b) (H&E, 200×), BALF cellularity (c and d), BALF total protein levels (e), and BALF TNF-α levels (f). Data in a and b are presented as mean ± SEM and are from three combined experiments; n = 8–15 per group; *, p < 0.01, □ vs ■. Data in c, d, and f are presented as mean ± SEM and are from one of three comparable experiments; n = 3–5 per group; *, p < 0.05, □ vs ■. Data in e are presented as mean ± SEM and are from two combined experiments; n = 4–8 per group; *, p < 0.01, □ vs ■.

FIGURE 4. In vivo neutralization of Mig and IP-10 after allo-SCT does not alter in vivo T cell expansion. Lethally irradiated bm1 mice were transplanted as described in Fig. 3 (syngeneic + control serum (□), allogeneic + control serum (■), and allogeneic + Mig/IP-10 antiserum (□)). T cell expansion (a) and serum cytokine levels (b) for IFN-γ and TNF-α were measured 7 days after BMT. Data are presented as mean ± SEM and are from one of two comparable experiments; n = 3–5 per group; +, p < 0.05, □ vs □ and ■.

effective than when both chemokines were blocked simultaneously (~60%; see Fig. 3).

Allo-SCT with CXCR3-deficient donor leukocytes reduces the severity of IPS

CXCR3 is the primary receptor for Mig and IP-10, and as shown in Fig. 2, is expressed on nearly all donor-derived CD8− cells infiltrating the lung after allo-SCT. Therefore, we next examined whether the deficiency of CXCR3 on donor leukocytes would also impair T cell migration into the lung during the development of IPS. Lethally irradiated bm1 mice received SCT from either allogeneic (B6 wild-type or B6 CXCR3−/−) or syngeneic (bm1) donors as described in Materials and Methods. Animals were monitored until day 28 at which point the extent of lung injury was examined in surviving animals. As shown in Fig. 5, CXCR3−/− SCT resulted in a significant reduction in the severity of IPS as measured by lung pathology (Fig. 5a), total BALF cellularity (Fig. 5b), numbers of BALF CD8+ T lymphocytes, serum IFN-γ levels, and lymphocyte cytotoxic function following the transplantation of bm1 mice with cells from either CXCR3−/− or CXCR3+/- donors. Consistent with our previous work using a similar SCT model (22), the absolute numbers of donor CD8+ T cells in the spleen were significantly increased on day 7 following CXCR3−/− SCT compared with wild-type controls, whereas serum levels of IFN-γ measured at this point did not differ between allogeneic groups (Fig. 6, c and d). Next, we assessed lectin-directed CTL activity of donor T cells by using a redirected CTL assay described in Materials and Methods. Splenic

CXCR3-deficient donor T cells exhibit enhanced expansion in secondary lymphoid tissue and normal cytolytic function after allo-SCT, despite decreased allospecific proliferation in vitro

The reduction in lung injury seen after allogeneic CXCR3−/− SCT could have also been secondary to alterations in T cell function, migration, or both. Although we hypothesized that the reduction in IPS severity was secondary to a defect in the recruitment of CXCR3−/− donor cells to the lung, previous reports have demonstrated that in vitro mixed lymphocyte responses to both MHC and minor histocompatibility complex differences are diminished when T cells are either cocultured with anti-CXCR3 mAb or are themselves deficient in CXCR3 expression (39, 57). To assess the possible contribution of altered T cell function to decreased lung inflammation in our model, we first measured the responses of naive B6 wild-type and B6 CXCR3−/− donor T cells to bm1 stimulators in vitro and found that allospecific proliferation and IFN-γ secretion were significantly decreased in CXCR3-deficient T cells (Fig. 6, a and b). Similar results were obtained when B6D2F1 stimulators were used (data not shown).

To determine whether the differences observed in vitro were relevant in our in vivo system, we measured splenic T cell expansion, serum IFN-γ levels, and lymphocyte cytotoxic function following the transplantation of bm1 mice with cells from either CXCR3−/− or CXCR3+/- donors. Consistent with our previous work using a similar SCT model (22), the absolute numbers of donor CD8+ T cells in the spleen were significantly increased on day 7 following CXCR3−/− SCT compared with wild-type controls, whereas serum levels of IFN-γ measured at this point did not differ between allogeneic groups (Fig. 6, c and d). Next, we assessed lectin-directed CTL activity of donor T cells by using a redirected CTL assay described in Materials and Methods. Splenic
T cells were harvested from bm1 SCT recipients on day 7 and cocultured with either EL-4 or P815 target cells in the presence of Con A. CTL function was comparable between groups when using either EL-4 target cells (Fig. 6e) or P-815 targets (data not shown). Equivalent results were also obtained when effectors were generated in vivo after transplanting B6 wild-type or CXCR3−/− cells into C3.5W/Sn5 recipient mice (data not shown). Alloantigen-dependent CTL activity was assessed using a third established SCT system; B6D2F1 mice received SCT from either allogeneic B6 wild-type or B6 CXCR3−/− donors, and splenic T cells isolated on day 815 were cocultured with either syngeneic EL-4 or allogeneic P-815 target cells without the addition of Con A. As shown in Fig. 6f, no differences in allospecific CTL function was observed between CXCR3-deficient and wild-type cells. These results demonstrate that the impaired ability of CXCR3−/− T cells to respond to alloantigens in vitro is overcome in the in vivo setting: CXCR3−/− T cells are capable of responding to host Ag with the same vigor as wild-type T cells after SCT.

The recruitment of CD8+ T cells to the lung is predominantly mediated by CXCR3 and not CCR5

The incomplete abrogation of IPS observed after either the in vivo neutralization of Mig and IP-10 or the deficiency of CXCR3 on donor T cells suggested that other chemokine receptor:ligand interactions may be operative in this setting. Specifically, CCR5 and its principal ligands, RANTES (CCL5) and MIP-1α (CCL3), can also contribute to effector T cell recruitment to allogeneic target organs (58–60), and the expression of each is increased in the lung after allo-SCT (35, 60). In a final set of experiments, we tested whether the combined blockade of CCR5 and CXCR3 receptor:ligand interactions would prove more effective in reducing donor T cell migration to the lung than targeting CXCR3 alone. B6 mice received allo-SCT from allogeneic B6 wild-type or B6 CCR5−/− donors, and were subsequently treated with either polyclonal Abs to both Mig and IP-10 or with control serum as described in Materials and Methods. Syngeneic SCT recipients treated with control serum were again included, and the development of lung injury in each group was assessed 28 days after SCT. As expected, recipients of allo-SCT from wild-type donors that received anti-Mig and IP-10 polyclonal Abs showed a significant reduction of lung pathology, BALF cellularity, and numbers of BALF CD8+ T cells (Fig. 7). Neutralization of Mig and IP-10 also resulted in a reduction in IPS severity in recipients of allogeneic donor cells, but the decrease in lung pathology and T cell recruitment was less than that seen in recipients of allo-SCT from wild-type donors (Fig. 7). By contrast, SCT with allogeneic CCR5−/− donor cells followed by the administration of control serum had no effect on lung injury. Collectively, these results demonstrate that donor CD8+ T cell recruitment to the lung is primarily dependent on CXCR3 receptor:ligand interactions and does not involve CCR5.

Discussion

IPS remains a frequently fatal complication following allo-SCT. Risk factors consistently include conditioning with total body irradiation, acute GVHD, and advanced recipient age (3, 4, 7, 62–65). Experimental and clinical studies suggest that synergy between the intensity of SCT conditioning regimens and alloreactive T cell responses may significantly contribute to the deleterious outcome of IPS after allo-SCT (9, 10, 66). The pathophysiology of IPS is complex, and both animal and human data support the involvement of inflammatory cytokines and cellular effectors in the evolution of this process (11–19). In particular, alloreactive Th1/ Tc1 lymphocytes have been shown to be critical to the early inflammatory events that contribute to pulmonary injury after allo-SCT (11–13, 35). However, the mechanisms that govern lymphocyte infiltration into the lung remain poorly understood. The recruitment of cellular effectors to sites of inflammation is

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**Table 1. In vivo neutralization of Mig or IP-10 reduces lung injury after allo-SCT**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pathology</th>
<th>Percent Reduction</th>
<th>BALF Cellularity</th>
<th>BALF CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syn + control</td>
<td>14</td>
<td>1.0 ± 0.6</td>
<td>NA</td>
<td>0.76 ± 0.14</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Allo + control</td>
<td>11</td>
<td>5.1 ± 0.5</td>
<td>NA</td>
<td>1.73 ± 0.14</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Allo + anti-Mig</td>
<td>11</td>
<td>3.2 ± 0.4**</td>
<td>39 ± 6.0</td>
<td>1.14 ± 0.17*</td>
<td>0.13 ± 0.02**</td>
</tr>
<tr>
<td>Allo + anti-IP-10</td>
<td>10</td>
<td>3.1 ± 0.4**</td>
<td>38 ± 6.3</td>
<td>1.45 ± 0.32</td>
<td>0.17 ± 0.04**</td>
</tr>
</tbody>
</table>

* Lethally irradiated bm1 mice received BMT from either syngeneic or allogeneic wild-type B6 donors as described in Fig. 1. Syngeneic recipients and a subset of allogeneic recipients were treated with polyclonal Abs to either murine Mig or IP-10 as described in Materials and Methods. Animals were analyzed on day 28 for lung histopathology and BALF cellularity. Data are presented as mean ± SEM and are from two combined experiments; n = 4–11 per group.

Percentage of reduction of injury was calculated as 100 – (l lung injury seen in polyclonal Ab-treated animals/lung injury seen in control – treated animals), and averaged from two independent experiments, NA. Not applicable.

**p < 0.01, and *p < 0.05, allogeneic + anti-Mig or allogeneic + anti-IP-10 vs allogeneic + control.**
strongly dependent upon the establishment of chemokine gradients in the inflamed tissue, and interactions between CXCR3 and CCR5 and their respective ligands have been shown to specifically contribute to the migration of Th1/Th1 lymphocytes (24, 58, 59, 67–70). Using an established murine SCT model, we tested the hypothesis that interactions between CXCR3 (expressed on activated donor T cells) and its ligands, Mig and IP-10, play an important role in lung injury seen after allo-SCT. The model used represents a fully ablative T cell expansion (2) and serum cytokine levels for IFN-γ/H9253 (2) and serum cytokine levels for IFN-γ/H11006 (2) are presented as meanSEM; data are from four wells per group and represent one of two comparable experiments; *p < 0.05, B6 wild-type (●) vs CXCR3+/−(○). c and d, Lethally irradiated bm1 mice received BMT from either syngeneic (bm1, □), allogeneic wild-type B6 (●), or allogeneic CXCR3+/−(○) donors as described in Materials and Methods. T cell expansion (c) and serum cytokine levels for IFN-γ (d) were measured 7 days after transplant. Data are presented as mean±SEM; n = 6 per group; *p < 0.05, □ vs ●. e, Alloantigen-independent, lectin-rediected (■). B6wt → EL-4 + Con A; ●, B6 CXCR3+/− → EL-4 + Con A; ▲, B6wt → EL-4. ●, B6 CXCR3+/− → EL-4) CTL activity was assessed as described in Materials and Methods. f, Alloantigen-dependent (■). B6wt → P815; ●, B6 CXCR3+/− → P815; ▲, B6wt → EL-4; ●, B6 CXCR3+/− → EL-4) CTL activity was assessed as described in Materials and Methods. Data are presented as mean±SE; effector T cells were pooled from six mice per group.

FIGURE 6. Allospecific T cell responses and CTL function of CXCR3-deficient cells. a and b, In vitro proliferation and IFN-γ proliferation was assessed in mixed lymphocyte reactions using bm1 stimulator cells. Data are presented as mean±SEM; data are from four wells per group and represent one of two comparable experiments; *p < 0.05, B6 wild-type (●) vs CXCR3+/−(○).

FIGURE 7. Donor CD8+ T cell recruitment to the lung is mediated by CXCR3 but not CCR5. Lethally irradiated bm1 mice received BMT from either syngeneic bm1 (●), allogeneic wild-type B6 (■), or allogeneic B6 CCR5−/− (horizontally and vertically lined columns) donors. One subset of recipients after B6 wild-type (●) or B6 CCR5−/− allo-SCT (horizontally lined columns) was treated with rabbit-antiserum to Mig and IP-10, and the other subset of recipients after B6 wild-type (●) or B6 CCR5−/− allo-SCT (vertically lined columns) was treated with control serum as described in Materials and Methods. Animals were analyzed on day 28 for lung histopathology scores (a) and BALF cellularity (b and c). Data are presented as mean±SEM and are from one experiment; n = 6–8 per allogeneic groups; n = 4 in syngeneic controls; *p < 0.05, horizontally lined columns vs ●, +, p < 0.05, ○ vs horizontally lined columns; #, p = 0.08, vertically lined columns vs horizontally lined columns.

Clinical and preclinical studies suggest that CXCR3 and its ligands significantly contribute to the cellular infiltration that results in heart, lung, and skin allograft rejection (39, 42, 54–56, 71–73). However, the functional relevance of each of the three CXCR3 ligands, Mig, IP-10, and I-TAC, is dependent upon the disease studied and the experimental models used (31, 54, 74–77). For example, Mig and IP-10, rather than I-TAC, seem to be involved in the development of bronchiolitis obliterans syndrome after lung transplantation (54) and acute cardiac allograft rejection (31, 75). Intragraft expression of CXCR3 and IP-10 has been shown to directly correlate with episodes of heart and lung rejection in human tissue (55, 56), whereas Mig has been identified as the dominant factor directing CXCR3+ T cells into both cardiac and tracheal allografts in murine models (42, 72). We found that in contrast to the significant increase of Mig and IP-10 in the BALF after allo-SCT, BALF levels of the third ligand, I-TAC, did not differ between syngeneic and allogeneic recipients at 1 wk after transplant. In fact, I-TAC levels were decreased in the BALF 4 wk after allo-SCT when fulminant lung injury is evident. Based upon this information, we focused our immunoneutralization studies on Mig and IP-10. Our group has recently shown that neutralizing Mig and IP-10 alone or in combination or by using CXCR3-deficient SCT donors significantly reduces the severity of IPS.

In addition to reducing donor T cell recruitment and lung histopathology, abrogation of CXCR3 receptor:ligand interactions by both strategies used resulted in a significant decrease in BALF TNF-α levels. Alloreactive T cells are significant producers of TNF-α in the lung after allo-SCT, and our group has recently shown that neutralizing Mig and IP-10 alone or in combination or by using CXCR3-deficient SCT donors significantly reduces the severity of IPS.

Materials and Methods
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shown that allo-SCT with TNF-α-deficient T cells results in a significant reduction of BALF TNF-α levels and IPS severity (35). Specifically, we found that donor T cell-derived TNF-α directly induces the expression of the inflammatory chemokines Mig, MCP-1, RANTES, and MIP-1α in the lung (35) and regulates the subsequent recruitment of additional T cells along with monocytes and macrophages. Thus, the observed reduction in the number of donor T cells recruited to the lung in the current experiments can be directly linked to alterations in BALF TNF-α levels. Collectively, these data begin to establish a conceptual framework for understanding how alloreactive lymphocytes can directly modulate the soluble and cellular inflammatory microenvironment in the lung that contributes to the development of IPS.

Although the disruption of CXCR3:Mig/IP-10 receptor-ligand interactions significantly reduced the severity of IPS, lung histopathology was not completely abrogated by either the administration of anti-Mig or anti-IP-10 Abs, nor by using CXCR3-deficient SCT donors. The presence of residual lung histopathology after Ab neutralization may be explained by a combination of factors. Firstly, incomplete neutralization could be attributed to the development of a xenogenic (mouse anti-rabbit) protein response or to inadequate tissue penetration. The former is unlikely in light of the profound immunosuppression observed after allo-SCT where lymphocyte responses during GVHD may not return to normal for several months (78), and by the absence of evidence for serum sickness or increased lung pathology after syngeneic SCT, whereas the latter is possible but would be difficult to assess in a quantitative fashion. In either case, such as these confounding issues would be overcome by the use of CXCR3-deficient mice as SCT donors. Remaining lung histopathology may also be explained by the redundancy that exists in chemokine biology. We were specifically interested in the potential role of CCR5 in our model because this receptor is coexpressed on activated Tc1/Th1 lymphocytes, and interactions between CCR5 and its principal ligands, RANTES and MIP-1α, can contribute to effector T cell recruitment to allogeneic target organs in other systems (58–60). However, our data show that the absence of CCR5 on donor cells had no effect on lung injury after allo-SCT, and unexpectedly, neutralization of Mig and IP-10 was less effective in reducing IPS in recipients of CCR5−/− SCT. Collectively, these data underscore the role of CXCR3 in this IPS model and suggest that the absence of CCR5 may enhance, perhaps by a compensatory mechanism, the use of CXCR3 for lymphocyte recruitment.

The significant decrease in lung injury observed in this study after disrupting CXCR3 receptor-ligand interactions and the reduction of experimental allograft rejection observed in a similar context has been predominantly attributed to reduced effector cell recruitment rather than to specific alterations in T cell cellularity (42, 54, 56, 72, 73). However, significant decreases in allospecific proliferation have been observed in vitro for both CXCR3−/− T cells and for T cells treated with anti-CXCR3 Abs, and attributed to alterations in CD25 (IL-2Rα) induction (39, 57). Consistent with these studies, we found that the capacity for donor-type CXCR3−/− T cells to proliferate and secrete IFN-γ in response to a class I MHC difference in host-type stimulator cells in vitro was significantly decreased as well. However, T lymphocyte cytolytic function and the systemic levels of IFN-γ were comparable after allo-SCT with wild-type or CXCR3−/− cells, whereas the expansion of CXCR3−/− T cells in secondary lymphoid tissue was actually enhanced. Similarly, no differences in T cell expansion or systemic cytokine levels were noted when anti-Mig and IP-10 Abs were administered after allo-SCT (Fig. 4). Therefore, these findings demonstrate that reductions in allospecific proliferation observed in vitro are overcome after allo-SCT, perhaps secondary to the proinflammatory environment that characterizes the early post–CT period, and are conducive to T cell activation and the up-regulation of CD25 (79–82). Moreover, these data support the hypothesis that the reduction in IPS severity demonstrated herein was secondary to impaired leukocyte migration to the lung and not to alterations in T cell cellularity.

In summary, our data reveal a significant role for Mig and IP-10 in the recruitment of CXCR3+ donor T cells to the lung after allo-SCT. The reduction in IPS seen after allo-SCT with CXCR3−/− donor mice or in allogeneic wild-type recipients treated with neutralizing Abs against Mig and IP-10 was dependent upon decreased infiltration of donor effector cells in the lung and occurred in the absence of additional immunsuppression. Recently, a synergistic beneficial effect was observed when neutralizing Abs to Mig were administered along with cyclosporin A to prevent lung allograft rejection in mice (72). A similar effect was also seen during cardiac allograft rejection, when cyclosporine treatment was combined with the use of blocking Abs against CXCR3 (39). Because IPS regularly develops during systemic immunsuppression and is poorly responsive to additional immunsuppressive therapy, it is conceivable that strategies that inhibit CXCR3 receptor:ligand interactions may serve as effective non-cross-reactive adjuncts to standard therapy intended to prevent or treat IPS and GVHD-related injury to other target organs after allo-SCT.

References


