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IFN-γ Receptor-Deficient Donor T Cells Mediate Protection from Graft-versus-Host Disease and Preserve Graft-versus-Tumor Responses after Allogeneic Bone Marrow Transplantation

Kai Sun,† Hui-Hua Hsiao,*,†,† K Minghui Li,‡,§ Erik Ames,*, Myriam Bouchlaka,‡,§ Lisbeth A. Welniak,*, Takeshi Hagino,*, Jared Jagdeo,*,§ Chien-Chun Pai,* Mingyi Chen,§ Bruce R. Blazar,‡,*** Mehrdad Abedi,††,† and William J. Murphy*,††,‡

Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation. It has been previously reported that lung GVHD severity directly correlates with the expansion of donor Th17 cells in the absence of IFN-γ. However, the consequence of Th17-associated lung GVHD in the presence of IFN-γ has not been well characterized. In the current study, T cells from IFN-γ receptor knockout (IFN-γR−/−) mice, capable of producing IFN-γ but unable to signal in response to IFN-γ, have been used to elucidate further the role of IFN-γ in GVHD. We found the transfer of donor T cells from either IFN-γR−/− or IFN-γR−/− knockout (IFN-γR−/−) mice resulted in significant increases in donor Th17 cells in the lung. Marked increases in IL-4-producing Th2 cells infiltrating the lungs were also observed in the mice of donor IFN-γR−/− T cells. Notably, despite the presence of these cells, these mice did not show the severe immune-mediated histopathological lung injury observed in mice receiving donor IFN-γ−/− T cells. Increases in lung GVHD did occur in mice with donor IFN-γR−/− T cells when treated in vivo with anti-IFN-γ demonstrating that the cytokine has a protective role on host tissues in GVHD. A survival benefit from acute GVHD was also observed using donor cells from IFN-γR−/− T cells compared with control donors. Importantly, tumor-bearing mice receiving IFN-γR−/− T cells versus wild-type donor T cells displayed similar graft-versus-tumor (GVT) effects. These results demonstrate the critical role of IFN-γ on host tissues and cell effector functions in GVHD/GVT. The Journal of Immunology, 2012, 189: 000–000.

H uman and mouse CD4+ T cells that produce IL-17 upon stimulation are defined as Th17 cells (1–3) and are highly proinflammatory effector T cells. There has been recent interest in the nature of Th17 cells in inflammatory diseases, including the regulation of the expansion of pathogenic Th17 cells by T cell-derived and other immune cell-derived cytokines (1, 2, 4). TGF-β and IL-6 are important for the development of Th17 cells, and IL-21 and IL-23 are critical for the stabilization and expansion of this population (1, 2, 4, 5). There are a number of other cytokines implicated in the promotion and stabilization and expansion of this population (1, 2, 4, 5). Notably, despite the presence of these cells, these mice did not show the severe immune-mediated histopathological lung injury observed in mice receiving donor IFN-γ−/− T cells. Increases in lung GVHD did occur in mice with donor IFN-γR−/− T cells when treated in vivo with anti-IFN-γ demonstrating that the cytokine has a protective role on host tissues in GVHD. A survival benefit from acute GVHD was also observed using donor cells from IFN-γR−/− T cells compared with control donors. Importantly, tumor-bearing mice receiving IFN-γR−/− T cells versus wild-type donor T cells displayed similar graft-versus-tumor (GVT) effects. These results demonstrate the critical role of IFN-γ on host tissues and cell effector functions in GVHD/GVT.

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Abbreviations used in this article: BMC, bone marrow cell; BMT, bone marrow transplantation; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; TCD, T cell-depleted; WT, wild-type.
increased level of Th17 frequency is comparable to those with the transfer of T cells from donor IFN-γ−/− mice. However, these mice did not show the immune-mediated histopathological lung injury seen in the mice with donor IFN-γ−/− T cells. The increase in lung GVHD susceptibility occurred in mice with donor IFN-γ−/− T cells in vivo treatment with neutralizing Abs to IFN-γ, directly demonstrating the protective effect of donor-derived IFN-γ on host GVHD target tissues. Finally, use of IFN-γ−/− donor T cells, compared with wild-type or IFN-γ−/− T cells, resulted in an increased survival benefit due to protection from GVHD while graft-versus-tumor (GVT) responses in tumor-bearing mice were preserved. These results indicate that donor T cells play a predominant role in the control of Th17-associated GVHD/GVT in murine allogeneic BMT through the production of IFN-γ.

Materials and Methods

Animals

Female BALB/c (BALB/c, H2b) and C57BL/6 (B6, H2b) mice were purchased from Taconic Laboratories. Wild-type (WT) C57BL/6J (B6, H-2b), IFN-γ−/− (B6, H-2b), and IFN-γ−/− (B6, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved according to the guidelines of the animal care and use committee. Mice were between 8 and 13 wk of age at the start of the experiments.

Cell preparations

Bone marrow cells (BMCs) were collected from the backbones, femurs, and tibiae. Bone marrow and spleen cell suspensions were prepared by gently crushing the tissues to release the cells. Cell preparations were filtered to remove particulates and washed prior to use. For lung leukocyte isolation, the lungs from each mouse were excised, washed in RPMI 1640, minced with scissors, and digested enzymatically at 37°C for 45 min in 10 ml of digestion buffer per lung [PBS, 1 mg/ml bovine albumin (Sigma), 1 mg/ml collagenase type 4 (Worthington, Lakewood, NJ)]. The cell suspension and tissue fragments were further dispersed by repeated aspiration through the bore of a 10-ml syringe for 30 s. Cells were resuspended and filtered through a sterile 100-μm nylon screen (Nitek, Kansas City, MO). The filtrate was centrifuged for 30 min in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Leukocyte pellets were resuspended in 1 ml 5% FBS media, and total lung leukocyte numbers were enumerated on a Coulter Z1 cell counter (Beckman Coulter, Fullerton, CA).

In vivo studies

BALB/c (H2b) mice were used as recipients.Recipient mice received total body irradiation (400 cGy×2 split dose, total 800 cGy) from a 137Cs source. Irradiation was followed by the infusion of 5×10^5 to 10×10^6 allogeneic donor C57BL/6J (B6, H2b) T cell-depleted (TCD)-BMCs intravenously with or without 6×10^5 to 10×10^6 purified T cells or 3×10^5 splenocytes (as a source of allogeneic T cells) from WT C57BL/6J. IFN-γ−/−, or IFN-γ−/− mice. All moribund mice were humanely killed. All experiments were performed at least three times with at least five mice per group. For the in vivo neutralization of IFN-γ, anti-IFN-γ (R4-6A2) was given at 0.5 mg i.p. on days 0, 1, 8, and 10 after BMT. For tumor studies, BALB/c (H2b) mice received 3×10^5 A20 T cell lymphoma cells 6 d before irradiation and infusion of B6 (H2b) allogeneic donor cells. Necropsy was performed on euthanized mice to confirm tumor pathology. To evaluate the tumor burden and GVHD responses, we used tumor colony outgrowth assays. Cells extracted from the lung, liver, or bone marrow of recipient mice 34 d after i.v. infusion of A20 tumor cells were cultured in colony assay media (IMDM containing 15% FBS, 100 U/ml penicillin/streptomycin, 5×10^-5 M 2-mercaptoethanol, and 2.75% methylcellulose) with 1.05×10^5 cells per Petri dish (in triplicate). Tumor colonies were counted after 10 d of culture under a stereomicroscope.

In vitro T cell activation and polarization of IL-17–producing cells

Unfractionated spleen cells (2×10^5/well) or purified CD4+ cells (5×10^5/well) from WT (IFN-γ−/− and IFN-γ−/−), IFN-γ−/−, and IFN-γ−/− mice were cultured in 96-well plates for 3–5 d with 5 μg/ml each of anti-CD3 (14-2511), anti-CD28 (37.511; Invitrogen, Carlsbad, CA), and with 20 ng/ml recombinant mouse IL-6 (Peprotech) and 5 ng/ml recombinant human TGF-β (Peprotech, Rocky Hill, NJ) and then analyzed for intracellular cytokine expression.

Intracellular cytokine analysis

Leukocytes isolated from lungs and spleen cells of mice from in vivo experiments or in vitro polarized cells were restimulated with 160 ng/ml PMA, 1600 ng/ml ionomycin, and GolgiStop (BD Pharmingen, San Diego, CA) for 4 h. Cells were surface labeled with FITC anti-CD4 and PE cyanine 5 (PC5) anti-CD8 followed by fixation and permeabilization with IntraPrep (Beckman Coulter). Cells were stained intracellularly with PE-anti-IL-17 (clone TC11–18H10), PE–TNF-α (clone MP6-XT22), PE–IFN-γ (clone XMG1.2), or isotype PE–labeled rat IgG1 (BD Pharmingen). Labeled cells were acquired on a three-color FACScan flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA). All data sets were analyzed using FlowJo software (Tree Star, Ashland, OR).

Semitquantitative evaluation analyses of lung GVHD severity by histopathological examination of lung tissues

Paraffin-embedded lung sections (5 mm) were stained with H&E for histologic examination. Sections from individual mice were graded on a 0–4 scale by a board-certified pathologist in a blinded fashion to ensure unbiased observations. Lung pathology grading was based on a standard nomenclature for lung graft rejection (13) to establish the following index of injury: grade 0, normal lung tissue; grade 1, mild, 1- to 3-cell-diameter periluminal infiltrates (around airways and vessels) with 5–25% of the lung tissue involved; grade 2, moderate, 4- to 10-cell-diameter periluminal infiltrates with 25–50% of the lung tissue involved; grade 3, severe generalized (50% lung tissue) inflammation, including giant cell granulation tissue formation; grade 4, severe damage similar to grade 3 with more diffuse perivascular, interstitial, and air space infiltrates and prominent parenchymal alveolar pneumocyte damage usually associated with intraalveolar necrotic cells, macrophages, hyaline membranes, hemorrhage, and neutrophils. Images were acquired with an Olympus BX45 clinical microscope and a DP72 digital camera.

Statistics

One-way ANOVA or Student t test was performed to determine if the mean values were significantly different (p < 0.05) when appropriate.

Results

CD4+ T cells lacking the receptor for IFN-γ display comparable increased Th17 frequency to CD4+ T cells lacking IFN-γ after in vitro polarization

IFN-γ has been regarded as an important negative regulator for the development of Th17 cells (3). Although CD4+ T cells lacking IFN-γ have been shown to elevate the frequency of Th17 cells in vitro and in vivo (14–17), the potential role of T cells lacking the receptor for IFN-γ in the development of Th17 cells has not been well investigated. Therefore, we first stimulated T cells or unfractionated spleen cells from IFN-γ−/− or IFN-γ−/− mice in Ag-independent Th17 polarizing conditions. CD4+ T cells from WT, IFN-γ−/−, or IFN-γ−/− mice were stimulated in vitro with anti-CD3, anti-CD28, IL-6, and TGF-β for 3–5 d. Intracellular IL-17 production was determined by restimulating the cells with PMA and ionomycin. As expected, the lack of IFN-γ in CD4+ T cells (IFN-γ−/− T cells) resulted in a much greater frequency of Th17-producing cells (Fig. 1) compared with WT CD4+ T cells. The higher Th17 frequency within CD4+ T cells lacking IFN-γ receptor expression (IFN-γ−/− T cells) was comparable to the Th17 frequency of CD4+ T cells lacking IFN-γ, indicating the development of Th17 cells can also be downregulated by targeting IFN-γ receptor signaling. Moreover, an equivalent frequency of TNF-α+CD4+ T cells can be seen in CD4+ T cells from WT, IFN-γ−/−, and IFN-γ−/− mice, indicating the absence of IFN-γ or lack of IFN-γ responsiveness does not affect CD4+ T cell-derived TNF-α expression.

CD8+ T cells lacking the receptor for IFN-γ show comparable Te17 frequency to CD8+ T cells lacking IFN-γ after in vitro polarization

Although there has been great interest in the induction of IL-17 in CD4+ T cells, IL-17 production in CD8+ T cells has also been
Transfer of donor IFN-γ−/− and IFN-γR−/− CD4+ T cells for the development of IL-17–producing Th17/Tc17 cells.

Recent studies have demonstrated that transplantation of IFN-γ−/− donor T cells led to marked expansion of donor Th17 cells in the lungs, resulting in increased lung GVHD with severe immune-mediated histopathological lung injury during murine BMT (4). After observing that IFN-γR−/− T cells display comparable Th17 and Tc17 frequency as with IFN-γ−/− T cells after in vitro polarization, we then proceeded to determine if our in vitro observations with regard to donor Th17/Tc17 expansion could be mirrored in vivo. Using the well-characterized allogeneic BMT model, C57BL6 (B6)→BALB/c, we examined the lungs for donor Th17 cell accumulation in recipients of IFN-γ−/− or IFN-γR−/− T cells. Lethally irradiated BALB/c recipients were given allogeneic TCD-BMCs from WT B6 mice together with freshly isolated, noncultured spleen cells from WT, IFN-γ−/−, or IFN-γR−/− mice. On day 7 after allogeneic BMT, serum IFN-γ levels were significantly increased in the groups of recipients that received T cells from donor WT or IFN-γR−/− mice. However, serum IFN-γ levels were undetectable in the groups of recipients that received T cells from donor IFN-γ−/− mice. When day 12 after BMT, serum IFN-γ levels were increased in recipients that received T cells from donor IFN-γ−/− or IFN-γR−/− mice. Consistent with our in vitro observations, both IFN-γ−/− and IFN-γR−/− donor T cells showed increased Th17 cell infiltration into the lungs compared with WT donor T cells. As expected, the Th17 frequency and total Th17 numbers in the lungs of mice receiving IFN-γ−/− or IFN-γR−/− donor T cells were comparable (p > 0.05) (Fig. 4A, Supplemental Fig. 1A, 1B).

These results demonstrate that the regulation of Th17 cells in the lungs, resulting in increased lung GVHD with severe immune-mediated histopathological lung injury during murine BMT (4). After observing that IFN-γR−/− T cells display comparable Th17 and Tc17 frequency as with IFN-γ−/− T cells after in vitro polarization, we then proceeded to determine if our in vitro observations with regard to donor Th17/Tc17 expansion could be mirrored in vivo. Using the well-characterized allogeneic BMT model, C57BL6 (B6)→BALB/c, we examined the lungs for donor Th17 cell accumulation in recipients of IFN-γ−/− or IFN-γR−/− T cells. Lethally irradiated BALB/c recipients were given allogeneic TCD-BMCs from WT B6 mice together with freshly isolated, noncultured spleen cells from WT, IFN-γ−/−, or IFN-γR−/− mice. On day 7 after allogeneic BMT, serum IFN-γ levels were significantly increased in the groups of recipients that received T cells from donor WT or IFN-γR−/− mice. However, serum IFN-γ levels were undetectable in the groups of recipients that received T cells from donor IFN-γ−/− mice. When day 12 after BMT, serum IFN-γ levels were increased in recipients that received T cells from donor IFN-γ−/− or IFN-γR−/− mice. Consistent with our in vitro observations, both IFN-γ−/− and IFN-γR−/− donor T cells showed increased Th17 cell infiltration into the lungs compared with WT donor T cells. As expected, the Th17 frequency and total Th17 numbers in the lungs of mice receiving IFN-γ−/− or IFN-γR−/− donor T cells were comparable (p > 0.05) (Fig. 4A, Supplemental Fig. 1A, 1B).
lungs during murine BMT is tightly controlled by IFN-γ either by homing or expansion. We also tested whether Tc17 development in the lungs can be regulated in this model by IFN-γ. Unlike Th17 cells, which are permanently present in the lungs from mice that received IFN-γ−/− or IFN-γR−/− donor T cells, a higher frequency of Tc17 cells in the lungs from recipients of IFN-γ−/− or IFN-γR−/− donor T cells was transiently seen on day 7 after BMT only. No increases in donor Tc17 cell accumulation in the lungs from recipients of IFN-γ−/− or IFN-γR−/− T cells was observed on day 12 after BMT compared with recipients of WT T cells (Fig. 4B, Supplemental Fig. 1C, 1D).

Recipients of IFN-γR−/− donor T cells after allogeneic BMT exhibit a higher frequency of Th2 cells in the lungs

After observing that the transfer of donor IFN-γ−/− or IFN-γR−/− T cells results in marked expansion of Th17 cells in the lungs during murine BMT, we then proceeded to examine the accumulation of Th2 cells in lungs of mice receiving IFN-γ−/− or IFN-γR−/− T cells on day 12 because Th2 cells have been reported to be one of the major contributors to lung GVHD pathology. Surprisingly, at this time point, the results showed a marked expansion of Th2 (IL-4+CD4+ T) cells only in the lungs from the recipients of IFN-γR−/− T cells, but no increases in Th2 frequency in the lungs of recipients that received IFN-γ−/− T cells compared with the recipients that received WT T cells (Fig. 4B, Supplemental Fig. 1C, 1D).

**FIGURE 2.** IFN-γ−/− and IFN-γR−/− CD8+ T cells display a comparable increased frequency of Tc17 cells after in vitro polarization. Unfractionated spleen cells from WT, IFN-γ−/−, and IFN-γR−/− mice were stimulated with anti-CD3, anti-CD28, IL-6, and TGF-β for 3–5 d and analyzed by flow cytometry for intracellular IL-17, IFN-γ, and TNF-α expression in CD8+ T cells. (A) Representative dot plots illustrating the polarization of IFN-γ−/− and IFN-γR−/− CD8+ T cells to Tc17 phenotype. (B) The total number of Tc17 cells within CD8+ T cells after in vitro polarization. Statistically significant increases in Tc17 cells from IFN-γ−/− and IFN-γR−/− CD8+ T cells compared with WT CD8+ T cells: p < 0.05.

**FIGURE 3.** Recipients of IFN-γ−/− but not IFN-γR−/− donor T cells exhibit a diminished ability to produce IFN-γ after BMT. After lethal total body irradiation of 800 cGy (400 cGy × 2 split dose), BALB/c (H2d) recipients were infused with 10 million WT B6 (H2b) TCD-BMCs with 3 × 106 spleen cells from WT, IFN-γ−/−, and IFN-γR−/− mice. Blood was harvested from the recipients on day 7 after BMT to measure serum levels of IFN-γ−/−. The data represent mean ± SE of three mice.

Recipients of IFN-γR−/− donor T cells after allogeneic BMT exhibit a higher frequency of Th2 cells in the lungs

After observing that the transfer of donor IFN-γ−/− or IFN-γR−/− T cells results in marked expansion of Th17 cells in the lungs during murine BMT, we then proceeded to examine the accumulation of Th2 cells in lungs of mice receiving IFN-γ−/− or IFN-γR−/− T cells on day 12 because Th2 cells have been reported to be one of the major contributors to lung GVHD pathology. Surprisingly, at this time point, the results showed a marked expansion of Th2 (IL-4+CD4+ T) cells only in the lungs from the recipients of IFN-γR−/− T cells, but no increases in Th2 frequency in the lungs of recipients that received IFN-γ−/− T cells compared with the recipients that received WT T cells (Fig. 4B, Supplemental Fig. 1C, 1D).
the Th17 cells showed similar proliferation capabilities of these cells whether they were derived from IFN-γ<sup>−/−</sup> or IFN-γR<sup>−/−</sup> donor T cells suggesting the T cells were not anergic in either instance (Fig. 5B).

**Recipients of IFN-γR<sup>−/−</sup> donor T cells show significantly reduced lung GVHD after allogeneic BMT**

Donor Th17 or Th2 cells in the absence of IFN-γ have been attributed a pathogenic effect on lung GVHD (4, 18). After observing an expansion of Th17/Th2 cells in the lungs of mice receiving IFN-γR<sup>−/−</sup> donor T cells, we then assessed the extent of immune-mediated histopathological injury in the lungs of mice with IFN-γR<sup>−/−</sup> donor T cells compared with mice with IFN-γ donor T cells. We performed experiments involving the transplantation of donor T cells from WT, IFN-γ<sup>−/−</sup>, or IFN-γR<sup>−/−</sup> mice. Surprisingly, our results demonstrated that only IFN-γ<sup>−/−</sup> but not IFN-γR<sup>−/−</sup> donor T cells led to markedly increased immune-mediated histopathological lung injury in recipients after allogeneic BMT (Fig. 6A, 6B). These results demonstrate that recipients of IFN-γ<sup>−/−</sup> or IFN-γR<sup>−/−</sup> donor T cells have differential pathological features/outcomes, and Th17/Th2-associated immune-mediated histopathological lung injury in allogeneic BMT can be ameliorated with the presence of donor IFN-γ-producing Th1/Tc1 cells in the local tissue despite the increased presence of Th17/Th2 cells in the lungs. Histopathological examination of the gut tissue in recipients of IFN-γ<sup>−/−</sup> donor T cells at the same time point that lung tissue was evaluated (i.e., day 12 posttransplant) did not show any statistically significant increase in the gut GVHD score compared with recipients of WT or IFN-γR<sup>−/−</sup> donor T cells (Fig. 6C). Skin GVHD scores, however, were significantly higher in the recipients of IFN-γ<sup>−/−</sup> donor T cells (data not shown).

**In vivo administration of neutralizing IFN-γ Abs exacerbates lung GVHD pathology in recipients of IFN-γR<sup>−/−</sup> donor T cells**

Histopathologic examination had revealed a protection against Th2/Th17 cell-associated lung GVHD in recipients receiving IFN-γR<sup>−/−</sup> donor T cells. Therefore, we next tested if protection against Th2/Th17 cell-associated lung GVHD in mice with IFN-
Donor IFN-γR−/− T cells were IFN-γ dependent. We used an anti–IFN-γ mAb to neutralize IFN-γ in recipients given IFN-γR−/− donor T cells in vivo. To this end, the treated group of these recipients received anti–IFN-γ on days 0, 1, 8, and 10 after BMT. A control group received an isotype control Ab in place of anti–IFN-γ. We found that anti–IFN-γ treatment significantly exacerbated lung GVHD with augmented immune-mediated histopathological lung injury in mice with IFN-γR−/− donor T cells (Fig. 7A, 7B). Anti–IFN-γ Ab neutralization further increased the percentage of Th2/Th17 cytokine-expressing cells in the lung in IFN-γR−/− donor groups (Fig. 7C). These results directly demonstrated the protective effect of donor Th1/Tc1 cell-derived IFN-γ on host lung tissue despite the marked increases in Th17/Th2 T cells.

Donor IFN-γR−/− T cells result in increased protection from GVHD and improved GVT effects after allogeneic BMT

As the use of IFN-γR−/− donor T cells resulted in greater protection from lung GVHD, we then wanted to ascertain the effects of such donor T cells on separating GVHD from GVT. We hypothesized that IFN-γR−/− T cells should still mediate GVT due to unimpaired production of IFN-γ. We observed that overall increased effects on survival from GVHD occurred when using donor IFN-γR−/− T cells compared with that of recipients of WT T cells (Fig. 8A). We then injected the A20 lymphoma cell line into BALB/c recipients and performed an allogeneic BMT using IFN-γR−/− versus control donor T cells. Mice receiving no donor T cells succumbed rapidly from tumor, whereas recipients of donor T cells from WT mice and from IFN-γR−/− had increased survival (Fig. 8B). Tumor colony outgrowth assay studies in different tissues showed that such a survival benefit was solely related to protection from GVHD as comparable tumor outgrowth was observed in the recipients of donor IFN-γR−/− versus WT spleen cells (Fig. 8C).

Discussion

The novelty of the current study lies in the observations that the recipients of IFN-γR−/− donor T cells had comparable accumulation of Th17/Th2 cells in the lungs as that of recipients of IFN-γ−/− donor T cells in BMT, yet much less lung pathology was observed. This was also associated with decreased mortality from GVHD. Most importantly, GVT capability of IFN-γR−/− donor T cells was preserved compared with WT control donor T cells. In this study, we have focused on the effect of IFN-γ and its receptor on lung GVHD because of the marked differences in pathology observed with the different sources of donor T cells. Acute GVHD of the lungs in mice (sometimes called idiopathic
pulmonary syndrome) is a well-defined but less well understood process that has significant clinical relevance. Our model clearly shows that transfer of WT donor T cells, similar to the clinical practice, does not generally result in lung GVHD perhaps due to high levels of donor-derived IFN-γ. Similar to previous observations by other investigators (4, 12), we have clearly shown that in this model, in the absence of donor-derived IFN-γ, significant expansion of Th17 cells occurs, resulting in lethal lung GVHD. We observed similar expansion of not only Th17 but also Th2 cells in lung recipients of IFN-γ−/− T cells, suggesting that IFN-γ directly inhibits proliferation and shifts the cells to a Th2/Th17 profile. However, the same effector cells did not cause the typical lung pathology expected from lack of IFN-γ signaling. This can be simply explained by the presence of detectable levels of donor-derived IFN-γ in IFN-γ−/− (versus IFN-γ−/−) transplanted animals indicating that host tissues are also affected by IFN-γ. To test our hypothesis, we have clearly shown that the absence of lung pathology in IFN-γ−/− transplanted mice can be reversed simply by using an anti–IFN-γ neutralizing Ab. Our data using IFN-γ−/− donor T cells also suggest that the protective effect of IFN-γR from lung GVHD due to Th2/Th17 cells is mostly based on its effect on recipient cells. Although copresence of donor Th1/Tc1 cells in the lungs can also decrease the effect of GVHD, the data indicate that effects on host tissues can result in significant protective effects. This is consistent with studies by Burman et al., which also demonstrated the protective effects of IFN-γ on host tissues. IFN-γ has been demonstrated to upregulate PD-L1 markedly, which can contribute to T cell suppression, and this may be a potential mechanism underlying the protection seen (12, 19).

With respect to pathological Th17-associated GVHD induced by IFN-γ−/− donor T cells, our results confirmed those of Maurermann et al. (4), who demonstrated that WT CD4+ donor T cells induced minimal lung inflammation, whereas IFN-γ−/− CD4+ donor T cells mediated IL-17–dependent severe lung injury in a murine allogeneic BMT model. In their studies, WT BMC plus WT or IFN-γ−/− T cells from donor BALB/c mice were transplanted into lethally irradiated CB6F1 recipients. Enhanced immune-mediated histopathological injury was observed in the lungs, but not in other organs (liver, gut) in the recipients of allogeneic IFN-γ−/− T cells compared with WT T cells early posttransplant. Other investigators, however, have shown sometimes lower GVHD score in recipients of IFN-γ−/− T cells versus WT T cells. Such a discrepancy might be explained by the differences in timing of tissue harvest between different experiments. Maurermann et al. (4) also demonstrated that this immune-mediated histopathological lung injury is associated with an increased accumulation of IL-17A–producing CD4+ T cells in the lungs, and in vivo treatment with anti–IL-17 mAb reduced disease severity. By using a different strain combination (i.e., C57BL/6 mice transplanted into lethally irradiated BALB/c recipients), we observed similar immune-mediated histopathological lung injury and accumulation of IL-17–producing CD4+ T cells in recipients of IFN-γ−/− T cells in the lungs. More recently, Yi et al. (12) demonstrated that not only donor Th17 cells but also donor Th2 cells play a pivotal role in augmenting lung GVHD after murine BMT. In these studies, WT TCD-BMCs plus WT or IFN-γ−/− IL-17−/− CD4+ T cells from donor B6 mice were transplanted into lethally irradiated BALB/c recipients. The lung GVHD after allogeneic BMT was found to be associated with the expansion of tissue-localized Th2 cells. By using IFN-γR−/− donor CD4+ T cells, Yi et al. (12) also observed a similar augmentation in Th2 differentiation in the absence of IFN-γ signaling in donor CD4+.

FIGURE 6. Recipients of IFN-γR−/− donor T cells fail to develop Th2/Th17 cell-associated lung GVHD. BALB/c (H2b) recipients were infused with 10 million WT B6 (H2b) TCD-BMCs with 3 × 10^6 spleen cells from WT, IFN-γ−/−, and IFN-γR−/− mice. On day +12 post-BMT, the mice were humanely killed, and lungs were collected, processed, and stained with H&E. Photomicrographs show representative histopathologic examination of H&E-stained sections of paraffin-embedded lung (A) or gut (B) tissues from recipients of grafts containing WT, IFN-γ−/−, or IFN-γR−/− donor T cells (original magnification, ×100). (C) Semi quantitative histologic analysis (grade 0–4) of four examined recipients in each group is shown.
T cells. They reported that donor Th2 cell-associated lung GVHD was significantly prevented by the expression of the IFN-γ inducible B7-H1 (PD-L1) resulting in suppression of infiltrating donor Th2 cells in the lung tissues (12). Consistent with this report, we also observed a higher frequency of Th2 cells as well as Th17 in the lungs of mice with IFN-γR−/− donor T cells. In contrast, we did not observe a marked expansion of Th2 cells in the lungs on day 12 after BMT of IFN-γR−/− donor T cells, which differs from observations by Yi et al. (12). Although both studies use the B6 into BALB/c host model, Yi et al. (12) investigated CD4-selected T cells in the transplant and observed IL-4 expression on CD4+ T cells in the spleen 7 d after BMT. In contrast, our donor graft contained whole T cell populations (both CD4+ and CD8+ cells), and only transient expression of IL-4 on CD4+ T cells in the lung was observed on day 7 after BMT. These significant differences may have contributed to the contrasting results in the recipients of IFN-γ−/− donor T cells.

Multiple mechanisms may be involved in the augmented GVHD-associated acute lung inflammation in the absence of donor T cell-derived IFN-γ. Using WT, IFN-γ−/−, and IFN-γR−/− mice as donors or recipients, Burman et al. (11) demonstrated that the rapid mortality in recipients of IFN-γ−/− donor T cells was due to the development of severe lung GVHD, and donor T cell-derived IFN-γ protected against GVHD by the recipient pulmonary parenchymal cells. This could prevent donor leukocyte migration into the lungs and/or subsequent expansion specifically within this organ. In accordance with these findings, the previously mentioned study by Mauermann et al. (4) confirmed the crucial role of IFN-γ signaling on host lung parenchymal cells in protecting the mice from T cell-mediated lung injury. Moreover, they showed additional evidence that the severity of lung injury in recipients of IFN-γ−/− donor T cells directly correlated with the expansion of pathogenic Th17 cells in the lungs (4). They concluded in their report that one of the mechanisms regarding IFN-γ protection against acute lung inflammation is the negative regulation of the expansion of pathogenic Th17 cells through interaction with the IFN-γ receptor on the pulmonary parenchymal cell population. Consistently, in vitro-differentiated Th17 cells have been attributed to more severe pathologic lesions in the lungs during BMT (20) suggesting that the role of donor-derived IFN-γ on host tissues versus donor T cells may be opposing with regard to the various processes in GVHD.

Besides Th17 cells, Yi et al. (12) showed that donor Th2 cells have a pathogenic effect on lung GVHD in the absence of IFN-γ and IL-17. With the benefit of simultaneous data generated using both IFN-γ−/− and IFN-γR−/− donor T cells, our studies suggest that the role of IFN-γ on recipient parenchymal lungs is not the negative regulation of the expansion of pathogenic Th17 cells (as similar expansion occurs in the presence of IFN-γ in the recipient of IFN-γR−/− T cells) but an effect on the pathogenicity of these already expanded Th17 cells or perhaps alteration of the different T cell subpopulations with regard to cytokine profile.

IL-17-secreting CD8+ T cells, termed Tc17 cells, were shown to be capable of mediating inflammation and immune responses (8, 21). An additional finding of our study is the dramatic expansion of IL-17 on CD8+ T cells from IFN-γ−/− or IFN-γR−/− mice under Ag-independent Th17 polarizing conditions. This is consistent with our observations that CD8+ T cells from WT mice also produce the majority of IFN-γ, and thus IFN-γ may act as an autocrine suppressive signal by these cells. In our in vivo experiments, unlike Th17 cells that were present in the lungs from mice that received IFN-γ−/− or IFN-γR−/− donor T cells even at later time points, a higher frequency of Tc17 cells in the lungs from these recipients was momentarily observed on day 7.
but not at later time points after BMT. It has been shown by Mauermann et al. (4) that it was CD4+ but not CD8+ T cells from donor IFN-γR2−/− mice playing a major role in lung GVHD. Our findings may explain why only CD4+ but not CD8+ donor T cells from IFN-γR2−/− mice exacerbated IL-17–dependent severe inflammation in the lungs during murine BMT (4). The exciting finding of increased anti-tumor GVT effects using the IFN-γR2−/− donor T cells needs to be explored further. The critical role of cytokines such as IFN-γ on successful anti-tumor responses, especially during GVT, has been well documented. Therefore, introducing cells unable to signal through IFN-γ (i.e., IFN-γR2−/− cells) could prevent GVHD while preserving beneficial GVT responses as their production of IFN-γ is unabated. These data may open the door for novel means to promote GVT without GVHD clinically with the use of receptor silencing ex vivo approaches.

In summary, our studies provide in vivo evidence that demonstrates protection by donor T cell-derived IFN-γ and copresence of

**FIGURE 8.** Increased survival from GVHD and anti-tumor effects using donor T cells from IFN-γR2−/− mice. (A) BALB/c (H2b) recipients of 5 million WT B6 TCD-BMCs with or without either IFN-γR2−/− or WT control B6 purified T cells (6 × 105) on day 0 after lethal total body irradiation. Significant increases in survival were observed in recipients of IFN-γR2−/− pure T cells compared with that of recipients of WT control B6 pure T cells: p < 0.05 (log-rank test). (B) BALB/c (H2b) mice received 3 × 105 A20 cells on day −6. On day 0, after lethal total body irradiation, BALB/c (H2b) mice received 5 million B6 (H2b) TCD-BMCs with/without either IFN-γR2−/− or WT control B6 purified T cells (1 × 106). Representative data from one of three independent experiments are presented. Use of donor T cells from IFN-γR2−/− mice in allogeneic BMT results in significant increases in survival compared with that of mice that received WT control B6 donor T cells: p < 0.05 (log-rank test). (C) Tumor colony assay was performed by extracting cells from different organs and culturing them on methylcellulose as described in Materials and Methods. Representative data from one of two independent experiments are presented and show that infusion of IFN-γR2−/− donor T cells can result in similar GVT effect as WT donor cells, which is significantly better than that of TCD-BMCs only: p < 0.001.
donor Th1/Tc1 cells against GVHD by restraining pathogenic responses induced by Th17/Th2 cells in the lungs and further show that the decreased GVHD pathology does not adversely affect GVT responses suggesting that IFN-γR blockade or silencing on the donor T cells may be of potential use in allogeneic BMT.

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Disclosures

The authors have no financial conflicts of interest.

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10 ROLE OF IFN-γ AND GRAFT-VERSUS-HOST DISEASE


Supplemental Figure 1- Increased frequency of Th17 cells in the lungs of recipients of grafts containing either IFN-γ−/− or IFN-γR−/− donor T cells. BALB/c (H2d) mice received 10 million WT B6 (H2b) TCD-BMC with 3 x 10^6 SC from WT, IFN-γ−/− and IFN-γR−/− mice. On day 7 and day 12 post-BMT, the mice were humanely sacrificed and the lungs were collected and light density leukocytes from lungs were isolated. A-D, Representative dot plots illustrating the frequency of donor derived Th17 and Tc17 cells in the lungs of mice on day 7 or day 12 after murine BMT.
Supplemental Figure 1A

WT  Ifnγ⁺  IfnγR⁺

IL-17

IFN-γ

TNF-α

CD4⁺ T cells in the lungs of mice on day7 after BMT

Supplemental Figure 1B

WT  Ifnγ⁺  IfnγR⁺

IL-17

IFN-γ

TNF-α

CD4⁺ T cells in the lungs of mice on day12 after BMT