Biology of Blood and Marrow Transplantation 13:46-55 (2007)

1083-8791/07/1301-0001$32.00/0
doi:10.1016/j.bbmt.2006.09.014

An Essential Role for IFN-γ in Regulation of Alloreactive CD8 T Cells Following Allogeneic Hematopoietic Cell Transplantation

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Received May 21, 2006; accepted September 29, 2006

ABSTRACT

We previously found that CD8 T cells from IFN-γ gene knockout (GKO) donors induce more severe lethal GVHD compared with CD8 T cells from wild-type (WT) donors in fully MHC-mismatched strain combinations. In this study, we investigated the mechanisms by which IFN-γ inhibits GVHD in a parent → F1 (B6 → B6D2F1) allogeneic HCT (allo-HCT) model. IFN-γ was strongly protective against GVHD in this parent → F1 haplotype-mismatched allo-HCT model. Irradiated B6D2F1 mice that received GKO B6 CD4-depleted splenocytes developed lethal GVHD with severe lung and liver injury, whereas those receiving a similar cell population from WT B6 donors survived long term. Donor CD8 cells showed rapid activation, accelerated cell division, and reduced/delayed activation-induced cell death in allogeneic recipients in which donor cells were incapable of producing IFN-γ. In consequence, the numbers of activated/effector (ie, $CD25^+$, $CD62L^-$, and $CD44^{high}$) donor CD8 T cells in the recipients of GKO allo-HCT significantly exceeded those in mice receiving WT allo-HCT. These data show that IFN-γ negatively regulates the CD8 T cell response by inhibiting cell division and promoting cell death and suggest that blockade of IFN-γ could augment the severity of GVHD in patients undergoing allo-HCT.

KEY WORDS

Graft-versus-host disease • interferon-gamma • allogeneic hematopoietic cell transplantation • CD8 T cells

INTRODUCTION

IFN-γ is a potent proinflammatory cytokine that plays important and complex roles in innate and adaptive immune responses. The compromised immunity to multiple intracellular pathogens in IFN-γ- and IFN-γ receptor-deficient mice suggests an important role for IFN-γ in the induction of cellular immune responses [1,2]. A recent study using IFN-γ receptor-deficient mice showed that IFN-γ acts directly on CD8 T cells to stimulate the development of CTL responses after lymphocytic choriomeningitis virus infection [3]. However, increasing evidence has demonstrated that IFN-γ may also downregulate immune responses. IFN-γ plays an important role in the maintenance of T cell homeostasis and eliminates activated CD4 [4-8] and CD8 [8-10] T cells by inducing apoptosis. It has been shown that IFN-γ promotes cell death of activated T cells [11-14]. IFN-γ has also been reported to facilitate induction of long-term allograft survival by blockade of T cell costimulation pathways [15]. Further, recent studies have shown that IFN-γ is required for the function of alloantigen-specific regulatory T cells [16] and may inhibit CD8 memory T cell generation [17]. Although the mechanisms remain largely unknown, these studies indicate that IFN-γ has a complex role in the regulation of immune responses.

Activated T cells produce IFN-γ and the level of IFN-γ in patients undergoing allogeneic HCT (allo-HCT) may reflect ongoing GVH allogeneic responses [18-21]. Such a correlation between high levels of

IFN-γ and severe GVHD has led to a suggestion that IFN-γ may be involved in the pathogenesis of GVHD. Of note, this cytokine has been reported to contribute to gut injury [22,23] and lymphoid hypoplasia [24,25] in patients after allo-HCT. However, studies using anti-IFN-γ antibody and IFN-γ gene knockout (GKO) mice have demonstrated that this cytokine can inhibit the development of acute GVHD by promoting apoptosis of alloreactive CD4 T cells [26-29].

We recently observed that IFN-γ-deficient CD8 T cells induce more severe GVHD than do wild-type (WT) cells in fully MHC- plus minor antigen-mismatched allogeneic recipients [30]. In the present study, we explored the mechanisms of the regulation of alloreactive CD8 T cells by IFN-γ using a clinically relevant, parent → F1, allo-HCT model. We observed that IFN-γ plays a critical role in controlling donor CD8 T cell activation, proliferation, and survival in patients after allo-HCT. In the absence of IFN-γ, activation and expansion of alloreactive donor CD8 T cells was significantly augmented and apoptotic cell death of such cells was markedly reduced, resulting in increased accumulation of highly divided donor CD8 T cells. Further, IFN-γ-deficient, but not WT, donor CD8 T cells induced lethal GVHD characterized by severe damage to nonlymphoid target tissues.

METHODS

Animals

Female WT C57BL/6 mice (B6; H-2^b), IFN-γ GKO mice on the B6 background (B6.129S7-$Ifng^{tm1Ts}$; H-2^b), and B6D2F1 (H-$2^{b\times d}$) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Anti-L^d 2C TCR transgenic mice on the B6 background were generously provided by Dr Dennis Y. Loh [31]. IFN-γ-deficient 2C TCR transgenic (GKO 2C) mice on the B6 background were generated as previously described [30]. Mice were housed in a specific pathogen-free microisolator environment and fed autoclaved feed and autoclaved acidified water. Protocols involving animals were approved by the Massachusetts General Hospital subcommittee on research animal care.

Allo-HCT and Induction of GVHD

Recipient B6D2F1 mice were lethally irradiated (9.75 Gy) and reconstituted within 4-8 hours with 7×10^6 T cell (ie, CD4 and CD8)-depleted (TCD) BM cells (BMCs) and 3×10^7 CD4-depleted spleen cells from WT or GKO B6 mice or with 5×10^6 BMCs and 1.5×10^7 spleen cells from 2C or GKO 2C B6 mice. Mice that received TCD BMCs and TCD splenocytes from allogeneic donors were used as non-GVHD controls. CD4- and CD8-depleted cells were prepared with anti-CD4 or CD8α microbeads followed by negative selection using the MACS separation system according to the manufacturer's protocol (Miltenyi Biotec, Auburn, Calif).

Histopathology

GVHD target tissues (liver, lungs, and colon) were harvested at various times after HCT and fixed in Bouin solution or 10% formalin. Paraffin sections were prepared and stained with H&E. Pathologic GVHD was evaluated by a pathologist (R Bronson) without knowledge of the treatment the animals received based on the parameters listed in Table 1. Each parameter was scored using a score system ranging from 0 to 4: 0, normal; 0.5, focal and rare; 1, focal and mild; 2, diffuse and mild; 3, diffuse and moderate; and 4, diffuse and severe.

Flow Cytometric Analysis

To determine T cell activation, single-cell suspensions were prepared from spleen and lymph nodes and stained with fluorescent (FITC or PE)-conjugated mAb specific for activation/memory markers (CD25, CD44, and CD62L) and anti-CD8 mAb. NKT (NKT) cells and subsets were identified by staining the cells with FITC-conjugated anti-CD3, PE-conjugated anti-NK1.1, plus APC-conjugated anti-CD4 and/or CD8. Treg cells were determined by an APC-conjugated anti-Foxp3 mAb (eBioscience, San Diego, Calif). In patients after allo-HCT, donor cells were identified by staining with biotinylated anti-H-$2D^d$ mAb 34-2-12 [32] developed with PE-streptavidin or streptavidin-allophycocyanin. Nonspecific FcγR binding was blocked with anti-mouse FcγR mAb 2.4G2. Exclusion of dead cells was per-

Table 1. *Pathologic GVHD Criteria*

Colon	Lung	Liver
Crypt epithelial cell regeneration, apoptosis	Perivascular cell infiltrate	Portal tract inflammatory cell infiltrate
Crypt loss	Peribronchiolar cell infiltrate	Bile duct lymphocytic infiltrate
Surface colonocyte attenuation		Vascular endothelialitis
Inflammatory cell infiltrate		Parenchymal apoptosis
Mucosal ulceration		Parenchymal microabscesses
Thickness of mucosa		Parenchymal mitotic figures

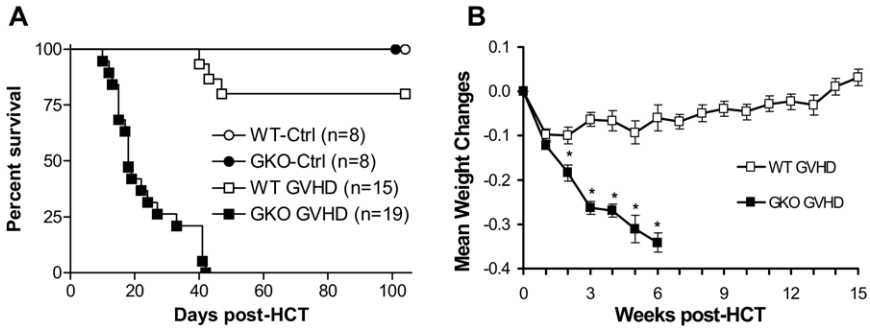

Figure 1. IFN-γ inhibits GVHD induced by donor CD8 T cells. Recipient B6D2F1 mice were lethally irradiated and reconstituted with 7×10^6 TCD BMCs and 3×10^7 CD4-depleted spleen cells from WT (WT GVHD; n = 15) or GKO (GKO GVHD; n = 19) B6 donors. Non-GVHD controls were lethally irradiated B6D2F1 mice that received TCD BMC alone or with TCD splenocytes from WT (WT-Ctrl; n = 8) or GKO (GKO-Ctrl; n = 8) donors. Data from 3 independent experiments are combined. The percentages of $CD8^+$ cells in WT and GKO allo-HCT inocula (ie, CD4-depleted splenocytes) used in these experiments were 17.9% and 17.7%, 16.1% and 15.7%, and 21% and 23%, respectively. Shown are (A) percent survival and (B) mean body weight changes (mean ± SDs) of the allo-HCT recipients. Mean weight changes = [(current weight/weight at day 0) − 1]. *$P < .05$.

formed by propidium iodine staining and live gating on propidium iodine-negative cells. Nonreactive FITC-conjugated and biotinylated mouse IgG2a mAb HOPC-1, PE- and allophycocyanin-labeled rat IgG2a mAbs were used as negative controls. In proliferation studies, donor spleen cells were incubated with 1 μM CFSE (Molecular Probes, Eugene, Ore) at 50×10^6 cells/mL for 15 min at 37°C in the dark. Cold medium with 2% FCS was added to stop the labeling reaction. Cells were then washed twice with fresh serum-free medium and resuspended at desired concentrations before transplantation. In apoptotic studies, cells were stained with APC-conjugated Annexin V and aminoactinomycin D. Apoptotic cells were defined by Annexin V-positive and aminoactinomycin D-negative population. Unless otherwise indicated, all antibodies used were purchased from BD PharMingen (San Diego, Calif).

Statistical Analysis

Statistical analysis of survival data was performed with the log-rank test. The Student *t* test was used to determine the level of significance of differences in group means. $P < .05$ was considered statistically significant in both types of analysis.

RESULTS

IFN-γ-deficient CD8 T Cells Induce Acute Lethal GVHD

To determine the role of IFN-γ in CD8-mediated GVHD, B6D2F1 mice were lethally irradiated and reconstituted with 7×10^6 TCD BMCs and 30×10^6 CD4-depleted spleen cells from WT or GKO B6 mice through the tail vein. As shown in Figure 1A, allo-HCT from GKO donors induced severe lethal GVHD, with >50% (10 of 19) mortality by 3 wk and 100% mortality by 6 wk ($P < .01$ versus recipients of WT allo-HCT). These mice developed severe clinical signs of acute GVHD, including massive weight loss (Figure 1B), ruffled fur, hunched posture, and diarrhea before death. In contrast, most mice (12 of 15) receiving a similar HCT inoculum from WT B6 donors survived long term. The recipients of WT allo-HCT showed only moderate (~10%) weight loss during the first week and gradually gained the weight back (Figure 1B). Control mice that received TCD allogeneic cells from WT or GKO B6 mice appeared healthy throughout the experiment (Figure 1). These results demonstrate that IFN-γ mediates significant protection against GVHD induced by CD8 T cells in F1 recipients of allo-HCT from parental donors, consistent with previous observations in fully MHC-mismatched strain combinations [30].

Increased Pathologic GVHD in Recipients of GKO Allo-HCT

Histologic examination was performed on tissues prepared from the recipient mice at days 2, 4, 5, 7, and 11 after HCT, and pathologic changes were scored blindly. WT and GKO allo-HCT groups showed minimal pathologic changes in lungs and liver at days 2 (not shown) and 4 (Figure 2A,B). By day 7 after HCT, severe inflammatory infiltrates were seen in the lungs and liver in the recipients of GKO allo-HCT, whereas significant inflammatory infiltrates were not detected in the same tissues from the recipients of WT allo-HCT until day 11 after HCT. Pathologic GVHD scores for both tissues harvested at days 5 and 7 were significantly higher in the recipients of GKO allo-HCT than in those receiving WT allo-HCT (Figure 2A,B). Further, the recipients of GKO allo-HCT showed markedly increased infiltration by CD8

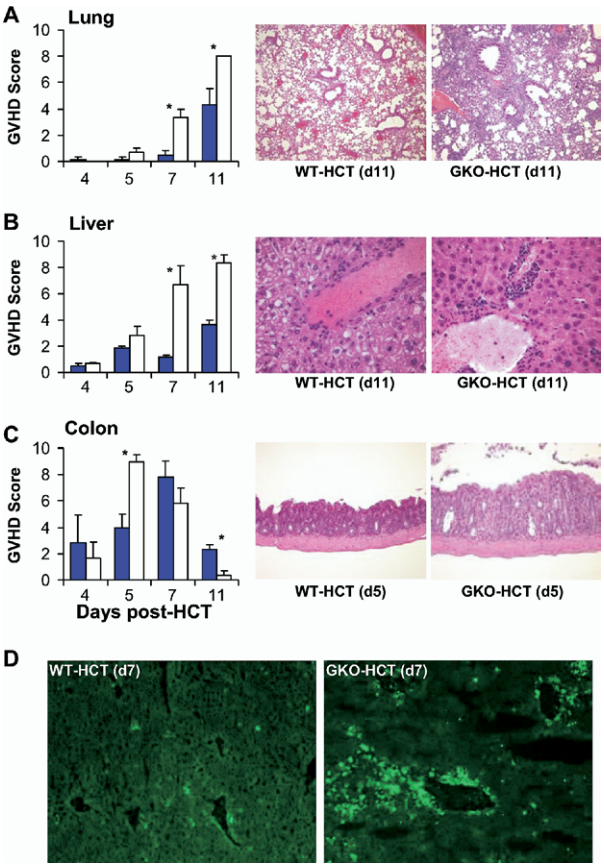

Figure 2. GKO allo-HCT induces more severe GVHD pathology. Shown are pathologic GVHD scores (mean ± SD, n = 3-6/group) and representative H&E photomicrographs of the lungs (A), liver (B), and colon (C) from the recipients of WT allo-HCT (solid columns) or GKO allo-HCT (open columns). *$P < .05$. D, Immunofluorescent staining of liver sections prepared from allo-HCT recipients at day 7 with anti-CD8. Three mice from each group were analyzed and representative photomicrographs are shown.

T cells in the liver compared with mice receiving WT allo-HCT (Figure 2D).

During the acute phase of GVHD progression, pathologic changes in the intestine included crypt destruction and mucosal alterations [33]. As shown in Figure 2C, by day 5 after HCT, the recipients of GKO allo-HCT developed severe colonic GVHD, as demonstrated by massive inflammatory cell infiltrates, crypt regeneration, epithelial cell apoptosis, surface colonocyte attenuation, and thickening of mucosa. Colonic GVHD in these recipients decreased after day 5 and subsided by day 11. Although a similar pathologic pattern was also seen in the recipients of WT allo-HCT, its development was delayed compared with that in recipients of GKO allo-HCT.

Augmented Activation and Expansion of Donor CD8 T Cells in Recipients of GKO Allo-HCT

To determine the mechanisms by which IFN-γ deficiency augments GVHD, we compared the activation and expansion of donor CD8 T cells in the spleens of lethally irradiated B6D2F1 mice that received TCD BMCs plus CD4-depleted splenocytes from WT or GKO B6 donors. The numbers of total donor CD8 T cells in the spleens of the recipients of GKO allo-HCT were significantly larger at days 4 and 5 but became similar or lower at day 7 than that in recipients of WT allo-HCT (Table 2). Because T cells gain CD25 expression and lose CD62L expression upon activation, we also compared the expression of these molecules on donor CD8 T cells. As shown in Figure 3A,B, the percentages of $CD25^+$ and $CD62^-$ donor CD8 T cells in the recipients of GKO allo-HCT were markedly greater than those in recipients of WT allo-HCT, suggesting an increased activation in the absence of IFN-γ. Further, GKO donor CD8 T cells acquired a "memory" phenotype (ie, $CD44^{high}$ and $CD45RB^{low}$) more rapidly than did WT donor CD8 T cells in allo-HCT recipients (Fig. 3A,B, and data not shown). Because nearly all GKO donor CD8 T cells lost expression of CD62L, a secondary lymphoid organ homing receptor [34], by day 5, and increased CD8 T cell infiltration was detected in the GVHD target tissues in the GKO allo-HCT group (Figure 2D); the reduction in donor CD8 T cells detected at day 7 in mice receiving GKO allo-HCT (Table 2) might be due in part to more rapid trafficking of activated CD8 T cells to GVHD target tissues.

We also performed a similar analysis in a 2C TCR transgenic allo-HCT model. The 2C cytotoxic T cell clone was derived from mice with $H\text{-}2^b$ that were immunized with $H\text{-}2^d$ cells [35]. Because most T cells in 2C TCR transgenic mice are $CD8^+$2C TCR^+ cells

Table 2. *Numbers of Donor CD8 T Cells in the Spleen of Allo-HCT Recipients at Indicated Times**

	Day 4			Day 5			Day 7		
Experiment	WT	GKO	*P*	WT	GKO	*P*	WT	GKO	*P*
1	1.0 ± 0.2	1.4 ± 0.2	>.05	3.8 ± 0.8	11.8 ± 2.5	<.05	28.4 ± 1.7	26.4 ± 1.3	>.05
2	0.7 ± 0.04	1.4 ± 0.1	<.05	3.9 ± 0.2	18.1 ± 1.3	<.001	N/A	N/A	N/A
3	2.0 ± 0.4	7.1 ± 0.7	<.01	6.2 ± 1.0	11.9 ± 2.7	>.05	15.0 ± 0.7	7.1 ± 1.3	<.01

N/A indicates not available.

*The number of donor CD8 T cells per spleen was determined by the product of the percentage of donor CD8 T cells measured by flow cytometric analysis and the total number of spleen cells harvested from each animal, and data are presented as group means ± SDs ($\times 10^6$) per spleen (n = 3/group in all 3 experiments).

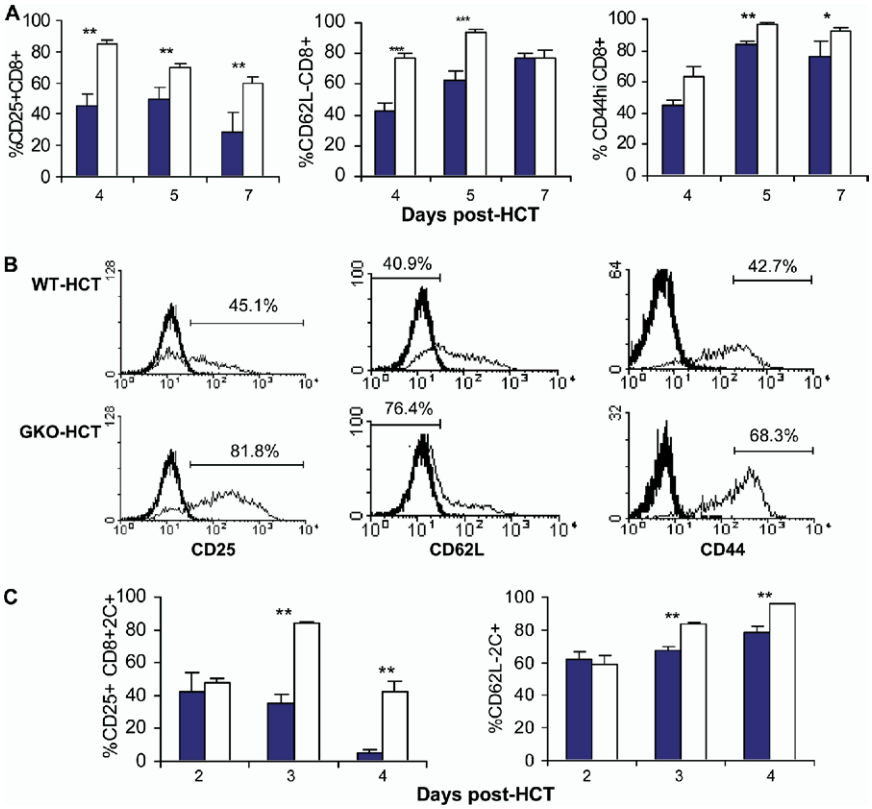

Figure 3. Augmented activation and expansion of donor CD8 T cells in the recipients of GKO allo-HCT. A, B, Expression of activation markers on donor CD8 T cells in the spleens of lethally irradiated B6D2F1 mice receiving allo-HCT from WT or GKO B6 donors (n = 6/group). A, Percentages of donor $CD25^+$, $CD62L^-$, and $CD44^{high}$ CD8 T cells at the indicated time points. WT and GKO donor CD8 T cells are presented as solid and open bars, respectively. B, Representative flow cytometric profiles display the expression of CD25, CD62L, and CD44 on gated donor CD8 T cells at day 4. Thick lines represent the isotype staining controls. Analysis gates were set based on staining profiles of appropriate isotype control mAs and naive splenocytes were stained with the same mAbs at the same time. C, Spleen cells were prepared from B6D2F1 recipients of allo-HCT from WT (solid bars) or GKO (open bars) 2C donors at the indicated time points, and the percentages of $CD25^+$ and $CD62L^-$ 2C T cells were analyzed by flow cytometric analysis. Data are shown as mean ± SD (n = 3/group). $^*P < .05$; $^{**}P < .01$.

that specifically recognize H-$2L^d$, administration of 2C T cells into H-$2L^{d+}$ mice allows the study of GVH reactivity of CD8 T cells. Lethally irradiated (9.75 Gy) B6D2F1 mice received 5×10^6 TCD BMCs and 15×10^6 spleen cells from WT 2C or GKO 2C TCR transgenic B6 mice. The percentages of $CD25^+$ and $CD62L^-$ 2C CD8 T cells were significantly higher in the B6D2F1 mice receiving GKO 2C cells than in those receiving WT 2C cells (Figure 3C). Together, these results indicate that the activation and expansion of GVH-reactive CD8 T cells are significantly increased in allo-HCT recipients when the donor cells are deficient in IFN-γ production.

Role of IFN-γ in Apoptosis and Cell Division of Alloreactive Donor CD8 T Cells

The total number of activated donor CD8 T cells could be determined by the combined effects of activation-induced cell death and cell division. When we assessed apoptosis of donor CD8 T cells in the recipient spleens in the B6 → B6D2F1 (Fig. 4A) and 2C → B6D2F1 (Figure 4B) models, we observed that donor CD8 T cell apoptosis was markedly reduced/delayed in the recipients of GKO allo-HCT compared with those receiving WT allo-HCT. In the B6 → B6D2F1 model, GKO donor CD8 T cell apoptosis was significantly reduced at earlier times, at days 4 and 5, but increased by day 7 (Fig. 4A). The later increase in apoptosis of GKO donor CD8 T cells may also contribute to the reduction in donor CD8 T cells in the spleen detected at day 7 in mice receiving GKO allo-HCT (Table 2). In the 2C → B6D2F1 allo-HCT model, significantly reduced apoptosis was detected at day 3 in GKO 2C T cells compared with WT 2C T cells (Fig. 4B).

To assess donor CD8 T cell division in allo-HCT recipients, lethally irradiated B6D2F1 mice received TCD BMCs and CFSE-labeled CD4-depleted spleen cells from WT or GKO B6 donors. Spleen and lymph node cells were prepared from the recipients at days 2 and 4 and analyzed for donor CD8 T cell divisions by flow cytometry. As shown

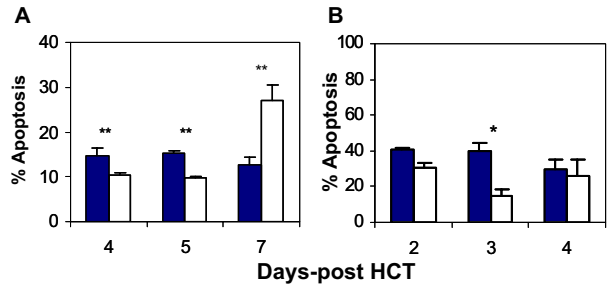

Figure 4. Effect of IFN-γ on apoptosis of donor CD8 T cells. Spleen cells were prepared from the recipients of WT (solid bars) or GKO (open bars) allo-HCT at the indicated time points, and the percentages of apoptotic donor CD8 (or 2C) T cells were determined by flow cytometric analysis. A, Percentages of apoptotic donor CD8 T cells in the spleens of B6 → B6D2F1 HCT recipients. B, Percentages of apoptotic donor 2C T cells in the spleens of 2C →; B6D2F1 HCT recipients. Data are shown as mean ± SD (n = 3-6/group). *$P < .05$; **$P < .01$.

in Figure 5A,B, the numbers of dividing donor CD8 cells, especially those with extensive cell divisions (≥8 divisions), were significantly increased in the mice receiving GKO allo-HCT compared with the recipients of WT allo-HCT.

Because of the presence of non-GVH-reactive CD8 T cells in the allo-HCT inoculum, it was difficult to conclude if the increased number of extensively divided donor CD8 T cells was due to augmented cell proliferation and/or reduced apoptosis of dividing donor T cells. Thus, we next compared cell division between GKO and WT 2C T cells in the 2C → B6D2F1 allo-HCT model. Similar to the B6 → B6D2F1 model, the numbers of host-reactive 2C T cells with ≥7 divisions were significantly larger in the recipients of GKO allo-HCT than in mice receiving WT allo-HCT (Figure 5C). However, the numbers of 2C T cells with <4 divisions were lower in mice receiving GKO allo-HCT than in those receiving WT allo-HCT (Figure 5C). Because GKO 2C T cells showed reduced apoptosis in allo-HCT recipients compared with WT 2C T cells (Figure 4B), the reduction in less divided GKO 2C T cells and the increase in GKO 2C T cells with ≥7 divisions is likely a consequence of accelerated proliferation of these

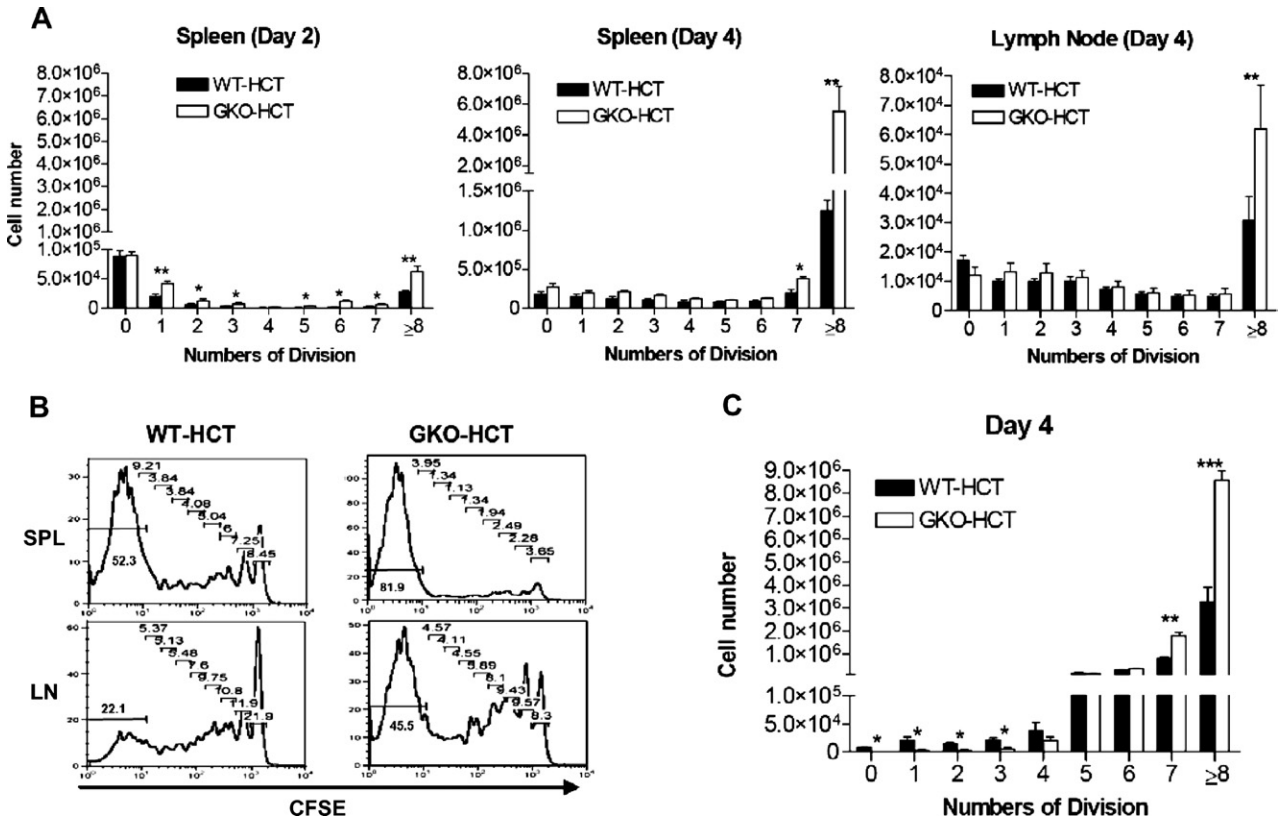

Figure 5. Effect of IFN-γ on division of donor CD8 T cells. A, B, B6D2F1 mice received BMCs and CSFE-labeled CD4-depleted spleen cells from WT (solid bars) or GKO (open bars) B6 donors (n = 3/group). Recipient spleen and lymph node cells were prepared at the indicated time points and analyzed for division of donor CD8 T cells. A, Numbers of donor CD8 T cells with each division in the spleen at days 2 and 4 and in the lymph nodes at day 4 after HCT. Axillary, brachial, and inguinal lymph nodes were harvested at the indicated time points, and pooled lymph node cells were analyzed. B, Representative histograms show CFSE levels in gated donor CD8 T cells at day 4. Numbers indicate percentages of cells with each number of cell divisions. C, B6D2F1 mice received BMCs and CFSE-labeled spleen cells from WT (solid bars) or GKO (open bars) 2C donors (n = 3/group). Data shown are percentages of donor 2C TCR^+ cells with different cell divisions (mean ± SD) in the recipient spleen at day 4 after HCT. *$P < .05$; **$P < .01$; ***$P < .001$.

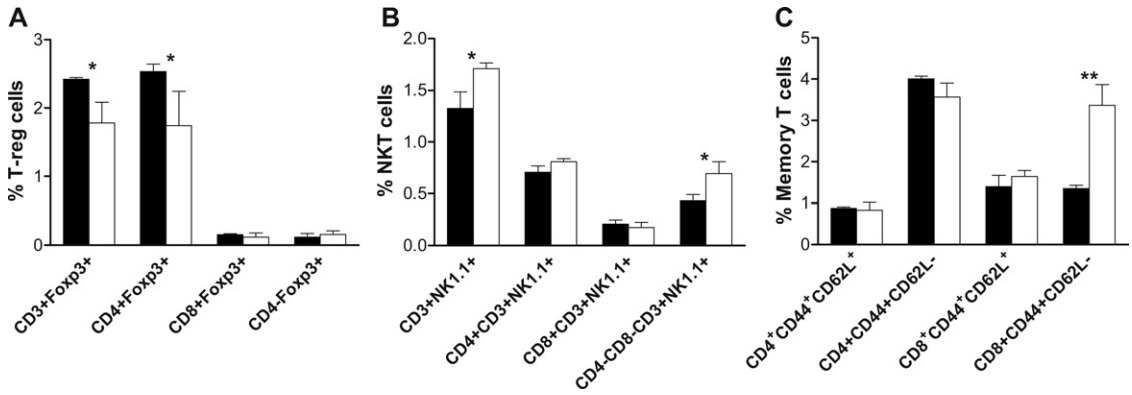

Figure 6. Comparison of Treg, NKT, and memory T cells in normal WT and GKO B6 mice. Spleen cells were prepared from naive WT (solid bars) and GKO (open bars) B6 mice (8 wk old; n = 3/group) and analyzed for Treg, NKT, and memory T cells by flow cytometry. A, Percentages of total ($CD3^+Foxp3^+$), CD4 ($CD4^+Foxp3^+$), CD8 ($CD8^+Foxp3^+$), and $CD4^-$ ($CD4^-Foxp3^+$) Treg cells. B, Percentages of total ($CD3^+NK1.1^+$), CD4 ($CD4^+CD3^+NK1.1^+$), CD8 ($CD8^+CD3^+NK1.1^+$), and double-negative ($CD4^-CD8^-CD3^+NK1.1^+$) NKT cells. C, Percentages of CD4 and CD8 central ($CD44^+CD62L^+$) and effector ($CD44^+CD62L^-$) memory T cells. Results are presented as mean ± SD. $^*P < .05$; $^{**}P < .01$.

cells. Together, these results indicate that IFN-γ promotes apoptosis and limits the proliferation of donor CD8 T cells in allo-HCT recipients.

T-reg, NKT, and Memory T Cell Contents in WT and GKO Mice

To determine whether the different ability to induce GVHD between WT and GKO allo-HCT might be attributable to differences in the contents of various T cell subsets in the HCT inocula, we compared the levels of Treg, NKT, and memory T cells in the spleen between WT and GKO B6 mice. In both groups, almost all splenic Treg (ie, $CD3^+Foxp3^+$) cells expressed CD4 (Figure 6A). The level of $CD4^+Foxp3^+$ Treg cells (most of these cells also express CD25) was significantly lower in the spleen of GKO mice compared with WT mice. However, the levels of $CD4^-$ (including $CD8^+$) cells expressing Foxp3 were comparable between WT and GKO mice and both were extremely low. Further, the levels of splenic CD4 and CD8 NKT (ie, $CD4^+CD3^+NK1.1^+$ and $CD8^+CD3^+NK1.1^+$) cells were similar between WT and GKO mice, but more double-negative NKT (ie, $CD4^-CD8^-CD3^+NK1.1^+$) cells were detected in the latter group (Figure 6B).

Interestingly, GKO mice showed a significant increase in CD8 T cells with an effector memory phenotype (ie, $CD44^+CD62L^-$) compared with WT mice (Figure 6C), consistent with the increase of alloreactive CD8 memory T cells in allo-HCT recipients (Figure 3). No significant difference was detected between WT and GKO mice in other memory T cell subsets, including CD4 and CD8 central memory (ie, $CD44^+CD62L^+$) T cells and $CD4^+$ effector memory T cells. Because memory T cells are markedly less potent than naive T cells in inducing GVHD [36-39], the higher incidence of GVHD observed in the recipients of GKO allogeneic cells is unlikely to be due to the inclusion of more CD8 effector memory T cells in the HCT inoculum.

DISCUSSION

This study provides evidence that IFN-γ plays a critical role in the regulation of CD8 T cell alloreactivity in vivo. Allo-HCT recipients of GKO CD8 cells developed severe acute GVHD and succumbed to death within 6 wk, whereas most of the mice receiving similar allo-HCT from WT donors survived long term (Figures 1 and 2). The augmented GVHD in recipients of GKO allo-HCT was associated with markedly increased expansion of alloreactive donor CD8 T cells. It has been reported that IFN-γ stimulates death of antigen-specific CD8 T cells in a *Listeria* infection model [14]. Our results demonstrate that IFN-γ also promotes death of alloreactive CD8 T cells in vivo, and that the increased donor CD8 T cell expansion and augmented GVHD in the recipients of GKO allo-HCT was, at least in part, due to the reduced/delayed death of host antigen-activated donor CD8 T cells.

IFN-γ appears to regulate a critical checkpoint of T cell proliferation. T cell proliferation is governed by the ordered activation of cyclin-dependent kinases (CDKs) [40]. Studies using gene-targeted knockout mice and transgenic mice have demonstrated that the CDK inhibitor, $p27^{Kip1}$, plays a critical role in controlling T cell proliferation, and the lack of $p27^{Kip1}$ expression results in a significant increase in T cell numbers [41,42]. A recent study has shown that naive CD8 T cells have high expression of $p27^{Kip1}$ and low CDK6 and CDK2 kinase activity, whereas G0/G1 memory CD8 T cells have low expression of $p27^{Kip1}$

and high CDK6 kinase activity, and the latter favors rapid cell division [43]. Further, IFN-γ has been shown to inhibit the proliferation of bronchial epithelial cells by preventing growth factor-induced downregulation of $p27^{Kip1}$ [44]. However, relatively little is known about the role of IFN-γ in regulating in vivo proliferation of activated CD8 T cells. In the present study, we observed that cell division rates and numbers of donor CD8 cells were markedly increased in the recipients of GKO allo-HCT compared with those in mice that received WT allo-HCT. Using the 2C allo-HCT model that allows specific assessment of the host-reactive CD8 T cells, we found that the lack of IFN-γ results in marked increases in the numbers of host MHC-reactive 2C T cells with ≥7 divisions, which was associated with a significant decrease in the numbers of less divided 2C T cells (Figure 5C). These results indicate that IFN-γ plays an important and previously unidentified role in controlling the proliferation of activated CD8 T cells in vivo, and that the lack of this cytokine accelerates the division of host antigen-reactive donor CD8 T cells in allo-HCT recipients. Because type 1 T cells, which are capable of producing IFN-γ, do not respond to IFN-γ due to the loss of IFN-γ receptor 2 expression [45,46], the role of IFN-γ in regulating cell division of host antigen-activated T cells might be mediated by an indirect mechanism. A recent study has shown that $CD11b^+$ cells responding to IFN-γ can limit CD8 T cell expansion and promote contraction of this population [47].

Histologic examinations revealed that mononuclear cell infiltration and tissue injury in GVHD target tissues was more severe in the recipients of GKO allo-HCT than in those that received WT allo-HCT. It is likely that the augmented donor T cell activation and expression due to reduced apoptosis and accelerated proliferation in the recipients of GKO allo-HCT may result in an overall increase in donor T cell infiltration in GVHD target tissues. The accelerated generation of effector/memory T cells in the recipients of GKO allo-HCT might also contribute to the rapid and severe tissue injury in these mice. Recently, IFN-γ has been shown to inhibit CD8 memory T cell generation in mice after dendritic cell vaccination [17]. In this study, we observed that the ratio of CD8 effector memory ($CD8^+CD44^+CD62L^-$) T cells in the spleen was significantly greater in GKO mice than in WT mice (Figure 6C). Further, GKO donor CD8 T cells lose CD62L expression more rapidly than WT CD8 T cells upon activation, and almost all donor CD8 T cells in the spleens of mice receiving GKO allo-HCT acquired an effector memory phenotype ($CD62L^-CD44^+$) by day 5 after HCT (Figure 3). These results indicate that the egress of activated donor CD8 T cells from lymphoid tissues may occur earlier in the recipients of GKO allo-HCT. The data also suggest that the absence of IFN-γ might provide conditions favoring the differentiation and/or expansion of CD8 effector memory T cells in allo-HCT recipients.

Treg cells have been shown to protect against GVHD [48,49]. Although IFN-γ plays an important role in the generation and function of CD4 Treg cells (Figure 6A) [16], the lack of this cytokine does not lead to reduction in $CD4^-$ Treg cells in the spleen (Figure 6A). Further, the level of $CD4^-$ Treg cells in the spleen is extremely low in WT and GKO mice. Because CD4-depleted allo-HCT was the procedure in our studies, these results suggest that the difference in GVHD between the recipients of WT and GKO allo-HCT was unlikely due to the difference in Treg cell contents in the donor inocula. NKT cells have also been shown to induce allograft tolerance [50] and inhibit GVHD [51,52]. IFN-γ seems to play an important role in NKT-induced allograft tolerance [50], but the GVHD-inhibitory effect of NKT cells has been shown to be dependent on their production of interluekin-4 [51]. Our results show that GKO mice have more $CD4^-CD8^-$ NKT cells in the spleen than WT mice (Figure 6B). Further functional analysis is needed to firmly determine the role of NKT cells in IFN-γ-mediated inhibition of GVHD.

IFN-γ is produced by multiple cell types, including type 1 T cells, NK/NKT cells, and dendritic cells (DCs) [53-56]. Recent studies have demonstrated that a novel DC subset, IFN-producing killer DCs (IKDCs), also produce IFN-γ [57,58]. These cells could serve as the major source of IFN-γ in some tumor models where the tumor cells are poorly recognized by NK cells [58]. However, the role of IKDCs in regulating allogeneic responses remains unknown. The present study demonstrates that donor-derived IFN-γ protects against GVHD, but has not identified the sources of donor IFN-γ that mediate the protective effect.

In summary, our results demonstrate that IFN-γ is an important regulatory cytokine during CD8 T cell activation in vivo. This cytokine inhibits the activation and expansion of donor CD8 T cells by promoting cell death and suppressing cell division and downregulates effector/memory CD8 T cell generation in allo-HCT recipients. Importantly, this cytokine mediates graft-versus-leukemic effects while inhibiting GVHD [30]. Our recent studies have indicated that IFN-γ is required for the optimal induction of GVH reactions that preferentially attack the host lymphohematopoietic cells (Wang H et al, manuscript in preparation), which has been shown to mediate graft-versus-leukemic effects without severe GVHD [59,60]. Further understanding of the molecular mechanisms involved in regulating T cell alloreactivity by IFN-γ will likely shed new light on the development of strategies in clinical GVHD prevention and therapy.

ACKNOWLEDGMENTS

We thank Dr Ronjon Chakraverty (University of Birmingham, United Kingdom) for critical review of the manuscript, Dr Xiaojian Wu for providing technical assistance, Mr Orlando Moreno for outstanding animal husbandry, and Ms Luisa Raleza for expert assistance with the manuscript. This work was supported by grants from American Cancer Society (RSG-03-227-01-LIB) and the National Institutes of Health (P01 CA111519 and R01 CA79989). WA was supported by National Institutes of Health Training Grant 5T32 HD07529-06. YGY was a recipient of the Orphan Medical Scholar Award from the American Society for Blood and Marrow Transplantation (2002-2004) and Outstanding Overseas Scholar Award from the Natural Science Foundation of China (NSFC 30328012).

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