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IFN-γ Production by Allogeneic Foxp3+ Regulatory T Cells Is Essential for Preventing Experimental Graft-versus-Host Disease

Christian Koenenecke,*+,† Chun-Wei Lee,*+,† Kristina Thamm,* Lisa Föhse,* Matthias Schafferus,‡ Hans-Willi Mittrücker,‡ Stefan Floess,§ Jochen Huehn,§ Arnold Ganser,† Reinhold Förster,* and Immo Prinz*

It is emerging that CD4+Foxp3+ regulatory T (Treg) cells can produce the proinflammatory cytokine IFN-γ when stimulated in a Th1 cytokine environment. In this study, we report that Foxp3+ Treg cells readily produced IFN-γ in vivo in a highly inflammatory model of graft-versus-host disease (GVHD) and during a Th1-dominated immune response to intracellular bacteria. Moreover, stimulation in vitro via TCR in the presence of IL-12 alone was sufficient to induce IFN-γ production by Treg cells in a dose-dependent manner. Transfer of donor Treg cells can prevent lethal GVHD; therefore, we used this model as a robust readout for in vivo Treg function. Interestingly, >50% of allogeneic donor, but not residual recipient Foxp3+ Treg cells produced IFN-γ after transplantation, suggesting that this cytokine production was alloantigen specific. These IFN-γ producers were stable Foxp3+ Treg cells because methylation analysis of the Foxp3 gene locus of transferred and reisolated Treg cells during GVHD showed a fully demethylated Treg-specific–demethylated region. Next, we addressed whether IFN-γ production was supporting or rather impairing the immunosuppressive function of Treg cells during GVHD. Blocking of IFN-γ with specific mAb completely abolished the beneficial effect of donor Treg cells. We could further show that only wild-type Treg cells, but not Treg cells from IFN-γ-deficient donor mice, prevented GVHD. This indicated that Treg cell-intrinsic IFN-γ production was required for their protective function. In conclusion, our data show that IFN-γ produced by Foxp3+ Treg cells has essential immune-regulatory functions that are required for prevention of experimental GVHD. The Journal of Immunology, 2012, 189: 2890–2896.

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D4+ Foxp3+ regulatory T (Treg) cells can regulate inflammatory responses and undisputedly play a central role in protecting tissues from immune damage. However, it is much less clear how Treg cell-mediated suppression works. Various mechanisms, including deprivation of IL-2, secretion of inhibitory cytokines, and modulation of DC function via CTLA-4, have been suggested (1, 2). This indicates that modes of Treg cell suppression are context dependent and may also help to explain the apparent heterogeneity in Treg cell phenotypes. In fact, recent studies revealed that Treg cells adopted a Th1-effector cell phenotype during an immune response to infection with Toxoplasma gondii that correlated with a reduced immunosuppressive function (3). Moreover, Treg cells can produce IFN-γ under Th1-polarizing conditions (4, 5). IFN-γ is considered an archetype inflammatory mediator, and the functional consequence of its production by Treg cells is unknown. First indications suggested that IFN-γ production by alloantigen-reactive CD25+ CD4+ Treg cells may be beneficial in preventing rejection of allogeneic skin grafts (6). Yet later the same authors proposed that IFN-γ was produced only transiently by induced Treg cells (7), and more recent reports were even concerned about a harmful conversion of Treg cells into “bad” IFN-γ–producing exFoxp3+ effector T cells that enhance inflammation and thereby may contribute to autoimmunity (8, 9).

In this study, we characterized the generation and function of stable IFN-γ–producing Treg cells in vitro and in vivo. In vitro TCR stimulation in the presence of IL-12 readily resulted in Foxp3+ IFN-γ producers, and in vivo such Treg cells were observed during a Th1 immune response against the intracellular bacterium Listeria monocytogenes and in acute graft-versus-host disease (GVHD). The functional relevance of IFN-γ production in Foxp3+ Treg cells was analyzed in a Th1-driven MHC major mismatch model of acute GVHD (C57BL/6→ lethally irradiated BALB/c). This model allows testing the functionality of Treg cells by a very strong readout, that is, the death or survival of recipient mice. Cotransplantation of bone marrow (BM) cells together with conventional T (Tconv) cells (including CD4+ and CD8+ T cells) from allogeneic donors (C57BL/6) causes lethal GVHD in recipients (BALB/c). The addition of Foxp3+ Treg cells at a 1:1 ratio relative to Tconv cells can prevent the disease and allows long-term survival of recipients (10, 11). In the current study, we show that the majority of allogeneic donor Foxp3+ Treg cells

Abbreviations used in this article: BM, bone marrow; BMT, BM transplantation; GVHD, graft-versus-host disease; TCD, T cell-depleted; Tconv, conventional T; Treg, regulatory T; TSDR, Treg-specific–demethylated region; WT, wild-type.

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produced IFN-γ after transplantation. Using either neutralizing anti–IFN-γ mAb or Treg cells from IFN-γ-deficient (Ifng−/−) donor mice, we demonstrate that IFN-γ secreted by Foxp3+ Treg cells critically contributes to prevent development of lethal GVHD. This indicates that IFN-γ production by allogeneic Treg cells is required for their immunosuppressive function during GVHD and hints at a more universal function of this cytokine beyond its role in classical Th1-type immune responses.

Materials and Methods
Abs, flow cytometric analysis, and cell sorting

Anti-CD4 Pacific Orange (RmCD4-2), anti-CD4 Pacific Blue (GR1.5), anti-CD4 Cy5 (RmCD4-2), anti-CD8 Pacific Orange (RmCD8), and anti-CD11c Pacific Orange (MEL-14) were purified from hybridoma supernatants and conjugated in house. Anti-Thy1.1 (clone HIS51) and anti-CD25 (clone PC61) Abs were purchased from BD Biosciences (San Jose, CA). Anti-CD44 PE (IM7.8.1) was purchased from InVitrogen (Camarillo, CA). Anti–IFN-γ (XM1.2) mAb and anti-TCRB (clone H57-597) were produced with rat hybridoma cell lines. All stainings were performed in PBS supplemented with 0.5% BSA (Biologicals, Berlin, Germany) in the presence of rat anti-mouse CD16/32 Fc block (2.4G2). For measurements of intracellular cytokines, T cells were stimulated with 50 ng/ml PMA (Calbiochem, La Jolla, CA) and SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). For activation, cells were placed in cell culture dishes that had been coated with 1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51) Abs (eBioscience). A quantity amounting to 40 ng/ml ionomycin (Invitrogen) for 1 h, followed by further 3-h incubation in the presence of 1 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO). Cells were fixed using a Fix/Perm buffer set (eBioscience, San Diego, CA), as described in the suppliers’ manual. For intracellular cytokine staining, we used anti-Foxp3 allophycocyanin (clone EJF-16s; eBioscience), anti–IL-17A PE (clone TC11-18H10; BD Biosciences), and anti–IFN-γ PE (XM1.2; BioLegend or eBioscience, both San Diego, CA). FACs data were acquired on LSRII (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR). FACs sorting was carried out at the Cell Sorting Core Facility of Hannover Medical School on the XDP machine (Beckman Coulter, Brea, CA).

Cell culture

Cell culture was performed in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% FCS (Biochrom), 25 mM HEPEs, 1 mM sodium pyruvate, 50 μM 2-ME, and 100 μM penicillin/streptomycin (clone EJF-16s; eBioscience). For activation, cells were cultured in cell culture dishes that had been coated with 1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51) Abs (eBioscience). A quantity amounting to 40 ng/ml mouse rIL-2 (R&D Systems, Minneapolis, MN) was added to all cultures, and as indicated, 0.1 or 1 ng/ml mouse rIL-12 (R&D Systems). On day 2 of culture, the stimulus was removed from the cells. After 5 d of cultivation, the medium was changed and the cells were subjected to intracellular staining.

Mice

Wild-type (WT) C57BL/6 Thy1.2 (H-2Kb) and BALB/c (H-2Kd) were purchased from Charles River Laboratories (Sulzfeld, Germany). C57BL/6 Foxp3GFP Thy1.1 (H-2Kb) reporter mice (12) and C57BL/6, 129S7-Ifng−/− (H-2Kb) mice (Ifng−/−) were bred at the central animal facility of Hannover Medical School under specific pathogen-free conditions. Foxp3gfp reporter mice. For preparation of TCD BM, BM cells were harvested from the femurs and tibias, stained with anti-TCRβ biotin (clone H57-597) and streptavidin-magnetic beads (Miltenyi Biotech). In some experiments, 1 μg anti–IFN-γ mAb (clone XM1.G2.1) or PBS was administered i.p. to transplant-recipient mice at day −1 before and day +3 after transplantation. After transplantation, mice were kept on antibiotic water (Cotrimoxazol; Ratiopharm, Ulm, Germany) for the first 21 d. Death of recipient mice without clinical signs of GVHD within 1 wk after transplantation was considered as non-GVHD mortality, and mice were excluded from the final analysis. Survival, weight loss, and clinical signs of GVHD were monitored daily. The outcome in independent survival experiments was similar irrespective of the applied isolation strategy (MACS or FACs).

L. monocytogenes infection of mice

C57BL/6-DEREG mice (14) were infected with a laboratory L. monocytogenes strain (15, 16). Mice received 2.5 × 104 bacteria via the lateral tail vein. The inoculum was always controlled by plating of serial dilutions. Mice were analyzed 8 d postinfection.

Treg-specific–demethylated region methylation analysis

CD4+Foxp3+ T cells from C57BL/6 Foxp3GFP Thy1.1 reporter mice were FACs sorted before allogeneic BM transplantation (BMT; input cells) or reisolated from recipients’ spleens and sorted 4 d after transplantation (output cells). DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and bisulfite sequencing of the Treg-specific–demethylated region (TSDR; Amplicon 2) was performed, as described previously (17).

Data analysis and statistics

Statistical analysis was performed with Prism 4 (GraphPad Software, La Jolla, CA). All significant values were determined using the unpaired two-tailed t test, and all error bars represent SEM or SD, as indicated in the figure legend. To analyze survival as shown in Figs. 5 and 6, log-rank test for statistical analyses was used. Statistical differences are shown as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Results

Treg cells produce IFN-γ in vivo

In an experimental model for acute GVHD, we observed high frequencies of IFN-γ+ Treg cells in allograft recipient mice. At day 4 after transplantation, >50% Thy1.1+ cells (derived from the donor Treg cell graft) readily produced IFN-γ, as measured by intracellular cytokine staining upon restimulation with PMA/ ionomycin (Fig. 1A, 1C). This excludes the possibility of conversion from Foxp3+ donor Treg cells to pathogenic Th17 cells and underlines the Th1-polarizing conditions in this in vivo model. Without alloantigen recognition, Foxp3+ donor T cells transferred to lethally irradiated syngeneic recipients barely produced any IFN-γ (Fig. 1C). Further evidence for the need of alloantigen recognition for IFN-γ production was found when Thy1.1+ allogeneic donor Treg cells (C57BL/6) were compared with residual Thy1.2+ recipient Treg cells (BALB/c) within the same mouse (Fig. 1D). In this

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study, IFN-γ production was largely confined to allogeneic donor Treg cells (Fig. 1D). These constituted ~20% of all IFN-γ-producing cells 4 d after transplantation, as demonstrated by transfer of donor Tconv and donor Treg cells (both C57BL/6) with separate Thy1 isoforms (Fig. 1E). To corroborate these findings in a different model, we analyzed Treg cells in a Th1 cytokine-dominated infection model with the intracellular pathogen <i>L. monocytogenes</i>. At the peak of the Th1 cell-dominated adaptive immune response, that is, at day 8 postinfection (15), we consistently found that 10–15% Foxp3<sup>+</sup> Treg cells in infected, but not in uninfected controls produced IFN-γ upon restimulation with PMA/ionomycin (Fig. 2A). However, the frequency of IFN-γ<sup>+</sup> Treg cells was lower than the respective proportion of IFN-γ<sup>+</sup> cells among Foxp3<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 2B). In summary, these results demonstrate that Treg cells can readily produce IFN-γ in vivo when stimulated in a Th1-type cytokine environment.

**IFN-γ production by stable Foxp3<sup>+</sup>CD4<sup>+</sup> T cells is stimulated by IL-12**

Our previous results suggested that only stable alloantigen-specific Foxp3<sup>+</sup> donor Treg cells exerted a beneficial effect on the outcome of GVHD (18). To assess whether the IFN-γ-producing Foxp3<sup>+</sup> Treg cells during GVHD were actually stable Treg cells, we analyzed the selective demethylation of an evolutionarily conserved element (17) within the Foxp3 locus-named TSDR of Treg cells before and after allogeneic transplantation. To this end, FACS-sorted donor Treg cells (input) were compared with donor Treg cells reisolated from recipients based on Foxp3EGFP reporter and Thy1.1 expression (output). We found that both the input and output populations presented a fully demethylated TSDR (Fig. 3). Given that the analyzed output population comprised a high proportion of IFN-γ-producing Foxp3<sup>+</sup> Treg cells (~50%; see also Fig. 1A, 1B), the IFN-γ-producing Foxp3<sup>+</sup>CD4<sup>+</sup> Treg cells in the applied GVHD model were indeed stable, but not transiently induced Treg cells. Next, we tested which Th1 stimuli would be required to drive IFN-γ expression by Treg cells. Titrating the master Th1-polarizing cytokine IL-12 to in vitro stimulated Treg cells, we observed that IL-12 alone was sufficient to induce IFN-γ production by Foxp3<sup>+</sup> Treg cells in a dose-dependent manner (Fig. 4A, 4B). This does not rule out that IL-12 can selectively expand IFN-γ-producing Treg cells. However, control experiments in which IFN-γ-secreting cells were excluded from the start population by IFN-γ secretion assay confirmed de novo induction of IFN-γ production by stimulated Foxp3<sup>+</sup> Treg cells in the presence of IL-12 (data not shown). In vitro culture in the presence of IL-12 also induced expression of the Th1 master transcription factor T-bet (Fig. 4C). Thus, IL-12 plays a pivotal role in controlling T-bet induction and IFN-γ production by Foxp3<sup>+</sup> Treg cells.

**Inhibition of IFN-γ with mAb blocks the beneficial effect of Treg cells in acute GVHD**

Next, we tested the hypothesis that IFN-γ would indeed contribute to effective immunoregulation by Treg cells in acute GVHD. In a first approach, we monitored survival of recipient mice that were cotransplanted with allogeneic Treg cells in the presence or absence of anti–IFN-γ blocking mAb. Thereby, we found a loss of protection conferred by Treg cell cotransfer in the anti–IFN-γ–treated as compared with the PBS-treated control group (Fig. 5A). Control mice receiving only Tconv cells died considerably faster with anti–IFN-γ mAb treatment (Fig. 5B), which is in line with the current literature showing that donor-derived IFN-γ can be protective in GVHD (19–21). However, anti–IFN-γ mAb treatment had no malicious effect for recipient mice transplanted with allogeneic Treg cells alone (Fig. 5B). Taken together, these
experiments suggested that systemic blocking of IFN-γ in acute GVHD did not turn allogeneic donor Treg cells into pathogenic effector cells, but, however, abolished their beneficial immunoregulatory effects.

Treg cell-intrinsic IFN-γ is critical for efficient prevention of GVHD

Next, we asked whether IFN-γ production by the Treg cells themselves was required for their suppressive function in vivo. Therefore, we purified Treg cells derived from WT and Ifng2/2 donors (both C57BL/6) to test their protective function in the GVHD model. Positive selection based on CD25 yielded percentages of 95% Foxp3+ cells for WT and Ifng2/2 mice (Fig. 6A). Moreover, samples from both genotypes contained a similar fraction of CD62L+ Treg cells (Fig. 6B), which were described to

FIGURE 2. Treg cells produce IFN-γ in L. monocytogenes infection. C57BL/6-DEREG mice were i.v. infected with 2.5 × 10⁴ L. monocytogenes. On day 8 postinfection, spleen cells of infected and control mice were stimulated in vitro for 4 h with PMA/ionomycin or with the peptide listeriolysin O₁₈₀₋₂₀₁ or remained without stimulation. Cells were analyzed by flow cytometry. (A) Bar graphs show frequencies of Treg cells with intracellular IFN-γ. (B) Bar graphs show frequencies of conventional CD4+ T cells with intracellular IFN-γ. Bars represent mean and SD of cells from three individually analyzed mice. The result is representative of three independent experiments. (C) Contour plots show concanaval intracellular IFN-γ and surface CD4 staining of three control (left) or three infected (right) mice, gated on Foxp3+ T cells.

FIGURE 3. Foxp3+ Treg cells show demethylated CpG motifs within the foxp3 locus before and after allogenic BMT. Foxp3+ or Foxp3+ CD4+ Thy1.1+ donor T cells from Foxp3EGFP mice were sorted by FACS, and methylation patterns of CpG-island amplicons of the foxp3 locus were analyzed before transplantation (input cells) or after reisolation from recipients 4 d after allogenic BMT (output cells). The methylation status of individual CpG motifs is color coded according to the panel on the right side. Two independent experiments are shown.

FIGURE 4. IFN-γ production of Treg cells in vitro. FACS-sorted CD4+ Foxp3+ T cells from Foxp3gfp-reporter mice were stimulated in vitro with plate-bound anti-CD3/anti-CD28 and IL-2 in the presence of increasing concentrations of mouse rIL-12. (A) Contour plots show intracellular IFN-γ and surface CD4 staining after 5 d of culture, gated on Foxp3+ T cells. (B) Bar graphs show mean and SD from triplicate wells representative of one of four individual cultures with similar results. (C) Expression analysis of T-bet in IFN-γ–producing CD4+Foxp3+ T cells after stimulation and culture, as described in (A), in the presence or absence of IL-12. Data are combined from two independent experiments.

Treg cell-intrinsic IFN-γ is critical for efficient prevention of GVHD

Next, we asked whether IFN-γ production by the Treg cells themselves was required for their suppressive function in vivo. Therefore, we purified Treg cells derived from WT and Ifng−/− donors (both C57BL/6) to test their protective function in the GVHD model. Positive selection based on CD25 yielded percentages of >95% Foxp3+ cells for WT and Ifng−/− mice (Fig. 6A). Moreover, samples from both genotypes contained a similar fraction of CD62L+ Treg cells (Fig. 6B), which were described to

FIGURE 5. Inhibition or absence of Treg-derived IFN-γ is critical for prevention of GVHD. (A) Survival curves of allogeneic BMT recipients treated with TCD BM alone (n = 3) or TCD BM and Tcvm cells with additional Treg cells, PBS, or anti–IFN-γ mAb. C57BL/6 (donor), BALB/c (recipient). Treg cells and Tcvm cells were purified using MACS isolation: Tconv + PBS (n = 12) versus Tconv + Treg + PBS (n = 12), p = 0.0128; Tconv + PBS (n = 12) versus Tconv + Treg + anti–IFN-γ mAb (n = 7), p = 0.6517; Tconv + Treg + PBS (n = 12) versus Tconv + Treg + anti–IFN-γ mAb (n = 7), p < 0.0001. Data are pooled from two independent experiments. To analyze survival, log-rank test for statistical analyses was used. *p ≤ 0.05, **p ≤ 0.001. (B) Survival curves of allogeneic BMT recipients (BALB/c) treated with TCD BM and Tcvm + anti–IFN-γ mAb (n = 11) or Tcvm + anti–IFN-γ mAb (n = 6) from C57BL/6 donors. Data are pooled from two independent experiments.
contain the protective fraction of Treg cells in this model (22). Strikingly, monitoring GVHD by survival analysis revealed that Ifng−/− Treg cells lacked the capacity to suppress pathogenic alloreactive donor T cells because their presence, in contrast to WT Treg cells, did not inhibit disease progression (Fig. 6C). In line with our results obtained from anti–IFN-γ treatment above, conventional Ifng−/− donor T cells induced an accelerated course of the disease (Fig. 6D), supporting the notion that IFN-γ derived from effector T cells is also critical for the course of acute GVHD (19–21). To exclude that the loss of suppressive function observed for Ifng−/− Treg cells may not be cell intrinsic, but rather caused by contamination of highly pathogenic conventional Ifng−/− donor T cells, we included a control group of mice receiving WT Treg cells plus an additional deliberate contamination of 2% Ifng−/− donor T cells (Fig. 6C). These recipient mice showed a similar prevention of the disease. Furthermore, IFN-γ deficiency alone did not turn the donor Treg cells into pathogenic effector T cells, because recipients that received only BM and Ifng−/− Treg cells did not develop GVHD (Fig. 6D).

**Discussion**

This study identified IFN-γ production as a novel effector mechanism of natural Treg cells in GVHD. IFN-γ production was intrinsic, albeit not exclusive, to Foxp3+ Treg cells and depended on allogeneic Treg stimulation. Of note, IFN-γ production by natural Foxp3+ Treg cells seems to occur only in Th1-type inflammatory environments, and is thus differentially regulated to other Treg cell-derived effector cytokines, such as the recently described TNF-α (23). To date, IFN-γ was reported to play manifold roles in the pathophysiology of acute GVHD (24–26). IFN-γ was considered a proinflammatory pathogenic factor because it is important for macrophage activation (27) and stimulates cytotoxic CD8+ T cells and NK cells. However, IFN-γ protected lethally irradiated mice after induction of CD4 T cell-mediated Th1-type experimental GVHD (20, 28). In experimental GVHD, IFN-γ mediated protective effects of IL-12 and IL-18 (19, 21, 29), inhibited cell division and promoted cell death of alloreactive donor T cells (30), and prevented tissue damage through interaction with recipient tissue (31). Although a dichotomous role of IFN-γ in inflammation and immunoregulation has been proposed (24, 25, 32, 33), it is still unclear whether Treg cell-derived IFN-γ has a direct function for their suppressive activity. A recent report by Dominguez-Villar et al. (4) identified human Treg cells expressing IFN-γ and reduced suppressive activity. These were increased in the peripheral blood of individuals with multiple sclerosis. In contrast, effective inhibition of allosresponses seems to depend on allogeneic-specific Treg cells (6, 34). It is now emerging that IFN-γ production by natural Treg cells can be important for their suppressive function in transplantation by autocrine activation through IFN-γ receptor signaling (35). Such autocrine activation may also explain why it is so important that IFN-γ is produced by the Treg cells themselves to inhibit acute GVHD. Furthermore, it was suggested recently that IFN-γ production identified Ag-specific Treg cells that may be especially effective in inhibiting immune response to viral infection (36).

Thus, it is presently not clear whether IFN-γ production by Treg cells is also important for their immunoregulatory function in other, nonallogeneic settings. However, our results showed that Treg cells produced IFN-γ at the peak of a Th1 immune response to the intracellular pathogen L. monocytogenes. Our in vitro stimulation data suggest that the Th1-polarizing cytokine IL-12 is also a key regulator of IFN-γ production by Foxp3+ Treg cells. Further studies will have to clarify whether Treg cell-derived IFN-γ in microbial infections supports their suppressive function, for example, by inducing Treg cells with optimized migratory properties for the suppression of Th1 responses (37) or whether Treg cells would adopt a Th1 effector phenotype, as suggested by Oldenhove et al. (3).

Based on the data of the current study, we propose that IFN-γ production indeed represents a mechanism of how Treg cells regulate immune responses in the lymphopenic and inflammatory environment of acute GVHD. But what would be the mechanism of how IFN-γ would suppress T cell responses? Principally, one would argue that IFN-γ derived from Treg cells should act in similar ways as IFN-γ from other cellular sources. It is conceivable that missing IFN-γ would favor a deviation of T cell responses from Th1 to other Th types, in particular toward Th17, and thereby contribute to or even exacerbate GVHD (38–40). Furthermore, it is emerging that efficient immune regulation by Treg cells requires the expression of transcription factors such as T-bet that are typically associated with effector T cell function (41). It is possible that IFN-γ would directly influence Treg function via induction of relevant chemokine receptors such as CXCR3, which was suggested to guide Treg cells to the target organs of GVHD (37, 42). Another mechanism how Treg-derived IFN-γ may be involved in suppression of GVHD could be via the induction of NO synthesis by macrophages, granulocytes, and potentially other cells (32, 43). Notably, a recent study suggested...
that in transplantation settings IFN-γ may lead to preferential expansion of Treg cells and death of effector cells (44). The an- swer how IFN-γ derived from Treg cells and other cellular sources share immune stimulatory and regulatory functions probably lies in the “where and when” of IFN-γ production by Treg cells as compared with Tconv cells.

We further support the notion that natural Treg cells are a stable lineage that retains its powerful immunosuppressive function even under the lymphopenic and inflammatory conditions of the employed model for acute GVHD (18, 45, 46). Thereby, the potentially proinflammatory cytokine IFN-γ was produced by transferred Treg cells that were not found to be “pathogenic Ex-Foxp3+ T cells,” as suggested by Zhou et al. (9), but by bona fide stable Treg cells with a fully demethylated TSDR (17). Rather, IFN-γ was required for the immunoregulatory function of the Treg cells in this experimental setting. By showing that IFN-γ-producing donor Treg cells may be beneficial for the graft recipient in acute GVHD, we rehabilitate those Treg cells that were suspected to turn into potentially pathogenic IFN-γ producers under lymphopenic or inflammatory conditions. In conclusion, our findings thus should be reassuring for those who observe IFN-γ production by Treg cells while establishing or already performing clinical applications of Treg infusion to prevent or treat human disease.

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Disclosures
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References


