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IL-10 exacerbates xenogeneic GVHD by inducing massive human T cell expansion

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Abstract

Although patients with GVHD have elevated serum levels of IL10, whether its role is protective or pathogenic remains unclear. Here, we used a humanized mouse model to study the role of IL-10 in GVHD. When human PBMCs were engrafted in NOD-scid IL2 γ ^{null} mice expressing human IL-10, the T cells underwent massive expansion resulting in lethality by day 21, whereas control mice survived for at least 40 days. Histopathology of the liver showed extensive mononuclear cell infiltration in IL-10 expressing but not in control mice. Corresponding to their aggressiveness, the T cells in the IL-10 group exhibited predominantly an effector memory phenotype (CD45RO⁺CD27⁻) while in control mice, the T cells were of transitional memory phenotype (CD45RO⁺CD27⁺). Further, IL-10 receptor blocking antibody was able to protect the animals from GVHD. Since our results demonstrate a direct pathogenic role for IL-10, blockade of IL-10 signaling may provide a therapeutic option for GVHD.

Keywords

GVHD; IL-10; PBMCs; NSG mice

1. Introduction

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality following allogeneic bone marrow transplantation (alloBMT). GVHD is mediated by donor T cells that expand upon encountering genetically disparate antigens and act in concert with proinflammatory cytokines to initiate a dynamic course of tissue destruction. Proinflammatory cytokines can also exacerbate GVHD by driving T cell expansion and

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trafficking to target organs. Hence it is no surprise that suppressing cytokines like IFN- γ , TNF- α and IL-1 that are known to contribute to GVHD pathogenesis has been a subject of intense investigation [1]. We have recently reported that expression of human IL-2 by hydrodynamic delivery of plasmids in NOD-scid IL2 γ c^{null} (NSG) mice accelerates human PBMC-induced xenogeneic GVHD [2].

In contrast, the role of the immunoregulatory cytokine IL-10 in GVHD pathogenesis remains ambiguous [3], [4] and [5]. Data from several studies suggest that there is a strong correlation between the severity of clinical GVHD and serum IL-10 levels [6]. As IL-10 is an immunosuppressive cytokine, this may represent a compensatory increase to suppress the exaggerated T cell response. In keeping with this view, elevated levels of IL-10 in recipients prior to BMT or presence of genetic polymorphisms in recipients associated with higher production of IL-10, correlate with reduced incidence or severity of GVHD [7] and [8]. However, on the flipside, recent studies have shown that the kinetics of IL-10 expression is highly dependent on clinical activity of GVHD [9]. IL-10 has also been suggested to be a critical mediator for the development of autologous GVHD [10]. In mouse studies, the outcome has varied depending on the dose and timing of IL-10 administration. Prior IL-10 treatment was beneficial, whereas post-transplant administration was found to have a deleterious effect. Similarly, low dose was found to be protective, while high doses exacerbated GVHD [4]. Collectively these data highlight the pleiotropic, sometimes opposing, nature of the effects of IL-10 on various aspects of GVHD pathogenesis. Here, we used a clinically relevant humanized mouse model to delineate the role of IL-10 in modulating GVHD. Our results suggest an unexpected role of IL-10 in driving the expansion of xenoreactive human T cells and enhancing the severity GVHD in NSG mice.

2. Methods

2.1 Animals and hydrodynamic injection of plasmids

NSG mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in specific pathogen free condition at the Paul L. Foster School of Medicine, TTUHSC animal facility. All experiments were done with 6-8 week old mice using study protocols approved by the TTUHSC IRB and IACUC committees. Human IL-10 expressing plasmid was obtained from InvivoGen (San Diego, CA, USA) and hydrodynamic injection was performed as previously reported [11].

2.2 Induction of xenogeneic GVHD and neutralization of IL-10

Human PBMCs were isolated from blood collected from healthy adult volunteers under an institutional review board (IRB) approved protocol. Six to eight week old control and hIL-10 expressing NSG mice were injected with PBMCs (1×10^7 or 3×10^7) through the tail vein. The animals were bled periodically by retro-orbital puncture to monitor human T cell reconstitution and observed daily for symptoms of GVHD such as hunched back, ruffling of hair, diarrhea and weight loss. Animals were sacrificed when they lost 20% of their body weight. For IL-10 neutralization experiments, 100 μ g of anti-IL-10R (R&D systems) antibody was injected twice a week starting 6 days after human cell reconstitution for a total of 5 injections.

2.3 ELISA, flow cytometry and cytokine bead array

To determine IL-10 levels in the serum, animals were bled periodically through retro-orbital puncture. IL-10 and IFN- γ serum levels were determined using commercial ELISA kits according to the manufactures instructions (Biolegend Inc, San Diego, CA, USA). For flow cytometric analysis, various fluorescent conjugated antibodies to human CD3, CD4, CD8, CD27, CD45, CD45 RO, Ki-67 and pSTAT3 were used (BD Biosciences San Diego, CA) or Tonbo Biosciences (San Diego, CA). Single cell suspensions were prepared from blood and spleen by standard procedures, cells were surface stained with appropriate combinations of mAbs and used for flow cytometric analysis directly or after intracellular staining with pSTAT3 or Ki-67 antibody. To test IL-10 responsiveness single cell suspensions prepared from spleen were treated with 100 ng/ml of recombinant hIL-10 (R&D systems) for 15 minutes before staining for CD3, CD4, CD8 and pSTAT3. To measure serum levels of IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ and TNF- α human Th1/Th2/Th17 Cytokine Bead Array (BD Pharmingen, San Diego, CA) was used according to manufactures instructions.

2.4 Cell sorting, RNA isolation and real time PCR

Single cell suspensions isolated from mouse splenocytes were stained with anti-human CD4, CD8 and CD45 antibodies and sorted using the FACS Aria machine (BD Biosciences). Sorted CD4⁺ and CD8⁺ cells with over 95% purity were pooled together and total RNA isolated from the cells with the RNeasy kit (Qiagen). cDNA was prepared using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen). qRT-PCR was carried out with the 7900 HT Fast Real Time PCR system using SYBR green detection master mix (Applied Biosystems, Foster City, CA). Primer pairs were as follows: IFN- γ (sense: 5'-TGTGGAGACCATCAA GGAAGAC-3', ant-sense, 5'-IFN- γ -R: CTTTGCGTTGGACATTCAAG-3') and β -actin (Sense: 5'-AGAGCTACGAGC TGCCTGAC-3', anti-sense 5'-TTCGTGGATGCCACAGGAC-3'). Bcl-2 primers were obtained from Qiagen.

2.5 *In-vitro* cytokine production assay

Cytokine production was quantified using the BD Cytofix/ Cytoperm Kit and GolgiStop™ (BD Biosciences), according to the manufacturer's instructions. Splenocytes were recovered from mice injected with PBMCs in presence and absence of hIL-10. After lysing the red blood cells, 1×10^6 cells were mock-stimulated or stimulated with phorbol myristate acetate (PMA) (0.5 mg/ml) and ionomycin (0.5 mg/ ml) in the presence of GolgiStop™ (0.1 mg/ml) for 6 h at 37°C in 5% CO₂. Cells were surface stained first with anti-human CD45, CD4 and CD8 antibodies to identify human T cells. The cells were then fixed and permeabilized using Cytofix/Cytoperm solution and stained with mAb to IL-2, IFN- γ or TNF- α (BD Biosciences).

2.6 Histological analysis

For histological examination, liver tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μ m) were cut and stained with hematoxylin and eosin.

3. Results and Discussion

It has been previously reported that NSG mice reconstituted with 1×10^7 human PBMCs remain free of GVHD for around 30 days [12] and [13]. We wished to express human IL-10 in this mouse model to test its role in GVHD. First, we injected NSG mice hydrodynamically with either control or hIL-10-encoding plasmid. Mock treated NSG mice were also used as control in some experiments. Control and IL-10 expressing NSG mice were transplanted with 1×10^7 human PBMCs and monitored for GVHD progression over time. As shown in Fig. 1A, more than 90% of IL-10 expressing animals succumbed to GVHD between 15 and 21 days, whereas control animals survived symptom free for up to 40 days when the experiment was terminated. This IL-10 induced GVHD acceleration was also evident when GVHD was induced with 3×10^7 PBMCs (Fig. 1A).

To understand the basis for the accelerated GVHD progression in presence of IL-10, we first analyzed the kinetics of human cell engraftment. In keeping with the known immunosuppressive role of the cytokine, very low numbers of CD45⁺/CD3⁺ human T cells were seen at earlier time points in animals expressing IL-10 as compared to controls (Fig. 1B). Surprisingly, after this initial delay, in the same animals, there was a massive expansion of human T cells over the course of just one week. Consistent with GVHD induction, morbid mice developed hunched posture, inactivity and weight loss (Fig. 1C). Analysis of the absolute number of human T cells at the time of euthanasia revealed a five-fold increase in T cell numbers in IL-10 expressing mice as compared to controls (Fig. 1D). We also evaluated the serum IL-10 level over time. The data showed high levels of IL-10 at 2 days post IL-10 plasmid injection (~ 800 ng/ml), but gradually declined thereafter and was nearly 200 fold lower (~ 4 ng/ml) at the time of euthanasia (Supplementary Fig. 1). However, as expected, at the time of euthanasia, IL-10 levels in the serum of these animals were significantly higher compared to mock treated animals (Fig. 1E). Histopathological analysis of liver showed marked mononuclear cell infiltration and associated organ destruction in IL-10 expressing animals, compared to limited infiltration observed in control animals (Fig. 1F).

We also investigated the serum levels of human Th1, Th2 and Th17 cytokines in the animals. Cytokine bead array analysis showed a similar pattern for most cytokines in both groups, with high levels of IFN- γ and undetectable levels of IL-2, IL-4, TNF- α , and IL-17A (data not shown). The high level of IFN- γ was confirmed by ELISA, which was also observed when the experiment was repeated with a higher dose (3×10^7) of PBMCs (Fig. 2A). However, when RNA extracted from purified splenic human T cells from the animals were subjected to qRT-PCR analysis, the data showed a 2 fold lower IFN- γ mRNA level in IL-10 treated compared to control animals (Supplementary Fig. 2). The higher serum IFN- γ level observed in IL-10 treated animals likely reflects the contribution from the numerically higher numbers of T cells in this setting. Overall, the undetectable levels of either IL-4 or IL-17 in association with the presence of elevated levels of serum IFN- γ in both control and IL-10 treated animals points to a predominantly Th1 response in both groups.

It has been shown recently that T cells in models of xeno-GVHD exhibit an effector memory phenotype [12]. To test the influence of IL-10 on the phenotypic composition of the

responding cells in our model, we stained human cells from the animals with antibodies to human CD3, CD4, CD8, CD27 and CD45 RO. In IL-10 expressing mice, effector memory cells (CD45 RO⁺ CD27⁻) were preferentially expanded, whereas in control mice the T cells were mostly of transitional memory (CD45 RO⁺CD27⁺) phenotype (Fig. 2B and C). To further confirm the differentiation status of T cells expanding in the presence of IL-10, we evaluated intracellular IL-2, TNF- α and IFN- γ production by human CD4⁺ and CD8⁺ T cells from spleens of control and IL-10 treated NSG mice after *ex vivo* stimulation with PMA and ionomycin (Fig. 2D and E). Flow cytometric analysis revealed that compared to controls, CD4⁺ and CD8⁺ T cells recovered from IL-10 treated group produced higher levels of TNF- α (16.33 $\pm$ 0.99 vs 10.10 $\pm$ 1.43 and 8.683 $\pm$ 0.90 vs 1.760 $\pm$ 0.25 for CD4⁺ and CD8⁺ T cells respectively). On the other hand IL-2 expression was similar between the two groups for CD8⁺ T cells (2.84 $\pm$ 0.31 for control vs 2.5 $\pm$ 0.38 with IL-10) but was significantly lower in CD4⁺ T cells from IL-10 treated mice compared to controls (16.26 $\pm$ 1.93 vs 4.93 $\pm$ 0.70 with control and IL-10 respectively). IFN- γ expression in CD4⁺ and CD8⁺ T cells was found to be uniformly high in both groups (44.40 $\pm$ 4.32 vs 36.50 $\pm$ 2.94 and 30.40 $\pm$ 2.70 vs 29.83 $\pm$ 3.43 for CD4⁺ and CD8⁺ T cells respectively). Taken together, IL-10 appears to promote effector differentiation of T cells in this model.

The significant increase in the T cell numbers observed in the presence of IL-10 may be due to either increased T cell proliferation or decreased T cell apoptosis. It is now recognized that IL-10 is capable of directly affecting the growth and development of a variety of cells [14]. On the one hand, IL-10 can directly promote the death of inflammatory cells, including activated macrophages, T cells and neutrophils [15], [16] and [17]. However, in contrast to its proapoptotic function, IL-10 has also been shown to increase the expression of the anti-apoptotic molecule Bcl-2, that enhances cell survival [18] and [19]. Hence, we evaluated Bcl-2 expression on isolated T cells by RT-PCR. However, we could not detect any significant differences in Bcl-2 expression between control and IL-10 treated groups (data not shown). Next we determined if there is any difference in the proliferation capacity of the repopulating T cells between the two groups, by assessing the expression of Ki-67. As shown in Fig. 2F, Ki-67 expression was 2 fold higher in CD3⁺ T cells from IL-10 treated animals as compared to controls, which indicates that IL-10 promotes cell proliferation in this model.

As we observed initial inhibition of T cell expansion with IL-10 followed by the massive expansion, we also investigated whether this was due to outgrowth of clones that became unresponsive to the cytokine. STAT3 is activated downstream of IL-10 signaling [14] so, we evaluated its phosphorylation status after stimulating isolated human T cells with the cytokine *ex vivo*. T cells from both control and IL-10 expressing mice showed similar STAT3 phosphorylation pattern in response to IL-10 (Fig. 3A and B) which rules out the possibility of an outgrowth of IL-10 resistant T cells. We then tested whether the T cell expansion was driven by IL-10 itself. For this, we used a previously reported anti-IL-10R antibody to block the effect of the cytokine [20] and [21]. Human PBMC reconstituted IL-10 expressing animals were treated with anti-IL-10R or isotype control antibody twice a week for a total of 5 injections and effect on the course of GVHD was evaluated. As shown the Fig. 3C, 8 out of 10 animals succumbed to GVHD in the presence of IL-10 alone, whereas

only 1 out of 10 animals died when the cytokine binding was blocked with anti-IL-10R antibody. Other manifestations of GVHD including weight loss, T cell expansion and histopathological changes were also drastically reduced (Fig. 3D- F).

To our knowledge our results are the first to demonstrate that human IL-10 potently activates and expands pathogenic human T cells with capacity to induce xenogeneic GVHD. Xenogenic GVHD in severely immunocompromised NSG mouse model may not exactly mimic allogeneic GVHD in the clinical setting. However, our finding is consistent with several reports showing high serum levels of IL-10 in patients with GVHD [6]. The nanogram/ml range of serum IL-10 levels that we observed in the animals is comparable to that in patients with GVHD post BMT [6]. IL-10 is known to have pleiotropic effects that may serve to ameliorate or exacerbate GVHD [3], [4] and [5]. We noted a delayed kinetics of T cell expansion with IL-10, which suggests an initial suppressive effect. However, this was followed by massive increase in T cell numbers within a short span of one week. As human APCs are likely to be short-lived in this model, differential effects of IL-10 on APCs and T cells might partly account for this biphasic response. In fact, IL-10 is known to down regulate expression of accessory/adhesion molecules, chemokines and cytokines on monocytes/macrophages, which can inhibit their T cell-activating capacity [14]. As we observed a lag in expansion of both CD4⁺ and CD8⁺ T cells, it is possible that the suppression is mediated via APCs during initial priming. In this context it is important to note that IL-10 can act on both mouse and human APCs [22], [23] and [24]. It is possible that some CD4⁺ and CD8⁺ T cells that become activated despite the presence of suppressive APCs expand and undergo effector differentiation in presence of IL-10 leading to exacerbation of GVHD. This hypothesis is strongly supported by our ability to reverse GVHD progression by IL-10R blockade. In agreement with our data, studies in mice and humans with IL-10 signaling defect show increased rate of tumor development implying a positive role for IL-10 in development of anti-tumor T cell responses [25]. Further, IL-10 is known to induce intratumoral expansion of tumor-resident CD8⁺ T cells in mouse tumor models [26]. These reports corroborate our data suggesting a stimulatory role of IL-10 in the expansion and effector differentiation of activated CD4⁺ as well as CD8⁺ T cells leading to exacerbation of GVHD.

4. Conclusion

Our results demonstrate for the first time, a direct role for IL-10 in driving GVHD pathogenesis. These finding calls for caution against using rhIL-10 as an immunosuppressive cytokine to treat GVHD and in fact, indicate that blockade of IL-10 may provide a therapeutic option.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GVHD	Graft versus host disease
IL-10	Interleukin 10
PBMCs	Peripheral blood mononuclear cells
NSG Mice	NOD-scid IL2 γ ^{null} mice

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Highlights

- Human IL-10 has a direct pathogenic role in graft versus host disease.
- IL-10 exacerbates GVHD by inducing massive expansion of T cells.
- The pathogenic effects of IL-10 can be reversed *in vivo* by IL-10R blockade.
- Blockade of IL-10 signaling may provide a therapeutic option to treat GVHD.

Figure 1.

Human IL-10 accelerates the induction of xenogeneic GVHD in NSG mice. Kaplan-Meier survival curve comparing the survival of mock (n=4), IL-10 conditioned (n=15) or vector injected (n=5) NSG mice transplanted with 1×10^7 hPBMCs (A). In mock and IL-10 plasmid injected (n=8) animals, survival was also compared after transplantation with 3×10^7 PBMCs (A). Kinetics of human T cell engraftment in presence and absence of human IL-10 in NSG mice transplanted with 1×10^7 hPBMCs (B). In the same animals, weight loss was monitored as a marker of GVHD progression (C). IL-10 conditioned and control animals sacrificed between day 15-21 and day 22 respectively were analyzed for absolute human T cells ($CD45^+/CD3^+$) numbers in whole spleen (D) by flow cytometry and serum IL-10 levels (E) by ELISA. Representative hematoxylin and eosin stained sections of liver showing mononuclear cell infiltration in the presence and absence of IL-10 (F). Data are representative of at least two different PBMC donors and each symbol represents an individual mouse.

Figure 2.

Accelerated GVHD in presence of IL-10 is associated with enhanced effector differentiation. IFN- γ levels in sera of control and IL-10 expressing animals injected with either 1×10^7 PBMCs or 3×10^7 PBMCs measured by ELISA at the time of euthanasia (A). Flow cytometric analysis of CD45 RO and CD27 expression on human T cells isolated from NSG mice in presence and absence of IL-10 (B-C). Representative data for human CD4⁺ and CD8⁺ T cells (B) and cumulative data (C) for each group are shown. Intracellular cytokine expression of IL-2, TNF- α and IFN- γ in human CD4⁺ (D) and CD8⁺ (E) T cells were analyzed by flow cytometry after stimulating splenocytes from reconstituted NSG mice *ex vivo* with PMA and ionomycin for 6 hrs in presence of GolgiStopTM. Flow cytometric analysis of Ki-67 expression on human T cells isolated from NSG mice in the presence and absence of IL-10 (F). Data are representative of at least two different PBMC donors and each symbol represents an individual mouse.

Figure 3.

Blocking IL-10 receptor protects NSG mice from IL-10 mediated GVHD enhancement. Spleen cells isolated from 1×10^7 PBMCs transplanted NSG mice were exposed to recombinant IL-10 (100 ng/ml) for 15 min *ex vivo* and analyzed for pSTAT3 (Y705) levels on human T cells by flow cytometry. pSTAT3 levels on unstimulated and IL-10 stimulated CD4⁺ (A) and CD8⁺ (B) T cells are shown. Comparative Kaplan-Meier survival analysis of NSG mice (n=10) injected with 1×10^7 PBMCs and IL-10 plasmid in presence and absence of IL-10R neutralizing antibody (C). Mock treated animals (n=10) injected with 1×10^7 PBMCs served as controls (C). Weight loss as a marker of GVHD progression (D), absolute number of human T (CD45⁺/CD3⁺) cells in whole spleen (E) and representative histopathology sections of the liver (F) are shown for the recipient animals. Data are representative of at least two different PBMC donors and each symbol represents an individual mouse.