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Combination of IL-10 and IL-2 induces oligoclonal human CD4 T cell expansion during xenogeneic and allogeneic GVHD in humanized mice

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Abstract

IL-10 is a crucial anti-inflammatory cytokine which can also exert a seemingly divergent immunostimulatory effects under certain conditions. We found high levels of the cytokine in a xenogeneic GVHD model where NOD-scid IL2rycnull (NSG) mice were transplanted with human PBMCs in presence of IL-2. Presence of exogenous IL-10 altered the kinetics of IL-2 induced human T cell reconstitution *in vivo*, showing an initial delay, followed by rapid expansion. Further, compared to IL-2 alone, treatment with IL-2 in combination with IL-10 increased survival in most animals and completely protected ~20% of mice from GVHD. Additionally, IL-2 induced expansion of both CD4⁺ and CD8⁺

xenoreactive T cells whereas a combination of IL-2 and IL-10 resulted in selective expansion of CD4⁺ T cells only. TCR V β repertoire analysis of CD4⁺ T cells showed that in contrast to IL-2 alone, simultaneous presence of both cytokines drastically reduced the V β repertoire of the expanded CD4⁺ T cells. Highly restricted V β usage was also observed when the cytokine combination was tested in an allogeneic GVHD model where NOD-scid IL2 γ c^{null} mice expressing HLA-DR4 (NSG-DR4) were transplanted with purified CD4⁺ T cells from HLA-DR4 negative donors. Taken together, our results demonstrate that IL-10 can profoundly modulate the subset composition and repertoire of responding T cells during GVHD.

Keywords: Biological sciences, Immunology

1. Introduction

Graft-versus-host disease (GVHD) remains a major determinant of morbidity and mortality in patients receiving allogeneic bone marrow transplantation (BMT). Recognition of allo antigens by T cells results in their activation, proliferation and differentiation into cells that mediate GVHD. T cells in association with other cell types such as macrophages and NK cells mediate tissue destruction characteristic of GVHD by direct cytotoxicity and secretion of proinflammatory cytokines. Cytokines play a vital role in modulating the induction as well as the intensity of the GVHD response [1]. Amongst the cytokines that are elevated during GVHD, the roles of IL-1, TNF- α , IL-6 and IFN- γ in disease pathogenesis are well understood but the role of Interleukin 10 (IL-10) remains unclear [2]. IL-10 is an immunoregulatory cytokine produced by a variety of cell types, including T cells, B cells and monocytes. In monocytes, the cytokine exerts its immunosuppressive and anti-inflammatory effects primarily by interfering with antigen presentation, phagocytosis and production of proinflammatory cytokines. On the other hand, IL-10-mediated inhibition of T cell proliferation and cytokine synthesis is predominantly an indirect effect via inhibitory effects on antigen presenting cells, although there are also reports of direct suppression of T cell function [3]. In agreement with the prevailing notion of a predominantly immunoregulatory function of IL-10, polymorphisms in the IL-10 promoter region that predispose towards enhanced production of the cytokine are associated with protection from GVHD [4]. However, IL-10 is also found to be elevated in clinical GVHD with high serum IL-10 levels being associated with fatal outcome in patients after bone marrow transplantation [5, 6, 7]. Studies in mice receiving MHC disparate bone marrow transplants also indicate that high dose IL-10 can accelerate GVHD lethality [5]. Similarly, we have reported that exogenous IL-10 can exacerbate xenogeneic GVHD in NOD-scid IL2 γ c^{null} (NSG) mice transfused with human peripheral blood mononuclear cells (PBMCs) [8]. Taken together, these disparate

findings suggest that IL-10 can display both immunosuppressive and immunostimulatory functions during GVHD.

The TH1 cytokine Interleukin 2 (IL-2), which plays a critical role in allogeneic T cell expansion and the immunoregulatory cytokine IL-10 are both concurrently elevated in sera of patients suffering from GVHD [9]. It not clear whether IL-10 actively synergizes with IL-2 and other cytokines in driving the immune events responsible for GVHD or its presence merely denotes a compensatory increase to dampen the exaggerated T cell response. In this study, we evaluated whether presence of IL-10 could mitigate the pathogenic effect of IL-2 and thereby reduce the severity of GVHD in humanized NSG mice. Our results indicate a biphasic effect, with an initial suppression manifesting as a delayed kinetics of human T cell reconstitution followed by massive expansion, so that the pathogenic T cells reach even higher levels than what was attained in animals treated with IL-2 alone. Further, while treatment with IL-2 results in expansion of both CD4⁺ and CD8⁺ human T cells with a relatively broad TCR V β repertoire, simultaneous presence of both cytokines leads to a selective and massive expansion of CD4⁺ T cells with very limited TCR V β repertoire with near complete absence of CD8⁺ T cells. A highly restricted TCR V β usage was also observed in an allogeneic GVHD model where NOD-scid IL2ryc^{null} mice expressing HLA-DR4 (NSG-DR4) were transplanted with purified CD4⁺ T cells from HLA-DR4 negative donors. Our results thus suggest that in our GVHD model, IL-10 plays a suppressive role initially, possibly via APCs exerting a strong immunosuppressive effect on naïve T cells but overall, its immunostimulatory effect predominates, with profound consequences on subset composition and repertoire of responding T cells.

2. Results

2.1. IL-10 levels are elevated in sera of animals with xenogeneic GVHD induced in the presence of exogenous IL-2

We have shown earlier that the prior presence of human IL-2 dramatically accelerates the onset of GVHD in NOD-scid IL2ryc^{null} mice [10]. Here all the animals succumb to disease by around day 25 even with as few as 1 million PBMCs. We carried out a cytokine bead array to identify the serum cytokines that are elevated in animals with IL-2 accelerated GVHD. Animals not treated with IL-2 were not included in the analysis as they showed no human cell reconstitution or GVHD under the same conditions. As shown in Fig. 1A, we found elevated levels of IFN- γ , IL-10 and IL-8 in the serum. We also carried out time point analysis of IL-10 levels, which indicated a gradual increase, reaching peak levels by the time the animals succumbed to GVHD (Fig. 1B). Animals reconstituted with human PBMCs in the absence of IL-2 were also euthanized at the same time as the previous group and sera from these animals were used as control for the assay. As

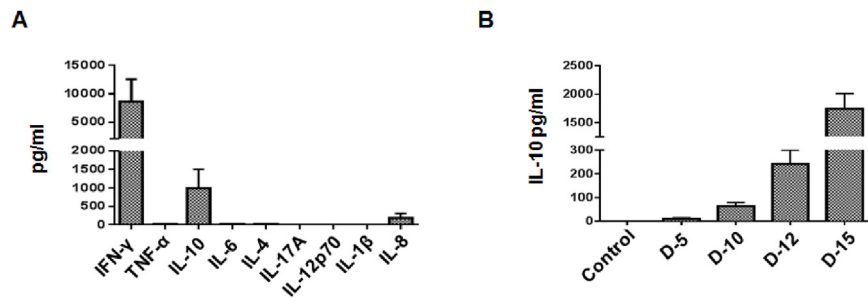


Fig. 1. High levels of IL-10 in the sera of Hu/PBL NSG mice with IL-2 induced accelerated GVHD. (A) Cytometric bead array analysis of Th1/Th2/Th17 and pro-inflammatory cytokines in the sera ($n = 5$) collected from animals at the time of euthanasia (B). Serum IL-10 levels at different time points post human cell reconstitution in the presence of IL-2 were tested by ELISA. Control represents sera collected on Day 15 from animal reconstituted with human PBMCs in the absence of IL-2. Error bars indicate standard deviation ($n = 6$). Data are representative of experiments from at least two different donors.

IL-10 is thought to play a key role in maintaining *in vivo* tolerance after allogeneic stem cell transplantation by suppressing the anti-host reactivity of donor-derived T cells, the results imply that levels of endogenous IL-10 attained in the animals may be insufficient to suppress xenoreactive T cell proliferation or its appearance late in the disease may be a factor for its inability to exert a protective effect.

2.2. IL-2 alone facilitates the expansion of CD4⁺ and CD8⁺ T cells whereas IL-2 combined with IL-10 preferentially expands CD4⁺ T cells

To test whether exogenous provision of IL-10 from day 1 could modulate GVHD by suppressing the IL-2-induced expansion of pathogenic T cells, human PBMCs were injected into animals expressing either IL-2 alone or with both IL-2 and IL-10. We have shown in previous studies that hydrodynamic injection of plasmids results in high levels of IL-2 and IL-10 in serum at day 2 post plasmid injection, but gradually declines thereafter [8, 10]. The nanogram/ml range of serum cytokine levels are comparable to levels seen in patients with GVHD post BMT [8, 10, 11]. As shown in Fig. 2A, a majority of animals in both groups (28 animals/group) succumbed to GVHD. However, presence of IL-10 increased survival ($p = 0.0219$) and completely protected ~20% of mice (6 animals) from GVHD mortality. We have earlier demonstrated that in presence of IL-10 alone, there was a slower kinetics of human cell reconstitution, followed by a massive expansion and associated disease pathology [8]. We found a similar delay in human cell reconstitution in animals expressing both IL-2 and IL-10 as compared to IL-2 alone (Fig. 2B). *In vitro* studies have demonstrated that α CD3 stimulation of PBMCs in presence of IL-10 selectively inhibits CD4⁺ but not CD8⁺ T cells [12]. Thus, we also analyzed the expression of CD4⁺ and CD8⁺ markers on the repopulating

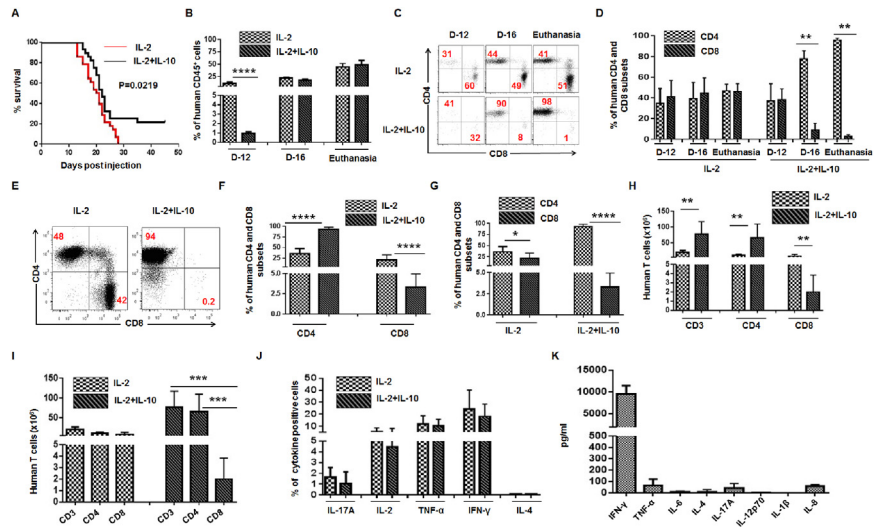


Fig. 2. A combination of human IL-2 and IL-10 selectively expands human CD4⁺ T cells during xenogeneic GVHD. (A) Kaplan-Meier survival curve comparing NOD-scid IL2 γ ^{null} mice injected with 2×10^6 human PBMCs in the presence of IL-2 alone (n = 28) or combination of IL-2 and IL-10 (n = 28). A log rank test was used to compare the distribution of survival between two groups. (B) Serial analysis of blood obtained by retro-orbital bleeding for kinetics of human CD45⁺ cell engraftment in presence IL-2 alone (n = 7) or both IL-2 and IL-10 (n = 7). (C, D) Representative flow cytometric analysis of blood (C) and cumulative data (D) for human CD4⁺ and CD8⁺ T cell subsets in animals with GVHD induced in the presence of IL-2 alone (n = 7) or with a combination of IL-2 and IL-10 (n = 7) at different time intervals post human PBMC reconstitution. (E-G) Representative flow cytometric analysis of spleen cells (E) and cumulative data (F,G) for human CD4⁺ and CD8⁺ T cell subsets in animals with GVHD induced in the presence of IL-2 alone (n = 10) or with a combination of IL-2 and IL-10 (n = 10). (H, I) Absolute number of human CD3⁺ (CD45⁺/CD3⁺), CD4⁺ (CD45⁺/CD3⁺/CD4⁺) and CD8⁺ (CD45⁺/CD3⁺/CD8⁺) T cells in whole spleen. (J) Intracellular expression of IL-17A, IL-2, TNF- α , IFN- γ and IL-4 in human CD4⁺ T cells were analyzed by flow cytometry after *ex vivo* stimulation of splenocytes with PMA and ionomycin for 6 h in the presence of GolgiStopTM (n = 5). Data are representative of experiments from at least two different donors. (K) Cytometric bead array analysis of Th1/Th2/Th17 and pro-inflammatory cytokines in the sera (n = 5) of animals (treated with IL-2 + IL-10) at the time of euthanasia. **p < 0.01; ***p < 0.001; ****p < 0.0001.

human T cells in the blood and spleen of these animals. Analysis of human cells in blood (CD45⁺) over time revealed a much slower kinetics of engraftment in presence of IL-10 compared to animals treated with IL-2 alone at the early time point of day 12. However this changed by day 16 with significant increase in human cell numbers in presence of IL-10, ultimately reaching similar levels as with IL-2 alone at the time of euthanasia (Fig. 2B). Contrary to what has been reported *in vitro*, subset analysis in presence of both cytokines, showed that the expansion over time was confined to the CD4⁺ T compartment only, with near complete absence of CD8⁺ T cells (Fig. 2C-D). Similarly, spleen cells harvested from euthanized animals showed expansion of both CD4⁺ and CD8⁺ T cell subsets in the presence of IL-2 alone, whereas when both cytokines were present, the subset composition was biased towards a preferential expansion of only CD4⁺ T cells

(Fig. 2E-G). The absolute number of human T cells in the spleen also showed massive expansion of human CD3⁺CD4⁺ T cell population in animals expressing both IL-2 and IL-10 whereas in IL-2 treated animals we observed comparable levels of both CD4⁺ and CD8⁺ subsets (Fig. 2H-I). To test the differentiation status of T cells expanding in the presence of IL-2 and IL-2/IL-10, we evaluated intracellular IL-2, TNF- α , IFN- γ , IL-17A and IL-4 production by human CD4⁺ T cells from spleens of these animals after *ex vivo* stimulation with PMA and ionomycin [8]. As shown in Fig. 2J, the cytokine profiles were very similar, with comparable levels of TNF- α and IFN- γ producing cells suggesting a predominantly TH1 response under both experimental conditions. This was also confirmed by measuring serum cytokine levels which showed increase in TH1 cytokines IFN- γ and TNF- α alongside IL-8 and IL-17 A (Fig. 2K).

2.3. IL-10 promotes expansion of CD4⁺ T cells with highly restricted V β usage

To understand the basis for the selective CD4⁺ T cell expansion in the combined presence of exogenous IL-2 and IL-10, we tested if this was due to a preferential expansion of residual T cells clones that escaped initial suppression by IL-10. For this, we performed a V β repertoire analysis of the expanded human CD4⁺ T cells. Purified CD4⁺ T cells from spleens of the animals were analyzed by flow cytometry using mAbs directed against 24 different human TCR V β subsets. As shown in Fig. 3, IL-2 expressing animals showed a broad V β representation suggesting a polyclonal expansion. In contrast, in the presence of both IL-2 and IL-10, the CD4⁺ T cell showed a highly restricted V β repertoire. Oligoclonal expansion of specific V β expressing CD4⁺ T cells ranging from 20–80% was present in all 10 animals treated with a combination of IL-2 and IL-10 but not in animals treated with IL-2 alone (Fig. 3A-B). However, the particular V β s expanded varied between animals although animals were from a single litter for each donor. This was found to be the case with PBMCs from multiple donors, which indicates that the observed preferential V β expansions in presence the cytokine combination may be a stochastic effect. To determine, whether this pronounced TCR V β skewing can be recapitulated *in vitro*, CD3 stimulated PBMCs were cultured for 20 days in presence of IL-2 alone and with both IL-2 and IL-10. We did not observe any skewing towards a particular TCR V β repertoire in these *in vitro* stimulated PBMCs (data not shown) which demonstrates that *in vitro* culture conditions may not recapitulate the complexities cytokine interactions with T cells *in vivo*.

2.4. Combination of IL-2 and IL-10 restricts CD4⁺ T cell V β repertoire in HLA-DR4 Tg NSG model of allogeneic GVHD

While repopulation of human PBMCs in NSG mice provides a robust GVHD model, the response is a xenogeneic activation to murine antigens which may differ

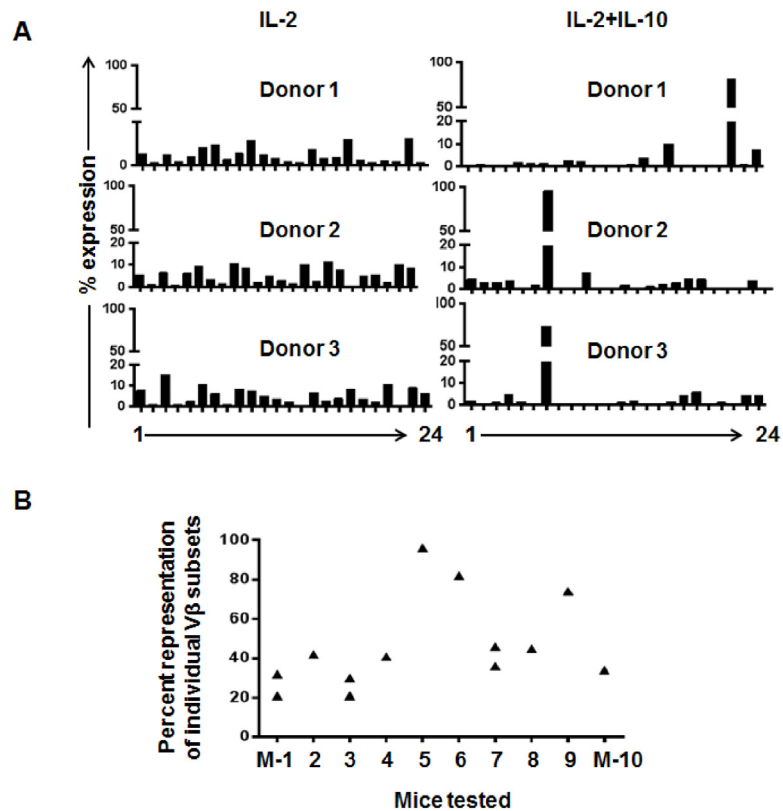


Fig. 3. Large oligoclonal expansions of human CD4⁺ T cells with very limited V β repertoire in animals with GVHD induced in the presence of a combination of human IL-2 and IL-10 but not with IL-2 alone. V β repertoire of CD4⁺ T cells purified from spleens of euthanized animals. (A) Spleen cells were analyzed for V β profile of expanded CD4⁺ T cells using beta mark TCR repertoire kit. Representative data of V β subset distribution on CD4⁺ T cells from groups of mice (10 animals/group) injected with PBMCs from 3 different donors in the presence of IL-2 or IL-2 + IL-10 are shown. Number 1–24 on the Y axis depicts V β s 5.3, 3, 7.1, 9, 17, 16, 18, 5.1, 20, 13.1, 13.6, 8, 5.2, 2, 12, 23, 1, 21.3, 11, 22, 14, 13.2, 4 and 7.2 in that order (B) The percentage of each V β subset showing over 20% expansion in animals with GVHD induced in presence of IL-2 + IL-10 combination (M1-M10 represents individual animals tested).

in many aspects from allogeneic response that drives human GVHD. Recently, NSG mice have been developed that lack murine MHC class-II but express a chimeric human HLA-DRB1*0401 [13]. This model allows induction of allogeneic response to human DR-4 after transplantation with purified CD4⁺ T cells from non DR-4 donors. Since xeno-GVHD is avoided, the model more closely resembles GVHD seen after allogeneic hematopoietic stem cell transplantation. We used this model to evaluate the effect of IL-2 and IL-10 on clonal expansion of allogeneic human CD4⁺ T cells *in vivo*. We first identified HLA-DR4 negative donors by PCR using genomic DNA isolated from PBMCs (Fig. 4A). Animals were injected with plasmids expressing either IL-2 alone or IL-2 and IL-10 in combination and transplanted with 3.5×10^6 purified CD4⁺ T-cells

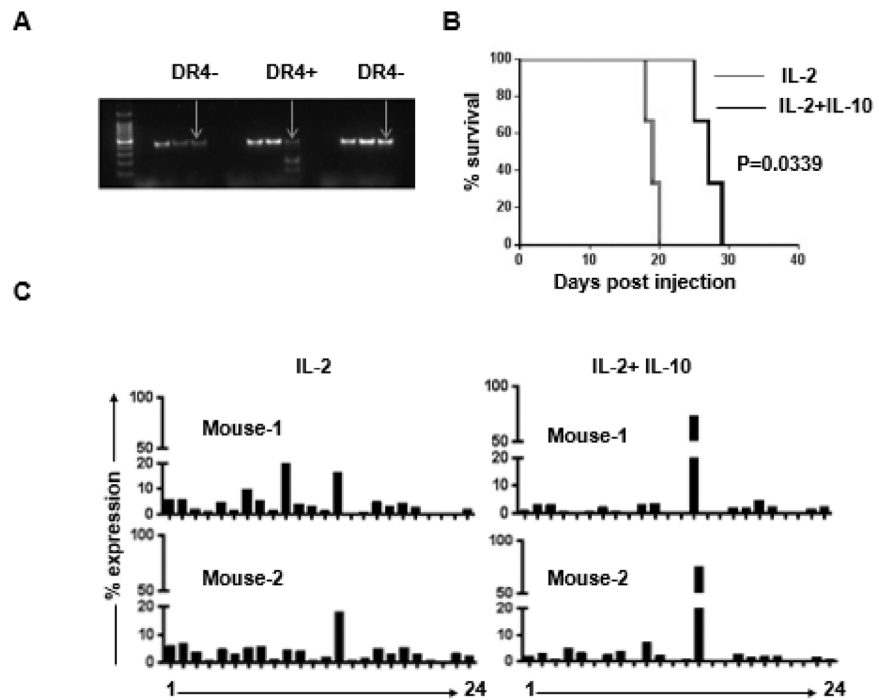


Fig. 4. Oligoclonal expansion of human CD4⁺ T cells is seen in NSG-DR4 mice after induction of allogeneic GVHD by transfer of purified DR4-negative CD4⁺ T cells in the presence both L-2 and IL-10, but not with IL-2 alone. (A) HLA-DR4 genotyping using DR low resolution primer set. Isolated genomic DNA was used for PCR using HLA-DR4 specific primer set obtained from olerup. Two bands corresponding to the length of 100 bp and 175 bp corresponds to HLA-DR4 positive individuals. (B) Kaplan-Meier survival curve comparing GVHD lethality in NSG-DR4 mice injected with 3.0×10^6 human HLA-DR4 negative CD4 T cells in the presence of IL-2 alone or both IL-2 and IL-10 ($n = 3$). Wilcoxon analysis was used to compare the distribution of survival between the two groups. (C) Percentage distribution of expanded V β subsets in animals treated with IL-2 alone or with IL-2 and IL-10 combination. Number 1–24 on the Y axis depicts V β s 5.3, 3, 7.1, 9, 17, 16, 18, 5.1, 20, 13.1, 13.6, 8, 5.2, 2, 12, 23, 1, 21.3, 11, 22, 14, 13.2, 4 and 7.2 in that order.

from HLA-DR negative donors to induce allogeneic GVHD. Here again we observed a delay in GVHD progression in presence of both IL-2 and IL-10 as compared to IL-2 alone group (Fig. 4B). Next, we carried out a TCR V β repertoire analysis (experiment was initiated with 3 animals/group but only 2 animals could be analyzed in each group as 1 animal from each group died during the course of experiment) with purified CD4⁺ T cells stained with the same panel of V β antibodies as before. As shown in Fig. 4C, CD4⁺ T cells from animals treated with IL-2 alone demonstrated a much broader TCR V β repertoire compared to animals treated with IL-2 and IL-10 combination. In the latter group nearly 75% of the CD4⁺ T cells expressed a single TCR V β . To further probe the oligoclonality of T cells expanded in the presence of IL-2 and IL-10, we PCR amplified the single TCR V β that was found to be dominant by flow cytometry in T cells from the 2 animals and subjected the cloned products to sequence analysis of the CDR3 region. A total of

19 clones were selected for each animal, sequenced and blasted with in the IMGT/V-QUEST (<http://www.imgt.org/IMGTvquest/share/textes/>). The majority of the CDR3 sequences within the amplified V β subtype were found to be identical (19/19 in mouse 1 and 18/19 in mouse 2), which further confirms the highly restricted clonality of the expanded T cells (Table 1).

3. Discussion

The main finding of this study is that in the NSG mouse model of human GVHD, the pleotropic immunoregulatory cytokine IL-10 has a complex and varied effect on IL-2 induced T cell expansion. Using two relevant humanized mouse models of xenogeneic and allogeneic GVHD, we show that when administered in combination with IL-2, IL-10 initially inhibits activation and expansion of responding T cells, but later, modulates the response to induce massive oligoclonal expansion of CD4⁺ T cells that culminates in GVHD lethality.

Cytokines play a major role in the induction and progress of GVHD [1]. After donor T cells get triggered by host allo antigens, IL-2 drives the expansion/differentiation of the cells into cytokine producing and/or cytotoxic effector cells. Other proinflammatory cytokines like IL-1, IL-6, IFN- γ and TNF- α also facilitate T cell activation and/or recruitment to host organs that are the sites of tissue destruction in GVHD [2]. Clinical GVHD is also associated with high serum levels of IL-10 but its relevance to disease pathogenesis and severity remains uncertain [5, 6, 7, 11]. Although this cytokine is known for its potent negative regulatory effect on alloreactivity, paradoxically, it can also exacerbate GVHD in experimental models.

Our data show that high levels of serum IL-10 correlate with the onset of GVHD in NSG mice transplanted with human PBMCs in the presence of IL-2. This is analogous to data in humans showing that high serum interleukin-10 levels after transplantation is associated with an increased risk of GVHD and a fatal outcome [11]. It has been suggested that increased IL-10 levels during GVHD may represent a late compensatory response that correlates with the severity of the disease, rather than a direct pathogenic effect [9]. In fact, single nucleotide polymorphisms in the IL-10 gene have been shown to significantly lower the risk

Table 1. CDR3 sequence analysis of the amplified V β subtype.

Animal	PCR Primer used	CDR3 Sequence	Frequency
Mouse-1	HBV2 (TCA ACC ATG CAA GCC TGA CC) and HBC rev (GGT GTG GGA GAT CTC TGC TTC)	SARATGTQGFDTQY	19/19
Mouse-2	HBV2 (TCA ACC ATG CAA GCC TGA CC) and HBC rev (GGT GTG GGA GAT CTC TGC TTC)	SASRPTGTSGSSYEQY SARDSSSYTEAF	18/19 1/19

of Grade III–IV GVHD after allogeneic bone marrow transplantation and this protection has been attributed to enhanced IL-10 production by APCs [4]. However, a follow up study that used transplants from unrelated instead of HLA-identical siblings used in the first study found that the risk of acute GVHD and its severity was not significantly affected by IL-10 gene variation [14]. Experimental studies in mice have shown that in fact, high doses of IL-10 can enhance GVHD lethality [5]. Our earlier studies also found that the presence of IL-10 can exacerbate xenogeneic GVHD in NSG mice although the effects are much less dramatic than with IL-2, requiring a five to ten fold higher dose of PBMCs and with a slower course of disease progression [8]. Another study found that donor but not host-derived IL-10 was important for T_{Reg} activity that was critical for suppression of GVHD [15]. Overall, it appears that IL-10 has complex and context-dependent functions that determine its beneficial or deleterious effects.

GVHD involves a cytokine cascade that includes IL-10. Most studies have looked at interventions with single cytokines which do not shed light on whether IL-10 acts in concert with other cytokines to enhance the allogeneic immune response responsible for GVHD or whether the combined strength of the proinflammatory responses far exceeds the anti-inflammatory capabilities of IL-10. Our study, using exogenous IL-2 and IL-10 in combination shows that the presence of IL-10 does not ameliorate GVHD severity or lethality. However, a closer look at the pattern of human T cell reconstitution in the xenogeneic and allogeneic mouse models using NSG and NSG-DR4 mice respectively, suggests that the cytokine can modulate the kinetics and quality of the T cell response. Unlike with IL-2 where human T cells became detectable in blood by 5 days, in presence of both IL-2 and IL-10, there was a significant reduction in human T cells in blood at initial time points, as we reported earlier in presence of IL-10 alone [8]. However, despite the slower kinetics, the timing of GVHD lethality was similar to that with IL-2 alone. As T cell suppression by IL-10 is thought to be predominantly through its action on APCs, the initial delay in human T cell reconstitution in presence of IL-10 could imply inhibition of T cell activation by APCs, which have a short life span after human PBMC transfer. However, we see a similar effect in our allogeneic NSG-DR4 mouse model, where GVHD is induced by injection of purified non-DR4⁺ CD4⁺ T cells in the absence of conventional APCs. It is possible that in this setting, IL-10 exerts its effect via mouse APCs expressing human HLA-DR4. As data suggests that activated T cells are relatively resistant to IL-10 mediated inhibition, unlike naïve T cells, which are highly susceptible [16], it is also possible that the expanded T cells represent activated alloreactive clones that escaped IL-10 inhibition and become activated, allowing them to undergo clonal expansion. Further, IL-10 is described to mediate its inhibitory effects on T cells, particularly CD4⁺ T cells by its ability to suppress IL-2 synthesis, an effect that may have been bypassed in presence of exogenous IL-2 [17]. IL-10 has also been reported to

protect activated T cells from apoptosis, thus once the responding T cells have overcome the initial suppression, the cytokine may even promote their growth and survival [18, 19].

In vitro studies have demonstrated that IL-10 exerts direct suppressive effect selectively on CD4⁺ cells but not on CD8⁺ T cells [12]. On the other hand, studies in an experimental mouse model has shown that high dose IL-10 administration enhanced both CD4⁺ and CD8⁺ T cell-mediated GVHD [5]. Our earlier studies on effect of exogenous IL-10 on xenogeneic GVHD in NSG mice showed a similar pattern of xenoreactive T cell expansion consisting of both CD4⁺ and CD8⁺ denomination [8]. However, in total contrast, while both CD4⁺ and CD8⁺ were expanded after human PBMC transfer in presence of IL-2 alone, IL-2 and IL-10 in combination induced a preferential expansion of CD4⁺ T cells with negligible levels of CD8⁺ T cells. A study in a transgenic mouse model of Sjogren's syndrome, where mouse IL-10 gene was expressed under the human salivary amylase promoter also found that infiltration in the glands consisted primarily of CD4⁺ T cells [20]. Additionally, we found that the subset bias of the responding CD4⁺ T cells was towards a predominantly of TH1 subtype with IL-2 alone or in combination with IL-10 as assessed by intracellular cytokine expression after mitogenic stimulation. When we probed their TCR diversity to uncover any distinctive effect of IL-10 on the repertoire, we found that the T cells exhibited oligoclonal V β expansions, unlike the more polyclonal response observed in the presence of IL-2. Even in the allogeneic GVHD model where CD4⁺ T cells alone were transferred to drive an anti-DR-4 response, we found a similar narrow oligoclonal response in the presence of IL-10 which was not the case with IL-2 alone. Further sequence analysis of the complementarity determining regions (CDR) (which determines site of contact with peptide presented by the HLA molecules) showed restricted clonality of the identified TCR V β . One explanation for the highly restricted V β usage is that they represent clonal expansion of residual cells that escaped initial inhibition by IL-10. Further, the dominant V β expansions differed amongst littermates although human PBMCs from a single donor was used for transplantation in for each experiment, which also suggests that the selection of particular V β was a stochastic effect and does not represent a selective outgrowth of immunodominant clones. Distinct oligoclonal T cell expansions have also been reported in human GVHD after stem cell transplantation [21, 22, 23, 24]. Whether IL-10 has a modulatory role with IL-2 in shaping the T cell repertoire in the GVHD response is not known but it is possible that increased bioavailability of the two cytokines could influence TCR repertoire at sites of GVHD activity. The data also demonstrate that one or two pathogenic T cell clones are sufficient to mediate GVHD. In summary, our studies highlight the complex pleotropic role of IL-10 in modulation of GVHD. Both inhibitory and stimulatory aspects of IL-10 function are evident during the course of xenogeneic and allogeneic GVHD in humanized

NSG mice. Our data points to a need for a better understanding of the molecular mechanism of the two disparate effects of IL-10 and calls for caution in using rhIL-10 as an immunosuppressive cytokine to treat GVHD and other T cell-mediated autoimmune conditions.

4. Methods

4.1. Animals

NSG and NSG-DR4 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in specific pathogen free conditions at the Paul L. Foster School of Medicine, TTUHSC animal facility. All experiments were performed with 6–8 week old mice according to guidelines established by the Animal Welfare act and the NIH guide for the care and use of laboratory animals. The study protocol was approved by the IACUC committee of Texas Tech University Health Sciences Center (TTUHSC).

4.2. Hydrodynamic injection of plasmids expressing hIL-2 and hIL-10

Human IL-2 gene construct was generated as described previously [10]. Human IL-10 expressing plasmid was obtained from Invivogen (San Diego, CA, USA) and purified using the endotoxin free maxi prep-kit from Qiagen (Valencia, CA, USA). For expressing IL-2 and IL-10 *in vivo*, animals were injected with 50 µg of plasmid in a total volume of saline equivalent to 8% of the body mass delivered within 5–8 seconds with a 27-gauge needle [25].

4.3. HLA-DR4 genotyping

HLA-DR4 negative individuals were identified by polymerase chain reaction (PCR). Briefly, genomic DNA was extracted from 1×10^6 donor PBMCs using DNeasy Blood & Tissue Kit (Qiagen) and PCR amplified using the DR low resolution kit from Olerup according to the manufacturer's instructions.

4.4. ELISA, flow cytometry and cytokine bead array

To determine IL-2 and IL-10 levels in the serum, animals were bled periodically through retro-orbital puncture. IL-2 and IL-10 serum levels were determined using commercial ELISA kit according to the manufactures instructions (Biolegend Inc, San Diego, CA, USA). For flow cytometric analysis mononuclear cells from blood and spleen of the animals were stained with appropriate fluorochrome-labeled monoclonal antibodies directed at human CD3, CD4, CD8 and CD45 (from BD Biosciences, San Diego, CA or Tonbo Biosciences, San Diego, CA). The beta mark TCR repertoire kit from Beckman Coulter was used for Human Vβ analysis.

All flow cytometry data were acquired on FACS Canto II instrument (BD) and analyzed with FlowJo software. Serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN- γ and TNF- α were measured using human Th1/Th2/Th17 and Inflammatory Cytokine Bead Array kits (BD Pharmingen, San Diego, CA) according to manufacturer's instructions.

4.5. Induction of xenogeneic and allogeneic GVHD

Human PBMCs were isolated from blood samples collected from healthy adult volunteers under an institutional review board (IRB) approved protocol. For xenogeneic GVHD induction, 6–8 week old NSG mice were injected with plasmids to express IL-2 or both IL-2 and IL-10 as described earlier [8, 10] followed by transfer of human PBMCs (2×10^6) by the i.v route. For allogeneic GVHD induction, NSG-DR4 animals expressing the cytokines were injected with 3×10^6 CD4⁺ T-cells from HLA-DR4 negative individuals. 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 euthanized when they lost 20% of their body weight. Single cell suspensions were prepared from blood and spleen for flow cytometric analysis.

4.6. Sequencing of TCR V β CDR3 region

Splenocytes from allo-GVHD mice were enriched for human T cells by negative selection with mouse/human chimera enrichment Kit (Stem Cell Technologies). Total RNA was isolated from the cells with the RNeasy kit (Qiagen). cDNA was prepared using SuperScript[®] III First-Strand Synthesis SuperMix (Invitrogen). PCR reaction was carried out with the Premix Ex Taq (TaKaRa) using previously published primers to amplify the relevant V β family or an unrelated V β as negative control [26]. PCR fragments were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and cloned into pCRTM4-TOPO vector (Life Technologies) and 19 positive clones selected from each sample were used for sequence analysis of the CDR3 region in the TCR V β amplicon.

4.7. Statistical analysis

Data were analyzed using prism 5.0 (GraphPad). For comparison of data with two variables, non-parametric Mann-Whitney test was used. For all statistical tests, p value < 0.05 were considered significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Declarations

Author contribution statement

Sojan Abraham, N Manjunath: Conceived and designed the experiments; Analyzed and interpreted the data.

Sojan Abraham, Hua Guo, Jang-Gi Choi, Chunting Ye, Midhun Ben Thomas, Nora Ortega, Guohua Yi: Performed the experiments.

Alok Dwivedi: Contributed reagents, materials, analysis tools or data.

Premlata Shankar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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