Human Regulatory T Cells Control Xenogeneic Graft-versus-Host Disease Induced by Autologous T Cells in RAG2$^{-/-}\gamma c^{-/-}$ Immunodeficient Mice

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Abstract

Purpose: Effective prevention of graft-versus-host disease (GvHD) is a major challenge to improve the safety of allogeneic stem cell transplantation for leukemia treatment. In murine transplantation models, administration of naturally occurring CD4$^+$CD25$^+$ regulatory T cells (Treg) can prevent GvHD. Toward understanding the role of human Treg in stem cell transplantation, we studied their capacity to modulate T-cell – dependent xenogeneic (x)-GvHD in a new model where x-GvHD is induced in RAG2$^{-/-}\gamma c^{-/-}$ mice by i.v. administration of human peripheral blood mononuclear cells (PBMC).

Experimental Design: Human PBMC, depleted of or supplemented with autologous CD25$^+$ Tregs, were administered in mice at different doses. The development of x-GvHD, *in vivo* expansion of human T cells, and secretion of human cytokines were monitored at weekly intervals.

Results: Depletion of CD25$^+$ cells from human PBMC significantly exacerbated x-GvHD and accelerated its lethality. In contrast, coadministration of Treg-enriched CD25$^+$ cell fractions with autologous PBMC significantly reduced the lethality of x-GvHD. Treg administration significantly inhibited the explosive expansion of effector CD4$^+$ and CD8$^+$ T cells. Interestingly, protection from x-GvHD after Treg administration was associated with a significant increase in plasma levels of interleukin-10 and IFN-γ, suggesting the *de novo* development of TR1 cells.

Conclusions: These results show, for the first time, the potent *in vivo* capacity of naturally occurring human Tregs to control GvHD-inducing autologous T cells, and indicate that this xenogeneic *in vivo* model may provide a suitable platform to further explore the *in vivo* mechanisms of T-cell down-regulation by naturally occurring human Tregs.

Allogeneic stem cell transplantation is a powerful approach in the treatment of hematologic malignancies as it mediates a curative graft-versus-tumor effect (1, 2). Unfortunately, the therapy is often complicated with life threatening graft-versus-host disease (GvHD; ref. 3). Recently, several murine studies indicated the possibility to prevent GvHD by administration of naturally occurring regulatory T cells (Treg), a small subset of CD4$^+$ T cells displaying constitutive expression of the interleukin 2 (IL-2) receptor α chain CD25 (4). It was reported that depletion of Tregs from grafts accelerated the lethality of GvHD, whereas coadministration of donor Tregs ameliorated or even prevented GvHD (5 – 8). Moreover, some studies showed prevention of GvHD without abrogating graft-versus-tumor effect after Treg administration (9 – 11). In humans, naturally occurring Tregs are present within the CD4$^+$CD25^{high} subset, express the transcription factor Foxp3 (12), and effectively suppress auto-, allo-, and antigen-specific T cells (13 – 15). Their role on GvHD and graft-versus-tumor effect is, however, not well understood. The available data on this issue have been generated exclusively by *in vitro* analyses of patient samples and are somewhat conflicting. Although some studies reported inverse correlations between Foxp3$^+$ CD4$^+$CD25^{high} Treg with acute or chronic GvHD (16, 17), other studies failed to show such an association (18) or showed the opposite (19). To date, no studies have been reported on the *in vivo* modulation of GvHD by (co)administration of human Treg with effector donor T cells.

In this study, we explored the *in vivo* capacities of human Tregs to control GvHD-inducing autologous T cells using a recently developed xenogeneic (x)-GvHD model in RAG2$^{-/-}$ common γ-chain (γc)$^{-/-}$ mice. In these mice, i.v. administration of human peripheral blood mononuclear cell (PBMC) induces x-GvHD associated with a cytokine storm, and dependent on the dose of CD4$^+$ and CD8$^+$ T cells (20). We

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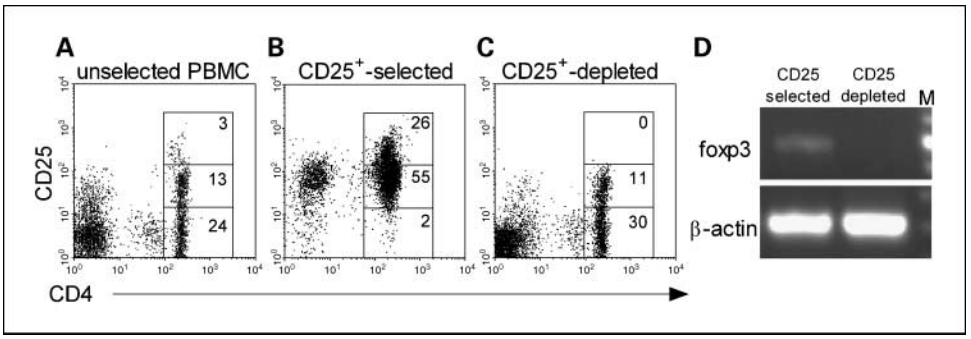

Fig. 1. Separation of $CD25^+$ and $CD25^-$ cells in human PBMC. A representative example of magnetic affinity cell sorting of human PBMC into $CD25^+$ and $CD25^-$ cell fractions. *A* to *C*, fluorescence-activated cell sorting analyses of cells in PBMC (*A*), $CD25^+$-selected (*B*), and $CD25^+$-depleted (*C*) fractions after labeling with CD4 (*X axes*) and CD25 (*Y axes*). The numbers indicate the percentage cells in the corresponding gates. *D*, the *Foxp3* gene expression in the $CD25^+$-selected and $CD25^+$-depleted fractions as determined by reverse transcription-PCR. Similar results were obtained in all experiments.

show that depletion of Treg from administered human PBMC significantly exacerbates the symptoms and accelerates the lethality of x-GvHD. In contrast, coadministration of Treg cell–enriched $CD25^+$ cell fractions prevented or significantly inhibited lethal x-GvHD. Protection from x-GvHD by Treg cells was associated with a significant reduction of human T cells from the circulation and with a significant increase in plasma levels of IL-10 and IFN-γ.

Materials and Methods

Mice and conditioning regimen. $RAG2^{-/-}\gamma c^{-/-}$ mice were bred in microisolator cages under specified pathogen-free conditions and received sterile water and irradiated pellets *ad libitum*. Female or male mice, ages 8 to 20 weeks, were used in the experiments as described previously (20). Briefly, 1 day before injection of human PBMC, mice were depleted of monocytes by i.v. injection of clodronate-containing liposomes. Two to five hours before injections, mice received total body irradiation with a single dose of 350 cGy gamma irradiation from a linear accelerator.

Separation of human PBMC into $CD25^+$ and $CD25^-$ fractions. Human PBMCs were isolated from buffy coats of blood bank donors by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. The percentages of $CD3^+CD25^+$ and $CD3^+CD25^-$ cells were determined by fluorescence-activated cell sorting analysis. Where indicated, a part of the isolated human PBMC was fractionated into $CD25^+$ and $CD25^-$ subsets using phycoerythrin-conjugated anti-CD25 antibodies (Becton Dickinson, San Jose, CA) and an immunomagnetic cell separation system (Auto-MACS, Miltenyi Biotech, Bergisch Gladbach, Germany). The cell fractions were phenotyped by fluorescence-activated cell sorting analysis. Results of a representative experiment are illustrated in Fig. 1. Unfractionated PBMC contained 3% of $CD4^+CD25^{high}$ T cells (Fig. 1A). After separations, the $CD4CD25^{high}$ cells were exclusively found in the "$CD25^+$-selected" fractions (Fig. 1B and C). In these fractions, non-$CD4^+$ cells are composed of 9% $CD8^+CD25^+$ and 8% $CD25^+$ B cells. No $CD14^+$ or $CD56^+$ cells were detected. The "$CD25^+$-depleted" fractions were devoid of $CD4^+CD25^{high}$ cells (Fig. 1C) and contained only low percentages of $CD4^+$ cells expressing intermediate CD25 levels. Both $CD25^+$-depleted cell fractions and unfractionated PBMC contained similar levels of $CD8^+CD25^-$ (19%), $CD14^+$ (15%), $CD19^+$ (12%), and $CD56^+$ (8%) cells.

Detection of Foxp3 gene transcripts in the $CD25^+$ and $CD25^-$ fractions. cDNA from $CD25^+$-selected and $CD25^+$-depleted cell fractions were amplified by PCR using a β-actin–specific primer set (5′-GTGCTATCCCTGTACGCCTCT-3′; 5′-AGGACTCCATGCCCAG-GAAGG-3′) and a Foxp3-specific primer set (5′-GAGAAGCTGAGTGC-CATGCA-3′; 5′-TTGATCTTGAGGTCAGGGGCCAGG-3′). The Foxp3 expression was predominantly found in the $CD25^+$-selected cell fraction (Fig. 1D). These results were confirmed using two other Foxp3 primer sets and in quantitative real time PCR (data not shown).

In vivo *administration of Treg cell–depleted ($CD25^-$) and Treg cell–enriched ($CD25^+$) human PBMC.* Unmodified PBMC, $CD25^+$-depleted, or $CD25^+$-selected cell fractions were resuspended in PBS + 0.1% human serum albumin and injected via the tail vein in a final volume of 0.2 mL. The counts of CD25-negative T cells in the PBMCs were used as reference to equalize the injected T-cell doses in different groups of mice. In repletion experiments, the $CD25^+$ cells were mixed with unmodified autologous human PBMC just before injection. The development of x-GvHD was monitored weekly as described (20). In short, mice were monitored for body weight, mobility (0, mobile; 1, limited mobility; 2, hardly mobile), and general appearance (0, normal fur; 1, ruffled fur; 2, ruffled fur + red swollen skin; 3, ruffled fur + red swollen skin + patchy alopecia). In case of severe x-GvHD, mice were sacrificed by cervical dislocation. x-GvHD diagnosis was given if the weight loss was >10%, and the scores for general appearance and mobility were at least 1.

In vivo *monitoring of human T cells and cytokines.* The engraftment and expansion of human $CD4^+$ and $CD8^+$ T cells, and the plasma levels of human cytokines, were monitored in 100 to 120 μL peripheral blood obtained weekly from the retro-orbital vein under anesthesia as described (20). T-cell subsets were monitored by fluorescence-activated cell sorting analyses. Where indicated, circulating human T cells were enumerated by addition of prelabeled reference beads (Flow Count Fluorospheres; Beckman-Coulter, Fullerton, CA) at 1,000 beads/μL in each sample. In these assays, the erythrocytes were removed by a lysis-no wash procedure and the sample volumes were adjusted to 500 μL before the addition of reference beads.

The concentrations of human cytokines IL-1, IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, and tumor necrosis factor-α were determined using a multiplex cytokine assay system (Bio-Plex, Bio-Rad Laboratories, Hercules, CA) using 10 μL murine plasma isolated from peripheral blood, as described previously (20). Data were analyzed using the Bio-Plex Manager software (Bio-Rad Laboratories) with five-parametric curve fitting.

Statistical analyses. Univariate analyses and survival analyses were conducted using GraphPad Prism (version 4.0).[3] Differences between groups were tested either in nonparametric nonpaired *t* tests or in log-rank tests. The differences were considered statistically significant if $P < 0.05$.

Results

Depletion of $CD25^+$ cells from infused human PBMC significantly exacerbates the symptoms and accelerates the lethality of x-GvHD in $RAG2^{-/-}\gamma c^{-/-}$ mice. In $RAG2^{-/-}\gamma c^{-/-}$ mice, the severity of x-GvHD induced by human PBMC is dependent on the administered T-cell dose (20). This allowed us to study the effect of Treg depletion on the severity of

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x-GvHD at different T-cell doses. Treg depletion with $CD25^+$ immunomagnetic beads completely removed $CD4^+CD25^{high}$ cells from the $CD25^+$-depleted fractions (Fig. 1). The $CD25^+$-depleted cells showed little or no Foxp3 expression, indicating the efficacy of the Treg cell removal (Fig. 1D). Test groups of mice received high, intermediate, and low doses of Treg-depleted PBMC containing 13×10^6, 8×10^6, and 4×10^6 $CD25^-$ T cells, respectively. Control groups received unmodified PBMC containing equivalent amounts of $CD25^-$ T cells. As expected, the lethality of x-GvHD correlated well with the administered T-cell dose in the control groups (Fig. 2A). At low doses only, a small fraction of control mice developed clinical x-GvHD (Fig. 2B). Depletion of $CD25^+$ cells had dramatic effects: The lethality was accelerated at high doses ($P < 0.001$), the incidence of lethal x-GvHD was increased at intermediate doses ($P = 0.025$), and the development of clinical x-GvHD was increased at low doses ($P = 0.037$; Fig. 2B). Peripheral blood

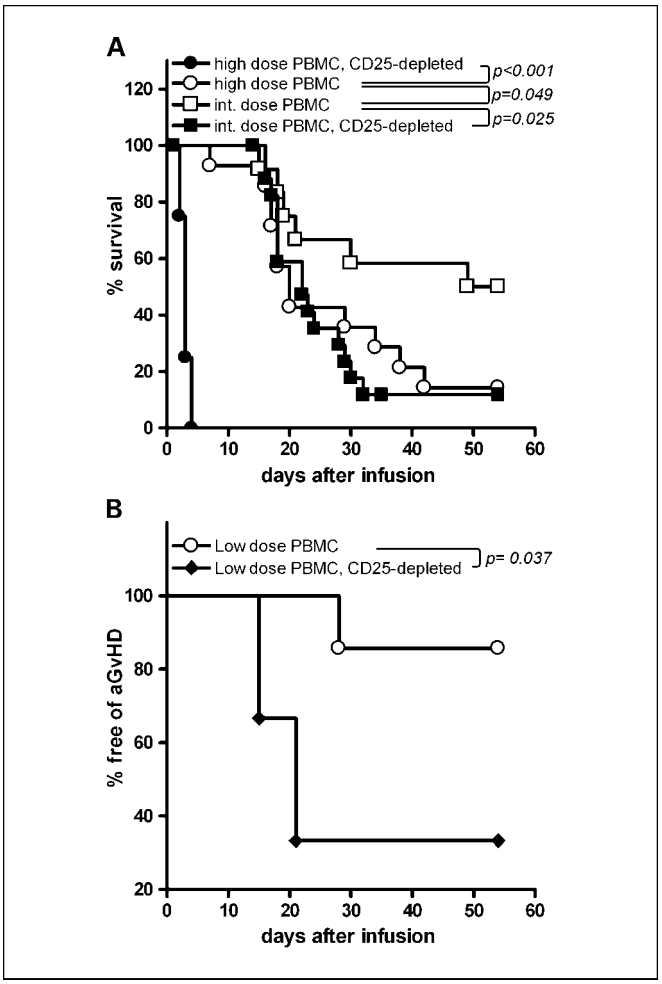

Fig. 2. The effect of $CD25^+$ depletion on x-GvHD induced by human PBMC. *A*, Kaplan-Meier survival estimates of $RAG2^{-/-}\gamma c^{-/-}$ mice that received a high dose of unmodified ($n = 13$) or CD25-depleted ($n = 4$) PBMC containing 13×10^6 $CD25^-$ T cells or intermediate dose of unmodified ($n = 12$) or $CD25^+$-depleted ($n = 20$) PBMC containing 8×10^6 $CD25^-$ T cells. Data are pooled from three independent experiments. The *P* values indicate the statistical differences between groups as calculated in log-rank tests. *B*, Kaplan-Meier estimates of mice remaining free of x-GvHD after injection of low dose of unmodified ($n = 7$) or $CD25^+$- depleted ($n = 6$) PBMC containing 4×10^6 $CD25^-$ T cells. Clinical scoring of GvHD is outlined in Materials and Methods. The *P* value indicate the statistical difference between the groups in a log-rank test.

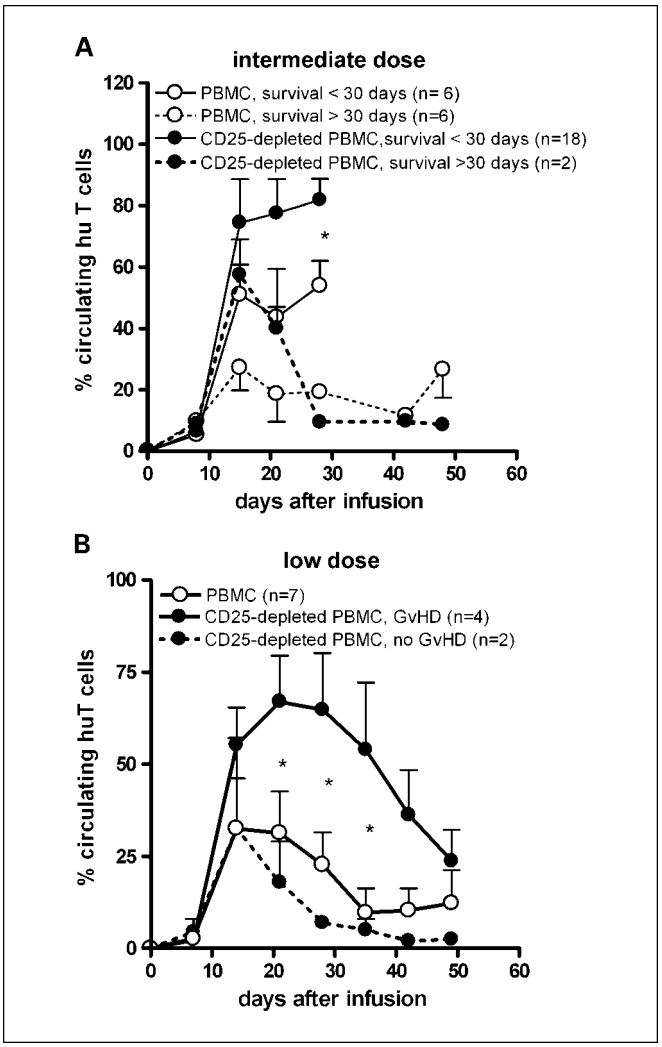

Fig. 3. Monitoring of the human T-cell engraftment and expansion after $CD25^+$ depletion. Mean percentages of circulating human T cells in mice treated with intermediate (*A*) or low (*B*) dose of unmodified or $CD25^+$-depleted PBMC. In all groups, mice with or without (lethal) GvHD are shown separately to illustrate the differences associated with the clinical outcome. Bars, SE. *, statistical analyses: In (*A*), the difference between CD25-depleted PBMC, survival <30 days (●), and PBMC, survival <30 days (○), at day 35 was significant ($P = 0.04$) in a two-tailed unpaired *t* test. In (*B*), the differences between the mice that received unmodified PBMC (○) and those that developed GvHD after $CD25^+$ depletion (●) were significant at days 21 ($P = 0.03$), 28 ($P = 0.04$), and 35 ($P = 0.03$) in two-tailed unpaired *t* tests.

analyses revealed that at intermediate doses, $CD25^+$ depletion increased the expansion rates of human T cells. Significant differences from the control group were visible at day 30 (Fig. 3A). More importantly, Treg depletion caused progressive expansion of human T cells in a significant higher number of mice (18 of 20) compared with the control group (6 of 6), which appears crucial for the development of lethal x-GvHD. At low doses, $CD25^+$ depletion significantly accelerated the expansion of human T cells in mice developing clinical x-GvHD. However, the mice did not develop lethal GvHD because at this low dose, human T cells did not show progressive expansion even after $CD25^+$ cell depletion (Fig. 3C). Analysis of the CD4/CD8 ratios revealed significant expansion of $CD4^+$ cells in the first week, followed by expansion of $CD8^+$ cells. There was no influence of $CD25^+$

depletion on the CD4/CD8 ratios at any time and treatment dose (data not shown).

Coadministration of human CD25$^+$ cells with autologous PBMC significantly reduces the incidence of lethal x-GvHD in the RAG2$^{-/-}\gamma c^{-/-}$ mice. Because the CD25$^+$ depletion experiments indicated a significant role for human Tregs on the development of x-GvHD, we evaluated whether Treg coadministration could prevent/ameliorate GvHD. High doses of PBMC containing 13 $\times$ 10^6 CD25$^-$ T cells were mixed with 4 $\times$ 10^6 to 6 $\times$ 10^6 CD25$^+$-selected cells, which were significantly enriched for CD4$^+$CD25^{high} Foxp3$^+$ T cells (Fig. 1B and D). Coadministration of Treg-enriched fractions significantly reduced the development of lethal x-GvHD (P = 0.025; Fig. 4A). In all mice that were protected from lethal-GvHD after Treg administration, expansion rates of human T cells were significantly lower compared with the control mice (Fig. 4B). Furthermore, extremely high CD4/CD8 ratios were observed in the first week, suggesting the preferential expansion of CD4$^+$CD25$^+$ Tregs (Fig. 4C).

Control of x-GvHD by naturally occurring Treg cells is associated with increased plasma levels of IFN-γ and IL-10. To gain more insight into the human Treg-mediated down-regulation of GvHD, we monitored plasma levels of several human cytokines after coadministration of high doses of PBMC plus 6 $\times$ 10^6 CD25$^+$ cells. Because a significant but partial protection from x-GvHD was observed (Fig. 5A), protected and unprotected mice were analyzed separately. In the protected mice, the numbers of circulating human T cells and the initial levels of IFN-γ and IL-10 were significantly lower compared with the control group and the unprotected mice (P < 0.001; Fig. 5B-D). In the protected group, plasma levels of IFN-γ and IL-10 increased significantly after week 3. In contrast, the levels of IFN-γ and IL-10 decreased gradually in the control group and in the unprotected mice. All other cytokines tested (IL-1, IL-2, IL-4, IL-5, IL-13, and tumor necrosis factor-α) were detected only at low levels in all subjects (data not shown).

Discussion

The new *in vivo* x-GvHD model in the RAG2$^{-/-}\gamma c^{-/-}$ mice enabled us to show, for the first time, the potent *in vivo* capacities of human Tregs to control GvHD-inducing autologous T cells: Depletion of CD25$^+$ T cells significantly exacerbated the symptoms and lethality of x-GvHD and coadministration of CD25$^+$ T cells significantly inhibited the peripheral human T-cell expansion and reduced the lethality of x-GvHD. Furthermore, we observed that protection from x-GvHD by CD25$^+$ cells was associated with significant increases in the plasma levels of IFN-γ and IL-10.

These results are consistent with several previous murine studies and suggest that human Tregs may indeed represent useful immunotherapeutic tools for GvHD prevention. Nevertheless, some precaution may be necessary in interpreting our results as we study the effects of human Tregs in a xenogeneic system. Although this T cell–mediated x-GvHD is associated with a cytokine storm and displays skin lesions similar to allo-GvHD (20), it may still be not entirely comparable with the clinical allo-GvHD. Our model has also some differences from murine BMT models. For instance, in our system, we enriched or depleted Tregs by a simplified procedure based on CD25$^+$

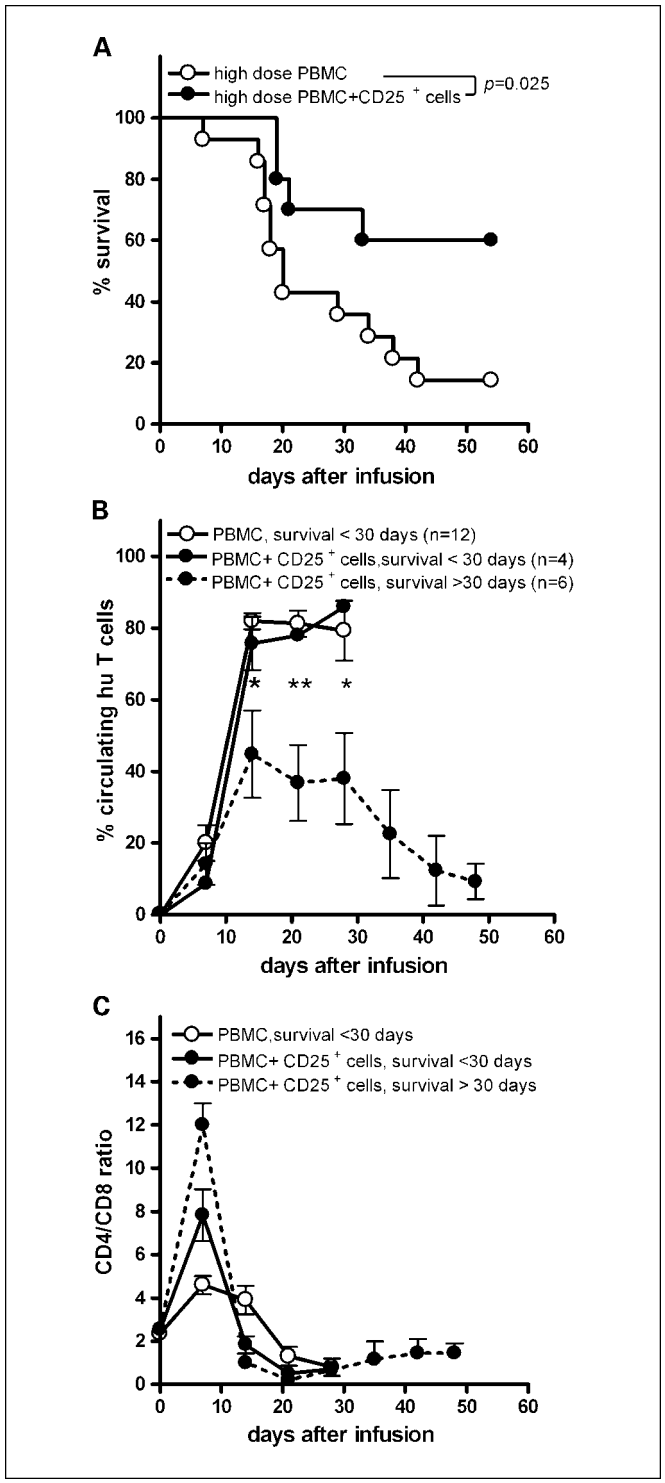

Fig. 4. The effect of CD25$^+$ cell coadministration on x-GvHD induced by autologous human T cells. *A*, Kaplan-Meier survival estimates of RAG2$^{-/-}\gamma c^{-/-}$ mice that received high dose of unmodified PBMC (13 $\times$ 10^6 CD25$^-$ T cells) only (n = 14) or PBMC plus CD25$^+$ cells, 4 $\times$ 10^6 to 6 $\times$ 10^6 cells/mice (n = 10). Data are pooled from three independent experiments. The P value indicate the statistical difference between groups as calculated in a log-rank test. *B*, mean percentages of circulating human T cells. *C*, CD4/CD8 ratios in mice treated with high dose of unmodified or CD25$^+$ cell added PBMC. Groups of mice with or without lethal GvHD (survival shorter or longer than 30 days) are shown separately to illustrate the differences associating with the clinical outcome. Bars, SE. *, ** statistical analyses: the differences between mice treated with unmodified PBMC, survival <30 days (○), and CD25$^+$ added PBMC, survival >30 days (●) were significant at days 14 (P = 0.03), 21 (P = 0.004), and 28 (P = 0.02) in two-tailed unpaired t tests.

selection. The effective separation of $CD4^+CD25^{high}$ and Foxp3-expressing cells indicated that this simplified procedure was sufficient to enrich or deplete the naturally occurring human Treg cells from human PBMCs. The $CD25^+$-selected cells contained some $CD8^+CD25^+$ T cells. However, it is unlikely that these cells can down-regulate x-GvHD, because we and others have shown in clinical studies that human $CD8^+CD25^+$ T cells, like many preactivated $CD4^+CD25^+$ cells, are associated with the development, rather than inhibition of GvHD (21, 22).

In our model, protection from lethal x-GvHD by Treg was associated with inhibition of the expansion of $CD4^+$ and $CD8^+$ human T cells. These results are consistent with a previous murine study in which inhibition of GvHD by Treg cells was shown to be due to a strong reduction in the expansion of $CD4^+$ and $CD8^+$ T cells rather than inhibition of their functional activities, like cytokine secretion (10). Thus, murine and human Tregs appear to down-regulate GvHD primarily by inhibiting the proliferation of T cells. However, the exact inhibitory mechanisms used by Tregs are not well known. In mice, the *in vivo* effects of Tregs are frequently associated with the regulatory cytokines IL-10 and/or transforming growth factor-β (23–27). It has been proposed that Tregs induce IL-10–producing TR1-like and/or transforming growth factor-β–producing Th3-like suppressor T cells (28). Remarkably, in our model, protection from x-GvHD was also associated with significant increases in plasma levels of IL-10. We also observed the production of IFN-γ, which is produced by human TR1 cells next to transforming growth factor-β and IL-10 (29). Thus, it seems relevant to further investigate the involvement of TR1 cells and TR1-associated cytokines in our model. For instance, it will be interesting to study whether administration of neutralizing anti-IL-10 and/or transforming growth factor-β antibodies will revert the inhibition of GvHD by Tregs. Nevertheless, such experiments are not only complex but also require high numbers of Tregs, which cannot be isolated from a single buffy coat. Therefore, we recently started generating Tregs by retroviral transduction of $CD4^+CD25^-$ cells with *Foxp3* genes

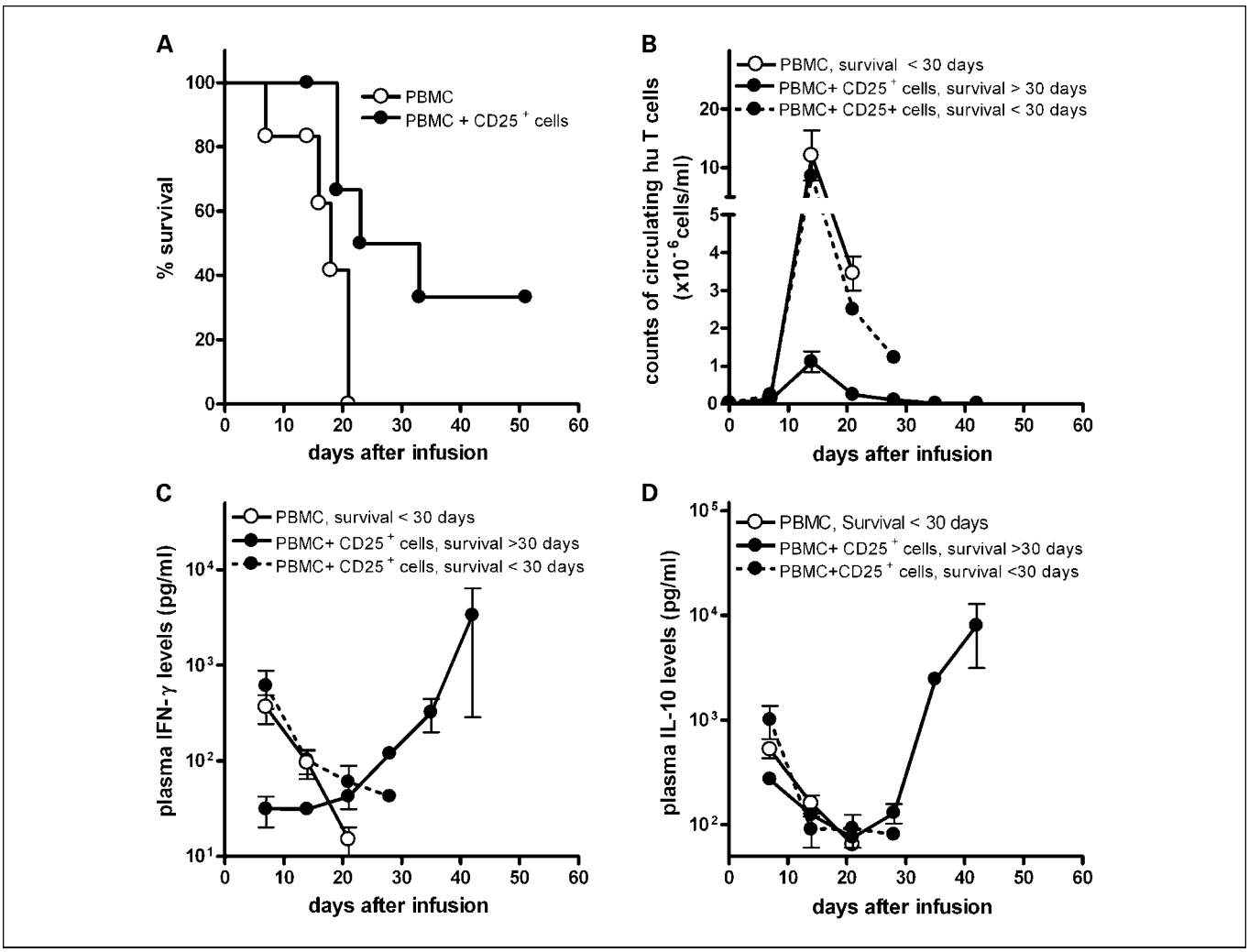

Fig. 5. Control of x-GvHD by infusion of naturally occurring Treg cells is associated with increased plasma IFN-γ and IL-10 levels. *A*, Kaplan-Meier survival curves of mice that received whole PBMC ($n = 6$) or $CD25^+$ T cell – added PBMC ($n = 7$) containing 13×10^6 $CD25^-$ T cells. The $CD25^+$ T cells were added at a dose of 6×10^6/mice. The differences between the groups were significant in a two-tailed log-rank test ($P = 0.02$). *B*, absolute numbers of circulating human T cells. *C* and *D*, the mean plasma IFN-γ (*C*) or IL-10 (*D*) levels in mice after administration of human PBMC. ○, x-GvHD mice that received whole PBMC ($n = 6$); -●-, mice that received $CD25^+$ cell – added PBMC but were not protected from x-GvHD ($n = 5$); ●, mice that received $CD25^+$ cell – added PBMC and were protected from lethal x-GvHD ($n = 2$). Bars, SE.

(30). These *in vitro* generated and expanded Tregs may enable us to address the *in vivo* down-regulatory mechanisms of Tregs more thoroughly. Generation of high numbers of Tregs may also enable us to study whether and at which conditions Treg may be used for the treatment of an established x-GvHD. However, the most intriguing question that still needs to be answered is whether the administration of human Treg will permit a graft-versus-tumor effect, as suggested by murine studies. We think our *in vivo* model is suitable to address this issue, as we recently succeeded in generating a human myeloma model by engrafting luciferase-positive human myeloma cell lines in the RAG2$^{-/-}\gamma c^{-/-}$ mice (31) and our preliminary results indicate the feasibility of converting this model into a DLI-based graft-versus-tumor model by infusion of human lymphocytes.

In conclusion, our current results show, for the first time, the potent *in vivo* capacity of human Treg to control x-GvHD–inducing autologous T cells. Further studies in this useful model may shed more light into the *in vivo* suppressor mechanisms of human Tregs and into their effects on the graft-versus-tumor effect.

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