ASBMT
American Society for Blood and Marrow Transplantation

Human CD4$^+$CD25$^+$ Cells in Combination with CD34$^+$ Cells and Thymoglobulin to Prevent Anti-hematopoietic Stem Cell T Cell Alloreactivity

Dolores Mahmud,[1] Benedetta Nicolini,[1] Lennert van den Dries,[1] Nadim Mahmud,[1,3] Mario Arpinati,[2] Damiano Rondelli[1,3]

Cotransplantation of human CD34$^+$ hematopoietic stem cells (HSC) and CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs) could prevent anti-HSC alloreactivity and reduce the risk of rejection in HLA mismatched transplants. To pursue this hypothesis we cocultured CD34$^+$ cells and CD4$^+$CD25$^+$ cells immunomagnetically isolated (Milteny) from human peripheral blood (unmanipulated or granulocyte-colony stimulating factor [G-CSF] mobilized) or cord blood. Enriched Tregs obtained from the same source (autologous) of CD34$^+$ cells showed greater inhibitory effect on T cell alloreactivity than third-party (allogeneic) Tregs. The immunosuppressive activity of Tregs was maintained after stimulation with allogeneic CD34$^+$ cells and Tregs did not modify the clonogenic activity of CD34$^+$ cells in vitro. Cotransplantation of Tregs with CD34$^+$ cells at 1:1 or 2:1 ratios in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice resulted in normal hematopoietic stem cell engraftment. Incubation with physiologic doses of rabbit antithymocyte globulin (rATG, thymoglobulin) did not affect the number of Tregs in 6-day culture. Upon exposure to thymoglobulin Tregs maintained their suppressive activity, increased expression of CCR7, and released multiple cytokines, primarily interleukin (IL)10. Our findings suggest that human autologous or allogeneic Tregs could be cotransplanted with CD34$^+$ cells after preparative regimens including thymoglobulin.

Biol Blood Marrow Transplant 17: 61-68 (2011)

KEY WORDS: CD34, Hematopoietic stem cells, Regulatory T cells, ATG, T cell alloreactivity

INTRODUCTION

After allogeneic hematopoietic stem cell transplantation (HSCT), graft rejection is more frequently associated with transplantation of T cell-depleted grafts, use of nonmyeloablative conditioning regimens, or use of cord blood (CB) stem cells [1]. T cell depletion by means of selection of CD34$^+$ cells has been utilized particularly in transplants from HLA mismatched donors to prevent graft-versus-host disease (GVHD) [2]. Rejection of CD34$^+$ cells can occur because of alloreactive host T cells. The allo-antigen presenting cell (APC) activity of CD34$^+$ cells was previously demonstrated in vitro and was shown to increase upon interaction with alloreactive T cells inducing the rapid upregulation of costimulatory molecules [3-5]. In addition, the crosstalk between CD34$^+$ cells and allo-T cells was shown to induce the release of cytokines such as tumor necrosis factor-alpha (TNF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF) by T cells that contributed to a rapid (5 day) differentiation of hematopoietic progenitors in CD11c$^+$CD86$^+$ APC [6]. Importantly, this CD34-derived APC subset demonstrated potent allostimulatory effect by means of direct, but also indirect antigen presenting capacity. Strategies to prevent anti-HSC alloreactivity have been investigated in the past, such as the use of a costimulatory blockade [7], which resulted in antigen-specific T cell unresponsiveness, or identification of nonimmunogenic subsets of CD34$^+$ cells, such as CD18$^-$ or CD40$^-$ progenitors [3-5]. However, none of these approaches are yet available in clinical transplant settings. An alternative strategy could be represented by the use of immunosuppressive cells, such as mesenchymal cells or regulatory T cells (Tregs) [8-10]. In particular, Tregs are of great interest in the transplant setting as a potential tool for inducing antigen-dependent T cell unresponsiveness [11,12].

From the [1]Section of Hematology/Oncology, University of Illinois at Chicago, Chicago, Illinois; [2]Department of Hematology/Oncology "Seragnoli," University of Bologna, Italy; and [3]University of Illinois at Chicago Cancer Center, Chicago, Illinois.

Financial disclosure: See Acknowledgments on page 67.

Correspondence and reprint requests to: Damiano Rondelli, MD, Section of Hematology/Oncology, University of Illinois at Chicago, 909 S. Wolcott Ave. (MC734), Chicago, IL 60612 (e-mail: drond@uic.edu).

Received May 28, 2010; accepted August 4, 2010

1083-8791/$36.00
doi:10.1016/j.bbmt.2010.08.004

In allogeneic HSCT from HLA matched unrelated [13] or mismatched related donors [2], the use of antithymocyte globulin (ATG) is associated with reduced GVHD. Recent studies demonstrated that rabbit ATG (thymoglobulin) spares and even expands $CD4^+CD25^+FoxP3^+$ cells [14,15]. In this study, we tested whether the combination of Tregs and $CD34^+$ cells can inhibit anti-stem cell alloreactivity while preserving stem cell activity. In addition, we tested whether Tregs' inhibition of anti-stem cell T cell alloreactivity would be affected by previous exposure to thymoglobulin, in an attempt to mimic pretransplant conditioning regimens including such agents.

MATERIALS AND METHODS

Cell Separation

CB or peripheral blood (PB) mononuclear cells (MNCs) and $CD34^+$ or $CD3^+$ cells were isolated as previously described [6]. $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were immunomagnetically isolated from unstimulated PB by a 2-step process including a CD4-positive selection followed by a CD25-negative selection using the Treg isolation kit (Miltenyi Biotec, Auburn, CA) or by a 3-step process including an initial monocyte depletion (CD14 separation kit, Miltenyi) when using granulocyte-colony stimulating factor (G-CSF) mobilized PB stem cells (PBSCs).

Flow Cytometry

Flow cytometric analysis was performed as previously described [6] using the following FITC, or PE, or PerCP-conjugated monoclonal antibodies (mAbs): CD34, CD3, CD4, CD25, CCR7, GITR, CD152, and CD62L. An anti-FoxP3 PE mAb was utilized for intracellular staining after permeabilization of the cell membrane according to the manufacturer's instruction (eBioscience, San Diego, CA).

T Cell Proliferation

In vitro liquid cultures with isolated MNC or purified $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were performed in the presence of rabbit ATG (Thymoglobulin, Genzyme, Cambridge, MA) at 100 μg/mL. Cells were cultured in medium containing RPMI-1640 (Cambrex, Baltimore, MD), 100 U/mL penicillin (Cambrex), 100 μg/mL streptomicin (Cambrex), and 10% AB human serum (HS, Hyclone, Logan, UT) that was not inactivated. Cultures were performed in round-bottomed 96-well plates for 12 days at 37°C in a 5% CO_2 humidified atmosphere. Daily T cell proliferation was assessed by 3H-thymidine uptake. In selected experiments, $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were cultured at 2.5×10^4 cells/100 μL with Thymoglobulin at 10 or 100 μg/mL for 6 days. Cells were then washed and viable cells were analyzed by trypan-blue exclusion assay.

Primary MLC

Irradiated stimulator (S) cells were mixed with responder (R) cells at 1:1 or 1:2 ratio for 6 days [6]. Purified $CD3^+$ and $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were used as responders. In selected experiments, MLC experiments were performed by adding thymoglobulin at 50, 100, 300, or 500 μg/mL to the culture. In these experiments, media contained 10% AB human serum that had not been inactivated. Proliferation was measured by ^{3}H-thymidine-uptake assay and stimulation index (SI) was calculated for each experiment as previously described [6].

Suppression Experiments

$CD4^+CD25^+$ and $CD4^+CD25^-$ cells, either freshly isolated or after stimulation with irradiated $CD34^+$ cells at 1:1 ratio for 6 days, were added back to a primary MLC at 1:1 ratio with R. In selected experiments, $CD4^+CD25^+$, or $CD4^+CD25^-$ cells as control, were incubated for 3 to 6 days with Thymoglobulin (Genzyme) at 100 μg/mL in medium containing noninactivated 10% human serum, and then added back to an MLC. The percentage of stimulation or suppression of T cell responses compared to the original MLC was calculated based on the SI obtained in each experiment.

CFU-C Assay

Purified $CD34^+$ were mixed with $CD4^+CD25^+$ or $CD25^-$ cells at 1:1 ratio for 24 hours and then were plated in methylcellulose medium with cytokines (Stem Cell Technologies, Vancouver, Canada) [6]. Colony forming cells (CFU-C), including granulocyte-macrophage CFU (CFU-GM), erythroid progenitors (burst-forming unit-erythroid [BFU-E]), and CFU-Mix were scored after 14 days of culture.

Cytokine Assay

$CD34^+$ cells (5×10^4 cells/well in 96-round bottom plate) were mixed with allogeneic $CD4^+CD25^+$ cells at 1:1 ratio in liquid culture w/wo addition of thymoglobulin at 100 μg/mL for 3 days in media containing noninactivated human serum. Control experiments included liquid cultures with Tregs and thymoglobulin. Supernatants were harvested (100 μL/well × 5 replicate wells) and anticytokines antibodies bound to microbeads were used in sandwich immunoassay to measure the levels of interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α, GM-CSF, interferon (IFN)-γ (Bio-Plex Cytokine assay, Bio-Rad, Hercules, CA), and TGF-β (TGF-β Multiplex kit, R&D Systems, Minneapolis, MN) according to manufacturers' instruction.

Transplantation

Human CB purified $CD34^+$ cells (2×10^5/animal) were mixed with allogeneic PB selected $CD4^+CD25^+$ cells at 1:0, 1:1, or 1:2 ratio and then transplanted into sublethally irradiated (300 cGy) nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice as previously described [6]. Six weeks after transplant total bone marrow (BM) cells were collected and analyzed to assess the engraftment of human $CD45^+$ cells of myeloid, lymphoid, and erythroid lineages by flow cytometry.

Statistical Analysis

t-Test analysis or analysis of variance (ANOVA) test were used for statistical analysis.

RESULTS

Anti-CD34 Cell Alloreactivity is Suppressed by Autologous or Allogeneic Tregs

In this study, we tested the hypothesis of whether human Tregs may be considered as a potential adoptive therapy in combination with purified HSCT. We initially separated human natural Tregs, defined as $CD4^+CD25^+$ cells, from CB, PB, or PBSC by means of a standard immunomagnetic method which is available also in clinical scale [16,17] and, therefore, could be of potential clinical benefit. After separation, $CD4^+CD25^+$ cell purity was on average 73% ± 9% (n = 11 experiments) (Figure 1A). Purity rates of Tregs obtained from CB, PBSC, or PB were comparable (not shown). Expression of intracellular FoxP3 and dim expression of CD127 on $CD4^+CD25^+$ cells were consistent with Treg phenotype (Figure 1B).

The function of these cells was then tested. Primary MLC were performed with irradiated $CD34^+$ cells isolated from CB or PBSC and allogeneic $CD3^+$ responder cells isolated from unmanipulated blood. Tregs or $CD4^+CD25^-$ control cells were isolated from PB, CB, or PBSC and could be allogeneic (PB) or autologous (CB or PBSC) to the $CD34^+$ cells. PB Tregs allogeneic to $CD34^+$ cells (ALLO PB) showed a 41% ± 16% inhibition of anti-$CD34^+$ cell T cell alloreactivity in MLC, compared to autologous CB Tregs (AUTO CB) (68% ± 14%) or autologous PBSC (AUTO PBSC) Tregs (76% ± 16%) ($P = .04$) (Figure 1C). As expected, in control experiments, addition of $CD4^+CD25^-$ cells that were allogeneic to $CD34^+$ stimulator cells and allo-responders increased anti-CD34 alloreactivity (ALLO PB), whereas when they had been isolated from the same source of $CD34^+$ cells did not modify the proliferation of allo-responders (AUTO CB, AUTO PBSC). To address the concern that Treg purity after immunomagnetic isolation was not sufficient and alloreactive T cells would be present, we tested whether prior incubation of the isolated Treg population with allostimulatory $CD34^+$ cells could hamper the suppressive effect of the cells by stimulating residual non-Treg T cells. After coculture with irradiated $CD34^+$ cells for 6 days at 1:1 ratio, Tregs were added back to a primary MLC with $CD34^+$ cells and third-party T cell responders. In Figure 2, we show that Tregs maintained

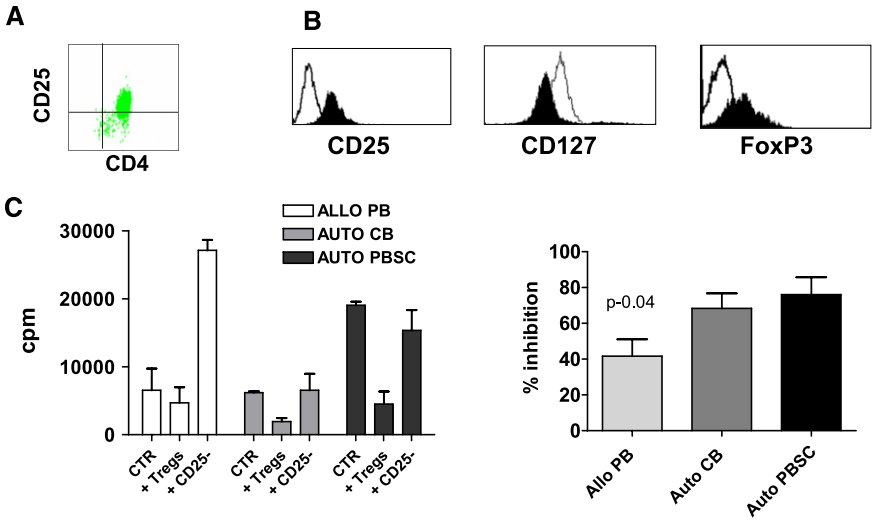

Figure 1. Tregs isolation and effect on $CD34^+$ cell allostimulatory activity. (A) Purity of $CD4^+CD25^+$ cells after immunomagnetic isolation (Treg isolation kit, Miltenyi) and (B) expression of CD25, CD127, and FoxP3 in $CD4^+CD25^+$ (filled histogram) or $CD4^+CD25^-$ (open histogram) cell fractions. (C) Tregs or $CD4^+CD25^-$ T cells isolated from allogeneic peripheral blood (ALLO PB), or autologous cord blood (AUTO CB), or autologous PBSC (AUTO PBSC) were added to an MLC with irradiated $CD34^+$ cells and allo-responder T cells. CD34: allo-responder, and responder: Treg or $CD4^+CD25^-$ cell ratios were: 1:2 and 1:1, respectively (n = 3 experiments for each condition). ^{3}H-thymidine uptake assay was utilized to measure T cell responses and mean ± SD cpm are shown (C, left). Addition of Tregs resulted in reduced alloreactivity compared to CTR experiments in each condition (ALLO PB: $P = .2$; Auto CB: $P = .05$, Auto PBSC: $P = .05$). Analysis of T cell inhibition (C, right) showed that Tregs obtained from the same stem cell product as $CD34^+$ cells (AUTO CB or AUTO PBSC) had a greater suppressive activity ($P = .04$ by ANOVA test). Suppression activity is indicated by the percentage of inhibition of responder cells.

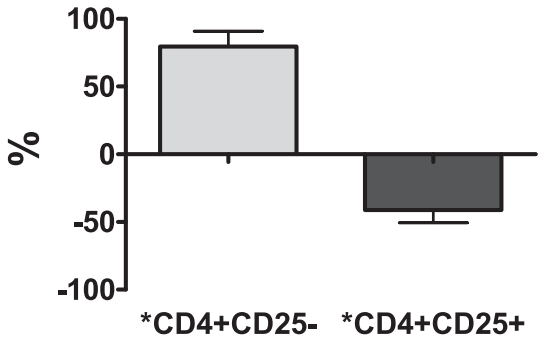

Figure 2. Unmodified suppressive activity of Tregs primed with allogeneic $CD34^+$ cells. Allogeneic PB Tregs that were initially cultured with irradiated $CD34^+$ cells for 6 days maintained their suppressive activity when added to a third party MLC at a 1:1 ratio with responder cells, as opposed to control $CD4^+CD25^-$ cells, which instead increased T cell alloreactivity (n = 3 experiments). The effect of Tregs or $CD4^+CD25^-$ cells is shown as percentage (%) of T cell proliferation or suppression compared with control MLC where allo-T cells are stimulated by $CD34^+$ cells. Results are shown as average ± SD of 3 separate experiments.

their capacity to partially inhibit alloproliferative responses as much as seen when using freshly unstimulated Tregs (Figure 1).

Tregs Do Not Affect In-Vitro and In Vivo $CD34^+$ Cell Clonogenic Activity

We then tested whether Treg cells that have potential clinical application may affect the stem cell function of $CD34^+$ cells. The short-term clonogenic activity of $CD34^+$ cells, measured as number of granulo-monocytic (CFU-GM), erythroid (BFU-E), or less committed mixed (CFU-Mix) colonies, was not affected in a standard 2 weeks CFU-C assay after initial 24 hours liquid culture of $CD34^+$ cells with Tregs or $CD4^+CD25^-$ cells as control (Figure 3A). To assess the long-term engraftment we cotransplanted $CD34^+$ cells and Tregs at 2 different doses in NOD/SCID mice. Similar to what was observed in vitro, the in vivo engraftment of $huCD34^+$ cells into NOD/SCID mice was not affected after cotransplanting $CD34^+$ cells and Tregs at 1:1 or 1:2 ratio (Figure 3B). The percentage of myeloid cells, myeloid DC, B cells, and erythroid cells was not different in the 2 groups compared to mice transplanted with $CD34^+$ cells alone (not shown).

In Vitro Effect of Thymoglobulin on T Cells

Clinical transplantation of $CD34^+$ cells in an HLA mismatch setting often includes the use of ATG in the conditioning regimen as a means of in vivo T cell modulation/depletion [2]. Based on ATG pharmacokinetics [18,19], any adoptive T cell therapy should be avoided for at least 2 weeks following treatment with ATG. A dose of thymoglobulin at 7 mg/kg of an adult patient's body weight, often used in clinical trials, corresponds approximately to a plasma concentration of 100 μg/mL, and this physiologic dose was also tested in a previous study [20]. We added doses of thymoglobulin from 50 to 500 μg/mL to MLC and assessed the effect on T cell alloreactivity in vitro. Experiments were performed with or without inactivation of complement in the media with no different results. We observed an increased T cell proliferation in primary MLC, where thymoglobulin was added at 50, 100, or 300 μg/mL, whereas a high dose of thymoglobulin at 500 μg/mL was highly suppressive (Figure 4A). To test if this observation was because of an antigen-independent direct effect of thymoglobulin on T cells, we incubated T cells with thymoglobulin at 100 μg/mL and measured T cell proliferation by ^{3}H-thymidine uptake assay every day for 12 days. In 1 representative experiment of 3 separate performed, shown in Figure 4B, thymoglobulin induced an initial proliferation of T cells, peaking on days 4 and 5, even when additional doses of thymoglobulin were added on days 3 and 6 of culture.

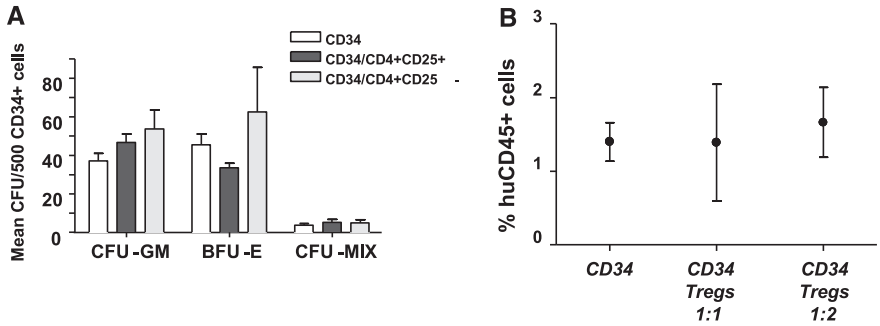

Figure 3. Clonogenic activity of $CD34^+$ cells in the presence of Tregs. (A) $CD34^+$ cells were mixed with $CD4^+CD25^+$ or $CD25^-$ T cells at a 1:1 ratio and then tested for CFU-C in semisolid media (n = 5 experiments). Differences were not statistically significant by ANOVA test. (B) Human $CD34^+$ cells were transplanted into NOD/SCID mice w/wo allogeneic Tregs at a 1:1 or 1:2 ratio, and 6 weeks later the marrow was harvested and tested by flow cytometry to assess human hematopoietic stem cell engraftment. The percentage of human $CD45^+$ cells was comparable in each group (n = 5 mice per group). Analysis of subsets of $huCD45^+$ cells did not show differences in myeloid or erythroid lineages in animals transplanted w/wo Tregs (not shown).

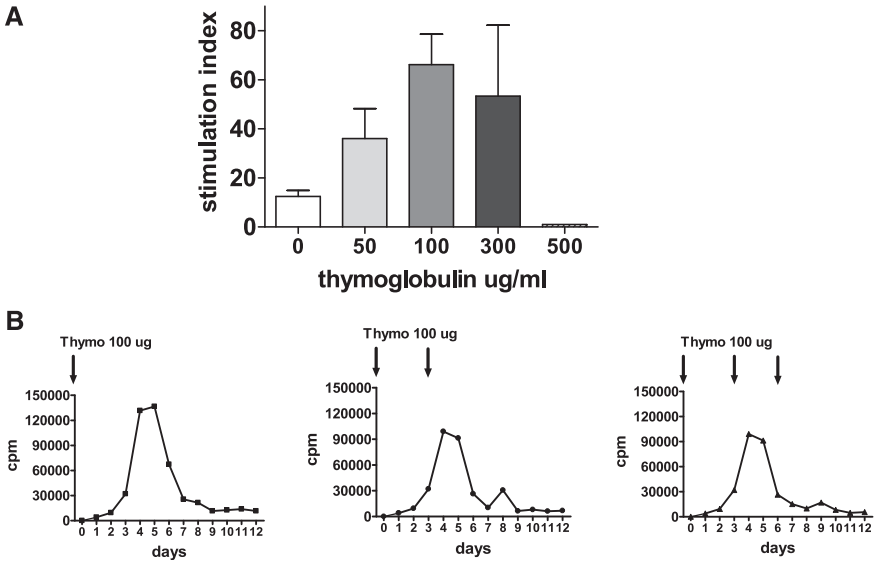

Figure 4. Effect of thymoglobulin on T cell proliferation in vitro. (A) Thymoglobulin was tested in vitro at 0 (control), 50, 100, 300, or 500 μg/mL, in primary MLC with MNC stimulator cells and allogeneic T cell responders at a 1:2 ratio in media containing human AB serum that was not inactivated. T cell alloreactivity was measured by ^{3}H-thymidine incorporation assay after 6 days of culture. Results are shown as mean S.I. ± SD of 5 separate experiments. T cell alloresponses in the presence of thymoglobulin at 50, 100, or 300 μg/mL were greater than in control MLC ($P = .02$), whereas thymoglobulin at 500 μg/mL completely inhibited T cell response ($P = .03$). (B) Thymoglobulin was added to liquid cultures of purified T cells at the physiologic dose of 100 μg/mL on day 1 only (B, left), days 1 and 3 (B, center), or days 1, 3, and 6 (B, right). T cell proliferation was measured daily for 12 days by ^{3}H-thymidine incorporation assay, and cpm values of 1 experiment representative of 3 are shown.

Suppression of Anti-CD34^{+} Cell Alloreactivity by Tregs Treated with Thymoglobulin

Based on the results earlier, we then tested whether thymoglobulin would still permit Tregs to reduce anti-CD34 cell alloreactivity. To this purpose, we initially incubated Tregs with thymoglobulin at 10 or 100 μg/mL for 3 or 6 days and then assessed cell viability in culture. As shown in Figure 5A, the number of Tregs did not significantly change over time compared to the baseline number (shown as dotted line), with a slight increase when a lower dose of thymoglobulin was utilized. More importantly, stimulation with thymoglobulin for 6 days did not modify the expression of FoxP3, whereas it increased the expression of CCR7, GITR, and CD152 on Tregs (Figure 5B). Finally, in experiments where allogeneic (third party) CD4^{+}CD25^{+} or CD4^{+}CD25^{-} cells pretreated with thymoglobulin for 3 days were added to a primary MLC with CD34^{+} cells and alloreactive T cells, Tregs maintained the capacity of inhibiting anti-HSC T-cell alloreactivity as opposed to CD4^{+}CD25^{-} control cells that showed little effect on the allo-responders (Figure 5C). Thymoglobulin effect on Tregs was similar when these cells had been obtained from third party PB or same PB as alloresponders (not show).

Thymoglobulin Stimulates Tregs-Mediated IL-10 Production

To assess whether thymoglobulin would have any effect on the interaction between Tregs and CD34^{+} cells, we utilized a Th1/Th2 Bioplex Cytokine assay (Bio-Rad) and a TGF-β Multiplex kit (R&D Systems) to measure the cytokine production after 3 days of co-culture of CD34^{+} cells and Tregs w/wo thymoglobulin. In experiments with CD34^{+} cells and Tregs without thymoglobulin, almost undetectable levels of cytokines were detected (Figure 6, left). On the contrary, in experiments where thymoglobulin was added to Tregs and CD34^{+} cells (Figure 6, center) or to Tregs alone (Figure 6, right), an increased production of cytokines, primarily IL-10, was observed. On the contrary, no detectable levels of TGF-β were found in any of the conditions described before (not shown). In control experiments on media containing only thymoglobulin no cytokines were found (not shown).

DISCUSSION

In this study, we show that an immunomagnetically enriched Treg cell population combined with CD34^{+} hematopoietic progenitors permits an efficient stem cell activity and inhibits the anti-stem cell alloreactivity of HLA mismatched T cells even in the presence of physiologic doses of thymoglobulin.

With an immunomagnetic cell separation method (Miltenyi) available also in clinical scale [16,17], Treg isolation required a 2-step process using unmanipulated PB or CB and a 3-step process with PBSC, and resulted in a purity of 73% on average. Selected Tregs expressed CD25 at different intensity, but were shown to express also FoxP3 and low levels of CD127. Our interest in testing these cells with CD34^{+} cells was based on

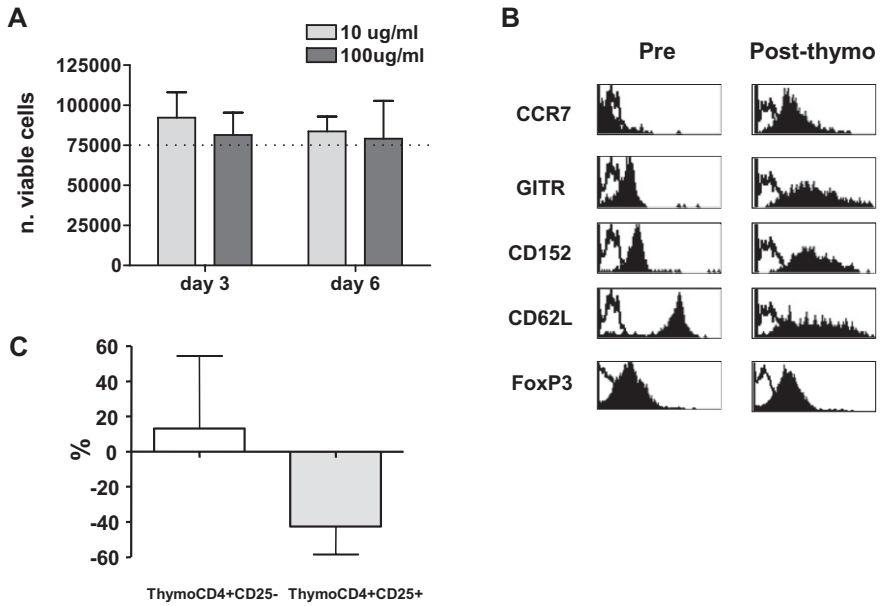

Figure 5. Thymoglobulin does not affect Tregs in vitro . (A) Liquid cultures of $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were performed in the presence of thymoglobulin at 10 or 100 μg/mL. Dotted line represents the baseline number of cells plated. After 3 and 6 days of culture, cells were harvested and counted by trypan blue exclusion to assess viable cells. Results are shown as average ± SD of 5 separate experiments. (B) Expression of CCR7, GITR, CD152, CD62L, and intracytoplasmic FoxP3 before (Pre) and after a 3-day culture with thymoglobulin (Post-thymo) was analyzed by flow cytometry in PB $CD4^+CD25^+$ Tregs. Histograms shown are representative of one of 3 separate experiments; (C) Allogeneic $CD4^+CD25^+$ cells, or $CD4^+CD25^-$ cells as control, were exposed to thymoglobulin for 3 days in vitro and then were added back to a primary MLC with irradiated $CD34^+$ stimulator cells and third-party T cells (n = 3 experiments). Thymoglobulin-exposed Tregs (Thymo-$CD4^+CD25^+$) suppressed the anti-HSC T cell alloreactivity as opposed to thymoglobulin-exposed $CD4^+CD25^-$ cells. Suppression activity is indicated by the percentage of inhibition of responder cells.

previous studies showing that human $CD34^+$ cells stimulate allogeneic T cell responses in vitro because of a subset of these progenitors with potent ability of presenting alloantigen and delivering costimulatory signals to $CD4^+$ and $CD8^+$ T cells [3,4]. Particularly, costimulatory molecules such as CD80, CD86, CD40, or ICOS-L are rapidly upregulated on $CD34^+$ cells in response to T cell contact and/or in the presence of TNF-α [5,21]. Futhermore, the allostimulatory activity of $CD34^+$ cells can be greatly enhanced upon crosstalking with allogeneic T cells that induce the differentiation of progenitors into professional APC in vitro and in vivo [6]. Here, we observed that Tregs obtained from the same source of $CD34^+$ cells, that is, PBSC or CB, suppressed anti-stem cell T cell alloreactivity by approximately 70%. The potent inhibitory effect detected despite a nonhighly purified Treg cell population is likely to be because of the fact that bystander residual non-Tregs were autologous to $CD34^+$ stimulator cells. However, third-party Tregs also inhibited T cell alloresponses by appoximately 40% to 50%, suggesting that residual bystander non-Tregs had very limited or no response capacity and did not prevent the Tregs from inhibiting allo-responders in MLC. This could be explained by a low sensitivity of the assay in measuring T cell proliferation. Alternatively, Tregs could induce the apoptosis of bystander CD4 effectors, as recently reported [22]. In a clinical setting, these findings suggest that a Treg product enriched in $CD4^+CD25^+$ cells would not increase the risk of GVHD. Moreover, the

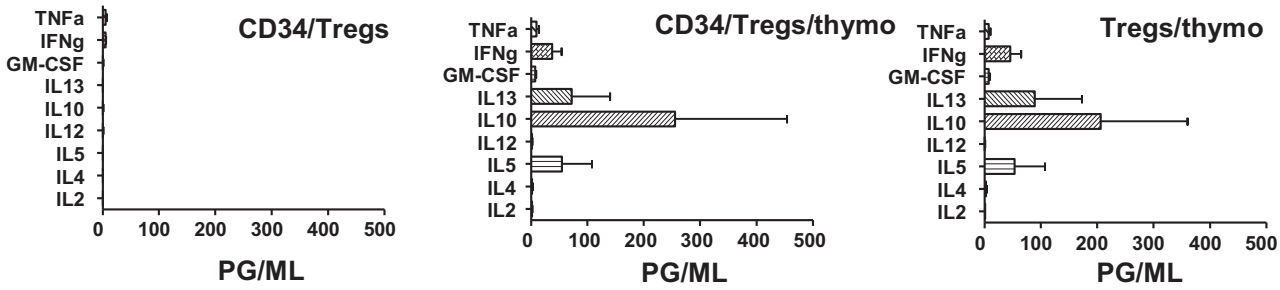

Figure 6. Thymoglobulin-induced IL10 production by Tregs. $CD4^+CD25^+$ Tregs were cultured with $CD34^+$ cells at a 1:1 ratio, with CD34 and thymoglobulin at 100 μg/mL, or with thymoglobulin alone. After 3 days supernatants were harvested and cytokine levels were measured by Bio-Plex Cytokine assay (n = 3 experiments). In the presence of thymoglobulin, Tregs produced mostly IL-10 and IL-13 independently of the allostimulatory effect of $CD34^+$ cells.

use of donor-derived Tregs in combination with $CD34^+$ cells could have the advantage of facilitating the immune reconstitution posttransplant. This effect, in fact, was demonstrated in a mouse model where Tregs improved the development of lymphoid cells derived from the graft by reducing GVHD, preserving thymic and lymph node structure, and accelerating the reconstitution of a diverse TCR-Vβ repertoire [23].

In our model, human Tregs did not affect the clonogenic activity of $CD34^+$ cells measured by CFU-C in vitro and BM engraftment after transplantation in NOD/SCID mice. In a recent study, Urbieta et al. [24] utilized BALB/C and B6 mice to test the effect of Tregs on hematopoiesis and found that Tregs activated with anti-CD3/CD28 beads and IL2 released TGF-β and suppressed CFU IL3/SCF in vitro and affected the recovery of neutrophils after T cell-depleted BM transplantation. This inhibitory effect was not observed when using freshly isolated Tregs. Although we did not test the effect of Tregs after in vitro activation with CD3/CD28 beads, our study seems to have some differences compared to the previously mentioned one. We used human Tregs with selected $CD34^+$ cells that were previously shown to potently stimulate CD28 receptor on T cells through CD80 and CD86. When we measured the production of cytokines in cultures of $CD34^+$ and Tregs, we could not detect any cytokines other than traces of TNF-α and IFN-γ, but no TGF-β. In addition, when $CD34^+$ cells and Tregs were transplanted in sublethally irradiated NOD/SCID mice, we assumed that Tregs would find multiple cytokines released by tissues upon radiation and by host cells. Nevertheless, Tregs did not affect the stem cell engraftment when tested at 2 different doses. We assume, therefore, that the clinical role of human Tregs in $CD34^+$ cells transplantation remains to be found, and it can be investigated in experimental clinical trials.

Because in HLA mismatched or haploidentical SCT the addition of ATG in the conditioning regimen is clinically relevant to reduce the risk of GVHD from donor T cells, and likely reduces the risk of rejection from host T cells, we addressed the question whether physiological doses of rATG (thymoglobulin) would affect the function of Tregs added to alloreactive T cells and $CD34^+$ cells. Indeed, recent studies proposed that thymoglobulin can expand Tregs by increasing the expression of FoxP3 not only on $CD4^+CD25^+$ but also on $CD4^+CD25^-$ cells, therefore increasing the pool of $CD4^+$ cells with inhibitory function [14,15]. Our approach differed from previous studies. We initially found that physiologic doses of thymoglobulin stimulated the proliferation in vitro of T cells within the first 3 to 5 days of culture. The mechanism for this finding remains uncertain, although it could be explained by recent findings showing high levels of type 1 and 2 cytokines released by $CD4^+$ cells stimulated with thymoglobulin [25]. In our experiments where thymoglobulin was tested in vitro for 6 days on purified T cell subsets, we observed the proliferation of $CD4^+CD25^-$ and $CD8^+$ cell fractions (not shown) but not of $CD4^+CD25^+$ Tregs, which remained unchanged compared to baseline numbers. However, Tregs that were cultured with thymoglobulin increased the expression of CD152 (CTLA4) and maintained their inhibitory effect on anti-stem cell T cell alloreactivity. In contrast, $CD4^+CD25^-$ cells showed only a very weak upregulation of FoxP3 (not shown) and no suppressive activity when added to allogeneic MLC.

A previous study suggested that among other immunologic effects, thymoglobulin can also reduce the chemotaxis of $CD4^+$ T cells to tissues with increased antigen presentation by blocking the binding of CXCR4 and SDF1 [20]. We observed an increased expression of CCR7 on isolated Tregs upon stimulation with thymoglobulin. CCR7 was previously shown to play a key role on the trafficking of naïve or central memory-like Tregs [26]. We, therefore, hypothesize that another immunomodulatory effect of thymoglobulin may be mediated by increasing the recirculation of Tregs in different tissues or lymphoid organs after transplantation. Our findings support other studies suggesting that thymoglobulin affects immune responses, at least initially, by an immunomodulatory rather than a cytotoxic effect. In fact, we demonstrated a direct effect of thymoglobulin on Treg ability to release multiple cytokines and particularly IL-10, IL-5, and IL-13, but not TGF-β. Importantly, this effect of thymoglobulin was observed also when Tregs were cocultured with allogeneic $CD34^+$ cells.

Overall, these results suggest that transplantation of $CD34^+$ cells and Tregs from the same donor might facilitate the engraftment by reducing the anti-stem cell response of host T cells. A direct effect of Tregs on donor T cells reducing GVHD would also be beneficial for a faster immune reconstitution [23]. Because the in vivo suppressive effect of expanded/induced Tregs remains unclear [27], the use of PBSC as a source of natural Tregs will facilitate the design of new clinical strategies of cotransplantation of $CD34^+$ and Tregs in T cell-depleted stem cell transplantation, as recently proposed [28] in a haploidentical transplantation study. Based on the effects of thymoglobulin on Tregs shown here, it could be also speculated that addition of Tregs to unmanipulated grafts in the presence of thymoglobulin may further enhance the anti-GVHD activity. However, this hypothesis is difficult to be tested in vitro and can only be validated in clinical trials.

ACKNOWLEDGMENTS

Financial disclosure: The study was partially supported by a grant of the Elsa U. Pardee Foundation to D.R. The authors thank Joseph Scholl, Genzyme Co., Cambridge, MA, for providing thymoglobulin for in vitro experiments.

AUTHORSHIP STATEMENT

D.M. performed in vitro and in vivo experiments, analyzed the data, and contributed to writing the manuscript. B.N. performed in vitro experiments and analyzed the data. L.W.J.V.D.D performed in vitro experiments. N.M. performed in vivo experiments and and contributed to writing the manuscript. M.A. contributed to designing the study and to writing the manuscript. D.R. designed the study, analyzed the data, and wrote the manuscript.

REFERENCES

1. Mattason J, Ringden O, Storb S. Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2008;14(Suppl 1):165-170.
2. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. *N Engl J Med*. 1998;339:1186-1193.
3. Rondelli D, Andrews RG, Hansen JA, Ryncarz R, Faerber MA, Anasetti C. Alloantigen presenting function of normal human CD34+ hematopoietic cells. *Blood*. 1996;88:2619-2675.
4. Rondelli D, Anasetti C, Fortuna A, et al. T cell alloreactivity induced by normal G-CSF-mobilized CD34+ blood cells. *Bone Marrow Transplant*. 1998;21:1183-1191.
5. Rondelli D, Lemoli RM, Ratta M, et al. Rapid induction of CD40 on a subset of granulocyte colony-stimulating factor-mobilized CD34(+) blood cells identifies myeloid committed progenitors and permits selection of non immunogenic CD40(−) progenitor cells. *Blood*. 1999;94:2293-2300.
6. Abbasian J, Mahmud D, Mahmud N, et al. Allogeneic T cells induce rapid CD34+ cell differentiation into CD11c+CD86+ cells with direct and indirect antigen-presenting function. *Blood*. 2006;108:203-208.
7. Arpinati M, Terragna C, Chirumbolo G, et al. Human CD34(+) blood cells induce T-cell unresponsiveness to specific alloantigens only under costimulatory blockade. *Exp Hematol*. 2003; 31:31-38.
8. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood*. 2005;105:2821-2827.
9. Tolar J, Hippen KL, Blazar BR. Immune regulatory cells in umbilical cord blood: T regulatory cells and mesenchymal stromal cells. *Br J Haematol*. 2009;147:200-206.
10. Joffre O, Santolaria T, Calise D, et al. Prevention of acute and chronic allograft rejection with CD4+CD25+FoxP3+ regulatory T Lymphocytes. *Nat Med*. 2008;14:88-92.
11. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531-562.
12. Albert MH, Liu Y, Anasetti C, Yu XZ. Antigen- dependent suppression of alloresponses by Foxp3-induced regulatory T cells in transplantation. *Eur J Immunol*. 2005;35:2598-2607.
13. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *Lancet Oncol*. 2009;10:855-864.
14. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+FoxP3 regulatory T cells. *J Am Soc Nephrol*. 2006;17:2844-2853.
15. Feng X, Kajigaya S, Solomou EE, et al. Rabbit ATG but not horse ATG promotes expansion of functional CD4+CD25 high FoxP3+ regulatory T cells in vitro. *Blood*. 2008;111: 3675-3683.
16. Hoffmann P, Boeld TJ, Eder R, et al. Isolation of CD4+CD25+ regulatory T cells for clinical trials. *Biol Blood Marrow Transplant*. 2006;12:267-274.
17. Di Ianni M, Del Papa B, Cecchini D, et al. Immunomagnetic isolation of CD4+CD25+FoxP3+ natural T regulatory lymphocytes for clinical applications. *Clin Exp Immunol*. 2009;156: 246-253.
18. Kakhniashvili I, Filicko J, Kraft WK, Flomenberg N. Heterogeneous clearance of antithymocyte globulin after CD34+ selected allogeneic hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. 2005;11:609-618.
19. Waller EK, Langston AA, Lonial S, et al. Pharmacokinetics and pharmacodynamics of anti-thymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. *Biol Blood Marrow Transplant*. 2003;9:460-471.
20. LaCorcia G, Swistak M, Lawendowski C, et al. Polyclonal rabbit antithymocyte globulin exhibits consistent immunosuppressive capabilities beyond cell depletion. *Transplantation*. 2009;87: 966-974.
21. Richter G, Hayden-Ledbetter M, Irgang M, et al. Tumor necrosis factor-alpha regulates the expression of inducible costimulator receptor ligand on CD34(+) progenitor cells during differentiation into antigen presenting cells. *J Biol Chem*. 2001; 276:45686-45693.
22. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol*. 2007;8:1353-1362.
23. Nguyen VH, Shashidhar S, Chang DS, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood*. 2008;11:945-953.
24. Urbieta M, Barao I, Jones M, et al. Hematopoietic progenitor cell regulation by CD4+CD25+ T cells. *Blood*. 2010 Mar 3 [Epub ahead of print].
25. Liu Z, Fang Y, Wang X, Wang P, Yun P, Xu H. Upregulation of molecules associated with T-regulatory function by thymoglobulin pretreatment of human CD4+ cells. *Transplantation*. 2008;86:1419-1426.
26. Menning A, Hopken HE, Siegmund K, Lipp M, Hamann A, Huehn J. Distinctive role of CCR7 in migration and functional activity of naïve and effector/memory-like Treg subsets. *Eur J Hematol*. 2007;37:1575-1583.
27. Koenecke C, Czeloth N, Bubke A, et al. Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. *Eur J Immunol*. 2009;39:3091-3096.
28. Di Ianni M, Falzetti F, Carotti A, et al. Adoptive immunotherapy with Tregs prevents GvHD and favours immune reconstitution after HLA haploidentical transplants for hematological malignancies. *Blood*. 2009;114:4 (abstr.).