

Original Article

Clinical grade isolation of regulatory T cells from G-CSF mobilized peripheral blood improves with initial depletion of monocytes

Pritesh Patel^{1,2*}, Dolores Mahmud^{1*}, Youngmin Park³, Kazumi Yoshinaga³, Nadim Mahmud^{1,2,3}, Damiano Rondelli^{1,2}

¹Division of Hematology/Oncology, University of Illinois at Chicago, Chicago, IL, USA; ²Cancer Center, University of Illinois, Chicago, IL, USA; ³Hospital Stem Cell Laboratory, University of Illinois Hospital and Health Sciences System, Chicago, IL, USA. *Equal contributors.

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Abstract: Clinical isolation of circulating CD4⁺CD25⁺ regulatory T cells (Tregs) from peripheral blood mononuclear cells is usually performed by CD4⁺ cell negative selection followed by CD25⁺ cell positive selection. Although G-CSF mobilized peripheral blood (G-PBSC) contains a high number of Tregs, a high number of monocytes in G-PBSC limits Treg isolation. Using a small scale device (MidiMACS, Miltenyi) we initially demonstrated that an initial depletion of monocytes would be necessary to obtain a separation of CD4⁺CD25⁺FoxP3⁺CD127⁻ cells from G-PBSC (G-Tregs) with a consistent purity >70% and inhibitory activity of T cell alloreactivity *in-vitro*. We then validated the same approach in a clinical scale setting by separating G-Tregs with clinically available antibodies to perform a CD8⁺CD19⁺CD14⁺ cell depletion followed by CD25⁺ cell selection (2-step process) or by adding an initial CD14⁺ cell depletion (3-step process) using a CliniMACS column. The 3-step approach resulted in a better purity (81±12% vs. 35±33%) and yield (66% vs. 39%). Clinically isolated G-Tregs were also FoxP3⁺CD127^{dim} and functionally suppressive *in-vitro*. Our findings suggest that a better and more consistent purity of Tregs can be achieved from G-PBSC by an initial single depletion of monocytes prior to selection of CD4⁺CD25⁺ cells.

Keywords: Regulatory T cells, G-CSF, CliniMACS, stem cell transplantation

Introduction

Regulatory T cells (Tregs) have been shown to inhibit donor T cell alloreactivity causing GVHD in stem cell transplants [1-3], as well as host T cell alloreactivity responsible for rejection in solid organ transplantation [4-6]. As only limited numbers of circulating CD4⁺CD25⁺FoxP3⁺ Tregs are found in normal peripheral blood, obtaining an adequate cell dose remains the major challenge to standard clinical application of Tregs. To address this problem, many studies have focused on either identifying the most efficient type of Treg [7-10], which would have sufficient inhibitory activity even in small numbers, or more commonly, on expanding Tregs *ex vivo* [11-13] or pharmacologically [14-16] to achieve adequate numbers of these cells for clinical applications. The pitfalls of these studies have been the loss of inhibitory activity upon expansion, or insufficient expansion.

The use of peripheral blood as a source of Tregs isolated in a clinically approved device has

been previously reported [1, 7, 10, 11, 17]. These studies have utilized the CliniMACS device and reagents with standard practice being to perform a 2 step procedure of initial double negative selection (anti-CD8, anti-CD19) followed by positive selection (anti-CD25). The use of Granulocyte Colony-Stimulating Factor mobilized peripheral blood stem cells (G-PBSC) as a potential source of CD4⁺CD25⁺ Tregs (G-Tregs) has not previously been reported. Based upon observations that the cell composition of G-PBSC grafts differs greatly from normal peripheral blood (PB), we hypothesized that modifications to the established cell separation method would be required to obtain high yields of inhibitory cells.

Methods

Flow cytometry

Flow cytometric analysis was performed on samples of peripheral blood products. The following fluorescein isothiocyanate (FITC), or phy-

Treg isolation from G-PBSC

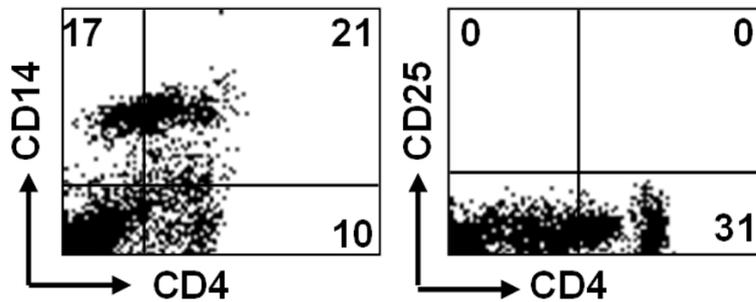


Figure 1. Flow cytometric analysis of CD4⁺CD25⁺ cells in G-PBSC. G-PBSC from a healthy donor were stained with anti-CD4, CD25 and CD14 antibodies and analyzed by flow cytometry. G-PBSC contained a significant number of CD14⁺ cells and very few CD4⁺CD25⁺ cells (n=5 with representative image shown).

coerythrin (PE), or peridin chlorophyll protein (PerCP) conjugated monoclonal antibodies (mAbs) were employed: CD34, CD14, CD4, CD25, CD127 and intracellular FoxP3 (Becton-Dickinson, San Jose, CA). Appropriate isotype controls were also utilized. Stained cells were washed twice in PBS and sample acquisition and analysis was performed on a FACSCalibur (Becton-Dickinson).

Small scale Treg separation

G-PBSC (All Cells, Alameda, CA) from healthy donors were incubated with an immunoconjugated CD34 monoclonal antibody and selected on a high gradient magnetic separation column (MidiMACS, Miltenyi Biotec, Auburn, CA). To assess the purity, aliquots of isolated CD34⁺ cells were re-stained with an anti-CD34 FITC-conjugated mAb (Becton-Dickinson) as described above. After selection, the CD34⁺ cell fraction was utilized to isolate Tregs using the Treg isolation kit (Miltenyi) as previously described [18]. This includes a 2 step process: 1) CD4⁺ cells are separated by negative selection utilizing a cocktail of biotin-conjugated mAbs against CD8, CD14, CD19, CD16, CD36, CD56, CD123, TCR α/δ and Glycophorin-A as a primary labeling reagent, and anti-biotin conjugated to microbeads, as secondary reagent. Therefore only the cells that do not express these markers, i.e. CD4⁺ cells, are eluted from the column; 2) CD4⁺ cells are incubated with microbeads conjugated to an anti-CD25 antibody (Miltenyi) and Tregs are isolated by positive selection (Treg isolation kit, Miltenyi). In selected experiments (3-step), we initially incubated CD34⁺ cells with an immuno-conjugated anti-CD14

antibody (Miltenyi) and isolated CD14⁻ cells prior to isolating the Tregs with the process described above.

Clinical scale Treg separation

Tregs were purified from G-PBSC (AllCells) obtained from healthy donors. In some cases CD34⁺ cells were initially isolated on a ClinMACS device (Miltenyi) by positive selection using the CD34-reagent kit (CliniMACS CD34, Miltenyi) as per manufacturer's instruc-

tion and Tregs were then isolated from the CD34⁻ cell fraction. In the first series of procedures Treg separation consisted of a 2 step isolation process using CliniMACS antibodies (anti-CD14, CD8, CD19 or CD25). CD34⁻ cells were incubated with anti-CD14, anti-CD8 and anti-CD19 antibodies and CD4⁺ cells were selected by negative selection. Following this step, the cells were then incubated with anti-CD25 antibody and the CD25⁺ cell fraction was positively selected. The second method utilized in other experiments consisted of a 3-step procedure including the initial depletion of monocyte from PBSC. PBSC were incubated with anti-CD14 mAb and the negative cell fraction was then collected in the CliniMACS. These CD14⁻ cells were then incubated with anti-CD8 and anti-CD19 antibodies and a negative selection (second step) obtained a cell population enriched in CD4⁺ cells. Finally cells were then incubated with anti-CD25 antibody and Tregs were positively isolated as CD4⁺CD25⁺.

Mixed lymphocyte reaction

Freshly isolated, purified CD34⁺ cells were washed twice, irradiated (3,000 cGy) and tested as stimulators (S) in primary mixed lymphocyte culture (MLC) as previously described [18]. Cells were resuspended in medium containing RPMI-1640 (Lonza, MD), 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% heat inactivated AB human serum inactivated at 56 $^{\circ}$ for 30 min. 5×10^4 purified T cell responders (R) were mixed with irradiated stimulators at 1:2 S/R ratio in round-bottomed 96-well plates for six days at 37 $^{\circ}$ C in a 5% CO₂ humidified atmosphere. Cells were then pulsed

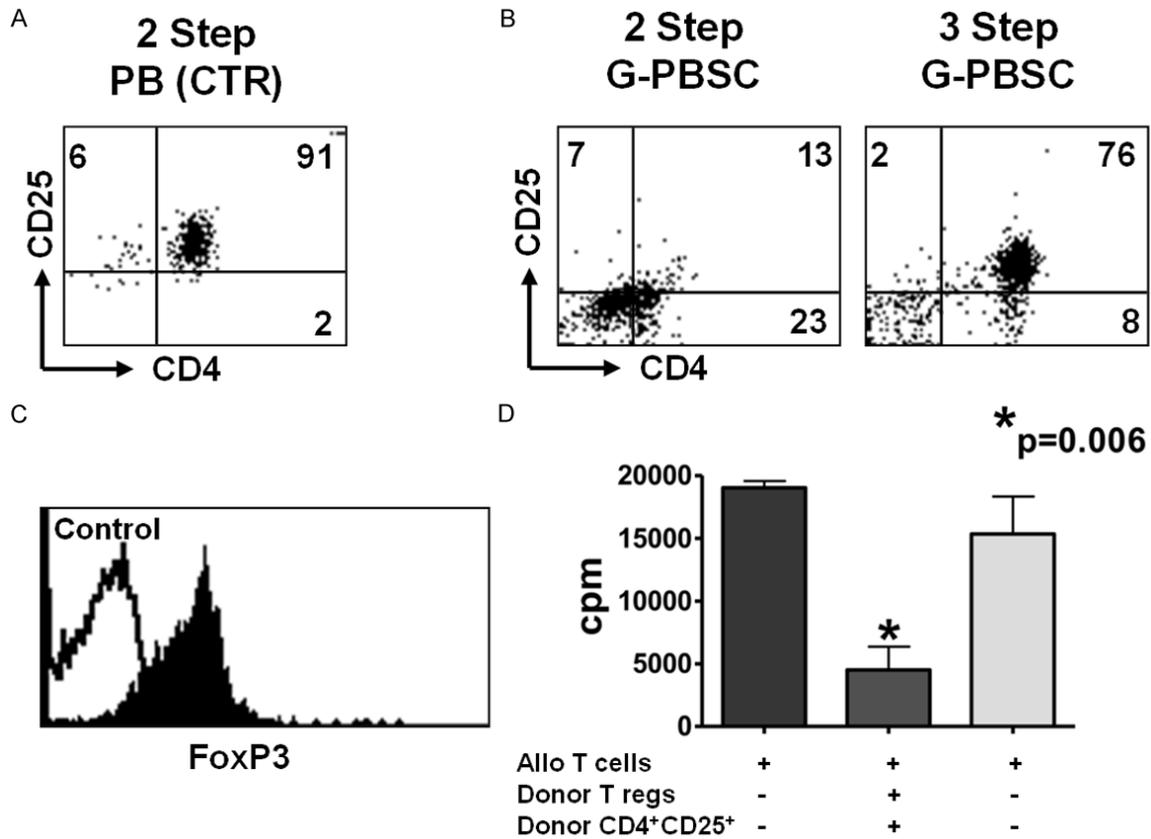


Figure 2. Efficient 3-step small scale isolation of Tregs from G-PBSC which show the ability to suppress T cell alloreactivity. Comparison of 2-step separation of unmanipulated peripheral blood and 2 vs 3 step separation of G-PBSC using a MidiMACS small-scale device (n=2 for 2 step G-PBSC and 3 for 3 step). A. CD4⁺ cells were initially negatively selected from unmanipulated peripheral blood cells after incubating the cells with a cocktail of lineage-specific antibodies conjugated with microbeads (Treg cocktail, Miltenyi) and then passing them through an immunomagnetic column. The CD4⁺ cells were then incubated with an anti-CD25 antibody conjugated with microbeads and positively selected as CD4⁺CD25⁺. The 2 step process in peripheral blood yielded a high purity of Tregs. B. Tregs were separated from G-PBSC by the same 2-step process. Without initial depletion of monocytes the Treg purity was low. The 3-step process using G-PBSC that included an initial depletion of CD14⁺ cells prior to the other 2 steps described above greatly improved purity. C. G-Tregs isolated using the 3-step process expressed FoxP3. D. G-Tregs isolated via the 3 step MidiMACS process show the ability to suppress anti-CD34 alloreactivity in MLC when Tregs are added in a 1:1 ratio with responders (n=3). CD4⁺CD25⁺ cells (control) do not affect alloreactivity.

with 1 μ Ci/well 3H-thymidine for 18 hours before harvest on day 6. Stimulation index (SI) was calculated for each individual experiment as: SI = cpm (T cell responders + stimulators)/cpm (T cell responders). In addition, Tregs were added to the MLC at various ratios of Treg: T cell responder in order to test suppressive function.

Statistical analysis

T-test or analysis of variance (ANOVA) was used for statistical analysis. All analysis was performed using GraphPad Prism (GraphPad, La Jolla, Ca).

Results

High monocyte content in G-PBSC interferes with isolation of functional Tregs

To test the efficiency and purity of Treg isolation from PB and G-PBSC we initially utilized a small-scale device. We isolated Tregs from PB using the standard isolation kit (Miltenyi) with a 2-step process. This yielded a CD4⁺CD25⁺ population with purity >90% (**Figure 2A**). Then we applied the same method to isolate Tregs from G-PBSC. Starting from on average $4.0 \pm 1.8 \times 10^8$ unseparated G-PBSC (n=3), we initially positively selected $2.9 \pm 0.6 \times 10^6$ CD34⁺ cells

Treg isolation from G-PBSC

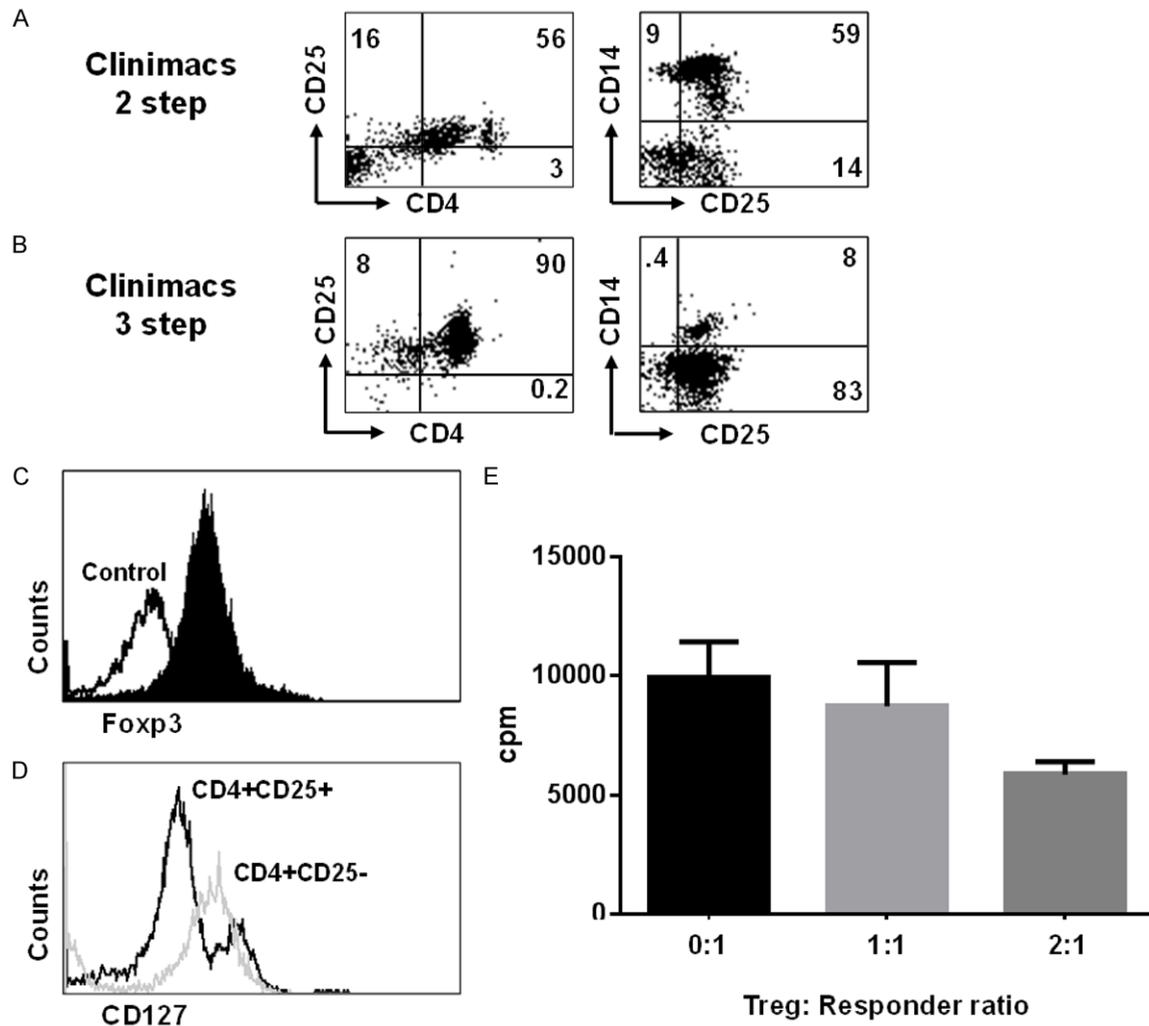


Figure 3. Clinical grade isolation of Tregs from G-PBSC. G-PBSC products obtained from healthy individuals were utilized to isolate Tregs in a clinically approved process (CliniMACS, Miltenyi). A. A 2-step process, which combined CD14⁺ depletion with CD8 and CD19 negative selection, resulted in a final product with a large number of CD14⁺ cells and low purity of Tregs. B. A 3-step process where an initial negative depletion of CD14⁺ preceded the isolation of CD4⁺ cells by negative selection of CD8 and CD19 cells, and the positive selection of CD25⁺ cells resulted in a high percentage of CD4⁺CD25⁺. C. 3-step G-tregs were FoxP3⁺. D. 3 step G-tregs were CD127^{dim}. E. Tregs isolated via the 3 step CliniMACS process showed the ability to suppress anti-CD34⁺ cell T cell alloreactivity in MLC in a dose dependent fashion. The figure shows the effect of addition of CD4⁺CD25⁻ cells as responders (control). In addition we added Tregs to responders at a ratio of 1:1 or 2:1. The addition of G-Tregs lead to a dose dependent decrease in alloreactivity, which trended towards statistical significance ($p=0.07$). This analysis was likely under powered due to the low number of samples.

(>90% purity). Subsequently, using the 2-step procedure on the CD34⁺ fraction, we consistently obtained a Treg fraction with <10% purity (Figure 2B). Based upon the high content of CD14⁺CD4^{dim} monocytes in G-PBSC (Figure 1), we hypothesized that the first step of the 2-process was not efficient at depleting a large number of monocytes. In addition, as monocytes may express CD25 (Figure 3A), residual monocytes could be positively selected in the second

step. Therefore we added an initial depletion of monocytes with an anti-CD14 antibody alone, before the standard isolation process. This obtained a 90±4% pure CD14⁺ product with an overall yield of 14±4%. This subsequently improved the efficiency of the final separation step. Overall the 3-step process allowed us to obtain 1.4±0.9 × 10⁶ CD4⁺CD25⁺ cells with purity improving from 6±8% to 69±5% ($p=0.001$). Intracellular expression of FoxP3

Treg isolation from G-PBSC

Table 1. Clinical-grade separation of Tregs from G-PBSC

	2 Step	3 step
PBSC ($\times 10^9$)	33.0 \pm 5.8	15.0 \pm 0.1
CD4 ⁺ CD25 ⁺ PRE (%)	0.58 \pm 0.0	1.0 \pm 0.1
CD4 ⁺ CD25 ⁺ POST (%)	35.0 \pm 33	81.0 \pm 12
CD4 ⁺ CD25 ⁺ abs number ($\times 10^6$)	71 \pm 95	105.0 \pm 14
Yield (%)	39 \pm 52	66 \pm 6.0

Cells obtained from unmanipulated G-PBSC were utilized to isolate Tregs comparing a 2 step versus the 3 step CliniMACS separation (n=3 for each method). The 3 step process resulted in a higher purity, less variability in the purity, and higher overall yield than the 2 step process.

was also detected in purified CD4⁺CD25⁺ cells by flow cytometry (**Figure 2C**). These findings showed that in a small-scale device after isolation of CD34⁺ cells, adequate numbers of Tregs could be obtained from the CD34⁺ cell fraction of G-PBSC by using a three-step process.

To test the regulatory function of G-Tregs we utilized a model of anti-CD34⁺ cell T cell alloreactivity previously described [18]. Primary MLC with irradiated CD34⁺ cells and allogeneic T cells were performed with further addition to the cell culture of G-Tregs or CD4⁺CD25⁻ cells as control. Addition of G-Tregs at 1:2 Treg: responder ratio resulted in 76 \pm 17% inhibition of anti-CD34 T cell alloreactivity (cpm: 19000 \pm 530 vs 4590 \pm 1880, n=3) (**Figure 2D**). As expected, control CD4⁺CD25⁻ cells did not show suppressive activity. Therefore, Tregs isolated from G-PBSC by a 3-step procedure suppressed *in vitro* T cell alloreactivity against CD34⁺ cells.

A 3-step isolation with the CliniMACS device improves Treg isolation from G-PBSC

In order to validate the results obtained on a small scale model in a clinical grade device, we compared a 2 step procedure (CD19 and CD8 negative selection followed by CD25 positive selection) with a 3 step procedure including an initial CD14 cell depletion using the CliniMACS device (Miltenyi) after staining the cells with CliniMACS antibodies approved for clinical use. As expected, both groups had initial low Treg numbers prior to cell separation (0.58% and 1%). The purity of Tregs following a 2-step separation was on average only 33%, as opposed to 81% using the 3-step process (**Table 1**). In fact, the 2-step process resulted in a high degree of variability in Treg purity due to the fact that in 2

of 3 experiments a large proportion of the final product contained CD14⁺CD25⁺ monocytes (**Figure 3A**). The additional initial step of CD14 depletion resulted in a decrease in the number of monocytes from 9% to 0.4%, which led then to a higher purity of Tregs (**Figure 3B**). To confirm that CD4⁺CD25⁺ cells isolated with the CliniMACS using the 3-step process were regulatory T cells, cells were then demonstrated to be CD127^{dim} (**Figure 3D**) and FoxP3⁺ (**Figure 3C**). In addition, we tested the ability of these Tregs to suppress anti-CD34 T cell alloreactivity using the same methodology as for MidiMACS separated Tregs. Using a Treg: T-responder ratio of 1:1 and 2:1, we were able to show inhibition in proliferation in a dose dependent fashion (**Figure 3E**). These findings suggest that although the purity achieved with CliniMACS was <90%, the Treg product obtained with the 3 step process could suppress T cell alloreactivity.

Discussion

Here we show that clinical grade isolation of G-Tregs (CD4⁺CD25⁺FoxP3⁺) from G-PBSC obtained from a healthy donor achieved a better purity (>80%) and a greater yield when an additional step of initial monocyte depletion with anti-CD14 antibody was used. In addition, we were able to show continued suppressive activity of the isolated clinical G-Tregs.

Here we initially tested two different approaches to achieve a better purity of Tregs from G-PBSC, based on the observation that a large amount of CD14⁺ cells are present in the leukapheresis product and that monocytes have a weak expression of CD4 but can also express CD25 [19]. Our findings in small-scale experiments indeed confirmed that the standard immunomagnetic methodology to isolate Tregs would yield a low fraction of CD4⁺CD25⁺FoxP3⁺ cells. Prior descriptions of Treg separation with the CliniMACS device were performed on unmanipulated blood and were based on double negative selection (CD8, CD19) followed by CD25 positive selection [2, 7]. The Tregs obtained rarely had purity greater than 60% and when the CD25^{bright} fraction of the Treg product was considered, purity would drop further [20, 21]. Because there are no prior reports of clinical grade isolation of Tregs from G-PBSC and the expected absolute number of T cells, and therefore of Tregs, would be higher in G-PBSC,

Treg isolation from G-PBSC

we then tested whether our findings in a small scale using a cocktail of many antibodies could be reproduced in a clinical grade method with the limited reagents available. Likely because of the large amount of monocytes in the apheresis product, when we combined the CD14 antibody with CD8 and CD19 antibodies for a first-step negative depletion on the CliniMACS we could not achieve a satisfactory depletion of monocytes, and after CD25⁺ selection the purity of Tregs was only 35±33%. On the contrary, an initial single depletion of CD14⁺ cells (1st step), followed by negative depletion of CD8⁺ and CD19⁺ cells (2nd step) and positive selection of CD25⁺ cells (3rd step) resulted in a more consistent and efficient isolation of T cells enriched in Tregs (purity: 81±12%). We believe that this type of study has not been done in the past due to the prohibitive cost of clinical reagents. This was also the reason for the limited number of experiments in our study. One important consideration is that with the clinical grade antibodies used here the final clinical Tregs product included the CD25^{intermediate} fraction, which may not retain the same degree of suppressive activity as the CD25^{bright} one. This may explain a slightly lower suppressive activity of the Tregs isolated on the CliniMACS compared to cells separated with the MidiMACS device. However, Tregs isolated from G-PBSC did not show any proliferative response. Other groups have described refinements to the separation process, such as using different bead density and flow rates [7] which may enhance CD25^{bright} numbers, and could potentially be applied also to the 3 step process described here when using G-PBSC grafts.

Improving methodologies of separation of Tregs from G-PBSC will have a beneficial impact on future studies in graft engineering and cell therapy to prevent T cell alloreactivity [22-24]. Using a 3-step isolation procedure, we were able to display a consistently high number of Tregs similar to the number of CD34⁺ cells. Based on these findings we envision the design of clinical trials where donor Tregs and CD34⁺ cells could be collected from the same initial product and infused in patients at 1:1 ratio. In particular, co-transplantation of donor CD34⁺ and G-Tregs could be exploited in HLA mismatched transplantation, infusion of donor Tregs in steroid-resistant GVHD, infusion of donor Tregs in rejection of organ transplant from living donor-

or infusion of Tregs for the treatment of autoimmune diseases [25, 26].

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Damiano Rondelli, Division of Hematology/Oncology, University of Illinois at Chicago, 840 S. Wood St, 820-E, Chicago IL 60612, USA. Tel: 312-996-6179; Fax: 312-413-7963; E-mail: drond@uic.edu

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Treg isolation from G-PBSC

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