MACS&more

Excerpt from MACS&more Vol 15 – 2/2013

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Clinical-grade separation and expansion of regulatory T cells for clinical studies on cell therapy in solid organ transplantation





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Introduction

Organ transplantation is the treatment of choice for patients with end-stage organ failure (e.g. kidney). The use of broad-spectrum immunosuppressive drugs results in accelerated mortality, is toxic to transplants, and does not prevent chronic rejection. Thus, the establishment of clinical tolerance to engrafted tissues to minimize or eliminate immunosuppression is a key research goal.

Naturally occurring, thymus-derived, $CD4^+CD25^+$ $FoxP3^+$ regulatory T (Treg) cells play a critical role in shaping many types of immune responses. They are important not only in maintaining peripheral tolerance to self-antigens and controlling autoimmune diseases, they can also limit immune responses to foreign antigens such as alloantigens. A great deal of experimental data suggests that adoptive therapy of *ex vivo* freshly isolated or *in vitro* expanded Treg cells may successfully promote tolerance. Although numerous preclinical animal models have shown that Treg cells can successfully prevent graft rejection, it still remains to be proved that this approach can be successfully translated into the clinical setting. A number of clinical trials using Treg cells are underway to control GvHD following bone marrow transplantation and as a potential treatment for type 1 diabetes. The application of Treg cells is also a promising strategy for the treatment of other autoimmune diseases and in the prevention of transplant rejections in solid organ transplantation. Development of cellular therapies exploiting the immunoregulatory attributes of Treg cells and their introduction to clinical practice would significantly increase the chance for long-term graft survival without need of life-long immunosuppression.

The aim of this study was to develop an optimal GMP-compatible protocol for the generation and expansion of Treg cells that will be used in clinical studies to prevent graft rejection in solid organ transplantation.

Materials and methods

Separation of Treg cells

200 mL of whole blood from a healthy donor and a renal failure patient was volume reduced using the Sepax® 2 device (Biosafe). $CD4^+CD25^+$ T cells were separated on a CliniMACS® Plus Instrument (Miltenyi Biotec) in two steps: depletion of $CD8^+$ cells followed by enrichment of $CD25^+$ cells.

Buffy coat was diluted to 100 mL with CliniMACS PBS/EDTA buffer supplemented with 0.5% human serum albumin and incubated with CliniMACS CD8 Reagent (Miltenyi Biotec) for 30 min at room temperature with constant agitation. Excess beads were removed by centrifugation. Cells were resuspended in 100 mL of buffer in a bag, which was then attached to a CliniMACS Tubing Set LS. $CD8^+$ cells were removed using the "depletion 2.1" program of the CliniMACS Plus Instrument. The cell fraction depleted of $CD8^+$ cells was then diluted with cold CliniMACS Buffer to 380 mL and incubated with CliniMACS CD25 Reagent (Miltenyi Biotec) for 15 min at 4 °C with constant agitation. Excess beads were again removed by centrifugation, and the cells were resuspended in 100 mL of buffer. The cell suspension was enriched for $CD25^+$ cells using the CliniMACS Tubing Set and the "enrichment 3.2" program. All reagents and consumables used were of clinical grade and processing steps were performed in a closed system using bags, which were attached and detached by means of a sterile welder.

Purity and identity of separated Treg cells

Analysis of cell surface and intracellular molecules to confirm the identity and purity of the separated cell population was performed by multicolor staining using the following monoclonal anti-human antibodies: CD4-PerCP/Cy5.5, CD25-PE, CD8-APC, and FOXP3-FITC. Cells were incubated 30 min with antibodies against the cell surface markers (CD4, CD8, and CD25), washed,

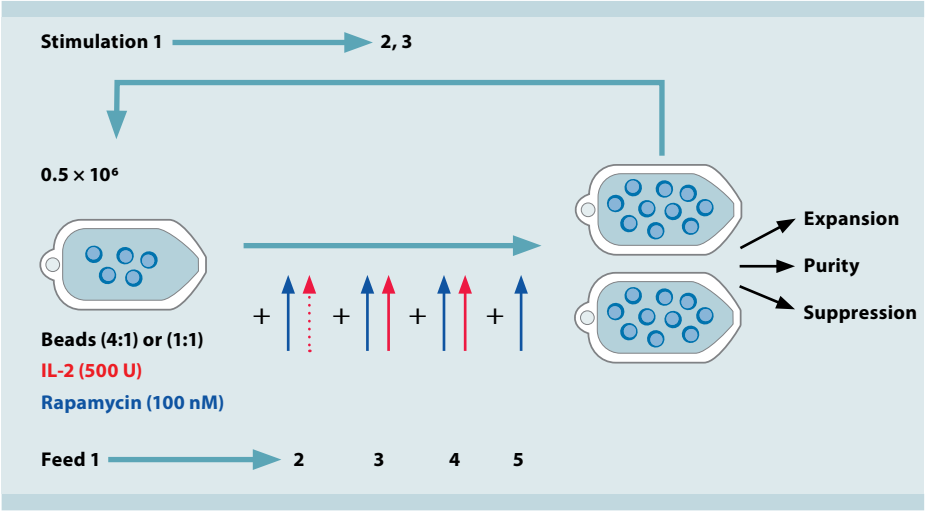

Figure 1 **Stimulation and feeding regime for expansion of Treg cell lines. For details see the text.**

and then incubated 30 min with fixation/permeabilization buffer. Subsequently, cells were washed with permeabilization buffer, incubated with an antibody against the intracellular marker FOXP3 for another 30 min, and analyzed by flow cytometry using the FlowJo® software.

Expansion of Treg lines

Human $CD4^+CD25^+$ T cells were seeded in MACS® GMP Cell Expansion Bags at 0.5×10^6 cells/mL in TexMACS™ GMP Medium (both Miltenyi Biotec) supplemented with 5% human serum containing 100 nM rapamycin (Rapamune®). Cells were activated with anti-CD3- and anti-CD28-coated beads (MACS GMP ExpAct Treg Kit, Miltenyi Biotec) at a bead-to-cell ratio of 4:1. IL-2 (500 IU/mL, Proleukin®) was added at feed 3 after activation and replenished at feed 4 for cycle 1 and feeds 1–4 for cycles 2 and 3. Cells were re-stimulated 2–3 days after the fifth feed. The incubation time between each feed was 2–3 days. At the day of restimulation, cells were pooled, fresh beads (1:1), rapamycin, and IL-2 were added, and the suspension seeded into bigger expansion bags (250, 500, or 1000 mL). Expanded cells were harvested at the end of cycle 3 and used for further analysis. The expansion process is depicted in figure 1.

Suppression assay

The functionality of the expanded Treg lines was assessed by an *in vitro* suppression assay. $CD4^+CD25^-$ T cells (Teff) were labeled with 2.5 nM CFSE and cocultured at different ratios with Treg cell lines. For cell activation anti-CD3/CD28-coated beads were used. Cells were harvested after 5 days. Proliferation of CFSE-labeled Teff cells was analyzed by flow cytometry. The suppressive ability of Treg cell lines was assessed as the percentage of decrease of Teff cell proliferation in the presence of Treg cells.

Results and discussion

The results shown in this report are a part of the scale-up process in the context of a clinical study. The goal was to obtain GMP-compatible Treg cell populations that are highly pure, functional, and available in a number sufficient to generate doses for patient treatment plus reference samples for quality control purposes. Data shown here are generated using samples from one healthy donor and one patient suffering from kidney failure.

Recovery and expansion of Treg cells isolated from blood of a healthy donor and a patient

The final goal of this study is the use of a single dose of Treg cells (derived from the patient) to be injected *in vivo* to induce prolongation of organ allograft survival. To ensure a successful preparation of a Treg cell dose a sufficient starting number of Treg cells needed to be purified and expanded. In this study Treg cells were separated from 200 mL of blood from a healthy donor and a patient in a waiting list for a kidney transplant (ESKF). The two preparations were compared with regard to cell recovery and the cells' ability to expand. While a total of 29×10^6 Treg cells were obtained from the blood of the healthy donor a considerably lower number of Treg cells was recovered from the patient blood (1×10^6). We think that this may be an isolated case and is probably patient dependent. However, we were able to demonstrate that the expansion ability of Treg cells obtained was comparable between the patient (1×10^9) and the healthy donor (1×10^{11}). For both preparations a sufficient number of Treg cells was obtained to create a cell dose of 3×10^6/kg of patient's body weight and quality

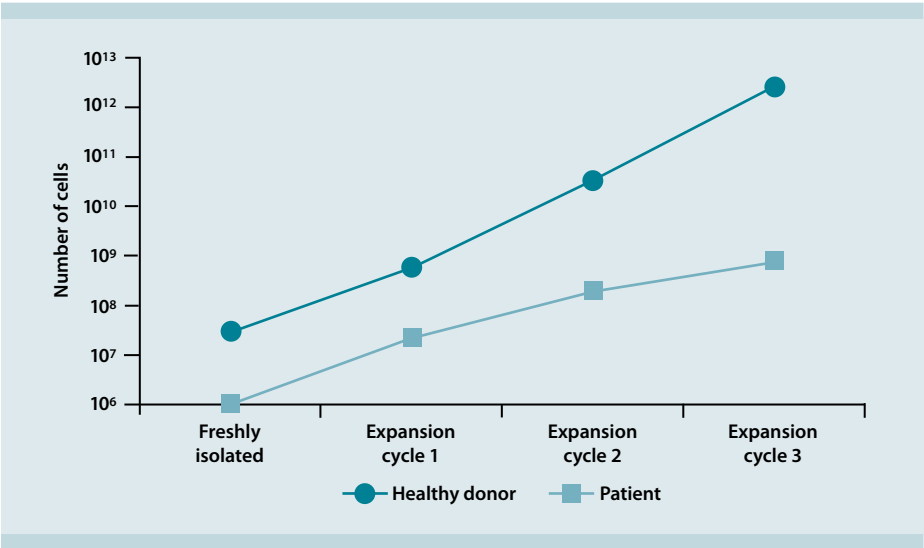

Figure 2 **Expansion of Treg cell lines generated from blood of a healthy donor and a patient.** Cells were cultured as described in materials and methods. Cell numbers were determined prior to cell culture (freshly isolated) and after one, two, and three expansion cycles.

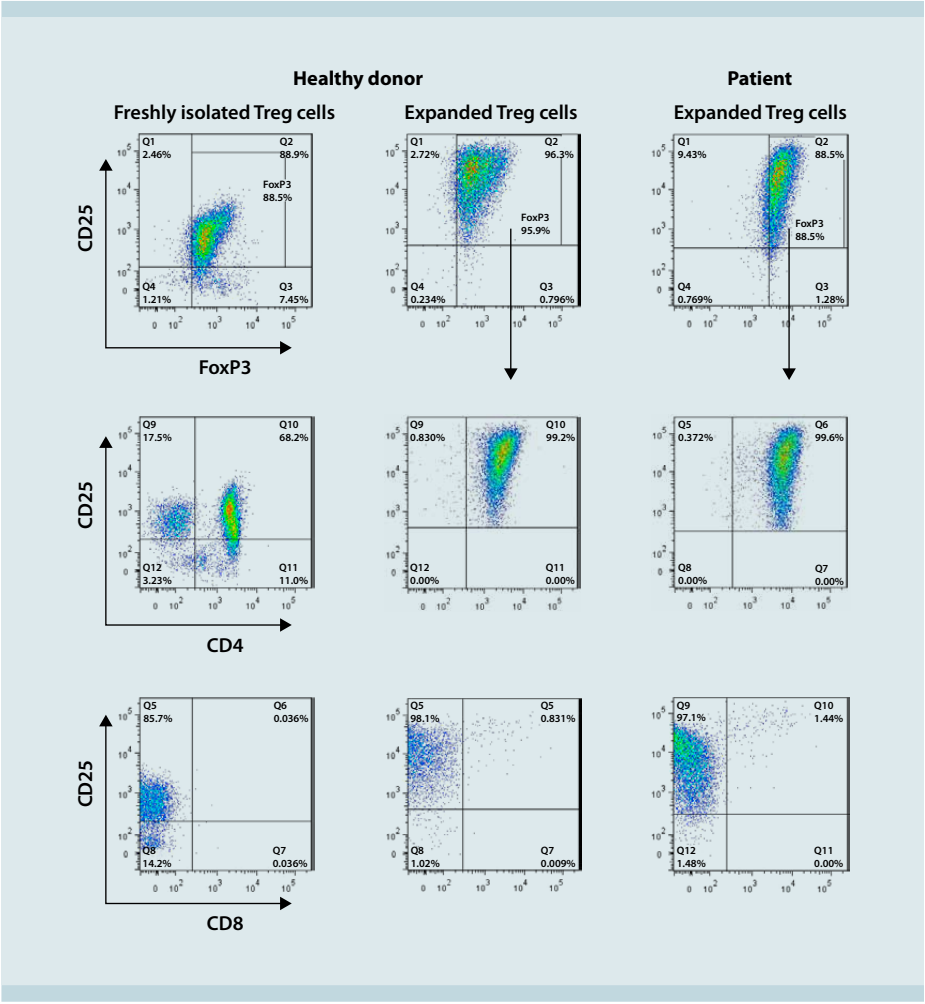

Figure 3 **Identity and purity of Treg cells generated from blood of a healthy donor and a patient.** Freshly isolated Treg cells from a healthy donor and expanded Treg cell lines from a healthy donor and a patient were labeled with fluorochrome-conjugated antibodies and analyzed by flow cytometry.

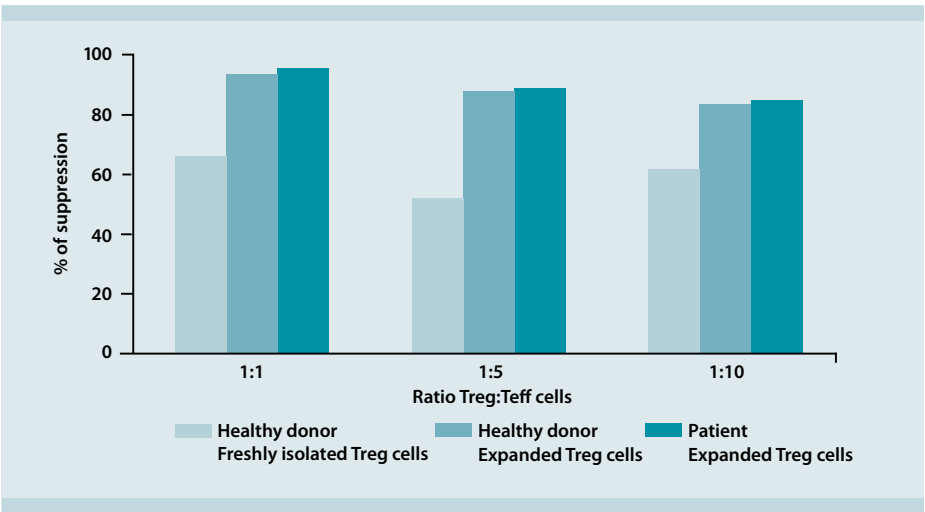

Figure 4 **Suppressive ability of freshly isolated and expanded Treg cells from healthy donor and patient.** The assay was performed as described in the materials and methods section. Three different ratios of Treg:Teff were used. Data indicate the percentage of suppression, i.e., the decrease of Teff cell proliferation in the presence of Treg cells.

control samples. The expansion profile is shown in figure 2.

Identity and purity of freshly isolated and expanded Treg cells

The identity and purity of freshly isolated and expanded Treg cells was confirmed by flow cytometry. Cells were stained for the cell surface markers CD4, CD25, and CD8, and intracellular staining was performed to show expression of FOXP3.

The quality of the cell separation process was very good, and the percentages of contaminating non-Treg cells were low in both preparations. Flow cytometric analysis of the Treg cells obtained from the healthy donor demonstrated that the percentage of cells expressing CD25 and FOXP3 cells was 89%; 68% of the cell population were $CD4^+CD25^+$ (fig. 3, left). However, during the course of expansion the proportion of $CD4^-$ cells decreased and by the time of final harvest they were all eliminated (fig. 3, middle). The contamination with $CD8^+$ cells in freshly isolated Treg cells was minimal at 0.04% (fig. 3 left). We did not perform staining of Treg cells from the patient sample prior to cell culture as the number of freshly isolated cells was too low. The gating strategy for the analysis of cells after the final harvest was slightly different (fig. 3). The identity of the cells and the purity of the final formulation product complied with set release criteria for a Treg cell therapy product, which are ≥60% $CD4^+CD25^+FoxP3^+$cells and ≤10% $CD8^+$ cells: The results demonstrated that at the end of the culture 96% of the total live cells were $CD25^+FoxP3^+$ in the cell line from the healthy donor and 89% from the patient, and of these cells 99% were also $CD4^+$. The percentage of contaminating $CD8^+$ cells in the sample from the healthy donor was 0.8% and from the patient 1.4% (fig. 3, middle and right). Purities were well within the range of the release criteria.

Functionality of freshly isolated and expanded Treg cell lines

We have tested the suppression effect of freshly isolated and expanded Treg cells from a healthy donor and a patient sample. Our data show that the percentage of suppression in the sample from the healthy donor considerably increased from 66% (before expansion) to 94% (after expansion) at a 1:1 ratio of Treg:Teff

cells. The same results were seen also at other Treg:Teff cell ratios. This increased suppressive ability directly correlated with increased cell purity at the end of expansion. Due to the cell number restriction at the start of cell culture, we were unable to test the suppressive function of freshly isolated Treg cells from the patient sample. However, expanded Treg cell lines from the patient showed the same suppressive ability as Treg cell lines from the healthy donor with percentages of suppression between 85% and 96% at all Treg:Teff cell ratios (fig. 4).

Conclusion

- Using the GMP-compatible protocol for the separation and expansion of regulatory T cells we were able to isolate Treg cells of high purity.
- Treg cells were expanded to a clinically relevant number while maintaining their functional ability.
- The expanded Treg cells fulfill all the requirements of set release criteria and are therefore suitable for use as cell therapy product in a phase I clinical trial.

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