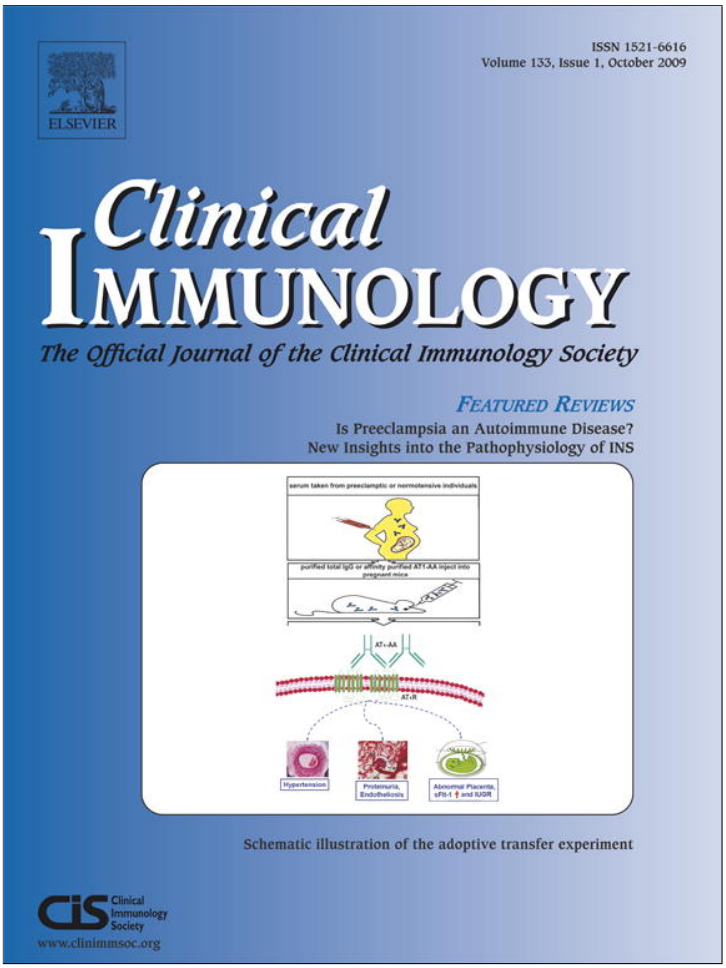

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RAPID COMMUNICATION

First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127− T regulatory cells

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Abstract Here, we describe a procedure and first-in-man clinical effects of adoptive transfer of ex vivo expanded CD4+CD25+CD127− T regulatory cells (Tregs) in the treatment of graft versus host disease (GvHD). The cells were sorted from buffy coats taken from two family donors, expanded ex vivo and transferred to respective recipients who suffered from either acute or chronic GvHD. The therapy allowed for significant alleviation of the symptoms and reduction of pharmacologic immunosuppression in the case of chronic GvHD, while in the case of grade IV acute GvHD it only transiently improved the condition, for the longest time within all immunosuppressants used nonetheless.

Introduction

Translating the knowledge on immune tolerance gained in basic studies into clinical practice holds promise of better and safer therapies in medicine. T regulatory cells (Tregs), originally described in mice as CD4+CD25+ T cells [1], are recognized as one of the major tools in this research. Adoptive transfer of these cells has been found to be extremely successful in many diseases in animal models [2].

Graft versus host disease (GvHD) is recognized as the most suitable candidate to be tried as the first disease in translation studies in human setting [2]. We and others found that the low number of Tregs in the periphery after bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) is associated with the risk of GvHD [3,4]. Moreover, the onset of GvHD is correlated with the number of Tregs in leukapheresis product infused during PBSCT [3]. For a long time, adoptive transfer of Tregs was not considered in the clinic as it is known from animal studies that only the transfer of a high number of these cells, at least equal to the number of effectors in the body, may impose tolerance [5]. At the same time, Tregs compose a very small

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subset in human peripheral blood and therefore a low number is available after sorting. Only recently, the application of T cell expanders – plastic beads coated with stimulatory antibodies – made rapid expansion of Tregs possible [6]. Other important achievements that paved the way for clinical application of Tregs were additional markers that detailed Tregs phenotype. The expression of FoxP3 [7] and low expression of IL7 receptor (CD127) [8–10] on Tregs are probably the most useful.

Here, we describe the method and first two patients with GvHD who received the treatment with ex vivo expanded Tregs in our trial.

Methods

According to the approval from the Ethics Committee, the transfer could only be applied as adjuvant therapy, if routinely approved immunosuppression was ineffective in stopping the progress of GvHD. Both, donors and recipients signed informed consent prior to the therapy.

The donors underwent standard clinical and laboratory examination in order to be included. Blood count within the norm and no signs and symptoms of infectious, autoimmune and oncologic diseases were required to perform autotransfusion. Neither the donors nor recipients could be the carriers of HBV, HCV, HIV, and *Treponema pallidum*.

Each time, a half liter of peripheral blood was taken from the donor during standard donation in the blood bank (Supplement 1). The blood was then separated into red cells, fresh plasma and leukocyte buffy coat. Red cells were stored for the transfusion back to the donor, if necessary.

Buffy coats were sorted to CD4+CD25highCD127– Tregs, which were subsequently expanded ex vivo prior to adoptive transfer to the recipients, as already described [11]. Briefly, negative immunomagnetic sorting (StemCell Technologies, Canada) was applied in order to achieve CD4+ T cells (96–99% purity). CD4+ T cells were then stained with the following cocktail of monoclonal antibodies (mAb) purchased from BDBiosciences, Poland (fluorochrome and clone in the brackets): anti-CD3 (PacificBlue or PE-Cy7, UCHT1), anti-CD4 (APC, RPA-T4), anti-CD8 (PerCP, SK1), anti-CD19 (PerCP, 4G7), CD14 (PerCP, MφP9), anti-CD16 (PerCP-Cy5.5, 3G8), anti-CD25 (FITC, M-A251), anti-CD127 (PE, hIL-7R-M21), and 7-AAD Via-probe. The cells were sorted with FACSARIA sorter (BDBiosciences) to the following phenotype of Tregs: CD3(+) CD4(+)CD25(high)CD127(−)doublet(−)lineage(−)dead(−). In addition, applying FACS sorter allowed for the exclusion of cell conglomerates (different subsets of cells that stick to the cells of interest and contaminate them when sorted erroneously together) thanks to the electronic doublet discrimination and "purity" mode of the sorter (droplets containing cells of interest together with other cells are not sorted). The purity was also improved with "dump" channel (cell suspension is stained with antibodies for lineages other than CD4+, such as anti-CD8, anti-CD19, anti-CD14, anti-CD16, and dead cell dye 7-AAD; fluorescence of all these reagents is detected in the same photomultiplier – "dump channel" – and lineage+dead+ cells are excluded from further sorting). Each time all removable parts of the sample line were replaced with sterile single-use parts and the sorter was primed in "aseptic sort" mode.

Sorted Tregs (1×10^5 per well) were then cultured for a maximum of 3 weeks in 96-well U-bottom plates in RPMI 1640 medium supplemented with 10% of complement-

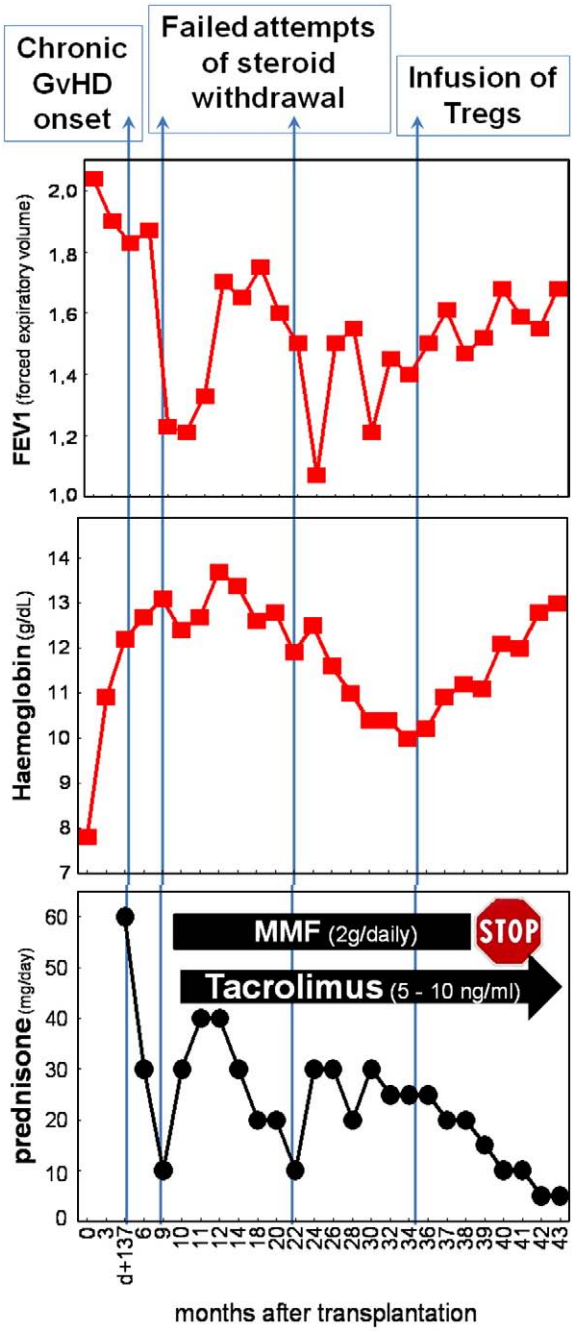
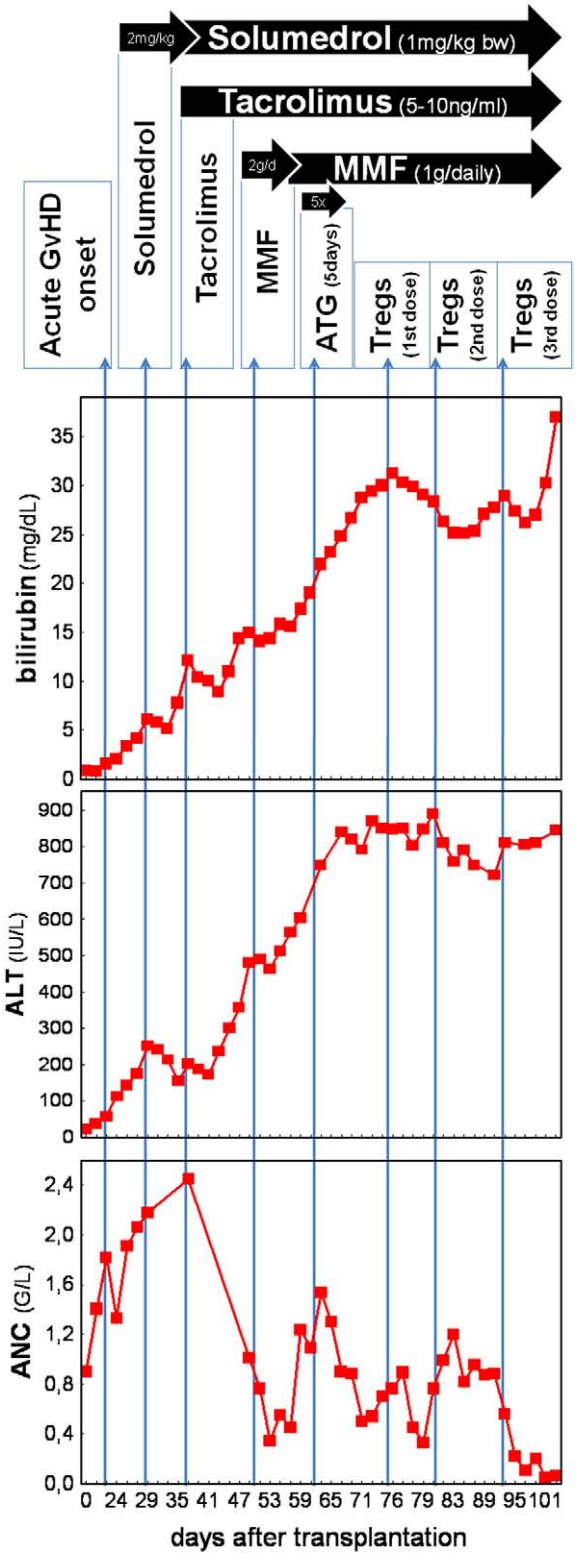


Figure 1 Adoptive transfer of expanded Tregs in chronic GvHD. The main clinical episodes are shown at the top and referred to the laboratory data and time with arrows. Patient developed the symptoms at the day +137. Two attempts of steroid withdrawal (months +9 and +22) failed due to severe lung obturation. Successful waning of steroids and cessation of MMF associated with improvement in lung function and haemoglobin levels were only possible after administration of expanded Tregs (month +35). The figure shows parameters of lung function (FEV1 – forced expiratory volume), haemoglobin levels and immunosuppression administered throughout the treatment.

inactivated autologous fresh plasma from autotransfusion with high concentration of interleukin 2 (1000 U/ml, Proleukin, Chiron, USA) and anti-CD3/anti-CD28 beads (T cell Expander, Invitrogen, USA) in 1:2 ratio. Passages of expanding Tregs were made every two days during the first week and every day during the second and third weeks of the culture. Every week, the cultures were monitored for the quality of Tregs and microbial contamination. The quality check consisted of FoxP3 staining and suppression assay (sample of Tregs is mixed with autologous effectors and the suppression of function of the latter is measured, either inhibition of proliferation or IFNγ production) [11,12]. Microbial safety check consisted of detection of DNA of HBV, HCV and, HIV and microbial contamination in the supernatants of Tregs cultures using standard microbiology cultures, separately for aerobic and anaerobic pathogens.

Prior to adoptive transfer Tregs were washed out completely from expander beads using magnetic field and placed in RPMI 1640 with 10% autologous plasma without IL2. After three days in resting conditions, the cells were washed out again, suspended in 250 ml of 0.9% NaCl (Polfa, Poland) and transferred in slow infusion to the patient under conditions of standard blood transfusion. The dose checked in presumptive studies in healthy volunteers was 1×10^5cells/per kg body weight (bw). High dose in acute GvHD was additionally approved by the Ethics Commitee.



Results

Chronic GvHD

A Caucasian woman, aged 44, underwent BMT due to myelodysplastic syndrome (MDS RCMD, IPSS INT-2) in 2005. Transplanted marrow was taken from HLA-matched sibling donor. Myeloablative regimen consisted of busulfan and cyclophosphamide and GvHD prophylaxis consisted of cyclosporine and methotrexate (Fig. 1).

At the day +137 the patient developed symptoms of chronic *de novo* GvHD (skin, liver and lungs) [13]. Obliterative lung changes were the most prominent problem. Definitive diagnosis of bronchiolitis obliterans could not be established in CT and the patient refused open lung biopsy. Administered prednisone (60 mg/daily; 1.2 mg/kg bw) resolved most of the symptoms. Due to diabetes (patients started therapy with insulin), advanced osteoporosis (multiple rib fractures) and anaemia two attempts of steroids withdrawal were made around the day +270 and +660. Unfortunately, both attempts failed because of severe bronchial obturation that occurred soon after the dose reduction. Moreover, in order to stop the

Figure 2 Adoptive transfer of expanded Tregs in acute GvHD. The main clinical episodes are shown at the top and referred to the laboratory data and time with arrows. Patient developed the symptoms at the day +22. In the following days patient was treated with increasing immunosuppression with consecutively added drugs: solumedrol (added at the day +29, 2 mg/kg bw, dose reduced to 1 mg/kg bw at the day +37), tacrolimus (day +37, under monitoring of serum concentration between 5 and 10 ng/ml), MMF (day +49, 2 g/daily, dose reduced to 1 g/daily at the day +52) and, ATG (day +61, 5-day course). Finally, patient received three times (days +75, +82 and, +93, around 6×10^7 of Tregs per infusion) adoptive transfer of expanded Tregs. The figure shows the levels of bilirubin, Alanine transaminase (ALT) and the number of neutrophils (ANC) throughout the treatment.

progression of symptoms after the first attempt, stronger triple-drug suppression was necessary [prednisone, tacrolimus and mycophenolate mofetil (MMF)]. This triple therapy was maintained for approximately 2 years. In May 2008 patient received adoptive transfer of T regulatory cells taken from the donor and expanded ex vivo beforehand. The yield of Tregs immediately after sorting was 4×10^5 cells and after 2 weeks of expansion single dose of 1×10^5cells/kg bw was administered (90% of the cells were FoxP3+). Injection allowed for complete withdrawal of MMF and significant reduction in the dose of prednisone (5 mg/daily; 0.1 mg/kg bw). Moreover, despite withdrawal of bronchodilatators, lung function improved. Blood haemoglobin increased to normal, insulin is now withdrawn, musculoskeletal pain stopped and body weight backed to normal. The percentage of CD4+FoxP3+ T cells doubled in peripheral blood (from 2.5% prior to the transfer to 5% six months after the transfer) and the level of cytokines in serum decreased [IL6 from 2.95 to 0, IL10 from 4.7 to 3.1, IL7 from 2.45 to 0, CCL21 from 197 to 50 — measured with multicytokine flexset kit BDBiosciences, Poland or ELISA R&D, France, all values in pg/ml].

Acute GvHD

A Caucasian male, aged 40, with blast crisis in the course of chronic myelocytic leukemia (CML Philadelphia-positive) was treated with imatinib in order to achieve second chronic phase. It was then followed by allogeneic PBSCT from HLA-matched sibling donor. Engraftment was achieved at the day +15 (Fig. 2).

The increase in the level of transaminases, bilirubin and diarhoea started from the day +22. At the day +29 patient fulfilled the criteria of acute GvHD grade II [14]. Temporary improvement of the condition was achieved with methylprednisolone (2 mg/kg bw), but then the symptoms exaggerated to the grade IV. At day +37 tacrolimus (serum level 5–10 ng/ml) was introduced, which resulted in brief alleviation of the symptoms (grade III GvHD) followed by further deterioration. At the day +49 MMF (2 g/daily) was added to before mentioned therapy. At the same time, patient started developing pancytopenia (MMF was reduced to 1 g/daily) and severe infections (candidemia and CMV reactivation).

At the day +61 further deterioration was noted. Applied 5-day course of ATG failed to stop the progression of disease. At this point, Tregs were taken from the donor and sorted for expansion (initial yield from the sorter was 3×10^5 of Tregs). Three infusions of expanded Treg cells were administered at the day: +75 (90% of the cells were FoxP3+), +82 (70% were FoxP3+), and +93 (40% were FoxP3+). The total number of cells infused was around 3×10^6cells/kg bw (three infusions, each around 6×10^7 of Tregs). A slight decrease in the level of laboratory parameters with moderate improvement of patient's condition was noted after first infusion of Treg and this plateau was then continued after two subsequent doses. Unfortunately, no more Tregs were available, the condition further deteriorated and the patient died at the day +112 from multiorgan dysfunction. No changes in the level of FoxP3+ cells were noted and the cytokines were undetectable.

Discussion

Our trial proved that the adoptive transfer of expanded Tregs might be a good option as an adjuvant therapy in chronic and acute GvHD. The presented technique is relatively easy and allows obtaining high number of Tregs within days.

When the adoptive transfer of Tregs was considered in the patient with chronic GvHD, the major goal was the waning of steroids, which was supposed to alleviate some adverse effects of these drugs such as diabetes, musculoskeletal pain, and progression of osteoporosis. Interestingly, we achieved not only a reduction of steroids, but also significant improvement in the bronchial obturation to the level that bronchodilatators could be ceased. Taking into account that earlier attempts to withdraw steroids failed, we assume that this time the reduction was possible thanks to the transferred Tregs. Importantly, the transfer was associated with the increase in the level of Tregs and decrease in the level of cytokines in the peripheral blood. The results were less optimistic in the case of acute GvHD. However, it has to be highlighted that Tregs were administered relatively late. Yet, they were able to impose temporary stabilisation of the symptoms, the longest among all used immunosuppressants. In the future studies we should probably consider earlier application or the administration of expanded Tregs in the prophylaxis of GvHD. This case also illustrates the problem with expression of FoxP3 in long-term ex vivo cultures of Tregs. The expression fades with the time either due to the intrinsic features of *foxp3* gene or to the contamination of sorted Tregs with effectors [15]. Indeed, in our study the third infusion in acute GvHD patient was the least effective as the level of FoxP3+ cells dropped down already in the culture.

The purity of transferred Tregs seems to be crucial for the procedure as the contamination with effectors hampers both expansion phase [11] and protection from GvHD [5]. The transfer of effectors together with Tregs may simply make the procedure ineffective. In our hands, satisfactory purity for expansion could be obtained only with FACS sorter thanks to some technical features characteristic for this apparatus, as described in Methods section. The major drawback of this approach is that currently available equipment is not GMP-approved and can be applied in a very limited range of cases, like those presented. The alternative might be immunomagnetic separation. Unfortunately, despite promising reports [16,17], it is hardly possible that the discrimination of effectors similar to FACS-sorting can be achieved with immunomagnetic techniques.

Yet another problem with this therapy is polyclonality of expanded Tregs. However, the time during which sorted cells keep their phenotype ex vivo and the necessity of the administration of high number of the cells limit the application of manoeuvres selecting antigen-specific Tregs reported in experimental studies.

There are many aspects of the therapy that should improve its effectiveness. Nevertheless, even at this stage, we may state that the transfer of pure Tregs expanded with presented method exerts some suppressive effect and should be considered as an adjuvant therapy in GvHD. Furthermore, introduction of this therapy in other diseases with immune background should be granted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2009.06.001.

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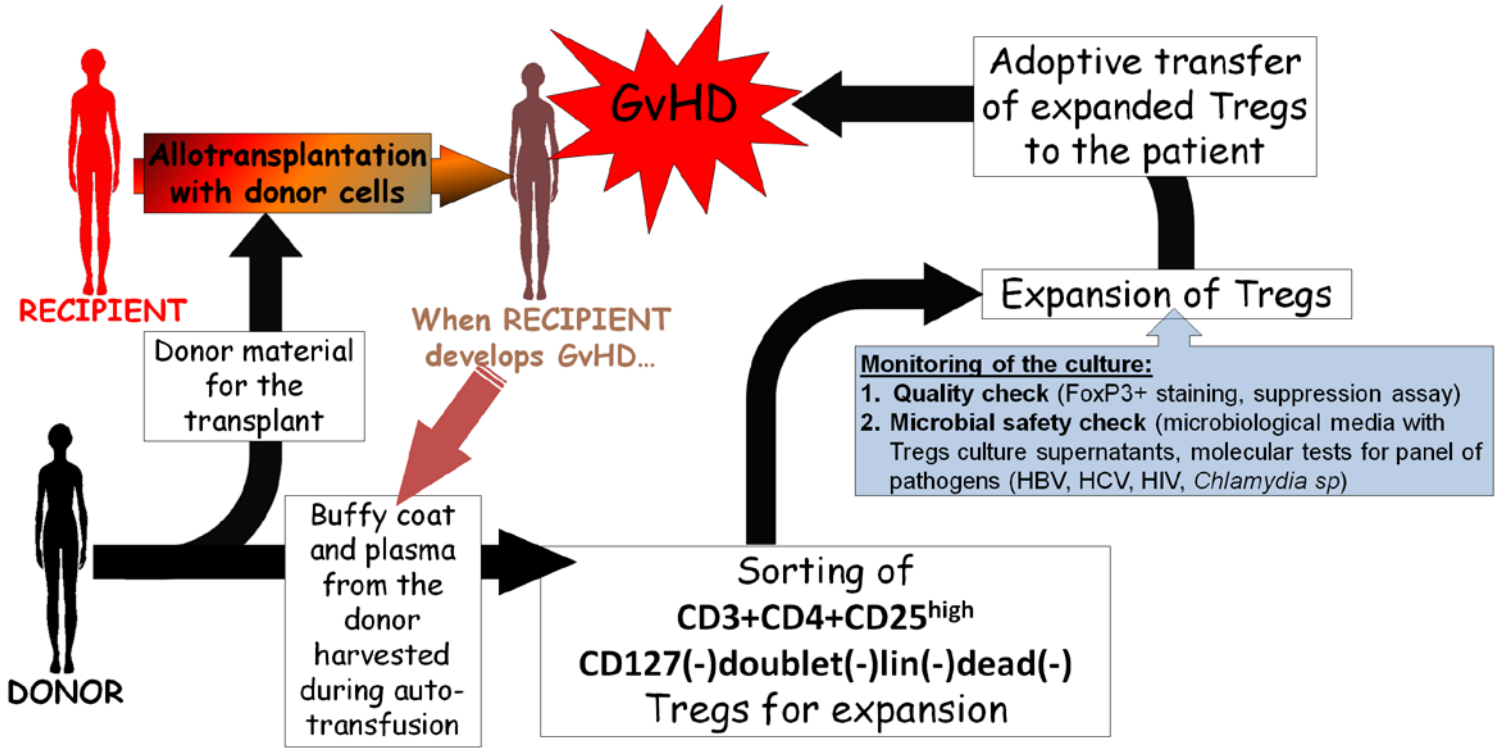
Legend

Supplement 1

The scheme of the procedure applied in the study

Patients, who developed GvHD after the transplantation, receive sorted Tregs [CD3+CD4+CD25(high)CD127(-)doublet(-) lineage(-) dead(-)] expanded in autologous plasma with high dose of IL2 and anti-CD3/anti-CD28 antibodies from respective family donors. Tregs undergo careful monitoring of the quality and microbial contamination before the adoptive transfer is performed.



Supplement 1