Clinical-scale selective depletion of alloreactive T cells using an anti-CD25 immunotoxin*

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Allogeneic hematopoietic stem cell transplantation is the treatment of choice for many hematological malignancies. Its efficacy is limited by graft-versus-host disease (GVHD), the leading cause of post-transplant morbidity and mortality. GVHD is mediated by a subpopulation of T cells in the stem cell graft. *Ex vivo* T cell depletion of all T cells of the graft can prevent development of GVHD but can lead to a delay in immune reconstitution and an increase of potentially lethal opportunistic infections and leukemic relapses. Hypothetically, an approach that enables a selective depletion of the alloreactive donor T cells that cause GVHD while preserving third party (anti-leukemic and anti-microbial) reactivity would be optimal for recipients of HSCT. Our preliminary data demonstrated that an anti-CD25 immunotoxin, which reacts with a cell surface activation antigen, can selectively deplete alloreactive donor T cells activated by non-leukemic recipient white blood cells while preserving the beneficial third-party reactivity *in vitro*.

In this report we describe a method for clinical-scale *ex vivo* selective depletion of alloreactive donor T cells using the anti-CD25 immunotoxin, RFT5-SMPT-dgRTA. Two logs of alloreactive T cells could be selectively depleted while preserving third party reactivity. This method was reproducible in 10 pre-clinical experiments with 8 HLA-mismatched healthy volunteer pairs and 2 HLA-matched sibling donor/patient pairs.

Key words: T cell, immunotoxin, graft-versus-host disease, allogeneic hematopoietic stem cell transplantation.

Allogeneic HSCT is an effective therapy for a variety of malignant and non-malignant diseases [7]. However, efficacy is often limited by GVHD and infections, both of which are the major cause of mortality and morbidity associated with allogeneic HSCT. Acute GVHD is initiated by donor-derived alloreative T cells which are stimulated by major and/or minor histocompatibility antigens on cells from the recipient [4]. These activated T cells recruit effector cells which cause dysregulated production of inflammatory cytokines and damage to target tissues, including the skin, liver and gastrointestinal tract [5]. In clinical trials using T cell depletion to prevent GVHD in HLA-matched related donors, a dose of T cells $<10^5$/kg of the recipient's body weight has rarely been associated with severe GVHD [8]. Depletion of all T cells from the graft prevented the development of GVHD but also led to a delay in immune reconstitution and an increase in potentially lethal opportunistic infections and leukemic relapses [2, 8]. Our previous results as well as results of other authors have demonstrated that selective depletion of activated alloreactive T cells using an anti-CD25 immunotoxin (IT) is highly effective at eliminating alloreactivity while preserving third-party (anti-leukemia and anti-microbial) reactivity *in vitro* [9, 10]. Here we investigated the same approach using a medium-scale up (10^8 responder cells) and clinical-scale up (10^9 responder cells) to test the feasibility, specificity, and effectiveness of allodepletion. The information will be central in translating this approach into clinical practice.

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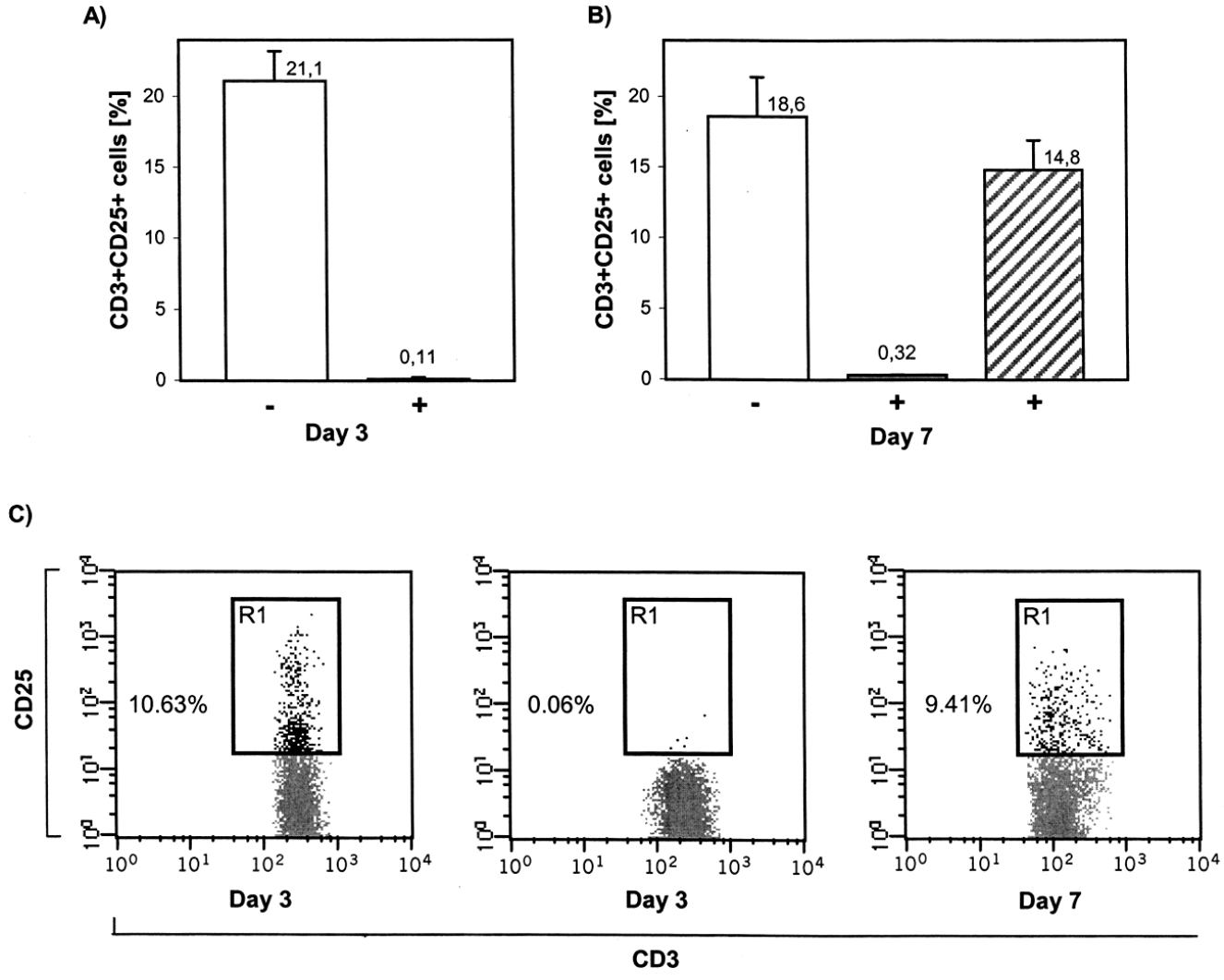

Figure 1. Selective depletion of alloreactive T cells.

Responder cells were incubated with irradiated stimulator cells in an MLR for 7 days as described in the Methods. After 24 hours, 2 μg/ml of the IT and 6 mM NH_4Cl were added to the MLR for 24 hours. Cells incubated with the IT were washed and incubated for another 24 hours with 1 μg/ml of the IT and 6mM NH_4Cl. On Day 3 cells were washed, counted and restimulated for 4 days with either irradiated PBMCs from the same or another HLA-mismatched individual (third party). Responder $CD3^+CD25^+$ cells were counted by FACS before the MLR (4.4–6.7%, median 5.6%, data not shown in the figure), after 72 hours of incubation (Day 3), and after 7 days of incubation (Day 7).

A) and B) represent the mean (+SD) of 8 HLA-mismatched healthy volunteer pairs. White bars: cells without the IT and NH_4Cl treatment (–); grey bars: IT plus NH_4Cl-treated cells (+); stripped bars: IT plus NH_4Cl-treated cells restimulated with third party PBMCs. C) represents FACS analysis of one of the two donor/patient pairs. The percentages of $CD3^+CD25^+$ cells (in R1 rectangles) are shown in black. Left panel: Day 3 without the IT and NH_4Cl treatment; middle panel: Day 3 after the IT plus NH_4Cl treatment; right panel: Day 7 after the IT plus NH_4Cl treatment and restimulation with third party PBMCs. The data (Day 3 and Day 7) for the second donor/patient pair did not differ more then 11% from the first one.

Material and methods

Anticoagulated peripheral blood specimens were obtained from healthy volunteer donors. Shortly before HSCT an apheresis was performed on 2 patients who were in complete clinical and hematological remission of their disease (one acute myeloid leukemia and one acute lymphocytic leukemia). Apheresis was also performed on their HLA-matched sibling donors as part of their peripheral blood stem cell collection for HSCT. All 10 healthy volunteers and both sibling donors and patients signed informed consent. PBMCs were obtained by Ficoll-hypaque (Sigma, St. Louis, MO, USA) density gradient separation and used immediately in a MLR without previous cryopreservation. MLRs were performed in complete media (CM) which consisted of X-VIVO 15 (Biowhittaker, Walkersville, MD, USA) supplemented with 5% AB human serum. First, 5

MLRs using healthy volunteer PBMCs were performed with 10^8 responder cells. Then 3 MLRs using healthy volunteer PBMCs were performed with 10^9 responder cells. Finally 2 MLRs using HLA-matched donor PBMCs as responder cells were performed using 1.4–1.5x10^9 responder cells. PBMCs from patients or volunteers served as stimulator cells after irradiation with 25 Gy and all irradiated cells were dead after 24 hours of incubation as demonstrated by trypan blue and propidium iodide staining (data not shown). A ratio 5:1 responder/stimulator cells were used in all MLRs. Cells were incubated for 7 days at 37 °C in 5% CO_2 in CM in 75 cm^2 or 175 cm^2 flasks (Corning, Corning, NY, USA) on a rocker at a concentration 5x10^6/ml responder cells. After 24 hours, cells were subjected to selective depletion of alloreactive responder cells using an anti-CD25 IT [3, 9]. Briefly, cells were mincubated with 2 μg/ml IT for 24 hours, washed once, and incubated mwith 1 μg/ml IT for another 24 hours. 6 mM NH_4Cl was used as an IT-enhancing agent during both IT incubations. The doses of the IT and NH_4Cl have been described previously [9, 11]. Non-IT treated MLRs served as a control. FACS analysis of alloreactive cells was performed using FITC-labeled anti-human CD3 and phycoerythrin-labeled anti-human CD25 monoclonal antibodies (BD Pharmingen, San Diego, CA, USA).

Results

Based on previous small-scale experiments used to optimize the dose and timing of both the IT and NH_4Cl [9], we evaluated 5 HLA-mismatched healthy volunteer pairs in a medium-scale MLR using 10^8 responder cells. Responder cells were activated by allogeneic stimulator cells and their peak activation (19.6–26.5%, mean 23.1% of $CD3^+CD25^+$ responder cells) occurred after 72 hours. If the IT and NH_4Cl were added for 24 hours after the first 24 hours of the MLR, the remaining population represented 0.15–0.98% (mean 0.44%) of $CD3^+CD25^+$ responder cells. The recovery percentage of viable responder cells was 62.3–89.7% (mean 78.2%). The depletion of alloreactive cells increased following another 24 hour exposure to the IT and NH_4Cl resulting in 0.00–0.24% (mean 0.10%) of $CD3^+CD25^+$ responder cells with 64.7–86.5% (mean 75.4%) viability. Further exposure to the IT and NH_4Cl did not improve the selective depletion but instead decreased the total number of viable cells (data not shown). Next step was to determine whether restimulation of the allodepleted cells on Day 3 with irradiated cells from the same individual demonstrated any residual alloreactivity. Only minimal increases in $CD3^+CD25^+$ responder cells were observed on Day 7 (0.05–0.43%, mean 0.29%), but third party reactivity was preserved, reaching 10.3–19.6% (mean 15.5%) on Day 7 when activated on Day 3 with HLA-mismatched PBMCs from another healthy donor.

After selective depletion experiments using the medium-scale MLR, the same protocol was used on a clinical-scale and included cells from three healthy volunteer pairs and two donor/patient pairs. The results are shown in Figure 1 and demonstrate a potent and sustained effect of selective depletion of alloreactive $CD3^+CD25^+$ responder T cells and good preservation of a third party reactivity. The viability of selectively depleted cells on Day 3 of the MLR reached 67.3–83.7% in healthy donor pairs, and 69.4% and 75.1% in donor/patient pairs. In all cases, > a 2-log depletion of alloreactive cells was documented.

Discussion

It has been well documented in clinical studies that levels of alloreactive T cells of less then 10^5/kg of recipient body weight are rarely associated with severe GVHD in patients transplanted with cells from HLA-matched sibling donors [6], but delayed immune reconstitution has become a problem, leading to higher rates of severe opportunistic infections and relapses of the malignant disease [2, 6]. Based on our results, the selective *ex vivo* depletion with this IT appears to be an effective strategy for eliminating or minimizing the dose of alloreactive cells (<10^5/kg of recipient body weight) while preserving third-party reactivity in 10^7/kg donor T cells. In case of HLA-mismatched or HLA-matched unrelated donors the threshold of alloreactive T cells for GVHD development is less then 10^4/kg.

In a study with 15 pediatric patients undergoing HSCT form HLA-matched unrelated or HLA-mismatched related donors, using the same approach but ower doses of allodepleted donor T cells (1–8x10^5/kg), there was suppression of severe GVHD, but infections and relapses remained a problem leading to deaths in 5 of 15 patients [1]. Another similar approach that we have tested pre-clinically [11] employed IT-mediated selective depletion of alloreactive cells starting with total of 10^8/kg donor T cells, thus resulted in <3.2x10^6 alloreactive cells/kg of the recipient's body weight. GVHD could be controlled by the administration of cyclosporin A. In summary, the optimal dose of allodepleted T cells must be carefully evaluated in clinical studies. There may be a better opportunity for HSCT from HLA-matched sibling donors who can tolerate higher doses of donor T cells then in case of HSCT from HLA-mismatched related of HLA-matched unrelated donors.

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