Tregs prevent GvHD and promote immune reconstitution in HLA-haploidentical transplantation

Running title: Tregs in haploidentical transplantation

Mauro Di Ianni1,2, Franca Falzetti1, Alessandra Carotti1, Adelmo Terenzi1, Flora Castellino4
Elisabetta Bonifacio1, Beatrice Del Papa1, Tiziana Zei3, Roberta Iacucci Ostini1, Debora Cecchini1,
Teresa Aloisi1, Katia Perruccio1, Loredana Ruggeri1, Chiara Balucani1, Antonio Pierini1, Paolo
Sportoletti1, Cynthia Aristei1, Brunangelo Falini1, Yair Reisner3, Andrea Velardi1, Franco Aversa1
and Massimo F Martelli1

1Hematology and Clinical Immunology Section, Department of Clinical and Experimental
Medicine, University of Perugia, Italy.

2Chair of Hematology, Department of Internal Medicine and Public Health, University of L’Aquila,
Italy.

3 Weizmann Institute of Science, Immunology Department, Rehovot, Israel.

4 Translational Medicine, Novartis Vaccines and Diagnostic, Italy

Correspondence: Mauro Di Ianni, Chair of Hematology, Department of Internal Medicine and
Public Health, University of L’Aquila, 67100 L’Aquila, Italy Phone: (39)075-5783604; fax (39)
075-5783219; email: mauro.diianni@cc.univaq.it
Abstract

Hastening post-transplant immune reconstitution is a key challenge in HLA-haploidentical haematopoietic stem cell transplantation (HSCT). In experimental models of mismatched HSCT T regulatory cells (Tregs), when coinfused with conventional T cells (Tcons), favoured post-transplant immune reconstitution and prevented lethal GvHD. The present study evaluated the impact of early infusion of Tregs, followed by Tcons, on GvHD prevention and immunological reconstitution, in 28 patients with high risk haematological malignancies who underwent HLA-haploidentical HSCT. We show for the first time in humans that adoptive transfer of Tregs prevented GvHD in the absence of any post-transplant immunosuppression, promoted lymphoid reconstitution, improved immunity to opportunistic pathogens and did not weaken the Graft vs Leukaemia effect. This study provides evidence that Tregs are a conserved mechanism in human beings.
Introduction

A viable option for high-risk, acute leukaemia patients without matched donors is haematopoietic stem cell transplantation (HSCT) from HLA-haploidentical three-loci mismatched family members who are promptly available for almost all patients (1,2). Until the 1990s full-haplotype mismatched T cell replete transplants were unsuccessful because donor alloreactive T cells triggered a high incidence of severe graft versus host disease (GvHD) despite post-transplant immune suppression (3,4). The breakthrough came with the use of a megadose of extensively ex vivo T-cell depleted peripheral blood haematopoietic progenitor cells and a highly myeloablative conditioning regimen containing anti-thymocyte globulin (ATG) which exerts additional T cell depletion in vivo. This approach ensures a high rate of primary engraftment in the absence of GvHD (5) with over 40% long-term event free survival and excellent quality of life (1,2). However extensive ex vivo and in vivo T cell depletion delays recovery of immune responses against pathogens, leading to a high incidence of life-threatening infections (1,2).

Strategies to hasten post-transplant immune reconstitution without triggering GvHD, have included infusion of donor T cells after engineering with a suicide gene (6), photodynamic purging (7) or use of an anti-CD25 monoclonal antibody (8) to remove alloreactive cells. An alternative strategy might be based on donor CD4+CD25+ T regulatory cells (Tregs). In murine models of HSCT across MHC barriers, CD4+CD25+ Tregs suppressed lethal GvHD (9) and favoured post-transplant immune reconstitution when coinfused with conventional T cells (Tcons) (10).

The main obstacle to clinical application of human Tregs is their paucity in peripheral blood. Although ex vivo expanded polyclonal (11) or recipient-specific Tregs (12) were proposed to circumvent this potential barrier, we opted for closed, automated immunoselection (13) of naturally occurring Tregs. Here, for the first time in humans, we show early infusion of freshly isolated donor Tregs, followed by Tcons at time of full haplotype mismatched HSCT, prevented GvHD while favouring Tcon-mediated post-transplant immune reconstitution.
Patients and methods

Study design, conditioning regimen, stem cell mobilization and supportive care

The Umbria Regional Hospital Ethics Committee (CEAS Umbria) approved, in 2008, the protocol entitled “Adoptive Immunotherapy with Natural Regulatory T cells (Treg) and Effector T Cells in Allogeneic Haematopoietic Stem Cell Transplantation from 2-3 Loci Mismatched HLA-Haploidentical Family Donors for Patients with High Risk Haematological Malignancies” (Protocol No 01/08). Written informed consent was obtained for all patients and donors in accordance with the Declaration of Helsinki. Inclusion criteria: acute myeloid or lymphoid leukaemia in remission at high risk of relapse; acute leukaemia with primary induction failure, in chemoresistant relapse or in relapse after autologous transplant; high-grade Hodgkin’s or non-Hodgkin’s lymphoma in relapse after three lines of chemotherapy and autologous transplant; age range 18 to 65 years of age; no major lung, liver, renal and cardiac dysfunction; no major psychiatric disturbances; lack of an HLA-identical sibling or a matched, unrelated Registry donor. Patient population: 28 consecutive patients (11 male; 17 female; median age 41, range 21-60) were enrolled. Twenty-two had acute myeloid leukaemia (AML) (10 in first complete remission [CR], 10 in ≥ second CR, 2 in relapse), 5 had acute lymphoid leukaemia (ALL) (4 in first CR; 1 in relapse) and 1 had high-grade non-Hodgkin Lymphoma (NHL) in relapse. All patients who were transplanted in CR1 were at high risk of relapse (5 with FLT-3/ITD; 3 with t(9:22); 2 with complex karyotypes; 1 secondary AML; 1 biphenotypic; 1CR after second-line induction; 1 with CNS and skin localization at diagnosis). Donor eligibility: family member with one haplotype identical to patient’s, able to donate haematopoietic stem cells after treatment with G-CSF and undergo leukapheresis sessions for collecting haematopoietic stem cells, Tregs and Tcons. Primary endpoints: to demonstrate that, in the HLA-haploidentical transplant setting and without any post-transplant immunosuppression, Tregs inhibit effector T-cell alloreactivity, thus eliminating the risk of GvHD; to demonstrate that Tregs do not cross-inhibit conventional pathogen-specific T-cell responses and that adoptive transfer of T regulatory cells and conventional T cells hastens immune reconstitution. Secondary
endpoints: to reduce the incidence and severity of bacterial, viral and fungal infections and consequently, transplant related mortality (TRM); to demonstrate this strategy does not increase the incidence of post-transplant leukaemia relapse. Transplantation Procedure: the transplant timeline schema is shown in Figure 1. Briefly, the conditioning regimen included total body irradiation (8 Gy on single fraction at fast dose rate with lung shielding) on day –10, thiotepa (4 mg/kg/day) on days –10 and -9, fludarabine (40 mg/sqm/day) from days –10 to –6; cyclophosphamide (35 mg/kg/day) on days –7 and –6. After conditioning, patients received an infusion of freshly isolated donor Tregs (day - 4). Immediately afterwards, donors were treated with G-CSF to mobilise peripheral blood progenitor cells. After collection, CD34+ cells were positively immunoselected using the CliniMACS device (Miltenyi Biotec GmbH, Bergisch Gladbach Germany) (1,2) and freshly infused on day 0 (mean CD34+ dose 9.4x10^6/kg ± 3.4 with a mean of 0.8x10^4/kg ±0.4 contaminating T cells). On the same day patients also received an infusion of Tcons that had been collected before donors were treated with G-CSF, separated from peripheral blood mononuclear cells by negative selection using CliniMACS CD19 Reagent (Miltenyi Biotec) and cryopreserved. No post-transplant GvHD prophylaxis whatsoever was given.

The interval between the Treg and Tcon infusions was in accordance with animal data indicating that Tregs needed to be administered first to provide greatest protection against GvHD (14) .

Even though in vitro assays showed Tregs inhibited MLR at a Tcon:Treg ratio of 1:2, for safety reasons 0.5x10^6/kg Tcons were administered with 2x10^6/kg Tregs in the first group of 4 patients. Onset of acute GVHD was the indication for stopping Tcon dose escalation. As none of these 4 patients developed acute GvHD, Tcons were then escalated to 1x10^6/kg in the next 17 patients while the Tregs remained unchanged. Tcons were increased to 2x10^6/kg in the last 5 patients who received 4x10^6/kg Tregs. Two patients did not receive Tcons because FoxP3+ expression in the Treg preparation was <50%.
Patients were managed in positive pressure airflow rooms. Red cell and platelet transfusions were given according to institutional policy. Prophylaxis against viral and fungal infections consisted of acyclovir (10 mg/kg every 8 hours daily) or ganciclovir (10 mg/kg/die) and liposomal amphotericin-B (2 mg/kg daily) from day –10 until the end of neutropenia. Prophylaxis after neutrophil recovery consisted of thrimethoprim-sulfamethoxazole (from day +50 until day + 150) and acyclovir (until +6 months after HSCT).

Tregs separation and analysis

After obtaining informed consent from donors, mononuclear cells were obtained by apheresis using a continuous-flow cell separator (Cobe SPECTRA, Cobe BCT Inc., Lakewood, CO). CD4+CD25+ regulatory T cells were isolated using large scale (CliniMACS TM Instruments, Miltenyi Biotec) separation systems. Clinical-grade reagents were used (GMP conditions). CD4+CD25+ regulatory T cells were isolated in a two-step procedure. Briefly, cells were washed, adjusted to 87.5 mL in PBS, EDTA and 2% human albumin (HA), labelled with CliniMACS CD8 and CD19 microBeads (Miltenyi Biotec) for 30 minutes at room temperature on an orbital shaker, re-washed and re-suspended in 100 ml PBS/EDTA/2% HA. CD8 and CD19 labelled cells were depleted by the 2.1 depletion program on the CliniMACS instrument (Miltenyi Biotec). The negative cell fraction was suspended in 190 ml PBS/EDTA/2% HA, labelled with 7.5 ml CD25 microbeads (CliniMACS) for 30 minutes at room temperature on an orbital shaker, washed, and re-suspended in 100 ml PBS/EDTA/2% HA. CD25+ cells were isolated by automatic positive selection cycles using the 3.1 enrichment program on the CliniMACS device. Aliquots before and after each labelling, depletion and enrichment steps were immunophenotyped. Phenotypes were determined using direct immunofluorescence with a panel of monoclonal antibodies (MoAbs) directed against the following antigens: CD45, CD3, CD4, CD8, CD14, CD19, CD16, CD56, CD11b, CD45RA, CD45RO, CD62L, CD127 (Coulter Corporation, Hialeah, FL, USA) CD25 (conjugated to biotin, mouse IgG2b, clone 4E3), GITR, CTLA-4, CD49d, CD39 (Miltenyi) and FoxP3 (e-Bioscience, San Diego,
Mouse IgG 2-APC (e-Bioscience) was used as control for FoxP3 analysis. Dot plots of combined stainings with CD25/CD127/FoxP3 and CD25/CD45RA/FoxP3 were additionally performed on a different series of 3 donors. Cells were analysed by Cytomics FC500 Cytometer (Coulter Corporation, Hialeah, FL, USA). Release criteria were ≥90% CD4+/CD25+ and ≥ 50% FoxP3+ cells in the Treg product.

The suppressive capacity of the immunoselected population was established as follows. CFSE labeled CD4+/CD25- (Tcons) were in cultured in 96 –well plates at 2×10^4 cells/well with PHA (4 mcg/ml) (Biochrom AG, Berlin, Germany) in presence of varying amounts of CD4+/CD25+ (Tregs). The suppressive capacity of Tregs towards responder cells in co-culture (Tcons:Tregs ratio 1:1 or 1:2) was expressed as the relative inhibition of the percentage of CFSElow cells [100x (1-%CFSElow CD4+/CD25-T cells in coculture / %CFSElow CD4+/CD25-T cells alone)] for CFSE based measurement of proliferation.

Chimerism analysis and immunological studies

Chimerism was assessed on DNA extracted from peripheral blood samples by multiplex fluorescent short-tandem repeat analysis (AmpFISTR Profiler Plus PCR kit; Applied Biosystems, Foster City, CA). Peripheral blood was collected in ethylenediaminetetraacetic acid for lymphocyte analysis weekly for the first two months and then monthly. Flow cytometric immunophenotyping analyzed lymphocyte subsets, including CD20+, CD8+CD16+CD56+, CD3+CD4+, CD3+CD8+, CD4+CD45RA+, CD4+CD45RO+, and CD8+CD45RA+, CD8+CD45RO+, CD4+CD25+. All antibodies were obtained from Coulter Corporation, Hialeah, FL, USA. Cells were analysed by Cytomics FC500 Cytometer (Coulter Corporation, Hialeah, FL, USA).

T-cell receptor third complementarity-determining region spectratyping

Spectratyping was performed on recipients’ peripheral blood every 30 days post transplant. CDR3 size distribution of 26 TCR Vβ families was determined by RT-PCR as previously described (15).
In brief, RNA was extracted and cDNA synthesized using MMLV-RT reverse transcriptase (Nanogen Advanced Diagnostic, Turin, Italy). PCR was performed with a specific forward primer for one of the TCR Vβ families along with a constant Cβ reverse primer labelled with fluorescent FAM (16). RT-PCR products were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) using GeneScan software. Normal TCR Vβ CDR3 was characterized by a Gaussian distribution, containing 8–10 peaks for each Vβ subfamily. The overall complexity of TCR Vβ subfamilies was determined by spectratype scoring as previously described (17).

Limiting dilution assay to evaluate pathogen-specific responses in CD4⁺ T cells.

Patient PBMC to be used as antigen-presenting cells were irradiated with 20 Gy and plated in 96 U bottomed plates at the concentration of 2 x 10⁶/ml, i.e. 2 x 10⁵ cells/well, in RPMI-1640 medium with 10% donor plasma, and pulsed with CMV, Aspergillus fumigatus (A. fumigatus) conidia, Candida albicans (C. albicans) yeasts, Toxoplasma gondii (T. gondii), Herpes Simplex Virus (HSV), Adenovirus (ADV) and Varicella Zoster Virus (VZV) antigens (18). Patient cells (the selection criteria was a percentage of CD3+ cells equal to 5-10% of the PBL) to be used as responder cells were plated on irradiated, antigen-pulsed cells at cell concentrations ranging from 10,000 to 125 cells/well for CMV-, C. albicans-, T. gondii, HSV, ADV and VZV-specific responses, and from 100,000 to 3,500 for A. fumigatus-specific responses. CMV, T. gondii, HSV and ADV antigens are from Microbix Biosystems Inc., (Toronto, Canada) as full proteins inactivated using gamma radiations. As VZV antigens, we used the commercial vaccine (Varivax, Sanofi Pasteur, Lyon, France). Heat-inactivated A. fumigatus conidia and C. albicans yeasts were provided by the Microbiology Division, University of Perugia. On day +14 of culture, IL-2 was added at the final concentration of 100 IU/ml. On days 14-18, wells containing growing clones were counted by screening plates with an inverted microscope. Clones were assessed for CD3, CD4, CD8, αβ and γδ TCR phenotype as described. After resting in IL-2-free medium for 24 hours,
pathogen-specificity was assessed by re-stimulating clones with pathogen-pulsed irradiated APC for 3 days. DNA synthesis was measured by H\(^3\)-thymidine uptake, as described. To consider thymidine incorporation of a clone Ag-reactive as Ag-specific, cpm should be between the value of the clone stimulated with autologous APC alone and the value of the clone stimulated with PHA.

*Limiting dilution assay to evaluate pathogen-specific responses in CD8+ T cells.*

Patient PBMC to be used as antigen-presenting cells were irradiated with 20 Gy and plated in 96 U bottomed plates at the concentration of 2 x 10^6/ml, i.e. 2 x 10^5 cells/well, in RPMI-1640 medium with 10% donor plasma, and pulsed with CMV, T. gondii, HSV, ADV, and VZV (18). Patient CD8+ cells were separated by negative selection using anti-CD4 and anti-CD16 immunomagnetic beads (Miltenyi Biotec). Patient CD8+ cells to be used as responder cells were plated on irradiated, antigen-pulsed cells at cell concentrations ranging from 40,000 to 1,250 cells/well. On day +1 of culture, IL-2 was added to a final concentration of 0.5 IU/ml. On days +10 and +14 IL-2 concentrations were raised to respectively, 1.0 IU/ml and 20.0 IU/ml. Between days +18 and +21 wells containing growing clones were counted by screening plates with an inverted microscope. Clones were assessed for CD3, CD4, CD8, \(\alpha\beta\) and \(\gamma\delta\) TCR phenotype as described. To consider thymidine incorporation of a clone Ag-reactive as Ag-specific, cpm should be between the value of the clone stimulated with autologous APC alone and the value of the clone stimulated with PHA.

*KIR phenotyping and NK cell functional analysis*

The alloreactive NK cell subsets in donors and monthly in recipients post transplants were phenotyped using different combinations of GL-183 (anti-KIR2DL2/L3/S2), EB6B (anti-KIR2DL1/S1) and Z27 (anti-KIR3DL1/S1) mAbs conjugated with the fluorochrome PE, #143211 (anti-KIR2DL1), DX9 (anti-KIR3DL1) and Z199 (anti-NKG2A) mAbs conjugated with FITC. Samples were analyzed by cytofluorimetry on a FACSCanto with the Diva program (Becton Dickinson). Functional analysis of alloreactive NK cell subsets in donors and monthly in recipients post transplants were assessed in cytotoxicity assays against recipient target cells and/or KIR ligand
mis-matched vs the donor, to determine the frequency of alloreactive NK clones. PBMCs depleted of T cells by negative anti-CD3 immunomagnetic selection (Miltenyi, Bergisch Gladbach, Germany) were plated under limiting-dilution conditions, activated with phytohemagglutinin (PHA; Biochrom KG, Berlin, Germany), and cultured with interleukin-2 (Chiron BV, Amsterdam, Netherlands) and irradiated feeder cells. Cloning efficiencies ranged from 1 in 5 to 1 in 10 plated NK cells. Cloned NK cells were screened for alloreactivity by standard 51Cr release cytotoxicity at an effector-to-target ratio of 10:1 against allogeneic, KIR ligand-mismatched PHA lymphoblasts. Clones exhibiting greater than 30% lysis were scored as alloreactive.
Results

Treg and Tcon characteristics

The phenotype of the immunoselected CD4+/CD25+ Tregs (purity 92.7%±2.1) was CD25high 33.6%±13.1; CD25int 52.6%±8.9; CD25low 4.7%±2.7; FoxP3 69.2%±13.9; CD127 16.6%±6.8, (mean±SD). CD45RO+ cells were predominant while the CD45RA+ cells were around 10%. CD62L expression was enriched as well as CD39 (Figure 2). Dot plots of combined stainings with CD25/CD45RA/FoxP3 and CD25/CD127/FoxP3 showed that 3.7%±4.1 of cells were FoxP3+/CD45RA+ and 94.3%±4.4 were FoxP3+/CD127+ (Figure 3). The in vitro suppressive capacity was 67%±22 (±SD) (ratio Tcons:Tregs 1:2).

The Tcon composition was: CD3 93.16%± 4.82; CD4 60.19% ± 7.24; CD8 32.22% ± 6.48; CD25 4.85% ± 2.17..

Engraftment and GvHD

Twenty-six of the 28 patients achieved primary, sustained full-donor type engraftment. Neutrophils reached 1x10^9/L at a median of 15 days (range 11-39). Platelets reached 25x10^9/L and 50x10^9/L medianly at 13 and 15 days, respectively (ranges 11-48, and 13-60). Only 2/26 evaluable patients developed ≥ grade II acute GvHD and both were among the 5 patients who had received 4x10^6/kg Tregs and 2x10^6/kg Tcons. At a median follow-up of 11.2 months (range 3.6-21.4) no patient has developed chronic GvHD.

Post transplant immune recovery

There was a rapid, sustained increase in peripheral blood T cell sub-populations. CD4 and CD8 counts reached 50/µL medianly on, respectively, days 33 (range 19-63) and 27 (range, 13-87); 100/µL medianly on days 42 (range 28-135) and 38 (range 19-95); 200/µL on days 67 (range 40-
146) and 48 (range 21-95) (Figure 4A). A wide T-cell repertoire developed rapidly (Figure 4B). Naïve and memory T cell subsets increased significantly over the first year post-transplant, demonstrating sustained immune recovery over time (Figure 4C). B cell reconstitution was rapid and sustained. CD20+ cells reached a mean of 132/µL three months post-transplant (Figure 4D). Immunoglobulin serum levels normalized within 3 months.

Compared with our standard haploidentical transplant, specific CD4+ and CD8+ for opportunistic pathogens such as Aspergillus, Candida, Cytomegalovirus (CMV), Adenovirus, Herpes Simplex Virus, Varicella Zoster Virus, Toxoplasma emerged significantly earlier (at each time point p<0.0001) (Figure 5A). Fewer episodes of CMV reactivation occurred (Figure 5B) and no patient developed CMV disease. CMV clearance was often associated with expansion of specific CD8 cells. Treg cell counts were monitored at different time-points for over 1 year. Only a median of 7.5 (range 1-19) CD4/CD25high cells/µl were found in the peripheral blood after 6 months post transplants and a median of 11 (range 5-14) CD4/CD25high cells/µl after 1 year.

To further investigate immune competence 7 patients (3-14 months after stem cell transplantation) were vaccinated against pandemic influenza with 2 doses of MF59-H1N1california. Five of seven patients achieved protective antibody titers (titer ≥40 in hemoagglutinin inhibition and microneutralization) and marked increases in H1N1 California specific CD4+ T cells.

Adoptive immunotherapy with Tregs did not impair NK cell reconstitution/maturation (Figure 6) which was faster with enhanced donor alloreactive NK cell repertoires against KIR-ligand mismatched targets. In particular NK cells expressing single KIR responsible for NK cell alloreactivity were higher and reconstituted faster (KIR2DL2/3 p<0.01 at 1, 3 and 5 months) in Treg-haploidentical transplantation than in standard haploidentical transplants (Figure 6A vs 6B). The frequency of alloreactive NK clones directed against KIR ligand mis-matched recipient target cells was also higher (p=NS) (Figure 6C vs 6D).
Outcomes

Thirteen of 26 patients died due to veno-occlusive disease (VOD) (3), multi-organ failure (MOF) (1), adenoviral infection (1), adenoviral infection and GvHD (1), GvHD (1), bacterial sepsis (1), systemic toxoplasmosis (1), fungal pneumonia (3) and CNS aspergillosis (1). One AML patient who had been transplanted in chemoresistant relapse from a non-NK alloreactive donor, relapsed 6 months post-transplant. At a median follow-up of 12 months (range 9-21) 12/26 (46.1%) patients are alive and disease free.
Discussion

In HLA-haploidentical transplantation without post-transplant immunosuppression, extensive ex-vivo T cell depletion (3.5 - 4 \times 10^{10}) of the graft is mandatory to prevent acute and chronic GvHD. In children with combined immunodeficiency who received HLA-haploidentical bone marrow transplant, 4 \times 10^4 T cells/Kg was identified as the GvHD threshold dose (19). In adults with acute leukaemia, after a conditioning regimen that included TBI, cyclophosphamide, thiotepa and antithymocyte globulin (ATG), 1-5 \times 10^5/Kg T cells contaminating the CD34+ cell inoculum were associated with an 18% incidence of grade II-IV GvHD (5). To reduce the incidence of GvHD, the number of T lymphocytes in the graft was lowered to one-tenth (1-3.5 \times 10^4 T cells/Kg). Acute GvHD developed in 8/141 evaluable patients (<5%)(1,2).

In assessing the real threshold dose, the effects of anti-T antibodies in the conditioning regimens do, however, need to be factored in. ATG, with its 6 day plasma half-life might have exerted an in vivo cytotoxic effect against donor T lymphocytes and thus helped prevent GvHD. Similar considerations can be applied to OKT3, which was used in children (20). Interestingly, adding back 3 \times 10^4/Kg donor T lymphocytes to improve post-transplant immune-reconstitution 2-3 months after transplantation, which is beyond the time frame of the ATG half-life, was associated with high risk of severe GvHD (1,2).

The present study demonstrates for the first time that adoptive immunotherapy with freshly purified CD4^+CD25^+ Tregs counteracted the GvHD potential of a high number of donor Tcons in HLA-haploidentical HSCT. The striking finding was the absence of GvHD in patients who received up to 1 \times 10^6 Tcons/Kg (2 logs more than in the above mentioned studies) following an infusion of 2 \times 10^6 Tregs. It is worth noting that no post-transplant immunosuppression was given and that, as the conditioning regimen contained cyclophosphamide instead of ATG, there was no ATG-related lympholytic action on effector T cells. When 2 \times 10^6/Kg Tcons were infused two out of five patients
developed GvHD. Consequently we can conclude that $1 \times 10^6$ /Kg Tcons is the GvHD threshold dose in this type of HLA-haploidentical transplant setting.

However we may hypothesize that the real number of infused T cons was even greater, since the immune-selected CD4+CD25+ cells contained about 30% FoxP3- cells, which are potentially effector T cells. Conversely, the number of Tregs might also have been more in vivo. They could have proliferated in the post-conditioning pro-inflammatory environment of the T cell depleted host in the 4-day interval before the Tcons were infused. In fact, pre-clinical studies had demonstrated that in vivo Treg activation and expansion permitted subsequent infusion of more Tcons without triggering GvHD (14).

One issue is whether adoptive immunotherapy with Tregs compromised general immunity, blunting responses to infectious agents. In animal models, infusion of recipient-type specific Tregs (12) and of polyclonal purified Tregs+Tcons (10) promoted immune reconstitution and protected mice from lethal CMV infection (10). In the present clinical setting naturally occurring, polyclonal purified Tregs did not inhibit expansion of co-infused Tcons with a broad TCR repertoire. CD4+ and CD8+ cell counts achieved sustained levels quickly, unlike standard T-cell depleted mismatched HSCT with CD4+ positive cell counts below 100 and 200/mmc for 10 and 16 months respectively (1). High frequencies of pathogen-specific CD4+ and CD8+ T cell precursors were detected as early as two, rather than 9-12 months post-transplant (18). Strikingly, prevention of CMV disease was markedly improved, with no CMV-related deaths. They had been the major cause of mortality, accounting for 40% of non-leukaemic deaths, in our previous study (2). We may hypothesize that, as in animal models, in a post-conditioning inflammatory environment Tregs are activated by recipient antigen-presenting cells, block alloreactive T cells in an antigen-specific fashion and allow expansion of non-alloreactive T cells which ensure long-term immunity.

In adults with high risk acute leukemias, HSCTs from alternative sources (matched unrelated donor, unrelated cord blood and full-haplotype mismatched family member) are still associated with quite high TRM (21, 22, 2). In our previous series of full-haplotype mismatched HSCTs mortality from
causes other than leukaemia relapse was 40% (1) and 36.5% (2). In the present study, the 50% transplant related mortality (TRM) needs to be viewed in light of these outcomes as well as the clinical characteristics of this cohort. Four of the 13 deaths were due to regimen related toxicity and three of these patients had been heavily pre-treated with several lines of polychemotherapy including gemtuzumab ozogamicin. The 4 patients who died of aspergillosis had fungal localisation in the lungs (3 patients) and central nervous system (1 patient) before transplantation. No fatal infections occurred after the first 2 months post-transplantation, confirming the good recovery of immune response against pathogens.

Another crucial point about adoptive immunotherapy with Tregs is whether the graft-versus-leukaemia (GvL) effect is maintained, as reported in some animal models (12, 23). Surprisingly in patients at very high risk of relapse, only 1 relapse has occurred at a median follow-up of 12 months in a patient with acute myeloid leukaemia who was transplanted in chemoresistant relapse from a non-NK alloreactive donor. Although no definitive conclusions can be drawn, the low incidence of relapse may be due to the powerful regeneration of donor-vs-recipient alloreactive NK repertoires in patients who received a transplant from a potentially NK alloreactive donor (24). Moreover, in all patients, the high number of infused Tcons are hypothesized to exert a GvL effect by recognizing leukaemia-associated antigens. In any case, the Treg infusion is clearly not associated with increased incidence of leukaemia relapse.

In conclusion, combining donor Tregs and Tcons in this first clinical trial prevented GvHD, enhanced immune recovery, thus providing evidence that Treg properties are a conserved mechanism in human beings.
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Authorship

M.D.I., F.F., and M.F.M. designed the study and oversaw the results; M.D.I. and M.F.M. drafted the paper; B.F., Y.R., A.V., F.A. contributed to the design and interpretation of the study; A.C., A.T., T.A., A.P., P.S. and C.A. provided clinical data; F.C., E.B., B.D.P., T.Z., R.I.O., D.C., K.P., L.R. and C.B performed the in vitro studies and interpreted the results.

Conflict of interest disclosure

The authors declare no competing financial interests.
References


Figure legends

**Figure 1. Overall view of the protocol.** The conditioning regimen includes Total Body Irradiation (TBI) 8 Gy in a single fraction at 16 cGy/m, Thiotepa (TT) 4 mg/kg/day, Cyclophosphamide (CTX) 35 mg/kg/day and Fludarabine 40 mg/sqm/day. On day -4 the patient received freshly isolated donor Tregs, followed by a megadose of CD34+cells and Tcons (day 0). No post-transplant immunosuppression was given whatsoever.

**Figure 2. Immunophenotype of the immunoselected Treg.** The phenotype belongs to one representative case. (A) CD4/CD25 purity. (B) CD25 expression within final fraction. FoxP3, CD127, CD45RO, CD45RA, CD39 and CD62L expressions are also shown.

**Figure 3. Immunophenotype of the immunoselected Treg.** Dot plots of combined stainings with CD25/CD45RA/FoxP3 and CD25/CD127/FoxP3 are shown. Positive cells are boxed and the percentage of cells is shown.

**Figure 4. Immunological reconstitution after Tregs-based transplantation.** (A) CD4 (left) and CD8 (right) recovery kinetics. (B) Post-transplant TCR Vβ spectratyping complexity score over time. (C) Naïve and Memory T cell recovery. (D) CD20+ recovery kinetic.

**Figure 5. Immunological reconstitution after Tregs-based transplantation.** (A) Reconstitution of pathogen-specific T-cell responses. Right panels: post-transplant frequencies of CD4+ and CD8+ pathogen-specific T-cell clones in the Treg haploidentical transplantation vs standard haploidentical transplant (left panels). Sample numbers were at least three for each time points. CD4+ pathogen-
specific T-cell clones in the Treg haploidentical transplantation at month +1 (ASP=2.6±1.2; CAND=5.3±2.1; CMV=115±61; ADV=156±78; HSV=32±4; TOXO=65±26; VZV=48±34); at months +3 (ASP=37.5±34; CAND=69.5±42; CMV=68±48; ADV=422±333; HSV=79±16; TOXO=170±57; VZV=48±34); at months +6 (ASP=91.6±29; CAND=133±91; CMV=259±245; ADV=303±216; HSV=137±90; TOXO=263±156; VZV=337±280); at months +12 (ASP=210.5±190.2; CAND=139±104; CMV=359±288; ADV=160±103; HSV=222±173; TOXO=277±218; VZV=155±111) (p versus standard haploidentical transplants at each time point =<0.0001). CD8+ pathogen-specific T-cell clones in the Treg haploidentical transplantation at month +1 (CMV=1.2±3; ADV=0; HSV=1.5±1; TOXO=3±0.4; VZV=2±2); at month +3 (CMV=4.4±3.5; ADV=0; HSV=1.6±1.5; TOXO=1.5±1.2; VZV=4.2±4); at month +6 (CMV=4.3±2.3; ADV=4±3.4; HSV=4.8±4.5; TOXO=3.6±2; VZV=4.2±4.1); at month +12 (CMV=11±1; ADV=11±1; HSV=9±2; TOXO=10.3±1.5; VZV=11±1) (p versus standard haploidentical transplants at each time point =<0.0001). (B): Episodes of CMV reactivation are significantly lower (p<0.05) after “haplo transplants with Tregs” (right panel) as compared to “standard haplo” transplants (left panel).

Figure 6. Regeneration of alloreactive NK cell repertoires against KIR-ligand mis-matched target. Panels A and B: Immunophenotype of NK cell repertoire in donors and recipients after Tregs (A) and standard (B) HLA-haploidentical transplantation identified potentially alloreactive NK cells which express single KIR. KIR2DL2/3 p<0.01 at 1, 3 and 5 months
Panels C and D: Cytotoxicity assay identified alloreactive NK cells which killed KIR ligand-mismatched PHA lymphoblasts in donors and recipients after Tregs (C) and standard (D) HLA-haploidentical transplantation (p=NS).
Figure 1
Figure 2

CD4
Figure 3

- CD25 vs. CD4: 96.6%
- FoxP3 vs. CD45RA: 9.6%
- CD45RA vs. CD127: 87.8%
Tregs prevent GvHD and promote immune reconstitution in HLA-haploidentical transplantation

Mauro Di Ianni, Franca Falzetti, Alessandra Carotti, Adelmo Terenzi, Flora Castellino, Elisabetta Bonifacio, Beatrice Del Papa, Tiziana Zei, Roberta Iacucci Ostini, Debora Cecchini, Teresa Aloisi, Katia Perruccio, Loredana Ruggeri, Chiara Balucani, Antonio Pierini, Paolo Sportoletti, Cynthia Aristei, Brunangelo Falini, Yair Reisner, Andrea Velardi, Franco Aversa and Massimo F Martelli