HLA-haploidentical transplantation with regulatory and conventional T cell adoptive immunotherapy prevents acute leukemia relapse

Massimo F Martelli¹, Mauro Di Ianni¹,²*, Loredana Ruggeri¹*, Franca Falzetti¹, Alessandra Carotti¹, Adelmo Terenzi¹, Antonio Pierini¹, Maria Speranza Massei¹, Lucia Amico¹, Elena Urbani¹, Beatrice Del Papa¹, Tiziana Zei¹, Roberta Iacucci Ostini¹, Debora Cecchini¹, Rita Tognellini³, Yair Reisner⁴, Franco Aversa⁵, Brunangelo Falini¹, and Andrea Velardi¹

¹Division of Hematology and Clinical Immunology, Department of Medicine, University of Perugia, Italy. ²Hematology Section, Department of Life, Health and Environmental Sciences, University of L’Aquila, Italy. ³Blood Bank, Ospedale Santa Maria della Misericordia, Perugia, Italy. ⁴Weizmann Institute of Science, Immunology Department, Rehovot, Israel. ⁵Hematology and BMT Unit, Department of Clinical and Experimental Medicine, University of Parma, Italy

* M. Di Ianni and L. Ruggeri contributed equally to this study.

Correspondence: Mauro Di Ianni, Hematology Section, Department of Life, Health and Environmental Sciences, University of L’Aquila, Italy. E-mail: mauro.diianni@cc.univaq.it

Running head: T regulatory cells in haploidentical transplant
Key points:
-Haploidentical transplantation with regulatory and conventional T cell adoptive immunotherapy prevents high-risk acute leukemia relapse
-The graft-vs-leukemia effect is separated from graft-vs-host disease even across major HLA-barriers
ABSTRACT

Post-transplant relapse is still the major cause of treatment failure in high-risk acute leukemia. Attempts to manipulate alloreactive T cells to spare normal cells while killing leukemic cells have been unsuccessful. In HLA-haploidentical transplantation we reported that donor-derived T regulatory cells (Tregs), co-infused with conventional T cells (Tcons), protected recipients against graft versus host disease (GvHD). The present phase II study investigated whether Treg-Tcon adoptive immunotherapy prevents post-transplant leukemia relapse.

43 adults with high-risk acute leukemia (AML 33; ALL 10) were conditioned with a TBI-based regimen. Grafts included CD34⁺ cells (mean 9.7x10⁶/kg), Tregs (mean 2.5x10⁶/kg) and Tcons (mean 1.1x10⁶/kg). No post-transplant immunosuppression was given. 95% patients achieved full-donor type engraftment. 15% developed ≥ grade II acute GvHD. The probability of disease-free survival was 0.56 at a median follow-up of 46 months. The very low cumulative incidence of relapse (0.05) was significantly better than in historical controls. These results demonstrate the immunosuppressive potential of Tregs can be used to suppress GvHD without loss of the benefits of GvL activity. Humanized murine models provided insights into the mechanisms underlying separation of GvL from GvHD, suggesting the GvL effect is due to largely unopposed Tcon alloantigen recognition in bone marrow.
INTRODUCTION

In patients with acute leukemia (AL) at high risk of relapse because of unfavorable cytogenetics, molecular markers and disease status (CR≥2), the most powerful post-remission therapy is hematopoietic stem cell transplantation (HSCT) from a matched sibling (MSD) or unrelated donor (MUD) (1,2). When patients do not have a MSD or MUD, unrelated cord blood (UCB) (3) and haploidentical-related donor (haplo) (4) HSCTs are emerging as alternatives.

In eradicating malignancy, i.e. the so-called graft-vs-leukemia effect (GvL), clinical observations (5, 6) and experimental models (7-9) established that the allogeneic immune system played a crucial role. Donor T cells recognize host allo-antigens on leukemic cells, although hematopoietic-specific and leukemia-specific responses may also occur (10). They also mediate graft-vs-host disease (GvHD), a major cause of morbidity and mortality after HSCT.

In T cell replete HSCT pharmacological immunosuppression for GvHD prophylaxis and treatment is non-specific and only partially successful. More importantly, it may compromise the T-cell induced GvL effect. Indeed relapse is still the major cause of treatment failure in high risk AL patients (11-14).

To date, attempts to manipulate alloreactive T cells to spare normal cells while killing leukemic cells have been largely unsuccessful. In the search for strategies to separate GvHD and the GvL effect and prevent disease recurrence, attention focused on a thymic-derived CD4+CD25+ FoxP3+ regulatory T-cell subpopulation (Tregs) which physiologically helps maintain immunological self-tolerance and immune homeostasis (15, 16). Evidence from murine models of bone marrow transplantation across major histocompatibility class I and II barriers showed that co-infusion of conventional T lymphocytes (Tcons) with Tregs, whether freshly isolated (17-19), ex vivo expanded polyclonal (20) or recipient-type (21), suppressed lethal GvHD without impairing Tcon activity against malignant diseases (22,23). In high-risk AL patients undergoing full-haplotypemismatched transplantation without any post-transplant immunosuppression we demonstrated that
adoptive immunotherapy with donor FoxP3+ T regs (2x10^6/kg) and broad repertoire Tcons (1x10^6/kg) almost completely prevented acute and chronic GvHD and favoured post-transplant immunological reconstitution (24).

To address the issue of leukemia relapse, the present study investigated whether Treg-Tcon adoptive immunotherapy provided a powerful Tcon-mediated GvL effect in the absence of GvHD.
PATIENTS AND METHODS

Patient inclusion criteria

The protocol was approved by Umbria Regional Hospital Ethical Committee and registered as 0108. Adults (18-65 years) with high risk AL were eligible for haplo-HSCT with Treg-Tcon adoptive immunotherapy if they did not have an MSD or MUD. Before enrolment all patients provided written informed consent in accordance with the Declaration of Helsinki.

Donors

A healthy family member with one HLA-haplotype identical to the patient’s, who was able to donate haematopoietic stem cells after treatment with G-CSF and undergo leukapheresis sessions for collecting hematopoietic stem cells, Tregs and Tcons. An NK alloreactive donor was preferentially selected when available (Table 1).

Transplantation Procedure

Figure 1 illustrates the transplant timeline schema. Briefly, the conditioning regimen included total body irradiation (TBI), thiotepa, fludarabine. The first 25 patients also received cyclophosphamide. To reduce extra-hematological toxicity and the risk of veno-occlusive disease instead of cyclophosphamide the other 18 were given alemtuzumab (8 patients) or thymoglobulin (12 patients) when alemtuzumab was withdrawn for patients with haematological diseases. Anti-T antibodies were administered 21 days before transplant to prevent interference with Treg-Tcon adoptive immunotherapy. All patients received freshly isolated donor Tregs on day -4, followed by a megadose of purified CD34+ cells and Tcons on day 0. The 4-day interval between Treg and Tcon infusions was in accordance with animal data indicating that early Treg administration provided greatest protection against GvHD (18). No pharmacological post-transplant GvHD prophylaxis was given. Patients were managed according to our standard haplo-HSCT protocol (25).
Graft processing

Tregs and Tcons were collected from donors before they underwent G-CSF treatment for CD34+ cell collection. 2 total blood volumes from a single leukapheresis procedure were processed with COBE Spectra (Terumo BCT, CO, USA). Tcons were separated from peripheral blood mononuclear cells (PBMCs) by negative selection using CliniMACS CD19 reagent (Miltenyi Biotec), and cryopreserved (24). Naturally occurring CD4+CD25+ FoxP3+ Tregs were selected by means of a fully automated immunomagnetic procedure (Miltenyi Biotec GmbH, Bergisch Gladbach Germany) by depleting the leukapheresis product of CD8+/CD19+ cells and then positively immunoselecting CD25+cells (24). The initial leukapheresis products contained a median of 12.1x10^9 (range 5.4-18x10^9) nucleated cells. After magnetic cell separation a median of 272x10^6 (range 142-412x10^6) Tregs was recovered.

Immediately after Treg and Tcon collection, donors were treated with G-CSF to mobilize peripheral blood progenitor cells. After collection, CD34+ cells were positively immunoselected using the CliniMACS device (Miltenyi Biotec) (25). The target numbers of Tregs, Tcons and CD34+ cells were achieved for all patients (see below). Phenotypes were determined using direct immunofluorescence with a panel of monoclonal antibodies as described before (24). Overall 3 days of apheresis were needed for each donor (1 for Treg and Tcon collection; 2 for CD34+ cells).

Suppression assays

Treg suppressive capacity was established as follows. Carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled CD4+/CD25- T lymphocytes were cultured in 96-well plates at 2x10^4 cells/well with phytohemagglutinin (PHA; 4 μg/mL; Biochrom) in the presence of varying numbers of CD4+/CD25+ (Tregs). For a CFSE based measurement of proliferation, the suppressive capacity of Tregs toward responder cells in coculture (a Tcon:Treg ratio of 1:1 or 1:2) was expressed as the
relative inhibition of the percentage of CFSE\text{low} as follows: $100 \times (1 - \frac{%\text{CFSE}^{\text{low}}\text{CD4+CD25- T cells in coculture}}{%\text{CFSE}^{\text{low}}\text{CD4+CD25-T cells alone}})$.

**Chimerism analysis and immunological studies**

Chimerism was assessed on DNA extracted from peripheral blood samples by multiplex fluorescent short-tandem repeat analysis. Peripheral blood was collected weekly for the first two months and then monthly for cytometric lymphocyte subset immunophenotyping. Alloreactive NK cell subsets were phenotyped in donors and monthly in recipients after transplant. Function was analysed in cytotoxicity assays against KIR ligand mis-matched recipient target cells to determine the frequency of alloreactive NK clones (24).

**End-points and definitions**

Primary end-point: to assess the impact of Treg/Tcon immunotherapy in haploidentical transplantation on the cumulative incidence of post-transplant leukemia relapse. Disease status was assessed in bone marrow in all transplant recipients on days 30, 60, 90, 120, 180, 240 and 360, and then annually. Relapse was defined as disease recurrence according to the following: marrow morphology, flow cytometry, cytogenetics including fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR) for disease markers. Secondary end-points: full donor type engraftment; probability of disease-free survival (DFS) based on patients who were alive, without evidence of disease. Incidence of grades II-IV and III-IV aGvHD on day +100; chronic GvHD at 1 year; cumulative incidence of non-relapse mortality (NRM), i.e. death by any cause in the absence of disease relapse; GvHD mortality included patients with a history of GvHD who died from infections or organ failure during immunosuppressive therapy.
**Historical controls**

We compared outcomes of the present Treg-Tcon transplants with 114 high risk CR1, ≥CR2 AL patients (50 ALL; 64 AML; median age 37 years) (Table 1), from previous haploidentical-HSCT trials (25-27) All had received a similar conditioning regimen with TBI, thiotepa, cyclophosphamide/fludarabine and thymoglobulin which, unlike the present series, was administered during conditioning and not 21 days earlier. The inoculum included a megadose (mean CD34+ cells 10x106/kg±2.1) of haematopoietic progenitor cells. The contaminating T lymphocytes ranged in number from 1x104-1x105 /Kg body weight.

**Statistical analyses**

Cumulative incidence estimates (evaluated by the Gray test) were used for relapse and non-leukemic mortality, as they are competing risks. The Kaplan-Meier method evaluated EFS. The log-rank test assessed impact of disease (AML v ALL), disease status, gender, age, pre-transplant donor-recipient pair CMV, donor-recipient NK cell on EFS. Multivariate analysis (Cox regression model) also investigated the impact of these factors on non-leukemic mortality, relapse and EFS. Two-sided *P* values <.05 were considered significant.

**Mouse models**

Colonies of NOD-scidIl2rgtm (NSG) mice were bred at Perugia University Animal House. All experiments were performed in accordance with the National Ethic Approval Document for animal experimentation.

NSG mice were irradiated with 3.5 Gy and infused i.v. with human primary AML cells (7x10⁶/mouse), human common Ph¹ ALL cell line (SUP-B15, 2x10⁶/mouse) or human Burkitt-like cell line (Namalwa, 2x10⁶/mouse). After primary AML leukemia engraftment, mice were co-infused with human Tcons (3x10⁶/mouse) and Tregs (3x10⁶/mouse). In mice treated with lymphoblastic cell lines Tregs and T cons were infused together with leukemic cells. Controls were
either untreated or infused with human Tcons (3x10^6/mouse) or Tregs (3x10^6/mouse). Tregs and Tcons came from the same donor and were HLA-mismatched with the primary leukemias and leukemias cell lines. At different time-points mice were evaluated for GvHD (by observation score and histopathology), leukemia infiltration and T cell homing (by flow cytometry with a combination of anti–human monoclonal antibodies: CD45, CD25, FoxP3, CD34, CD13, (eBioscience, California, USA), CD3, CD56, CD8, CD4 and CD14, (Miltenyi, Koln, Germany), CD33, CD19, CD20, CD10 (Beckman Coulter, Marseilles, France), CD117, CD7, Kappa, Lambda, IgM (Invitrogen, California, USA). Each group was comprised of 5 mice. All experiments were repeated in duplicate. Results of duplicated experiments were combined.

Human CD3 T cells were purified from murine bone marrow by anti-human CD3 conjugated microbeads (Miltenyi) and assessed in a chromium release cytotoxicity assay with 5:1 ratio as effectors against targets (leukemia and autologous to leukemia PHA blasts).
RESULTS

Patients and graft characteristics

43 consecutive AL patients, (median age 40 years; range 18-65) were treated between September 2008 and December 2012. 33 had AML (18 CR1, 15 ≥ CR2), 10 had ALL (7 CR1; 3 in ≥ CR2). Twenty-four of these patients have already been reported in a previous paper (24). All patients who were transplanted in CR1 were at high risk of relapse (8 FLT-3/ITD; 8 complex karyotypes; 4 with t(9:22); 2 primary induction failure; 1 secondary AML; 1 CR after second-line induction; 1 with central nervous system (CNS) and skin involvement at diagnosis).

All patients received a full haplotype mismatched graft which included CD34+ cells (mean 9.7x10^6/kg±3.1; mean contaminating T cells: 0.8x10^7/kg ±0.4), Tregs (mean 2.5x10^6/kg ±1) and Tcons (mean 1.1x10^6/kg ±0.6). The Treg phenotype was Foxp3+ 81.01%±16.47; Helios/FoxP3+ 54%±8.4; CD127+ 11.72%±7.653. CD45RO+ cells were 90% and CD45RA+ cells 10%. Treg suppressive capacity in vitro was 67%±22 (±SD) (ratio Tcons:Tregs 1:2). Infused Tcons were 90.72%±9.6 CD3+; 57.77%±8.85 CD4+; 31.21% ± 8.59 CD8+.

Table 1 shows the demographic characteristics and disease status of the present series of patients vs the historical control group. Median age, the proportion of AML vs ALL cases and remission status were similar in the two cohorts. All patients in both cohorts who underwent transplantation in CR1 had high-risk features. Median time from diagnosis to transplantation was 6 months [range, 3-48 months present cohort] vs 5 months [range, 2-60 months historical controls]; P = 0.4). For patients who underwent transplantation in CR2, duration of remission was 6.5 months [range, 1-60 months present series] vs 7 months [range, 2-55 months historical controls]; P = 0.6).

Engraftment and post-transplant immune recovery

41/43 patients achieved primary, sustained full-donor type engraftment. Neutrophils reached 1x10^9/L (median 16 days; range 10-39). Platelets reached 20x10^9/L and 50x10^9/L (medians 13 and 15 days, respectively; ranges 8-48 and 13-60). The incidence of sustained engraftment was similar
for Treg/Tcon vs historical control patients (95% vs 90%; $P = .8$). Likewise, time to neutrophil and platelet recovery was not different between Treg/Tcon and historical control ($P = .11$).

There was a rapid, sustained increase in peripheral blood T cell sub-population recovery: CD4+ and CD8+ cells reached 50/µL at a median of, respectively, 30 days (range 16-65) and 25 (range, 12-90); 100/µL at a median of 40 (range 25-150) and 45 days (range 18-100); 200/µL at a median of 55 (range 45-160) and 60 days (range 50-140). Compared with standard haploidentical transplantation, specific CD4+ and CD8+ for opportunistic pathogens such as A fumigatus, C albicans, CMV, ADV, HSV, and VZV toxoplasma emerged significantly earlier (at each time point $P < .0001$) (24).

NK cells reached 200/µL at a median of 25 days (range 19-35) and 400/µL at a median of 45 days (range 22-70). Post-transplant alloreactive NK clones against KIR ligand mismatched targets had a higher frequency than in historical controls (15±6 vs 7±5 at one month post-transplant).

**GvHD and NRM**

Only 6/41 patients (15%) developed ≥ grade II acute GvHD. In 2, GvHD responded rapidly to a short course of immunosuppression, only one of these patients developed chronic GvHD. Even though $1.1\times10^6$/kg ±0.6 Tcons were infused, the incidence of ≥ grade II aGvHD was similar ($P=0.2$) to the 11% in historical controls. They had received extensively T cell depleted grafts and ATG which exerted additional in vivo T cell depletion. Three patients (7.5%) died of GvHD (1 grade II aGvHD, 2 grade III-IV aGvHD) compared with 10 (9%) in the historic cohort.

Overall in the present series, the cumulative incidence of NRM was 0.40 which fell to 0.21 in 18 patients who received anti-T antibodies in the conditioning. Causes of non-relapse death were: veno-occlusive disease in 3 patients who were heavily pre-treated until a few days before transplantation, GvHD (3), adenoviral infection (2), bacterial sepsis (2), HHV6 infection (2), multi-organ failure (1), systemic toxoplasmosis (1). Lung (3) and CNS (1) aspergillosis in patients with invasive fungal disease before transplantation.
Relapse and DFS

At a median follow-up of 46 months (range 18-65 months), only 2/41 evaluable patients have relapsed. Both had NPM+FLT3+ AML in molecular relapse at time of HSCT and had received a transplant from non-NK alloreactive donors. Risk of relapse was not related to modification of the treatment plan over time. In 114 historical controls, relapses occurred in 11/50 with ALL, in 10/32 with AML transplanted from non NK alloreactive donors and in 4/32 AML patients who were transplanted from NK alloreactive donors. Fig 2 reports relapse rates in the Treg-Tcon treated patients and historical controls who underwent haplo-HSCT from NK alloreactive and non-NK alloreactive donors.

In the Treg-Tcon cohort the cumulative incidence of relapse was significantly lower than in historical controls (0.05 vs 0.21; P=0.03). Multivariate analysis identified Treg-Tcon adoptive immunotherapy as the only predictive factor associated with a reduced risk of relapse (relative risk 0.06; 95% CI, 0.02-0.35; P = 0.02).

23/41 patients are alive and well with DFS probability of 0.56 at 18 months minimum follow-up. DFS in the control cohort was 0.39 (p=0.07) indicating a trend towards better survival under the Treg/Tcon protocol.

Murine studies

All mice that received myeloid or lymphoblastic leukemias, Tregs and Tcons were rescued from leukemia and survived without GvHD. Cytofluorimetric analysis of all organs and tissues showed disease eradication. All mice that received leukemic cells with or without Tregs died of leukemia within 60-75 days. Those that received leukemia cells plus Tcons developed severe GvHD and died within 60 days ( p<0.002 mice with Treg/Tcon infusions vs all others).
Human T cells from bone marrow in mice that were rescued from leukemia by Treg-Tcon adoptive immunotherapy exhibited similar cytotoxicity against both leukemic and PHA blast cells, autologous to leukemia (Fig 3). This experiment was repeated twice.
DISCUSSION

The present study is the first to analyze the impact of Treg-Tcon adoptive immunotherapy on AL disease eradication. Infusion of freshly isolated FoxP3+Tregs in this large series of high-risk patients undergoing full-HLA haplotype mismatched transplantation controlled the alloreactivity of up to 1x10^6/kg T lymphocytes which is about 2 log more than the threshold dose for GvHD. Indeed, nearly 90% of patients were protected against GvHD, thus confirming previous results (24). Brunstein et al. who employed ex vivo expanded polyclonal Tregs as supplemental GvHD prophylaxis in double-unit UCB transplantation, also reported a significantly reduced incidence of grade 2 to 4 GvHD (28).

Present results and Brunstein et al., suggest that adoptive immunotherapy with Tregs does not compromise general immunity or blunt responses to infectious agents. Immunological reconstitution in our transplant recipients was stronger and faster than in the historical controls. Prevention of CMV disease improved markedly, with no CMV-related deaths which had been one of the major causes of mortality in the historical control group.

Overall NRM is still unsatisfactory in the present series but it is interesting to note it dropped sharply in patients who received anti-T antibodies in the conditioning regimen. Better outcomes may have been linked to cyclophosphamide suspension as less extra-medullary toxicity was associated with lack of severe VOD and multi-organ failure. In the future to reduce the risk of post-transplant infection-related morbidity and mortality, it might be worth increasing the number of Tcons in the graft by means of CD3+CD45RO+ cells (29), thus approaching what is termed a “designed” graft for haploidentical transplantation (30).

One major concern about the use of Tregs in HSCT is their potential to suppress anti-neoplastic immune responses, as suggested by some studies in solid tumors (31, 32) and hematological malignancies (33, 34). For example, Tregs accumulated in leukemic sites in mice, preventing adoptively transferred anti-AML T cells from proliferation and cytolysis (35). Conversely,
interleukin-2 diphtheria toxin, which depleted Tregs expressing CD25, increased the number of cytotoxic T lymphocytes at tumour sites and resulted in tumour regression (36).

On the other hand, in mismatched transplant mouse models adoptive immunotherapy with Tregs and Tcons protected mice from GvHD without impairing Tcon control of neoplastic cell line expansion, such as A20 leukemic cells of BALB/c origin (22, 23) BCL1 lymphoma (22) and P815 mastocytoma (23). The present study shows for the first time in humanized mouse models that human Treg-Tcon immunotherapy eradicated human primary myeloid leukemia and lymphoblastic leukemia cell lines without triggering GvHD.

In the present series of AL patients, infusion of Tcons under the Treg protective umbrella, in the absence of post-transplant pharmacological immunosuppression guaranteed almost total control of AML and ALL relapse. The cumulative incidence of post-transplant leukemia relapse was 0.05 at a medium follow-up of 46 months, which is extremely low considering these patients were at high-risk of relapse according to cytogenetics, molecular markers and disease stage at transplant. In our historical controls relapse rates were over 30% in high-risk ALL patients and in AML patients who were not transplanted from NK alloreactive donors. Similarly, relapse rates ranged from 28% to 40% in recent clinical trials of T cell replete HSCT, independently of whether the donor was a MSD, MUD, UCB or haplo (11-14). In the present series the T-cell dependent GvL effect was so powerful that it masked the anti-AML activity exerted by post-transplant generation of alloreactive donor-vs-recipient NK cell repertoires (37-39).

The mechanisms underlying Treg suppression of GvHD with no loss of GvL activity are still obscure. In animal models Edinger et al observed that Tregs inhibited early expansion of alloreactive donor T cells and their capacity to induce GVHD but did not inhibit co-transplanted Tcon activation and cytotoxicity against leukemia and lymphoma cells in vitro and in vivo. Thus GvL activity appears to rely mainly on Tcon activation rather than expansion and consequently requires transplantation of sufficient Tcons (22).
In our murine models human T cells that were harvested from bone marrow killed human leukemia cells and autologous PHA blasts in vitro, thus confirming their cytotoxicity activity was preserved and suggesting the T cell dependent GvL effect is mainly due to alloantigen recognition.

The GvL effect in the absence of GvHD could also be related to Treg migratory properties which are linked to expression of homing molecules for diverse sites. Tracking Treg in vivo dynamics in animal models of incompatible transplantation showed that upon infusion of polyclonal Tregs alloantigen-specific Treg were activated and expanded in lymph nodes and then migrated to peripheral tissues (skin, gut, liver, lung) (18). Thus alloantigen-specific Tregs controlled Tcon alloreactivity not only in lymph nodes but in non-lymphoid tissues which are GvHD targets.

In the clinical transplant setting, one might hypothesize the GvL effect is due to largely unopposed Tcon alloreactivity in bone marrow. In fact, few infused donor Tregs are likely to migrate there since they are almost 100% RO+ with very low CXCR4 expression. In humans Booth et al. showed CD45RO+ Tregs home to skin whereas CD45RA+ Tregs expressing CXCR4 localize preferentially in bone marrow (40). There, according to Fujisaki et al, they provide immune privilege for the hematopoietic stem cell niche, thus protecting hematopoietic stem/progenitor cells (HSPCs) against Tcon alloreactivity. Interestingly, in this mouse transplant model Treg depletion in bone marrow was associated with T con-induced loss of HSPCs (41). Similarly, in our setting, failure of donor Tregs to home to the bone marrow might have exposed leukemic stem cells to donor T cell alloreactivity.

Whatever the mechanisms, with Treg adoptive immunotherapy there is no need for post-transplant pharmacological prophylaxis against GvHD which, as we know, could impair the GvL effect. Thus, without the risk of Tcon activity being inhibited, 1x10⁶/kg T effector cells are sufficient to eradicate minimal residual disease. Consequently, the 30-35% incidence of relapse, which is generally reported by conventional T cell replete HSCT in high-risk AL patients, is practically eliminated.

Present results also demonstrate that adoptive immunotherapy with Tregs does not require ex vivo Treg expansion systems (42, 43) which are cumbersome, expensive and require GMP facilities.
In conclusion we are confident the innovative use of Tregs and Tcons is a major advance on conventional HSCT which, as leukemia immunotherapy, is associated with severe side effects and is only partially efficacious. In the future the present strategy might be applied to exploit the GvL effect of Tcons that recognize minor histocompatibility antigens in matched donor HSCT.
Acknowledgements

We would like to thank Dr. Geraldine Anne Boyd for editorial assistance. This work was supported by a Translational Research Grant from the Leukemia and Lymphoma Society and by funding from Italian Association for Cancer Research (AIRC), Associazione Umbra Leucemie e Linfomi (AULL), Italian Ministry of Further Education (PRIN no, 2010NECHBX_005), Italian Ministry of Health (ERA-NET TRANSCAN, no. PER-2011-2353844) and the European Union 7th Framework Program (as part of the “Nano II” project, no: 229289). L.R. is a Leukemia and Lymphoma Society Scholar in Clinical Research.

Authorship

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

Conflict of interest disclosure: the authors declare no competing financial interests.

Correspondence: Mauro Di Ianni, Hematology Section, Department of Life, Health and Environmental Sciences, University of L’Aquila, Italy. E-mail: mauro.diianni@cc.univaq.it. Tel. (39) 0862-433556; fax (39) 0862-368642.
References


33. Kelley TW, Parker CJ. CD4(+)CD25(+) FoxP3(+) regulatory T cells and hematological malignancies. *Front Biosci (Schol Ed).* 2010; 2:980-982.


## Patient Demographics

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Treg-Tcon</th>
<th>Historical controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>43</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>20/23</td>
<td>63/51</td>
<td>P= 0.5</td>
</tr>
<tr>
<td>Age in years (median and range)</td>
<td>40 (range 18-65)</td>
<td>37 (range 8-66)</td>
<td>P= 0.8</td>
</tr>
<tr>
<td>Median time and range from diagnosis to transplant, CR1</td>
<td>6 months (3-48)</td>
<td>5 months (2-60)</td>
<td>P= 0.4</td>
</tr>
<tr>
<td>Median length of remission and range, CR2</td>
<td>6.5 months (1-60)</td>
<td>7 months (2-55)</td>
<td>P= 0.6</td>
</tr>
<tr>
<td>Disease and status</td>
<td>ALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I CR</td>
<td>7</td>
<td>27</td>
<td>P= 0.08</td>
</tr>
<tr>
<td>≥II CR</td>
<td>3</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I CR</td>
<td>18</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>≥II CR</td>
<td>15</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>NK cell alloreactivity</td>
<td>ALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10</td>
<td>50</td>
<td>P= 0.07</td>
</tr>
<tr>
<td>No</td>
<td>3 (30%)</td>
<td>19 (38%)</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>33</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14 (43%)</td>
<td>32 (50%)</td>
<td></td>
</tr>
<tr>
<td>High-risk CR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Induction failure</td>
<td>25 (58%)</td>
<td>67 (59%)</td>
<td>P= 0.8</td>
</tr>
<tr>
<td>- Secondary</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>- t(9;22)</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>- Bilineage leukemia</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>- Complex caryotype</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>- FLT3 ITD</td>
<td>8</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>- WBC&gt; 50000/mmc at diagnosis for ALL</td>
<td>4</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Not applicable
Legends to the Figures

Figure 1

Conditioning regimen and inoculum. Panel A: 25 patients (Sept 2008-Oct 2009): 8 Gy in 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/m²/day) from days –10 to –6; cyclophosphamide (35 mg/kg/day) on days –7 and –6. Panel B: 18 patients (May 2010-Dec 2012): Alemtuzumab lot # 84039D 20 mg/ m², Thymoglobulin 3-7 mg/Kg

Figure 2

Cumulative incidence of post-transplant leukemia relapses in historical controls (panels A and C) and in the present cohort of Treg-Tcon patients (panels B and D). Relapse rates were evaluated separately for transplants from NK alloreactive (solid line) versus non-NK alloreactive (dotted line) donors. In historical AML patients the cumulative incidence of relapse was significantly lower for those who were transplanted from NK alloreactive donors (0.32 versus 0.03; P= 0.03) while no difference was observed in historical ALL patients (0.31 and 0.29 respectively). In Treg-Tcon haplo transplants leukemia relapse was markedly reduced in all patients, independently of NK alloreactivity (panels B and D).

Figure 3

Co-infusion of human conventional T cells (Tcons) and human regulatory T cells (Tregs) exert a GVL effect without GvHD in mice with human leukemia. Panel A: NSG mice were given 3.5 Gy TBI and then infused with 7x10⁶ primary human AML cells. 30 days after leukemia engraftment, mice were treated with 3x10⁶ Tcons and/or 3x10⁶ Tregs. Untreated (○) or Treg-infused (▲) mice died of leukemia; Tcon-treated mice (●) died of GvHD; mice coinfused with 3x10⁶ Tcons and 3x10⁶ Tregs (■) survived without GvHD. Panel B: Human T cells harvested from bone
marrow of mice treated with the Tcon plus Treg combination exerted allogeneic lysis against leukemia (black bar) and PHA blasts (white bar) autologous to leukemia to an effector to target ratio = 5:1 in a Chromium releasing cytotoxicity assay. **Panel C:** Mice were infused with $2 \times 10^6$ Ph+ cell line cells (SUP-B15) and treated with $3 \times 10^6$ Tcons and $3 \times 10^6$ Tregs (■) survived leukemia without GvHD. Untreated mice and those infused with $3 \times 10^6$ Tregs (▲) died of leukemia within 60 days; mice infused with $3 \times 10^6$ Tcons (●) died of GvHD. **Panel D:** Similar outcomes were obtained with Burkitt’s cell line (Namalwa). All experiments were conducted in duplicate in groups of 5 mice.
TBI based conditioning

A
8 Gy TBI
Thiotepa
Fludarabine
Cyclophosphamide

B
Alemtuzumab
or
Thymoglobulin
8 Gy TBI
Thiotepa
Fludarabine

CD34+
10x10^6/Kg

T regs
2x10^6/Kg

Tcons
1x10^6/Kg

No post-transplant immunosuppression

days

Figure 1
Figure 2

(A) T cell depleted haplo

(A) AML

(B) Treg-Tcon haplo

(B) AML

(C) ALL

(D) ALL

Years post transplant

Years post transplant
Figure 3
HLA-haploidentical transplantation with regulatory and conventional T cell adoptive immunotherapy prevents acute leukemia relapse

Massimo F. Martelli, Mauro Di Ianni, Loredana Ruggeri, Franca Falzetti, Alessandra Carotti, Adelmo Terenzi, Antonio Pierini, Maria Speranza Massei, Lucia Amico, Elena Urbani, Beatrice Del Papa, Tiziana Zei, Roberta Iacucci Ostini, Debora Cecchini, Rita Tognellini, Yair Reisner, Franco Aversa, Brunangelo Falini and Andrea Velardi