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Clinical-Grade–Expanded Regulatory T Cells Prevent Graft-versus-Host Disease While Allowing a Powerful T Cell–Dependent Graft-versus-Leukemia Effect in Murine Models

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A B S T R A C T

We developed a good manufacturing practices–compatible expansion protocol to improve number and purity of regulatory T cells (Tregs) available for clinical trials. Six clinical-grade separation procedures were performed, followed by expansion with high-dose interleukin (IL)-2, anti-CD3/anti-CD28 TCR stimulation, and rapamycin for 19 days achieving a median of 8.5-fold (range, 6.25 to 13.7) expansion. FOXP3 expression was stably maintained over the culture period, while the percentage of CD127 was significantly reduced. The in vitro suppression assay showed a strong Mixed Lymphocytes Reaction inhibition. In vitro amplification did not induce any karyotypic modification. To evaluate the graft-versus-host disease (GVHD)/graft-versus-leukemia (GVL) bifunctional axis, expanded Tregs and conventional T cells (Tcons) were tested in NOD/SCID/IL2Rgnull mice injected with primary acute myeloid leukemia (AML) cells, AML cell line, acute lymphoid leukemia Philadelphia cell line, or Burkitt-like lymphoma cell line. All mice that received leukemia cells together with expanded Tregs and Tcons were rescued from leukemia and survived without GVHD, showing that Treg expansion procedure did not compromise GVHD control and the strong Tcon-mediated GVL activity. This report might represent the basis for treating high-risk leukemia and/or relapsed/refractory leukemia patients with high-dose Treg/Tcons.

INTRODUCTION

The pivotal role of $CD4^+/CD25^+/FoxP3^+$ regulatory T cells (Tregs) in immune responses generated interest in their adoptive transfer for clinical application [1,2]. In the setting of high-risk acute leukemia (AL) patients undergoing full haplotype-mismatched transplantation, adoptive immunotherapy with Tregs and conventional T cells (Tcons) in a 2:1 ratio (2 million freshly isolated Tregs followed by 1 million Tcons) prevented acute and chronic graft-versus-host disease (GVHD) without any pharmacological post-transplantation immunosuppression. Additionally, post-transplantation immunological reconstitution improved and a powerful graft-versus-leukemia (GVL) effect was observed [3,4]. Even though naturally occurring Tregs were easily immunoselected from peripheral blood in these studies [5], a major limitation of this approach was their relatively low number, which ex vivo Treg expansion could easily overcome. With more Tregs infused, the Tcon dose could correspondingly increase, in the hope of providing a stronger GVL effect and more rapid immune recovery.

To this aim, starting from immunoselected Tregs, we established good manufacturing practice (GMP)–compatible polyclonal expansion protocols that resulted in a large number of Tregs with a stable and suppressive phenotype and normal karyotype. In a humanized murine model, adoptive immunotherapy with expanded Tregs and Tcons eradicated AL in the absence of GVHD. These results opened the road to clinical trials with ex vivo–expanded Treg/Tcon adoptive

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immunotherapy for patients with high-risk AL and/or relapsed/refractory leukemia who otherwise have very poor prognosis, regardless of form of stem cell transplantation.

MATERIALS AND METHODS

Treg/Tcon Separation

Tregs and Tcons were collected from donors before they underwent granulocyte colony–stimulating factor treatment for $CD34^+$ cell collection. Two total blood volumes from a single leukapheresis procedure were processed with COBE Spectra (Terumo BCT, Lakewood, CO). Tcons were obtained from peripheral blood mononuclear cells and cryopreserved [5]. 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. An aliquot of 4×10^6 immunoselected cells was used for expansion studies.

Treg Expansion

Tregs were plated at 1×10^6/mL in TexMACS GMP Medium (Miltenyi Biotec), supplemented with 5% human AB serum, containing rapamycin 100 nM (MACS GMP Miltenyi Biotec) according to previous reports [6-8].

Cells were activated with antiCD3/CD28 beads (GMP GradeExpAct Treg Kit Miltenyi Biotec) at a bead to cell ratio of 4:1. On day +2 after activation, rapamycin and IL-2 at 1000 IU/mL (MACS GMP Miltenyi Biotec) were added and replenished every 2 days. Beads were removed by magnetic adherence (MACSiMAG Separator Miltenyi Biotec) each 7 days after activation and fresh anti-CD3/CD28beads (1:1 ratio), rapamycin, and IL-2 were added. After 19 days of expansion, the beads were removed and Tregs were analyzed for phenotype and functional analysis. Tregs rested for 1 day in medium containing a low concentration of IL-2 (20 IU/mL) before being intravenously injected into the mice.

Suppression Assay

Treg suppressive capacity was established as follows. Cryopreserved Tcons were thawed and labeled with 2.5 uM carboxyfluorescein diacetate succinimidyl ester (CFSE; Life, Life Technologies, Eugene, OR). Then, 1.5×10^5 Tcons were cultured alone or cocultured with Tregs at ratio 1:1 in 96-well plates and incubated at 37°C for 5 days in presence of phytohemagglutinin (4 μg/mL; Biochrom GmbH, Berlin, Germany). For a CFSE-based measurement of proliferation, the suppressive capacity of in vitro–expanded Tregs toward Tcons in coculture was expressed as the relative inhibition of the percentage of $CFSE^{low}$ cells as follows: 100 × (1-%$CFSE^{low}$Tcons in cocolture/% $CFSE^{low}$ Tcons alone).

Immunophenotyping of Treg and Tcons

Phenotypes were determined using direct immunofluorescence with a panel of monoclonal antibodies directed against the following antigens: CD45, CD3, CD4, CD8, CD14, CD19, CD16, CD56, CD11b, CD127 (Coulter Corporation, Hialeah, FL), CD25 (conjugated to biotin, mouse IgG2b, clone 4E3), FoxP3 (eBioscience, San Diego, CA). Mouse IgG 2-APC (eBioscience) was the control for FoxP3 analysis. Cells were analyzed by Cytomics FC500 Cytometer (Coulter Corporation).

Cytogenetics Analysis

Twenty million expanded Treg cells were cultured for additional 72 hours with phytohemagglutinin-L, 4.8 μg/mL, Biochrom) and IL-2 (1000 IU/mL, Roche, Basel, Switzerland). Karyotype was performed after G-banding with Wright stain in 20 metaphases; 100 unbanded metaphases were evaluated for gaps, breaks, and fragments.

DNA Demethylation Analysis of the Treg-Specific Demethylation Region (TSDR)-FoxP3 Gene Locus

Genomic DNA was isolated from Tregs on day 0 and after 19 days expansion using Maxwell 16 Instrument (Promega Corporation, Madison, WI). Bisulfite treatment of 1 γ genomic DNA was performed using methylation kits (EpiTect Fast DNA Bisulfite Kit Qiagen, Hilden, Germany) according to the manufacturer's instructions. Methylation-specific quantitative PCR was performed using Power SYBR Green PCR reagent (Thermo Scientific, Bartlesville, OK) and primers for methylation-specific and demethylation-specific *FOXP3*, as previously described [9] (*FOXP3*-TSDR demethylation-specific: Forward 5'-TAGGGTAGTTAGTTTTTGGAATGA-3'; Reverse 5'-CCATTAACATCATAACAACCAAA-3'. *FOXP3*-TSDR methylation-specific: Forward 5'-CGATAGGGTAGTTAGTTTTCGGAAC-3'; Reverse 5'-CATTAACGTCATAACGACCGAA-3'). The demethylation rate of *FOXP3*-TSDR was computed using a formula as previously described [10]: $100/(1 + 2^{[CtTG-CtCG]}) \times 100\%$ where Ct TG represents the cycle threshold achieved with TG (demethylated) primers and Ct CG (methylated) primers.

Mouse Models

NOD/SCID/IL2Rgnull (NSG) mice were bred and maintained in the Perugia University animal house. All experiments were performed in accordance with the Nationals Ethics Approval Document of animal experimentation. NSG mice were irradiated with 2.5 Gy and infused with human primary acute myeloid leukemia (AML) cells (10×10^6/mouse) or human AML cell line (3×10^6/mouse), or human Philadelphia chromosome–positive acute lymphoblastic leukemia (ALL), ALL cell line (SUP-B15, 2×10^6/mouse) or human Burkitt-like cell line (Namalwa, 1×10^6/mouse).

Twenty days after primary AML leukemia engraftment, mice were treated with 3×10^6/mouse Tcons and/or 3×10^6/mouse in vitro–expanded Tregs. In mice treated with lymphoblastic or myeloid cell lines, Tregs and Tcons were infused with leukemic cells. Controls were either untreated or infused mice with human Tcons or Tregs (3×10^6/mouse). All experiments were repeated in duplicate and each group comprised 5 mice.

Statistical Analysis

Student's *t* test was used to compare variables and was applied by GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA) and are presented as X-Y scatter or Kaplan-Meier curves. Kaplan-Meier method evaluated murine survival. All *P* values are 2-sided and were considered significant at $P < .05$.

RESULTS

Treg Selection

Six procedures were performed. The initial leukapheresis products contained a median of 11×10^9 (range, 8.5×10^9 to 12.9×10^9) nucleated cells. In the starting fraction, the median number of $CD4^+/CD25^+$ was 858×10^6 (range, 499×10^6 to 1005×10^6), while the median number of $CD4^+/CD25^{high}$ was 72×10^6 (range, 32×10^6 to 1820×10^6). After magnetic cell separation, a median of 277.5×10^6 (range, 240×10^6 to 311×10^6) was recovered in the final cell fraction ($CD4^+/CD25^+$) with a mean purity of 96.37% ± 1.6.; $CD4^+/CD25^+/FoxP3^+$ cells were 98.66% ± .77 while the percentage of $CD4^+/CD25^+/CD127^+$ cells were 26.96% ± 5.5. When $CD4^+/CD25^{high}$ are considered, a median of 129×10^6 (range, 45×10^6 to 151×10^6) was recovered. The final fraction contained no B cells and $CD8^+$ cells; $CD56^+$ cells were 1.38% ± 2.6 and $CD11b^+$ cells were .5% ± .38. Treg suppressive capacity was 61.35% ± 18.4 (ratio 1:1).

Treg Expansion

An aliquot of 4×10^6 immunoselected Tregs were cultured with antiCD3/CD28, IL-2, and rapamycin for 19 days. Tregs were harvested weekly for up to 19 days and the expression of CD4, CD25, FOXP3, CD127, CD3, CD56 and CD19 was evaluated.

After 7 days culture, the number of cells increased to $7.1 \times 10^6 \pm 1.2$; on day +14, the mean number of cells was $19.6 \times 10^6 \pm 6.8$ (day 7 versus day 14: $P = .0016$) while on day +19 the number of cells was $34.16 \times 10^6 \pm 11.4$ (day 14 versus day 19: $P = .0319$; day 7 versus day 19: $P = .0001$).

The expansion achieved after 19 days of culture was a median of 8.5 fold (range, 6.25 to 13.7 fold). Phenotypic analysis showed that no significant differences were detected in the percentage of Tregs expressing CD4, CD25, and FOXP3. At the end of culture period, the percentages of $CD127^+$ and $CD45RA^+$ cells were reduced from 30.79% ± 7.8 versus 3.94% ± 2.3 ($P = .0001$) and 24.5% ± 12.8 versus 3.2% ± 2.8, respectively, while the percentage of $CD45RO^+$ increased to 96.6% ± 3.3 from 83.71% ± 10 (day +19 versus day 0). No $CD56^+$ or $CD20^+$ cells were detected at the end of the expansion procedure, while the percentage of $CD8^+$ cells increased from .0342% ± .030 to 8.5% ± 2.1. The immune suppressive capacity of expanded Treg populations (on day

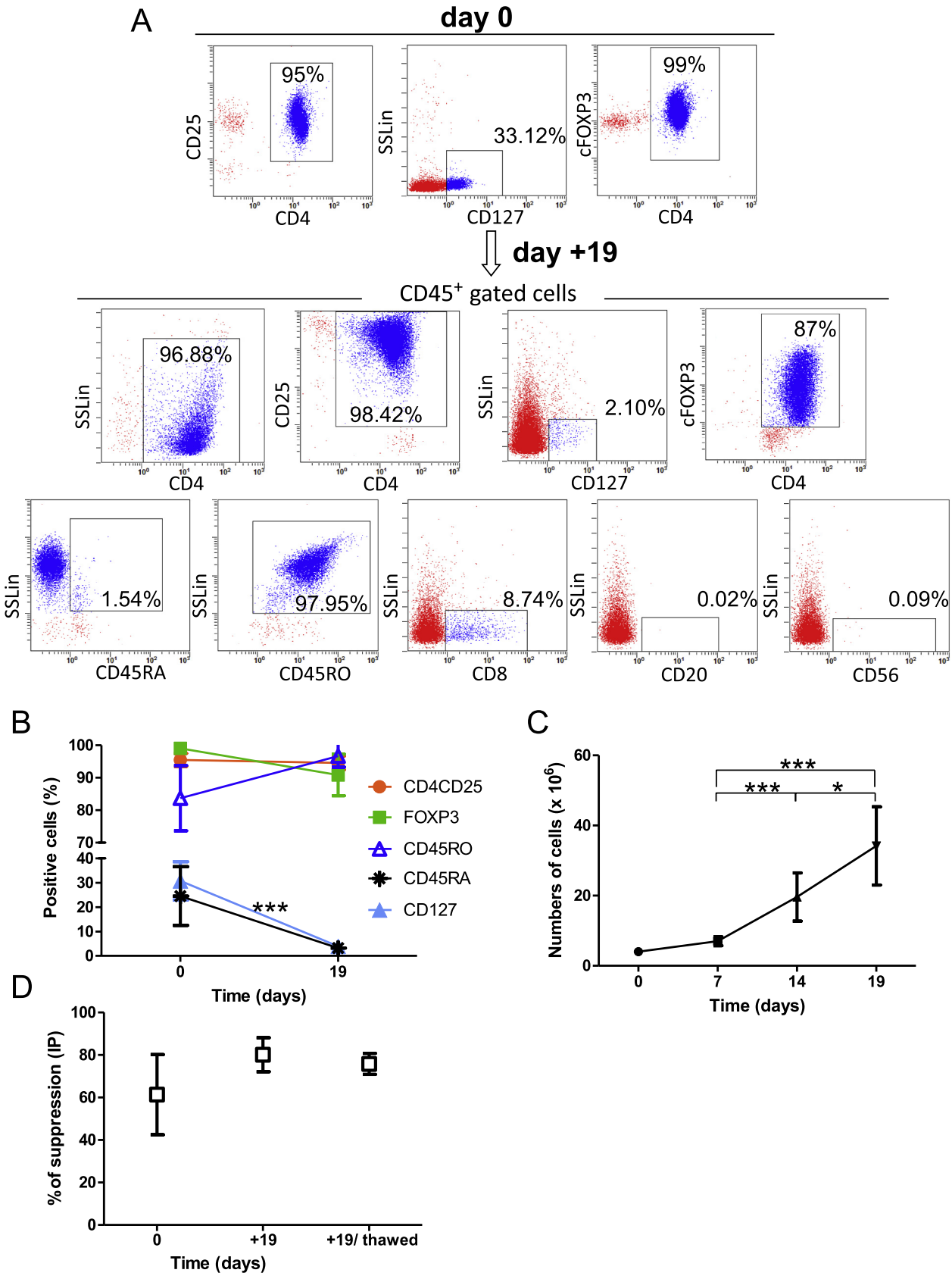

Figure 1. Immunophenotype, expansion, and suppressive activity of the immunoselected Tregs. (A) $CD4^+/CD25^+$, $CD127^+$, and $FoxP3^+$ cells on day 0. On day +19, $CD4^+/CD25^+$, $CD127^+$, $CD45RA^+$, $CD45RO^+$, $FoxP3^+$, $CD56^+$, $CD20^+$ and $CD8^+$ cells are shown (gating on $CD45^+$). Six different experiments were performed. (B) Percentage of expression of common Tregs markers of cells on day 0 and on day +19. Six different experiments were performed. (C) Cell numbers during in vitro expansion. Counts were performed weekly. Six different experiments were performed. $^*P < .05$, $^{***}P < .001$. (D) Percentage of suppression assay (IP). IP represents the percentage of inhibition of Tcon proliferation.

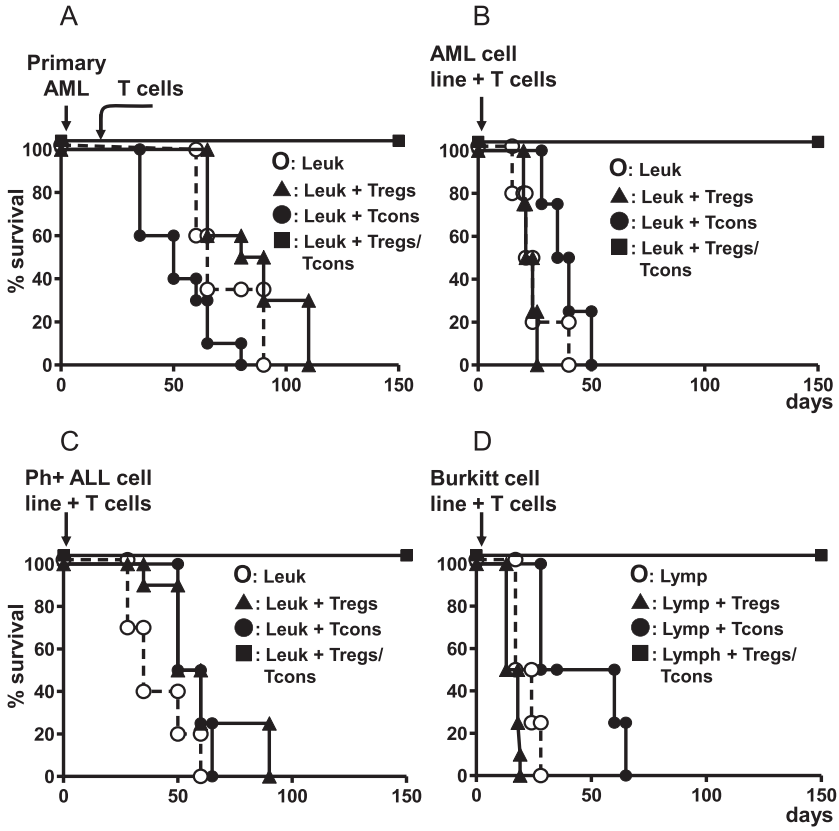

Figure 2. Tcons exert GVL without GVHD when infused with in vitro–expanded Tregs. (A) NSG mice were given 2.5 Gy TBI and then infused with 10×10^6 primary human AML cells. Twenty days after leukemia engraftment, mice were treated with 3×10^6 Tcons and/or 3×10^6 in vitro expanded autologous to T cons Tregs. Untreated (o) or Treg-infused (▲) mice died of leukemia; Tcon-treated mice (●) died of GVHD; mice coinfused with 3×10^6 Tcons and 3×10^6 Tregs (■) survived without GVHD. (B) Mice infused with 3×10^6 KG1 human AML cell line and treated with 3×10^6 Tcons and 3×10^6 Tregs (■) survived leukemia without GVHD. Untreated mice (o) and those infused with 3×10^6 Tregs (▲) died of leukemia within 40 days; mice infused with 3×10^6 Tcons (●) died of GVHD. (C) Mice infused with 2×10^6 Ph + cell line cells (SUP-B15) and left untreated (o) died of leukemia like mice treated with 3×10^6 Tregs only (▲), mice infused with 3×10^6 Tcons (●) cleared leukemia but died of GVHD, mice treated with 3×10^6 Tcons and 3×10^6 Tregs (■) survived leukemia without GVHD. (D) Similar outcomes were obtained with 1×10^6 Burkitt's cell line (Namalwa). Untreated mice (o) and those infused with 3×10^6 Tregs (▲) died of lymphoma within 25 days; mice infused with 3×10^6 Tcons died of GVHD without lymphoma (●). Mice treated with 3×10^6 Tcons and 3×10^6 Tregs (■) survived without evidence of lymphoma. All experiments were conducted in duplicate in groups of 5 mice.

+19) was tested in an inhibition assay showing the mean inhibition percentage of Tcons was 89.25 ± 2.8 (Tregs:Tcons, 1:1). The suppressive ability of expanded Tregs was re-evaluated at the day +20 before the Tregs were intravenously injected into the mice and showed that Treg suppressive functions was maintained. Moreover, phenotypic analysis and the immune suppressive capacity of expanded Treg populations were also analyzed after freezing and thawing, showing the maintenance of FoxP3 expression (92.95 ± 11.59%) and of the suppressive capacity (Figure 1D).

Cytogenetic Analysis

The karyotype was 46,XY in all cells. No gaps, breaks, or fragments were found.

DNA Demethylation Analysis of the TSDR-FoxP3 Gene Locus

We analyzed the hypomethylation of the TSDR of the FoxP3 gene as hallmark of Treg. Analysis of 3 independent donors revealed that TSDR demethylation was maintained after the expansion period compared to day 0 (44.16 ± 24% versus 46.6 ± 9.4%).

Mouse Models

We infused 2.5 Gy–irradiated NSG mice with 10 millions of primary AML cells (Figure 2A), 3×10^6 AML cell line (Figure 2B), 2×10^6 ALL Philadelphia cell line (Figure 2C), or 1×10^6 Burkitt-like lymphoma cell line (Figure 2D). All mice that received primary human AML cells or myeloid or lymphoblastic leukemias and expanded Tregs with Tcons were rescued from leukemia and survived without GVHD. All untreated or Treg-infused mice died of leukemia within 30 to 110 days, depending to the type of leukemia or lymphoma. Those that received leukemia cells plus Tcons died of severe GVHD within 70 days ($P < .001$) (Figure 2).

DISCUSSION

Using the same approach as Romano et al. and Scotta et al. [6-8] the present study showed that anti-CD3/CD28 coated beads, a high dose of IL-2, and rapamycin induced 8.5-fold polyclonal expansion of Tregs, reaching its maximum after 19 days culture. No significant differences were detected in CD4, CD25, and FOXP3 expression, showing this expansion technique preserved the Treg phenotype [6-8].

During expansion, contaminating CD127 cells, which have the potential to trigger GVHD, were significantly reduced

compared with freshly isolated Tregs from leukapheresis products, which contained CD127-contaminating cells ranging from .18% to 32.6% in number, as they varied with the donor [5]. In our view, having achieved this grade of purity with ex vivo Treg expansion, it is reasonable to expect the risk of GVHD will be eliminated.

Large-scale translation of our 8.5-fold median amplification would permit infusion of up to 20×10^6/kg body weight (bw) Tregs, in combination with up to 10×10^6/kg bw Tcons. The 2:1 Treg:Tcon ratio was shown to be safe in terms of GVHD prevention in our mouse models and clinical trials [3,4].

Functional studies showed that expanded Tregs had a strong in vitro suppressive capacity while, as expected, in vivo experiments with expanded Tregs eliminated the risk of GVHD in xeno-transplantation models using NSG mice. As Tregs exert their main suppressive activity in the early phase after transplantation [11], expanded Tregs are functional even after 19 days of in vitro expansion.

We also showed that human primary myeloid leukemia and lymphoblastic leukemia cell lines were eradicated. Since similar results were achieved with freshly isolated Tregs, ex vivo expansion did not compromise the strong Tcon-mediated GVL activity in the absence of GVHD.

Ex vivo–expanded Tregs have been used in hematopoietic stem cell transplantation in 2 relevant clinical settings. In matched hematopoietic stem cell transplantation, Theil et al. [12], used a similar methodology as ours (immune-magnetic Treg isolation and polyclonal expansion in presence of CD3/CD28 beads, IL-2, and rapamycin) to treat 5 patients with refractory chronic GVHD. They received up to 4.72×10^6/kg Tregs in combination with low-dose IL-2. The benefit was unclear as some patients improved but others did not, but the study illustrated the opportunities and risks of adoptive Treg treatment.

In cord blood transplantation, ex vivo Treg expansion relied on isolating Tregs from a third umbilical cord blood unit that was 4/6 to 6/6 HLA matched to the patient [13,14]. Tregs were expanded in cultures stimulated with K562 cells that had been modified to express the high-affinity Fc receptor (CD64) and CD86, the natural ligand of CD28 (KT64/86). In our opinion, the present methodology, as also used by Thiel, is easy to perform and appears to be more widely applicable in most transplantation centers than the technique that Brunstein et al. have already used. In their report, up to 100×10^6/kg bw were infused in 11 patients with AL, in whom the incidence of GVHD was significantly lower than in controls (9% versus 45%). However, these patients also received pharmacological immune suppression with sirolimus and mycophenolate mofetil.

On the basis of the above clinical results, it is still not clear whether infusing more Tregs will translate into better clinical outcomes than adoptive immunotherapy with freshly isolated Tregs [3,4]. Consequently, we are planning to compare the use of expanded Tregs versus freshly isolated Tregs in a prospective randomized trial of haploidentical transplantation.

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