Role of donor regulatory T-cell adoptive immunotherapy in B-cell immunity after hematopoietic cell transplantation

CD4$^+$/FOXP3$^+$ regulatory T cells (Treg) play a critical role in self-tolerance by suppressing the function of other immune cells and preventing excessive immune responses.[1]

Mouse models and clinical studies in allogeneic hematopoietic cell transplantation (HCT) show that donor Treg control graft-*versus*-host disease (GvHD).[2-5] In a recent series of patients with acute leukemia undergoing T-cell-depleted haploidentical HCT (haplo-HCT), infusion of donor Treg allowed for the subsequent safe infusion of donor conventional T cells (Tcon) and resulted in high engraftment rates, a low incidence of chronic GvHD (2%) and relapse (4%), and a 70% chronic GvHD/relapse-free survival.[5] Co-infusion of donor Treg and Tcon in the absence of pharmacological immunosuppression allowed fast post-transplant immune reconstitution that resulted in a low incidence of life-threatening infections.[5]

Mouse Treg also shield hematopoietic stem cells (HSC) from self- or allo-reactive T cells in the bone marrow (BM) and facilitate donor HSC engraftment.[6,7] Moreover, mouse BM Treg trigger interleukin-7 production by ICAM1$^+$ perivascular stromal cells and thereby promote HSC differentiation towards B-cell lymphopoiesis.[7]

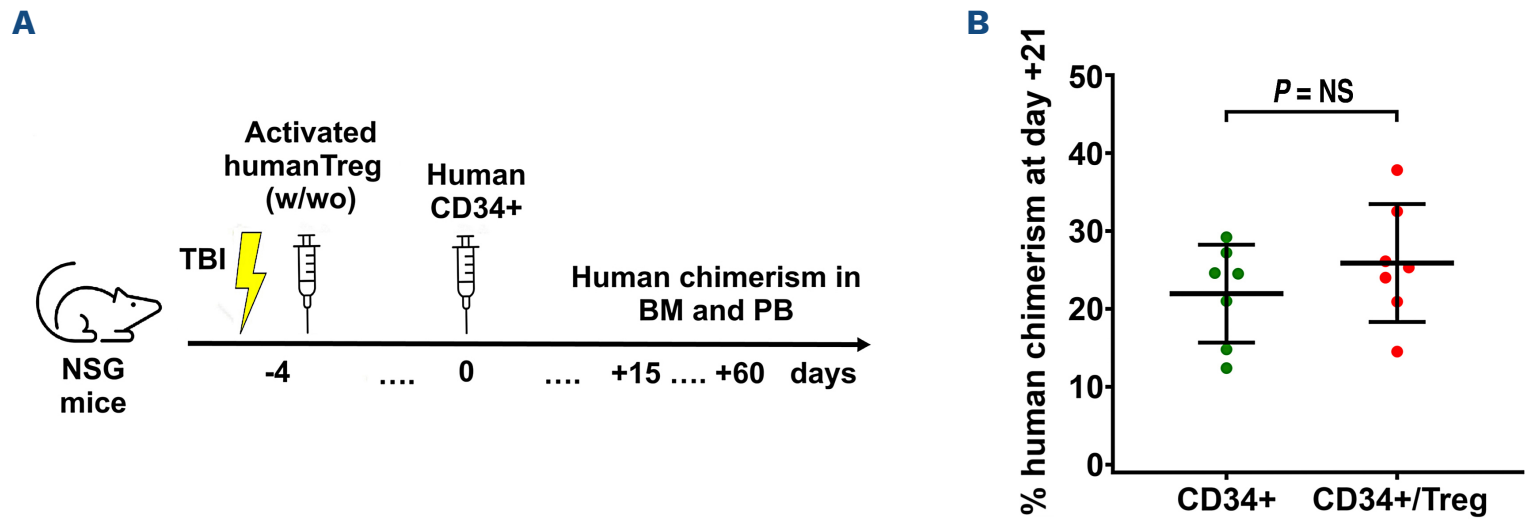
In the present study, we show that also human Treg promote donor HSC engraftment, lymphopoiesis, and B-cell reconstitution in preclinical xenotransplantation models and in patients undergoing haplo-HCT with Treg/Tcon adoptive immunotherapy (Treg/Tcon haplo-HCT).

We first investigated the impact of human Treg on early engraftment of HLA-matched CD34$^+$ cells and stem cell-derived immune reconstitution in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Charles River, Wilmington, MA, USA). NSG mice were bred at the animal facility of the University of Perugia. Experiments were approved by the Italian competent authority, the Ministry of Health. Mice were infused with as few as 1x10^6 human CD34$^+$ cells with or without activated Treg from the same healthy donor (Figure 1A). Donor CD4$^+$CD25$^+$ Treg were selected by immunomagnetic depletion of CD8$^+$/CD19$^+$ cells and subsequent positive selection of CD25$^+$ cells (CliniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). On average, the final Treg product was composed of 71%±8.5% CD4$^+$CD25$^+$CD127$^-$FOXP3$^+$ cells without contaminant B cells. To obtain donor Tcon, CD3$^+$ T cells were separated from the leukapheresis product by Ficoll gradient. After administration of granulocyte colony-stimulating factor, donor CD34$^+$ cells were collected from two or three leukaphereses and positively immunoselected (CliniMACS).[5] Treg were activated with anti-CD3/CD28 Dynabeads at a 1:1 ratio (Thermo Fisher Scientific, Waltham, MA, USA) and 50 IU/mL of recombinant human interleukin-2 (Miltenyi Biotec) for 4 days, to prolong their persistence *in vivo* after infusion. NSG mice were sublethally irradiated (2 Gy) and injected intravenously with 1x10^6 CD34$^+$ cells at day 0, with or without 0.5-1x10^6 activated Treg from the same donor at day -2.

Human chimerism in BM tended to be higher when CD34$^+$ cells were co-infused with Treg (Figure 1B). In the presence of Treg, CD34$^+$ cells preferentially accumulated in the epiphysis where engraftment occurs,[8] as shown by immunohistochemical analyses (Figure 1C, D, *Online Supplementary Figure S1*). Accordingly, the number of human CD34$^+$ cells in the BM of mice infused with Treg was lower, suggesting that CD34$^+$ cells underwent differentiation (Figure 1E). Indeed, Treg promoted human HSC-derived early immune reconstitution in peripheral blood (PB) (which was maintained for as long as it was monitored, i.e., 2 months) (Figure 1F). Immune reconstitution in mice co-infused with Treg consisted initially of myeloid cells and later of lymphoid cells (Figure 1G). Conversely, immune reconstitution was not detectable in mice infused only with CD34$^+$ (<1% of human chimerism in PB) (Figure 1F). Therefore, in the above model human Treg facilitated early HSC-derived immune reconstitution.

To assess whether Treg infusion could also favor peripheral donor B-cell reconstitution, we used an additional model that includes infusion of human PB mononuclear cells as a source of Tcon and B cells (about 5x10^5) (as performed in our clinical Treg/Tcon-based haplo-HCT). Sublethally irradiated NSG mice were infused with 3x10^6 PB mononuclear cells at day 0 with or without 3x10^6 Treg from the same donors at day -2 (Figure 2A). In such a model of GvHD, not only did Treg protect mice from GvHD lethality induced by Tcon[4] (*data not shown*), but their infusion was also associated with an increase in the number of human B cells in BM and spleen. This effect could be observed as early as 7 days after infusion and was more pronounced in the spleen (Figure 2B). This experiment demonstrated that donor Treg infusion allows for an early expansion of co-infused donor peripheral B cells.

We therefore investigated B-cell reconstitution in 44 consecutive patients (median age 54 years) who underwent Treg/Tcon haplo-HCT between October 2016 and July 2019 (data retrieved from the Umbria Region Institutional Review Board-approved clinical trial with identification code 02/14, public registry #2384/14, *clinicaltrials.gov #NCT03977103*)



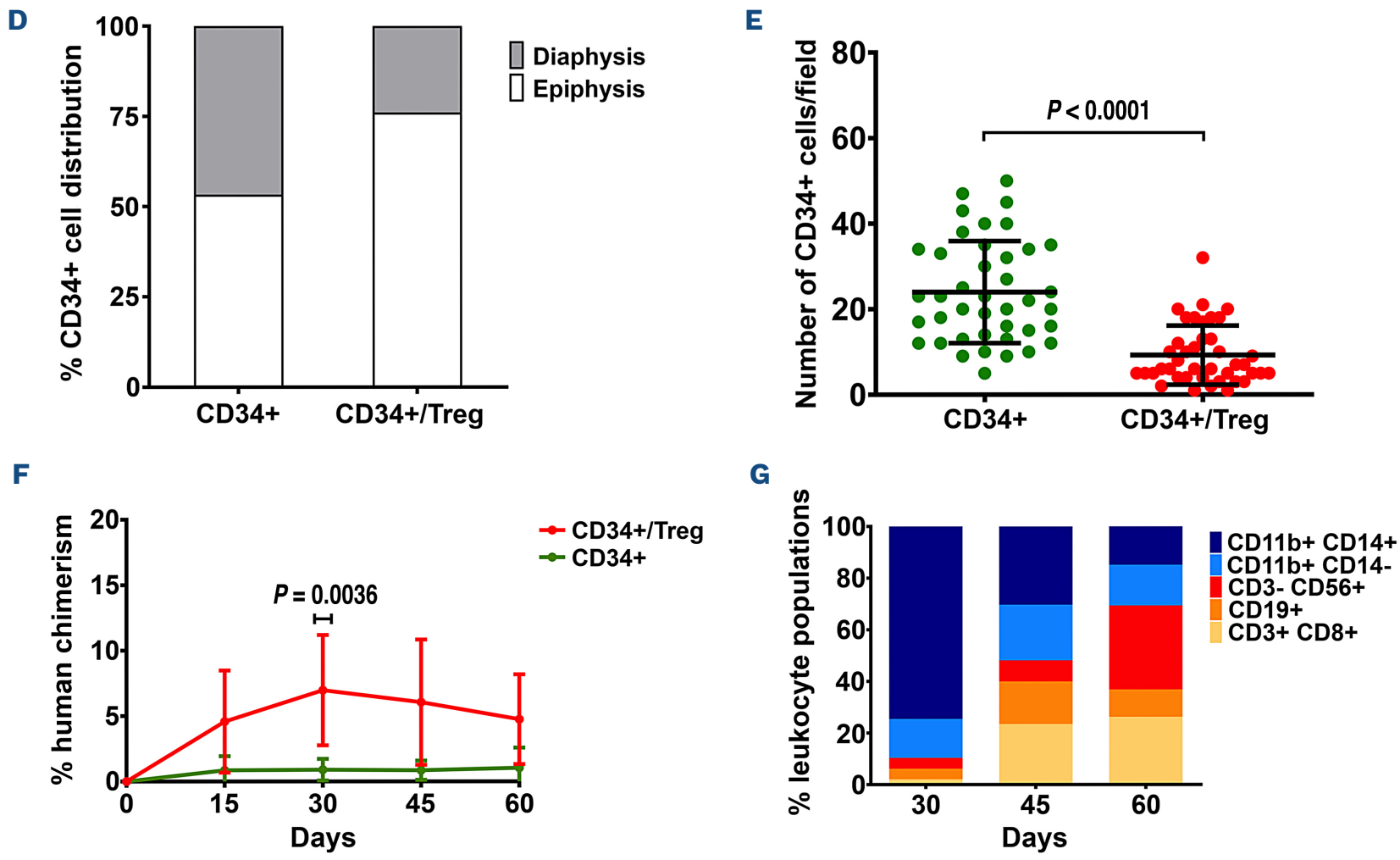


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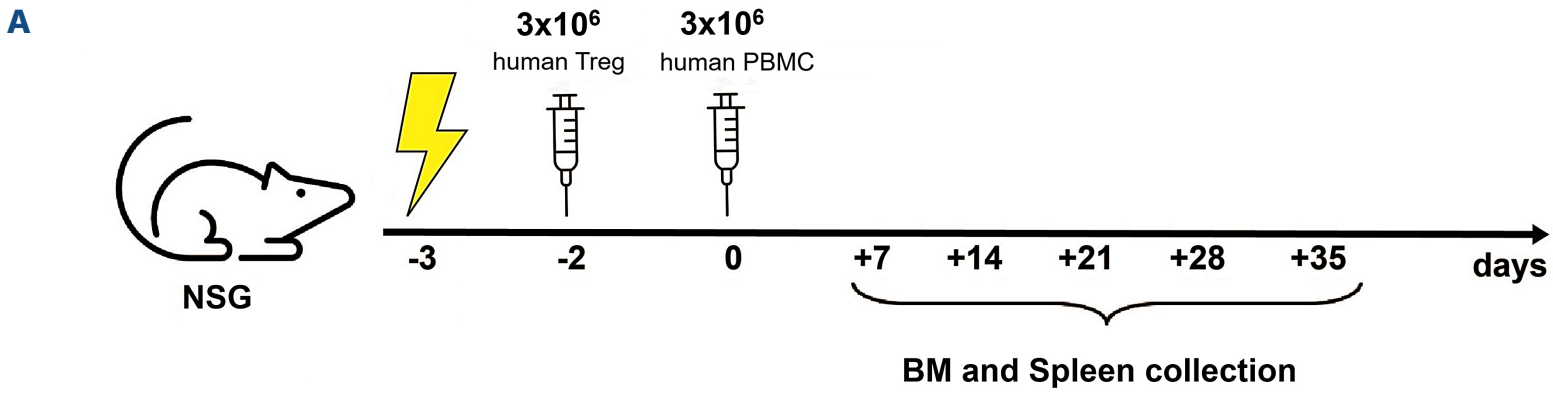
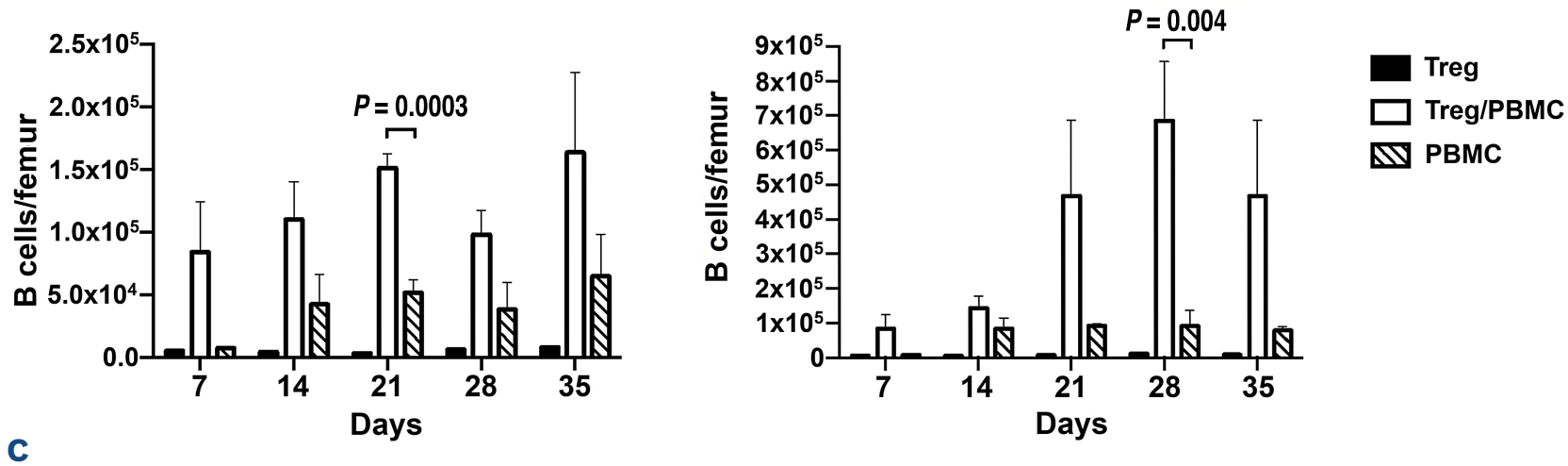
Figure 1. Human T-regulatory cells facilitate engraftment of HLA-matched CD34⁺ cells in a xenogeneic mouse model. (A) Schematic representation of the xenogeneic mouse model used to assess the impact of human T-regulatory cells (Treg) on early engraftment of HLA-matched CD34⁺ cells from the same healthy donor. (B) Human chimerism in the bone marrow 21 days after the infusion of CD34⁺ cells with (red symbols) or without (green symbols) Treg, as evaluated by flow cytometry with FACSCanto and FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA). Data are expressed as mean percentage of human CD45⁺ cells/total CD45⁺ cells ± standard deviation (SD) of seven mice per group. Human CD3⁺CD4⁺ T cells including infused FOXP3⁺ Treg were gated out. *P*=0.18 in a paired *t* test. (C) Representative immunohistochemical staining of diaphyses and epiphyses of femurs harvested 45 days after the infusion of CD34⁺ cells with (right) or without (left) Treg. Human hematopoietic progenitor cells show blue cytoplasmic staining (anti-human antibody CD34 class II, blue arrows) and human Treg show brown nuclear staining (anti-human FOXP3 antibody, brown arrows). Magnification X400. Representative 40X magnification fields are shown in *Online Supplementary Figure S1*. (D) Distribution of CD34⁺ cells in diaphysis *versus* epiphysis of femurs harvested 45 days after the infusion of CD34⁺ cells with or without Treg, as evaluated by immunohistochemistry. Data are expressed as mean percentage of human CD34⁺ cells ± SD of two mice per group. (E) Number of human CD34⁺ cells in femurs harvested 45 days after the infusion of CD34⁺ cells with (red symbols) or without (green symbols) Treg, as evaluated by immunohistochemistry. Data are expressed as mean number per 10X field ± SD of two mice per group. *P*<0.0001 in a paired *t* test. (F) Human chimerism in peripheral blood (PB) of mice infused with CD34⁺ cells with (red symbols and line) or without Treg (green symbols and line), as evaluated by flow cytometry at the indicated time points after the infusion of CD34⁺ cells. Data are expressed as mean percentage of human CD45⁺ cells/total CD45⁺ cells ± SD of seven mice per group. *P*=0.0036 in a multiple *t* test with the Sidak-Bonferroni method at the indicated time point. Human CD3⁺CD4⁺ T cells including infused FOXP3⁺ Treg were gated out. (G) Normalized percentage of human leukocyte populations in PB of mice infused with CD34⁺ cells and Treg, as evaluated by flow cytometry at the indicated time points after the infusion of CD34⁺ cells. The following human leukocyte populations were analyzed in PB: CD11b⁺CD14⁺ monocytes, CD11b⁺CD14⁻ neutrophils, CD3⁻CD56⁺ NK cells, CD19⁺ B cells, CD3⁺CD8⁺ T cells. Data are expressed as mean percentage of leukocyte populations of seven mice per group. Human CD3⁺CD4⁺ T cells including infused FOXP3⁺ Treg were gated out. Graphical and statistical analyses were performed with GraphPad Prism software (Dotmatics, Boston, MA, USA). TBI: total body irradiation; w/wo: with/without; BM: bone marrow; NS: not statistically significant.

(*Online Supplementary Table S1*). Forty patients received myeloablative conditioning with radiotherapy and chemotherapy. Irradiation consisted of total body irradiation (13.5 Gy) for patients up to age 50 years and total marrow/lymphoid irradiation (marrow 13.5 Gy, lymph node 11.5 Gy) for patients aged 51 to 65 years. Chemotherapy consisted of thiotepa (5 mg/kg for identical or 7.5 mg/kg, total dose), fludarabine (150 mg/m^2, total dose), and cyclophosphamide (20 mg/kg for identical or 30 mg/kg, total dose). Four patients received myeloablative conditioning with chemotherapy only (thiotepa, fludarabine and treosulfan) because ineligible for radiotherapy. Patients received an infusion of 2×10^6/kg donor Treg on day –4 followed by 1×10^6/kg Tcon on day –1. Around 10×10^6/kg positively purified CD34⁺ hematopoietic progenitor cells were infused on day 0. No pharmacological GvHD prophylaxis was administered after transplantation. The patients' demographics and outcomes are reported in *Online Supplementary Table S1*.

BM and PB samples were collected and analyzed to quantify B-cell subsets. We analyzed the absolute number of CD34⁺CD38⁺CD10⁺ common lymphoid progenitors, CD10⁺CD19⁻ pre-pro-B cells, CD10⁺CD19⁺ pre-B and pro-B cells, CD38⁺CD19⁺CD5⁻IgM⁺ immature B cells, CD38⁺CD19⁺CD5⁺IgM⁺ transitional B cells, CD19⁺CD20⁺ mature B cells and CD138⁺CD19⁺CD56⁻ plasma cells. We found that, early after transplant, the different subsets of B-cell precursors in the BM of patients were more represented with respect to those of healthy donors, except for transitional B cells and plasma cells (Figure 2C). B-cell counts were >100/mm^3 in PB between 3 and 4 months after transplantation (Figure 3A). Total IgM also reached normal levels 3 months after transplantation (96±67 mg/dL), and IgM production tended to be faster than in seven patients who contemporarily underwent T-cell-depleted haplo-HCT with no T-cell add-backs (Figure 3B).[9] There was no difference in B-cell reconstitution according to the conditioning regimen used (*data not shown*), confirming previous findings.[5] We also assessed pathogen-specific B-cell responses, by evaluating cytomegalovirus (CMV)-specific IgM production (note that these patients were investigated before the introduction of CMV prophylaxis with letermovir). Production of CMV-specific IgM (at a median of 99 days) was detected in seven of 16 evaluable patients who experienced CMV reactivation after Treg/Tcon haplo-HCT, while it was undetectable in the seven patients who experienced CMV reactivation after T-cell-depleted haplo-HCT (1/7 patients died of CMV disease). Notably, patients who produced CMV-specific IgM had a lower incidence of a second CMV reactivation (Figure 3C). No patient died of CMV disease after Treg/Tcon haplo-HCT.

B-cell response to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection or vaccination was evaluated in a series of 29 patients (median age 55 years) who underwent Treg/Tcon HCT, 16 from a haploidentical and 13 from an HLA-identical donor, between January 2019 and February 2022 (*Online Supplementary Table S2*). Twenty-six patients (90%) produced anti-SARS-CoV-2-specific IgG after vaccination at a median of 10.5 months after transplant (range, 5-26 months; 26/29 patients), or after infection (3/29 patients). Only one of the 16 patients who were transplanted from a haploidentical donor did not produce anti-SARS-CoV-2-specific IgG. These results show that Treg/Tcon adoptive immunotherapy was associated with fast reconstitution of functional B cells after





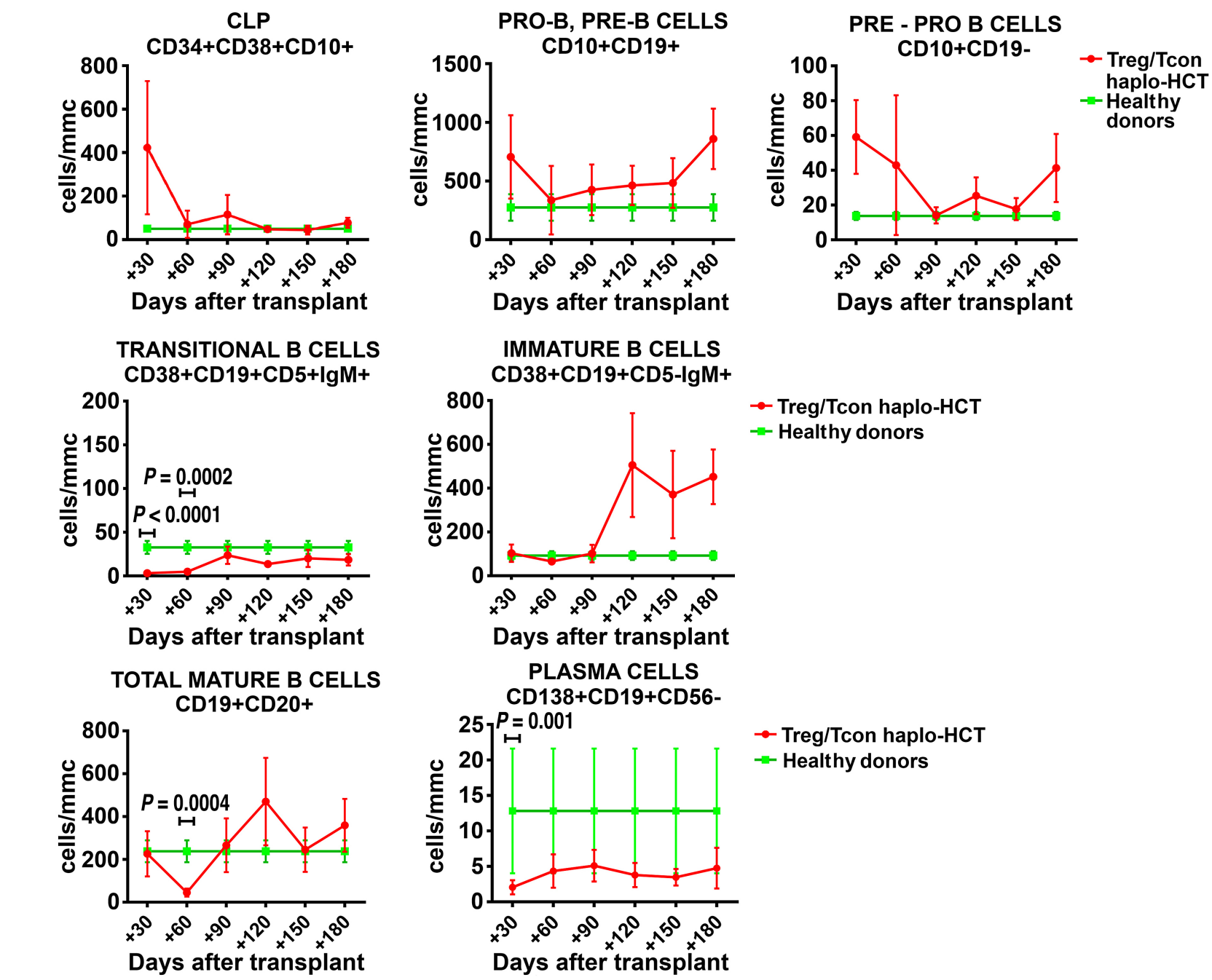


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Figure 2. B-cell reconstitution in lymphoid tissues after haploidentical hematopoietic cell transplantation with T-regulatory/T-conventional-cell immunotherapy. (A) Schematic representation of the xenogeneic mouse model used to assess the role of T regulatory cells (Treg) in the expansion of co-infused mature B cells. In the experimental arm, sublethally irradiated (2 Gy) NSG mice were infused with $3x10^6$ human peripheral blood mononuclear cells (PBMC) as a source of conventional T cells (Tcon) (about 60%) and B cells (about 15-20%) at day 0 with $3x10^6$ human $CD4^+CD25^+$ Treg from the same donors at day -2. NSG mice which received only Treg or only PBMC served as control groups. (B) Absolute number of human B cells harvested from a femur or spleen of mice infused with Treg or Tcon, or co-infused with Treg and Tcon, as evaluated by flow cytometry at the indicated time points after the infusion of Tcon. Data are expressed as mean ± standard deviation of three mice per group. P<0.005 in a multiple *t* test with the Sidak-Bonferroni method at the indicated time points. (C) Absolute number of common lymphoid progenitors and of the indicated B-cell precursors and populations in the bone marrow of 35 Treg/Tcon haploidentical cell transplantation patients after transplant (red symbols and line) and of five healthy transplant donors (green line), as evaluated by flow cytometry at the indicated time points after transplant. *P*<0.005 in a multiple *t* test with the Sidak-Bonferroni method at the indicated time points. BM: bone marrow; CLP: common lymphoid progenitors; haplo-HCT: haploidentical hematopoietic cell transplant.

haplo-HCT.

Previous studies showed that mouse Treg support an immunological niche for HSC in the BM, by shielding HSC from self- or allo-reactive T cells and by promoting engraftment. The present data in immunodeficient mice show that human Treg are also capable of promoting early engraftment and differentiation of $CD34^+$ cells. Indeed, Treg adoptive transfer boosted $CD34^+$ cell-derived early immune reconstitution with evidence of expansion of human lymphocytes in PB of the mice 2 months after $CD34^+$ cell infusion (Figure 1F, G). As Treg were obtained from the $CD34^+$ cell donor, these effects were independent of a Treg-mediated

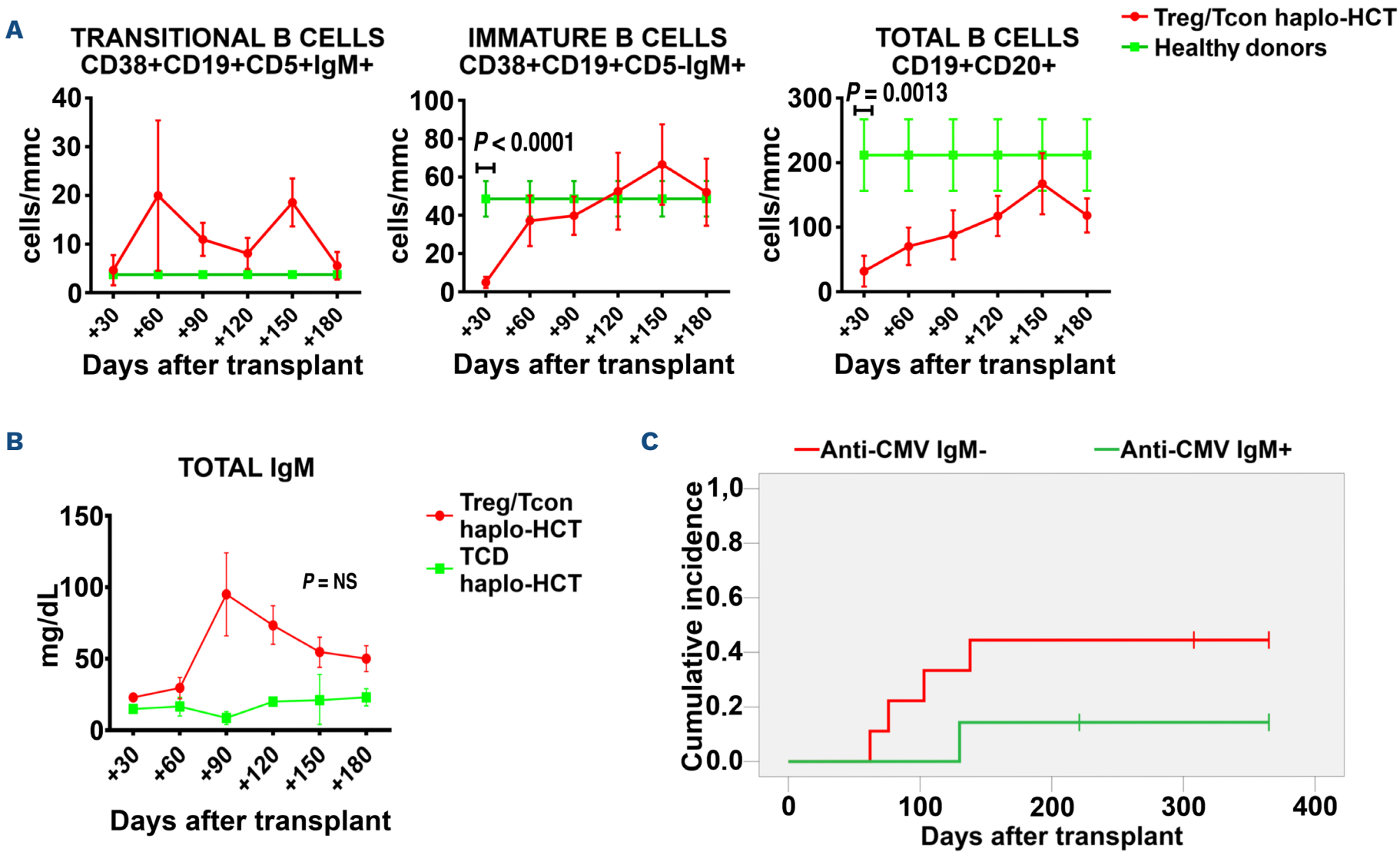

Figure 3. Peripheral B-cell reconstitution and immunity after haploidentical hematopoietic cell transplantation with T-regulatory/T-conventional-cell immunotherapy. (A) Absolute numbers of transitional B cells, immature B cells, total mature B cells in peripheral blood of 39 patients who underwent T regulatory (Treg)/conventional T (Tcon) cells haploidentical hematopoietic cell transplantation (haplo-HCT) patients, as evaluated by flow cytometry at the indicated time points after transplant. *P*<0.005 in a multiple *t* test with the Sidak-Bonferroni method at the indicated time points. (B) Concentration of total IgM measured in the serum of 39 Treg/Tcon haplo-HCT patients (red symbols and line) and seven contemporary T-cell-depleted haplo-HCT patients (green symbols and line) at the indicated time points after transplant. (C) Cumulative incidence of a second cytomegalovirus (CMV) reactivation in Treg/Tcon haplo-HCT patients according to positivity (7 patients, green line) or negativity (9 patients, red line) of anti-CMV specific-IgM in serum after the first post-transplant CMV reactivation. CMV reactivation was defined as the presence of >1,000 copies of CMV DNA in peripheral blood. The cumulative incidence graph was produced with IBM SPSS Statistic software (IBM Armonk, NY, USA). NS: not statistically significant; TCD: T-cell-depleted.

immune suppressive allogeneic reaction, nor were they linked to any T-cell-mediated attack against infused CD34+ cells (as mice were immunodeficient and no human Tcon add-backs were infused). Therefore, such findings suggest that Treg support BM hematopoiesis independently of their immune-suppressive function.

Interestingly, Treg adoptive transfer accelerated reconstitution of B-cell precursors and mature B cells, in line with previous observations in mice that showed BM Treg promote differentiation of HSC towards B-cell lymphopoiesis. Early detection of BM B cells in patients who received transplant with Treg/Tcon adoptive immunotherapy in the absence of post-transplant immunosuppression also suggests that such an approach favors stem cell-derived immune rebuilding.

Furthermore, Treg infusion facilitated mature B-cell peripheral expansion in xenogeneic mouse models (in the absence of human HSC, as shown in Figure 2A, B). Taken together, such findings might explain the early BM and PB functional B-cell reconstitution that was observed in patients.

We show that Treg-mediated acceleration in B-cell reconstitution exerts clinically relevant protective effects in haplo-HSC transplanted patients, as indicated by the early production of CMV-specific IgM, by the protection against repeated CMV reactivations and CMV mortality and by the 90% seroconversion rate after SARS-CoV-2 vaccination. It is of note that Dhakal and colleagues reported a seroconversion rate of 69% at a median of 26 months after transplantation in a cohort of 71 allogeneic HCT recipients.[10] Moreover, a pooled analysis of studies on the effectiveness of SARS-CoV-2 vaccination after allogeneic HCT reported seroconversion rates ranging from 28% to 96%.[11] Furthermore, B-cell reconstitution in this cohort of patients appeared to be faster than in studies of haplo-HCT with high-dose post-transplant cyclophosphamide,[12-15] and was comparable with B-cell reconstitution reported after matched-HCT with post-transplant cyclophosphamide.[16] Taken together these findings suggest that donor B-cell reconstitution plays a relevant role in protecting patients from post-transplant infections, especially the ones that could benefit from early vaccination.

While further studies are required to elucidate the underlying mechanisms, at least two factors can contribute to boost B-cell immunity after transplant: namely the role of donor Treg which promote immune reconstitution and induce expansion of mature B cells infused with the graft, and the absence of pharmacological immune suppression to prevent GvHD. Finally, the present results support the adoption of early vaccination programs after Treg/Tcon haplo-HCT.

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Disclosures

No conflicts of interest to disclose.

Contributions

SP performed experiments, analyzed experimental data, and collected and analyzed clinical data. RL performed experiments and analyzed data. VV collected clinical data and performed experiments. EH performed experiments. TZ and RIO isolated hematopoietic stem cells and lymphocytes from transplant donors. BB and AT performed immunohistochemistry studies. RS, FZ and ST provided clinical care. MPM, AC, AV and LR provided clinical care, supervised the study and reviewed the manuscript. AM performed experiments, analyzed data, designed the study and wrote the manuscript. AP designed the study and wrote the manuscript.

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Data-sharing statement

Additional reagents, data and anonymized clinical data that support the findings of this study will be made available upon reasonable request to the corresponding author by non-profit organizations for 1 year after the publication of the manuscript.

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