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

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Author contributions: S.P. performed experiments, analysed experimental data, and collected and analysed clinical data; R.L. performed experiments and analysed data; V.V. collected clinical data and performed experiments; E.H. performed experiments; T.Z. and R.I.O performed isolation of hematopoietic stem cells and lymphocytes from transplant donors; B.B and A.T. performed immunohistochemistry; R.S., F.Z. and S.T. carried out clinical care; M.P.M., A.C., A.V. and L.R. carried out clinical care, supervised the study and reviewed the manuscript; A.M. performed experiments, analysed data, design the study and wrote the manuscript; A.P. design the study and wrote the manuscript.

CD4+/FOXP3+ regulatory T-cells (Tregs) 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 Tregs control graft-versus-host disease (GvHD) (2-5). In a recent series of acute leukemia patients undergoing T-cell-depleted haploidentical HCT (haplo-HCT), infusion of donor Tregs allowed for the subsequent safe infusion of donor conventional T cells (Tcons) and resulted in high engraftment rates, low incidence of chronic GvHD (2%) and relapse (4%), and a 70% chronic GvHD/relapse-free survival (5). Co-infusion of donor Tregs and Tcons in the absence of pharmacologic immunosuppression allowed fast post-transplant immune reconstitution that resulted in low incidence of life-threatening infections (5).

Mouse Tregs also shield HSCs from self- or allo-reactive T cells in the bone marrow (BM) and facilitate donor HSCs engraftment (6,7). Moreover, mouse BM Tregs trigger IL-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 Tregs 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 Tregs on early engraftment of HLA-matched CD34+ cells and stem cell-derived immune reconstitution in NSG mice. NSG mice were bred at the animal facility of University of Perugia. Experiments were approved by the Italian competent authority Ministero della Salute. Mice were infused with as few as $1x10^6$ human CD34+ cells with or without activated Tregs from the same healthy donor (Figure 1A). Donor CD4+CD25+ Tregs 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 Tcons, CD3+ T cells were separated from the leukapheresis product by Ficoll gradient. After G-CSF administration, donor CD34+ cells were collected from 2 to 3 leukaphereses and positively immunoselected (CliniMACS) (5). Tregs were activated with anti-CD3/CD28 Dynabeads at a 1:1 ratio (Thermo Fisher Scientific, Waltham, MA, USA) and 50 UI/ml of recombinant human IL-2 (Miltenyi Biotec) for 4 days, to prolong their persistence in vivo after infusion. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Charles River, Wilmington, MA, USA) were sublethally irradiated (2 Gy) and injected intravenously with $1x10^6$ CD34+ cells at day 0, with or without $0.5\text{-}1x10^6$ activated Tregs from the same donor at day -2.

Human chimerism in BM tended to be higher when CD34+ cells were co-infused with Tregs (Figure 1B). In the presence of Tregs, CD34+ cells preferentially accumulated in the epiphysis where engraftment occurs (8), as shown by immunohistochemical analyses (Figure 1C-D, Supplementary Figure 1). Accordingly, the number of human CD34+ cells in the BM of mice infused with Tregs was lower, suggesting CD34+ cells underwent differentiation (Figure 1E). Indeed, Tregs promoted human HSC-derived early immune reconstitution in PB, (which was maintained for as long as it was monitored, i.e., 2 months) (Figure 1F). Immune reconstitution in mice co-infused with Tregs was initially composed 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 Tregs facilitated early HSC-derived immune reconstitution.

To assess whether Tregs infusion could also favour peripheral donor B cell reconstitution, we used an additional model that includes infusion of human peripheral blood mononuclear cells (PBMCs) as a source of Tcons and B cells (about $5x10^5$) (as performed in our clinical Treg/Tcon-based haplo-HCT). Sublethally irradiated NSG mice were infused with $3x10^6$ PBMCs at day 0 with or without $3x10^6$ Tregs from the same donors at day -2 (Figure 2A). In such a model of GvHD, not only Tregs did protect mice from GvHD lethality induced by Tcons (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). Such experiment demonstrates that donor Tregs 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 from October 2016 to 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., Supplementary table 1). 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/m2, 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 Tregs on day −4 followed by 1×10^6/kg Tcons 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 post-transplantation. Patient demographics and outcomes are reported in supplementary table 1.

BM and PB samples were collected and analysed to quantify B cells subsets. We analysed 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 respect those of healthy donors, except for transitional B cells and plasma cells (Figure 2C). B cell counts were >100/mmc in PB between 3 and 4 months after transplant (Figure 3A). Total IgM also reached normal levels 3 months after transplant (96±67 mg/dL), and IgM production tended to be faster compared with that of 7 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 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 7/16 evaluable patients who experienced CMV reactivation after Treg/Tcon haplo-HCT, while it was undetectable in the 7 patients who experienced CMV reactivation after T-cell depleted haplo-HCT (1/7 patient died of CMV-disease). Notably, patients who produced CMV-specific IgM had lower incidence of a second CMV reactivation (Figure 3C). No patient died of CMV disease after Treg/Tcon haplo-HCT.

B cell response to 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, from January 2019 to February 2022 (Supplementary table 2). 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 1 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 haplo-HCT.

Previous studies showed mouse Tregs support an immunological niche for HSCs in the BM, by shielding HSCs from *self*- or *allo*-reactive T cells and by promoting engraftment. The present data in immunodeficient mice show that human Tregs are also capable of promoting early engraftment and differentiation of CD34+ cells. Indeed, Tregs 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 Tregs were obtained from the CD34+ cell donor, these effects were independent of Treg-mediated 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 Tcons add-backs were infused). Therefore, such findings suggest Tregs support BM hematopoiesis independently of their immune-suppressive function.

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

Furthermore, Tregs infusion facilitated mature B cell peripheral expansion in xenogeneic mouse models (in the absence of human haematopoietic stem cells, 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 Tregs-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 transplant 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 appears to be faster compared with studies of haplo-HCT with high-dose post-transplant Cyclophosphamide (PtCy) (12-15), and it is comparable with B cell reconstitution reported after matched-HCT with PtCy (16). Taken together these findings suggest 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 Tregs 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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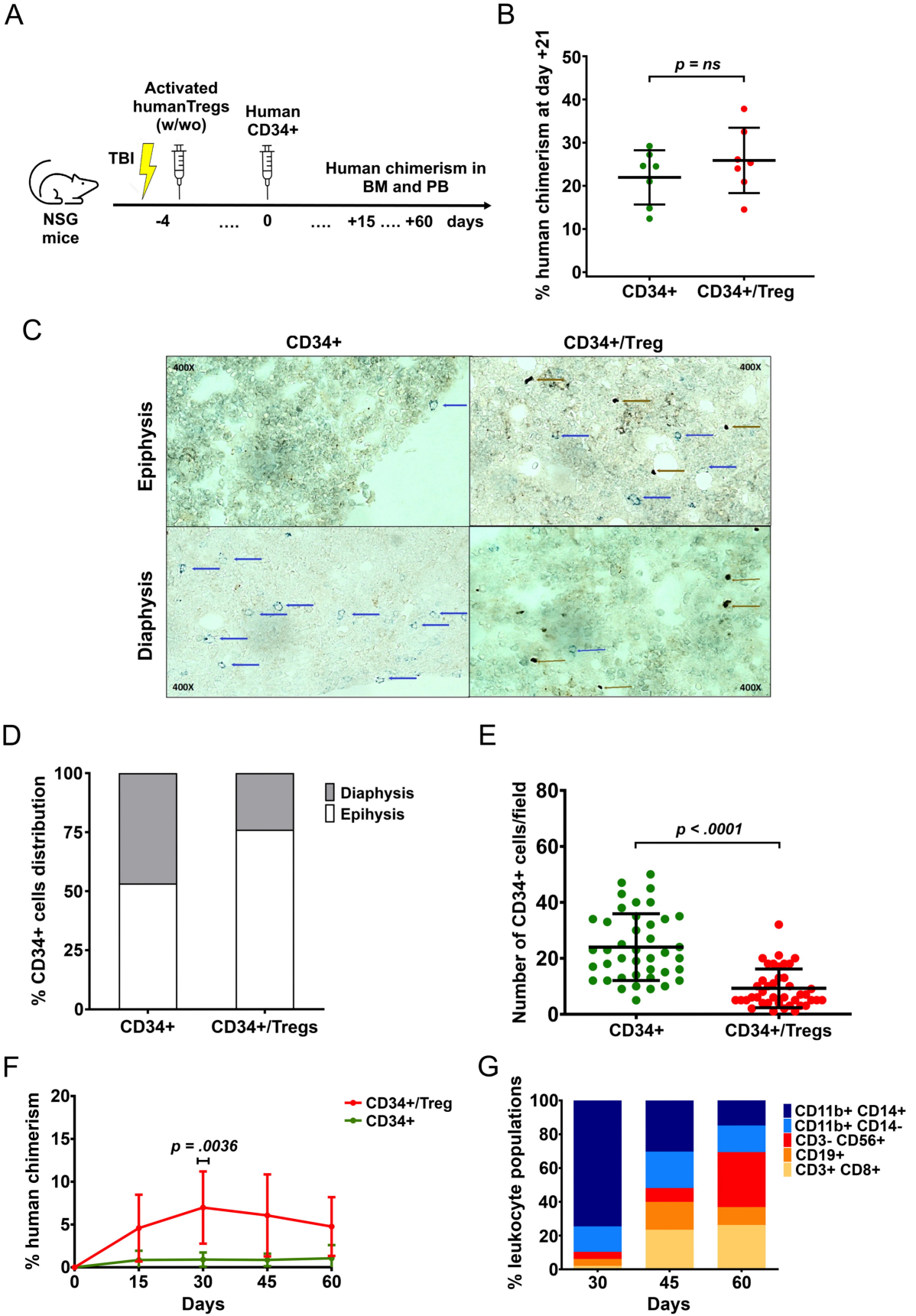
Figure legends

Figure 1. Human Tregs facilitate engraftment of HLA-matched CD34+ cells in a xenogeneic mouse model. (A) Schematic representation of the xenogeneic mouse model used to assess impact of human Tregs on early engraftment of HLA-matched CD34+ cells from the same healthy donor. (B) Human chimerism in the BM 21 days after the infusion of CD34+ cells with (red symbols) or without Tregs (green symbols), as evaluated by flow cytometry with FACSCanto and FACSDiva Software (BD Biosciences, Franklin Lakes, NJ, USA). Data are expressed as mean % of human CD45+ cells/total CD45+ cells ± SD of seven mice per group. Human CD3+CD4+ T cells including infused FOXP3+ Tregs were gated out. *p value = .18* in a paired T test. (C) Representative immunohistochemical staining of diaphysis and epiphysis of femurs harvested 45 days after the infusion of CD34+ cells with (right) or without Tregs (left). Human hematopoietic progenitors cells have a blue cytoplasmic staining (anti-human antibody CD34 Class II, blue arrows) and human Tregs have a brown nuclear staining (anti-human FOXP3 antibody, brown arrows). 400x magnification fields are shown; representative 40x magnification fields are shown in supplementary Figure 1. (D) Distribution of CD34+ cells in diaphysis versus epiphysis of femurs harvested 45 days after the infusion of CD34+ cells with or without Tregs, as evaluated by immunohistochemistry. Data are expressed as mean % of human CD34+ cells ± SD of 2 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 Tregs (green symbols), as evaluated by immunohistochemistry. Data are expressed as mean number for 10X field ± SD of 2 mice per group. *p value* < 0.0001 in a paired T test. (F) Human chimerism in PB of mice infused with CD34+ cells with (red symbols and line) or without Tregs (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 % of human CD45+ cells/total CD45+ cells ± SD of seven mice per group. *p value* = .0036 in a multiple T test with Sidak-Bonferroni method at the indicated time point. Human CD3+CD4+ T cells including infused FOXP3+ Tregs were gated out. (G) Normalized % of human leukocyte populations in PB of mice infused with CD34+ cells and Tregs, as evaluated by flow cytometry at the indicated time points after the infusion of CD34+ cells. The following human leukocyte populations were analysed in PB: CD11b+CD14+ monocytes, CD11b+CD14- neutrophils, CD3-CD56+ NK cells, CD19+ B cells, CD3+CD8+ T cells. Data are expressed as mean % of leukocyte populations of seven mice per group. Human CD3+CD4+ T cells including infused FOXP3+ Tregs were gated out.
Graphical and statistical analyses were performed with GraphPad Prism Software (Dotmatics, Boston, MA, USA).

Figure 2. B cell reconstitution in lymphoid tissues after haplo-HCT with Treg/Tcon immunotherapy. (A) Schematic representation of the xenogeneic mouse model used to assess the role of Tregs 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 (PBMCs) as a source of Tcons (about 60%) and B cells (about 15-20%) at day 0 with $3x10^6$ human CD4+CD25+ Tregs from the same donors at day -2. NSG mice which received only Tregs or only PBMCs served as control groups. (B) Absolute number of human B cells harvested from a femur or spleen of mice infused with Tregs or Tcons, or co-infused with Tregs and Tcons, as evaluated by flow cytometry at the indicated time points after the infusion of Tcons. Data are expressed as mean ± SD of 3 mice per group. *p value* < .005 in a multiple T test with Sidak-Bonferroni method at the indicated time point. (C) Absolute number of common lymphoid progenitors (CLP) and of the indicated B cell precursors and populations in the BM of 35 Treg/Tcon haplo-HCT patients after transplant (red symbols and line) and of 5 healthy transplant donors (green line), as evaluated by flow cytometry at the indicated time points after transplant. *p value* < .005 in a multiple T test with Sidak-Bonferroni method at the indicated time points.

Figure 3. Peripheral B cell reconstitution and immunity after haplo-HCT with Treg/Tcon immunotherapy.

(A) Absolute number of transitional B cells, immature B cells, total mature B cells in PB of 39 Treg/Tcon haplo-HCT patients after transplant, as evaluated by flow cytometry at the indicated time points after transplant. *p value* < .005 in a multiple T test with 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 7 contemporary T-cell depleted (TCD) haplo-HCT patients (green symbols and line) at the indicated time points after transplant. (C) Cumulative incidence of a second 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 >1000 copies of CMV DNA in PB. Cumulative incidence graph was performed with IBM SPSS Statistic Software (IBM Armonk, NY, USA).


A
Activated humanTregs (w/wo)
Human CD34+
TBI
Human chimerism in BM and PB
NSG mice
-4
....
0
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+15 .... +60 days
B
p = ns
% human chimerism at day +21
CD34+
CD34+/Treg
C
CD34+
CD34+/Treg
Epiphysis
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% CD34+ cells distribution
Diaphysis
Epihysis
CD34+
CD34+/Tregs
E
p < .0001
Number of CD34+ cells/field
CD34+
CD34+/Tregs
F
CD34+/Treg
CD34+
p = .0036
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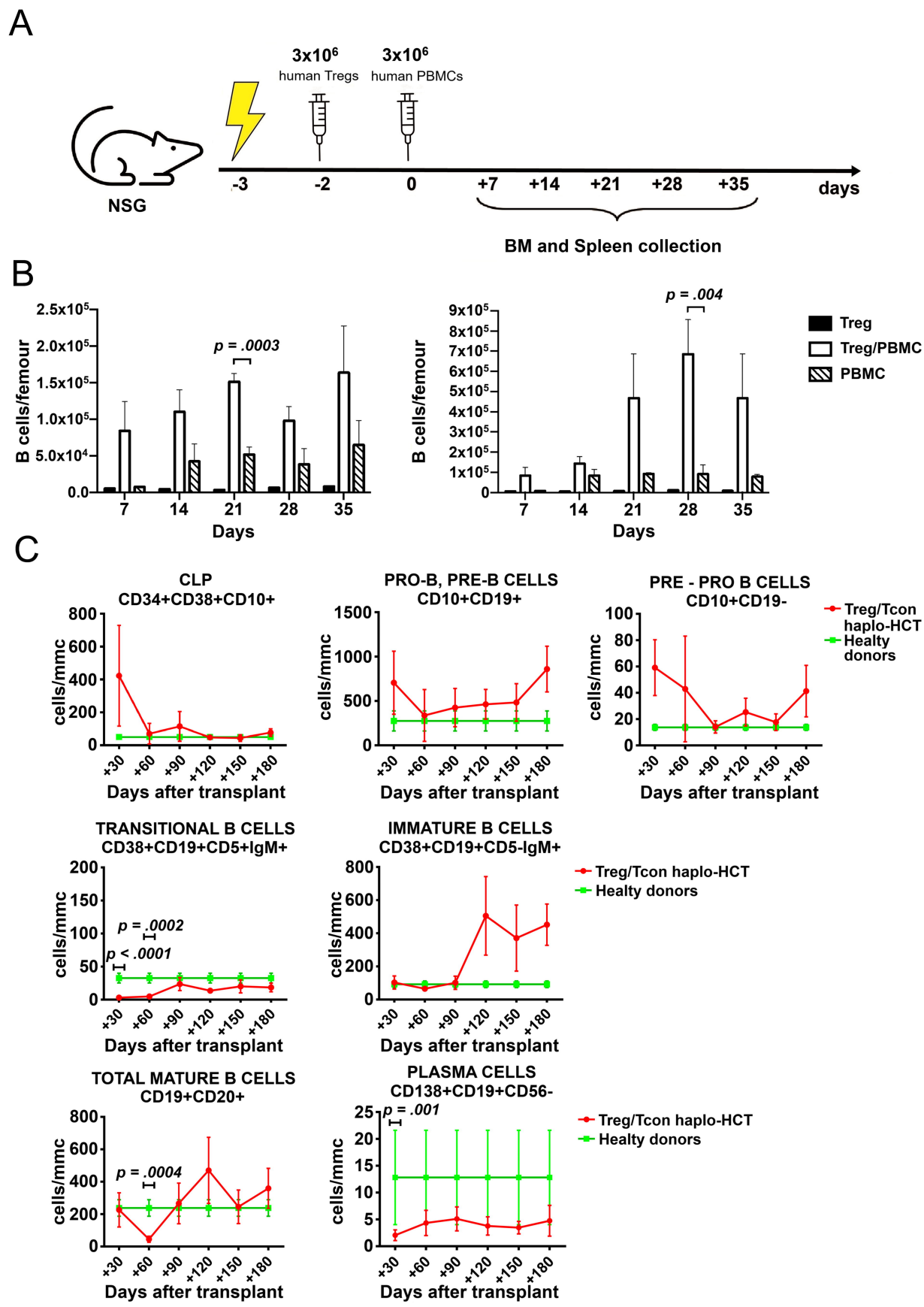
CD11b+ CD14-
CD3- CD56+
CD19+
CD3+ CD8+
Days


A
3x10⁶ human Tregs
3x10⁶ human PBMCs
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-3
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0
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+14
+21
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+35
days
BM and Spleen collection
B
B cells/femour
p = .0003
p = .004
Days
Treg
Treg/PBMC
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C
CLP
CD34+CD38+CD10+
PRO-B, PRE-B CELLS
CD10+CD19+
PRE - PRO B CELLS
CD10+CD19-
cells/mmc
Days after transplant
Treg/Tcon haplo-HCT
Healty donors
TRANSITIONAL B CELLS
CD38+CD19+CD5+IgM+
p = .0002
p < .0001
IMMATURE B CELLS
CD38+CD19+CD5-IgM+
TOTAL MATURE B CELLS
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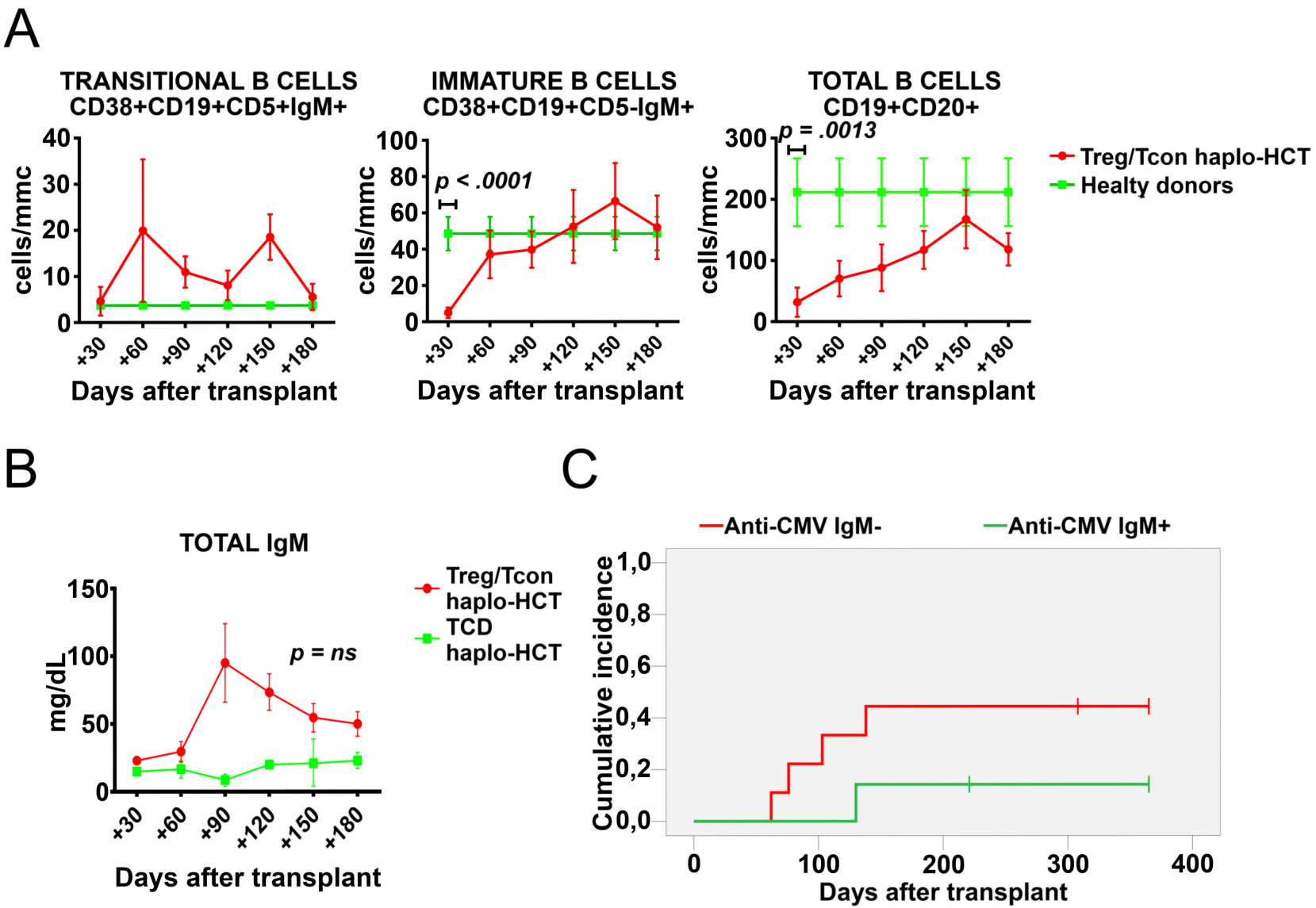
PLASMA CELLS
CD138+CD19+CD56-
p = .001


A
TRANSITIONAL B CELLS
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30
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+30
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+120
+150
+180
Days after transplant
IMMATURE B CELLS
CD38+CD19+CD5-IgM+
cells/mmc
100
80
60
40
20
0
p < .0001
+30
+60
+90
+120
+150
+180
Days after transplant
TOTAL B CELLS
CD19+CD20+
cells/mmc
300
200
100
0
p = .0013
+30
+60
+90
+120
+150
+180
Days after transplant
Treg/Tcon haplo-HCT
Healty donors
B
TOTAL IgM
mg/dL
150
100
50
0
p = ns
+30
+60
+90
+120
+150
+180
Days after transplant
Treg/Tcon haplo-HCT
TCD haplo-HCT
C
Anti-CMV IgM-
Anti-CMV IgM+
Cumulative incidence
1,0
0,8
0,6
0,4
0,2
0,0
0
100
200
300
400
Days after transplant

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Supplementary Table 1. Diagnosis and outcomes of HCT recipients

HCT Protocol	**Treg/Tcon haplo-HCT**	**T cell-depleted haplo-HCT**
Number of patients	44	7
Diagnosis		
Acute Myeloid Leukemia	32	3
Acute Lymphoblastic Leukemia	9	4
Multiple Myeloma	2	-
Myelodysplastic Syndrome	1	-
Outcomes		
Grade II-IV Acute GvHD	15	0
Chronic GvHD	1	0
Relapse	10	1
Non-relapse mortality	10	5

Supplementary Table 2. Diagnosis and outcomes of HCT recipients evaluated for SARS-CoV-2 vaccination or infection

HCT Protocol	**Treg/Tcon haplo-HCT**	**Treg/Tcon HLA identical-HCT**
Number of patients	16	13
Diagnosis		
Acute Myeloid Leukemia	13	12
Acute Lymphoblastic Leukemia	2*	1
Myelodysplastic Syndrome	1	0
Outcomes		
Grade II-IV Acute GvHD	4	3
Extensive Chronic GvHD	0	0
Relapse	1	0
Non-relapse mortality	0	0

Post-transplant anti-SARS-CoV-2 specific-IgG were evaluated in serum samples of patients who experienced SARS-CoV-2 infection or were vaccinated with two doses of Pfizer (New York, NY, USA)-BioNTech SE (Mainz, Germany) or Moderna (Cambridge, MA, USA) SARS-CoV-2 vaccine after Treg/Tcon HCT.

***** One of these patients was affected by a bilinear acute leukemia.

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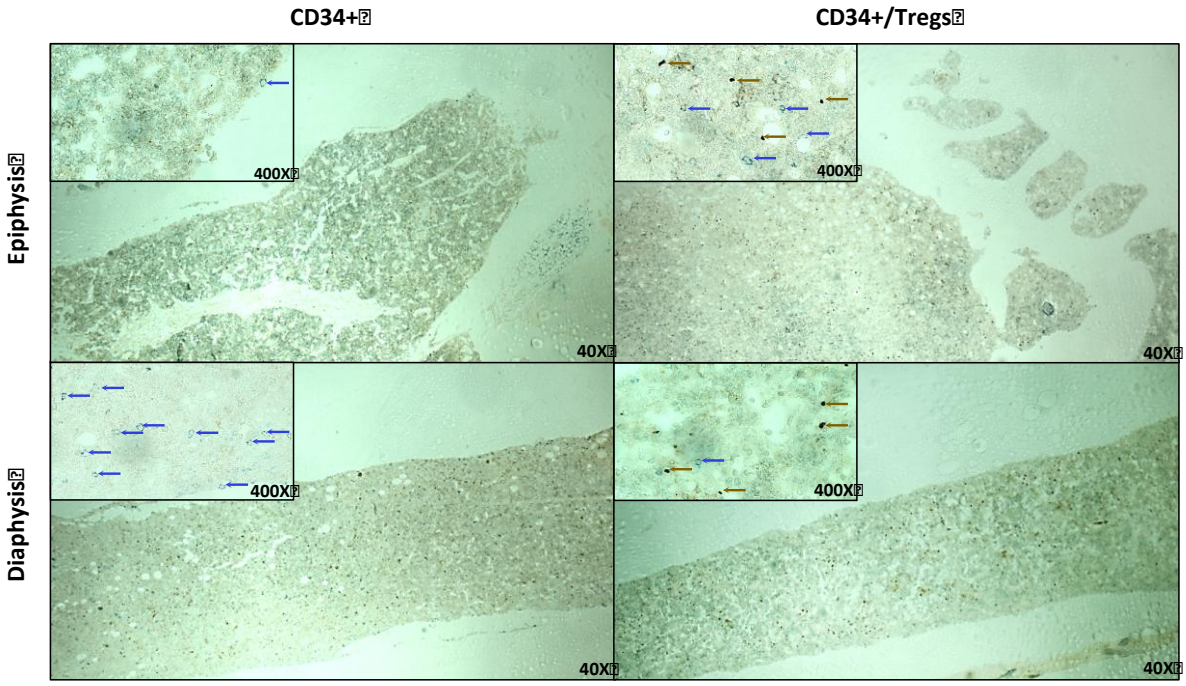
Supplementary Figures



Supplementary Figure 1. Representative immunohistochemical staining of diaphysis and epiphysis of femurs harvested from NSG mice 45 days after the infusion of CD34+ cells with (right) or without Tregs (left). Human hematopoietic progenitor cells have a blue cytoplasmic staining (blue arrows, anti-human antibody CD34 Class II, clone QBEnd 10, Agilent Dako, Santa Clara, CA, USA) and human Tregs have a brown nuclear staining (brown arrows, anti-human FOXP3 antibody, clone SP97, Thermo Fisher Scientific, Waltham, MA, USA). Target cells were detected with the REAL Detection System, Alkaline Phosphatase/RED (rabbit/mouse, Agilent Dako, Santa Clara, CA, USA) and the REAL Detection System, Peroxidase/DAB (rabbit/mouse, Agilent Dako, Santa Clara, CA, USA). 400X and 40X magnification fields are shown.