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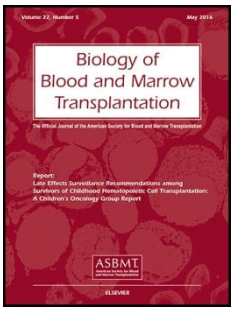

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T cell-mediated rejection of human CD34+ cells is prevented by costimulatory blockade in a xenograft model

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Highlights

- Engraftment of CD34+ cells before T cell-mediated rejection in a xenograft model.
- Abatacept allows CD34+ cell engraftment in xenograft model with allogeneic T cells.
- Boost of CD34+ cells improves hematopoiesis after HSCT with costimulatory blockade.

ABSTRACT

A xenograft model of stem cell rejection was developed by co-transplantating human CD34+ and allogeneic CD3+ T cells into NOD-scid γ-chain^{null} (NSG) mice. T cells caused graft failure when transplanted at any CD34:CD3 ratio between 1:50 to 1:0.1. Kinetics

experiments showed that two weeks after transplantation CD34+ cells engrafted the marrow and T cells expanded in the spleen. Then at four weeks only memory T cells populated both sites and rejected CD34+ cells. Blockade of T cell costimulation was tested by injecting the mice with abatacept (CTLA4-IgG1) from day -1 to +27 (Group A), or from day -1 to +13 (Group B), or from day +14 to +28 (Group C). On day +56, groups B and C had rejected the graft while in group A graft failure was completely prevented, although with lower stem cell engraftment than in controls (p=0.03). Re-transplantation of group A mice with same CD34+ cells obtained a complete reconstitution of human myeloid and B cell lineages and excluded latent alloreactivity. In this first xenograft model of stem cell rejection we showed that transplantation of HLA mismatched CD34+ cells may be facilitated by treatment with abatacept and late stem cell boost.

Keywords: CD34; stem cell transplantation; rejection; abatacept; T cell

INTRODUCTION

Allogeneic transplantation of selected CD34+ progenitor cells can be performed to eliminate the risk of severe acute graft-versus-host disease (GVHD) since donor T cells are largely depleted during stem cell isolation. This strategy has been utilized in HLA matched transplants (1,2) but mostly in transplants from HLA incompatible (haploidentical) donors in patients with hematologic malignancies or with non-malignant disorders(3-6). However, the conditioning treatment with chemotherapy and radiation therapy necessary to suppress the host immune system leads to a high risk of infectious complications after transplantation, due to the prolonged time necessary to achieve an adequate immune reconstitution. An obstacle to achieving immune tolerance after transplantation of selected CD34+ cells is related to the fact that these cells represent a heterogeneous cell population, including a subset of progenitors with antigen presenting cells (APC) activity (7-11) and rapidly differentiating into monocytic/dendritic cells upon encountering allogeneic T cells (12).

However, there is no in-vivo experimental model to test anti-CD34+ cell immune alloreactivity. NOD/SCID mice that bear a targeted mutation in the IL-2 receptor common gamma chain (IL-2Rγ^{null},or NSG) and lack B, T and natural killer (NK) cells, have been utilized to expand human T cells in xenogeneic GVHD models (13,14), as well as to engraft CD34+ cells to test their in-vivo repopulating ability (15,16). Based on the ability of NSG mice to engraft both stem cells and T cells, we developed a new model to investigate the in-vivo rejection of human CD34+ cells after co-transplantation with HLA incompatible T cells. Our model allowed us also to analyze the kinetics of stem cell and T cell in-vivo engraftment or expansion. In addition we addressed the question whether costimulatory blockade using a CTLA4-IgG1 molecule (abatacept) (17) could prevent rejection of human CD34+ cells, as previously suggested by findings in mouse models of MHC mismatched bone marrow transplant (18-20), as well as in-vitro data with human T cells (21). Results shown here suggest that early and prolonged blocking of T cell costimulation followed by a stem cell boost may facilitate the engraftment of incompatible CD34+ cells.

MATERIALS AND METHODS

Flow Cytometry. The monoclonal antibodies (clone name) utilized were the following: fluorescein isothiocyanate (FITC), or phycoerythrin (PE), or peridinin chlorophyll protein (PerCP) CD45 (2D1), CD34 (581/CD34) , CD33 (HIM3-4), CD14 (M5E2), CD19 HIB19), CD3 (UCHT1), CD4 (RPA-T4), CD8 HIT8a), CD25 (M-A251), CD56 (B159) Becton-Dickinson, San Jose, CA); CD1c (BDCA1) (AD5-8E7) and BDCA2 (REA693) (Miltenyi Biotec, Auburn, CA); CD45RO-allophycocyanin (UCHL1), CD45RA-P450 (HI100), CCR7-PE-Cy7 (3D12); and appropriate isotype controls. Stained cells were analyzed on a FACSCaliburTM (Becton Dickinson) or a Beckman Coulter flow cytometer (Beckman Coulter, Brea, CA).

Cell separation. CD34+ cells were purified from cord blood (CB) or mobilized peripheral blood stem cells (PBSC) by MidiMACS high gradient magnetic separation column (Miltenyi Biotec) as previously described (12). PB CD3+ T cells, and in selected experiments CD4+ or CD8+ T cells, were immunomagnetically purified on a MidiMACS column by negative selection as previously described (8). Aliquots of isolated CD34+ or T cells were restained with appropriate mAbs and analyzed by flow cytometry showing on average 95±1.6% cell purity.

Immunofluorescence (IF) microscopy. CD34+ and allogeneic T cells were cultured for 3 days in RPMI and 10% AB human serum (HS, Hyclone, Logan, Ut), then washed in phosphate-buffered saline (PBS) before cytocentrifugation onto polylysine-coated microscope slides. Cells were fixed on slides for 20 min in 4% formaldehyde at room temperature followed by cold (-20 C) acetone treatment for 3 min. The cells were blocked with 5% normal goat serum (Santa Cruz, Dallas, TX) in PBS for 30 min followed by incubation with Alexa Fluor® 488 anti-human CD34 and Alexa Fluor® 594 anti-human CD3 antibodies (Biolegend, San Diego, CA) for 45 min, and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 1 min. The slides were mounted with prolonged anti-fading media (Molecular Probe) and the proteins were visualized using an Axioskop 2 (Carl Zeiss MicroImaging, Inc.) equipped with 40x Objective (Zeiss Ph2, Plan-NEOFLUAR, 40x/0.75, ∞/0.17).

CFU-C Assay. Purified CD34+ cells were plated at 0.5 x10^3 cells/plate in duplicate cultures with a cytokine enriched semi-solid medium (METHOCULT GF H4435, Stem Cell Technologies, Vancouver, BC, Canada), as previously described (12). CTLA4-IgG1 fusion molecule (Abatacept, Bristol Meyers Squibb, New York, NY) was added to the cultures at 0 (control), 100, 250 or 500 µg/ml. Colony-forming Unit in culture (CFU-C), including

Granulocyte- Macrophage CFU (CFU-GM), erythroid progenitors (burst-forming unit-erythroid, [BFU-E]) and CFU-Mix were scored after 14 days as previously described (12).

Transplantation. NSG mice purchased from Jackson Laboratories (Bar Harbor, ME) were housed in a strict barrier environment. The study was approved by UIC Animal Care Committee and performed in accordance with national guidelines of laboratory animal care. Human CD34+ cells ($2x10^5$/animal) were mixed at 1:0.1, 1:0.5, 1:1, 1:2, 1:5, 1:10 or 1:50 ratio with allogeneic PB selected T cells obtained from a healthy donor and then injected i.v. into separate cohorts of sublethally irradiated (300cGy) NSG mice. The HLA typing of CD3+ T cells and three separate batches of CD34+ cells used in these experiments was: CD3: HLA A (30,32); B (13,14); C (06,08); DRB1 (13,07); CD34 sample 1: HLA A (02,26); B (35,37); C (06,04); DRB1 (08,10); CD34 sample 2: HLA A (01,80); B (08,52); C (07,16); DRB1 (03,13); CD34 sample 3: HLA A (74,29); B (58,78); C (10,16); DRB1 (17,01).

In selected experiments, CD34+ cells were co-transplanted with CD4+ or CD8+ T cells at 1:1 ratio. Control mice were injected with CD34+ cells or T cells ($2x10^5$/animal) alone. At different time-points after transplant, ranging between 1 to 8 weeks, the animals were sacrificed and femurs and tibias were flushed with Hanks balanced salt solution (HBSS; Biowhittaker, Walkersville, MD) with 2% fetal bovine serum (FBS) (Hyclone Laboratories) and 0.02% sodium azide. Single-cell suspension of spleen cells was prepared by mechanical homogenization of spleen, filtered through 40um cell strainer, followed by depletion of erythrocytes using red blood cell lysing buffer (Sigma, St Louis, MO). Cells were resuspended in RPMI with 2% FBS and preincubated with 1 mg/ml human gamma globulin (Bayer, Elkhart, IN) to block human Fc receptors. Murine Fc receptors were blocked by a second incubation of the cells in 2.4G2 (an anti-mouse Fc receptor mAb) (BD Pharmingen, San Diego, CA). Human cell engraftment was assessed by measuring the expression of anti-huCD45 PerCP. Analysis of lineage specific markers on marrow or spleen

cells was performed on gated huCD45+ cells. Mice were evaluated for xenogeneic GVHD by measuring the body weight, fur changes, hunched posture and mobility at different time points, ranging from 1 to 8 weeks after transplant.

Costimulatory blockade. In-vivo costimulatory blockade was tested in NSG mice transplanted with CD34 and allogeneic T cells at 1:1 ratio by injecting abatacept intra-peritoneum (i.p.) at 250 µg/mouse every other day from day -1 up to day 28 post-transplant. Since the average mice weight was 20-25g, the dose of abatacept injected was approximately 10-12.5 mg/kg per dose. Engraftment of human cells was then measured in the marrow and spleen at day 28 or day 56 post-transplant. In selected experiments, mice that had been treated with abatacept until day 28, were then re-transplanted with additional CD34+ cells at 2 x 10^5/mouse on the same day, and then sacrificed on day 56 to assess the engraftment of huCD45+ cell subsets.

Secondary MLC. In selected experiments, splenocytes of NSG mice were centrifuged over Ficoll-Hypaque and rested for 24 hrs at 37°C. Then they were analyzed by flow cytometry and co-cultured for 3 days in secondary mixed leukocyte cultures (MLC) (10) at 5x10^4 CD45+CD3+ cells/well with irradiated CD34+ cells (2.5x10^4/well) from same donor used for in-vivo transplant, or for 6 days with 3rd party PB MNC (5x10^4/well). Cells were pulsed with 1 mCi /well 3H-thymidine for 18 hours before harvest on day 3 or 6.

Statistical analysis. *t*-test or ANOVA test were performed to compare 2 or greater series of data, respectively. All statistical tests were performed by using Graph Pad Prism version 6.0 (GraphPad Inc., San Diego, CA).

RESULTS

T cell-mediated rejection of human CD34+ cells in NSG mice. Marrow and spleen of NSG mice can be successfully engrafted with human hematopoietic cells within 4-8 weeks after transplant. Here we analyzed the engraftment of human CD34+ cells that were transplanted with or without HLA incompatible CD3+ T cells. Seven groups of mice (n=5-10/group) were transplanted with CD34+ and allogeneic CD3+ T cells at a CD34:CD3 ratio of 1:50, 1:10, 1:5, 1:2, 1:1, 1:0.5, 1:0.1. Control mice were transplanted with either CD34+ (ratio: 1:0) or CD3+ cells alone (ratio: 0:1) at $0.2x10^6$ cells/mouse. As expected, xenogeneic GVHD occurred only in mice receiving higher doses of T cells, whereas mice with CD34+ alone or with T cells at $\leq 0.2x10^6$ cells/mouse (CD34:CD3 cell ratio $\geq$ 1:1) did not develop GVHD (not shown). Co-transplantation of CD34+ cells and allogeneic T cells at any CD34:T cell ratio tested, from 1:50 to 1:0.1, resulted in a significantly reduced number of huCD45+ cells in the bone marrow compared with control animals transplanted with CD34+ cells alone ($p<0.001$) (Figure 1-A, left). On the contrary, the spleen of mice receiving CD34+ and T cells at any tested ratio had similar or increased numbers of huCD45+ cells compared to control mice (Figure 1-A, right). Phenotypic analysis of huCD45+ marrow and spleen cells of mice transplanted with CD34:T cell ratios from 1:50 to 1:0.5 showed the presence of >98% CD3+ cells (Figure 1-B). These findings were consistent with expansion of T cells and rejection of CD34+ cells. Mice transplanted with the highest doses of T cells (CD34:T cell ratio = 1:10 and 1:50) died earlier than the others due to GVHD (not shown). This may explain an overall lower expansion of huCD45+CD3+ cells in the spleen of these mice. The largest absolute number of splenic T cells was detected in mice receiving CD34+ and T cells at 1:1 ratio and had rejection but no GVHD (4.0±2.0 CD45+CD3+ cells/mouse). Mice that were transplanted with very low numbers of T cells at a 1:0.1 CD34:CD3 ratio showed <50% marrow engraftment of cells derived from CD34+ cells than control mice (Figure 1-A). However, the phenotype of huCD45+ cells in the marrow and spleen of these mice (Figure 1-C) showed a mix of myeloid cells (CD33+), monocytes (CD14+), myeloid dendritic cell

(DC) (CD1c+ or CD1a+), B lymphocytes (CD19+), plasmacytoid DC (CD19-BDCA2+), and CD34+ cells, and also the expansion of allogeneic T cells. On the contrary, control mice transplanted without T cells (Figure 1-D) showed the engraftment of a large portion of B cells and a high number of CD34+ cells. These findings suggest that even low doses of allogeneic T cells completely prevented or severely affected the engraftment of CD34+ cells.

CD4+ T cells are potent effectors of CD34+ cells rejection. Expansion of T cells after transplantation prevented CD34+ cells to engraft NSG mice. A normal CD4:CD8 ratio and comparable percentages of activated CD4+CD25+ T cells or CD3+CD56+ NK T cells were detected in mice with rejection independently of the initial CD34:CD3 ratio (Suppl. Figure 1).

We then transplanted CD34+ cells and purified allogeneic CD4+ or CD8+ T cells at 1:1 ratio to assess whether they could independently reject CD34+ cells. Six weeks after transplant, the bone marrow of mice transplanted with CD34+ and CD4+ cells showed only an expansion of T cells with full rejection of stem cells (Figure 2, left). In mice transplanted with CD8+ cells, we found only 9% huCD45+ cells that consisted 80% of T cells and 20% of myeloid and B cells derived from CD34+ cells (Figure 2, center). Only in mice transplanted with CD8+ cells we detected a subset of reactive CD8bright/CD4dim T cells (22). These cells were excluded to be derived from CD34+ cells by fluorescence-in-situ-hybridization (FISH) analysis for chromosomes x and y in mice transplanted with sex mismatched CD34+ and T cells (data not shown). Although both CD4 and CD8+ cells impaired CD34+ engraftment, CD4+ cells seemed more potent and expanded a greater number of CD45RA+ naive T cells (Figure 2, right) ($p=0.03$).

Rejection of incompatible CD34+ cells occurs after initial engraftment. Based on our results showing rejection of stem cells at 4-6 weeks after co-transplantation of CD34+ and T cells at 1:1 ratio, we tested whether T cells directly prevented the homing of CD34+ cells in the marrow or, alternatively, deleted the stem cells after an initial engraftment. We performed kinetics experiments where marrow and spleen of mice transplanted with CD34+ and allogeneic T cells at 1:1 ratio were analyzed after 1, 2 and 4 weeks after transplant (Figure 3) (n=5-7 mice per group). At 1 week after transplant we could detect only 0-0.5% huCD45+ cells both in the marrow and spleen, and cells were not sufficient for definitive phenotypic analysis of myeloid or T cell subsets (not shown). In mice sacrificed at 2 weeks after transplant, the marrow showed on average 3.1±1% huCD45+ cells, with 97±1% CD33+ myeloid cells, 2±1% CD34+ cells and only 1.3±0.9% CD3+ T cells. Control animals transplanted with CD34+ cells alone had a greater engraftment of huCD45+ cells (11±3%), with 85±1% CD33+ and 12±2% CD34+ cells, while control animals transplanted with T cells alone had only <0.5% huCD45+ cells in the marrow at this time point (Figure 3, left). On the contrary, the spleen of mice transplanted with CD34+ and T cells showed 2.1±2% huCD45+ cells that were predominantly T cells (91±17%), (Figure 3, right). As expected, week 4 results confirmed the full rejection of CD34+ cells, shown in Fig.1, with the presence of >98% T cells both in the marrow and the spleen. These results demonstrated that in the first two weeks after transplant a portion of CD34+ cells engrafted the bone marrow, while T cells expanded primarily in the spleen. Then between week 2 and 4 following transplantation allogeneic T cells caused a complete rejection of CD34+ cells.

Costimulatory blockade with abatacept prevents stem cell rejection. Our previous in-vitro studies demonstrated that in-vitro costimulatory blockade with CTLA4-Ig and anti-CD40L antibody prevented the APC activity of CD34+ cells and induced antigen specific T cell anergy (20). The costimulatory molecule utilized here, abatacept, is currently used in the

immunosuppression therapy of patients with autoimmune diseases. In in-vitro co-cultures with CD34+ cells and T cells abatacept did not prevent cell binding, nor affected the colony formation activity (CFU-C) of CD34+ cells (Figure 4).

Based on the findings that graft rejection was documented only after the first two weeks following co- transplantation of CD34+ and allogeneic T cells at CD34:CD3 1:1 ratio, we designed an experiment to test if three different schedules of treatment with abatacept could differently impact CD34+ cell rejection. Mice in Group A were injected every other day with abatacept at 250 µg i.p. from day -1 to day +27 post-transplant, mice in Group B from day -1 to day +13 and mice in group C from day +14 to day +28 (Figure 5-A). On day +56 the mice were sacrificed to examine marrow and spleen cells by flow-cytometry. In mice treated with abatacept for 4 weeks (Group A) CD34+ cells were not rejected since >97% of huCD45+ cells in the marrow and 90% in the spleen were CD3 negative (Figure 5-B) and their phenotype showed B cells, myeloid cells, dendritic cells and a small fraction of CD34+ cells (Figure 5-C). However, the overall marrow engraftment of huCD34+ cells (huCD45: 2±1%) was lower than in control animals transplanted with CD34+ cells alone and treated with abatacept (huCD45: 16±4%) ($p=0.03$). Mice in Group B and Group C, instead, showed an expansion of mostly CD45+RO+ memory T cells in the marrow and spleen (Figure 5-C), with comparable absolute numbers of splenic CD3+ T cells (Figure 5-D). Groups B and C mice also had a greater fraction of myeloid CD1c+ DC (3.1±2.8% and 2.7±2.5%, respectively) compared to Group A mice (1±1%)($p=0.02$). T cells immunomagnetically isolated from the spleen of mice in Groups B and C were then tested as responders in a 3 day secondary MLC against the same CD34+ cells that had been utilized in the transplant, or third party MNCs (Figure 5-D). As expected, T cell responders from both Group B and Group C mice

showed a greater proliferative response against CD34+ cells than third party cells, suggesting that a short and/or delayed treatment with costimulatory blockade did not prevent the development of an antigen-specific T cell alloreactivity.

Boost of CD34+ cells after treatment with abatacept achieves full engraftment

Since mice in Group A did not show rejection but the overall CD34+ cell engraftment was lower than in control animals that did not receive T cells, we hypothesized that the reduced marrow engraftment four weeks after stopping abatacept could be due to either a latent rejection caused by alloreactive T cells outside the spleen, or the reduction of the pool of early hematopoietic progenitors in the marrow after transplant. To test this hypothesis, we repeated the transplant experiment as in Group A mice, with abatacept injection for 4 weeks, and then we re-challenged the mice on day 28 with a second transplant of CD34+ cells (Figure 6-A) without any further treatment. Then, four weeks following the boost of CD34+ cells (day +56) the stem cell engraftment in the marrow increased to 36±13% huCD45+ cells without any evidence of T cells, and to 10±4% huCD45+ in the spleen, with only 1±1% T cells (Figure 6-B). The increased engraftment of huCD45+ cells also resulted in a significant increase in the absolute number of marrow CD34+ cells 4 weeks after the boost, compared with mice that not received the boost (Figure 6-C) ($p=0.002$), suggesting a fully reconstituted hematopoiesis. This is also supported by the phenotype of CD45+ cell subsets shown in one case representative of 5 mice after the boost (Figure 6-D). Altogether these findings suggest that a partial depletion of CD34+ may have occurred early after transplant. In fact, a re-challenge with CD34+ cells did not elicit a secondary in-vivo T cell response but, on the contrary, restored full hematopoietic function.

DISCUSSION

In this study we utilized a xenograft stem cell transplant to develop the first experimental model of rejection of human CD34+ hematopoietic stem cells mediated by allogeneic T

cells. This model also allowed us to show that in-vivo blockade of T cell costimulation with CTLA4-Ig for 4 weeks after transplant allowed the engraftment of CD34+ cells in the presence of HLA incompatible T cells.

T cell alloreactivity induced by human CD34+ cells was previously only demonstrated *in vitro*[7-9]. Here we hypothesized that a new xenograft model testing human stem cell repopulating activity in the presence of allogeneic T cells could allow us to analyze the in-vivo T cell alloreactivity against human CD34+ hematopoietic progenitors. We based our hypothesis on previous experiments showing that CD34+ cells transplanted into NSG mice recapitulate the long-term clonogenic function of hematopoietic stem cells (15,16), and studies of xenogeneic GVHD performed by injecting high dose of human T cells into NSG mice (13,14). Our model showed that co-transplantation of CD34+ cells and allogeneic T cells at different ratios, ranging from 1:50 (very high dose of T cells) to 1:0.1 (very low dose of T cells), resulted in complete or partial stem cell graft failure. Also, both CD4+ and CD8+ T cells could cause CD34+ cell rejection. A possible limitation of this model could be that human T cells are likely to be activated by xenogeneic mouse antigens, responsible for xenogeneic GVHD. Although this could not be avoided completely, all the experiments designed to study the kinetics of rejection, as well as the role of costimulatory blockade, were done co-transplanting CD34 and T cells at 1:1 ratio which resulted in graft rejection but never caused xenogeneic GVHD.

The risk of graft rejection is rare in HLA matched allogeneic hematopoietic stem cell transplant (HSCT) and is instead higher in HLA mismatched umbilical CB transplants (23) or HLA mismatched transplants from unrelated donors (24). Also, rejection usually manifests either as failure to engraft donor cells after transplant (primary graft failure), or as secondary graft loss after initial engraftment. This was tested in our model by kinetics experiments. Interestingly, two weeks after transplant huCD45+ cells detected in the bone marrow were >90% myeloid cells, whereas those in the spleen were >90% T cells. The marrow

engraftment of CD34+ cells was then lost at 4 weeks and only T cells were detected in the marrow and spleen. Therefore, it appears that homing of CD34+ cells in the marrow occurred within the first two weeks, prior to the migration of activated T cells from the spleen that then depleted the stem cells within two more weeks.

In haploidentical transplants of CD34+ cells, it has been suggested that in addition to T cells, NK cells could also play a role in rejection, especially in the setting of a killer inhibitory receptor (KIR) epitope- matched donor (25). Although in our model we did not test NK cells, we recently demonstrated that while CD34+ cells can directly activate the NKG2D receptor on NK cells and stimulate their proliferation and release of cytokines *in-vitro*, they do not stimulate an NK cell-mediated CD34+ cell cytolysis (26).

Based on our findings, we then tested whether blocking T cell activation within the first two weeks after transplant could prevent graft failure. The concept of a time-specific immunosuppression has been already successfully applied in preventing GVHD in transplants from incompatible (haploidentical) donors by using high dose cyclophosphamide to eliminate donor alloreactive T cells on day 3 and 4 after transplant (27,28). Since our previous *in-vitro* data demonstrated that allo-T cells stimulated by CD34+ cells became anergic in the presence of costimulatory blockade (21), we tested whether rejection of CD34+ cells could be prevented by abatacept (CTLA4-IgG1). The dose of abatacept utilized here (10-12.5 mg/kg/dose) was similar to what has been used in a previous GVHD mouse model (18). Moreover, in a recent clinical trial to prevent acute GVHD, patients received abatacept at 10 mg/kg/dose for 4 times within 28 days in addition to standard cyclosporine/methotrexate prophylaxis (29). It is not known at this time if a similar regimen may be sufficient to prevent graft failure in a T cell depleted transplant. In fact, based on our findings, even a small fraction of alloreactive T cells could cause stem cell rejection. In our xenograft model we showed that after 14 injections of abatacept within 28 days almost no T cells could be found in the marrow or spleen. On the contrary, mice treated for only 2 weeks

still had T cells with strong alloreactivity against CD34+ cells. Treatment with abatacept every other day for 4 weeks prevented the activation and expansion of alloreactive T cells. In addition, based on our findings this may have also limited the differentiation of CD34+ cells into professional APC such as CD1c+ DC. However, because we observed a lower overall engraftment of CD34+ cells in mice treated with abatacept, we hypothesized that abatacept could have only delayed T cell expansion, and a latent T cell alloreactivity would persist due to lymphocytes in lymphoid organs not examined in these experiments. To address this hypothesis we tested whether a secondary T cell response could be elicited by re-challenging residual T cells with the same CD34+ cells initially transplanted. Mice were re-injected with CD34+ cells right after stopping abatacept on day 28, and then sacrificed four weeks later. This boost of CD34+ cells resulted in a full huCD45+CD34+ cell engraftment without T cell expansion in the marrow. This finding could suggest that a partial reduction of the pool of early progenitors occurred after first transplant, possibly due to an initial T cell-induced differentiation (12), and this pool was then reconstituted by newly transplanted CD34+ cells from the same donor. In alternative, a veto effect of CD34+ cells on a very small number of residual T cells, previously described in-vitro (30), could be also hypothesized to explain a persistent suppressive effect on residual alloreactive T cells. However, our findings seem to reproduce what is observed in the clinical setting when a boost of donor CD34+ cells restores the hematopoiesis of patients with a persistent low blood cell count after allogeneic stem cell transplantation, in the absence of clinical signs of viral infections, GVHD or decrease in donor cell chimerism (31,32).

Based on these observations, a clinical use of costimulatory blockade starting at the time of transplant and continued for at least one month may be efficacious in clinical settings with higher risk of graft failure, such as: a) T cell depleted allogeneic HSCT; b) T cell repleted HSCT with non-myeloablative conditioning regimens; c) HSCT in aplastic anemia patients; d) solid organ transplant combined with HSCT to induce stable donor cell chimerism and

immune tolerance. Because the effect of CTLA4-Ig may depend on the presence of regulatory T cells (Tregs) (33), we hypothesize that abatacept could also be exploited in combination with other immunotherapies that preserve or expand the number of Tregs, such as thymoglobulin (34) or rapamycin (35) or high dose cyclophosphamide post-transplant (36).

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose.

AUTHORSHIP CONTRIBUTION

AO, BN, JLMF and DR contributed to the study design; AO, DM, BN, NM, EB and VS performed all the experiments, AO, DM, BN, VS, PRP, NM and DR analyzed the data; AO, VS, JLMF and DR contributed to the writing of the manuscript; PRP, DM, NM, MA assisted in the critical review of the manuscript and all authors approved the final version of the manuscript for submission.

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Figure 1: In-vivo human stem cell rejection in NSG mice transplanted with huCD34+ cells and allo-T cells. (A) Engraftment of huCD45+ cells in the marrow and spleen 4-6 weeks after co-transplantation with human CD34+ and allo-T cells at different ratios. (n=5-10 mice per group) (B) Engraftment of huCD45+CD3+ T cells in the marrow and spleen of mice co-transplanted with human CD34+ and allo-T cells at different ratios. Human cell engraftment consisted of >97% T cells in mice transplanted with CD34+ and T cells at 1:0.5, 1:1, 1:2, 1:5, 1:10 and 1:50 ratio. Subset analysis of huCD45+ cells engrafted in the marrow and spleen of NSG mice co-transplanted with human CD34+ and allo-T cells at 1:0.1 ratio (C) and in control animals transplanted with CD34+ cells alone (D). The proportion of engrafted human cell subpopulations was examined 6 weeks after transplantation. The data shown represents mean values ± SD. Statistical differences among groups were calculated by ANOVA test and p-values are indicated when <0.05.

Figure 2: CD4 and CD8 T cells in the rejection of CD34+ cells in NSG mice. Mice that were co-transplanted with CD34+ cells and allogeneic CD4+ T cells at 1:1 ratio (n=6) were sacrificed at 6 weeks and showed >97% huCD45+ T cells in the bone marrow, (left), while mice transplanted with CD8+ T cells showed >80% huCD45+ T cells and with a small fraction of myeloid and B cells (center). The analysis of huCD45RA+ naïve T cells recovered in mice transplanted with CD4+ or CD8+ T cells (right) showed a significantly greater percentage of naïve T cells within CD4+ T cells (p=0.03). The data is represented as mean value ±SD.

Figure 3: Engraftment of CD34+ cells in the bone marrow and allo-T cells in the spleen 2 weeks after transplantation in NSG mice. NSG mice were co-transplanted with CD34+ and allo-T cells at 1:1 ratio, or with CD34+ or CD3+ cells alone as control, and cells obtained from the marrow or spleen were analyzed by flow cytometry at 1, 2 or 4 weeks after transplant. In mice co-transplanted with CD34+ and T cells, at 1 week post-transplant

the huCD45+ cells were <0.5% (not shown) and at 4 weeks the engrafted cells were >98% T cells, as shown in Figure 2. The overall engraftment of huCD45+ cells and huCD45+ myeloid (CD33), or T (CD3) or CD34+ cells in the marrow (left) and spleen (right) of NSG mice co-transplanted with CD34+ and allo-T cells at 1:1 ratio, or controls is shown as mean value ±SD (n=5-8 mice per group).

Figure 4: CD34+ cells bind T cells and have normal clonogenic activity in the presence of abatacept. A) CD34+ and allo-T cells (CD3+) were cultured in-vitro for 6 days alone or in combination at 1:1 ratio, with or without abatacept. After staining the cells with anti-CD34 or anti-CD3 antibodies, cells were analyzed by immunofluorescence microscopy. Isolated CD34+ cells were found to be bound with T cells in cultures with or without abatacept. B) Purified CD34+ cells were mixed with abatacept at 0 µg (control), 100 µg, 250 µg or 500 µg for 24 hours in liquid culture and then tested for their CFC activity in semisolid medium for 2 weeks. The results show the mean number ± SEM of colonies/10^3 cells plated (n=3 experiments). Differences in the number of colony units with or without abatacept were not statistically significant.

Figure 5: Long-term engraftment of huCD34 cells after transplantation with allo-T cells and injection with CTLA4-Ig (abatacept). (A) NSG mice co-transplanted with CD34+ and allo-T cells at 1:1 ratio were injected i.p. with abatacept (n=6) (left quadrants) at 250 µg i.p. every other day from d-1 to d+27, (Group A), or from day -1 to 13 (Group B), or from day 14 to day 28 (Group C), and then were sacrificed on day 56 to analyze the bone marrow and the spleen. Control mice were animals transplanted with CD34+ cells and T cells without abatacept. B) The engraftment of huCD45+ and CD3+ cells is shown in one representative case for each group: control mice (CTR), Group A, Group B and Group C. The circled area represents the huCD45+CD3- cells derived from CD34+ transplanted cells. C) A complete

analysis of human CD45+ cells and lineage specific cell subsets detected on day 56 after transplant in the marrow of mice from each group is shown. Values are shown as mean ± SD. D) Absolute number of T cells (huCD45+CD3+) detected in the spleen of mice in Group A, B and C. Values are shown as mean±SD. The dotted line indicates the number of T cells initially transplanted ($1x10^5$/mouse). E) T cells obtained from the spleen of mice that received abatacept for the first two weeks (Group B) or the second two weeks (Group C) after transplant were pooled and tested as responders in a secondary MLC against the original CD34+ cells that had been previously frozen, or against 3rd party MNC. T cell proliferation of pooled T cells from mice in group B and C was measured by 3H-Thymidine uptake after 4 days of culture and the results are shown as mean ± SD cpm from samples tested in triplicate. The figure is representative of 3 separate experiments.

Figure 6: Boost of CD34+ cells after treatment with abatacept restores full stem cell engraftment. A) A scheme of the transplant model of CD34+ and allogeneic T cells injected IV on day 0, treatment with abatacept IV every other day from day -1 to day +27, and boost of CD34+ cells on day 28 is shown. B) Engraftment after re-transplantation of CD34+ cells without immunosuppression at day 56 restored engraftment of large number of CD45+ CD3- cells. C) Absolute number of CD34+ cells detected on day 56 in the marrow of mice initially transplanted with allogeneic T cells and treated with abatacept for 4 weeks, and mice that also received a CD34+ cell boost on day 28. In these latters the number of human CD34+ cells detected in the marrow on day 56 was significantly higher (p=0.002). D) Phenotype of marrow cells in mice re-transplanted with CD34+ cells at day 56. The figure is representative of 5 separate animals.

Suppl. Figure 1: Splenic T cell subsets in NSG mice transplanted with huCD34+ cells and allo-T cells. Mice that rejected the graft after co-transplantation of CD34+ cells and

increasing doses of CD3+T cells (ratios 1:1 to 1:50) were sacrificed 6 weeks after transplant. Splenic huCD45+CD3+ cells were analyzed and showed comparable percentages of CD4+, CD8+, CD4+CD25+, CD56+ and CD4+CD8+ T cells.

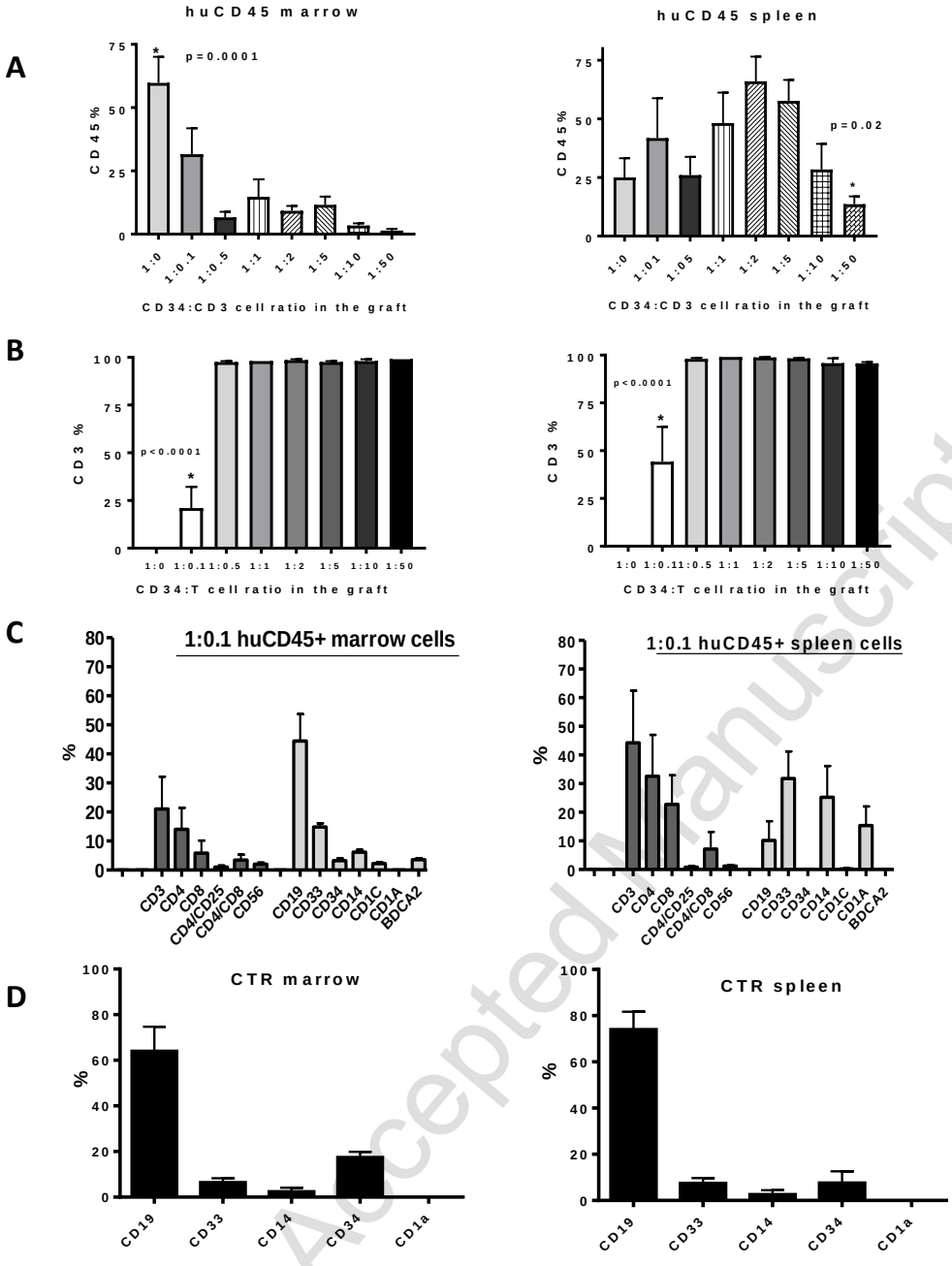


Figure 1

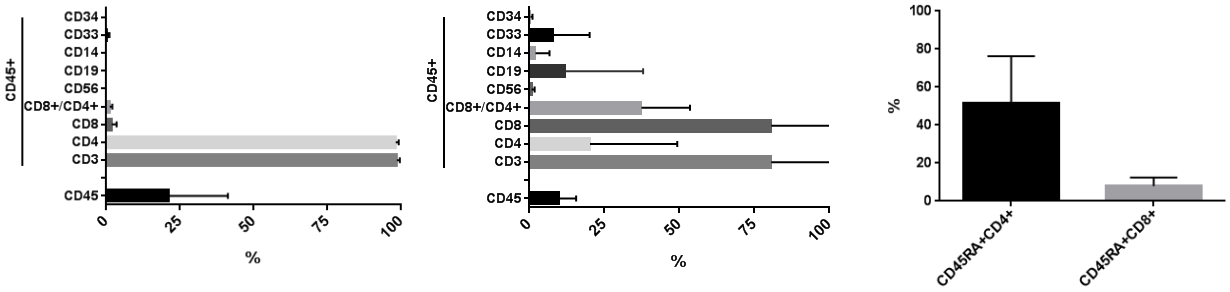
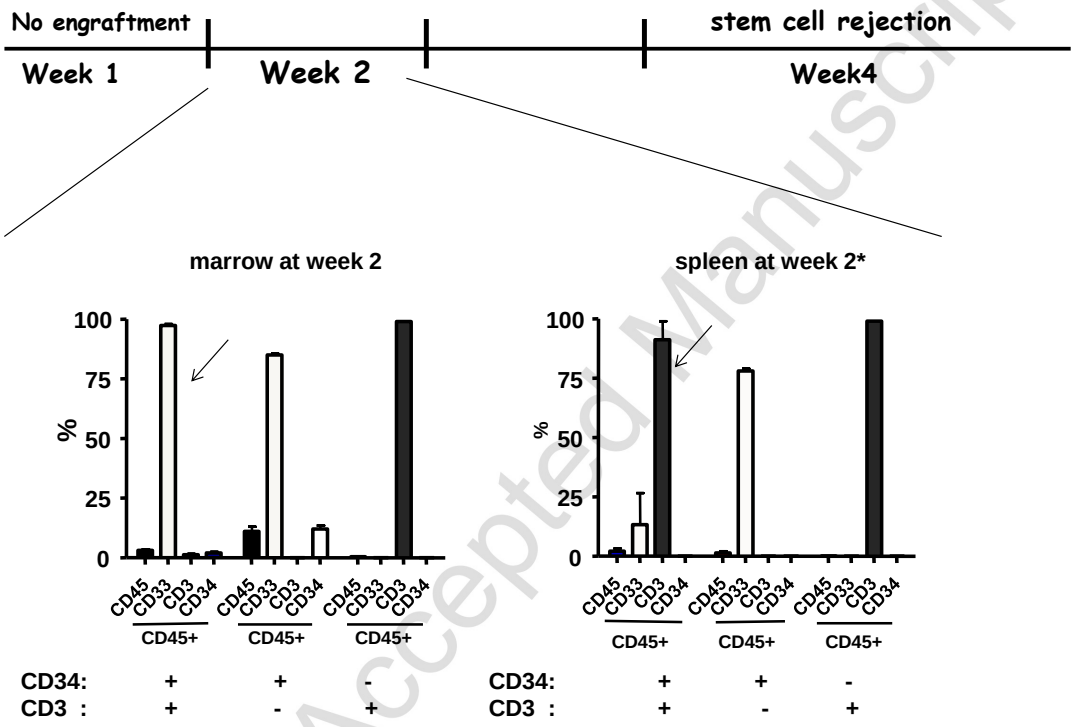


Figure 2.



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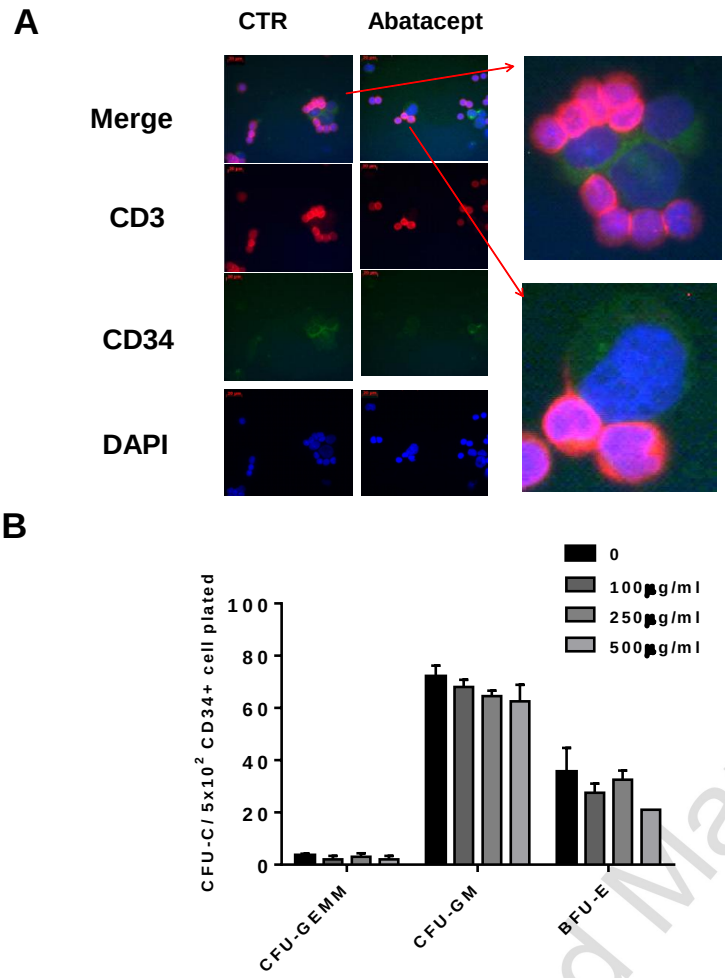

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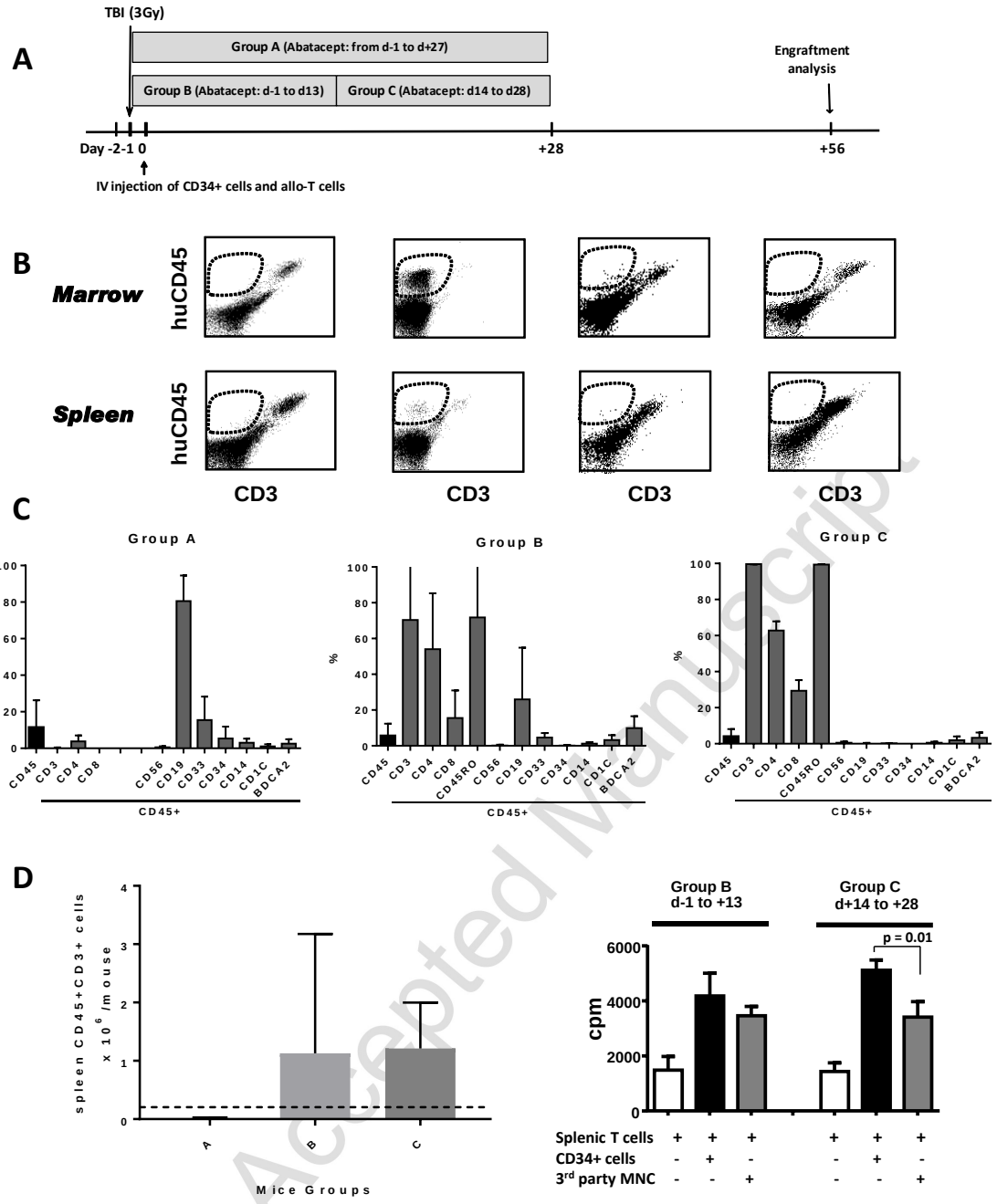

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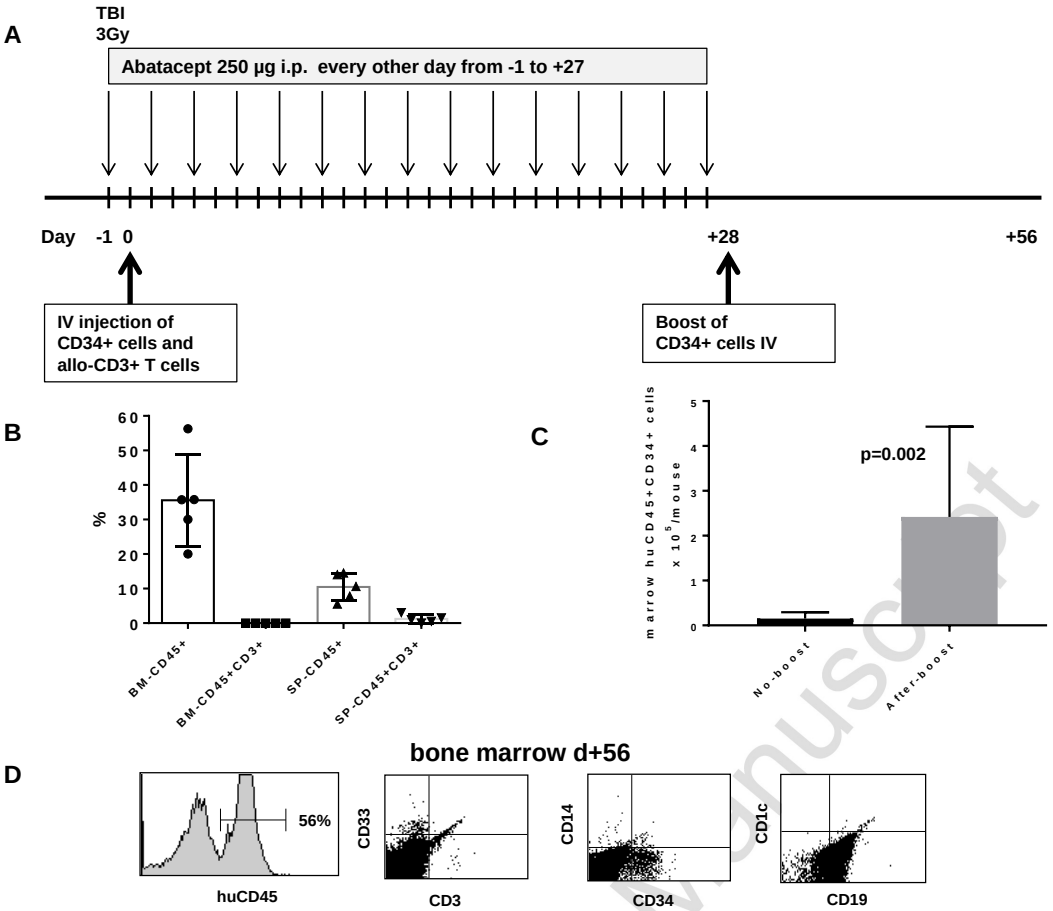
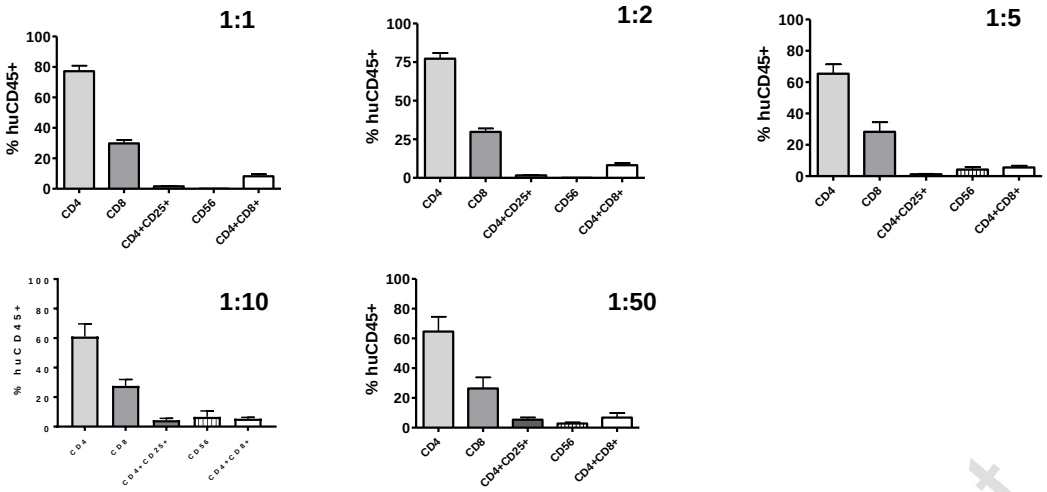


Figure 6.



Suppl. Figure 1.