Title: Bone marrow neutralizes regulatory T-cells

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Statement of Translational Relevance

CD4⁺CD25⁺ regulatory T-cells (Tregs) are appealing tools for the prevention of Graft-versus-Host Disease (GvHD) after allogeneic stem cell transplantation, mainly because murine models suggest that adoptive transfer of these cells will not hamper the Graft versus Tumor (GvT) effect. Nonetheless, it is currently unknown whether human Tregs can be safely applied in different types of hematological malignancies. Addressing this issue in a humanized murine model, we now discovered that ex vivo cultured human Tregs do not suppress anti-tumor immunity in the bone marrow, but readily suppress the anti-tumor immunity if the tumor is located outside the bone marrow. Further exploration of this remarkable phenomenon revealed that bone marrow stromal cells reverse the suppressive activity of Tregs and promote interleukin-17 expression in these cells via the production of interleukin-1β and interleukin-6. These results provide new insights into the Treg immunobiology and indicate the conditional benefits of future Treg-based therapies.
Abstract

Purpose: Regulatory T-cells (Tregs) are potent tools to prevent Graft-versus-Host Disease (GvHD) induced after allogeneic stem cell transplantation or donor lymphocyte infusions. Toward clinical application of Tregs for GvHD treatment, we investigated the impact of Tregs on the therapeutic Graft-versus-Tumor (GvT) effect against human Multiple Myeloma (MM) tumors with various immunogenicities, progression rates and localizations in a humanized murine model.

Experimental Design: Tumor bearing immunodeficient Rag2−/−γc−/− mice bearing various human MM tumors were treated with human PBMC alone or together with autologous ex vivo cultured Tregs. Mice were analyzed for the in vivo engraftment, homing of T cell subsets, development of GvHD and GvT. In additional in vitro assays Tregs that were cultured together with BM stromal cells were analysed for phenotype and functions.

Results: Treatment with PBMC alone induced variable degrees of anti-tumor response, depending on the immunogenicity and the growth rate of the tumor. Co-infusion of Tregs did not impair the anti-tumor response against tumors residing within the bone marrow (BM), irrespective of their immunogenicity or growth rates. In contrast, Tregs readily inhibited the anti-tumor effect against tumors growing outside the BM. Exploring this remarkable phenomenon, we discovered that BM stroma neutralizes the suppressive activity of Tregs in part via production of interleukin-1β/interleukin-6. We furthermore found in vitro and in vivo evidence of conversion of Tregs into interleukin-17-producing T cells in the BM environment.

Conclusions: These results provide new insights into the Treg immunobiology and indicate the conditional benefits of future Treg-based therapies.
Introduction

Naturally occurring regulatory T-cells (Tregs) possess excellent capacities to suppress T cells mediating GvHD after allogeneic Stem Cell Transplantation (allo-SCT) and donor lymphocyte infusions (DLI) (1-6). Indeed, the first clinical trial showed the successful control of GvHD by co-infusion of CD4⁺CD25⁺ Tregs, hereby encouraging the further clinical application of Tregs in this setting (6). Nonetheless, when allo-SCT and DLI are applied as treatment of hematological cancers, a potential pitfall may be the suppression of the beneficial Graft-versus-Tumor (GvT) effects. Although some murine studies demonstrated the maintenance of the GvT effect after co-infusion of Tregs with effector T cells (7, 8) the impact of human Treg infusions on GvT remains largely unexplored (6). In particular, it is unknown whether Tregs will permit GvT against different types of hematological tumors, with different immunogenicities, growth patterns and locations. Furthermore, it needs to be clarified why Tregs allow GvT in the allo-SCT setting (7, 8), whereas they seriously impair the anti-tumor immunity in several models for solid tumors (9-15). Toward application of human Tregs in clinical trials, we were prompted to gain thorough insight in tumor-related conditions, such as tumor immunogenicity and tumor location, which might influence the effect of Tregs on GvT. To address this, we applied a humanized GvT model, in which infusion of human PBMC in immune deficient RAG2⁻/⁻γc⁻/⁻ mice carrying human tumors induces a T-cell-mediated GvT effect, but also lethal xenogeneic(x) GvHD (16, 17). Previously, we have optimized this model to accommodate various multiple myeloma (MM) tumors with different immunogenicities growth rates and growth locations, i.e. either primarily inside or outside the bone marrow (BM) (16). Furthermore, we have shown that freshly isolated human Tregs can effectively control the development of xGvHD in this model (1). Although there are many differences between xGvHD and human allo GvHD, the successful control of xGvHD by human Tregs in this model provided us a highly useful readout to measure the in vivo functionality of Tregs. Thus using this platform, we now explored the impact of human Tregs on MM tumors with different immunogenicities, growth rates and localizations. Since clinical application of
Tregs requires *ex vivo* expanded Tregs (18), we deliberately used *ex vivo* cultured Tregs, and tested their influence on GvT induced by autologous T-cells.

We demonstrate that fully functional *ex vivo* cultured human Tregs - as evident by suppression of xGvHD-, do not suppress the GvT effect against MM tumors that reside within the BM, largely independent of their immunogenicity or growth rates. However, human Tregs readily inhibited the GvT effect against tumors residing outside the BM. Further exploration provided evidence that this remarkable phenomenon may be due to the capacity of BM to neutralize the suppressive activity of Tregs, in part by converting them into interleukin-17 (IL-17)-producing T cells.
Materials and methods

Isolation of human BM cells, blood cells, and expansion of human Tregs

Human PBMCs (huPBMCs) for isolation of Tregs, in vitro suppression assays and infusion into mice were isolated from healthy donors by Ficoll density gradient centrifugation (16). PBMC and BM cells from MM patients for in vitro studies on BM and Treg characterization in clinical samples were collected with approval of the Medical Ethical Committee of the University Medical Center Utrecht. Human CD4⁺CD25⁺ Tregs were isolated from PBMCs using immunomagnetic Treg isolation beads (Miltenyi Biotec) according to the manufacturer’s protocol, unless indicated otherwise. Isolated Tregs were ex vivo expanded by stimulation with CD3/CD28 T-cell expander beads (Invitrogen) according to the manufacturer’s protocol in X-VIVO-15 medium (Lonza) containing 5% human serum (Sanquin), 120 U/ml IL-2 (Proleukin) and 100 mg/ml kanamycin (Invitrogen) for 10-14 days. At the start of culture 5ng/ml recombinant TGF-β1 (Peprotech) was added to stabilize expression of FOXP3 and suppressive function(19). Using this protocol, Tregs expanded more than 100-fold after one round of expansion ex vivo (Supplementary Figure S1A). Expanded Tregs displayed potent suppressor activity in vitro (Supplementary Figure S1B). The expanded Treg cultures contained 70% +/- 3% Foxp3+ T cells which were also negative for CD127 prior to application in our assays. The cultures also contained 4.5 +/- 3.6 % IL-17 producing cells as detected after stimulation with PMA/Ionomycin (see also ref 20).

To analyze the effect of BM stromal cells and MM cells on Tregs, the ex vivo cultured Tregs were further expanded in the presence of irradiated BM stromal cells (passage 2-5 of stromal cells derived from MM-patients and the stromal cell line HS-5 (ATCC); 1 cell per 2 Tregs) and irradiated MM (U266, LME-1) cells (1 cell per Treg) during 10-14 days. IL-6 and IL-1β were neutralized with anti-IL-6 antibody (eBioscience) and recombinant human IL-1receptor antagonist (IL-1RA) (R&D systems) as described elsewhere (21).
In vitro analysis of Treg function

In vitro suppression of T-cell proliferation by Tregs was tested either in standard $^3$H-tritium based proliferation assays (Supplementary Figure S1) or in assays with CFSE-labeled responder T-cells (Tresp) (figure 4A) after stimulation of the responder cells with CD3/CD28 expander beads in 96-wells flat bottom plates (Corning) as previously reported(22). Flow cytometry of CFSE-based proliferation assays was carried out with a FACSCanto II equipped with a HTS-plate reader (Becton Dickinson). Suppression of proliferation was calculated as follows: % suppression = 100% * ([proliferation Tresp alone) - (proliferation Tresp in the presence of Tregs)]/ (proliferation Tresp alone).

Mice

Immune deficient RAG2$^{-/-}$γc$^{-/-}$ mice (16) were housed Specified-Pathogen-Free at the Central Animal Facility of the University of Utrecht. All experiments were conducted with permission from the local Ethical Committee for Animal Experimentation in accordance with national law.

Induction and monitoring of GvT and xGvHD

The inoculation of RAG2$^{-/-}$γc$^{-/-}$ mice with Luciferase-transduced tumor cells, induction of GvT and xGvHD by injection with human PBMC and monitoring of xGvHD were carried out as described in previous studies (7, 16, 17). BLI was used to quantitatively monitor tumor progression of tumor load inside and outside the BM as has been described in detail previously (16, 23). Briefly, mice received i.v. human MM cells (U266 (5x10$^6$/mouse), RPMI-8226 (5x10$^6$/mouse) or LME-1(1x10$^6$/mouse))(16, 23). After 2-5 weeks, when tumors were visible by BLI, mice were either left untreated (i.v. PBS-infusion) as control, or i.v. infused with human PBMC containing 1x10$^7$ CD3$^+$ T-cells to induce GvT and xGvHD, or i.v. infused with human PBMC containing 1x10$^7$ CD3$^+$ T-cells + 10$^7$ expanded autologous CD4$^+$CD25$^+$ Tregs. The day before infusion of T-cells, macrophages were depleted with clodronate-liposomes to propagate human T-cell-engraftment (16, 17).
Human CD4$^{+}$ and CD8$^{+}$ T cells were monitored in blood and BM of treated mice by flow cytometry as described previously\cite{1, 17, 17}. Tregs were phenotyped by flow cytometry using a human FOXP3 staining set (eBioscience). Cells were analyzed with a FACSCalibur (Becton Dickinson) and quantified using Flow-Count\textsuperscript{TM} Fluorospheres (Beckman Coulter). BLI data were analyzed with M3 Vision software (Biospace lab).

**Phenotype analysis of Tregs after co-culturing with BM stromal cells and MM cells**

After expansion in the presence of BM cells and/or MM cells, Tregs were stimulated with 1ng/ml PMA (AppliChem) and 1μM ionomycin (Boehringer Mannheim) for five hours to stimulate cytokine production for intracellular analysis of IL-17. After two hours 1:1000 Brefeldin A (eBioscience) was added to allow intracellular accumulation of IL-17. Intracellular staining for IL-17 (anti-IL-17A, 64CAP17, eBioscience) and FOXP3 (anti-FOXP3, PCH101, eBioscience) were done according to the manufacturer’s protocol. Flow cytometry for phenotype analysis was done with a FACSCalibur (Becton Dickinson).

**Analysis of cytokine secretion by adherent accessory cells and MM cells**

Human MM cells (U266), BM stromal cells, epithelial cells (HEK 293) and human umbilical vein endothelial cells (HUVEC) were cultured in RPMI medium containing 10% human serum and penicillin/streptomycin (Invitrogen). MM cells were seeded at 1x10\textsuperscript{5}/ml, adherent accessory cells were seeded at 5x10\textsuperscript{5}/ml. After three days supernatant was taken for analysis of secreted cytokines. Secreted IL-1β and IL-6 were quantified using a flow cytometry-based Human Inflammatory Cytokine Cytometric Bead Array (CBA) according to the manufacturer’s protocol (Becton Dickinson). Samples were analyzed with a FACSCalibur (Becton Dickinson).
Statistical analyses

Univariate- and survival analyses of in vivo experiments were executed with GraphPad Prism (version 4.0) and significance of differences were tested with Mann-Whitney U or log-rank tests. Data on paralysis, T-cell counts and in vitro T-cell function and -phenotype were analyzed with a two-tailed t-test (GraphPad Prism). \( P<0.05 \) was considered significant.
Results

Human Tregs remain functional after ex vivo expansion, but permit GvT against MM residing in the BM, largely independent of the tumor-immunogenicity

Since therapeutic application of human Tregs requires high numbers of cells, we expanded freshly isolated CD4^+CD25^+ Tregs for our studies (Supplementary Figure S1A). After confirming their suppressive capacity in vitro (Supplementary Figure S1B), we used them in our in vivo model to evaluate their impact on GvT against MM tumors with different properties. We started the in vivo studies using the U266-derived tumors, which are highly immunogenic with moderate growth rate, and exclusively grow in the BM (16). As expected, infusion of only huPBMC in mice carrying U266-derived MM tumors resulted in high numbers of CD4^+ and CD8^+ T-cells in circulation within two weeks after infusion (Figure 1A). These mice succumbed into lethal xGvHD (Figure 1B), but their tumors were cleared (Figure 2A). Co-infusion of ex vivo expanded Tregs significantly suppressed the numbers of T-cells in peripheral blood, and significantly inhibited lethal xGvHD (Figure 1A, 1B), demonstrating that ex vivo expanded human Tregs maintained their in vivo regulatory function. In contrast, infused Tregs did not compromise the GvT effect against U266-tumors (Figure 2A). These results illustrated for the first time that not only murine Tregs but also human Tregs can permit the therapeutic GvT effect. Ex vivo analysis of T-cells isolated from the BM revealed that infused Tregs were able to display effective homing to BM (Figure 2A inset), but apparently did not diminish the T-cell numbers in this primary site of U266-tumors.

T-cell responses against less immunogenic antigens are more prone to suppression by Tregs (24). Therefore, in the same experiment setting we also questioned whether human Tregs would abrogate GvT against a less immunogenic tumor, derived from human MM cell line RPMI-8226, which also grow primarily in the BM (16). As expected, we observed a less prominent GvT effect against RPMI-tumors (16). Co-infusion of human Tregs had only a slight but not a significant inhibitory effect on GvT (Figure 2B). These results, although not entirely ruling out the possibility, indicated that the immunogenicity of the tumor was not a major factor influencing the impact of Tregs on allogeneic
GvT. Finally, when we used a murine BM tumor (A20 lymphoma) in similar settings, we observed that effector cells in human PBMC could also mediate a xenogeneic anti-tumor effect against these murine tumors (supplementary figure S2). However, this anti-tumor effect was also not downregulated by Tregs, which were otherwise capable of reducing the xGvHD.

**Tregs inhibit the GvT effect against tumors residing outside the BM**

Human MM tumors U266 and RPMI as well as the murine A20 tumor exclusively grow in the BM in our model. Thus, to explore whether tumor location would influence the impact of Tregs on GvT, we used the MM cell line LME-1, which establishes aggressive tumors not only inside but also outside the BM(16). Remarkably, we observed that the outcome of Treg-treatment depended on the tumor location: co-infusion of Tregs with PBMC resulted in significant outgrowth of tumors outside the BM (Figure 3A, 3B), while leaving the GvT effect against tumors residing inside the BM largely unaffected (Figure 3A, 3C). These results for the first time identified tumor location as an important factor influencing the clinical outcome of Treg treatment, and suggested that BM creates an environment preventing suppression by Tregs.

**BM stroma converts Tregs into non-suppressor T-cells**

Emerging evidence reveals that Tregs display plasticity to be converted into non-regulatory T-cells, mainly by pro-inflammatory cytokines IL-1β and IL-6 (21,26-29). Since Tregs did not suppress GvT in the BM (Figures 2 and 3), and since BM stromal cells abundantly secreted these cytokines (data not shown), we questioned whether BM stroma would convert Tregs into non-suppressor cells. To this end, in a first set of experiments we cultured Tregs in the presence of U266 cells, with or without addition of BM stromal cells. Tregs cultured in the presence of BM stromal cells displayed significantly reduced suppressive capacity as compared to control cultures (Figure 4A), remarkably without significant reduction in the FOXP3 expression (Figure 4B, 4C). Since in a number of studies neutralization of suppressive function of Tregs correlated with the induction of IL-17 expression (28),
we tested whether Tregs cultured with BM stromal cells produced IL-17. Indeed, in parallel with loss of suppressive capacity, a significantly increased proportion of FOXP3⁺ T cells cultured in the presence of BM stromal cells showed production of IL-17 (Figure 4D). Furthermore, neutralizing the activity of IL-1β and IL-6 in these cultures reverted the Treg-neutralizing effects of BM stroma in four of six donors, and the IL-17-inducing effect in all donors (p<0.05) (Figure 4D). Similar results were obtained using the LME-1 cells instead of U266 cells and using the Tregs from two additional donors (figure 4E). Finally, we addressed as to whether freshly isolated but unexpanded Tregs would display a similar behavior as the expanded Tregs in the presence of BM stromal cells. Culture of freshly isolated CD4⁺CD127dimFOXP3⁺ Tregs from 3 healthy donors in the presence of BM stromal cells alone or in the presence of MM cells revealed similar results: The significant induction of IL-17 expression within FOXP3⁺ cells in the presence of BM stromal cells revealed that the conversion into IL-17 producing cells is a property of both expanded and freshly isolated and unexpanded Tregs.

Since the in vitro assays suggested the conversion of Tregs into IL-17 producing non-suppressive cells in the BM microenvironment, we sought evidence for this in our LME-1 model, in which the GvT effect against extramedullary but not medullary tumors was abrogated. In a similar setting we treated the mice bearing LME-1 tumors with PBMC, PBMC+ Treg or no treatment. As expected, the Treg infusion controlled the xGvHD, reduced the CD4⁺ and CD8⁺ T cell numbers at week 3 but had a similar differential effect on GvT in the intra- and extramedullary sites (Supplementary figure S3). Hence we analyzed the conversion of Tregs into IL-17 producing cells within and outside the BM compartment (figure 5). Since large extramedullary tumors did not allow the isolation of sufficient numbers of human T cells, spleen was analyzed as a site outside the BM. In addition, we have injected large numbers of effector PBMC+ Tregs in subcutaneously generated LME-1 tumors. In all analyzed mice, the fraction of FOXP3⁺ cells producing IL-17 was higher in the BM than in the spleen. Furthermore, FOXP3⁺ Tregs recovered from subcutaneous tumors showed little or no IL-17 expression. These results indicated that the conversion of Tregs into IL-17 producing cells...
preferentially occurred in the BM compartment and substantiated the idea that permission of GvT by Tregs may indeed be related to the neutralization of their suppressive activity and conversion into IL-17-producing cells by the stromal cells in the BM compartment.

Finally, we sought evidence for the phenomenon of IL-17 propagation in Treg by the BM environment in humans. Therefore, we analyzed BM and blood samples from MM patients for the expression of IL-17 in the FOXP3+CD4+ Tregs (figure 6). In the BM, a significantly higher proportion of FOXP3+CD4+ T cells produced IL-17 as compared to peripheral blood, suggesting that IL-17 propagation found in our humanized mouse model may also apply to the human situation.
Discussion

In this study we demonstrate that fully functional ex vivo cultured human Tregs can permit the T donor T cell mediated therapeutic anti-tumor effect against MM. However, the maintenance of the anti-tumor effect by Tregs appears a conditional phenomenon as it did not occur outside the BM. Our analyses towards understanding the mechanism of this remarkable finding provide evidence that BM stromal cells can generate a microenvironment that neutralizes the suppressor activity of Tregs. The neutralization of Tregs by BM stromal cells was in part due to the secretion of IL-1β and IL-6, which appeared to convert a fraction of Tregs into IL-17-producing cells.

Our findings may have important implications for the clinical application of human Tregs in the allo-SCT/DLI setting. First, by partly confirming the previous findings in murine models (7, 8) our results indeed encourage the co-infusion of naturally occurring Tregs in GvHD treatment. However, we now propose that Treg treatment will be appropriate and safe only in case of hematological malignancies progressing exclusively in the BM. This conclusion is supported by the distinct outcome of Treg administration on the GvT effect against LME-1 tumors growing in- and outside the BM. Actually, this novel finding of conditional regulation by Tregs in our model fits well with unexplained and apparently conflicting observations in previous studies. For instance, in several solid tumor models, where tumors usually reside outside the BM, Tregs were consistently shown to hamper anti-tumor immunity(9-11, 30). In contrast, in murine allo-transplantation models, in which hematological tumors often reside in the BM, Tregs did not hamper the GvT effect (7, 8). In fact, in some allo-transplantation models GvT was abrogated when hematological tumors were put outside the BM (8, 12). None of these studies, however, attributed a role for the BM environment in the clinical outcome, probably because they were not executed in a controlled manner (same tumor, inside and outside the BM at the same time) as we did. Now, our results combined with these previous observations, strongly suggest that BM microenvironment may contribute importantly to the outcome of Treg therapy. The mechanism of this may be the capacity the BM stromal cells to neutralize the suppressor function of Tregs.
Although we did not directly demonstrate the in vivo Treg-neutralization, several indications made us to draw this conclusion: the first indication is the observation that Tregs, while capable of controlling x-GvHD and down regulating the GvT effect outside the BM, and while capable of homing to BM, did not affect the development of GvT in this latter compartment. This suggested a conditional loss of Treg function in the BM compartment. This suggestion is further supported by the in vitro demonstration that BM stromal cells significantly reduced the suppressive functions of Tregs. Since the loss of suppressive functions of Tregs is frequently linked to the conversion into IL-17 producing T cells (21, 27-29, 31) we also tested this possibility. Indeed, we found that a fraction of FOXP3⁺ T cells produced IL-17 in the presence of BM stromal cells. In further analysis, we found in vivo evidence for the conversion of Tregs into IL-17 producing cells in the BM compartment. In addition, in an analysis of BM and PBMC samples obtained from MM patients, BM was found to contain more FOXP3⁺ T-cells expressing IL-17-producing cells as compared to PBMC. This finding suggests that IL-17 propagation by BM demonstrated in our humanized mouse model is relevant for the clinical human situation, and therefore warrants further studies to unravel the exact mechanisms of action and the role of IL-17⁺ Tregs in the clinical setting. Thus, in the light of these results, it is tempting to speculate that our study represents one of the first examples for the clinical relevance of the recently emerging concept of Treg-plasticity, i.e. the conversion of Tregs into non-suppressor IL-17 producing T-cells under the influence of certain cytokines, in particular IL-6 and IL-1β (21, 27-29,31). The fact that neutralizing the activity of IL-1β and IL-6 in our in vitro assays reverted the Treg-neutralizing and the IL-17-inducing effects of BM stroma further substantiates this conclusion. However, it needs to be noted that, in our assays conversion to IL-17⁺ cells occurred in a small fraction of FOXP3⁺ T-cells. It is therefore likely that other mechanisms of Treg neutralization exist, and are worth investigating in future studies. Also noteworthy, the reduced suppressive function in our experiments was not accompanied by reduced frequency of FOXP3⁺ cells. This result is consistent with recent studies indicating that FOXP3 is not the only factor that determines the suppressive function of Tregs (32-36). Furthermore, this finding
demonstrates that the loss of suppressive function in our in vitro cultures was not due to the outnumbering of FOXP3⁺Tregs by residual FOXP3⁻ non-Tregs.

Our results may have specific implications for MM, which is a typical BM-residing tumor, but can also manifest at extramedullary sites. First of all, the treatment of MM by allogeneic transplantation and DLI is possible, but there is currently much reluctance to apply this mode of cellular immunotherapy due to severe toxicity related to GvHD. In the light of our results, we anticipate that MM patients with exclusively medullary tumors may significantly benefit from Treg therapy combined with DLI (3).

Here, an apparent controversial issue is the fact that, the IL-6 and IL-1β produced by BM stroma may also support growth of MM and are thought to induce resistance to conventional therapies (36). Furthermore, IL-17 producing T cells are also thought to promote MM growth (38). However, our results suggest that these pathogenic effects may be effectively compensated by neutralization of Tregs by providing an optimal platform for cytotoxic T cells to mediate the GvT effect.

Secondly, the differential Treg activity in medullary vs extramedullary sites may also help us to understand the mechanisms of extramedullary relapses, which comprises around 20% of all relapses after allo SCT (39). However, since the majority of the patients display systemic relapses, and isolated extramedullar relapses occur only in a minority (2%) of the patients (39), it should be noted that differential Treg activity in medullary vs extramedullary sites is certainly not the main reason why relapse remains the most important cause of treatment failure.

Since in our model the neutralization of Tregs was mainly dependent on BM stromal cells, rather than the tumor itself, our results may be also relevant for other type of tumors residing in the BM. Furthermore, it seems also conceivable that other stromal cells can convert Tregs into non-suppressor cells. Stromal cells of other tissues may also be capable of producing IL-6 and IL-1β to neutralize Tregs. This may be in particular relevant for a number of autoimmune diseases such as rheumatoid arthritis and diabetes, because such a mechanism may also explain why Tregs in the inflamed organs of these autoimmune diseases are dysfunctional and tend to differentiate into Th17 cells (40,41).
Altogether, our results demonstrate that BM may provide a favorable environment for effector T-cells to mediate an adequate GvT effect by neutralizing Tregs. Our results also provide new opportunities for further exploitation and regulation of Tregs in the treatment of cancer and autoimmune diseases.

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**Supplementary information**

Supplementary information is presented in Figures S1 and S2.
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Figure legends

Figure 1. Human Tregs remain capacity to suppress xGvHD after ex vivo expansion.

Human PBMC were infused with or without co-infusion of expanded human CD4+CD25+ Tregs to treat immune deficient mice carrying human multiple MM tumor cells in the bone marrow. Two weeks after infusion of PBMC (n=8) or PBMC + Treg (n=9), numbers of human CD4+ and CD8+ cells in blood were counted (values show arbitrary units compared to standard number of count-beads added to sample) (A). Suppression of GvHD by Tregs is shown as mice surviving PBMC-induced lethal GvHD after infusion of PBMC (n=13) or PBMC + Treg (n=15) (B). Each figure represent one of two independent experiments, and in each experiment PBMC/Tregs (autologous combinations per recipient mouse) from two different human cell donors were tested (each of which infused in half of number of mice per group). Error bars indicate SEM. * p<0.05.

Figure 2. Ex vivo cultured human Tregs allow allogeneic GvT induced by human PBMC against multiple MM in the bone marrow.

Human PBMC were infused with or without co-infusion of expanded human CD4+CD25+ Tregs to treat immune deficient mice that carry human multiple MM tumor cells in the BM. GvT against high-immunogenic MM tumor cell line U266 (Figure A; PBMC:n=13, PBMC + Treg:n=15, untreated PBS control: n=14) and low-immunogenic cell line RPMI (Figure B; PBMC:n=8, PBMC + Treg:n=9, untreated PBS control: n=6) was monitored by bioluminescence-imaging (BLI) of luciferase expression by Luciferase-gene marked tumor cells. The inset bar in Figure A shows frequencies of human CD4+ and CD8+ cells in BM and the frequency of FOXP3+ in the CD4+ population in the BM two weeks after infusion of PBMC (black bars, n=2) or PBMC + Treg (white bars, n=2). Figures represent one of two independent experiments, and in each separate experiment PBMC/Tregs (autologous combinations per recipient) from two different human cell donors were tested (each of which infused in half of number of mice per group). Error bars indicate SEM.
**Figure 3. Human Tregs suppress GvT against tumors growing outside the bone marrow.**

Human PBMC were infused with or without co-infusion of expanded human CD4^+CD25^+ Tregs into immune-deficient mice carrying *Luciferase*-transduced LME-1 multiple MM (MM) tumors growing both outside (extramedullary) and inside (intramedullary) the bone marrow (n=4/group). Extramedullary and intramedullary tumor load was analyzed by BLI as done in previous studies(16, 22) and indicated by gates (A). Figure A exemplifies tumor load in different groups at week 6 after treatment. Mean extramedullary tumor load (B) and mean intramedullary tumor load (C) per group was analyzed using gates shown in Figure 3A weekly. Error bars indicate SEM. * p<0.05.

**Figure 4. Bone marrow stroma induces a non-suppressive phenotype and IL-17-production in human Tregs via the IL-6/IL-1β axis.**

Tregs were cultured with anti-CD3/anti-CD28 and IL-2 in the absence (neutral (-) condition) or presence of either multiple MM (MM) ) (U266 in A-D; U266 and LME-1 in E), or MM + bone marrow (BM) stromal cells. After one round of stimulation, Tregs were analyzed for their loss of capacity to suppress T-cell proliferation (n=6 donors) (A), for the mean FOXP3-expression expressed as mean fluorescence intensity (MFI) (n=5 Treg donors, mean±SEM for neutral condition: 136±54)(B) , for the percentage of cells producing FOXP3 (n=5 Treg donors, mean±SEM for neutral condition: 48±6) (C), and for IL-17-expression within the FOXP3^+ population (n= Treg 5 donors, mean± SEM for MM+BM condition: 4.5±0.7) (D). All values are normalized to the neutral (-) condition to overcome bias by variation between experiments. Blocking anti-IL-6 antibody (αIL-6) and IL-1RA, antagonizing IL-1β activity, was added to assess the involvement of IL-6 and IL-1β in both loss of suppressive function (A) and induction of IL-17-production (D) in Tregs. Figure E shows for two different Treg donors IL-17^+ cells within the FOXP3^+ population after culture with U266 or with LME-1 in the presence/absence of BM. Error bars indicate SEM. * p<0.05. (F) Conversion of freshly isolated CD4^+CD25^+CD127^dim FOXP3^+ Tregs from 3 healthy donors upon culture with BM stromal cells and MM cells **,** *** P<0.005 and
0.0005, respectively. The frequency of IL-17⁺ cells within the FOXP3⁺ cells is depicted after normalization for the control culture. For this experiment CD4⁺ and CD127dim cells were isolated using MACS. Virtually all CD4⁺CD127dim cells were also Foxp3⁺.

**Figure 5. Bone marrow propagates IL-17 in human Tregs in vivo.**

To assess the in vivo impact of bone marrow on IL-17 production by Tregs, T cells were isolated from BM or from spleen of LME-1 tumor carrying mice, which were treated with human PBMC alone or in combination with Treg (n=4) at day 14 of the treatment. To isolate sufficient number of T cells from extramedullar tumors, the effector cells and Tregs were injected into subcutaneously generated tumors in separate mice (n=6). IL-17 production in the FOXP3⁺ Treg fractions was analyzed. Error bars indicate SEM. *, ** p<0.05, and <0.005 respectively (unpaired t test).

**Figure 6. Bone marrow propagates IL-17 in Tregs in clinical samples**

IL-17 production in the FOXP3⁺ Treg fraction of multiple MM patients was analyzed in peripheral blood (PB; n=14), as extramedullary organ, and compared to bone marrow (BM; n=14). * p<0.05.
FIGURE 1

A

B

PBMC

PBMC + Treg

PBMC

PBMC + Treg

P = 0.0069

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FIGURE 2

A

B
FIGURE 5
FIGURE 6

[Graph showing IL-17 expression in Treg (% in FoxP3+ T-cells) for human PB and BM]
Human regulatory T-cells do not suppress the anti-Tumor immunity in the Bone Marrow: a role for Bone Marrow Stromal cells in Neutralizing Regulatory T cells

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