Human Regulatory T Cells Do Not Suppress the Antitumor Immunity in the Bone Marrow: A Role for Bone Marrow Stromal Cells in Neutralizing Regulatory T Cells

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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 tumors with various immunogenicities, progression rates, and localizations in a humanized murine model.

Experimental Design: Immunodeficient Rag2⁻/⁻γc⁻/⁻ mice, bearing various human multiple myeloma tumors, were treated with human peripheral blood mononuclear cell (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 bone marrow stromal cells were analyzed for phenotype and functions.

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

Conclusions: These results provide new insights into the Treg immunobiology and indicate the conditional benefits of future Treg-based therapies. Clin Cancer Res; 19(6); 1–9. ©2012 AACR.

Introduction

Naturally occurring Tregs possess excellent capacities to suppress T-cells–mediating GVHD after allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusions (DLI; refs. 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 hematologic cancers, a potential pitfall may be the suppression of the beneficial graft-versus-tumor (GVT) effects. Although some murine studies showed 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 hematologic 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 antitumor 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 peripheral blood mononuclear cell (PBMC) in immune-deficient RAG2⁻/⁻γc⁻/⁻ mice carrying human tumors induces a T-cell–mediated GVT effect, but also lethal...
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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 hematologic malignancies. Addressing this issue in a humanized murine model, we now discovered that ex vivo cultured human Tregs do not suppress antitumor immunity in the bone marrow, but readily suppress the antitumor 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 (IL-17) expression in these cells via the production of IL-1β and IL-6. These results provide new insights into the Treg immunobiology and indicate the conditional benefits of future Treg-based therapies.

Materials and Methods

Isolation of human bone marrow 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 bone marrow cells from multiple myeloma patients for in vitro studies on bone marrow and Treg characterization in clinical samples were collected with approval of the Medical Ethical Committee of the University Medical Center Utrecht (the Netherlands). Human CD4⁺CD25⁺ Tregs were isolated from PBMCs using immunomagnetic Treg isolation beads (Miltenyi Biotech) 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 to 14 days. At the start of culture, 5 ng/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 1 round of expansion ex vivo (Supplementary Fig. S1A). Expanded Tregs displayed potent suppressor activity in vitro (Supplementary Fig. S1B). The expanded Treg cultures contained 70 ± 3% Foxp3⁺ T cells, which were also negative for CD127 before application in our assays. The cultures also contained 4.5 ± 3.6% IL-17-producing cells as detected after stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin (see also ref. 20).

To analyze the effect of the bone marrow stromal cells and multiple myeloma cells on Tregs, the ex vivo cultured Tregs were further expanded in the presence of irradiated bone marrow stromal cells (passage 2–5 of stromal cells derived from multiple myeloma patients and the stromal cell line HS-5 [American Type Culture Collection, Manassas, VA]; 1 cell per 2 Tregs) and irradiated multiple myeloma (U266, LME-1) cells (1 cell per Treg) during 10 to 14 days. IL-6 and IL-1β were neutralized with anti–IL-6 antibody (eBioscience) and recombinant human IL-1 receptor antagonist (IL-1RA; R&D systems) as described elsewhere (21).

In vitro analysis of Treg function

In vitro proliferation of T-cell proliferation by Tregs was tested either in standard ³H-tritium–based proliferation assays (Supplementary Fig. S1) or in assays with CFSE-labeled responder T cells (Tresp) (Fig. 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 an 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 bone marrow as has been described in detail previously (16, 23). Briefly, mice received i.v. human multiple myeloma cells (U266 [5 × 10⁶/mouse], Roswell Park Memorial Institute [RPMI]-8226 [5 × 10⁶/mouse] or LME-1 [1 × 10⁶/mouse]; refs. 16 and 23). After 2 to 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 1 × 10⁷ CD3⁺ T cells to induce GVT and xGVHD, or i. v. infused with human PBMC containing 1 × 10⁷ CD3⁺ T cells + 10² 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 bone marrow of treated mice by flow cytometry as described previously (1, 17, 17). Tregs were phenotyped by flow cytometry using a human FOXP3 staining set (eBioscience). Cells were analyzed with a FACS Calibur (Becton Dickinson) and quantified using Flow-Count Fluospheres (Beckman Coulter). BLI data were analyzed with M3 Vision software (Biospace lab).

**Phenotype analysis of Tregs after coculturing with bone marrow stromal cells and multiple myeloma cells**

After expansion in the presence of bone marrow cells and/or multiple myeloma cells, Tregs were stimulated with 1 ng/ml PMA (AppliChem) and 1 μmol/L ionomycin (Boehringer Mannheim) for 5 hours to stimulate cytokine production for intracellular analysis of IL-17. After 2 hours, 1:1,000 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-FOX3, PCH101; eBioscience) were done according to the manufacturer’s protocol. Flow cytometry for phenotype analysis was done with a FACS Calibur (Becton Dickinson).

**Analysis of cytokine secretion by adherent accessory cells and multiple myeloma cells**

Human multiple myeloma cells (U266), bone marrow stromal cells, epithelial cells (HEK293), and human umbilical vein endothelial cells (HUVEC) were cultured in RPMI medium containing 10% human serum and penicillin/streptomycin (Invitrogen). Multiple myeloma cells were seeded at 1 × 10⁷/mL, adherent accessory cells were seeded at 5 × 10⁴/mL. After 3 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 T-cell phenotype were analyzed with a 2-tailed t test (GraphPad Prism). P < 0.05 was considered significant.

**Results**

Human Tregs remain functional after ex vivo expansion, but permit GVT against multiple myeloma residing in the bone marrow, largely independent of the tumor immunogenicity

Because therapeutic application of human Tregs requires high numbers of cells, we expanded freshly isolated CD4⁺CD25⁺ Tregs for our studies (Supplementary Fig. S1A). After confirming their suppressive capacity in vitro (Supplementary Fig. S1B), we used them in our in vivo model to evaluate their impact on GVT against multiple myeloma 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 bone marrow (16). As expected, infusion of only huPBMC in mice carrying U266-derived multiple myeloma tumors resulted in high numbers of CD4⁺ and CD8⁺ T cells in circulation within 2 weeks after infusion (Fig. 1A). These mice succumbed into lethal xGVHD (Fig. 1B), but their tumors were cleared (Fig. 2A). Coinfusion of ex vivo expanded Tregs significantly suppressed the numbers of T cells in peripheral blood, and significantly inhibited lethal xGVHD (Fig. 1A and B), showing 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 (Fig. 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 bone marrow revealed that infused Tregs were able to display effective homing to bone marrow (Fig. 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 multiple myeloma cell line RPMI-8226, which also grow primarily in the bone marrow (16). As expected, we observed a less prominent GVT effect against RPMI-tumors (16). Coinfusion of human Tregs had only a slight but not a significant inhibitory effect on GVT (Fig. 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 bone marrow tumor (A20 lymphoma) in similar settings, we observed that effector cells in human PBMC could also mediate a xenogeneic antitumor effect against these murine tumors (Supplementary Fig. S2).

Figure 1. Human Tregs remain capacity to suppress xGVHD after ex vivo expansion. Human PBMC were infused with or without coinfusion of expanded human CD4+CD25+ Tregs to treat immune-deficient mice carrying human multiple myeloma tumor cells in the bone marrow. A, 2 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 with standard number of count-beads added to sample). B, 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). Each figure represents 1 of 2 independent experiments, and in each experiment PBMC/Tregs (autologous combinations per recipient mouse) from 2 different human cell donors were tested (each of which infused in half of number of mice per group). Error bars indicate standard error of the mean (SEM); *P < 0.05.

Figure 2. Ex vivo cultured human Tregs allow allogeneic GVT induced by human PBMC against multiple myeloma in the bone marrow. Human PBMC were infused with or without coinfusion of expanded human CD4+CD25+ Tregs to treat immune-deficient mice that carry human multiple myeloma tumor cells in the bone marrow. GVT against high-immunogenic multiple myeloma tumor cell line U266 (A; PBMC: n = 13, PBMC + Treg: n = 15, untreated PBS control: n = 14) and low-immunogenic cell line RPMI (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 A shows frequencies of human CD4+ and CD8+ cells in bone marrow and the frequency of FOXP3+ in the CD4+ population in the bone marrow 2 weeks after infusion of PBMC (black bars, n = 2) or PBMC + Treg (white bars, n = 2). Figures represent 1 of 2 independent experiments, and in each separate experiment PBMC/Tregs (autologous combinations per recipient) from 2 different human cell donors were tested (each of which infused in half of number of mice per group). Error bars indicate SEM.
However, this antitumor 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 bone marrow**

Human multiple myeloma tumors U266 and RPMI as well as the murine A20 tumor exclusively grow in the bone marrow in our model. Thus, to explore whether tumor location would influence the impact of Tregs on GVT, we used the multiple myeloma cell line LME-1 (25), which establishes aggressive tumors not only inside but also outside the bone marrow (16). Remarkably, we observed that the outcome of Treg-treatment depended on the tumor location: coinfusion of Tregs with PBMC resulted in significant outgrowth of tumors outside the bone marrow (Fig. 3A and B), while leaving the GVT effect against tumors residing inside the bone marrow largely unaffected (Fig. 3A and C). These results for the first time identified tumor location as an important factor influencing the clinical outcome of Treg treatment, and suggested that bone marrow creates an environment preventing suppression by Tregs.

**Bone marrow stroma converts Tregs into nonsuppressor T cells**

Emerging evidence reveals that Tregs display plasticity to be converted into nonregulatory T cells, mainly by proinflammatory cytokines IL-1β and IL-6 (21, 26–29). Because Tregs did not suppress GVT in the bone marrow (Figs. 2 and 3), and because bone marrow stromal cells abundantly secreted these cytokines (data not shown), we questioned whether bone marrow stroma would convert Tregs into nonsuppressor cells. To this end, in a first set of experiments we cultured Tregs in the presence of U266 cells, with or without addition of bone marrow stromal cells. Tregs cultured in the presence of bone marrow stromal cells displayed significantly reduced suppressive capacity as compared with control cultures (Fig. 4A), remarkably without significant reduction in the FOXP3 expression (Fig. 4B and C). Because 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 bone marrow 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 bone marrow stromal cells showed production of IL-17 (Fig. 4D). Furthermore, neutralizing the activity of IL-1β and IL-6 in these cultures reverted the Treg-neutralizing effects of bone marrow stroma in 4 of 6 donors, and the IL-17-inducing effect in all donors (P < 0.05; Fig. 4D). Similar results were obtained using the LME-1 cells instead of U266 cells and using the Tregs from 2 additional donors (Fig. 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 bone marrow stromal cells. Culture of freshly isolated and unexpanded Tregs from 3 healthy donors in the presence of bone marrow stromal cells alone or in the presence of multiple myeloma cells revealed similar results: The significant induction of IL-17 expression within FOXP3+ cells in the presence of bone marrow stromal cells revealed that the conversion into IL-17–producing cells is a property of both expanded and freshly isolated and unexpanded Tregs.

Because the *in vitro* assays suggested the conversion of Tregs into IL-17–producing nonsuppressive cells in the bone marrow 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 Fig. S3). Hence we analyzed the conversion of Tregs into IL-17–producing cells within and outside the
Because large extra-medullary tumors did not allow the isolation of sufficient numbers of human T cells, spleen was analyzed as a site outside the bone marrow. 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 bone marrow 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 bone marrow 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 bone marrow compartment.

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**Figure 4.** Bone marrow stroma induces a nonsuppressive phenotype and IL-17 production in human Tregs via the IL-6/IL-1\(\beta\) axis. Tregs were cultured with anti-CD3/anti-CD28 and IL-2 in the absence [neutral (-) condition] or presence of other multiple myeloma (U266 in A–D; U266 and LME-1 in E) or multiple myeloma + bone marrow stromal cells. After 1 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 FOX3 (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 multiple myeloma + bone marrow 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 (aIL-6) and IL-1RA, antagonizing IL-1\(\beta\) activity was added to assess the involvement of IL-6 and IL-1\(\beta\) in both loss of suppressive function (A) and induction of IL-17 production (D) in Tregs. E, for 2 different Treg donors IL-17\(^+\) cells within the FOXP3\(^+\) population after culture with U266 or with LME-1 in the presence/absence of bone marrow. Error bars indicate SEM; *, \(P < 0.05\); **, \(P < 0.01\); and ***, \(P < 0.005\), respectively. The frequency of IL-17\(^+\) cells within the FOXP3\(^+\) cells is depicted after normalization for the control culture. For this experiment, CD4\(^+\) and CD127\(\text{dim}\) cells were isolated using MACS. Virtually all CD4\(^+\)CD127\(\text{dim}\) cells were also Foxp3\(^+\).
In the bone marrow, a significantly higher proportion of and compared with bone marrow (combination with Treg (carrying mice, which were treated with human PBMC alone or in cells were isolated from bone marrow or from spleen of LME-1 tumor analyzed. Error bars indicate to the human situation.

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 bone marrow 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 **, P < 0.005, respectively (unpaired t test).

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

Figure 6. Bone marrow propagates IL-17 in Tregs in clinical samples. IL-17 production in the FOXP3+ Treg fraction of multiple myeloma patients was analyzed in peripheral blood (PB; n = 14), as extramedullary organ, and compared with bone marrow (n = 14); *, P < 0.05.

Discussion

In this study we show that fully functional ex vivo cultured human Tregs can permit the T donor T-cell–mediated therapeutic antitumor effect against multiple myeloma. However, the maintenance of the antitumor effect by Tregs seems to be a conditional phenomenon as it did not occur outside the bone marrow. Our analyses toward understanding the mechanism of this remarkable finding provide evidence that bone marrow stromal cells can generate a microenvironment that neutralizes the suppressor activity of Tregs. The neutralization of Tregs by bone marrow stromal cells was in part because of 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 coinfusion of naturally occurring Tregs in GVHD treatment. However, we now propose that Treg treatment will be appropriate and safe only in case of hematologic malignancies progressing exclusively in the bone marrow. This conclusion is supported by the distinct outcome of Treg administration on the GVT effect against LME-1 tumors growing inside and outside the bone marrow. 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 bone marrow, Tregs were consistently shown to hamper antitumor immunity (9–11, 30). In contrast, in murine allo-transplantation models, in which hematologic tumors often reside in the bone marrow, Tregs did not hamper the GVT effect (7, 8). In fact, in some allo-transplantation models GVT was abrogated when hematologic tumors were put outside the bone marrow (8, 12). None of these studies, however, attributed a role for the bone marrow environment in the clinical outcome, probably because they were not executed in a controlled manner (same tumor, inside and outside the bone marrow at the same time) as we did. Now, our results combined with these previous observations strongly suggest that bone marrow microenvironment may contribute importantly to the outcome of Treg therapy. The mechanism of this may be the capacity the bone marrow stromal cells to neutralize the suppressor function of Tregs.

Although we did not directly show 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 downregulating the GVT effect outside the bone marrow, and while capable of homing to bone marrow, did not affect the development of GVT in this latter compartment. This suggested a conditional loss of Treg function in the bone marrow compartment. This suggestion is further supported by the in vitro demonstration that bone marrow stromal cells significantly reduced the suppressive functions of Tregs. Because the loss of suppressive functions of Tregs is frequently linked to

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the conversion into IL-17–producing T cells (21, 27–29, 26), we also tested this possibility. Indeed, we found that a fraction of FOXP3⁺ T cells produced IL-17 in the presence of bone marrow stromal cells. In further analysis, we found in vitro evidence for the conversion of Tregs into IL-17–producing cells in the bone marrow compartment. In addition, in an analysis of bone marrow and PBMC samples obtained from multiple myeloma patients, bone marrow was found to contain more FOXP3⁺ T-cells–expressing IL-17–producing cells as compared with PBMC. This finding suggests that IL-17 propagation by bone marrow showed 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, that is the conversion of Tregs into nonsuppressor IL-17–producing T cells under the influence of certain cytokines, in particular IL-6 and IL-1β (21, 26, 27–29). 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 bone marrow 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 (31–35). Furthermore, this finding shows that the loss of suppressive function in our in vitro cultures was not because of the outnumbering of FOXP3⁺ Tregs by residual FOXP3⁻ non-Tregs.

Our results may have specific implications for multiple myeloma, which is a typical bone marrow–residing tumor, but can also manifest at extramedullary sites. First of all, the treatment of multiple myeloma by allogeneic transplantation and DLI is possible, but there is currently much reluctance to apply this mode of cellular immunotherapy because of severe toxicity related to GVHD. In the light of our results, we anticipate that multiple myeloma 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 bone marrow stroma may also support growth of multiple myeloma and are thought to induce resistance to conventional therapies (36). Furthermore, IL-17–producing T cells are also thought to promote multiple myeloma growth (37). 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.

Second, the differential Treg activity in medullary versus extramedullary sites may also help us to understand the mechanisms of extramedullary relapses, which comprises around 20% of all relapses after allo-SCT (38). However, because the majority of the patients display systemic relapses, and isolated extramedullar relapses occur only in a minority (2%) of the patients (38), it should be noted that differential Treg activity in medullary versus extramedullary sites is certainly not the main reason why relapse remains the most important cause of treatment failure.

Because in our model the neutralization of Tregs was mainly dependent on bone marrow stromal cells, rather than the tumor itself, our results may be also relevant for other type of tumors residing in the bone marrow. Furthermore, it seems also conceivable that other stromal cells can convert Tregs into nonsuppressor 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 (39, 40).

Altogether, our results suggest that bone marrow 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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