CD4+CD25+FOXP3+ T regulatory cells reconstitute and accumulate in the bone marrow of patients with multiple myeloma following allogeneic stem cell transplantation

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Very little is known about the number and function of immunosuppressive CD4+CD25+FOXP3+ T regulatory cells (Treg) in the human bone marrow and it is unclear whether bone marrow-residing Treg are capable of regenerating following allogeneic stem cell transplantation. This is particularly surprising since the bone marrow represents a major priming site for T-cell responses and Treg play important roles in the prevention of T-cell-mediated graft-versus-host disease and in promoting tumor escape from T-cell-dependent immunosurveillance.

Design and Methods
Applying flow cytometry, real-time polymerase chain reaction, and functional assays, we performed the first study on bone marrow and peripheral blood Treg in healthy donors as well as multiple myeloma patients before and after allogeneic stem cell transplantation.

Results
We found that, following the allogeneic transplantation, donor-derived CD4+CD25+FOXP3+ Treg expanded faster than conventional CD4+ T cells, leading to an accumulation of Treg in the bone marrow of transplanted patients who lack relevant thymic function. The reconstituted bone marrow-residing CD4+CD25+FOXP3+ Treg of myeloma patients after allogeneic stem cell transplantation consisted preferably of CD45RA-CCR7- memory T-cells and contained low numbers of T-cell receptor excision cycles, indicating that Treg had indeed expanded outside the thymus. Importantly, bone marrow-residing Treg of newly diagnosed and myeloma patients after allogeneic stem cell transplantation expressed high levels of transforming growth factor β and cytotoxic T-lymphocyte antigen 4, and showed a strong inhibitory function.

Conclusions
We suggest that allogeneic stem cell transplantation provides a short but significant window of opportunity for CD8+ T cells before an exuberant regeneration of immunosuppressive Treg sets in. Later after transplantation, bone marrow-residing Treg probably contribute to suppressing graft-versus-host disease but may also undermine persistent immune control of multiple myeloma.

Key words: multiple myeloma, immunology, tumor immunology, T cells, T regulatory cells, transplantation

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Introduction

Multiple myeloma is a clonal B-cell malignancy characterized by an accumulation of mature plasma cells in the bone marrow leading to bone destruction and bone marrow failure. Myeloma remains incurable and even with high-dose chemotherapy and autologous stem cell transplantation (SCT) patients show a median survival of only 3 to 5 years.[1] Following allogeneic SCT lower relapse rates have been reported, probably due to a graft-versus-myeloma effect mediated by donor lymphocytes.[2] A proportion of patients treated with allogeneic SCT even achieve molecular remission resulting in long-term freedom of disease.[3] Unfortunately, these therapeutic improvements have been hampered by a high treatment-related mortality[4] based on the fact that immune responses derived from the allogeneic graft are not strictly myeloma-specific and are, therefore, associated with immune-mediated side effects.

For more than two decades, one principal goal in the field of allogeneic SCT has been to segregate beneficial graft-versus-myeloma effects from life-threatening graft-versus-host disease (GVHD). We reasoned that induction chemotherapy might play an important role in the generation of graft-versus-myeloma effects since it has been shown that chemotherapy-induced lymphodepletion is capable of boosting antitumor immunity.[5] The eradication of the immunosuppressive influence of regulatory T cells has been proposed as one mechanism underlying this paradigm.[6]

Prevention of immune reactivity to self-antigens is primarily achieved through negative selection in the thymus, however, some autoreactive T cells escape into the periphery and several mechanisms are implemented to keep such anti-self T cells in check. It has recently become clear that peripheral tolerance is largely maintained by immunosuppressive regulatory T cells (Treg), such as $CD4^+CD25^+$ T cells, which typically co-express transcription factor forkhead box P3 (FOXP3).[7] Animal models have shown that $CD4^+CD25^+$ Treg have the potential to prevent GVHD following allogeneic SCT by inhibiting pathogenic T cells.[8] Accordingly, the numbers of Treg seem to be reduced in the peripheral blood of patients suffering from GVHD[9] and increased $CD4^+$ $CD25^+$ $FOXP3^+$ Treg numbers in donor-derived stem cell transplants result in a diminished risk of GVHD.[10,11]

Unfortunately, in addition to their role in suppressing autoimmune responses, Treg also represent a main obstacle to an effective anti-tumor T-cell response.[12] Patients with solid tumors have increased numbers of Treg in their peripheral blood and tumor-infiltrating Treg are associated with reduced survival in cancer patients.[12] In animal models, elimination of Treg led to increased tumor-specific immune responses[13] and to an enhanced T cell-mediated rejection of established tumors.[14] Accordingly, depletion of $CD4^+CD25^+$ Treg seems to enhance anti-tumor immunity in cancer patients.[15]

Practically nothing is known about the presence and function of Treg in the human bone marrow, especially after allogeneic SCT. This seems surprising since studies have emphasized the role of the bone marrow as a major priming site for T-cell responses[16,17] and T cells directed against solid tumors are enriched within this compartment.[18,19] Furthermore, bone marrow-residing memory T cells are involved in the control of dormant hematologic malignancies,[20] and, in patients with myeloma, such bone marrow-infiltrating lymphocytes have the potential to target myeloma cells and their precursors.[21] Based on these findings and the fact that the bone marrow represents the immediate tumor environment of myeloma, we conducted the first study focusing on marrow-residing $CD4^+CD25^+$ $FOXP3^+$ Treg.

$CD4^+CD25^+FOXP3^+$ Treg have traditionally been thought to be generated exclusively in the thymus and there is still no definitive answer to the question whether human $CD4^+CD25^+FOXP3^+$ Treg can expand in the periphery or whether peripheral non-regulatory T cells can convert into human Treg *in vivo*.[22] In our patients, donor-derived stem cell preparations containing conventional T cells as well as $CD4^+CD25^+FOXP3^+$ Tregs were transferred into recipients who were lacking relevant thymic function. While thymic involution begins in early childhood, the thymus may retain some low-level activity during adult life in supporting T-cell differentiation.[23] High-dose chemotherapy, however, has devastating effects on the thymus[24] and GVHD in patients following allogeneic SCT further contributes to the destruction of thymic function.[25] Overall, it seems highly unlikely that the thymus has the capacity to make a significant contribution to the reconstitution of naïve T cells in elderly patients who have undergone allogeneic SCT. Any significant expansion of Treg in these patients should, therefore, be based on the proliferation of donor Tregs or on the conversion of donor-derived conventional T cells into Treg.

In this study, we performed the first systematic analysis of Treg numbers and function in the human bone marrow examining myeloma patients treated with allogeneic SCT. For comparison, Treg of healthy bone marrow donors and newly diagnosed myeloma patients were analyzed and peripheral blood Treg of the same patients were examined in parallel. The potential of Treg to reconstitute following allogeneic SCT was explored and the immunosuppressive function of marrow-derived Treg was investigated.

Design and Methods

Patients and healthy stem cell donors

Forty consecutive post-allogeneic SCT myeloma patients, 17 newly diagnosed myeloma patients, 24 healthy bone marrow donors and 15 blood donors were studied. Healthy subjects and myeloma patients, who were admitted for treatment at the University Medical Center Hamburg-Eppendorf, gave informed consent in accordance with the Declaration of Helsinki of 1975, as revised in 2000. All patients treated with allogeneic SCT had undergone previously received conventional chemotherapy and autologous SCT. Patients received pretransplant conditioning with melphalan (140 mg/m^2) and fludarabine (90-150 mg/m^2) within

a prospective trial as recently reported.[26] GVHD prophylaxis consisted of antithymocyte globulin (60 mg/kg) in the case of unrelated SCT, short course methotrexate on days +1, +3, and +6, and cyclosporine A (3 mg/kg) until day +180. The study protocol was approved by the local ethics committee (Hamburger Ärztekammer; decision number OB-038/06).

Bone marrow and blood samples

Bone marrow and blood samples from myeloma patients were obtained during routine diagnostic procedures. Samples obtained from consenting healthy donors were part of marrow or blood donations. Mononuclear cells were isolated by density gradient centrifugation and underwent immediate analysis by flow cytometry or were cell-sorted for extraction of RNA or genomic DNA.

Flow cytometry

Fresh bone marrow for flow cytometry was available from 40 post-allogeneic SCT myeloma patients, 17 newly diagnosed myeloma patients, and 15 healthy bone marrow donors. Peripheral blood was available from 18 of the post-transplant patients and 14 of the newly diagnosed patients. Mononuclear cells were stained using monoclonal antibodies to CD4, CD25, CCR7 (R&D Systems, Minneapolis, MN, USA), CD8, and CD45RA (BD Biosciences, San Jose, CA, USA) and appropriate IgG isotype controls. Co-staining of intracellular FOXP3 was performed applying anti-FOXP3 monoclonal antibody PCH101 (eBioscience, San Diego, CA, USA). Samples were analyzed using a FACSCalibur cytometer and CELLQuest software (BD Biosciences).

Purification of CD4+CD25+ Treg and inhibition assay

$CD4^+$ T cells were positively selected using magnetic beads (Dynal, Oslo, Norway). $CD4^+CD25^+$ cells were isolated from $CD4^+$ cells using anti-CD25 beads (Miltenyi Bergisch Gladbach, Germany). Autologous bone marrow or peripheral $CD4^+$ T cells (2.5×10^4) were stimulated with 5 µg/mL soluble anti-CD3 (BD Biosciences) and 0.5 µg/mL anti-CD28 (eBioscience) antibodies. As feeder cells, 5×10^5 irradiated autologous peripheral blood mononuclear cells, which had been depleted of $CD3^+$ T cells, were added. To determine the inhibitory capacity of Treg, 2.5×10^4 $CD4^+$ $CD25^+$ T cells were added to each well. Cells were co-cultured in 96-well round-bottomed plates in a final volume of 200 µL complete RPMI containing 10% human serum for 5 days. Cell proliferation was measured using the Biotrak™ cell proliferation enzyme-linked immunosorption assay system (Amersham Biosciences, Piscataway NJ, USA). Responder cells were pulsed with 10 µM bromodeoxyuridine for the last 18 hours of culture. Following fixation, peroxidase-labeled anti-bromodeoxyuridine was added and absorbance was read at 450 nm using a microtiter plate spectrophotometer (SLT Labinstruments, Salzburg, Austria).

Real-time polymerase chain reaction

T-cell subpopulations were isolated from total bone marrow mononuclear cells using a FACSAria cell sorter (BD Biosciences). Extraction of genomic DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extraction of RNA and reverse transcription were performed using the RNeasy Mini Kit (Qiagen) and AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time polymerase chain reaction was performed as described previously.[27] Primer sequences for target genes and the housekeeping gene glyceraldehydephosphate dehydrogenase (*GAPDH*) are given in *Supplementary Table 1*.

Statistical analysis

The Mann-Whitney U test was used to calculate differences between groups of patients and Wilcoxon's test was applied to determine significant differences between immune parameters within the same groups of patients. Spearman's rank correlation was used to analyze correlations between patients' characteristics and immunological parameters. Results were considered statistically significant if $p<0.05$.

Results

Patients' characteristics

Reflecting the general myeloma population, newly diagnosed and post-allogeneic SCT patients showed a male predominance; the typical patient was around 60 years old and IgGκ was the most common idiotype (*Supplementary Table 2*). The only significant differences between these two groups were a lower number of bone marrow-infiltrating plasma cells ($p=0.001$) and a higher serum albumin ($p=0.003$) in the post-transplant myeloma patients.

The myeloma patients who had undergone allogeneic SCT had all received the same induction chemotherapy. At the time of analysis, all transplanted patients had fully engrafted and showed complete chimerism. The median interval between transplantation and analysis was 37 months (range, 4-110 months). Thirty-one (77.5%) of the post-allogeneic SCT patients were in complete or near-complete remission (immunofixation-positive), seven (17.5%) had suffered a relapse, and two (5.0%) showed progressive disease. Most of the transplanted patients (n=33; 82.5%) were off immunosuppressive medication and did not display any signs of GVHD. However, one patient suffered from acute GVHD (grade II) and six patients had limited (n=3) or extensive (n=3) chronic GVHD.

CD4+FOXP3+ Treg reconstitute in the bone marrow of myeloma patients after allogeneic SCT

Since it was unknown whether human bone marrow-derived Treg would show a pattern of reconstitution comparable to that observed for non-regulatory T cells, we first examined whether the time passed since transplantation was related to the number of Treg present in the bone marrow of our post-transplant myeloma patients. Plotting the percentages of bone marrow-residing $CD8^+$ T cells against

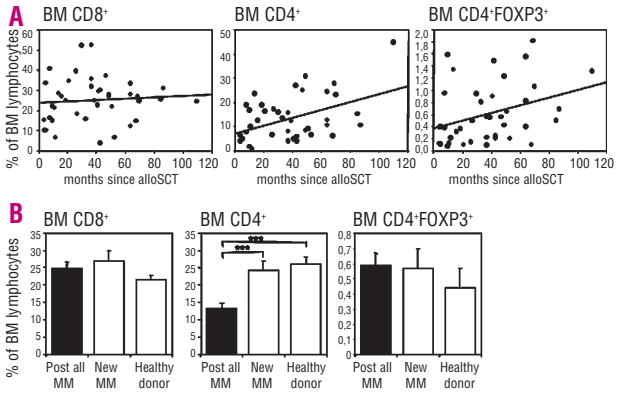

Figure 1. CD4⁺FOXP3⁺ Treg reconstitute in the bone marrow of myeloma patients after allogeneic SCT. Percentages of bone marrow-residing CD8⁺ T cells, CD4⁺ T cells, and CD4⁺FOXP3⁺ Treg were determined in 17 newly diagnosed myeloma patients (MM), in 40 myeloma patients following allogeneic SCT (Post MM) and in 15 healthy bone marrow donors using flow cytometry. Data are given as percentage of total bone marrow (BM) lymphocytes plotted against the number of months since allogeneic SCT (A). Mean percentages of each T-cell subset were also compared between groups (B). Bars show mean values ± standard error of mean. Asterisks indicate statistically significant differences between groups (*p<0.001).**

the number of months that had passed since transplantation did not show a significant association between the two parameters. In contrast, percentages of marrow-residing conventional CD4⁺ T cells correlated strongly (p=0.01, r=0.46) with the time passed since transplantation. The same was true for CD4⁺FOXP3⁺ Treg which seemed to have undergone a significant (p=0.02, r=0.37) expansion in the bone marrow of patients following allogeneic SCT (Figure 1A). Importantly, conventional T cells as well as Treg were both donor-derived, as indicated by donor-specific real-time polymerase chain reaction analysis (*data not shown*).

Next, we analyzed whether immune reconstitution had led to complete replenishment of the bone marrow with donor-derived Treg and conventional T cells at the median time of 37 months post-transplantation. We observed comparable frequencies of bone marrow-residing CD8⁺ T cells in post-allogeneic SCT patients, newly diagnosed patients, and healthy bone marrow donors (Figure 1B), indicating that CD8⁺ T cells had already fully reconstituted in our transplanted patients, a finding supporting earlier studies showing a very rapid expansion of CD8⁺ T cells during the first weeks following allogeneic SCT.[28] Such a rapid expansion of CD8⁺ very early after transplantation would also explain why a relation between the number of these cells and the time passed since transplantation was not detectable at our study's median follow-up time of 37 months post-transplantation.

When we analyzed percentages of bone marrow-residing CD4⁺ T cells in all three groups, we found markedly decreased numbers of these cells in the transplanted patients compared to newly diagnosed patients and healthy controls (Figure 1B), supporting previous studies showing diminished numbers of conventional CD4⁺ T cells for several years after allogeneic SCT. Surprisingly, however, this was not the case for donor-derived CD4⁺FOXP3⁺ Treg, which, in contrast to their non-regulatory CD4⁺counterpart, had already reached levels comparable to those in healthy volunteers, indicating a profound difference between the two types of CD4⁺ cells regarding their potential to expand after myeloablative therapy in these patients without significant thymic function.

Immune reconstitution results in the accumulation of CD4⁺CD25⁺FOXP3⁺ Treg in the bone marrow of myeloma after allogeneic SCT

Analyzing whether the preferential reconstitution of Treg might have led to an accumulation of these cells within the bone marrow of post-allogeneic SCT myeloma patients, we indeed observed a strong enrichment of CD4⁺FOXP3⁺ Treg in the transplanted patients compared to in newly diagnosed patients and healthy controls (Figure 2). As shown in the representative dot plot in Figure 2, we generally found only a minority of all bone marrow-resident CD25⁺ T cells to be positive for FOXP3, while nearly all FOXP3⁺ T cells simultaneously expressed CD25. In contrast to FOXP3-CD25⁺ T cells, which are probably activated T cells, these triple-positive CD4⁺CD25⁺FOXP3⁺ cells most likely represent *true* Tregs and were also strongly enriched among the CD4⁺ T cells of the post-transplant myeloma patients (Figure 2).

In accordance with the idea that Treg might contribute to the suppression of GVHD in patients after allogeneic SCT, we observed lower percentages of CD4⁺FOXP3⁺ Treg in the

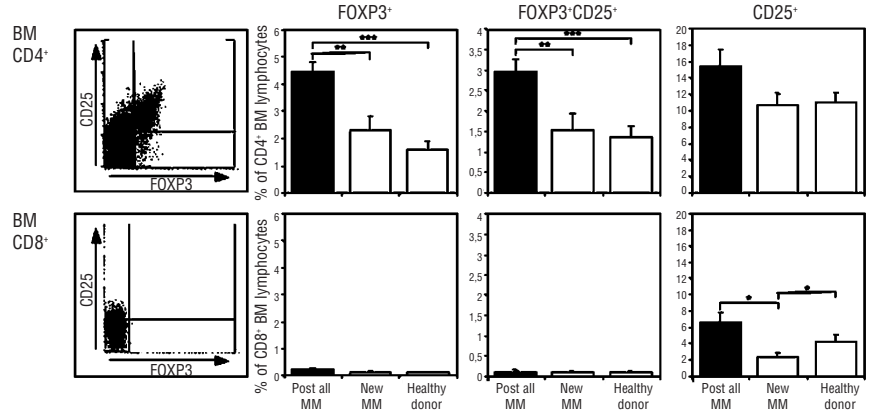

Figure 2. Accumulation of CD4⁺FOXP3⁺ Treg in the bone marrow of myeloma patients after allogeneic SCT. Percentages of bone marrow-residing CD4⁺ (upper row) and CD8⁺ (lower row) T cells expressing FOXP3 and/or CD25 were determined using flow cytometry in 40 myeloma patients following allogeneic SCT, 16 newly diagnosed myeloma patients, and 15 healthy donors. Data are given as percentages of all CD4⁺ and CD8⁺ T cells expressing the given combination of antigens. Bars show mean values + standard error of mean. Asterisks indicate statistically significant differences between groups (*p<0.05, **p<0.01, ***p<0.001).

bone marrow of our patients with GVHD than in those without GVHD (*data not shown*). However, given the small number of patients suffering from allo-immune phenomena, this finding failed to reach statistical significance (p=0.13). Interestingly, the presence of progressive disease or relapse of myeloma did not have any influence on the numbers of Treg present in the patients' bone marrow, supporting the hypothesis that bone marrow-residing Treg might contribute to the prevention of GVHD while allowing immune-mediated graft-versus-myeloma effects.

It has recently been reported that FOXP3+ Treg are also present among CD8+ T cells.[29] However, we never observed protein expression of FOXP3 in bone marrow CD8+ T cells (Figure 2B), suggesting that T cells with a regulatory function are restricted to the CD4+ subpopulation. In contrast, we did observe significant expression of CD25 on marrow-residing CD8+ T cells (Figure 2), but, as in the case of CD4+ T cells, there were no differences regarding CD25 expression between groups, supporting the idea that CD25-expressing CD4+ and CD8+ T cells mostly represented activated non-regulatory T cells.

Comparing Treg numbers in the bone marrow with those in the peripheral blood, we observed that Treg had indeed reconstituted in the periphery and that, in general, the percentages of these cells were higher in the peripheral blood than in the bone marrow (Figure 3). Importantly, however, donor-derived CD4+FOXP3+ Treg in post-allogeneic SCT myeloma patients were much more likely to remain in the bone marrow than the same cells in the control group (Figure 3), indicating a promoting effect of allogeneic SCT on Treg homing into the bone marrow compartment.

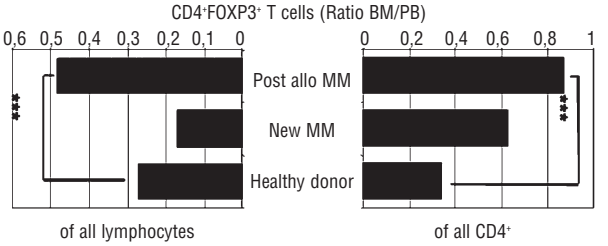

Figure 3. Ratio of bone marrow/peripheral blood CD4+FOXP3+ Treg as an indicator of Treg homing into the bone marrow compartment. Percentages of bone marrow-residing and corresponding peripheral blood CD4+FOXP3+ Treg were determined using flow cytometry in 14 patients with newly diagnosed myeloma and in 18 myeloma patients following allogeneic SCT. The ratio of bone marrow CD4+FOXP3+ Treg/peripheral blood CD4+FOXP3+ Treg was calculated as an indicator of Treg homing to the bone marrow compartment. Since peripheral blood mononuclear cells were not available from healthy bone marrow donors, peripheral blood mononuclear cells of 15 consecutive healthy blood donors were analyzed for comparison. Bars show mean ratios. Asterisks indicate statistically significant differences between groups (*p<0.001).**

Bone marrow-residing CD4+CD25+FOXP3+ Treg express high levels of transforming growth factor β (TGF-β) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) and exhibit full inhibitory function

Since a recent study indicated a diminished function of peripheral CD4+CD25+ Treg in patients with myeloma,[30] we next investigated the functional properties of the Treg in the bone marrow of our post-transplant patients. We found that bone marrow-residing Treg generally exerted a very strong inhibitory effect on the proliferation of autologous non-regulatory CD4+ T cells. In some cases, the inhibitory capacity of bone marrow Treg even seemed to be stronger in both groups of myeloma patients than in healthy donors (Figure 4A). Bone marrow CD4+CD25+ Treg from myeloma patients were as potent in suppressing the proliferation of conventional CD4+ as were CD4+CD25+ Treg derived from the peripheral blood of the same patient (*Supplementary Figure 1A*).

In order to identify some of the possible mediators of the immunosuppressive function of bone marrow Treg, we analyzed, for the first time, the expression of a number of key molecules which have been linked to the inhibitory function of these cells, in Treg residing in the human bone marrow. When we analyzed FACS-sorted bone marrow CD4+CD25+ T cells for their expression of a variety of genes using real-time polymerase chain reaction, we found that these cells were significantly enriched for Treg because only CD4+CD25+ T cells expressed high levels of FOXP3 and CD25 (*Supplementary Figure 1B*). Since it has been suggested that TGF-β1, CTLA-4, and interleukin 10 (IL-10) contribute to the immunosuppressive effects exerted by Treg *in vivo*,[22] we quantitatively analyzed the expression of these genes in Treg. While we did not observe any difference in the expression of IL-10 between CD4+CD25+FOXP3+ Treg and their CD4+CD25-FOXP3- counterparts, we found that only bone marrow Treg expressed significant levels of CTLA-4 while conventional CD4+CD25- T cells did not (*Supplementary Figure 1B*). The most striking hallmark of bone marrow-residing CD4+CD25+FOXP3+ Treg was, however, their strong expression of TGF-β1 which approached the expression levels of the *GAPDH* housekeeping gene (*Supplementary Figure 1B*). We, therefore, conclude that CTLA-4 and especially TGF-β1 might represent mediators of the immunsuppressive function of Treg residing in the bone marrow compartment.

Reconstituted bone marrow CD4+FOXP3+ Treg of post-allogeneic SCT myeloma patients represent memory T cells which have expanded outside the thymus

Finally, we investigated whether a peripheral expansion of Treg was responsible for the regeneration and the accumulation of Treg in the bone marrow compartment. T-cell receptor (TCR) diversity expressed on T-cell precursors is created in the thymus through recombination of gene segments encoding the variable parts of the TCR α and β chains. During these processes, by-products of the rearrangements are generated in the form of TCR excision circles (TREC). As these molecules are lost upon further mitotic cell division, their quantification is considered a valuable tool to measure the proliferative history of T cells and their developmental proximity to the thymus.[31]

In our current study, we investigated for the first time

TREC levels in bone marrow-residing Treg and other T-cell subpopulations in order to determine which cell types had undergone significant numbers of cell divisions in the periphery of myeloma patients following allogeneic SCT. We used $CD3^+CD4^-CD8^-$ cells as positive controls since these lymphocytes represent *early* T cells which have only recently been generated from T-cell precursors and which accumulate in the bone marrow.[32] As expected, $CD4^-CD8^-$ T cells showed very high numbers of TREC (*Supplementary Figure 2*), indicating that these T cells had indeed undergone only minimal numbers of cell divisions since they had been generated. In contrast, conventional $CD4^+$ and $CD8^+$ T cells showed very low TREC concentrations (*Supplementary Figure 2A*) based on the fact that these lymphocytes had expanded in the periphery following allogeneic SCT. Notably, we observed equally low levels of TREC in bone marrow-infiltrating $CD4^+CD25^+$ Treg (*Supplementary Figure 2A*), supporting the idea that following allogeneic SCT these cells had undergone as many peripheral cell divisions as their conventional T-cell counterparts.

The bone marrow is a site where memory T cells preferentially accumulate[33] and T cells can be divided into different memory subclasses based on their expression of CCR7.[34] Naïve and central memory T cells are CCR7-positive and home efficiently into lymphoid tissue whereas CCR7-negative effector memory T cells have the potential to migrate into peripheral sites of inflammation. Analyzing the expression of CCR7 on regular $CD4^+$ and $CD4^+FOXP3^+$ Treg we did not observe any differences between the groups (*Supplementary Figure 2B*). We suggest that this may be due to the fact that CCR7 is not only expressed on naïve T cells but also on antigen-experienced *central memory* T cells.[34] We did, however, observe a highly increased prevalence of CCR7-negative $CD8^+$ T cells in the bone marrow of all myeloma patients regardless of their treatment status (*Supplementary Figure 2B*), indicating that the presence of the disease in the bone marrow might cause a shift towards an effector phenotype among $CD8^+$ T cells.

Finally, we analyzed bone marrow-residing T cells for expression of CD45RA, which can be found on naïve T cells that have recently emigrated from the thymus but not on T cells that have evolved into memory-type T cells following release into the periphery.[35,36] While there was no difference between groups regarding $CD8^+$ T-cell expression of CD45RA, we observed a significantly reduced number of CD45RA-positive bone marrow $CD4^+$ T cells in post-allogeneic transplant patients compared to in newly diagnosed myeloma patients and healthy donors (*Supplementary Figure 2B*) demonstrating the defective thymus-dependent production of naïve $CD4^+$ T cells in these patients.

In all groups analyzed, bone marrow-residing $CD4^+FOXP3^+$ Treg were less likely to express CD45RA than their conventional $CD4^+$ counterparts. However, the expression of this marker was even further reduced following allogeneic (*Supplementary Figure 2B*), suggesting that the Treg reconstitution we had observed in the bone marrow of our patients following allogeneic SCT was indeed based on the expansion of donor-derived memory-type T cells and not on the *de novo* production of thymus-derived CD45RA-expressing Treg.

Discussion

To contribute to resolving the questions of whether in humans conventional T cells can convert into Treg and whether $CD4^+CD25^+FOXP3^+$ Treg have the capacity to proliferate and expand outside the thymus, we analyzed bone marrow-residing and peripheral Treg of post-allogeneic SCT myeloma patients who are unlikely to have relevant thymic function. In these patients, immune reconstitution following allogeneic SCT is characterized by a rapid expansion of peripheral $CD8^+$ memory T cells and a delayed recovery of $CD4^+$ T cells for several years post-transplantation.[28,37,38] Peripheral naïve T cells are generally reconstituted very late after transplantation[37,38] and, at least in the case of $CD4^+$ T cells, this regeneration of antigen-inexperienced cells is largely thymus-dependent.[39,40]

When we analyzed percentages of allogeneic $CD8^+$ T cells at a median time of 37 months after allogeneic SCT, we found that these cells had already fully reconstituted. The subset of conventional bone marrow $CD4^+$ T cells expanded during the years following allogeneic SCT although the numbers of these cells remained significantly reduced, suggesting that the patients' thymus was not capable of restoring these T cells and that peripheral expansion was not able to compensate fully for this deficit. In marked contrast, $CD4^+CD25^+FOXP3^+$ Treg, which also expanded following allogeneic SCT, were already completely reconstituted. In our patients, donor-derived stem cell preparations were transferred into recipients who had undergone myeloablative treatment and were devoid of relevant thymic function. The expansion of Treg in our patients must, therefore, have been based on the expansion of donor Treg or on the conversion of donor-derived non-regulatory T cells into $CD4^+CD25^+FOXP3^+$ Treg. In addition, we observed preferential expression of a memory phenotype by bone marrow-residing Treg. These observations further support the conclusion that the Treg expansion we observed was based on the division of memory-type Treg or on the conversion of conventional memory $CD4^+$ T cells into Treg. Finally, in conjunction with the above-mentioned findings, our observation of minimal levels of TREC in bone marrow-residing $CD4^+CCD25^+FOXP3^+$ Treg leads us to the conclusion that these Treg do indeed have the potential to expand outside the thymus as has been indicated for Treg in the peripheral blood.[11,41]

In our post-allogeneic SCT myeloma patients, Treg reconstitution resulted in an accumulation of donor-derived $CD4^+CD25^+FOXP3^+$ cells in the bone marrow. Our data suggest that one reason for this might be enhanced homing of Treg into the bone marrow compartment following allogeneic SCT where Treg might contribute to keeping GVHD under control.[42] It is not unlikely that bone marrow-residing Treg have the capacity to prevent GVHD without infiltrat-

ing the respective target organ.[43] In mice the ability of Treg to enter priming sites of pathogenic T cells within lymphatic tissue determines their ability to suppress GVHD[44] and the bone marrow compartment as a major site for T-cell priming might be an ideal place for Treg to act protectively against GVHD.

Alternatively, bone marrow-residing myeloma cells might use Treg to evade immunosurveillance. Thus, solid tumors seem to actively attract FOXP3-expressing Treg into their environment where they undermine the induction of tumor-specific immunity.[12] However, the fact that the vast majority of our post-transplant patients showed no signs of GVHD and were in complete remission despite the accumulation of fully inhibitory Treg in their bone marrow might suggest that these cells allow for a graft-versus-myeloma effect while preventing harmful allo-immunity.

Two groups have recently examined numbers and function of Treg in the peripheral blood of myeloma patients with somewhat conflicting results.[30,41] While there were important differences in the approaches of both groups, which might explain the differing results, both groups observed an increase in $CD4^+CD25^+$ T cells in the peripheral blood of myeloma patients. In addition, Prabhala *et al.* analyzed a few patients for $CD4^+FOXP3^+$ Treg showing decreased proportions of these cells in multiple myeloma. While our focus was on the bone marrow compartment and not on the peripheral blood, we did not observe any differences in the percentages of Treg in the bone marrow or in the periphery between newly diagnosed myeloma patients and healthy donors (*data not shown*). One explanation for these divergent results might be that we did not use CD25 as a Treg marker but analyzed protein expression of FOXP3. The identification of FOXP3, which is not only the most reliable marker for Treg to date but also represents a crucial developmental factor for these cells, has been a major advancement.[22] Moreover, our data suggest that CD25 cannot be used as a Treg marker for the quantitative analysis of bone marrow-residing Treg since it is also unspecifically expressed by a large number of conventional bone marrow-residing $CD4^+$ and $CD8^+$ T cells.

When we examined the function of bone marrow-derived Tregs of our post-allogeneic SCT myeloma patients, we found that $CD4^+CD25^+FOXP3^+$ Treg expressed high levels of CTLA-4 and the immunosuppressive cytokine TGF-β1 and efficiently suppressed the proliferation of conventional T cells. While this is the first study to examine Treg function in the bone marrow of myeloma patients, others observed a decreased inhibitory function of Tregs from the peripheral blood of these patients.[30] Our results are, however, in agreement with those of Beyer *et al.* who detected a normal suppressor function of $CD4^+CD25^+$ T cells in patients with myeloma.[41] As has been pointed out previously,[41] the differing findings can best be explained by differences in the experimental approaches used by the respective groups. While Prabhala *et al.* used whole peripheral blood mononuclear cells as proliferating cells, accepting variations in the composition of mononuclear cells subtypes between groups, we and others have used highly purified $CD4^+$ or $CD4^+CD25^-$ T cells as the stimulated cell fraction.

The most promising concept for active immunotherapy of myeloma might be the combination with allogeneic SCT. This setting offers the advantages of an immune system unaffected by negative influences of the tumor. Furthermore, active immunotherapy following allogeneic SCT can build on anti-tumor immunity generated as a part of graft-versus-myeloma effects.[45] We suggest that allogeneic SCT provides a short but significant window of opportunity for $CD8^+$ T cells before an exuberant regeneration of immunosuppressive Treg sets in. Future therapeutic approaches in myeloma should focus on keeping Treg under control while amplifying and shaping allogeneic SCT-induced graft-versus-myeloma effects, e.g. by applying polyvalent cancer vaccines and/or tumor-specific adoptive immunotherapy. Such approaches could help to boost and broaden a post-transplant anti-myeloma immune response and might contribute to preventing recurrences in patients with multiple myeloma.

Authorship and Disclosures

DA designed the research, analyzed the data, and wrote the paper; YC performed research, contributed vital new analytical tools, and analyzed the data; TL performed research and analyzed the data; JP, CF, JA, KB, CW, TE and ARZ performed the research; BF and CB analyzed the data; NK designed the research and analyzed the data. The authors reported no conflict of interest.

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