Tumor-Specific Regulatory T Cells from the Bone Marrow Orchestrate Antitumor Immunity in Breast Cancer

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Abstract

Endogenous antitumor effector T-cell responses and immune-suppressive regulatory T cells (Treg) critically influence the prognosis of patients with cancer, yet many of the mechanisms of how this occurs remain unresolved. On the basis of an analysis of the function, antigen specificity, and distribution of tumor antigen–reactive T cells and Tregs in patients with breast cancer and transgenic mouse tumor models, we showed that tumor-specific Tregs were selectively activated in the bone marrow (BM) and egressed into the peripheral blood. The BM was constantly depleted of tumor-specific Tregs and was instead a site of increased induction and activity of tumor-reactive effector/memory T cells. Treg egress from the BM was associated with activation-induced expression of peripheral homing receptors such as CCR2. Because breast cancer tissues express the CCR2 ligand CCL2, the activation and egress of tumor antigen–specific Tregs in the BM resulted in the accumulation of Tregs in breast tumor tissue. Such immune compartmentalization and redistribution of T-cell subpopulations between the BM and peripheral tissues were achieved by vaccination with adenoviral vector-encoded TRP-2 tumor antigen in a RET transgenic mouse model of spontaneous malignant melanoma. Thus, the BM simultaneously represented a source of tumor-infiltrating Tregs and a site for the induction of endogenous tumor-specific effector T-cell responses, suggesting that both antitumor immunity and local immune suppression are orchestrated in the BM.

Introduction

Spontaneous T-cell responses against tumors have emerged as crucial events that determine patient prognosis. High effector and memory T-cell infiltrates in many tumors, including breast cancer, correlate with improved clinical outcome and treatment sensitivity (1–6), particularly in tumors where clonal T-cell expansion has occurred (7). Tumor-specific effector (Teff) and memory T (Tmem) cells are detectable in the peripheral blood (PB) of many patients with cancer, proportions of which are functionally active (33%–53%; refs. 8–13) and correlate with long-term survival (14–17). However, the conditions that regulate Teff and Tmem cell induction are poorly understood and limited by immune-suppressive cells, including regulatory T cells (Treg; ref. 12). Tregs prevent the induction of autoreactive and tumor-reactive T-cell responses and are thereby essential in maintaining peripheral tolerance against self-antigens (18). Tumor antigen (TA)–specific Tregs have been identified in the PB of tumor patients (19) and can efficiently suppress Teff cells responses in colorectal and in breast cancer (12, 20). Increased Treg infiltration in tumors is

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).
also associated with poor outcomes in many cancers, including breast cancer (21–23).

Treg and Teff cell responses in patients with cancer are viewed as opposing forces where Tregs suppress tumor-specific T-cell responses resulting in uncontrolled tumor progression, or where T-cell–based tumor immune surveillance occurs resulting in tumor control and improved survival (24–26). However, as both TA-reactive T cells and Tregs can be detected in the PB of patients with cancer (20, 27), it remains unclear how tumor-reactive Teff/Tmem cells can be spontaneously generated despite the presence of tumor-specific Tregs.

An important site of tumor-specific T-cell–activation and accumulation is the bone marrow (BM; ref. 28). Naïve T cells encounter TAs imported and presented by BM-resident antigen-presenting cells, leading to their priming, expansion, acquisition of effector/memory function, and egress into circulation (29, 30). Accordingly, TA-reactive T cells are enriched in the BM of patients with cancer (28). BM is a preferred organ of Treg recirculation and accumulation, contributing to peripheral tolerance against self-antigens and TAs in patients with cancer (31). Increased Treg frequencies are found in BM of tumor-bearing mice (31). Together these findings suggest a tight spatial and or chronologic regulation of antigen-specific Treg and Teff cell responses in the same patient, with potential implications for peripheral tumor immune surveillance. Thus, the potential presence and numbers of tumor-specific Tregs in BM of patients with cancer and their relation to TA-specific T-cell responses require further characterization.

Here, we studied the relationship between BM-resident and circulating TA-specific Tregs, their impact on Teff and Tmem cell responses, and their impact on systemic and intratumoral antitumor immunity in breast cancer. On the basis of functional and phenotypic studies of tumor-specific Tregs and Teff cells from 56 patients with primary breast cancer and an autochthonous mouse tumor model, we provided evidence that spontaneous Teff cell responses against TAs occur in the BM due to a selective depletion of the local population of TA-specific Tregs. After T-cell receptor (TCR) stimulation, these cells expressed peripheral homing receptors, such as CCR2, which enabled their egress from the BM into the PB. Accordingly, the occurrence of TA-specific T eff cells in the BM was associated with their functional inhibition induced by TA-specific PB Tregs and a strong accumulation of Tregs in the tumor tissue.

Materials and Methods

Patients and donors

PB and BM samples were collected from 56 patients with breast cancer who did not receive neoadjuvant therapy and 30 healthy donors after approval of the institution’s ethics committee (#70/99) upon signed consent. All investigations were performed in accordance with the principles embodied in the Declaration of Helsinki. BM (40 mL) was aspirated under local anaesthesia from both iliac crests and 40 mL blood was collected by venipuncture. Samples were processed immediately after collection and mononuclear cells were collected after Ficoll gradient centrifugation (27).

Mice

Mice (C57BL/6 background) expressing the human ret transgene in melanocytes under the control of mouse metallothionein-1 promoter-enhancer (32) were kindly provided by Dr. I. Nakashima (Department of Biomedical Sciences, Chubu University, Aichi, Japan). The RET transgenic mouse model of spontaneous malignant melanoma that overexpresses the tumor antigen TRP-2 were used. Tumors appeared spontaneously at the age of 20–70 days and approximately 25% of the RET transgene mice developed macroscopically visible skin tumors. Mice were housed under specific pathogen-free conditions in the animal facility of the German Cancer Research Center. Experiments were performed in accordance with government and institutional regulations. BM, spleen, lymph nodes, and tumor were collected and immediately transferred into serum-free RPMI medium and stored on ice. After removal of necrotic tissue and fat, tumor biopsies were cut into small pieces and filtered through a cell strainer. Spleens and lymph nodes were dissociated and also filtered. Samples from these three tissues were depleted of RBC by ammonium chloride lysis, washed twice, and used for FACS staining as described previously (33).

Antigens

We designed 8 long peptides derived from 5 tumor-associated antigens commonly overexpressed in breast cancer [(Heparanase150–163,9,10) Mammaglobin-14, Mammaglobin-141–150, Mucin-1137–157, Her2/neu51–60 (12, 35, 36), and p53118–137 (12, 37)] using the SYFFPETHI-database (12, 38), which predicts presentation on a wide range of HLA types containing at least one well-defined HLA-A*0201 T-cell epitope. As a negative control antigen, we used human IgG (Endobulin, Baxter). In addition, lysates of the breast cancer cell lines [MCF-7 (39), KS24.22 (40)] and pro-monocytic leukemia cell line (U937) generated by five freezing and thawing cycles were used as source of breast tumor or control antigens as described previously (15). Briefly, cells were lysed by repeated freezing (at −80°C) and thawing (at 37°C). Five freeze–thaw cycles resulted in complete disruption of the cells into fragments. Supernatants were prepared by spinning the cell lysates at 1,500 rpm for 10 minutes. The supernatants were filtered through 0.22-μm Millipore filters and either used immediately or frozen at −80°C until future use.

Dendritic cell generation and activation

Dendritic cells (DC) were differentiated using standard protocols as described previously (12, 15). In brief, adherent cells from BM and blood samples were cultured for 7 days in serum-free X-VIVO 20 (Lonza) containing 50 ng/mL recombinant human GM-CSF, and 1,000 U/mL IL4 with standard incubation conditions at 37°C and 5% CO2. DCs were enriched using anti-CD3, anti-CD56, and anti-CD19–coupled magnetic beads (purity of HLA-DR+/CD11c− DC was >85%; Supplementary Fig. S1) and pulsed overnight with long peptides, cell lysates, or control antigens (200 μg/106 cells/mL) in cytokine free X-VIVO medium or supplemented with FCS (10%)

T-cell culture and isolation

T-cells isolated from BM and blood samples were cultivated for 7 days in RPMI1640 (Sigma-Aldrich) supplemented with 10% AB serum (PAN Biotech), 100 U/mL IL2 (PromoCell), and 60 U/mL IL4 (with standard incubation conditions at 37°C and 5% CO2) and purified (percentage of purity >90%) by using T-cell Negative Isolation Kit (Dynal), Life Technologies (11344D) as per the manufacturer’s instructions. In some experiments, Tregs were

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depleted from purified T cells using the CD4+CD25+ Treg Isolation Kit (Miltenyi Biotec, 130-091-01, as per the manufacturer’s instructions). Percentage of purity was as follows: >80% CD25+Foxp3- Tregs (gated on CD3+CD4+ T cells) and >95% of CD25+CD127- Tregs (gated on CD25+Foxp3+ Tregs; Supplementary Fig. S2).

ELISpot analysis

IFN-γ-secreting T cells (specific to peptides mentioned in the “Antigens” section) were determined using the IFN-γ ELISpot Kit (Mabtech; ref. 15) and analyzed with ImmunoSpot Image Analyzer (Cellular Technology). Antigen-pulsed DCs were incubated with autologous T cell at 1:10 ratio for 40 hours with standard incubation conditions at 37°C and 5% CO2. In some cases, autologous Tregs were added at different ratios. Individual patients were designated as responders (ELISpot positive) if the spot numbers in wells with the tested tumor antigens were significantly (significance was calculated on the basis of t tests) higher than control wells, otherwise patients were denoted as nonresponders (ELISpot negative). When significant tumor reactivity was detected, the frequency of antigen-specific T cells was calculated as follows: (spots in test wells – spots in control wells)/T cells per well. Positive tests were denoted as response rates in some graphs.

Flow cytometry

HLA-II tetramer staining was performed as described previously (20). Mammaglobin-specific HLA class-II and control CLIP tetramers were obtained from the tetramer core facility at Emory University (Atlanta, GA). Live cells were distinguished using Live/Dead Fixable Yellow Dead Cell Stain (Thermo Fisher Scientific)/Zombie-NIR (BioLegend) for 15 minutes at 4°C, washed, and subsequently blocked with human immunoglobulin (Kiovig-100 μg/mL, Baxter) for 15 minutes at 4°C. Cells were then incubated with phycoerythrin (PE)-labeled tetramers loaded with mammaglobin or CLIP peptide (6 μg/mL) in 50 μL FACS buffer for 2 hours at 37°C followed. Cells were washed and stained with antibodies purchased from BD Biosciences unless otherwise stated: anti-CD3-Alexa-Fluor700/PE (clone UCHT1), anti-CD4-Pacific-PerCP-cy7.5/APC-Cy7 (clone SK3/RPA-T4), anti-CD25-PerCP-eFluor710/APC Cy7 (clone 4E9, ebioscience)/clone M-A251), and anti-CD127-H7R-M2 antibodies for 20 minutes at 4°C. In some experiments, migratory markers were additionally analyzed using anti-CD62L (clone Dreg 56), anti-CCR7 (clone 4B12, BioLegend), anti-CXCR4 (clone 12G5, BioLegend), anti-CXCR3 (clone 1G6), anti-CCR5 (clone 2D7), anti-Beta1 (clone T52/16, BioLegend), anti-PSGL (clone KPL-1), anti-LFA-1 (clone m24, BioLegend), anti-CCR6 (clone 11A9) and anti-CCR4 (clone 1G1), anti-CCR2 (clone K036C2), and anti-LTB4R (clone 202/7B1, R&D Systems). For intracellular Foxp3 staining (performed after the surface staining mentioned above), cells were permeabilized with Fixation/Permeabilization Concentrate and Diluent (ebioscience) followed by staining with anti-Foxp3/APC antibody (clone 236A/E7, ebioscience). For mouse experiments, single-cell suspensions were treated with Fc-block (BD Biosciences) and live cells were distinguished using Zombie-NIR (BioLegend) as described above followed by staining with mAbs for 30 minutes at 4°C. The following antibodies (purchased from BD Biosciences) were used: CD3-PerCP-Cy5.5 (clone 17A2)/FITC (clone 48-2B), CD4-PE-Cy7/V500 (clone RM4-5), CD45.2-PE (clone 104), CD25-APC-Cy7 (clone PC61)/APC-R700 (clone PC61), CCR2-APC (clone 475301)/BV605 (clone 475301), LAG3-PE-Cy7 (clone CB97W), LAP-PerC-eFl710 (clone TW7-16B4), CD69-biotin (H1.2F3), and streptavidin-IFITC. Cells were washed and intracellular Foxp3-PE (clone IFJ-166)/AF647 (clone MF23) staining was performed as described above. Cells were acquired using the FACSCanto II/Lyric flow cytometer (BD Biosciences) and analyzed with the FlowJo software.

Assessment of Treg infiltration in tumor tissue

Tumor areas on formalin-fixed paraffin-embedded (FFPE) breast tumor sections were demarcated by hematoxylin and eosin (H&E)–stained reference slides and subsequently scraped into ependorf tubes manually (27). Tumor-infiltrating Tregs were analyzed by epigenetic PCR of Treg-specific demethylated region (TSDR) on DNA isolated from FFPE-patient breast tumor sections at Epiontis GmbH located in Berlin as described previously (27, 41). Proportions of Tregs in tumors were quantified on the basis of GAPDH plasmid units obtained in each assay. Alternatively, intratumoral Tregs were detected microscopically on acetone-fixed cryosections. Slides were rehydrated in 1× Tris-buffered saline with 0.5% Tween-20 (TBST) and then stained with the following antibodies diluted 1:50 in TBST: anti-CD4 polyclonal goat-IgG (R&D Systems) and anti-FoxP3 (clone 236A/E7, Thermo Fisher Scientific) mAbs that were subsequently detected using secondary antibodies from Thermo Fisher Scientific (Alexa Fluor 488 chicken anti-goat and Alexa Fluor 594 chicken anti-mouse) as described previously (27). 4′,6-Diamidino-2-phenylindol (DAPI) staining was used as a master marker for cell identification on the basis of nuclei detection (100 μL of 1 μg/mL DAPI). Slides were then scanned on Olympus IX51 microscope. Tregs present in the tumor were detected on the basis of the FoxP3 and CD4 positivity.

Treg suppression assay

Treg suppression assay was performed using the Treg Suppression Inspector kit (Miltenyi Biotec, 130-092-909) according to the manufacturer’s instructions. [3H] thymidine (1 μCi/well, Amersham) was given for the last 16–18 hours of incubation and proliferation of T cells was measured by determining the amount of incorporated [3H] in counts per minute (cpm) using a scintillation counter (Liquid Scintillation Counter 1450, PerkinElmer) in triplicate wells as described previously (12).

Analysis of antigen-specific Treg activity

A total of 2.5 × 10^5 isolated T cells (from PB and BM samples) were cocultured with 5 × 10^5 antigen-pulsed autologous DCs in cytokine-free X-VIVO 20 medium for 24 hours with standard incubation conditions at 37°C and 5% CO₂. Meanwhile Treg-depleted T cells (1 × 10^5/mL) were stimulated for 12–16 hours with 1 μg/mL plate-bound anti-CD3 (clone OKT3-Thermo Fisher Scientific) antibody. Plates were coated using 1 μg/mL CD3 (clone OKT3-Thermo Fisher Scientific) antibody in PBS overnight for 12–16 hours at 4°C. The solution was removed the next day, washed with PBS thrice, and the coated plates were used for stimulation. Afterwards, polyclonally activated Treg-depleted T cells were washed in X-vivo 20 medium and added into Treg wells for 72 hours. Cell proliferation was measured by thymidine incorporation counts as described above. The presence of antigen-specific Tregs was designated if count numbers in the presence of test antigens were significantly lower than in the presence of control antigen as determined by t tests.
Analysis of Treg migration

Treg migration was assessed by loading Tregs purified from the blood of healthy individuals (100,000 unstimulated or polyclonally activated Tregs in 100 µL X-vivo 20 medium, using 1 µg/mL plate-coated CD3 antibodies) on transwells (5 µm-pore size, Corning) coated with 10 µg/mL collagen IV (42). RhuCCL2 (R&D Systems) was added to the X-vivo 20 medium in bottom wells in a volume of 500 µL. The numbers of cells that migrated to the bottom wells within 4 hours (at 37°C) were determined by flow cytometry and normalized to a fluorescent bead control (CountBright Absolute counting beads, Thermo Fisher Scientific).

Analysis of CCL2 in plasma

Plasma from PB and BM of patients was collected by centrifugation of samples at 3,000 rpm for 15 minutes at 4°C. The clear supernatant was taken for quantifying CCL2 by luminex bead-based analysis using the Bio-Rad Singleplex Set (171-BK36MR2) as per the manufacturer’s instructions and analyzed by Bio-Plex Manager software (version 6.0).

Mouse immunization

RET Transgenic mice with macroscopic tumors were injected at the base of the tail (s.c.) with a mixture of 100 µg CTL epitope (TRP-2180-188–derived peptide SVYDFFVWL) and 140 µg Th epitope (HBV core antigen128-140–derived peptide TPRPARPPNA-PIL) emulsified in Incomplete Freund adjuvant (IFA). Ratio of IFA:Antigen (diluted in PBS) was 1:1 and volume of injection was 100 µL. Recombinant adenosin encoding human tyrosinase-related protein-2 (Ad5.TRP-2) and control vector encoding PIL emulsified in Incomplete Freund adjuvant (IFA) were kindly provided by Dr. T. T. Manager software (version 6.0).

Statistical analyses

Data distribution was denoted by mean or mean with SEM. Paired or unpaired two-tailed Student t test, Fisher’s exact test or χ² test were used. The correlation (r) between two datasets were computed using nonparametric Spearman or parametric Pearson test. Asterisks indicate significant differences between the groups compared. (*) P < 0.1; * * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. GraphPad Prism (versions 5, 6, 8.0.1) software was used for analysis.

Results

Tumor-specific T-cell responses were controlled by PB Tregs but not BM Tregs

We first assessed whether, and to what extent, TA-reactive T cells in the BM were controlled by Tregs. To this end, we determined the frequencies of BM-resident T cells that secreted IFNγ after ex vivo TAA-specific stimulation, after depletion or in the presence of CD4+ CD25 + Tregs, or after depletion in a short-term IFNγ ELISPot assay. Autologous DCs loaded with well-defined synthetic long peptides derived from Heparanase, Mucin-1, Mammaglobin A, p53, and Her2/neu, all containing multiple epitopes presented by several different HLA alleles (12, 20), or with a respective control peptide derived from human IgG (12) were used for T-cell stimulation. Likewise, processed PB T cells from the same patient cohort were also assessed for comparison with BM T cells. TAA-specific T-cell responses were defined by significantly increased IFNγ spot numbers in triplicate wells comparing the test antigens to the negative control antigen.

Primary data from one representative patient (Fig. 1A) and cumulative data from altogether 54 patients with primary breast cancer (Fig. 1B–F) revealed significantly increased frequencies (Fig. 1B and C) and response rates (Fig. 1D–F) of TAA-reactive T cells in the BM compared with the PB of patients with breast cancer and in comparison with T cells from healthy individuals (Fig. 1C). While Treg depletion did not considerably increase TAA-responsiveness of BM T cells, it caused a consistent increase in TAA-specific T-cell responsiveness of PB T cells (Fig. 1A–F; Supplementary Fig. S3). Thus, our data demonstrated that functionally potent TAA-specific T cells were enriched at similar frequencies in both PB and BM of patients with breast cancer, however, TAA-specific responses of PB T cells, but not BM T cells, were strongly inhibited by Tregs.

The BM of patients with breast cancer was selectively depleted of TAA-specific Tregs

To explore potential reasons underlying the differential impact of Tregs in the BM and PB, we examined the presence and suppressive activity of TA-reactive Tregs in both compartments. To this end, we isolated Tregs from PB and BM by CD4+ CD25 + magnetic beads and confirmed their suppressive capacity toward conventional T (Tcon) cells after polyclonal activation (Fig. 2A). We next added increasing amounts of BM-derived Tregs to BM-derived Tcon cells and assessed their IFNγ secretion in response to breast cancer cell line–derived antigens or control antigens (derived from the leukemia cell line U937) in an IFNγ ELISPot assay. We detected a dose-dependent suppression of TAA-specific IFNγ secretion by TAA-stimulated Tregs (Fig. 2B). These data suggested that TAA-reactive Tregs, generated during breast cancer development reside in the blood and BM and can efficiently suppress TAA-reactive T cell responses when present at sufficient numbers.

To identify which breast tumor antigens were recognized by breast tumor-reactive Tregs, we used a functional assay based on the significantly increased suppressive activity of TCR-stimulated Tregs compared with unstimulated Tregs (12). One representative experiment with PB-derived Tregs from a patient with breast cancer demonstrated the presence of mammaglobin-1 and MUC1–reactive Tregs (Fig. 2C). Cumulative data for Tregs derived from PB (n = 18) and BM (n = 13) of patients with primary breast cancer revealed the presence of TAA-reactive Tregs in both PB and BM; however, different detection rates of PB (62%) and BM samples (38%) and significantly increased T-cell–suppressive activity of TA-reactive Tregs in the blood compared with BM (Fig. 2D and E) demonstrated that TAA-reactive Tregs were reduced in BM compared with PB. Although target specificities of TAA-reactive Tregs showed variability in different samples, Tregs reactive against all tested antigens were detectable within the patient cohort (Fig. 2F). Among them, the breast gland-specific antigen mammaglobin-1 was recognized most frequently by Tregs from patients (60%); in contrast, TAA-reactive Tregs were never detectable in healthy individuals (Fig. 2F). Taken together, Tregs reactive against various TAA were detectable in the PB and
BM of patients with breast cancer, but both their frequency and suppressive activity were significantly lower in the BM.

These differences between BM and PB could be caused by increased numbers of TAA-specific Tregs in the peripheral blood and/or by an increased functional activity of PB-derived Tregs. To determine this mechanism, we first quantified the frequencies of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>/</sup>C<sub>0</sub> Tregs among total CD4<sup>+</sup> T cells in the PB and BM of healthy individuals and patients with breast cancer (Fig. 3A). Unexpectedly, compared with healthy donors, patients with breast cancer showed a selective reduction of Tregs in their BM (Fig. 3B) and a similar decrease of Tregs was detectable in the BM of patients with colorectal cancer, suggesting that the loss of BM-resident Tregs may be a general characteristic of patients with cancer (Supplementary Fig. S4).

We addressed the question whether this reduction was based on a selective depletion of TAA-specific Tregs. We thus quantified mammaglobin-1–specific CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>/</sup>C<sub>0</sub> Tregs using HLA-II tetramers loaded with mammaglobin-derived HLA-II restricted peptide mam34-48(20) (Fig. 3C). Approximately
Tumor-Specific Tregs Induce Antitumor Immunity

**Figure 2.**
TAA-specific Tregs in BM and PB of patients with breast cancer. **A,** Inhibition of Tcon cell proliferation (measured by a [3H] thymidine incorporation assay) by cocultured Tregs isolated from the blood of a representative breast cancer patient after activation with anti-CD3 antibodies. The respective ratios of CD4⁺CD25⁺ Tregs:CD4⁺CD25⁻ Tcon cells in each well are shown on the x-axis. Each well contained 5.0 × 10⁴ Tcon cells, with the exception of the ratios 0:1 (no Tregs; white bar) and 1:0 (no Tcon cells). Means and SEM of [3H] thymidine incorporation (cpm) after 16 hours are shown. Asterisks indicate statistical significance for comparison of respective test group with control group without Tregs (white bar). Shown is one representative experiment out of 4 different T-cell donors. **B,** Dose-dependent inhibition of IFNγ secretion from breast tumor–reactive BM Tcon cells by BM-derived Tregs from a representative breast cancer patient. BM-derived Tcon cells were depleted of Tregs cocultured with purified BM-derived Tregs at indicated Treg:Tcon ratios. Autologous DCs present in the wells were pulsed with breast tumor antigens (lysate of breast cancer cell line KS24.22; black bars) or with antigens derived from the leukemia cell line U937 (source of breast cancer unrelated antigens; white bars). Mean ± SEM of IFNγ spot numbers in triplicate wells is shown. Shown is one representative experiment out of 4 different T-cell donors. **C,** Representative experiment of Treg specificity displaying selective suppression of Tcon cell proliferation from PB (black bars) and BM (white bars) with DCs pulsed with breast tumor antigens (lysate of breast cancer cell line KS24.22; black bars) or with antigens derived from the leukemia cell line U937 (source of breast cancer unrelated antigens; white bars). **D,** Proportions of breast cancer patients possessing TAA-reactive Tregs in their PB (n = 18; white bar) or BM (n = 13; black bar). **E,** Within the same group of patients, PB (n = 18) or BM (n = 13), the amount of TAA-specific Treg-mediated suppression of Tcon cell proliferation from PB (62 tests) and BM (34 tests) is shown. This was calculated as a percentage reduction in the proliferation of Tcon cells cocultured with Tregs, stimulated with individual TAAs versus control peptide. **F,** Patients with breast cancer and healthy donors were tested for the presence of TAA-specific Tregs in the PB. Data represent the percentage of individuals with Tregs specific for the respective antigen (patients with breast cancer, n = 14–56; healthy donors, n = 10–14). (Unpaired two-tailed Student t test (A–C), paired two-tailed Student t test, and χ² test (D and F) were used.) n.s., not significant.
0.2% of the Tregs in the PB specifically recognized mammaglobin-1, while mammaglobin-specific Tregs were hardly detectable in BM of the same patients (Fig. 3D). Interestingly, TA-specific Teff cells were not reduced, but rather increased in BM (Fig. 3E). Thus, in contrast to tumor-specific Teff cells, tumor-specific Tregs were selectively depleted from the BM of patients with breast cancer which may, at least partially, account for the observed overall reduction of Tregs in the BM.

Migration of CCR2+ Tregs from BM correlated with T-cell suppression

To assess potential reasons for the selective egress of Tregs from the BM, we studied the expression of homing receptors on PB and BM Tregs of healthy individuals and patients with breast cancer (Supplementary Fig. S5). Among them, CCR2 and CCR6 were selectively upregulated only in the BM of patients with cancer (Fig. 4A and B). Both chemokine receptors are employed by T cells
for homing to peripheral sites of inflammation (44) and CCR2 mediates egress of monocytes out of the BM (45) and recruitment of Tregs from lymph node to tumors (46). By multicolor flow cytometry, we studied CCR2 and -6 expression on mammaglobin-specific CD4+ Treg and Tcon cell populations. TAA-specific BM Tregs had upregulated CCR2 expression as assessed by mean fluorescence intensity (MFI) values compared with tetramer-negative BM Tregs, and compared with Tet+ /Tet− Tcon cells (Fig. 4C-E). We noticed a significant elevation in CCL2 concentrations in the PB compared with the BM in patients with cancer, whereas the opposite was observed in healthy donors (Supplementary Fig. S6). In the same samples, we observed that the expression of another chemokine receptor CCR5 was increased in PB T cells but did not differ between Treg and Tcon cell subsets (Supplementary Fig. S7), thus showing that tumor-specific BM Tregs selectively upregulated CCR2. CCR2 was generally coexpressed with CCR6 on BM Tregs (Supplementary Figs. S8 and S10). In contrast to CCR2, CCR6 was increased on both TAA-specific Tregs and Tcon cells in the PB and on TAA-unspecific...
PB T cells (Supplementary Fig. S9). As Treg trafficking to the BM is largely mediated through the chemokine receptor CXCR4 (31), we assessed its expression on Tregs. Whereas all BM-resident Tregs expressed some degree of CXCR4 (Supplementary Fig. S3), we observed a selective increase of CXCR4 expression in TAA-specific BM Tregs (Fig. 4F). Thus, the small population of TAA-specific Tregs in the BM was characterized by high CXCR2 and CXCR4 expression.

We wondered whether the selective upregulation of CCR2 expression in BM Tregs was triggered by TCR stimulation. Polyclonal activation of purified Tregs from healthy individuals with anti-CD3 mAbs induced CCR2 expression (Fig. 5A), indicating that CCR2 delineates a population of TCR-stimulated Tregs. Accordingly, TCR-activated Tregs, but not unstimulated Tregs, migrated along gradients toward the CCR2 ligand CCL2 (Fig. 5B), which also orchestrates the emigration of CCR2+ monocytes out of the BM (47, 48). Notably, increased BM expression of CCR2 correlated with reduced frequencies of CCR2-positive Tregs (Fig. 5C), and reduced frequencies of CCR2-positive BM Tregs were associated with an increase of CCR2-expressing Tregs in PB (Fig. 5D). We further observed a strong negative correlation between increasing levels of CCR2 in BM Tregs and reduced frequencies of total Tregs in the BM (Fig. 5E). These data suggest that Treg reduction in the BM of patients with cancer was based on a selective migration of activated, TA-specific CCR2+ Tregs into the PB.

CCR2 plays an important role in redirecting activated T cells into inflamed tissues expressing the chemokines CCL2, CCL8, CCL7, CCL13, CCL11, CCL24, and CCL26 (49–54). Because CCL2 is expressed at high levels in many cancers, including breast cancer (55, 56), we hypothesized that an increased CCR2 expression on Tregs in the PB correlated with increased Treg accumulation in the breast tumor tissue. We indeed detected significant correlations between CCR2 expression on circulating Tregs and Treg infiltration of breast tumors (Fig. 5F and G, respectively).

Taken together, our observations suggest that in PB, TAA-specific Tregs were enriched in the population of total CCR2+ Tregs. We therefore hypothesized that the suppression of TAA-specific T cell effector cells in the PB of patients with cancer was determined by this subpopulation rather than by the total Treg population. Indeed, the occurrence and extent of TAA-specific T effector cell responses assessed by IFNγ ELISPOT assay decreased with increased numbers of CCR2+ Tregs in the blood of the patients but not with increased numbers of total Tregs (Fig. 6A and B). Next, we studied whether the migration of highly suppressive, activated CCR2+ Tregs from the BM to the tumor tissue might be associated with the induction of TAA-specific effector T-cell responses in the BM. Indeed, we observed a significant correlation between the development of TAA-specific T-cell responses in the BM and an accumulation of Tregs in breast cancer tissue (Fig. 6C).

Tumor vaccination induced migration of BM Tregs to tumor tissue

To evaluate a putative TA-triggered mechanism of initial activation and migration of tumor-specific Tregs from the BM to tumors, we applied a RET transgenic mouse model of spontaneous malignant melanoma (33). Similar to patients with cancer, there were reduced numbers of Tregs in the BM of tumor-bearing mice compared with wild-type (WT) littermates (Fig. 7A; Supplementary Fig. S11A). In addition, decreased Treg frequencies in the BM correlated with the increased accumulation of Tregs in tumors (Fig. 7B). In contrast to the BM, Treg frequencies were unaltered in spleen and lymph nodes of tumor-bearing mice (Fig. 7C; Supplementary Fig. 11B), demonstrating that upon tumor development Treg depletion selectively occurred in the BM. As with the BM-resident Tregs in patients with breast cancer, BM-resident Tregs in ret tumor-bearing mice showed increased expression of CCR2, suggesting their activation (Fig. 7D; Supplementary Fig. S11C). We observed significantly increased expression of the activation marker Lymphocyte activation gene-3 (LAG3; Supplementary Fig. S11D; refs. 57, 58) and of Latency-associated peptide (LAP; Supplementary Fig. S11E), indicating recent antigen-specific TCR activation (58, 59) in BM Tregs of tumor-bearing animals compared with WT animals (Fig. 7D). CCR2 induction and antigen-specific activation were selectively found in BM-resident Tregs and tumor-resident Tregs, but not in Tregs from spleen or lymph nodes (Fig. 7E) or with conventional T cells (Supplementary Fig. S12). CCR2+ LAP+ Tregs were present at high levels only in the BM and tumor, but not in spleen and lymph nodes (Fig. 7F). Sixty-eight percent (n = 7; mean + SD: 67.64 + 24.67) and 81% (n = 8; mean + SD: 80.96 + 21.76) of CCR2+ Tregs coexpress LAP in the BM and tumor, respectively. These findings indicated predominant antigen-specific activation of Tregs in the BM of tumor-bearing mice and suggested that the BM may represent a reservoir for tumor infiltrating activated Tregs. To assess whether Treg migration from the BM to tumor tissue was indeed triggered by an antigen-specific activation, we vaccinated tumor-bearing animals subcutaneously with adenalinaric vectors encoding for the full-length TRP-2 antigen or with a mock vector control. Treg numbers in the BM were reduced, CCR2 on BM Tregs was upregulated, and there were increased Tregs in the PB after TRP-2-specific vaccination (Fig. 7G–I) compared with mice treated with a vector control. In vaccinated animals, we observed that increased proportions of Tregs in the tumor correlated with a pronounced reduction of Tregs in BM (Fig. 7I). Thus, migration of Tregs from the BM was triggered by antigen-specific activation and contributed to increased Treg accumulation in tumor tissue.

Discussion

Here, we demonstrated the presence of TAA-reactive Tregs in the BM of patients with breast cancer and characterized their predominant target specificities. Because breast tumor-reactive Tregs were not detectable in the BM of healthy individuals, we concluded that their induction and expansion occurred in the context of breast tumor development. We previously reported that the BM could be a secondary lymphoid organ in which naïve T cells are primed against TAA through TAA-loaded antigen-presenting cells (29) that migrate from the PB to particular niches within the BM parenchyma (30). Such mechanisms might also apply to naïve Tregs resulting in their clonal expansion. Here, we showed that only TAA-specific Tregs in the BM consistently upregulated CCR2, which suggested that its expression may depend on signals mediated through the TCR. We indeed found that TCR stimulation induced CCR2 expression in BM Tregs. Thus, in the BM, TAA-specific Tregs appear to be consistently activated by TAs. CCR2 was reported to mediate the migration of
Figure 5. Correlation of CCR2 expression and Treg accumulation in BM, PB, and breast tumor tissue. A, CCR2 expression in purified unstimulated (n = 17, white bar) Tregs or after stimulation with anti-CD3 antibodies (n = 4, black bar). Mean ± SEM values are shown. B, Mean ± SEM proportions of purified Tregs (unstimulated or polyclonally activated) from the PB (n = 4) of healthy individuals migrating through transwell membranes are shown. Transwell chambers contained 25 ng/mL (gray circles), 100 ng/mL CCL2 (black circles), or cell culture medium without CCL2 as control (white circles). n.s., not significant. C, Frequencies of CCR2⁺ Tregs from the BM of patients with breast cancer with increased (n = 5, white circles) or decreased (n = 8, gray circles) CCR2 expression levels on BM Tregs as determined by MFI. Low = samples with MFI values of CCR2 expression on CCR2⁺ BM Tregs below median; high = samples with MFI values of CCR2 expression on CCR2⁺ BM Tregs above median. D, Frequencies of CCR2⁺ Tregs in the PB of patients with breast cancer with increased (n = 6, white circles) or decreased (n = 7, gray circles) frequencies of CCR2⁺ Tregs in the BM. Low = samples with frequencies of CCR2⁺ BM Tregs below median (gray circles); high = samples with frequencies of CCR2⁺ BM Tregs above median (white circles). E, Inverse correlation between CCR2 expression levels on BM Tregs and the overall proportions of Tregs in the BM of patients with breast cancer (n = 14). F and G, Correlation between Treg infiltration into tumors and the frequencies of CCR2⁺ Tregs (F) or CCR2 expression levels on Tregs (G) in the blood of patients with breast cancer. Data from 17 different patients are shown in F and G (due to logarithmic representation, four values are not visualized in G). R, correlation coefficient. Unpaired two-tailed Student t test was used for A due to insufficient number of paired samples; paired two-tailed Student t test (B), Fisher exact test (C and D), nonparametric Spearman correlation test (E), and Pearson correlation test (F and G) were also used. Each symbol represents values from 1 patient (C–G).
monocytes from the BM to the PB (47) and thus might also mediate Treg migration. Interestingly, the subpopulation of TAA-specific Tregs remaining in the BM-expressed CCR2 together with CXCR4, which retains immune cells within the BM (60). In contrast to Tregs, TAA-specific Tcon cells expressed lower levels of CCR2 in the BM, indicating that TCR stimulation did not induce CCR2 upregulation in BM Tcon cells or that their stimulation was inhibited by local TAA-specific Tregs.

Although the BM has been described as a site of Treg accumulation (31), we here show that Tregs are strongly reduced in BM of patients with cancer as compared with healthy individuals. This depletion particularly affected TAA-specific and CCR2-expressing Tregs, suggesting that the phenomenon of selective Treg reduction in the BM of patients with cancer is induced by tumor antigen-specific stimulation.

Reduced numbers of CCR2+ Tregs in the BM were associated with increased frequencies of CCR2+ Tregs in the PB. Similarly, tumor-bearing mice contained reduced Treg frequencies in their BM. These Tregs showed increased expression of CCR2 together with markers of antigen-specific activation, which was not the case for Tregs residing in other lymphoid organs such as the spleen or lymph node. Similar levels of CCR2 and respective activation markers were found in tumor-resident Tregs as was found in BM Tregs. We also demonstrated that experimental tumor-specific activation decreased Tregs in the BM, increasing Treg in the PB. Taken together, these data suggest that in patients with breast cancer, TAA-specific stimulation of Tregs in the BM induces their migration from the BM into the PB.

The accumulation of TAA-specific CCR2+ Tregs in the peripheral blood correlated with reduced T effector cell reactivity against TAA, which resulted in significantly lower antitumor responsiveness of T effector cells compared with their BM-derived counterparts. This reduced TAA response by T effector cells in the blood was likely caused by TAA-specific Tregs since their depletion completely restored TAA responsiveness to levels observed for the BM T effector cells. Thus, the activation of TAA-specific Tregs in the BM and their migration to the PB deprived the BM of an important mechanism of self-tolerance, which resulted in the spontaneous induction of TAA-specific T effector-cell responses. This generated a population of activated, TAA-specific Tregs that systemically circulated through the body.

While the PB is most likely not a site of TAA-specific T effector cell or Treg activity, it transports T cells to target organs. Because TAA-specific Tregs in the BM and PB showed significant overexpression of CCR2 and CXCR4, they possessed the capacity to migrate toward gradients of their chemokine ligands CCL2, -8, and -20 (44). We demonstrated here that CCL2 induced directed chemotaxis of activated but not of resting Tregs.

Cells of the innate immune system express chemokines at sites of inflammation and in human breast cancer (50, 51, 53, 55, 56).

**Figure 6.**
Correlation of CCR2 expression in Tregs and TAA-specific T-cell responses in patients with breast cancer. A, TAA-reactive T-cell responses in PB T cells (PBTC) were associated with low numbers of CCR2+ Tregs in the blood. Mean ± SEM frequencies of total Tregs among CD4+ T cells (left graph) or of CCR2+ Tregs among total Tregs (right graph) from patients with breast cancer are shown with (ELISpot result pos, white bars) or without (ELISpot result neg, black bars) breast TA-reactive T-cell responses as determined by IFNγ ELISpot analysis. B, Inverse correlation between the frequencies of CCR2+ Tregs in the PB of breast cancer patients and numbers of TAAs recognized by PB T cells in IFNγ ELISpot analysis (n = 11). Each symbol represents values from 1 patient. C, TAA-reactive T-cell responses in BM T cells (BMTC) were associated with increased Treg infiltration in the corresponding breast tumors. Mean ± SEM values of proportions of total Tregs among CD4+ T cells are shown in patients with breast cancer with (ELISpot result pos, white bar, n = 16) or without (ELISpot result neg, black bar, n = 12) breast TA-reactive BM T-cell responses as determined by IFNγ ELISpot analysis. Unpaired two-tailed Student t test (A and C) and nonparametric Spearman correlation test (B) were used.
Figure 7.
Analysis of Tregs in melanoma-bearing RET transgenic mice. A, Percentage of CD4+CD25+FoxP3+ Tregs (within CD3+CD4+ T cells) in the BM of tumor-bearing (n = 27 Ret tumor mice) and nontransgenic littermates (n = 15 wt mice) represented as mean ± SEM. B, Percentages of Tregs (within CD3+CD4+ T cells from n = 20 mice) in BM were plotted against the frequency of Tregs in the skin tumor from the same mouse. The correlation between the two variables was calculated using a linear regression analysis. C, Proportions of Tregs from spleens and lymph nodes of WT (n = 3) and Ret tumor mice (spleen, n = 7; lymph node, n = 8). D, Expression levels of CCR2, LAG3, and LAP on BM Tregs from WT (n = 3) or Ret tumor mice (n = 7) expressed as MFI and percentage. E, MFI expression of CCR2, LAG3, and LAP on Tregs from the spleen (n = 7), lymph node (n = 8), BM (n = 7), and tumor (n = 8) from Ret tumor mice expressed as MFI and percentage. F, Proportions of Tregs coexpressing CCR2 and LAP in the spleen (n = 7), lymph node (n = 8), BM (n = 7), and tumor (n = 8) of Ret tumor mice (G–I). Transgenic mice were immunized with the adenovirus construct encoding human TRP-2 (AdTRP-2; immunized) or with the control vector encoding EGFP (control). Percentage of Tregs in the BM (n = 5 mice/group). G, Expression of CCR2 (data were expressed as MFI ratio [MFI of experimental samples/MFI of respective negative controls]; n = 5 mice/group and percentage of Tregs in the PB (n = 15 mice/group; H) from control and immunized groups (I). Data are shown as mean ± SEM. J, Percentages of Tregs in the BM (within CD3+CD4+ T cells from n = 12 mice) were plotted against amounts of Tregs in the tumor from the same mouse. The correlation between two variables was calculated using a linear regression analysis. Unpaired Student t test (A, C, D, F, and G), paired two-tailed Student t test (E), one-tailed Mann–Whitney test (H), or parametric Pearson correlation test (B and J) were used. LN, lymph node; ret tu, Ret tumor.
Thus, activated breast TAA-specific Tregs expressing CCR2 and -6 possessed a strong propensity for selective migration toward breast cancer tissue to encounter their cognate antigen and subsequently suppressed the activity of Teff cells. In accordance to this, we demonstrated that tumor-specific vaccination of tumor-bearing mice induced CCR2 expression on Tregs in the BM and caused a significant accumulation of these cells in the tumor tissue that correlated with a decreased Treg frequency in the BM of the same mice. We detected a significant accumulation of tumor-infiltrating Tregs in those patients with breast cancer who had developed spontaneous TAA-specific Teff-cell responses in the BM. The accumulation of Tregs in tumors and their local immune-suppressive activity correlates with poor patient outcome in many cancers, including breast cancer (21, 23). Therefore, mechanisms of tumor-specific Treg activation and migration into the circulation and their immigration into tumor represent critical hurdles for cancer therapy. Tumor-infiltrating Tregs can migrate from tumor draining lymph node in a CCR2-dependent mechanism (46). Our data suggested that BM represents an additional, novel reservoir for tumor-infiltrating Tregs and thus contributes to local immune suppression in tumors.

Our data suggest that activation of TAA-specific Tregs in the BM results in a net reduction of Tregs from this compartment and their migration to tumors allowing initiation of TAA-specific Teff-cell responses in the BM and their migration to the PB. This mechanism may efficiently inhibit the local activity of TAA-specific Teff cells by the accumulation of activated, TAA-specific Tregs in the tumor tissue. This mechanism of immune compartmentalization, which is characterized by overall shifts of larger immune cell subpopulations between the BM and peripheral tissues, can be activated by vaccination with tumor antigens, even under nontolerogenic conditions such as vaccination with a viral vector–encoded TAA. Our findings may provide an explanation why systemic T-cell immunity protects from tumor relapse (after tumor resection), while it appears incapable of protecting from local tumor progression (61).

Disclosure of Potential Conflicts of Interest
H.-H. Böhm is a scientist at Merck KGaA. No potential conflicts of interest were disclosed by the other authors.

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References


59. Tran DQ, Anderson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. Blood 2009;113:5125–33.


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Tumor-Specific Regulatory T Cells from the Bone Marrow Orchestrate Antitumor Immunity in Breast Cancer

Yingzi Ge, Hans-Henning Böhm, Anchana Rathinasamy, et al.


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