CXCL12 Mediates Immunosuppression in the Lymphoma Microenvironment after Allogeneic Transplantation of Hematopoietic Cells

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Abstract

Clinical studies indicate a role of allogeneic hematopoietic cell transplantation (alloHCT) for patients with refractory or recurrent B-cell lymphoma (BCL) indicative of a graft-versus-tumor effect. However, the relevance of local immunosuppression in the BCL microenvironment by donor-derived regulatory T cells (Treg) after alloHCT is unclear. Therefore, we studied Treg recruitment after alloHCT in different murine BCL models and the impact of lymphoma-derived chemoattractive signals. Luciferase transgenic Tregs accumulated in murine BCL microenvironment and microarray-based analysis of BCL tissues revealed increased expression of CXCL9, CXCL10, and CXCL12. In vivo blocking identified the CXCR4/CXCL12 axis as being critical for Treg attraction toward BCL. In contrast to Tregs, effector T cells displayed low levels of CXCR4 and were not affected by the pharmacologic blockade. Most important, blocking CXCR4 not only reduced Treg migration toward tumor tissue but also enhanced antitumor responses after alloHCT. CXCL12 production was dependent on antigen-presenting cells (APC) located in the lymphoma microenvironment, and their diphtheria-toxin receptor (DTR)-based depletion in CD11c.DTR-Tg mice significantly reduced Treg accumulation within BCL tissue. CXCL12 was also detected in human diffuse, large BCL tissues indicative of its potential clinical relevance. In conclusion, we demonstrate that Tregs are recruited toward BCL after alloHCT by infiltrating host APCs in a CXCL12-dependent fashion. Blocking CXCR4 enhanced antitumor effects and prolonged survival of tumor-bearing mice by reducing local Treg accumulation, indicating that CXCR4 is a potential target to interfere with tumor escape after alloHCT.

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Introduction

Allogeneic hematopoietic cell transplantation (alloHCT) was shown to be an effective treatment option in patients with refractory or recurrent non-Hodgkin lymphoma (1–3). CD4+FoxP3+ regulatory T cells (Treg) interfere with antitumor immunity in the presence of autologous tumor (4). The immnosuppressive effect of Treg in the tumor microenvironment was shown not to occur randomly but to be due to the specific recruitment of Tregs (4) or the conversion of conventional T cells into Tregs (5). Increased numbers of Tregs within the tumor tissue, in the tumor draining lymphatic tissue, or in the peripheral blood were found in human samples from solid tumors (4, 6, 7), non-Hodgkin B-cell lymphoma (BCL; ref. 8), Hodgkin lymphoma (9), late stage myelodysplastic syndrome (10), and acute myeloid leukemia (11). Within the tumor microenvironment, Tregs were shown to suppress natural killer (NK) cell–mediated cytotoxicity by affecting NKG2D receptor expression (12), interfering with dendritic cell (DC)-mediated cytotoxicity (13) and T-cell maturation (14).

Following reduced intensity conditioning, residual active BCL tissue is still present in the recipient and could attract functionally immunosuppressive Tregs to the BCL microenvironment, resulting in local immunosuppression (15, 16). Therefore, the goal of this study was to delineate factors that impact Treg migration and accumulation in the presence of active BCL tissue. We employed in vivo bioluminescence imaging (BLI)–based Treg tracking methodologies (17) in combination with neutralization or blockade of different
chemokines that were identified by microarray analysis. Our data clearly indicate that the CXCL4/CXCL12 axis is critical for Treg accumulation within the tumor microenvironment and has a major impact on antitumor effects after alloHCT that could be exploited in the clinical situation.

**Methods**

**Mice**

C57Bl/6 (H-2Kb, Thy-1.2), FVB/N (H-2Kq, Thy-1.2), and Balb/c (H-2Kd, Thy-1.2) mice were purchased from the local stock of the animal facility at Freiburg University. Only gender-matched combinations (6 and 12 weeks old) were used for transplant experiments. Luciferase (lac+) transgenic C57Bl/6 mice have been previously described (18). All animal protocols (G07-19, G08-8) were approved by the University Committee on the Use and Care of Laboratory Animals at Albert-Ludwigs University. CD11c.DTR-Tg mice (19) were kindly provided by A. Diefenbach.

**Human subjects**

All samples were collected after approval of the study protocol by the Ethics Committee of the Albert-Ludwigs University and after written informed consent. Diffuse, large B-cell lymphoma (DLBCL) samples were collected in a prospective manner.

**Bone marrow transplantation model and histopathologic scoring**

Bone marrow transplantation (BMT) experiments were performed as previously described (20). Briefly, recipients were injected intravenously with 5 x 10^6 wild-type (wt) bone marrow cells after lethal irradiation with 900 cGy. The following transplant models were employed: C57Bl/6→Balb/c and FVB/N→C57Bl/6. Slides of liver, small bowel, and large bowel samples collected on day 7 were stained with hematoxylin and eosin (H&E) and scored by an experienced pathologist (U.V. Gerlach) according to a previously published histopathologic scoring system (21).

**B-cell lymphoma models**

BCL cells were injected subcutaneously into the shaved right flank on the day of alloHCT after irradiation. When indicated, wt or lac+ transgenic A20 BCL (Balb/c background, 5 x 10^6), or the 291PC Burkitt lymphoma (C57Bl/6, 2 x 10^5 and 5 x 10^5), was employed. The Burkitt lymphoma cell line 291PC was established from the human λ-c-myc transgenic mouse (22). Primary lymphoma cells from 1 C57Bl/6 λ-hu-c.myc transgenic mouse were cultured on irradiated MRC5 feeder cells in Iscoves’s MDM medium supplemented with 20% fetal calf serum (FCS). The animals were sacrificed when the tumor reached a diameter above 15 mm or showed ulceration.

**Generation of luciferase-expressing 291PC Burkitt lymphoma cells**

The retrovirus encoding the luciferase–aminoglycoside phosphotransferase (neomycin resistance) fusion gene (Luci–Neo) was constructed from the luciferase gene of pUHC 13-3 (pTRE Luc) and the neomycin resistance gene from pcDNA 3.1 (Invitrogen), using L1_eGFP_IRES as a backbone (23). The transduction of the 291PC cells using a VSV-G (BD Clontech) pseudotyped retrovirus was performed according to the instructions from the manufacturer. After selecting successfully transduced cells using 1 to 3 mg/mL of neomycin, their luciferase activity was tested with a standard luciferase assay (Promega E4550) in a Luminometer (BMG Lumistar; BMG Labtech GmbH).

**In vivo chemokine neutralization experiments**

AMD3100 (Sigma) was administered at a previously reported dosage of 5 mg/kg for 7 days after Treg infusion (24). Anti-CXCL3 (clone:CXCR3-173; Biolegend) was injected intraperitoneally at a dosage of 200 μg per mouse, which was previously shown to have in vivo activity (25). Control groups received matched isotype antibody (Ab) at the same dosage (clone:HTK888; Biolegend).

**In vivo BLI**

**In vivo BLI** was performed as previously described (17). Mice were imaged using an IVIS100 charge-coupled device imaging system (Xenogen) for 5 minutes or Berthold Nightowl (Bert-Hold Technologies). Cell expansion was quantified in photons/s/cm² with Living Image 3.0 Software (Calipers).

**In vitro proliferation assays**

CD4^+ T cells from C57Bl/6 mice and irradiated splenocytes from Balb/c mice were purified by MACS-positive selection (Miltenyi). CFSE labeling of cells was done with Vybrant CFDA SE (Molecular Probes) as previously described (26). To test the suppressor activity of CD4^+ T cells, these cells were isolated from either spleen or lymphoma tissues as indicated in the respective experiments.

**Coculture experiments**

Bone marrow DCs were prepared basically as described (27), except that interleukin 4 (IL-4) was not used. A total of 5 x 10^4 Balb/c DCs per well, together with 2 x 10^5 A20 lymphoma cells, were cultivated using supernatant from A20 lymphoma cells in 96-well plates in a total volume of 200 μL at 37°C, 5% CO_2. Supernatants were collected after 24 hours for chemokine measurement.

**Migration assay**

For Treg migration studies, 24-well, flat-bottomed plates with a pore size of 5 μm (Costar; Corning) were employed. RPMI medium with 2% FCS was used in the transwells. CXCL12 (R&D Systems) was added to the medium at a concentration of 100 ng/mL. After incubation for 2 hours at 37°C, the number of cells migrated across the transwell was quantified by fluorescence-activated cell sorting (FACS) analysis.

**Preparation of tumor-infiltrating lymphocytes**

Tumors were removed, homogenized, and digested in RPMI 1640, 20% FCS, and 400 units/mL of collagenase.
Flow cytometry

All Abs were purchased from BD Pharmingen, Biologend, eBiosciences, and R&D and used as FITC, PE, Alexa647, or eFluor450 conjugates. The following Abs were used: CD3 (17A3), CD4 (GK 1.5/RM4-5), CD8 (53-6.7), CD25 (PC61), CD11c (HL3/N418), CD19 (6D5), Thy-1.1 (H1S51), Foxp3 (FJK-16s), CXCR3 (CXCR3-173), CXCR4 (TG12), H-K^q (AF6-88.5), H-K^d (GFI 1.1), H-K^b (KH114), NK1.1 (PK136), CD11b (M1/70), PDGFR (APB5), CD34 (MEC 14.7), and CXCL12 (79018). Data were acquired with a CyanADP flow cytometer (Beckman Coulter) and then analyzed with Flowjo 7/8 software (Tristar).

Conventional histology and immunohistochemistry

Fresh frozen sections of 5-µm thickness were mounted on microscope slides (Superfrost/Plus; R. Langenbrink). Evaluation of tissue sections was performed on a Zeiss Axioplan 2 microscope. The standard objectives used were 20×/numerical aperture 0.45 and 40×/numerical aperture 0.60. Digital photographs from the microscope were obtained using a Spot digital camera. For immunoenzymatic staining, the tissue was fixed for 10 minutes in acetone (Sigma) and the primary biotinylated Ab was applied (anti-CXCL12; R&D systems clone: 79014). For visualization, streptavidin alkaline phosphatase–coupled Ab and corresponding substrate (Vector Labs) or the DAB-system (Dako Cytomation) was used.

ELISA-based cytokine analysis for CXCL-12

Cell culture supernatants were obtained on microplate slides (Superfrost/Plus; R. Langenbrink). Evaluation of tissue sections was performed on a Zeiss Axioplan 2 microscope. The standard objectives used were 20×/numerical aperture 0.45 and 40×/numerical aperture 0.60. Digital photographs from the microscope were obtained using a Spot digital camera. For immunoenzymatic staining, the tissue was fixed for 10 minutes in acetone (Sigma) and the primary biotinylated Ab was applied (anti-CXCL12; R&D systems clone: 79014). For visualization, streptavidin alkaline phosphatase–coupled Ab and corresponding substrate (Vector Labs) or the DAB-system (Dako Cytomation) was used.

Luminometric analysis of different tissues

Organs were collected from Balb/c recipients on day 21 after transplantation, homogenized, and their protein content determined by Bradford assay. Luminometric analysis was performed as previously described (28).

Microarray analysis

RNA was isolated from A20 tumor cells grown in cell culture or from A20 tumor grown in vivo following alloHCT. RNA samples with an RNA integrity number greater than 7 were further processed with the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay as described by the manufacturer. We used the Genedata Expressionist software (version 5.1.2) for further data analysis. CEL files were imported into the Refiner module of Expressionist where quantile normalization and probe summarization were performed using the Refiner RNA condensing algorithm. To identify differentially expressed genes between the A20 cultured cell and the A20 grown in vivo, the unpaired Bayes t test (CyberT; ref. 29), with the Bayes confidence estimate value set to 10 and a window size of 101 genes, was performed with the Analyst module of Expressionist. To control the false discovery rate, the Benjamini–Hochberg q-value was calculated. We then used the "N-fold regulation" activity of Analyst to calculate the median ratio between the A20 without stroma (w/o stroma) group and the A20 with stroma in vivo group.

Statistical analysis

Differences in animal survival (Kaplan–Meier survival curves) were analyzed by log-rank test. For comparison of proliferation of conventional luc transgenic T cells, cytokines, mean fluorescence, and graft-versus-host disease (GvHD) histopathology scores between experimental groups, the Student’s t test was used. P < 0.05 was considered to be statistically significant.

Results

Donor-type Tregs accumulate within malignant BCLs after alloHCT and are functionally suppressive

To study their migratory pattern, Tregs were isolated from luciferase transgenic donors and 10^6 cells were administered to the recipients on day 7 after alloHCT. We observed that Tregs migrated to and accumulated in lymphoma tissue (Fig. 1A, left), which was not the case when benign B cells were injected into the same anatomic area (data not shown). As the purity of adoptively transferred Treg was greater than 90% CD4^+Foxp3 cells (data not shown), at most 10% of the transferred luc^+ cells could be conventional T cells (Tconv), which would be equivalent to 1 × 10^5 T cells. To study whether this amount of Tconv would display the same trafficking pattern, 1 × 10^5 luc^+CD4^+CD25^+ cells were adoptively transferred on day 7 after alloHCT and followed by BLI. In contrast to Tregs, CD4^+CD25^+ cells accumulated in secondary lymphoid organs, GvHD target organs including the abdominal area, skin (ears), and also the A20 BCL region (Fig. 1A, right). On the basis of this obvious difference, we hypothesized that the Treg trafficking pattern mirrored specific recruitment and was not simply due to random accumulation. A comparable Treg recruitment phenomenon was observed in the murine 291PC Burkitt lymphoma model as shown in later studies. To further determine the amount of Treg infiltration in different tissues independent of their depth within the body, which could in turn influence signal intensity, we isolated individual organ and evaluated their luminescence intensity normalized to the protein content of the tissue (Fig. 1B). When luc^+ Tregs were transferred, the highest luciferase activity was found within the A20 BCL tissue (Fig. 1B), corresponding to the BLI data. To determine whether or not the observed BLI
A signal at the A20 BCL sites was derived from functionally suppressive Tregs, CD4 T cells from the A20 BCL or the spleen were used as suppressors in vitro. We observed the strongest suppressor activity when CD4 T cells isolated from A20 BCL tissue were used (Fig. 1C), which was compatible with FACS analysis, indicating that more than 66% of the CD4+ population isolated from the A20 BCL after alloHCT were donor derived (Thy1.1) and Foxp3 positive when Tregs were transferred (Fig. 1D).

Chemokine production within the lymphoma at the time point of Treg transfer

To screen for factors that may be causative for the observed Treg accumulation within the A20 BCL in vivo, we performed comparative microarray analysis of A20 BCL cells. We compared A20 BCL grown in vitro (w/o stroma) to A20 BCL grown in Balb/c mice following alloHCT and containing a tumor stroma or benign B cells (Fig. 2A). On using a fold-change threshold value of greater than 3 and a q-value of less than 0.05, we observed the strongest suppressor activity when CD4 T cells isolated from A20 BCL tissue were used (Fig. 1C), which was compatible with FACS analysis, indicating that more than 66% of the CD4+ population isolated from the A20 BCL after alloHCT were donor derived (Thy1.1) and Foxp3 positive when Tregs were transferred (Fig. 1D).
0.05, 652 genes fulfilled these criteria (619 upregulated and 43 downregulated in A20 BCL + stroma vs. A20 BCL w/o stroma). As we were mainly interested in the chemokine profile, we selected all genes that had an annotation as “chemokine” or “chemokine receptor.” This resulted in 58 genes, of which 11 fulfilled these criteria. We selected the 2 receptor/ligand(s) combinations CXCR3/CXCL9/10 and CXCR4/CXCL12 for later functional in vivo analysis, based on their significantly higher expression in A20 BCL + stroma (Fig. 2B) and the high expression of CXCL12 on CD4⁺Foxp3⁺ cells prior to their adoptive transfer (Fig. 2C). In contrast to CXCR4, CXCR3 was sparsely expressed on sorted CD4⁺Foxp3⁺ cells (Fig. 2C). Therefore, we included CXCR3 blocking in the following functional studies as a negative control.

Interference with CXCR4 but not CXCR3 blocks Treg migration toward A20 BCL

To systematically investigate the relevance of the receptor/ligand(s) pairs for Treg migration, we blocked their function with an Ab against CXCR3 or with the CXCR4 inhibitor AMD3100, which has been shown to prevent CXCR4 engagement (30). Consistent with the increased expression of CXCL12 RNA in A20 BCL tissue, CXCL12 protein could be detected by immunohistochemistry in different murine BCLs (A20, 291PC) at significant levels (Fig. 3A, left). Within the tumor, we could identify antigen-presenting cells (APC), fibroblast cells, and endothelial cells as CXCL12-producing cells (Fig. 3A, middle). AMD3100 but not anti-CXCR3 or PBS was found to reduce the accumulation of luc⁺ Treg toward established A20 BCL after alloHCT, as shown for individual time points (Fig. 3B, top), or when plotted as photons per second over time for the lymphoma area (Fig. 3B, bottom). This observation was consistent with enhanced Treg migration upon CXCL12 stimulation in vitro (Fig. 3C). Because recent evidence suggests a therapeutic role for AMD3100 in non-Hodgkin lymphoma (31), the drug could reduce A20 BCL viability and thereby have an indirect impact on Treg trafficking. However, we found that AMD3100 did not interfere with the viability of the murine A20 BCL during the observation period (data not shown). To study whether or not AMD3100 affected Treg-mediated GvHD protection, the inhibitor was given in combination with PBS. Importantly, neither the protective effect of Treg on survival (Fig. 3D, top) nor that on GvHD severity (Fig. 3D, bottom) was affected when AMD3100 was given. Interestingly, CXCL12 expression was also found in 9 of 15 human DLBCL samples of which 1 representative example is shown (Fig. 3A, right).

Host-derived APCs residing within the BCL tissue are critically involved in CXCL12 production, and their targeted deletion abrogates Treg recruitment

In vitro CXCL12 production by A20 cells alone was either low or absent. However, it could be significantly increased when APCs were included in the culture (Fig. 4A). Immunohistochemical staining of A20 BCL tissue isolated after alloHCT showed the presence of CD11c⁺ APCs within A20 BCL tissue (Fig. 4B, top). The majority of APCs residing in the tumor microenvironment were of recipient (H2-Kd) origin (Fig. 4B, bottom). On the basis of the observation that CXCL12 was not produced by the A20 BCL cells themselves, and that in vitro APCs were the major source of the chemokines, we aimed to assess the relevance of the BCL infiltrating, host-derived APCs for Treg migration in a functional in vivo study. Therefore, CD11c⁺DTR Tg mice (19) were used as alloHCT recipients, and CD11c depletion by diphtheria toxin (DT) yielded potent APC reduction when given 2 days prior and 2 days after alloHCT (data not shown). In these experiments, the Burkitt-type 291PC BCL was used, as it was on the same genetic background as the CD11c⁺DTR Tg mice (both C57Bl/6). The absence of host APCs caused a reduction in Treg recruitment signal toward the established 291PC BCL, as shown for an individual time point (Fig. 4C, left) and in the quantification of the luc signal over the 291PC BCL region (Fig. 4C, right). In keeping with the functional studies, CXCL12 was not detectable in the 291PC BCL tissue of CD11c-depleted CD11c⁺DTR Tg mice (Fig. 4D). These data are indicative of host APCs playing a major role after alloHCT in the process of CXCL12-mediated chemoattraction of Treg toward the 291PC BCL tissue. These data confirm the presence and biological activity of host APCs following alloHCT (32).

Low level of CXCR4 expression allows effector cells to infiltrate BCL tissue independent of CXCR4 neutralization

Because the usage of AMD3100 affected Treg migration to BCL tissue, we next studied the impact of AMD3100 on the immune compartment within the 291PC BCL. The numbers of CD4, CD8 T cells, and NK cells in the BCL tissue were not significantly different whether or not AMD3100 was administered (Fig. 5A). This obvious difference in comparison with Tregs was based on the low to absent CXCR4 expression on the surface of conventional CD4 T cells, CD8 T cells, and NK1.1 cells among the lymphocytes isolated from the spleen (Fig. 5B). These data indicate that cytotoxic T cells and NK cells were not prevented from reaching the 291PC tissue when CXCR4 was blocked.

Impact of CXCR4 neutralization on antitumor effects and survival

To study the effects of CXCR4 blocking by AMD3100 on the rejection of the BCL by allogeneic T cells, growth of luciferase transgenic 291PC lymphoma after alloHCT was monitored by BLI in a longitudinal fashion. The addition of donor type T cells decelerated the growth of the lymphoma, although in all groups several mice developed strong tumor growth (Fig. 6A). The strength of the antitumor effect was reduced when Tregs were adoptively transferred and only PBS treatment was given (Treg/PBS). The group that received Tregs and AMD3100 displayed significantly less tumor growth than the group receiving Treg/PBS (P = 0.026; Fig. 6A). Reduced tumor growth translated into improved survival of the Treg/AMD3100 group as compared with the Treg/PBS group (P = 0.04; Fig. 6B). These data indicate that AMD3100 enhances antilymphoma effects postallografting most likely by blocking Treg recruitment toward the lymphoma tissue.
Figure 2. Differential chemokine expression within the A20 BCL and corresponding receptors on Tregs. A, RNA was isolated from A20 BCL tissue grown for 7 days in vivo (+ stroma) or from A20 BCL grown in vitro cultures (w/o stroma). Experiment names are aligned as columns and genes as rows. The gene expression values are color-coded, and the color bar on the left displays the coding for the expression values in a logarithmic scale. B, absolute gene expression for the indicated chemokines is displayed as RMA signal values. The P values are indicated for the individual comparisons on top of each bar diagram. C, surface expression of the corresponding receptors, CXCR3 and CXCR4, after gating on CD4+ cells prior to adoptive transfer is displayed. Top, representative FACS plots; bottom, percentages of CD4+Foxp3+CXCR3+ and CD4+Foxp3+CXCR4+ are displayed for sorted CD4+CD25+ cells. Each data point represents an individual donor mouse (n = 9; *, P < 0.0001).
**Figure 3.** Impact of in vivo CXCR4 blocking on Treg recruitment to A20 BCL tissue. A, indicated tissues (SB, small bowel; LB, large bowel; ILN, inguinal lymph nodes; and spleen, liver, 291PC lymphoma, A20 lymphoma) were isolated on day 7 after alloHCT and analyzed by immunohistochemistry for the expression of CXCL12. Left, number of CXCL12+ cells/high-power field (12 high-power fields per organ analyzed). The P values are indicated for the individual comparison in the figure. Middle, A20 lymphoma tissue was isolated on day 15 after alloHCT performed as described for the C57Bl/6 > BALB/c combination. Pregating was on CXCL12+ cells. Displayed are APCs (CD11c+CD11b–, CD11c–CD11b–, CD11c–CD11b+ MΦ), fibroblast cells (PDGFR+CD19–), and endothelial cells (CD34+CD19–). A total of 7 or more individual mice were analyzed, and the experiment was performed twice. Right, representative human DLBCL tissue stained for CXCL12 (red) as described earlier (original magnification ×200). A total of 15 individual lymphoma patients were analyzed (9/15 positive). B, Treg in vivo migration was assessed as described for Fig. 1. AMD3100 (AMD, n = 9, 5 mg/kg of bodyweight), or PBS (n = 6) was injected daily from day 7 to 13. Top, representative time points of BLI. Bottom, signal intensity derived from lymphoma area (red circle) region (y-axis) and time (x-axis) is plotted for the indicated groups. Experiments were repeated 3 times with total number of mice indicated earlier (SB, n = 15/LB, n = 12). C, in vitro migration of Tregs was assessed in a transwell chamber with or w/o CXCL12 as a stimulus (*, P < 0.05). The experiment was repeated 3 times. A representative experiment is shown. D, top, survival of C57Bl6 recipients that received lethal irradiation (9 Gy) and 5 × 10⁶ bone marrow cells (H-2Kq) and where indicated T cells (day 2, intravenously 1 × 10⁶) and Tregs (day 0, intravenously 5 × 10⁵). AMD3100 (5 mg/kg of bodyweight) or PBS were injected intraperitoneally day 0 to 6. Bottom, 7 days after transplantation, mice from the indicated groups (each n = 3) were sacrificed and analyzed for evidence of GvHD (AMD = AMD3100).
Figure 4. In vivo depletion of APCs reduces Treg migration to and CXCL12 production in the lymphoma. A, amounts of CXCL12 within the supernatant of A20 cells alone, APCs alone, or a coculture of both, or the combination of A20 culture supernatant and APCs is displayed; *, P < 0.05. B, CD11c+ APCs are detected in the A20 BCL in vivo. Top, staining for CD11c-DAB/hematoxylin or CD11c-Alexa-488/DAPI (original magnification ×1,000). Bottom, representative FACS plot of cells isolated from the A20 BCL tissue 20 days after alloHCT (each n = 5), percentages of recipient (H-2Kd)/donor-type (H22-Kb) CD11c+ APCs within tumor and spleen. C, 291PC cells (2 × 10^5) were given subcutaneously in the right flank after irradiation of C57Bl/6 alloHCT recipients (H2-Kb). Recipients were either wt (n = 7) or CD11c.DTR Tg with (n = 5)/w/o DT (n = 3) treatment as indicated; luc+ Tregs were given day 7 after alloHCT. Left, 2 representative time points. Right, photons derived from Tregs within the A20 BCL (red circle) area (wt vs. DT, P = 0.066; w/o DT vs. DT, P = 0.08). The experiment was repeated twice. D, 291PC Burkitt lymphoma was isolated on day 13 after alloHCT performed as described under C and analyzed by immunohistochemistry for CXCL12. Left, representative immunohistologic staining for CXCL12, original magnification 100×, counterstaining with hematoxylin. Right, quantification of CXCL12+ cells within high-power fields for each group (n = 11; *, P < 0.05).
Discussion

The failure of immune responses to eliminate tumors has been convincingly attributed to active suppression mediated by Tregs recruited toward the tumor (8) or its draining lymph nodes (33). In contrast, the link between donor-derived Treg accumulation in BCL and the local effector immune response after alloHCT is poorly understood.

By tracking Tregs in vivo, we observed their migration to different BCL tissues after alloHCT, suggesting their immunomodulatory effect at this site. Mechanistically, we could show that A20 BCL tissue displayed increased expression of CXCL12 and that this chemokine was functionally relevant for Treg recruitment whereas CXCL9–11, which binds to CXCR3, was not. CXCL12 was dependent on the presence of tumor-infiltrating host APCs as shown by immunohistologic analysis. The in vivo depletion of host-type CD11c+ cells in CD11c-DTR-Tg recipients was sufficient to reduce Treg accumulation in 291PC BCL tissue. These findings indicate that host APCs that survive irradiation (34) infiltrate BCL tissue and are essential for Treg recruitment. Selective recruitment of CXCR4-expressing Tregs to the tumor may be the underlying cause for the high expression of CXCR4 on tumor Tregs.

Under physiologic conditions, CXCR4+ Tregs are recruited to the bone marrow where significant levels of CXCL12 are produced (35). Our observations that Tregs accumulate in BCL tissue after alloHCT are compatible with studies indicating their recruitment toward the bone marrow compartment in patients with bone marrow infiltration by multiple myeloma (36). In solid tumors, it has been previously shown that the chemokine is involved in Treg recruitment toward pleural mesothelioma (37).

Furthermore, it was reported that the CXCR4/CXCL12 axis is crucial for tumor trafficking of plasmacytoid DCs in patients with ovarian cancer (38). Apart from cell recruitment, CXCL12 induced a regulatory phenotype in conventional CD4 T cells involved in experimental autoimmune encephalomyelitis (39), suggesting its immunomodulatory role. Besides their endogenous CXCL12 production, APCs residing within the lymphoma microenvironment may indirectly enhance the production of the chemokine by stroma cells including fibroblasts and endothelial cells. It was previously shown that different cell populations, including tumor cells (40, 41), stromal fibroblast cells (42), and vascular endothelial cells (43, 44), can contribute to the production of CXCL12.

Figure 5. Effector cell expression of CXCR4 and accumulation independent of CXCR4 blocking. A, 291PC lymphoma cells (2 × 10^6) were given subcutaneously in the right flank after irradiation and BMT of C57Bl6 alloHCT recipients (H2-Kb). Tconv (1 × 10^6, H2-Kb) were given when the tumor was established following alloHCT. Tumor-infiltrating cell populations were isolated 5 days after T-cell transfer from lymphoma tissue. Each data point represents an individual mouse (PBS: n = 7; AMD: n = 6). No significant difference was noted between the PBS and the AMD3100 group (AMD = AMD3100). B, CXCR4 expression in different cell populations isolated from spleen of untreated mice was measured. Left, representative FACS plots for NK1.1+, CD4+, CD4/FoxP3+, and CD8+ T cells with respect to CXCR4 expression. Right, percentage of CXCR4+ cells within the NK1.1+, CD4+, CD4/FoxP3+, and CD8+ populations.
CXCR4 blocking did not affect A20 BCL rejection by conventional T cells and also allowed for protection from GvHD. This would suggest that blocking the CXCR4/CXCL12 axis may have clinical potential in modifying the immune response after alloHCT. Such modification could be particularly important in the setting of residual tumor burden after alloHCT, and in light of the observation that high Treg counts were associated with increased relapse rates in patients with chronic myeloid leukemia after alloHCT (45).

This hypothesis is supported by our finding that Tregs influenced 291PC lymphoma rejection when transferred after alloHCT. This is in line with previous reports on the impaired rejection of P815 mastocytoma cells when Tregs were given following alloHCT (46). One could speculate that this impairment of tumor control due to transfer of Tregs after allografting is caused by rapid tumor growth as well as the subcutaneous location, which may support immune escape, as has been described for immune-privileged regions such as the eye (47) or the skin (48). In such settings, Tregs may tip the balance toward the failure of T cell–mediated tumor rejection despite the strength of alloreactivity. In light of the current use of AMD3100 in patients undergoing hematopoietic cell transplantation (49) and our finding that CXCL12 is detectable in human DLBCLs, AMD3100 treatment may be a justified addition to adoptively transferred Tregs.

In conclusion, we observed that Tregs are recruited toward BCL after alloHCT and that this immunosuppressive process is dependent on BCL-infiltrating host-type APCs. Affecting Treg recruitment toward BCL tissue through the blockade of the CXCR4/CXCL12 axis by the use of AMD3100 enhances antilymphoma effects, which may have important clinical implications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

C. Dürr designed the experiments, performed experiments, analyzed data, and helped to write the manuscript. D. Pfeifer supervised and analyzed microarray experiments. R. Claus performed and analyzed experiments. A. Schmitt-Graeff supervised immunohistochemistry of human lymphoma samples; U.V. Gerlach performed histopathologic scoring; R. Graesser helped to generate expression of luc:291PC Burkitt lymphoma cells; A. Gerbitz provided Burkitt lymphoma cells, helped to design tumor experiments, and to write the manuscript; R.S. Negrin helped to design experiments and to write the manuscript; and R. Zeiser designed the studies, analyzed data, and wrote the manuscript. All authors have read and agreed to the final version of the manuscript.

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