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Macrophage inflammatory protein-2 (MIP-2)/CXCR2 blockade attenuates acute graft-versus-host disease while preserving graft-versus-leukemia activity

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ABSTRACT

Allogenic bone marrow transplantation (BMT), an important treatment for hematological malignancies, is often complicated by graft-versus-host disease (GVHD). Suppression of GVHD is associated with the unwanted diminishment of the graft-versus-leukemia (GVL) response. The aim of this study was to maintain the benefits of GVL during GVHD suppression through isolated blockade of T-cell migration factors. To this end, we developed a murine model of B-cell leukemia, which was treated with BMT to induce GVHD. Within this model, functional blockade of MIP-2/CXCR2 was analyzed by observing proteomic, histologic and clinical variables of GVHD manifestation. Luminex assay of collected tissue identified several cytokines [granulocyte colony-stimulating factor (G-CSF), keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), and interleukin-23 (IL-23)] that were upregulated during GHVD, but reduced by neutralizing the MIP-2/CXCR2 axis. In addition, donor T-cell blockade of CXCR2 combined with recipient administration of anti-MIP-2 caused a significant decrease in GVHD while preserving the GVL response. We propose that blocking the MIP-2/CXCR2 axis represents a novel strategy to separate the toxicity of GVHD from the beneficial effects of GVL after allogenic BMT.

1. Introduction

Allogenic bone marrow transplantation (BMT) is an important therapy for several hematologic malignancies [1]. One primary therapeutic benefit of BMT in this setting is the resultant graft-versus-leukemia (GVL) effect, caused by T cells and natural killer cells derived from the grafted stem cell population that attack malignant cells in the host. However, the same process drives graft-versus-host-disease (GVHD), a potentially fatal complication of BMT that causes acute damage to the gastrointestinal (GI) tract, liver, skin, and lungs [2]. Strategies that effectively separate the beneficial effects of GVL from the adverse effects of GVHD are necessary to reduce the mortality and morbidity of BMT.

We previously showed that during GVHD, donor T cells sequentially migrate to target organ locations, and that this is required for GVHD progression at those organs [3,4]. This led to the hypothesis that inhibition of donor T-cell recruitment to these sites would abrogate GVHD-mediated pathology. We further posed that this approach would not significantly alter donor T-cell function, thus preserving beneficial GVL effects [5,6].

Herein we compared the abilities of several cytokines and chemokines released from the small intestine, liver, and skin to selectively recruit T cells to these organs during the acute phase of GVHD [7]. In addition, we evaluated the effect of blocking macrophage inflammatory protein-2 [MIP-2, also known as chemokine (C-X-C motif) ligand 2 (CXCL2)], a chemokine commonly expressed by GVHD target organs (intestine, liver). For consistency, we will further use the name CXCL2. Finally, we assessed whether simultaneous neutralization of the CXCL2 reciprocal receptor, chemokine (C-X-C motif) receptor 2 (CXCR2), which is expressed in donor T cells, affected the prevalence and characteristics of GVHD and GVL after BMT. Blockade of the CXCL2/CXCR2 axis synergistically attenuated GVHD lethality without loss of the protective effects that characterize GVL. These findings suggest that inhibition of the CXCL2/CXCR2 axis interrupts donor T-cell migration to select organs, comprising a novel and effective strategy to attenuate GVHD after allogenic BMT while maintaining the GVL response.

2. Materials and methods

2.1. Animals

Female BALB/c (H-2Kd) mice were purchased from OrientBio (Eumsung, Korea). EGFP-transgenic mice, originally produced by

Abbreviations: GVHD, graft-versus-host disease; BMT, bone marrow transplantation; GVL, graft-versus-leukemia; KC CXCL1, keratinocyte-derived chemokine; MIP-2 CXCL2, macrophage inflammatory protein-2; CXCR2, chemokine (C-X-C motif) receptor 2; PKH, Paul Karl Horan; BU, busulfan; CY, cyclophosphamide.

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Dr. M. Okabe, Osaka University, were obtained from RIKEN BRC [BRC No. C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131Osb]. All animals were maintained under pathogen-free conditions on a 12 h light/dark cycle with free access to food and water. All procedures were approved by the Animal Care and Use Committee of the Ewha Womans University, School of Medicine (ESM 11-0166).

2.2. Induction of GVHD

Recipient female BALB/c mice received busulfan (BU, 80 mg/kg) daily for 4 days, followed by cyclophosphamide (CY, 200 mg/kg) daily for 2 days via intraperitoneal injection. After a day of resting (day-1), BMT was performed on day 0 according to the following procedure. Bone marrow cells (BMCs) were obtained from the femur and tibia of male EGFP-transgenic donor mice, and a single cell suspension was prepared. A spleen (SP) single-cell suspension was prepared by disrupting the spleen. Recipient mice were injected with 2×10^7 BMCs combined with 3×10^7 SP cells, in a total volume of 250 μl, via lateral tail vein injection. Mice transplanted with BMCs alone were compared as healthy control group.

2.3. Assessment of GVHD

Recipient mice were examined daily during the experimental period. Tissue samples from liver, small intestine, and colon were stained with hematoxylin and eosin (H&E) and analyzed to confirm the presence of GVHD. Three weeks after BMT, tissue samples derived from colon, small intestine, lung, and liver were procured, fixed in formalin, and embedded in paraffin. Sections (5 μm) were stained with H&E and examined under an Olympus BX51 light microscope (Olympus, Japan).

2.4. Tumor induction

A20 murine B-cell lymphoma cells (ATCC No. TIB-208) were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.05 mM 2-mercaptoethanol for 7–10 days before BMT. Prior to injection, the cells were labeled with Paul Karl Horan 26 (PKH 26) using a red fluorescence cell linker kit (Sigma Aldrich, Cat. No. MINI26). On the day of BMT (day 0 of the GHVD induction schedule), BMT cohorts were given 1×10^6 A20 cells via lateral tail vein injection.

2.5. Multiplex analysis

On day 3, mice were sacrificed and tissue samples (liver and colon) were immediately harvested and lysed.

The Luminex Xmap Technology (Millipore, MILLIPLEX™) uses proprietary techniques to internally color-code microspheres with two fluorescent dyes [8,9]. Using precise concentrations of these dyes, 100 distinctly colored bead sets can be created with individual beads coated with a specific capture antibody [10,11]. The following cytokines were measured; Panel 1: G-CSF, GM-CSF, IFNγ, interleukin (IL)-1α, IL-2, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17a, IP-10, KC, MCP-1, MIP-1α, MIP-1β, RANTES, TNFα, and CXCL2; Panel 2: IL-22, IL-25, and TARC/CCL17; Panel 3: IL-23, IL-33/NF-HEV (mature), and TIMP-1 [12].

2.6. Blockade of CXCR2 during BMT

Anti-CXCR2 antibody (R&D Systems, MAB2164) was used to block surface-expressing CXCR2 on the spleen cells before transplantation. The cells were pre-incubated with antibody (10 μg/ml per 10^6 cells/ml) at 37 °C for 1 h at room temperature. Rat IgG-2a (R&D Systems) was used as a control antibody. In addition, recipient-derived CXCL2 was neutralized using anti-CXCL2 antibody (R&D Systems, MAB 452). Anti-CXCL2 or control antibody (200 μg rat IgG-2b, R&D Systems) was administered intraperitoneally 2 h before BMC and SP cell transplantation, and again 2 days after the procedure. Recipient mice were monitored for survival, clinical signs of GVHD, and the extent of inflammation [13–15].

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

To confirm the expression of BCL-2 in the A20 cell line, total RNA was extracted from A20 cells using a TRIzol® RNA extraction kit (Invitrogen). Total RNA (1 μg) was transcribed into complementary DNA using a reverse transcription system (Promega). The primers used for PCR were: 5′-TAC CGT CGT GAC TTC GCA GAG-3′ (forward) and 5′-GGC AGG CTG AGC AGG GTC TT-3′ (reverse) for BCL-2 (350 bp). As control for RNA input, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was evaluated using the primers 5′-GTC TTC TCC ACC ATG GAG AAG GCT-3′ (forward) and 5′-CAT GCC AGT GAG CTT CCC GTT CA-3′ (reverse, 395 bp). To quantify BCL expression in recipient mice, total RNA extracted from BMCs or peripheral blood (PB)-derived mononuclear cells (MNCs) was assayed by real-time PCR [16,17]. Real-time PCR using a SYBR Green detection kit (Applied Biosystems) was conducted using 50 ng of cDNA with an ABI PRISM 7000 sequence detection system (Applied Biosystems).

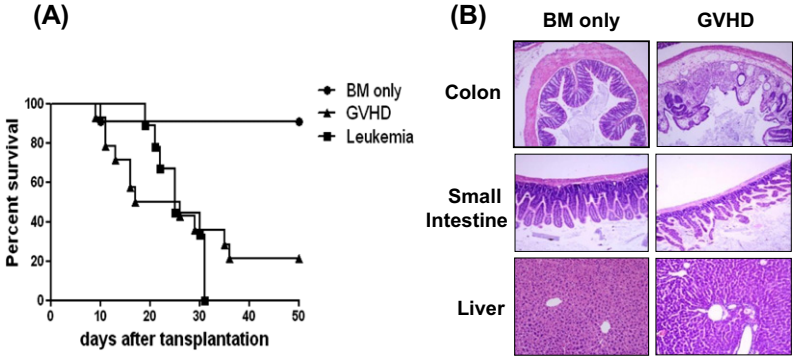

Fig. 1. Induction of GVHD. Female BALB/c mice conditioned with 80 and 200 mg/kg of BU and CY, respectively, were administered PKH (Sigma–Aldrich) labeled 1×10^6 A20 cells and then transplanted the next day with either 2×10^7 allogenic bone marrow (BM) cells alone (leukemia) or in combination with 3×10^7 spleen (SP) cells (GVHD). Mice transplanted with 2×10^7 allogenic BM alone (BM only) following BU-CY preconditioning were used as control group. (A) Survival rate analysis of different treatment groups were performed using Kaplan–Meier estimates (*P-value < 0.05). (B) Histologic analysis of colon, small intestine, and liver from representative tumor bearing BALB/c recipients 3 weeks after transplantation with B6 BM and SP cells (GVHD) or from BALB/c recipients transplanted with BM only (magnification 200×).

2.8. Flow cytometry

On day 21 after GVHD induction, MNCs derived from bone marrow or peripheral blood were collected to quantify the degree of red fluorescence-expressing cells. Data were acquired on a FACSCalibur system (BD Bioscience) and analyzed using CellQuest software (BD Bioscience).

2.9. Statistical analysis

Values were expressed as mean ± standard error of the mean (SEM). Survival curves were plotted using Kaplan–Meier estimates. Non-parametric Mann–Whitney test and two-way analysis of variance (ANOVA) were conducted using GraphPad Prism 5 (GraphPad Software Inc.). A P value <0.05 was considered statistically significant.

3. Results

3.1. Clinical manifestations of GVHD

A tumor-bearing mouse model of GVHD was established as shown in Fig. 1. Leukemia was induced in mice conditioned with BU-CY by injection with A20 cells. Subsequently, allogenic BMCs for leukemia or BMCs plus SP cells for GVHD were transplanted. While leukemic mice showed drastic mortality within 30 days, the addition of SP cells to the BMT procedure significantly

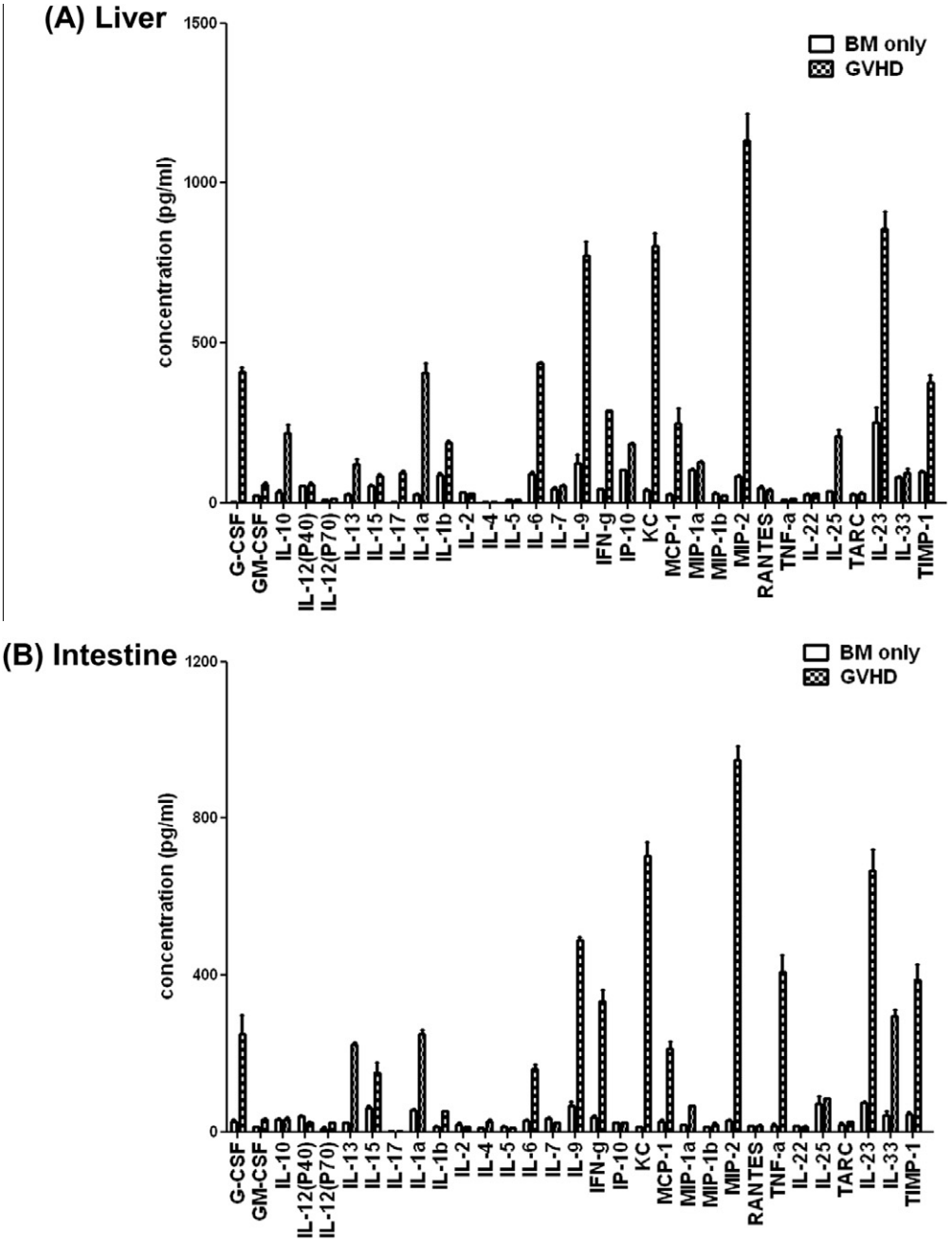

Fig. 2. Increased cytokines and in target organs during GVHD. Small intestine, liver, and skin tissue was isolated from GVHD mice 3 days after transplantation of BM plus SP cells, and tissue lysates were evaluated for cytokine and chemokine levels using the Luminex multiplex array (data not shown). Four compounds were found to be most strongly upregulated [G-CSF, KC, MIP-2 (CXCL2) and IL-23].

attenuated this, even though there was an early onset of death in all animals (Fig. 1A). Major target organs of GVHD, including intestine and liver, showed clear histopathological evidence of GVHD. At day 21 post-SP transplantation, mucosal crypts in the GI tract (small intestine and colon) were severely disturbed, showing hyperplasia and hyperchromatic nuclei with numerous cells exhibiting apoptotic characteristics. Liver samples at day 21 post-SP transplantation showed extramedullary hematopoiesis and inflammatory infiltrates in the portal triad (Fig. 1B).

3.2. Cytokine and chemokine expression in GVHD target organs

On day 3 of GVHD induction, we harvested GVHD target organs (liver and colon) and prepared tissue lysates (five mice per group), measuring cytokine and chemokine protein levels using the Luminex multiplex assay. Thirty-one different compounds were measured; the most frequently upregulated proteins were G-CSF, keratinocyte-derived chemokine (KC; CXCL1), MIP-2 (CXCL2), and IL-23 (Fig. 2A and B). Interestingly, KC and CXCL2 are homologue proteins that act on the same receptor (CXCR2), which implies that targeting CXCR2 might have a duel effect, addressing two proteins that are both upregulated during GVHD in our model.

3.3. Blockade of the CXCR2/MIP-2 (CXCL2) axis attenuates the GVHD response

Because recruitment of T cells into the recipient target organ is critical to the maximal induction of GVHD, we studied chemokine production in the BMT recipients. As observed with the multiplex assay, the target organs strongly produced CXCL1 and CXCL2, which share a common receptor, CXCR2. We tested how CXCR2 inhibition on donor T cells affects the induction and pathogenesis of GVHD. In addition, we blocked host-derived CXCL2 by administering anti-CXCL2 to the recipient. Recipient mice were transplanted with SP cells that had been treated with anti-CXCR2 antibody. As shown in Fig. 3A, the use of the anti-CXCR2 antibody delayed the onset of GVHD and improved survival compared to mice treated with control antibody. Because CXCR2 is activated by its ligand CXCL2, it is possible that the beneficial effect of anti-CXCR2 could be synergistically enhanced by co-inhibiting

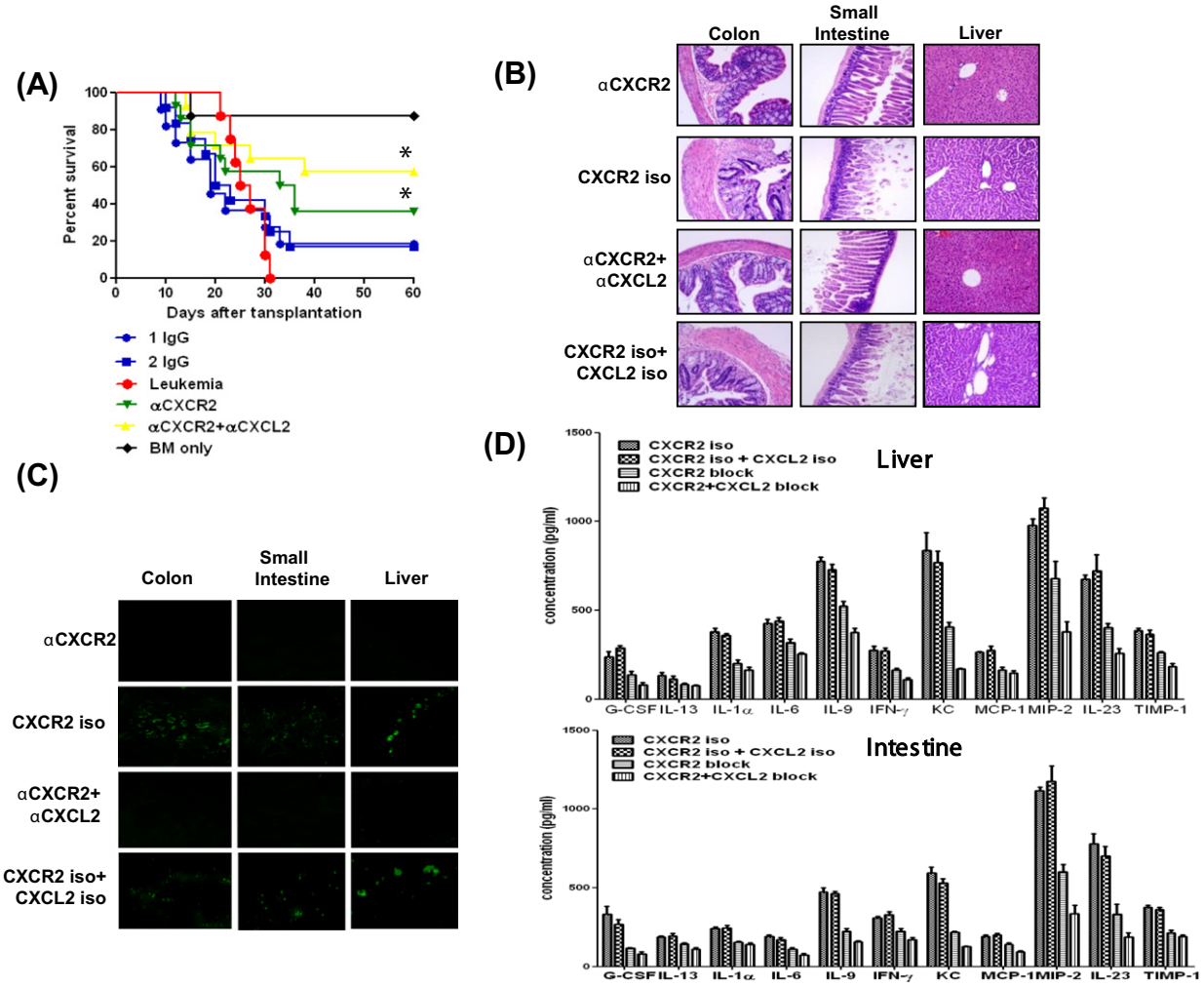

Fig. 3. Antibody blockade of CXCR2 signaling reduces severity of GVHD. To test the effect of blockade of CXCR2 signaling, SP cells were pre-incubated with anti-CXCR2 (10 μg/ml per 1×10^6 cells/ml) at 37 °C for 1 h. Recipient mice from the GHVD group also received anti-CXCL2 neutralizing antibodies intraperitoneally 2 h before BMC and SP cell transplantation, and again 2 days after transplantation (200 μg/injection), in order to eliminate host-derived CXCL2. Isotype antibodies (IgG) were used as controls. Blocking of CXCR2 signaling significantly reduced mortality compared to controls (*P-value < 0.05). Overall survival is shown in panel A and histological sections of colon, small intestine, and liver from representative BALB/c recipients in each experimental group are shown in panel B (magnification ×200). (C) GFP positive donor SP cells were observed in target organs taken from isotype-injected GVHD mice, whereas the presence of SP cells was diminished in groups treated with blocking antibody (magnification ×400). (D) Eleven cytokines/chemokines (G-CSF, IL-13, IL-1α, IL-6, IL-9, IFN-γ, KC, MCP-1, CXCL2, IL-23, and TIMP-1) that were upregulated in liver and intestine at the initial GVHD stage were reduced by neutralizing the CXCL2/CXCR2 interaction.

CXCL2. To determine whether CXCL2 blockade in the host alone would be sufficient to reduce GVHD, we administered anti-CXCL2 to recipient mice prior to SP transplantation. We found that the combined blockade of CXCR2 and CXCL2 caused an additional reduction of GVHD, evidenced by enhanced survival (Fig. 3A) and attenuated histopathologic determinants of GHVD (Fig. 3B). Interestingly, the presence of GFP-expressing donor cells in target organs was prevented by CXCR2/CXCL2 blockade (Fig. 3C). These data indicate that preventing the interaction of CXCL2 with its receptor CXCR2 can effectively reduce GVHD.

3.4. Blockade of the CXCR2/CXCL2 axis decreases inflammation in target organs

Because we observed significant inflammation in liver and intestine during GVHD, we assessed the levels of cytokines and chemokines under CXCL2/CXCR2 axis blockade. Interestingly, inhibition of CXCL2/CXCR2 axis attenuated overall inflammation, and specifically the levels of G-CSF, IL-13, IL-1α, IL-6, IL-9, IFN-γ, KC, MCP-1, CXCL2, IL-23, and TIMP-1, which were upregulated in liver and intestine during the initial stage of GVHD (Fig. 3D). This suggests that blocking the recruitment of donor T cells by specific ligand/receptor interference can reduce inflammation in this setting.

3.5. Blockade of the CXCR2/CXCL2 axis retains the GVL effect

We tested whether inhibition of the CXCL2/CXCR2 axis affects the ability of donor T cells to mount an anti-tumor response *in vivo*. We used an A20 B-cell lymphoma model that is sensitive to T cell-mediated elimination.

The BU-CY preconditioned recipient mice were injected with red fluorescence-labeled A20 cells. The next day the animals were transplanted with allogeneic BM cells (for leukemia) or BM cells in combination with SP cells (for GVHD). Twenty-one days after transplantation, MNCs were isolated from BM and PB to compare the prevalence of red fluorescent cells. The proportion of red fluorescence-expressing cells was significantly decreased by inhibition of the CXCR2/CXCL2 axis (Fig. 4A), as well as the expression of oncogene BCL-2 (Fig. 4B). Thus, while CXCL2/CXCR2 axis blockade attenuated GVHD, it did not hinder the ability of donor T cells to mediate the GVL response.

4. Discussion

Allogenic BMT is frequently complicated by GVHD, which is caused by alloreactive T cells from the graft [3,18] and promotes an immune reaction against the immune-suppressed host. In the setting of leukemia, part of this pathologic process has a beneficial effect, termed GVL, through eradication of residual host malignancy [2,19]. Unfortunately, detrimental aspects of GVHD are closely intertwined with therapeutic GVL responses, complicating the management of hematological malignancies.

The pathophysiology of acute GVHD is divided into three phases [20,21]. In the initial phase, the pre-transplantation drug regimen promotes inflammation as well as expression of major histocompatibility complex (MHC) and co-stimulatory molecules in antigen-presenting cells (APCs) [10]. In the second phase, termed the

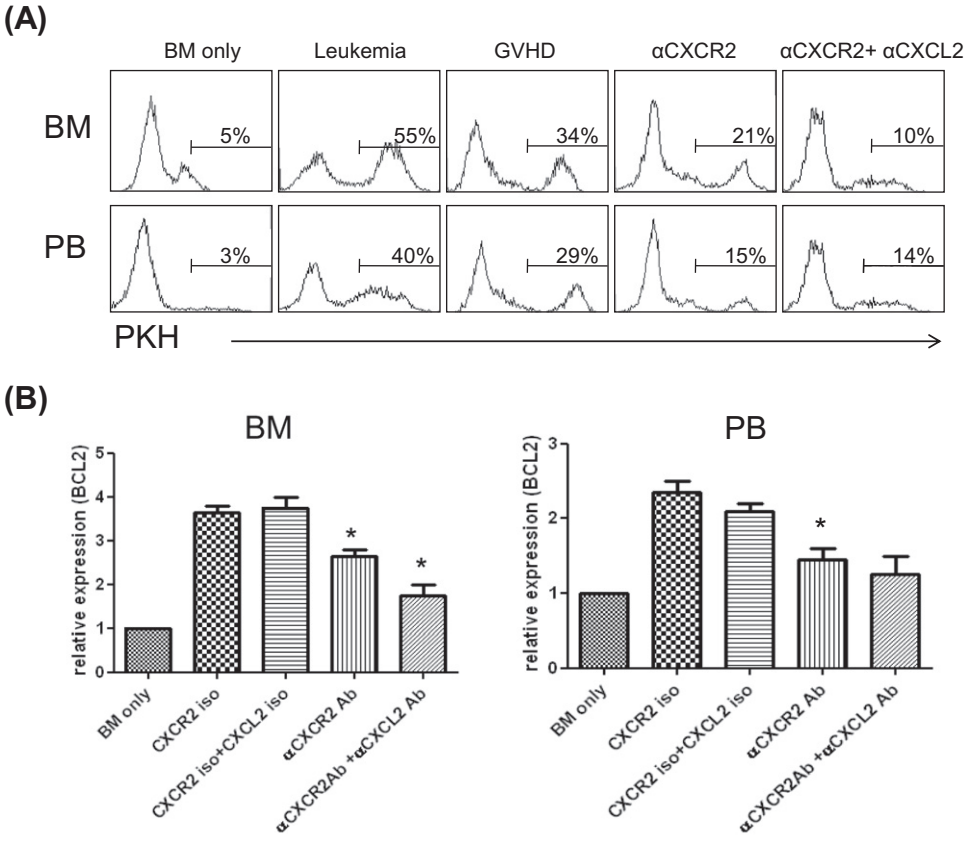

Fig. 4. Antibody blockade of CXCR2 signaling preserves the GVL effect. On day 21 after SP cell transplantation, BM and PB cells from recipient mice were isolated and analyzed for red fluorescence (PKH26 positive A20 cells) by flow cytometry. Also expression levels of Bcl2 were determined by real-time PCR. (A) Co-inhibition of CXCL2 and CXCR2 significantly attenuated the proportion of PKH26 positive A20 cells in both BM and PB. (B) Bcl2 expression in BM and PB was also reduced by inhibition of CXCL2/CXCR2 ligation compared to each isotype treatment (*P-value < 0.05).

activation phase, donor T cells interact with APCs, inducing activation and proliferation. The activated T cells differentiate into effector cells and secrete cytokines, targeting major organ systems such as the GI tract, liver, skin, and lungs. During the third phase, these targeted structures undergo apoptosis, mediated by inflammatory cytokines including tumor necrosis factor-alpha. In addition, inflammatory processes are induced by endotoxins that enter via the gastrointestinal mucosa, which is interrupted by the chemoradiation regimen that preludes BMT [22]. The present study focused on the migration of donor T cells during the activation phase of GVHD and the mediators affecting this process [23,24].

Although the GVL effect is one of the most potent demonstrations of adoptive immunotherapy, it naturally coexists with the presence of GVHD [9], because both responses are primarily mediated by donor T cells [25,26]. In the current study, we demonstrated that GVHD can be significantly attenuated without a corresponding loss in GVL effect by blocking the CXCL2/CXCR2 interaction.

Lymphocyte trafficking is tightly regulated through expression of specific adhesion molecules and chemokine receptors on the lymphocyte surface, combined with ligand expression for these receptors by target tissues. We previously tracked migration of enhanced green fluorescent protein (eGFP) transgenic donor T cells after transplantation using a bio-imaging system in a fully MHC-mismatched murine allogenic BMT model [27]. Interestingly, donor T cells partitioned to lymphoid tissues within hours after transplantation. In the following 2–3 days post-transplantation, allogenic T cells expanded within lymphoid tissues. Between 3 and 21 days after transplantation, allogenic T-cell numbers increased in GVHD target organs, including the GI tract, liver, and skin.

Based on our previous observations of T-cell migration in mice models of GVHD [27], we hypothesized that the separation between GVHD and GVL is caused by selective recruitment of T cells into GVHD target organs. We further hypothesized that this selective recruitment is due to specific expression of cytokines and chemokines, and the presence of their receptors on tumor cells, donor T cells, and the cells of recipient GVHD target organs.

This study was performed in three steps: first, a GVHD murine model was generated using myelo-ablative chemotherapy followed by BM cell and T-cell infusion, which was confirmed by histology and survival rates. Secondly, we identified IL-23, KC, CXCL2, and G-CSF as cytokines and chemokines overexpressed in GVHD target organs. The T-cell receptor CXCR2, a ligand for both CXCL2 and KC, was identified as a major player in GVHD [6]. Further, blocking the identified cytokines and chemokines in a murine GVHD/leukemia model isolated the GVL response in the presence of GVHD: simultaneous blocking of CXCL2 and KC inhibited GVHD, while GVL was preserved. Moreover, blocking CXCL2 and its receptor CXCR2 synergistically decreased GVHD severity, as evidenced by improved histological scoring. Importantly, this CXCL2/CXCR2 axis blockade did not interrupt the GVL response, as observed through decreased red fluorescence as well as lower BCL2 expression in A20 tumor cells that were retrieved from recipients post-transplantation.

Our data suggest that blocking T-cell migration to GVHD target organs inhibits GVHD while maintaining GVL. This could be achieved through specific blockade of CXCL2 and its receptor CXCR2. Herein we conclude that blocking CXCR-2 in allogenic donor T cells and CXCL-2 in recipient GVHD target organs resulted in a significantly decreased GVHD response in our model, while T-cell function as well as the GVL effect remained largely intact. Targeting CXCL2 and CXCR2, possibly in combination with other integrin or chemokine receptor antagonists, provides a novel therapeutic approach to GVHD management in the setting of hematological malignancy.

Acknowledgments

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