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Differential Roles for CCR5 Expression on Donor T Cells during Graft-versus-Host Disease Based on Pretransplant Conditioning

Christian A. Wysocki,* Susan B. Burkett,* Angela Panoskaltsis-Mortari,† Suzanne L. Kirby,* Andrew D. Luster,‡ Karen McKinnon,* Bruce R. Blazar, † and Jonathan S. Serody2*

The coordinated expression of chemokines and receptors may be important in the directed migration of alloreactive T cells during graft-vs-host disease (GVHD). Recent work demonstrated in a murine model that transfer of CCR5-deficient (CCR5−/−) donor cells to nonconditioned haploidentical recipients resulted in reduced donor cell infiltration in liver and lymphoid tissues compared with transfer of CCR5+/+ cells. To investigate the function of CCR5 during GVHD in conditioned transplant recipients, we transferred CCR5−/− or wild-type C57BL/6 (B6) T cells to lethally irradiated B6D2 recipients. Unexpectedly, we found an earlier time to onset and a worsening of GVHD using CCR5−/− T cells, which was associated with significant increases in the accumulation of alloreactive CD4+ and CD8+ T cells in liver and lung. Conversely, the transfer of CCR5−/− donor cells to nonirradiated recipients led to reduced infiltration of target organs, confirming previous studies and demonstrating that the role of CCR5 on donor T cells is dependent on conditioning of recipients. Expression of proinflammatory chemokines in target tissues was dependent on conditioning of recipients, such that CXCL10 and CXCL11 were most highly expressed in tissues of irradiated recipients during the first week post-transplant. CCR5−/− T cells were shown to have enhanced migration to CXCL10, and blocking this ligand in vivo improved survival in irradiated recipients receiving CCR5−/− T cells. Our data indicate that the effects of inhibiting CCR5/ligand interaction on donor T cells during GVHD differ depending on conditioning of recipients, a finding with potentially important clinical significance. The Journal of Immunology, 2004, 173: 845–854.
donor T cells was critical in the recruitment of alloreactive CD8+ T cells into the lung and liver in a class I MHC-disparate transplant (21). Interestingly, the recruitment of CD4+ T cells was increased at these sites in the absence of CCL3 production by donor T cells and correlated with earlier mortality in MHC class II-disparate recipients. Furthermore, in a completely MHC-mismatched model of idiopathic pneumonia syndrome, the absence of CCL3 production by donor T cells led to increased T cell accumulation in both spleen and lung (24), suggesting that other proinflammatory cytokines may overcompensate for the absence of CCL3 in certain target organs.

A critical role for the chemokine receptor, CCR5, in the migration of alloreactive T cells in nonirradiated haploidentical recipients was recently shown (25, 26). However, a specific role for CCR5 in transplants using conditioning therapy, commonly used for human allogeneic bone marrow transplant recipients, was not demonstrated. Thus, we were interested in determining the function of CCR5 on donor T cells in the setting of intensive conditioning therapy. Unexpectedly, we found that the administration of CCR5-/- donor T cells led to enhanced GVHD and earlier lethality in three different conditioned model systems. In this study we show that the activity of CCR5 during GVHD is dependent on the inflammatory milieu and provide a potential mechanism for the enhanced GVHD found using CCR5-/- T cells that involves enhanced migration of these cells to CXCL10.

**Materials and Methods**

**Mice**

Donor mice consisted of male C57BL/6j mice (H2b; termed B6; The Jackson Laboratory, Bar Harbor, ME) and CCR5-/- mice, which have been described previously (42). We performed backcrosses of CCR5-/- mice for 10 generations on the B6 genetic background to generate donor mice. In some experiments donors were CCR5-/- or B6 mice expressing the enhanced GFP (eGFP) protein. Generation of eGFP-expressing B6 was previously described (21), and these were crossed with our CCR5-/- strain to generate eGFP-expressing CCR5-/- mice.Recipient mice were male (B6 x DBA/2) F1, mice, referred to as B6D2 (H2b, H2d; The Jackson Laboratory, BALB/c (H2d), and B10.BR (H2k). Within each experiment, all recipients were the same age, which ranged from 6–12 wk. Donor mice also ranged from 6–12 wk of age. In some in vitro assays of T cell function, donors were female B6 and CCR5-/- mice. Donors were age- and gender-matched in each experiment.

**Preparation of cells for transplant**

On the day of transplantation, bone marrow was collected from both B6 donors and syngeneic controls by euthanizing animals with CO2, and collecting femurs and tibiae. These bones were teased apart, treated with ACK lysis buffer, and washed in PBS/2% fetal bovine serum (FBS) (Life Technologies). Cells were collected, and erythrocytes were lysed with PBS/2.5% FBS (Life Technologies). Cells were collected into 1 ml of PBS/2.5% FBS, 2.5% d-glucose, and 4 μM 2-mercaptoethanol (2-ME) for 10 min at 4°C until staining. The cell suspensions were washed three times in PBS/2% FBS. Erythrocytes were removed using ammonium chloride, potassium carbonate (ACK) lysis buffer. Cells were washed in PBS/0.5% FBS and incubated with anti-CD90 mAb-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and T cells were depleted by separation on a magnetic column with collection of the column flow-through. These cells were washed and suspended at 3 x 10^6/ml in injectable, nonbacteriostatic 0.9% NaCl solution. The efficiency of T cell depletion was >95%, as measured using flow cytometry. Donor T cells were isolated on the day of transplantation by collecting the spleens from B6, CCR5-/-, or syngeneic control mice, treating cell suspensions with ACK lysis buffer to remove erythrocytes, and incubating with anti-CD90 mAb-coated microbeads as described above. T cells were isolated via magnetic column selection using the manufacturer’s instructions. These cells were suspended in injectable 0.9% NaCl at 5 x 10^7/ml. The purity of selected T cells was ~90%. Alternatively, in several experiments unselected splenocytes were used. These were prepared as described above, except that after the ACK lysis step, cells were washed, counted, and resuspended at 5 x 10^6/ml in 0.9% injectable saline.

**Bone marrow transplantation**

B6D2 and B10.BR recipients were lethally irradiated with 850–950 cGy from a 54.3 Cs source (86.3 cGy/min.), BALB/c recipients were lethally irradiated with 950 cGy of irradiation. After irradiation, mice were kept in autoclaved cages, fed gamma-irradiated food, and received water (pH 2) treated with 2 g/l neomycin sulfate. The following day, mice received 3 x 10^7 T cell-depleted bone marrow cells (either B6 or syngeneic cells) and 1–5 x 10^7 magnetically selected CD90+ splenic T cells (B6, CCR5-/-, or syngeneic T cells) or 5 x 10^6 unsplenocytes, in a total volume of 0.2 ml, by tail vein injection. In one set of experiments, groups of transplant recipients received i.p. injections of either hamster anti-mouse CXCL10 mAb (43) or hamster IgG control Ab (The Jackson Laboratory). These mice received 100 μg of Ab in 200 μl of PBS i.p. on days 1, 3, and 5. In another set of experiments, nonirradiated B6D2 recipients received 5 x 10^7 unsplenocytes alone in a volume of 0.2 ml by tail vein injection.

**GVHD grading**

Mice were observed twice weekly for signs of GVHD, and a previously described clinical scoring system was used to assess disease severity (44).

**Histopathology**

Samples of each organ were removed at the time of death (days 3, 7, and 18), placed into Omnifix (F. R. Chemical, Mount Vernon, NY), and paraffin-embedded. These were sectioned with a microtome. The sections were stained with H&E, and a histopathology team was designated for evidence of GVHD using a quantitative assessment, as described previously (45). The sections were evaluated by one of us (A.P.-M.), who was blinded to the treatment given.

**Analysis of cytokines in tissue homogenates**

Transplanted mice were killed on day 7. Whole liver, lung, and colon were collected into 1 ml of PBS/2.5% d-glucose, 2.5% d-glucose, and 4 μM 2-mercaptoethanol (2-ME) (Roche, Indianapolis, IN) and homogenized using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). Homogenates were incubated on ice for 30 min, cleared by centrifugation, and immediately analyzed for IL-2, IL-4, IL-5, IFN-γ, and TNF-α using the mouse Th1/Th2 cytokine bead array (BD Pharmingen, San Diego, CA). Homogenates were analyzed undiluted, and diluted 1/5 in assay dilution buffer (BD Pharmingen). The assay bead array analysis was performed and analyzed according to the manufacturer’s protocol.

**Isolation of leukocytes from tissues**

Mice were killed on days 3, 7, and 18 and perfused through the heart with 10 ml of PBS. Spleen, liver, lung, colon, and kidney were excised and weighed. Livers, lungs, colons, and kidneys were digested in a solution of 1 mg/ml collagenase A (Roche) and 75 U of DNase I (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 containing 0.1 M CaCl2 with 0.01% FeCl3. Deseeded tissues were treated with ACK lysis buffer to remove RBCs and were passed through 100-μm pore size cell strainers. Leukocytes were isolated by centrifugation in a solution of 40% Percoll (Sigma-Aldrich) in RPMI 1640/5% newborn calf serum. The pelletted cells were washed in PBS/2% FBS and stored overnight at 4°C before Ab staining for flow cytometric analysis. Spleens were teased apart, treated with ACK lysis buffer, and washed in PBS/2% FBS, and the cells were stored at 4°C until staining.

**Abs and flow cytometry**

Cells isolated as described above were blocked for 15 min at 4°C in mouse whole IgG (Sigma-Aldrich), followed by Ab staining for 15 min at 4°C. Stained cells were washed once with PBS/2% FBS before fixing in 1% formaldehyde. Abs used for FACS were obtained from BD Pharmingen and included anti-H2K b -FITC (clone AF6-88.5, mouse IgG2a, anti-H2K d -FITC (SF1-1.1, mouse IgG2a, anti-mCD3e-PerCP (145-2C11, hamster), and anti-mCD4-PerCP (RM4-5, rat IgG2a), and anti-mCD8α-PerCP (53-6.7, rat IgG2a). Isotype controls were obtained from BDPharmingen. FACS analysis was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Three-color analysis was performed using FlowJo analysis software (Tree Star, Ashland, OR), in which cells staining with the PerCP-labeled CD marker mAb were gated, the expression of H2K b and H2K d was determined on the gated cells, and the number of PerCP-positive H2K b-positive H2K d-negative cells (donor T cells) in the sample was quantified. In some experiments, when eGFP-expressing donor cells were used, no anti-H2 Abs were used to distinguish donor cells. The eGFP+ cells staining positively for CD markers were quantified.

In vivo activated T cells were stained for CXCR3 expression using a purified rat anti-mouse CXCR3 mAb (clone 220803; R&D Systems, Minneapolis, MN) or rat anti-mouse IgG2a isotype control (R&D Systems), followed by secondary staining with PE-conjugated goat anti-rat Ig (BD Pharmingen).
Real-time RT-PCR analysis of chemokine ligand and receptor expression

Total RNA was prepared from freshly harvested tissues and fresh or cultured T cells using TRIzol (Invitrogen, San Diego, CA) according to the manufacturer’s protocol. Synthesis of cDNA from total RNA was performed using random hexamers, Moloney murine leukemia virus reverse transcriptase, and RNasin (Promega, Madison, WI). Real-time RT-PCR was performed using the ABI PRISM 7900 sequence detection system (PerkinElmer, Foster City, CA). Primers and/or probes were designed to span exons, as previously described. CCL3, CCL4, CXCL10, and CXCR3 probes were labeled at the 5’ end with the reporter dye FAM and at the 3’ end with the quencher dye TAMRA. The 18S rRNA probe was labeled at the 5’ end with the reporter dye TET and at the 3’ end with TAMRA. Probes were synthesized by Integrated DNA Technologies (Coralville, IA), and primers with the reporter dye TET and at the 3’ orientation: CCL3 forward, CGCCAATTCATCGTTGACTA; CCL3 reverse, AGATCTGCGTTCTCAGTTA; CCL4 forward, CCGTGCTGACCAGACTT; CCL4 reverse, AGACTGCTGCTGCTCATAGTA; CCL4 probe, CCAAATGGCG TCTGACCTCCAC; CCLX10 forward, GAAGATCGTCGACTCGAAT GAA; CCLX10 reverse, CTTCAAGTCAAGAGCTCCTT; CCLX10 probe, TGAATCCGGAATCTAAGACCATCAA; CCLX11 forward, CTCCTTATGTTCA; CCLX11 reverse, CTCTTGCTACTCTGCTGCTT; CCLX11 probe, CGCTGTCTTTGCATCGGCCC; CXCR3 forward, AGCCTTCCTTATGTTCA; CXCR3 reverse, CAGAGAAGTCGCT; CXCL11 probe, CGCTGTCTTTGCATCGGCCC; CXCR3 forward, AGCCTTCCTTATGTTCA; CXCR3 reverse, CAGAGAAGTCGCT; CXCL11 probe, CGCTGTCTTTGCATCGGCCC; CXCR3 forward, AGCCTTCCTTATGTTCA; CXCR3 reverse, CAGAGAAGTCGCT; CXCL11 probe, CGCTGTCTTTGCATCGGCCC;

T cell apoptosis assays

Mixed lymphocyte cultures were prepared as described above, using irradiated B6D2 stimulator cells and B6/eGFP and CCR5−/− eGFP responders at a 1:1 ratio. These were incubated for 3–6 days, and at these time points, cells were collected and stained with PE-conjugated annexin V and the vital dye 7-aminoactinomycin D. The proportions of GFP+ cells staining positively for annexin V-PE and negatively for 7-AAD were determined by flow cytometry.

CTL assays

Effector cells (splenic CD90+ T cells) were prepared as described above from B6 and CCR5−/− mice. These were mixed in a 1:1 ratio with irradiated B6D2 splenocytes and were cultured for 5 days in the MLR culture medium described above at a concentration of 1 × 10^6 cells/ml. Target cells were PR815 (H2b, EL-4 (H2b), and YAC-1 cells labeled with 100 μCi of ^65Cu (Amersham Pharmacia Biotech) at 37°C for 90 min and washed extensively. Effector cells and targets were mixed at E:T cell ratios of 50, 25, 12.5, and 6.25:1 and cultured for 6 h at 37°C. Chromium released into the medium in these cultures was compared with spontaneous release by target cells alone and with maximum release by targets treated with 10% Triton X-100 (Sigma-Aldrich). Chromium release was quantified by scintillation counting using the CountPro counter (PerkinElmer): % specific lysis = ([experimental release − spontaneous release]/maximum release − spontaneous release) × 100.

Statistical analyses

Estimates of the probability of survival for all groups were determined using the method described by Kaplan and Meier (47). Groups were compared for differences in histopathology scores using the rank-sum test and for differences in cytokine production, cell infiltration, relative chemokine expression, and chemotaxis using the Student’s t test. For all tests, p ≤ 0.05 was considered significant.

Results

Survival and GVHD disease severity

To examine the importance of CCR5 expression on donor T cells in GVHD, haploidentical parent-to-F1 transplants were performed in which heavily irradiated B6D2 mice were given 3 × 10^6 T cell-depleted bone marrow cells from B6 donors supplemented with 2.5 × 10^6 splenic T cells from either CCR5−/− (B6 background) or B6 mice. Interestingly, although at this T cell dose all the mice receiving B6 T cells survived (Fig. 1A), mortality was 50% for mice receiving 2.5 × 10^6 CCR5+/− T cells (p = 0.05). Mortality was also increased in hamster IgG-treated B6D2 recipients of 1 × 10^6 CCR5−/− vs B6 donor T cells (Fig. 6). These data correlated with statistically greater clinical GVHD scores in B6D2 recipients receiving 2.5 × 10^6 T cells from CCR5−/− compared with B6 donors beginning on day 17 (Fig. 1B). When evaluated in B6D2 mice receiving a dose of T cells that induced GVHD in all animals (5 × 10^6), recipients of CCR5−/− T cells again suffered significantly earlier mortality compared with recipients of B6 T cells (p = 0.02; Fig. 1C).

To determine whether the effect found using CCR5−/− T cells was dependent on the GVHD model used, we performed transplants in which lethally irradiated BALB/c or B10.Br recipients received T cells from CCR5−/− or wild-type B6 mice (complete MHC-mismatched models). In all experiments, recipient mice had earlier mortality after the receipt of CCR5−/− T cells compared with wild-type B6 T cells (see Table I). Thus, the effect of the administration of CCR5−/− T cells was not limited to a single strain combination.

Histopathology

Histopathology in liver, lung, colon, spleen, and kidney sections was studied in B6D2 recipients of CCR5−/− or B6 unselected
spontaneous T cell cytotoxicity by the method of 51Cr release assay (data not shown). Thus, alterations in proliferation, induction of apoptosis, or cytotoxicity are unlikely to explain our in vivo findings.

Infiltration of GVHD target organs by CCR5−/− donor cells is dependent on conditioning of recipient mice

A recent study by Murai et al. (26) using nonirradiated B6D2 recipient mice demonstrated decreased, rather than increased, infiltration of target organs by CCR5−/− cells, compared with CCR5+/+ B6 donor splenocytes, on day 14 post-transplant. As this study used a different strain of CCR5−/− mice than ours, we sought to determine whether the apparent discrepancy between our data and those of the previous study was due to strain differences or transplant conditioning. We performed transplants in B6D2 recipients using 5 × 10⁷ unselected splenocytes from B6 and CCR5−/− donors using the method used by Murai et al. (26), which does not include recipient conditioning, and assessed donor T cell infiltrates at day 14. As shown in Fig. 4A, infiltration of liver, lung, spleen, and Peyer’s patches by CCR5−/− donor cells was moderately decreased compared with B6 in the absence of irradiation. However, in B6D2 recipients that were conditioned with 850 cGy of irradiation, we confirmed our earlier findings of an increased number of CCR5−/− compared with B6 donor T cells in the liver and lung (Fig. 4B). The increases observed in liver and lung in irradiated recipients at this time point were similar to our findings on day 18 in the previous experiment (Fig. 3), in which we demonstrated significantly increased CCR5−/− T cell infiltrates in the liver, but only a modest increase in CD8⁺ CCR5−/− T cells in the lung.

Table I. Median survival with range for different recipient strains receiving B6 TCD bone marrow and 5 × 10⁷ T cells from either CCR5−/− or B6 donors

<table>
<thead>
<tr>
<th>Group</th>
<th>B6D2b</th>
<th>B10.Ba</th>
<th>BALBc/129d</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>39 (21–50)</td>
<td>19 (17–25)</td>
<td>31 (19–41)</td>
</tr>
</tbody>
</table>

a n = 5–6 mice/group, with the exception of B6D2 (n = 14/group).
b p = 0.017.
c p = 0.008.
d p = 0.056.
week post-transplant in nonirradiated recipients, and interestingly, recipients. The CCR5 ligands were induced during the second significantly different from peak levels seen on day 7 in irradiated signi

crease on day 7. The CXCR3 ligands continued to increase through the expression of the CXCR3 ligands again preceded that of the CCR5 ligands did not decrease during the second week, with the exception of CCL3 expression in the lung, which declined 

than the peak expression observed in the irradiated recipients. Chemokine expression was not induced in the liver and lung of either nonirradiated or irradiated recipients of syngeneic T cells, with the exception of a modest, but significant, increase in CXCL11 expression in the liver in irradiated syngeneic transplant recipients on day 3.

In the spleen, where we had not seen significant increases in CCR5<sup>−/−</sup> T cell infiltrates in irradiated allogeneic transplant recipients (but had seen decreased CCR5<sup>−/−</sup> T cell infiltrates in nonirradiated recipients), we observed an earlier decline in CXCR3 ligands in irradiated recipients, with expression peaking on day 3 rather than on day 7. We again observed significantly higher peak levels of CCL3 and CCL4 in nonirradiated recipient spleens on day 14. Again, no significant expression of chemokines was observed in spleen in either irradiated or nonirradiated syngeneic controls, with the exception of modest induction of CXCL10 on day 3 in irradiated syngeneic controls. Thus, the combination of irradiation and allogeneic T cells led to earlier expression of both CXCR3 and CCR5 ligands in the liver and lung compared with transfer of allogeneic T cells alone. Transfer of allogeneic T cells to nonconditioned recipients resulted in later induction of these chemokines, but interestingly, higher relative expression of CCR5 ligands than in irradiated recipients. The spleen was unique in irradiated recipients, in that the expression of CXCR3 ligands did not continue to increase through the first week as they did in liver and lung, but declined between days 3 and 7.

Role of CXCL10 in migration of CCR5<sup>−/−</sup> T cells and GVHD severity

To determine whether the increased expression of CXCL10 early post-transplant could play a role in enhanced GVHD found using CCR5<sup>−/−</sup> T cells, we evaluated the in vitro migration of wild-type and CCR5<sup>−/−</sup> cells. We activated B6 and CCR5<sup>−/−</sup> T cells in culture using methods shown previously to induce the expression of both CXCR3 and CCR5 (46). The optimal concentration of CXCL10 for the migration of B6 T cells was determined to be 100 ng/ml (data not shown). At this concentration of CXCL10, CCR5<sup>−/−</sup> T cells had significantly increased migration compared with B6 T cells (Fig. 6A). This increase in migratory response to CXCL10 was not associated with increased expression of CXCR3 by CCR5<sup>−/−</sup> T cells, as determined by both real time RT-PCR and staining for cell surface protein (Fig. 6B and C). To assess the in vivo relevance of this finding, B6D2 recipients were lethally irradiated and given B6 bone marrow with B6 or CCR5<sup>−/−</sup> T cells. Included were groups of recipients treated with an anti-CXCL10 mAb. B6D2 recipients given CCR5<sup>−/−</sup> T cells and four doses of a hamster IgG control Ab had 43% mortality compared with 0% in those receiving B6 T cells and IgG control Ab. Administration of four doses of 100 μg of anti-murine CXCL10 mAb prevented mortality completely in B6D2 recipients of CCR5<sup>−/−</sup> T cells (Fig. 6D). Thus, the enhanced GVHD found using CCR5<sup>−/−</sup> donor T cells was abrogated by blocking the function of CXCL10.

Discussion

The chemokine system is quite complex, with a large number of ligands, some with overlapping functions, that bind to a diverse array of receptors. Despite this, recently Murai et al. (25, 26) have shown that the absence of CCR5 on donor T cells led to a marked decrease in the migration of alloreactive T cells into the gastrointestinal tract and decreased inflammation in the liver in B6D2 mice that did not receive conditioning therapy. In this study we sought to determine whether blocking the function of CCR5 had a similar effect in lethally irradiated recipient mice. Interestingly, we show that in conditioned recipients, eliminating the expression of CCR5

**FIGURE 2.** Quantitative histopathologic assessment of GVHD in B6D2 recipients of B6 and CCR5<sup>−/−</sup> splenocytes on days 3, 7, and 18. Quantitative assessment of histopathologic lesions in tissues of B6D2 transplant recipients was performed by one of us (A.P.-M.), who was blinded to the identity of the transplanted mice. Tissues were evaluated from six mice per group. □, Tissues from mice receiving B6 donor cells; ■, tissues from mice receiving CCR5<sup>−/−</sup> donor cells. Colons were equally sampled between the groups to include sections of both distal and proximal colon. Scores were assigned to each section based on a previously published set of criteria (45). Values presented are the mean ± SEM for each group. *, p < 0.05; **, p < 0.01. Score range for each tissue, 0–4.
on donor T cells led to more rapid and severe GVHD than in recipients of wild-type T cells. This was found in three different transplant models, confirming that this finding was not specific to a particular donor/recipient strain combination. The increased GVHD severity in recipients of CCR5/H11002/H11002 donor T cells correlated with increased T cell infiltrates in the liver and lung and increased tissue pathology in the lung. Significantly, we found that administration of CCR5/H11002/H11002 donor T cells led to increased liver and lung infiltration only in irradiated transplant recipients, and this was associated with markedly increased expression of the CXCR3 chemokine ligands, CXCL10 and CXCL11, at these sites during the first week post-transplant. Lastly, we have shown a novel role for CCR5 in regulating the response of activated T cells to one of the CXCR3 ligands, in that elimination of CCR5 expression resulted in enhanced T cell migration to CXCL10.

Murai et al. (26) described decreased proportions of eGFP-expressing CCR5/H11002/H11002 splenocytes infiltrating liver, spleen, mesenteric lymph node, and intestinal epithelium on day 14 post-transplantation. To confirm that the differences in our data were due to differences in pretransplant conditioning, we performed transplants in nonirradiated B6D2 recipients and assessed donor cell infiltrates on day 14 post-transplant, as did Murai et al. (26). Our data confirm that reported by Murai et al. (26), as in the absence of irradiation, transplantation of CCR5/H11002/H11002 T cells led to fewer donor T cells isolated from the liver, lung, and Peyer’s patch, whereas in lethally irradiated recipients there was an increase in CCR5/H11002/H11002 T cells at these sites. These data demonstrate that differences in pretransplant conditioning altered the effect of eliminating CCR5 expression from donor cells on the migration and/or expansion of these cells. This finding has clear significance, as in the clinical setting, conditioning of allogeneic bone marrow transplant recipients with TBI and/or chemotherapy is necessary for efficient engraftment. Thus, our work suggests that the use of inhibitors of CCR5 could exacerbate, not ameliorate, GVHD in patients that receive fully myeloablative conditioning therapy.

To understand the mechanism behind the differential role of CCR5 in T cell migration during GVHD in irradiated and nonirradiated recipients, we analyzed the expression of several proinflammatory chemokines in GVHD target organs at various time points after transplant. We focused our analysis on the liver and lung, where we had seen significant differences in B6 vs CCR5/H11002/H11002 T cell infiltrates. The expression of CCL3, CCL4, CXCL10, and...
CXCL11 were significantly increased in liver and lung from irradiated recipients compared with nonirradiated recipients during the first 7 days post-transplant. Although delayed compared with irradiated recipients, the expression of the CCR5 ligands, CCL3 and CCL4, was more prominent in these tissues in nonirradiated recipients on day 14 post-transplant. Consistent with a role for these ligands in GVHD pathogenesis in nonirradiated recipients, recruiting T cell subsets were identified by staining for CD4 and CD8. Donor and recipient cells were distinguished either by staining for H2Kb and H2Kd or by eGFP expression in donor cells (these data are pooled from two separate experiments; \( n = 8 \) in each group). B. Irradiated (850 cGy) B6D2 recipients of B6 bone marrow and eGFP+ B6 (●) or eGFP+ CCR5\(^{+/-}\) (□) splenocytes were killed on day 14, and infiltrates were quantified in target tissues as described above (\( n = 4 \) in each group). * \( p < 0.05 \).

**FIGURE 4.** Infiltration of B6 and CCR5\(^{+/-}\) donor cells in tissues of nonirradiated recipients. A. Nonirradiated B6D2 recipients of B6 (●) and CCR5\(^{+/-}\) (□) unselected splenocytes were killed on day 14. Liver, lung, spleen, and Peyer’s patches were collected. Liver, lung, and spleen were weighed. Leukocytes were isolated from all the above tissues, and infiltrating T cell subsets were identified by staining for CD4 and CD8. Donor and recipient cells were distinguished either by staining for H2K\(^{\alpha}\) and H2K\(^{\delta}\) or by eGFP expression in donor cells (these data are pooled from two separate experiments; \( n = 8 \) in each group). B. Irradiated (850 cGy) B6D2 recipients of B6 bone marrow and eGFP+ B6 (●), or eGFP+ CCR5\(^{+/-}\) (□) splenocytes were killed on day 14, and infiltrates were quantified in target tissues as described above (\( n = 4 \) in each group). * \( p < 0.05 \).

lung of irradiated recipients correlated with elevated levels of CXCR3 ligands that persisted throughout the first week post-transplant in those tissues. In contrast, we did not see increased CCR5\(^{-/-}\) T cell infiltrates in the spleen, where levels of these ligands decreased substantially after day 3. The decreased expression of ligands that bind to CXCR3 in lethally irradiated recipients after day 7 in liver and lung and after day 3 in spleen could be due to an influx of donor T cells that either dilute recipient cells expressing these ligands or destroy them, which would be consistent with previous work on the time for turnover of recipient APCs post-transplantation (50, 51). The observation of only modest induction of CXCL11 in liver and CXCL10 in spleen in irradiated recipients of syngeneic transplants confirms our previous observations that induction of these ligands early after transplantation requires both conditioning effects and transfer of allogeneic T cells. Because CXCR3 ligands were more prominently expressed during the first week post-transplant in target tissues of irradiated recipients, we hypothesized that the difference in GVHD severity and tissue infiltrates in recipients of CCR5\(^{-/-}\) vs B6 donor T cells could result from differences in the activity of these ligands on B6 and CCR5\(^{-/-}\) T cells. We therefore assessed the ability of B6 and CCR5\(^{-/-}\) T cells to migrate in response to CXCL10. CCR5\(^{-/-}\) T cells migrated more efficiently to a physiologically relevant dose of CXCL10 compared with B6 T cells. Interestingly, this was not associated with increased expression of CXCR3 in CCR5\(^{-/-}\) T cells. Furthermore, Ab-mediated neutralization of CXCL10 in vivo during the induction of GVHD prevented increased mortality in recipients of CCR5\(^{-/-}\) donor T cells. These data suggest that CCR5 may be involved in modulating the response of T cells to CXCR3 ligands. The capacity of CCR5 to reduce the responsiveness of lymphocytes to non-CCR5-binding chemokines through heterologous desensitization has been demonstrated in other studies (36, 37), although this is the first demonstration that eliminating CXCR3 expression increases T cell sensitivity to a CXCR3 ligand. Previous investigators have shown that Th1 cells express CXCR3 and CCR5 preferentially (32, 33). As acute GVHD in the B6 into B6D2 model is mediated by Th1 cytokine-expressing T cells (5), our in vitro and in vivo data would suggest that CCR5 may play an important role in down-modulating the response to ligands that bind CXCR3 on Th1/Tc1 cells. We are currently evaluating this hypothesis.

We have demonstrated a correlation between donor T cell infiltration and tissue pathology in the lung, which is consistent with previous studies (24). However, concordant with previous findings from our group (21) and others (52), significant increases in CCR5\(^{-/-}\) donor T cell infiltrates in the liver did not lead to significant increases in pathology, as assessed by quantitative histopathological scoring. The difference between B6 and CCR5\(^{-/-}\) CD8\(^{+}\) T cell infiltrates increased between days 7 and 18; thus, it is possible that the differences in liver GVHD scores may have been significant if evaluated beyond day 18. Alternatively, the liver has been shown to trap systemically activated CD8\(^{+}\) T cells via adhesion molecule expression on sinusoidal endothelium and to induce their apoptosis, possibly through cytokine production by bone marrow-derived Kupffer cells and dendritic cells (53). Thus, the increased number of CD8\(^{+}\) T cells found in the liver may reflect the trapping of previously activated alloreactive T cells, which do not cause tissue damage characteristic of GVHD.

A specific role for the chemokine receptor, CXCR3, in mediating graft rejection after heart transplantation has been conclusively demonstrated by Hancock et al. (54, 55). However, the complexity of the chemokine system has made it difficult to identify a specific function for chemokine ligands and receptors during GVHD. Most studies that have identified a role for a specific ligand or receptor
have relied on recipient mice that have not been conditioned or that have received sublethal conditioning (20–22, 25, 26). Our work suggests a potential explanation for these findings. Our group has recently shown that donor cells infiltrate widely into peripheral lymphoid tissues immediately post-transplantation, followed 48–72 h later by migration of activated T cells to GVHD target organs (56). This could lead to increased expression of chemokine ligands, such as CXCL10 and CXCL11 by recipient cells, induced by the combination of irradiation and production of IFN-γ by activated allogeneic donor T cells interacting with professional recipient APCs. The local production of CXCL10 and CXCL11 would lead to the recruitment and expansion of donor T cells in these GVHD target organs. This would correspond with our finding that the infiltration of CCR5−/− and B6 T cells does not differ on day 3, but is significantly different by day 7 post-transplantation. Later autocrine production of CCR5-binding chemokines by recruited T cells responding to alloantigens within target organs may be critical in amplifying this response. In support of this model, our group has identified allogeneic donor T cells as the major source of the CCR5 ligand CCL3 during GVHD in settings of both class I and class II MHC mismatch and has demonstrated that CCL3 production by donor T cells in target organs such as liver and lung is not detectable until day 6 post-transplant (21). In the absence of conditioning therapy, autocrine production by donor T cells may be the predominant source of chemokines; therefore, CCR5 ligands may play a more significant role. Thus, approaches that do not target both these axes may show little effect in MHC-mismatched bone marrow transplants using lethal irradiation, and our data indicate that inhibiting only one axis may potentiate the other. This hypothesis would be consistent with recent data from Duffner et al. in which they were able to show a significant role for CXCR3 in the migration of donor T cells to the small bowel in a minor mismatch model, but not in the B6 into B6D2 model (23). In this model, MHC mismatch induces more significant T cell expansion and subsequent production of CCR5-binding chemokines by these cells. In the absence of CXCR3 expression, T cells may have a greater response to CCR5-binding chemokines. Additionally, CXCR3- and/or CCR5-binding chemokines may be

FIGURE 5. Real-time RT-PCR analysis of chemokine expression in GVHD target tissues from irradiated vs nonirradiated transplant recipients. Tissues from irradiated (○) and nonirradiated (■) allogeneic transplant recipients and irradiated (△) and nonirradiated (▲) syngeneic transplant recipients were collected on days 3, 7, and 14 post-transplant, and total RNA was isolated immediately. Real-time PCR analysis of CCL3, CCL4, CXCL10, and CXCL11 expression was conducted on cDNA prepared from these RNA samples. The relative expression of these chemokines was calculated as described in Materials and Methods. Chemokine expression in organs of untreated B6D2 mice was also assessed and is represented as the day 0 point of each plot. All reactions were performed in duplicate. Values presented represent the mean ± SEM. Irradiated allogeneic transplant recipients on days 3 and 7 (n = 5) and day 14 (n = 3) are shown. Nonirradiated allogeneic transplant recipients on days 3, 7, and 14 (n = 3); irradiated syngeneic transplant recipients on days 3, 7 (n = 3), and 14 (n = 2); and nonirradiated syngeneic transplant recipients on days 3 and 7 (n = 2) and day 14 (n = 1) are shown. Untreated B6D2 mice were used as controls (n = 2). Asterisks denote significant differences in chemokine expression between irradiated and nonirradiated allogeneic transplant recipients at each time point; exclamation points denote significant differences between irradiated and nonirradiated allogeneic transplant recipients in peak expression of chemokines (●, p < 0.05; **, p < 0.001; †, p < 0.05; ††, p < 0.001).
important only in the migration of T cells into the small bowel, liver, and lung; other ligands and receptors may be critical in the migration of alloreactive T cells to the colon and skin.

In summary, we have found that the absence of CCR5 on donor T cells can enhance or diminish T cell migration into specific GVHD target organs dependent on whether recipient mice receive conditioning therapy. This suggests that targeting CCR5 will only be effective clinically in the absence of myeloablative conditioning therapy. Additionally, we have found that CCR5 inverted T cells have enhanced migration to the CXCR3 ligand, CXCL10, demonstrating a novel role for CCR5. Future work will determine the mechanism for this finding.

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