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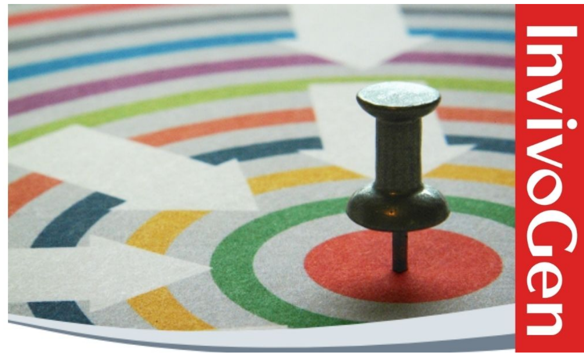

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J Immunol (2004) 173 (2): 845–854.
https://doi.org/10.4049/jimmunol.173.2.845

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Differential Roles for CCR5 Expression on Donor T Cells during Graft-versus-Host Disease Based on Pretransplant Conditioning[1]

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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.**

Graft-vs-host disease (GVHD)[3] is due to the recognition by alloreactive T cells of minor or major Ags presented by MHC proteins (1–4). This recognition leads to the elaboration of cytokines and the destruction of recipient APCs and other cells by these cytokines (5–11) and cytolytic proteins, such as perforin or FAS (2, 12–19). Before tissue destruction, T cells must be recruited to specific organs in which GVHD occurs such as the gastrointestinal tract, liver, lung, and skin. Recent work suggests that chemokines may play an important role in this recruitment (20–26).

Chemokines are small chemotactic cytokines that mediate their activity by binding to G protein-coupled receptors (GPCRs) (27) and fall into four families, C, CC, CXC, and CX_3C, based on the number and position of N-terminal cysteine residues (28). Receptors for these molecules are named accordingly, with CC chemokines binding CC receptors (CCR) and CXC chemokines binding CXCR (28). The system is complex, with multiple chemokine ligands binding any one receptor, and in most cases, multiple receptors binding the same chemokine. Specificity is imparted upon the system through differential expression of chemokine ligands and receptors by particular cell types and/or in response to specific stimuli. For example, naive T cells express CCR7, a receptor for the chemokines CCL19 and -21, which are expressed in secondary lymphoid tissues (29–31). Activation and differentiation to Th1/T cytotoxic type I (Tc1) cells induces down-regulation of CCR7, and up-regulation of CXCR3 and CCR5, receptors for chemokines CXCL9, CXCL10, and CXCL11 and CCL3, CCL4, and CCL5, respectively (28, 32, 33). Interaction of chemokines with receptors results in a series of intracellular events leading to the activation of integrins, firm arrest of rolling leukocytes, and actin polymerization (27). Additionally, signaling events involving receptor phosphorylation lead to desensitization of receptors by uncoupling from G proteins and can lead to internalization of receptors through a β-arrestin-dependent process (34). Cross-talk between chemokine receptors can occur through heterologous desensitization, a process through which activation of one receptor leads to the ligand-independent phosphorylation and decoupling of other GPCRs (34–38).

The involvement of chemokines in the recruitment of effector T cells to target organs during GVHD is a relatively new finding with potential therapeutic importance. Expression of proinflammatory chemokines, such as CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, in target organs during GVHD has been demonstrated in a number of experimental models (21, 39–41), although specific roles for most of these ligands during GVHD have not been determined. Our group demonstrated that the production of CCL3 by

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Received for publication January 8, 2004. Accepted for publication May 4, 2004.

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[1] This work was supported by Grants CA58223 and 102052 (to J.S.S.) and Grants AI34495, HL55209, and HL66308 (to B.R.B.) from the National Institutes of Health and a grant from the Lineberger Comprehensive Cancer Center (to J.S.S.).

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; eGFP, enhanced GFP; GPCR, G protein-coupled receptor; ACK, ammonium chloride, potassium carbonate; Tc, T cytotoxic.

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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 chemokines 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 ($H2^b$; 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×DBA/2) F_1 mice, referred to as B6D2 ($H2^{bxd}$; The Jackson Laboratory), BALB/c ($H2^d$), and B10.BR ($H2^k$). Within each experiment, all recipient mice 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 CO_2 and collecting femurs and tibiae. These bones were flushed with 1–2 ml/bone RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FBS (Life Technologies). Cells were collected, and 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 3×10^7/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 5×10^7/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 5×10^8/ml in 0.9% injectable saline.

Bone marrow transplantation

B6D2 and B10.BR recipients were lethally irradiated with 850–950 cGy from a ^{137}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 3×10^6 T cell-depleted bone marrow cells (either B6 or syngeneic cells) and $1–5 \times 10^6$ magnetically selected $CD90^+$ splenic T cells (B6, $CCR5^{-/-}$, or syngeneic T cells) or 5×10^7 unselected splenocytes, 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, 1, 3, and 5. In another set of experiments, nonirradiated B6D2 recipients received 5×10^7 unselected splenocytes 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 individual sections were evaluated 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 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 4 mM Pefabloc SC (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 cytokine bead array assay 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 1640/5% newborn calf serum. Digested 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 pelleted 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 1.0% formaldehyde. Abs used for FACS were obtained from BD Pharmingen and included anti-$H2K^b$-FITC (clone AF6-88.5, mouse IgG2aκ), anti-$H2K^d$-PE (SF1-1.1, mouse IgG2aκ), anti-mCD3ϵ-PerCP (145-2C11, hamster IgG group 1κ), anti-mCD4-PerCP (RM4-5, rat IgG2aκ), and anti-mCD8α-PerCP (53-6.7, rat IgG2aκ). Isotype controls were obtained from BD/Pharmingen. 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 vitro 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).

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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 exon/exon junctions. CCL3, CCL4, CXCL10, CXCL11, 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 were synthesized by the University of North Carolina oligonucleotide synthesis facility. The primer and probe sequences were as follows (in the 5′ to 3′ orientation): CCL3 forward, CGCCAATTCATCGTTGACTA; CCL3 reverse, AGATCTGCCGGTTTCTCTTA; CCL3 probe, CTTTGCTCCCAGC CAGGTGTCATTT; CCL4 forward, CTGTGCTCCAGGGTTCTC; CCL4 reverse, AGACTGCTGGTCTCATAGTA; CCL4 probe, CCAATGGGC TCTGACCCTCCCAC; CXCL10 forward, GAGATCATTGCCACGAT GAA; CXCL10 reverse, CTCCAGTTAAGGAGCCCTTT; CXCL10 probe, TGAATCCGGAATCTAAGACCATCAA; CXCL11 forward, CTCAAGG CTTCCTTATGTTCA; CXCL11 reverse, CTTTCTCGATCTCTGCCATTT; CXCL11 probe, CGCTGTCTTTGCATCGGCCC; CXCR3 forward, AGC-CATGTACCTTGAGGTTA; CXCR3 reverse, CAGAGAAGTCGCT CTCGTTT; CXCR3 probe, AGATGCCTCGGACTTTGCCTTTCTT; 18S rRNA forward, GCTGCTGGCACCAGACTT; 18S rRNA reverse, CGGC TACCACATCCAAGG; and 18S rRNA probe, CAAAATTACCCACTC CCGACCCG. Standard curves were generated for each primer and probe set, using dilutions of separate plasmids containing a cloned copy of each amplicon. PCR was conducted in a total volume of 15 μl with 2× Platinum Quantitative Supermix-UDG (Invitrogen). Thermocycler parameters included 2 min at 50°C, 2 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 56°C for 1 min and 30 s. Values for chemokine and CXCR3 expression in each sample were calculated based on standard curves, and these were normalized by dividing the copies of chemokine message by the copies of 18S rRNA. To assess differences between time points, relative chemokine expression was calculated by dividing the normalized values for each experimental sample at each time point by the mean normalized value for irradiated allogeneic recipients on day 3. Relative CXCR3 expression was assessed by dividing normalized values for each sample by the mean normalized value for freshly isolated B6 T cells.

Chemotaxis assays

Splenic CD90$^+$ T cells were isolated from B6 and CCR5$^{-/-}$ donors, and these cells were stimulated to induce the expression of both CCR5 and CXCR3, as previously described (46). Briefly, cells were cultured at 1.5 × 10^6/well for 48 h in 24-well plates (Costar, Acton, MA) in the presence of immobilized anti-CD3ϵ and anti-CD28 Abs (5 and 2 μg/ml, respectively; BD Pharmingen), followed by removal from immobilized Abs, and an additional 48 h in culture in the presence of 1 ng/ml recombinant murine IL-12 (PeproTech, Rocky Hill, NJ). Culture medium consisted of RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate (Life Technologies), 1× nonessential amino acids (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies), and 50 μM 2-ME (Sigma-Aldrich). After this culture period, 2 × 10^5 T cells were placed on top of the membrane of a Chemotx plate (NeuroProbe, Cabin John, MD), and 100 ng/ml recombinant murine CXCL10 or CCL4 (PeproTech) was aliquoted into the lower wells. Chemotaxis was allowed to occur at 37°C in 5% CO_2 for 30 min. Each sample was assayed in duplicate. The membrane was then removed, and the cells in the lower well were quantified via MTT (Sigma-Aldrich).

MLRs

Responder cells (B6 and CCR5$^{-/-}$ T cells or B6/eGFP and CCR5$^{-/-}$/eGFP T cells) were prepared as described above, using magnetic beads to select for CD90$^+$ splenocytes. Responders (5 × 10^5) were mixed with irradiated (21 Gy) B6D2 splenic stimulators at four different stimulator: responder ratios (0.5, 1, 2, and 4) and plated in 96-well, round-bottom plates (Costar, Cambridge, MA). These reactions were performed in triplicate and incubated 4–5 days at 37°C in 5% CO_2. Culture medium consisted of Iscove's medium (Life Technologies), supplemented as described above. During the last 16–20 h of incubation, 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) was added. Cells were harvested onto filters using a FilterMate harvester (PerkinElmer, Boston, MA), and [^{3}H]thymidine incorporation was measured by scintillation counting via a TopCount counter (PerkinElmer, Boston, MA).

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 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 P815 (H2^d), EL-4 (H2^b), and YAC-1 cells labeled with 100 μCi of ^{51}Cr (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 TopCount 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, $p \leq 0.05$ 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-F$_1$ 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. 1*A*), 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. 1*B*). 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. 1*C*).

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

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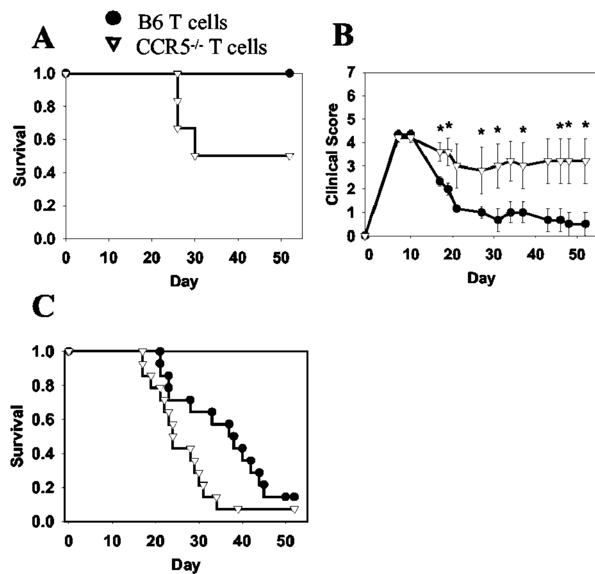

FIGURE 1. Survival of mice receiving allogeneic bone marrow transplants plus CCR5$^{-/-}$ or B6 T cells. *A*, B6D2 mice were irradiated the evening before transplant of 3×10^6 T cell-depleted B6 bone marrow cells plus 2.5×10^6 donor T cells, as described in *Materials and Methods*. Mice were observed for 52 days post-transplant ($n = 6$ animals/group). $p = 0.05$ for the difference in mean survival. *B*, Clinical GVHD scores of irradiated B6D2 mice receiving 2.5×10^6 donor T cells, as described above, were evaluated at the time points shown, using a validated set of criteria referenced in *Materials and Methods*. Data shown represent the mean ± SEM at each time point. *, $p < 0.05$. *C*, Irradiated B6D2 recipients of 3×10^6 T cell-depleted B6 bone marrow cells plus 5×10^6 T cells were monitored for survival. This figure represents pooled data from two experiments ($n = 14$ animals treated/group). $p = 0.02$.

Table I. *Median survival with range for different recipient strains receiving B6 TCD bone marrow and 5×10^6 T cells from either CCR5$^{-/-}$ or B6 donors*[a]

Group	*B6D2*[b]	*B10.BR*[c]	*BALB/c*[d]
CCR5$^{-/-}$	26 (17–39)	12 (10–15)	18 (12–25)
B6	39 (21–50)	19 (17–25)	31 (19–41)

[a] $n = 5$–6 mice/group, with the exception of B6D2 (n = 14/group).
[b] $p = 0.017$.
[c] $p = 0.008$.
[d] $p = 0.056$.

splenocytes and syngeneic controls (Fig. 2). Histopathology on day 3 was unremarkable in all groups, with the exception of findings in the spleen and colon that are typically found after irradiation. On days 7 and 18, there were changes in the liver, lung, colon, and spleen of mice receiving either CCR5$^{-/-}$ or B6 splenocytes indicative of acute GVHD. There was a statistically significant difference in the GVHD score in the lungs of mice receiving CCR5$^{-/-}$ compared with B6 splenocytes on days 7 ($p = 0.03$) and 18 ($p = 0.007$). GVHD scores in the liver assessed on day 18 were modestly, but not significantly, higher in recipients of CCR5$^{-/-}$ vs B6 splenocytes. Pathology in the spleens was similar between the two groups. Consistent with studies in other murine GVHD models, scoring of GVHD in the kidney sections from all transplant groups was minimal even at the latest time point (data not shown), when pathology in other tissues was most significant. Thus, the accelerated GVHD severity and mortality in recipients of CCR5$^{-/-}$ T cells was found to correlate significantly with more severe histopathologic lesions arising early in the course of disease in the lungs of mice receiving CCR5$^{-/-}$ cells as well as a trend toward more severe lesions in the liver later post-transplant.

Quantification of cells infiltrating GVHD-affected organs

We next evaluated donor T cell infiltrates in GVHD target organs on days 3, 7, and 18 by flow cytometry. Interestingly, as shown in Fig. 3, we found significantly increased donor T cell infiltrates in organs from mice receiving CCR5$^{-/-}$ compared with B6 splenocytes. The most significant increases were found in CD8$^+$ T cells in the liver and lung on day 7 and in the liver on day 18. However, significant differences were also found for CD4$^+$ T cells on day 7 in the liver and lung. These data correlated with increased histopathology scores in the lung after receipt of CCR5$^{-/-}$ vs B6 T cells. Significant increases in CCR5$^{-/-}$ donor T cell infiltrates were also observed in the kidney at early time points (data not shown), although the number of cells present was very modest. Infiltration of CCR5$^{-/-}$ donor T cells in the spleen was not significantly increased at any time point compared with that of B6 donor cells, which correlated with the histopathology data. No significant differences in T cell infiltrates in the colon between mice receiving B6 or CCR5$^{-/-}$ cells were observable at any time point. As previous studies demonstrated enhanced production of cytokines such as IFN-γ, IL-4, and GM-CSF by CCR5$^{-/-}$ effector cells (48, 49), we assessed cytokine levels in the above target organs via the cytokine bead array assay. Although significant levels of Th1/Tc1 cytokines (IFN-γ and TNF-α), but not Th2/Tc2 cytokines (IL-4), were observed in all target organs, no significant differences existed between recipients of B6 and CCR5$^{-/-}$ donor cells (data not shown).

To address potential hypotheses for the difference in number of CCR5$^{-/-}$ donor T cells in the lung and liver compared with B6, we evaluated the proliferation, apoptosis, and cytotoxicity of CCR5$^{-/-}$ and B6 T cells in vitro. No increase was found in proliferation in response to B6D2 alloantigens by MLR, survival using annexin V staining of apoptotic cells, or CTL activity by ^{51}Cr 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$^{-/-}$, 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 5×10^7 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. 4*A*, 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. 4*B*). 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.

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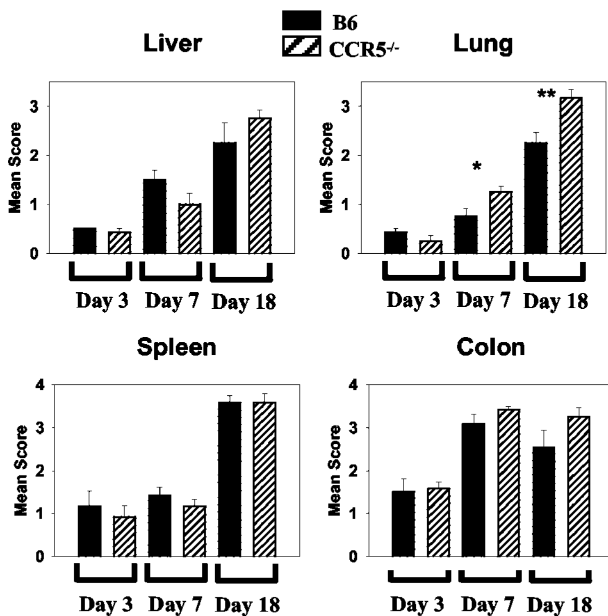

FIGURE 2. Quantitative histopathologic assessment of GVHD in B6D2 recipients of B6 and CCR5$^{-/-}$ splenocytes on days 3, 7, and 18. Quantitative assessment of histopathologic lesions in tissues of B6D2 transplant recipients was performed by one of us (A.P.-M.), who was blinded to the identity of the transplanted mice. Tissues were evaluated from six mice per group. ■, Tissues from mice receiving B6 donor cells; ▨, tissues from mice receiving CCR5$^{-/-}$ 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.

Differential expression of chemokines in target tissues during GVHD based on pretransplant conditioning

We next addressed the expression of several proinflammatory chemokines at days 3, 7, and 14 post-transplant in both irradiated (950 cGy) and nonirradiated B6D2 recipients of B6 transplants, syngeneic transplants (B6D2), and untreated controls. Total RNA was collected from livers, lungs, and spleens from recipients, and real-time RT-PCR was performed on cDNA generated from these RNA samples. As illustrated in Fig. 5, in the liver and lung, where we had observed significant differences in B6 vs CCR5$^{-/-}$ T cell infiltrates in irradiated recipients, the expression of all chemokines assayed occurred earlier in irradiated recipients of allogeneic T cells compared with nonirradiated recipients. In the liver and lung of irradiated recipients, the CXCR3 ligands, CXCL10 and CXCL11, were induced earliest, beginning on day 3 post-transplant, whereas the CCR5 ligands, CCL3 and CCL4, began to increase on day 7. The CXCR3 ligands continued to increase through the first week post-transplant, and their expression either reached a plateau or declined during the second week. The expression of the CCR5 ligands did not decrease during the second week, with the exception of CCL3 expression in the lung, which declined slightly. In nonirradiated recipients receiving allogeneic T cells, the expression of the CXCR3 ligands again preceded that of the CCR5 ligands, beginning on day 7. Levels of CXCR3 ligands were highest on day 14 in these recipients, but peak levels were not significantly different from peak levels seen on day 7 in irradiated recipients. The CCR5 ligands were induced during the second week post-transplant in nonirradiated recipients, and interestingly, peak expression observed on day 14 was in most cases (again with the exception of CCL3 expression in the lung) significantly higher 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$^{-/-}$ T cell infiltrates in irradiated allogeneic transplant recipients (but had seen decreased CCR5$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ T cells, we evaluated the in vitro migration of wild-type and CCR5$^{-/-}$ cells. We activated B6 and CCR5$^{-/-}$ 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$^{-/-}$ T cells had significantly increased migration compared with B6 T cells (Fig. 6*A*). This increase in migratory response to CXCL10 was not associated with increased expression of CXCR3 by CCR5$^{-/-}$ T cells, as determined by both real time RT-PCR and staining for cell surface protein (Fig. 6, *B* 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$^{-/-}$ T cells. Included were groups of recipients treated with an anti-CXCL10 mAb. B6D2 recipients given CCR5$^{-/-}$ 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$^{-/-}$ T cells (Fig. 6*D*). Thus, the enhanced GVHD found using CCR5$^{-/-}$ 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

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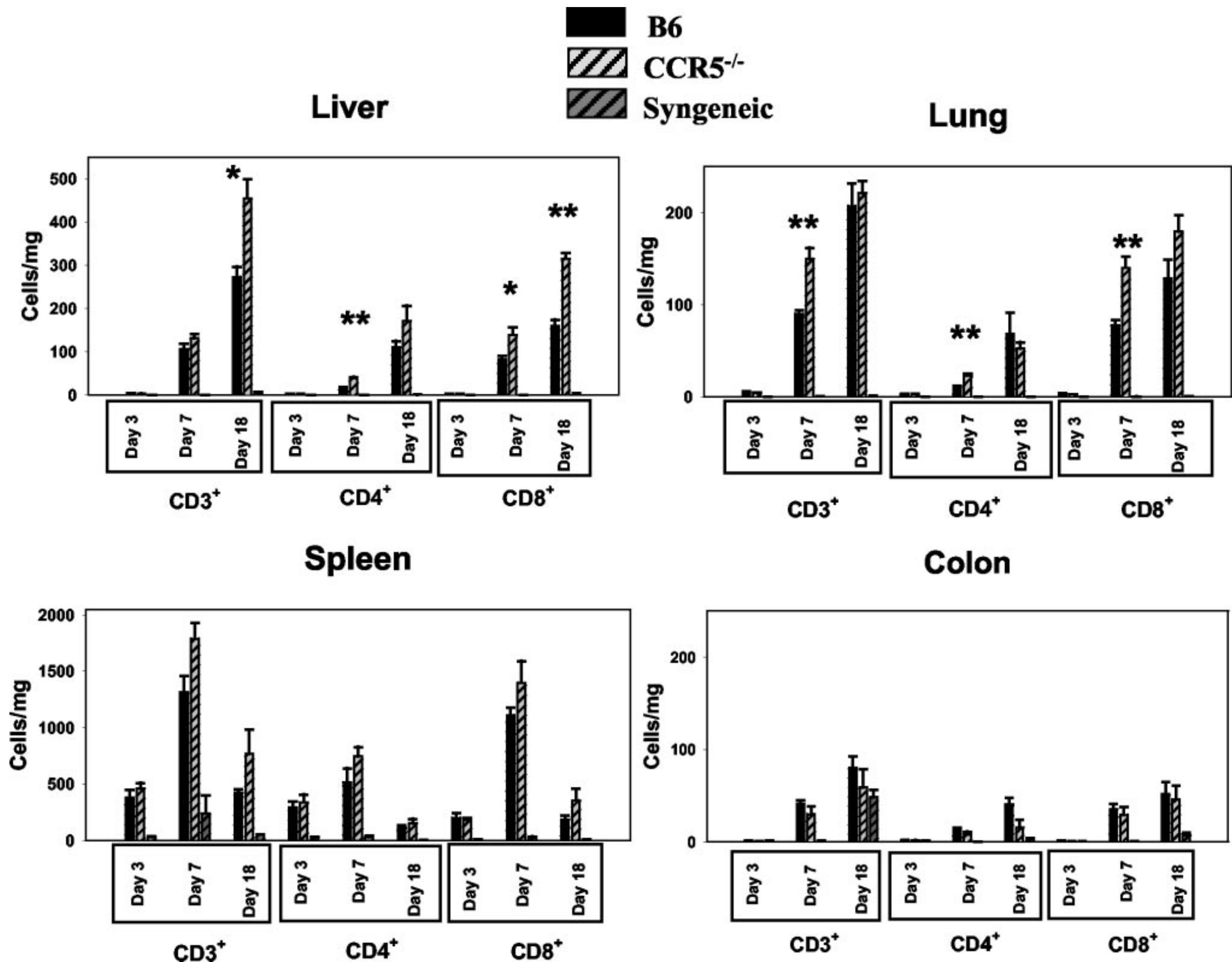

FIGURE 3. T cell infiltrates in GVHD target organs. B6D2 recipients of B6 (■) or CCR5$^{-/-}$ (▨) splenocytes (n = 3) and syngeneic controls (▨; n = 2) were killed on days 3, 7, and 18 post-transplant. Liver, lung, colon, kidney, and spleen were collected and weighed, and infiltrating leukocytes were isolated. T cell subsets infiltrating these organs were determined by staining for CD3, CD4, and CD8. The donor or recipient identity of these cells was determined by staining for H2K^b and H2K^d. The methods used for flow cytometric analysis are described in the text. The mean number of cells per milligram of tissue with the SEM are shown. This experiment was performed twice, with representative data from one experiment shown. *, $p < 0.05$; **, $p < 0.01$.

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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ donor 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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ T cell infiltrates. The expression of CCL3, CCL4, CXCL10, and

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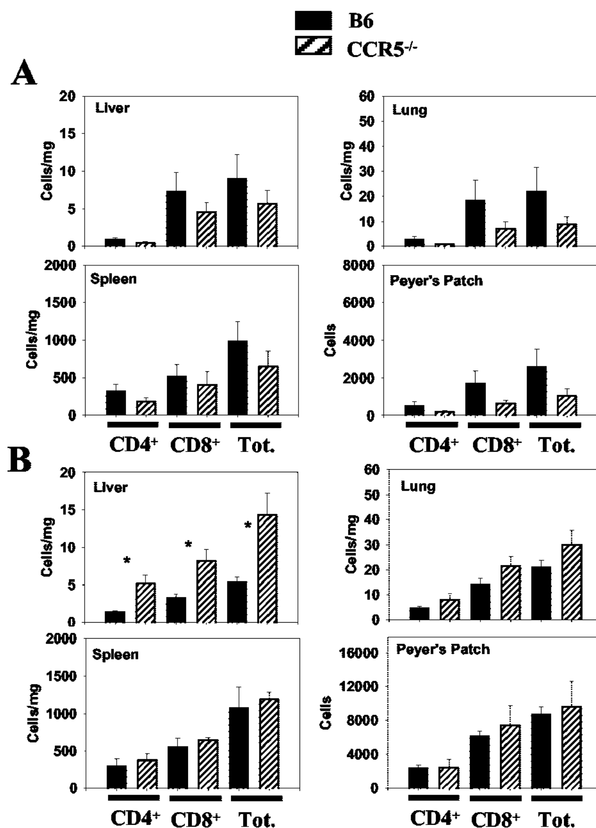

FIGURE 4. Infiltration of B6 and CCR5$^{-/-}$ donor cells in tissues of nonirradiated recipients. *A*, Nonirradiated B6D2 recipients of B6 (■) and CCR5$^{-/-}$ (▨) unselected splenocytes were killed on day 14. Liver, lung, spleen, and Peyer's patches were collected. Liver, lung, and spleen were weighed. 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^b and H2K^d 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). *, $p < 0.05$.

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, recruitment of T cells into these target organs was largely dependent upon CCR5 expression on donor T cells, as the infusion of CCR5$^{-/-}$ donor T cells resulted in lower levels of infiltration. Importantly, we found a marked increase in the IFN-inducible CXCR3 ligands, CXCL10 and CXCL11, in the liver and lung of irradiated compared with nonirradiated recipients during the first week post-transplant. Interestingly, the expression of CXCR3 ligands in the spleens of irradiated recipients declined rapidly during the first week post-transplant. This is consistent with previous observations in our laboratory that CXCR3 ligands such as CXCL9 and CXCL10 are expressed early after transplant due to the combined effects of radiation and transfer of allogeneic T cells (J. Serody, unpublished observations) and suggests that they are expressed primarily from recipient cells post-transplant. The increased infiltration of CCR5$^{-/-}$ T cells observed in the liver and 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 CCR5 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

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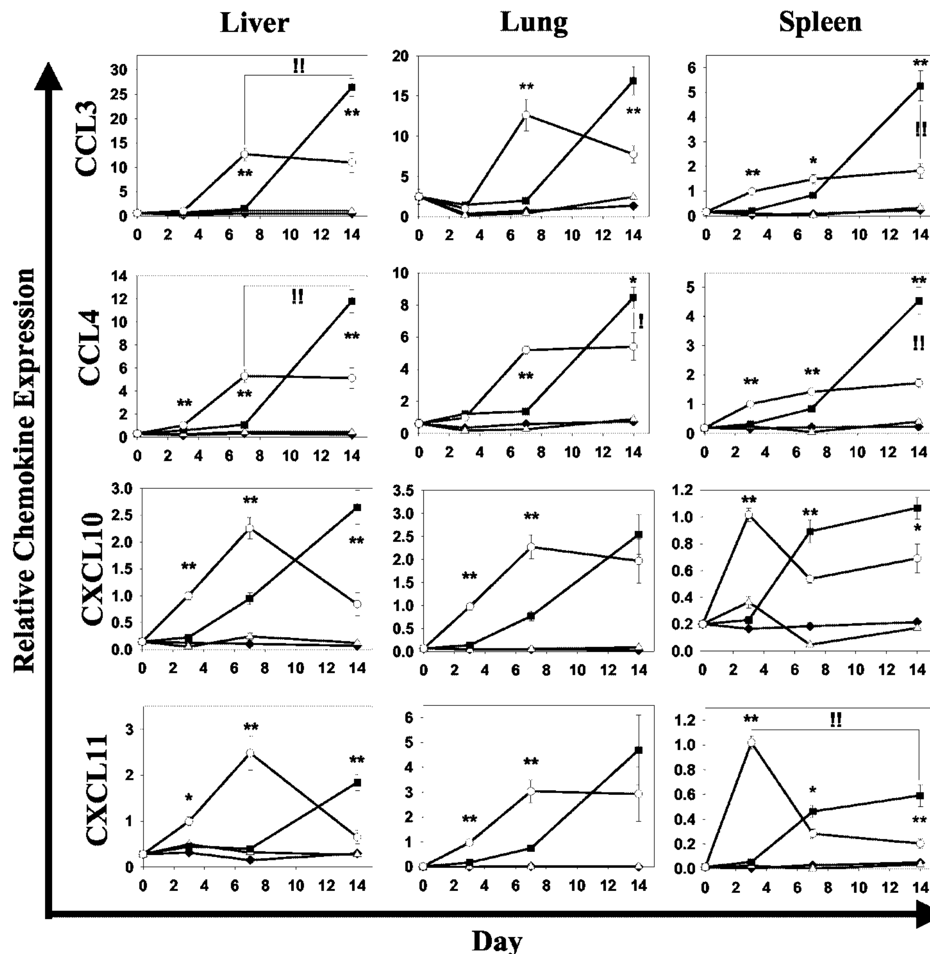

FIGURE 5. Real-time RT-PCR analysis of chemokine expression in GVHD target tissues from irradiated vs nonirradiated transplant recipients. Tissues from irradiated (○) and nonirradiated (■) allogeneic transplant recipients and irradiated (△) and nonirradiated (♦) syngeneic transplant recipients were collected 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 (*, $p < 0.05$; **, $p < 0.001$; !, $p < 0.05$; !!, $p < 0.001$).

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

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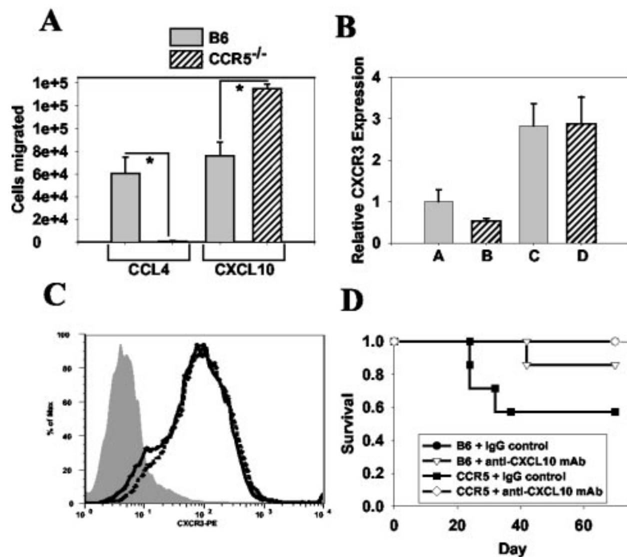

FIGURE 6. The role of CXCL10 in migration of $CCR5^{-/-}$ T cells and in GVHD in recipients of $CCR5^{-/-}$ T cells. *A*, B6 (■) and $CCR5^{-/-}$ (▨) CD90-selected T cells were activated in culture, as described, to induce the expression of CXCR3 and CCR5, and their ability to migrate in response to 100 ng/ml murine CCL4 and CXCL10 was assessed in vitro in a standard chemotaxis assay. The figure shows data pooled from two separate experiments. $CCR5^{-/-}$ T cells did not migrate in response to CCL4, confirming the phenotype of these cells in vitro. Values presented are the mean ± SEM. $*$, $p = 0.01$. *B*, Real-time RT-PCR analysis of CXCR3 expression in B6 (■) and $CCR5^{-/-}$ (▨) T cells. *Bars A* and *B*, Freshly isolated T cells. *Bars C* and *D*, Cultured cells used in the above chemotaxis assays. Calculation of relative CXCR3 expression is described in *Materials and Methods*. The figure shows pooled data from two separate experiments. *C*, Flow cytometric analysis of CXCR3 expression on B6 (solid line) and $CCR5^{-/-}$ (dashed line) cultured T cells. The gray histogram represents isotype control staining. Cells were stained with a rat anti-mouse CXCR3 mAb as described in *Materials and Methods*. The cells represented are from one of the experiments described above, demonstrating enhanced chemotaxis of $CCR5^{-/-}$ T cells to CXCL10. D, Irradiated B6D2 recipients received 3×10^6 T cell-depleted bone marrow cells and 1×10^6 B6 or $CCR5^{-/-}$ T cells with either anti-murine CXCL10 Ab or an isotype hamster IgG control Ab (100 μg i.p. on days −1, 1, 3, and 5) and were monitored for survival. $n = 7$ mice/group.

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 $CCR5^{-/-}$ 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.

Acknowledgments

We thank Dr. Judith Ng-Cashin for advice and critical reading of the manuscript, and Dr. Jennifer Kuhns, Katherine Chwastiak, Robin Craven, and Ivana Ferrer for technical assistance.

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