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Memory T cells migrate to and reject vascularized cardiac allografts independent of the chemokine receptor CXCR3¹

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Abstract

Background—Memory T cells migrate to and reject transplanted organs without the need for priming in secondary lymphoid tissues, but the mechanisms by which they do so are not known. Here we tested whether CXCR3, implicated in the homing of effector T cells to sites of infection, is critical for memory T cell migration to vascularized allografts.

Methods—CD4 and CD8 memory T cells were sorted from alloimmunized CXCR3^{−/−} and wildtype B6 mice and co-transferred to congenic B6 recipients of BALB/c heart allografts. Graft-infiltrating T cells were quantitated 20 and 72 hours later by flow cytometry. Migration and allograft survival were also studied in splenectomized alymphoplastic (*aly/aly*) recipients, which lack secondary lymphoid tissues.

Results—We found that polyclonal and antigen-specific memory T cells express high levels of CXCR3. No difference in migration of wildtype vs CXCR3^{−/−} CD4 and CD8 memory T cells to allografts could be detected in either wildtype or *aly/aly* hosts. In the latter, wildtype and CXCR3^{−/−} memory T cells precipitated acute rejection at similar rates. Blocking CCR5, a

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

chemokine receptor also upregulated on memory T cells, did not delay graft rejection mediated by CXCR3^{-/-} memory T cells.

Conclusions—CXCR3 is not critical for the migration of memory T cells to vascularized organ allografts. Blocking either CXCR3 or CXCR3 and CCR5 does not delay acute rejection mediated by memory T cells. These findings suggest that the mechanisms of memory T cell homing to transplanted organs may be distinct from those required for their migration to sites of infection.

Keywords

T lymphocyte; chemokine receptor; transplantation; rejection

Introduction

Memory T cells constitute a significant proportion of the human alloreactive T cell repertoire and are implicated in the pathogenesis of both acute and chronic allograft rejection (1,2). An important advantage of memory T cells over their naïve counterparts is their ability to migrate directly to the transplanted organ and cause rejection without the need for homing to secondary lymphoid tissues (3,4). Recent evidence suggests that memory T cells act as sentinels that enter the allograft at an early time point after transplantation and contribute to the initiation of the rejection response (5). The mechanisms responsible for the migration of memory T cells to the transplanted organ, however, are not known.

The principal chemokine receptor required for the homing of effector T cells to sites of autoimmunity or infection is CXCR3 (6,7). CXCR3 is the sole receptor for the IFN γ -induced chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), which are expressed in inflamed tissues including allografts (8). CXCR3 is absent on naïve T cells but is strongly upregulated on activated T cells (9). The role of CXCR3 in allograft rejection is controversial (10). While some investigators demonstrated attenuated acute or chronic cardiac allograft rejection in CXCR3^{-/-} recipients (11,12), others failed to show significant alterations in the rejection process in the absence of CXCR3 signaling (13–15). These studies focused on the role of CXCR3 in the primary rejection response in immunologically naïve mice. Thus, the role of CXCR3 in rejection mediated by memory T cells remained undefined. Here we addressed this question by studying the migration and function of adoptively transferred wildtype (*wt*) and CXCR3^{-/-} memory T cells in mouse cardiac allograft models.

Results

Chemokine receptor and adhesion molecule expression on memory T cells

Since the requirements for the migration of memory T cells to transplanted allogeneic tissues are not known, we evaluated chemokine receptor and adhesion molecule expression on memory CD4 and CD8 T cells harvested from alloimmunized mice (B6 mice immunized with BALB/c splenocytes). Chemokine receptor expression on memory T cells was determined in both the polyclonal and antigen-specific (CD8⁺H60-multimer⁺) populations and compared to that on naïve T cells. The H60-multimer identifies CD8 T cells specific for the immunodominant H60 minor histocompatibility antigen present in mice on the BALB but not B6 background. As shown in Fig. 1a, polyclonal CD4 and CD8, and antigen-specific CD8 memory *wt* T cells expressed very high levels of CXCR3 compared to naïve T cells. CCR5 and CXCR5 were also higher on memory than naïve T cells (Fig. 1a), but no differences in CCR4 and CCR6 expression were observed (plots not shown). The adhesion molecule VLA-4 (heterodimer of CD49d and CD29), which is important for the transendothelial migration of activated T cells into non-lymphoid tissues, was upregulated

on the memory population (Fig. 1a). No differences in chemokine receptor or VLA-4 expression were detected between effector (T_{EM} , $CD44^{high}CD62L^{low}$) and central (T_{CM} , $CD44^{high}CD62L^{high}$) H60-specific memory CD8 T cells (Fig. 1a). Except for the complete absence of CXCR3, CXCR3^{-/-} memory T cells expressed similar levels of chemokine receptors and adhesion molecules (Fig. 1b), produced similar levels of IFN γ upon *ex vivo* recall (Fig. 1c), and had similar proportions of T_{EM} and T_{CM} subsets as their *wt* counterparts (Fig. 1d). To test whether chemokine receptor expression changes after memory T cells interact with their cognate antigens, we sorted $CD4^{+}$ and $CD8^{+} CD44^{high}$ memory T cells from immunized *wt* and CXCR3^{-/-} mice and evaluated chemokine receptor expression 24 hours after *in vitro* restimulation with syngeneic or allogeneic splenocytes. As shown in Fig. 1e, no significant difference in chemokine receptor expression was observed between memory T cells restimulated with syngeneic or allogeneic splenocytes in either the *wt* or CXCR3^{-/-} groups, indicating that memory recall did not alter chemokine receptor expression. In addition to establishing that CXCR3 is significantly upregulated on memory T cells and remains upregulated after recall, the data presented in Fig. 1 demonstrate that *wt* and CXCR3^{-/-} memory T cells have comparable phenotype and *ex vivo* function without evidence of compensatory increase in key chemokine receptors in the absence of CXCR3.

Migration of memory T cells to cardiac allografts is independent of CXCR3

To investigate whether CXCR3 is required for the migration of memory T cells to vascularized allografts, we co-transferred CFSE-labeled B6 *wt* (Thy1.1, CD45.2) and CXCR3^{-/-} (Thy1.2, CD45.2) CD4 and CD8 memory ($CD44^{high}$) T cells in equal numbers to congenic B6 *wt* recipients (CD45.1, Thy1.2) two to three days after heart transplantation. Allografts were harvested 20 or 72 hours after cell transfer and the transferred *wt* and CXCR3^{-/-} memory T cells that infiltrated the graft tissue were identified by flow cytometry as shown in Fig. 2a. We found similar numbers of *wt* and CXCR3^{-/-} CD4 and CD8 memory T cells in the cardiac allografts at both 20 and 72 hours ($p > 0.05$), with more transferred T cells detected at the later time point (Fig. 2b). Recovery of *wt* and CXCR3^{-/-} memory T cells from the grafts could be largely attributed to their migration there because none had divided at 20 hours and only a minority had begun dividing by 72 hours (Fig. 2b, CD8 CFSE dilution plots shown). Both T_{EM} and T_{CM} memory phenotype cells were detected in the allografts and no differences in migration could be discerned between *wt* and CXCR3^{-/-} T_{EM} and T_{CM} subpopulations (plots not shown). Immunofluorescence microscopy of allograft tissue confirmed the presence of the transferred, congenic memory T cells within the myocardium (Fig. 2c).

To rule out the contribution of the recipient's secondary lymphoid tissues to the activation of transferred memory T cells and their subsequent migration to the graft tissue, we repeated the memory T cell co-transfer experiment described above except that splenectomized alymphoplasia (*aly/aly*-spleen) mice, which lack all secondary lymphoid tissues, were used as hosts. Since *aly/aly*-spleen mice do not reject cardiac allografts (3), this experiment also allowed us to rule out any effect the host's anti-graft T cell response may have on the migration of transferred memory T cells. As shown in Fig. 2d, *wt* and CXCR3^{-/-} CD4 and CD8 memory T cells transferred to *aly/aly*-spleen recipients were recovered in similar numbers from the cardiac allografts ($p > 0.05$), indicating that CXCR3 is not required for direct migration of memory T cells to vascularized organ transplants. In contrast to memory T cells, naïve *wt* memory T cells did not migrate significantly to cardiac allografts at 72 hours after transfer (Fig. 2d). Analysis of CFSE dilution at 72 hours after transfer confirmed that *wt* and CXCR3^{-/-} memory T cells begin to proliferate in the graft in the absence of host secondary lymphoid tissues (Fig. 2d).

Finally, *wt* and CXCR3^{-/-} CD4 and CD8 memory T cells were readily detected in the spleen and lymph nodes of *wt* hosts and the bone marrow, lung and liver of both *wt* and *aly/*

aly-spleen hosts (data not shown), providing further evidence that CXCR3 expression on memory T cells is not required for their migration to lymphoid and non-lymphoid tissues under homeostatic conditions.

CXCR3 and CCR5 are not required for allograft rejection mediated by memory T cells

To test whether CXCR3-independent migration of memory T cells to the graft has functional consequences, we studied cardiac allograft survival in *aly/aly*-spleen recipients after the transfer of either *wt* or CXCR3^{-/-} CD4 and CD8 memory T cells. As previously reported (3), *aly/aly*-spleen mice that did not receive exogenous T cells or those that received naïve T cells did not reject cardiac allografts (MST > 100 days) (Fig. 3a). The transfer of *wt* or CXCR3^{-/-} memory T cells to *aly/aly*-spleen recipients precipitated acute allograft rejection with the same tempo (MST = 18 and 20.5 days, respectively, $p > 0.05$) (Fig. 3a).

Since CCR5 is upregulated on memory T cells (Fig. 1a & b) and prolonged allograft survival has been reported in CCR5^{-/-} recipients in some studies (16), we tested whether combined CCR5 and CXCR3 blockade delays memory T cell-mediated rejection. As shown in Fig. 3a, daily administration of anti-CCR5 monoclonal antibody to *aly/aly*-spleen mice that received CXCR3^{-/-} memory T cells for 15 days did not significantly alter the tempo of acute rejection (MST = 10 days, $p > 0.05$ compared to recipients that received either *wt* or CXCR3^{-/-} memory T cells). Histological analysis of heart allografts rejected by *wt*, CXCR3^{-/-}, or CXCR3^{-/-} + anti-CCR5 memory T cells confirmed the presence of acute cellular rejection, characterized by mononuclear interstitial cell infiltration and active arteritis in all groups (Fig. 3b). The infiltrate consisted primarily of lymphocytes and macrophages.

Discussion

We investigated in this study whether the inflammatory chemokine receptor, CXCR3, is required for the migration of memory T cells to transplanted organs. We found that CD4 and CD8 memory T cells migrate to newly transplanted, vascularized, cardiac allografts and cause acute rejection independent of CXCR3. Moreover, CCR5 blockade did not delay allograft rejection mediated by CXCR3^{-/-} memory T cells. These findings add to a mounting body of evidence that CXCR3 and CCR5, often implicated in the homing of effector T cells to sites of infection or autoimmunity, are in fact not essential for allograft rejection (13–15,17).

While some studies have found prolonged survival of fully MHC-mismatched cardiac allografts in CXCR3^{-/-} recipients or attenuated chronic vasculopathy in multiple minor antigen mismatched grafts (11,12), others did not find a significant role for CXCR3 in allograft rejection (13–15). Factors that could explain these contradictory results include the use of genetically deficient mice vs. CXCR3 blocking agents or differences in the background of knock-out mice used. Importantly, these reports studied the role of CXCR3 in the primary immune response while our study directly addressed the requirement of CXCR3 for the migration and function of memory T cells. By adoptively transferring CXCR3^{-/-} and *wt* memory T cells to cardiac allograft recipients, we extend the observation that CXCR3 is not essential for rejection mediated by memory T cells.

Memory T cells play a key immune surveillance function that protects the host against infection and cancer. In the context of organ transplantation, memory T cells survey and enter non-self tissues early after transplantation where they cause acute rejection and/or potentiate the innate and adaptive immune responses to the graft (3,5). In contrast, naïve T cells are dependent on activation within secondary lymphoid tissues, a process that could require several days, before they differentiate into effector T cells with non-lymphoid tissue

homing capability. The role of memory T cells in transplant rejection is particularly relevant in humans where alloreactive memory T cells are quite prevalent, even in individuals not previously exposed to alloantigens (1). Therefore, understanding how memory T cells migrate to transplanted organs is critical for developing targeted strategies that interrupt the rejection response at its earliest stages. Our findings that CXCR3 is not essential for memory T cell homing to the graft and that blocking CCR5 in the absence of CXCR3 does not impair acute rejection mediated by memory T cells rule out these chemokine receptors as likely targets of anti-rejection therapy.

Due to the redundancy of the chemokine and chemokine receptor families, it is possible that other chemokine receptors direct the migration of memory T cells to non-lymphoid tissues. We observed upregulation of CXCR5 on memory T cells and others have reported expression of additional chemokine receptors (for example, CCR1, CCR2, and CCR6) on activated T cells (18). Given that only modest enhancement of allograft survival has been reported in CCR1^{-/-} and CCR2^{-/-} recipients (18), we suspect that no single chemokine receptor will provide the dominant mechanism by which effector or memory T cells reach a transplanted organ. This raises the possibility that generalized blockade of chemokine receptor function will be necessary to prevent rejection, or that T cells home to non-self tissues in a chemokine receptor-independent fashion. Both hypotheses remain to be tested.

Materials and Methods

Mice

C57BL/6J (B6) (Thy1.2, CD45.2, H-2^b), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1, Thy1.2, H-2^b), B6.PL-Thy1a/CyJ (Thy1.1, CD45.2, H-2^b) and BALB/cJ (BALB/c) (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CXCR3^{-/-} mice on the B6 background (Thy1.2, CD45.2, H-2^b) were provided by Dr. Craig Gerard (Children's Hospital, Boston). Alymphoplasia mice (Map3k14^{aly/aly}, Thy1.2, H-2^b) were purchased from CLEA (Osaka, Japan) and bred onto a B6 CD45.1 congenic background (hereafter referred to as *aly/aly*). All animals were maintained under specific pathogen free conditions.

Surgical procedures and mouse treatment

Splenectomies and heterotopic transplantation of primarily vascularized cardiac allografts were performed as previously described (8). Allograft rejection was defined as cessation of palpable heartbeat and was confirmed by histological analysis. Where indicated, recipients were injected with 100 µg of monoclonal rat anti-mouse CCR5 antibody (C34-3448, BD Pharmingen) i.p. on day -1 and then daily until rejection or day +14 after transplantation. This antibody dose has been shown to inhibit progression of insulinitis in non-obese diabetic mice (19). All procedures were performed per IACUC guidelines.

Generation, isolation, and adoptive transfer of memory T cells

B6 *wt* (Thy1.1, CD45.2) and CXCR3^{-/-} (Thy1.2, CD45.2) mice were immunized i.p. on days 0 and 21 with 2×10^7 BALB/c splenocytes. Six weeks later spleen and lymph node (inguinal, mesenteric, brachial and axillary) cells were enriched for T cells by negative selection using MACS (AutoMACS Pro, Miltenyi) or EasySep (Stem Cell Technologies) kits and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 0.5 µM) (Invitrogen) prior to high-speed sorting on a BD Aria Plus for CD4⁺CD44⁺CFSE⁺ and CD8⁺CD44⁺CFSE⁺ memory T cells (> 96% purity). To study lymphocyte migration, 6×10^6 *wt* (Thy1.1, CD45.2) and 6×10^6 CXCR3^{-/-} (Thy1.2, CD45.2) CD4 and CD8 memory T cells (3×10^6 cells of each subset) were co-transferred i.v. into B6 (Thy1.2, CD45.1) or splenectomized *aly/aly* (*aly/aly*-spleen, Thy1.2, CD45.1) mice that had received a BALB/c cardiac allograft 2 – 3 days earlier. To study allograft rejection, same numbers of *wt* and

CXCR3^{-/-} memory T cells were transferred separately into *aly/aly*-spleen mice 2 – 3 days after heart transplantation.

T cell recovery and enumeration after adoptive transfer

Adoptive hosts were sacrificed 20 or 72 hours after T cell transfer. Mice were perfused with 20 – 40 ml of PBS + 0.5% heparin via the left ventricle until the fluid exiting the right ventricle did not contain any visible blood. Cells from the spleen, lymph nodes, liver, lung, bone marrow and cardiac allograft were isolated as previously described (4). Briefly, tissues were homogenized using a GentleMACS tissue processor (Miltenyi, CA) followed by hypotonic RBC lysis when necessary. Liver, lung and cardiac allograft tissues were digested at 37°C in buffer containing Collagenase IV (350 U/ml) and DNase I (20 ng/ml), and lymphocytes were isolated by gradient centrifugation using Lympholyte M (CedarLane Labs, NC). Total recovered cells were counted and the transferred memory T cells enumerated by flow cytometry by gating on the CD45.2⁺Thy1.1⁻ and CD45.2⁺Thy1.1⁺ populations after live/dead cell discrimination and exclusion of non-T cells (CD11b⁺, CD11c⁺, CD45R/B220⁺, CD49b⁺, F4/80⁺, CD16/32⁺, and Ly-76⁺).

Flow cytometry and intracellular cytokine staining

Fluorochrome- or biotin-tagged antibodies were purchased from BD Pharmingen, eBioscience, BioLegend or R&D Systems: CD4 (RM4-5), CD8α (53-6.7), CD62L (MEL-14), CD44 (1M7), CD90.1 (OX-7), CD45.1 (A20), CD45.2 (104), CD16/32 (2.4G2), CD45R/B220 (RA3-6B2), CD49b (DX5), F4/80 (BM8), Ly-76 (TER-119), CD11b (M1/70), CD11c (HL3), and IFNγ (XMG1.2). Fixable live/dead Aqua cell stain (405nm excitation) and Cell Tracker Violet (405nm excitation) was purchased from Invitrogen. Soluble, fluorochrome-tagged MHC class I H-2K^b/peptide multimers using the H60 peptide LTFNYRNL were provided by the NIAID Tetramer Facility (Atlanta, GA). Multimer staining was carried out according to NIAID recommendations by staining lymphocytes for appropriate surface markers for 10 min at 4 °C followed by staining with multimer for 30 min at 4 °C. Intracellular cytokine staining was performed as previously described (4). Briefly, lymphocytes from previously immunized mice were stimulated *ex vivo* with Balb/c splenocytes (1:1) for 16 hours in the presence of Brefeldin A. Cells were then washed, stained for surface markers, fixed, permeabilized with 0.25% saponin, and incubated with anti-IFNγ antibody for 1hr at RT. Flow acquisition was performed on LSRII analyzers (BD Biosciences), and data analyzed using Flowjo software (Treestar Corp.). Chemokine receptor expression was determined 24 hours after *in vitro* restimulation by following the same procedures described above with the exception of Brefeldin A.

Histology and immunofluorescence

Allografts were procured from mice after extensive cardiac perfusion and were processed immediately. For vibratome sections, tissue was incubated in 3% low melting point agarose at 37°C for 15 min, followed by embedding in 6% low melting point agarose on ice. 200 μm tissue sections were cut and fixed for 2 hours in 4% paraformaldehyde at 4°C, blocked with 2% BSA, biotin and streptavidin overnight, and incubated with primary (CD45.2-biotin (eBioscience) and rat anti-mouse CD31 (BD Pharmingen)) and secondary (anti-rat Alexa Fluor 555 (Invitrogen) and Streptavidin-Cy5 (Jackson ImmunoResearch)) antibodies overnight. DAPI staining was carried out for 30 min at a 200 nM concentration. Tissue sections were imaged with an Olympus FV-1000 confocal microscopy system. Images were analyzed using Volocity LE software (Improvision, Perkin-Elmer).

Statistical analysis

Statistical significance in allograft survival experiments was calculated using the log-rank test and for all other experiments using the unpaired t-test (Graphpad Prism v5.0 software). Significance was set at $p < 0.05$.

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Figure 1. Phenotype and *ex vivo* function of *wt* and *CXCR3*^{-/-} memory T cells

Splenic T cells were harvested from *wt* (a) or *CXCR3*^{-/-} (b) B6 mice > 6 wks after immunization with BALB/c splenocytes. Chemokine receptor and integrin (VLA-4) expression was analyzed by flow cytometry after gating on the CD4⁺ and CD8⁺ naïve (CD44^{low}CD62L^{high}) and polyclonal memory (CD44^{high}) T cell populations, or on the CD8⁺H60⁺ CD44^{high} antigen-specific T_{EM} (CD62L^{low}) and T_{CM} (CD62L^{high}) memory subsets. Plots shown are representative of 3 experiments. (c) IFN γ production by *wt* and *CXCR3*^{-/-} polyclonal CD8⁺ memory T cells 16 hours after *ex vivo* restimulation with allogeneic (allo) or syngeneic (syn) splenocytes (n = 3, mean $\pm$ SD). (d) T_{CM} and T_{EM} phenotype of *wt* and *CXCR3*^{-/-} CD8⁺H60⁺ antigen-specific memory T cell populations. Plots shown are representative of 3 experiments. (e) Memory CD4 and CD8 T cells were sorted from B6 mice > 6 wks after immunization with BALB.B splenocytes and restimulated *in vitro* with either syngeneic (B6) or allogeneic (BALB.B) splenocytes for 24 hours. Chemokine receptor expression was analyzed by flow cytometry after gating on polyclonal CD4⁺ and antigen-specific CD8⁺H60⁺ CD44^{high} memory T cells. Stimulators were gated out by use of congenic markers or CellTracker Violet labeling.

Figure 2. Migration of *wt* and *CXCR3*^{-/-} memory T cells to cardiac allografts
Sorted *wt* (CD45.2, Thy1.1) and *CXCR3*^{-/-} (CD45.2, Thy1.2) polyclonal CD4 and CD8 memory T cells were co-transferred to congenic (CD45.1, Thy1.2) recipients 2 days after cardiac allograft transplantation. Allografts were harvested at 20 and 72 hours after cell transfer. Co-transferred *wt* and *CXCR3*^{-/-} memory T cells that infiltrated the graft were identified and quantitated by flow cytometry according to the gating strategy shown (a). (b) Quantitation of transferred *wt* and *CXCR3*^{-/-} memory T cells recovered from allografts removed from *wt* recipients (2 independent experiments; n = 3 mice/experiment; mean ± SD). Proliferation of recovered CD8 memory T cell populations determined by CFSE dilution is shown in the histograms. (c) Immunofluorescence staining of cardiac allograft tissue demonstrating the presence of *CXCR3*^{-/-} (CD45.2) memory T cells within the myocardium 6 days after transfer to congenic *wt* (CD45.1) recipients. Transferred T cells appear green (CD45.2⁺), endothelial cells red (CD31⁺), and nuclei blue (DAPI). White bar = 50 μm (magnification = 60x). (d) Quantitation of transferred naïve *wt* T cells as well as *wt* and *CXCR3*^{-/-} memory T cells as described under (b) except that recipients were *aly/aly*-spleen mice (2 independent experiments; n = 3 mice/experiment; mean ± SD). n.s. = not significant

Figure 3. Cardiac allograft rejection by memory T cells is independent of CXCR3 and CCR5
 CD4 and CD8 memory T cells were transferred to *aly/aly*-spleen recipients 2 days after cardiac transplantation and allograft survival was determined by palpation (a). Control mice did not receive any exogenous T cells or received naïve T cells. Rat anti-mouse CCR5 antibody was administered daily for 15 days starting on the day prior to transplantation. No significant difference in allograft survival was observed among the groups that received memory T cells. (b) Representative cardiac allograft histology (H&E staining) from the indicated groups showing extensive cellular infiltrate and myocyte destruction with active arteritis (magnification = 30x).