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Antibody-Mediated Rejection of Cardiac Allografts in CCR5-Deficient Recipients

Taiji Nozaki,§ Hiroyuki Amano,§ Alice Bickerstaff,¶ Charles G. Orosz,¶ Andrew C. Novick,* Kazunari Tanabe,§ and Robert L. Fairchild³*†‡

Rejected MHC-mismatched cardiac allografts in CCR5−/− recipients have low T cell infiltration, but intense deposition of C3d in the large vessels and capillaries of the graft, characteristics of Ab-mediated rejection. The roles of donor-specific Ab and CD4 and CD8 T cell responses in the rejection of complete MHC-mismatched heart grafts by CCR5−/− recipients were directly investigated. Wild-type C57BL/6 and B6.CCR5−/− (H-2b) recipients of A/J (H-2a) cardiac allografts had equivalent numbers of donor-reactive CD4 T cells producing IFN-γ, whereas CD4 T cells producing IL-4 were increased in CCR5−/− recipients. Numbers of donor-reactive CD8 T cells producing IFN-γ were reduced 60% in CCR5−/− recipients. Day 8 posttransplant serum titers of donor-specific Ab were 15- to 25-fold higher in CCR5−/− allograft recipients, and transfer of this serum provoked cardiac allograft rejection in RAG-1−/− recipients within 14 days, whereas transfer of either serum from wild-type recipients or immune serum from CCR5-deficient recipients diluted to titers observed in wild-type recipients did not mediate this rejection. Wild-type C57BL/6 and B6.CCR5−/− recipients rejected A/J cardiac grafts by day 11, whereas rejection was delayed (day 12–60, mean 21 days) in μMT−/−/CCR5−/− recipients. These results indicate that the donor-specific Ab produced in CCR5−/− heart allograft recipients is sufficient to directly mediate graft rejection, and the absence of recipient CCR5 expression has differential effects on the priming of alloreactive CD4 and CD8 T cells. The Journal of Immunology, 2007, 179: 5238–5245.

Although the majority of graft rejection episodes are mediated by donor Ag-primes T cells, acute humoral rejection (AHR) is an increasingly observed problem in clinical transplantation (1–3). The presence of alloantibodies is associated with more frequent and severe rejection episodes, as well as poorer graft function and survival. The development of donor-reactive IgG responses markedly increases the risk of acute rejection in cardiac transplant patients (4, 5). Circulating anti-HLA Abs are also associated with late loss of kidney, heart, lung, and liver grafts, including the development of chronic rejection (6, 7). Graft endothelial cells are the primary targets of donor-reactive Abs during AHR, and C4d staining of capillaries in endomyocardial biopsies correlates with the presence of circulating anti-donor Abs and ongoing AHR (8, 9). The use of C4d staining in graft biopsies has improved diagnosis of AHR and led to the recognition that the incidence of AHR is more prevalent than previously thought (10–12). This diagnostic tool has led to the realization that in cardiac transplant patients the incidence of AHR without an accompanying cellular rejection may be as high as 15% (13). Importantly, graft rejection mediated by donor-specific Ab is less responsive to conventional immunosuppressive therapy than T cell-mediated acute rejection episodes (14, 15).

T cell-mediated rejection of allografts is initiated by the infiltration and activation of donor Ag-specific effector T cells in the graft. Trafficking of T cells and other leukocytes to inflammation in peripheral tissues including allografts is directed in part by leukocyte expression of chemokine receptors responsive to chemokines produced at the inflammatory site (16, 17). A major approach to investigate the role of chemokines and their receptors in directing Ag-primes T cells to tissue sites during elicitation of immune responses has involved the use of mice with targeted gene deletions in specific chemokines or chemokine receptor genes. Several studies have reported increased or long-term survival of MHC-mismatched heart and islet allografts in CXCR3−/− and CCR5−/− recipients (18, 19). A recent report from this laboratory indicated that complete MHC-mismatched cardiac allograft survival was only extended 2–3 days in CCR5−/− recipients when compared with survival in wild-type recipients (20). However, cardiac allograft rejection in CCR5-deficient recipients was accompanied by low cellular infiltration into the graft, but intense deposition of C3d in the large vessels and parenchymal capillaries of the graft as well as the presence of high serum titers of donor-specific Ab. These results were consistent with the diagnostic characteristics of AHR (2). The goal of the current study was to directly test the ability of the donor-specific Ab generated in CCR5−/− allograft recipients to mediate rejection of cardiac allografts and to test the requirement for this Ab in the rejection of heart allografts observed in CCR5−/− recipients. The results indicate that the high titers of Ab induced in CCR5−/− cardiac allograft recipients are
sufficient to provoke rejection of the grafts supporting the use of CCR5−/− allograft recipients as a novel model of AHR. Although effector CD8 T cell responses are substantially decreased in CCR5-deficient allograft responses, delayed rejection is observed in the absence of Ab production, indicating the ability of suboptimal donor-reactive T cell responses to mediate rejection in the absence of the Ab.

Materials and Methods

Animals

C57BL/6 (H-2b), A/J (H-2a), B6.RAG-1(−/−), and B6.mMT−/− mice were obtained through C. Reeder at the National Cancer Institute (Frederick, MD). CCR5−/− mice were obtained from The Jackson Laboratory. CCR5−/− and mMT−/− mice were crossed to generate the B6.mMT−/−/CCR5−/− double-knockout mice. Male mice of 8–12 wk of age were used in all experiments.

Antibodies

The following Abs were used for immunohistochemistry, immunofluorescence, and flow cytometry during these studies: FITC-conjugated goat anti-mouse IgG plus IgM Ab, purified rat anti-mouse CD4 (GK 1.5) mAb, and purified rat anti-mouse CD8 (53-6.7) mAb (BD Pharmingen); FITC-conjugated rabbit anti-C3d Ab (DakoCytomation); rat anti-mouse macrophage (F4/80) mAb (Serotec); and anti-mouse Ly-6G mAb, RB6.8C5, purified from culture supernatants on Sepharose G columns.

Heterotopic heart transplantation

Heart transplantations were performed by microsurgical methods reported by Corry et al. (21). Briefly, the donor aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava using microsurgical techniques. After anastomosis, the heart was perfused with recipient’s blood and resumed contraction. The strength of the heart graft was monitored by abdominal palpation each day until rejection, which was considered as total cessation of contraction and was confirmed visually by laparotomy. For some experiments, groups of wild-type C57BL/6 and CCR5−/− heart allograft recipients were treated with 400 μg/d anti-CD4 mAb i.p. on days −3, −2, −1, +4, and +8, and every 4 days thereafter until graft rejection.

Flow cytometric analysis

Titers of donor-specific Abs in cardiac allograft recipient serum were determined using a flow cytometry-based method, as previously described (20). Briefly, thymocytes from naive A/J and C57BL/6 mice were suspended in HBSS and incubated with serial 4-fold dilutions of sera from wild-type C57BL/6 or CCR5−/− allograft recipients or from naive-wild-type mice. After 1 h on ice, the cells were washed and resuspended in staining buffer (Dulbecco’s PBS with 2% FCS/0.02% NaN3) containing FITC-conjugated goat Abs specific for mouse IgG and IgM, and purified rat anti-mouse IFN-γ or IL-4 mAb (BD Pharmingen). After an additional 30 min on ice, the cells were washed and analyzed by flow cytometry. The mean channel fluorescence was measured for each sample, and the dilution of allograft recipient serum that returned the mean channel fluorescence to that observed when the A/J thymocytes were stained with the 1/4 dilution of naive C57BL/6 serum was divided by two and recorded as the titer.

Immunohistochemistry

Cross-sections of the center of cardiac grafts obtained at the time of rejection were frozen at −80°C in OCT compound (Sakura Finetek). Sections were cut at 7–8 μm and placed onto slides. For immunohistochemical staining, sections were air dried for 30 min and fixed in cold acetone for 10 min. Slides were washed with PBS and incubated for 30 min at room temperature with the following primary Abs diluted 1/50 in PBS: anti-CD4 mAb (GK 1.5), anti-CD8α mAb (53-6.7), anti-Ly-6G (RB6) mAb, and anti-F4/80 mAb. After rinsing with PBS, the slides were incubated for 20 min with biotinylated goat anti-rat IgG diluted 1/100. Streptavidin-HRP (DakoCytomation) was then applied for 20 min, followed by substrate-chromagen 3,3’-diaminobenzidine (Sigma-Aldrich), and finally washed with dH2O. The slides were counterstained with hematoxylin, dehydrated, and mounted. For immunofluorescent staining of C3d, FITC-conjugated rabbit anti-C3d Ab (1/100 dilution) was added to the slides after fixation. The slides were incubated for 30 min, washed, mounted with VECTASHIELD (Vector Laboratories), and viewed under a fluorescent microscope.

FIGURE 1. Temporal production of donor-reactive Ab in wild-type C57BL/6 and B6.CCR5−/− allograft recipients. Groups of four wild-type C57BL/6 and B6.CCR5−/− mice received heterotopic heart transplants from A/J donors, and serum was collected on days 4, 6, and 8 posttransplant in each individual recipient. Serial dilutions of the serum were tested for reactivity to syngeneic and A/J thymocytes to determine the titers of donor-reactive Ab. The individual titers for each individual recipient at each day posttransplant are indicated, and the bar in each group indicates the mean titer. ∗, p < 0.01.

Serum transfer

Sera of wild-type C57BL/6 and CCR5−/− heart allograft recipients were collected on day 8 posttransplant and pooled, and 400 μl was injected i.v. to a RAG-1−/− mouse, which had received an A/J heart graft 2 days before the serum transfer.

ELISPOT assays

Alloantigen-specific T cell priming of heart graft recipients was assessed by enumerating donor-specific T cells producing IFN-γ and IL-4 by ELISPOT assay, as previously described (20, 22). Briefly, ELISPOT plates were coated with purified rat anti-mouse IFN-γ or IL-4 mAb (BD Pharmingen), incubated overnight at 4°C, and then blocked with 1% BSA/PBS. Spleen cells were prepared from graft recipients or from naive mice, and the CD4+ and CD8+ T cells were separated by negative selection with Dynabeads (Invitrogen Life Technologies) and used as responder cells. Mitomycin C-treated donor and recipient spleen cells were used as stimulator cells in each assay. Responder cells were cultured with stimulator cells in serum-free HL-1 medium (BioWhittaker) with 1 mM L-glutamine for 24 h at 37°C in 5% CO2. Plates were extensively washed to remove the cells, and biotinylated rat anti-mouse IFN-γ or IL-4 mAb (BD Pharmingen) was added. After overnight incubation at 4°C, plates were washed with 0.05% Tween 20/PBS, and alkaline phosphatase-conjugated goat anti-biotin Ab (Sigma-Aldrich) was added to each well. The plates were incubated for 1.5 h at room temperature and washed with PBS, and NBT-5-bromo-4-cloro-3-indolyl substrate (Bio-Rad) was added. The resulting spots were counted on an ImmunoSpot Series I analyzer (Cellular Technology).

Results

Kinetics of donor-specific Ab production in CCR5−/− allograft recipients

We have previously demonstrated that the absence of CCR5 in cardiac allograft recipients leads to an increased alloantibody response with intense deposition of complement split products in rejected grafts (20). To further investigate the induction of the alloantibody response in CCR5-deficient recipients, the temporal production of donor-specific Ab in wild-type and CCR5−/− recipients of MHC-mismatched A/J cardiac allografts was compared (Fig. 1). Four days after transplantation, donor-specific Ab titers were at low levels in both wild-type and CCR5−/− recipients (36.0 ± 16.0 vs 40.0 ± 0.0). By day 6, Ab titers began a marked increase in the CCR5−/− recipients (466.7 ± 266.7) and were at high levels (9000 ± 4000) by day 8 posttransplant. In wild-type recipients, donor-specific Ab titers remained low on day 6 (68.0 ± 19.6) and increased on day 8 (340 ± 98.0) posttransplant, but were well below the titers observed in the serum of CCR5−/− recipients.
To test the relationship of serum anti-donor Ab titer and reactivity of the Ab in the allograft, A/J heart grafts were retrieved from wild-type and CCR5−/− recipients at each day of the analysis, and prepared sections were stained to detect the complement split product C3d as an indication of Ab deposition in the graft (Fig. 2). At day 4 posttransplant when titers in both groups of recipients were low, there was little evidence of C3d deposition in

FIGURE 2. Deposition of C3d in allografts increases with donor-reactive Ab titers. Groups of wild-type C57BL/6 (a, c, and e) and B6.CCR5−/− (b, d, and f) mice received heterotopic heart transplants from A/J donors, and the allografts were retrieved on days 4 (a and b), 6 (c and d), and 8 (e and f) posttransplant. Prepared frozen sections were stained with FITC anti-C3d Ab and viewed by immunofluorescent microscopy to detect deposition of C3d. Isografts from wild-type C57BL/6 and B6.CCR5−/− recipients did not stain for C3d (data not shown). Original magnification, ×200.

FIGURE 3. Allograft rejection mediated by transfer of sera from CCR5−/− allograft recipients. Groups of wild-type C57BL/6 and B6.CCR5−/− mice received heterotopic heart transplants from A/J donors, and serum was collected from each recipient on day 8 posttransplant and pooled for each group. Groups of five RAG-1−/− mice received A/J heart transplants, and on day 2 posttransplant were injected with 400-μl aliquots of the pooled serum from wild-type or CCR5−/− recipients or CCR5−/− recipient serum that had been diluted 1/15. Grafts were monitored daily by palpation, and rejection was confirmed visually by laparotomy.

FIGURE 3. Allograft rejection mediated by transfer of sera from CCR5−/− allograft recipients. Groups of wild-type C57BL/6 and B6.CCR5−/− mice received heterotopic heart transplants from A/J donors, and serum was collected from each recipient on day 8 posttransplant and pooled for each group. Groups of five RAG-1−/− mice received A/J heart transplants, and on day 2 posttransplant were injected with 400-μl aliquots of the pooled serum from wild-type or CCR5−/− recipients or CCR5−/− recipient serum that had been diluted 1/15. Grafts were monitored daily by palpation, and rejection was confirmed visually by laparotomy.

FIGURE 4. Leukocyte infiltration into Ab-mediated rejected cardiac allografts in RAG-1−/− recipients. On day 14, rejected heart allografts from RAG-1−/− recipients of immune CCR5−/− serum (a and b) and surviving allografts from recipients of immune wild-type serum (c and d) were retrieved. Frozen sections were prepared and stained to detect neutrophil (a and c) and macrophage (b and d) infiltration into the grafts. Original magnification, ×100.
transplant, and pooled aliquots were transferred i.v. to RAG-1/H11002. The ability of the donor-specific Ab produced in CCR5/H11002 allograft rejection
CD4 rejection, a 1/15 dilution of the CCR5 differences in titer of the immune sera accounted for the allograft 15-fold higher than the wild-type recipient sera. To test whether
allografts from CCR5-deficient recipients. Serum from CCR5/H11002 cardiac allograft recipients mediates allograft rejection
The ability of the donor-specific Ab produced in CCR5−/− heart
graft recipients to mediate rejection of the allografts was directly tested using a serum transfer approach. Sera were collected from CCR5−/− and wild-type heart allograft recipients on day 8 posttransplant, and pooled aliquots were transferred i.v. to RAG-1−/− mice that had received A/J cardiac graft 2 days before the serum transfer (Fig. 3). All allografts survived for >100 days in RAG-1−/− recipients that received the wild-type immune serum. In contrast, transfer of the CCR5−/− immune serum mediated rejection of all A/J hearts in the RAG-1−/− recipients within 12 days. The donor-specific Ab titer in the pooled CCR5−/− recipient sera was 15-fold higher than the wild-type recipient sera. To test whether differences in titer of the immune sera accounted for the allograft rejection, a 1/15 dilution of the CCR5−/− immune sera was transferred to RAG-1−/− recipients of A/J heart allografts. This dilution abrogated the ability of the sera to reject the allografts.

Rejected allografts from the RAG-1−/− mice that had received the CCR5−/− immune sera and allografts from the RAG-1−/− mice that had received the wild-type immune sera were retrieved at day 14 posttransplant, and prepared sections were examined for leukocyte infiltration (Fig. 4). Intense neutrophil and macrophage infiltration throughout the graft parenchyma was observed in the allografts rejected by the CCR5−/− immune sera, whereas grafts from the RAG-1−/− mice receiving the wild-type immune sera had little evidence of neutrophil and macrophage infiltration.

Depletion of CD4 T cells prolongs MHC-mismatched allograft survival in CCR5-deficient vs wild-type recipients
The induction of donor-specific T cell immunity in CCR5−/− cardiac allograft recipients was then investigated. First, the development of donor-reactive CD4 and CD8 T cell populations in the

the allografts. By day 6 posttransplant, clear deposition of C3d was observed in the large vessels and the parenchymal capillaries of allografts retrieved from the CCR5-deficient recipients and the intensity of staining increased in allografts retrieved at day 8. In contrast, low deposition of C3d was observed at day 6 posttransplant in allografts from wild-type recipients, and this deposition was restricted to the large vessels. Although the intensity of this deposition increased by day 8 and expanded to a few of the parenchymal capillaries, the intensity of staining remained considerably less in allografts from wild-type recipients than that observed in the allografts from CCR5-deficient recipients.

Serum from CCR5−/− cardiac allograft recipients mediates allograft rejection
The ability of the donor-specific Ab produced in CCR5−/− heart graft recipients to mediate rejection of the allografts was directly tested using a serum transfer approach. Sera were collected from CCR5−/− and wild-type heart allograft recipients on day 8 posttransplant, and pooled aliquots were transferred i.v. to RAG-1−/− mice that had received A/J cardiac graft 2 days before the serum transfer (Fig. 3). All allografts survived for >100 days in RAG-1−/− recipients that received the wild-type immune serum. In contrast, transfer of the CCR5−/− immune serum mediated rejection of all A/J hearts in the RAG-1−/− recipients within 12 days. The donor-specific Ab titer in the pooled CCR5−/− recipient sera was 15-fold higher than the wild-type recipient sera. To test whether differences in titer of the immune sera accounted for the allograft rejection, a 1/15 dilution of the CCR5−/− immune sera was transferred to RAG-1−/− recipients of A/J heart allografts. This dilution abrogated the ability of the sera to reject the allografts.

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FIGURE 5. Priming of donor-reactive CD8+ and CD4+ T cells from C57BL/6 and B6.CCR5−/− allograft recipients. Negatively isolated CD8+ T cells (a) and CD4+ T cell suspensions (b and c) were prepared from naïve C57BL/6 mice and from wild type (WT) C57BL/6 and B6.CCR5−/− cardiac allograft recipients, and analyzed by ELISPOT assay to enumerate donor-reactive cells producing IFN-γ (a and b) and IL-4 (c). The results are representative of three individual experiments. *, p < 0.05.

FIGURE 6. CD4-independent rejection of cardiac allografts in wild-type and CCR5−/− recipients. Groups of five wild-type C57BL/6 and B6.CCR5−/− mice were treated with 400 μg/day rat IgG or anti-CD4 mAb, and received heterotopic heart transplants from A/J donors. Grafts were monitored daily by palpation, and rejection was confirmed visually by laparotomy.

FIGURE 7. Mononuclear cell infiltration into rejected cardiac allografts in anti-CD4 mAb-treated wild-type and CCR5−/− recipients. At the time of rejection, grafts were retrieved from wild-type (a, c, and e) and CCR5−/− (b, d, and f) recipients treated with depleting CD4 mAb. Sections were stained with H&E (a and b), anti-CD8 mAb (c and d), or CD4 mAb (e and f). Infiltration into rejected allografts from nontreated wild-type and CCR5−/− recipients is shown in Fig. 10. Original magnification, ×200.
spleens of wild-type and CCR5−/− recipients of A/J allografts was tested by ELISPOT assay on the day that the donor-specific Ab titers began to increase, day 6 posttransplant (Fig. 5). The numbers of donor-reactive CD8 T cells producing IFN-γ were significantly decreased in the spleens of the CCR5−/− vs wild-type recipients. In contrast, the numbers of CD4 T cells producing IFN-γ were similar in the spleens of both groups of recipients. Furthermore, there was a marked increase in the number of donor-reactive CD4 T cells producing IL-4 in the spleens of the CCR5-deficient allograft recipients, whereas the numbers of these CD4 T cells were barely above naive levels in wild-type recipients.

Because the increased CD4 T cell IL-4-producing response was consistent with the increased production of donor-specific Ab observed in the CCR5−/− recipients, the rejection of the allografts in wild-type and CCR5−/− recipients treated with CD4 T cell-depleting Ab was then tested. Groups of recipients were treated with control rat IgG or anti-CD4 mAb on 3 consecutive days before the transplant, as well as every 4 days after the transplant. The presence of CD4 T cells in the peripheral blood was monitored at several time points after transplantation, and CD4 T cells were completely absent in the blood of recipients treated with anti-CD4 mAb. Depletion of CD4 T cells in wild-type recipients extended allograft survival from day 7–8 to day 13–25 with a mean graft survival time of 20.0 ± 1.8 days (Fig. 6). In CCR5−/− recipients, CD4 T cell depletion extended the survival of 75% of the allografts from day 10–11 to day 30–51 (mean 33.3 ± 7.7 days). At the time of rejection, allografts in CD4 T cell-depleted wild-type and CCR5−/− recipients were heavily infiltrated with CD8 T cells, and the presence of CD4 T cells was not observed (Fig. 7). Consistent with the depletion of CD4 T cells, rejected allografts from CD4 T cell-depleted CCR5−/− recipients did not have detectable deposition of C3d, whereas rejected allografts from control Ab-treated CCR5−/− recipients exhibited intense C3d deposition (Fig. 8).

FIGURE 8. Absence of C3d deposition in rejected cardiac allografts from CD4-depleted wild-type C57BL/6 and CCR5−/− recipients. At the time of rejection, grafts were retrieved from B6.CCR5−/− recipients treated with control IgG (a) or anti-CD4 mAb (b) and from wild-type recipients (c) treated with anti-CD4 mAb. Prepared frozen sections were stained with FITC anti-C3d Ab and viewed by immunofluorescent microscopy to detect deposition of C3d. Original magnification, ×200.

Absence of Ab production and CCR5 delays cardiac allograft rejection

As a final approach to investigating the role of donor-specific Ab in the rejection of cardiac allografts in CCR5−/− recipients, the CCR5−/− mice were crossed with B cell-deficient μMT−/− mice to generate μMT−/−/CCR5−/− mice. The rejection of heart allografts was then compared in μMT−/− and μMT−/−/CCR5−/− recipients. Similar to the rejection times of A/J heart allografts in wild-type C57BL/6 and CCR5−/− recipients, rejection of the heart allografts in μMT−/− mice occurred between days 7 and 10 (Fig. 9). Survival in the μMT−/−/CCR5−/− recipients was extended, with 60% of the grafts surviving to day 14–60 (mean graft survival 21 ± 6.8 days). Rejected allografts from wild-type recipients had the characteristic intense perivascular accumulation of mononuclear cells, and this accumulation was markedly decreased in...
rejected allografts from the CCR5"/" and μMT"/"/CCR5"/" recipients (Fig. 10). In particular, immunohistochemical analyses indicated little obvious difference in CD4+ T cell infiltration into rejecting allografts from the three different recipients. However, rejecting allografts from wild-type recipients exhibited intense perivascular CD8+ T cell infiltration as well as throughout the graft parenchymal tissue, and this infiltration was clearly decreased in rejected allografts from both CCR5"/" and μMT"/"/CCR5"/" recipients.

As a potential cause for the delayed allograft rejection in the μMT"/"/CCR5"/" recipients, alloreactive T cell priming in the spleens of wild-type, CCR5"/", and μMT"/"/CCR5"/" recipients was compared on day 8 posttransplant by enumerating donor-specific T cells producing IFN-γ in ELISPOT assays. As previously observed, the number of donor-reactive T cells producing IFN-γ in CCR5"/" recipients was almost one-third that observed in wild-type recipients (Fig. 11). In the μMT"/"/CCR5"/" recipients, these numbers were even lower on day 8 posttransplant, but increased by day 12 to the levels observed in the CCR5"/" recipients at day 8 posttransplant.

![Figure 10](http://www.jimmunol.org/)

**FIGURE 10.** Mononuclear cell infiltration into rejected cardiac allografts from C57BL/6, B6.CCR5"/", and B6.μMT"/"/CCR5"/" recipients. Groups of wild-type C57BL/6 (a, d, and g), B6.CCR5"/" (b, e, and h), and B6.μMT"/"/CCR5"/" (c, f, and i) mice received heterotopic heart transplants from A/J donors. At the time of rejection, grafts were retrieved and sections were prepared and stained with H&E (a–c), anti-CD4 mAb (d–f), or CD8 mAb (g–i). Original magnification, ×200.

**FIGURE 11.** Priming of donor-reactive T cells from C57BL/6, B6.CCR5"/", and B6.μMT"/"/CCR5"/" cardiac allograft recipients. ELISPOT assays were performed on days 8 and 12 posttransplant to enumerate donor-reactive IFN-γ-producing T cells in the spleens of wild-type, B6.CCR5"/", and B6.μMT"/"/CCR5"/" (dKO) cardiac allograft recipients. Similar results were obtained in two individual experiments performed.

### Discussion

Acute cellular rejection of heart allografts in rodent models and in clinical transplantation is characterized by intense CD8 T cell graft infiltration (23, 24). At the time allografts are rejected, the recipients typically produce low levels of donor-specific Abs, which is indicated by dim staining of the complement split product C3d in the large vessels of the graft. Recent studies from this laboratory indicated marked decreases in donor-reactive T cell priming and T cell and macrophage graft infiltration in CCR5"/" recipients of MHC-mismatched cardiac allografts when compared with rejecting allografts in wild-type recipients (20). Despite these differences, wild-type and CCR5"/" recipients reject the allografts at similar times. Rejected allografts from CCR5-deficient recipients, however, had intense deposition of C3d in the large vessels and parenchymal capillaries that was accompanied by serum titers of donor-reactive Abs that were 15-fold higher or more than those induced in wild-type recipients. These results suggested that the rejection of MHC-mismatched cardiac allografts in CCR5"/" recipients might be mediated by donor-specific Ab.

The production of donor-reactive Ab was evident in the serum of CCR5"/" recipients as early as day 6 posttransplant and increased with time posttransplant to much higher titers than observed in wild-type recipients. Importantly, the increase in Ab titer with time posttransplant correlated with the intensity of C3d deposition in the allografts. Passive transfer of serum from CCR5-deficient allograft recipients provoked rejection of donor heart allografts in RAG-1"/" recipients within 14 days of Ab transfer. Two studies have reported the ability of transferred donor-reactive IgG2a mAb or serum Abs to mediate rejection of heart allografts in B cell-deficient μMT"/" mice (25, 26). The contribution of the alloreactive T cell response to graft rejection in these models was not directly tested, but is likely to have played a critical role because graft rejection was provoked in one of the studies by transfer of immune serum from wild-type recipients with rejected allografts (25). In the current study, transfer of immune sera from wild-type recipients or dilution of the CCR5-deficient recipient serum failed to induce rejection of the allografts in RAG-1"/" mice. Collectively, these experiments directly demonstrate that the amount of donor-reactive Ab produced in CCR5"/" cardiac rejection.
allo graft recipients is sufficient to mediate graft rejection, and that this rejection is dependent on the high titers of the Ab.

In the absence of recipient T cells, rejection mediated by the transferred Ab was associated with intense perivascular infiltration of neutrophils and macrophages as well as throughout the graft parenchyma. Such margination of neutrophils and macrophages in allografts is a diagnostic characteristic of acute humoral rejection (2, 14, 27, 28). In vitro studies have indicated the ability of endothelial reactive Abs to stimulate production of neutrophil and macrophage chemoattractants, including the chemokines CXCL1/KC and CCL2/MCP-1 (1, 29). Consistent with these studies, heart allograft rejection in RAG-1−/− recipients provoked by transfer of the immune serum from CCR5−/− recipients is associated with high levels of CXCL1/KC in the graft and in the serum (T. Nozaki, unpublished results). CXCL1 binding to CXCR2 on tissue-infiltrating neutrophils induces degranulation and the release of a myriad of proteases and oxidants that mediate graft endothelium and parenchymal tissue damage (30, 31). As has been shown in many studies, Ab-mediated activation of complement and formation of the membrane attack complex is also a major cause of graft endothelium damage (28, 32–34).

The role of donor-reactive Ab in allograft rejection in CCR5−/− recipients was also investigated by generating recipients deficient in both B cells and CCR5. Cardiac allografts in these recipients had slightly longer times of survival than was observed in wild-type, CCR5−/−, or μMT−/− recipients, but most of the grafts were rejected by μMT−/−/CCR5−/− recipients within 20 days. These results suggest that other mechanisms compensate for the absence of Ab production to mediate rejection of the allografts in CCR5−/− recipients. In contrast to cardiac allografts in wild-type recipients, however, rejected allografts in the μMT−/−/CCR5−/− recipients had substantial decreases in CD8 T cell infiltration. We attempted to test the role of CD8 T cells in the rejection of cardiac allografts in the μMT−/−/CCR5−/− recipients by depleting the recipients of CD8 T cells. This strategy, however, completely abrogated rejection of the grafts in μMT−/−/CCR5−/− and μMT−/− recipients (T. Nozaki, unpublished results). Studies from several laboratories have indicated the need for a threshold frequency of donor-reactive effector T cells to reject allografts (35, 36). The rejection of allografts in the μMT−/−/CCR5−/− recipients suggests that the low number of CD8 T cells primed in CCR5-deficient recipients eventually reaches the necessary threshold to reject the grafts. In support of this, depletion of CD4 T cells in CCR5−/− and wild-type recipients extended survival, but did not prevent rejection. The observed rejection of cardiac allografts in both wild-type C57BL/6 and B6.CCR5−/− mice treated with depleting anti-CD4 mAb is a different result than reported by several investigators (37–39). However, the amounts and/or frequency of mAb administered to graft recipients in these previous studies are not the same as those used in the current studies. It is also worth noting that there was no evidence of allograft-infiltrating CD4 T cells or the production of donor-specific Ab in either the wild-type or CCR5-deficient recipients consistent with the rejection of the allografts in the absence of CD4 T cells.

Our previous studies had indicated marked decreases in the number of donor-reactive T cells producing IFN-γ in CCR5−/− recipients of cardiac allografts. In the current studies, this decrease is shown to be restricted to the CD8, and not the CD4, T cell compartment. Priming of these CD8 T cells was further decreased in the μMT−/−/CCR5−/− recipients, but reached levels observed in the CCR5−/− recipients with time posttransplant. These results indicate the requirement for CCR5 expression to achieve optimal priming levels of donor-reactive CD8 T cells. It is unlikely that this requirement for CCR5 expression is mediated through delivery of helper signals to the alloreactive CD8 T cells during priming as CD4-mediated help for the Ab response was delivered in the CCR5-deficient recipients. Because CD8 T cells up-regulate expression of CCR5 during priming to cardiac and skin allografts (20), these receptors may function to provide additional signals to the CD8 T cells during priming to donor Ags.

In contrast to the decreased priming of alloreactive CD8 T cells, the levels of donor-reactive CD4 T cells producing IFN-γ were similar in wild-type and CCR5-deficient recipients of cardiac allografts, and donor-reactive CD4 T cells producing IL-4 were markedly increased in CCR5−/− recipients. Similar increases in donor-reactive T cells producing IL-4 in CCR5−/− recipients of islet allografts have been reported, although donor-reactive Ab production was not tested during this response (40). The increased development of IL-4-producing CD4 T cells in CCR5−/− recipients is in line with the increased donor-specific Ab produced and as an underlying mechanism promoting this increased Ab response. CCR5 expression has been noted on B cells in 3-mo-old NOD, but not BALB/c, mice (41). We have not observed CCR5 expression on splenic B cells in wild-type allograft recipients, suggesting that the increased Ab responses induced in CCR5-deficient allo graft recipients are not mediated by the absence of negative signaling through CCR5 on B cells. The production of donor-specific Ab was completely inhibited by treatment with anti-CD154 mAb, indicating that the high titers of donor-specific Ab produced required the delivery of CD4 T cell-mediated help through CD154. Disruption of this help by peritransplant treatment with anti-CD154 mAb also resulted in a substantial extension of graft survival (i.e., from day 8–10 to day 15–30) in both wild-type and CCR5−/− recipients (T. Nozaki, unpublished results).

Overall, the results of the current studies indicate that the absence of CCR5 in cardiac allograft recipients has opposite effects on alloreactive CD4 and CD8 T cell responses. The CD8 T cell response in CCR5−/− allograft recipients is markedly diminished, suggesting that expression of CCR5 may be required to achieve optimal levels of priming. The priming of alloreactive CD4 T cells to IFN-γ-producing cells is not affected by the absence of CCR5, and development to IL-4-producing cells is enhanced. This suggests the absence of a component regulating the donor-reactive CD4 T cell response in CCR5−/− recipients. During initial studies, we have observed similar numbers of CD4+CD25+FoxP3+ T regulatory (Treg) cells in the spleens of wild-type C57BL/6 and B6.CCR5−/− mice. However, several studies have indicated the activation-induced expression of CCR5 on a small component (~15%) of the CD4+CD25+ Treg compartment and the absence of Treg-mediated regulation of effector T cell responses in acute graft-vs-host and parasite infections in CCR5−/− mice (42, 43). The results of the current study suggest these cells as potential regulators of alloreactive CD4 T cell responses, and that the absence of the CCR5+ cells results in unregulated helper signals that underline the high levels of donor-specific Ab induced. Studies to further investigate the CCR5-mediated regulation of alloreactive CD4 and CD8 T cell responses are in progress.

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Disclosures
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References


