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CCR2 Regulates Monocyte Recruitment As Well As CD4⁺ T_H1 Allorecognition After Lung Transplantation

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Abstract

Graft rejection remains a formidable problem contributing to poor outcomes after lung transplantation. Blocking chemokine pathways have yielded promising results in some organ transplant systems. Previous clinical studies have demonstrated upregulation of CCR2 ligands following lung transplantation. Moreover, lung injury is attenuated in CCR2-deficient mice in several inflammatory models. In this study, we examined the role of CCR2 in monocyte recruitment and alloimmune responses in a mouse model of vascularized orthotopic lung transplantation. The CCR2 ligand MCP-1 is upregulated in serum and allografts following lung transplantation. CCR2 is critical for the mobilization of monocytes from the bone marrow into the bloodstream and for the accumulation of CD11c⁺ cells within lung allografts. A portion of graft-infiltrating recipient CD11c⁺ cells expresses both recipient and donor MHC molecules. Two-photon imaging demonstrates that recipient CD11c⁺ cells are associated with recipient T cells within the graft. While recipient CCR2 deficiency does not prevent acute lung rejection and is associated with increased graft infiltration by T cells, it significantly reduces CD4⁺ T_H1 indirect and direct allorecognition. Thus, CCR2 may be a potential target to attenuate alloimmune responses after lung transplantation.

Keywords

Allorecognition; CD4⁺ T cells; chemokine receptors; lung transplantation; monocytes

Introduction

Recruitment of host leukocytes, a critical step in allograft rejection, is regulated by chemokines and their receptors (1). Chemokine production following transplantation occurs in two stages and the early antigen-independent chemokine cascade, regulated by surgical trauma, donor brain death and ischemia-reperfusion injury leads to recruitment of neutrophils and macrophages (2-4). The later antigen-dependent cascade directs trafficking of activated T cells into the grafts. The potential of targeting chemokine receptors in

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ameliorating rejection has been studied in several experimental transplantation models, but varies by the grafted organ (1,5-10).

CC chemokine receptor 2 (CCR2) is expressed on many hematopoietic cells and its main ligand is monocyte chemoattractant protein-1 (MCP-1) (11-15). In various pulmonary inflammation models, interactions between CCR2 and its ligands contribute to the development of lung injury (16-18). Elevated serum levels of CCR2 ligands in the early postoperative period have been associated with a higher risk of rejection in human lung recipients and heterotopic tracheal transplant experiments have suggested that CCR2 inhibition could reduce graft injury following lung transplantation (3,19). However, the role of CCR2 in vascularized lung transplantation remains unexplored.

Here, we report that CCR2 is important for the mobilization of monocytes from the bone marrow into the periphery, but not for the recruitment of monocytes from the blood into lung grafts. Recipient CCR2 deficiency is associated with a marked reduction of CD11c⁺ antigen presenting cells (APCs) within lung allografts. Graft-infiltrating recipient CD11c⁺ APCs can express both donor and recipient MHC molecules and we demonstrate by two-photon imaging that recipient T cells are associated with recipient CD11c⁺ APCs within lung allografts. While recipient CCR2-deficiency does not prevent acute lung rejection, it significantly attenuates CD4⁺ T_H1 direct and indirect allorecognition.

Materials and Methods

Animals

Male C57BL/6 (B6) (H-2K^b), Balb/c (H-2K^d), B6 CD45.1⁺ and B6 CCR2-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). B6 mice expressing enhanced yellow fluorescent protein driven by a CD11c promoter (CD11c-EYFP⁺) were a gift from M. Nussenzweig (Rockefeller University, NY). Left lung transplants were performed as previously described (20-22). Briefly, cuffs are placed on the donor bronchus (20 G), pulmonary artery (24 G) and vein (20 G). The cuffed donor bronchus and vessels are inserted into the respective recipient structures and secured with 10-0 ligatures.

Flow cytometry

Lungs, spleen and lymph nodes were prepared for flow cytometry as previously described (20,23). Blood, collected by cardiac puncture and bone marrow, isolated from femurs, were depleted of red cells with ACK lysing buffer. Cells were preincubated with anti-CD16/32 (clone 93) for 15 minutes and then stained with fluorochrome-labeled anti-Ly-6C (AL-21), anti-CD11b (M1/70), anti-CD11c (HL3), anti-I-A^b (AF6-120.1), anti-I-A^d (39-10-8), anti-CD45 (30.F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD90.2 (30-H12), anti-CD4 (RM4-5), anti-CD8 (53-6.7) antibodies and isotype controls (BD Biosciences, San Jose, CA, USA; eBioscience, San Diego, CA).

Adoptive transfer of monocytes

Bone marrow from wild-type or CCR2-deficient B6 mice was depleted of MHC class II⁺(I-A^b) (AF6-120.1) and CD11c⁺ (HL3) cells and selected for monocytes with antibodies to Gr-1 (Ly6C/G) (RB6-8C5) and CD115 (AFS98) (Miltenyi Biotec, Auburn, CA, USA) (24). B6 CD45.1⁺ recipients of Balb/c lungs received 1×10^7 carboxyfluorescein succinimidyl ester (CFSE)-labeled (Molecular Probes, Eugene, OR) CD45.2⁺Gr-1⁺CD115⁺ monocytes isolated from wild-type or CCR2-deficient B6 mice intravenously at the time of transplantation. Recruitment of CFSE-labeled CD45.2⁺ monocytes was analyzed 4 days later.

ELISA

Frozen lung specimens were homogenized and centrifuged at 10,000 rpm for 5 minutes. Lysates and serum samples were normalized for protein concentration with a colorimetric BCA Protein Assay kit (Pierce, Rockford, IL, USA). MCP-1 (BD Biosciences, San Diego, CA) serum and tissue levels and IL-12p40 (eBioscience) tissue levels were read at 450 nm on an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Histology

Grading for rejection was performed in a blinded fashion by a pathologist (25).

Immunofluorescent staining

Lungs were prepared for immunofluorescent staining as previously described (21). Unlabeled anti-CD4 (H129.19), anti-CD8 (53–6.7) (BD Biosciences) and anti-CD68 (FA-11) (AbD Serotec, Raleigh, NC) antibodies were visualized using donkey anti-rat IgG conjugated with indocarbocyanine (Roche, Indianapolis, IN). No detectable staining was observed with isotype-matched or species-specific control antibodies.

Two-photon imaging

Four days after Balb/c → B6 CD11c-EYFP⁺ lung transplantation, we injected 10⁷ CMTMR-labeled (Invitrogen, Carlsbad, CA) B6 CD90⁺ T cells into the recipients and imaged the grafts with a two-photon microscope 30 h later (23).

Recall assay

CD45.1⁺ CD11c⁺ cells from Balb/c → CD45.1⁺ B6 allografts were sorted flow cytometrically 7 days after transplantation. A total of 10⁶ CD45.1⁺ CD11c⁺ cells were injected into naïve B6 or Balb/c mice. Seven days later splenic CD4⁺ T cells were positively isolated with magnetic beads from these sensitized hosts as well as control naïve B6 and Balb/c mice and stimulated with T cell depleted B6 or Balb/c splenocytes at a T cell:splenocyte ratio of 1:3 for 48 h. Cultures were then stimulated with 20 ng/mL of PMA, 1 μM ionomycin and 2 μM Golgi Plug (BD Pharmingen) for 4 h and stained with antibodies specific for CD90.2 (30-H12) CD4 (RM4–5), IFN-γ (XMG1.2) and isotype controls.

ELISPOT

CD4⁺ T cells, purified from spleens of untransplanted wild-type B6 or CCR2-deficient B6 mice or from Balb/c lungs 7 days after transplantation into wild-type B6 or CCR2-deficient B6 mice were cultured with irradiated Balb/c stimulators or B6 stimulators that had been pulsed for 24 h with Balb/c allopeptide derived from equivalent numbers of freeze-thawed splenocytes in plates coated with capture mouse IFN-γ (BD Biosciences) or IL-17-specific antibodies (eBioscience). T cells were cultured with stimulators at 1:3 ratios. After 40 h, cells were incubated with biotinylated IFN-γ- or IL-17-specific antibody.

Statistical analysis

Significant differences ($p < 0.05$) were calculated using the Student's *t*-test.

Results

MCP-1 production is increased after lung transplantation

We first analyzed MCP-1 levels in the recipient serum at various time points after syngeneic (B6 → B6) and allogeneic (Balb/c → B6) lung transplantation (Figure 1A). Serum levels of MCP-1 were increased in syngeneic and allogeneic graft recipients as early as 24 h

following transplantation and were comparable up to 3 days. However, MCP-1 serum levels in allogeneic recipients were significantly higher than in syngeneic recipients at 5 days following transplantation. At all time points examined, the serum levels of MCP-1 were significantly higher in CCR2-deficient B6 recipients of Balb/c allografts compared to B6 wild-type recipients of either Balb/c or B6 grafts.

Analysis of lung graft tissue expression of MCP-1 at 7 days after transplantation demonstrated significantly higher levels in allogeneic when compared to syngeneic grafts (Figure 1B). The expression of MCP-1 was significantly higher in allografts that had been transplanted into CCR2-deficient B6 mice when compared to allografts that had been transplanted into wild-type B6 mice.

Patterns of graft-infiltrating APCs are regulated by CCR2

Having shown that MCP-1 was upregulated after syngeneic and allogeneic transplantation, we next asked whether CCR2 controlled lung recruitment of bone marrow derived APCs. We analyzed lung grafts 7 days after transplantation into either wild-type or CCR2-deficient recipients. In comparison to naïve untransplanted wild type mice CD11c⁺ cells were not significantly increased in syngeneic lung grafts (Figure 2A). There were significantly more CD11c⁺ cells in allogeneic compared to syngeneic grafts. We observed a significant reduction in lung allograft-infiltrating CD11c⁺ cells in CCR2-deficient compared to wild-type recipients.

We and others have shown that two populations of CD11c⁺ APCs in the lung can be distinguished by the expression of CD11b (20,26). CD11c⁺CD11b^{hi} cells have been characterized as dendritic cells whereas CD11c⁺CD11b^{lo} cells have been reported to be macrophages (26,27). Unlike the case for syngeneic grafts, the majority of CD11c⁺ cells expressed high levels of CD11b in wild-type recipients of allogeneic grafts (Figure 2B). A significantly lower percentage of lung allograft-infiltrating CD11c⁺ cells expressed CD11b in CCR2-deficient recipients when compared to wild-type recipients of lung allografts.

We also compared levels of IL-12p40, which is known to be produced by dendritic cells and macrophages under inflammatory conditions (28). In comparison to B6 wild-type recipients that received syngeneic lung grafts, IL-12p40 levels were significantly higher in allogeneic lung grafts (Figure 2C). Notably, allogeneic lung grafts expressed significantly lower levels of IL-12p40 in CCR2-deficient recipients than in wild-type recipients. Thus, CCR2 regulates the patterns of CD11c⁺ APC accumulation and expression of IL-12p40 in lung allografts.

CCR2 is critical for the mobilization of monocytes from bone marrow after lung transplantation

As macrophages and dendritic cells differentiate from monocytes, we next analyzed circulating monocytes in the peripheral blood of lung recipients. We defined monocytes as CD11b⁺ Ly6C^{hi} cells that lack expression of Ly6G and CD11c (29-31). Seven days after transplantation, more CD11b⁺ Ly6C^{hi} monocytes accumulated in the peripheral blood of wild-type B6 recipients of allografts compared to B6 recipients of syngeneic grafts (Figure 3). The percentage of CD11b⁺ Ly6C^{hi} monocytes in the blood of CCR2-deficient allograft recipients was significantly lower than that of wild-type recipients of either syngeneic or allogeneic lungs. We also observed significant reductions in CD11b⁺ Ly6C^{hi} monocytes within the spleen and mediastinal lymph nodes of CCR2-deficient allograft recipients compared to wild-type recipients of syngeneic or allogeneic grafts. We next analyzed the monocyte population in the bone marrow. Compared to wild-type recipients of either syngeneic or allogeneic grafts, the bone marrow of CCR2-deficient allograft recipients was

enriched for CD11b⁺ Ly6C^{hi} monocytes. Thus, CCR2 controls the egress of CD11b⁺ Ly6C^{hi} monocytes from the bone marrow into the periphery following lung transplantation.

Recruitment of monocytes from peripheral blood to lung grafts is not regulated by CCR2

Attenuation of graft-infiltrating APC accumulation in CCR2-deficient recipients raised the possibility that CCR2 could control monocyte chemotaxis from the peripheral blood into lung grafts. To address this issue, we performed adoptive transfer studies with CD115⁺ Gr1⁺ bone marrow-derived monocytes. At the time of transplantation with Balb/c lungs recipient CD45.1⁺ B6 mice received either wild-type CD45.2⁺ B6 or CCR2-deficient B6 (CD45.2⁺) CFSE-labeled bone marrow monocytes intravenously. Four days after transplantation, we analyzed transplanted lungs, native lungs and the spleen. We observed comparable recruitment of adoptively transferred CFSE⁺ CD45.2⁺ cells into lung allografts irrespective of CCR2 expression indicating no defect in chemotaxis from the peripheral blood to inflammatory sites (Figure 4A, B). The vast majority of both wild-type and CCR2-deficient monocytes differentiated into CD11c⁺ cells (Figure 4A). Only very few adoptively transferred CFSE-labeled CD45.2⁺ cells were detected in the native right lungs or the spleen indicating their selective recruitment to inflammatory sites (data not shown). Thus, the recruitment of monocytes from the blood to lung allografts and their differentiation toward CD11c⁺ cells does not require CCR2.

Recipient CCR2 deficiency does not prevent acute lung rejection

Syngeneic B6 lung transplants have no sign of inflammation 7 days after transplantation (Figure 5A). Syngeneic grafts remain ventilated and virtually free of inflammation as far as 6 months after engraftment (data not shown). On the other hand, Balb/c lungs transplanted into nonimmunosuppressed B6 hosts are acutely rejected 7 days after engraftment. Having shown that CCR2 controls bone marrow egress of monocytes and the accumulation of CD11c⁺ cells in allografts, we next assessed whether Balb/c lungs were acutely rejected when transplanted into CCR2-deficient recipients. Similar to the case for wild-type B6 recipients, Balb/c lungs transplanted into CCR2-deficient B6 recipients demonstrated acute rejection 7 days after transplantation. Rejection grades of Balb/c lungs did not differ after transplantation into wild-type or CCR2-deficient recipients (Figure 5B). To assess T-cell infiltration, we performed immunostaining for CD4⁺ and CD8⁺ T cells in syngeneic and allogeneic grafts (Figure 5A). Irrespective of CCR2 expression in the recipients, immunostaining demonstrated that allografts were infiltrated by CD4⁺ and CD8⁺ T cells to a larger extent than syngeneic grafts and that CD8⁺ T cells predominated over CD4⁺ T cells in both wild-type and CCR2-deficient lung allograft recipients (Figure 5A). We next quantified T cells within allografts by flow cytometry and observed significantly more CD4⁺ and CD8⁺ T lymphocytes after transplantation into CCR2-deficient hosts (Figure 5C). We also stained grafts for CD68, a heavily glycosylated transmembrane protein that is specifically expressed by tissue macrophages (32) (Figure 5A). Compared to syngeneic grafts, we observed more CD68⁺ cells in allografts that had been transplanted into wild-type or CCR2-deficient recipients. Thus, recipient CCR2 deficiency is associated with increased infiltration of T cells into allografts and does not prevent acute lung rejection.

Indirect and direct CD4⁺ T_H1 alloresponses are significantly reduced in CCR2-deficient lung recipients

We have recently reported that T cells can be primed within lung allografts, which is associated with their clustering around donor CD11c⁺ cells (23). Having demonstrated that recipient CD11c⁺ cells accumulate in lung allografts, we wanted to determine whether these cells also interact with recipient T cells within the allograft. Four days after transplantation of Balb/c lungs into B6 CD11c-EYFP⁺ mice, we injected CMTMR-labeled B6 T cells into recipients and analyzed the grafts by two-photon imaging 30 h later (Figure 6). We observed

that many recipient T cells were associated with recipient CD11c⁺ cells. Based on the markedly reduced accumulation of CD11c⁺ cells in CCR2-deficient recipients, we next analyzed IFN- γ recall responses by graft-infiltrating CD4⁺ T cells after indirect allostimulation, a pathway triggered through recipient APCs processing alloantigen. The frequency of CD4⁺ T cells isolated from Balb/c lungs 7 days after transplantation into wild-type B6 hosts producing IFN- γ after indirect stimulation was significantly higher than that of CD4⁺ T cells isolated from Balb/c lungs after transplantation into CCR2-deficient B6 recipients (Figure 7).

We further analyzed the phenotype of graft-infiltrating recipient CD11c⁺ cells. We transplanted Balb/c lungs into CD45.1⁺ B6 recipients and examined the graft-infiltrating CD45.1⁺ CD11c⁺ cells 7 days later. In addition to expressing high levels of recipient (I-A^b) MHC class II, a notable fraction of these cells also carried donor (I-A^d) MHC class II molecules on their surface (Figure 8A). We observed no significant staining for donor MHC class II molecules on CD45.1⁺ CD11c⁺ cells within the spleen or native right lung. The phenotype of these recipient graft-infiltrating CD11c⁺ cells with expression of donor MHC class II molecules raised the possibility that they could contribute to CD4⁺ T-cell activation through the direct allorecognition pathway. To test this, we isolated CD45.1⁺ CD11c⁺ cells from lung allografts of Balb/c $\rightarrow$ CD45.1⁺ B6 transplants 7 days after engraftment. We injected these cells into either naïve B6 or Balb/c mice. Seven days later, we tested their recall responses to stimulation with T cell depleted Balb/c (I-A^d) or B6 splenocytes (I-A^b), respectively. These graft-infiltrating recipient CD11c⁺ cells elicited comparable and enhanced IFN- γ responses by CD4⁺ T cells to I-A^d- or I-A^b-bearing splenocytes suggesting that recipient CD11c⁺ cells play a role in CD4⁺ T-cell activation via direct allorecognition (Figure 8B).

Based on these findings, we hypothesized that—in addition to nearly abolishing indirect CD4⁺ allorecognition—recipient CCR2 deficiency could also regulate direct alloimmune responses. We observed that the frequency of allograft-infiltrating CD4⁺ T cells that produce IFN- γ after direct stimulation with Balb/c splenocytes was significantly higher after transplantation into wild-type B6 recipients compared to CCR2-deficient B6 recipients (Figure 9A). There were no significant differences in IFN- γ production between CD4⁺ T cells isolated from naïve wild-type or CCR2-deficient B6 mice after stimulation with Balb/c splenocytes (Figure 9A). Only very few CD4⁺ T cells isolated from wild-type or CCR2-deficient lung recipients produced IL-17 after stimulation with Balb/c splenocytes (Figure 9B).

Discussion

Rejection rates of lungs are higher than those of other organs and it is thus imperative to define cellular and molecular pathways that regulate lung rejection (33,34). Here, we observed that CCR2 regulates the infiltration of CD11c⁺ APCs into vascularized lung grafts and plays a previously unrecognized role in mediating direct alloimmune responses.

In the lung, MCP-1 can be produced by both stromal cells and hematopoietic cells (35,36). In humans, MCP-1 levels are elevated in the bronchoalveolar fluid during acute lung rejection and clinical improvement of acute rejection is associated with decreases in MCP-1 (3,37). Consistent with previous observations that MCP-1 can be upregulated following alloantigen-independent injury, we found that serum MCP-1 levels were elevated in recipients of both syngeneic and allogeneic grafts as early as 24 h after transplantation (Figure 1A) (4,38-41). However, MCP-1 levels were significantly higher in acutely rejected allografts than in syngeneic grafts (Figure 1B). We observed significantly higher serum and graft levels of MCP-1 protein in CCR2-deficient recipients when compared to wild-type

recipients of either syngeneic or allogeneic grafts (Figure 1A, B). Elevated levels of MCP-1 in CCR2-deficient mice have been observed in pulmonary infection models (42,43). A recent study suggested that recruitment of CCR2-expressing monocytes to inflamed lungs leads to consumption of local MCP-1 providing a putative mechanism for the increased serum and tissue MCP-1 levels in our CCR2-deficient lung allograft recipients (44).

It has recently been demonstrated in infectious and hypercholesterolemia models that CCR2 is critical for the egress of monocytes from the bone marrow into the bloodstream (11,29). We observed a significant increase in the bone marrow and a marked reduction in the peripheral blood CD11b⁺ Ly6C^{hi} monocyte population in CCR2-deficient recipients indicating that CCR2 is important for monocyte egress from the bone marrow after lung transplantation (Figure 3). While inflammatory stimuli such as sepsis or burns have been shown to be associated with bone marrow monocytopoiesis, to our knowledge this is the first description of an increase in bone marrow monocytes after organ transplantation (29,45).

Seven days after transplantation, we observed an increase in CD11c⁺ cells in allografts compared to syngeneic grafts and lungs of untransplanted mice (Figure 2A). There was a marked reduction in CD11c⁺ cells in allografts in CCR2-deficient recipients. Moreover, in CCR2-deficient recipients, we observed a more pronounced reduction among the subset of CD11c⁺ cells that expressed high levels of CD11b (Figure 2B). While both lung macrophages and dendritic cells express CD11c, dendritic cells are CD11b^{hi} while macrophages are CD11b^{lo} (26,27). A recent report proposed that CCR2⁺ inflammatory monocytes initially differentiate into a cell expressing intermediate levels of CD11c and MHC class II and high levels of CD11b after entering virally infected lungs (16). Subsequently, these cells mature into either CD11c⁺ MHC class II^{hi} dendritic cells or CD11c⁺ MHC class II^{lo} exudate macrophages. Similar to our observations, the CD11b^{hi} subset of lung-infiltrating monocyte-derived APCs was most profoundly decreased in CCR2-deficient animals. These findings could at least in part be explained by previous observations that CCR2 regulates dendritic cell maturation (46). Alternatively, reduced local inflammatory cytokine levels resulting from attenuated T-cell activation in CCR2-deficient recipients could account for relative reductions in CD11b expression on graft-infiltrating APCs.

While the reduction in graft-infiltrating CD11c⁺ cells in CCR2-deficient animals could have been solely due to deficient mobilization of monocytes from the bone marrow, there also existed the possibility that CCR2 is important to recruit monocytes from the blood into allografts. Whether CCR2 is critical for the recruitment of monocytes from the blood to inflammatory sites has been controversial. Following intravenous infection with *Listeria monocytogenes*, CCR2 was not required for the migration of monocytes from the bloodstream into peripheral tissues (29). Alternatively, during peritoneal inflammation, wild-type monocytes were recruited more efficiently from the blood into inflamed sites than CCR2-deficient monocytes (11). We show that monocyte migration from the bloodstream into allografts is CCR2 independent (Figure 4). Thus, the relative paucity of blood monocytes in CCR2-deficient recipients likely results from a combination of deficient bone marrow egress and efficient recruitment of monocytes from the blood into the allograft.

Requirements for chemokines in shaping alloimmune responses differ between various grafts. While interfering with CCR2 has a comparatively minor effect on the survival of cardiac allografts, experimental and clinical studies have shown that this chemokine pathway plays an important role in pancreatic islet transplantation (10,47,48). Human lung transplant recipients undergoing acute or chronic rejection have higher levels of CCR2 ligands in their bronchoalveolar lavage fluid compared to healthy recipients and airway

obliteration is attenuated in heterotopic tracheal transplants when CCR2 signaling is abrogated (3). Lungs are comparatively rich in hematopoietic cells and therefore direct allorecognition triggered through donor APCs may play a more dominant role at early time points. We have recently demonstrated that recipient T cells associate with graft-resident donor CD11c⁺ APCs early after transplantation and that, unlike the case for other organs, T-lymphocyte activation can take place within the lung (23,49). Seven days after transplantation, only a small percentage of graft-resident hematopoietic cells express donor, but not recipient MHC class II in wild-type or CCR2-deficient recipients (Balb/c → B6 0.97% ± 0.39; Balb/c → CCR2^{-/-} 1.31% ± 0.32; p = 0.52) indicating comparable mobilization of passenger leukocytes.

CCR2-deficient mice are susceptible to infections that require a T_H1 response for resolution (12,50-52). Decreased IFN- γ production in CCR2-deficient mice is thought to be a consequence of suboptimal T-cell activation due to defects in trafficking of hematopoietic APCs (51). Lower graft levels of IL-12p40 in CCR2-deficient recipients are likely a consequence of the reduced recruitment of CD11c⁺ APCs (Figure 2C) (28). IL-12p40 can associate with IL-12p35 to form IL-12p70, which plays an important role in T_H1 differentiation. The reduction in recruitment of CD11c⁺ cells to allografts in CCR2-deficient recipients is associated with a significantly reduced frequency of graft-infiltrating CD4⁺ T cells producing IFN- γ after both indirect and direct stimulation (Figures 2A, 7 and 9A). At later time points, recipient T cells also associate with recipient CD11c⁺ APCs within the graft, a portion of which expresses both donor and recipient MHC molecules (Figures 6 and 8). This finding is consistent with previous reports that dendritic cells can acquire intact MHC molecules from other cells (53,54). Therefore, our observations suggest that the semidirect allorecognition pathway, where recipient T cells can be activated through the direct pathway after recognition of intact donor MHC molecules on recipient APCs is attenuated in CCR2-deficient recipients (53).

Despite markedly attenuated recruitment of recipient APCs, lungs were acutely rejected after transplantation into CCR2-deficient recipients (Figure 5A). Thus, donor cells are likely sufficient to activate alloreactive T cells. To this end, our previous findings that nonhematopoietic cells can prime CD8⁺ T cells and our recent observation that lungs are acutely rejected independent of CD4⁺ T cells raise the possibility that activation of CD8⁺ T cells by pulmonary stromal cells suffices to trigger lung rejection (55,56). Another important consideration is that reduced CD4⁺ direct and indirect T_H1 alloresponses in CCR2-deficient hosts could be compensated for by increased numbers of graft-infiltrating T lymphocytes (Figure 5C). This may at least in part be due to elevated local levels of CCR2 ligands such as MCP-1, which can signal independent of CCR2 and has been shown to inhibit growth factor withdrawal-mediated apoptosis of CD8⁺ T cells (57,58).

In conclusion, CCR2 plays a critical role in the recruitment of CD11c⁺ APCs to lung allografts and regulates indirect CD4⁺ T_H1 alloresponses. Moreover, graft-infiltrating recipient CD11c⁺ APCs can acquire donor MHC molecules and contribute to direct CD4⁺ T_H1 alloresponses. However, recipient CCR2 deficiency does not prevent acute lung rejection and is associated with increased graft infiltration by T cells. Therefore, in combination with other complementary strategies CCR2 may be a suitable target in attenuating alloimmune responses triggered through recipient APCs following lung transplantation.

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Figure 1. Measurement of serum and graft tissue MCP-1 levels following lung transplantation
 (A) Serum levels of MCP-1 are expressed as a mean $\pm$ S.E.M. (B6 $\rightarrow$ B6 [n = 6]; Balb/c $\rightarrow$ B6 [n = 5]; Balb/c $\rightarrow$ CCR2^{-/-} [n = 6]; *p < 0.05; **p < 0.01). In resting wild-type B6 and CCR2-deficient B6 mice serum MCP-1 levels were 72 $\pm$ 12 pg/mL and 82 $\pm$ 25 pg/mL, respectively. (B) Lung tissue MCP-1 levels in resting wild-type B6 (n = 3) and CCR2-deficient B6 mice (n = 3) as well as in lung grafts 7 days after B6 $\rightarrow$ B6 (n = 5), Balb/c $\rightarrow$ B6 (n = 5) and Balb/c $\rightarrow$ CCR2^{-/-} (n = 6) lung transplants. MCP-1 levels are expressed as a mean $\pm$ S.E.M. (*p < 0.05 Balb/c $\rightarrow$ B6 vs. B6 $\rightarrow$ B6 and **p < 0.01 Balb/c $\rightarrow$ B6 vs. Balb/c $\rightarrow$ CCR2^{-/-}).

Figure 2. Analysis of patterns of APC accumulation, phenotype and IL-12p40 levels in lung grafts following transplantation

(A) Quantification of CD11c⁺ events in the lungs of wild-type B6 mice (n = 4), CCR2^{-/-} mice (n = 4) and in the grafts of either B6 → B6 (n = 8), Balb/c → B6 (n = 7) or Balb/c → CCR2^{-/-} (n = 12) lung recipients 7 days after transplantation. Lungs were digested and stained with antibodies specific for CD11c and analyzed by FACS. CD11c⁺ events were expressed as a mean percentage ± S.E.M. (*p < 0.05 Balb/c → B6 vs. Balb/c → CCR2^{-/-}) gated on live cells by forward and sidescatter. There was no statistically significant difference between CD11c⁺ cells in wild-type B6 mice and B6 → B6 syngeneic recipients (p = 0.26). (B) B6 → B6 (n = 8), Balb/c → B6 (n = 7) or Balb/c → CCR2^{-/-} (n = 12) lung grafts were digested 7 days following transplantation and stained with antibodies specific for CD11c and CD11b. FACS analysis was conducted on live events and represented as relative mean ratio ± S.E.M. (*p < 0.05 Balb/c → B6 vs. Balb/c → CCR2^{-/-}) of CD11c⁺CD11b^{hi} events versus CD11c⁺CD11b^{lo} events. (C) Assessment of IL-12p40 levels in wild-type B6 mice (n = 3) or CCR2^{-/-} mice (n = 3) and in the grafts of either B6 → B6 (n = 5), Balb/c → B6 (n = 8) or Balb/c → CCR2^{-/-} (n = 6) lung recipients 7 days after transplantation. IL-12p40 levels are expressed as a mean ± S.E.M. (*p < 0.05 Balb/c → B6 vs. Balb/c → CCR2^{-/-}).

Figure 3. Analysis of monocyte accumulation in the bone marrow, peripheral blood and secondary lymphoid organs of lung graft recipients

Bone marrow (BM), peripheral blood (PBL), spleen (SPLN) and mediastinal lymph nodes (LN) were isolated from wild-type B6 mice (n = 5), CCR2^{-/-} mice (n = 5) and from B6 → B6 (n = 6), Balb/c → B6 (n = 5) or Balb/c → CCR2^{-/-} (n = 6) lung recipients 7 days after transplantation. Tissues were stained for CD45, CD11b and Ly6C and analyzed by FACS. Representative FACS plots for lung graft recipients (left) are depicted as percentages of CD11b⁺ Ly6C^{hi} cells per live CD45⁺ cells. Quantification of CD11b⁺ Ly6C^{hi} cells (right) for indicated tissues of resting mice or transplant recipients 7 days after engraftment are shown as a mean percentage ± S.E.M. (*p < 0.05; **p < 0.01 Balb/c → B6 vs. Balb/c → CCR2^{-/-}) of live CD45⁺ gate.

Figure 4. Recruitment of CCR2-deficient and B6 monocytes from peripheral blood to lung allografts

Wild-type B6 (CD45.2⁺) or CCR2-deficient (CD45.2⁺) magnetic bead-purified bone marrow-derived monocytes were labeled with CFSE and then injected into B6 CD45.1 recipients of Balb/c lung allografts at the time transplantation. Lung allografts were digested 96 h following transplantation and stained with antibodies specific for CD45.2 and CD11c and analyzed by FACS. (A) Representative FACS plots (left panel) are gated on live events by forward and sidescatter. Histograms (right panel) are gated on live CD45.2⁺ CFSE⁺ events and show expression of CD11c. Shaded histogram indicates isotype control staining. (B) Quantification of lung graft monocyte accumulation is represented as the mean percentage of CFSE⁺ CD45.2⁺ events $\pm$ S.E.M. (Balb/c $\rightarrow$ B6, $n = 7$ and Balb/c $\rightarrow$ CCR2^{-/-}, $n = 6$, $p = 0.42$) gated on live cells.

Figure 5. Histology, T cell and macrophage accumulation in lung grafts

(A) Sectioned graft tissue from indicated lung recipients were stained with hematoxylin and eosin (H&E), CD4⁺, CD8⁺ and CD68⁺-specific antibodies. Sections are shown at 100× magnification for H&E staining and at 200× magnification for immunostains. (B) Graft sections from indicated transplant combinations 7 days after transplantation were scored by a blinded pathologist and represented as a mean rejection score ± S.E.M. (B6 → B6 [n = 6], Balb/c → B6 (n = 7), Balb/c → CCR2-deficient B6 [n = 7] *p = 0.66 for Balb/c → B6 vs. Balb/c → CCR2^{-/-}) on a scale of A0 to A4 (25). (C) Flow cytometric analysis of absolute numbers of CD4⁺CD90⁺ and CD8⁺CD90⁺ T cells per 0.1 g tissue weight in Balb/c lungs 7 days after transplantation into wild-type (n = 10) or CCR2-deficient (n = 5) B6 recipients (**p < 0.01).

Figure 6. T-cell clustering around APCs of recipient origin in lung allografts

(A) Balb/c → B6 CD11c-EYFP⁺ graft showing adoptively transferred CMTMR-labeled B6 T cells (red), recipient CD11c-EYFP⁺ cells (green) and collagen fibers (blue; second harmonic signal). Maximum intensity projection of 21 z-steps with 2.5 μm distance between each plane representing a volume of 130 $\mu\text{m} \times 110 \mu\text{m} \times 50 \mu\text{m}$ (x,y,z). Scale bar equals 25 μm . Blue arrows identify T cells, which are in direct contact with EYFP-expressing cells. (B–D) Direct T cell—CD11c-EYFP⁺ cell contacts have been identified by examination of three z-steps crossing the EYFP⁺ cell of interest.

Figure 7. Attenuated indirect allorecognition in CCR2-deficient lung graft recipients
 IFN- γ ELISPOT analysis conducted on co-cultures containing B6 stimulators pulsed with Balb/c antigen and CD4⁺ T-cell responders isolated from grafts of Balb/c $\rightarrow$ B6 (n = 5) or Balb/c $\rightarrow$ CCR2-deficient B6 (n = 5) lung recipients 7 days following transplantation. Quantification is depicted above a representative plate as the mean number of spots $\pm$ S.E.M. (*p < 0.05) per 200,000 CD4⁺ T cells.

Figure 8. Phenotypic and functional characterization of graft-infiltrating recipient CD11c⁺ cells (A) I-A^b and I-A^d expression on live CD45.1⁺ CD11c⁺ cells in lung allograft, spleen and native right lung 7 days after Balb/c → CD45.1⁺ B6 lung transplantation. Results are representative of three independent experiments. (B) IFN-γ responses to stimulation with T cell depleted B6 splenocytes of CD4⁺ T cells isolated from spleens of naïve Balb/c mice or Balb/c mice 7 days after sensitization with CD45.1⁺CD11c⁺ cells isolated from Balb/c → CD45.1⁺ B6 lung allografts, purified 7 days after transplantation (top panel). IFN-γ responses to stimulation with T cell depleted Balb/c splenocytes of CD4⁺ T cells isolated from spleens of naïve B6 mice or B6 mice 7 days after sensitization with CD45.1⁺CD11c⁺ cells isolated from Balb/c → CD45.1⁺ B6 lung allografts, purified 7 days after transplantation (bottom panel). Results are representative of two independent experiments.

Figure 9. Blunted Th1 direct allorecognition in CCR2-deficient lung graft recipients
 (A) IFN- γ and (B) IL-17 ELISPOT analysis conducted on co-cultures of Balb/c stimulators with CD4⁺ T-cell responders isolated from the spleens of resting wild-type B6 (n = 3) or CCR2-deficient B6 mice (n = 3) or the grafts of Balb/c $\rightarrow$ B6 (n = 4) and Balb/c $\rightarrow$ CCR2^{-/-} (n = 4) lung recipients. The positive control for the IL-17 ELISPOT analysis was conducted on PMA and ionomycin-stimulated CD4⁺ T cells isolated from the spleens of resting B6 mice (n = 4). Spot quantification is depicted above a representative plate as the mean number of spots $\pm$ S.E.M. (**p < 0.01) per 200,000 CD4⁺ T cells.