Role of CXCL9/CXCR3 Chemokine Biology during Pathogenesis of Acute Lung Allograft Rejection[1]

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Acute allograft rejection is a major complication postlung transplantation and is the main risk factor for the development of bronchiolitis obliterans syndrome. Acute rejection is characterized by intragraft infiltration of activated mononuclear cells. The ELR-negative CXC chemokines CXCL9, CXCL10, and CXCL11) are potent chemoattractants for mononuclear cells and act through their shared receptor, CXCR3. Elevated levels of these chemokines in bronchoalveolar lavage fluid have been associated with human acute lung allograft rejection. This led to the hypothesis that the expression of these chemokines during an allogeneic response promotes the recruitment of mononuclear cells, leading to acute lung allograft rejection. We performed studies in a rat orthotopic lung transplantation model of acute rejection, and demonstrated increased expression of CXCL9 and CXCL10 paralleling the recruitment of mononuclear cells and cells expressing CXCR3 to the allograft. However, CXCL9 levels were 15-fold greater than CXCL10 during maximal rejection. Inhibition of CXCL9 decreased intragraft recruitment of mononuclear cells and cellular expression of CXCR3, resulting in lower acute lung allograft rejection scores. Furthermore, the combination of low dose cyclosporin A with anti-CXCL9 therapy had more profound effects on intragraft leukocyte infiltration and in reducing acute allograft rejection scores. This supports the notion that CXCL9 interaction with cells expressing CXCR3 has an important role in the recruitment of mononuclear cells, a pivotal event in the pathogenesis of acute lung allograft rejection. *The Journal of Immunology,* 2003, 171: 4844–4852.

L ung transplantation is a therapeutic option for patients with end-stage pulmonary disorders. Unfortunately, acute lung allograft rejection is a common complication with an incidence and severity that exceed all other solid organ transplantations (1, 2). Studies suggest at least one episode of acute rejection occurs in 70–80% of lung transplantation recipients during the first postoperative year, with the majority of lung transplantation recipients experiencing at least one episode of rejection during the life of the allograft (1, 2). Chronic lung allograft rejection, known as bronchiolitis obliterans syndrome (BOS),[3] is the leading cause of morbidity and mortality postlung transplantation (1, 2). The number of episodes and severity of acute lung allograft rejection correlate with the development of chronic (BOS) lung allograft rejection (1–3).

Acute lung allograft rejection is characterized by an infiltration of mononuclear cells that ultimately contributes to loss of lung architecture and function. However, the mediators that orchestrate the recruitment of leukocytes to the allograft have not been fully elucidated. CXCL9, CXCL10, and CXCL11 are IFN-inducible ELR (glutamic acid-leucine-arginine)-negative CXC chemokines, and are potent chemoattractants for mononuclear cells, specifically activated T cells, B cells, and NK cells (4–6). All three chemokines share an ability to signal through a G protein-coupled receptor, CXCR3 (7, 8). CXCR3/CXCR3 ligand biology has been shown to be important during rejection in murine models of cardiac allograft rejection (9, 10). In addition, elevated levels of CXCR3 ligands have been associated with both acute and chronic human lung allograft rejection (11, 12). Furthermore, we have shown that inhibition of CXCR3/CXCR3 ligand biology reduced fibroplasia during murine chronic (BOS) rejection (12). Because of these findings, we hypothesized that ELR-negative CXC chemokines are important in acute lung allograft rejection, the main risk factor for the development of BOS.

Our studies extend the results of previous murine and human studies and demonstrate proof of concept that CXCL9 interaction with CXCR3 is directly involved in the pathogenesis of acute rejection by reducing the infiltration of mononuclear cells and cells expressing CXCR3. These findings may ultimately result in novel therapies designed to modulate CXCL9/CXCR3 biology and impact on the development of acute lung allograft rejection.

Materials and Methods

Reagents

Polyclonal goat anti-murine CXCL9-specific antiserum was produced by the immunization of goats with the murine chemokine rCXCL9 (R&D Systems, Minneapolis, MN). The goat was immunized in multiple intradermal sites with CFA, followed by at least three boosts in IFA (12). The

polyclonal Abs were purified from this high titer serum using a protein A-agarose column (Pierce, Rockford, IL), as previously described (13). Direct ELISA was used to evaluate antisera titers, and sera were used for Western blot, ELISA, and neutralization assays when titers had reached greater than 1/1,000,000. The specificity of anti-CXCL9 Ab was assessed by Western blot analysis against a panel of human, murine, and rodent recombinant cytokines. The anti-CXCL9 Ab was specific in our sandwich ELISA without cross-reactivity to a panel of cytokines, including human and rodent IL-1Ra, IL-1, IL-2, IL-6, IL-4, TNF-α, IFN-γ, CXCL10, CXCL1/2/3, CCL4, CCL2, CCL5, CCL3, CXCL1, CXCL8, CXCL5, and CXCL3 (12). Furthermore, the anti-CXCL9 had a specific neutralizing capacity for murine CXCL9 by inhibiting splenocyte chemotaxis response to murine CXCL9. In contrast, anti-CXCL9 did not inhibit chemotaxis in response to CXCL10, CXCL12, or CCL5 (12).

Orthotopic single lung transplantation model

To assure for immunogeneic standardization and histoincompatibility, these studies used inbred, unmodified, specific pathogen-free rats, Brown Norway (RT1^n) and Lewis (RT1^1). Orthotopic rat left lung transplantation was performed, as previously described by us, using a modification of Prop et al. and Maarck and Wildevuur (14–16) with over 85% postoperative survival. The 15% mortality associated with this procedure was directly attributable to surgical mortality and usually occurred within 8 h posttransplantation. Animals weighed between 200 and 300 g. Preoperative anesthesia consisted of 0.5 mg atropine (s.c.); anesthesia was induced with 4% halothane in a mixture of 40% O_2/60% NO_2. The animal's trachea was intubated with an 18-gauge endotracheal tube. Anesthesia was maintained throughout the operation using 0.25–1% halothane in 40% O_2/60% NO_2 mixture through a Harvard rodent ventilator. The animal's body temperature was maintained using a warming mattress. Intravascular volume was assured by 1.5 ml i.p. infusion of 0.9% normal saline every hour perioperatively. The operation on the donor animal was performed by removing the anterior chest wall and infusing 1000 U of heparin into the right atrial appendage of the heart and allowing time to circulate. The right ventricle was then flushed with 50 ml of normal saline after transection of the pulmonary vein, followed by extraction of the left lung. The operation on the recipient animal was performed via a left posterior-lateral thoracotomy in the fourth or fifth intercostal space. The left lung hilum was identified, dissected, and cross-clamped. The recipient's left native lung was removed. The reimplantation of the donor's lung entailed the successive anastomosis of the following structures: 1) pulmonary vein and artery were anastomosed with interrupted 10-0 Ethilon, followed by removal of the clamp and restoration of perfusion, and 2) the bronchus was anastomosed with interrupted 9-0 Ethilon, followed by reinflation at a maximal pressure of 25–30 cm H_2O. The allograft/isograft ischemic time was maintained at less than 120 min and recorded for each recipient animal. The thorax was closed over an 18-gauge chest tube, which was removed before the animal recovering from anesthesia (17–21). Postoperatively, the animals were kept in an oxygenated cage for the first 24 h. The animals were allowed to eat and drink ad lib through the course of the experiment. At time of sacrifice, animals were euthanized with 50 mg of ketamine (i.p.).

In vivo neutralization of endogenous CXCL9 and the administration of cyclosporin A (CsA)

In separate experiments, animals received by (i.p.) injection 20 mg of goat polyclonal IgG anti-CXCL9 or an equivalent quantity of control goat IgG (Sigma-Aldrich, St. Louis, MO) every other day beginning at postoperative day 0 until sacrifice. Moreover, to create experiments analogous to the clinical setting, we titrated the lowest dose of CsA (mixed in Cremophor EL castor oil; Sigma-Aldrich) given (i.p.) (0.50, 1.00, 1.50, 1.75, and 1.85 mg/kg/day) to attenuate acute lung allograft rejection at day 6, as compared with control (Cremophor EL). Then experiments were performed with the combination of low dose CsA (1.85 mg/kg/day) injected (i.p.) everyday starting at postoperative day 0 until sacrifice plus anti-CXCL9, or control Ab given every other day starting at postoperative day 0 until sacrifice, or day 14, whichever came first.

Histopathology

A random 5-μm paraffin-embedded tissue section for six different lung allografts was stained with H&E and reviewed using a quantitative histopathologic acute lung rejection scoring system derived from modifications of previous solid organ rejection and acute lung injury scoring systems (22–24). The histopathology was blindly reviewed, and all qualitative histological changes were noted. In addition, four easily identifiable pathologic processes were scored on a scale of 0–4: 0 = normal; 1 = infrequent mild infiltrates and/or involves <25% of the allograft; 2 = frequent mild

to moderate dense infiltrates and/or involves 25–50% of the allograft; 3 = severe dense and diffuse infiltrates with extension into the alveolar space and septae with frequent confluence and/or involves 50–75% of the allograft; and 4 = very severe diffuse infiltrates with complete extension into the interstitial and alveolar spaces and/or involves >75% of the allograft. The four pathologic processes are: 1) perivascular inflammation; 2) peribronchiolar inflammation; 3) alveolar inflammation; and 4) alveolar wall thickness. An overall total score of acute rejection was obtained based on the summation of all scores from the four pathological processes to generate a cumulative total histopathological score. All scores are presented as a mean ± SEM generated from the cohort of allografts at each time point.

CXCL9 and CXCL10 ELISAs

CXCL9 and CXCL10 protein from bronchoalveolar lavage fluid (BALF) and whole lung homogenates were quantitated using a modification of a double-ligand method, as previously described (12, 16, 22, 25).

FACS analysis of infiltrating leukocyte populations

Lung single-cell suspension preparations were made using a method previously described (22, 26). Single-cell suspensions were dually stained with Tri-color-conjugated mouse anti-rat CD45 (Caltag, Burlingame, CA) and PE-conjugated mouse anti-rat CD3, CD4, CD8, NK1.1 (BD PharMingen, San Diego, CA), Pan B (B cell) (Caltag), and for FITC-conjugated anti-rat ED-1 (Serotec, Oxford, U.K.), which was applied only after the cells were permeabilized with cytofix/cytoperm (BD PharMingen). Single-cell suspensions were analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences), as previously described (16).

Total RNA isolation and real-time quantitative PCR

Total cellular RNA was isolated, as previously described (12, 16, 22). Total RNA was determined, reverse transcribed into cDNA, and amplified using TaqMan reverse-transcription reagents (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using specific TaqMan primers and probes, using the ABI Prism 7700 Sequence Detector and SDS analysis software (Applied Biosystems). Negative controls were the following: no reverse transcription; no template cDNA. The primers and probe sequences (forward primer, reverse primer, TaqMan probe) used were: CXCL9 primers forward, TGT GGA GTT CGA GGA ACC CT; reverse, TGC CTT GGC TGG TGC TG; probe, 6FAMTGA TAA GGA ATC AGC GAT GCT CCT GCAMRA; CXCL10 primers forward, AGA ACG GTG CGC TGC AC; reverse, CCT ATG GCC CTG GGT CTC A; probe, 6FAMTGC ATC GAC TTC CAT GAA CAG CCG TAMRA; CXCR3 primers forward, CTG CTG CCC AGT GGG TTT; reverse, GTT GAT GTT GAA CAG GGC ACC; probe, 6FAMCGG CTC TGG TCT CTG CAA AGT GGCMRA; and housekeeping gene 18S TaqMan predeveloped assay reagent (Applied Biosystems).

Quantitative analysis of gene expression was done using the comparative C_T (ΔC_T) methods, in which C_T is the threshold cycle number (the minimum number of cycles needed before the product can be detected) (27). The arithmetic formula for the ΔC_T method is described as the difference in threshold cycles for a target (i.e., CXCL9) and an endogenous reference (i.e., our housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e., CXCL9 in allografts at day 6) and relative to a calibration normalized to an endogenous reference (i.e., CXCL9 in syngeneic controls at day 6) is given by $2^{-\Delta\Delta CT}$ (27). The following is an example for comparing CXCL9 expression from allografts and syngeneic controls at day 6. Both CXCL9 from the allografts and syngeneic controls at day 6 are normalized to 18S: $\Delta\Delta C_T = \Delta C_T$ (CXCL9 from allograft at day 6 normalized to endogenous 18S) $-\Delta C_T$ (CXCL9 from syngeneic control at day 6 normalized to endogenous 18S). The calculation of $2^{-\Delta\Delta CT}$ then gives a relative value when comparing the target to the calibrator, which we designate in this context as fold increase of allograft to syngeneic controls of the target mRNA relative quantification.

Statistical analysis

Data were analyzed on a computer using the Statview 4.5 statistical package (Abacus Concepts, Berkeley, CA). All animal group comparisons were evaluated by the unpaired t test. Data were expressed as mean ± SEM.

Results

Rat orthotopic single left lung transplantation involves a temporal increase in infiltrating leukocytes during acute rejection

Human lung transplantation typically involves an initial reimplantation response (i.e., ischemia-reperfusion), which usually is followed by at least one episode of acute rejection during the life of the allograft (1–3). Translational studies were performed using a fully mismatched, reproducible, immunogenetically standardized, rat orthotopic single left lung transplantation model, with similar characteristics of human lung allograft rejection. The full kinetics of the severity of acute rejection in this rat model was assessed over a 6-day time course. Lewis rats were subjected to lung transplantation with allografts from Brown Norway rats or from syngeneic donors. Animals were sacrificed on days 1, 4, and 6 posttransplantation, and lungs were harvested for histopathologic scoring system based on a modification of other rejection and acute lung injury scores performed (22–24). Allografts and syngeneic controls at day 1 both demonstrated an early reimplantation response consisting of diffuse interstitial/alveolar leukocyte infiltration, hemorrhage, and congestion with alveolar wall thickening. There was no significant difference in perivascular, peribronchiolar, alveolar inflammation, and alveolar wall thickening, with overall total rejection scores of 5.65 ± 0.38 vs 5.66 ± 0.58, $p = 0.97$, respectively (Fig. 1). However, allografts at day 4 demonstrated early rejection with frequent mild to moderate dense perivascular, peribronchiolar, and alveolar inflammation and alveolar wall thickening, as compared with syngeneic controls demonstrating a marked decrease in leukocyte infiltration (perivascular/peribronchiolar/alveolar) and alveolar wall thickening. The overall total scores of allografts and syngeneic controls at day 4 were 7.14 ± 0.39 vs 2.33 ± 0.40, $p < 0.0001$, respectively (Figs. 1). Allografts at day 6 demonstrated more aggressive rejection with >50% of the allograft involving severe dense perivascular and peribronchiolar infiltrates that extended into alveolar septae/space with frequent confluent areas of inflammation. In contrast, day 6 syngeneic controls demonstrated minimal in leukocyte infiltration and alveolar wall thickening. The overall total scores of allografts and syngeneic controls at day 6 were 11.26 ± 0.21 vs 2.00 ± 0.82, $p < 0.0001$, respectively (Fig. 1).

CXCL9 and CXCL10 are elevated during rat acute lung allograft rejection

Human studies using BALF have demonstrated an association between elevated levels of CXCL9, CXCL10, and CXCL11 during acute lung allograft rejection (11, 12). In these studies, human CXCL9 and CXCL10 levels were far greater than CXCL11 (11, 12). The two known rat IFN-inducible ELR-negative CXC chemokines CXCL9 and CXCL10 (to our knowledge, rat CXCL11 has not been described) were evaluated using real-time quantitative PCR on whole lung homogenates. There was a temporal increase in expression of both CXCL9 and CXCL10 from the allografts, as compared with syngeneic controls, with the greatest differences seen at the time point of maximal intragraft leukocyte infiltration at day 6 (Fig. 2I). Interestingly, the expression of CCL9 was over 3-fold greater than CXCL10 at day 6 (Fig. 2I). Because
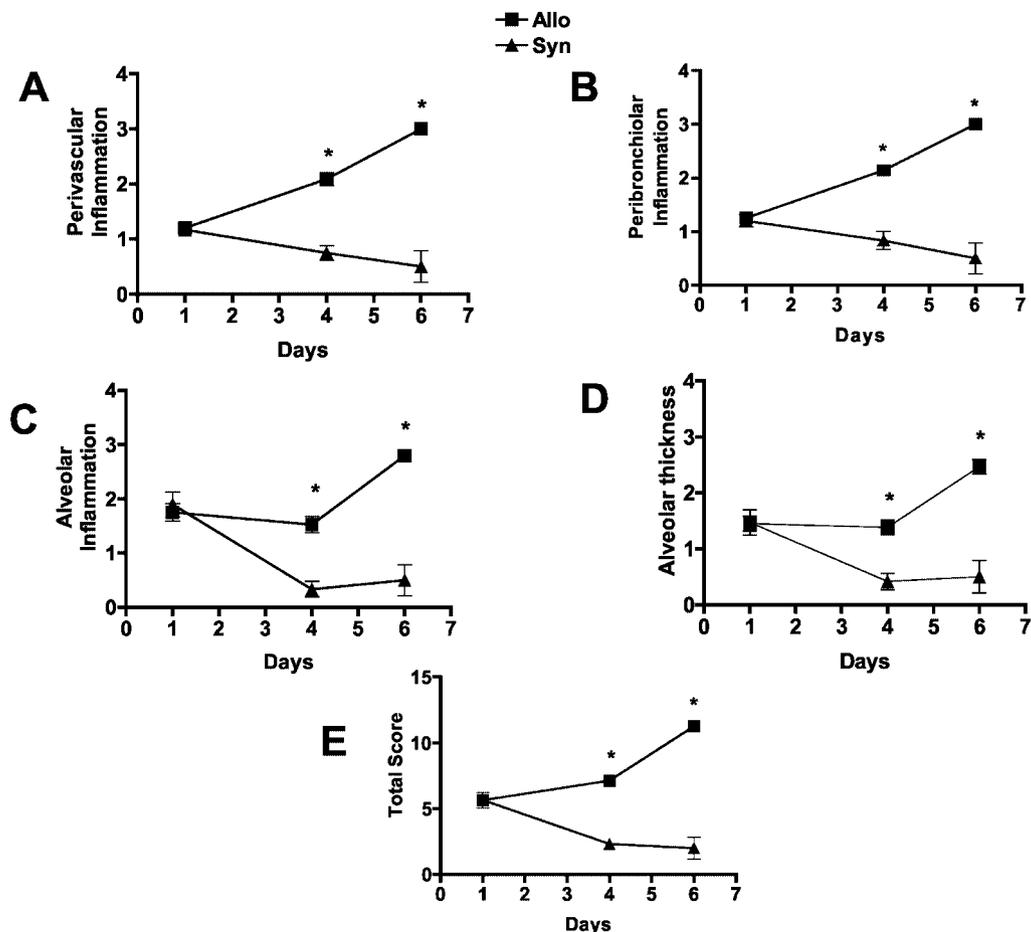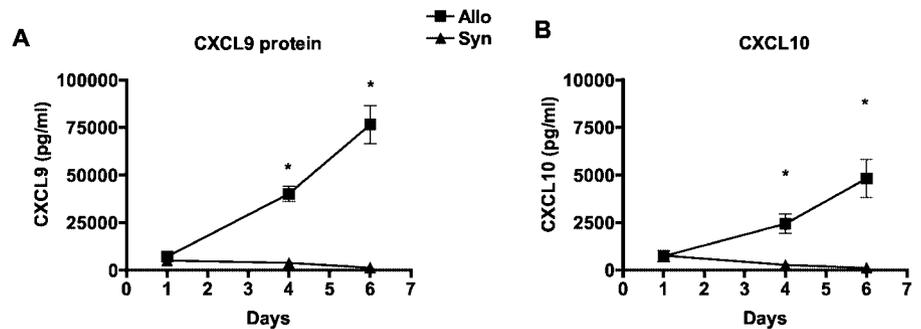

FIGURE 1. Temporal increases in *A*, perivascular; *B*, peribronchiolar; *C*, alveolar inflammation; *D*, alveolar wall change; and *E*, cumulative total acute rejection score in lung allografts over a 6-day time course ($n = 6$ left transplanted lungs evaluated at each time point; *, $p < 0.05$).
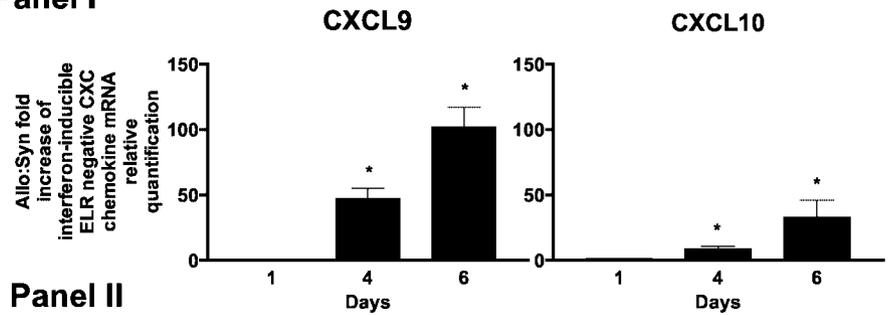
FIGURE 2. CXCL9 and CXCL10 expression is markedly elevated in lung allografts during acute rejection. *I*, Real-time quantitative PCR determination of CXCL9 and CXCL10 mRNA expression presented as a fold increase of chemokine expression from the allografts, as compared with syngeneic controls at days 1, 4, and 6. *II*, Whole lung homogenate protein levels measured by ELISA, of CXCL9 and CXCL10 from lung allografts and syngeneic controls at days 1, 4, and 6 ($n = 6$ lungs at each time point; *, $p < 0.05$).

all of the human lung rejection studies of CXCL9 and CXCL10 were performed on BALF, rat BALF was analyzed during the rejection process. Again, both CXCL9 and CXCL11 protein levels were temporally elevated in BALF (data not shown) at similar magnitudes analogous to previous human data (11, 12). Although BALF samples the peribronchial and alveolar airspaces, it may actually underestimate the total lung or interstitial levels of these CXC chemokines. Therefore, we evaluated whole lung homogenates for protein levels of these IFN-inducible ELR-negative CXC chemokines. There were elevations of both CXCL9 and CXCL10 in lung allograft homogenates, as compared with controls (Fig. 2*III*). However, CXCL9 was over 15-fold greater than CXCL10 at the time point of maximal rejection, day 6 (Fig. 2*II*).

CXCR3 expression parallels the production of CXCR3 ligands during the pathogenesis of acute lung allograft rejection

Rat CXCR3 is the shared receptor for the ligands CXCL9 and CXCL10 (28). Kinetics of CXCR3 expression was evaluated over the 6-day time course using real-time quantitative PCR of whole lung homogenates. There was no significant difference in the expression of CXCR3 at day 1 between allografts and syngeneic control. Conversely, days 4 and 6 demonstrated an increase in CXCR3 mRNA expression from allografts, as compared with syngeneic controls (Fig. 3), and its expression paralleled CXCR3 ligand and leukocyte recruitment in the allograft (Figs. 1–3).

In vivo depletion of endogenous CXCL9 reduces intragraft recruitment of mononuclear cells during acute allograft rejection

CXCL9 expression was markedly greater than CXCL10 during acute lung rejection. Based on these data, we determined whether inhibiting endogenous levels of CXCL9 with specific anti-CXCL9 Abs would alter intragraft recruitment of mononuclear cells. These experiments involved Lewis rats as the recipients of Brown Norway lungs. The recipients received specific anti-CXCL9 or control Abs at days 0, 2, and 4. Investigation of mononuclear cell recruitment was performed by FACS analysis on single-cell suspensions of whole lung allograft digests at day 6. There was a marked at-

tenuation of infiltrating CD3, CD4, CD8, NK cells, B cells, and mononuclear phagocytes in allografts from animals treated with anti-CXCL9, as compared with control Abs (Fig. 4*IA*). Moreover, real time PCR analysis of whole lung allografts from both treatment groups demonstrated a significant reduction in CXCR3 expression at day 6 in those animals treated with anti-CXCL9, as compared with control Abs (Fig. 4*IB*).

In vivo neutralization of endogenous CXCL9 attenuates acute lung allograft rejection scores

Finding significant reductions of intragraft mononuclear cells and expression of CXCR3 in the anti-CXCL9 group led us to determine whether this had any effects on allograft rejection. Quantitative histopathological scoring confirmed the FACS analysis, demonstrating significant reductions in perivascular, peribronchiolar, and alveolar leukocyte infiltration (Fig. 4, *IA*, *II*, and *III*). Overall total rejection scores for the anti-CXCL9 and control Ab groups were 6.66 ± 0.745 vs 11.11 ± 0.60, $p < 0.0001$, respectively (Fig. 4*II*).

To extend our studies beyond day 6, we assessed long-term effects of the anti-CXCL9 Ab on acute lung allograft rejection scores. Allograft recipients were treated either with anti-CXCL9 or control Abs every other day beginning at day 0 until sacrifice. Quantitative scoring demonstrated significant reductions in perivascular, peribronchiolar, alveolar inflammation and alveolar thickness in the anti-CXCL9 group, as compared with the control group with total scores: day 8, 8.00 ± 0.76 vs 14.33 ± 0.803, $p < 0.0001$, and day 10, 10.00 ± 0.00 vs 16.00 ± 0.00, $p < 0.003$, respectively (Fig. 4, *II* and *III*). Because there was significant rejection day 10 in the anti-CXCL9 group, we aborted further time point analyses with this treatment strategy alone.

Calcineurin inhibitors such as CsA have led to improved survival of solid organ transplants (29). However, with significant side effects related to these reagents, any reduction in their dose would be important for the diminution of transplant recipient morbidity (29). For these reasons, we titrated (0.5, 1.0, 1.50, 1.75, and 1.85 mg/kg/day) the lowest dose of CsA required to inhibit allograft rejection at day 6, the time when anti-CXCL9 therapy maximally decreased acute lung allograft rejection scores. The CsA
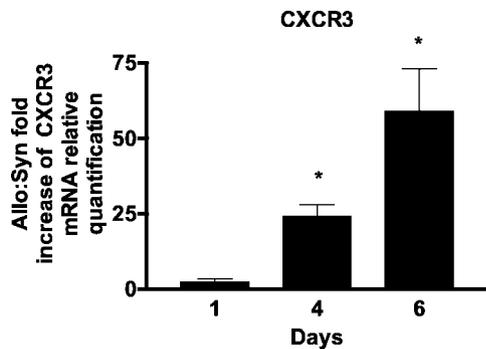
CXCR3

FIGURE 3. CXCR3 mRNA expression in the lung is elevated during acute allograft rejection. Real-time quantitative PCR determination of CXCR3 mRNA expression presented as a fold increase of CXCR3 expression from the allografts, as compared with syngeneic controls at days 1, 4, and 6. ($n = 6$ lungs at each time point; $*$, $p < 0.05$).

dose of 1.85 mg/kg/day given at day 0 and daily until sacrifice at day 6 or 8 reduced rejection as compared with the control-treated group (Cremofor EL castor oil), with overall total histopathologic scores 11.6 ± 0.40 vs 6.14 ± 1.20, $p = 0.0035$, respectively (Fig. 5). However, by day 8, no significant reduction in histopathological rejection score was detected (Fig. 5). With this low dose of CsA attenuating rejection only at day 6, separate experiments were designed to evaluate the combination of CsA and anti-CXCL9 Abs. Recipients received anti-CXCL9 or control Abs every other day beginning at day 0 through day 16 or until sacrificed, plus low dose CsA (1.85 mg/kg) injected everyday beginning at day 0 until the animal was sacrificed. We chose to evaluate lung allografts starting at day 10 with the combination regimen (anti-CXCL9 plus CsA) because the anti-CXCL9 therapy alone at day 10 was less effective in reducing rejection scores than earlier time points day 6 and 8. There were profound reductions in histopathologic scores in the anti-CXCL9 plus CsA group, as compared with control Ab plus CsA out to day 14 (Fig. 6). In addition, there were significant reductions in histopathologic scores out to day 20, but not to the same magnitude as the earlier time points (Fig. 6). The reason that multiple time points of histopathologic scoring are necessary in this model system of orthotopic lung allograft rejection is that there is no noninvasive method to adequately and quantitatively assess rejection, whereas in the cardiac transplantation models, rejection can be assessed by cardiac palpation.

Discussion

Human acute lung allograft rejection is characterized by a perivascular and peribronchiolar mononuclear cell infiltration that intensifies to dense leukocyte infiltrates extending into the interstitium and alveolar space with eventual alveolar, pneumocyte, and parenchymal damage. However, the exact mechanisms leading to leukocyte recruitment to the lung allograft have not been fully elucidated. Studies have established an association between elevated levels of biologically active IFN-inducible ELR-negative CXC chemokines in human BALF from patients undergoing acute and chronic lung allograft rejection (11, 12). In these studies, the levels of CXCL9 and CXCL10 were not only a magnitude greater than CXCL11, but were better predictors of rejection (12). Translational experiments using a fully mismatched rat orthotopic single left lung transplantation model were performed to further evaluate the role of these chemokines during acute lung allograft rejection. This model system was characterized and demonstrated an initial ischemia-reperfusion injury at day 1 for both allografts and syn-

geneic controls. However, at days 4 and 6, there was a temporal increase in an intense perivascular/bronchiolar inflammatory process, followed by parenchymal and alveolar wall damage.

Both CXCL9 and CXCL10 expression paralleled the inflammatory process during acute lung allograft rejection. Unfortunately, CXCL11 has not been isolated and cloned to our knowledge in rats, and therefore was not evaluated in this model system. Interestingly, rat CXCL9 mRNA expression from lung allografts was >3-fold higher than CXCL10. Because protein is the important biological end product of mRNA expression, protein levels of CXCL9 and CXCL10 from allografts were analyzed. Both CXCL9 and CXCL10 levels were elevated in BALF, and were comparable to human BALF during acute lung rejection (11, 12). However, whole lung allograft homogenates demonstrated CXCL9 levels were >15-fold greater than CXCL10 at the time of maximal allograft rejection. In general, studies have found that chemokine gene expression is usually associated with protein secretion to the extracellular space (30). Moreover, the secreted chemokine protein, due to heparin-binding properties, is able to bind to components of the extracellular matrix and at the same time demonstrate biological function (30). These findings are potentially consistent with why BALF may underestimate the magnitude of expression of a number of factors during lung allograft rejection.

Other animal models of solid organ rejection, including skin, heart, and trachea, have also demonstrated augmented levels of both CXCL9 and CXCL10 (10, 12, 31–34). However, depending on the type of organ transplanted or the strain of mice used, the kinetics and magnitude of CXCL9 and CXCL10 differ (10, 12, 31–34). For example, in a fully mismatched murine model of cardiac rejection (A/J donor hearts to C57BL/6 recipients), CXCL9 expression temporally increases with maximal levels seen before peak rejection. In contrast, CXCL10 expression in the same model system is minimally increased during rejection (33). Furthermore, the peak protein levels of both CXCL9 and CXCL10 were maximal at day 3, with CXCL9 2- to 3-fold greater than CXCL10 in this model system (33). In another fully mismatched cardiac rejection model (BALB/c to C57BL/6), CXCL10 expression was greater and increased sooner than CXCL9 during the rejection process (34). Furthermore, in a murine model of BOS, CXCL9 and CXCL10 from the allografts were temporally elevated with similar levels (12). Together these studies demonstrate different magnitudes and kinetics of CXCL9 and CXCL10 for different models of allograft rejection. Importantly, our study involves an orthotopic animal models system that is performed in the same manner as a human single lung transplantation, and is a very robust rejection process as compared with the other murine models that are performed in a heterotopic position.

Not all solid organ transplantations are the same. The lung is considered one of the most immunogeneic solid organs to be transplanted (35). This most likely has to do with the lung's major role in host defense involving inhaled Ags and pathogens and its large content of mononuclear cells (35). In fact, lung transplantation recipients have a dramatically higher morbidity and mortality as compared with other solid organ transplantation patients (1, 2). For example, postlung transplantation survival is only 42% as compared with 70% for liver, kidney, and cardiac transplantation at 5 years (1, 2). Moreover, the actual incidence and severity of acute lung allograft rejection are greater than other solid organ transplants (1, 2).

All solid organ transplants do not respond to antirejection medications in the same manner. Induction therapy with antithymocyte globulin has no effect on the development of chronic lung rejection
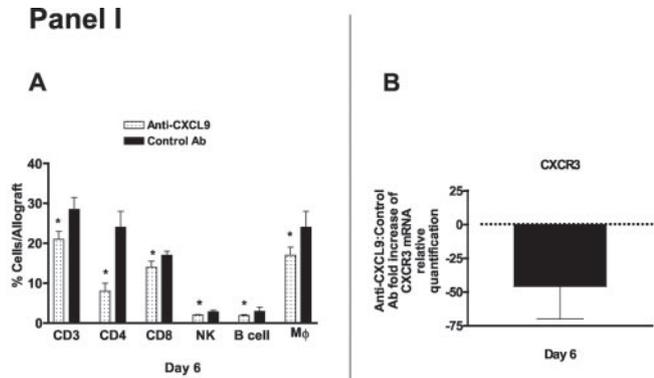
FIGURE 4. *I*, Neutralization of CXCL9 reduces infiltrating lung allograft mononuclear cells and the expression of CXCR3. *A*, FACS analysis of percentage of leukocyte cell surface markers CD3, CD4, CD8, NK cells, B cells, and Mϕ (mononuclear phagocytes) from allografts treated with anti-CXCL9 vs control Abs ($n = 6$ lungs at each time point; *, $p < 0.05$). *B*, Real-time quantitative PCR determination of CXCR3 mRNA expression presented as fold decrease of CXCR3 expression from lung allografts of animals treated with anti-CXCL9, as compared with control Ab ($n = 6$ lungs at each time point; *, $p < 0.05$). *II*, Neutralization of CXCL9 inhibits leukocyte infiltration in acute lung allograft rejection. Quantitative histopathologic scores from days 6–10 of allografts from animals treated with anti-CXCL9, as compared with control Abs. *A*, Perivascular; *B*, peribronchiolar; *C*, alveolar inflammation; *D*, alveolar wall change; and *E*, cumulative total acute rejection score in lung allografts ($n = 6$ lung allografts evaluated at each time point; *, $p < 0.05$). *III*, Representative photomicrographs with H&E ($\times 400$) of the histopathology of lung allograft rejection from animals treated with anti-CXCL9, as compared with control Ab at days 6, 8, and 10.
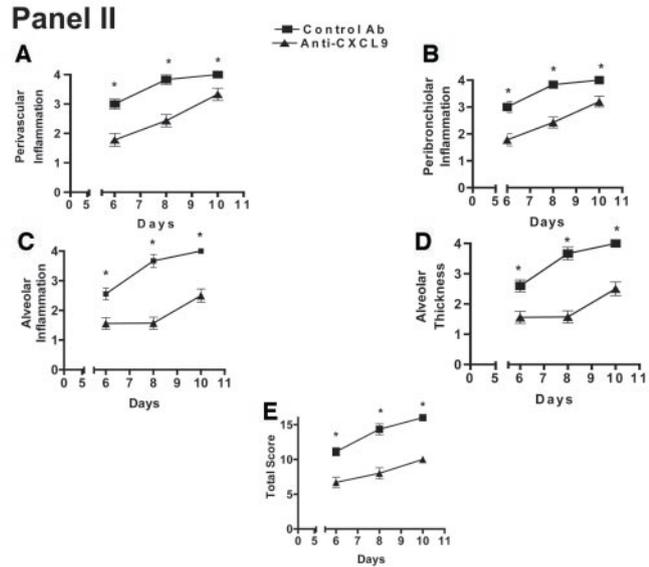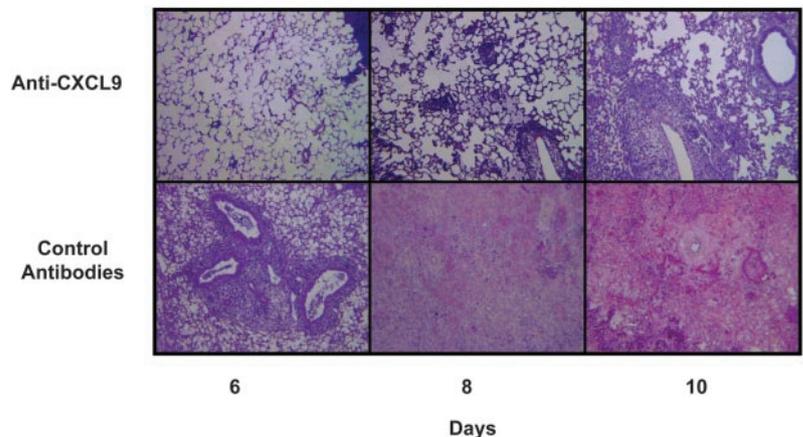
or lung transplantation recipient survival (36). However, when similar studies have been performed with renal transplantation recipients, there were both a decrease in chronic rejection and prolongation of graft survival (37–39). Moreover, a pooled analysis of three studies with renal transplantation recipients has demonstrated a significant reduction in acute rejection with mycophenolate mofetil as compared with azathioprine or placebo (40). However, when similar studies were performed with lung transplantation recipients, there was no difference in acute rejection (41). Furthermore, it is now known that different organs transplanted differ in their propensity for rejection and have distinct cellular require-

ments for rejection (42, 43). These differences make the type of transplanted organ used in studies of allograft rejection an important variable in dictating the immune requirements for rejection.

Paralleling leukocyte infiltration into the allograft and CXCL9 and CXCL10 levels was a temporal increase in CXCR3 expression. These data are supported by human studies that have found leukocytes in BALF from patients undergoing rejection with increased cell surface expression of CXCR3 (11). In addition, in a murine model system of BOS, infiltrating lymphocytes express CXCR3 and are associated with the development of fibro-obliteration (12). Taken together, these studies suggest that IFN-inducible ELR-negative CXC chemokines
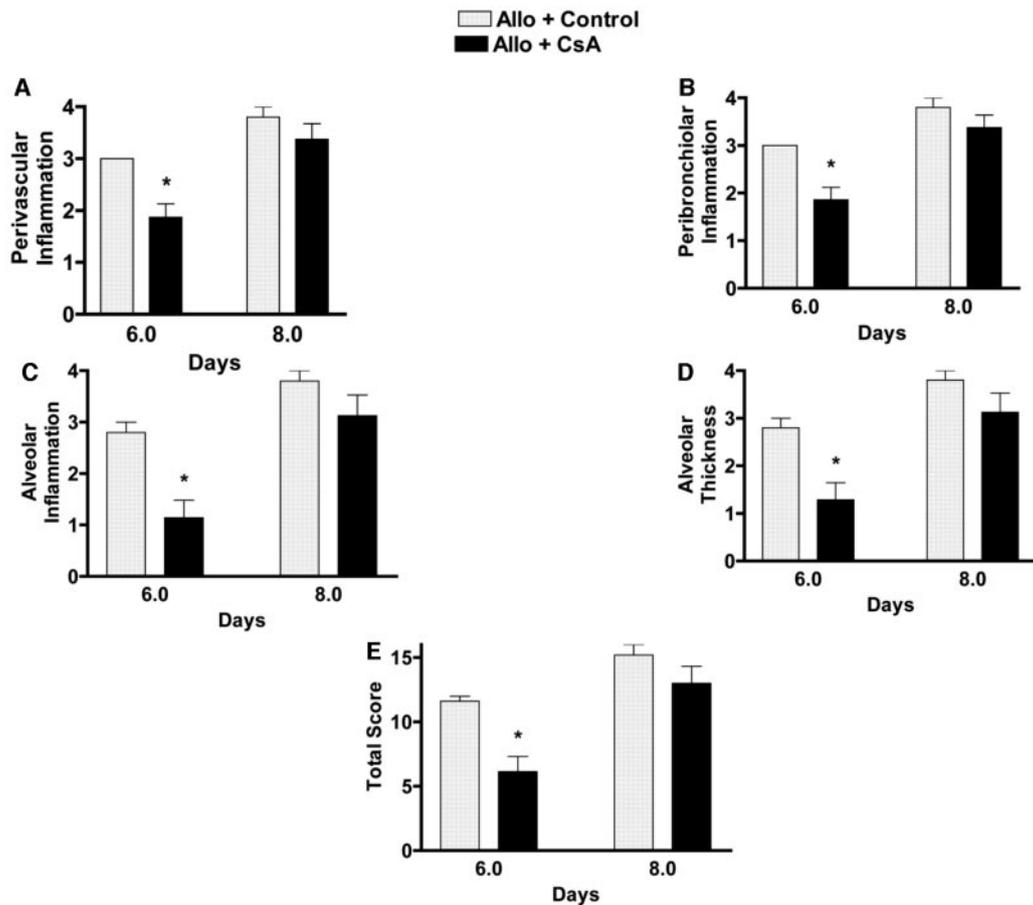
FIGURE 5. CsA given at a dose of 1.85 mg/kg/day beginning at day 0 and daily until sacrifice on day 6 or 8 attenuates acute lung allograft rejection scores at day 6, but not day 8. Quantitative histopathologic scores of lung allografts treated with low dose (1.85 mg/kg/day) CsA, as compared with control (Cemorphor EL oil) at days 6 and 8. *A*, Perivascular; *B*, peribronchiolar; *C*, alveolar inflammation; *D*, alveolar wall change; and *E*, cumulative total acute rejection score in lung allografts ($n = 6$ lung allografts at each time point; $*$, $p < 0.05$).

are important in the recruitment of mononuclear cells expressing CXCR3 during the pathogenesis of rejection.

With CXCL9 markedly greater than CXCL10, we determined the effects of neutralizing CXCL9 on allograft rejection. Allograft recipients that received anti-CXCL9 had a significant reduction of intragraft mononuclear cells (i.e., CD3, CD4, CD8, NK cells, B cells, and mononuclear phagocytes) confirmed by histopathological assessment. In addition, the anti-CXCL9-treated group had a significant reduction in expression of intragraft CXCR3. This attenuation of intragraft mononuclear cells and CXCR3 expression was accompanied by decreased parameters of acute lung allograft rejection that remained significant until day 10. These results are similar to other studies of skin, cardiac, and chronic (BOS) rejection, in which neutralization of CXCL9 reduced the recruitment of CXCR3-expressing leukocytes and impaired allograft rejection (12, 31, 33, 44). However, our findings expand on the results from these other studies by demonstrating that blocking CXCL9/CXCR3 interaction not only inhibits CD3, CD4, and CD8 cell, but also NK and B cell recruitment into the allograft. Future studies of B and NK cells will determine their specific roles during the acute lung allograft rejection process in this model system. Moreover, blocking CXCL9/CXCR3 biology in the heterotopic models of rejection has different effects on lymphocyte recruitment (12, 31, 33, 44). For example, in a murine cardiac allograft model (A/J to C57BL/6), neutralization of CXCL9 leads to more profound reduction in CD8, as compared with CD4 cells (33). In contrast, using a murine cardiac or BOS model (BALB/c to C57BL/6),

CXCR3 blocking leads to equal reductions in both CD4 and CD8 cells (10, 12). In our current study, neutralization of CXCL9 leads to more profound reductions in CD4 cells, as compared with CD8 cells. The different effects on CD4 and CD8 cells are most likely due to the different type of organ transplant model and animal strains used. Taken together, these studies demonstrate the importance of CXCL9/CXCR3 interactions during both the acute and chronic allograft rejection, and suggest CXCL9 may be a critical ligand in promoting the continuum of acute to chronic rejection.

Interestingly, we found that lung allografts from animals depleted of CXCL9 demonstrated reductions in mononuclear phagocytes. However, mononuclear phagocytes do not express CXCR3 (5, 7, 45). The reduction in intragraft mononuclear phagocytes may be indirect and due to reduced numbers of lymphocytes expressing CXCR3 interacting with other immune and/or nonimmune cells promoting a proinflammatory cytokine/chemokine cascade in the recruitment of mononuclear phagocytes.

Clinically, all lung transplantation recipients are placed on CsA or one of its analogues. Unfortunately, CsA has several side effects, necessitating limiting its dose to reduce toxicity. A low dose of CsA (1.85 mg/kg/day) was found to attenuate acute rejection scores at day 6; however, by day 8, no significant effect was found. This is supported by other studies of rat solid organ transplantation in which CsA given alone can decrease allograft rejection scores and prolong allograft survival (46–48). To simulate a clinical scenario, low dose CsA in combination with anti-CXCL9 was evaluated as a treatment strategy to attenuate lung allograft rejection.

FIGURE 6. Treatment with the combination of anti-CXCL9 (given every other day beginning at day 0 and until sacrifice or at day 14) and CsA (given everyday beginning at day 0 and until sacrifice) is synergistic for reducing acute lung allograft rejection scores. Quantitative histopathologic scores of lung allografts from animals treated with combined anti-CXCL9 Abs and low dose CsA, as compared with control Abs and low dose CsA at days 14–20. *A*, Perivascular; *B*, peribronchiolar; *C*, alveolar inflammation; *D*, alveolar wall change; and *E*, cumulative total acute rejection score in lung allografts ($n = 6$ lung allografts evaluated at each time point; $*$, $p < 0.05$).
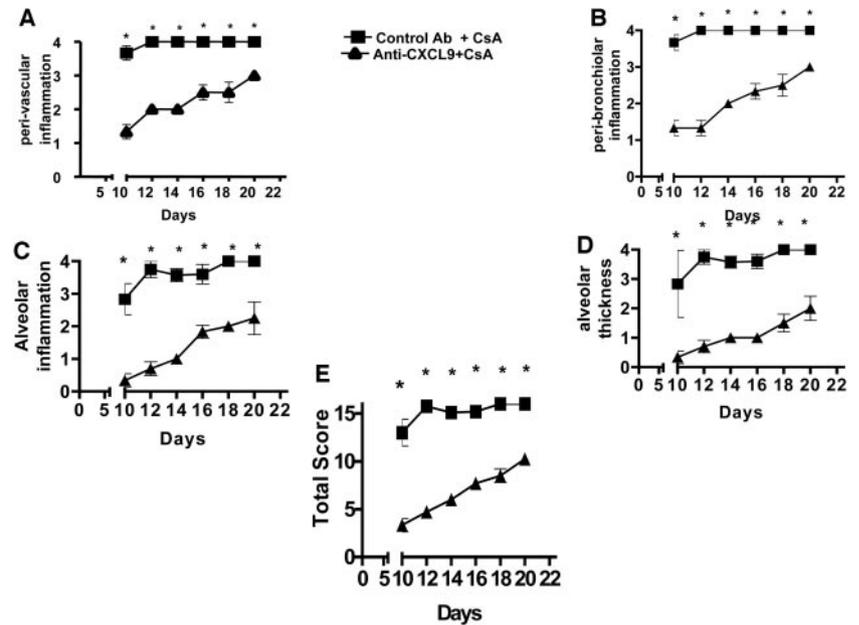
We found prolonged impairment in parameters of acute allograft rejection for 20 days. The reason for this synergistic effect may be due to CsA ability to inhibit cytokine mRNA transcription, particularly the synthesis of IL-2 and the expression of IFN-γ, CCL5, and CCL2 (49–52). Therefore, depletion of CXCL9 in combination with the effect of CsA has a profound synergistic consequence on leukocyte trafficking to the allograft. This is supported by other studies in which the addition of a short course of CsA (10 mg/kg/day for 14 days) to CXCR3$^{-/-}$ recipients of cardiac allografts prolonged allograft survival (10, 53). However, we expanded on these results by demonstrating that a very low dose of CsA for a prolonged period of time can act synergistically with the inhibition of just one chemokine ligand, CXCL9, instead of inhibiting multiple chemokine ligands shared by the same receptor CXCR3. This may preserve some of the transplant recipients' innate/adaptive host response in regard to infectious diseases.

In conclusion, we demonstrated that the inhibition of CXCL9 attenuates acute lung allograft rejection scores by reducing the recruitment of allograft mononuclear cells expressing CXCR3. The combination anti-CXCL9 and low dose CsA had profound and synergistic effects on attenuating allograft rejection scores. Future studies will determine whether strategies of inhibiting combined CXCR3/CXCR3 ligands and other chemokines/chemokine receptors in combination with CsA will ultimately lead to additive or syngeneic reductions in the pathogenesis of acute lung allograft rejection.

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