Chemokines and Transplant Vasculopathy

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Abstract—Transplant vasculopathy (TV) remains the leading cause of late death among heart transplant recipients. Transplant vasculopathy is characterized by progressive neointimal proliferation, leading to ischemic failure of the allograft. Multiple experimental and clinical studies have shown that injury to the graft at various stages of transplantation can be a risk factor for development of transplant vasculopathy. The hallmark of cardiac allograft injury is the infiltration of leukocytes. Recruitment of leukocytes requires intercellular communication between infiltrating cells, endothelium, parenchymal cells, and components of extracellular matrix. These events are mediated via the generation of adhesion molecules, cytokines, and chemokines. The chemokines, by virtue of their specific cell receptor expression, can selectively mediate the local recruitment/activation of distinct leukocytes/cells, allowing for migration across the endothelium and beyond the vascular compartment. This report provides a comprehensive review of the chemokines that participate in the development of transplant vasculopathy. (Circ Res. 2008;103:454-466.)

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Nearly 2200 patients underwent heart transplantation in the United States in 2006.1 Advances in perioperative care and immunosuppression have led to a 1-year survival exceeding 85% at most transplant centers.1 Beyond the first year, there is a steady attrition of survival that has remained virtually unchanged in the past decade. The leading cause of midterm and late death among heart transplant recipients is transplant vasculopathy (TV). The incidence of angiographically visible TV in heart transplant recipients exceeds 50% at 5 years.2 TV is characterized by diffuse intimal proliferation of coronary arteries and veins of the donor heart, leading to ischemic failure of the allograft. Because of denervated state, many heart transplant recipients do not experience typical anginal symptoms and may present with congestive heart failure symptoms or arrhythmic sudden death. Treatment options in patients with advanced TV are limited because of diffuse nature of disease. Surgical or percutaneous revascularization procedures have limited applicability and/or success. Retransplantation is an option in selected patients; however, inferior results and limited donor availability pose an ethical dilemma.3 Hence, TV remains a common and frequently fatal, yet poorly treatable, complication of heart transplantation.

The pathological hallmarks of endstage TV include an expanded neointima made up of smooth muscle cells, infiltrating mononuclear cells, and extracellular matrix. In early stages of TV in experimental models, marked inflammation involving the intima and adventitial layers of the vessel wall...
is a common observation. Analysis of explanted human hearts with end-stage TV show that the neointima is composed of 2 distinct layers: a subendothelial layer composed of loose connective tissue and extracellular matrix with a significant number of infiltrating mononuclear cells. The second layer of the neointima, which is closer to the internal elastic lamina is made up of predominantly smooth muscle cells. Immunohistochemical analysis of the coronary arteries of the explanted human hearts with TV show that the majority of infiltrating cells are CD3+ cells with a CD4/CD8 ratio of approximately 2. Macrophages (CD68+ cells) account for 8% to 15% of infiltrating cells of the expanded intima. B cells (CD220+ cells) are encountered infrequently.

Although the pathogenesis of TV is not completely defined, it is believed that TV is caused by repeated episodes of injury and repair to the graft vasculature. Injury to the graft begins with the donor brain death. Many other forms of injury are inflicted on the allograft throughout all stages of transplantation (ie, caused by cold ischemic preservation, surgical trauma, reperfusion, immune-mediated responses, infections, or metabolic derangements). These forms of injury may indeed overlap and yield synergistic damage to the allograft. The importance of these events in the development of TV is supported by multiple clinical and experimental studies. Early allograft dysfunction in kidney and lung transplantation are known risk factors for chronic rejection (equivalent to TV). Acute cellular rejection in heart transplantation has also been shown to contribute to the development of chronic rejection. Despite this association, it is important to acknowledge that acute rejection and TV are likely caused by distinct processes. Acute rejection is characterized by host immune responses against graft parenchymal or vascular cells, whereas the pathological hallmark of TV is concentric intimal expansion, with relative sparing of the parenchyma. Additionally, the existing immunosuppressive therapies are quite effective against acute rejection, but have failed to significantly alter the incidence and/or course of TV. There is also experimental and clinical evidence that the chemotactic mediators of mononuclear cell recruitment in acute rejection may be different from those in TV.

The late phase of TV is dominated by chemotaxis of smooth muscle cells, which yield the expanded intima. Several reports have shown that the majority of intimal smooth muscle cells in experimental TV are host-derived. In contrast, the smooth muscle cells of the neo-intimal lesions in human TV have been shown to be of donor origin. The origin of intimal smooth muscle cells and the mediators of their recruitment are thoroughly discussed in this thematic series and elsewhere and, therefore, are not discussed further in this review. There is also emerging evidence that bone marrow-derived progenitor cells can migrate to the areas of vascular damage and differentiate into smooth muscle cells and endothelial cells. CXC ligand CXCL12/stromal cell-derived factor (SDF)-1 has been shown to play a unique role in recruitment of bone marrow stem cells. CXCL12/SDF-1 is released by a variety of cell types during tissue injury and is thought to mediate recruitment of CXCR4-expressing bone marrow stem cells to the sites of injury to facilitate tissue repair (ie, differentiation into smooth muscle cells and endothelial cells). The role of stem cells in TV and their homing mechanisms will be the subject of another article in this thematic series.

This report focuses on the role of chemokines in recruitment of leukocytes that participate in early graft dysfunction, acute rejection, and TV. As noted above, these early stages of injury (early graft dysfunction and acute rejection) are known to contribute to the late development of TV. For purposes of clarity, each class of chemokine–chemokine receptor axis are discussed separately.

**Chemokine/Chemokine Receptors**

Chemokines are a group of low-molecular-weight (8- to 11-kDa) cytokines that mediate cellular trafficking. The chemokine superfamily is divided into 4 subfamilies (C, CC, CXC, and CX3C) based on the presence of a conserved cysteine residue at the NH2 terminus. The C subfamily consists of XCL1 and XCL2, which attract lymphocytes. CC chemokines predominantly recruit mononuclear cells. CXC chemokines are further subdivided on the basis of the presence or absence of the sequence glutamic acid-leucine–arginine (ELR) near the NH2 terminal. ELR+ CXC chemokines are neutrophil chemoattractants with angiogenic properties. ELR− CXC chemokines are chemoattractants of lymphocytes with angiogetic properties. The fourth subclass of chemokines is CX3C subfamily; CX3CL1 is the only known member of this subfamily.

All chemokine actions are mediated through 7-transmembrane-spanning G protein–coupled receptors. These heterotrimeric G proteins are composed of α (defines the identity of the protein), β, and γ subunit. The chemokine receptors generally undergo internalization and phosphorylation following ligand binding. Interaction of a ligand with its receptor leads to exchange of GTP for GDP and the dissociation of the α subunit from the βγ subunit. The dissociated Gi and G0/γ can activate downstream signal transduction events.

In addition to their trafficking properties, chemokines have also been shown to have several extrachemotactic properties, such as cellular activation and differentiation. It has been shown that CCL3, CCL4, and CCL5 stimulate T-cell differentiation into Th-1 subtype. This effect is mediated both directly and indirectly via interleukin (IL)-12. In contrast, CCL2 polarizes T-lymphocyte differentiation to Th-2 subtype by suppressing IL-12 expression and stimulating IL-4 expression.

In addition, CCL2 also appears to have a direct effect on T-lymphocyte differentiation toward Th-2 subtype. Among CXCR3-binding chemokines, CXCL10 has been shown to enhance generation of tumor-specific T cells and protective immunity in an IL-12 gene therapy model.

Neutralization of CXCL10 in a murine model of Toxoplasma gondii infection results in impairment of antigen-specific T-cell generation. CXCL10 has also been shown to be important in generation of T-lymphocyte effector functions. Another study examined the extrachemotactic properties of CXCL9. CXCL9 was shown to stimulate T-lymphocyte proliferation and increase the number of interferon (IFN)-γ–producing T cells both in vivo and in vitro. These stimulatory effects are independent of IL-2, but are controlled by IFN-γ, and are found to occur in major histocompatibility complex
(MHC) class I and II and totally mismatched models. These studies highlight the extrachemotactic properties of some chemokines that is similar to the role of several classic cytokines.

**CC Chemokine/CC Chemokine Receptor**

**CC Chemokine Biology During Early Cardiac Allograft Dysfunction**

Early cardiac allograft dysfunction is attributable to several perioperative factors such as donor brain death, hypotension, cold storage, and reperfusion. These processes culminate in ischemia/reperfusion injury, an alloantigen-independent mechanism that is linked to the development of TV. Cold storage itself is associated with oxidative stress, cell death, and the release of inflammatory mediators, which activate both donor passenger and recipient leukocytes. The reperfusion of blood results in reoxygenation of ischemic tissue, which generates reactive oxygen intermediates. These reactive oxygen intermediates cause more cellular damage and the activation of nuclear factor-kB, an important transcription factor for chemokine production. The hallmark of early injury attributable to ischemia/reperfusion is inflammatory cell recruitment, and the CC chemokines appear to play a role in this process.

By using a rat heterotopic cardiac transplantation model, Tanaka et al demonstrated that longer cardiac allograft cold ischemia times caused a temporal increase in oxygen-derived free radicals, caspase activity, and tumor necrosis factor-α levels, that subsequently resulted in augmented cardiomyocyte apoptosis. They also found a significant upregulation of CCL2 expression in the cardiac allografts undergoing ischemia/reperfusion injury that paralleled allograft inflammation and tissue damage. Importantly, the increased levels of CCL2 during ischemia/reperfusion injury were associated with subsequent development of vessel fibrointimal thickening and luminal narrowing, the hallmarks of cardiac allograft TV. Yun et al have also shown that ischemia/reperfusion injury in murine cardiac allografts augmented CCL5 expression, which then paralleled intragraft leukocyte recruitment. Similar elevations in CCL2 expression were seen in a rat model of lung allograft ischemia/reperfusion injury, and in vivo neutralization of CCL2 led to a reduction in lung injury. These results suggest that ischemia/reperfusion injury in experimental models is associated with upregulation of CC chemokines (CCL2 and CCL5) that may participate in inflammatory cell recruitment to the graft and the subsequent graft injury.

**CC Chemokine Biology in Acute Rejection**

Multiple clinical studies have shown that acute allograft rejection episodes are a strong predisposing risk factor for the development of TV. Allograft rejection may be initiated by inherent nonspecific injury imposed on the allograft (such as ischemia/reperfusion, infectious diseases, etc), which activates components of innate immunity, causing an abnormally high inflammatory state within the allograft. These nonspecific inflammatory signals can activate resident donor antigen-presenting cells (APCs) and recipient APCs to mature and emigrate to draining lymphoid tissue. Within lymphoid tissue, APCs prime recipient T cells into alloreactive effector T cells. These alloantigen-primed effector T cells then traffic to the allograft and cause rejection. Important for the trafficking of: (1) APCs from the allograft to the draining lymph node; (2) the unprimed T cell to the APCs within the lymph node; and (3) the primed T cells from the lymph node to the allograft, are chemokines. Histopathologically, the hallmark of acute cardiac allograft rejection is the extravasation of mononuclear cells into the cardiac parenchyma resulting in damage and dysfunction (Figure 1).
Because CC chemokines are important in the recruitment of mononuclear cells, several investigators have examined the role of CC chemokines during cardiac allograft rejection.

By using a fully mismatched murine heart transplant model (BALB/c to C57BL/6/129), Gao et al found that the expression of CCL3 and CCL5 in the allografts were markedly upregulated. Additionally CCR1, the shared receptor for both CCL3 and CCL5, was found on mononuclear cells infiltrating cardiac allografts during acute rejection. To evaluate the effects of inhibiting the interactions of CCL3 and CCL5 with CCR1 during cardiac allograft rejection, they performed studies using a genetic approach. They used CCR1−/− mice on a C57BL/6 background as recipients of the BALB/c cardiac allografts and found a moderate increase in allograft survival from 7 to 14 days. Moreover, when given a short course of cyclosporin A (CsA), the CCR1−/− recipients accepted their allografts for greater than 100 days. Mechanistically, the allografts from CCR1−/− mice given CsA demonstrated a reduction in infiltrating leukocytes and cytokine/chemokine/receptor expression (ie, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IFN-γ, CCL2, CCL5, CCL3, CCL4, CXCL1, CCL11, CXCL10, CCR1, CCR2, and CCR5). Importantly, deletion of CCR1 on murine leukocytes had minimal effects on T-cell proliferation, suggesting CCR1−/− mice have a defect in cellular recruitment with impaired cytokine/chemokine amplification.

Horuk et al also evaluated the role of CCR1 during acute cardiac allograft rejection using a rat cardiac transplantation model. They inhibited the CC chemokine–CCR1 interaction through the use of a CCR1 antagonist called BX471. BX471 given alone to Lewis rat recipients improved cardiac allograft survival by only 2 days. The combination of CsA plus the BX471 had more dramatic results and improved cardiac allograft survival by 11 days. Mechanistically, they showed that the BX471 did not affect the pharmacokinetics of CsA but did attenuate the infiltration of mononuclear cells and reduced leukocyte adhesion to activated microvascular endothelium, via the downregulation of leukocyte β2 integrin and CD11b. These studies suggest that inhibiting the CC chemokine interaction with CCR1 reduces leukocyte integrin expression, decreasing the ability of these chemokines to facilitate the recruitment of mononuclear cells to the allograft during rejection (Figure 2).

Based on the finding of elevated levels of CCL3, CCL4, and CCL5, as well as their shared receptor, CCR5, during acute cardiac rejection, Gao et al evaluated the specific role of CCR5 during cardiac allograft rejection. Similar to CCR1, they found that CCR5 was expressed on graft infiltrating mononuclear cells. Using either in vivo neutralization studies of CCR5 or CCR5−/− recipient animals, they demonstrated that the allografts had a marked reduction in infiltrating/activated mononuclear cells (ie, cells expressing IL-2 receptor). More importantly, they demonstrated that both strategies of ablating CCR5 signaling could prolong allograft survival from 7 to 20 days. Even more profound results were seen when CsA was given to the CCR5−/− recipients (ie, permanent allograft survival without signs of leukocyte infiltration, interstitial fibrosis, or the development of TV). Interestingly, when individual ligands of CCR5 were depleted, there was no significant prolongation of allograft survival. More specifically, when CCL5−/− or CCL3−/− animals were used as recipients of cardiac allografts, there was no significant attenuation of cardiac allograft rejection. This led the authors to conclude that individual ligand inhibition has a negligible effect on rejection because it leaves the action of redundant chemokine ligands intact. However, others have shown in vivo depletion of CCL5 inhibits cardiac allograft rejection, as well as acute lung allograft rejection. The differences between these studies (anti-CCL5 antibody therapy given to allograft recipients versus CCL5−/− recipient mice) may be explained by anti-CCL5 antibody therapy neutralizing endogenous production of CCL5 from both donor and recipient cells. This strategy inhibits CCL5 interaction with all cells (both donor and recipients). This is in contrast to using the CCL5−/− recipient mice, which involves only the inhibition of CCL5 expressed from recipient cells, thus allowing donor-derived CCL5 to interact with donor and recipient cells expressing its receptors (ie, CCR1, CCR3, and CCR5). The interaction of donor-derived CCL5 may be enough to drive alloimmune injury/rejection. Future experiments involving CCL3, CCL4, and CCL5 knockout donors transplanted into CCL3, CCL4, and CCL5 knockout recipients, respectively, will be required to determine the role of individual CC chemokines, as compared to their appropriate receptor(s) biology during cardiac allograft rejection.

Amano et al also determined a role for CCR5 expressing cells in acute rejection of cardiac allografts using a fully mismatched murine heart transplant model (A/J to C57BL/6/129). Using fluorescence-activated cell-sorting analysis on cardiac allografts, they demonstrated that 50% of the infiltrating CD4 cells and 32% of the infiltrating CD8 cells expressed CCR5. Surprisingly, by immunohistochemical studies, they did not find infiltrating macrophages expressing CCR5. Furthermore, they found that allografts from the CCR5−/− recipients had a 4-fold reduction in infiltrating CD8 and CD4 cells and a 2-fold reduction in infiltrating macrophages, which led to prolongation of allograft survival from 9 to 11 days. These authors also indirectly evaluated the role of CCR5 expression on recipient T cells during cardiac allograft rejection. They found that splenocytes from CCR5−/− recipients of allografts had a significant reduction in IFN-γ– but not IL-4–producing splenocytes. In addition, they evaluated the role of passenger dendritic cells (DCs) in priming recipient T cells by injecting A/J donor DCs into CCR5−/− mice (naïve nontransplanted) and found a reduction in IFN-γ–producing splenocytes. Furthermore, when BALB/c donor hearts were depleted of passenger leukocytes and then transplanted into CCR5−/− mice, their splenocytes demonstrated a reduction in IFN-γ expressing cells. Collectively, these data suggest that: (1) CCR5 expression is important for polarizing alloreactive T cells toward a type I immune response, which has been previously shown to be important for acute rejection; and (2) CCR5 expression on T cells is important in trafficking cells to the lymphoid tissue to interact with donor/passerenger-derived DCs and recipient DCs, which are known to release CCR5 ligands (ie, CCL3 and CCL5).

Humoral rejection is known to mediate cardiac allograft dysfunction. Development of donor-specific antibodies
has emerged as a risk factor for acute cardiac rejection and TV.57–59 Using a murine heart transplant model (A/J to C57BL/6/129), Nozaki et al noted that the donor hearts were infiltrated with less mononuclear cells in CCR5⁻/⁻ recipients, yet there was only a modest prolongation of allograft survival.60 Notably, they found that the allografts from the CCR5⁻/⁻ mice had increased deposition of C3d in the large vessels and parenchymal capillaries. These recipients had high-serum titers of donor-specific antibody. In fact, sera from CCR5⁻/⁻ recipients transferred to RAG-1⁻/⁻ recipients led to deposition of allo-antibodies on donor endothelia. This interaction resulted in upregulation of chemokines and recruitment of macrophages/neutrophils to the allograft, which is among the pathological hallmarks of humoral rejection, a risk factor for the development of TV.57–59

Other studies involving solid organ transplant have also demonstrated a role for CCR5/ligand interactions during acute allograft rejection. For instance, survival of pancreatic islet cells were prolonged from 10 to 38 days in CCR5⁻/⁻ recipients.61 CCL5 has also been associated with renal allograft rejection.62–65 More specifically, CCL5 was found to be important in upregulating leukocyte integrins, allowing for firm adhesion of leukocytes to activated renal allograft endothelium (Figure 2). Inhibition of CCL5 interaction with its receptors using a CCL5 antagonist (ie, Met-RANTES) led to a reduction in renal graft injury.65 Animal studies have also demonstrated a correlation between elevated levels of CCL5 from rejecting lungs and the recruitment of intragraft mononuclear cells expressing CCR1 and CCR5.66–68

Studies involving human cardiac allograft recipients have also demonstrated a possible relationship between CC chemokines and acute cardiac rejection. Fahmy et al noted an association between increased expression of CCL5 and CCR5 in endomyocardial biopsies with acute cardiac allograft rejection in the first 3 months after transplantation.69 Additionally, other studies involving human heart transplant recipients found that CCL5 and CCR5 expression as well as the number of CCL5 positive cells (ie, CD45RO⁺ and mononuclear phagocytes) correlated with the presence and grade of acute rejection.70,71 Simeoni et al examined the role of CC chemokine polymorphism in acute rejection of cardiac allografts.72 Using a cohort of 158 heart transplant recipients, the authors examined CCR5 haplotypes E, F, and G (all of the −2459A allele known to cause increased CCR5 expression on leukocytes)73–75 and the CCL5 −403A variant (which has been shown to cause an increase in expression of CCL5).76 They demonstrated an association for the genetic polymorphism of CCR5 E haplotype and acute rejection episodes. Furthermore, because multiple genetic polymorphisms can coexist, they also evaluated the interplay of different alleles involving

Figure 2. Chemokines are presented to rolling leukocytes by stimulated endothelial cells. These chemokines activate leukocytes, upregulating integrins and allowing adherence to endothelium. CX3CL1, by itself, can cause leukocyte firm adhesion.
receptors/chemokines and acute rejection. CCR5 E haplotype and the CCL5 –403A allele were associated with an increased risk of developing acute rejection episodes after 3 months after transplantation. These human studies suggest that increased expression of CCL5 interacting with cells expressing high levels of CCR5 may be a risk factor for acute cardiac rejection.

CCL2 is known to be produced de novo by human islets, and its expression is increased in the presence of tumor necrosis factor-α and IL-1β.27 CCR2 is the receptor for CCL2, CCL7, CCL13, and CCL12, which are potent chemotactants for mononuclear cells and may be important for the promotion of a Th-2 response.77 By using a murine model of cardiac allograft rejection (BALB/c to C57BL/6), Abdi et al found that CCR2+/− recipients had a survival benefit from 8 to 12 days.78 CCR2−/− recipients had decreased IFN-γ- and increased IL-4-producing splenocytes when exposed to donor cells ex vivo. In addition, recovered allografts from CCR2−/− recipients expressed increased levels of IL-4, IL-5, and IL-10 and decreased levels of IFN-γ and IL-12. Moreover, the CCR2−/− mice failed to generate effector CD8+ cells. These same investigators used a fully mismatched heterotopic islet cell allograft rejection model (pancreatic islet allografts placed under the kidney capsules of recipient mice rendered diabetic by streptozotocin) and found increased expression of CCR2 and its ligands CCL2 and CCL12 during rejection.79 When islet cells were transplanted into CCR2−/− recipients, there was a prolonged allograft survival from 12 to 24 days. Furthermore, 25% of the CCR2−/− recipients exhibited long-term graft survival. Similarly, when using the same islet cell transplant model system, Lee et al demonstrated decreased rejection in CCR2−/− recipients. Mechanistically, they found that the interaction of CCL2/CCR2 contributed to alloreactive T-cell clonal expansion/proliferation and differentiation. In addition, further islet allograft survival was achieved by blockade of the CCL2/CCR2 pathway in conjunction with low-dose rapamycin therapy via a downregulation of the programmed death-1 (PD-1: PD-1/PD-L1) pathway.77 Collectively, these studies suggest that the abrogation of CCR2/ligand biology may cause a type 2 polarized immune response and a reduction in rejection of transplanted organs.

CCL17 and CCL22 bind to CCR4, a highly expressed receptor on type 2 cells that is used for homing of memory T cells.79,80 This receptor has also been identified on monocytes, Langerhans DCs, monocytes, NK cells, and platelets. Alferink et al demonstrated a significant prolongation of cardiac allograft survival when fully mismatched hearts were transplanted into immunosuppressed CCL17+/− recipients.81 Follow-up studies by Huser et al using CCR4−/− recipient mice also demonstrated prolonged cardiac allograft survival and even more profound effects with the addition of galium nitrate.82 Mechanistically, these studies suggest that inhibition of CCR4/ligand biology increases cardiac allograft survival by attenuating the interaction of DCs and T cells in secondary lymph nodes and by inhibiting the recruitment of CCR4 expressing monocytes and NK cells to the allograft (Figure 1).

One of the best inducers of rodent tolerance is the inhibition of CD40–CD154 interactions in conjunction with a donor-specific transfusion.83,84 In fact, this treatment leads to well-functioning allografts despite intragraft infiltrating mononuclear cells. This paucity of allograft injury suggests an active process is regulating mononuclear cells within the allograft (ie, Treg cells). This is consistent with data demonstrating the presence of Foxp3+ Treg cells in tolerized skin allografts after anti-CD4 antibody therapy. Foxp3 is a gene that encodes a forkhead/winged helix transcription factor, Scurfin, which is required for Treg cell development and thus is a marker for Treg cells. Based on these data, Lee et al evaluated the presence of Treg cells (allograft Foxp3 expression) in tolerized (anti-CD154 with a donor-specific transfusion) allografts, as compared to untreated allografts, as well as isografts and naïve hearts.83 They found a hierarchy of Foxp3 and CCR4 expression (anti-CD154 with a donor-specific transfusion >> untreated allografts >> isografts > naïve hearts). Importantly, cardiac allografts from CCR4−/− recipients treated with anti-CD154 with a donor-specific transfusion therapy had significant reductions in Foxp3 expression and rejected their cardiac grafts at a normal rate. This suggests that CCR4, in this model system, is critical for Treg cell recruitment. Overall, the studies mentioned above suggest that CCR4/ligand biology has multiple functions during allograft rejection (important in DC–T cell attraction for T cell priming, recruits injurious CD4 and NK cells, yet can recruit protective Treg cells). It will be important to determine the specific detrimental and protective mechanism of CCR4/ligand biology during the pathogenesis of TV.

CC Chemokine Biology in TV

The importance of CCR5 in experimental TV is further highlighted by the several studies. Previous studies have demonstrated that a brief course of CD4 monoclonal antibody therapy induces donor-specific cardiac allograft tolerance in the fully mismatched (BALB/c to C57BL/6) models; however, these allografts do develop TV.88,89 When CD4 monoclonal antibody therapy was given to CCR1−/− recipients, the
vessels were protected from TV. Confirmatory studies were performed using the class II mismatched model (bm12 to C57BL/6 mice) that reproducibly develops TV in 24 to 40 days. In this model system, the allografts from CCR1−/− recipients were free of TV out to 100 days. Similar results were found with the CCR5−/− recipients, demonstrating a dramatic reduction in TV. Using an antagonist of chemokine receptors CCR1 and CCR5, Yun et al demonstrated a significant decrease in the severity of intimal lesions in a murine model of TV. The authors showed that CCR1 and CCR5 blockade attenuated TV in this experimental model via: (1) reduction of CD4 and CD8 lymphocytes and macrophages recruitment; and (2) suppression of donor-specific proliferative responses. In a primate model, CCR5 blockade with cyclosporine was associated with prolonged allograft survival, delayed antibody production, and attenuation of TV.

Using an in vivo model of human allogeneic T cell–mediated vascular injury (intimal lesions resembling TV), Burns et al showed that chemokine receptors CCR5 and CXCR3 are expressed by the T cells infiltrating the intima and adventitia of human coronary arteries. CCR5 and CXCR3-binding chemokines were also produced by the graft vascular cells. Interestingly, despite production of chemokine ligands by the medial compartment of the vessel wall, there was a paucity of infiltrating T cells, suggesting the notion that the arterial media may be a site of immunologic privilege. The role of CCR5 and CXCR3 has recently been examined in a robust murine heart transplant model. Combined blockade of CCR5 and CXCR3 resulted in indefinite survival of the donor hearts. Importantly, the long-surviving donor hearts did not display any manifestations of TV. Additional studies suggested that the mechanism of the beneficial effects of combined CCR5 and CXCR3 blockade in this model may be attributable to induction of CD25 regulatory T cells and attenuation of alloantigen-specific T-lymphocyte proliferation. The role of chemokine receptor blockade in controlling TV is intriguing and deserves further investigation.

Several clinical studies have examined the expression of CCR5 and its corresponding ligands in human TV. In a study of explanted human hearts with TV, 50% to 75% of infiltrating cells in the intimal and adventitial layers of the vessel wall expressed CCR5 and CXCR3. Moreover, CCR5 and CXCR3-binding ligands were found on a large number of mononuclear cells and vessel wall cells, in proximity to the CCR5- and CXCR3-expressing cells. More recently, Hagemeijer et al demonstrated that a large number of CD4+ T cells in the neointima of human TV are skewed toward an activated memory Th-1 phenotype and express CCR5, CXCR3, and CX3CR1. This population of T cells may be responsible for the chronic inflammatory responses commonly seen in TV. This study also showed a significant population of CD4+ T cells expressing CCR3 and CCR8 (Th-2 phenotype). The presence of this population in human TV may explain the proliferative response/matrix deposition/remodeling, which are also characteristic features of TV.

CCL5, a ligand for CCR5, has also been shown to be upregulated in both human and experimental chronic lung allograft dysfunction, also known as bronchiolitis obliterans syndrome (BOS). Neutralization of endogenous CCL5 in the experimental model reduced the numbers of graft infiltrating CD4 T cells, preserved allograft airway lumen patency, and attenuated early epithelial injury. Presently, there are no available studies examining the role of CCR2/ligand biology in cardiac TV. However, in lung transplantation, CCL2 levels in bronchoalveolar lavage fluid from patients with acute rejection and BOS were markedly elevated, biologically active, and localized to airway epithelium and mononuclear cells. Translational studies, using a murine model of BOS, were consistent with human data demonstrating CCL2 localizing to airway columnar epithelium and infiltrating mononuclear cells. Furthermore, CCL2 levels paralleled the recruitment of mononuclear cells and cellular expression of its receptor, CCR2. A genetic approach was used to determine the effects of inhibiting CCR2/ligand biology on BOS. Allografts from CCR2−/− mice demonstrated significant reductions in mononuclear phagocytes that were not accompanied by significant reductions in lymphocytes. Histopathologic assessment of these allografts demonstrated significantly less matrix deposition, airway obliteration, and epithelial injury. These findings suggest that CCR2-expressing cells are pivotal during the pathogenesis of BOS. These results were corroborated in a rat heterotopic subcutaneous model of BOS. Importantly, these studies demonstrate that CCR2/ligand biology is important in the continuum of acute to chronic allograft rejection.

Unfortunately, to date, there are no studies that demonstrate whether specific receptors/CC chemokines polymorphisms are associated with TV. However, there are studies involving human chemokine receptor polymorphisms that impact on long-term renal allograft survival. CCR5Δ32 is a nonfunctional mutant allele of CCR5 and, in a multicenter study, was found to be associated with prolonged renal allograft survival. However, a more recent study demonstrated that recipients with the CCR5Δ32 mutation developed acute cellular rejection after renal transplant at a higher rate than control recipients. More specifically, despite the lack of a functional CCR5, dense T-cell infiltration into the allograft was detectable. This indicates that alternative chemokine pathways can promote T-cell trafficking during acute rejection. Thus, the association of CCR5Δ32 to the long-term renal allograft survival may be explained by the effects of CCR5 on memory CD8 T-cell generation, which likely influences chronic rejection.

These studies suggest that although CC chemokines may be a potential target to inhibit inflammatory/fibroproliferative disorders such as TV, the inhibition of multiple chemokine pathways simultaneously after transplantation may be more fruitful.

**CX3C Chemokine/CX3C Chemokine Receptor**

CXCL1 is involved in direct leukocyte activation, chemotaxis, and adhesion through its interaction with CXCR1. CXCL1 has been associated with murine acute cardiac rejection: CXCL1 was localized to endothelium, epicardium, endocardium, myocardium, and infiltrating mononuclear cells in the allografts. In vivo antibody depletion of CXCL1 increased allograft survival without gross disruption in graft leukocyte infiltration. Interestingly, when CXCR1−/−
recipients were used, there was no effect on allograft survival, even though there was a reduction in graft-infiltrating NK cells. The addition of CsA to the CX3CR1−/− mice prolonged allograft survival by 18 days. Together, these studies suggest that NK cells of the innate immune system and T cells of the adaptive immune system act synergistically to cause rejection and CXCL1 (both soluble and tethered from recipient/donor cells)/CXCR1 interaction is important for the recruitment of NK cells to the allograft (Figure 2). CXCL1 has also been found on the endothelium, intimal, and adventitia of human coronary arteries with TV, in close association with CXCR1-expressing cells. However, there are no experimental or clinical studies demonstrating a cause and effect relationship.

The human CX3CR1 V249I allele has been shown to have a reduced number of CX3CL1 binding sites and binding affinity on peripheral blood mononuclear cells. The CX3CR1 V249I allele in recipients with CCR5 E haplotype had a reduction in acute rejection episodes, suggesting a cooperative action of these 2 receptors in inflammatory cell recruitment.

**CXC Chemokine/CXC Chemokine Receptor**

CXC chemokines are divided based on the presence or absence of the sequence glutamic acid–leucine–arginine (ELR) near the amino terminal. ELR+ chemokines recruit neutrophils and have angiogenic properties. ELR− CXC chemokines attract lymphocytes with angiostatic properties.

**CXC Chemokine Biology During Early Cardiac Allograft Dysfunction**

ELR+ CXC chemokines, known chemoattractants for neutrophils, have been shown to participate in ischemia/reperfusion injury in solid organ transplantation. In both a murine cardiac allograft and isograft models, as well as a rat lung transplant model, increased levels of ELR+ CXC chemokines were detected, correlating with neutrophil infiltration. Neutralization of CXC chemokines in the heart model prolonged allograft survival by 15 days and decreased inflammatory cell recruitment. In the lung model, inhibition of CXCR2/CXCR2 ligand interaction was associated with a reduction in neutrophil infiltration and graft injury. Elevated levels of ELR+ CXC chemokines in bronchoalveolar lavage fluid of patients after lung transplantation have also been associated with ischemia/reperfusion injury. Collectively, these studies suggest that ELR+ CXC chemokines and their interaction with their respective receptors may play a role in neutrophil recruitment and mediate early allograft injury, which is a known risk factor for development of TV.

ELR− CXC chemokines include CXCL9, CXCL10, and CXCL11; they bind to their shared receptor CXCR3, which is expressed predominantly on Th-1 cells, some B cells, and natural killer cells. ELR− CXC chemokines participate in lymphocyte recruitment in virtually all stages after transplantation. Zhai et al found increased expression of CXCL9, CXCL10, and CXCL11 in a rat ischemia/reperfusion model, correlating with T-cell recruitment. In vivo neutralization of CXCR3 led to reduction in CXCR3+ CD4 T-lymphocyte recruitment, amelioration of reperfusion injury, and improved allograft survival. CXCL10 has also been shown to be elevated in ischemia/reperfusion model in a murine cardiac allograft and isograft model.

**CXC Chemokine Biology in Acute Rejection**

In reference to acute cellular rejection, several experimental and clinical studies have implicated the ELR− CXC chemokines/CXCR3 axis. Hancock et al have shown that full MHC-mismatched donor hearts had prolonged survival in CXCR3−/− mice; addition of cyclosporine led to permanent graft survival without manifestations of TV. Studies using blocking antibodies to CXCR3 have also yielded similar results. However, several recent reports have not been able to reproduce similar beneficial effects. Two independent groups have reported that CXCR3−/− and control mice reject full MHC-mismatched donor hearts at the same rate. These studies also showed that T-cell infiltration into the rejected hearts were similar in the 2 groups. Using a multiple minor antigens mismatched heart model, Kwan et al have recently shown that the acceptance of the graft, severity of rejection, and infiltration of T cells are similar in CXCR3−/− recipients versus control recipients. On the other hand, graft survival in animals experiencing acute rejection was moderately prolonged in CXCR3−/− recipients (13.9 ± 3.5 versus 21.3 ± 11.0 days, P < 0.05). Alloantigen-specific CD8 lymphocytes were found predominantly in the splenic and peripheral blood compartments of CXCR3−/− mice and not in the donor heart. Furthermore, CXCR3−/− recipients with long-term graft survival had less infiltration with CD8 lymphocytes and displayed less TV. These observations suggest that CXCR3 may play a role in trafficking of alloreactive T-lymphocytes in alloimmune responses.

In contrast to the above, the impact of neutralization of individual CXCR3-binding chemokines on experimental acute rejection is modest, at best. Cardiac allografts had similar survival in CXCL10−/− recipients compared to control recipients. Interestingly, when CXCL10−/− hearts were used as donors, the grafts survived to more than 40 days, suggesting that donor-derived CXCL10 is important in recruitment of lymphocytes to the graft. When comparing CXCL10 to CXCL9 in a mouse heterotopic heart transplant model, CXCL9 expression was 5-fold greater than CXCL10 and neutralization of CXCL9 was more potent in prolonging allograft survival. Translational studies also have shown a role in CXC–CXCR3 axis in acute lung rejection. In a rat heterotopic lung transplant model, CXCL9 and CXCL10 expression was elevated in acute rejection and paralleled inflammatory cell recruitment. Neutralization of CXCL9 reduced infiltration of mononuclear cells and attenuated acute rejection. Addition of low does of CsA prolonged allograft survival further. The modest importance of CXCL9 and CXCL10 in acute rejection has also been corroborated in experimental murine skin graft models, as well as islet graft models.

In addition to the above experimental studies, several clinical reports have examined the role of ELR− CXC chemokine–CXCR3 axis in acute rejection. In human heart transplantation, CXCL10 expression has been shown to be upregulated in endomyocardial biopsies of patients undergoing acute rejection.
Interestingly, in 4 patients who developed TV, there was a trend toward persistent endomyocardial infiltration with CXCR3 T cells. Additionally, bronchoalveolar lavage fluid from lung transplant recipients with acute rejection demonstrate elevated levels of CXCL10, CXCL9, and CXCL11 levels and increased number of CXCR3 lymphocytes. Colocalization studies of human lung biopsies showed that the CXCL10 is likely produced by lung macrophages/epithelial cells, leading to recruitment of CXCR3 cells.

CXC Chemokine Biology in TV

ELR CXC chemokines have been implicated in the development of BOS (chronic rejection of lung transplantation). ELR CXC chemokines not only mediate neutrophil recruitment but can also promote angiogenesis. Their shared endothelial cell receptor is CXCR2. Elevated levels of multiple ELR CXC chemokines correlated with the presence of BOS. “Proof-of-concept” studies using a murine model of BOS demonstrated not only an early neutrophil infiltration but also marked vascular remodeling in the tracheal allografts. In addition, ELR CXC chemokines were persistently expressed in the tracheal allografts even in the absence of significant neutrophil infiltration and were temporally associated with vascular remodeling during fibro–obliteration of the tracheal allograft. Furthermore, treatment with anti-CXCR2 antibody inhibited early neutrophil infiltration and later vascular remodeling, which resulted in the attenuation of murine BOS. A more profound attenuation of fibro–obliteration was seen when CXCR2 mice received CsA. This supports the notion that the CXCR2/CXCR2 ligand biological axis has a bimodal function during the course of BOS: early, it is important for neutrophil recruitment, and, later, during fibro–obliteration, it is important for vascular remodeling independent of neutrophil recruitment.

There is limited experimental or clinical information on the role of ELR CXC chemokines in the development of TV. Using a mouse model of TV, Yun et al observed: (1) there was sustained upregulation of CXCL9 gene transcript and protein level, paralleling T-lymphocyte recruitment and preceding TV development; and (2) neutralization of CXCL9 reduced CD4 lymphocyte recruitment and attenuated TV lesions but was insufficient to prevent intimal lesions. In a mouse model of BOS, Belperio et al demonstrated increased expression of ELR CXC chemokines, paralleling CXCR3 mononuclear cell recruitment. Neutralization of CXCR3 or its ligands CXCL9 and CXCL10 reduced CXCR3 cell recruitment and attenuated the severity of BOS. On the other hand, Medoff et al could not demonstrate any beneficial effect from CXCL9 or CXCL10 gene deletion in a mouse model of BOS. The discrepancy between this study and the multiple studies previously cited might be attributable to the nonconditional knockout mice that were used as recipients.

There is limited clinical information on the role of ELR CXC chemokine in TV. Heart transplant recipients with TV have been shown to have elevated plasma CXCL11 level when compared to heart transplant recipients without TV. CXCL11 was also present on endothelial cells of graft vessels with TV; CXCR3 mononuclear cells were found within TV lesions, suggesting a causative role in their recruitment. In 11 heart transplant recipients, Zhao et al showed an association between upregulation of CXCR3 ligands and TV. The existing body of experimental and clinical knowledge suggest that the CXC–CXCR3 axis is important in recruit-
ment of mononuclear cells in the continuum from early perioperative injury to the alloimmune mediated injury to the graft (Figure 3). Although proof-of-concept clinical studies are lacking, it is generally accepted that deletion or neutralization of individual CXC chemokine ligands may have minimal or at best modest effect on allograft survival and/or development of TV. In contrast, their common receptor, CXCR3, is more important in recruitment of mononuclear cells and its deletion/neutralization may serve as a strategy to control leukocyte trafficking after transplantation.

**Conclusion**

In summary, several human studies have suggested a role for chemokines in TV. Furthermore, translational studies in animal models of allograft dysfunction have demonstrated proof of principle that chemokine–chemokine receptor interactions play a pivotal role in mediating the leukocyte infiltration that leads to the continuum of early allograft dysfunction to TV. The future studies of chemokine–chemokine receptor interactions will lead to the development of new paradigms to understand the pathogenesis of TV. Furthermore, they should pave the way for the development of pharmaceutical agents that will target chemokine biology and provide new treatments that will ultimately enhance long-term cardiac allograft survival.

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**Disclosures**

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Belperio and Ardehali Chemokines and Transplant Vasculopathy 465


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