Published in final edited form as:

Clin Lab Med. 2008 September ; 28(3): 441–vii. doi:10.1016/j.cl.2008.07.004.

Chemokine-Directed Strategies to Attenuate Allograft Rejection

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The major cause of allograft injury and failure (ie, rejection) is the consequence of the coordinated trafficking of donor-reactive T cells and the expression of their effector functions at the vascular endothelial surface and within graft parenchymal tissues. The trafficking of antigen-primed T cells and other leukocyte populations to tissue sites of inflammation requires slowing of the cells to roll on the vascular endothelium followed by arrest [1]. The stopped cells are then often directed through the endothelial barrier and into the tissue parenchyma. Leukocyte rolling and arrest occur under vascular shear stress and are under the control of specific adhesion molecules complementarily expressed by the leukocytes and the endothelium. Although rolling is primarily mediated by endothelial expressed selectins interacting with binding proteins expressed by the leukocytes, arrest is mediated by the synergistic activities of two sets of molecules on the leukocytes and their respective ligands on the endothelium and the tissue parenchyma: (1) integrins and other adhesion molecules, and (2) chemokines and other chemoattractant molecules. This article focuses on the induced expression and role of chemokines in the graft and expression of chemokine receptors by leukocytes infiltrating grafts to cause injury. Emphasis is placed on strategies that have been designed to block the functions of these receptors and inhibit leukocyte interaction with the graft in an attempt to attenuate graft injury and improve long-term graft outcome.

The chemokines and chemokine receptors

The chemokines are a family of approximately 50 cytokines that direct cell migration during inflammatory situations and also function to position leukocytes in the bone marrow, thymus, and peripheral lymphoid tissues during the development of lymphoid architecture [2,3]. The chemokines are divided into four families based on conserved cysteine residues in the amino terminal end of the molecule. These four families include the CXC, the CC, the CX₃C, and the C chemokines. For the purposes of this article, individual chemokines are referred to by the currently recommended nomenclature rather than the older designations. Table 1 provides the list of representative chemokines, their old names, their new designations, and sources of their production.

Chemokines mediate their function by binding to 7 transmembrane-spanning receptors expressed on leukocytes and other cells. There are 20 human chemokine receptors that are differentially expressed on leukocyte populations and direct the movement or activation of the receptor-bearing leukocytes to the chemokines produced in the tissue site. The chemokine

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receptors are coupled to G proteins that are activated following receptor–ligand interactions to mediate polymerization/depolymerization of actin and in this way regulate cell motility. The chemokine proteins possess heparin-binding properties and, in most cases, are not presented to receptor-bearing cells as soluble proteins but as multimers noncovalently linked to proteoglycans on the surfaces of cells [4,5]. This manner of solid phase presentation is likely to localize chemokine gradients at particular tissue sites for the leukocytes expressing the specific chemokine receptor.

Several principles concerning the expression and activation of chemokine receptors are worth noting. First, leukocytes of the innate immune system, primarily neutrophils, macrophages, eosinophils and mast cells, constitutively express specific chemokine receptors. The production of specific chemokine ligands directs these sentinel/circulating leukocytes to tissue sites of inflammation, allowing penetration of the endothelial and epithelial barriers. Second, chemokine receptor engagement of its ligand results in G protein coupled signals through the GTPases Rho and Rap1 that induce conformational changes in integrins on the cell surface of the leukocyte, resulting in integrin activation with the consequence being firm adhesion of the leukocyte on the surface of the endothelial barrier [6-9]. Third, ligand engagement of chemokine receptors on granulocytes induces granule release [10,11]. For example, CXCR1 engagement of CXCL8 (ie, IL-8) stimulates neutrophils to release azurophilic and tertiary granules containing reactive oxygen species, cytokines, and proteases, mediators of tissue injury. Fourth, naïve T and B lymphocytes express specific sets of chemokine receptors that direct their positioning in lymphoid tissues. On cellular activation such as that occurring during interaction with antigen or antigen-presenting cells, the B and T cells are stimulated to express different sets of chemokine receptors. In conjunction with activation induced changes in the expression of adhesion molecules, the lymphocytes are directed out of the lymphoid tissue and into the vasculature.

Chemokines and chemokine receptors in allograft rejection

The appearance of specific sets of chemokines and chemokine receptor pairs in allografts during rejection reflects the different immune compartments (ie, nonadaptive inflammatory versus donor-specific/adaptive responses) that mediate graft injury. The first inflammatory processes are induced by the surgical tissue trauma and ischemia-reperfusion injury imposed on the graft, inherent processes of transplantation. This early inflammation includes the production of acute phase cytokines (IL-1, IL-6 and TNF α) and reactive oxygen species and the activation of complement [12-15]. These inflammatory mediators rapidly stimulate the endothelium and other cells in the graft to produce chemokines that direct neutrophils and macrophages into the grafts. The chemokines involved in this trafficking include the major neutrophil chemoattractant in humans IL-8 (mice lack both the gene encoding IL-8 and its receptor, CXCR1), and CXCL1 and CXCL2, the major neutrophil chemoattractants in mice, and the monocyte/macrophage chemoattractants CCL2, CCL3, and CCL4. The expression of these chemokines at early times posttransplant is observed in both iso- and allografts with equivalent kinetics and levels and during reperfusion of ischemic organs in experimental ischemia/reperfusion models. In animal transplant models, the early posttransplant induction of neutrophil and macrophage chemoattractant chemokines, particularly CXCL1, CXCL2, CCL2 and CCL3, is mirrored by the temporal infiltration of neutrophils and macrophages into skin and heart iso- and allografts of various major and minor histocompatibilities [16-21]. In clinical renal grafts, induction of IL-8 mRNA is observed within 30 minutes of graft declamping with much higher levels observed in cadaver versus living donor grafts [22]. High levels of IL-8 are also observed during reperfusion of lung transplants [23]. The direct correlation between ischemic time and early IL-8 mRNA levels in renal and lung grafts is consistent with the higher level of tissue injury observed following prolonged ischemic times [24-28]. Increased IL-8–mediated neutrophil infiltration and activation is likely to play a role

in the association between prolonged ischemia and the increased incidence of delayed graft function and poorer graft outcome.

One critical consequence of the cytokines and other proinflammatory mediators produced early in the allograft is the activation of interstitial dendritic cells to emigrate from the graft and into graft recipient lymphoid tissues. These mediators, particularly TNF α and possibly heat shock/stress proteins binding to TLR4, induce the expression of CCR7 on the dendritic cells, and after the dendritic cells have left the graft and entered the vasculature, this receptor guides the dendritic cells into the CCL19/CCL21 rich T cell zones of the lymphoid tissue draining the graft where interaction with naïve T cells is promoted [29-31]. Productive interaction with donor antigen-reactive T cells through the direct alloantigen presentation pathway results in the activation of the T cells and their development to effector T cells. Similar mechanisms are likely to mediate recipient-derived monocyte infiltration into the allograft and, following their maturation into dendritic cells, trafficking back to the lymphoid tissues to activate T cells through the indirect pathway of alloantigen presentation [32].

Before donor-reactive T cell activation through the direct and indirect antigen presentation pathways, the T cells in the lymphoid tissue express adhesion molecules and chemokine receptors that promote their positioning in the T cell rich zones of the lymphoid tissue and their interactions with mature/activated antigen-presenting dendritic cells [33]. During activation the T cells down-regulate adhesion molecules and chemokine receptors promoting retention in lymphoid tissues and up-regulate adhesion molecules (selectin-binding proteins and integrins) and chemokine receptors that facilitate their trafficking to sites of inflammation. Under the influence of the high levels of inflammation accompanying a transplant, most donor antigen-activated T cells express CXCR3 and CCR5 [34,35].

A second set of inflammatory processes is initiated as these donor antigen-primed T cells leave the lymphoid tissue, enter the vasculature, and are directed to the allograft. The T cells interact with donor antigens expressed by the graft endothelium that activate the T cells to produce cytokines, including IFN- γ . This IFN- γ , in turn, stimulates cells in the graft to produce chemokines including CXCL9, CXCL10, and CCL5, that amplify the infiltration of donor antigen-primed effector T cells and other CXCR3 and CCR5 expressing leukocytes into the graft. Similar to chemokines, IFN- γ and other cytokines bind to proteoglycans and on binding to cytokine receptors expressed on the infiltrating leukocytes stimulate the cells including neutrophils and macrophages to produce these T cell chemoattractant chemokines in the graft parenchymal tissue.

In addition to effects on receptor expressing leukocytes, CXC chemokines have important effects on endothelial cells. The CXC chemokines that have an amino terminal ELR motif (ELR⁺) have angiogenic effects on endothelial cells, likely through binding to CXCR2 on endothelial cells [36]. In contrast, CXC chemokines lacking this motif (ELR⁻) inhibit angiogenesis. In humans, there are two alternative splice isoforms of CXCR3, CXCR3A and CXCR3B. CXCR3A is expressed on mononuclear leukocytes and on T cells differentiating to the IFN- γ producing functional phenotype. CXCR3B is constitutively expressed on endothelial cells and engagement results in growth arrest. The role of ELR⁺ and ELR⁻ CXC chemokines has been widely studied in tumorigenesis [36]. ELR⁺ chemokines are produced early in allografts in response to the inflammation of ischemia reperfusion and ELR⁻ chemokines are produced at high levels during T cell attack on the graft. The angiogenic and angiostatic effects of these chemokines on the endothelium of allografts are relatively unknown at this time.

As with the induction of chemokines in grafts of experimental models, the production of chemokines directing neutrophils and macrophages into grafts is observed early following reperfusion of clinical transplants including heart and kidney grafts. Furthermore, chemokines

and chemokine receptors associated with antigen primed T cell infiltration are detectable during acute rejection episodes and during the development of vasculopathy and fibrosis in clinical heart, kidney, and lung transplants. Because the expression levels of many of these chemokines are significantly associated with rejection there is considerable interest in the use of these chemokines as biomarkers to indicate the presence of ongoing rejection. The application of this approach in heart transplants is limited to endomyocardial biopsies in which the presence of CXCR3 and its ligands, CXCL9 and CXCL10, and CCR5 and CCL5 have been found to significantly correlate with the presence and International Society for Heart and Lung Transplantation grade of rejection [37-39]. In renal transplant patients, mRNA levels of CXCL9 and CXCL10 mRNA in urine sediments and protein levels in the urine have significant correlations with ongoing rejection [40,41]. Similarly, the detection of elevated levels of these and other chemokines in the bronchial alveolar lavage fluid of lung transplant patients is associated with the development of bronchiolar obliterans syndrome, the major cause of lung transplant loss [42-44]. Considerable effort continues to identify chemokines that predict imminent rejection or the development of chronic injury in grafts.

Experimental strategies testing the role of chemokine/chemokine receptor pairs in allograft rejection

The observed induction of specific chemokines in experimental and clinical allografts during cell-mediated injury raised the hypothesis that these cytokines play a role in directing leukocyte infiltration into the allografts. This hypothesis predicted that interference with the function of specific chemokine/chemokine receptors would inhibit donor antigen-primed T cell infiltration into allografts, improving graft survival. Initial approaches to investigate this role of chemokines tested the effect of specific anti-chemokine antibodies on cellular infiltration into grafts and on graft outcome.

Many studies testing chemokine antagonism have been directed at components mediating ischemia-reperfusion injury with the expectation that graft survival would be improved by attenuating this early posttransplant tissue injury. In a mouse model, recipient treatment at the time of reperfusion of major histocompatibility complex (MHC)-mismatched cardiac allografts (A/J → C57BL/6) with a single dose of rabbit anti-CXCL1/KC antiserum extended allograft survival up to 2 weeks beyond the survival observed in control serum treated recipients [45]. This treatment also markedly decreased mononuclear cell infiltration into the allografts when examined at day 7 posttransplant without affecting the priming of donor-reactive T cells. Similarly, A/J heart allografts in C57BL/6 recipients treated with goat antiserum to CXCR2 also had prolonged survival of 7 to 10 days, which is explained, at least in part, by the ability of the antiserum to deplete recipient neutrophils [46]. Consistent with these experiments, A/J allograft survival in CXCR2^{-/-} recipients was extended for about 7 days when compared with allografts in wild-type recipients. Interference with CXCL1/CXCR2 also inhibited neutrophil infiltration and decreased lung allograft injury in orthotopically transplanted MHC-mismatched lung allografts in a rat model [42]. These results suggested the importance of early neutrophil allograft infiltration in mediating early graft injury during ischemia/reperfusion injury and facilitating subsequent donor antigen-primed T cell infiltration into the grafts. In support of this hypothesis, antibodies to CXCL1 and CXCL2 synergized with short-term T cell costimulatory blockade to prolong long-term survival (>100 days) of MHC-mismatched cardiac allografts in a murine model [46].

During development of donor antigen-activated T cells to IFN- γ -producing/cytotoxic cells, CXCR3 and CCR5 expression is induced on the T cells [34,35,47]. This expression suggested that these receptors and their ligands might be effective targets to develop reagents that would inhibit donor antigen-primed T cell infiltration into allografts. These targets were further suggested by the presence of CXCR3 and CCR5 ligands during acute cell-mediated rejection

of allografts in animal models and in clinical transplants [17,19,21,37-39]. Early studies in murine models indicated the ability of antibodies to CXCL9 to promote long-term survival of class II MHC mismatched skin allografts and to extend the survival of MHC-mismatched heart allografts up to 12 days longer than in control antibody-treated recipients [18,48]. Transplantation of MHC-mismatched hearts from CXCL10^{-/-} donors also resulted in a greater than 30 day extension in survival versus grafts from wild-type donors whereas no advantage in survival was observed when heart allografts from wild-type recipients were transplanted to CXCL10^{-/-} recipients [49]. More excitement in the field was generated by the report that MHC-mismatched cardiac allografts from BALB/c donors survived up to 60 days posttransplant in CXCR3^{-/-} (versus 7–10 days in wild type) recipients and that addition of low-dose cyclosporin A completely abrogated rejection in the CXCR3-deficient recipients. [50]. Unfortunately, these results were not reproduced in several other laboratories testing MHC-mismatched heart allograft survival in the same and in independently generated CXCR3^{-/-} recipients indicating that recipient expression of CXCR3 is not required to reject cardiac allografts [51-53]. The reasons for the discrepancies between these two sets of studies are unclear at this time.

Use of CCR5^{-/-} mice as recipients of MHC heart allografts has also been reported to result in extended graft survival and when combined with cyclosporin A results in long-term allograft survival [54]. These data parallel a clinical study reporting longer survival of kidney transplants in patients who are homozygous for the gene encoding a form of CCR5 (CCR5Δ32) that is unable to bind its ligands [55]. Subsequent clinical studies have been unable to confirm this advantage in kidney or liver transplant patients who had homozygous expression of CCR5Δ32 [56,57]. In addition, recent studies in mouse models have indicated no extension in MHC-mismatched cardiac or renal allograft survival in CCR5^{-/-} recipients [58,59]. These allografts are rejected, however, with low mononuclear cell infiltration but intense antibody deposition in graft capillaries indicating antibody mediated rejection. The anti-donor antibody response in CCR5^{-/-} cardiac and renal allograft recipients is 15- to 40-fold higher than that observed in wild-type recipients indicating a dysregulation of the antibody response in the absence of recipient CCR5 expression. A marked increase in donor-reactive CD4 T cells producing IL-4 is likely to contribute to the dysregulated antibody response in the CCR5-deficient recipients [60].

Because antagonism of single chemokines/chemokine receptor pairs has had, for the most part, moderate effects in attenuating T cell infiltration and promoting allograft survival, attempts to target two receptors in allograft recipients have also been reported. Treatment of CCR5^{-/-} recipients with a goat antiserum to CXCR3 resulted in long-term (>100 days) survival of MHC-mismatched heart allografts [61]. The CXCR3 antiserum does not deplete activated T cells in the recipients but does have an effect on the magnitude of donor-reactive T cell priming. Whether the absence of rejection in this model is mediated by an effect on T cell priming or is mediated by effects on primed T cell trafficking to the graft are not clear. Treatment of C57BL/6 recipients of BALB/c heart allografts with a nonpeptide antagonist of both CCR5 and CXCR3 prolongs graft survival for only a few days [62].

In certain instances, the induction of particular chemokines in allografts may actually be beneficial to graft survival. In wild-type C57BL/6 mice, peritransplant treatment with anti-CD154 mAb plus donor spleen cells (DST) results in long-term survival of MHC-mismatched (BALB/c) heart allografts and this survival is associated with increased expression of the T regulatory cell (Treg) marker FoxP3, the chemokine receptor CCR4, and its ligand CCL22 in the allograft at day 7 posttransplant [63]. In contrast, long-term allograft survival is not observed in CCR4^{-/-} recipients and the rejecting hearts have low levels of FoxP3 and CCL22 expression. These data suggest that anti-CD154 mAb/DST-induced CCR4⁺ Tregs are directed from the spleen to the allograft under the influence of CCL22. In support of this, anti-CD154

mAb mediated long-term survival of MHC-mismatched heart allografts is abrogated by administering the TLR9 agonist CpG at the time of transplantation and is associated with decreased levels of CCR4 ligands and FoxP3⁺ Tregs in the allografts on day 7 posttransplant [64]. Collectively, these studies indicate that the induction of CCR4 ligands in allografts may serve to direct the infiltration of CCR4 expressing Tregs into the allograft and inhibit rejection of the allograft. Mechanisms by which CCR4 ligands are induced in allografts are unknown and may prove to be a necessary component of strategies to induce long-term allograft survival.

Transition to the development of receptor antagonists for therapy in transplantation

The efficacy of anti-chemokine and anti-chemokine receptor antibodies in attenuating autoimmune disease and allograft rejection has spurred a great amount of research and development of chemokine receptor antagonists. This development has included potential chemokine receptor antagonists targeted at components of the innate and the adaptive immune systems.

Initial strategies have focused on the efficacy of Met-RANTES in which the addition of a methionine residue to the amino terminus of RANTES alters the chemokine into a CCR1/CCR5 antagonist [65]. In a rat chronic renal allograft rejection model (Fisher → Lew), daily treatment of recipients with Met-RANTES decreased T cell and monocyte infiltration into the allografts as well as the intensity of vascular and tubular injury when examined on day 7 posttransplant [66]. When this study was extended to an acute rejection renal allograft model (BN → Lew) treatment with Met-RANTES synergized with cyclosporin A to decrease interstitial inflammation when compared with allograft recipients treated with either agent alone. Similarly, in a mouse model of chronic rejection development in class II MHC mismatched heart allografts (B6.H-2^{bm12} → C57BL/6), daily treatment with Met-RANTES beginning on day 4 posttransplant reduced the infiltration of T cells and macrophages into the grafts and attenuated the development of vasculopathy when examined on day 56 posttransplant [67].

More recent effort has turned from modification of chemokine proteins to the synthesis of receptor antagonists. Recipient treatment with a nonpeptide antagonist of CCR1, BX471, beginning at day 20 posttransplant in the Fischer → Lew rat renal model reduced mononuclear cell infiltration and glomerular and tubular injury when grafts were examined on day 42 [68]. Initiation of treatment at the time of transplantation did not have a marked effect on cell infiltration or other inflammatory events associated with acute rejection when examined at day 10 posttransplant. Consistent with these results, monotherapy with BX471 had little effect on the survival of MHC-mismatched heart grafts in a rat model but was modestly synergistic with low doses of cyclosporin in extending survival from day 12 to day 18 [69]. In a similar vein, a nonpeptide allosteric inhibitor of CCR5 had no effect on the survival of heterotopically transplanted heart allografts in a nonhuman primate study [70]. Monotherapy with the antagonist did decrease infiltration of CCR5-expressing T cells and macrophages into the heart allografts. Furthermore, the antagonist synergized with cyclosporin to reduce anti-donor antibodies and graft vasculopathy in the recipients with a general trend in improved graft survival in the small number of recipients examined.

Several molecules targeting CXCR3 have been developed and are only beginning to be tested for effects on allograft survival in various animal models [71,72]. Use of a small molecular CXCR3 antagonist, MRL-957, in a mouse heterotopic heart transplant model resulted in little improvement in the survival or histopathology of MHC-mismatched BALB/c allografts in C57BL/6 recipients transgenically expressing human CXCR3 [52]. Treatment of BALB/c murine recipients of C57BL/6 MHC-mismatched heart allografts from days 1 to 7 with a small molecule TAK-779 that binds to both CCR5 and CXCR3 resulted in prolongation of allograft

survival (from day 8 to day 13) and reduced infiltration of CD4 and CD8 T cells into the allografts when examined at day 7 posttransplant [62]. When the use of this compound was extended to the chronic heart allograft rejection model, B6.H-2^{bm12} → C57BL/6, treatment every day for 6 weeks significantly attenuated the development of vasculopathy and fibrosis in the graft at day 42 posttransplant.

In addition to molecules that are targeted to receptors on antigen-activated T cells, several antagonists of chemokine receptors expressed by neutrophils have been developed and are under current testing [73]. Given the key role of neutrophils in mediating tissue injury during ischemia-reperfusion injury and the impact of this injury on graft function and long-term outcome, use of such antagonists to block neutrophil infiltration into allografts is likely to have some benefit. A small molecule inhibitor of CXCR1 and CXCR2, repertaxin, has been developed and is extremely efficacious in inhibiting neutrophil infiltration into rat livers and tissue injury following 1 hour of ischemia [74,75]. Repertaxin is now in clinical trials for testing its effect on ischemia/reperfusion injury in transplanted organs.

Summary

The different types of immune injury imposed on tissue allografts induce unique sets of chemokines. The use of chemokine receptor antagonists to inhibit T cell graft infiltration and improve graft function and survival has not been, for the most part, an effective strategy in experimental transplantation, particularly when this strategy is applied in recipients receiving complete MHC mismatched grafts. In contrast, use of anti-chemokine/chemokine directed strategies often works well in extending allograft survival when MHC mismatches are minimized. This finding raises the likelihood of multiple and redundant mechanisms directing T cells and other leukocytes into grafts, particularly in face of the stronger immune responses associated with MHC-mismatched grafts. The real usefulness of the chemokine/chemokine receptor system in transplantation may be as biomarkers to identify ongoing donor-nonspecific and donor-specific immune responses in the graft.

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Table 1

Representative chemokines and their receptors

Chemokine	Old Name	Receptors	Cellular targets
CXCL1	Gro α	CXCR20	Neutrophils, EC
CXCL2	MIP-2	CXCR2	Neutrophils, EC
CXCL5	LIX	CXCR2	Neutrophils
CXCL8	IL-8	CXCR1/CXCR2	Neutrophils, EC
CXCL9	Mig	CXCR3	Th1 cells, EC
CXCL10	IP-10	CXCR3	Th1 cells, EC
CXCL11	I-TAC	CXCR3	Th1 cells, EC
CXCL12	SDF-1	CXC4	Bone marrow cells, Hematopoietic cells
CXCL13	BCA-1	CXCR5	B cells, T helper cells
CCL1	TCA-3	CCR8	Th17 and Th2 cells
CCL2	MCP-1	CCR1	Monocytes/macrophages T cells
CCL3	MIP-1 α	CCR3/CCR5	Monocytes/macrophages T cells, neutrophils
CCL4	MIP-1 β	CCR5	Monocytes/macrophages T cells, neutrophils
CCL5	RANTES	CCR1, CCR3, CCR5	Monocytes/macrophages T cells, neutrophils
CCL11	Eotaxin	CCR3	Eosinophils
CCL17	TARC	CCR4	Th2 cells
CCL19	ELC	CCR7	DC, naïve T cells
CCL21	SLC	CCR7	DC, naïve T cells
CCL22	MDC	CCR4	Th2 cells, DC
CCL27	CTACK	CCR10	CD4 T cells

Abbreviation: EC, Endothelial cells.