A Role for Fractalkine and Its Receptor (CX_3CR1) in Cardiac Allograft Rejection[1]

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The hallmark of acute allograft rejection is infiltration of the inflamed graft by circulating leukocytes. We studied the role of fractalkine (FKN) and its receptor, CX_3CR1, in allograft rejection. FKN expression was negligible in nonrejecting cardiac isografts but was significantly enhanced in rejecting allografts. At early time points, FKN expression was particularly prominent on vascular tissues and endothelium. As rejection progressed, FKN expression was further increased, with prominent anti-FKN staining seen around vessels and on cardiac myocytes. To determine the capacity of FKN on endothelial cells to promote leukocyte adhesion, we performed adhesion assays with PBMC and monolayers of TNF-α-activated murine endothelial cells under low-shear conditions. Treatment with either anti-FKN or anti-CX_3CR1-blocking Ab significantly inhibited PBMC binding, indicating that a large proportion of leukocyte binding to murine endothelium occurs via the FKN and CX_3CR1 adhesion receptors. To determine the functional significance of FKN in rejection, we treated cardiac allograft recipients with daily injections of anti-CX_3CR1 Ab. Treatment with the anti-CX_3CR1 Ab significantly prolonged allograft survival from 7 ± 1 to 49 ± 30 days ($p < 0.0008$). These studies identify a critical role for FKN in the pathogenesis of acute rejection and suggest that FKN may be a useful therapeutic target in rejection. ***The Journal of Immunology*****, 2000, 165: 6067–6072.**

Acute allograft rejection is characterized by an intense cellular immune response marked by influx of circulating leukocytes into the transplant. The accumulation of activated immune cells into the allograft is essential to the pathogenesis of tissue injury. Recruitment of leukocytes into sites of inflammation involves a tightly regulated series of molecular interactions that includes the initial capture and rolling of cells mediated by selectins, followed by firm arrest on endothelium, a process mediated by integrins (1). In the course of these events, the leukocyte becomes activated through stimulation of G protein-coupled chemokine receptors, resulting in enhanced integrin adhesiveness and activation-dependent stable arrest (2). The recruitment process culminates in diapedesis across the endothelium and migration into the inflamed tissue. Although expression of various adhesion molecules, such as ICAM-1 and E-selectin, is up-regulated in rejection (3), the functional importance of enhanced adhesion molecule expression has not been established. Likewise, expression of a range of chemokines has been demonstrated in rejecting transplants (4–8), but their contribution to the pathogenesis of rejection is not yet clear.

Recently, the human chemokine fractalkine (FKN)[3] and its murine homologue neurotactin have been identified as novel chemokines with a unique transmembrane chemokine/mucin hybrid structure (9, 10). FKN not only promotes leukocyte activation, but, unlike other chemokines, can mediate each individual step of the leukocyte adhesion cascade (11). Specifically, FKN interacts with its unique receptor, CX_3CR1, to affect firm adhesion of resting monocytes, resting and activated $CD8^+$ T lymphocytes, and activated NK cells. Like all the other known chemokine receptors, CX_3CR1 belongs to the large family of G protein-coupled receptors (12). Although little is known about CX_3CR1 signaling mechanisms, calcium flux that is triggered by CX_3CR1 is pertussis toxin sensitive, suggesting that it couples to $G_i\alpha$ (12).

Human CX_3CR1 mRNA is expressed in T lymphocytes, monocytes, and NK cells (12), and the rat homologue RBS11 is found in leukocytes, microglia, and astrocytes (13–15). The equivalent murine receptor, mCX_3CR1, is found in PBL and multiple organs, with particular abundance in brain and lung (16). Given that CX_3CR1-bearing leukocyte subsets are precisely the ones found within acutely rejecting allografts, we postulated that FKN might play a functional role in rejection. We tested this hypothesis using mouse models. Our studies demonstrate that 1) FKN expression is enhanced on rejecting mouse cardiac allografts; 2) enhanced FKN expression on activated mouse endothelial cells promotes increased leukocyte adhesion; and 3) inhibition of FKN-CX_3CR1 signaling with an anti-CX_3CR1 Ab significantly prolongs survival of mouse cardiac allografts in a vigorous model of acute rejection. Thus, the FKN-CX_3CR1 pathway for leukocyte trafficking and activation plays an integral role in the pathogenesis of allograft rejection.

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[3] Abbreviation used in this paper: FKN, fractalkine.

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Materials and Methods

Cells and culture conditions

Mouse PBMC were isolated from heparinized whole blood by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, NC) as previously described (17). Contaminating RBC were lysed, and PBMC were washed three times with PBS. After the final wash step, PBMC were resuspended in warm FCS and incubated at 37°C with 10 μCi of ^{51}Cr/million PBMC for 1 h. Unincorporated ^{51}Cr was removed by washing, and the labeled cells were resuspended in medium. Py-4-1 endothelial cells, derived from hemangiomas of polyomavirus transgenic mice (18), were grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS.

Antibodies

Abs used in these studies included purified polyclonal rabbit anti-mouse FKN directed against the chemokine domain of mouse FKN (Torrey Pines Biolabs, San Diego, CA) and purified polyclonal rabbit anti-rat CX_3CR1 Ab (Torrey Pines Biolabs) (13, 14), each used at concentration of 20 μg/ml. The secondary Ab used in these studies was biotin-conjugated goat anti-rabbit IgG adsorbed for human, mouse, and rat serum proteins (Jackson ImmunoResearch Laboratories, West Grove, PA), used at a final concentration of 10 μg/ml. The tertiary reagent was streptavidin-FITC (PharMingen, San Diego, CA), used at 5 μg/ml. For flow cytometry studies, described below, the Abs used were FITC-conjugated CD3e (145-2C11), CD4 (L3T4), CD8a (53-6.7), IgM (R6-60.2), Pan-NK (DX5), NK1.1 (PK136), and CD11b (Mac-1, M1/70), all from PharMingen, and F4/80 from Serotec (Raleigh, NC).

Flow cytometry

Py-4-1 cells were stimulated for 4 h with recombinant murine TNF-α 100 U/ml (Genentech, San Francisco, CA) and then lifted from the cell culture flask using enzyme-free cell dissociation buffer (Life Technologies). Cells were washed in PBS containing 2% bovine calf serum and then incubated with polyclonal anti-FKN Ab at 4°C for 45 min. Cells were washed and then incubated with biotinylated goat anti-rabbit IgG for 45 min at 4°C. After another wash, the cells were incubated with streptavidin-FITC for 45 min at 4°C. Following a final wash, the cells were fixed in 2% formaldehyde in PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

In vitro adhesion assays

Leukocyte adhesion assays were performed as described previously with minor modifications (17). Briefly, Py-4-1 cells were grown to confluence in 24-well culture plates, activated with TNF-α, washed, and incubated at 37°C for 20 min with medium or anti-FKN Ab. ^{51}Cr-labeled PBMC (0.5×10^6/well) were resuspended in 400 μl of medium alone or medium containing anti-CX_3CR1 Ab at 20 μg/ml. The leukocyte suspension was added to the endothelial monolayer under gentle rocking conditions (10 cycles/min). After 30 min the medium was decanted, and the wells were gently washed. Adherent cells were lysed by treating with 1% Triton in PBS. Total binding was determined by measuring individual well-associated cpm using a gamma counter. To determine whether treatment with anti-CX_3CR1 Ab altered the phenotype of PBMC bound to the activated endothelium, a parallel set of experiments was performed in which endothelial monolayers were overlaid with nonradiolabeled PBMC under the conditions described above. At the conclusion of the assay, cells were gently lifted from culture wells using enzyme-free cell dissociation buffer (Life Technologies), and FACS analysis performed, gating on the leukocyte rather than endothelial cell population. Cell surface markers evaluated included CD3, CD4, CD8, IgM, NK1.1, pan-NK, and F4/80.

Mouse cardiac transplantation

Heterotopic murine cardiac transplants were performed as described previously (19). Recipient C57BL/6 (H-2^b) mice were anesthetized using isoflurane and prepared by separating the vena cava and aorta between the renal vasculature and the iliac bifurcation. The donor heart was dissected from an MHC-mismatched (DBA/2 $\times$ BALB/c)F_1 (H-2^d) mouse, and an end-to-side anastomosis was created between the recipient aorta and the donor heart ascending aorta. A similar vascular anastomosis was created between the donor superior vena cava and the inferior vena cava of the recipient. Surgical mortality of the recipients was ~10%. Allograft survival was determined by directly palpating the cardiac impulse through the abdominal wall, with graft failure defined as the cessation of a palpable heartbeat. For immunostaining experiments, cardiac allografts and isografts from untreated recipients were harvested at 3 and 7 days posttransplantation.

Anti-CX_3CR1 Ab treatment

Heterotopic mouse heart transplants were performed as described above. On the day before transplantation, 75 μl of undiluted CX_3CR1 antiserum or control rabbit serum was injected i.p. into prospective recipient C57BL/6 (H-2^b) mice. Thereafter, recipient mice received similar injections each day for a period of up to 4 wk following transplantation, after which time the therapy was discontinued. Cardiac allograft survival was determined as described above. To further examine the effect of anti-CX_3CR1 on the nature of rejection, we evaluated the histopathology of cardiac allografts in the experimental groups. On day 3 or 7 after transplantation, the allografts were removed and placed in 10% buffered formalin. The hearts were then sectioned and stained with hematoxylin and eosin, and the slides were reviewed by a pathologist (D.N.H.) who was masked to the treatment groups.

Immunohistochemistry

The ventricles were cut in cross-section and fixed in formalin. Formalin-fixed hearts were paraffin-embedded, sectioned, and mounted on slides. Slides were subsequently deparaffinized by immersing in xylene and graded ethanols. Immunohistochemical staining for FKN was performed using the pressure cooker method for Ag retrieval. Briefly, slides were incubated in an epitope decloaking chamber (Biocare Medical, Walnut Creek, CA) at 120°C and ≤15–20 psi for ~25 min according to the manufacturer's specifications. Slides were demarcated using a lipophilic pen and were incubated for 30 min at room temperature with normal goat serum. Slides were washed four times with PBS containing 0.5% BSA, then incubated with either anti-FKN Ab or normal rabbit serum at room temperature for 45 min. Slides were washed four times with PBS containing 0.5% BSA, then incubated with biotin-conjugated goat anti-rabbit IgG at room temperature for 30 min. Slides were washed again, then incubated with ABC Elite developing reagent (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. This was followed by another wash and incubation with Tris-HCl containing diaminobenzidine and hydrogen peroxide (0.6%) for 3 min. Slides were then placed under running water for 2 min, stained with hematoxylin for 15 s, washed under running water for 2 min, and then placed in water containing NH_4OH (1/500) for 15 s. Slides were sequentially immersed in graded ethanols and xylene, then mounted using Acrytol (Surgipath, Richmond, IL).

Statistical analysis

Results are expressed as the mean ± SEM unless otherwise indicated. Student's *t* test was used to determine the level of significance of differences in mean values between treatment groups. For nonparametric analysis of the allograft survival study, a Mann-Whitney *U* test was used.

Results

Activation-induced expression of FKN by Py-4-1 cells

Since the endothelium of the allograft represents the initial site of interactions with leukocytes from the recipient, we examined expression of FKN in the mouse endothelial cell line Py-4-1. As shown in Fig. 1, low levels of FKN expression could be detected in quiescent Py-4-1 cells by flow cytometry using anti-FKN Ab. Exposure of Py-4-1 cells to TNF-α, a proinflammatory cytokine present at high levels in rejecting allografts, caused a marked upregulation of FKN expression. These data suggest that in the mouse, similar to previous reports in human cells, endothelial activation by proinflammatory cytokines such as TNF-α is associated with enhanced cell surface expression of FKN.

Murine PBMC adhesion to Py-4-1 cells

To determine the functional consequences of enhanced FKN expression in activated Py-4-1 cells, we examined binding of freshly isolated mouse PBMC to confluent Py-4-1 monolayers under low-shear conditions. Quiescent endothelial monolayers bound PBMC at low levels, but binding increased by 4-fold in response to activation with TNF-α (data not shown). To define the contribution of the FKN- CX_3CR1 pathway to leukocyte adhesion to the endothelial cell line, PBMC were incubated with anti-CX_3CR1 Ab before

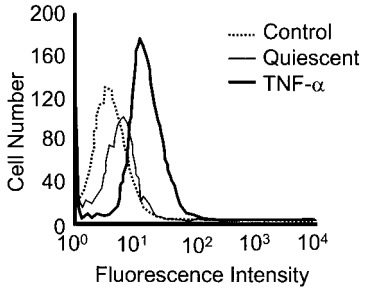

FIGURE 1. Expression of FKN by TNF-activated Py-4-1 cells. Confluent Py-4-1 monolayers were cultured for 4 h with either medium alone or TNF-α, incubated with anti-FKN Ab, stained with biotin-conjugated goat anti-rabbit IgG and streptavidin-FITC, then analyzed by flow cytometry. Staining of quiescent (thin line) and TNF-α-activated (bold line) Py-4-1 cells is indicated. The dashed line shows staining of activated Py-4-1 cells with unreactive isotype-control IgG Ab. Data are representative of three individual experiments.

the assay. The anti-CX_3CR1 Ab caused a modest (12%), but significant, reduction in leukocyte binding ($p < 0.01$). Preincubation of PBMC with control rabbit IgG had no effect on binding to endothelial cells (Fig. 2). Pretreatment of Py-4-1 cells with an anti-FKN Ab caused a more marked (33%) reduction in PBMC binding ($p < 0.01$). Combined treatment with the two Abs further reduced PBMC binding (62%; $p < 0.01$). Thus, a significant portion of PBMC binding to activated murine endothelial cells is mediated by the FKN-CX_3CR1 pathway.

To determine the effects of CX_3CR1 Ab blockade on the phenotype of PBMC bound to the activated endothelium, a parallel set of experiments was performed in which endothelial monolayers were overlaid with nonradiolabeled PBMC. At the conclusion of the assay, cells were gently lifted from culture wells using enzyme-free cell dissociation buffer, and FACS analysis was performed, gating on the leukocyte, rather than the endothelial, cell population. Using this approach, we found that a range of PBMC bound to activated endothelium, including T lymphocytes, B lymphocytes, NK cells, and monocytes. Although pretreatment with anti-CX_3CR1 Ab generally caused a proportional reduction in PBMC of each class that was bound to endothelium, the proportion of $CD8^+$ lymphocytes were reduced by almost 20% ($p < 0.001$).

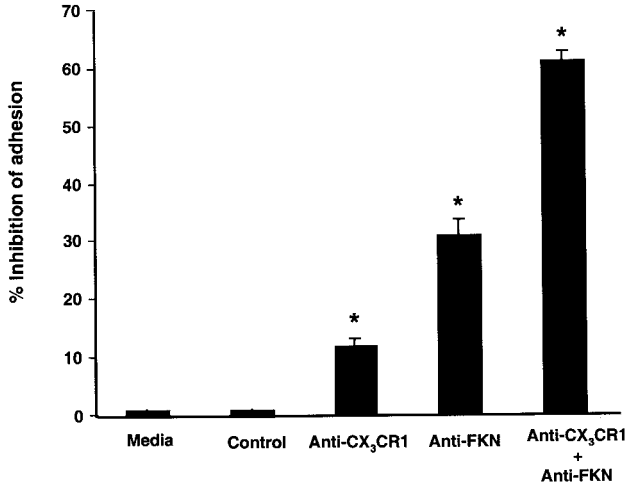

FIGURE 2. Inhibition of mouse PBMC binding to Py-4-1 cells. Confluent Py-4-1 monolayers were cultured with either medium alone or TNF-α for 4 h. Py-4-1 cells were incubated with anti-FKN Ab, whereas ^{51}Cr-labeled PBMC were incubated with a control Ab or a function-blocking Ab reactive with CX_3CR1. PBMC were subsequently incubated with the Py-4-1 monolayers at room temperature in the presence of the indicated Abs. Nonadherent PBMC were removed by washing. Adherent cells were lysed, and total binding was determined by measuring individual well-associated counts per minute using a gamma counter. Values are the average counts per minute of triplicate wells in one of two separate experiments. Results are expressed as the percent inhibition of adhesion compared with total binding observed with TNF-α-activated Py-4-1 cells. *, Differences between Py-4-1 cells that were untreated (no mAb) and all other groups were significant ($p < 0.01$).

FKN expression in rejecting cardiac allografts

To determine whether FKN expression might be regulated by an inflammatory response in vivo, we examined expression of FKN protein in a mouse model of cardiac transplant rejection. We examined FKN expression in mouse cardiac allografts at early and late time points during rejection. FKN expression in allografts was compared with that in nonrejecting isografts in tissue sections using polyclonal rabbit Ab against mouse FKN. Negligible FKN staining was detected in nonrejecting isografts at any time (Fig. 3, *C* and *F*). In contrast, on day 3 after transplant when leukocyte infiltrates were first detectable, FKN was prominently expressed diffusely on endothelium of cardiac allografts (Fig. 3*A*). FKN was also detected on epicardium and endocardial surfaces. By day 7, further enhancement of FKN expression was detected, including apparent expression on myocardium (Fig. 3*D*). No staining was observed with nonimmune rabbit serum (Fig. 3, *B* and *E*). Thus, enhanced FKN expression accompanies cardiac allograft rejection.

Prolonged in vivo survival of allografts treated with anti-CX_3CR1 Ab

To determine the functional importance of the up-regulation of FKN expression in allograft rejection, we treated recipient animals with a neutralizing anti-CX_3CR1 Ab. Beginning on the day before transplantation, recipient animals received daily i.p. injections of 75 μl of anti-CX_3CR1 Ab or nonimmune rabbit serum for up to 4 wk. In the animals that received rabbit serum ($n = 9$), the mean survival of cardiac allografts was 7 ± 1 days (Fig. 4). Treatment with anti-CX_3CR1 Ab significantly prolonged graft survival to a mean of 49 ± 30 days (Fig. 4; $p < 0.0008$). Although all the control allografts underwent rejection on day 7 or 8, the survival of anti-CX_3CR1-treated grafts ranged from 12–91 days (Table I). In long-surviving anti-CX_3CR1-treated animals, Ab administration was discontinued after 4 wk, and these grafts continued to survive for a mean of 33 ± 23 days, resulting in total mean survival of 61 ± 29 days (Fig. 4). Therefore, treatment with anti-CX_3CR1 Ab with no additional immunosuppression induces a significant prolongation of cardiac allograft survival. These findings indicate a unique role for the FKN-CX_3CR1 pathway in promoting allograft injury.

To determine whether increased allograft survival correlated with reduced leukocyte infiltration of the graft, additional experiments using the cardiac transplant model were performed. We examined the effects of anti-CX_3CR1 Ab on the development of histopathologic lesions during the early stages of graft rejection, before there was significant graft loss in the control group. On days 3 and 7 after transplantation, allografts were harvested from recipients that had received anti-CX_3CR1 or control serum. Allograft sections were stained and evaluated without knowledge of the treatment groups. At 3 days, patchy inflammation was visible in all the allografts, but no obvious differences were seen between the experimental and control groups. At 7 days, a mixture of vascular and interstitial inflammation as well as patchy myocyte necrosis were observed in both groups. However, no systematic differences were detected at the level of light microscopy between treatment

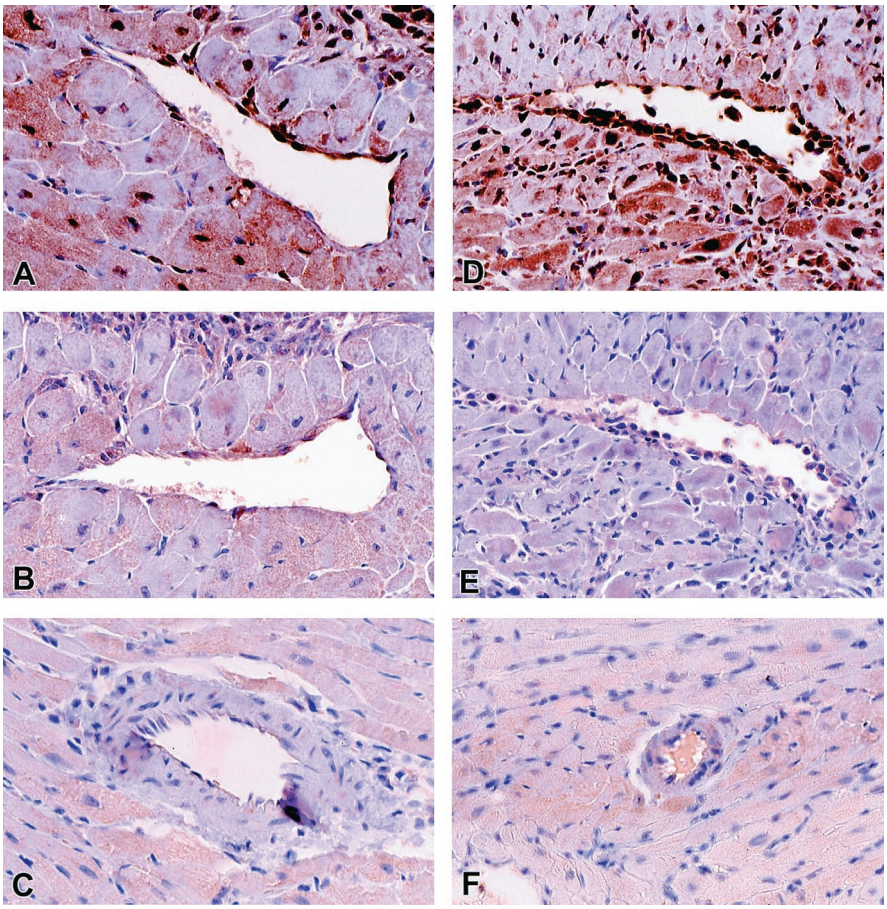

FIGURE 3. Expression of FKN in rejecting murine cardiac allografts. Sections from rejecting mouse cardiac allografts or control isografts harvested 3 or 7 days posttransplantation were incubated with anti-FKN polyclonal Ab as outlined in *Materials and Methods*. Slides were counterstained with hematoxylin (magnification, ×100). Sections from tissues derived 3 days posttransplantation were stained with anti-FKN Ab (*A*) or control rabbit serum (*B*). Sections derived from rejecting allografts 7 days posttransplantation were similarly stained with anti-FKN Ab (*D*) or control rabbit serum (*E*). For comparison, tissue derived from nonrejecting isografts was stained with anti-FKN Ab (*C*) or control rabbit serum (*F*).

and control groups. Thus, the protective effect of anti-CX_3CR1Ab in rejection occurs without marked alteration in the character of the cellular infiltrate, at least in the early stages of rejection. These data suggest that FKN may promote allograft injury through direct effects on immune cell functions, but further studies are necessary to elucidate the precise mechanisms responsible.

Discussion

Acute rejection of a vascularized organ graft is characterized by an intense inflammatory response. This response is focused within the confines of the graft, directly impairing its function and eventually resulting in destruction of the transplanted tissue. The inflammatory response associated with rejection is triggered by recognition of alloantigens by recipient T cells. This activation of allospecific T cells initiates the expansion and mobilization of a complex cellular immune response. Through a series of tightly integrated cellular and molecular interactions, diverse populations of immune cells enter the graft where they exert their effector functions. Most current anti-rejection therapies target T cell activation and proliferation. Interventions directed toward other parts of this response, such as the process of trafficking of leukocytes to the graft, are relatively unexplored.

A general understanding of the process of leukocyte recruitment into sites of inflammation has been developed using simple in vitro and in vivo models (1). This process consists of systematic, stepwise interactions between adhesion molecules expressed on endothelium and the circulating leukocyte. During its later phases, chemokine signaling through G protein-coupled receptors facilitates firm adhesion and migration of the leukocyte across the endothelium (2, 20, 21). Signals from chemokine receptors promote and facilitate leukocyte activation and thus may also contribute to the intensity of the inflammatory response. Although it is generally assumed that this paradigm explains the recruitment and accumulation of immune cells within rejecting tissues, the relative contributions of each component of this recruitment process to allograft rejection have not been explicitly characterized.

We have studied the potential role of a recently identified member of the chemokine family, FKN, in rejection. FKN is unusual among chemokines in that it is a membrane-bound molecule with a chemokine domain coupled to a mucin stalk (22). In a mouse endothelial cell line, we found that expression of FKN is markedly up-regulated when the cells are activated by TNF-α. These findings are consistent with previous studies of Bazan and associates using HUVEC, where TNF-α induced expression of FKN mRNA and protein (9). Since TNF-α is highly expressed in rejecting allografts, this regulatory pathway for FKN expression may have direct relevance in rejection.

Human FKN mRNA expression is present in normal heart, brain, kidney, lung, pancreas, and colon (9). Likewise, in normal mice, neurotactin mRNA is especially prominent in brain and, to a lesser extent, in kidney, lung, and heart (10). The studies by Harrison et al. (14) further pinpoint the site of FKN expression in the CNS of the rat to neurons. Similar to our studies in cultured endothelial cells, studies by Pan et al. (10) have suggested that inflammatory stimuli may also enhance FKN expression in vivo. These authors found increased levels of neurotactin staining on vascular endothelium in brain following LPS treatment (10). Enhanced neurotactin expression in activated microglia from mice with severe experimental autoimmune encephalomyelitis has also been documented. We found only negligible levels of FKN expression in nonrejecting cardiac isografts. In contrast, expression of FKN was markedly up-regulated in allografts as early as 3 days

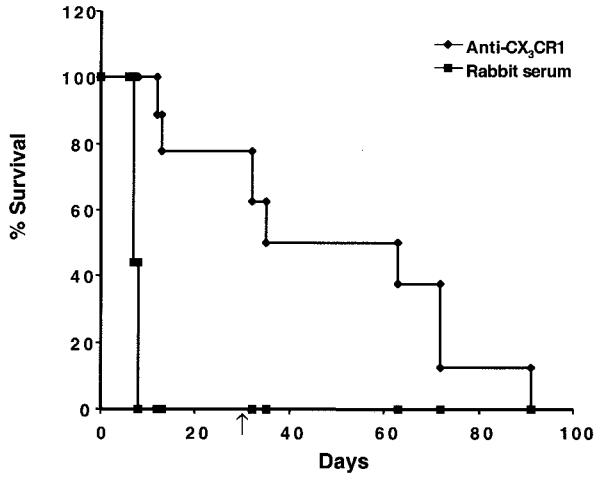

FIGURE 4. Survival of murine cardiac allografts following recipient treatment with anti-CX_3CR1 Ab treatment. Heterotopic mouse heart transplants were performed as described in *Materials and Methods*. On the day before transplantation, recipient C57BL/6 (H-2^b) mice were injected i.p. with 75 μl of anti-CX_3CR1 Ab or control rabbit serum. Thereafter, recipient mice received similar daily injections for a period of 4 wk, after which time ($\uparrow$), Ab therapy was discontinued. Cardiac allografts were monitored daily, and the length of survival of each transplant noted. Results are expressed as the daily percent survival of allografts.

after transplantation. FKN expression became more prominent as the rejection process progressed. The most intense staining for FKN was present on endothelial surfaces, where it might be involved in the early events associated with trafficking of circulating leukocytes into the graft. However, definite staining was also found on epicardial surfaces and cardiac myocytes in ventricular tissue. Although FKN expression in myocytes has recently been described (23), its functional significance is not clear.

FKN has dual functions in leukocyte migration: as a chemokine and also as a mediator of leukocyte capture and firm adhesion (11, 12). For example, under physiologic conditions of flow, FKN and CX_3CR1 interact to mediate sequential steps of the leukocyte adhesion cascade, including rapid capture, stable arrest, and activation (11). Firm leukocyte adhesion mediated by FKN-CX_3CR1 is not sensitive to pertussis toxin, suggesting that it does not require integrin activation. Although most previous studies of FKN-dependent leukocyte adhesion have been performed using human systems, we find that neurotactin has a similar role in the mouse. In our studies a substantial portion (>60%) of PBMC binding to TNF-α-activated murine endothelial cells is mediated by an FKN-CX_3CR1-dependent pathway. Although anti-FKN Ab blockade inhibited binding by 33%, anti-CX_3CR1 treatment only blocked 12% of adhesion, suggesting incomplete blockade of this pathway. In contrast, blocking both arms of the FKN-CX_3CR1pathway using Ab therapy dramatically decreased leukocyte-endothelial binding by 62%. Taken together, these data suggest that up-regulation of FKN on endothelial surfaces of a rejecting allograft should have potent effects to promote leukocyte adhesion.

The propensity for a given cell population to be influenced by FKN is determined by expression of CX_3CR1, the receptor for FKN. For example, in microchemotaxis assays, soluble FKN is a potent chemoattractant for CX_3CR1-bearing leukocytes (12). CX_3CR1 expression has been detected in a range of leukocyte populations, including $CD14^+$ monocytes, $CD8^+$ lymphocytes, and $CD16^+$ NK cells (12). These cells are all typically present among the infiltrating cell population observed in rejecting allografts. Similar to other chemokine receptors, such as CCR1 and CCR2 (24), CX_3CR1 expression in T lymphocytes is up-regulated by cytokines such as IL-2 (12). Since the microenvironment of the rejecting allograft is rich in proinflammatory cytokines, the capacity for FKN responses may be maximized during rejection.

Table I. *Allograft survival with anti-CX_3CR1 Ab treatment*[a]

Treatment	Length of Graft Survival (days)
Anti-CX_3CR1	12, 13, 32, 35, 63, 72, 72, 91
Rabbit serum	7, 7, 7, 7, 7, 8, 8, 8, 8

[a] Survival of murine cardiac allografts following recipient treatment with anti-CX_3CR1 Ab. Heterotopic mouse heart transplants were performed as described in *Materials and Methods*. On the day before transplantation, recipient C57BL/6 (H-2^b) mice were injected i.p. with 75 μl of anti-CX_3CR1 Ab or control rabbit serum. Thereafter, recipient mice received similar daily injections for a period of 4 wk, after which time Ab therapy was discontinued. Cardiac allografts were monitored daily, and the length of survival for each transplant was noted.

To determine whether enhanced expression of FKN plays a role in allograft rejection, we treated cardiac transplant recipients with a neutralizing Ab against CX_3CR1. In a vigorous model of allograft rejection, blockade of the FKN-CX_3CR1 pathway significantly increased allograft survival. In comparing the individual animals, the impact of anti-CX_3CR1 Ab treatment was variable. The reasons for this variability are not clear, but they may relate to variations in the degree of CX_3CR1 inhibition that was achieved or to interactions with other factors, such as ischemic injury. Nonetheless, as depicted in Table I, a definite beneficial effect of therapy was seen in each animal that received anti-CX_3CR1 Ab. These data indicate that the FKN-CX_3CR1 pathway has a unique function to promote allograft rejection.

Our findings add to the accumulating body of evidence suggesting an important role for chemokines in the pathogenesis of allograft rejection. For example, enhanced expression of a number of C-C chemokines has been demonstrated in murine allograft models as well as in rejecting transplants in humans (5). Because of the range of chemokines that are expressed in rejection and the substantial overlap in functions between chemokine family members, some authors suggested that inhibition of individual chemokines or chemokine receptors was unlikely to have a substantive impact on the course of rejection. Nonetheless, several reports have identified specific chemokines and chemokine receptors that appear to have unique and nonoverlapping roles in the pathogenesis of allograft rejection. For example, the absence of CCR1 significantly prolonged allograft survival in a murine model of acute cardiac allograft rejection (25). However, the effects of CCR1 deficiency were more marked across isolated class I or class II MHC differences. Our data suggest that the FKN-CX_3CR1 pathway also has a nonredundant role in allograft rejection. The efficacy of anti-CX_3CR1 Ab in prolonging the survival of cardiac allografts with complete MHC disparity between donor and host suggests that the FKN-CX_3CR1 pathway may be a useful therapeutic target for antirejection therapies.

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References

1. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell 76:301.*
2. Campbell, J. J., J. Hedrick, A. Zlotnik, M. A. Siani, D. A. Thompson, and E. C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science 279:381.*
3. Solez, K., L. C. Racusen, F. Abdulkareem, E. Kemeny, E. von Willebrand, and L. D. Truong. 1997. Adhesion molecules and rejection of renal allografts. *Kidney Int. 51:1476.*

4. Fairchild, R. L., A. M. VanBuskirk, T. Kondo, M. E. Wakely, and C. G. Orosz. 1997. Expression of chemokine genes during rejection and long-term acceptance of cardiac allografts. *Transplantation 63:1807.*
5. Gladue, R. P., and T. M. Coffman. 2000. Potential roles for chemokines in transplant rejection. In *Chemokines in Disease: Biology and Clinical Research*, C. A. Hebert, ed. Humana Press, Totowa, NJ, p. 159.
6. Nadeau, K. C., H. Azuma, and N. L. Tilney. 1995. Sequential cytokine dynamics in chronic rejection of rat renal allografts: roles for cytokines RANTES and MCP-1. *Proc. Natl. Acad. Sci. USA 92:8729.*
7. Pattison, J., P. J. Nelson, P. Huie, I. von Leuttichau, G. Farshid, R. K. Sibley, and A. M. Krensky. 1994. RANTES chemokine expression in cell-mediated transplant rejection of the kidney. *Lancet 343:209.*
8. Strehlau, J., M. Pavlakis, M. Lipman, M. Shapiro, L. Vasconcellos, W. Harmon, and T. B. Strom. 1997. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc. Natl. Acad. Sci. USA 94:695.*
9. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX_3C motif. *Nature 385:640.*
10. Pan, Y., C. Lloyd, H. Zhou, S. Dolich, J. Deeds, J. A. Gonzalo, J. Vath, M. Gosselin, J. Ma, B. Dussault, et al. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. [Published erratum appears in *1997 Nature 389:100.] Nature 387:611.*
11. Fong, A. M., L. A. Robinson, D. A. Steeber, T. F. Tedder, O. Yoshie, T. Imai, and D. D. Patel. 1998. Fractalkine and CX_3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J. Exp. Med. 188:1413.*
12. Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T. J. Schall, and O. Yoshie. 1997. Identification and molecular characterization of fractalkine receptor CX_3CR1, which mediates both leukocyte migration and adhesion. *Cell 91:521.*
13. Feng, L., S. Chen, G. E. Garcia, Y. Xia, M. A. Siani, P. Botti, C. B. Wilson, J. K. Harrison, and K. B. Bacon. 1999. Prevention of crescentic glomerulonephritis by immunoneutralization of the fractalkine receptor CX_3CR1. *Kidney Int. 56:612.*
14. Harrison, J. K., Y. Jiang, S. Chen, Y. Xia, D. Maciejewski, R. K. McNamara, W. J. Streit, M. N. Salafranca, S. Adhikari, D. A. Thompson, et al. 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX_3CR1-expressing microglia. *Proc. Natl. Acad. Sci. USA 95:10896.*
15. Maciejewski-Lenoir, D., S. Chen, L. Feng, R. Maki, and K. B. Bacon. 1999. Characterization of fractalkine in rat brain cells: migratory and activation signals for CX_3CR1-expressing microglia. *J. Immunol. 163:1628.*
16. Combadiere, C., J. Gao, H. Tiffany, and P. Murphy. 1998. Gene cloning, RNA distribution, and functional expression of mCX_3CR1, a mouse chemotactic receptor for the CX_3C chemokine fractalkine. *Biochem. Biophys. Res. Commun. 253:728.*
17. Robinson, L. A., L. Tu, D. A. Steeber, O. Preis, J. L. Platt, and T. F. Tedder. 1998. The role of adhesion molecules in human leukocyte attachment to porcine vascular endothelium: implications for xenotransplantation. *J. Immunol. 161:6931.*
18. Heyward, S. A., N. Dubois-Stringfellow, R. Rapoport, and V. L. Bautch. 1995. Expression and inducibility of vascular adhesion receptors in development. *FASEB J. 9:956.*
19. Nataraj, C., M. Oliverio, R. Mannon, P. Mannon, L. Audoly, S. Amuchastegui, P. Ruiz, O. Smithies, and T. Coffman. 1999. Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *J. Clin. Invest. 104: 1693.*
20. Bargatze, R. F., and E. C. Butcher. 1993. Rapid G-protein-regulated activation event involved in lymphocyte binding to high endothelial venules. *J. Exp. Med. 178:367.*
21. Honda, S., J. Campbell, D. Andrew, B. Engelhardt, B. Butcher, R. Warnock, R. Ye, and E. Butcher. 1994. Ligand-induced adhesion to activated endothelium and to vascular cell adhesion molecule-1 in lymphocytes transfected with the N-formyl peptide receptor. *J. Immunol. 152:4026.*
22. Fong, A. M., H. P. Erickson, J. P. Zachariah, S. Poon, N. J. Schamberg, T. Imai, and D. D. Patel. 2000. Ultrastructure and function of the fractalkine mucin domain in CX_3C chemokine domain presentation. *J. Biol. Chem. 275:3781.*
23. Harrison, J. K., Y. Jiang, E. A. Wees, M. N. Salafranca, H. X. Liang, L. Feng, and L. Belardinelli. 1999. Inflammatory agents regulate in vivo expression of fractalkine in endothelial cells of the rat heart. *J. Leukocyte Biol. 66:937.*
24. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med. 184:569.*
25. Gao, W., P. S. Topham, J. A. King, S. T. Smiley, V. Csizmadia, B. Lu, C. J. Gerard, and W. W. Hancock. 2000. Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J. Clin. Invest. 105:35.*