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Blocking the Monocyte Chemoattractant Protein-1/CCR2 Chemokine Pathway Induces Permanent Survival of Islet Allografts through a Programmed Death-1 Ligand-1-Dependent Mechanism

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Islet allografts are subject to rapid rejection through host cellular immune responses involving mononuclear cell recruitment and tissue injury. Interruption of leukocyte recruitment through chemokine receptor targeting is of therapeutic benefit in various experimental models, but little is known about the contribution of chemokine pathways to islet allograft rejection. We found that murine islets produce monocyte chemoattractant protein-1 (MCP-1; CCL2) in vitro and that islet allograft rejection was associated with intragraft expression of MCP-1 and its receptor, CCR2. We therefore investigated whether MCP-1 and CCR2 are required for the rejection of fully MHC-disparate islet allografts. Wild-type mice treated with blocking anti-MCP-1 mAb plus a brief, subtherapeutic course of rapamycin had long-term islet allograft survival, in contrast to the effect of treatment with either mAb or rapamycin alone. CCR2−/− mice treated with rapamycin also maintained islet allografts long-term. Both MCP/CCR2- and rapamycin-sensitive signals were required for maximal proliferation of alloreactive T cells, suggesting that MCP-1/CCR2 induce rejection by promoting alloreactive T cell clonal expansion and homing and migration. Prolonged islet allograft survival achieved by blockade of the MCP-1/CCR2 pathway plus rapamycin therapy was accompanied by a mononuclear cell infiltrate expressing the inhibitory receptor, programmed death-1 (PD-1), and its ligand (PD-L1, B7-H1), and prolongation of islet allograft survival was abrogated by anti-PD-L1 mAb therapy. These data show that the blockade of MCP-1 binding to CCR2 in conjunction with subtherapeutic immunosuppression can have profound effects on islet allograft survival and implicate the expression of the PD-1/PD-L1 pathway in the regulation of physiologic responses in vivo. The Journal of Immunology, 2003, 171: 6929–6935.

Chemokines are small m.w. chemotactic cytokines involved in cell recruitment, activation, and differentiation; they act by binding to G protein-coupled, seven-transmembrane chemokine receptors (1–3). Although various mRNA encoding essentially all the main chemokines and their receptors can be detected during clinical allograft rejection, experimental studies demonstrate key roles for specific chemokine/chemokine receptor pathways in mediating leukocyte recruitment and rejection of vascularized cardiac allografts (4, 5). The most potent effects are seen by targeting CXCR3 (6) or its ligand, inducing proinflammatory protein-10 (7), with additional, albeit lesser, benefits seen by targeting CCR5 (8). In contrast, only modest effects were seen with targeting of CCR1 (9), CCR2 (4), or CX3CR1 (10), and blockade of several other chemokines had no therapeutic benefit in the vascularized cardiac allograft model (4). One important question arising from such work is to what extent are such data universal, or might they depend upon the type of graft transplanted?

Islet allografting has recently become a therapeutic option for patients with type 1 diabetes, although there are many limitations to its widespread use, including development of allograft rejection. In one mechanistic study, to our knowledge, concerning the roles of chemokines and their receptors in islet allograft rejection, Abdi et al. (11) found that islet allografts placed under the kidney capsules of CCR5−/− mice had a 3- to 4-fold prolongation of allograft survival compared with wild-type recipients. However, no studies have demonstrated that CCR5 or its chemokine ligands are expressed by human islets or after human islet allografting. In fact, one group reported that no mRNA for macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, or RANTES (all chemokine ligands for CCR5) was detected in resting human islets or upon their stimulation (12). Conversely, the chemokine, monocyte chemoattractant protein-1 (MCP-1; CCL2), is known to be produced de novo by human islets, and its expression is increased by exposure to IL-1β, TNF-α, or LPS in vitro (12, 13). Furthermore, low levels of MCP-1 secretion after human islet allografting were found to be a relevant factor in long-term graft survival and insulin independence (12).

MCP-1 is the main monocyte chemoattractant in vivo (14–16). MCP-1 can also attract memory T cells and activated NK cells in...
vitro (17, 18) and is key to the development of Th2 responses (19). The receptor for MCP-1, CCR2 (20), is highly expressed on monocytes and activated T cells, including memory T cells and Th1 and Th2 cells (3). CCR2−/− mice demonstrate a defect in Th1-type responses (21, 22) and a corresponding susceptibility to infections with intracellular pathogens (23, 24) and resistance to development of experimental autoimmune encephalomyelitis (25, 26). In this study we investigated the effects of targeting MCP-1 or its receptor on islet allograft survival in murine recipients rendered diabetic using streptozotocin.

Materials and Methods

Mice

MCP-1−/− (16) and CCR2−/− (27) mice, back-crossed more than eight generations on a C57BL/6 background, and wild-type C57BL/6 (H-2b), BALB/c (H-2k), and C57BL/6×DBA F1 (H-2b/d) mice from The Jackson Laboratory (Bar Harbor, ME) were housed in specific pathogen-free conditions. C57BL/6 mice given a single injection i.p. of clinical grade streptozotocin (Zanosar; 180–225 mg/kg; Pharmacia and Upjohn, NJ) were considered diabetic when glucose levels of >300 mg/dl were obtained on 2 consecutive days. Studies were performed with a protocol approved by the institutional animal care and use committee of Children’s Hospital of Philadelphia.

Islet isolation, in vitro culture, and transplantation

The pancreata of female BALB/c retired breeders were infused via the common bile duct with collagenase-P (Roche, Indianapolis, IN; 2 mg/ml, 5 ml) and digested for 8 min at 37°C. Islets were purified on a Ficoll gradient (1000 g, 10 min) using a stereomicroscope, and 500 islets (100 islets/ml) were cultured in 5 ml of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Islets cultured at 37°C in 5% CO2 and 95% humidified air were stimulated with one of the following agents (R&D Systems, Minneapolis, MN): LPS (1 μg/ml), IFN-γ (100 U/ml), IL-1 (100 ng/ml), or TNF-α (100 ng/ml). After 24 h, they were harvested and used for real-time quantitative RT-PCR (qRT-PCR).

For islet allografting, 400–600 islets from BALB/c donors were placed under the kidney capsule of each diabetic C57BL/6 recipient. Primary function was defined as a reduction of blood glucose to <200 mg/dl post-transplant, and rejection as a rise in blood glucose to >300 mg/dl. Allografted mice (six to eight per group) were untreated or were treated from the day of transplant with rapamycin (Sigma-Aldrich, St. Louis, MO; 0.2 mg/kg/d for 14 days); the hamster anti-mouse MCP-1 mAb, 2H5 (28) (200 μg i.p. three times per week for 2 wk), the hamster anti-B7-H1 mAb 10B5 (29) (100 μg i.p. three times per wk for 2 wk), or hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). As indicated, long-term graft function was confirmed by nephrectomy and development of hyperglycemia.

Quantitative RT-PCR (qRT-PCR) studies

RNA was extracted from cultured islets and islet allografts using TRIzol reagent (Life Technologies), and RT of RNA samples (2 μg) using random hexamers was performed with an ABI PRISM 5700 unit (Applied Biosystems, Foster City, CA). Specific primer and probe sequences for target genes were used for qRT-PCR amplification of total cDNA (50 ng) with an ABI PRISM 5700 machine (Taquin Pardar; Applied Biosystems). Relative quantitation of target cDNA was determined by arbitrarily setting the control value to 1. Changes in the cDNA content of a sample were expressed as fold increases above the set control value. Differences in cDNA input were corrected by normalizing signals obtained with specific primers to ribosomal RNA. Nonspecific amplification was excluded by performing RT-PCR reactions in the absence of target cDNA.

Histopathology and immunopathology

Islet grafts (n = 4/group/time point) were harvested at rejection or >100 days post-transplant, and H&E-stained paraffin sections were used to assess islet graft function, monocyte infiltration, and fibrosis. Corresponding cryostat sections were labeled using mAbs directed against macrophages and T, B, and NK cells (BD Pharmingen, San Diego, CA); PD-1, PD-1 ligand-1 (PD-L1), and PD-L2 (eBiosciences, San Diego, CA); and CCR2 and CCR5 (30), as previously described (31, 32). Insulin was detected in paraffin sections using a guinea pig Ab (Dako, Carpinteria, CA) (31). The specificity of labeling was assessed using isotype-matched mAbs or purified IgG.

Flow cytometry of adoptively transferred CFSE-labeled cells and in vitro studies

Alloreactive T cells were generated by i.v. injection of 20 million CFSE-labeled C57Bl/6 (H-2b) spleen and lymph node cells into C57Bl/6×DBA F1 (H-2b/d) recipients, a parent→F1 MHC mismatch in which only donor cells respond (33). Spleens were harvested from F1 mice after 3 days, and spleenocytes were incubated with CD4-PE, CD8-PE, anti-PD-L1-PE or anti-PD-L2-PE, and biotin-conjugated anti-H-2k or anti-H-2d mAb (BD Pharmingen). Donor alloreactive T cells were distinguished from recipient T cells by gating on H-2k− and H-2d−negative cells (FACScalibur; BD Biosciences, Mountain View, CA), and T cell proliferation was assessed using CFSE division profiles.

For intracellular cytokine staining, spleenocytes from MCP-1−/−, CCR2−/−, or wild-type controls were activated in vitro using CD3 mAb alone or CD3 plus CD28 mAb, as described previously (31). After 48 h, cells were harvested and analyzed by flow cytometry for the expression by CD4 or CD8+ T cells of CD25, PD-1, PD-L1, and PD-L2.

Statistics

Islet allograft survival was used to generate Kaplan-Meier survival curves and comparison between groups was performed by log-rank analysis.

Results

MCP-1 is up-regulated in murine islets by inflammatory stimuli

Given data that MCP-1 is produced by human islets de novo and its expression increased by inflammatory cytokines in vitro, we analyzed murine islet MCP-1 mRNA expression by qRT-PCR. Like human islets (12), murine islets cultured in vitro for 24 h expressed MCP-1 mRNA, and exposure to LPS, IL-1β, TNF-α, or IFN-γ led to a 4- to 12-fold increase in MCP-1 mRNA expression (Fig. 1a). Islet MCP-1 mRNA expression was also increased post-transplant. Levels of MCP-1 mRNA in islets harvested 7 days post-transplant were increased ∼130-fold compared with levels in corresponding islet isografts (Fig. 1b), although the relative contributions of inflammatory cells and islets cells to MCP-1 production were not determined. If MCP-1 were to play a role in islet allograft rejection, local expression of CCR2 would also be anticipated. We therefore assessed intragraft mRNA levels of CCR2 plus those of two other chemokine receptors previously implicated in host alloresponses, CXCR3 (6) and CCR5 (8, 11). Levels of CCR2, CCR5, and CXCR3 mRNA were each markedly increased in allografts vs isografts at 7 days post-transplant (Fig. 1c). Immunohistologic studies of corresponding islet allografts showed localization of CCR2 to host mononuclear cells (Fig. 1d). Infiltrating mononuclear cells also showed staining for CCR5 and CXCR3 (data not shown), although in each case the extent of receptor by T cells vs macrophages was not determined.

Beneficial effect of targeting MCP-1/CCR2 pathway in conjunction with low dose rapamycin

Our findings of MCP-1 expression by islets and recruitment of CCR2+ host mononuclear cells during islet allograft rejection led us to ask whether this pathway was of practical therapeutic consequence with regard to controlling or preventing islet allograft rejection. Moreover, given concerns that gene-targeted mice may have compensatory responses associated with specific gene loss, we sought to target the MCP-1/CCR2 pathway in both knockout and wild-type recipients.
CCR2−/− and wild-type C57BL/6 mice rendered diabetic by streptozotocin received fully MHC-disparate islet allografts from BALB/c donors (Fig. 2a). Islet allograft survival in CCR2−/− mice was not significantly different from that in CCR2+/+ controls \((p > 0.05)\). However, although rapamycin therapy (0.2 mg/kg/d for 14 days) prolonged islet graft survival only a few extra days in wild-type recipients, the same protocol in CCR2−/− recipients led to permanent engraftment \((>120\) days; \(p < 0.005)\). Untreated wild-type mice or those receiving control hamster IgG or hamster anti-MCP-1 mAb rejected islet allografts within 2 wk (Fig. 2). Consistent with the effects of targeting CCR2 in conjunction with rapamycin therapy, anti-MCP-1 mAb plus rapamycin therapy led to permanent \((>120\) days) engraftment, whereas controls given rapamycin plus hamster IgG were rejected within 20 days \((p < 0.005)\).

**MCP-1 and CCR2 contribute to alloreactive T cell clonal expansion and differentiation in vivo**

As rejection is a CD4+ T cell-dependent event, we analyzed the capacity of T cells from MCP-1−/− or CCR2−/− mice to undergo activation in vitro and in vivo. We first assessed whether T cells from each strain had comparable levels of expression of CD25...
after stimulation in vitro with CD3/CD28 mAbs. Both CD4^+ and CD8^+ T cells from MCP-1^+/^- or CCR2^+/^- mice showed decreased induction of CD25 after 48 h in culture compared with cells from wild-type controls (Fig. 2b), suggesting that this pathway might contribute to full T cell activation. We next determined whether the lack of MCP-1 or CCR2 affected the alloactivation of CD4^+ T cells using an in vivo MLR model in which CFSE-labeled C57BL/6 (H-2b) splenocytes were adoptively transferred into semiallogeneic B6D2F1 (H2^bd) mice. Fig. 3 shows the data for CD4^+ T cell responses; comparable effects in each case were seen with CD8^+ T cells, although the overall magnitude of their proliferation in this model was less than that for CD4^+ T cells (data not shown). Compared with controls, CD4^+ T cells from MCP-1^+/^- mice exhibited a modest decrease in alloreactive CD4^+ T cell clonal expansion, which was accentuated by the use of rapamycin (Fig. 3a). Similarly, anti-MCP-1 mAb decreased the alloactivation of wild-type CD4^+ T cells, and this effect was again accentuated by rapamycin (Fig. 3b). CD4^+ T cells from CCR2^+/^- also proliferated less than wild-type cells (Fig. 3c), and responses were markedly attenuated by rapamycin therapy (Fig. 3d). Likewise, effector cytokine production by alloactivated T cells was affected; transfer of CCR2^+/^- donor CD4^+ T cells led to decreased production of IL-2 and IFN-\(\gamma\), as measured by flow cytometric analysis of intracellular cytokine expression, compared with controls (Fig. 3e).

**FIGURE 3.** Effects of MCP-1 and CCR2 on the alloactivation of CD4^+ T cells in vivo (3 days postadoptive transfer). a, CFSE-labeled cells from MCP-1^+/^- mice and WT mice were adoptively transferred to F1 mice. In contrast to cells from wild-type or MCP-1^+/^- donors, the use of rapamycin (RPM; green) reduced the number of CD4^+ T cells undergoing maximal cell division, whereas the use of RPM and MCP-1^+/^- donor cells (gold) resulted in the least response to alloantigen. b, Neutralization of MCP-1 (blue) or use of RPM (green) in F1 animals after adoptive transfer of CFSE-labeled wild-type donor cells reduces T cell alloactivation and proliferation, whereas the combination of RPM and anti-MCP-1 mAb (gold) has a still further and markedly inhibitory effect. c, CD4^+ cells from CCR2^+/^- donors (green) demonstrate decreased responses to alloantigen in vivo compared with cells from wild-type donors. d, Use of RPM largely suppresses the proliferative response of CD4^+ T cells from CCR2^+/^- donor mice. Alloactivated CD4^+ T cells from CCR2^+/^- donor mice (green) exhibit decreased IL-2 (e) and IFN-\(\gamma\) (f) intracellular cytokine staining compared with cells from wild-type controls.

**PD-1 pathway and islet allografts**

We investigated the intragraft features associated with prolongation of islet allograft survival in wild-type recipients treated with anti-MCP-1 mAb plus rapamycin (Fig. 4a). Allografts were well preserved and showed dense insulin staining despite the presence of a prominent peri-islet mononuclear cell infiltrate. Infiltrating CD45^+ leukocytes consisted of T cells and macrophages and showed expression of components of the PD-1 pathway, including PD-1, PD-L1, and PD-L2/B7-DC (37, 38). This led us to assess the expression of PD-1 and PD-L1 by T cells activated in vitro and in the parent→F1 CFSE model.

PD-1 was not expressed by resting CD4^+ or CD8^+ T cells, but was induced upon activation by CD3/CD28 mAbs (Fig. 4b). Similarly, although both cell types cultured under basal conditions expressed only trace levels of PD-L1, strong up-regulation was observed after cell activation with CD3/CD28 mAbs (Fig. 4b). PD-1 and PD-L1 were also up-regulated on CD4^+ and CD8^+ T cells after alloactivation in vivo, as assessed by comparison with the levels of expression by nondividing transferred cells, and expression appeared to be a function of cell division (Fig. 4c). Moreover, the expression of PD-L1 on alloactivated wild-type donor
CD4+ T cells tended to increase for at least five successive divisions, whereas PD-L1 expression continued to rise beyond five divisions in the case of alloactivation of MCP-1−/− donor T cells, with or without rapamycin therapy (Fig. 4d).

**Anti-PD-L1 mAb revokes long-term graft survival**

We next considered whether the increased PD-L1 expression on alloreactive T cells seen after targeting the MCP-1-CCR2 pathway was of functional significance by testing the effects of PD-L1 blockade in vivo. Administration of a neutralizing anti-PD-L1 mAb from the time of engraftment to islet allograft recipients treated with anti-MCP-1 mAb plus rapamycin led to rejection by 20–25 days post-transplant (Fig. 5a). In parallel studies, treatment with anti-PD-L1 mAb resulted in increased production of IL-2 by CD4+ cells and increased IFN-γ production by CD8+ cells after alloactivation in vivo compared with corresponding untreated or rapamycin-treated controls (Fig. 5b).
Discussion

This study showed that the MCP-1/CCR2 pathway is up-regulated during islet allograft rejection, and that targeting this pathway, in conjunction with a brief course of rapamycin, could induce long-term islet allograft survival through a PD-L1-dependent mechanism.

Chemokines are involved in various biologic processes beyond cell migration, including cell activation and differentiation, and previous studies have analyzed the relative importance of the main chemokines and their receptors in mediating vascularized cardiac allograft rejection (4, 5). In this study subrenal capsular islet allografts were rejected within 2 wk of transplantation in conjunction with a host mononuclear cell in grafts were rejected within 2 wk of transplantation in conjunction with a host mononuclear cell in vascularized cardiac allografts in wild-type mice treated with anti-MCP-1 mAb plus rapamycin (Fig. 5a) compared with islet survival in mice receiving anti-MCP-1 plus RPM (red). b, Effects of anti-PD-L1 mAb therapy on production of IL-2 and IFN-γ by CD4+ and CD8+ T cells undergoing alloactivation in vivo in the presence of anti-PD-L1 mAb or RPM or no therapy. The mean fluorescence of IL-2 and IFN-γ labeling is shown after permeabilization and intracellular cytokine staining and is representative of three experiments.

The critical importance of the PD-1 pathway in achieving long-term engraftment in our model was shown by the rejection of islet allografts in recipients treated with anti-PD-L1 mAb (Fig. 5a) and by concomitant adoptive transfer studies showing that anti-PD-L1 mAb enhanced IL-2 and IFN-γ production by T cells undergoing alloactivation in vivo (Fig. 5b). There is growing evidence that PD-L1 expression contributes to the maintenance of host unresponsiveness in the periphery as well as at sites of inflammation (32, 40–42). However, it is unclear in any of this work at which sites the blockade of PD-L1 is effective, given the capacity of various leukocytes (43–45), endothelial cells (46, 47), and dendritic cells (44, 48) to express this molecule upon immune activation.

Further studies are required to assess the extent to which targeting MCP-1 or CCR2 in conjunction with rapamycin can induce actual tolerance, because challenge with second-donor grafts or third-party grafts has not yet been investigated. Similarly, whether there is any direct link between MCP-1 expression and inhibition of PD-1 or PD-L1 induction warrants analysis. Lastly, in a broader context, the current studies illustrate the importance of dissecting the involvement of chemokines and their receptors in mediating islet allograft rejection. Such work appears highly relevant if efforts to promote long-term engraftment without ongoing immunosuppression and associated toxicity are to achieve success.

References
