Role of Donor-Derived Monocyte Chemoattractant Protein-1 in Murine Islet Transplantation

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Monocyte chemoattractant protein-1 (CCL2/MCP-1) is a proinflammatory chemokine produced by several cell types, including pancreatic islets. High levels of donor-derived CCL2 have been associated with poor islet allograft outcome in patients with type 1 diabetes; however, the causal relationship is unknown. The constitutive and inducible expression of chemokines and their receptors by pancreatic islets in vitro were investigated, specifically the role of donor-derived CCL2 in marginal mass murine islet transplantation. The results showed that inflammatory cytokine stimulation of islets induced de novo expression of CCL2, CCL5/RANTES, CXCL9/MIG, and CXCL10/IP-10 and increased expression of CXCL2/macrophage-inflammatory protein-2. CCL2 mRNA and protein were highly expressed within 2 d in cultures. Transplantation of islets with high levels of CCL2 into syngeneic recipients led to a significantly greater influx of CCR2+ cells and higher expression of monocyte/macrophage-associated inflammatory cytokines compared with low CCL2-donor islets. The level of pretransplantation CCL2 inversely correlated (P < 0.0001) with isograft function. In contrast, in CCR2−/− recipients, this correlation was not present. A direct toxic effect of CCL2 on islets was excluded by assessing cell viability and insulin release in vitro. In conclusion, CCL2 secreted by islets plays an important role in the immediate islet graft function. Strategies to decrease islet-derived CCL2 release may increase the success of islet transplantation.


In the immediate period after islet transplantation, the marginal islet mass is further compromised by β cell damage caused by ischemia-reperfusion and the inflammatory response. In addition to adhesion molecules, such as selectins and integrins, the complex process of extravasation and influx of leukocyte subsets into the site of tissue injury is mediated, to a significant extent, by the expression of specific chemokines and chemokine receptors (1). Monocyte chemoattractant protein-1 (CCL2/MCP-1) is a proinflammatory chemokine produced by monocytes/macrophages and lymphocytes in addition to many other cell types, including endothelial cells, mesangial cells, renal tubular cells, and fibroblasts, in response to cytokines, oxidized LDL, and LPS (2–4). Several studies have now shown that CCL2 is also strongly expressed by human and rodent pancreatic islets under various conditions (5–11). The biologic actions of CCL2 are mainly mediated through the interaction with the CC chemokine receptor 2 (CCR2), the other ligands for which include CCL8/MCP-2, CCL7/MCP-3, and CCL12/MCP-5. CCR2 receptors are found on the surface of inflammatory mononuclear cells, endothelial cells, and fibroblasts (12). Through binding to the CCR2 receptor, its ligands signal monocytes, natural killer, and activated and memory T cells to migrate to sites of injury as part of the inflammatory response (13,14).

We hypothesized that the local generation of chemokines by islet cells may be important in the initiation and regulation of inflammatory processes during insulitis and islet graft destruction. This hypothesis is supported by recent data that demonstrated that high levels of CCL2 released by human islet cells may play an important role in the clinical outcome of islet allografts in patients with type 1 diabetes, because high levels of donor-derived CCL2 secretion were associated with poor islet graft outcome (6). Despite recent publications on CCL2 secretion by pancreatic islets (5–11), no mechanistic data outlining the role of donor-derived CCL2 are available. In the present study, we specifically investigated the role of donor-derived CCL2 in murine islet transplantation.

Materials and Methods

Mice and Diabetic Model

Animals were treated in strict compliance with regulations established by the Institutional Animal Care and Use Committee. Mice were born and housed under specific pathogen-free conditions. The recipients were rendered diabetic by a single intraperitoneal injection of 180 mg/kg streptozotocin (Sigma, St. Louis, MO) and considered diabetic when the tail-vein blood glucose concentration as determined by One-Touch glucometer (Lifescan, Milpitas, CA) was >300 mg/dl for 2 consecutive days. Inbred C57BL/6 (H-2b) mice (Jackson Laboratories, Bar Harbor, ME) were used at 10 to 12 wk of age (25 to 30 g). The CCR2 gene knockout mice (background C57BL/6, Fl0) were provided by...
W.A. Kuziel (Department of Microbiology, Institute for Cellular and Molecular Biology, University of Texas, Austin, TX), and deletion was confirmed by genotyping (15).

**Islet Isolation, Culture, and Transplantation**

Islet isolation and their culture and transplantation were previously described in detail (5). Briefly, 3 ml of cold Hank’s buffer/collagenase P solution (1.5 mg/ml; Roche Diagnostics, Mannheim, Germany) was infused into the pancreatic duct in situ, and the removed pancreas was digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). For marginal mass syngeneic transplantation, digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). For marginal mass syngeneic transplantation, digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). For marginal mass syngeneic transplantation, digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). For marginal mass syngeneic transplantation, digested at 37°C for 20 min. Islets were purified on a discontinuous Ficoll gradient (Sigma). 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As islets are composed of different residential cell populations, an established β cell line (β-TC3) was also studied to delineate the contribution of this cell type. Immediately after the isolation and purification procedure, islets expressed the transcripts of specific chemokine genes, namely CCL6, CCL7, CCL17, CCL19, CCL21, CCL28, CXCL2, CXCL5, CXCL11, and CXCL15. The expression of CCL2 was induced after 24 h in culture. In addition, most of the chemokines identified immediately after isolation were further upregulated after 24 h in culture. The same chemokine mRNA expression pattern was observed in β-TC3 in long-term culture. Transcripts for the chemokine receptors CCR7 and CXCR5 were found in both β-TC3 and isolated islets (Table 1).

One mechanism by which invading mononuclear cells destroy β cells is by local production of cytokines such as TNF-α, IL-1β, and IFN-γ. To study whether proinflammatory conditions result in the induction of chemokines in islets, we exposed cells in culture to a combination of TNF-α, IL-1β, and IFN-γ for 24 h at a concentration used previously (10,11). Cytokine stimulation of islets led to an induction of CCL2, CCL5/RANTES (regulated on activation normal T cell expressed and secreted), CXCL2/MIP-2, CXCL9/MIG, and CXCL10/IP-10. With the exception of CXCL2, all genes were also induced in the β-TC3 cell line (β-TC3), suggesting a source other than β cells within the islets for CXCL2 (Table 2). These findings were confirmed by QPCR (data not shown).

To determine whether CCL2 mRNA expression was associated with protein synthesis and release, we determined CCL2 levels in culture medium by ELISA and found them to correlate well with the pattern of mRNA expression. Islets that were cultured up to 2 d had a progressive increase in CCL2 production (Figure 1). These in vitro data suggest that islets and β-TC3 cells constitutively expressed the mRNA for several chemokines and chemokine receptors, and exposure to proinflammatory cytokines upregulated additional potent chemokines, with the potential to attract cells expressing CCR2, CCR5, and CXCR3, such as activated T cells and monocytes/macrophages.

### Table 1. Chemokine and chemokine receptor gene expression in murine β-TC3 cells and islets immediately after isolation (0 h) and after 24 h in culture, without the addition of cytokines, detected by cDNA array

<table>
<thead>
<tr>
<th>Gene</th>
<th>Other Name</th>
<th>β-TC3 0 h</th>
<th>β-TC3 24 h</th>
<th>Islets 0 h</th>
<th>Islets 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>TCA3</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>CCL2</td>
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<td>–</td>
<td>–</td>
<td>17</td>
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<td>MIP-1α</td>
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<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>CCL4</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
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<tr>
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<tr>
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<tr>
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### Table 2. IL-1β, IFN-γ, and TNF-α induced chemokine gene expression in islets and cultured β-TC3 cells detected by cDNA array

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>β-TC3</th>
<th>Islets</th>
</tr>
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<tbody>
<tr>
<td>CC</td>
<td>CCL2</td>
<td>MCP-1</td>
</tr>
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<td>RANTES</td>
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</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td></td>
</tr>
</tbody>
</table>

*aData are presented as fold variation in gene expression compared with their respective controls (untreated cells). A ≥twofold change of gene expression comparing stimulated to unstimulated cells was considered significant. When genes were not detectable in unstimulated controls, the fold change was expressed as >+2, +5, or +10 depending on expression level.*
High Levels of Pretransplantation CCL2 Effectively Recruit CCR2+/H11001 Monocytes/Macrophages and T Cells

To assess whether donor-derived CCL2 has the potential to attract CCR2+/H11001 cells in vivo, we analyzed intragraft mRNA expression in isografts by QPCR and localized it with in situ hybridization. By using the isografts, we were able to determine recruitment independent of allogeneic stimulus. High levels of pretransplantation CCL2 by islets resulted in greater intragraft CCR2 mRNA expression (Figure 2). In situ hybridization localized a significant CCR2+/H11001 mononuclear cellular infiltrate surrounding the islet cells (Figure 3). Molecular markers for leukocytes suggested increased recruitment of monocytes/macrophages (CD64, CD68) and T cells (CD3) into the CCL2-expressing donor grafts (Figure 2), with early recruitment of monocytes/macrophages on day 1 followed by late recruitment of T cells on day 4.

Intragraft Cytokine mRNA Expression Pattern

Expression of seven cytokines was analyzed on days 1 and 4 posttransplantation of cultured and uncultured islets to assess their local production. There was no detectable expression of IL-2 and IFN-γ (data not shown). Both cytokines are mainly produced by T cells and natural killer cells after their activation, suggesting that significant T cell activation does not occur within 4 d posttransplantation. In addition, there was no difference of IL-10 expression in the two groups and time point's analyzed (data not shown).

In contrast, there was evidence for production of monocyte/macrophage-derived cytokines. Cultured islets showed higher expression of IL-1β, TNF-α, TGF-β1, and MIP-1α when compared with freshly isolated islets after transplantation (Figure 4). MIP-1α is a product of monocytes and macrophages and primary stimulator of other proinflammatory cytokines such as IL-1, IL-6, and TNF-α. This is consistent with the pattern of cytokine production seen in cultured transplanted islets. These

Figure 1. Effect of islet cell culture on CCL2 secretion. CCL2 secretion of 13 different islet preparations immediately after isolation (0 h), 1 and 2 d of culture. CCL2 concentration is given as pg/ml supernatant and pg/islet.

Figure 2. Isograft CCL2, CCR2, monocyte/macrophage marker CD64 and CD68, and T cell marker CD3 mRNA expression. Grafts were analyzed by QPCR 1 and 4 d after transplantation of cultured or freshly isolated islets (*P < 0.05 freshly isolated versus cultured islets).
Figure 3. CCR2 was localized by in situ hybridization using an antisense riboprobe, demonstrating mRNA expression (in situ signal indicated by the deposition of silver grains) by infiltrating mononuclear cells. As negative controls, serial sections were hybridized with the CCR2 sense probe, demonstrating a very small number of nonspecifically deposited silver grains. Original magnification ×400.

Figure 4. Isograft cytokine mRNA expression. Grafts were analyzed by QPCR 1 and 4 d after transplantation of cultured or freshly isolated islets (*P < 0.05 freshly isolated versus cultured islets).

Data suggest that mainly monocyte/macrophage-derived cytokines and not T cell–derived cytokines are likely mediators for the impaired islet graft function.

Effect of Islet-Derived CCL2 on Islet Isograft Function

We next examined whether the islet cell culture or the infiltrating mononuclear cells are detrimental to islet grafts using a marginal mass transplantation model in streptozotocin-induced diabetic syngeneic C57BL/6 mice. In this model, the degree of glucose reduction, expressed as percentage reduction of the pretransplantation glucose concentration, reflects islet graft function in vivo. The mean glucose reduction over 4 d posttransplantation was significantly lower when islets were transplanted after 2 d of culture compared with freshly isolated islets grafts (−40 ± 15.9 versus −55 ± 7.7%; P = 0.038). We then examined whether pretransplantation CCL2 levels ranging from 0.46 pg/islet (22.9 pg/ml) to 4.12 pg/islet (206 pg/ml) correlated with islet graft function. We found that the glucose reduction 1 d posttransplantation inversely correlated with the level of CCL2 secretion of islets (P < 0.0001; Figure 5). This effect was independent of the time interval analyzed (mean posttransplantation glucose reduction day 1 to 2 r = −0.923 (95% confidence interval [CI], −0.98 to −0.74), day 1 to 3 r = −0.921 (95% CI, −0.98 to −0.74), and day 1 to 4 r = −0.915 (95% CI, −0.98 to −0.72). Isografts that were transplanted to CCR2−/− recipients demonstrated reduction of glucose independent of the level of CCL2 production by islets (Figure 5). Although the CCR2−/− animals were significantly protected from this effect, damage to the islets could not be completely accounted for by MCP-1/CCR2 interaction because a minimal decrease in percentage of glucose reduction was seen in the absence of the receptor, suggesting a role for other cytokines or chemokines.

CCL2 Is Not β Cell Toxic and Does not Impair Insulin Release In Vitro

We and others have shown that islet cell expression of CCR2 mRNA can be induced under various conditions (5,19). Using the more sensitive QPCR assay (data not shown), we demonstrated that islet cells constitutively expressed small amounts of CCR2 mRNA. Therefore, to determine whether the detrimental effect of high levels of CCL2 secretion was due to direct toxicity, we measured in vitro glucose-stimulated insulin release and β cell viability, in the presence of recombinant CCL2 at concentrations similar to those produced by cultured islets (Figure 1). Islet cell insulin release at basal (5.5 mM) or high glucose (16.7 mM) conditions was unaffected by rCCL2 at a range of concentrations (100 to 1000 pg/ml; Figure 6). β-TC3 cells that were incubated with rCCL2 concentrations ranging from 100 to 10⁵ pg/ml were also evaluated for cell viability using the MTT method. We found that rCCL2 had no direct effect on β-TC3 cell proliferation or viability (Figure 7). These results strongly suggest that CCL2 itself had no deleterious effect on β cell viability or endocrine function.

Discussion

Highly differentiated pancreatic β cells have prominent chemokine-producing capabilities and, therefore, the potential to induce or alter inflammatory as well as noninflammatory processes (5–11). Murine islet cells are not passive bystanders of their own destruction but have the potential to secrete CCL2, and this study is the first to show that this is detrimental for immediate posttransplantation graft function. The important role of the CCR2 pathway in conjunction with low-dose immunosuppression has been demonstrated in a murine allogeneic islet transplantation model (18,20). However, few data are available to support the role of donor-derived chemokines in transplantation (21).

Our data suggest that islets have the capacity to behave in a similar manner to cells of the immune system, recruiting leukocytes from the blood stream, resulting in the amplification of
the mononuclear cell infiltration within the islet itself. This supports the data of a recent study in islet transplant patients with type 1 diabetes that found a correlation with high basal donor islet CCL2 production before transplantation with a poor islet graft function in wild type recipients. This correlation was absent when the CCR2 pathway was disrupted by transplanting CCR2<sup>−/−</sup> mice. Data in the literature are conflicting as to whether islets expressing CCL2 constitutively may be due to the differences in species and technique sensitivities. The data that islets express and secrete CCL2 as soon as they are cultured are consistent with an inflammatory function for CCL2 in this setting, and migration studies with isolated monocytes have shown that CCL2 secreted by islets is biologically active (6,7). Our data that in vitro culture of islets augmented CCL2 secretion are in agreement with previous data on human islets cultured up to 6 (6) or 11 days after isolation (22). Our in vivo experiments showed that the inflammatory mediator CCL2 exerts a biologic effect by providing a gradient to attract CCR2<sup>+</sup> cells to the site of inflammation. Moreover, the amount of CCL2 produced negatively correlated with islet graft func-

Figure 5. Effect of islet-derived CCL2 on islet isograft function after marginal mass transplantation of streptozotocin-induced diabetic mice. (A) Linear regression of pretransplant CCL2 concentration with glucose reduction 1 d posttransplantation. Linear coefficient (r) and its 95% confidence interval and P-value are shown. High pretransplant CCL2 significantly correlated with poor islet graft function in wild type recipients. This correlation was absent when the CCR2 pathway was disrupted by transplanting CCR2<sup>−/−</sup> mice. (B) Recipients divided into low and high donor-derived CCL2 and the effect on glucose reduction. CCL2 cutoff value was the 50th percentile (82 pg/ml).

Figure 6. Effect of CCL2 on islet and β-TG insulin secretion. Cells were cultured for 12 h with 0 (vehicle control), 100, and 1000 pg/ml rCCL2 and then exposed to 5.5 mM or 16.7mM glucose for 1 h. Supernatants were analyzed for insulin content with ELISA. Data are shown as mean insulin secretion ± SEM.

Figure 7. Effect of CCL2 on β-TG cell viability was measured by MTT metabolism. Data are % of control mean ± SEM of three experiments performed in quintuplicate. β-TG were plated in 96-well plates and cultured for 48 hours in the presence of rCCL2 at various concentrations.
tion, a correlation absent when the CCR2 pathway was disrupted.

Our data suggest that the primary cause of transplanted islet dysfunction is antigen-nonspecific inflammation at the graft site mediated by the monocyte/macrophage-associated inflammatory products IL-1β, TNF-α, and MIP-1α. Recent data also documented significant expression of macrophage-associated inflammatory genes, including IL-1β, IL-6, TNF-α, and CCL2, in islets from a brain-dead donor (23), and these islet grafts were found to have a decreased functionality in vivo and in vitro (24). Together, the data suggest that donor-derived CCL2 leads to monocyte/macrophage infiltration, further cytokine secretion, and activation of the innate and adaptive immune response to cause graft dysfunction.

Of note, transgenic mice with targeted CCL2 expression for β cells develop intense insulitis, with a predominance of macrophages but no diabetes (25). This suggests that the local production of CCL2 is not sufficient to cause tissue destruction but might contribute to the recruitment of a first wave of mononuclear cells, and the final result depends on the presence of other chemokines. Our data with early monocyte/macrophage infiltration followed by T cells would be consistent with that hypothesis. In addition, in our marginal mass model, which resembles clinical islet transplantation, mild insulitis might affect islet graft function. Our results exclude, however, that CCL2 is directly β cell toxic, as it did not affect β cell viability or the release of insulin, confirming previous studies with human islets (6).

Our data have several potential clinical implications. First, they provide evidence that islet cell culture affects islet graft function in vivo. Transplantation of islets immediately after isolation was done in the original description of the Edmonton protocol (26) and may explain the high success rate not reached in other sites despite the same immunosuppressive protocol (27). At this point, however, it is unknown whether islet cell culture has a negative impact on graft function in immunosuppressed recipients. Second, measurement of pretransplantation CCL2 level may be a useful tool to preselect islets to increase engraftment success rates. Third, strategies to prevent in vitro CCL2 secretion might offer new approaches in clinical islet transplantation. Of note, NF-κB was found to play an important role for CCL2 expression in β cells and might be a potential target for pretransplantation, ex vivo gene therapy (9). In addition, it was previously shown that the in vitro CCL2 expression in human islets can be decreased by cyclosporine and the vitamin B derivative nicotinamide (28).

Using a cDNA array approach, we also identified the induction of potent chemokines CCL5, CXC9, CXCL10, and CXCL2 after treatment with cytokines. It is interesting that CXCL9 and CXCL10 are not only chemoattractant but also angiostatic and, therefore, of possible importance during islet transplantation, in which revascularization of the islets is required for successful engraftment (29). However, the role of these chemokines in the recruitment of inflammatory cells and angiogenesis is unclear and needs to be studied further. We have also shown that islets as well as β-TC3 cells expressed the chemokine receptor CCR7, along with both its ligands CCL19/SLC and CCL21/MIP-3β.

Expression of both CCL19 and CCR7 was also found in glomerular mesangial cells, where this pathway was important for cell survival, suggesting a homeostatic or regulatory role for chemokines expressed by nonlymphoid tissue (30).

In conclusion, high pretransplantation CCL2 concentration has a negative impact on immediate islet graft function as a result of increased inflammatory cell recruitment. Therefore, strategies to decrease CCL2 release by islet preparations or blocking the CCL2/CCR2 pathway in the recipient might increase the success of islet engraftment and long-term insulin independence in human islet transplantation.

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References


