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The Chemokine Binding Protein M3 Prevents Diabetes Induced by Multiple Low Doses of Streptozotocin

Andrea P. Martin,* Jennifer M. Alexander-Brett,† Claudia Canasto-Chibuque,* Alexandre Garin,* Jonathan S. Bromberg,**‡ Dave H. Fremont,† and Sergio A. Lira2*

Multiple injections of low-dose streptozotocin (MLDS) induce lymphocytic insulitis and diabetes in rodents. To test whether the influx of inflammatory cells was associated with changes in the expression of chemokines, we measured the expression of all known chemokine ligands by real-time quantitative PCR in isolated islets. With the exception of CCL20 and CCL19, chemokines were not significantly expressed in islets from wild-type mice before MLDS treatment. Ten days after treatment, the expression of several chemokines, including CXCL9, CCL1, CXCL10, and CCL21, was dramatically up-regulated. The expression of CCL1, CXCL9, and CCL21 protein was confirmed by immunohistochemistry and was mostly associated with the infiltrating cells. The mouse herpesvirus 68-encoded chemokine decoy receptor M3 can broadly engage these chemokines with high affinity. To test whether a blockade of chemokine function would alter the onset or magnitude of insulitis and diabetes, we used transgenic mice expressing M3 in β cells (rat insulin promoter (RIP)-M3 mice). RIP-M3 mice were normoglycemic and responded normally to glucose challenge but were remarkably resistant to diabetes induced by MLDS. Islets from MLDS-treated RIP-M3 mice had fewer inflammatory cells and expressed lower levels of chemokines than those from MLDS-treated controls. The role of M3 in chemokine blockade during insulitis was further supported by in vitro experiments demonstrating that multiple chemokines up-regulated during islet inflammation are high-affinity M3 ligands that can be simultaneously sequestered. These results implicate chemokines as key mediators of insulitis and suggest that their blockade may represent a novel strategy to prevent insulitis and islet destruction.


Type 1 diabetes is an autoimmune disease characterized by a local inflammatory reaction in and around islets that is followed by selective destruction of insulin-secreting β cells (1). The factors leading to the destruction of the islets are still not known, but it is well accepted that immune-based mechanisms involving macrophages and T cells are responsible for the death of the β cells (2, 3).

Experimentally induced insulin-dependent diabetes mellitus in rodents with multiple low doses of streptozotocin (MLDS)3 is a widely used model that has clinical and histoimmunological features similar to those of human disease, with T cells and macrophages playing a major pathogenic role (4). When administered in animals at multiple low doses, the β cell toxin streptozotocin is thought to induce initial cell damage by alkylating the DNA in islet cells (5) and/or by spontaneously releasing NO (6). Subsequently, as a result of a novel β cell Ag expression, mononuclear cells will infiltrate the islets and start a multifactorial process (7) that will lead to islet destruction and diabetes.

The trafficking of macrophages and T cells has been shown to be regulated by chemokines, small m.w. chemoattractant proteins that interact with G protein-coupled receptors present at the cell surface of these leukocytes to regulate their migration, differentiation, and function (8–10). The migration of macrophages and T cells is controlled by multiple chemokines, including CCL2, CCL21, CCL19 (9, 11, 12), CXCL10, and CXCL9 (13). The migration of monocytes and macrophages is mediated by CCL2 and its receptor, CCR2 (14). Both homeostatic and inflammatory T cell trafficking are under the control of chemokines. The homeostatic migration of T cells is regulated by CCL21 and CCL19. Both CCL19 and CCL21 interact with a common receptor, CCR7, which is expressed by naive and memory T cells (15). The influx of effector T cells into inflamed areas is regulated by multiple chemokines, including the IFN-γ-inducible chemokines CXCL10 and CXCL9 and their receptor CXCXR3 (13).

Chemokines are not highly expressed by the normal islets, but chemokine expression has been detected in islets cultured in vitro. Primary cultures of murine and human pancreatic islets express and secrete CCL2 (16). CCL2 expression in the pancreas parallels disease progression in NOD mice (17, 18). A recent study has suggested an important role for CCL2 in the clinical outcome of islet transplantation in patients with type I diabetes (16). Low CCL2 secretion by islets before transplantation was the most relevant factor for predicting long-lasting insulin independence. CCL21 is not normally expressed by pancreatic islets, but its expression has been observed in the pancreas of NOD mice (19, 20). The expression of CCL21 by the islets has been proposed.

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2 Abbreviations used in this paper: MLDS, multiple low-doses of streptozotocin; h, human; MHV-68, mouse herpesvirus 68; m, mouse; Q-PCR, quantitative real time PCR; RIP, rat insulin promoter; RU, resonance unit; SPR, surface plasmon resonance; STZ, streptozotocin; tg, transgenic; VEGF, vascular endothelial growth factor; wt, wild type.

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to be an important factor contributing to autoimmunity (21). Moreover, β cells secrete CXCL9 and CXCL10 during virus-induced diabetes (13, 22).

Interference with the chemokine system has been shown to attenuate or prolong the development of experimental models of diabetes. For instance, animals lacking the chemokine receptor CXCR3 show a delayed onset of type 1 diabetes subsequent to a viral infection (13). In addition, Abs neutralization of a macrophage-derived chemokine (CCL22) causes a significant reduction of CCR4⁺ T cells within the pancreatic infiltrates and inhibits the development of insulitis and diabetes in NOD mice (23). Finally, the treatment of NOD mice with a neutralizing anti-CCR5 Ab inhibits β cell destruction and diabetes (24). In none of these models, however, is there a complete abrogation of disease, which suggests that other factors (including additional chemokines) may be relevant for pathogenesis.

Testing a possible role for multiple chemokines in the pathogenesis of diabetes has been hampered by the lack of pharmacological agents with broad specificity and by the fact that many chemokine receptor genes are clustered in the genome, which precludes the generation of compound mutants by conventional breeding techniques. A novel tool for testing chemokine function is M3, a chemokine-binding protein encoded by the mouse herpesvirus 68 (MHV-68). M3 blocks chemokine receptor-binding interfaces and displaces and removes chemokines from inflammatory sites (25). Recent studies from our laboratory show that the expression of M3 by β cells blocks lymphocyte recruitment induced by transgenic (tg) expression of CCL21 (26), CCL2, and CXCL13 (27).

In this study we show that chemokines are expressed in the islets of mice treated with MLDS before the development of diabetes. Furthermore, we show that the expression of the promiscuous chemokine decoy receptor M3 in the islets reduces the leukocyte infiltration and islet destruction induced by MLDS. These results indicate that chemokines are important determinants of diabetes and suggest that multichemokine blockade may be a novel approach to prevent insulitis and diabetes.

Materials and Methods

Transgenic mice

Rat insulin promoter (RIP)-M3 tg mice were described previously (26). B6D2F1 mice were obtained from Charles River Laboratories. All mice were housed under specific pathogen-free conditions in individually ventilated cages at the Mount Sinai School of Medicine Animal Facility (New York, NY). All experiments were performed following institutional guidelines.

Streptozotocin treatment in vivo

To induce diabetes, mice (6–10 wk of age) were injected i.p. with streptozotocin (STZ) (40 mg/kg freshly dissolved in cold 0.1 M citrate buffer (pH 4.5); Calbiochem, EMD Biosciences) for 5 consecutive days as previously described (28). Blood glucose was monitored weekly over the following 35 or 70 days using a Ascensia Elite XL one-touch blood glucometer (Bayer). Animals were considered diabetic when their blood glucose for 60 min at 37°C in an atmosphere of 95% O₂ and 5% CO₂. For insulin release, islets from control and tg animals were incubated for 240 min using an Ascensia Elite XL one-touch blood glucometer (Bayer).

Histology

Tissues for light microscopic examination were fixed by immersion in 10% phosphate-buffered formalin and then processed for paraffin sections. Tissue sections, 5-μm sections were cut and stained with H&E. For immunohistochemical staining fresh-frozen sections were first fixed with ice-cold acetone for 20 min and dried and stored at -20°C. Slides were incubated for 1 h at room temperature with purified primary Abs followed by incubation with the appropriate labeled secondary Abs for 30 min. Primary Abs used were anti-CD45 (catalog no. 550539), CD3 (catalog no. 553058), CD11c (catalog no. 553799), CD11b (catalog no. 553308), and CD3 (catalog no. 550274) from BD Biosciences, rat anti-insulin (catalog no. MAB1417), anti-CCL1 (catalog no. MAB845), anti-CCL2 (catalog no. AF455) from R&D Systems, anti-CXCL9 (provided by J. Farber and H. Zhang, National Institute of Allergy and Infectious Diseases), and guinea pig polyclonal anti-insulin (catalog no. A0564) from DakoCytomation. The secondary Abs used were Alexa Fluor 488 goat anti-rat IgG (catalog no. A-11006), Alexa Fluor 594 goat anti-rat IgG (catalog no. A-11077), and Alexa Fluor 594 goat anti-rabbit IgG (catalog no. A-11037) from Molecular Probes and FITC anti-Guinea Pig IgG (catalog no. 127065160) from Jackson ImmunoResearch Laboratories.

To study the cellular changes promoted by STZ treatment we performed semiquantitative analysis on insulin/CXCL4-stained sections, assessing 20–80 islets per animal. Three grades of infiltration were based on the number of CD45⁺ cells in or around the islet: 1 (10–20 cells), 2 (20–50 cells), and 3 (>50 cells). At least 20 sections were evaluated per mouse and per day in a blinded fashion. Data are presented as mean insulin scores ± SD for the indicated experimental groups.

Intraperitoneal glucose tolerance test

A 16-h fast, glucose (1.5 g/kg body weight in saline (0.9% NaCl)) was administered i.p. The blood glucose was monitored at 0, 30, 60, 120, and 240 min using an Ascensia Elite XL one-touch blood glucometer (Bayer).

Isolation of pancreatic islets of Langerhans

Islets of Langerhans were isolated as previously described (29). Briefly, the common bile duct was clamped distal to the pancreatic duct junction at its hepatic insertion. The proximal common bile duct was then cannulated using a 27-gauge needle, and the pancreas was infused by retrograde injection of 2 ml of ice-cold collagenase solution (1.0 mg/ml; Sigma-Aldrich) in HBSS (Invitrogen Life Technologies). Pancreatic tissue was recovered and subjected to a 12-min digestion at 37°C. Subsequently, ice cold HBSS was added and the suspension was vortexed at full speed for 10 s. Islets were hand picked under a dissection microscope. Islets were used immediately after isolation to obtain RNA or protein.

Western analysis

To analyze Glut-2 expression, islets from control and tg mice were sonicated in freshly prepared lysis buffer (5% SDS, 80 mMOL/L Tris-HCl (pH 6.8), 5 mMOL/L EDTA, and 0.5 mMOL/L PMSF). The supernatants containing the cell lysate were separated by centrifugation and the protein concentrations were determined using the MicroBCA assay (Pierce). Forty micrograms of protein from each sample was added to loading buffer and analyzed using 10% SDS-polyacrylamide gels. Proteins were transferred from the gels to Immobilon-P membranes (Millipore) using standard techniques. Blots were incubated with Abs against Glut-2 (Santa Cruz Bio-technology) and actin (Sigma-Aldrich) and then with a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) and a peroxidase-conjugated rabbit anti-mouse IgG (Abcam), respectively.

To analyze M3 expression, 200 islets from control and tg animals were incubated for 24 h in glucose-free medium and supernatants were collected. Twenty micrograms of protein from each sample was processed as described above. Blots were incubated with primary Abs against M3 (26) and a peroxidase-conjugated goat anti-rabbit IgG (Abcam). Chemiluminescence was detected using the Western Lightning Western Blot Chemiluminescence Reagent Plus (enhanced luminol) (PerkinElmer).

Islet glucose-stimulated insulin release

The insulin secretion assay was performed as described by Eizirik et al. with some modifications (30). Briefly, 20 islets from each group were set in quintuplicate in a 24-well plate and incubated in CMRL 1066 medium (Cellgro, Mediatech) supplemented with 1.7 mM glucose or with 16.7 mM glucose for 60 min at 37°C in an atmosphere of 95% O₂ and 5% CO₂. For insulin content, fresh islets were collected, washed with 1 ml of PBS, and sonicated in acid ethanol to extract insulin. All of supernatants and extractions were kept at -20°C. Insulin released into supernatants and insulin content was measured by ELISA (ALPCO Diagnostic).

Quantitative real-time PCR (Q-PCR)

Total RNA was extracted from pooled islets using the RNeasy maxi kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed for 3 μg of RNA. Q-PCR was conducted in duplicate from 25 ng of cDNA and with each primer at 0.4 μM in a 30-μl final reaction volumes of 1X SYBR Green PCR Master Mix (Applied Biosystems). PCR cycling conditions were 50°C for 2 min, 95°C for 15 min, and
Table I. Sequences of primers used to study mRNA chemokine expression by Q-PCR (5' to 3')

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Surface plasmon resonance (SPR)-based competition assay

SPR experiments were conducted by using a Biacore 2000 biosensor at 25°C under conditions of 20 mM HEPES (pH 7.4), 150 mM sodium chloride, 0.005% Triton X-100. The M3 and human (h)CCL2 used in this analysis were produced as described elsewhere (J. M. Alexander-Brett and D. H. Fremont, submitted for publication). Murine (m)CXCL10, mCCL21, and vascular endothelial growth factor (VEGF) were purchased from BioSource, and murine CX3CL1 were purchased from Xenotech, submitted for publication. Murine (m)CXCL10, mCCL21, and hVEGF (each at 50 nM) serving as positive and negative controls, respectively.

Briefly, the competition assay measures the ability to bind the second M3 site, with CXCL1 and VEGF (each at 50 nM) serving as the positive and negative controls, respectively.

Analysis

Data are expressed as means ± SEM. An unpaired t test, one-way ANOVA, and repeated measures ANOVA were used to determine statistical significance. Differences were considered significant when p < 0.05.
Several sections of pancreata from control mice (whether MLDS led to changes in chemokine protein expression. Higher levels than at day 0) (Fig. 1). CXCL10, and CCL1 (which were expressed at 31- to 178-fold higher levels of some chemokines, most notably CXCL9, were detected 3–5 days after the initiation of treatment (data not shown). No significant differences in chemokine expression were expressed at very low levels before treatment (data not shown). Further analysis showed that CCL1 and CXCL9 expression is mostly associated with the inflammatory cells. White arrowheads point to the coexpression of chemokines and the cell marker analyzed in each case. In B–J immunostaining was performed in pancreata 10 days after of MLDS treatment. Scale bars, 25 μm.

FIGURE 1. MLDS treatment induces the expression of chemokines and the recruitment of inflammatory cells to the islets of Langerhans. A. Fold increase in chemokine mRNA levels in islets of Langerhans after MLDS. Values were determined by Q-PCR before treatment and at day 10. Shown are chemokines for which there was at least a 3-fold difference in expression levels. B and C, Insulin/CD45 (green/red) staining at day 0 (B) and day 10 (C). D–F, CCL1 (red) together with CD45 (green; D), CD11b (green; E) and CD11c (green; F) staining. G, Immunostaining for CCL21 (red). H, CCL21 (red) and CD31 (green) staining. I and J, CXCL9 (red) together with CD11b (green; I) and CD11c (green; J) staining. Note that chemokine expression was detected 3–5 days after the initiation of treatment (data not shown). No significant differences in chemokine expression were detected 3–5 days after the initiation of treatment (data not shown). However, there were striking differences in the expression levels of some chemokines, most notably CXCL9, CXCL10, and CCL1 (which were expressed at 31- to 178-fold higher levels than at day 0) (Fig. 1A).

We then used immunohistochemical analysis to determine whether MLDS led to changes in chemokine protein expression. Several sections of pancreata from control mice (n = 8) before and 10 days after initiation of MLDS were analyzed using Abs against some of the chemokine ligands that were found to be highly upregulated in the Q-PCR analysis (CXCL9, CCL1, and CCL21). To determine the cellular source of chemokines, we used Abs against insulin, the pan-leukocyte marker CD45, the myeloid marker CD11b, dendritic cells (CD11c), T cells (CD3), and endothelial cells (CD31). Few CD45+ cells were found within islets of Langerhans before treatment (Fig. 1B). However, by day 10 we observed many CD45+ inflammatory cells within the islets (Fig. 1C). At this point, we detected strong staining for the chemokines studied (Fig. 1, D–J). As shown in Fig. 1, CCL1 and CXCL9 were localized to the infiltrating cells (within CD45+ cells; Fig. 1D and data not shown). Further analysis showed that CCL1 and CXCL9 were expressed mainly by CD11b+ and CD11c+ cells (Fig. 1, E–F and I–J, respectively) but not for T cells or endothelial cells (data not shown). CCL21 was found in areas where there was an accumulation of inflammatory cells (Fig. 1G) mainly expressed by endothelial (CD31+) cells (Fig. 1H). These results validate the results obtained by Q-PCR analysis and indicate that MLDS treatment induced the expression of chemokines primarily in cells infiltrating the islets of Langerhans.

Expression of M3 does not affect the ability of β cells to produce or secrete insulin

Next, we tested the hypothesis that an islet-specific chemokine blockade affected the development of diabetes induced by MLDS. To this end we used mice expressing the chemokine-binding protein M3 in the islets (RIP-M3 mice) (26). We have previously shown that mice expressing M3 in the islets develop normally and are normoglycemic. Furthermore, we found that the constitutive expression of M3 in the pancreas of the RIP-M3 mice did not affect the development of lymphoid or nonlymphoid tissues. To rule out that expression of M3 affected β cell function we performed both in vitro and in vivo experiments.

First we confirmed that islets from tg animals secreted M3 in vitro. Isolated islets from tg RIP-M3 mice secreted immunoreactive 44-kDa M3 protein after 24 h of culture (Fig. 2A). No immunoreactivity was found in the medium from control islets. Densitometric scanning of the bands showed that islets from homozygous (tg/tg) mice released approximately double the amount of M3 as heterozygous (tg/wt) islets (data not shown, n = 4 experiments).

To determine whether the expression of M3 by β cells affected glucose homeostasis, we analyzed the insulin content and the secretion of insulin after a glucose challenge in islets from controls...
and RIP-M3 tg mice. There was no significant difference in the insulin content between islets from control and RIP-M3 mice (n = 15 per group, p = 0.65; one-way ANOVA). C, Isolated islets were cultured with 1.7 or 16.7 mM glucose for 1 h. The insulin concentration in supernatants was determined by ELISA. The results represent three separate experiments under similar experimental conditions (p = 0.75; one-way ANOVA). D, Intraperitoneal glucose tolerance test. Glucose was injected i.p. into fasting non-tg and tg mice (8 wk old) and blood glucose was measured every 30 min (p = 0.97; one-way ANOVA). E, Blood glucose levels after the administration of a high dose of STZ in RIP-M3 tg/wt (n = 5, ○), RIP-M3 tg/tg (n = 5, ◇) and non-tg (wt) littermates (n = 5, □) (p = 0.78; one-way ANOVA).

**Figure 2.** Expression of M3 does not alter the function of β cells. A, Western blot analysis of M3 protein in supernatants of cultured islets. B, Insulin content measured by ELISA in freshly isolated islets from control and RIP-M3 mice (n = 15 per group, p = 0.65; one-way ANOVA). C, Isolated islets were cultured with 1.7 or 16.7 mM glucose for 1 h. The insulin concentration in supernatants was determined by ELISA. The results represent three separate experiments under similar experimental conditions (p = 0.75; one-way ANOVA). D, Intraperitoneal glucose tolerance test. Glucose was injected i.p. into fasting non-tg and tg mice (8 wk old) and blood glucose was measured every 30 min (p = 0.97; one-way ANOVA). E, Blood glucose levels after the administration of a high dose of STZ in RIP-M3 tg/wt (n = 5, ○), RIP-M3 tg/tg (n = 5, ◇) and non-tg (wt) littermates (n = 5, □) (p = 0.78; one-way ANOVA).

Expression of M3 in β cells blocks the development of diabetes induced by MLDS

To test the effect of M3 on the development of diabetes we treated 6–10 wk-old male RIP-M3 mice and control littermates with either STZ (40 mg/kg) or vehicle for 5 consecutive days. As expected (32), four weeks after the beginning of treatment 85% of the control mice treated with MLDS were diabetic. At this point only 35% of the RIP-M3 tg/wt mice and, remarkably, none of the homozygous RIP-M3 mice were diabetic (Fig. 3A). After 70 days all control mice were diabetic but only 60% of heterozygous mice and none of the homozygous RIP-M3 mice developed disease (n = 5 per group).

To exclude the possibility that RIP-M3 mice were resistant to the effects of STZ, we analyzed the expression of Glut-2 (the transporter for STZ (33)) in islets of RIP-M3 tg/wt, tg/tg, and control mice by Western blotting. The levels of Glut-2 protein in the islets of normal and tg mice were comparable (Fig. 3, C and D), suggesting that the expression of M3 did not alter the mechanism of STZ uptake into the cell.

**Figure 3.** Expression of M3 in β cells blocks the development of diabetes induced by MLDS. A, Diabetes incidence in animals treated with multiple low doses of STZ. Shown is the cumulative incidence of diabetes in RIP-M3 tg/wt (n = 25, ○) and RIP-M3 tg/tg (n = 13, ◇) mice and their non-tg (wt) littermates (n = 24, □). Animals were considered diabetic if blood glucose levels were higher than 250 mg/dl in two consecutive measurements. B, Western blot analysis of islet extracts from controls and RIP-M3 mice (n = 6) using Glut-2 Ab together with actin Ab as an internal control. C, Densitometric scanning of Western blots. The results are representative of two separate experiments (p = 0.44; one-way ANOVA).
Expression of M3 in β cells prevents the recruitment of inflammatory cells

Recent studies from our laboratory show that islet-specific expression of M3 blocks leukocyte recruitment induced by islet-specific expression of CCL21 (26), CCL2, and CXCL13 (27).

To test the hypothesis that M3 expression blocked STZ-induced diabetes by preventing chemokine-induced recruitment of inflammatory cells, we treated mice (n = 3 per group) with MLDS and then sacrificed at 7, 14, and 21 days after the first injection. The infiltration of CD45+ cells into the islets of control mice was first seen on day 7 (Fig. 4, A and B), and the number of inflammatory cells and the frequency of infiltrated islets increased thereafter. By day 21 most control islets were infiltrated by mononuclear cells and had lost normal morphologic integrity. In contrast, a small number of infiltrating cells was found in RIP-M3 tg/wt mice only after 21 days of treatment and the morphological appearance of the islets was normal (Fig. 4B).

To determine whether the M3 blockade of inflammatory cell influx altered the mRNA chemokine expression, we performed Q-PCR on total RNA isolated from islets before and after MLDS. After 10 days of treatment, several chemokines were up-regulated in control mice. However, there was no increase in chemokine mRNA expression in islets from tg mice (data not shown). These results are consistent with the view that the main source of chemokines at this point were the infiltrating cells.

M3 binds multiple chemokines expressed during insulitis with high affinity

Inflammatory stimuli generally result in the up-regulation of groups of chemokines rather than a single species, as reflected by the elevated expression of numerous chemokines in the MLDS model described here. Previous studies have indicated that M3 binds to both murine and human chemokines from all four structural classes, thus acting as a broad-spectrum chemokine scavenger (34, 35). However, the M3 affinities for several chemokines that are relevant to the MLDS model have not been reported. To establish whether M3 binds to up-regulated inflammatory and homeostatic chemokines, we measured its affinity for mCCL21 and mCXCL10. Binding studies were conducted using a SPR-based competition assay (J. M. Alexander-Brett and D. H. Fremont, submitted for publication), which yielded affinities of 12 ± 1 and 320 ± 30 pM for mCCL21 and mCXCL10, respectively (Fig. 5A). It was also of interest to assess whether a single M3 dimer could...
simultaneously engage two distinct chemokines. This question was addressed using a two-site SPR binding assay in which M3 was immobilized to the sensor chip via interaction with the high-affinity chemokine hCCL2, and chemokine binding to the second site was demonstrated by injecting several chemokines over the M3-CCL2 coupled sensor chip (shown schematically in Fig. 5B). The chemokines mCCL21, mCXCL10, and hXCL1 all bind the available M3 second site, indicating that M3 is capable of simultaneously bind relevant chemokines of different structural classes at opposite ends of the dimer (Fig. 5B). The lack of binding for the negative control protein VEGF demonstrates the specificity of this interaction.

Discussion
Autoimmune diseases are thought to result from complex interactions between genetic and environmental factors. Although the mechanisms by which autoimmune diseases evolve in individuals are inevitably comprised of numerous variables, there is evidence that infection may represent a key element in the development of certain autoimmune responses leading to tissue-specific destruction (36–39). Leukocytes are frequently involved in the response to infection and in autoimmune responses, but information on mechanisms leading to their recruitment and the emergence of the autoimmune attack is lacking.

In diabetes, an accumulation of cells around the islets precedes the onset of clinical disease. We hypothesized that chemokines are critical determinants of the influx of leukocytes into the islets and, thus, constitute one of the major components in the pathogenesis of diabetes. To start addressing the role of chemokines in diabetes, we have examined in a comprehensive and quantitative fashion the expression of chemokines in the MLDS model. Our results show that of all the murine chemokine ligands, only two (CCL20 and CCL19) were expressed, albeit at low levels, in pancreatic islets of B6D2F1 mice before MLDS treatment. MLDS treatment induced the high expression of several chemokines, including CXCL9, CXCL10, and CCL2, before the onset of diabetes. The expression of these chemokines was likely triggered by IFN-γ and TNF-α (40), cytokines whose expression has been documented in islets before the onset of MLDS-induced diabetes (41). The early expression of these cytokines likely influenced the production of chemokines by resident inflammatory cells, endocrine cells, and endothelial cells and promoted the infiltration of the islets, first by macrophages and subsequently by lymphocytes (31). The infiltrating cells themselves represent a major source of chemokines, as suggested by our immunohistochemical analysis. We propose that this initial burst in the expression of chemokines contributes to the further recruitment of inflammatory cells and the destruction of the islets. Further studies focusing on the temporal analysis of chemokine mRNA and protein will be required to evaluate the role of chemokines in the onset of diabetes triggered by MLDS.

Although the changes in chemokine expression suggest a link with pathogenesis, they do not prove it. The results shown here indicate that chemokines are critical for pathogenesis. The expression of a chemokine scavenger in the islets dramatically reduced cellular infiltration and blocked the development of diabetes. This effect was more pronounced in the islets from homozygous RIP-M3 mice, which expressed higher levels of M3 than the islets of heterozygous mice. We believe that the blockade in cellular recruitment was caused by M3 inactivation of one or more chemokines expressed during the course of MLDS treatment. The crystal structure of M3 shows that M3 dimerizes and generates a binding site for the N-terminal region of chemokines that precludes the binding to their receptors (42). M3 also uses electrostatic mimicry to directly inhibit chemokine-glycosaminoglycan interactions for a diverse array of chemokines (D. H. Alexander and J. M. Fremont-Brett, 2006, submitted for publication). Glycosaminoglycan association is important for chemokine function, being a prerequisite for chemokine uptake, transcytosis to the apical side of the endothelial cell, and appropriate solid-phase presentation to the passing leukocytes (43). In vivo, M3 reduces mononuclear cellular responses after MHV-68-induced meningitis in mice (44) and blocks leukocyte recruitment to the pancreas induced by CCL21 (26), CXCL13, and CCL2 (27), respectively.

We suggest that these scavenging properties of M3 are essential for the reduced inflammation observed in the RIP-M3 tg mice. Many of the chemokines up-regulated in the MLDS model are known to bind M3 with high affinity (34, 35). In this study we show that M3 binds the murine chemokines CCL21 and CXCL10.

**FIGURE 5.** High-affinity M3 binding of relevant chemokines. A, Solution binding affinity of murine CCL21 and CXCL10 to M3. Representative titration curves are shown with equilibrium chemokine binding ($R_{eq}$) to surface-bound M3 bound plotted as a function of coincjected M3 concentration with corresponding fits to the data. Thus, M3 chemokine binding affinities are determined by the competitive inhibition of chip binding. B, The schematically depicted second-site binding assay was used to assess the ability of M3 dimers to simultaneously bind distinct chemokines. M3 is loaded with CCL2 with a 2:1 stoichiometry and bound to the chip via interaction with biotinylated hCCL2. Chemokine binding to the second free M3 chemokine binding site is qualitatively assessed by sensorgram response, shown here for 50 nM injections of CXCL10, CCL21, hXCL1 ($K_D$ = 500 pM), and VEGF used as a negative control.
with picomolar affinity (12 and 320 pM, respectively) (Fig. 5). Further, we show that M3 is able to engage either of these chemokines while simultaneously binding CCL2 at opposite ends of the dimer structure, suggesting that M3 can effectively inhibit mixed populations of chemokines as observed in the insulinosis models. As such, M3 may disrupt the proper presentation and function of CCL2 (42), CXCL10, and CCL21, important players regulating the migration of monocytes and T cells that are critical for the development of diabetes (19, 23). Although our results suggest that the primary site of chemokine blockade induced by M3 is the endocrine pancreas, we cannot rule out that the trace amounts of M3 present in pancreatic lymph nodes and the bloodstream (not shown) may contribute to the effects seen here.

In conclusion, we demonstrate that chemokine expression is a hallmark of insulinitis promoted by MLDS treatment. This altered expression is not limited to one or a few chemokines; several chemokines are expressed at the same time, a pattern not limited to one or a few chemokines; instead, expression is not limited to one or a few chemokines; instead, certain chemokines. Further work will be directed to investigate whether M3 can block diabetes in autoimmune settings and to test whether it can prevent the rejection of transplanted islets.

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Disclosures
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