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The Chemokine Decoy Receptor M3 Blocks CC Chemokine Ligand 2 and CXC Chemokine Ligand 13 Function In Vivo

Andrea P. Martin,* Claudia Canasto-Chibuque,* Limin Shang,* Barrett J. Rollins,† and Sergio A. Lira2*

Chemokines and their receptors play a key role in immune homeostasis regulating leukocyte migration, differentiation, and function. Viruses have acquired and optimized molecules that interact with the chemokine system. These virus-encoded molecules promote cell entry, facilitate dissemination of infected cells, and enable the virus to evade the immune response. One such molecule in the murine gammaherpesvirus 68 genome is the M3 gene, which encodes a secreted 44-kDa protein that binds with high affinity to certain murine and human chemokines and blocks chemokine signaling in vitro. To test the hypothesis that M3 directly interferes with diverse chemokines in vivo, we examined the interaction of M3 with CCL2 and CXCL13 expressed in the pancreas of transgenic mice. CCL2 expression in the pancreas promoted recruitment of monocytes and dendritic cells; CXCL13 promoted recruitment of B and T lymphocytes. Coexpression of M3 in the pancreas blocked cellular recruitment induced by both CCL2 and CXCL13. These results define M3 as a multichemokine blocker and demonstrate its use as a powerful tool to analyze chemokine biology. The Journal of Immunology, 2006, 177: 7296–7302.

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Leukocyte migration is a complex biological function mediated by multiple factors, including chemokines and their receptors (1). Chemokines are small, secreted proteins that bind to G protein-coupled receptors on the surface of leukocytes and regulate both their trafficking through lymphoid organs and their influx into inflamed areas. Impaired function of chemokines or their receptors has been associated with increased susceptibility to infections and autoimmune diseases (2).

Recent studies have shown that viruses have developed strategies to escape immune responses by subverting (or evading) chemokine function (3, 4). One such example is provided by a natural rodent pathogen, murine gammaherpesvirus 68 (MHV-68),3 which is closely related to Kaposi’s sarcoma-associated herpesvirus (or HHV8) and EBV. Intranasal infection by MHV-68 causes viral pneumonitis, followed by an infectious mononucleosis-like syndrome with splenomegaly and lymphocytosis (5–7). CNS infection by MHV-68 is associated with up-regulation of several chemokines involved in macrophage and lymphocyte trafficking (8). MHV-68 encodes a 44-kDa protein, M3, that is abundantly secreted from virally infected cells during acute infection (9). Infection with a MHV-68 virus deficient in M3 results in increased infiltration of the CNS by macrophages and lymphocytes, suggesting that M3 disrupts leukocyte migration and thus favors immune evasion.

M3 binds C, CC, CXC, and CX3C chemokines (10, 11) and disrupts chemokine signaling and chemotaxis in vitro (11). Systemic expression of M3 reduces intimal hyperplasia subsequent to femoral artery injury (12) and prolongs aortic allograft transplantation (13), processes known to be affected by chemokines. Only one study to date has examined the ability of M3 to block specific chemokines in vivo. M3 blocks CCL2-mediated chemotaxis of CCR7-expressing cells in vitro and drastically reduces lymphocyte recruitment induced by this chemokine in a transgenic model (14). Thus, it remains unclear whether M3 blocks other CC or CXC chemokines in vivo.

In this study, we show that M3 blocks leukocyte recruitment induced by CCL2, and by CXCL13. These results define M3 as a multichemokine blocker in vivo and demonstrate its use as a powerful tool to analyze chemokine biology.

Materials and Methods

Mice

RIPCXCL13 mice were generated by microinjection of the RIPCXCL13 transgene into B6D2F1 mouse eggs (The Jackson Laboratory) and transferred into oviducts of ICR foster mothers (Charles River Laboratories), according to published procedures (15). The RIPCXCL13 transgene was constructed as follows: a 3.45 kb, BstXI fragment of CXCL13 genomic DNA was isolated from a bacterial artificial chromosome clone and cloned into the EcoRV/SalI sites of the rat insulin promoter (RIP)-TNF-α-pBS plasmid (16). TNF-α was released from RIP-TNF-α-pBS by EcoRV/SalI digestion, and replaced with the CXCL13 genomic DNA. RIPCCL2 mice were generated using a transgene described in Grewal et al. (17) using conventional techniques (15). Five independent lines were established. RIPM3 transgenic mice were described previously (14).

Identification of the transgenic mice was accomplished by PCR amplification of mouse tail DNA. Specifically, the primers used for detection of mouse tail DNA. Specifically, the primers used for detection of the RIPCXCL13 transgene were: 5′-AGACTCCACGGGTAGTTGG-3′ and 5′-GAGGCACGTGGCAGGAG-3′. For the RIPCCL2 transgene, the primers were: 5′-AACCTTGATGCAATACCGAC-3′ and 5′-GGCCAGAGCATCAACTG-3′. PCR amplification of the mouse insulin promoter gene, used as an internal control, was amplified with the following primers: 5′-ACCTGTCATCACCTCCTTATTCCG-3′ and 5′-CGGAGGAGCAGGAGACAC-3′. Endogenous low density lipoprotein gene was amplified with the following primers: 5′-CGGCTCTCATCGACTTGT-3′. PCR conditions were as follows:
94°C, 30 s; 60°C, 30 s; 72°C, 60 s. All mice were housed under specific-pathogen-free conditions in individually ventilated cages at the Mount Sinai School of Medicine Animal Facility. All experiments were performed following institutional guidelines.

**Histology**

Tissues for light microscopic examination were fixed by immersion in 10% phosphate-buffered formalin and then processed for paraffin sections. Routinely, 5-μm sections were cut and stained with H&E. For immunostaining, fresh frozen sections were first fixed with ice-cold acetone for 20 min, 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 (no. 550539), CD3 (no. 550275), CD11c (no. 550283), B220 (no. 550286) and CD11b (no. 553308) from BD Biosciences/BD Pharmingen; anti-F4/80 (no. MCA497) from Serotec; anti-CCL2 (AF479) and anti-CXCL13 (AF470) from R&D Systems; and guinea pig polyclonal anti-insulin (no. A0564) from DakoCytomation. Secondary Abs used were Alexa Fluor 488 and 594 goat anti-rat IgG (nos. A-11006 and A-11007), Alexa Fluor 488 goat anti-rabbit IgG (no. A-11034), and Alexa Fluor 594 donkey anti-goat IgG (no. A-11058) from Molecular Probes; and Cy3 goat anti-Armenian hamster (no. 127-165-160) and FITC anti-guinea pig (no. 706-095-148) from Jackson ImmunoResearch Laboratories.

To determine the degree of islet infiltration we analyzed histological sections of the pancreas of different animals. Sections were stained with H&E or with Abs against CD45 and insulin. Between 40 and 100 islets were examined for each mouse. Insulitis was scored as follows: no lesions; small or peri-insular leukocytic aggregates, usually periductal infiltrates; medium or moderate insulitis with mononuclear cells infiltrating <50% of the islet architecture; and large or severe insulitis with >50% of the islet tissue infiltrated by mononuclear cells. Data are presented as mean insulitis score ± SD for the indicated experimental group.

**Isolation of pancreatic islets of Langerhans**

Islets of Langerhans were isolated as previously described (18). 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 (In vitroLog Life Technologies). Pancreatic tissue was recovered and subjected to a 14-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. In other experiments, islets were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, 20 mM HEPES, 1% penicillin-streptomycin, 1% l-glutamine, and 2.5 ml of 7.5% NaHCO3 (all from Invitrogen Life Technologies) at 37°C, 5% CO2. Supernatants and serum from transgenic mice were stored at −20°C until assayed. CCL2 and CXCL13 concentrations were measured by ELISA following the manufacturer’s guidelines (R&D Systems).

**FACS analysis**

Islets were isolated from different lines of transgenic mice as described above. Isolated islets were passed through a 40-μm diameter mesh to obtain a single-cell suspension. Islet cell suspensions were centrifuged at 1400 rpm for 5 min at 4°C and resuspended in FACS staining buffer (PBS containing 2% FCS and 0.01% sodium azide). Cells were incubated for 20 min at 4°C with 5 μg/ml Fc block (BD Pharmingen) and then stained with directly conjugated primary mAbs (BD Pharmingen). Samples were analyzed in a FACS Canto instrument (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star).

**Quantitative real-time PCR**

Total RNA was extracted from islets using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed from 2 μg of RNA. Quantitative real-time PCR was conducted in duplicates from 25 ng of cDNA and with 0.4 μM of each primer in a 30-μl final reaction volume of 1× SYBR Green PCR Master Mix (Applied Biosystems) using the ABI PRISM 7700 instrument. PCR cycling conditions were as follows: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 1 cycle of 60°C for 1 min. Relative expression levels were calculated as 2−ΔΔCt with ubiquitin as endogenous control. The primers used were as follows: M3, forward GAGGGCCAGTCTCAAAATGTG and reverse GCTGACCCCAACAGGTTAG; CCL2, forward GCTGGAGCATCCACGTGTT and reverse ATCTGTGCGGTGAATGATAGCA; CXCL13, forward CATAGTCGATCAGTTACGC and reverse TCTTG TCCCCATGCAAACCTCA; preproinsulin, forward GGGGACAGGTT ACCCTCAGA and reverse TGATCTCACAAGCCCAGCTCT; and ubiquitin, forward TGGCTATTAAATTTACGTTCTGCAT and reverse GCAATGGTCTAGAGTGCAAGATGAA.
Statistical analysis

Data are expressed as means ± SD. Paired, unpaired Student’s t test and one-way ANOVA were used to determine statistical significance. Differences were considered significant when \( p < 0.05 \).

Results

Expression of CCL2 by pancreatic \( \beta \) cells promotes migration of mononuclear cells into the islets of Langerhans

Eleven transgenic founders were generated by microinjection of the RIPCCL2 transgene (17) into fertilized mouse eggs. Five transgenic lines were established from these founders. All transgenic mice developed normally and were fertile. To examine whether CCL2 was produced by the transgenic islets, we measured CCL2 secreted in the supernatants of cultured pancreatic islets from transgenic mice and controls. As shown in Fig. 1A, islets from control mice secreted small amounts of CCL2 (0.03 ± 0.006 ng/ml/islet). In contrast, RIPCCL2 transgenic islets secreted 20- to 700-fold more CCL2 than control islets (Fig. 1A).

We next asked whether the expression of CCL2 promoted recruitment of leukocytes into the islets of Langerhans. To this end, we analyzed leukocyte infiltration in the pancreas of RIPCCL2 and wild-type animals by immunostaining using an anti-CD45 Ab (Fig. 1B). Few CD45<sup>+</sup> cells were found in the islets of wild-type mice. In marked contrast, large number of CD45<sup>+</sup> cells were found in pancreatic islets of RIPCCL2 mice. The most pronounced infiltrates were observed in animals of lines 1 and 254, which expressed the highest levels of CCL2 (Fig. 1B).

To study in detail the effect of CCL2 expression in islets of Langerhans, we expanded lines 251 and 254. Analysis of H&E-stained sections of transgenic pancreas showed that pancreata of animals from line 251 had a mild insulitis, with mononuclear cells predominantly accumulating in the periphery of the islets (Fig. 1C, upper panel). Pancreata from mice in line 254 showed a severe insulitis, with mononuclear cells accumulating in the periphery and within the islets (Fig. 1C, upper panel). The frequency and the size of these infiltrates did not change during the life of the animals (data not shown). Next, we phenotyped the leukocyte infiltrates using Abs against common surface markers. In agreement with Grewal et al. (17), most of the CD45<sup>+</sup> cells in RIPCCL2 islets were monocytes or macrophages (Fig. 1C, middle panel). Next in abundance in the infiltrates were CD11c-positive cells (dendritic cells (DCs); Fig. 1C, bottom panel). There were also a few scattered T and B cells (data not shown). We observed a direct correlation between the levels of CCL2 expressed and the number of cells in the infiltrates. Islets from mice of line 254 showed more monocytes and DCs than islets from mice of line 251 and control islets (Fig. 1B and C and not shown). These results indicated that CCL2 promoted recruitment of monocytes and DCs into the islets and that this effect was dependent on the amount of CCL2 produced.

Expression of M3 in \( \beta \) cells blocks migration of monocytes and DC induced by CCL2 expression

To test the hypothesis that M3 expression blocks CCL2 function in vivo, we crossed RIPCCL2 mice from both lines with mice expressing M3 in the islets (RIPM3 mice) (14). Double transgenic mice are referred to as RIPM3/CCL2 mice.

We examined histological sections of pancreata of control, single transgenic mice (RIPCCL2) and double transgenic mice (RIPM3/CCL2) of lines 251 and 254. Semiquantitative analysis of infiltrated islets was performed in pancreata from adult mice (6–12 wk old). No infiltrates were found in islets of control or RIPM3 transgenic mice regardless of age (\( n = 8 \) each; data not shown). As expected, mononuclear infiltrates of varying sizes were found in
the pancreatic islets of RIPCCL2 transgenic mice. 65 ± 10% of the islets of RIPCCL2 mice from line 251 (n = 10) had infiltrates (Fig. 2A), mostly arranged around the islets (Fig. 2B). In striking contrast, none of islets from RIPM3/CCL2 mice of the same line (n = 15) had infiltrates (Fig. 2A, B and C) resembling islets of control mice (data not shown).

To determine whether M3 could block leukocyte recruitment induced by higher amounts of CCL2, we analyzed pancreata from mice of line 254, which produced 15-fold more CCL2 than pancreata from mice of line 251. Semiquantitative analysis of infiltration in islets of Langerhans showed that 97 ± 2% of islets of RIPCCL2 mice and 96 ± 2% of the islets of RIPM3/CCL2 mice were infiltrated (n = 9 each; Fig. 2D). In both cases, these infiltrates contained large clusters of CD45⁺ cells around the islets. But, in contrast to RIPCCL2 254 islets (Fig. 2E), CD45⁺ cells were rarely found in the center of the RIPM3/CCL2 254 islets (Fig. 2F).

Taken together, these results indicate that expression of M3 in pancreatic islets blocked the accumulation of mononuclear cells induced by the ectopic expression of CCL2, and that this effect was more pronounced in the line expressing lower levels of CCL2 in the pancreas.

**Coexpression of M3 in β cells does not affect CCL2 production**

A possible explanation for the results described above would be that expression of M3 negatively affected the expression of CCL2, thus accounting for reduced leukocyte recruitment. To test whether expression of M3 affected the expression of CCL2 and vice versa, we analyzed CCL2, M3, and preproinsulin mRNA expression in isolated islets from control, single transgenic mice, and RIPM3/CCL2 mice of both lines. All mice showed comparable levels of preproinsulin mRNA (Fig. 3A), suggesting that the presence of the transgenes did not affect global islet function. No CCL2 mRNA expression was detected in freshly isolated islets from control and RIPM3 mice. As expected, mice from RIPCCL2 lines 251 and 254 expressed CCL2 mRNA at very different levels, but transcription of CCL2 was not affected by the introduction of the M3 transgene. There were also no differences between the levels of M3 mRNA detected in single or double transgenic islets (Fig. 3A). These results indicate that the transcription of M3 and CCL2 genes is not affected in islets of double transgenic mice.

We next examined whether M3 and CCL2 proteins were produced by double transgenic islets. Immunostaining of pancreata from animals from RIPM3/CCL2 line 251 showed that CCL2 and M3 proteins were produced by the islets of Langerhans (data not shown). Within the islets, M3 (Fig. 3C) and CCL2 (Fig. 3D) were expressed by insulin-producing β cells (Fig. 3B). Both M3 and CCL2 proteins were expressed by the same cells (data not shown).

Increased production of M3 or reduced production of CCL2 protein could explain the decrease in leukocyte recruitment in double transgenic mice. To test whether coexpression of M3 and CCL2 affected their secretion, we measured their levels in serum. M3 was detected at equivalent amounts in the serum of RIPM3 or RIPM3/CCL2 mice, but not in the serum of control mice (Fig. 3E). Similarly, CCL2 was detected in the serum of single- or double-positive mice, and at very low levels in the serum of controls (Fig. 3F). Of note, the levels CCL2 were higher in the serum of double transgenic than in the single transgenic mice (Fig. 3F).

Altogether, these results indicate that coexpression of M3 and CCL2 in the islets does not cause changes in the transcription, nor changes in protein production that could explain the reduced recruitment of leukocytes to the islets.

**FIGURE 3.** Coexpression of M3 in β cells does not affect CCL2 production. A. Relative expression of M3, CCL2, and preproinsulin mRNA in pancreatic islets of control mice, RIPM3, RIPCL3, and RIPM3/CCL2 mice from both lines (normalized to ubiquitin; n = 2 experiments). B–D. Representative picture of immunostaining for insulin (blue, B), M3 (green, C), and CCL2 (red, D) in the pancreata of RIPM3/CCL2 transgenic mice. E. Western blot analysis of the serum from control, RIPM3, and RIPM3/CCL2 mice of both lines (n = 6) using M3 Ab. The results are representative of two separate experiments. F. CCL2 levels in serum from mice of RIPM3/CCL2 lines and control littermates (n = 12 mice in each group; error bars, SD). Scale bars, 25 μm.

**M3 blocks organized accumulation of lymphocytes promoted by expression of CXCL13 within the islets of Langerhans**

Expression of CXCL13 in pancreatic islets promotes recruitment of B and T cells (19). To generate transgenic mice expressing CXCL13 in the pancreas, we injected a transgene encoding a genomic segment of CXCL13 under the control of the RIP. A total of six founders were generated from these injections and two transgenic lines were derived from them. CXCL13 expression was detected by immunostaining in the pancreatic islets of both transgenic lines (Fig. 4A), but not in islets of control mice (data not shown). CXCL13 protein colocalized with insulin, indicating specific expression by β cells (Fig. 4B). To confirm that CXCL13 could induce lymphocyte recruitment in vivo, we examined H&E-stained paraffin sections of transgenic pancreata at different ages. In agreement with the findings reported by Luther et al. (19), mononuclear infiltrates of varying sizes were found in the pancreatic islets of transgenic mice regardless of age. One transgenic line was expanded for the analysis shown in this study. In this RIPCLX13 line, ~30 ± 14% of the islets were infiltrated by mononuclear cells (10 ± 9% of the islets had large infiltrates, 8 ± 5% medium, and 12 ± 7% small infiltrates) (Fig. 4C).
To further characterize the cellular infiltrates, we performed immunohistochemical staining using Abs against several leukocyte cell surface makers. We observed that the majority of the infiltrating cells were B220<sup>H11001</sup>B lymphocytes and CD3<sup>H11001</sup>T cells. The infiltrates appeared to be organized into separate compartments with T cells at the center of the infiltrates. CD11c<sup>H11001</sup> cells, and very small numbers of F4/80<sup>H11001</sup> cells were also found among the cellular infiltrates (data not shown).

To evaluate the effect of M3 expression on the migration of cells induced by CXCL13 in vivo, we crossed RIPCXCL13 mice with RIPM3 mice to obtain RIPM3/CXCL13 mice that simultaneously expressed M3 and CXCL13 in the islets. Next, we evaluated H&E-stained paraffin sections of pancreata from control, single transgenic mice, and RIPM3/CXCL13 mice. No infiltrates were found in islets of control or RIPM3 transgenic mice regardless of age (n = 16 in each group; data not shown). As described above, mononuclear infiltrates of varying sizes were found in ~30% of the pancreatic islets of RIPCXCL13 mice (n = 23). In marked contrast, mononuclear infiltrates were found in <10% of the RIPM3/CXCL13 islets, and the size of the infiltrates was significantly reduced (n = 26; Fig. 4C). Furthermore, there were also changes in the distribution of the infiltrates. Mononuclear cells occupied the center of islets in RIPCXCL13 animals (Fig. 4D), whereas they accumulated predominately near ducts or in the periphery of the islets of the RIPM3/CXCL13 animals (Fig. 4E).

Flow cytometric analysis confirmed the histological findings. Control mice had on average 3 x 10<sup>3</sup> CD45<sup>H11001</sup> cells in the pancreatic islets, whereas the number of CD45<sup>H11001</sup> cells from islets of RIPCXCL13 mice was increased 15-fold (45 x 10<sup>3</sup> cells). Coexpression of M3 with CXCL13 reduced the number of CD45<sup>H11001</sup> cells by 85% (6.5 x 10<sup>3</sup> cells) (n = 4 mice in each group; Fig. 4F). Despite the notable decrease in the number of leukocytes, the infiltrates in RIPM3/CXCL13 mice were still dominated by B cells (~3 x 10<sup>3</sup> cells, 7-fold fewer B cells than were found in RIPCXCL13 mice). CD3<sup>H11001</sup> T cells represented a very small fraction of the infiltrates (0.7 x 10<sup>3</sup> cells; Fig. 4F).

To exclude the possibility that expression of both transgenes in islets affected the production of CXCL13 or M3, we measured the levels of preproinsulin, M3, and CXCL13 mRNA in the islets of control, RIPM3, RIPCXCL13, and RIPM3/CXCL13 mice by quantitative real-time PCR. As shown in Fig. 4G, all mice showed equivalent levels of preproinsulin mRNA. CXCL13 was not expressed in isolated islets from control and RIPM3 mice. The levels of M3 and CXCL13 mRNA observed in the double transgenic animals were comparable to those detected in the single transgenic animals, indicating that transcription of both transgenes was not...
affected by their coexpression. To test whether CXCL13 and M3 proteins were released from the islets we measured CXCL13 and M3 levels in the serum of control, RIPM3, RIPCXCCL13, and RIPM3/CXCL13 mice. CXCL13 levels were higher in RIPCXCCL13 and RIPM3/CXCL13 mice than in controls or RM3 mice. The levels of CXCL13 in RIPCXCCL13 and RIPM3/CXCL13 mice were not statistically different (Fig. 4H). M3 was also detected in the serum of transgenic mice (data not shown), indicating that expression of M3 did not reduce the production of CXCL13 by the islets or vice versa.

These results indicate that expression of CXCL13 in islets of Langerhans is sufficient to promote recruitment and topological segregation of B and T cells, and that these activities can be significantly blunted by coexpression of M3 by the islets.

Discussion
In this study, we have tested the ability of M3, a chemokine-binding protein encoded by MHV-68 to block recruitment of leukocytes induced by two different chemokines in vivo. Our results show that M3 blocks leukocyte recruitment induced by CCL2 and CXCL13 in vivo.

M3 binds several murine and human chemokines with high affinity in vitro. Crystallographic analysis shows that M3 dimerizes and generates a binding site for the N-terminal region of chemokines, which prevents them from binding to their cognate receptors (20, 21). Furthermore, M3 also inhibits the interaction of chemokines with glycosaminoglycans (22), a prerequisite for chemokine uptake, transcytosis to the apical side of the endothelial cell, and appropriate solid-phase presentation to the passing leukocytes (23). These two general properties render M3 an effective chemokine scavenger or decoy receptor. To date, the ability of M3 to bind and block chemokine function in vivo has not been comprehensively studied. Results presented in this study indicate that M3 can inhibit the activity of two different chemokines and suggest that it may work as a multichemokine blocking agent in vivo.

We first tested whether M3 could block function associated with the chemokine CCL2. CCL2 is a potent monocyte chemotactant secreted by a variety of cell types in response to proinflammatory stimuli (24). The main receptor for CCL2 is CCR2, which is expressed on peripheral blood monocytes and activated T cells (25). CCL2 has been implicated in a number of inflammatory diseases, including multiple sclerosis (26), chronic kidney disease (27), atherosclerosis, and rheumatoid arthritis (28). In this study, we show that when coexpressed with CCL2 in pancreatic islets, M3 inhibited CCL2-induced accumulation of mononuclear cells. Given that the coexpression of M3 and CCL2 in β cells did not alter the transcription or secretion of both proteins, the decrease in the number of mononuclear cells was most likely due to chemokine blockade in situ. A similar effect was observed when M3 was coexpressed with CCL2 in transgenic islets (14). This blockade was less pronounced in the presence of higher levels of CCL2 (RIPM3/CCL2 line 254). The center of the islets appeared less infiltrated in RIPM3/CCL2 than in RIPCXCCL2 254 animals, but the number of islets presenting peri-islet infiltrates and the size of these infiltrates were similar between these two groups. The fact that the blocking effect of M3 was maximum at the center of the islets may be related to the fact that the highest concentration of β cells occurs in this area (29, 30). We presume that the recruitment of cells into the perivascular space was due to the increased amount of CCL2 produced by the islets of animals in line 254.

Although M3 binds C, CC, CXC, and CX3C chemokines, it displays selectivity within the CXC class, which may be functionally relevant for viral pathogenesis (31). CXCL13 is a chemokine constitutively expressed in lymphoid tissues. CXCL13 binds CXCR5, a receptor expressed by naïve B cells and by a subpopulation of activated CD4+ T cells (32). This ligand/receptor pair facilitates basal lymphocyte trafficking and homing through lymphoid organs, a process essential for adaptive immune responses. CXCL13 expression can also be regulated by inflammatory cytokines, and its expression in ectopic lymphoid follicle-like aggregates has been reported in several chronic inflammatory conditions (33–38). When coexpressed with CXCL13 in pancreatic islets, M3 inhibited CXCL13-induced accumulation of mononuclear cells. The number of islets infiltrated, and the total number of cells per islet, was significantly reduced in animals expressing both proteins. Furthermore, M3 expression disturbed the organization of the infiltrates promoted by CXCL13 expression. The lymphocytes did not segregate in specific areas, and tended to accumulate in the periphery of the islets or closer to the ducts (data not shown). The decreased infiltration was not due to reduced production of the chemokine, because both RIPCXCCL13 and RIPM3/CXCL13 transgenic mice showed similar amounts of CXCL13 mRNA in the pancreas and CXCL13 protein in the serum.

The ability of M3 to block CCL2 and CXCL13 may have implications for MHV-68 pathogenesis. Because CCL2 and CXCL13 are expressed during MHV-68 infection, it is possible that M3 favors immune evasion by directly blocking their functions (39). It should be kept in mind that the inhibitory properties of M3 may depend on the local concentrations of chemokines and M3, as suggested by the in vitro studies and by the results shown in this study. It is unclear at this point whether M3 forms complexes with chemokines in serum and what is the fate of these complexes. We are currently addressing these questions and examining whether the formation of these complexes contributes to the increased levels of CCL2 in the serum of RIPM3/CCL2 mice.

In summary, our results indicate that M3 is an efficient chemokine scavenger for both CCL2 and CXCL13 in vivo. These results suggest that M3 may be an efficient multichemokine blocker in vivo. We are currently testing whether M3 expression in the pancreas affects the development of insulin-dependent diabetes, an inflammatory disease characterized by expression of multiple chemokines in the islets (40–42).

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Disclosures
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References