Islet expression of M3 uncovers a key role for chemokines in the development and recruitment of diabetogenic cells in NOD mice.

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ABSTRACT

Objective: Type 1 diabetes is an autoimmune disease characterized by a local inflammatory reaction in and around islets followed by selective destruction of insulin-secreting β-cells. We tested the hypothesis that chemokines affect different mechanisms responsible for the development of diabetes in NOD mice.

Research Design and Methods: We examined chemokine expression in islets of NOD mice and tested their functional relevance to development of diabetes using transgenic mice expressing the mouse herpesvirus 68 (MHV68)-encoded chemokine decoy receptor M3 (NOD-M3 mice) in insulin-secreting β-cells.

Results: Multiple chemokines were expressed in pancreatic islets of NOD mice before development of diabetes. Islet-specific expression of the pan-chemokine inhibitor M3 dramatically reduced leukocyte infiltration and islet destruction and completely blocked development of diabetes in NOD-M3 mice. M3 blocked diabetes by inhibiting the priming of diabetogenic cells in the pancreatic lymph nodes and their recruitment into the islets. This effect was specific to the pancreatic islets because M3 expression did not affect other ongoing autoimmune processes.

Conclusions: These results demonstrate that chemokines mediate afferent and efferent immunity in Type 1 diabetes, and suggest that broad chemokine blockade may represent a viable strategy to prevent insulitis and islet destruction.

NONSTANDARD ABBREVIATIONS.
CCL, CXCL: chemokine ligand.
CCR, CXCR: chemokine receptor.
CFSE: carboxyfluorescein succinimidyl ester.
DCs: dendritic cells
LNs: lymph nodes
PNAd: peripheral lymph node addressin.
PPLNs: peripancreatic lymph nodes.
RIP: rat insulin promoter.
Type 1 diabetes is an autoimmune disease characterized by a local inflammatory reaction in and around islets followed by selective destruction of insulin-secreting β cells (1). The factors leading to the destruction of the islets are still unknown, but it is thought that immune-based mechanisms are responsible for the death of the β-cells (2). T cells are critical in the pathogenesis of diabetes. Transfer of T cells isolated from diabetic NOD mice into irradiated NOD recipients leads to diabetes (3). Furthermore, NOD-nu/nu mice, that lack the thymus, do not develop insulin or diabetes (4). Other studies support a role for macrophages in induction of diabetes. Depletion of macrophages with clodronate-loaded liposomes leads to disappearance of dendritic cells, macrophages and lymphocytes from the endocrine pancreas of NOD mice and causes a delay in the onset of diabetes (5). Moreover, macrophage depletion affects development of β cell–cytotoxic T cells and prevents autoimmune diabetes (6).

Several lines of evidence suggest a role for chemokines in the pathogenesis of diabetes. Chemokines are found in freshly isolated islets or are produced by cultured pancreatic beta cells, including CCL5, CXCL9, CXCL10 (7; 8), and CCL2 (7; 9). CCL2 expression in the pancreas also parallels disease progression in NOD mice (10; 11). CCL21 is not expressed by pancreatic islets normally, but it has been observed in the pancreas of NOD mice (12) and may be an important factor contributing to autoimmunity (13). Animals lacking the chemokine receptor for CXCL10, CXCR3, show delayed onset of diabetes subsequent to a viral infection (7). Also, antibody neutralization of the macrophage-derived chemokine CCL22 causes a significant reduction of CCR4-positive T cells within the pancreatic infiltrates and attenuates disease onset and insulitis frequency in an adoptive transfer model (14). In addition to a role in recruitment of pathogenic T cells (14; 15), chemokines have been implicated in recruitment of regulatory CD4+CD25+ T cells to the pancreatic islets (16). However, despite suggestive evidence that chemokines are expressed during conditions leading to diabetes there are no studies to date that conclusively demonstrate that chemokine blockade prevents diabetes.

Here we show that several chemokines are expressed before development of diabetes and that islet-specific expression of the pan-chemokine blocker M3 abrogates inflammatory cell infiltration of the islets and completely blocks development of diabetes in NOD mice.

**RESEARCH DESIGN AND METHODS**

**Mice.** NOD and NOD.SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME). RIP-M3 mice, described previously (17), were backcrossed from a mixed B6D2 (C57Bl/6 x DBA/2) background onto the NOD background for 11 generations and onto B6 background for more than 11 generations. BDC2.5 NOD mice were kindly provided by Dr. Ralph...
Steinman (The Rockefeller University, New York). In all experiments, transgenic mice were compared to their corresponding littermates. 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.

**Diabetes.** The blood glucose was monitored weekly using a one-touch blood Ascensia Elite XL glucometer (Bayer, Elkhart, IN). Animals were considered diabetic when their blood glucose levels were greater than 250 mg/dl in two consecutive daily measurements.

**Histology.** For immunohistochemical staining, slides were incubated for 1 hr at room temperature with purified primary antibodies followed by incubation with the appropriate labeled secondary antibodies for 30 min. Primary antibodies used were anti-CD45, CD3, CD31, CD11c, B220, CD11b from BD Biosciences Pharmigen (San Diego, CA); F4/80 from Serotec; anti-CCL1, anti-CCL21, anti-CCL2, and anti-insulin from R&D Systems (Minneapolis, MN), rabbit anti-CXCL9 (kindly provided by J. Farber, NIH), anti-CCL3, CCL4, CCL11, CCL22 and CXCL10 (provided by S. Kunkel), and guinea pig polyclonal anti-insulin from DAKOCytomation (Carpinteria, CA). Secondary antibodies used were Alexa Fluor 488- and Alexa Fluor 594-goat anti-rat IgG from Molecular Probes (Eugene, OR), and Cy3-goat anti-Armenian hamster, FITC-donkey anti-Guinea Pig IgG and Cy5-goat anti-rat from Jackson ImmunoResearch (West Grove, PA).

To determine the degree of islet infiltration in NOD and NOD-M3 female mice, and in NOD.SCID mice in transfer experiments, groups of mice were analyzed at 10 or 30 wk of age or 9 wk after transfer respectively, assessing at least 80-100 islets per animal. Insulitis was scored as follows: grade 0, no lesions; grade 1, peri-insular leukocytic aggregates, usually periductal infiltrates; grade 2, <25% islet destruction; and grade 3, >25% islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as mean insulitis score ± SD for the indicated experimental groups.

**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-V solution (1 mg/ml; Sigma, St. Louis, MO) in HBSS. Pancreatic tissue was recovered and subjected to a 15 min digestion at 37°C. Ice cold HBSS was added and the suspension was vortexed at full speed for 10 s. Islets were hand-picked under a dissecting microscope.

**Flow cytometry analysis.** Isolated islets or lymph nodes (LNs) were incubated in 5 mg/ml collagenase D (Roche Applied Science, Indianapolis, IN) at 37°C for 45 min. Samples were strained through a 70-µm diameter nylon mesh to obtain a single-cell suspension,
centrifuged, and washed in PBS. All cell suspensions were resuspended in FACS staining buffer (PBS containing 2% fetal calf serum and 0.01% sodium azide). Islet suspension cells were incubated for 20 min at 4°C with 5 µg/ml Fc block (BD PharMingen, San Diego, CA) and then stained with directly conjugated primary mAbs (BD PharMingen, San Diego, CA). Samples were analyzed in a FACSCanto instrument (Becton Dickinson, San Jose, CA). Data were analyzed using the FlowJo software (Tree Star).

**Adoptive transfer studies.** Splenocytes (20 x 10^6 cells) obtained from newly diabetic NOD female mice were injected i.v. into 5- to 6-wk-old NOD.SCID and NOD-M3.SCID mice. In other experiments splenocytes (20 x 10^6 cells) obtained from diabetic NOD and non-diabetic NOD-M3 female mice were injected i.v. into 5-6-wk-old NOD.SCID mice. BDC2.5 (6-11 wk of age) splenocytes (5 x 10^7) were incubated with 10 mM CFSE for 10 min at 37°C. The reaction was quenched with 10 ml of cold PBS and followed by washing twice in PBS. The 2.5 x 10^7 CFSE-labeled total splenocytes in 100 µl of sterile PBS were injected i.v. per recipient (4- to 6-wk-old NOD and NRM3 mice). At day 4 after transfer, peripancreatic and cervical LNs were removed and a single cell suspension was done per each LNs and each recipient mouse. For monitoring the extent of cell death in the adoptively transferred cells, LN cells from the recipients were also stained for PI, CD3, CD4 and Vβ4 antibodies (BD Biosciences Pharmigen, San Diego, CA).

**Statistical analysis.** One-way ANOVA and Unpaired T-test were used to determine statistical significance. Differences were considered significant when P < 0.05.

**RESULTS**

**NOD islets express several chemokines.** In NOD mice infiltration of mononuclear cells into pancreatic islets begins as early as 4-6 wks of age, accumulating in the periphery (peri-insulitis) of a few pancreatic islets (19). The autoimmune destruction of the islets is extensive by 10 wks of age, 8-12 wks before the onset of overt diabetes (20; 21). To analyze the expression of chemokines in islets of NOD female mice, we performed immunostaining using antibodies against CXCL9, CXCL10, CCL1, CCL3, CCL4, CCL22 and CCL24, insulin, CD31 and CD45. By 4-6 wks of age (n=5), the islets of Langerhans had a small number of CD45+ cells that localized to the periphery of the islets (Fig. 1A and B). All chemokines studied were expressed in islets with peri-insulitis. CCL1, CCL3, CCL4, CCL22, CXCL9 and CXCL10 were primarily expressed by infiltrating cells (Fig. 1C, D, E, G, I and J, respectively). CXCL10 was also expressed by β cells (Fig. 1J), in agreement with a previous study (22). CCL24 was expressed by infiltrating cells (Fig. 1H) and by CD31+ endothelial cells in the islets (Online Appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]). As described before (12), CCL21 was expressed in para-ductal areas and its expression was increased in areas rich in inflammatory cells (Figure 1F). Analysis of pancreata of
10-wk-old mice \((n=5)\) showed increased in the expression of all chemokines studied compared to the earlier time points (data not shown). At this point, large infiltrates occupying the whole islet were observed. Some of these aggregates had T and B cells (Fig. 1K) and peripheral lymph node addressin (PNAd) positive high endothelial vessels that coexpressed CXCL9 (Fig. 1L). Taken together, these results show that numerous chemokines are expressed in NOD islets and suggest that the expression of several chemokines may be necessary to drive the recruitment of inflammatory cells required for the development of diabetes.

**Expression of M3 in NOD islets prevents development of insulitis and diabetes.** To examine if chemokine blockade would affect diabetes development in NOD mice, we backcrossed mice expressing M3, a viral chemokine binding protein, in \(\beta\) cells (17) onto the NOD background. N11 female NOD-M3 and their NOD littermates were monitored weekly for the development of hyperglycemia. By 45 wks of age, more than 80% of the NOD female mice \((n=61)\) were diabetic (Fig. 2A). Remarkably, expression of M3 in islets of NOD mice \((\text{NOD-M3 littermates, } n=66)\) completely abrogated the development of diabetes (Fig. 2A, \(P<0.0001\)). Furthermore, NOD-M3 mice did not develop disease over the next 15 wks \((n=16, \text{Fig. 2A})\).

To investigate the effect of M3 expression by \(\beta\) cells on insulitis we examined pancreata from NOD and NOD-M3 female mice at 10 wks of age \((n=5 \text{ per group})\). Semi-quantitative analysis of islet infiltrates showed that, as expected, most \((95\%)\) islets from NOD nondiabetic mice had peri-insulitis and in some cases developed a destructive inflammatory infiltrate (Fig. 2B and D), with marked loss of \(\beta\)-cell mass. In contrast, islets from NOD-M3 mice were virtually devoid of infiltrating cells and showed a normal complement of insulin producing cells (Fig. 2C and D, \(P=0.004\)). None of the NOD-M3 females that were more than 60 wks of age \((n=13)\) showed insulitis however, only 12±10% of islets had peri-insulitis and in some cases inflammatory cells were present in the exocrine tissue (Online Appendix Fig. 2).

Lymphocytes are also often observed in the salivary and lacrimal glands of NOD mice (23). We analyzed the presence of infiltrates in salivary and lacrimal glands from NOD and NOD-M3 mice at 10 wks of age and onwards. CD45+ infiltrates were found in salivary glands of both NOD \((20/20)\) and NOD-M3 \((22/22)\) mice (Fig. 2E and F respectively). Small infiltrates were also found in lacrimal glands of NOD \((3/5)\) and NOD-M3 \((2/5)\) mice. Overall, these findings indicate that \(\beta\)-cell expression of M3 prevented islet mononuclear infiltration and diabetes development, but did not prevent cellular infiltration into other organs.

**Expression of M3 in pancreas impedes infiltration of diabetogenic cells into the islets.** Since the absence of mononuclear cells in the pancreas of NOD-M3 mice could be due to a blockade of tissue accumulation of diabetogenic cells, we tested if M3 blocked entry of diabetogenic cells into the islets. To generate mice deficient in mature
T and B cells that also expressed M3 in islets we crossed NOD-M3 mice to NOD-SCID mice (referred as NOD-M3.SCID mice). When splenocytes from newly diabetic NOD females were transferred into young NOD-SCID mice, all NOD-SCID mice (9/9) developed diabetes within 7 wks of transfer (Fig. 3A). Although autoreactive T cells within NOD splenocytes could also induce diabetes in all NOD-M3.SCID recipient mice (11/11) (Fig. 3A), the development of diabetes was significantly delayed ($P=0.0086$). To test if the delay in diabetes development was caused by a reduced influx of the cells into the islets we examined pancreata of the recipients 2 wk after transfer. NOD-SCID recipients had insulitis and peri-insulitis in 75% islets (Fig. 3B and D). In contrast, NOD-M3.SCID mice ($n=5$) had mild infiltrates of leukocytes in ~25% of the islets and a normal complement of insulin-producing cells (Fig. 3C and D). However, at later time points infiltrates in islets from NOD-M3.SCID mice were comparable to those observed in NOD-SCID recipients (data not shown). These results indicate that M3 expression delayed the insulitis and islet destruction induced by diabetogenic splenocytes derived from NOD mice.

Because M3 expression in the islets delayed but did not completely prevent diabetes after transfer of diabetogenic cells, we considered the possibility that M3 expression also inhibited development of diabetogenic cells in NOD-M3 mice. To determine whether functional diabetogenic splenocytes could be detected in NOD-M3 mice, we harvested spleen cells from NOD and NOD-M3 mice and transferred them into young NOD-SCID female mice. After transfer of NOD splenocytes all NOD-SCID recipients (7/7) became diabetic in 8 wks. No diabetes was observed in the recipients up to 16 wks after transfer of splenocytes from NOD-M3 mice (0/19) (Fig. 3E). Histological analyses of the pancreata from the recipients showed that 100% the islets of the animals transferred with splenocytes from NOD mouse had massive cellular infiltration and few remaining insulin-producing cells (Fig. 3F and H). Animals that received NOD-M3 splenocytes had diffuse, low-grade infiltrates of leukocytes in the islets and a normal complement of insulin-producing cells (Fig. 3G and H). Interestingly, most of the NOD-SCID mice that received NOD-M3 splenocytes had infiltration in the islets, but only in 60% of them. Furthermore, these infiltrates were less severe than those observed in recipients of NOD cells (Fig. 3H). Lymphocytic infiltrates consisting mainly of $CD3^+$ T cells and $B220^+$ B cells, were present in the salivary glands of NOD-SCID mice transplanted with both splenocytes from NOD and NOD-M3 mice (Online Appendix Fig. 3A and B), suggesting that the presence of M3 blocked development of diabetogenic cells, but did not affect autoimmune disease to salivary glands. Taken together these results indicate that islet-specific expression of M3 delays insulitis induced by adoptively transferred NOD splenocytes, and impairs the development of diabetogenic cells.

Expression of M3 in pancreas reduces local inflammation that leads to a defective generation of diabetogenic cells. The
initiating events in diabetes are not fully understood, but β cell stress and death during early islet restructuring are thought to provide autoantigens, which induce the formation of diabetogenic cells in peripancreatic lymph nodes (PPLNs) (24; 25). Therefore, we asked whether expansion of islet-specific CD4+ T cells was affected in NOD-M3 mice. To this end, we took advantage of BDC2.5 NOD TCR-transgenic mice, which carry the rearranged TCR α (Vα1) and β (Vβ4) chain genes from a diabetogenic, β cell-specific, CD4+ T cell clone isolated from a diabetic NOD mouse (26). Adoptively transferred CarboxyFluorescein Succinimidyl Ester (CFSE)-labeled naive BDC2.5 splenocytes were analyzed for their proliferation in PPLNs and irrelevant cervical LNs on the basis of CFSE dilution on day 4 post-transfer into NOD and NOD-M3 recipients. BDC2.5 cells showed significantly lower levels of proliferation in PPLNs of NOD-M3 recipients (13±6% n=12, Fig. 4A, lower right panel), while NOD recipients displayed a significantly higher (P <0.0001) percentage of proliferating BDC2.5 T cells (35±11% n=8, Fig. 4A, lower left panel). BDC2.5 cells did not proliferate in cervical lymph nodes from NOD and NOD-M3 recipients (Fig. 4A, upper panel).

Dendritic cells (DCs) are believed to be essential in the initial activation of islet-specific T cells in PPLNs (27). Since islet-specific BDC2.5 cells CD4+ T cells proliferated less when transferred into NOD-M3 mice, it could be argued that the observed differences could be due to alterations in the number of DCs in PPLNs. To test this hypothesis, we compared the composition of DC subsets in PPLNs from 30-40 days old NOD (n=7) and NOD-M3 (n=8) mice. To investigate the different DC subsets, single cell suspensions were stained with anti-CD11c and CD11b antibodies and analyzed by flow cytometry. We observed that the relative number of DCs in PPLNs from NOD-M3 mice was reduced and had significantly less CD11c+CD11b+ cells than NOD mice (0.48±0.08% in NOD mice vs. 0.3±0.02% in NOD-M3 mice, P =0.02; Fig. 4B). This difference was more evident when we analyzed the absolute number of CD11c+CD11b+ DCs (2.1±0.6 x 10^3 cells in NOD mice vs. 2.1±0.6 x 10^3 cells in NOD-M3 mice; P =0.0011; Fig. 4C). The relative and absolute numbers of the other two populations of DCs (CD11c+CD11b⁻ and CD11c⁻CD11b⁺) were not affected (Fig. 4B and C). There were no differences among these DC subsets in cervical LNs (Online Appendix Fig. 4A and B). Since DCs capture antigen in tissues and then migrate into draining lymph nodes to present their antigens to T cells, we asked whether the presence of M3 in the islets would alter the composition of DCs present in the islets. We isolated islets from NOD and NOD-M3 mice and stained single cell suspensions with different surface markers. As shown in Figure 4D-F, we observed a significant reduction in the relative number of CD11c⁺CD11b⁺ cells in islets of NOD-M3 mice (22.9±3.4% in NOD mice vs. 11.1±1.5% in NOD-M3 mice; P =0.0054 Fig. 4E), as well as in the absolute number of this DC subset (Fig. 4F). NOD-M3 islets showed 249±47 CD11c⁺CD11b⁺ cells/100 islets, whereas the number of CD11c⁺CD11b⁺ cells from islets of
NOD mice was increased 4-fold (965±85 cells/100 islets) \( (P < 0.0001; \text{Fig. 4F}) \). Interestingly, there was no difference in the number of CD11c\(^+\)CD11b\(^+\) DCs in the blood of NOD-M3 mice when we compared with blood of NOD mice (Online Appendix Fig. 4C). To investigate whether this difference was driven solely by the expression of M3, we measured the number of CD11c\(^+\)CD11b\(^+\) DCs in islets of mice that are not prone to autoimmunity. To this end, we isolated islets from B6 and 6RIP-M3 mice (RIP-M3 mice (17) crossed over 10 times into the B6 background). We found that the expression of M3 in a non-autoimmune background does not alter the relative or the absolute numbers (Fig. 5A and B) of any DC subset analyzed. Taken together, these results indicate that M3 expression by \(\beta\) cells in NOD mice decreases inflammation in the islets, impairs the migration of a subset of DCs into the islets and PPLNs, and reduces the proliferation of diabetogenic cells in the draining lymph nodes.

**DISCUSSION**

In this study we examined the role of the chemokine system in an experimental model of diabetes. We show that chemokine expression precedes development of diabetes and that islet-specific blockade of chemokine function reduces the local inflammation that results in a defective development and pancreatic accumulation of diabetogenic cells. These results thus, constitute formal demonstration that chemokines are critical determinants of autoimmune diabetes.
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partially prevents diabetes (32). Neutralization of CCL22 with an antibody causes a significant reduction of CCR4-positive T cells within the pancreatic infiltrates and partially inhibits the development of diabetes in adoptive transfer model in NOD-SCID (14). Furthermore, treatment of NOD mice with a neutralizing anti-CCR5 antibody reduces incidence of diabetes (33). In all these studies, the individual treatments reduce inflammatory infiltrates, but do not completely block diabetes development, suggesting additional requirements.

To test the hypothesis that diabetes is caused by the combined activities of several chemokines we used M3, a chemokine binding protein encoded by the mouse herpesvirus 68 (MHV68) that binds several murine and human chemokines with high affinity (34; 35). M3 interacts with the N-loop of chemokines and mimics elements of GPCR recognition to block interaction with cognate receptors by a competitive inhibition mechanism (36; 37). In vivo, M3 reduces mononuclear cellular responses after MHV68-induced meningitis in mice (38). Systemic expression of M3 reduces intimal hyperplasia subsequent to femoral artery injury (39) and aortic allograft transplantation (40). Finally, and perhaps more important in the context of these studies, expression of M3 in transgenic islets inhibits recruitment of leukocytes induced by islet-specific expression of CCL21 (17), CXCL13 and CCL2 (41), and prevents insulitis and diabetes induced by multiple low doses of streptozotocin (18). Here we show that the scavenging properties of M3 are essential for the reduced inflammation observed in the NOD-M3 transgenic mice. Many of the chemokines upregulated in the NOD model, such as CCL2, CCL3, CCL4, CCL5 and CCL21, bind to M3 with high affinity (34; 35).

We suggest that M3 blocks pancreatic autoimmunity by disrupting both the influx and generation of diabetogenic cells. Our results indicate that local M3 expression blocks influx of diabetogenic cells. Adoptive transfer of NOD splenocytes into NOD-SCID and NOD-M3-SCID recipients causes diabetes. However, the course of the disease is delayed in the NOD-M3-SCID mice, suggesting a partial blockade in the recruitment of diabetogenic cells. The failure of M3 to prevent development of diabetes in this context could be due to either the amount or kind of chemokines produced relative to those present in NODM3 islets during development. Experiments addressing these hypotheses are currently underway. Finally, the failure of splenocytes from NOD-M3 mice to transfer disease suggests that either β-cell specific T-cells are not formed in the NOD-M3 animals or that such cells may be very reduced in number and incapable of inducing disease.

The activation of naive T cells requires not only TCR stimulation but also the simultaneous delivery of a costimulatory signal by a specialized antigen presenting cells in draining lymph nodes. Activation of transgenic, islet-specific CD4+ T cells (BDC2.5 cells) as well as diabetogenic CD8+ T cells occurs in the pancreatic lymph nodes at 3–4 wk of age (24; 27). Interference with priming and diabetes occurs if PPLNs are ablated (42; 43) or if important cellular constituents are
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ablated (44). In this context, DCs are believed to be essential in the initial activation of islet-specific T cells in PPLNs (27). Both CD11c+CD11b+ and CD11c+CD11b- (or CD11c+CD8+) DCs have been shown to be responsible for presentation of islet antigens in the draining PPLNs (45; 46). Recently, Harbers and colleagues have shown that T cell proliferation is abolished in DC-depleted RIP-mOVA/CD11c-DTR mice, and that T cell proliferation can be reverted when mice are reconstituted with CD11c+ splenic cells (47), confirming the importance of DCs in islet antigen presentation. Here we show that NOD-M3 mice have a reduced number of CD11c+CD11b+ cells in both islets and PPLNs at 4 wk of age. As dendritic cell migration is highly dependent on chemokines, it is likely that blockade of chemokine function promoted by M3 directly interfered with dendritic cell migration from the blood into islets and draining lymph nodes. Finally, the effects of M3 in our system appear to be local. We have previously shown that M3 is highly expressed by the islets. It is detected in the supernatants of transgenic islets cultured in vitro (17; 18) and low levels are present in circulation (41). Thus, the pancreatic levels of expression of M3 are sufficient to block diabetes, but do not affect disease development in the salivary and lacrimal glands.

In summary, expression of the chemokine decoy receptor M3 in β cells prevented development of insulitis and autoimmune diabetes. To date most antagonists targeting single chemokine receptors have not been successful in treating autoimmune diseases in the clinic (48). Our results suggest that chemokine receptor antagonists that can block multiple receptors may be a viable strategy to ameliorate autoimmune diabetes. This concept can be further tested in experimental models using broad-spectrum chemokine antagonists in both prophylactic and therapeutic contexts.

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Competing interest’s statement.
The authors declare that they have no competing financial interests.
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FIGURE LEGENDS

Figure 1. Expression of chemokines in islets of Langerhans in NOD mice. (A-J) Immunostaining for insulin (green) and CD45 (A-B), CCL1 (C), CCL3 (D), CCL4 (E), CCL21 (F), CCL22 (G), CCL24 (H), CXCL9 (I) and CXCL10 (J) (red) was performed in pancreata from NOD mice at 4-6 weeks of age. Note that chemokine expression is mostly associated with the inflammatory cells. (K) Pancreata from NOD mice at 10 weeks of age shows a significant infiltrate formed by T (CD3, red) and B (B220, blue) cells and presence of PNAd positive cells (green). (L) CXCL9 is expressed by the PNAd+ cells. Scale bars= 10µm.

Figure 2. Expression of M3 in β-cells of NOD mice blocks development of diabetes but not autoimmunity against salivary glands. (A) Cumulative incidence of diabetes in NOD non-transgenic littermates (n=61) and NOD-M3 (n=66) mice until 45 wks of age and NOD-M3 (n=16) mice until 60 wks of age. (B,C) Representative picture of immunostaining for CD45 (red) and insulin (green) in pancreata from non-transgenic littermates (B) and NOD-M3 (C) mice at 10 wks of age. (D) Insulitis score of pancreata from NOD and NOD-M3 mice, 10 wks of age (n=5/group). (E,F) Representative picture of immunostaining for CD45 (red) in salivary glands from non-transgenic littermates (D) and NOD-M3 (E) mice at 10 wks of age. Scale bars= 25µm.

Figure 3. Islet-specific expression of M3 delays migration of diabetogenic cells into the islets and impairs their development. (A) Cumulative incidence of diabetes in NOD.SCID (n=9) and NOD-M3.SCID (n=11) recipients of splenocytes from diabetic NOD mice. (B,C) Representative picture of immunostaining for CD45 (red) and insulin (green) in pancreata from NOD.SCID (B) and NOD-M3.SCID (C) recipients of splenocytes from diabetic NOD mice 14 days after transfer. (D) Insulitis score of pancreata from NOD.SCID and NOD-M3.SCID recipients of splenocytes from diabetic NOD mice 14 days after transfer (n=5/group). (E) Cumulative incidence of diabetes in NOD.SCID recipients of splenocytes from diabetic NOD (n=7) and NOD-M3 (n=19) mice. (F,G) Representative picture of immunostaining for CD45 (red) and insulin (green) in pancreata from NOD.SCID recipients of splenocytes from NOD (F) and NOD-M3 (G) mice 16 wks after transfer. (H) Insulitis score of pancreata from NOD.SCID recipients of splenocytes from NOD (n=7) and NOD-M3 (n=19) mice 16 wks after transfer. Scale bars= 25µm.

Figure 4. Expression of M3 in pancreas reduces local inflammation that leads to defective generation of diabetogenic cells. (A) Proliferation profiles of CFSE-labeled BDC2.5 (Vβ4+CD4+) T cells recovered 4 days after transfer from cervical LNs and PPLNs of 4-wk-old NOD (n=7) and NOD-M3 (n=12) mice. Representative plots from four separate experiments are shown. The CFSE profile of Vβ4+CD4+ T cells in NOD and NOD-M3 recipients is represented in the left and right, respectively. (B,C) DC subsets
in PPLNs of 30-40-days old NOD (n=7) and NOD-M3 (n=8) mice were. Values indicate the relative (B) or absolute number (C) of each subset (analysis was performed in alive (PI-) cells; average ± SD of three independent experiments, *P=0.02; **P=0.0011). (D) Representative dot-plot of the analysis performed in islets isolated from NOD and NOD-M3 mice stained with anti-CD11c (x axis) and CD11b (y axis) antibodies. Cells were gated in PI-/CD45^+ cells. (E,F) DC subsets in isolated islets from 4-5-wk-old NOD (n=8) and NOD-M3 (n=9) mice. Values indicate the relative (E) or absolute number (F) of each subset (analysis was performed in alive (PI-) cells, CD45^+ cells; average ± SD of three independent experiments, **P=0.0054; ***P<0.0001). Absolute number of DC subsets in isolated islets represents the absolute number of DCs per 100 islets.

**Figure 5. DC subsets are similar in islets isolated from B6 and RIP-M3 mice.** (A,B) DC subsets in islets isolated from 4-8-wk-old B6 (n=5) and 6RIP-M3 (n=6) mice. Values indicate the relative (A) or absolute number (B) of each subset (analysis was performed in alive (PI-), CD45^+ cells; average ± SD of three independent experiments). Absolute number of DC subsets in isolated islets represents the absolute number of DCs per 100 islets.
FIGURE 1
FIGURE 2

A

B

C

D

E

F

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FIGURE 3

A. Kaplan-Meier survival analysis of diabetes-free status in NOD→NOD-SCID (n=9) and NOD→NOD-M3-SCID (n=11) mice.

B, C. Fluorescence images showing beta-cell aggregates in NOD→NOD-SCID and NOD→NOD-M3-SCID mice, respectively.

D. Bar chart showing the percentage of insulin-positive beta cells in NOD→NOD-SCID and NOD→NOD-M3-SCID mice.

E. Kaplan-Meier survival analysis of diabetes-free status in NOD→NOD-SCID (n=7) and NOD-M3→NOD-SCID (n=19) mice.

F, G. Fluorescence images showing beta-cell aggregates in NOD→NOD-SCID and NOD-M3→NOD-SCID mice, respectively.

H. Bar chart showing the percentage of insulin-positive beta cells in NOD→NOD-SCID and NOD-M3→NOD-SCID mice.
FIGURE 4

A. BDC 2.5 cells → NOD mice, BDC 2.5 cells → NOD-M3 mice

Cerv LNs

- Cerv LNs
- # Cells
- PP LNs

B. PP LNs

- CD11c+ CD11b+
- CD11c+ CD11b
- CD11c+ CD11b+

C. Positive cells (10^6)

- CD11c+ CD11b+
- CD11c+ CD11b
- CD11c+ CD11b+

D. NOD

- NOD
- NOD-M3

E. ISLETS

- NOD
- NOD-M3

F. Positive cells (10^6)

- CD11c+ CD11b+
- CD11c+ CD11b
- CD11c+ CD11b+
FIGURE 5

(A) N.S.  
CD11c⁺ CD11b⁺  
CD11c⁺ CD11b⁻  
CD11c⁻ CD11b⁺  

(B) N.S.  
CD11c⁺ CD11b⁺  
CD11c⁺ CD11b⁻  
CD11c⁻ CD11b⁺  

Positive cells (%)  
Positive cells/100 islets