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CXC Chemokine Ligand 10 Neutralization Suppresses the Occurrence of Diabetes in Nonobese Diabetic Mice through Enhanced β Cell Proliferation without Affecting Insulitis

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We have shown that neutralization of IFN-inducible protein 10/CXCL10, a chemokine for Th1 cells, breaks Th1 retention in the draining lymph nodes, resulting in exacerbation in Th1-dominant autoimmune disease models induced by immunization with external Ags. However, there have been no studies on the role of CXCL10 neutralization in Th1-dominant disease models induced by constitutive intrinsic self Ags. So, we have examined the effect of CXCL10 neutralization using a type 1 diabetes model initiated by developmentally regulated presentation of β cell Ags. CXCL10 neutralization suppressed the occurrence of diabetes after administration with cyclophosphamide in NOD mice, although CXCL10 neutralization did not significantly inhibit insulitis and gave no influence on the trafficking of effector T cells into the islets. Because both CXCL10 and CXCR3 were, unexpectedly, coexpressed on insulin-producing cells, CXCL10 was considered to affect mature and premature β cells in an autocrine and/or paracrine fashion. In fact, CXCL10 neutralization enhanced proliferative response of β cells and resultantly increased β cell mass without inhibiting insulitis. Thus, CXCL10 neutralization can be a new therapeutic target for β cell survival, not only during the early stage of type 1 diabetes, but also after islet transplantation. *The Journal of Immunology,* **2004, 173: 7017–7024.**

The chemokine family is comprised of ~50 proteins, and the biological function of most chemokines has been extensively studied (1, 2). There are two major subfamilies of chemokines, termed CXC and CC according to the arrangement of the first two conserved cysteines that are separated by one amino acid and are adjacent, respectively (1, 2). Although CXC chemokines containing the glutamic acid-leucine-arginine (ELR)[2] motif preceding the first conserved cysteine have a chemoattractant activity for neutrophils (3), IFN-inducible protein 10/CXCL10, monokine induced by IFN-γ/CXCL9, and IFN-inducible T cell α chemoattractant/CXCL11, all of which lack the ELR motif, are selective chemoattractants for activated T lymphocytes (4–7), especially Th1 cells (8–10). Furthermore, it was reported that the common receptor for these three ELR$^-$ CXC chemokines, named CXCR3, is differentially expressed on Th1 and T cytotoxic type 1 cells generated in vitro, although CCR5 is also characteristic of them (8, 9, 11).

Because gene expression of these three ELR$^-$ CXC chemokines is induced in tissue-, cell type-, and stimulus-specific fashions (10, 12–14), the effect of CXCL10 neutralization differs depending on the pathogens or immunogens and the target organs affected. We have shown that CXCL10 is selectively induced in dendritic cells (DCs) in the draining lymph nodes (LNs) to retain Th1 cells, maintaining DC-T cluster formation in acute experimental autoimmune encephalomyelitis (EAE) (15) and heat-killed *Propionibacterium acnes*-induced hepatitis (16). Although CXCL10 neutralization supplies Th1 cells into the circulation by breaking Th1 retention in the draining LN, increased or decreased Th1 accumulation in the target organs depends on the expression of the other two CXCR3 ligands, especially CXCL11 (15–17), whose in vitro chemotactic activity is 100-fold stronger than that of CXCL9 (10). Thus, increased Th1 accumulation by CXCL10 neutralization exacerbates EAE (15) and heat-killed *P. acnes*-induced hepatitis (16), in which CXCL11 is markedly produced in the target organs, whereas decreased accumulation of effector T cells results in the decreased ability to kill the pathogens in mice with *Toxoplasma gondii* (17) or HSV infection (T. Kaburaki, H. Yoneyama, K. Matsushima, and S. Narumi, manuscript in preparation), in which CXCL11 is induced only slightly or not at all. Whereas all of the four Th1 disease models are induced by administration with external Ags, there have been no studies on the role of CXCL10 neutralization on the development of Th1-dominant autoimmune disease models induced by constitutive intrinsic self Ags.

Type 1 diabetes is a T cell-dependent autoimmune disease, but the underlying mechanisms that initiate disease and destroy pancreatic β cells in genetically susceptible individuals remain unclear. NOD mice naturally develop an insulin-dependent diabetes that is similar to human type 1 diabetes (18). It has been shown that autoimmune diabetes is initiated by developmentally regulated presentation of β cell Ags in the pancreatic LNs where those Ags are transported (19). Then, immune cells infiltrate pancreatic islets progressively until diabetes culminates (20). Although immunohistochemical studies showed that macrophages and T lymphocytes are the predominant immune cell phenotypes in the inflamed islets and that they may promote β cell destruction (21–23), Th1 cells were suggested to be crucial in β cell destruction (24). In contrast, Th2 cells were suggested to counteract the destructive

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[2] Abbreviations used in this paper: ELR, glutamic acid-leucine-arginine; DC, dendritic cell; LN, lymph node; EAE, experimental autoimmune encephalomyelitis; CY, cyclophosphamide; GAD65, glutamic acid decarboxylase 65; PTHrP, parathyroid hormone-related protein; pAb, polyclonal Ab; HEV, high endothelial venule.

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process and thus prevent occurrence of diabetes (25). Cyclophosphamide (CY), an immunomodulating agent, when administered to 9- to 10-wk-old NOD mice, induces a rapid influx of immune cells into the pancreatic islets, resulting in an accelerated and synchronous onset of diabetes (26). Although the mechanism of CY action is not yet clear, this agent is able to enhance the immune response under appropriate conditions, either by an induced rebound in the proliferation of effector T cells sensitive to the internal self Ags, or by preferentially eliminating Th2 cells and/or regulatory T cells.

We have recently observed the elevated serum levels of CXCL10 and found a positive correlation between the serum CXCL10 levels and the numbers of IFN-γ-producing $CD4^+$ cells in the peripheral blood, which recognize glutamic acid decarboxylase 65 (GAD65), one of β cell-derived specific self Ags, in both patient groups of "classical" type 1 diabetes and so-called "slowly progressive insulin-dependent diabetes mellitus" or "latent autoimmune diabetes in adults (LADA)" (27, 28). Furthermore, it has been speculated that the increased serum CXCL10 levels and GAD65-reactive IFN-γ-producing $CD4^+$ cells are important for rapid disease progression in anti-$GAD65^+$ diabetic patients with residual β cell function (28). Here, we would like to clarify the role of CXCL10 neutralization in the development of type 1 diabetes in NOD mice after disease acceleration with CY.

Materials and Methods

Neutralizing mAb against mouse CXCL10

Two mAbs (αrIPa and αrIPb), whose IgG subclass is IgG1, against rat CXCL10 were obtained by immunizing mice with the fusion protein of Fc (Ig constant region) and rat CXCL10, one amino acid of which was replaced with that of mouse CXCL10, and then they were screened by measuring the binding to the fusion protein in which Aic2A (common β subunit of mouse IL-3, IL-5, and GM-CSF receptors) was substituted for Fc (5, 6). In the present study, αrIPb was used, which was confirmed to neutralize not only rat CXCL10- (6) but also mouse CXCL10-induced chemotaxis of splenocytes obtained from mice treated with Con A and intracellular calcium influx of Chinese hamster ovary-K1 cells transfected stably with murine CXCR3 (5, 16). A total of 100 and 200 μg/ml of this mAb could block the activity of murine CXCL10 (100 ng/ml) by 50% and 100%, respectively, in chemotaxis. The specificity of this mAb for CXCL10 was confirmed immunologically against murine CXCL9, CCL3, CCL21, and CCL22 (16).

Diabetes acceleration after the administration of CY in NOD mice

Nine-week-old female nondiabetic NOD mice were injected i.p. with CY on day 0 (200 mg/kg body weight) and treated with anti-CXCL10 mAb (6, 15, 16, 29) (200 μg/mouse) or the IgG subclass-matched control Abs (anti-human parathyroid hormone-related protein (PTHrP) mAb, which is not cross reactive to mouse PTHrP, from Dr. T. Tamatani, JT Pharmaceutical Frontier Research Laboratories, Yokohama, Japan; and anti-rotavirus mAb from Dr. H. Kawachi, Niigata University Graduate School of Medical and Dental Sciences, Niipota, Japan) every other day from day −1 to day 13. All animals were assessed for glycosuria daily in the evening, and plasma glucose was examined in the evening when glycosuria was observed. Diabetes was confirmed when glycosuria was positive and plasma glucose levels in the evening were higher than 200 mg/dl. All animal experiments complied with the standards set out in the guidelines of Keio University and the University of Tokyo.

Evaluation of insulitis

The degree of insulitis was assessed by histological score. The pancreas from each NOD mouse (10 mice per group) was obtained 10 days after CY injection, immediately before the mice began to exhibit overt diabetes. The pancreas was fixed in 10% buffered formalin and embedded in paraffin, and sections were stained with H&E. A minimum of 15 islets from more than three sections of each pancreas cut at intervals of 150 μm was scored independently and blindly by three observers using the following rankings: 0, normal; 1, peri-insulitis (mononuclear cells surrounding islets and ducts, but no infiltration of the islet architecture); 2, moderate insulitis (mononuclear cells infiltrating <50% of the islet architecture); and 3, severe insulitis (mononuclear cells infiltrating >50% of the islet architecture).

Immunostaining

The pancreas from each NOD mouse (five mice per group) was obtained 10 days after CY injection at a similar time when mice were sacrificed for evaluation of insulitis. For the in vivo proliferation assay, mice were injected with BrdU (500 μg/mouse; Sigma-Aldrich, St. Louis, MO) 1 h before they were killed. Each pancreas was inflated with warm OCT compound and snap-frozen in liquid nitrogen. As well as evaluation of insulitis, a minimum of 15 islets from more than three sections of each pancreas cut at intervals of 150 μm was examined to count the cells that were positively stained.

To identify the cell type of pancreatic islet-infiltrating leukocytes, the following anti-mouse mAbs were used: CD3ε (clone 145-2C11), CD4 (RM4-5), CD8a (53-6.7), and pan-NK cells (DX5) (all from BD Pharmingen, San Diego, CA) and F4/80 (Cl:A3-1 from Serotec, Oxford, U.K.). As a secondary Ab, an HRP- (BioSource, International, Camarillo, CA) or an alkaline phosphatase-labeled anti-rat Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) was used.

To clarify the localization of CXCL10, CXCL9, and CXCR3, double immunofluorescence staining was performed. For immunostaining for CXCL10, CXCL9, and CXCR3, goat polyclonal Abs (pAbs) from Santa Cruz Biotechnology (Santa Cruz, CA) were used. Each goat pAb against CXCL10, CXCL9, or CXCR3 has been demonstrated to detect each target protein specifically (16, 29). Acetone-fixed 4-μm fresh frozen tissue sections were incubated with each goat pAb, followed by FITC-labeled anti-goat IgG. At the same time, they were incubated with rabbit anti-insulin pAb (Santa Cruz Biotechnology), rat anti-PECAM-1, and CD4 mAbs (BD Pharmingen), followed by Alexa-594-labeled anti-rabbit and rat Ig (Molecular Probes, Eugene, OR), respectively. Then they were observed by fluorescence microscopy (16, 29).

To detect replicating cells, a BrdU staining kit (Zymed Laboratories, South San Francisco, CA) was used, according to the manufacturer's instructions (16, 29). Before BrdU staining was performed, acetone-fixed 4-μm fresh frozen tissue sections were immunostained with rabbit anti-insulin or amylin pAb (Biogenesis, Poole, U.K.) and/or rat anti-CD3ε mAb using indirect immunoalkaline phosphatase or immunoperoxidase methods mentioned above, followed by further fixation with 1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) in PBS for 9 min. To further confirm whether $BrdU^+$ cells were β cells or T lymphocytes, triple immunofluorescence analysis for BrdU, insulin, and CD3 was performed using Alexa-488-labeled streptavidin, Alexa-546-labeled anti-rabbit, and Alexa-647-labeled anti-rat IgG, respectively (Molecular Probes).

Furthermore, to quantitate insulin content, we calculated the ratio of insulin-containing cell volume to the whole islet using the spectral confocal scanning system (TCS SP2 AOBS; Leica Microsystems, Tokyo, Japan) after immunofluorescent staining for insulin.

Quantitative RT-PCR

Total RNA was extracted from the pancreatic LNs and the pancreas by homogenization using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) and was reverse transcribed. Thereafter, cDNA was amplified and quantitatively determined using the ABI 7700 sequence detector system (Applied Biosystems, Foster City, CA) with a set of primers and probes corresponding to CXCL10, CXCL9, CXCL11, CXCR3, IFN-γ, TNF-α, IL-4, IL-10, TGFβ, and GAPDH as previously described (10, 16, 30, 31). Each gene expression was normalized to that of GAPDH.

Statistics

The incidence of diabetes in NOD mice after CY administration was compared between groups using Fisher's exact test. The other mean values among groups with or without CY administration and treated with anti-CXCL10 mAb or the control mAb were compared using ANOVA followed by Scheffe's test. A p value of <0.05 was considered significant.

Results

CXCL10 neutralization suppresses the incidence of diabetes

To clarify whether neutralization of CXCL10 affected the insulitis and the incidence of diabetes in NOD mice after disease acceleration with CY, female 9- to 10-wk-old NOD mice were injected i.p. with CY (200 mg/kg body weight) on day 0 and were treated i.p. with anti-CXCL10 mAb (6, 15, 16, 29) (200 μg/mouse) or the IgG subclass-matched control mAb, anti-human PTHrP mAb, every other day from day −1 to day 13. Then, we checked the cumulative incidence of diabetes by examining urine and plasma glucose levels. Treatment with anti-CXCL10 mAb significantly

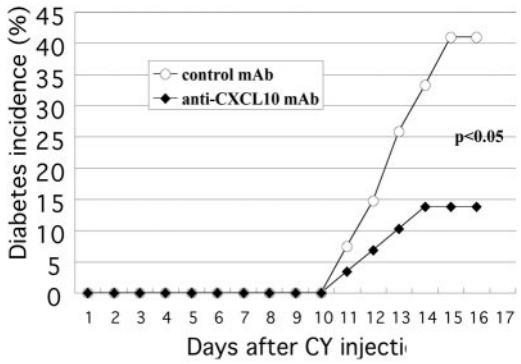

FIGURE 1. CXCL10 neutralization suppresses the incidence of diabetes in NOD mice after CY administration. The cumulative incidence of CY-induced diabetes was monitored by measuring urine and plasma glucose levels after CY injection in each group of NOD mice that were treated with neutralizing mAb against CXCL10 ($n = 29$) or the control mAb (the IgG subclass is the same as anti-CXCL10 mAb) ($n = 27$).

inhibited the incidence of diabetes, compared with the control mAb treatment, although the rapidity of the onset, which was observed at least 10 days after the CY administration, was the same in both groups (Fig. 1). Most blood glucose levels measured in the evening in mice that were judged as diabetic were >250 mg/dl. The administration with the control mAb did not show significantly different influences on the incidence of diabetes, compared with the injections with anti-rotavirus mAb or phosphate-buffered salt solution substituted for anti-PTHrP mAb (data not shown).

To clarify whether the lower incidence of diabetes induced by CXCL10 neutralization resulted from inhibited development of insulitis due to decreased infiltration of T lymphocytes into pancreatic islets, we investigated insulitis scores and the cell numbers of T lymphocytes, macrophages, and NK cells infiltrating in the islets from the NOD mice treated with neutralizing anti-CXCL10 mAb and with the control mAb on day 10 immediately before the mice began to exhibit overt diabetes (Figs. 2 and 3). First, islet histology showed that islets from NOD mice treated with anti-CXCL10 mAb were apparently larger than those from mice treated with the control mAb (Fig. 2). Against our expectation, however, the insulitis scores and the numbers of all cell types of inflammatory cells infiltrated into the pancreatic islets in NOD mice treated with anti-CXCL10 mAb were not significantly different from those mice treated with the control mAb (Figs. 2 and 3), although CXCL10 neutralization seemed to inhibit insulitis slightly (Fig. 2). Furthermore, we could not detect any significant difference in gene expression of IFN-γ, IL-4, IL-10, TNF-α, and TGFβ in addition to CXCR3 in the pancreas between both mouse groups (Table I). The finding that CXCL10 neutralization suppressed the incidence of diabetes despite the unchanged insulitis scores and T cell infiltration into the pancreatic islets suggested other mechanisms different from the effect of CXCL10 neutralization on T cell trafficking.

Islet β cells coexpress both CXCL10 and CXCR3

To investigate why no effect of CXCL10 neutralization on T cell trafficking into the target organ was seen in the autoimmune diabetes model, differing from the four Th1 disease models induced by administration with external Ags as mentioned in the *Introduction*, and to clarify the new function of the CXCL10/CXCR3 system other than chemotaxis, we examined the gene expression patterns of CXCL10, CXCL9, CXCL11, and their common receptor CXCR3 in the pancreases and the draining LN (the pancreatic LN) of NOD mice at various time points (days 0, 4, 7, and 10) after disease acceleration with CY (Fig. 4). In the pancreatic LNs, CXCL10 gene expression began to be induced by day 7, and was markedly induced on day 10, immediately before diabetes began to be overt, whereas gene expression of CXCL9 and CXCL11 was only slightly induced (Fig. 4*A*). CXCR3 mRNA expression in the pancreatic LNs was also induced in a similar manner to that of CXCL10 after administration with CY. Although gene expression patterns of CXCL10 and CXCL11 were similar to those seen in the draining LNs in EAE (15), heat-killed *P. acnes*-induced hepatitis

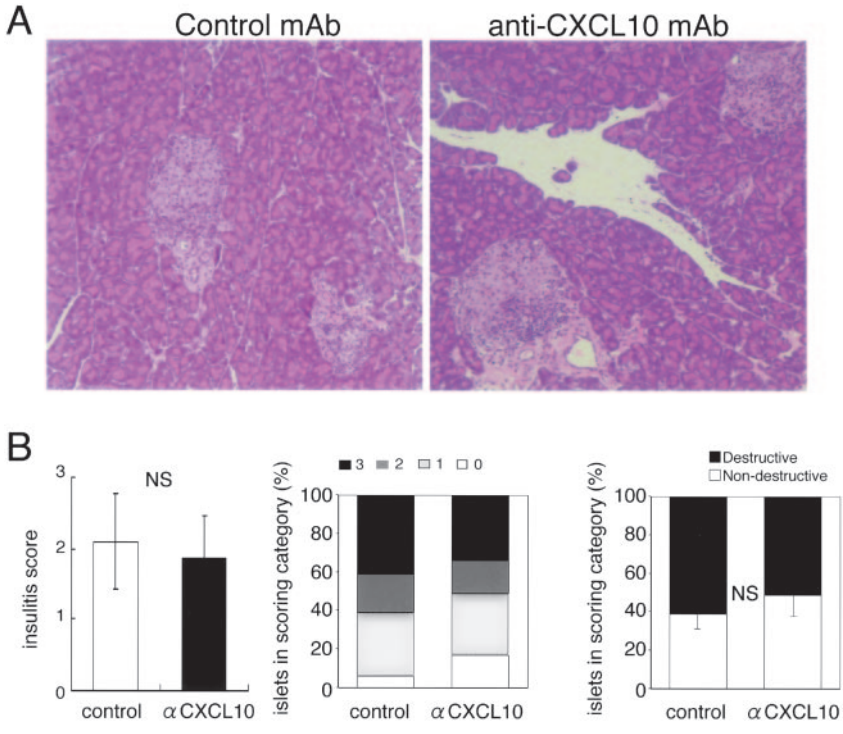

FIGURE 2. The representative islet histology and insulitis scores in NOD mice treated with anti-CXCL10 or the control mAb after disease acceleration with CY. NOD mice were injected with CY and treated with anti-CXCL10 mAb or the control mAb (10 mice per group). Ten days after CY injection, mice were sacrificed. The pancreases were fixed in 10% buffered formalin and embedded in paraffin, and sections were stained with H&E. More than three sections of each pancreas were examined for scoring insulitis (magnification, ×200). The insulitis scores are presented not only as means but also as proportions of islets in each scoring category (0, 1, 2, and 3), or in nondestructive (scores of 0 and 1) and destructive (scores of 2 and 3) islet histology. The values are shown as mean ± SEM. Similar results were obtained in two separate experiments.

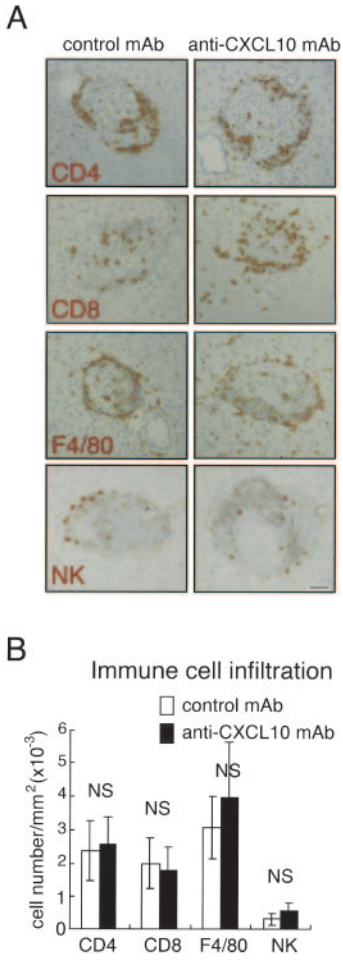

FIGURE 3. The numbers of immune cells infiltrating into pancreatic islets in NOD mice treated with anti-CXCL10 or the control mAb after disease acceleration with CY. NOD mice were injected with CY and treated with anti-CXCL10 mAb or the control mAb (five mice per group). Ten days after CY injection, mice were sacrificed, and more than three sections of each pancreas were examined for counting the immune cells infiltrating into islets, using immunostaining analysis for CD4, CD8, F4/80, and pan-NK (magnification, ×200). The numbers of positive cells in each islet from each pancreas were counted and divided by the area of the islet. Then, the average of each immune cell number was obtained from each mouse and each was compared among the groups. The values are shown as mean ± SEM. Similar results were obtained in two separate experiments.

(16), and HSV infection ((T. Kaburaki, H. Yoneyama, K. Matsushima, and S. Narumi, manuscript in preparation), CXCL9 expression, which is induced in high endothelial venules (HEVs) (32, 33), was only slightly induced, in contrast with that in the Th1 disease models immunized with external Ags (15, 33). In contrast, in the pancreas, induction of CXCL10 and CXCL9 was apparently seen by day 7 and was further increased on day 10, although CXCL9 mRNA induction was delayed compared with CXCL10 (Fig. 4*B*). However, CXCL11 gene expression was scarcely detected in the pancreas, as observed in the target organs in mice with protozoan (17) or viral infection (T. Kaburaki, H. Yoneyama, K. Matsushima, and S. Narumi, manuscript in preparation), where decreased accumulation of inflammatory cells was observed. CXCR3 mRNA expression in the pancreas was also induced gradually since day 7 after administration with CY, as seen in the pancreatic LN.

We further investigated the cellular localization of CXCL10, CXCL9, and CXCR3 in the pancreas on day 10 after administration of CY (Fig. 5). An immunostaining study showed that although CXCL10 and CXCL9 were detected at lower levels in the islet in NOD mice without CY administration, they were markedly augmented after the disease acceleration with CY (Fig. 5, *A* and *B*). Interestingly, the augmented CXCL10 expression was observed on the islet β cells producing insulin (Fig. 5*A*), but not on the vascular vessels or islet-infiltrating cells. In contrast, CXCL9 production was detected in the vascular endothelial cells, which were shown as PECAM-1$^+$ cells, lining capillaries in the islets in addition to those outside the islets, but not in the islet parenchymal cells (Fig. 5*B*).

CXCR3 expression was observed on a few CD4$^+$ T cells surrounding the islets in NOD mice without CY administration (Fig. 5, *C* and *D*). Although some islet-infiltrating T lymphocytes expressed CXCR3 after the disease acceleration with CY, CXCR3 expression was markedly exhibited on the islet β cells similar to CXCL10 (Fig. 5, *A* and *C*), as seen in the epithelial cells in the murine acute colitis model (29), in which CXCL10 neutralization protected mice from acute colitis through enhanced crypt cell proliferation without affecting the inflammatory infiltrates (29).

Table I. *Gene expression of cytokines and CXCR3 in the pancreases on day 10 following administration with CY in NOD mice treated with anti-CXCL10 or the control mAb*[a]

Gene Expression Examined	Treatment	
	Control mAb	Anti-CXCL10 mAb
IFN-γ	0.618 ± 0.275	0.474 ± 0.281
TNF-α	0.425 ± 0.132	0.954 ± 0.171
IL-4	0.0526 ± 0.0077	0.0291 ± 0.0083
IL-10	0.0955 ± 0.0258	0.2360 ± 0.0901
TGFβ	3.13 ± 1.02	2.18 ± 0.58
CXCR3	0.906 ± 0.372	0.681 ± 0.141

[a] The pancreases were obtained from NOD mice treated with anti-CXCL10 (n = 5) or the control (n = 5) mAb on day 10 following CY injection. Total RNA from each mouse was prepared and reverse transcribed. Thereafter, cDNA was amplified and quantitatively determined using the ABI 7700 sequence detection system (Applied Biosystems). Each gene expression was normalized with that of GAPDH. The values (×10^2) are shown as the means ± SEM. Similar results were obtained in two separate experiments.

CXCL10 neutralization enhances the proliferation of islet β cells

The coexpression pattern of CXCL10 and its receptor CXCR3 on the islet β cells suggested that CXCL10 functions on the islet β cells in an autocrine and/or paracrine fashion. Furthermore, it was

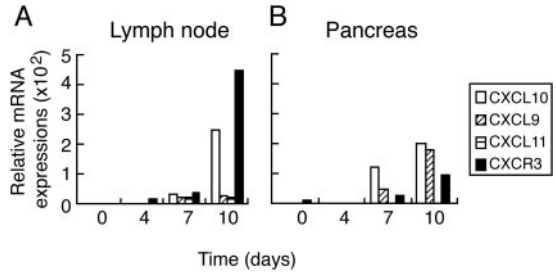

FIGURE 4. Gene expression of CXCR3 and its three ligands in the pancreatic LNs (*A*) and the pancreas (*B*) after administration with CY in NOD mice. The pancreatic LNs and the pancreases were obtained from NOD mice on days 0, 4, 7, and 10 after CY injection. Total RNA from four mice on each day was prepared, pooled, and reverse transcribed. Thereafter, cDNA was amplified and quantitatively determined using the ABI 7700 sequence detection system (Applied Biosystems). Each amount of CXCR3 and its three ligands was normalized with that of GAPDH. Similar results were obtained in two separate experiments.

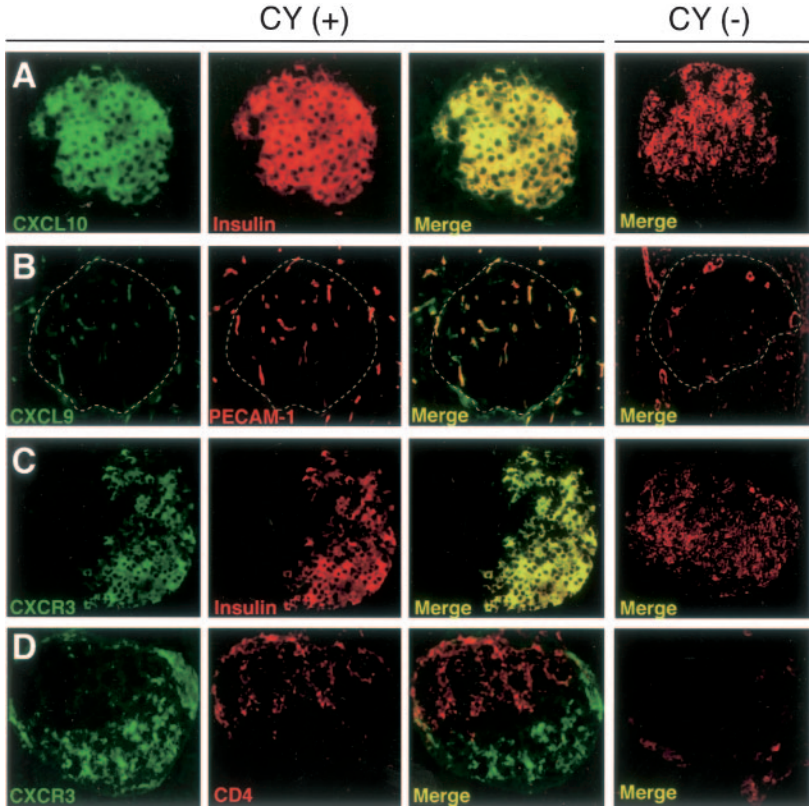

FIGURE 5. Islet β cells coexpress both CXCL10 and CXCR3 in NOD mice after disease acceleration with CY administration. The pancreases were obtained from NOD mice 10 days after administration with or without CY, and double immunofluorescence staining was performed for CXCL10/insulin (*A*), CXCL9/PECAM-1 (*B*), CXCR3/insulin (*C*), and CXCR3/CD4 (*D*).

demonstrated that CXCL10 possesses the capacity to suppress the proliferation of intestinal epithelial cells (29), hemopoietic progenitor cells (34), and vascular endothelial cells (35). As expected from the expression pattern of CXCL10 in the islets after CY administration, we did not detect significant alterations of PECAM-1$^+$ endothelial venules in the islets by treatment with anti-CXCL10 mAb (data not shown). Therefore, we hypothesized that CXCL10 neutralization enhanced the proliferation of islet β cells and increased β cell mass, resulting in the protection of NOD mice from the onset of diabetes.

We examined the effect of CXCL10 neutralization on the proliferation of islet β cells by in vivo BrdU labeling 1 h before killing on day 10 after CY administration with or without the treatment with anti-CXCL10 mAb (Fig. 6). BrdU$^+$ islet cells were regarded as proliferative cells. Whereas the replicating islet cells tended to be decreased after the development of insulitis, CXCL10 neutralization exhibited a marked increase of BrdU$^+$ cells in the islets (Fig. 6, *A–C* and *F*). Although some BrdU$^+$ cells in the islets from mice treated with anti-CXCL10 mAb were also stained with anti-insulin Ab, some markedly and some faintly (Fig. 6*D*), most of them looked like islet cells morphologically (for example, the nuclei were not round) and were stained clearly with anti-amylin Ab and not with anti-CD3 mAb (Fig. 6, *D* and *E*), showing that such BrdU$^+$ cells were confirmed as immature β cells and/or islet cells (36) and not islet-infiltrating T lymphocytes. Thus, most T cells staining positive for BrdU could be excluded in Fig. 6*F*. The mottled insulin-staining pattern, observed in the islets with insulitis after CY administration obtained from NOD mice treated with CXCL10 neutralization, is similar to that of the animals in the recovery period after the administration with streptozotocin, an islet toxin (37). It suggests that the islets in both circumstances include a considerable number of immature islet cells before and after the replication. In contrast, CXCL10 neutralization did not affect the apoptosis of islet β cells according to TUNEL (data not shown).

Lastly, to quantitate insulin content, we calculated the ratio of insulin-containing cell volume to the whole islet using the spectral confocal scanning system (TCS SP2 AOBS; Leica Microsystems) after immunofluorescent staining for insulin. The β cell mass in the pancreas from the NOD mice was decreased after the disease acceleration with CY and was restored to the levels in NOD mice without CY administration by CXCL10 neutralization (Fig. 7), corresponding to the result of the BrdU$^+$ replicating islet cell number (Fig. 6).

Discussion

We have demonstrated here that CXCL10 neutralization suppresses the occurrence of diabetes after disease acceleration with CY administration in NOD mice, through enhanced proliferation of β cells, resulting in increased insulin content in the islets without inhibiting insulitis. However, the possibility would be completely undeniable that the number of β cell-specific autoaggressive T cells infiltrating into the islets is reduced by CXCL10 neutralization, although it is usually accompanied with reduced migration of total immune cells (38).

The trafficking of inflammatory cells into the target organs caused by CXCL10 neutralization in the autoimmune diabetes model in this study differed not only from the animal models of EAE (15) and heat-killed *P. acnes*-induced hepatitis (16), in which T lymphocyte accumulation was increased, but also from the mice with protozoan (17) or viral infection (T. Kaburaki, H. Yoneyama, K. Matsushima, and S. Narumi, manuscript in preparation), in which the accumulation was decreased. Although the gene expression patterns of CXCR3 ligands in the pancreas (Fig. 4*B*) suggested that CXCL10 neutralization would inhibit the accumulation of effector T cells, CXCL10 neutralization, in fact, did not (Fig. 3).

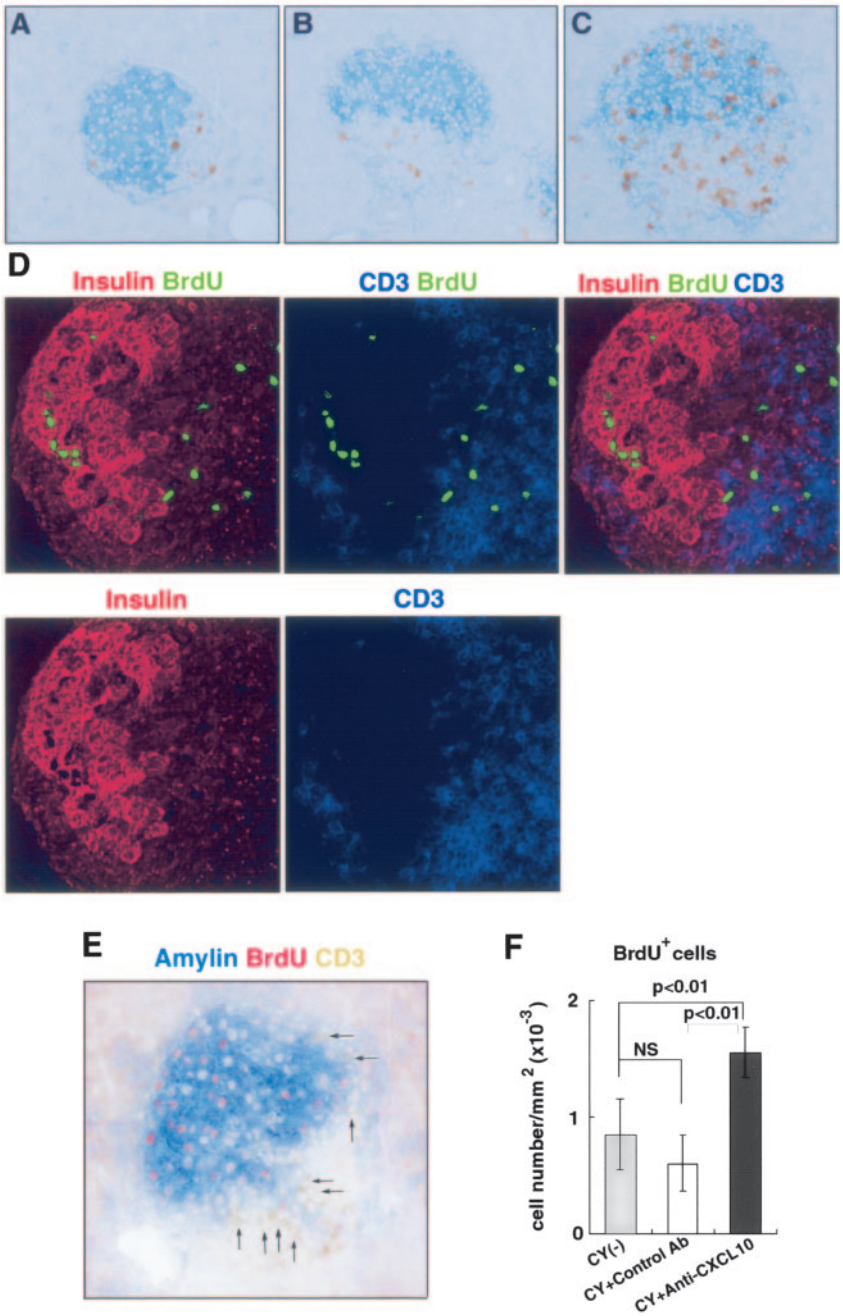

FIGURE 6. CXCL10 neutralization enhances the proliferation of islet β cells. NOD mice were partitioned into three groups. The first group of NOD mice was injected i.p. with PBS only ($n = 5$) (*A*). The other two groups of NOD mice were injected with CY and treated with anti-CXCL10 mAb ($n = 5$) (*C*) or the control mAb ($n = 5$) (*B*). Ten days after CY injection, mice were injected with BrdU and sacrificed 1 h later. More than three sections of each pancreas were examined to count the number of replicating cells using a BrdU staining kit (Zymed Laboratories), and $BrdU^+$ cells are colored brown (*A–C*). At the same time, the pancreases were stained with anti-insulin Ab, and positive cells were colored blue (*A–C*) (magnification, ×200). Furthermore, to identify $BrdU^+$ cells, triple immunofluorescence analysis for insulin (red), BrdU (green), and CD3 (blue; magnification, ×400) (*D*) and triple immunohistochemical analysis for amylin (blue), BrdU (red), and CD3 (brown; magnification, ×200) (*E*) were performed using the pancreases from mice injected with CY and treated with anti-CXCL10 mAb. The arrows indicated in *E* show $CD3^+$ T lymphocytes, in which BrdU was not detected. The number of $BrdU^+$ islet cells in each islet was counted and divided by the area of the islet. Then, the average of the positive cell number was obtained from each mouse and was compared among the three groups (*F*). The values are shown as mean ± SEM. Similar results were obtained in two separate experiments.

However, in the draining LNs, whereas CXCL10 produced by DCs in the paracortex areas (16) was induced in a manner similar to that of acute Th1 disease models immunized with external Ags (15, 16), CXCL9 produced by HEVs (32, 33) was only slightly induced in NOD mice (Fig. 4), in contrast with the animal models of EAE (15), heat-killed *P. acnes*-induced hepatitis (16, 33), and HSV infection (33). In our recent study on HSV infection, CXCL9 induced in HEVs is indispensable for plasmacytoid DC precursors to migrate into the draining LNs from circulation (33). Although the function of such plasmacytoid DCs is under investigation, the presence or absence of them in the draining LNs may determine the expression pattern of chemokine receptors and, resultantly, the CXCR3 dependency of the effector T cells in the retention in the draining LNs and the migration into the target organs. Alternatively, in the autoimmune disease model induced by constitutive intrinsic self Ags, many more effector T cells may be mobilized in a CXCR3-independent manner from the spleen than from the draining LNs, overwhelming the suppressive effect of CXCL10 neutralization on the CXCR3-dependent T cell accumulation in the islets. In this context, Cameron et al. (39) reported that expression of MIP-1α/CCL3 in the islets and diabetogenic splenic T cells with CCR5, one of the CCL3 receptors, contributes to the development of destructive insulitis, which naturally occurs in NOD mice. In contrast, although it has been recently reported that CXCL10 but not CXCL9 caused CXCR3-mediated T cell infiltration into the islets in which β cells were artificially manipulated to produce glycoprotein of lymphocytic choriomeningitis virus (38, 40), the effector T cells were generated through the acute infection with lymphocytic choriomeningitis virus. Taken together, whether $CXCR3^+$ or $CCR5^+$ effector T cells are preferably induced in Th1-dominant autoimmune disease models may depend, at least in part, on whether the immunogens are external or intrinsic self Ags or on whether the occurrence of insulitis is acute or chronic. In the present study, both $CXCR3^+$ and $CXCR3^-$ (some would be $CCR5^+$) effector T

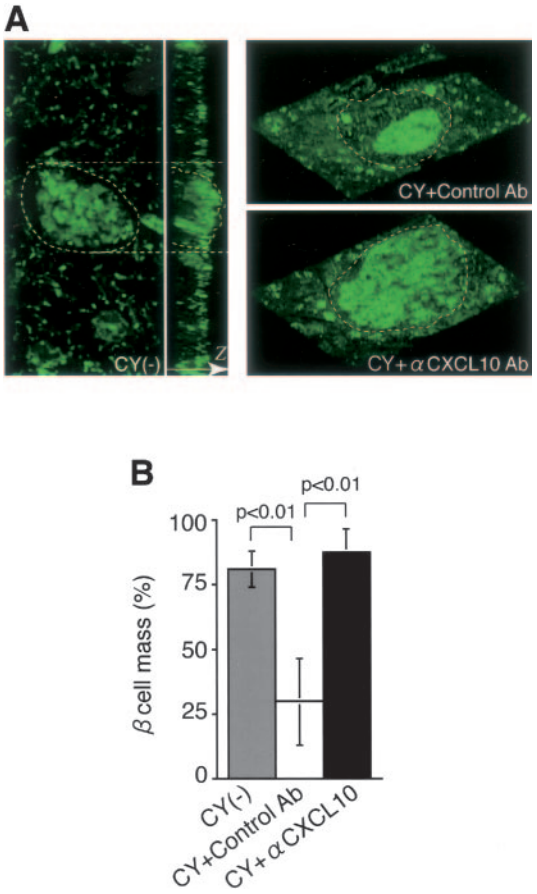

FIGURE 7. CXCL10 neutralization increases β cell mass. NOD mice were partitioned into three groups. The first group of NOD mice was injected i.p. with PBS only ($n = 5$). The other two groups of NOD mice were injected with CY and treated with anti-CXCL10 mAb ($n = 5$) or the control mAb ($n = 5$). Ten days after CY injection, mice were sacrificed, and more than three sections of each pancreas were examined to quantitate insulin content. We calculated the ratio of insulin-containing cell volume to the whole islet using the spectral confocal scanning system (TCS SP2 AOBS) (Leica Microsystems), after immunofluorescent staining for insulin (*A*). The average of the β cell mass (%) obtained from each mouse and was compared among the groups (*B*). The values are shown as mean ± SEM. Similar results were obtained in two separate experiments.

cells were considered to be generated in NOD mice laid under drastic immunomodulation by CY injection without immunization by external Ags. Thus, CXCL10 neutralization could affect insulitis and T cell infiltration into the islets less after disease acceleration with CY administration in NOD mice (Figs. 2 and 3). Whatever causes insulitis, CXCL10 neutralization would not exacerbate, but rather would inhibit, the insulitis because the islets do not produce CXCL11 (Fig. 4), in contrast with EAE (15) and heat-killed *P. acnes*-induced hepatitis (16).

CXCL10 expression was exhibited on the islet insulin-producing cells, whereas CXCL9 production was observed on the vascular endothelial cells lining capillaries in the islets, in addition to those outside the islets (Fig. 5). The restrictive production of CXCL10 and CXCL9 by the parenchymal cells and the vascular endothelial cells, respectively, was also shown in the intestine with acute colitis in our recent study on a mouse model of ulcerative colitis (29). Furthermore, in inflamed LNs, as mentioned above, it was reported that CXCL9 is selectively detected in a subset of HEVs (32, 33), whereas CXCL10 is selectively produced by DEC-205$^+$ mature DCs after the immunization with heat-killed *P. acnes* (16). This restrictive expression pattern of CXCL10 and CXCL9 differs from that seen in the organs infected with live organisms, in which CXCL9 in addition to CXCL10 is produced in parenchymal cells (14). In contrast, CXCL11 expression is induced only slightly or not at all in the organs, including the CNS infected with live organisms (17), although it is apparently detected in the CNS in the EAE model (15).

The islet β cells exhibited coexpression of CXCR3 and CXCL10 (Fig. 5, *A* and *C*). NOD mouse-derived β cell lineage also induces gene expression of CXCR3 (Y. Kanazawa and A. Shimada, unpublished observations), which is consistent with the present in vivo study. The augmented coexpression of CXCL10 and its receptor CXCR3 in the islet insulin-producing cells after insulitis acceleration suppresses proliferative response of β cells in an autocrine and/or paracrine fashion (Figs. 6 and 7). This is consistent with our previous study on a murine model of ulcerative colitis, in which both CXCL10 and CXCR3 were induced in epithelial cells and inhibited proliferative response of epithelial cells in an autocrine and/or paracrine fashion (29). Furthermore, we have confirmed that the recovery of the β cell mass after streptozotocin-induced islet injury, in which T cells are not infiltrated, is enhanced by CXCL10 neutralization (T. Kaburaki, H. Yoneyama, K. Matsushima, and S. Narumi, manuscript in preparation). It has been recently reported that an alternatively spliced variant (named CXCR3-B) of CXCR3 (renamed CXCR3-A) mediates the inhibition of vascular endothelial cell growth induced by CXCR3 ligands (41). CXCL10-inhibited proliferation of β cells and intestinal epithelial cells could be caused through CXCR3-B, although further detailed study is necessary to clarify it.

It was suggested that β precursor cells in adults are located, as in embryos, in the pancreatic duct and that these cells migrate into the tissue parenchyma, where they are differentiated and generate new islets. This compensatory islet growth has been demonstrated after partial pancreatectomy (42). In addition to the duct, mature islets also have been considered to contain precursor cells, which are seen in streptozotocin-induced islet injury (37). In the present study in NOD mice after CY-induced acceleration of diabetes, the β cell proliferation enhanced by CXCL10 neutralization was suggested to correspond to the latter case.

For as yet unknown reasons, the pancreatic β cell has a remarkably limited proliferation potential, and the production of new β cells by division is normally ~3% per day, a figure that rapidly declines with increasing age (43). In fact, β cell regeneration is not a noteworthy feature in either human or animal models for type 1 diabetes, similar to type 2 diabetes (44–46). In CY-induced diabetes in NOD mice, the number of proliferative β cells was not greater, but rather was less than that of NOD mice without disease acceleration by CY administration (Fig. 6). The limited proliferative potential of β cells was suggested to be due, at least in part, to the induction of CXCL10 by β cells in insulitis, although some growth stimuli for premature β cells were suggested to be induced. Therefore, CXCL10 neutralization enhanced β cell proliferation considerably in pancreatic islets with insulitis (Figs. 6 and 7).

The β cell mass is a major determinant of the total amount of insulin that can be secreted by the pancreas. Because CXCL10 neutralization enhances β cell proliferation in insulitis, resulting in increased β cell mass, and never increases the infiltration of effector T cells, CXCL10 can be a new therapeutic target for β cell survival, not only during the early stage of type 1 diabetes, but also after transplantation of islets or islet stem cells.

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