Suppression of Th1 cell activation and prevention of autoimmune diabetes in NOD mice by local expression of viral IL-10

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Abstract
Insulin-dependent diabetes mellitus in the NOD mouse model is caused by the T cell-mediated autoimmune destruction of pancreatic β cells. Viral IL-10 (vIL-10), encoded in the Epstein–Barr virus genome, shares many of the anti-inflammatory properties of cellular IL-10, but lacks its immunostimulatory properties. In the present study, we generated transgenic (Tg) NOD mice in which vIL-10 was produced exclusively in pancreatic islets and investigated the effect of vIL-10 on the development of diabetes. The accumulation of lymphocytes around islets was more prominent, but the invasive insulitis decreased in the vIL-10 Tg mice. The incidence of diabetes was markedly reduced in the vIL-10 Tg mice, in clear contrast to the accelerated diabetes seen in the murine IL-10 Tg NOD mice. IL-12p40 and IFN-γ mRNA levels were decreased in pancreata of the vIL-10 Tg mice, although CD4 mRNA level was markedly increased. These results suggest that locally produced vIL-10 induced leukocyte migration, but inhibited the activation of Th1, probably through suppressing the production of IL-12. They indicate that vIL-10 may well be superior to cellular IL-10 in the treatment of autoimmune diabetes. The vIL-10 Tg NOD mice should provide a useful tool for understanding the differential action of vIL-10 versus cellular IL-10.

Introduction

Human insulin-dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of insulin-producing β cells in the pancreatic islets (1). NOD mice spontaneously develop a form of autoimmune diabetes that shares many immunogenetic features with human IDDM (2,3) and thus are used as an excellent model for IDDM in various studies. Autoimmune diabetes is characterized by mononuclear cell infiltration into the pancreatic islets, termed insulitis. These mononuclear cells consist of CD4+ and CD8+ T cells, B cells, NK cells, and macrophages. Immunological studies have revealed a vital role for CD4+ T cells, which selectively produce IFN-γ, IL-2 and tumor necrosis factor (TNF)-β, and promotes cell-mediated immunity (4–6), in the development of autoimmune diabetes in NOD mice (7,8).

Cytokines, which are produced at the site of T cell activation, regulate the differentiation and proliferation of T cells, and also play important roles in the development of autoimmune diseases. IL-10, first identified as cytokine synthesis inhibitory factor (CSIF) (9), is known to suppress the development of Th1 cells by inhibiting the functions of antigen-presenting cells (APC). IL-10 decreases the antigen-presenting capacity of APC by down-regulating the expression of MHC class II molecules (10) and co-stimulatory molecules such as CD80 and CD86 (11–13). IL-10 also inhibits IL-1, TNF-α and IL-12 production by APC (14–16). Furthermore, IL-10 has direct effects on CD4+ T cells and suppresses IL-2 secretion (17,18). Because of these immunosuppressive abilities, IL-10 has been considered to be an effective agent for treating Th1-mediated autoimmune diseases. In fact, systemic administration of IL-10 to NOD mice prevented the onset of autoimmune diabetes (19,20) and we have also previously reported that systemic delivery of IL-10 by intramuscular injection of an
IL-10 expression plasmid vector reduced the development of autoimmune diabetes in NOD mice (21).

However, studies of transgenic (Tg) NOD mice expressing IL-10 in pancreatic islets revealed opposing effects of IL-10 on the development of autoimmune diabetes (22,23): locally produced IL-10 unexpectedly accelerated the development of insulitis and diabetes in these Tg NOD mice. Although the precise mechanisms leading to the acceleration of diabetes in IL-10 Tg NOD mice have not been clarified, the immunostimulatory activities of IL-10 have been implicated. In addition to its immunosuppressive activities, IL-10 is known to have immunostimulatory activities, such as the induction of MHC class II molecules on B cells, the enhancement of growth and differentiation of activated B cells, the co-stimulation of proliferation of thymocytes and mast cells (24–27), and the enhancement of differentiation and cytotoxicity of CD8+ T cells (28,29).

It has been reported that an open reading frame of the Epstein–Barr virus (EBV) genome, the BamHI C fragment rightward reading frame 1 (BCRF1), encodes an IL-10-like molecule, termed vIL-10 (30, 31). The protein sequences of vIL-10 and human IL-10 are 84% identical (31,32), and they share many of the same immunosuppressive properties (31). Interestingly, vIL-10 lacks most of the immunostimulatory properties of IL-10 (24,26,32). In fact, the rejection of allogeneic and syngeneic tumors expressing vIL-10 was suppressed in mice, although IL-10 accelerated tumor rejection (33,34). Thus, vIL-10 seems to protect T_h1 cell-mediated immune reactions more specifically than does IL-10. These findings suggest that the islet-specific production of vIL-10 rather than IL-10 in NOD mice could clarify the effects of T_h1 inhibition on the development of autoimmune diabetes without the bias caused by the immunostimulatory activities of IL-10.

In the present study, we produced Tg NOD mice expressing vIL-10 under the control of the rat glucagon promoter (Glu-vIL-10 Tg), and examined the effects of vIL-10 on the development of insulitis and on the incidence of autoimmune diabetes. This promoter has been shown to direct pancreatic $\alpha$ cell-specific expression in Tg mice and we previously used it to express cellular IL-10 in NOD Tg mice (23). We reasoned that Tg mice, in which vIL-10 expression is directed to $\beta$ cells, should enable us to examine the paracrine effects of vIL-10 on autoimmune destruction of $\beta$ cells without directly manipulating $\beta$ cells. The results showed that the incidence of both invasive insulitis and diabetes was markedly reduced in these Tg mice.

**Methods**

**Mice**

NOD/Shi mice were purchased from Clea Japan (Osaka, Japan) and were kept under specific pathogen-free conditions in our animal facility at Osaka University Medical School.

**Generation of Glu-vIL-10 Tg mice**

The Glu-vIL-10 transgene was constructed by inserting the vIL-10 coding sequence derived from pcDSRx-BCRF1 into the unique EcoRI site of the transgene vector, which contains the rat glucagon promoter and the rabbit $\beta$-globin gene sequences from the second exon to the polyadenylation signal (23) (Fig. 1A). The transgene vector was digested with Xhol and the resulting 2.6 kb transgene fragment was purified for microinjection.

The Glu-vIL-10 transgene fragment was injected into the pronuclei of one-cell embryos of NOD mice to produce Glu-vIL-10 Tg NOD mice (Glu-vIL-10 Tg), as described (35). The Tg founder mice were identified by PCR analysis of tail DNA. The primers used for mouse screening were as follows: forward primer, 5’-GCTGGTTATGCTGATTGC-3’; backward primer, 5’-ACGACTGAAAGGACTCTTTAG-3’. These primers amplify the transgene sequences between the third exon of the $\beta$-globin gene and the vIL-10 coding sequence. Trans-
genic mice were bred with NOD mice and gender-matched non-Tg littermates were used as controls.

RT-PCR
Total RNA was extracted from the major organs of mice by the guanidine thiocyanate–cesium chloride centrifugation method (36). The RNA was reverse-transcribed using an oligo(dt) primer and Superscript II reverse transcriptase (Gibco/BRL, Gaithersburg, MD). The reverse-transcribed samples were subjected to PCR for the Glu-vIL-10 transgene mRNA with the following primers: forward primer, 5'-AGCCTGGATCCGGAACCTCTT-3'; backward primer, 5'-ACGACTGAGGCATCTTCTTAG-3'. To standardize the relative amount of cDNA template in each sample, RT-PCR for the HPRT gene mRNA was performed in parallel. Primers for the HPRT mRNA detection were as follows: forward primer, 5'-CTCCTGCTCAAAATGTTTG-3'; backward primer, 5'-TCCTTTCTGTCTAGCTAGGG-3'; forward primer, 5'-TGGGCTGCTCAAACTTTCAGAATCT-3'; backward primer, 5'-GTCGTAGCAAACCACCAAGTGGA-3'.

To determine the levels of mRNA in pancreas, total RNA was extracted from the whole pancreas of female Tg (n = 5) and non-Tg (n = 5) at 13 weeks of age, and RT-PCR was performed with the following primers: CD4 forward primer, 5'-GGAATGCTAGCCGGTCTCCT-3'; CD4 backward primer, 5'-CTCACAGGTCGAAGATTTGTG-3'; CD8 forward primer, 5'-CTGATGCTGCCAGTGCTTCCT-3'; CD8 backward primer, 5'-CCTTCTGTGCTGACTAGGG-3'; CD14 forward primer, 5'-TGGGGTCTCAAAATTTCCAAGTG-3'; CD14 backward primer, 5'-CTCCGAAATAGAATCTCAAGG-3'; CD19 forward primer, 5'-CTGGCCTGCAAACTTCCAATG-3'; CD19 backward primer, 5'-GACCCCAAAAGCTGGAGCCCG-3'.

Isolation of pancreatic islets and Western blotting
Mice were anesthetized with an i.p. injection of pentobarbital at a lethal dose. Pancreatic islets were isolated by stationary collagenase (Sigma-Aldrich, St Louis, MO) digestion of the pancreas followed by Ficol 400 (Pharmacia Biotech, Uppsala, Sweden) density-gradient purification (38). One hundred islets were cultured in RPMI 1640 medium containing 10% FCS at 37°C.

Isolated islets and other organs were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 0.1% SDS and 0.1% sodium deoxycholate) with protease inhibitors. Protein concentrations were determined using a protein assay kit (BioRad, Hercules, CA). These lysates and supernatants from the cultured islets were mixed with a one-third volume of SDS sample buffer (75 mM Tris-HCl, pH 6.8, 6% SDS, 15% glycerol, 15% 2-mercaptoethanol and 0.015% bromophenol blue) and heated at 98°C for 5 min. These samples were subjected to SDS–PAGE on a 15% polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Bedford, MA). To estimate the amount of vIL-10, known amounts of recombinant human IL-10 (Immugenex, Los Angeles, CA) and recombinant vIL-10 (PharMingen, San Diego, CA) were simultaneously loaded onto the gel. The membrane was incubated at room temperature for 3 h with biotinylated rat anti-IL-10 mAb, JES3-12G8 (PharMingen), which reacts with both human IL-10 and vIL-10 but not with murine IL-10. After washing, the membrane was incubated at room temperature for 30 min with streptavidin–horseradish peroxidase conjugate (PharMingen) and processed for autoradiography using chemiluminescence techniques (ECL kit; Amersham, Arlington Heights, IL), according to the manufacturer’s instructions.

ELISA of viral IL-10
Serum samples obtained from mice were assayed for vIL-10 using ELISA as follows. Plates (96-wells) were coated with 2 µg/ml of the rat anti-IL-10 mAb, JES3-9D7 (PharMingen), at 4°C overnight, washed with PBS containing 0.05% Tween 20 (PBS/Tween) and blocked by incubation with PBS containing 1% BSA at room temperature for 1 h. After washing with PBS/Tween, appropriately diluted samples were added to the wells. Plates were incubated at 4°C overnight and washed with PBS/Tween. Biotinylated rat anti-IL-10 mAb (JES3-12G8; 2 µg/ml) was added to the wells and the plates were incubated at room temperature for 3 h with agitation. After washing with PBS/Tween, diluted streptavidin–horseradish peroxidase conjugate was added to the wells. Plates were incubated at room temperature for 30 min and washed with PBS/Tween. Substrate (o-phenylenediamine) was added to the wells and absorbance at 490 nm was measured on a microplate reader. Recombinant vIL-10 (PharMingen) was used as a standard and the linear range of this ELISA system was from 30 to 2000 pg/ml.

Diagnosis of diabetes and evaluation of insulin
Female Glu-vIL-10 Tg (F1 and F2) and non-Tg mice were screened weekly for the presence of glucosuria from 10 to 40 weeks of age, using TesTape A (Eli Lilly, Indianapolis, IN). Glucosuric mice were monitored daily and diabetes was diagnosed when mice were glucosuric for 3 consecutive days.
Pancreata were removed from female Glu-vIL-10 Tg and non-Tg at 13 weeks of age, and were fixed with 20% formalin. After embedding the samples in paraffin, 3-μm sections of each pancreas were prepared for hematoxylin & eosin staining. More than 25 islets from each pancreas were examined by light microscopy. To assess the progression of insulitis, each islet was assigned to one of the following four grades: 0, no insulitis; 1, non-invasive peri-insulitis; 2, mild insulitis (<25% of islet area contains infiltrating cells); 3, severe insulitis (≥25% of islet area contains infiltrating cells).

**Immunohistochemistry**

Pancreata of female Glu-vIL-10 Tg (n = 4) and non-Tg (n = 4) were subjected to immunohistochemical analysis at 15 weeks of age. Samples were embedded in OCT compound (Tissue TEK; Miles, Elkhart, IN) and frozen in liquid nitrogen. Consecutive sections (10 μm thick) were cut with a cryostat, placed on 3-aminopropyltriethoxysilane-coated slides and fixed in ice-cold acetone for 10 min. Samples were washed in PBS and then incubated with FITC-conjugated rat anti-mouse CD4 mAb (RM4-5), CD8 mAb (53-6.7) or B220 mAb (RA3-6B2) (PharMingen) at room temperature for 1 h. Following subsequent washes in PBS, specimens were counterstained with hematoxylin and observed with a fluorescence microscope (Olympus, Tokyo, Japan).

**Induction of diabetes by cyclophosphamid**

Male Glu-vIL-10 Tg and non-Tg were injected i.p. with 200 mg/kg of cyclophosphamide (Sigma-Aldrich) at 10 weeks of age. Two weeks later, a second injection was performed at the same dose. All mice were tested for glucosuria twice a week and were diagnosed as diabetic when they were glucosuric for 3 consecutive days.

**Statistical analysis**

Statistical analysis was performed using the log-rank test, the Mann–Whitney U-test and the unpaired Student’s t-test with the Stat View version 4.5 program for the Macintosh (Abacus Concepts, Berkeley, CA). P values for significance were set to 0.05.

**Results**

**Establishment of Glu-vIL-10 Tg mice**

We have produced Tg NOD mice (Glu-vIL-10 Tg) expressing vIL-10 under the control of the rat glucagon promoter (Fig. 1A). Two founder Tg mice (lines 4 and 7) carrying the Glu-vIL-10 transgene were generated and their offspring, obtained by mating the founder with normal NOD mice, were used in the subsequent experiments. The non-Tg NOD littermates were matched for gender and used as controls.

The tissue specificity of the Glu-vIL-10 transgene expression was examined by RT-PCR. Glu-vIL-10 mRNA was detected not only in the pancreas, but also in the lung, small intestine and colon of Glu-vIL-10 Tg (Fig. 1B, top). Control HPRT mRNA was detected in all of the organs examined (Fig. 1B, bottom). It is probable that the Glu-vIL-10 transgene was expressed in the small intestine and colon because the glucagon promoter is active in the intestinal neuroendocrine L cells, which produce GLP-1 (glucagon-like peptide-1) as a cleavage product of preproglucagon (39). The reason for the Glu-vIL-10 transgene expression in the lung is not known. To examine the relative expression levels of vIL-10 in these organs, lysates from islets, lung, small intestine and colon from Glu-vIL-10 Tg were subjected to Western blot analysis using an mAb that reacts with vIL-10 but not with murine IL-10. Although the same amounts of protein were loaded, only the lysate from islets produced the appropriate band of ~16 kDa detected with this mAb (data not shown). These results suggested that vIL-10 was produced at high levels in the pancreatic islets of Glu-vIL-10 Tg, and that production of vIL-10 in the small intestine, colon and lung was very low.

**Secretion of vIL-10 from the islets of Glu-vIL-10 Tg**

We next determined the levels of vIL-10 in the systemic circulation. Serum samples from 10-week-old Glu-vIL-10 Tg and non-Tg were assayed for vIL-10 by ELISA. The serum levels of vIL-10 in Glu-vIL-10 Tg of lines 4 and 7 were 22.5 ± 5.7 ng/ml (n = 3) and 6.5 ± 1.3 ng/ml (n = 4) respectively (values are mean ± SD). The level of vIL-10 in the serum of non-Tg was less than the detection limit of the assay (<30 pg/ml).

**Incidence of spontaneous diabetes in Glu-vIL-10 Tg mice**

To evaluate the effect of vIL-10 on the development of autoimmune diabetes, female Glu-vIL-10 Tg and non-Tg were monitored for glucosuria weekly up to 40 weeks of age. In Glu-vIL-10 Tg from lines 4 and 7, the cumulative incidence of diabetes was markedly reduced at 40 weeks of age, although a few mice had become diabetic at about 15 weeks of age in both lines (Fig. 2). Statistical analysis demonstrated a highly significant difference in the incidence of spontaneous diabetes between Glu-vIL-10 Tg and non-Tg (log-rank test, P < 0.001 for both lines).

**Effects of vIL-10 on insulitis**

To investigate the mechanisms underlying the preventive effect of locally produced vIL-10 on autoimmune diabetes, we performed histological examination of pancreata and evaluated the levels of insulitis. At 13 weeks of age, the pancreata of Glu-vIL-10 Tg were markedly infiltrated by inflammatory cells, which, however, remained in the outside of the islets. Although the accumulation of infiltrating cells around vessels and ducts was severe, and some islets suffered from invasive insulitis, most of the islets remained intact in Glu-vIL-10 Tg (Fig. 3A and B). On the other hand, most of the islets of non-Tg were infiltrated by mononuclear cells at the same age (data not shown). The difference in the progression of insulitis between Glu-vIL-10 Tg and non-Tg at 13 weeks of age is compared in Table 1. The percentage of peri-insulitis (non-invasive insulitis) in Glu-vIL-10 Tg was much higher, and the percentages of mild insulitis and severe...
insulitis in Glu-vIL-10 Tg were significantly lower than those in non-Tg. At 51 weeks of age, we still found intact islets surrounded by inflammatory cells and adipose tissue in Glu-vIL-10 Tg (Fig. 3C and D).

To examine the effects of vIL-10 on the population of lymphocytes infiltrating the islets, we performed immunohistochemical analysis of pancreata collected from Glu-vIL-10 Tg and non-Tg at 15 weeks of age, using mAb against the lymphocyte surface markers CD4, CD8 and B220. The results revealed that infiltrating cells in the pancreas of Glu-vIL-10 Tg were dominated by CD4+ T cells and B cells (Fig. 4A and C). A few CD8+ T cells were observed in and around the islets of Glu-vIL-10 Tg (Fig. 4B), whereas they were detected generally in the islets of non-Tg (Fig. 4E). CD4+ T cells and B cells tended to accumulate around the islets and around the blood vessels respectively in the pancreas of Glu-vIL-10 Tg.

To quantify the population of infiltrating cells in pancreas, we performed quantitative RT-PCR analysis for CD4, CD8, CD14 (macrophage marker) and CD19 (B cell marker) mRNA. The CD4 mRNA level in the pancreas of Glu-vIL-10 Tg was significantly increased compared with that of non-Tg, while there were no significant differences in the CD8, CD14 and CD19 mRNA levels between Glu-vIL-10 Tg and non-Tg (Fig. 5A). These results supported that the proportion of CD4+ T cells in the infiltrating cells in the pancreas of Glu-vIL-10 Tg was reduced.

Cytokine expression in the infiltrating cells

To examine the effects of locally produced vIL-10 on the cytokine expression in the infiltrating cells around pancreatic islets, we performed quantitative RT-PCR analysis by the real-time PCR method (37). IL-12p40 mRNA level in the pancreas of Glu-vIL-10 Tg was significantly decreased compared with that of non-Tg (Fig. 5B), though there were no differences in the levels of IL-1β and TNF-α mRNA between Glu-vIL-10 Tg and non-Tg (data not shown). Furthermore, although infiltration of CD4+ T cells was much severer in the pancreas of Glu-vIL-10 Tg than non-Tg as mentioned above, the IFN-γ mRNA level was rather decreased (Fig. 5B). These results suggested that locally produced vIL-10 inhibited the production of IL-12, a potent differentiation and activation factor for T_h1 cells, leading to the suppression of IFN-γ production from CD4+ T cells.

Cyclophosphamide-induced diabetes in Glu-vIL-10 Tg mice

Cyclophosphamide treatment accelerates the development of autoimmune diabetes in NOD mice by activating autoreactive T cells (40). To determine whether there were autoreactive T cells in Glu-vIL-10 Tg, we administered cyclophosphamide to male Glu-vIL-10 Tg and non-Tg, and examined the incidence of diabetes. Although the development of spontaneous diabetes was markedly reduced, cyclophosphamide accelerated diabetes in both lines of Glu-vIL-10 Tg. The cumulative incidence of diabetes induced by cyclophosphamide in Glu-vIL-10 Tg was even significantly higher than in non-Tg (line 7, log-rank test, P < 0.05) (Fig. 6). These results suggested that in Glu-vIL-10 Tg there were a considerable number of autoreactive T cells kept in an inactive state, which could be reversed by cyclophosphamide.

Discussion

In the present study, we have generated the Tg NOD mice (Glu-vIL-10 Tg), which express vIL-10 in pancreatic islets, and demonstrated that locally produced vIL-10 could prevent the development of autoimmune diabetes in NOD mice. We also demonstrated that IL-12p40 was significantly reduced and IFN-γ was not increased in the Glu-vIL-10 Tg, although the infiltration of CD4+ T cells around the pancreatic islets was severe. These results indicate that the prevention of autoimmune diabetes in Glu-vIL-10 Tg is due to the local suppression of T_h1 in the pancreas, probably through reducing the production of IL-12. Recent studies have revealed that vIL-10 down-regulates CD40 on APC (41), which mediates an important signal to induce IL-12 production in APC (42,43). These inhibitory effects of vIL-10 on APC are probably involved in the molecular mechanisms of IL-12 reduction in Glu-vIL-10 Tg. Since the levels of IL-4 mRNA were not increased in Glu-vIL-10 Tg (data not shown), it is not likely that T_h2 instead of T_h1 was stimulated in the pancreata of Glu-vIL-10 Tg. The levels of vIL-10 in the systemic circulation were as high as 5–30 ng/ml. Therefore, we cannot exclude the possibility that vIL-10 produced in the pancreatic islets influenced the systemic immune regulation, leading to the suppression of diabetes in Glu-vIL-10 Tg.

Although the development of spontaneous diabetes was
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Fig. 3. Histological examination of pancreata in female Glu-vIL-10 Tg mice. Marked accumulation of infiltrating cells around blood vessels and islets was observed at 13 weeks of age (A and B). There were some intact islets in the degenerative pancreatic tissue even at 51 weeks of age (C and D).

Table 1. Severity of insulitis in Glu-vIL-10 Tg mice and their non-Tg littermatea

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<tr>
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<td>0</td>
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<tr>
<td>Tg</td>
<td>8.4 ± 5.6</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>9.5 ± 4.8</td>
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<tr>
<td>P value</td>
<td>NS</td>
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aInsulitis in female Glu-vIL-10 Tg mice of line 4 (n = 5) and their female non-Tg littermates (n = 5) was examined at 13 weeks of age. Insulitis of individual islets was graded from 0 to 3 as follows: 0, no insulitis; 1, non-invasive peri-insulitis; 2, mild insulitis; 3, severe insulitis. More than 25 islets per mouse were assessed and the percent distribution among the grades was determined. Values represent the average percentages (mean ± SD). P values denote the significance level between Tg and non-Tg for each grade in the unpaired Student’s t-test.

markedly reduced in Glu-vIL-10 Tg, this reduction was reversed by the administration of cyclophosphamide. Cyclophosphamide accelerates development of diabetes probably by activating autoreactive T cells (40). Therefore, we suggest that locally produced vIL-10 does not prevent the generation of pathogenic T cells, including T₃₁, but induces a hyporesponsive state in these T cells. Such a local effect of vIL-10 on T cells is consistent with the previous report that vIL-10 induced local anergy to allogenic and syngenic tumors, which could be reversed by administrating IL-2 or IL-12 in vivo (33).

Recently, we have reported that the oxidative stress plays an essential role in the destruction of pancreatic β cells in NOD mice (44). Since vIL-10 inhibits the production of reactive oxygen species by macrophages (45), it is also possible that pancreatic β cells in Glu-vIL-10 Tg were protected from oxidative stress. Additionally, another T cell subset may be involved in the protection. Recently, Groux et al. reported that IL-10 drives the generation of a CD4⁺ T cell subset, designated T regulatory cell type 1 (T₃₁), which suppresses antigen-specific immune responses and actively down-regulates a pathological immune response in vivo (46). Buer et al. also reported another subset of regulatory T cells (47). Thus, it is possible that vIL-10 induced a similar type of regulatory T cells in islet-infiltrating cells and that these cells actively prevented the destruction of β cells. Experiments are in progress to examine the generation of T cells with immunoregulatory activity in Glu-vIL-10 Tg.

In Glu-vIL-10 Tg, invasive insulitis was markedly reduced, but the accumulation of inflammatory cells around islets, and especially around blood vessels, was more extensive. A similar accumulation of inflammatory cells around islets was reported by Wogensen et al. for the pancreata of Tg BALB/c mice expressing IL-10 in pancreatic islets (48). They suggested that IL-10 may induce increased expression of MHC class II molecules and adhesion molecules on vascular
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Fig. 4. Immunohistochemical analysis for cell surface markers of the lymphocytes infiltrating the islets. Pancreas of a line 4 female Glu-vIL-10 Tg mouse (A–C) and a non-Tg littermate (D–F) at 15 weeks of age were subjected to immunohistochemistry. Consecutive sections were stained with FITC-conjugated anti-mouse CD4 mAb (A and D), anti-mouse CD8 mAb (B and E) and anti-mouse B220 mAb (C and F). Infiltrating cells in the pancreas of the Glu-vIL-10 Tg mouse were dominated by CD4+ T cells and B cells (A and C). A few CD8+ T cells were observed in or around the islets of the Glu-vIL-10 Tg mouse (B), whereas they were detected generally in the islets of the non-Tg littermate (E).

endothelial cells, resulting in the homing of inflammatory cells. It is possible that vIL-10, like IL-10, can induce leukocyte migration in vivo.

In Tg NOD mice expressing murine IL-10 in islets, autoimmune diabetes is markedly accelerated (22,23). Recently, Balasa et al. reported that acceleration of diabetes in their IL-10 Tg mice was prevented by treatment with an anti-CD8 mAb (49). They suggested that the IL-10 expressed in pancreatic islets contributes to the pathology of diabetes via a CD8+ T cell pathway, eliminating the requirement for B cells and CD40–CD40 ligand co-stimulation. Although IL-10 suppresses the APC-dependent activation of CD8+ T cells, a high dose of IL-10 was shown to directly co-stimulate the proliferation of CD8+ T cells and augment their cytotoxic activity in vitro in the absence of APC (28,29). This stimulatory effect of IL-10 on CD8+ T cells was also suggested in vivo by the ability of an IL-10 Tg mouse line to limit the growth of immunogenic tumors by a CTL-mediated mechanism (50). On the other hand, in our Glu-vIL-10 Tg, the incidence of spontaneous diabetes was markedly reduced and the infiltration of CD8+ T cells into the pancreas was reduced. The infiltrating cells were mainly composed of CD4+ T cells and B cells. Furthermore, RT-PCR analysis of pancreata showed that the CD8 mRNA levels were not increased in Glu-vIL-10 Tg, compared with non-Tg. These observations suggest that the stimulatory activity of vIL-10 on CD8+ T cells may be very low compared with IL-10 or even absent, although no studies have yet tested the direct effects of vIL-10 on CD8+ T cells. vIL-10 is secreted from EBV-infected B cells and suppresses immune reactions to the virus-infected cells (31,51,52). Therefore, it is probable that vIL-10 lacks the ability to co-stimulate the proliferation and differentiation of CD8+ T cells, which play important roles in the immune responses to viral infection. The differentiation and prolifera-
tion of T\(_h\)1 cells were probably suppressed through the inhibition of APC functions in both IL-10 and vIL-10 Tg NOD mice, but CD8\(^+\) T cells might be activated directly by overexpressed IL-10 in the IL-10 Tg NOD mice. CD8\(^+\) T cells have been reported to be a strong effector in the destruction of pancreatic \(\beta\) cells in NOD mice (53,54). Thus, we speculate that the difference between vIL-10 and IL-10 in the direct effects on CD8\(^+\) T cells led to the opposite results in the development of autoimmune diabetes in these Tg NOD mouse lines.

In this study, we demonstrate that both T\(_h\)1 activation and autoimmune diabetes in NOD mice were effectively prevented by local production of vIL-10, in clear contrast to the accelerated diabetes seen in IL-10 Tg NOD mice. The present study indicates the superiority of vIL-10 over IL-10 for the treatment of T\(_h\)1-mediated autoimmune diseases. Additionally, the vIL-10 Tg mice should provide a useful tool for understanding the differential action of vIL-10 versus IL-10 in vivo.

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<td>EBV</td>
<td>Epstein–Barr virus</td>
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<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
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<td>Tg</td>
<td>transgenic</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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References

the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J. Exp. Med. 174:915.
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