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Systemic Administration of IL-18 Promotes Diabetes Development in Young Nonobese Diabetic Mice

Yoichi Oikawa,* Akira Shimada,† Akira Kasuga,‡ Jiro Morimoto,* Tadashi Osaki,§ Hideaki Tahara,‡ Tatsushi Miyazaki,‖ Fumi Tashiro,‖ Eiji Yamato,‖ Jun-ichi Miyazaki,‖ and Takao Saruta*

IL-18 is now identified as a pleiotropic cytokine that acts as a cofactor for both Th1 and Th2 cell development. Type 1 diabetes is considered a Th1-type autoimmune disease, and to date, the suppressive effect of exogenous IL-18 on the development of diabetes has been reported in 10-wk-old nonobese diabetic (NOD) mice. In the present study we administered exogenous IL-18 systemically in 4-wk-old NOD mice using i.m. injection of the IL-18 expression plasmid DNA (pCAGGS-IL-18) with electroporation. Contrary to previous reports, the incidence of diabetes development was significantly increased in NOD mice injected with pCAGGS-IL-18 compared with that in control mice. Systemic and pancreatic cytokine profiles deviated to a Th1-dominant state, and the frequency of glutamic acid decarboxylase-reactive IFN-γ-producing CD4⁺ cells was also high in the IL-18 group. Moreover, it was suggested that the promoting effect of IL-18 might be associated with increased peripheral IL-12, CD86, and pancreatic IFN-inducible protein-10 mRNA expression levels. In conclusion, we demonstrate here that IL-18 plays a promoting role as an enhancer of Th1-type immune responses in diabetes development early in the spontaneous disease process, which may contribute to elucidating the pathogenesis of type 1 diabetes. The Journal of Immunology, 2003, 171: 5865–5875.

Interleukin-18 is a recently discovered cytokine that was initially identified by Okamura et al. (1) as a potent IFN-γ-inducing factor; it shares biological similarity with IL-12 (a strong Th1 inducer). Indeed, in combination with IL-12, IL-18 plays an important role in the Th1-mediated immune response by its ability to induce IFN-γ production by activated macrophages (1–5). In contrast, it has been recently reported that with IL-2 (but not with IL-12), IL-18 has the potential to induce a Th2 cytokine (IL-13) in T cells and NK cells in vitro (6). Moreover, current evidence suggests that IL-18 can potentially induce other Th2 cytokines (IL-4, IL-5, IL-10) and IgE and IgG1 production (7–10). Therefore, IL-18 can act as a cofactor for both Th1 and Th2 cell development, which are dependent on the surrounding cytokine milieu (11).

Type 1 diabetes is considered to be a T cell-mediated autoimmune disease (12), based upon studies of nonobese diabetic (NOD) mice (13, 14). The NOD mouse, an excellent model for human type 1 diabetes, spontaneously develops autoimmune diabetes. In this model, infiltration of dendritic cells and macrophages, followed by T cells, is observed at 3–4 wk of age (called peri-insulitis; described as the initiation phase in this manuscript) (15–18). After T cell-mediated destruction of pancreatic β cells at 4–6 mo of age, NOD mice develop overt diabetes (19). Although the critical islet-associated Ags responsible for the initiation and development of insulitis are not completely identified, reactivity to some candidate autoantigens, including epitopes of insulin (20, 21), heat shock protein (22–24), and glutamic acid decarboxylase (GAD) (25, 26), has been reported. In addition, it has been reported that immunization of NOD mice with insulin (27–30) or GAD (31–34) can prevent diabetes development and reduce the response to other autoantigens.

Current evidence suggests that the onset of diabetes in NOD mice is closely related to an imbalance between Th1 and Th2 cells, such as a functional shift of Th2-type (protective) CD45RBlo CD4⁺ T cells to Th1-type (pathogenic) cells (35–37). Moreover, insufficient CD28/B7 (for example, CD28/CD86) co-stimulation is expected to affect the Th1 or Th2 development of uncommitted CD4⁺ T cells and mediate the onset of diabetes in NOD mice (38).

IL-18 is considered to be associated with the development of diabetes in NOD mice. Indeed, the IL-18 gene is located near Idd12, which is one of the genes related to diabetes development in the NOD mouse (39). In addition, in parallel with IL-12 mRNA expression, IL-18 mRNA expression is increased in the pancreas of NOD mice after the induction of diabetes by cyclophosphamide (40). In contrast, conflicting with these previous reports, we recently discovered that i.p. administration of exogenous IL-18 to NOD mice (10–12 wk of age) suppressed the induction of diabetes by cyclophosphamide (41). Similarly, Rothe et al. (42) reported a suppressive effect of IL-18 on the spontaneous development of diabetes in NOD mice (daily i.p. injection of IL-18 from 10 wk of age). However, there has been no report on the effect of exogenous IL-18 in very young (∼4 wk of age; initiation phase) NOD mice to date.

It is possible that differences in the dose, timing, or route of IL-18 administration will result in different effects on the development of diabetes in the NOD mouse as well as other cytokines (IL-10, IL-12) as previously reported (43–48). Therefore, even if

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2 Abbreviations used in this paper: NOD, nonobese diabetic; GAD, glutamic acid decarboxylase; HPRT, hypoxanthine phosphoribosyltransferase; IP-10, IFN-inducible protein-10; PTH, parathyroid hormone; TA, tibialis anterior.

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exogenous IL-18 can suppress the development of diabetes in NOD mice, further assessment of the safety and efficacy of IL-18 administration is required before its application to human type 1 diabetes. Recently, Miyazaki et al. (49) established i.m. injection of cytokine plasmid cDNA with electroporation, which is an effective means of long term systemic delivery of cytokine. Thus, in the present study we used this procedure and investigated the effect of systemic IL-18 delivery on diabetes development in very young (4 wk of age) NOD mice. To our surprise, we demonstrated here that there was a significantly higher incidence of spontaneous diabetes development in IL-18-treated NOD mice compared with controls. Under the influence of exogenous IL-18 administration by this procedure, the insulitis score shifted to a higher grade, and both systemic and pancreatic cytokine profiles deviated to a Th1-dominant state in IL-18-treated mice compared with controls. In addition, the IFN-inducible protein-10 (IP-10; a Th1-associated chemokine) mRNA expression level was significantly elevated in IL-18-treated mice. Furthermore, besides the higher frequency of peripheral GAD-reactive, IFN-γ-producing CD4+ cells observed. IL-12(p40) and CD86 (costimulatory molecule) mRNA expression levels in both splenocytes (including macrophages and/or activated lymphocytes) and pancreatic lymph nodes were markedly increased in the IL-18-treated group, suggesting that the altered APC functions induced by exogenous IL-18 may contribute to the differentiation of uncommitted cells to pathogenic Th1 cells such as GAD-reactive, IFN-γ-producing CD4+ T cells and accelerate the destruction of pancreatic β cells.

Materials and Methods

Mice

Four-week-old female NOD mice were purchased from CLEA Japan (Tokyo, Japan). They were kept under specific pathogen-free conditions in the animal facility of Tokyo Denryoku Hospital. Urinary glucose analysis was performed using Tes-Tape (Shionogi, Osaka, Japan) twice a week starting at 10 wk of age. Plasma glucose levels were determined using Gluast-Ace (Sanwa Kagaku, Nagoya, Japan) when glucosuria was detected, and mice were considered to be diabetic after two consecutive blood glucose values >250 mg/dl. In our colony the cumulative incidence of spontaneous diabetes development of female NOD mice reaches ~70% by 40 wk of age.

Plasmid vectors

Plasmid pCAGGS-IL-18 was constructed by inserting mouse IL-18 cDNA into the unique EcoRI site between the CAG (cytomegalovirus immediate-early enhancer-chicken β-actin hybrid) promoter and the 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector (Fig. 1). To facilitate the secretion of bioactive mouse IL-18, the leader sequence of the human prepro-parathyroid hormone (preproPTH) gene was ligated upstream of mature mouse IL-18 cDNA (50). Plasmids were grown in Escherichia coli HB101, prepared using plasmid purification columns (Qiagen, Hilden, Germany) according to the supplier’s protocol and were further purified by ethidiu bromide CsCl equilibrium density gradient centrifugation. The quantity and quality of the purified plasmid DNA were assessed by OD at 260 and 280 nm and also by electrophoresis in 1% agarose gel. The IL-18 expression capacity of the resulting pCAGGS-IL-18 plasmid DNA was confirmed by transient transfection into cultured cells, followed by measurement of IL-18 in the culture supernatant by ELISA (data not shown).

Intramuscular injection with electroporation

Four-week-old NOD mice were anesthetized with pentobarbital, and 50 μg of pCAGGS-IL-18 or pCAGGS plasmid DNA (control) was injected into the bilateral tibialis anterior (TA) muscles (total, 100 μg of plasmid DNA/mouse) using an insulin syringe with a 27-gauge needle with the electroporation method described below. The same injections were sequentially 2 wk later (at 6 wk of age). For electroporation a pair of electrode needles with a 5-mm gap was inserted into the muscle to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator. Three pulses of 100 V each were delivered to each injection site at a rate of one pulse per second, each pulse lasting for 50 ms. Then, three pulses of the opposite polarity were applied (49).

Detection of IL-18 mRNA at the injection site of pCAGGS-IL-18 plasmid DNA

Muscles injected with pCAGGS-IL-18 or pCAGGS plasmid DNA were removed 2 days after DNA injection, frozen in liquid nitrogen, and stored at –80°C. Total RNA was prepared from the muscle samples using the guanidinium isothiocyanate method. RNA was reverse transcribed with ReverTra Ace (Toyobo, Osaka, Japan) and oligo(dT) at 42°C for 1 h. The reverse-transcribed samples were then subjected to PCR using Taq polymerase (Takara, Otsu, Japan). For IL-18 and hypoxanthine phosphoribosyltransferase (HPRT) mRNA detection, specific oligonucleotide primers were designed as follows: 5′-IL-18 primer, TCTGAGCTGACCGCTTACCTC; 3′-IL-18 primer, CTGCGGTCTGACAGTGAA; 5′-HPRT primer, CTGGAAAGTGGATACG; and 3′-HPRT primer, TGCCCTAAAGCTATAGTG. These primers were designed to encompass the intron sequences, so that the appropriate PCR products from injected plasmid DNA could be distinguished from the products amplified from contaminating genomic DNA. The PCR consisted of 94°C for 2 min and 30 cycles at 94°C for 15 s, 55°C for 30 s, followed by extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gel.

Histology

The pancreas was removed from each mouse, fixed in 10% formaldehyde, and embedded in paraffin. Thin sections at five levels, 100 μm apart, were cut for staining with H&E to evaluate the islet-infiltrating immune cells by light microscopy. At least 25 islets from each mouse were observed and scored by two independent blinded observers using the following criteria: grade 0, islets free of insulitis; grade 1, islets with lymphocyte infiltration in <25% of the area; grade 2, lymphocyte infiltration in 25–50% of the area; grade 3, lymphocyte infiltration in 50–75% of the area; grade 4, lymphocyte infiltration in >75% of the area or small retracted islets. The histological score gives the mean infiltration grade of the islets analyzed.

Polyclonal stimulation of splenocytes

The spleen and pancreatic lymph nodes from each mouse were removed aseptically and minced. After lysing RBC, cells were resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Life Technologies) and penicillin/streptomycin (Life Technologies). The cells (1 × 10^6) resuspended in 200 μl of culture medium were transferred to each well of a round-bottom, 96-well plate. Then, anti-CD3 Ab (145-2C11; BD PharMingen, San Diego, CA) was added.
added to each well (final concentration, 5 µg/ml). No stimulant was added to control wells. Then the cells were cultured for 72 h at 37°C in a humidified 5% CO₂ atmosphere. The supernatant was collected at the end of culture and frozen at −80°C until cytokine assay.

**Cytokine measurement by ELISA**

Each cytokine (IFN-γ, IL-4, IL-10) was measured by ELISA as previously described (51). Briefly, a flat-bottom, 96-well plate was coated with anti-IFN-γ Ab (BD Pharmingen) or anti-IL-4 (JESS-2A5; BD Pharmingen) Abs in PBS with overnight incubation at 4°C. Collected supernatants and standards (purified recombinant IFN-γ, IL-4, IL-10; BD Pharmingen) were added and incubated for another 2 h. After washing with 0.1% Tween 20/PBS, biotinylated anti-IFN-γ (XM11.3, BD Pharmingen), anti-IL-4 (BVD6-24G2; BD Pharmingen), or anti-IL-10 (SXC-1; BD Pharmingen) Abs were added, followed by another 1-h period of incubation. AB solution (Vestacastin ABC kit; Vector Laboratories, Burlingame, CA) was then added, and the plate was incubated for another 30 min. Finally, the color reagent ABTS (Sigma-Aldrich, St. Louis, MO) was added, and the OD was measured with an ELISA reader (Bio-Rad, Richmond, CA) at 405 nm. The amount of cytokine present was determined from standard curves using purified recombinant cytokines. The detection limits for these three cytokines were all <20 pg/ml. Intra- and interassay variations in the assay of each cytokine were <5% (3 and 4%, respectively). The mouse serum IL-18 level was analyzed using a murine IL-18 ELISA kit (MBL, Nagoya, Japan) according to the manufacturer's instructions, with a detection limit of 12.5 pg/ml.

**Semiquantitative PCR analysis of cytokines, IP-10 and CD86 mRNA**

Total RNA was extracted from the pancreas, spleen, and pancreatic lymph nodes collected from each mouse using the RNeasy Mini Kit (Qiagen). The extracted RNA was reverse transcribed using a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the manufacturer’s instructions. The reverse-transcribed samples were frozen at −80°C until semiquantitative PCR assay.

Semiquantitative PCR was conducted for IFN-γ, IL-4, IL-10, IL-12(p40), IL-10, CD86, and β-actin (internal control) in an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) as previously reported (52). Briefly, the probes were labeled with the fluorescent reporter dye FAM (covalently linked to the 5'-end of the oligonucleotide) and the quencher TAMRA. The primer and probe sequences used were as follows: 5' IFN-γ primer, CAGCAACAAGGAGGGCAA; 3' IFN-γ primer, GCTTCCTGAGGTCTCATTCC; IFN-γ probe, AGGTCAACA ACCACAGGTTC-AGGC; 5' IL-4 primer, TCTCATGAGGCTGAC AGACTCT; 3' IL-4 primer, GCTTTATGCTGCTACAGGAAT; IL-4 probe, CTGCACTGATAGTGGT-ACAACTCC; 5' IL-10 primer, CACAGGAGCTCAGTAC; 3' IL-10 primer, GTCAGAACGGC AGCTAGACA; IL-10 probe, AAGAGAGCTCATTCTAGC-CTGGC TCA; 5' IL-12(p40) primer, CTCTCTAATTTTGAGAACCCAAT; 3' IL-12(p40) primer, CTCTCATATTTTTTTTCAGAGCTC; 5'-end probe, ACTCCG-GAGGTTCCGTGCTCA; 5' IF-10 primer, CGTTTCTT CAGGGCTCATTCC; 3' IF-10 primer, TGGGTCTAGATGCAGGTTC AG; IP-10 probe, AAGCTTGGAA-TCATCCCTGCAGGCT; 5' CD86 primer, TGGTTCCTGAGACTCATTGG; 3' CD86 primer, CGTT ACCTGATGCTAATTGG; CD86 probe, CGGCA-GAGGTTTGGCA GGAACTAC; 5' β-actin primer, CACCTGCAACTTCTGATTG GGA; 3' β-actin primer, TCCAGCTTTCTCTTTGGGTA; and β-actin probe, TGTA-GTTCTGATGGCAGGCTATTAGGCCC. All reactions were performed using the TaqMan Universal MasterMix according to the manufacturer’s instructions (Applied Biosystems). The mRNA level obtained was expressed relative to the β-actin PCR product amplified from the same sample (sample PCR product/β-actin PCR product) × constant.

**Ag-specific stimulation and intracellular cytokine staining for flow cytometry**

Ag-specific cytokine responses were assessed using an intracellular cytokine staining system as previously described (53). Briefly, the spleen and pancreatic lymph nodes from each mouse were removed aseptically and minced. After lysing RBC, cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin/streptomycin. The cells (1 x 10^6) resuspended in 200 µl of culture medium were transferred to each well of a round-bottom, 96-well plate. Then, recombinant GAD65 produced in yeast (RSR, Cardiff, U.K.) or regular insulin (Novo Nordisk Pharma, Copenhagen, Denmark) was added to each well (final concentration of each stimulant, 5 µg/ml). The endotoxin level of recombinant GAD65 was <0.1 endotoxin units/ml (at an Ag concentration of 10 µg/ml) by a chromogenic assay method (54). No stimulant was added to control wells. The cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for a total of 72 h with the last 4 h including a final concentration of 10 µg/ml brefeldin A (Sigma-Aldrich). After incubation, 150 µl of a serial sample was transferred to a 5-ml polystyrene round-bottom tube (BD Biosciences, Franklin Lakes, NJ). Then 5 µl of CD4-CyChrome Ab (H129.19; BD Pharmingen) was added, and the tubes were incubated at room temperature for 15 min. FACs lysing solution (4 ml; BD Biosciences) was added, and the tubes were vortexed gently and incubated at room temperature for another 10 min. After centrifuging the tubes at 1600 rpm for 5 min, the supernatant was removed, and the cells were washed with 0.1% BSA/PBS. Then 500 µl of FACs permeabilizing solution (BD Biosciences) was added, and the tubes were incubated for 10 min at room temperature in the dark. After washing twice with 0.1% BSA/PBS, 3 µl of IFN-γ-FITC Ab (IgG1; BD Pharmingen) and 5 µl of IL-4-PE Ab (IgG2b; BD Pharmingen) or isotype controls (IgG1-FITC and IgG2b-PE; BD Pharmingen) were added, and the tubes were incubated for 30 min at room temperature in the dark. After washing, the prepared cells were analyzed with an Epics Altra (Coulter, Hialeah, FL). To confirm that our intracellular cytokine staining system was working, the OVA-specific cytokine response was assessed as a positive control. Briefly, 10-wk-old C57BL/6 mice were immunized with OVA (Sigma-Aldrich) in combination with IFA (Sigma-Aldrich) and immunized at 14 days after immunization. The spleen lymphocytes were collected and stimulated with OVA (10 µg/ml) for 72 h. Afterward, the procedure as described above and confirmed that the system was working.

**Statistical analysis**

Results are presented as the mean ± SE. χ² test was used to compare the incidence of diabetes. Differences in serum IL-18 concentrations and IL-12 mRNA and CD86 mRNA expression levels between postinjection days were analyzed using Student’s t test. Differences in insulin spot size, cytokine production levels, mRNA expression levels, and the number of GAD-reactive IFN-γ-producing CD4⁺ cells between groups were analyzed using the Mann-Whitney U test for nonparametric unpaired observations. The correlations between the pancreatic IP-10 mRNA expression level and the insulitis score, between the pancreatic IFN-γ mRNA expression level and the insulitis score, and between the pancreatic IP-10 mRNA expression level and the pancreatic IFN-γ mRNA expression level were analyzed by means of Pearson’s correlation coefficient (r). The correlations between the population of GAD-reactive, IFN-γ-producing CD4⁺ cells and the insulitis score, between the population of GAD-reactive, IFN-γ-producing CD4⁺ cells and the insulin spot size, between the pancreatic IFN-γ mRNA expression level and the pancreatitis score were analyzed using Spearman’s correlation coefficient (ρ). A value of p < 0.05 was considered statistically significant.

**Results**

Incidence of diabetes development after i.m. injection of pCAGGS-IL-18 plasmid DNA

First, 50 µg of pCAGGS-IL-18 (IL-18 group; n = 28) or pCAGGS plasmid DNA (control group; n = 31) was injected into the bilateral TA muscles (50 µg/mouse; total, 100 µg of plasmid DNA/mouse) of 4-wk-old female NOD mice with electroporation. The same injections were repeated 2 wk later (at 6 wk of age). At several time points through 22–28 wk of age, the cumulative incidence of diabetes was significantly increased in the IL-18 group compared with the control group and the untreated female NOD mouse group (n = 20; Fig. 2). These findings indicate that systemic administration of IL-18 to young NOD mice results in the acceleration of diabetes development. However, the final cumulative incidence in the control untreated group reached the same level as that in the IL-18 group.

To determine whether this accelerating effect was influenced by age at the time of plasmid DNA injection, similar injections of pCAGGS-IL-18 plasmid DNA were given to NOD mice at 8 and 10 wk of age sequentially and showed no apparent effect on diabetes development (data not shown).
IL-18 plasmid DNA (IL-18 group) was significantly elevated at 2–7 days after pCAGGS-IL18 plasmid DNA injection. The serum IL-18 level in mice injected with pCAGGS-IL-18 plasmid DNA was below the detection limit (12.5 pg/ml) as was that in untreated NOD mice. The IL-18 level in pCAGGS-injected mice (control group) was below detection at 2, 7, and 14 days after plasmid DNA injection; peak levels were observed 3–7 days after injection of plasmid DNA into muscle; peak levels were observed 3–7 days after injection and gradually decreased thereafter (Fig. 4).

Detection of IL-18 mRNA at the injection site of pCAGGS-IL-18 plasmid DNA

We determined the presence of IL-18 mRNA transcribed from the i.m. injected pCAGGS-IL-18 plasmid DNA by RT-PCR. IL-18 mRNA was detected only in muscles injected with pCAGGS-IL18 plasmid DNA (Fig. 3, lane 3) and not in those injected with pCAGGS plasmid DNA (Fig. 3, lane 4). Control HPRT mRNA was detected at the sites of both injections (Fig. 3, lanes 1 and 2).

Detection of IL-18 in serum after i.m. injection of pCAGGS-IL-18 plasmid DNA

To determine whether i.m. injection of pCAGGS-IL-18 plasmid DNA yielded a significant level of IL-18 in the systemic circulation, 6-wk-old NOD mice were injected with pCAGGS-IL18 or pCAGGS plasmid DNA into the bilateral TA muscles (total, 100 μg of plasmid DNA/mouse), and the serum IL-18 level was determined by ELISA. Data are shown as the mean ± SE.

Effect of i.m. injection of pCAGGS-IL-18 plasmid DNA on the severity of insulitis

To investigate the mechanism of the accelerating effect of i.m. injection of pCAGGS-IL-18 plasmid DNA on autoimmune diabetest, each non-diabetic mouse from the IL-18 and control groups was killed at 8, 12, 17, and 30 wk of age, and the degree of insulitis was evaluated. Each insulitis score at 8, 12, and 17 wk of age (i.e., prediabetic stage) was significantly higher in the IL-18 group compared with the control group (Fig. 5), then the insulitis score in the control group reached the same level as that in the IL-18 group at 250 mg/dl. The incidence of diabetes was significantly increased in the IL-18 group compared with the control group (Fig. 2), then the incidence in the control group; untreated female NOD mice (untreated group; n = 20).

* p < 0.05 vs control group; † p < 0.05 vs untreated group (by χ² test).

FIGURE 2. Cumulative incidence of diabetes in plasmid DNA-injected NOD mice. Female NOD mice were sequentially injected with pCAGGS (control group; n = 31) or pCAGGS-IL-18 (IL-18 group; n = 28) in the bilateral TA muscles (total, 100 μg of plasmid DNA/mouse) with electroporation at 4 and 6 wk of age. These mice were monitored for glucosuria twice a week, starting at 10 wk of age. Plasma glucose levels were determined when glucosuria was detected, and mice were considered diabetic after two consecutive blood glucose values ≥250 mg/dl. The incidence of diabetes was significantly increased in the IL-18 group compared with the control group at 4 and 6 wk of age. These mice were monitored for glucosuria twice a week, starting at 10 wk of age. Plasma glucose levels were determined when glucosuria was detected, and mice were considered diabetic after two consecutive blood glucose values ≥250 mg/dl.

FIGURE 3. RT-PCR detection of IL-18 mRNA in muscle injected with plasmid DNA. Muscles injected with pCAGGS-IL-18 (a, lanes 2 and 4) or pCAGGS plasmid (a, lanes 1 and 3) were removed 2 days after DNA injection. cDNA products prepared from RNA of the muscle samples were subjected to PCR. IL-18 mRNA was detected only in muscle injected with pCAGGS-IL-18 (lane 4, arrow), and control HPRT mRNA was detected in both groups (lanes 1 and 2). For IL-18 and HPRT mRNA detection, specific primers were designed to encompass the intron sequences. The IL-18 forward primer was designed to hybridize with the sequence immediately downstream of the transcriptional start site of the CAG promoter (b), and the IL-18 primer pair could amplify only IL-18 cDNA derived from pCAGGS-IL-18, but not from the endogenous IL-18 gene. The PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide (a). Lane M, m.w. size marker.

FIGURE 4. Time course of serum IL-18 level after i.m. injection of plasmid DNA. Six-week-old NOD mice were injected with pCAGGS (control group; n = 10) or pCAGGS-IL-18 (IL-18 group; n = 13) in the bilateral TA muscles with electroporation, and serum IL-18 levels were examined by ELISA. Data are shown as the mean ± SE. * p < 0.02 vs day 7; † p < 0.002 vs day 0 or 14 (by Student’s t test).
30 wk of age. This finding reflects the cumulative incidence of diabetes, as shown in Fig. 2, and indicates that the pancreas in the IL-18 group was markedly infiltrated by inflammatory cells in a relatively early stage of the disease process compared with the control group. Therefore, exogenous IL-18 may facilitate diabetogenic lymphocytes to destroy pancreatic \( \beta \) cells.

**Systemic and pancreatic cytokine profiles**

To examine the effect of IL-18 on the systemic Th1/Th2 cytokine balance, we evaluated the cytokine production of splenocytes induced by polyclonal (anti-CD3 Ab) stimulation. There was no significant difference in the population of splenic CD4\(^+\) and CD8\(^+\) cells between the IL-18 and control groups (data not shown). As shown in Fig. 6a, IFN-\( \gamma \) production was significantly increased in the IL-18 group compared with the control group at 8 wk (\( p < 0.05 \)) and 12 wk (\( p < 0.01 \)) of age. In pancreatic lymph nodes a similar tendency was observed (Fig. 6b). There was no significant difference in the cytokine profiles of spleen and pancreatic lymph nodes between the control group and untreated NOD mice (data not shown), and each cytokine production level from cells without anti-CD3 Ab stimulation was below the detection limit (\(< 20 \text{ pg/ml}\)) in all tested mice regardless of pretreatment. To investigate the effect of IL-18 on the local (pancreas) cytokine milieu, we evaluated the cytokine (IFN-\( \gamma \), IL-4, and IL-10) mRNA expression levels in the pancreas using semiquantitative PCR analysis. As shown in Fig. 7a, the pancreatic IFN-\( \gamma \) mRNA expression level in the IL-18 group was significantly higher than that in the control group at 8 wk (\( p < 0.05 \)) and 17 wk (\( p < 0.01 \)) of age, whereas no significant difference in pancreatic IL-4 and IL-10 mRNA expression levels was observed between the two groups (data not shown regarding IL-10). As a result, the local (pancreatic) cytokine profile tended to deviate to a Th1-dominant state in the IL-18 group compared with the control group. In pancreatic lymph nodes as well, a similar tendency was observed (Fig. 7b). Moreover, there was a positive correlation between the pancreatic IFN-\( \gamma \) mRNA expression level and the insulitis score (\( r_p = 0.563; p < 0.001 \)) in the prediabetic stage. These findings suggested that exogenous IL-18 administration to 4-wk-old NOD mice might promote the infiltration of diabetogenic Th1 cells, but not regulatory cells (Th2 or T regulatory 1 cells) into the islets and facilitate the destruction of pancreatic \( \beta \) cells.

**Elevated local (pancreatic) IP-10 mRNA expression level in IL-18-treated NOD mice**

IFN-inducible protein-10 (IP-10), which is a chemokine that promotes the migration of activated Th1 cells, is now considered to be associated with the pathogenesis of autoimmune diseases (53, 57). To investigate the relationship between local (pancreatic) IP-10 and insulitis under the influence of exogenous IL-18, we assessed the IP-10 mRNA expression level in the pancreas using semiquantitative PCR analysis. As shown in Fig. 8, the pancreatic IP-10 mRNA expression level was significantly increased in the IL-18 group compared with the control group at 8 wk (\( p < 0.01 \)) and 17 wk (\( p < 0.05 \)) of age. In addition, the pancreatic IP-10 mRNA expression level was positively correlated with both the insulitis score (\( r_p = 0.517; p < 0.01 \)) and the pancreatic IFN-\( \gamma \) mRNA expression level (\( r_p = 0.510; p < 0.01 \)) in the prediabetic stage. These findings indicate that a high expression level of local IP-10 in the prediabetic stage is closely related to the severity of insulitis; thus, the
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FIGURE 7. Cytokine mRNA expression levels in pancreas (a) and pancreatic lymph nodes (b) in IL-18-treated NOD mice and control mice. IFN-γ and IL-4 mRNA expression levels from each group were evaluated at 8 (control group, n = 5; IL-18 group, n = 7), 12, 17, and 30 wk of age (control group, n = 5; IL-18 group, n = 5 at each time point) using semiquantitative PCR analysis. The mRNA level was expressed relative to the β-actin PCR product amplified from the same sample. Data are shown as the mean ± SE. For pancreatic lymph nodes, the lymphocytes pooled at each time point were used for analysis. *, p < 0.05; †, p < 0.01 (by Mann-Whitney U test).

High level of local IP-10 in the IL-18 group reflects the progression of insulitis mediated by exogenous IL-18 administration.

High frequency of GAD-reactive, IFN-γ-producing CD4+ cells in IL-18-treated NOD mice

To ascertain whether IL-18 has an effect on the development of islet-associated Ag-reactive lymphocytes, we evaluated the population of GAD- or insulin-reactive cytokine (IFN-γ or IL-4)-producing CD4+ cells using an intracellular cytokine-staining system with flow cytometry. As expected, the population of splenic (i.e., peripheral) GAD-reactive, IFN-γ-producing CD4+ cells was significantly increased in the IL-18 group compared with the control group at 8, 12, and 17 wk of age (Fig. 9a). In pancreatic lymph nodes a similar tendency was observed (Fig. 9b). Moreover, the population of GAD-reactive, IFN-γ-producing CD4+ cells was positively correlated with insulitis score (ρ = 0.510; p < 0.01), the pancreatic IFN-γ mRNA expression level (ρ = 0.429; p < 0.03), and the pancreatic IP-10 mRNA expression level (ρ = 0.479; p < 0.01) in the prediabetic stage. Regarding GAD-reactive, IL-4-producing CD4+ cells (Fig. 9, a and b) and insulin-reactive cytokine (IFN-γ or IL-4)-producing CD4+ cells, no significant difference between the two groups was observed (data not shown). In the absence of Ags (GAD and insulin), each population of IFN-γ-producing CD4+ T cells and IL-4-producing CD4+ T cells in the spleen was below the detection limit (<5 counts/50,000 cells in our intracellular cytokine measurement system) in all mice examined. These results indicate that exogenous IL-18 may facilitate the development of GAD-reactive, Th1-type CD4+ cells and contribute to promoting destructive insulitis.

Elevated peripheral IL-12(p40) and CD86 mRNA expression levels in IL-18-treated NOD mice

Recently, it has been reported that the differentiation and matura-

tion of naive CD4+ cells into functional Th1 or Th2 cells require appropriate CD28/B-7 costimulation between APCs and naive cells, and that aberrant (or insufficient) CD28/CD86 costimulation in young NOD mice is associated with the onset of autoimmune diabetes (38). Moreover, it was recently reported that the activation of naive diabetogenic T cells in pancreatic lymph nodes, but not in islets, is closely related to the initiation of (peri-)insulitis (58). Accordingly, to evaluate the effect of IL-18 on APC function, we examined IL-12(p40) and CD86 mRNA expression levels of splenocytes (as a representative of peripheral inflammatory cells) and pancreatic lymph nodes using semiquantitative PCR analysis. As shown in Figs. 10 and 11, IL-12(p40) and CD86 mRNA expression levels in both splenocytes and pancreatic lymph nodes were significantly increased in the IL-18 group compared with the control group; IL-12(p40) and CD86 mRNA expression levels in pancreatic lymph nodes increased linearly through 4–8 wk of age in the IL-18 group (Fig. 11), whereas splenic IL-12(p40) and CD86 mRNA expression levels in the IL-18 group peaked at 7
days after injection and then gradually decreased to basal levels at 14 days after injection (Fig. 10). Intriguingly, the changes in IL-12(p40) and CD86 mRNA expression levels after pCAGGS-IL-18 injection were obviously correlated with the serum IL-18 level (compare Figs. 4 and 10). These findings suggest that exogenous IL-18 administration may have an effect on APC function (i.e., IL-12 production and CD28/CD86 costimulation) in the periphery and pancreatic lymph nodes of 4-wk-old NOD mice, resulting in the development of uncommitted CD4+ cells into diabetogenic Th1 cells (such as GAD-reactive, IFN-γ-producing CD4+ cells).

Discussion
The results presented here demonstrate that exogenous IL-18 administration to very young (4-wk-old) NOD mice accelerates the development of diabetes. Based on previous reports, IL-18 is considered a pleiotropic cytokine, which enhances innate immunity and both Th1- and Th2-driven immune responses depending on the surrounding cytokine milieu (11). Regarding the effect of exogenous IL-18 administration on diabetes development in NOD mice, we recently found that i.p. administration of exogenous IL-18 to NOD mice (10–12 wk of age) suppressed the induction of diabetes by cyclophosphamide (41). Moreover, we found that IL-18 administration affected the systemic cytokine profile, which shifted to a more Th2-dominant state, suggesting that exogenous IL-18 may regulate autoimmunity against pancreatic β cells through an IL-18-mediated, systemic, Th2-dominant cytokine balance (41). These reported findings suggested the involvement of IL-18 in Th2-type responses in vivo. Similarly, Rothe et al. (42) reported that daily i.p. administration of IL-18 to NOD mice (starting at 10 wk of age) had a suppressive effect on spontaneous diabetes development. In their experimental system the pancreas of IL-18-treated NOD mice expressed more IL-4 mRNA than that of non-treated mice, so they concluded that exogenous IL-18 might change the islet-infiltrating T cell phenotype from Th1 to Th2 cells. However, conflicting with their findings, we demonstrated an
accelerating effect of IL-18 administration on diabetes development in NOD mice in the present study (Figs. 2 and 5). We found that both systemic and pancreatic cytokine profiles deviated to a Th1-dominant state in IL-18-treated mice compared with controls (Figs. 6 and 7). These findings appear to be consistent with the originally identified characteristic of IL-18 as an enhancer of Th1-driven immune responses (1). The discrepancies in the results between the present study and the previous two reports (41, 42) are attributable to differences in the method of IL-18 administration, the level of serum IL-18, and the age of the animals at the time of IL-18 administration.

With regard to the method of IL-18 administration, we introduced i.m. injection of IL-18-expressing plasmid cDNA in this study. This technique is considered simple, inexpensive, and safe compared with gene transfer systems using viral vectors (59–62). However, application of this method has been limited by the relatively low expression level of the transferred gene (62, 63). Miyazaki et al. (49) recently established a novel gene (cytokine-expressing plasmid cDNA) transfer system with electroporation, which is a more effective means of achieving stable and long term systemic delivery of cytokines, leading to higher serum concentrations of cytokines. As shown in Fig. 4, a significant level of serum IL-18 was maintained for 2 wk, and the peak level reached ~130 pg/ml with a single i.m. pCAGGS-IL-18 plasmid DNA injection using this system. In contrast, i.p. administration of large amounts of IL-18 (0.3 μg/mouse) made the serum IL-18 level extremely high (150 ng/ml, on the average), resulting in the induction of Th2-like effects of exogenous IL-18 on diabetes development in NOD mice (41, 42). In addition, Th2-like effects of IL-18 have been reported in in vitro systems (6, 9) and in vivo systems using IL-18 transgenic mice (64) or a high dose of exogenous IL-18 administration (7, 8, 10); all of the reports were assessed under nonphysiological conditions (i.e., extremely high concentrations of IL-18). Such an extremely high and nonphysiological cytokine level in serum may cause inappropriate systemic effects and lead to difficulty in interpreting the effect on diabetes development. In fact, diabetes was not prevented in pCAGGS-IL-18 plasmid DNA-injected older (8- and 10-wk-old) NOD mice in our system. Therefore, we speculate that the preventive effect of exogenous IL-18 on spontaneous diabetes development, as previously reported (41, 42), may be attributable to the extremely high level of serum IL-18. Current reports suggest that a polarized Th1-type response influences the development of type 1 diabetes (35–37), and that islet-associated Ag-reactive Th1-type CD4+ cells and CD8+ cells are pathogenic T cells (25, 26, 65–69), which are directly associated with pancreatic β cell destruction by cytotoxic effects through Fas-Fas ligand interaction and the release of granzymes, perforin, TNF-α, and IFN-γ, etc. (70). In the present study we demonstrated that exogenous IL-18 administration had an effect on the systemic and pancreatic cytokine balance, which preferentially deviated to a Th1-dominant state (Figs. 6 and 7). Moreover, GAD-reactive, IFN-γ-producing CD4+ cells were significantly increased in the IL-18 group compared with the control group (Fig. 9). At the initiation phase (~4 wk of age), islet-associated Ag-reactive, uncommitted T cells may be stimulated by APCs and differentiate into either pathogenic Th1-type cells (under the influence of IL-12) or protective Th2-type cells (under the influence of IL-4). Thus, we speculate that exogenous IL-18 administration in the initiation phase is likely to act preferentially as a cofactor and enhancer of Th1 cell differentiation, probably together with elevated IL-12 production from APC (Figs. 10a and 11a). To our knowledge, there has been no report to date that exogenous IL-18 induces endogenous IL-12 production, so further studies are required to elucidate the relationship between exogenous IL-18 and endogenous IL-12.

To confirm whether the Th1-type effect is influenced by age at the time of DNA injection, we gave the same injections of pCAGGS-IL-18 plasmid DNA to NOD mice at 5 and 10 wk of age sequentially as described, and no significant effect on the systemic and pancreatic (Th1/Th2) cytokine balance and the population of GAD- or insulin-reactive cytokine (IFN-γ and IL-4)-producing CD4+ cells was observed at an older age (data not shown). Boitard et al. (71) previously reported that some regulatory factors (or CD4+ lymphocytes as reported by Boitard et al.) overcome some diabetogenic factors (or lymphocytes) in ~8-wk-old NOD mice. Indeed, as shown in Fig. 9c, the number of splenic IL-4-producing, GAD-reactive CD4+ cells was greater than the number of IFN-γ-producing, GAD-reactive CD4+ cells in the control (i.e., pCAGGS treatment) group at 8 wk of age. In addition, we previously reported that the GAD-reactive, Th2-type response (IL-10 production) was significantly elevated in 10-wk-old NOD mice (51). These two reports (51, 71) suggest that regulatory functions may be most efficient around 8–10 wk of age. Considering our previous finding that IL-10 production from splenocytes by polyclonal (anti-CD3 Ab) stimulation was significantly increased in i.p. IL-18-injected NOD mice (10–12 wk of age) compared with control mice (41), we can speculate that exogenous IL-18 administration may also affect regulatory cells as a cofactor for the Th2 response at an older age (~10 wk of age) and ultimately offset both Th1 and Th2
responses reciprocally. Although the detailed mechanism is unknown, these findings suggest that the higher IL-18 delivery at the early stage of insulitis, but not the late stage, contributes to inducing islet infiltration and pancreatic β cell destruction and accelerating diabetes development in NOD mice.

We have recently discovered that IP-10, a chemokine that promotes the migration of activated Th1 cells (72–74), is expressed in insulitis lesions in NOD mice (J. Morimoto, H. Yoneyama, A. Shimada, S. Yamada, Y. Oikawa, K. Matsushima, T. Saruta, and S. Narumi, unpublished observations). In fact, IP-10 is expressed in pancreatic β cells that are bordered by infiltrating lymphocytes (Y. Oikawa, unpublished observations). The areas of IP-10-expressing β cells in peri-insulitis gradually widen as insulitis progresses, suggesting that the pancreatic IP-10 expression level is associated with the state of insulitis in the prediabetic stage. Although there is no evidence regarding the reciprocal relationship between IP-10 expression in islets and diabetes development, we recently observed that anti-IP-10 Ab treatment suppressed diabetes development in NOD mice after cyclophosphamide administration (J. Morimoto, H. Yoneyama, A. Shimada, S. Yamada, Y. Oikawa, K. Matsushima, T. Saruta, and S. Narumi, unpublished observations). Moreover, in humans, we have also reported that the serum IP-10 level is elevated in type 1 diabetic patients (53). Based on these findings, it is possible that local (pancreatic) IP-10 expression is related to the etiology and activity of autoimmune insulitis. In this study we demonstrated that the pancreatic IP-10 mRNA expression level was significantly increased in the IL-18 group compared with the control group (Fig. 8). In addition, the pancreatic IP-10 mRNA expression level was positively correlated with the insulin score, the pancreatic IFN-γ mRNA expression level, and the population of peripheral GAD-reactive, IFN-γ-producing CD4+ cells in the prediabetic stage. Based on these findings, there is a possibility that the elevated pancreatic IP-10 expression in the IL-18 group more efficiently promotes the migration of diabeticogenic Th1 cells into insulitis lesions and the progression of β cell destruction. To our knowledge, there is no previous report on the relationship between IL-18 and IP-10, and further studies are needed to elucidate the detailed mechanism.

APC in NOD mice are considered to have abnormal differentiation and function, contributing to the development of pathogenic (Th1-type) CD4+ and CD8+ T cells (75–78). For example, macrophages prepared from NOD mice are capable of secreting elevated levels of IL-12, which could facilitate the preferential differentiation of Th1-type and T cytotoxic 1 cells compared with those from nonautoimmune strains of mice (75). In addition, the association between the CD28/B7 pathway and the activation of naive or memory T cells has been investigated in autoimmune diseases, including autoimmune diabetes (38). In the present study we demonstrated that the expression levels of IL-12(p40) and CD86 mRNA in splenocytes were significantly increased in the IL-18 group compared with the control group (Fig. 10). Considering that the number of GAD-reactive IFN-γ-producing CD4+ cells was increased in IL-18-treated young NOD mice (Fig. 9), we speculate that elevated IL-12 production and up-regulation of CD86 in APC may have promoted the differentiation of uncommitted β cell-reactive CD4+ T cells in the IL-18 group. Regarding the CD86 molecule, these findings are consistent with the reports by Bluestone and colleagues (38), who demonstrated, using the NOD mouse, that the CD28/CD86 pathway is closely related to the pathogenesis of diabetes. For example, Salomon et al. (79) recently demonstrated that CD86-deficient NOD mice, which developed peri-insulitis, but not progression to intrainsulitis, were protected from overt diabetes. In addition, Lenschow et al. (80) previously demonstrated that treatment of NOD mice with anti-CD86 mAb early in the disease process (between 2 and 7 wk of age) prevented the development of diabetes. These two reports suggest that CD28/CD86 activation plays a primary role in the initiation of diabeticogenic cells very early in the disease course in this model (~4 wk of age; i.e., in the initiation phase) (38).

According to the previous report by Hoglund et al. (58), naive (or uncommitted) β cell-reactive T cells are first activated by islet-associated APCs in the pancreatic lymph nodes (but not in the islets) in the initiation phase, and the activated T cells sequentially migrate to the islets, leading to the initiation of (peri-)insulitis. Considering that the expression levels of IL-12(p40) and CD86 mRNA in pancreatic lymph nodes were markedly increased in IL-18-treated young NOD mice (Fig. 11, a and b), we can speculate that exogenous IL-18 administration in the initiation phase may have a certain influence on APC function especially in pancreatic lymph nodes, contributing to the initiation and/or activation of diabeticogenic cells. However, there is no evidence that IL-18 directly affects APC function; thus, we believe that APC function may have been enhanced by the increased level of IFN-γ via exogenous IL-18 administration rather than by a direct effect of systemic IL-18 delivery.

In conclusion, we demonstrated in this study that IL-18 has an accelerating effect as an enhancer of Th1-type immune responses on diabetes development early in the spontaneous disease process, which may contribute to elucidating the pathogenesis of type 1 diabetes. To date, several reports have shown that exogenous IL-18 administration prevents diabetes development in NOD mice, and that IL-18 treatment may be useful for the prevention of type 1 diabetes development in humans as well in the future. However, we emphasize that, to the contrary, IL-18 treatment is capable of promoting diabetes development, so that the method (route) of IL-18 administration, the level of serum IL-18, and the timing of IL-18 administration have to be carefully assessed. Therefore, further prudent investigations are required before application of IL-18 administration to human type 1 diabetic patients.

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**References**

IL-18 PROMOTES DIABETES DEVELOPMENT IN NOD MICE


