IL-18 Inhibits Diabetes Development in Nonobese Diabetic Mice by Counterregulation of Th1-Dependent Destructive Insulitis

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IL-18 Inhibits Diabetes Development in Nonobese Diabetic Mice by Counterregulation of Th1-Dependent Destructive Insulitis

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The development of type 1 diabetes in animal models is T cell and macrophage dependent. Islet inflammation begins as peripheral benign Th2 type insulitis and progresses to destructive Th1 type insulitis, which is driven by the innate immune system via secretion of IL-12 and IL-18. We now report that daily application of IL-18 to diabetes-prone female nonobese diabetic mice, starting at 10 wk of age, suppresses diabetes development ($p < 0.001$, 65% in sham-treated animals vs 33% in IL-18-treated animals by 140 days of age). In IL-18-treated animals, we detected significantly lower intraislet infiltration ($p < 0.05$) and concomitantly an impaired progression from Th2 insulitis to Th1-dependent insulitis, as evidenced from IFN-$\gamma$ and IL-10 mRNA levels in tissue. The deficient progression was probably due to lesser mRNA expression of the Th1 driving cytokines IL-12 and IL-18 by the innate immune system ($p < 0.05$). Furthermore, the mRNA expression of inducible NO synthase, a marker of destructive insulitis, was also not up-regulated in the IL-18-treated group. IL-18 did not exert its effect at the levels of islet cells. Cultivation of islets with IL-18 affected NO production or mitochondrial activity and did not protect from the toxicity mediated by IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$. In conclusion, we show for the first time that administration of IL-18, a mediator of the innate immune system, suppresses autoimmune diabetes in nonobese diabetic mice by targeting the Th1/Th2 balance of inflammatory immune reactivity in the pancreas. The Journal of Immunology, 1999, 163: 1230–1236.

Materials and Methods

Animals

Female NOD mice, which were originally obtained from Prof. C. Y. Wu in 1990 (University of Beijing, Beijing, China), were bred in the animal house at Katholike University and maintained under conventional conditions (19). The diabetes incidence of female mice was 75%. The colony was screened regularly for viral or bacterial infections. Mice were fed a standard diet (Hope Farms, Woerden, The Netherlands) and tap water ad libitum. One group of animals ($n = 44$) was treated daily with 0.3 $\mu$g of IL-18 (Fujisaki Institute, Fujisaka, Japan) plus 2 mg of OVA as a carrier protein (OVA, grade VI, Sigma, Deisenhofen, Germany) per animal daily starting at 10 wk of age. The control group ($n = 45$) was treated daily with 2 mg of OVA only. BALB/c mice were purchased from Charles River (Wiga, Sulzfeld, Germany) and treated daily with 0.3 $\mu$g of IL-18 plus 2 mg of OVA. Urinary glucose analysis was done daily starting at 8 days posttreatment in both animal groups; hyperglycemia was confirmed by blood glucose determination (Glucocard, Menarini, Florence, Italy). Animals were regarded as diabetic when blood glucose levels were found to be $>16.7$ mmol/l (300 mg/dl). Groups of six normoglycemic animals were killed before and 14 and 21 days after starting the injection of IL-18. Mice were sacrificed under anesthesia, and the pancreas was excised and cut in half.

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mRNA analysis

Total RNA was isolated from fresh pancreatic tissue by guanidinium thiocyanate-phenol-chloroforom extraction (17). Isolated RNA quality was verified by running a 1% agarose gel with 4% formaldehyde. All isolated pancreas RNA samples showed intact 18S and 28S rRNA bands without visible degradation of the mRNA, similar to the total RNA of spleens, which was always isolated in parallel. Determination and quantification of specific mRNA was performed by RT-PCR as described elsewhere (17, 18). Specific primers for β-actin, IFN-γ, and IL-10 were purchased from Clontech (Palo Alto, CA). The specific primers for inducible NO synthase (iNOS), IL-12p40, IL-12p35, and IL-18 were used as described previously (5, 17, 20). PCR products were subjected to electrophoresis on a 1.2% agarose gel with 4% formaldehyde. All isolated RNA samples were subjected to electrophoresis on a 1.2% agarose gel with 4% formaldehyde. Relative PSL values generated in these experiments did not differ significantly.

Isolation and exposure of pancreatic islets

Pancreatic islets were prepared from C57BL/6J mice from our own breeding colony at the Diabetes Research Institute as described previously (21). Briefly, islets were isolated from the pancreas by injection of a collagenses solution into the duct (Serva, Heidelberg, Germany; 0.48 U/mg in HBSS). After incubation for 25 min (37°C), the islets were enriched on a Ficoll density gradient (Ficoll 400, Pharmacia, Freiburg, Germany) followed by hand picking. For cytokine exposure, 20 freshly isolated islets were seeded per well of a half area 96-well microtiter plate in 150 μl of RPMI 1640 medium supplemented with 1 mmol/l pyruvate, 2 mmol/l-glutamine, 10% FCS, 2 g/l NaHCO3, 2.38 g/l HEPES (Serva), 25 mg/ml ampicillin, 120 mg/ml penicillin, 270 mg/l streptomycin (Serva), and 10% FCS (Sigma). After 1 day of precultivation (37°C, 5% CO2), the recombinant mouse cytokines IL-1β (Endogen, Woburn, MA, 50 U/ml), TNF-α (Genzyme, Cambridge, MA, 500 U/ml), and IFN-γ (Genzyme, 100 U/ml) were added; the incubation was continued for 72 h (37°C, 5% CO2). Mouse rIL-18 (1000/ml) was added at the beginning of or 4 h before exposure to IL-1β/TNF-α/IFN-γ.

Nitrite determination

The release of NO from islets was assessed by determining the concentration of accumulated NO2⁻ in the culture supernatant using the Griess reagent (22). At the end of the experiment, 50 μl of the culture supernatants was removed and added to 50 μl of a 1:1 mixture of a sulfanilamide solution (Sigma, 0.03 g in 10 ml 2.5% H3PO4) and a naphthylethylenediamine solution (Sigma, 0.03 g in 10 ml 2.5% H3PO4) in the wells of a 96-well, flat-bottom microtiter plate. The OD of the resulting solution was determined photometrically at 550 nm, and the NO2⁻ concentrations were quantified from a standard curve obtained with NaNO2.

MTT assay

The mitochondrial activity of the islets was assessed by a colorimetric test that detects the conversion of the tetrazolium salt MTT (Sigma) into its formazan product (23) by enzymes of the respiratory chain. After the end of the cytokine exposure period, the islet culture supernatant was replaced by an MTT solution (1 mg/ml in RPMI 1640 medium with supplements). After 4 h of incubation (5% CO2, 37°C), the formazan crystals were dissolved in 50 μl of isopropanol and the OD of the resulting blue solution was determined photometrically (540 nm, reference wavelength of 650 nm). The residual mitochondrial activity of the islets was calculated as a percentage of the untreated sample.

Histology

Pancreatic tissue was snap frozen in liquid nitrogen, and cryostat sections were stained with hematoxylin-eosin for evaluation of infiltrating immune cells by light microscopy. Analysis was done from all islets in different sections at intervals of 200 μm, yielding a total of ≥20 islets per animal. Grade 0 was defined as no infiltration, grade 1 as two to five mononuclear cells around the islet, grade 2 as more than five mononuclear cells surrounding the islet without intraislet infiltration, grade 3 as <20% of the intraislet area infiltrated, and grade 4 as >20% of the intraislet area infiltrated. Slides were coded, and insulin and glucagon stains were evaluated independently by two observers. The histology score gives the mean infiltration grade of the islets analyzed.

Statistical analysis

The mean radioactive signals of the RT-PCR products, the results of the NO2⁻ accumulation, and the mitochondrial activity were compared by the Wilcoxon U test, Student’s t test, or the rank correlation assay of Spearman. Statistical analysis for incidence of diabetes was performed by Kaplan-Meier survival analysis; the degree of insulinis was performed by the χ² test.

Results

Treating NOD mice with IL-18 suppressed diabetes development in 9 of 27 animals, in contrast to control-treated NOD mice (17 of 26 animals; p < 0.001). Interestingly, there was also a delayed onset of diabetes development in IL-18-treated NOD mice (Fig. 1).
FIGURE 3. RT-PCR analysis of the Th1-type cytokine IFN-γ and the Th2-type cytokines IL-10 and IL-4 in the pancreata of NOD mice at days 14 and 21. A, The relative quantities of the RT-PCR signal for IFN-γ (A), IL-10 (B), IL-4 (C), IFN-γ/IL-10 (D), and IFN-γ/IL-4 (E) of individual control NOD (■) and IL-18-treated NOD (●) mice are shown as determined by PSL followed by normalization to the signals of RT-PCR of the β-actin mRNA of individual mice. The single bars give the mean values of each group.
In the sham-treated group, the first animals became diabetic at day 70 of age, whereas in the IL-18-treated group, the first animal developed diabetes significantly later, at day 96 (p < 0.005). Treating non-diabetes-prone BALB/c mice did not induce diabetes development (0 of 30 mice).

To examine the impact of IL-18 treatment on prediabetic islet infiltration, we analyzed six animals per group at days 0, 14, and 21 after the start of IL-18 treatment. Before IL-18 administration, most islets of the NOD mice showed intrasial infiltration (54%; Fig. 2), with a mean insulitis score of 2.4 (Fig. 2). A substantial progression of insulitis was seen at 14 and 21 days after starting treatment in control-treated, normoglycemic NOD mice. Nearly all islets exhibited an advanced insulitis grade with intrasial infiltration (78% and 68%, Fig. 2). Interestingly, in normoglycemic, IL-18-treated NOD mice, there was no progression of islet infiltration over time. The percentage of islets with intrasial infiltration did not increase (31%; 32%; Fig. 2) but was significantly decreased compared with 70-day-old NOD mice (p < 0.05). The insulitis score of the IL-18-treated animals was significantly less after 14 days (p < 0.005) and 21 days (p < 0.05) of treatment in contrast to the sham-treated animals (Fig. 2).

The impact of IL-18 administration on the cytokine gene expression pattern was analyzed. Control-treated NOD mice showed up-regulated expression of the mRNA for the Th1-specific cytokine IFN-γ at 21 days after the start of the experiment (p < 0.05, Fig. 3A). There was also a significant increase in IFN-γ mRNA expression in normoglycemic, IL-18-treated NOD mice (p < 0.01); however, there was no difference between the IL-18-treated group and the control group (Fig. 3A). The gene expression of the Th2-type cytokine IL-10 was not changed in IL-18-treated NOD and control-treated NOD mice within 14 and 21 days after starting treatment of the animals (Fig. 3B). A significant impact of the treatment with IL-18 became evident when calculating the ratio of mRNA levels of IFN-γ vs IL-10 in individual animals. Control-treated NOD mice showed an increased IFN-γ/IL-10 ratio, indicating a shift toward Th1-type reactivity in the pancreas at 14 and 21 days after the start of treatment, whereas IL-18 treatment prohibited such shift toward Th1-type reactivity (Fig. 3C; 14 days, p < 0.05; 21 days, p < 0.01; 21 days, p < 0.05 when excluding the highest values). The expression of the Th2 cytokine IL-4 was higher in IL-18-treated mice than in sham-treated mice at 21 days after treatment of the animals (p < 0.05, Fig. 3C). As a consequence, treatment with IL-18 also prevented progression toward Th1-biased insulitis when considering the ratio of IFN-γ vs IL-4 mRNA levels in the pancreas (Fig. 3E; 21 day, p < 0.002).

The analysis of the mRNA expression of the innate immunity cytokines IL-12 and IL-18 is shown in Fig. 4. At 14 and 21 days after starting with animal treatment, the ratio of IL-12p40 vs IL-12p35 mRNA levels of individual animals was calculated. The group treated with IL-18 did not show an up-regulation of mRNA expression of IL-12 (Fig. 4B) and IL-18 (Fig. 4A). The control-treated NOD mice showed a significant up-regulation for these two cytokines at 21 days after the start of treatment, in contrast to IL-18-treated NOD mice (p < 0.05; Fig. 4, A and B), and expression of these cytokines significantly correlated in individual animals at 21 days after the start of treatment (IL-12p40/p35, p < 0.01). Furthermore, there was a significant correlation of intrainsulitis and IL-18 (p < 0.01) or IL-12 mRNA expression (p < 0.05) at 21 days after the start of treatment.

We also examined the mRNA expression of iNOS as a parameter closely associated with β cell destruction (5). iNOS mRNA expression increased by 14 days posttreatment in the control NOD group, whereas iNOS expression in the IL-18-treated NOD group was significantly reduced at 14 days (p < 0.005) and 21 days (p < 0.05) after the start of treatment in comparison with the control groups after 14 and 21 days (Fig. 5A). Furthermore, a high expression of iNOS in the pancreas correlated with high intrainsulitis at 21 days after the start of treatment (p < 0.01). In addition, animals with a high ratio of Th1- vs Th2-associated cytokines also showed high iNOS expression (Fig. 5B). The correlation between iNOS mRNA levels and the ratio of IFN-γ/IL-10 mRNA was calculated as r = 0.83, p < 0.05 for all animals; when the highest values were excluded, the correlation was calculated as r = 0.77, p < 0.05. The correlation between iNOS mRNA levels and the ratio of IFN-γ/IL-4 was calculated as r = 0.94, p < 0.01 (Fig. 5C).

Finally, we investigated whether IL-18 is able to exert direct effects on islets. Pancreatic islets from normal C57BL/6J mice were exposed to IL-18 in the absence or presence of the inflammatory cytokine IL-1β alone or in combination with TNF-α and IFN-γ. After 72 h of exposure, IL-18 alone at a dose of 1000 U/ml
showed neither an effect on the NO release nor an effect on the residual mitochondrial activity of the islets (Fig. 6, A and B). Islets exposed to 50 U/ml IL-1β released a significantly increased amount of NO (3.3 ± 0.6 nmol/l NO₂⁻, \( p < 0.001 \) compared with the untreated control), which induced stimulation of the respiratory activity (152.1 ± 16.6%, \( p < 0.001 \) compared with the untreated control). As expected, exposure to a combination of the inflammatory mediators IL-1β, TNF-α, and IFN-γ resulted in a further, significantly increased release of high amounts of NO (Fig. 6A, 6.0 ± 1.8 nmol/l NO₂⁻, \( p < 0.01 \) compared with the NO₂⁻ concentration detected after IL-1β exposure) that strongly depressed the mitochondrial activity of the islets (Fig. 6B, 36.8 ± 10.6%, \( p < 0.001 \) compared with the untreated control). However, no effect was seen when IL-18 was added to the islets at the beginning or at

Table I. Effects of treatment with IL-18 on the development of insulitis in NOD mice

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<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Days Posttreatment</th>
<th>No Insulitis (grade 0)</th>
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<th>Intraisulitis (%)(grade 3 and 4)</th>
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</table>

\( a \) Shown are the mean infiltration grades of the untreated and IL-18-treated animals. Grade 0 was defined as no perinsulitis, grade 1 as two to five mononuclear cells around the islet, grade 2 as more than five mononuclear cells surrounding the islet without intraislet infiltration and intraisulitis, grade 3 as <20% of the intraislet area infiltrated, and grade 4 as >20% of the intraislet area infiltrated.
inflammatory cytokines. After 72 h of incubation, the concentration of NO, which was identified as a major cell toxic mediator in ex- 

inflammation toward the Th1 type, as evidenced by an increase of IFN-γ over IL-10 and IL-4 mRNA levels. However, animals treated with IL-18 did not show such progression. Therefore, we conclude that exogenous IL-18 interfered with inhibition of the natural Th1 shift in NOD mice, leading to a dampening of Th1-dependent destructive insulitis. This conclusion concurs with the results of semiquantitative graded insulitis, which showed a significantly lesser intraislet infiltration in mice receiving IL-18.

The reduced aggressiveness of the insulitis process in mice treated with IL-18 is also recognizable from a significantly decreased expression of iNOS mRNA. iNOS mRNA is induced in macrophages, endothelial cells, and β cells by inflammatory cytokines such as IFN-γ, IL-1β, and TNF-α, whereas Th2 cytokines are inhibitory (29–32). A close correlation between pancreatic iNOS mRNA levels and destructive intraislet infiltration has been reported by our group (5, 33). Furthermore, iNOS expression is a marker of macrophage activation. The decreased expression of iNOS mRNA in the pancreas of NOD mice receiving IL-18 indicates a dampening of the diabetogenic inflammatory process.

Interestingly, the systemic administration of IL-18 downregulated the proinflammatory activities of the innate immune system, as demonstrated by a lesser gene expression of IL-12 and of IL-18 itself. This may be due to the stimulation of Th2-type cytokines by IL-18 in cells of the innate immune system, as described recently (34). The suppression of the Th1-driving activities of the innate immune system by exogenous IL-18 provides a possible mechanism for the lack of progression toward destructive intraisletis. Another possible mode of action is that IL-18 acts directly on pancreatic islets and modulates their functional activity under inflammatory cytokines. IL-1β, TNF-α, and IFN-γ induced the release of NO, which was identified as a major β cell toxic mediator in experimental systems of the pathogenesis of type 1 diabetes (35). As expected, low concentrations of IL-1β induced the release of moderate amounts of NO and exerted a stimulatory effect on islet metabolism (36, 37). Exposure to a combination of the cytokines IL-1β, TNF-α, and IFN-γ resulted in the formation of high amounts of NO, which led to a strong reduction of the islet respiratory activity indicative of the islet cell toxic effect of high NO concentrations (38, 39). However, the addition of IL-18 had no

Discussion

Our results show that the administration of IL-18 during the prediabetic phase significantly decreased the incidence of diabetes in NOD mice. It is probable that this effect is due to the counterregulation of the immune system. Instead of inducing a Th1 response, there is still a Th2 response in IL-18-treated NOD mice, which will not lead to destructive insulitis (24). Similar effects have been observed for systemically administered IL-12 and TNF-α (12, 25–27). Especially in the case of IL-12 treatment, it is known that low doses of IL-12 suppress diabetes development, whereas high doses accelerate diabetes development (12, 25). During preparation of this manuscript, parallel work by Tokui et al. (28) was published in an abstract form; this work also describes an inhibitory effect of IL-18 administration on the cyclophosphamide-accelerated disease process in NOD mice. The latter finding suggests that the effect of exogenous IL-18 on disease progression is quite robust and is not dependent upon a selective treatment protocol or on the NOD mouse colony.

Previous studies linked a shift toward Th1-dominated insulitis progression from benign toward destructive insulitis and subsequent diabetes onset. Therefore, we analyzed mice before and at 14 and 21 days after treatment with IL-18 for progression toward Th1 insulitis. In the case of diabetes acceleration by systemically administered IL-12, NOD mice developed diabetes within this time course (12). Cytokine mRNA expression was analyzed in the total pancreas, because islet isolation in our hands introduces a bias due to poor islet yield in animals with advanced stages of insulitis and due to the preferential loss of periductular and perisinular over intrainsular leukocytes during the isolation procedure. Previous studies have shown a close correlation between cytokine mRNA levels in the total pancreas and the mean insulitis score in individual animals (29). Furthermore, we were also able to demonstrate a close correlation between total pancreas mRNA levels and immunohistochemical staining of cytokines in islets of the same pancreas (5). Analysis of pancreatic RNA at 70, 84, and 91 days revealed a progression of inflammation toward the Th1 type, as evidenced by an increase of IFN-γ over IL-10 and IL-4 mRNA levels. However, animals treated with IL-18 did not show such progression. Therefore, we conclude that exogenous IL-18 interfered with inhibition of the natural Th1 shift in NOD mice, leading to a dampening of Th1-dependent destructive insulitis. This conclusion concurs with the results of semiquantitative graded insulitis, which showed a significantly lesser intraislet infiltration in mice receiving IL-18.

4 h before the exposure to the inflammatory cytokines. IL-18 was unable to modulate the cytokine-induced alterations of NO release and the metabolic activity.

FIGURE 6. Lack of effect of IL-18 on NO release (A) and mitochondrial activity (B) of islets exposed to inflammatory cytokines. Mouse islets (20 islets in 160 μl) were incubated in the absence or in the presence of IL-1β (50 U/ml) or a mixture of the cytokines IL-1β (50 U/ml), TNF-α (500 U/ml), and IFN-γ (100 U/ml) (Cyt.Mix). The exposure to IL-18 (100 ng/ml) was started at the same time or at 4 h before the addition of the inflammatory cytokines. After 72 h of incubation, the concentration of NO3 accumulated in the culture supernatant was determined by the Griess reaction; the residual mitochondrial activity was assessed by the MTT assay as described in Materials and Methods. Data show the means ± SD from three to five experiments performed in triplicate. **, p < 0.001 compared with the untreated samples.
effect on the stimulatory or inhibitory effects on NO release and on mitochondrial activity induced by the inflammatory cytokines. These results imply that IL-18 has no direct effect on islet cells, because the cytokine can neither induce the protection of islets nor induce sensitization to increase their susceptibility toward the damaging effects of inflammatory mediators.

In conclusion, we have shown that exogenously administered IL-18 interferes with diabetes development and limits Th1 reactivity. Furthermore, the decreased expression of iNOS in the pancreas of IL-18-treated mice indicated a lower activation of inflammatory cells, which promote β cell survival, whereas IL-18 itself did not show any effect on insulin-producing β cells. These findings demonstrate for the first time a modulatory function of IL-18 in autoimmune disease and underscore the potent instructive role of the innate immune system on adaptive T cell-dependent immune responses.

Acknowledgments

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References