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

Helga Rothe, Andreas Hausmann,* Kristina Casteels, Hakuri Okamura, Masashi Kurimoto, Volker Burkart,* Chantal Mathieu,† and Hubert Kolb*

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-γ 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-1β, TNF-α, and IFN-γ. 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.

Type 1 diabetes is an immune disease, in which mononuclear cells infiltrate into pancreatic islets. In animal models with spontaneous diabetes development (the Bio/Breeding rat and the nonobese diabetic (NOD) mouse), it could be demonstrated that APCs, like macrophages and dendritic cells, are the first infiltrating cells, followed by T cell infiltration and finally by B cell infiltration (1–4). Concomitantly, a progression of islet inflammation from benign perinsulitis to destructive intrainsulitis is observed. Perinsulitis is characterized by a dominance of Th2 cells, whereas the destructive state of intrainsulitis is characterized by a dominance of Th1 cells and activities. Furthermore, a predominance of Th1-type reactions has been postulated for NOD mice (5–9). IL-12 and IL-18, produced by the first infiltrating cells (macrophages), are known to drive the differentiation of Th cells toward the Th1 type (10–13).

IL-18 is a single chain cytokine that is produced by cells of the innate immune system and induces Th1 cell proliferation in vitro (13, 14). Protein structure similarities to IL-1 are observed (15), and the latter is known to have direct effects on pancreatic islet cells, such as inducing cell death via the induction of NO (16). In the animal model of the cyclophosphamide accelerated and synchronized diabetes development of the NOD mouse, IL-18 gene expression is up-regulated early on in the pancreas as well as in the periphery and is thought to drive the shift from Th2-dependent to Th1-dependent insulitis (17, 18). Interestingly, the gene is located on chromosome 9 in or near Idd2 and is therefore discussed as being a candidate gene for this diabetes-associated gene locus (17). In our present study, we analyzed further the possible role of the innate immune system in autoimmune diabetes of NOD mice by administrating IL-18 to the prediabetic NOD mice. To our knowledge, this is the first attempt to interfere in autoimmune disease by this innate cytokine.

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 Katholieke 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 μg of IL-18 (Fujiyama 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 μg 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.
longitudinally for histological examination and mRNA analysis. The principles of laboratory animal care were followed (National Institutes of Health publication no. 85-23, revised 1985).

mRNA analysis

Total RNA was isolated from fresh pancreatic tissue by guanidinium thiocyanate-phenol-chloroform extraction (17). Isolated RNA quality was verified by running a 1.2% agarose gel with 4% formaldehyde. All isolated pancreas RNA samples showed intact 18S and 28S RNA 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 2% agarose gel followed by hybridization with specific 32P-labeled probes binding at the sites between the primer sequences. Signals were quantified by measuring the 32P-stimulated luminescence (PSL) with a phosphor imager (Fuji, BAF1000, Raytest, Staubenhardt, Germany). The relative PSLs of PCR products were calculated by normalization of the measured PSL to the β-actin signal (5, 17). RT-PCR analysis of mRNAs was repeated by varying the cycle number for specific mRNA amplification (n = 29, 32, 35) and for β-actin mRNA amplification (n = 20, 23, 26, 29, 32). The relative PSL values generated in these experiments did not differ by >25%.

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 collagenase 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 mM/l pyruvate, 2 mM/l-glutamine, 10 mM l-nonsessental amino acids (100x), Life Technologies Europe, Heidelberg, Germany), 2 g/l NaHCO3, 2.58 g/l HEPES (Serva), 25 mg/ml ampicillin, 120 mg/ml penicillin, 270 mg/ml 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 (10000/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 re-
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 intraislet 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 intraislet 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 intraislet 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-$\gamma$ at 21 days after the start of the experiment ($p < 0.05$, Fig. 3A). There was also a significant increase in IFN-$\gamma$ 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-$\gamma$ vs IL-10 in individual animals. Control-treated NOD mice showed an increased IFN-$\gamma$/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-$\gamma$ 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 intraislet insulitis 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 $\beta$ 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 at 14 and 21 days (Fig. 5A). Furthermore, a high expression of iNOS in the pancreas correlated with high intraislet insulitis 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-$\gamma$/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-$\gamma$/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-1$\beta$ alone or in combination with TNF-$\alpha$ and IFN-$\gamma$. 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>
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<th>Animals</th>
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<th>No Insulitis (grade 0)</th>
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<th>Intrainsulitis (%) (grade 3 and 4)</th>
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*Shown are the mean infiltration grades of the untreated and IL-18-treated animals. Grade 0 was defined as no perisulitis, grade 1 as two to five mononuclear cells around the islet, grade 2 as more than five mononuclear cells surrounding the islet without intrasulitis infiltration and intrainsulitis, grade 3 as <20% of the intrasulitis area infiltrated, and grade 4 as >20% of the intrasulitis area infiltrated.
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 perinsular 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 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.

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 pancreata 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 intraisulitis. 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 pre-diabetic 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

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-1(β (50 U/ml) or a mixture of the cytokines IL-1(β (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.

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.
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 pancreata 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