Suppression of cyclophosphamide induced diabetes development and pancreatic Th1 reactivity in NOD mice treated with the interleukin (IL)-12 antagonist IL-12(p40)₂

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Summary The macrophage product interleukin (IL)-12 is known to drive Th1 reactions in physiological and pathological immune responses. Here we report that treatment with the homodimeric IL-12p40 subunit, an antagonist of the bioactive IL-12p35/p40 heterodimer, suppresses diabetes development in cyclophosphamide-injected NOD mice. Female mice of 70 days old received cyclophosphamide (250 mg/kg) to accelerate and synchronize diabetes development, and daily injections of 1 µg IL-12(p40)₂. While there was no delay of the first diabetes cases, the incidence of overt diabetes was significantly decreased in treated mice (46 vs 23 %, $p < 0.05$). Analysis of mRNA expression in the pancreas showed that administration of the IL-12 antagonist had dampened interferon-gamma gene expression, decreased the ratio of

interferon-gamma/IL-10 mRNA levels and in parallel suppressed the expression of the inducible nitric oxide synthase. At the same time intra-islet infiltration was significantly decreased ($p < 0.001$). Interestingly, the administration of IL-12(p40)₂ also affected IL-12 gene expression, by downregulation of p35 mRNA. We conclude that IL-12 p40 homodimer suppresses diabetes development in the NOD mouse by dampening islet inflammation via selective downregulation of Th1 type responses. The naturally occurring IL-12 antagonist IL-12(p40)₂ represents a new and specific Th1 directed approach to prevent autoimmune diabetes. [Diabetologia (1997) 40: 641–646]

Keywords NOD mouse, IL-12 antagonist, Th1/Th2 balance, inducible NO synthase, insulinitis.

The disease process in diabetes prone nonobese diabetic (NOD) mice can be accelerated by a single injection of cyclophosphamide (CY). Concomitantly, early peri-insular infiltration of islets progress to destructive intra-insulitis [1–3]. At the immunoregulatory level a shift from T-helper type 2 (Th2) towards Th1 type reactivity is noted, as evidenced by a marked increase of IFN-γ gene expression [3–5]. We have reported previously that the increase in Th1

reactivity in islets is preceded by a rise in IL-12 gene expression, suggesting that IL-12 mediates the accelerating effect of CY [6]. Indeed, the disease promoting effects of CY are suppressed in IL-12 knockout mice and daily injection of IL-12 to young NOD mice was found to expedite diabetes development [7, 8].

IL-12 is a 75 kDa heterodimeric cytokine produced from macrophages and other antigen presenting cells. The p35 subunit is ubiquitously expressed in many cell types where the p40 subunit is inducible [9]. The production of biologically active recombinant IL-12 requires the coexpression of the IL-12 p40 and p35 subunits [10–12]. Although initially described as a cytokine activating spontaneously cytotoxic lymphocytes the significance of IL-12 lies in its effect on T-helper cells [12]. IL-12 conditions naive T-helper cells to differentiate along the Th1 pathway, and stimulates Th0 or Th2 cells to transiently produce

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Abbreviations: HBSS, Hank's balanced salts solution; NOD, non-obese diabetic; CY, cyclophosphamide; IFN-γ, interferon-gamma; PSL, ³²P stimulated luminescence; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcriptase-polymerase chain reaction; FCS, fetal calf serum.

interferon-gamma (IFN γ) [12]. Furthermore, IL-12 is known to be involved in infectious and organ specific autoimmune diseases [6, 7, 13, 14]. The p40 subunit is able to form a homodimer, which has been shown to antagonize IL-12 activities in vitro and hence may reflect a natural suppressor of Th1 activation in vivo [15, 16]. In our present study we examined the inhibitory effect of the IL-12 antagonist IL-12 (p40)₂ on the development of the CY accelerated diabetes of the NOD mice in vivo.

Materials and methods

Animals. Female NOD/Bom mice were purchased from Bomholtgard Breeding Centre (Ry, Denmark) at 9 weeks of age and maintained in our animal facility under conventional conditions with standard diet (Sniff, Soest, Germany) and tap water ad libitum (mean incidence: 0% at age 80 days, > 90% at age 250 days). One group of animals ($n = 31$) was treated daily with 0.5 μ g IL-12(p40)₂ + 2 μ g ovalbumin (OVA, grade VI; Sigma, Deisenhofen, Germany) daily for 3 days before treatment of the animals with CY and thereafter daily with 1 μ g IL-12 (p40)₂ + 2 μ g OVA for 4 weeks. The control animal group was treated with 2 μ g OVA only. All mice received CY (250 mg/kg i.p.) at 70 days of age. Urinary glucose analysis was performed daily starting 8 days after CY treatment and hyperglycaemia was confirmed by blood glucose determination. Animals were regarded as diabetic when blood glucose levels were found to be above 16.7 mmol/l as determined by the hexokinase method. Groups of 5–6 normoglycaemic animals were killed before and 10 days after injection of CY. The later point of time is close to the onset of overt diabetes in a fraction of mice (Fig. 1). Mice were killed under anaesthesia, the pancreas was excised and cut in half longitudinally for histological and mRNA analysis. The principles of laboratory animal care were followed (NIH publication No. 85–23, revised 1985).

IL-12 homodimer. IL-12(p40)₂ was generously provided by E. Nickbarg (Genetics Institute, Cambridge, Mass., USA). Purified IL-12(p40)₂ was prepared as described previously [16] and was essentially free (> 98%) of p40 monomer.

mRNA analysis. Total RNA was isolated from fresh pancreatic tissue by guanidinium thiocyanate-phenol-chloroform extraction [3]. Isolated RNA quality was verified by running a 1.2% agarose gel with 4% formaldehyde. All isolated RNA showed intact 18 S and 28 S rRNA bands without visible degradation of the mRNA, like total RNA of spleens which was always isolated in parallel. Determination and quantification of specific mRNA was performed by reverse transcriptase polymerase chain reaction (RT-PCR) as described elsewhere [3, 18]. Specific primers for β -actin, IFN- γ , IL-4, IL-10 and TGF- β were purchased from Clontech (Clontech Laboratories Inc., Palo Alto, Calif., USA). The specific primers for inducible nitric oxide synthase (iNOS), IL-12 p40 and IL-12 p35 were used as described elsewhere [3, 5]. The first cycle of each PCR was started for 3 min at 95°C denaturation time. In the next cycles denaturation was performed for 1 min. Primer annealing was done for 1 min at 60°C (β -actin, IFN- γ , IL-4, IL-10, TGF- β), 58°C (IL-12p35), 55°C (IL-12p40) or 50°C (iNOS). The elongation steps were done at 72°C for 80 s (β -actin, IFN- γ , IL-4, IL-10, TGF- β), for 90 s (iNOS), 40 s (IL-12p35) or 30 s (IL-12p40). PCR products were subjected to electrophoresis on a 2% agarose

Fig. 1. Diabetes incidence of IL-12(p40)₂ treated animals versus control group after CY treatment. Shown are the percentages of diabetic animals in the control group (■) and the IL-12(p40)₂ treated group (▲) after CY treatment

gel followed by hybridization with specific ³²P labelled probes binding at the sites between the primer sequences. Signals were quantified by measuring the ³²P stimulated luminescence (PSL) by a phosphorimager. Relative PSL of PCR products was calculated by normalization of the measured PSL to the strength of β -actin signal [3, 18]. RT-PCR analysis of mRNAs was repeated by varying the cycle number for specific mRNA amplification ($n = 29, 32, 35$) and for β -actin message amplification ($n = 20, 23, 26, 29, 32$). Relative PSL values generated in these experiments did not differ more than 25%.

Isolation and stimulation of peritoneal macrophages. Peritoneal macrophages were isolated by lavage of the peritoneum with 4-ml ice-cold HBSS (Hank's balanced salt solution; Gibco, Heidelberg, Germany). The cells were washed three times with 10 ml PBS (Gibco) and separated by adherence on FCS (fetal calf serum, Gibco) coated petri dishes (37°C, 5% CO₂). After 1 h the non-adherent cells were flushed off and the adherent cell population was detached by incubation for 7 min in ice-cold Ca²⁺ - Mg²⁺ - free HBSS. The detached cells were washed three times in ice-cold PBS, resuspended in RPMI 1640 + 10% FCS at 10⁶/ml, followed by culture in 12-well microtiter wells. The cells were stimulated with 1 μ g lipopolysaccharide (LPS from *Salmonella minnesota*, Sigma) for 0, 24 h.

Nitrite and TNF production by macrophages. Supernatants of the macrophage cultures were collected and nitrite levels were determined by the Griess method as described previously [19]. In the same supernatants TNF α levels were measured by a L929 bioassay [20].

Histology. Pancreatic tissue was snap frozen in liquid nitrogen and cryostat sections were stained with haematoxylin-eosin for evaluation of infiltrating immune cells by light microscopy. Analysis was done for all islets in different sections at intervals of 100 μ m, yielding a total of at least 20 islets per animal. Three grades of infiltration were defined: grade 1: no periductular infiltrates or less than 5 immune cells peri- and intra-insular with or without periductular infiltrates; grade 2: periductular and peri-insular infiltrates with or without intra-islet infiltration of less than 20% of the islet area; grade 3: intra-islet infiltration of more than 20% of the islet area. Slides were coded and insulitis grades rated independently by two observers.

Fig. 2 A–C. RT-PCR analysis of Th1 cytokine IFN-γ and Th2 cytokine IL-10 in pancreas of NOD mice before and 10 days after CY ± 12(p40)₂ treatment **A:** Relative quantities of RT-PCR signal for IFN-γ as determined by ³²P stimulated luminescence followed by normalization to the signals of RT-PCR of β-actin mRNA of individual mice. Single bars give mean values of each group. **B:** Relative quantities of RT-PCR signal for IL-10 calculated as described above. Single bars give mean values of each group. **C:** Ratios of relative mRNA quantities of RT-PCR signals for IFN-γ/IL-10 of individual mice. The single bars give mean value of each group

Statistical analysis Mean radioactive signals of RT-PCR products were compared by Wilcoxon U-test, Student's *t*-test or rank correlation assay of Spearman (*R*). Statistical analysis for incidence of diabetes and degree of insulinitis was performed by chi²-test.

Results

Treatment of NOD mice with CY induced overt diabetes in 27 of 59 mice (Fig. 1). All cases of diabetes occurred during days 8–13 after CY, demonstrating

synchronization of the disease process. NOD mice treated daily with IL-12(p40)₂ showed suppression of diabetes incidence (*p* < 0.05, Fig. 1). There was no delay of diabetes development in the IL-12(p40)₂ treated mice compared to the control group.

Prior to CY administration NOD mice were devoid of insulinitis in the majority of islets (Table 1). Substantial progression of insulinitis was seen 10 days after CY treatment in normoglycaemic mice. Nearly all islets exhibited an advanced insulinitis grade with intra-islet infiltration (97%, *p* < 0.0001 compared to day 0). In the IL-12(p40)₂ treated group progression of insulinitis towards intrainsular infiltration was also noted, but it was less pronounced 10 days after CY treatment, with 25% rather than 3% of islets with grades 1–2 (*p* < 0.001 compared to animals treated with CY alone, Table 1).

Analysis of the cytokine mRNA expression pattern in these animals showed significant differences between the normoglycaemic IL-12(p40)₂ treated and control animals. The CY-treated control animals showed upregulated gene expression in the Th1 specific cytokine IFN-γ. Treatment with IL-12(p40)₂ dampened the rise in IFN-γ mRNA levels (*p* < 0.05, Fig. 2A). Gene expression of the Th2 cytokine IL-10

Table 1. Islet infiltration grades

Treatment	Time point (CY = 0 days)	Number of mice		Insulinitis grades (%)		
		Mice	Islets	1	2	3
None	Day 0	5	109	68	25	7
CY	Day 10	6	192	0	3	97
CY + IL 12(p40) ₂	Day 10	6	153	4	21	75

Islet infiltration grades were analysed by eosin-haematoxylin staining of snap frozen pancreata

Fig. 3. RT-PCR analysis of IL-12 p40 and p35 subunits in pancreas of NOD mice before and 10 days after CY $\pm$ IL-12(p40)₂ treatment. Shown are relative quantities of RT-PCR signal for p40 ($\blacktriangledown$) and p35 ($\bullet$) as determined by 32 P stimulated luminescence followed by normalization to signals of RT-PCR of β -actin mRNA of individual mice. Single bars give mean values of each group

was also induced by CY but mean mRNA levels were not significantly affected by administration with IL-12p40 homodimer. The most striking effects were noted for the ratio of IFN- γ compared to IL-10 mRNA levels. Treatment with IL-12(p40)₂ strongly decreased Th1/Th2 mRNA ratios (Fig. 2C, $p < 0.01$).

The analysis of IL-12p35 and p40 mRNA is shown in Figure 3. CY induced a fivefold increase of IL-12p35 and an eightfold increase of IL-12p40 mRNA. Concomitant administration of the IL-12 antagonist decreased p35 mRNA levels ($p < 0.05$) but did not affect p40 mRNA levels. Next, the ratio of IL-12p40 compared to p35 mRNA levels of individual animals

was calculated. The group treated with IL-12 antagonist had a lower p35/p40 ratio ($p < 0.05$).

We also examined the mRNA expression of the iNOS as a parameter closely associated with beta-cell destruction [3, 17]. iNOS mRNA expression increased after CY treatment in both animal groups, but there was significantly less iNOS mRNA expression in the IL-12(p40)₂ treated animal group (Fig. 4A, $p < 0.05$). Animals with high iNOS expression also showed a high ratio of Th1 over Th2 cytokine expression (Fig. 4B). The correlation between iNOS mRNA levels and the ratio IFN- γ /IL-10 mRNA was calculated as $R = 0.98$, $p < 0.05$.

Finally, we tested whether administration of IL-12(p40)₂ had any general effects on macrophage function. Peritoneal macrophages were harvested 10 days after CY treatment and challenged in vitro with 1 μ g LPS/ml. Macrophages from NOD mice which had received CY plus IL-12(p40)₂ responded similarly to those from the CY control group with substantial nitrite production ($2.9 \pm 4.8 \mu\text{mol/l NO}_2^-$ [CY] vs $2.9 \pm 4.8 \mu\text{mol/l NO}_2^-$ [CY + (p40)₂], $n = 6$) and TNF α release (50.8 ± 25.8 U [CY] vs 40.0 ± 21.2 U TNF [CY + (p40)₂], $n = 6$) at 24 h.

Discussion

Administration of IL-12(p40)₂ significantly decreased the incidence of CY-induced diabetes. It is probable that this effect is due to the inhibitory effect of the p40 homodimer on IL-12 functions as shown in vitro [15]. During preparation of this manuscript, parallel work by Trembleau et al. [21] has been published in abstract form which also describes an inhibitory effect of IL-12(p40)₂ on the disease process in NOD mice. IL-12(p40)₂ acts as a specific antagonist by binding to the IL-12 receptor without inducing appropriate signals [16, 22]. If exogenous IL-12(p40)₂ exhibited similar properties in vivo, effects on IL-12 driven IFN- γ production and the Th1/Th2 cytokine balance are to be expected. Previous studies linked a shift towards Th1 dominated insulinitis with progression from benign towards destructive insulinitis and subsequent diabetes onset. We therefore analysed

Fig. 4 A, B. RT-PCR analysis of iNOS in the pancreas of NOD mice before and 10 days after CY $\pm$ IL-12(p40)₂ treatment. **A:** Relative quantities of RT-PCR signal for iNOS as determined by 32 P stimulated luminescence followed by normalization to the signals of RT-PCR of β -actin mRNA of individual mice. Single bars give the mean values of each group. **B:** Correlation of individual iNOS and IFN- γ /IL-10 mRNA expression in pancreas of NOD mice. Shown are individual data for NOD mice

mice before and after CY-accelerated progression towards Th1 insulinitis. Indeed, we observed less IFN- γ gene expression and a decreased IFN- γ /IL-10 mRNA ratio in the pancreas of NOD mice receiving injections of p40 homodimer in addition to CY.

Cytokine gene expression was analysed in total pancreas RNA because islet isolation in our hands introduces a bias due to poor islet yield in animals with advanced stages of insulinitis and the preferential loss of periductular and peri-insular over intra-islet leukocytes during the isolation procedure. Previous studies have shown a close correlation between pancreatic mRNA levels and immunostaining of cytokines in islet sections [3]. In addition, we observed a close correlation between cytokine mRNA levels in total pancreas with the mean insulinitis score in individual animals [18] in another animal model of spontaneous diabetes development, the BioBreeding rat.

We conclude that exogenous IL-12(p40)₂ interfered with activation of Th1 immune reactivity in the pancreas of CY-treated NOD mice, leading to dampening of Th1-dependent destructive insulinitis. The conclusion concurs with results of semiquantitative grading of insulinitis which shows a significant decrease of intra-islet infiltration in mice receiving IL-12(p40)₂.

The lesser aggressiveness of the insulinitis process in mice treated with the IL-12 antagonist is also recognizable from a significantly decreased expression of iNOS mRNA. iNOS mRNA is induced in macrophages, endothelial cells and beta cells by inflammatory cytokines such as IFN- γ , IL-1 and TNF α , while Th2 cytokines are inhibitory [23–25]. A close correlation between pancreatic iNOS mRNA levels and destructive intra-islet infiltration has been reported by our group [3, 18]. An interrelationship between the cytokine network and iNOS gene expression is demonstrated here by a significant correlation of individual iNOS mRNA levels with the IFN- γ /IL-10 mRNA ratio in pancreatic RNA. A causal relationship between IFN- γ /IL-10 mRNA ratio and iNOS expression seems probable. Studies in vitro and in vivo have shown that IFN- γ induces iNOS expression while IL-10 is suppressive [26, 27]. Nitric oxide is regulated as a mediator of beta-cell destruction in rodent models of type 1 diabetes [28, 29]. In addition, iNOS expression is a marker of macrophage activation. Taken together, the decreased expression of iNOS mRNA in the pancreas of NOD mice receiving IL-12(p40)₂ indicates a dampening of the diabetogenic inflammatory process.

Interestingly, the administration of IL-12(p40)₂ also acted back on IL-12 gene expression. While levels of p40 mRNA remained unaffected there was significant suppression of p35 gene expression. The p35 subunit of the bioactive heterodimer appears to mediate much of the signalling in terms of inducing IFN- γ production in IL-12 receptor positive Th1 or

Th0 cells [30, 31]. Hence, the downregulation of p35 message in response to IL-12(p40)₂ treatment indicates a decrease of bioactive IL-12 in NOD mouse pancreas. This regulatory action of the p40 homodimer on p35 gene expression may result from a direct effect on macrophages or may be a consequence of decreased Th1 reactivity and the resulting lower IFN- γ /IL-10 ratio. IFN- γ is a potent inducer, IL-10 is a suppressor of IL-12 production [32]. However, IL-10 has been reported to suppress both p35 and p40 gene transcription [33], which has not been observed here. We conclude that the IL-12 antagonist not only competes with bioactive IL-12 for Th1 cell activation but also suppresses further release of IL-12 inhibiting IL-12p35 gene transcription.

Our studies render an in vivo role of the p40 homodimer in regulating IL-12 bioactivity and Th1 responses possible. Further support has come from a pilot trial of IL-12(p40)₂ treatment in collagen arthritis of mice, where a delay of clinical symptoms and a decrease of disease incidence was noted [34]. A role for IL-12(p40)₂ as a natural antagonist of IL-12 in vivo is also supported by reports that p40 is produced in excess over IL-12 [32–35] and for longer periods than bioactive IL-12 is demonstrable [36, 37]. In situ hybridization of spleens of LPS-injected mice showed that most of the p35 and p40 mRNA is localized in different areas [38]. Naturally occurring p40 homodimer has been purified and shown to inhibit IL-12-induced T-cell proliferation.

In conclusion, we have shown that exogenous IL-12(p40)₂ represents a new and specific approach to interfere with the development of IL-12 dependent autoimmune type 1 diabetes. Since IL-12(p40)₂ also occurs naturally, treatment with this IL-12 antagonist probably makes use of physiological feedback mechanisms to restrict Th1 reactivities. Furthermore, the decreased expression of iNOS in the pancreata of IL-12(p40)₂ treated mice indicates a lower activation stage of macrophages and other inflammatory cells, which will promote beta-cell survival.

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