Cell. Mol. Life Sci. 62 (2005) 2658–2668
1420-682X/05/222658-11
DOI 10.1007/s00018-005-5259-0

Cellular and Molecular Life Sciences

Research Article

Interleukin-17 stimulates inducible nitric oxide synthase-dependent toxicity in mouse beta cells

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Received 14 June 2005; received after revision 17 September 2005; accepted 21 September 2005
Online First 2 November 2005

Abstract. The influence of the proinflammatory cytokine interleukin (IL)-17 on inducible nitric oxide (NO) synthase (iNOS)-mediated NO release was investigated in the mouse insulinoma cell line MIN6 and mouse pancreatic islets. IL-17 markedly augmented iNOS mRNA/protein expression and subsequent NO production induced in MIN6 cells or pancreatic islets by different combinations of interferon-γ, tumor necrosis factor-α, and IL-1β. The induction of iNOS by IL-17 was preceded by phosphorylation of p38 mitogen-activated protein kinase (MAPK), and inhibition of p38 MAPK activation completely abolished IL-17-stimulated NO release. IL-17 enhanced the NO-dependent toxicity of proinflammatory cytokines toward MIN6 cells, while IL-17-specific neutralizing antibody partially reduced the NO production and rescued insulinoma cells and pancreatic islets from NO-dependent damage induced by activated T cells. Finally, a significant increase in blood IL-17 levels was observed in a multiple low-dose streptozotocin model of diabetes, suggesting that T cell-derived IL-17 might be involved in NO-dependent damage of beta cells in this disease.

Key words. IL-17; iNOS; nitric oxide; beta cells; T cells; autoimmunity; diabetes.

The interleukin (IL)-17 family is composed of six homodimeric cytokines (IL-17A–E), which are mainly produced by activated and memory T lymphocytes [1]. Its prototypical member IL-17A (referred to as IL-17 in the text) exerts pleiotropic activities, including the induction of the major proinflammatory mediators IL-6, IL-1, tumor necrosis factor (TNF)-α and prostaglandin E2 in fibroblasts, endothelial cells and macrophages [2, 3], as well as the induction of C-X-C chemokines, IL-8 and macrophage inflammatory protein-2 (MIP-2), resulting in subsequent recruitment of neutrophils [4]. Systemic and/or local upregulation of IL-17 has been demonstrated in autoimmune diseases such as rheumatoid arthritis [5], multiple sclerosis [6], inflammatory bowel disease [7] and systemic lupus erythematosus [8]. One of the key effector molecules presumably involved in inflammatory destruction of self tissue in these pathological conditions is nitric oxide (NO) [9–12], a highly reactive free radical generated by inducible NO synthase (iNOS) in cytokine-stimulated macrophages and resident cells [13]. While unable to induce iNOS in human and rodent macrophages [3, 14], IL-17 readily stimulates iNOS-dependent NO release in human chondrocytes and murine osteoblasts, astrocytes, endothelial cells or bone marrow cells [14–19]. It therefore seems conceivable, as we have recently proposed, that IL-17-triggered iNOS activation in resident cells might contribute to tissue destruction in various autoimmune/inflammatory disorders [20].

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Type 1 diabetes mellitus (T1DM) is an immune-mediated disease resulting from the T cell-orchestrated destruction of the insulin-producing beta cells of the islets of Langerhans in the pancreas [21]. The disease is characterized by two distinct phases: insulitis, when a mixed population of leukocytes invades the islets; and diabetes, when most beta cells have been destroyed and insulin production becomes insufficient to control blood glucose levels [22]. An earlier theory suggested that resident and/or islet-infiltrating macrophages activated by T cell products may directly induce beta cell death by releasing cytotoxic levels of NO within the islets [23, 24]. A more recent hypothesis, on the other hand, proposes that pancreatic beta cells themselves, stimulated by both T cell- and macrophage-derived cytokines, might be the primary source of NO that will eventually lead to their apoptotic or necrotic suicide, by inhibiting mitochondrial enzymes and directly damaging the DNA [25–27]. The NO-dependent autotoxicity observed in proinflammatory cytokine [interferon (IFN)-γ, TNF-α, IL-1]-treated rodent beta cell lines and highly purified beta cells [28–30], as well as the absence of beta cell damage in IFN-γ + IL-1-treated islets containing IFN-γ-unresponsive beta cells [31] apparently support the latter model. Although IL-17 has gained increased attention as a mediator of iNOS expression in a variety of cell types, its possible involvement in the induction of iNOS in beta cells has not been assessed thus far. A rationale for such an approach has been provided by recent finding that the progression of insulitis to diabetes in nonobese diabetic (NOD) mice correlates with increased IL-17 transcripts in pancreas and augmented IL-17 blood levels [32].

In the present study, we investigated for the first time the effects of IL-17 on iNOS expression, NO release, and cell viability in cultures of the mouse insulinoma cell line MIN6 and pancreatic islets.

Materials and methods

Cell cultures and reagent. Mouse recombinant IL-17 was obtained from R&D (Minneapolis, Minn.), while rat (IgG1, κ) anti-mouse IL-17 monoclonal antibody (Ab) and isotype-matched rat anti-human IL-7 Ab (no cross-reactivity with mouse IL-7) were from Pharmingen (San Jose, CA). According to the manufacturer, anti-mouse IL-17 Ab showed no cross-reactivity with any of the mouse cytokines tested [IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-12, IL-15, granulocyte/macrophage colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α]. Mouse recombinant IFN-γ, TNF-α, IL-1β, and other reagents were from Sigma (St. Louis, MO.), except if specifically stated. The mouse insulinoma cell line MIN6, established by Dr. J.-I. Miyazaki [33], was kindly donated with Dr. Miyazaki's permission by Dr. Karsten Buschard (Bartholin Instituttet, Copenhagen, Denmark). MIN6 cells were cultured in tissue culture flasks (Sarstedt, Numbrecht, Germany) until reaching approximately 80% confluence, when they were detached with a solution of trypsin (0.25%) and EDTA (0.02%) in PBS. Cells were washed and seeded for NO production into 96-well flat-bottom plates (6×10^4/well) in HEPES-buffered RPMI-1640 medium supplemented with 5% fetal calf serum (FCS), L-glutamine, 2-mercaptoethanol and antibiotics (culture medium). Pancreatic islets were isolated from CBA mice (2–4-months old), using the collagenase digestion technique. The pancreata were minced and subsequently incubated with collagenase type XI solution (2 mg/ml) in PBS at 37 °C for 10 min with gentle shaking. After incubation, cold PBS supplemented with 5% FCS and 0.05% L-glucose was added to stop the digestion. The tissue was layed over a Percoll gradient and left for 15 min to allow the islets to fall to the bottom of the tube. The islets were then collected, washed twice and incubated overnight in culture medium with 10% FCS. Afterward, the islets were collected, counted and seeded for the experiments into 96-well flat-bottom plates (1×10^4/well) in culture medium (10% FCS). Mononuclear cells (MNCs) from spleens of CBA mice were obtained by centrifugation of splenocyte suspension over Ficoll separation medium (ICN, Costa Mesa, Calif.). MNCs (2.5×10^5/well) were co-incubated with MIN6 cells, or 48-h conditioned supernatant from MNC cultures was used for stimulation of MIN6 cells or pancreatic islets.

ELISA detection of IL-17 in multiple low-dose streptozotocin-induced diabetes. Diabetes was induced in 2 to 4-month-old male Dark Agouti rats or CBA mice by daily intraperitoneal injection of streptozotocin in citrate buffer pH 4.5 (20 mg/kg or 40 mg/kg, respectively) for 5 consecutive days. Ten or 15 days (for rats and mice, respectively) after the last streptozotocin injection, the animals were sacrificed for blood collection. The ELISA for serum IL-17 was performed with anti-mouse IL-17 paired Abs (R&D Systems) according to the manufacturer's instructions. The sensitivity limit of the assay was < 5 pg/ml.

Nitrite measurement. Nitrite accumulation, an indicator of NO production, was measured in cell culture supernatants using the Griess reagent. The supernatants (50 µl) were mixed with an equal volume of Griess reagent (a mixture at 1:1 of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) and the absorbance at 570 nm was measured in a microplate reader. The nitrite concentration was calculated from an $NaNO_2$ standard curve.

Determination of iNOS mRNA by RT-PCR. For RNA isolation, MIN6 cells were seeded in 24-well plates (3×10^5/well). Total RNA from cell cultures was isolated with

TRI reagent, according to manufacturer's instructions. RNA was reverse transcribed using Moloney leukemia virus reverse transcriptase and random primers (Pharmacia, Uppsala, Sweden). PCR amplification of cDNA with primers specific for iNOS and β-actin as a house keeping gene was carried out in the same tube in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) thermal cycler as follows: 30 s of denaturation at 95 °C, 30 s of annealing at 52 °C, and 30 s of extension at 72 °C. The number of cycles (30 for iNOS and 25 for β-actin) ensuring nonsaturating PCR conditions was established in preliminary experiments. For iNOS, the primers were: sense, 5'-AAGTCAAATCCTACCAAAGTGA-3': antisense, 5'-CCATAATACTGGTTGATGAACT-3' and the PCR product was 409 bp. The primers for β-actin were: sense, 5'-TCCTTCTTGGGTATGG-3', antisense, 5'-ACGCAGCTCAGTAACAG-3' and the PCR product was 358 bp. The PCR products were visualized by electrophoresis through a 3% agarose gel stained with ethidium bromide. Gels were photographed and results analyzed by densitometry using Scion Image Beta 2 software.

Cell-based ELISA for iNOS. The expression of iNOS protein in MIN6 cells (6×10^4/well) was determined exactly as described in the original protocol for the cell-based ELISA [34]. Rabbit anti-mouse iNOS Ab (Santa Cruz Biotechnology, Santa Cruz, Calif. at 1:10 000 dilution was used as a primary Ab, while the detecting Ab was HRP-conjugated anti-rabbit IgG (U.S. Biochemical Corporation, Cleveland, Ohio) at 1:2500 dilution. The data obtained by measuring the light absorbance at 492 nm were corrected for differences in cell number by staining the wells with crystal violet after the ELISA procedure, as described in the original protocol [34].

Western blot analysis of p38 mitogen-activated protein kinase activation. For the western blot analysis, MIN6 cells were grown to 80% confluence in 30-mm petri dishes. After treatment, cells were collected by scraping, washed with cold PBS, and lysed in ice-cold buffer containing 0.1 M NaCl, 0.01 M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 100 µg/ml PMSF and 1 µg/ml aprotinin. After centrifugation at 3200 g for 5 min (4 °C), the concentration of proteins in cell lysate supernatants was measured by the Bradford assay [35]. The samples were mixed with 6× gel-loading buffer (0.3 M Tris-Cl pH 6.8, 10% SDS, 30% glycerol, 0.84 mM 2-mercaptoethanol, and 0.2% bromophenol blue), and the mixture was boiled for 5 min. SDS-PAGE electrophoresis of samples containing 50 µg of proteins was performed through a 5% stacking and 12% resolving gel in electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% SDS). The resolved proteins were then transferred onto a Hybond nitrocellulose membrane (Amersham Life Sciences; Amersham, UK) at 1 mA/cm^2 of membrane, using transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 15% methanol). After overnight blocking at 4 °C with 5% non-fat milk in PBS containing 0.1% Tween 20, blots were incubated for 1 h at room temperature with rabbit anti-mouse p38 MAPK (1:250) or rabbit anti-mouse phospho-p38 MAPK (1:250) (both from Cell Signalling Technology, Beverly, Mass.). The membranes were then thoroughly washed with PBS/Tween, and incubated for 1 h at room temperature with HRP-conjugated anti-rabbit IgG (1:2500 in blocking buffer). After washing, peroxidase activity corresponding to the 38-kDa p38 mitogen-activated protein kinase (MAPK) band was visualized by TMB liquid substrate for membranes, the blots were photographed and results analyzed by densitometry using Scion Image Beta 2 software.

Cell viability. Cellular respiration, as an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan. At the end of the incubation, the MTT solution was added to cell cultures at a final concentration of 0.5 mg/ml and cells were incubated for an additional 1 h. Thereafter, medium was removed and cells were lysed in DMSO. The conversion of MTT to formazan was monitored by an automated microplate reader at 570 nm.

Statistical analysis. To analyze the significance of the differences between various treatments we used ANOVA followed by Student-Newman-Keuls test ($p < 0.05$ was considered significant). For the analysis of IL-17 blood levels, a Mann-Whitney rank sum test was used ($p < 0.05$ was considered significant).

Results

IL-17 is increased in multiple low-dose streptozotocin-induced diabetes. Before assessing the effect of IL-17 on beta-cell NO release, we sought to validate such an approach by examining the levels of IL-17 in experimental diabetes induced in rats and mice by administration of multiple low doses of streptozotocin. In this model, a limited initial damage induced by low subtoxic doses of streptozotocin triggers immune, mainly T cell- and macrophage-mediated destruction of Langerhans islets in which NO apparently plays a significant role [36]. In our experiments, the ELISA revealed that 10 or 15 days after the last injection of streptozotocin, the IL-17 concentration in the serum of streptozotocin-treated rats (fig. 1A) or mice (fig. 1B), respectively, was significantly increased in comparison with that of healthy controls (fig. 1). The hyperglycemia (glucose concentration > 11 mmol/l) occurred on day 15 in rats and day 21 in mice, as determined by blood glucose analysis

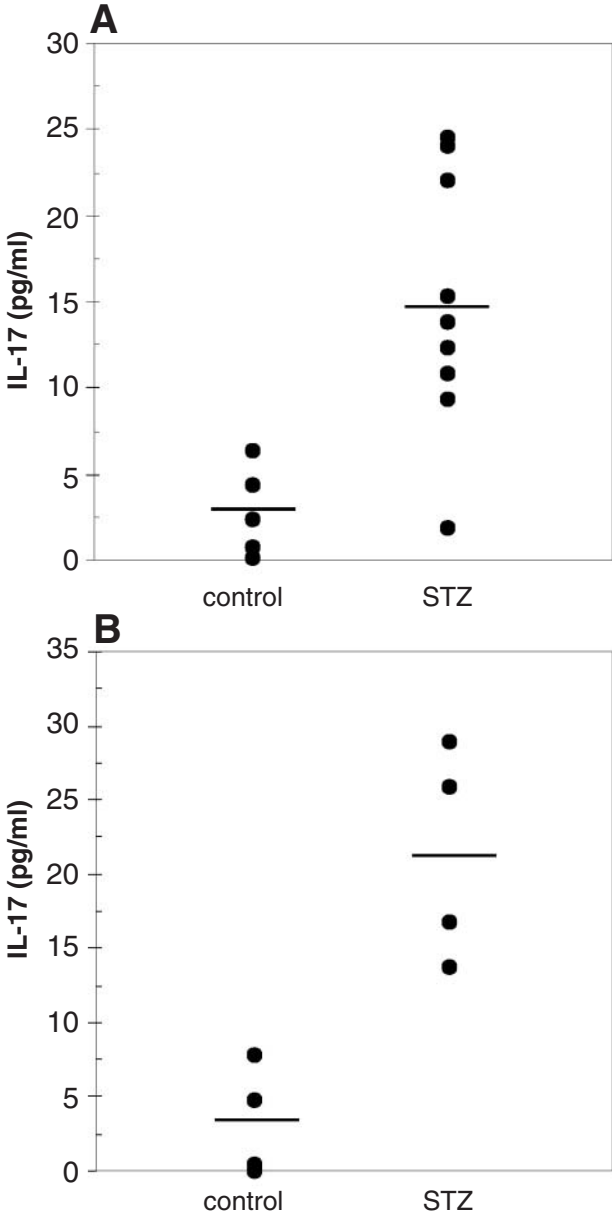

Figure 1. Blood IL-17 levels in streptozotocin-induced diabetes. Autoimmune diabetes was induced by multiple low doses of streptozotocin (STZ). Serum of nine streptozotocin-treated and five control rats (**A**), or four diabetic and four control mice (**B**), was analyzed for the presence of IL-17 by ELISA. Horizontal lines represent mean values ($p < 0.05$, Mann-Whitney test).

in the animals enrolled in the same experiment but not sacrificed for the collection of serum (data not shown). Therefore, the increase in serum IL-17 seems to precede the clinical manifestation of disease in multiple low-dose streptozotocin-induced diabetes.

IL-17 stimulates proinflammatory cytokine-induced NO production in MIN6 cells. After confirming that IL-17 is upregulated in experimental diabetes, we wanted to examine if stimulation of NO production might be a mechanism by which IL-17 could contribute to beta cell destruction during T1DM. Cells of the mouse insulinoma cell line MIN6 were treated for 48 h with recombinant IL-17 in the absence or presence of various proinflammatory cytokines (IFN-γ, IL-1, TNF-α) or their combination. While IFN-γ, IL-1 and TNF-α were ineffective alone, they efficiently stimulated NO production in MIN6 cells if applied in different combinations (fig. 2A). IL-17 was not able to induce NO production in MIN6 cells, either on its own or in combination with IFN-γ, IL-1 or TNF-α (fig. 2A). However, IL-17 significantly upregulated NO release induced by any combination of proinflammatory cytokines, and its stimulating effect was evident even if all three cytokines (IFN-γ, IL-1 and TNF-α) were combined (fig. 2A). The IL-17-mediated enhancement of NO production was not due to the presence of lipopolysaccharide (LPS) or some other contaminant in the IL-17 preparation, as it was completely abolished with the IL-17-specific neutralizing Ab, but not with the isotype-matched control Ab (fig. 2A). While IFN-γ + IL-1-induced NO release was prevented with 5 μg/ml IL-1R antagonist (22.7 ± 1.5% of control, n = 3), IFN-γ + TNF-α + IL-17-mediated NO production was not significantly affected by antagonizing endogenous IL-1 (96.6 ± 5.8% of control, n = 3). The experiments in which IL-17, IFN-γ and TNF-α were used at different concentrations confirmed that the NO-inducing effects of cytokines, including that of IL-17, were dose dependent (fig. 2B, C). Finally, the action of IL-17 was also time dependent, as a constantly growing increase of NO production was observed in IL-17-co-stimulated cells throughout 72 h of incubation (Fig 2D). The increase in NO synthesis was not due to stimulation of cell proliferation, as IL-17 did not significantly increase the cell number after 48 or 72 h of incubation (data not shown). Results similar to those presented in figure 2B–D were obtained if IL-1 was used instead of TNF-α (data not shown). Based on these observations, the IL-17 concentration of 10 ng/ml and incubation period of 48 h (for nitrite determination) were used in some of the subsequent experiments. It should be noted that IL-17 was able to stimulate NO release only if applied simultaneously with the proinflammatory cytokines, but not if added after the cells had been pre-treated with IFN-γ + TNF-α or IFN-γ + IL-1 (10 ng/ml each), and washed before addition of IL-17 (data not shown).

IL-17 is involved in T cell-dependent NO synthesis in MIN6 cells. Our next aim was to demonstrate the ability of IL-17 to enhance beta cell NO production in a biologically more relevant experimental setting. To establish the relative contribution of IL-17 in the immune cell-mediated NO synthesis in beta cells, we co-cultivated MIN6 cells with spleen MNCs. In accordance with the proposed T cell origin of IL-17, spleen MNCs produced large

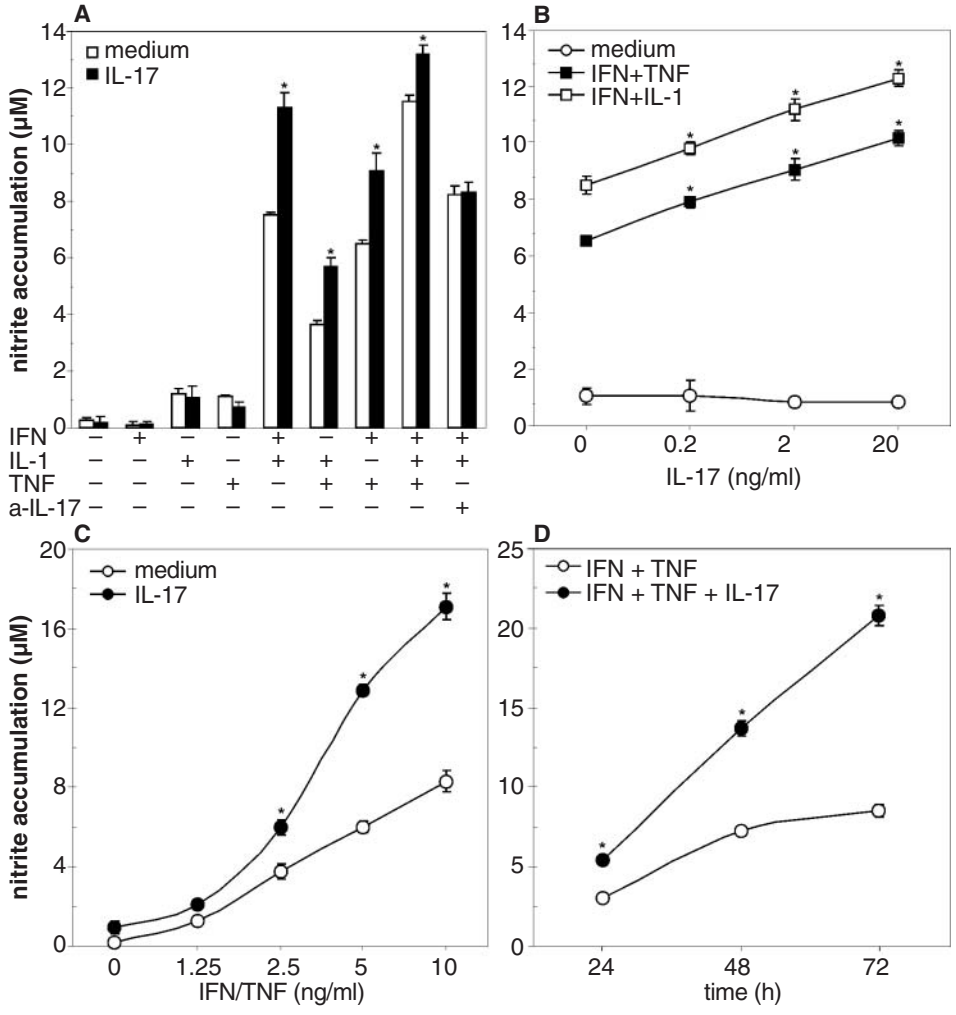

Figure 2. IL-17 stimulates cytokine-induced NO production in MIN6 cells. (**A**) MIN6 cells (6×10^4/well) were incubated for 48 h with 10 ng/ml of IL-17, IFN-γ, TNF-α, IL-1, or 2 μg/ml of anti-IL-17 Ab (a-IL-17) in various combinations. (**B**) MIN6 cells were stimulated for 48 h with IFN-γ + TNF-α or IFN-γ + IL-1 (10 ng/ml each), in the presence or absence of different doses of IL-17. (**C**) MIN6 cells were incubated for 48 h with IL-17 (10 ng/ml), in the presence or absence of different concentrations of IFN-γ and TNF-α. (**D**) MIN6 cells were treated for various time periods with IFN-γ + TNF-α, in the presence or absence of IL-17 (10 ng/ml each). The results of nitrite accumulation from a representative of three experiments are presented as the mean ± SD of triplicate measurements (*$p < 0.05$).

amounts of IL-17 (0.8–1.0 ng/ml) upon stimulation with the polyclonal T cell activator Con A. Con A-treated co-cultures of spleen MNCs and MIN6 cells generated high amounts of NO that were partly, but significantly reduced with a neutralizing anti-IL-17 Ab (fig. 3A). The anti-IL-17 Ab had no effect on NO release in Con A-stimulated MNC cultures in the absence of MIN6 cells, indicating that the neutralization of IL-17 in co-cultures of MNC and MIN6 cells selectively affected the NO production in the latter cells. Such an assumption was confirmed by the ability of anti-IL-17 to reduce in a dose-dependent fashion the NO release induced in the MIN6 cell line by the conditioned supernatant from Con A-treated MNC cultures (fig. 3B). Thus, IL-17 seems to be among the T cell mediators required for maximal induction of NO synthesis in pancreatic beta cells.

IL-17 stimulates iNOS expression and p38 MAPK activation in MIN6 cells. Aminoguanidine, a fairly selective inhibitor of the iNOS isoform [37], completely abolished IFN-γ + IL-1 + IL-17-triggered NO release in MIN6 cell cultures (not shown) suggesting that the observed effect of IL-17 was exerted through induction of iNOS expression. Accordingly, RT-PCR analysis revealed that although ineffective alone, IL-17 was able to stimulate IFN-γ + IL-1-induced expression of iNOS mRNA in MIN6 cells, but not in the presence of anti-IL-17 Ab (fig. 4A). In line with the results of nitrite measurements, the supernatant of Con A-treated spleen MNCs also induced the expression of iNOS mRNA in MIN6 cells, while the inhibitory action of the anti-IL-17 confirmed the participation of IL-17 in this effect (fig. 4A). The IL-17-mediated potentiation of IFN-γ + IL-1-triggered iNOS mRNA expression correlated with the presence of an increased amount of iNOS protein, as confirmed by cell-based ELISA (Fig 4B). The NO-inducing effect of IL-17 was apparently dependent on p38 MAPK activity, as SB203580, a selective inhibitor of p38 MAPK activation [38], completely diminished IL-17-mediated potentiation of NO release (fig. 4C) without reducing cell

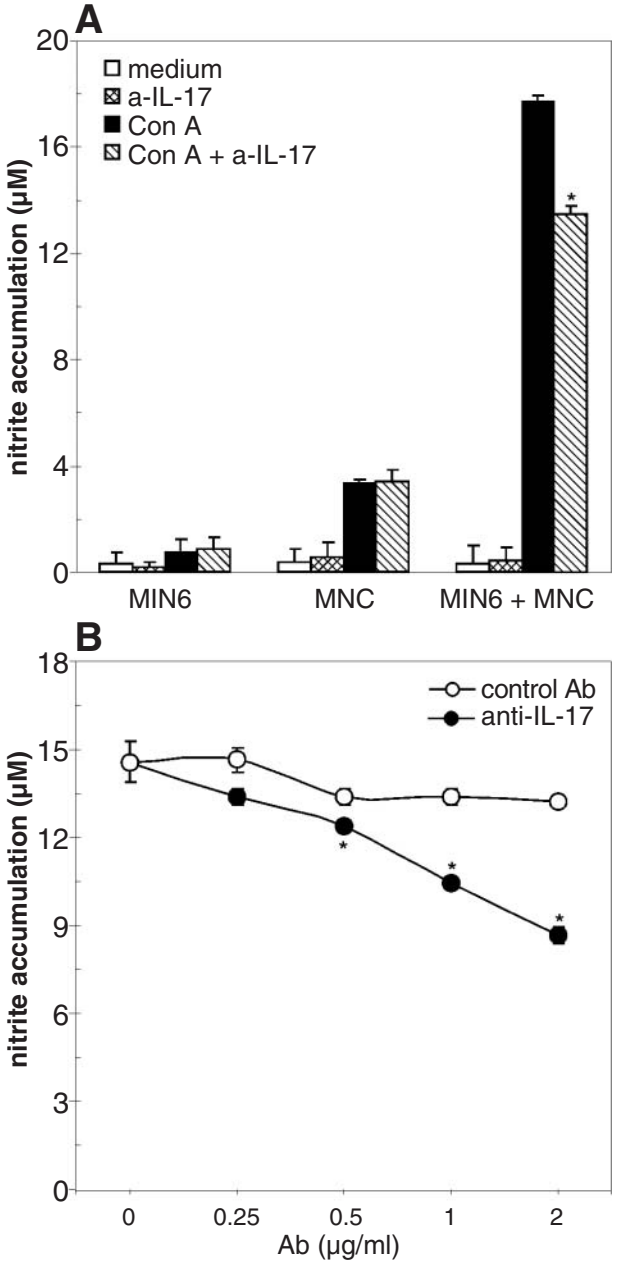

Figure 3. IL-17 contributes to spleen MNC-induced NO production in MIN6 cells. (**A**) MIN6 cells (6×10^4/well) and spleen MNCs (2.5×10^5/well) were incubated separately or together, with or without Con A (5 μg/ml) and/or anti-IL-17 (1 μg/ml). (**B**) MIN6 cells were incubated in conditioned medium (25%) from 48-h Con A-treated spleen MNC cultures, in the presence or absence of various concentration of neutralizing anti-IL-17 Ab or irrelevant isotype-matched control Ab. Nitrite accumulation was measured after 48 h of incubation and the results from the representative of five (**A**) or four (**B**) experiments are presented as the mean ± SD of triplicate measurements (*$p < 0.05$).

viability (determined by trypan blue exclusion; data not shown). However, since SB203580 also downregulated IFN-γ + IL-1- and IFN-γ + TNF-α-induced NO synthesis in MIN6 cells (fig. 4C), it was not possible to conclude whether IL-17 itself was able to activate p38 MAPK, or whether some other IL-17-triggered signals co-operated for iNOS expression with p38 MAPK induced by IFN-γ/TNF-α or IFN-γ/IL-1 treatment. To resolve this issue, the ability of IL-17 to activate p38 MAPK was assessed by western blot analysis of the phosphorylated, active form of the kinase. In accordance with the results obtained with SB203580, the phosphorylation of p38 MAPK was readily induced either by IL-17 or IFN-γ/TNF-α treatment, which collaborated in an additive fashion to further induce p38 MAPK activation (fig. 4D). Taken together, these data indicated that IL-17 might stimulate iNOS expression in MIN6 cells at least partly via activation of p38 MAPK.

IL-17-induced NO is toxic for beta cells. In addition to inducing the release of high amounts of NO, the treatment with the combination of IFN-γ, IL-1 and IL-17 was apparently toxic for MIN6 cells, as the MTT assay revealed a significant reduction in their mitochondrial respiration (fig. 5A). The observed toxicity was mediated by cytokine-induced NO, since the aminoguanidine-mediated blockade of NO release was accompanied by the complete recovery of cellular respiration (fig. 5A). Neutralization of IL-17 reduced the NO production and partially recovered the mitochondrial respiration (fig. 5A), suggesting that IL-17 participated in the NO-dependent toxicity. When different concentrations of IL-17 were added to IFN-γ + IL-1-stimulated MIN6 cells, a clear negative correlation ($r = -0.97$, $p < 0.05$; linear regression) was observed between the dose-dependent increase in NO production and the concomitant reduction in cellular respiration (fig. 5B), thus supporting the contribution of IL-17-triggered NO release to cytokine-induced toxicity. To further corroborate this assumption, the effect of a neutralizing anti-IL-17 Ab on the mitochondrial respiration was assessed in MIN6 cells treated with the supernatant of Con A-activated spleen MNCs. While the Con A supernatant-induced NO production was accompanied by a decrease in cellular respiration, the neutralization of IL-17 partly, but significantly reduced both the NO release and the toxicity associated with administration of soluble MNC products (fig. 5C). Finally, to rule out the possibility that the observed effects were somehow specific for the MIN6 cell line, the influence of IL-17 on NO production and cellular respiration was investigated in mouse pancreatic islets. Similarly to results obtained with MIN cells, mouse pancreatic islets readily upregulated their NO production in response to IL-17 applied together with IFN-γ, TNF-α, or IL-1 in various combinations (fig. 6A). Moreover, the NO production and toxicity induced in pancreatic islets by soluble products of Con A-activated spleen MNCs were both partly reduced with anti-IL-17 Ab (fig. 6B). Therefore, IL-17-mediated induction of NO release was

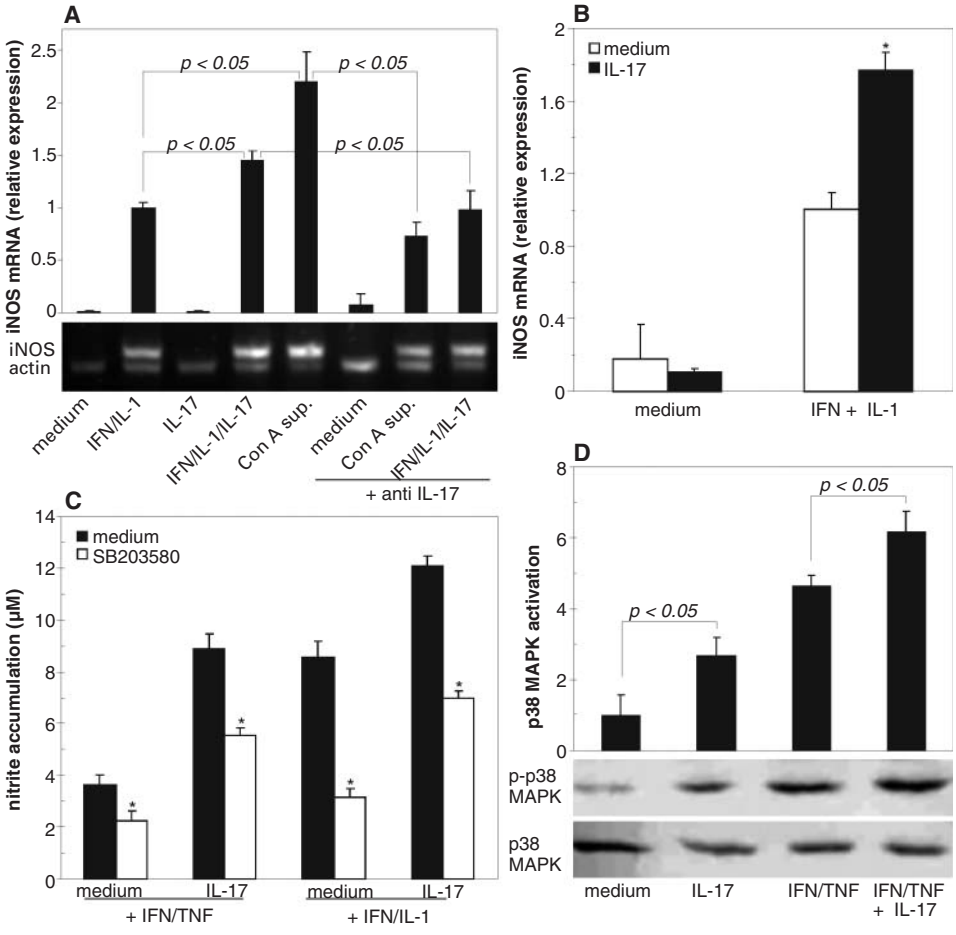

Figure 4. IL-17 stimulates the expression of iNOS mRNA/protein and activates p38 MAPK in MIN6 cells. (**A**) MIN6 cells (6×10^4/well) were treated with IFN-γ/IL-1 ± IL-17 (10 ng/ml each), or with 25% supernatant of Con A-activated spleen MNCs, in the presence or absence of anti-IL-17 (1 μg/ml). After 6 h, RNA was isolated and RT-PCR for iNOS/β-actin performed. The gel from the representative experiment is presented, while the results (iNOS/actin ratio, normalized to 1 for IFN-γ + IL-1) are means ± SD of three experiments. (B) Cell-based ELISA for iNOS was performed in MIN6 cells (6×10^4/well) after 24 h of incubation with IFN-γ + IL-1 (each 10 ng/ml), in the presence or absence of IL-17 (10 ng/ml). The results from the representative of three experiments are presented as iNOS expression relative to the value obtained after IFN-γ + IL-1 stimulation (arbitrarily set to 1). The data are means ± SD of triplicate cultures (*$p < 0.05$). (**C**) MIN6 cells (5×10^4/well) were treated for 48 h with IFN-γ/IL-1 or IFN-γ/TNF-α ± IL-17 (10 ng/ml each), in the presence or absence of p38 MAPK inhibitor SB203580 (20 μg/ml). The results of nitrite measurement from the representative of two experiments are presented as the mean ± SD of triplicate cultures (*$p < 0.05$). (D) Phosphorylation of p38 MAPK was assessed by Western blot after 30 min incubation of MIN6 cells with IFN-γ/TNF-α and/or IL-17 (10 ng/ml each). The blot is from the representative experiment, while p38 MAPK activation (calculated as the p-p38/p38 signal ratio, arbitrarily set to 1 for untreated cells) is presented as the mean ± SD from three experiments.

apparently responsible for the toxicity of this T cell cytokine to pancreatic beta cells.

Discussion

In the present study we demonstrate for the first time the ability of the T cell cytokine IL-17 to upregulate the expression of iNOS mRNA and protein, and cause NO-dependent toxicity in cultures of the MIN6 beta cell line and mouse pancreatic islets. In light of the findings that IL-17 is markedly upregulated in two different models of experimental autoimmune diabetes [32 and the present study], these results strongly suggest that this proinflammatory cytokine could contribute to the pathology of diabetes through activation of suicidal NO release in beta cells. Our most recent preliminary findings, showing a positive correlation between circulating IL-17 levels and the severity of streptozotocin-induced diabetes (measured by blood glucose concentration), indeed support such a hypothesis (unpublished results).

The T cell- and macrophage-derived proinflammatory cytokines IFN-γ, TNF-α and IL-1 have been regarded as principal mediators of iNOS induction in various cell types, including beta cells of Langerhans islets of the pancreas [39]. In line with data previously obtained in human chondrocytes and murine osteoblasts, astrocytes or endothelial cells [14–18], the results of the present report indicate that IL-17 might also be an important co-stimulator of proinflammatory cytokine-induced NO generation in mouse beta cells. Although IL-17 can induce IL-1 secretion in human macrophages [3] and chondrocytes [15], or stimulate TNF-α-induced IL-1 expression in fibroblasts [40], the effect of IL-17 in our experiments was probably not mediated through

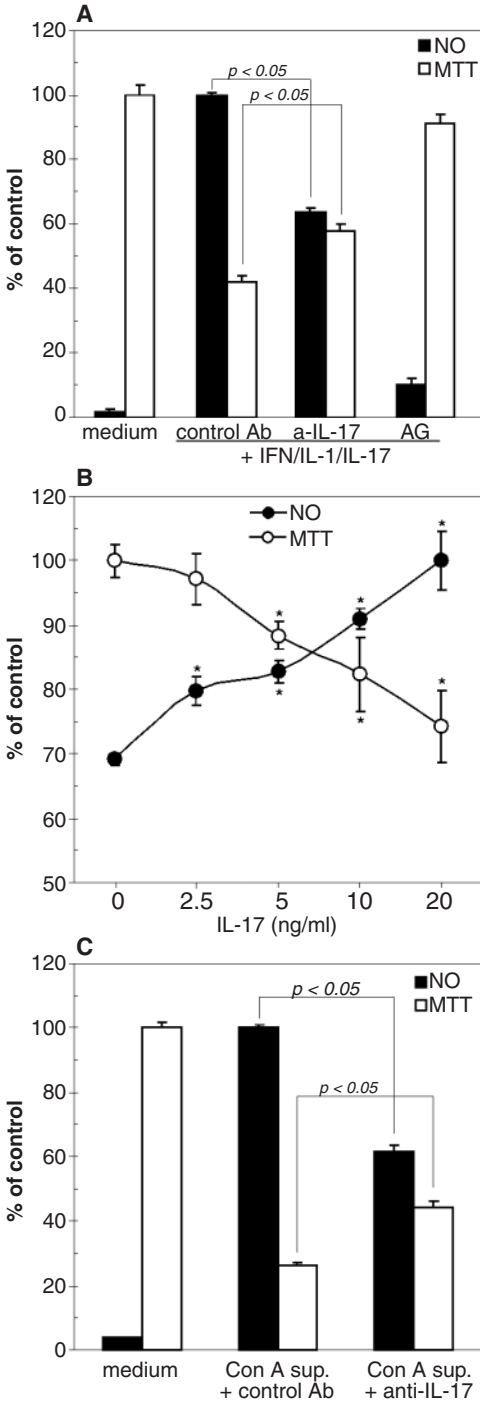

Figure 5. NO-dependent toxicity of IL-17 toward MIN6 cells. (**A**) MIN6 cells (6×10^4/well) were treated with IFN-γ + IL-1 + IL-17 (10 ng/ml each), in the presence or absence of control Ab (2 μg/ml), anti-IL-17 (2 μg/ml) or the iNOS inhibitor aminoguanidine (AG; 2 mM). (**B**) MIN6 cells were stimulated with IFN-γ + IL-1 (10 ng/ml each), in the presence or absence of various concentrations of IL-17. (C) MIN6 cells were treated with 25% supernatant from Con A-stimulated spleen MNCs, in the presence or absence of control Ab (2 μg/ml) or anti-IL-17 (2 μg/ml). (**A–C**) The results of the nitrite measurement and MTT assay, presented as % of control (maximal NO or MTT values)**,** are the mean ± SD of triplicate cultures from a representative of two (**A**), three (**C**), or four (**B**) experiments (*p < 0.05).

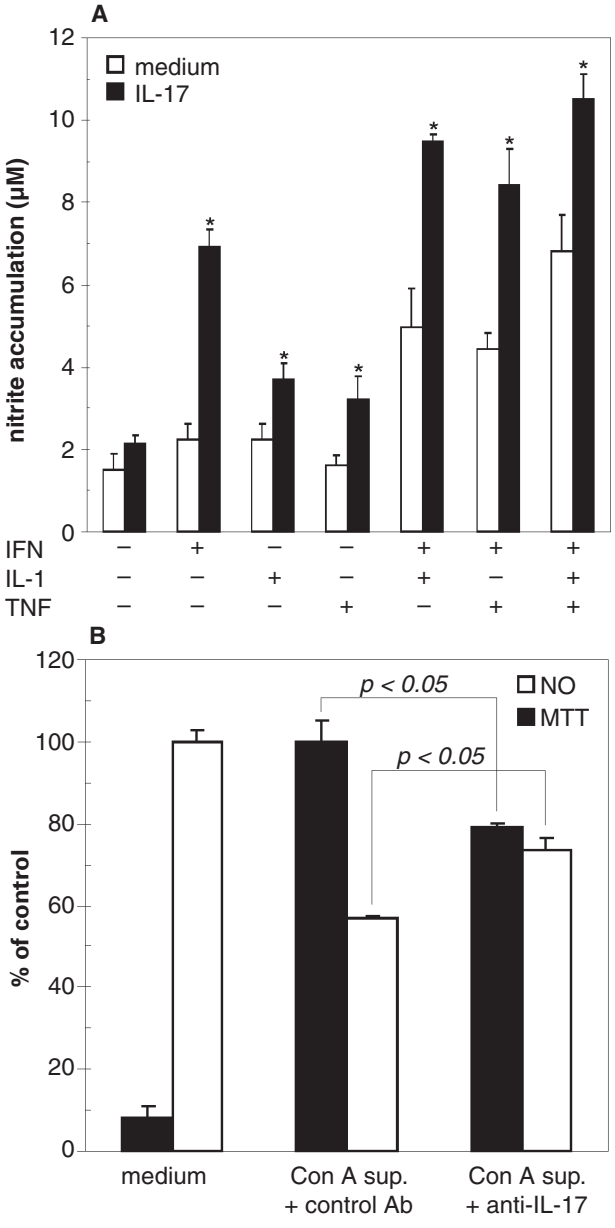

Figure 6. IL-17 stimulates NO production and NO-dependent toxicity in mouse pancreatic islets. Mouse pancreatic islets (1×10^4/well) were incubated for 48 h with IL-17, IFN-γ, TNF-α, and IL-1 (10 ng/ml each) in different combinations (**A**), or with a 25% supernatant of Con A-stimulated mouse spleen MNCs in the presence or absence of 2 μg/ml of control Ab or anti-IL-17 (**B**). The results of nitrite determination and MTT assay from the representative of three experiments are presented as the mean ± SD of triplicate cultures (*p < 0.05). The results in **B** are presented as % of control (maximal NO or MTT values).

induction of IL-1, as the IL-1R antagonist was unable to affect IL-17-mediated potentiation of NO synthesis in IFN-γ + TNF-α-treated MIN6 cells. However, the fact that combining IL-17 with a single proinflammatory cytokine (IFN-γ, TNF-α or IL-1) was sufficient to induce

NO in whole islet cultures, in contrast with no effect in MIN6 cells, raises the possibility that IL-17 might also stimulate the release of some NO-inducing molecule from non-beta cells (e. g. macrophages). Nevertheless, the NO-inducing effect of IL-17 in both insulinoma and whole islet cultures was preserved even if IL-1, IFN-γ and TNF-α were applied together, suggesting that IL-17-induced intracellular events leading to NO release did not completely overlap with those of the three classical iNOS inducers. The observed synergistic interaction of IL-17 with the proinflammatory cytokines did not simply stem from the ability of the latter to stimulate expression of the IL-17 receptor, as pre-treatment with IFN-γ/TNF/IL-1 failed to induce responsiveness to a subsequent addition of IL-17. Importantly, neutralization experiments with IL-17-specific Ab have confirmed that T cell-derived IL-17 is absolutely required for maximal iNOS induction and subsequent NO production induced by soluble products of activated MNCs in the MIN6 beta cell line and mouse pancreatic islets. Of note, similar results were obtained with the rat insulinoma cell line RINm5F (our unpublished observation), thus unequivocally establishing IL-17 as a non-redundant participant in the cytokine-induced beta cell NO release *in vitro*. The fact that the anti-IL-17-mediated drop in NO release was somewhat more pronounced than the corresponding recovery of cell viability (see fig. 5A, 5C) is consistent with the known ability of proinflammatory cytokines to kill beta cells through both NO-dependent and NO-independent mechanisms [41].

The activation of MAPK family member p38 MAPK is among the key intracellular events connecting the IL-17 receptor to transcription of IL-6, matrix metalloproteinase-9, cyclooxygenase-2, as well as the iNOS gene in various cell types [14–16, 18, 42–46]. Accordingly, IL-17 readily triggered phosphorylation of p38 MAPK in MIN6 cells, and pharmacological blockade of p38 MAPK activation markedly downregulated IL-17-stimulated NO release in our experiments. The downstream events following IL-17-triggered phosphorylation of p38 MAPK might involve activation of iNOS transcription factor AP-1 [20], which could then co-operate in the initiation of iNOS transcription with IFN-γ-induced interferon regulatory factor-1 (IRF-1) and IL-1/TNF-α-activated nuclear factor-κB (NF-κB), the key transcription factors for iNOS induction in pancreatic beta cells [47]. However, as the IFN-γ/TNF-α combination was quite sufficient for p38 MAPK phosphorylation in the MIN6 cell line, the putative role of this MAP kinase in IL-17-mediated co-stimulation of iNOS induction in beta cells appears to be a minor one at best. Since in various experimental settings, IL-17 was also able to activate p42/p44 MAPK, c-Jun N-terminal kinase (JNK) and Janus kinase (JAK), as well as IRF-1 and NF-κB [20], an exploration of the interplay between IL-17 and other proinflammatory cytokines in the activation of these iNOS-inducing signals in beta cells is currently underway in our laboratory.

There is a question of the possible biological consequences of IL-17-mediated induction of iNOS in beta cells. While the involvement of NO in human T1DM is still uncertain [27], the results of the studies with pharmacological inhibitors of NO production and iNOS-deficient mice [23, 48, 49] strongly argue for a role of NO in beta cell destruction during experimental autoimmune diabetes. In accordance with the currently dominant paradigm that proposes suicidal iNOS induction in beta cells as a main mode of endocrine pancreas damage in T1DM, the iNOS expression has been reported to increase in pancreatic beta cells of NOD mice [50], and beta cell-selective expression of an iNOS transgene was sufficient to induce diabetes in mice [51]. While IFN-γ, TNF-α and IL-1 have been regarded as the key cytokines involved in the iNOS induction and subsequent inhibition of insulin release and death of beta cells [39], our results introduce T cell-derived IL-17 as a potent and non-redundant co-stimulator possibly involved in the 'fine-tuning' of proinflammatory cytokine-induced iNOS expression and the ensuing damage in mouse beta cells. The partial recovery of cellular respiration achieved by IL-17 neutralization in MNC supernatant-treated insulinoma or pancreatic islet cell cultures suggests that IL-17 could indeed participate in T cell/macrophage-mediated beta cell destruction in T1DM. Such a scenario is supported by recent study in which the microarray analysis of pancreata from NOD mice revealed that transcription of genes encoding both IL-17 and its receptor is markedly increased during late-stage insulitis and overt diabetes [32]. Moreover, the finding by the same authors that IL-17 blood levels are also increased in diabetic NOD mice [32] have been confirmed in the present study in the streptozotocin model of T1DM. Finally, the IL-23-driven subset of IL-17-secreting T cells, designated $Th_{IL\text{-}17}$, has recently been shown to play a crucial role in development of central nervous system and joint autoimmune inflammation [52, 53]. In light of these data combined with the results of the present study, exploring the role of the pathogenic $Th_{IL\text{-}17}$ subset in autoimmune diabetes using IL-17 knockout animals or *in vivo* neutralization of this cytokine seems to be an inevitable step in deciphering the immunopathology of T1DM.

Acknowledgements. This work was supported by funds of The Ministry of Science and Environmental Protection of the Republic of Serbia (grants No. 1664 and 2020). The authors are grateful to Dr. F. Nicoletti (University of Catania, Catania, Italy) for providing IL-17 ELISA and anti-IL-17 Ab, and Dr. C. A. Dinarello (University of Colorado Health Sciences Center, Denver, Colo.) for the kind gift of IL-1R antagonist. We would also like to thank Dr. M. Mostarica Stojkovic and Dr. Z. Ramic (Institute of Microbiology and Immunology, School of Medicine, University of Belgrade) for helpful discussion.

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