IFN γ /TNF α synergism in MHC class II induction: effect of nicotinamide on MHC class II expression but not on islet-cell apoptosis

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Abstract

Aims/hypothesis. Interferon- γ (IFN γ) and TNF α synergistically induce pancreatic beta-cell apoptosis. Apart from their direct effect, we studied the possible indirect immunological role of IFN γ /TNF α synergism on pancreatic beta-cell death by investigating MHC class II induction by cytokines. The effect of nicotinamide on the cytokine-induced MHC class II expression and pancreatic beta-cell death was also studied.

Methods. Immunocytochemistry, flow cytometry and RNase protection assay were used to study MHC class II expression. Immunoblotting was done to study downstream signals of IFN γ . The effects of nicotinamide on islet-cell apoptosis and diabetes mellitus were examined using MTT assay and adoptive transfer model.

Results. IFN γ alone induced MHC class II expression on a small number of insulinoma cells. TNF α alone did not induce MHC class II expression, but en-

hanced IFN γ -induced MHC class II expression. MHC class II expression by cytokine(s) was due to the induction of class II transactivator (CIITA). Nicotinamide reduced MHC class II expression by cytokine(s) but did not protect insulinoma-cell apoptosis by IFN γ and TNF α in combination or protect against the development of diabetes mellitus after adoptive transfer of diabetogenic lymphocytes.

Conclusion/interpretation. IFN γ and TNF α synergistically induced MHC class II expression on insulinoma cells through the induction of CIITA; nicotinamide reduced the expression of cytokine-induced MHC class II expression on insulinoma cells through its effect on CIITA expression; and the preventive effect of nicotinamide on Type I (insulin-dependent) diabetes mellitus is probably due to its effect of MHC class II expression rather than that on islet cell apoptosis. [Diabetologia (2002) 45: 385–393]

Keywords IFN γ , TNF α , MHC, Type I diabetes mellitus, STAT1, IRF-1, CIITA, nicotinamide.

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Abbreviations: APC, Antigen-presenting cell; CIITA, class II transactivator; DTH, delayed-type hypersensitivity; FITC, fluorescein isothiocyanate; IFN, interferon; IRF, interferon regulatory factor; MTT, 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; NOD, non-obese diabetic; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride; STAT, signal transducer and activator of transcription; TCR, T-cell receptor

Islet cells of the pancreas are susceptible to extensive T-cell-mediated damage in Type I (insulin-dependent) diabetes mellitus. The Fas ligand is not the effector molecule for the majority of effector T-cells in pancreatic beta-cell apoptosis [1, 2]. Data indicates that interferon- γ (IFN γ) and tumour necrosis factor- α (TNF α) synergistically induced caspase-dependent apoptosis on mouse islet cells both in vitro and in vivo [3]. IFN γ seems to render otherwise resistant pancreatic islet cells susceptible to TNF α -mediated apoptosis by activating downstream signal transducer and activator of transcription-1 (STAT1) and IFN regulatory factor-1 (IRF-1). The effect of cytokines on islet cell-death has recently become an area of in-

tense research because of the importance of islet-cell death in diabetes mellitus, but the effect of cytokines on major histocompatibility complex (MHC) class II expression in pancreatic beta cells has been studied for many years [4, 5]. MHC class II molecules are surface glycoproteins which bind exogenous antigens and present them to T-cell receptors (TCR). Although most cells express MHC class I molecules, the expression of class II molecules is restricted to specialized antigen-presenting cells (APC). It has been suggested that the induction of MHC class II on nonlymphoid cells, such as pancreatic beta cells which do not normally express MHC class II molecules [6], could result in the initiation of autoimmunity [7]. Although controversies exist [8–10], it has been reported that islet beta cells from the patients with Type I diabetes mellitus or animals with autoimmune diabetes mellitus express MHC class II antigens [11–13]. However, *in vivo* role of specific cytokines in the induction of MHC class II molecules on pancreatic beta cells is not clearly defined [11, 12, 14]. More controversial is the immunological impact of such an induction of MHC class II molecules on nonlymphoid cells such as pancreatic beta cells. Ectopic expression of MHC class II molecules on pancreatic beta cells by transgenic technology did not lead to autoimmune diabetes mellitus, in contrast to the original suggestion that aberrant expression of MHC class II molecules on nonlymphoid cells would lead to self-antigen presentation by non-APC and autoimmune process [15, 16]. It led instead to immunological tolerance or ignorance depending on the amount and type of antigens expressed [17]. The expression of MHC class II molecules on pancreatic beta cells alone seems to be insufficient for the initiation of autoimmunity probably because nonlymphoid cells such as pancreatic beta cells lack co-stimulatory signals required for the initiation of immune responses [18, 19]. However, such findings leave open the possibility that aberrant MHC molecules on pancreatic beta cells do play an immunological role. They could facilitate apposition between pancreatic beta cells with MHC class II molecules and T cells bearing TCR and enhance the chance of bystander killing by cytokines in delayed-type hypersensitivity (DTH)-like model [3, 20].

We studied the possible indirect immunological role of IFN γ and TNF α synergism, which has been demonstrated to directly induce apoptosis of pancreatic beta cells, by investigating the cytokine-induced induction of MHC class II molecule and upstream signals. We also studied the effect of nicotinamide on MHC class II expression and effector phase of the development of diabetes mellitus. The reason was that nicotinamide has been reported to affect the downstream intracellular signals of IFN γ [21–23] and reduce the incidence of diabetes mellitus [24–27].

Materials and methods

Immunocytochemistry. MIN6N8 cells, SV 40-transformed insulinoma cells derived from non-obese diabetic (NOD) mice [28] (kindly provided by Dr Miyazaki, Osaka University, Japan), were cultured in DMEM (GibcoBRL, Rockville, Md., USA)-15 % FCS. Cells maintained on coverslips were cultured for 3 days in the medium containing cytokines. To induce surface MHC class II molecules, 100 U/ml rat IFN γ (kindly provided by Dr van der Meide, TNO Primate Center, The Netherlands) and 1000 U/ml murine TNF α (R&D Systems, Minneapolis, Minn., USA) were simultaneously added to the medium. After culture, cells were washed with PBS to remove potentially adhering apoptotic cells and fixed in methanol at -20°C for 20 min. The coverslips were incubated with 1.5 % goat serum for 30 min to block non-specific binding of IgG. To detect MHC class II (I-A) molecules of NOD mice origin, cells were incubated with 1:100 diluted monoclonal anti-mouse I-A antibody reactive to I-A^{b7} (Cedarlane, Ontario, Canada) at 4°C for 30 min. The coverslips were washed three times in PBS and then incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, Calif., USA) for 30 min followed by washing with PBS. After quenching of endogenous peroxidase activity with 1 % H_2O_2 in methanol, avidin-biotin-peroxidase complex (Vector Laboratories) in PBS was applied for 30 min. After extensive washing in PBS, the coverslips were incubated in AEC substrate solution for 4 min. The reaction was stopped by washing in PBS, and the coverslips were examined on a light microscope.

Flow cytometry. MIN6N8 cells treated with IFN γ and/or TNF α for 1–3 days were detached with 0.05 % trypsin-0.02 % EDTA and incubated with anti-mouse I-A antibody (Cedarlane, Ontario, Canada) for 30 min at 4°C . The cells were stained with 10 $\mu\text{g}/\text{ml}$ of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Zymed, San Francisco, Calif., USA). After washing in PBS-2 % FCS-0.02 % NaN_3 , flow cytometry was performed using FACStar (Becton-Dickinson, Sunnyvale, Calif., USA). In experiments assessing the effect of nicotinamide on MHC class II antigen induction, nicotinamide (5 or 20 mmol/l) (Sigma Chemicals, St. Louis, Mo., USA) was added to the medium containing cytokines.

RNAse protection assays. MIN6N8 cells were cultured for 2 days with or without cytokine(s)/nicotinamide (20 mmol/l). RNA was prepared from cultured MIN6N8 cells according to a protocol using acidic phenol. Briefly, cells were lysed in 4 mol/l guanidinium thiocyanate solution. After sequential addition of 2 mol/l sodium acetate, acidic phenol and chloroform, RNA in aqueous phase was precipitated with isopropanol. [^{32}P]dCTP-labelled riboprobes were prepared by *in vitro* transcription of linearised expression vectors harbouring bacteriophage promoters and *I-A*^{b7} (kindly provided by Dr H.O. McDevitt, Stanford University, Stanford, Calif., USA) [29] or murine class II transactivator (*CIITA*) cDNA (kindly provided by Dr C-H. Chang, University of Michigan, Ann Arbor, Mich., USA) [30], respectively. The riboprobes were hybridized to 5 μg of each RNA sample and control tRNA (Ambion, Austin, Tex., USA). The protected RNA hybrid and the respective probes were analysed on a 6 % polyacrylamide gel.

Western blot. MIN6N8 cells were cultured with or without cytokine(s) or nicotinamide (20 mmol/l) or both for 30 min or 36 h to detect phospho-STAT1 / IRF-1, respectively.

MIN6N8 cells were lysed in 20 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonylfluoride (PMSF), and

0.25% NP-40. A total of 50 μ g protein was then separated by 10% SDS-PAGE electrophoresis and was transferred electrophoretically to polyvinylidenedifluoride (PVDF) membranes. After blocking non-specific binding in 5% non-fat dried milk, membranes were incubated with antibody to phospho-Stat1(Tyr701) (New England Biolabs, Beverly, Mass., USA) or IRF-1 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at room temperature for 1 h. Then they were washed with PBS-0.15% Tween 20, and incubated with peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at room temperature. The bound antibody was visualized with an enhanced chemoluminescence kit (Amersham Pharmacia Biotech).

Cytotoxicity. For MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assays (Sigma), MIN6N8 cells were seeded into each well of 96-well plates. IFN γ (100 U/ml) and TNF α (1000 U/ml) treatment was carried out for 3 days with or without nicotinamide (5, 10, 20 mmol/l). Then, MTT (0.5 mg/ml) was added to each well. After incubation at 37°C for 4 h and aspiration of the culture medium, DMSO (Merck, Darmstadt, Germany) was added to dissolve insoluble crystals. Absorption at 540 nm was measured using an automated microplate reader (Thermomax, Molecular Devices, Sunnyvale, Calif., USA).

Adoptive transfer of diabetes mellitus. NOD mice were purchased from Jackson Laboratory (Bar Harbor, Me., USA) and were maintained in a specific-pathogen-free environment at the Samsung Biomedical Research Institute in Korea. The incidence of diabetes mellitus in female NOD mice was above 70% at 24 weeks of age. Animal care was in accordance with our institutional guideline. Splenocytes (2×10^7) from diabetic NOD mice were infused into the tail vein of each 8-week-old female NOD mouse after irradiation (775 rad). Mice were considered diabetic when their non-fasting blood glucose concentration was higher than 300 mg/dl at two successive determinations. Some mice were treated with a 10% aqueous solution of nicotinamide at a dose of 0.5 mg/g/day s.c. daily after adoptive transfer until the onset of diabetes mellitus. Control NOD mice were treated with PBS. The incidence of diabetes mellitus was about 80% within 3 weeks after the adoptive transfer and 100% within 4 weeks after the adoptive transfer in our previous experiments.

Statistical analysis. Results were expressed as means $\pm$ SEM. Student's *t* test was employed to compare the mean values between the groups. Results were considered statistically significant when *p* values were less than 0.05.

Results

Aberrant expression of MHC class II and CIITA on MIN6N8 cells by IFN γ and TNF α . MIN6N8 cells did not express detectable I-A antigens after in vitro culture for 3 days. Treatment with 100 U/ml IFN γ for 3 days induced I-A antigen expression on only some MIN6N8 cells ($\sim 9\%$) as identified by visual counting on a microscope or flow cytometry (Fig. 1B and Fig. 2A, B). TNF α alone did not induce detectable I-A expression on MIN6N8 cells at any concentration (Fig. 1C and Fig. 2B). The addition of 100 U/ml TNF α did not increase I-A expression induced by

IFN γ (Fig. 1D and Fig. 2A). However, the combination of 100 U/ml IFN γ and 1000 U/ml TNF α , employed to induce apoptosis of insulinoma cells or islet cells [3], resulted in the induction of I-A molecules on a much higher percentage ($\sim 30\%$) of MIN6N8 cells (Fig. 1E and Fig. 2A, B). The induction of I-A molecules by cytokines was evident after 2 days of the cytokine treatment and reached a maximal expression 3 days after the cytokine treatment (Fig. 2A). We then determined *I-A* mRNA expression in MIN6N8 cells using RNase protection assays. Treatment with 100 U/ml IFN γ only for 2 days induced some *I-A* mRNA expression and the addition of 1000 U/ml TNF α further increased the expression of *I-A* mRNA (Fig. 3, lane 2, 4), consistent with the I-A protein expression pattern. *I-A* mRNA was not detected in MIN6N8 cells treated with TNF α alone (Fig. 3, lane 3).

Next, we investigated the induction of CIITA by cytokines, which is the most critical step in the cytokine-induced MHC class II expression in lymphoid cells [30]. Consistent with the I-A expression pattern, IFN γ alone induced the expression of *CIITA* mRNA (Fig. 4, lane 2). The addition of 1000 U/ml TNF α further increased the expression of *CIITA* mRNA (Fig. 4, lane 4), suggesting that CIITA expression correlates well with MHC class II antigen expression and CIITA is induced by cytokines in non-lymphoid cells as well as in lymphoid cells.

IFN γ induced STAT1 phosphorylation and IRF-1 expression on MIN6N8 insulinoma cells, which are upstream signals of CIITA. TNF α did not induce phosphorylation of STAT1 or IRF-1 expression by itself, and did not affect IFN γ -induced phosphorylation of STAT1 or induction of IRF-1 as previously reported [3] (Fig. 5).

Nicotinamide and the inhibition of MHC class II and CIITA expression induced by IFN γ and TNF α . We then studied the effect of nicotinamide which has been reported to inhibit MHC expression and also inhibit the development of diabetes mellitus in NOD mice [5, 24]. Flow cytometric analyses showed that I-A expression by IFN γ alone or IFN γ and TNF α was inhibited by nicotinamide (Fig. 6). Compared with the percentage of I-A⁺ cells cultured with 100 U/ml IFN γ and 1000 U/ml TNF α without nicotinamide ($\sim 30\%$), I-A expression was lower in cultures containing 5 mmol/l (6.6%, $p < 0.05$), or 20 mmol/l (5.2%, $p < 0.05$) nicotinamide, respectively (Fig. 6). RNase protection assays also revealed that nicotinamide inhibited *I-A* mRNA induction by IFN γ both in the presence or absence of TNF α (Fig. 3, lane 5 $\sim$ 7).

Next, we examined the effect of nicotinamide on the CIITA expression. Twenty mmol/l nicotinamide significantly reduced the expression of CIITA by IFN γ alone or the combination of IFN γ and TNF α (Fig. 4, lane 5 $\sim$ 7), indicating that nicotinamide re-

Fig.1 (A–F). The expression of I-A antigen on MIN6N8 cells detected by immunocytochemistry after 3 days of culture. **A** Media only, **B** 100 U/ml IFN γ , **C** 100 U/ml TNF α , **D** 100 U/ml IFN γ + 100 U/ml TNF α , **E** 100 U/ml IFN γ + 1000 U/ml TNF α , **F** negative control. (Magnification $\times 400$)

duces MHC class II expression by inhibiting the expression of CIITA.

Nicotinamide did not affect STAT1 phosphorylation or IRF-1 induction by IFN γ alone or IFN γ /

TNF α , indicating that nicotinamide decreases the expression of CIITA and I-A without recourse to STAT1/IRF-1 signals (Fig.5).

Nicotinamide and the inhibition of apoptosis of insulinitoma cells and prevention of autoimmune diabetes mellitus induced by adoptive transfer. Because IFN γ and TNF α combination could directly impose apoptosis of islet cells [3] and also induce the expression of MHC class II on islet cells potentially leading to increased immune response against islet cells, we stud-

Fig. 2 (A, B). **A** Time course of I-A induction by cytokine(s). The percentage of I-A⁺ MIN6N8 cells was evaluated by flow cytometry. The data are means $\pm$ SEM from three independent experiments. ○ media; △ 100 U/ml IFN γ ; □ 100 U/ml IFN γ + 100 U/ml TNF α ; ◇ 100 U/ml IFN γ + 1000 U/ml TNF α . **B** Flow cytometric analysis of I-A expression on insulinoma cells after 3 days of culture. I-A expression was not detected in untreated MIN6N8 cells or those treated with 1000 U/ml TNF α alone. However, 100 U/ml IFN γ induced detectable expression of I-A on insulinoma cells, which was further increased by cotreatment with 1000 U/ml TNF α .

ied whether the reported preventive effect of nicotinamide on the development of Type I diabetes mellitus could be explained by its effect on apoptosis or MHC expression. MTT assays disclosed that 5 to 20 mmol/l nicotinamide did not affect the death of MIN6N8 by 100 U/ml IFN γ in combination with 1000 U/ml TNF α (Fig. 7), suggesting that the preventive effect of nicotinamide is not related to its possible effect on the effector phase of islet-cell death. We further studied if nicotinamide could affect the effector phase of islet-cell death by employing an in vivo adoptive transfer model. Daily administration

Fig. 3. The expression of I-A in MIN6N8 cells detected by RNase protection assays. I-A mRNA was detected in MIN6N8 cells treated with IFN γ or IFN γ and TNF α combination, which was inhibited by nicotinamide. Lane (1) media only, (2) 100 U/ml IFN γ , (3) 1000 U/ml TNF α , (4) 100 U/ml IFN γ + 1000 U/ml TNF α , (5) 100 U/ml IFN γ + 20 mmol/l nicotinamide, (6) 1000 U/ml TNF α + 20 mmol/l nicotinamide, (7) 100 U/ml IFN γ + 1000 U/ml TNF α + 20 mmol/l nicotinamide.

Fig. 4. The induction of *CIITA* in MIN6N8 cells detected by RNase protection assays. *CIITA* mRNA was detected in MIN6N8 cells treated with IFN γ or IFN γ and TNF α combination, which was also inhibited by nicotinamide. Lane (1) media only, (2) 100 U/ml IFN γ , (3) 1000 U/ml TNF α , (4) 100 U/ml IFN γ + 1000 U/ml TNF α , (5) 100 U/ml IFN γ + 20 mmol/l nicotinamide, (6) 1000 U/ml TNF α + 20 mmol/l nicotinamide, (7) 100 U/ml IFN γ + 1000 U/ml TNF α + 20 mmol/l nicotinamide.

of 0.5 mg/g nicotinamide did not inhibit diabetes mellitus after adoptive transfer of diabetogenic lymphocytes (Fig. 8), again suggesting that nicotinamide does not affect the effector phase of the development of diabetes mellitus by transferred diabetogenic lymphocytes and nicotinamide inhibits diabetes mellitus development probably through its effect on MHC expression.

Fig. 5. The induction of phosphorylated STAT1 (p-STAT1) and IRF-1 by cytokines. Phosphorylation of STAT1 and induction of IRF-1 were observed in IFN γ -treated MIN6N8 cells, which were not affected by TNF α or nicotinamide. Lane (1) media only, (2) 100 U/ml IFN γ , (3) 1000 U/ml TNF α , (4) 100 U/ml IFN γ + 1000 U/ml TNF α , (5) 100 U/ml IFN γ + 20 mmol/l nicotinamide, (6) 1000 U/ml TNF α + 20 mmol/l nicotinamide, (7) 100 U/ml IFN γ + 1000 U/ml TNF α + 20 mmol/l nicotinamide

Fig. 6. The effect of nicotinamide on IFN γ and TNF α -induced I-A expression. I-A expression was examined by flow cytometry after 3 days of culture in the presence of cytokine(s) with or without nicotinamide. The data are means $\pm$ SEM from three independent experiments. * p < 0.05 compared with untreated controls. I, IFN γ (U/ml); T, TNF α (U/ml). ■ 0 mmol/l nicotinamide; ▨ 5 mmol/l nicotinamide; □ 20 mmol/l nicotinamide

Discussion

In this study on the induction of MHC class II molecules by a cytokine combination, which also induces apoptosis on islet cells and insulinoma cells, insulinoma cells were employed instead of primary islet cells to avoid controversy regarding the source of MHC class II molecules. Previous studies showing the aberrant expression of MHC class II molecules did not clearly demonstrate their expression on islet cells because macrophages could not be ruled out as a source of MHC class II molecules [5, 31, 32]. MIN6N8 cells are representative insulinoma cells of NOD origin that produce and secrete insulin according to ambient glucose concentrations. Our results indicate that MIN6N8 insulinoma cells do not constitutively express MHC class II molecules and IFN γ alone induces MHC class II molecules in only a small percentage of the insulinoma cells. The addition of TNF α in-

Fig. 7. The effect of nicotinamide on MIN6N8 cell death by IFN γ and TNF α . Coculture with nicotinamide for 3 days did not reverse the cytotoxic effect of IFN γ /TNF α . Nic, Nicotinamide. The data are means $\pm$ SEM from three independent experiments. □ media; ■ 100 U/ml IFN γ + 1000 U/ml TNF α

Fig. 8. The effect of nicotinamide on the development of diabetes after adoptive transfer of diabetogenic splenocytes. The incidence of diabetes after adoptive transfer was not decreased by nicotinamide administration, compared to that in control mice treated with PBS. □ control (n = 7); ● nicotinamide (n = 7)

creased the expression of MHC class II molecules to a well detectable range. The expression of I-A molecules on MIN6N8 cells after treatment with IFN γ and TNF α combination was not due to non-specific adsorption of antibodies to dead insulinoma cells because most apoptotic cells after treatment with IFN γ and TNF α combination detach from coverslips, making it almost impossible to detect apoptotic cells on coverslips by Hoechst 33342 staining or by other methods (data not shown). We have detected apoptotic insulinoma cells after cytokine treatment mostly in the culture supernatant [3]. Our observation that nicotinamide reduced the expression of MHC class II molecules but did not affect insulinoma cell death by IFN γ and TNF α synergism also supports that the

expression of MHC class II molecules by IFN γ and TNF α synergism is not related to apoptosis of insulinoma cells by the cytokine combination. These results are consistent with a previous result employing primary mouse islets [5] or human islet cells [4, 8, 33]. In fact, the synergism between IFN γ and TNF α combination has been reported in a variety of biological phenomena besides induction of MHC expression [34]. Most notably, IFN γ /TNF α induces apoptosis of pancreatic beta cells in vitro and probably in vivo, leading to diabetes mellitus [3, 35, 36]. In our previous effort to identify the effector molecules in autoimmune diabetes mellitus, we found that IFN γ and TNF α but not each cytokine on its own, synergistically exerted caspase-dependent apoptosis on insulinoma cells and primary pancreatic islet cells. IFN γ and TNF α synergism in islet beta-cell death seems to be applicable in vivo because death of islet cells expressing IFN γ -inducible STAT1 or IRF-1 by adoptive transferred diabetogenic lymphocytes was inhibited by anti-TNF α antibody [3]. Activated CD4 + T lymphocytes, a major source of IFN γ , could act in collaboration with macrophages as a major source of TNF α for beta-cell death through DTH-like bystander killing in vivo [20, 37]. Thus, IFN γ and TNF α synergism seem to have a dual effect on the course of autoimmune diabetes mellitus: firstly, through the apoptosis of pancreatic beta cells; and secondly through the induction of MHC class II molecules. Previous reports have shown that transgenic expression of MHC class II molecules on pancreatic beta cells could not initiate autoimmunity [15, 16]. However, the effect of constitutive transgenic expression of MHC class II molecules from birth can be different from that of their de novo expression. It is also possible that non-lymphoid cells with aberrant MHC class II molecules contribute to the continuation or acceleration or both of autoimmune responses by enhancing T-cell-mediated recognition of their own antigens, even if they do not initiate autoimmunity [38]. Simple recognition of antigens by TCRs and further stimulation of 'activated' T cells could require much less stringent conditions than the initial sensitization of naive lymphocytes. Aberrant MHC class II molecules might also enhance apposition of islet cells and effector T cells which would lead to an increased chance of bystander killing by cytokines. Besides IFN γ and TNF α , death effector molecules such as perforin and granzyme [39–41] or Fas ligand could play a role in the early stage of diabetes mellitus development, although they might not be the dominant death effector molecules for the majority of CD4 + effector cells [1, 42]. Whatever the real in vivo death effector molecules might be, all such death effector molecules are antigen-non-specific whether they are on the cell surface or secreted (or cleaved). In this context, recognition of MHC class II molecules on islet beta-cells by T cells might facilitate ap-

proximation between islet cells and T cells and increase the chance of bystander target cell death by cytokines or the chance of target cell killing by membrane-bound death effector molecules such as perforin.

The expression of MHC class II molecules on islet cells by cytokine combination seems to be due to the induction of CIITA. *MHC class II* gene expression in immune cells are regulated by two factors known as CIITA and a Y box-binding protein [43, 44]. CIITA is a non-DNA-binding protein transactivator that functions as a molecular switch to control constitutive and IFN γ -inducible *MHC class II* gene expression in immune cells [44–49]. This report demonstrates that CIITA is induced by IFN γ treatment of MIN6N8 cells with or without TNF α indicating that CIITA is also involved in IFN γ -induced aberrant *MHC class II* gene expression in non-lymphoid cells as well as in lymphoid cells.

TNF α was shown to increase *I-A^{g7}* and *CIITA* mRNA induction by IFN γ . These results suggest that TNF α increases IFN γ -induced MHC class II expression by enhancing the expression of CIITA transcriptional factor. However, TNF α did not affect phosphorylation of STAT1 or the induction of IRF-1, two transcriptional factors binding to CIITA promoter [50]. Further studies will be necessary to clarify the mechanism of TNF α -mediated augmentation of *CIITA* transcription.

Previous reports demonstrated that in vitro treatment with high doses of nicotinamide protects islet cells against cytotoxicity by cytokines [51]. Furthermore, nicotinamide was effective in preventing diabetes mellitus in NOD mice and possibly in human Type I diabetes mellitus [24, 25, 52]. Nicotinamide has also been shown to reduce the downstream signals of IFN γ such as IRF-1 in isolated rat pancreatic islets [22]. Thus, it seemed reasonable to hypothesize that nicotinamide inhibits IFN γ -induced STAT1/IRF-1 activation necessary for TNF α -mediated apoptosis, leading to the inhibition of diabetes mellitus. However, nicotinamide did not reduce IFN γ -induced STAT1 or IRF-1 expression on insulinoma cells, contrary to previous reports [22]. It also failed to reduce islet cell death by cytokines in vitro and the incidence of diabetes mellitus after adoptive transfer in vivo. This discrepancy could be due to the difference in cell types employed. Instead, nicotinamide reduced the induction of CIITA and *I-A^{g7}* by IFN γ , which could explain how nicotinamide reduces the incidence of diabetes mellitus in humans and animal models of diabetes mellitus [5, 53]. This effect of nicotinamide on the expression of MHC class II molecules seems to be independent of its effect on STAT1 phosphorylation or IRF-1 induction because nicotinamide did not affect the activation/induction of STAT1 and IRF-1 while *CIITA* promoter has STAT1 or IRF-1 binding sites [50]. However, the inhibitory

effect of nicotimamide on the expression of I-A^{g7} might not be directly linked to the prevention of diabetes by nicotinamide because nicotinamide could have multiple actions at different levels of the immune or endocrine system and the immunological significance of aberrant MHC class II molecules on non-lymphoid cells is not well understood. Furthermore, our results regarding the mechanism of nicotinamide action were mostly obtained in vitro and might not be directly applicable to in vivo circumstances.

Taken together, the above findings suggest that IFN γ and TNF α synergistically induce MHC class II expression on insulinoma cells through the induction of CIITA as well as apoptosis; that nicotinamide reduces the expression of cytokine-induced MHC class II expression on insulinoma cells through its effect on CIITA expression; that the preventive effect of nicotinamide on Type I diabetes mellitus is probably due to its effect on MHC expression but not on islet cell apoptosis.

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