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NK3-Like NK Cells Are Involved in Protective Effect of Polyinosinic-Polycytidylic Acid on Type 1 Diabetes in Nonobese Diabetic Mice

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Type 1 diabetes in NOD mice is characterized by the uncontrolled Th1 immune responses and deficiency of regulatory or suppressor cells. Previous studies have shown that NOD mice treated with polyinosinic-polycytidylic acid (poly(I:C)) have a markedly reduced incidence of diabetes, but the underlying mechanisms remain unclear. In this study, we report that the prevention of diabetes by poly(I:C) is associated with the formation of Th2-enriched environment in spleen and pancreas. Further, we show that the prevention of diabetes and the formation of Th2-enriched environment depend on the presence of NK cells. Long-term poly(I:C)-treated NK cells exhibit a NK3-like phenotype, and are involved in the induction of Th2 bias of spleen cells in response to islet autoantigens via TGF-β-dependent manner. Therefore, NK cells mediate the protective effect of poly(I:C) possibly through the promotion of Th2 bias of immune responses. These findings suggest that NK cells can participate in the regulation of autoimmune diabetes.


Type 1 diabetes (T1D) is an autoimmune disease in which pancreatic islet β cells are destroyed by the immune system (1). The NOD mice spontaneously develop an autoimmune disease that strongly resembles human T1D. T1D is characterized by dysregulation of the immune system, including the deficiency of regulatory cells and the predominance of autoimmune Th1 immune responses (2). It is conceivable that enhancement of the function of regulatory cells will control the dysregulation of Th1 immune responses and prevent the onset of T1D. Indeed, several types of regulatory cells are implicated in the prevention of diabetes, including NKT cells, CD4+CD25+ T cells, and CD4+CD62L+ T cells (2–6).

Increasing evidence from human autoimmune diseases and animal models suggests the involvement of NK cells in immunoregulation or pathogenesis of autoimmune diseases (7, 8). However, the role of NK cells in T1D remains obscure. Several studies have documented that the decrease of NK cell number and impairment of NK cell function occur not only in NOD mice, but also in the peripheral blood of patients with T1D (9–11). The NOD.NK1.1 mice, which exhibit improved NK cell performance, have a reduced disease incidence (12). This is consistent with a recent study in which NK cells were implicated in the prevention of autoimmune diabetes mediated by CFA (13). However, in another study, NK cells were involved in the islet cell destruction in the BDC 2.5 TCR transgenic model in the C57BL/6 background (14).

Previous study has shown that NOD mice treated with polyinosinic-polycytidylic acid (poly(I:C)) have a markedly reduced incidence of diabetes, but the mechanisms remain unclear (15). Because poly(I:C) is a well-known activator of NK cells, we investigated the role of NK cells and found that NK cells played a critical role in this prevention.

Materials and Methods

Mice

NOD/lt mice (female or male, 6 wk old), C57BL/6 mice (female, 6 wk old), BALB/c mice (female, 6 wk old), and NOD-SCID mice (female, 4–6 wk old) were purchased from Shanghai Experimental Animal Center and maintained under specific pathogen-free conditions. Handling of mice and experimental procedures were conducted in accordance with experimental animals’ guidelines of University of Science and Technology of China and also with the laws on animal prevention of Anhui Province and China.

Reagents

Polyclonal rabbit anti-AsGM1 Ab and rabbit IgG were purchased from Wako Pure Chemical (16). Poly(I:C) sodium and Con A (Sigma-Aldrich) were dissolved in pathogen-free saline. Insulin was purchased from Sigma-Aldrich. Anti-CD3 PE-CY5 (145-2C11), anti-CD4 FITC (RM4-5), anti-DX5 PE (DX5), anti-DX5 FITC (DX5), anti-IL-4 PE (11B11), anti-IFN-γ PE (XMG1.2), anti-IL-4 neutralizing Ab (11B11), anti-IL-5 neutralizing Ab (TRFK5), and anti-IL-10 neutralizing Ab (JES5-16E3) were purchased from eBioscience. Anti-IL-5 PE (TRFK5) and anti-IL-10 PE (JES5-16E3) were purchased from BD Pharmingen. Biotinylated goat anti-mouse IL-13, biotinylated chicken anti-TGF-β1, anti-mouse IL-13 (38213), and anti-mouse TGF-β1(1D11) neutralizing Ab were purchased from R&D Systems.

In vivo treatments

For prevention of spontaneous T1D or the late treatment protocol, 50 μg/mouse poly(I:C) or control PBS was injected i.p. twice each week from 6 to 12 wk of age. For NK depletion in vivo, NOD mice were injected i.v. with 20 μl of anti-AsGM1 Abs or control rabbit IgG (the first injection of anti-AsGM1 Ab was 1 day before the first injection of poly(I:C)) once every 5 days from 6 to 12 wk of age. The elimination of NK cells in mice was confirmed by flow cytometry, and the majority (>95%) was depleted.
Evaluation of diabetes

Diabetes was assessed by monitoring glucose levels in the blood every week using SUPER GLUCOCARD II (ARKRAY) blood glucose meter. Mice with two consecutive blood glucose measurements \(>16.6\) mmol/L were considered diabetic.

ELISA

The serum or culture supernatant samples were kept at \(-80^\circ\text{C}\) until ready for cytokine or IgE measurements. Levels of IL-4, IFN-\(\gamma\), IL-5, IL-10, IL-13, and TGF-\(\beta\) were measured using commercially available ELISA kits from R&D Systems. IgE was measured using commercially available ELISA kits from RapidBio Laboratory.

Staining of intracellular cytokines

Mononuclear cells or purified NK cells were stimulated with PMA (30 ng/ml; Sigma-Aldrich) and ionomycin (1 \(\mu\)g/ml; Sigma-Aldrich). One hour later, monensin (5 \(\mu\)g/ml; Sigma-Aldrich) was added to prevent the secretion of induced cytokines into the supernatants. After 4 h of culture at \(37^\circ\text{C}\) and \(5%\) CO\(_2\), the cells were harvested and labeled by anti-CD4 FITC mAb (for staining of CD4\(^+\) T cells) or anti-CD3 PE-CY5 and anti-DX5 FITC (for staining of NK cells) for 30 min at 4\(^\circ\text{C}\), and fixed with PBS containing 2\% (w/v) paraformaldehyde for 20 min at room temperature. After being permeabilized with 100 \(\mu\)l of permeabilization buffer (BD Biosciences) containing mouse serum for 1 h at 4\(^\circ\text{C}\), the cells were incubated with anti-IFN-\(\gamma\) PE, anti-IL-10 PE, anti-IL-5 PE, anti-TGF-\(\beta\)-biotinylated, anti-IL-13-biotinylated, or anti-IL-4 PE mAb in permeabilization buffer for 1 h at room temperature. The samples staining for TGF-\(\beta\) or IL-13 were washed with permeabilization buffer twice and then incubated with PE-streptavidin (BD Biosciences) for 30 min. The samples were then washed with permeabilization buffer twice and analyzed by flow cytometry.

Purification of NK cells

NOD mice were pretreated with anti-CD4 (400 \(\mu\)g/mouse) and anti-CD8 (400 \(\mu\)g/mouse) Abs to deplete CD4\(^+\) and CD8\(^+\) T cells. Spleen cells were pooled and then passed through nylon wool columns to enrich NK cells 24 h later. The enriched NK cells were stained with anti-CD3 and anti-DX5 mAbs on ice for 30 min. Cells were washed three times with PBS, and then CD3\(^+\) DX5\(^+\) cells were sorted using a FACSVantage cell sorter (BD Biosciences). The purity of NK cells was confirmed by flow cytometry, and with >95% purified CD3\(^+\) DX5\(^+\) cells.
In vitro stimulation of NK cells

Purified NK cells (5 × 10^5/ml) from the spleen of PBS-treated NOD mice or poly(I:C)-treated NOD mice were incubated with poly(I:C) (25 μg/ml) or PBS in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, antibiotics, and 10 mM HEPES.

**In vitro neutralization assay**

For in vitro neutralization, control IgG, anti-IL-4 Ab (20 μg/ml), anti-IL-5 Ab (20 μg/ml), anti-IL-10 Ab (20 μg/ml), anti-IL-13 Ab (20 μg/ml), or anti-TGF-β Ab (20 μg/ml) was added to the culture.

**Preparation of islet cells**

Pancreata were treated with type IV collagenase (2 mg/ml; Sigma-Aldrich) at 37°C for 30 min with vigorous shaking in PBS supplemented with 5% FCS. Islets were further purified by discontinuous Percoll gradients. Briefly, the islet preparation was resuspended in 4 ml of 11% Percoll, and then layered on 2 ml of 21% Percoll. After centrifugation at 1800 rpm for 10 min, islets were harvested from 21–11% gradient interface. Thereafter, to obtain single cells, islet cells were dissociated by trypsin-EDTA (Invitrogen Life Technologies).

**Intrapancreatic cytokine ELISA**

Pancreata were isolated and snap frozen. Immediately before analysis, tissues were homogenized in antiprotease buffer (Invitrogen Life Technologies; 1 mg/ml). Then the homogenates were centrifuged to remove debris and passed through 1.2-μm filters. The filtrates were analyzed for cytokine concentrations by ELISA.

**Measurement of T cell responses**

For measurement of islet Ag-specific T cell responses, spleen cells (5 × 10^5/ml) from 6-wk female NOD mice were cocultured with mitomycin (Sigma-Aldrich; 50 μg/ml)-pretreated islet cells (10^4) or insulin protein (300 μg/ml) for 72 h at 37°C. As a control, the same number of responder cells was stimulated with Con A (2 μg/ml; Sigma-Aldrich) for 72 h. For evaluation of the inhibitory effects of NK cells on the islet Ag-specific T cell responses of spleen cells, NK (10^5/ml) cells were purified from NOD mice 1 wk after the last injection of poly(I:C) or PBS and then added in the cultures of spleen cells. Cytokines in the supernatants were measured by ELISA.

**Adoptive cell transfer**

Recipient female NOD/SCID mice, 4–6 wk of age, were injected i.v. with donor diabetic spleen cells (2 × 10^7) and purified NK cells (5 × 10^5) from poly(I:C)- or PBS-treated female NOD mice. Diabetic spleen cell donors were female NOD mice that typically exhibited blood glucose levels >16.6 mM for at least 1 wk. Multiple diabetic donor spleens were pooled to yield a sufficient number of cells for experiments.

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**FIGURE 4.** Poly(I:C)-induced prevention of T1D is associated with a Th2-enriched environment in NOD mice. Female NOD mice were treated with a series of poly(I:C), PBS, rabbit IgG plus poly(I:C), or anti-AsGM1 Ab plus poly(I:C) from 6 to 12 wk. Forty-eight hours after the last injection of poly(I:C), the serum IgE levels were determined by ELISA (A). Data shown represent mean ± SEM of five mice within each experimental group. B, The relative levels of cytokines in the pancreas of mice were determined by ELISA. Data shown represent mean ± SEM of five mice within each experimental group. C, Intracellular IL-4 and IFN-γ expression on CD4⁺ spleen cells was shown. Data from one of three representative experiments are shown.

**FIGURE 5.** Decrease of various cytokine expressions of spleen NK cells in NOD mice compared with male NOD mice and BALB/c mice. BALB/c, male NOD, and female NOD mice (n = 5) were administrated with PBS or poly(I:C), and then spleen cells were harvested at 6 h postinjection. Intracellular IL-4, IL-5, IL-10, IL-13, IFN-γ, and TGF-β expression on spleen NK cells was examined. Data from one of three representative experiments are shown.
Statistical analysis
A Student’s t test was used to calculate statistical significance where indicated, and a single factor ANOVA was used for multigroup comparison.

Results

Long-term treatment with poly(I:C) prevents spontaneous T1D in NOD mice

Female NOD mice, 6 wk of age, were treated with poly(I:C) or PBS, and blood glucose levels were monitored weekly between 12 and 30 wk of age. Consistent with previous studies (15), repeated injections of poly(I:C) both delayed and prevented diabetes in NOD mice (Fig. 1).

NK cells are involved in the prevention of T1D by poly(I:C) in NOD mice

Previous studies have documented that decrease of NK cell number and impairment of NK cell function occur in NOD mice (9, 10). Because poly(I:C) is a well-known activator of NK cells, we investigated whether the prevention of T1D in NOD mice by poly(I:C) was due to the effect of poly(I:C) on NK cells. As shown in Fig. 2A, the percentage of NK cells in the spleen of female NOD mice was lower compared with C57BL/6, BALB/c, and male NOD mice. Although long-term treatment with poly(I:C) increased the percentage of NK cells (Fig. 2B), the effect of poly(I:C) lasted only ~2 wk (Fig. 2C). To further investigate the role of NK cells, anti-AsGM1 Ab was injected in NOD mice to deplete NK cells. As shown in Fig. 3A, NK cell depletion abrogated the protective effect of poly(I:C) on T1D. Furthermore, to determine whether poly(I:C)-treated NK cells influenced diabetogenic T cells, we cotransferred spleen cells of untreated diabetic mice into NOD-SCID recipients with NK cells purified from poly(I:C)- or PBS-treated NOD mice. As shown in Fig. 3B, poly(I:C)-treated NK cells also almost prevented and delayed the onset of T1D. These results indicate that NK cells play a critical role in the prevention of T1D by poly(I:C) in NOD mice.

Poly(I:C)-induced prevention of spontaneous T1D is associated with a Th2-enriched environment in NOD mice

Because spontaneous T1D in NOD mice is characterized by uncontrolled Th1 immune responses (2), we then investigated whether poly(I:C)-induced prevention of T1D was associated with the immune deviation. At first, we measured serum IgE (as an indication of Th2 status) and found that long-term poly(I:C) treatment induced a significant increase in serum IgE level (Fig. 4A). Second, we found that the level of IFN-γ was decreased and the level of IL-4 was increased in the pancreas of poly(I:C)-treated NOD mice (Fig. 4B). Third, we examined the cytokine expression of spleen CD4+ T cells. As shown in Fig. 6.
4C, long-term poly(I:C) treatment decreased the expression of IFN-γ and increased the expression of IL-4 on spleen CD4+ T cells. These results indicate that long-term poly(I:C) treatment can modulate adaptive immune responses in NOD mice. Furthermore, our results showed that the effect of poly(I:C) was abrogated when NK cells were depleted with anti-AsGM1 Ab (Fig. 4). These results together demonstrate that poly(I:C)-induced prevention of T1D is associated with a Th2-enriched environment and NK cells are involved in this immune deviation.

**Long-term poly(I:C) treatment promotes spleen NK cells to exhibit a NK3-like phenotype**

Because poly(I:C)-treated NK cells are involved in the prevention of diabetes, we further investigated the effect of long-term poly(I:C) treatment on NK cells. First, we examined various cytokine expressions on NK cells from female NOD mice, male NOD mice, and BALB/c mice. As shown in Fig. 5, spleen NK cells of female NOD mice showed a lower intracellular cytokine expression, but did not exhibit significant bias toward secreting Th2- or Th3-type cytokines, compared with male NOD mice and BALB/c mice. Second, we investigated whether long-term poly(I:C) treatment influenced the cytokine expression of NK cells. NK cells were isolated from long-term poly(I:C)- or PBS-treated NOD mice and then stimulated with poly(I:C) in vitro. As shown in Fig. 6, ELISA and intracellular cytokine staining both showed that long-term poly(I:C)-treated NK cells produced more TGF-β and IL-10, and less IFN-γ than control with the challenge of poly(I:C) in vitro. These results suggest that long-term poly(I:C)-treated NK cells exhibit an induced NK3-like status.

**Poly(I:C)-treated NK cells promote spleen cells to display a bias toward Th2 immune responses in response to islet autoantigens**

To further explain why poly(I:C)-treated NK cells promoted the formation of Th2-enriched environment in NOD mice, we examined the direct effect of poly(I:C)-treated NK cells on responses of spleen cells against autoantigens. Spleen cells, when cocultivated with poly(I:C)-treated NK cells, produced increased amount of IL-4 and decreased amount of IFN-γ in response to the stimulation with pancreatic islet cells or insulin (Fig. 7). We therefore conclude that poly(I:C)-treated NK cells promote spleen cells to display a bias toward Th2 immune responses in response to islet autoantigens.

**TGF-β is involved in the modulation of Ag-specific T cell responses induced by poly(I:C)-treated NK cells**

Because poly(I:C)-treated NK cells significantly produced more TGF-β and IL-10, we examined the possible role of these cytokines in the promotion of Th2 bias of spleen cells in response to autoantigens. Anti-IL-4, anti-IL-5, anti-IL-10, anti-IL-13, or anti-TGF-β neutralizing mAb was added to the spleen cell cultures containing poly(I:C)-treated NK cells. As shown in Fig. 8, neutralization of TGF-β with mAb abolished the Th2 bias in response to autoantigens. Anti-IL-10 mAb also had a slight effect on the IFN-γ production, but little on IL-4 production. Other reagents, such as anti-IL-4, anti-IL-5, and anti-IL-13, had no effect on IFN-γ or IL-4 production. These results indicate that TGF-β plays a critical role in NK-mediated promotion of Th2 bias in response to autoantigens.

**FIGURE 7.** Poly(I:C)-treated NK cells modulate the islet Ag-specific T cell responses of spleen cells. Spleen (SP) cells (pooled from three mice) from female NOD mice were cocultured with the indicated autoantigens or Con A in the presence of purified NK cells (pooled from five mice) from poly(I:C)- or PBS-treated female NOD mice for 72 h. Cytokines in the culture supernatants were measured by ELISA. Data shown represent mean ± SEM of values from triplicate samples. Data from one of three representative experiments are shown.

**FIGURE 8.** Major role of TGF-β in the modulation of Ag-specific T cell responses induced by poly(I:C)-treated NK cells. Spleen (SP) cells (pooled from three mice) from female NOD mice were cocultured with insulin protein in the presence of purified NK cells (pooled from 10 mice) from poly(I:C)-treated female NOD mice for 72 h. Various cytokine-blocking reagents were added to the culture. Cytokines in the culture supernatants were measured by ELISA. Data shown represent mean ± SEM of values from triplicate samples. Data from one of two representative experiments are shown.
Discussion

The results presented in this study demonstrate that NK cells are involved in the formation of Th2-enriched environment and prevention of diabetes induced by poly(I:C), and suggest that NK cells mediate the protective effects of poly(I:C) possibly through the promotion of Th2 bias of immune responses. Long-term treatment with poly(I:C) induces NK cells to exhibit a NK3-like phenotype, and these induced NK3-like cells can promote spleen cells to display a bias toward Th2 immune responses in response to islet autoantigens. These results also suggest that TGF-β is involved in the Th2 bias of immune responses mediated by NK cells.

The role of NK cells in autoimmune diabetes remains controversial (8). On one hand, NK cells exhibit a pathogenic function in the last stage of diabetes in diabetes model induced by anti-CTLA-4 Ab (14). In contrast, NK cells can participate in the control of autoimmune diabetes by CFA (13). In this study, we report that NK cells are involved in the prevention of diabetes induced by long-term poly(I:C) treatment. These results suggest that NK cells play different roles in different stages of diabetes progression, because in poly(I:C)- or CFA-mediated prevention of diabetes, NK cells are activated in the early stage of diabetes.

Long-term poly(I:C)-treated NK cells can prevent the onset of diabetes in NOD mice, but it does not mean that NK cell activation will prevent the diabetes. Long-term poly(I:C)-treated NK cells induce Th2 bias of immune responses (Fig. 7), but activated NK cells by a single treatment of poly(I:C) do not (data not shown). In fact, NK cells from NOD mice do not exhibit significant bias toward secreting Th2- or Th3-type cytokines, compared with male NOD mice, C57BL/6 mice, and BALB/c mice (Fig. 5). However, long-term poly(I:C)-treated NK cells can produce more TGF-β than PBS-treated NK cells when challenged with poly(I:C) in vitro (Fig. 6).

Indeed, NK cells have also been reported to be implicated in other autoimmune diseases (7, 8). The protective or disease-promoting effects of NK cells are dependent on disease model. In patients with multiple sclerosis and lpr mice, which display a phenotype that resembles that of patients with systemic lupus erythematosus, NK cells play a protective role (17, 18). In EAE models, the role of NK cells is controversial, similar to diabetes. Zhang et al. (19) found that NK cells were protective in mouse model. However, Shi et al. (20) found that NK cells contributed to the disease promotion via IFN-γ-dependent manner in the same model.

Current dogma suggests Th cells can be classified into Th1 and Th2 subpopulations, distinguished on the basis of cytokine secretion (21, 22). Recently, other subtypes of T cells, with cytokine profiles distinct from either Th1 or Th2 cells, termed as Th3 cells, Th17 cells, and type 1 regulatory T cells, have been described (23–26). Th3 cells produce TGF-β with or without the production of IL-10 (27). On the contrary, type 1 regulatory T cell-mediated immunosuppression is mediated by the production of IL-10 (28). NK cells mainly produce type 1 cytokines. However, it has been reported that NK cells also produce type 2 cytokines and immunoregulatory cytokines, such as IL-10 and TGF-β (29, 30). Similar to T cells, IFN-γ-producing NK cells are called NK1 cells, whereas type 2 cytokine-producing NK cells are called NK2 cells (31–34). In this study, we suggest that long-term poly(I:C)-treated NK cells exhibit a NK3-like phenotype, which can produce more TGF-β and IL-10, and inhibit Th1 responses to islet autoantigens via TGF-β-dependent manner.

Poly(I:C) is a synthetic dsRNA analog recognized by TLR3 (35). It has been shown that TLR3 is expressed in dendritic cells, NK cells, fibroblasts, and intestinal epithelial cells, and more recently in human T lymphocytes (36–39). NK cells can be activated directly or indirectly (by APCs) in response to poly(I:C) (40–42). Although we found that the TLR3 mRNA levels on NK cells of long-term poly(I:C)-treated NOD mice were ~2-fold higher than those of PBS-treated NOD mice (data not shown), we didn’t know whether poly(I:C) affected the NK cells directly or indirectly following the effects of poly(I:C) on APCs, such as dendritic cells, in vivo.

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Disclosures

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References


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