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Regulation of Autoimmune Diabetes by Complete Freund’s Adjuvant Is Mediated by NK Cells

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Autoimmune (type 1) diabetes results from a loss of β cells that is mediated by self-reactive T cells. Previous studies have shown that a single injection of CFA prevents diabetes in nonobese diabetic (NOD) mice, but the mechanism(s) of protection remain unknown. We show here that NOD mice immunized with CFA have a markedly reduced incidence of diabetes and that this reduced incidence is associated with a decrease in the number of β cell-specific, autoreactive CTL. In addition, the adoptive transfer of diabetes into syngeneic NOD/SCID recipients was prevented by CFA immunization, and the protective effects of CFA were lost when cells expressing the NK cell marker, asialo GM1, were removed from both donor cells and recipient mice. Returning a population of CD3+DX5+ cells to the adoptive transfer restored the protective effects of CFA. Therefore, NK cells mediate the protective effects of CFA possibly through the down-regulation of autoreactive CTL and stimulation of NK cells.

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Materials and Methods

Mice

Female NOD mice were purchased from Taconic Farms (Germantown, NY), and NOD/SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in a specific pathogen-free facility at the animal care unit of the British Columbia Research Institute for Children’s and Women’s Health. The Animal Care Committee, Faculty of Medicine, University of British Columbia approved the care and use of all animals.

CFA immunizations and assessment of diabetes

Unless otherwise indicated, 5-wk-old NOD mice were administered a single 100-μl injection of CFA emulsion (Sigma, St. Louis, MO) in the base of their tails. Hyperglycemia was monitored by testing blood glucose once or twice weekly using test strips (LifeScan, Milpitas, CA). Mice with a blood glucose measurement of greater than 33 mM were considered diabetic and sacrificed.

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Abs, tetramers, and flow cytometry

The following mAbs were purchased from BD PharMingen (San Diego, CA): FITC-conjugated anti-CD3; PE-conjugated DX5; PE-conjugated CD11c; Cy-Chrome-conjugated anti-CD8; Cy-Chrome-conjugated anti-CD4, and PerCP-conjugated B220. The FITC-conjugated anti-CD8 and PE-conjugated CD11b were purchased from Cedarlane (Hornby, ON, Canada). PE-conjugated NRP-V7 tetramer was synthesized as previously described (12). Cells were washed in PBS and incubated with the indicated mAb conjugates for 30 min in a total volume of 25 μL of PBS containing 3% BSA. Cells were washed three times and resuspended in PBS containing 1% FCS and 1% paraformaldehyde. Immunostained cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

Immunostaining of NK cells and islet cells

Peripheral blood was collected from the tails of NOD mice before and 2, 6, and 24 h after CFA or PBS immunization. Following erythrocyte lysis, cells were incubated with Abs to DX5 and CD3 on ice for 30 min and washed with PBS containing 3% BSA twice. Immunostained cells were resuspended in PBS containing 1% FCS and paraformaldehyde and analyzed by flow cytometry as above. Tetramer staining of dispersed islet cells was performed as previously described (12).

Selective depletion of NK and T cells

Depletion of NK cells was performed both in vitro and in vivo using anti-asialo GM1 (Wako Bioproducts, Richmond, VA). In vitro NK cell depletion was performed by complement-mediated cytotoxicity to deplete NK cells. Briefly, 2 × 10⁷ spleen cells from diabetic NOD mice were incubated with a 1:200 dilution of rabbit anti-asialo-GM1 Ab for 1 h at 4°C under constant agitation. The treated spleen cells were washed and incubated with an appropriate dilution of rabbit complement (Sigma, St. Louis, MO) for 1 h at 37°C. After the cells were thoroughly washed, the resulting cell suspension was used as an NK-cell depleted suspension for adoptive transfer experiments. In vivo, NOD/SCID mice were injected i.v. with 50 μL of anti-asialo-GM1 or an equivalent amount of a rabbit serum (Sigma, St. Louis, MO) 1 day before adoptive transfer experiments. The efficacy of such treatment was evaluated using standard YAC-1 cellular cytotoxicity assays and by flow cytometry for NK cell markers. To deplete T cells in vitro, 2 × 10⁶ spleen cells were incubated with 5 μg/mL of Thy-1.2 Ab (BD PhaRingen) for 1 h at 4°C under constant agitation. The cells were then washed and incubated with an appropriate dilution of rabbit complement (Sigma) for 1 h at 37°C. After the cells were thoroughly washed, the resulting cell suspension was used as a T cell-depleted suspension for ELISPOT experiments.

ELISPot assays

ELISPsots were performed in 96-well MAIP S 4510 plates (Millipore, Watford, U.K.) using an IFN-γ ELISPOT kit from BD PhaRingen. ELISPOT membranes were scanned at high resolution and counted by two independent, blinded observers.

Purification of NK cells

Spleen cells were pooled from NOD mice and stained with anti-CD3, -CD8, and -DX5 mAbs on ice for 30 min. Cells were washed for three times with PBS, then CD3− CD8− DX5− cells were sorted using a FACSVantage SE Turbo cell sorter (Becton Dickinson) at the University of British Columbia multiuser flow cytometry facility. Purified cells were incubated in RPMI 1640 medium (Life Technologies, Grand Island, NY) plus 10% of FCS at 37°C overnight to detach Abs. Cells were then washed twice with PBS before adoptive transfer.

Adoptive transfer

Adoptive transfers were performed as previously described (6). Recipient female NOD/SCID mice, 4–8 wk of age, were injected i.v. with donor spleen cells (2 × 10⁶ viable cells) suspended in 200 μL of PBS. Diabetic spleen cell donors were female NOD mice that typically had exhibited blood glucose level greater than 33 mM for at least 1 wk. Multiple diabetic donor spleens were pooled to yield a sufficient number of cells for experiments.

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. A log-rank test was applied to compare survival curves.

Results

CFA immunization prevents diabetes in NOD mice

Female NOD mice, age 5 wk, were immunized with CFA or PBS (n = 10 per group), and blood glucose was monitored twice weekly between 12 and 32 wk of age. In agreement with previous studies (3–6), a single injection of CFA both delayed and prevented diabetes in NOD mice (Fig. 1). By 32 wk of age, nine PBS-injected mice were diabetic compared with three CFA-injected mice (p < 0.001).

CFA immunization prevents the accumulation of β cell-specific CTL in NOD mice

To determine the effect of CFA on a population of autoreactive β cell-specific CTL, the NRP-V7-reactive CTL in NOD mice were quantified following immunization. We have reported previously that NRP-V7-reactive CTL are readily detected in the spleens of prediabetic NOD mice (12). In the present study, female NOD mice (n = 5 per group) were immunized either with CFA or PBS at 5 wk of age and sacrificed between 14 and 18 wk of age. Spleen cells were then analyzed for the presence of NRP-V7-reactive CTL (Fig. 2A). Mice that had been immunized with CFA had a significantly lower proportion of NRP-V7-reactive CTL than PBS-injected mice (p < 0.05, Student’s t test), suggesting that CFA may prevent diabetes by regulating the number of diabetogenic NRP-V7 CTL. This effect was specific to the NRP-V7 CTL, as there was no change in the proportion of total CD8+ cells present in either CFA or PBS injected mice (Fig. 2B).

CFA induces NK cells to accumulate in blood and to secrete IFN-γ

CFA administration has been shown to stimulate IFN-γ secretion of NK cells in C57BL/6 mice (16). To determine the effects of CFA immunization on NK cells in NOD mice, animals were immunized with CFA or PBS and peripheral blood was assayed for the proportion of CD3− DX5− cells before (0) and at 2, 6, and 24 h after immunization (Fig. 3A). In NOD mice injected with CFA, there was a significant accumulation of CD3− DX5− cells, peaking at 6 h postimmunization (p < 0.05, single factor ANOVA). By 24 h, the proportion of CD3− DX5− cells in the peripheral blood had returned to pre-immunization levels. In contrast, there were no significant differences in the proportion of CD3− DX5− cells of PBS-immunized NOD mice. These data suggest that CFA acts to mobilize NK cells shortly after immunization. In addition, spleen cells obtained from mice immunized with CFA were analyzed by IFN-γ ELISPOT assay. Mice were sacrificed 4 h after injection with CFA, and the

FIGURE 1. Effect of CFA on incidence of diabetes in NOD mice. Female male NOD mice (n = 10) received a single injection of CFA (100 μL) into the tail base at 5 wk of age. Control mice (n = 10) received a single injection of PBS. Blood glucose was monitored weekly, and any animal with a reading of ≥33 mM was considered diabetic.
and the error bars refer to the SD generated from the repeated assays.

We hypothesized that CFA immunization may stimulate total number of CD8\(^+\) H11001/H11005 cells by increasing IFN-\(\gamma\) (16). Last, investigators have reported that CFA activates NK cells (DX5). Fig. 4A indicates that asialo GM1 would alter the outcome of adoptively transferred disease in NOD mice incubated with YAC-1 cells, cells from CFA-immunized mice incubated with PMA (5 ng/ml) and ionomycin (0.4 \(\mu\)g/ml), YAC-1 cells in medium alone, and T-depleted spleen cells from CFA-immunized mice in medium alone (CFA/SC). C. Summary of ELISPOT data. The mean number of spots produced by T-depleted spleen cells obtained from CFA-or PBS-immunized NOD mice is shown as spot forming units (SFU) per 5 \(\times\) 10\(^3\) cells (*, \(p < 0.01\)).

**FIGURE 2.** Effect of CFA on \(\beta\) cell-specific CTL. Female NOD mice were given a single injection of CFA (\(n = 20, 100 \mu l\)) or PBS (\(n = 20, 100 \mu l\)) at 5 wk of age. The mice were sacrificed at 14–18 wk of age, and the NRP-V7-reactive/CD8\(^+\) spleen cell population was determined (A). The total number of CD8\(^+\) cells in spleen was measured concurrently (B). *, \(p < 0.05\). The results are the mean value of five independent experiments, and the error bars refer to the SD generated from the repeated assays.

**FIGURE 3.** Effect of CFA on peripheral blood NK cells and IFN-\(\gamma\) secretion. A, Female NOD mice were given a single injection of CFA (\(n = 7, 100 \mu l\)) or PBS (\(n = 3, 100 \mu l\)) at 8 wk of age. Peripheral blood was collected before and at 2, 6, and 24 h after CFA or PBS injection. The NK cell population was determined by staining mononuclear cells with Abs to CD3 and DX5 surface markers. *, \(p < 0.05\). B, Female NOD mice were given a single injection of CFA (\(n = 7, 100 \mu l\)) or PBS (\(n = 6, 100 \mu l\)) and sacrificed 4 h after injection. Spleen cells were depleted of T cells before performing IFN-\(\gamma\) ELISPOT assays (500,000 effector cells per well and 10,000 YAC-1 target cells per well). Representative wells are shown (from left to right): T cell-depleted spleen cells from CFA- or PBS-immunized NOD mice incubated with YAC-1 cells, cells from CFA-immunized mice incubated with PMA (5 ng/ml) and ionomycin (0.4 \(\mu\)g/ml), YAC-1 cells in medium alone, and T-depleted spleen cells from CFA-immunized mice in medium alone (CFA/SC). C. Summary of ELISPOT data. The mean number of spots produced by T-depleted spleen cells obtained from CFA-or PBS-immunized NOD mice is shown as spot forming units (SFU) per 5 \(\times\) 10\(^3\) cells (*, \(p < 0.01\)).

CFA protection from diabetes is mediated by NK cells

The mechanism by which CFA prevents diabetes in NOD mice has been unclear, although the above data suggest that down-regulation of a population of autoreactive CTL or stimulation of NK cells may play a role. A previous study has suggested that the protective effect of CFA is dependent on a population of CD11b\(^+\) (Mac-1\(^+\)) spleen cells (4), but the identity of the CD11b\(^+\) cells was unknown. We hypothesized that CFA immunization may stimulate NK cells to mediate the protective effect. First, our data indicated a rapid effect of CFA on a CD3\(^-\)DX5\(^+\) subset of spleen cells (Fig. 3). Second, investigators have reported that CFA activates NK cells by increasing IFN-\(\gamma\) secretion (16). Last, NOD mice have been shown to carry defects in NK cell activity (14, 15). To ascertain a role for NK cells in the protective effect of CFA, we used an adoptive transfer model of diabetes. Spleen cells from diabetic NOD mice have previously been shown to passively transfer diabetes to irradiated NOD mice (17), and CFA has been shown to inhibit the transfer of diabetes (6). We designed experiments to transfer diabetes to NOD/SCID mice, to prevent the transfer of disease by CFA immunization and to determine the effect of NK cell depletion on diabetes outcome. To separate the effects of NK cells thoroughly from the experiment, we treated both NOD donor cells and NOD/SCID recipient mice with anti-asialo GM1. It was necessary to first determine the phenotypic specificity of this Ab by costaining spleen cells from NOD mice and NOD/SCID mice with anti-asialo GM1 Ab and markers for T cells (CD4 or CD8), macrophages and dendritic cells (DC; CD11b or CD11c), and for NK cells (DX5). Fig. 4A indicates that asialo GM1 is predominantly expressed by a population of CD11b\(^+\)DX5\(^+\) spleen cells. Thus, as has been previously described (18), asialo GM1-positive cells are NK cells that co-express DX5 and CD11b. In addition, because asialo GM1 is also expressed on a subset of activated CD8 T cells, we costained the (activated) \(\beta\) cell-specific CTL present in diabetic spleen cells with both NRP-V7 tetramer and CD8. Following anti-asialo GM1 depletion of spleen cells, there was no difference in the proportion of NRP-V7 cells present (Fig. 4B).

We next investigated whether NK cell depletion by anti-asialo GM1 would alter the outcome of adoptively transferred disease in
mice immunized with CFA. Recipient NOD/SCID mice were pretreated with either anti-asialo GM1 Ab (to deplete NK cells) or rabbit serum (control), then immunized with CFA after 24 h. Donor spleen cells pooled from diabetic NOD mice were also depleted of NK cells using asialo GM1 Ab. After NK cell depletion, spleen cells were i.v. injected to the NOD/SCID recipients, and the blood glucose of recipients was measured once weekly beginning on the day of adoptive transfer.

All PBS-immunized NOD/SCID recipients receiving spleen cells developed diabetes within 5 wk of adoptive transfer (Fig. 5). In contrast, the group of adoptively transferred mice that were immunized with CFA did not develop disease until between 6 and 10 wk following adoptive transfer, with two (33%) mice remaining diabetes-free beyond 10 wk ($p < 0.0001$, log-rank test). However, CFA-immunized mice pretreated with anti-asialo GM1, which received anti-asialo GM1-depleted spleen cells, developed hyperglycemia between 3 and 4 wk posttransfer. To confirm the specificity of the anti-asialo GM1 effect, a group of NOD/SCID recipients was pretreated with rabbit IgG and subsequently injected with CFA before the adoptive transfer of diabetic spleen cells. These mice developed diabetes at the same rate as mice treated with CFA alone (data not shown).

To verify that NK cells and not NKT cells expressing asialo GM1 were mediating the effects of CFA, a population of CD3+DX5+ cells (5 x 10^7 cells) obtained by sorting NOD spleen cells was returned to the anti-asialo GM1-treated donor spleen cells before adoptive transfer. These cells were greater than 99% CD3+DX5+ and thus did not include any NKT cells. The protective effect of CFA was restored in mice that received a “put-back” of CD3+DX5+ cells, with the onset of hyperglycemia delayed beyond 8 wk. This result was highly significant when compared with mice that did not receive a put-back of CD3+DX5+ cells ($p < 0.005$, log-rank test).

FIGURE 5. Prevention of diabetes by CFA is dependent on a population of asialo GM1-positive cells. Pooled spleen cells from diabetic NOD mice (2 x 10^7 cells) were adoptively transferred to NOD/SCID recipient mice that were immunized with PBS (■, n = 8), CFA (■, n = 8), or with CFA after depletion of NK cells from donor cells and recipient mice with anti-asialo GM1 (○, n = 10). Recipient mice immunized with CFA were pretreated with either 50 ml of rabbit anti-asialo GM1 Ab or rabbit serum IgG before adoptive transfer. One group of NOD/SCID recipient mice that was also pretreated with 50 ml of asialo GM1 Ab and immunized with 100 ml of CFA received adoptive transfer with asialo GM1-depleted diabetogenic spleen cells mixed with 5 x 10^6 CD3+DX5+ spleen cells (○, n = 4). Blood glucose was monitored weekly for all experiments, and diabetes was defined as a single reading of ≥33 mM.
CFA protection from diabetes is associated with a decrease in islet β cell-specific CTL

To determine whether the prevention of diabetes was associated with a decrease in the number of autoreactive T cells, the proportion of β cell-specific CTL in the islets of NOD/SCID adoptive transfer recipients was determined. Two weeks after adoptive transfer, islet cells were isolated from recipient mice that were pretreated with PBS, CFA, or CFA plus anti-asialo GM1 and stained with the NRP-V7 tetramer. The results show that CFA-immunized recipients had a lower proportion of NRP-V7-reactive CTL in islets than recipients that had not received CFA or that had received both CFA and asialo-GM1 depletion (Fig. 6). Together, these results indicate that CFA prevents diabetes by down-regulating autoreactive CTL and that NK cells play a central role in mediating these effects.

Discussion

In this study, we confirm that diabetes in NOD mice is delayed or prevented by a single injection of CFA into prediabetic mice. In addition, delay of disease is associated with the down-regulation of a major population of β cell-specific, autoreactive CTL. This latter finding was not unexpected as this population of NRP-V7-reactive cells has been shown to be an important predictor for the progression of disease in NOD mouse (12). Our experiments also indicate that CFA exerts a rapid stimulatory effect on NK cells and that a population of asialo GM1-expressing cells, likely NK cells, mediates protection from disease. The identity of these cells is supported by our experiment in which returning a population of CD3−DX5+ spleen cells to NOD/SCID mice restored the protective effects of CFA (Fig. 5). In addition, other reports show that CD11b-enriched cells increase in numbers after CFA immunization and that co-adoptive transfer of diabetic NOD spleen cells with a population of CD11b+ cells reduces the incidence of diabetes (4). Our staining results confirm that asialo GM1-positive NK cells express CD11b as well as DX5. Nonetheless, these experiments cannot specifically exclude an additional role for NKT cells.

Immunization of NOD mice with CFA produced an expansion of CD3−DX5+ cells in peripheral blood as early as 6 h after receiving a single administration of CFA. (The same pattern was also seen in spleen cells, data not shown.) While other groups have reported that BCG stimulates NK cells to proliferate (19), it is more likely, given the rapid accumulation of NK cells, that the increased proportion of cells seen following CFA immunization represents a trafficking phenomenon, rather than NK cell proliferation. Because CFA was administered s.c. in the tail base, NK cells may have been responding and trafficking from the spleen to the tail in response to bacterial stimulation. Nevertheless, in addition to cell mobilization, our results indicate that NK cells were stimulated by CFA injection to secrete IFN-γ, whereas PBS immunized were not, despite an equivalent number of splenic NK cells.

This finding suggests that a defect of IFN-γ secretion may exist in NOD NK cells, which may be overcome by administration of CFA, but additional experiments will be required to confirm this.

How might NK cells be stimulated by CFA? We suggest that the effects of CFA immunization may be mediated first by APCs, in particular DC and possibly by the intermediary actions of NKT cells. There are several lines of evidence to support this model. First, DC by virtue of their anatomical locations (mucosal and epithelial Ag exposure sites) and expression of innate receptors are one of the first cells to recognize and respond to foreign stimuli including vaccines and mycobacterial Ags (20, 21). Second, DC are known to express CD1d and to activate NKT cells in a CD1-dependent fashion (22). Finally, activated NKT cells rapidly “cross-talk” to stimulate NK cells, primarily through the secretion of IFN-γ (23). Moreover, NK cells themselves have recently been shown to directly suppress the afferent limb of the primary immune response (24), providing a potential mechanism by which NK cells may limit the expansion of β cell-specific CTL.

Alternatively, DC may activate NK cells directly without the need for a NKT cell intermediary, possibly through the secretion of IL-12 (25). For instance, Gerosa et al. have shown that in the presence of inflammatory mediators such as LPS or mycobacteria, human NK cells (CD3−CD16−CD56−) were activated by DC (26). Because NK cells are known to be both functionally and numerically deficient in NOD mice (as well as NOD/SCID mice) (14, 15, 27), we hypothesize that autoimmunity in these mice may be partly a consequence of the inability of NK cells to regulate DC. To address the mechanism by which NK cells fail to mediate CTL regulation, additional experiments focusing on the interaction of DC with NK and NKT cells in NOD mouse will be needed. Interestingly, defects in NK cells have previously been suggested in humans with type 1 diabetes (28–30). However, these observations have not recently been pursued in the context of current knowledge, and it may also be worthwhile to revisit these observations in light of our findings.

In summary, we report that a single administration of CFA decreases a major population of β cell-specific CTL resulting in the prevention or delay of diabetes. Moreover, removal of NK cells abolishes the protective effects of CFA while restoration of NK cells returns its protective effects, indicating that protection from disease is mediated by NK cells. Targeting NK cells represents a novel approach to the prevention of type 1 diabetes.

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References


