In vivo apoptosis of diabetogenic T cells in NOD mice by IFN-γ/TNF-α

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Abstract

Immunization with mycobacterial preparation such as Bacille Calmette–Guérin (BCG) or complete Freund’s adjuvant (CFA) prevents the onset and recurrence of type 1 diabetes in non-obese diabetic (NOD) mice. In this study, we explored the mechanism underlying the down-regulation of diabetogenic T cells by BCG treatment. We found that the potential of splenocytes from BCG-immunized diabetic NOD mice to adoptively transfer diabetes was significantly impaired. BCG immunization sequentially induced the production of TNF-α, IFN-γ and IL-4 by splenocytes, increased the expression of Fas high (Apo-1/CD95), Fas ligand (FasL, CD95L) and TNF receptor (TNFR) on T cells leading to T cell apoptosis. The primary role of IFN-γ and TNF-α in BCG-immunotherapy was demonstrated by (i) reversing the immune regulatory effect of BCG by in vivo treatment with neutralizing anti-cytokine antibodies, (ii) inducing effect similar to BCG by treatment with these cytokines. We show that Fas and TNF are two pathways in BCG-induced apoptosis of diabetogenic T cells, since in vitro blocking FasL or TNFR1 with antibody reduced T cell apoptosis and increased T cell proliferative response. In addition, TNF-α and agonistic anti-Fas antibody had a synergistic effect on the in vitro apoptosis of diabetogenic T cells. Our results suggest that BCG down-regulates destructive autoimmunity by TNF-α/IFN-γ-induced apoptosis of diabetogenic T cells through both Fas and TNF pathways. These studies provide a novel mechanism for blocking disease recurrence and immune modulating effect of BCG immunization in type 1 diabetes.

Introduction

We have previously shown that adjuvant containing mycobacteria such as CFA or BCG effectively prevents spontaneous diabetes (1–3), induced diabetes (4,5) and recurrence of diabetes (6,7) in NOD mice and Bio Breeding-Diabetes prone (BB-Dp) rats. BCG-induced protection against type 1 diabetes is attributed to the down-regulation of diabetogenic T cells both at the induction and effector phases of the disease. This treatment also induces regulatory cells that are sensitive to cyclophosphamide (8). CFA or BCG treatment in NOD mice has been shown to induce non-destructive insulitis (9). It is well established that a switch from Th1 to Th2 phenotype protects NOD mice from diabetes (10–12). Recent studies on NOD mice with cytokine gene deletions indicate that the immune response and cytokine switch after CFA or BCG therapy is probably an outcome rather than the cause of disease prevention (13).

In mice, the patterns of cytokine production after infection or immunization with mycobacteria are dependent on many factors, such as the route, the nature of mycobacteria and the mouse strains used (14,15). Both TNF-α and IFN-γ are the major Th1 cytokines produced early after mycobacterial infection, and this is followed by Th2-type cytokine production (16,17). We have reported that the production of IL-4 after BCG immunization in syngeneic islet transplanted NOD mice occurs late and is maintained (12). IFN-γ has been found to induce apoptosis of activated CD4 T cells in mice infected with mycobacteria (18). It also inhibits the development of diabetes by down-regulating anti-islet effector cells (19). In addition, the exacerbation of autoimmune encephalomyelitis in IFN-γ deficient mice is due to the failure of T cell apoptosis (20). Similarly, TNF-α has been shown to induce apoptosis in mature T cells (21) and in diabetogenic T cells of diabetic NOD mice (22). TNF-α suppresses spontaneous diabetes in NOD mice when given late but not early during the development of disease (23–25). Therefore, Th1-like cytokines might be a primary factor for CFA or BCG-induced down-regulation of
T cell apoptosis in BCG-immunized diabetic NOD mice

de destructive autoimmunity by activation-induced cell death (AICD) of diabetogenic T cells.

Fas–FasL pathway is well recognized as an efficient way to induce the apoptosis of activated Th1 and cytotoxic CD8 T cells (26–28). Moreover, TNF-α induces T cell apoptosis through TNFR, which plays a pivotal role in maintaining immune privilege of the eye through FasL-induced cell death promoted by TNF-α (29). In T cell receptor transgenic mice, it has been shown that both Fas and TNF are involved in AICD (30). Therefore, we postulate that Th1 cytokines may contribute to the deletion of diabetogenic T cells in BCG therapy by AICD through both Fas and TNF pathways.

In this study, the mechanism of BCG-immunization to down-regulate diabetogenic T cells in the spleen of diabetic NOD mice was determined. We investigated the splenocyte phenotype, T cell apoptosis, expression of Fas-FasL and TNFR, and the production of cytokines. Our study shows that the down-regulation of destructive autoimmunity against islet β cells in diabetic NOD mice by BCG immunization is due to TNF-α and IFN-γ-induced apoptosis of diabetogenic T cells through both Fas and TNF pathways.

Methods

Mice

NOD/LTJ mice were bred in specific pathogen-free condition at the Robarts Research Institute animal facilities (London, Canada). In this study, acutely diabetic NOD mice were defined by blood glucose level >16.5 mM for three consecutive days, and were used within 1 week. For adoptive transfer studies, 7-week-old NOD mice (irradiated for 750 rads 2 h before cell transfer) and NOD.SCID mice were used as recipients. Diabetic NOD mice were injected (s.c.) daily with insulin until the day of the experiment.

Antibodies and reagents

In this study, the following antibodies and reagents were used: PE-conjugated anti-Fas (Joe 2), FasL (Kay-10), CD4, CD8, TNF-α (TN3-19.12), IFN-γ (XMg1.2), IL-4 (11B11), anti-hamster IgG and rat IgG1; FITC-conjugated anti-CD4, CD8, CD11b and CD45RB mAbs (clone 16A); purified anti-Fas, FasL, TNFR1(p55), TNFR2 (p75) mAbs, mouse IgG 2b and hamster IgG1; 2 isotype control (NA/LE™); purified anti-IL-4, IFN-γ and TNF-α capture mAbs; biotin-conjugated detection mAbs and avidin-conjugated alkaline phosphatase (BD PharMingen, Ontario, Canada); purified anti-IL-4 mAb (11B11) was provided by Biological Response Modifiers Program, NCI-FCRDC (Bethesda, MD) for in vivo study. Anti-IFN-γ mAb (R46A2, ATCC) and control rat IgG used in vivo were purified in our laboratory. Mouse rIFN-γ (10⁷ U/μg) and rTNF-α (2.7 × 10⁹ U/μg) were purchased from R&D systems (Cedarlane, Ontario, Canada), mouse rIL-4 (5.6 × 10⁷ U/μg) was purchased from Becton Dickinson Labware, MA. Other reagents include: purified protein derivative (PPD) (Statens Seruminstitut, Denmark); FITC-TdT-mediated dUTP nick end labeling (TUNEL) (Roche, Mannheim, Germany); Humulin U (Eli Lilly Co., Indianapolis). Freeze-dried preparation of an attenuated strain of Mycobacterium bovis BCG (Aventis Pasteur, Toronto, Canada) was reconstituted with saline and stored in aliquots at −20°C.

Animal models for adoptive transfer of diabetes

Three experimental models were used as splenocyte donors in adoptive transfer of diabetes: (A) diabetic NOD mice were injected (i.p.) with (i) saline, (ii) 300 μg of BCG 6 days (BCG-6d) or (iii) 12 days (BCG-12d) before adoptive transfer of splenocytes; (B) for in vivo cytokine neutralizing assay, 2 days after BCG-immunization, diabetic NOD mice were injected (i.p.) with (i) 3.0 mg of anti-IL-4 mAb (11B11), (ii) 1.6 mg of anti-IFN-γ mAb (R4-6A2) or rat IgG as control five times at 2 days interval; (C) diabetic NOD mice were injected (i.p.) with rIL-4 (12.5 ng), rIFN-γ (2 μg) and/or rTNF-α (0.5 μg) in 0.25 ml of saline supplemented with 1% normal mouse serum daily for 10 days. In adoptive transfer experiments, 12 × 10⁶ splenocytes from the above groups of treated diabetic NOD mice were transferred (i.v.) into NOD.SCID mice or NOD mice irradiated with 750 rads 2 h before transfer (8). Urine glucose was tested with glucose test strips every other day and diabetes was verified by blood glucose level ≥16.5 mM.

T cell proliferation assay

Splenocytes (4 × 10⁶) were incubated with BCG (100 μg/ml) or PPD (20 μg/ml) for 4 days in flat-bottomed 96-well plates for T cell proliferation assay. In antibody blocking assays, splenocytes from BCG-immunized diabetic NOD mice were stimulated with PPD in the presence of anti-FasL, TNFR1, TNFR2 mAb, corresponding isotype controls (15 μg/ml) or medium for 4 days. 1 μCi of [3H]Tdr was added per well 16 h before harvesting, and incorporation was measured in a Liquid Scintillation Counter (PerkinElmer Wallac, St-Laurent, Canada).

Flow cytometric analysis

Phenotypic analysis. Splenocytes (10⁶) were directly stained with 0.5–1.0 μg of PE or FITC-conjugated mAb against CD4, CD8, CD11b, Fas, FasL, CD45RB alone or in different combinations. In TNFR1/TNFR2 analysis, cells were indirectly stained with first antibody followed by FITC-anti-hamster IgG and PE-anti-CD4 or anti-CD8 mAb. CD45RB<sup>high</sup> T cells were defined and analyzed based on fluorescence intensity.

Intracellular cytokine staining. Splenocytes were directly stained with CD4 or CD11b mAb, then fixed with 2% formaldehyde for 15 min and permeabilized by incubation with 0.5% saponin–PBS–2% BSA before staining with anti-IL-4, IFN-γ or TNF-α mAb. Appropriate isotypes of antibodies were used as controls. Ten thousand events were acquired for each sample on a FACScan and analyzed using CellQuest software (Becton Dickinson, MA).

ELISA for cytokine detection

For determination of cytokine secretion, splenocytes (2 × 10⁶) were cultured in 24-well plate in 1 ml medium in the presence of BCG (100 μg/ml) at 37°C. Culture supernatants were collected after 72 h. 96-well ELISA plates (Falcon, Becton Dickinson, NJ) were coated with anti-IL-4, TNF-α or IFN-γ
capture mAb (1 μg/ml) in NaHCO₃ buffer, pH 8.6. The levels of cytokine in supernatants were detected using a standard biotin–avidin system and developed with phosphatase substrate (P-nitrophenyl phosphate disodium, Sigma, Ontario, Canada). Optical densities were measured at 405 nm using an ELISA reader (Bio-Rad, Hercules, CA).

Measurement of apoptosis

T cell apoptosis was detected by TUNEL staining. Splenocytes under following conditions were tested: (i) splenocytes from diabetic NOD mice injected with saline, BCG or cytokine (IL-4, IFN-γ and/or TNF-α) were incubated with medium alone for 24 h; (ii) splenocytes from BCG-immunized diabetic NOD mice were incubated with PPD in the presence of blocking mAb to FasL, TNFR1, TNFR2 or isotype controls (15 μg/ml) for 24 h; (iii) splenocytes from diabetic NOD mice were incubated with IFN-γ and/or TNF-α for 48 h; (iv) splenocytes from diabetic NOD mice were incubated with combinations of agonistic anti-Fas mAb (0.5 and 15 μg/ml) and/or TNF-α (0, 200 and 1000 U/ml) for 48 h. Cells (2.5 x 10⁶) were first stained with PE-anti-CD4 or PE-anti-CD8 mAb, then fixed with 4% formaldehyde–PBS, permeabilized with 0.1% Na-citrate-0.1% Triton X-100 solution, and finally incubated with TUNEL kit or FITC alone as a negative control and analyzed by flow cytometry.

Statistical analysis

For statistical analysis, χ² test, Fisher Exact test and Student’s t-test were used. A P-value < 0.05 was considered statistically significant.

Results

BCG immunization of diabetic NOD mice impairs the ability of splenocytes to transfer diabetes

To test the ability to adoptively transfer diabetes, splenocytes isolated from both BCG and saline-treated diabetic mice were transferred to NOD.Scid mice. As shown in Fig. 1, the incidence of diabetes in saline, BCG-6d and BCG-12d groups was 25/26, 7/15 and 5/19, respectively, by 40 days (P < 0.0001) and 25/26, 11/15 and 8/19 by 60 days (P = 0.0003) after cell transfer. Histological examination showed that splenocytes from BCG-immunized diabetic mice induced much less insulitis in recipient NOD.Scid mice than those from the saline-treated group (data not shown). Therefore splenocytes from BCG-immunized diabetic NOD mice had a reduced ability to transfer disease.

BCG immunization decreases the proportion of T cells by inducing apoptosis

BCG immunization significantly decreased the proportions of CD4, CD8 and CD45RBlo T cells and increased CD11b positive macrophages in a time course study. The difference between BCG-6d and BCG-12d groups in CD8 and CD45RBlo T cells are also significant (Fig. 2A). CD45RBlo CD4 T cells are considered diabetogenic Th1 cells in diabetic NOD mice. Therefore, the reduction of both CD45RBlo CD4 and CD8 T cells in BCG-immunized NOD mice indicates the down-regulation of diabetogenic Th1 cells. In addition, the total numbers of splenocytes were only slightly increased in BCG-6d and BCG-12d groups (data not shown). TUNEL and T cell double staining showed that in vivo BCG treatment significantly increased CD4 and CD8 T cell apoptosis (Fig. 2B). The proportions of apoptotic CD4 and CD8 T cells were in the order BCG-6d > BCG-12d > saline group. Except for the TUNEL positive CD8 T cells of BCG-6d and 12d groups, the difference between saline and BCG groups, or BCG-6d and BCG-12d groups was significant (P < 0.05–0.01).

BCG immunization sequentially induces TNF-α, IFN-γ and IL-4 production

Intracellular and secreted TNF-α, IFN-γ and IL-4 in BCG-immunized diabetic NOD mice were analyzed by intracellular cytokine staining and ELISA, respectively. As shown in Fig. 3(A), intracellular cytokine expression revealed that in comparison with the saline group, the total number of TNF-α or IFN-γ positive splenocytes (macrophages plus CD4 T cells) was significantly increased in the BCG-12d group. But the highest number of TNF-α positive cells was found in the BCG-6d group. CD4 T cells of BCG-6d and 12d groups and macrophages of BCG-12d group had a significant increase of TNF-α expression. A significantly high expression of IFN-γ was observed only in the BCG-12d group. Interestingly, the major source of IFN-γ in the BCG-12d group was macrophages, and it correlated with the highest proportion of macrophages and lowest proportion of T cells in this group. IL-4 expression was increased to a higher level in the BCG-12d group. The patterns of cytokines secreted into supernatant were similar to the patterns of cytokine positive splenocytes, except for TNF-α in the saline group (Fig. 3B). Splenocytes from the saline group produced as much TNF-α as the BCG-6d group, when cultured with BCG but not medium alone (data not shown) for 3 days. Clearly, the peak of TNF-α production is earlier than that of IFN-γ, and T cells
are the major source. The level of IL-4 remained high in diabetic mice 15 days after BCG-immunization (data not shown). Our previous study showed that the production of IL-4 is persistently maintained at a higher level in diabetic NOD mice that have been immunized with BCG and grafted with syngeneic islet cells than in control mice (12). Therefore, BCG immunization induces an early proinflammatory Th1 response and a late Th2 response.

Enhancement of Th1 cytokine production is the primary cause for the impairment of diabetogenic T cells with BCG immunization

To explore the mechanisms underlying down-regulation of diabetogenic T cells by BCG-immunization, diabetic NOD mice were injected (i.p.) with neutralizing mAb to IFN-γ or IL-4 during BCG priming. Isotype-matched rat IgG and saline were used as controls. The incidence of diabetes in NOD.SCID mice transferred with splenocytes from anti-IL-4 mAb (Fig. 4A) or anti-IFN-γ mAb-treated mice (Fig. 4B) was examined. There was no significant difference in the incidence of diabetes between anti-IL-4 mAb-treated and control groups. In contrast, anti-IFN-γ mAb treatment partially abolished the effect of BCG on the impairment of diabetogenic T cells. A significant difference in the ability of splenocytes to transfer diabetes was found between anti-IFN-γ mAb and control rat IgG (P = 0.004) or BCG alone group (P = 0.011) 10 weeks after disease transfer. There was also no significant difference in the ability of splenocytes to transfer diabetes between saline and BCG + anti-IFN-γ mAb groups. Mechanistically, in vivo administration of neutralizing mAb to IFN-γ also reversed the effect of BCG on down-regulation of CD45RB<sup>high</sup> CD4 T cells, increased apoptosis of CD4 T cells and low T cell proliferative response to BCG (Table 1). These results suggest that the down-regulation of destructive autointiminity in BCG-immunized diabetic NOD mice is triggered by the early production of Th1 cytokines. Conversely, the delayed Th2-like response in these mice may reflect the function of resident and/or up-regulated Th2 cells following BCG-induced apoptosis of diabetogenic Th1 cells.

Fig. 2. Decreased proportion of T cells and increased apoptotic T cells in BCG-immunized diabetic NOD mice. (A) For phenotypic analysis, splenocytes (10<sup>6</sup>) were directly stained with FITC or PE-conjugated anti-CD11b, CD4 or CD8 mAb alone or anti-CD4, CD8 and CD45RB mAbs in combination. Results are representative of three experiments, and are presented as the mean (%) ± SEM of three mice. (B) For T cell apoptosis analysis, splenocytes from saline or BCG-treated (BCG-6d and BCG-12d) groups (n = 3) were cultured in medium for 24 h and then stained for TUNEL positive CD4 and CD8 T cells. The numbers shown in each representative dot plot graph are the mean (%) ± SEM for TUNEL positive CD4 or CD8 T cells, representative of two separate experiments. *P < 0.05–0.001 compared with saline group; †P < 0.05–0.02 compared with BCG-12d group.

Fig. 3. Changes of cytokine pattern in diabetic NOD mice immunized with BCG. (A) For analysis of intracellular cytokines (TNF-α, IFN-γ and IL-4), splenocytes freshly isolated from each group of mice (n = 3) were double stained for cytokine positive CD4 T cells and macrophages. Results are representative of two experiments and presented as mean (%) ± SEM of total cytokine positive cells (macrophages plus CD4 T cells). The horizontal lines within columns separate cytokine positive macrophages (upper) from CD4 T cells (bottom). (B) For analysis of released cytokine in supernatant, splenocytes from each group of mice (n = 5) were incubated with BCG (100 µg/ml) for 72 h. Supernatant was analysed by ELISA. Results are expressed as the mean (ng/ml for TNF-α and IFN-γ and U/ml for IL-4) ± SEM. *P < 0.05–0.0002 compared with saline group; †P < 0.02–0.001 compared with BCG-6d group.
Statistical analysis:

Experiments. Splenocytes and CD4 T cells, respectively, and are from two separate CD4 T cells. Results are expressed as percent positive cells in BCG + rat IgG and saline groups, respectively.

Two days later, mice were injected i.p. with 1.6 mg of rat IgG or anti-IL-4 mAb (3 mg; 11B11) (A) or anti-IFN-γ mAb (1.6 mg; R46A2) (B) every 2 days for a total of five injections. Rat IgG and saline were used as controls. Two days after the last injection, splenocytes were isolated from each group and 12 × 10^6 cells were transferred to NOD.SCID mice. Results are presented as cumulative incidence of immunized diabetic NOD mice (Table 1). Systemic administration of IFN-γ and TNF-α treatment was accompanied by reduced proportions of CD4 and CD8 T cells, which reached significance in all but the CD8 T cells from TNF-α group (Fig. 5A). Concordantly, systemic administration of IFN-γ or TNF-α alone or in combination increased T cell apoptosis (Fig. 5B). Increased CD8 T cell apoptosis was significant between saline and all three cytokine-treated groups, while significant increases in CD4 T cell apoptosis was only reached in mice treated with both IFN-γ and TNF-α. Higher level of apoptosis occurred synergistically with IFN-γ and TNF-α in combination, but was not statistically significant in comparison with IFN-γ or TNF-α alone. There was also a decrease in CD45RBlow CD4 and CD8 T cell populations in cytokine-treated groups (data not shown). Therefore, the finding that exogenous IFN-γ and/or TNF-α are able to replace BCG in the down-regulation of diabetogenic T cells in diabetic NOD mice further indicates a primary role for Th1 cytokines in BCG immunotherapy.

**Increased expression of Fas**

**High**, FasL and TNFR on T cells of BCG-immunized diabetic mice

Fas-FasL and TNFR-TNF-mediated signalings are two important pathways to induce T cell apoptosis (29,30). Consequently, Fas/FasL and TNFR expression was analyzed on CD4 and CD8 T cells in diabetic NOD mice 12 days after BCG immunization (Fig. 6). Fas is constitutively expressed on most types of cells. In this study, Fas�� is defined as high intensity of Fas expression (over expression). BCG immunization significantly increased Fas高/FasL expression on CD4 and CD8 T cells. At the same time, there was also a significant increase in TNFR1 and TNFR2 expression on CD4 T cells, but only TNFR1 expression was significantly increased on CD8 T cells. Therefore, the increase in apoptosis of diabetogenic T cells after BCG-immunization is accompanied by the up-regulation of Fas高, Fasl, and TNFR expression on T cells.

**IFN-γ and/or TNF-α incubation enhances apoptosis and Fas/FasL expression of diabetogenic T cells**

To further unravel the role of Th1 cytokine in BCG-induced apoptosis of diabetogenic T cells, changes in apoptosis and Fas/FasL expression were evaluated on T cells cultured in the presence of Th1 cytokines. Splenocytes from diabetic NOD mice were incubated in vitro with IFN-γ and/or TNF-α for 2 days, then double stained for TUNEL positive CD4 or CD8 T cells. Figure 7(A) shows concentration-dependent induction of T cell apoptosis by IFN-γ and/or TNF-α. A significant increase in apoptosis of CD4 T cells was found using the higher concentration of IFN-γ (100, 500 ng/ml) or TNF-α (5, 25 ng/ml) alone or in combination. Increased apoptosis in CD8 T cells was only found significant at the highest concentration of IFN-γ (500 ng/ml) and TNF-α (25 ng/ml) in combination. Parallel increases in Fas/FasL expression were observed on both CD4 and CD8 T cells incubated with the highest concentration of IFN-γ, TNF-α alone or in combination (Fig. 7B). A significant
increase in Fas/FasL expression on CD4 T cells was found in all three cytokine-treated groups. For CD8 T cells, a significant increase in Fas/FasL expression was only found in the presence of TNF-α alone or in combination with IFN-γ. The association of T cell apoptosis and Fas/FasL expression found in splenocyte culture in the presence of IFN-γ and/or TNF-α further suggests the primary role of Th1 cytokines in BCG-induced immune regulation in diabetic NOD mice.

Both Fas and TNF pathways are involved in BCG-induced apoptosis of diabetogenic T cells
As described above, BCG immunization up-regulates the expression of Fas/FasL and TNFR, which may lead to the apoptosis of diabetogenic T cells. To further elucidate the pathway through which T cell apoptosis was induced, anti-FasL or anti-TNFR mAb was added to splenocyte cultures from BCG-immunized diabetic NOD mice to block the corresponding ligand and receptor binding. Both TUNEL staining and T cell proliferation assays were carried out by incubating splenocytes with PPD for 1 and 4 days, respectively. In the presence of anti-FasL or anti-TNFR1 mAb, T cell apoptosis was significantly decreased (Fig. 8A), while the T cell proliferative response to PPD was increased (Fig. 8B). Addition of TNFR2 mAb to the culture had little effect. Thus, in vitro blocking the Fas–FasL or TNF–TNFR1 pathways rescues Th1 cells from BCG-induced apoptosis and increases T cell proliferative response accordingly. Our results indicate the involvement of both Fas–FasL and TNF–TNFR1 pathways in BCG-induced T cell apoptosis in diabetic NOD mice.

In vitro cooperative interaction between TNF and Fas pathways on the induction of diabetogenic T cell apoptosis
To further elucidate the relationship between Fas and TNF-mediated T cell apoptosis, splenocytes from diabetic NOD mice were incubated with sub-optimal concentrations of TNF-α (200, 1000 U/ml) or anti-Fas mAb (5, 15 µg/ml) alone or in combination of various concentrations. Results presented in Fig. 9 show that TNF-α or anti-Fas mAb alone did not induce significant T cell apoptosis in culture, whereas in combination, they had a synergistic effect on both CD4 and CD8 T cell apoptosis. A significant increase in CD4 T cell apoptosis was seen in cultures with a combination of 1000 U/ml TNF-α and 5 or 15 µg/ml anti-Fas mAb (P < 0.05–0.02). A similar pattern was seen in the CD8 T cell population, when high concentrations of both anti-Fas mAb and TNF-α were added to the culture. In low concentrations of TNF-α, apoptosis only increased in combination groups than in TNF-α alone (P < 0.05). The dose-dependent synergistic interaction between TNF-α and anti-Fas mAb in apoptosis of diabetogenic T cells provides an in vitro correlation to explain the in vivo effect underlying the down-regulation of destructive autoimmunity in diabetic NOD mice after BCG immunization.

Discussion
The mechanisms underlying the prevention of spontaneous diabetes, induced diabetes and recurrence of diabetes by mycobacterial preparation (CFA or BCG) are complex. It may involve the induction of regulatory cells, cytokine switch and T cell apoptosis (12,22,31). Diabetic NOD mice have a dominant population of diabetogenic T effector cells that can adoptively transfer disease in non-diabetic recipients [for review, see (32)]. On the other hand, down-regulation of diabetogenic T cells by BCG may also involve induction of T cell anergy, peripheral deletion and/or induction of regulatory cells.

In this study, we explored the mechanism of peripheral deletion by which BCG-immunized diabetic NOD mice become tolerant to syngeneic islet graft and their splenocytes lose the ability to transfer diabetes. We found significant changes in T cell and macrophage populations of diabetic NOD mice after BCG immunization. The down-regulation of CD45RB<sup>low</sup> T cells was accompanied by increased T cell apoptosis and the number of macrophages. In diabetic NOD mice, CD45RB<sup>low</sup> CD4 T cells have been shown to be diabetogenic Th1 cells (33). In pre-diabetic NOD mice, islet-infiltrating CD4<sup>high</sup> T cells are highly diabetogenic and the
majority of them express CD45RBlow, a memory T cell marker (34). It has also been shown that CD45RB low CD4 T cells are IFN-γ-secreting Th1 cells in long-lived immunity to mycobacteria (35). In addition, soluble FasL has been found to induce apoptosis in CD4+CD45RBlow 'memory' cells (36). Our data suggest that diabetogenic CD45RB low T cells undergo apoptosis upon BCG immunization. This directly impairs the ability of splenocytes to transfer diabetes in this study and prevents the recurrence of diabetes in an islet transplantation model by peripheral deletion of diabetogenic T cells (6,7). We also observed that the low T cell proliferative response is out of proportion to the low number of T cells induced by BCG. This suggests the possible involvement of regulatory cells such as T cells, macrophages and NK cells (8,37,38).

What triggers the apoptosis of diabetogenic T cells after BCG immunization? BCG has been shown to induce a strong Th1 response with secretion of TNF-α and IFN-γ both in mouse and human (39,40). A shift from a Th1 to Th2 cytokine occurs in the later stages following in vitro incubation of peripheral blood cells from healthy persons with BCG, and in CD4 T cells following mycobacterial infection (16,17), which supports our findings. In a previous study, IL-4 production occurs later and is maintained even for a few months after BCG-induced protection of syngeneic islet grafts when IFN-γ production is no longer significant (12). We also found that neutralizing IFN-γ but not IL-4 with mAb was able to abolish the protective effect induced by BCG. This suggests that Th1 cytokines play a major role in triggering BCG-induced down-regulation of diabetogenic T cells, and up-regulation of Th2 cytokines may be secondary to this effect. Similar findings have been reported in glial fibrillary acidic protein immunotherapy in NOD mice, in which the protection is relied upon up-regulation of IFN-γ production (41). It is speculated that BCG down-regulates both induction and effector phases of diabetogenic T cells by induction of Th1 apoptosis and activation of Th2 cells through IL-10 production. Similarly, we found that administration of IFN-γ and/or TNF-α to diabetic NOD mice mimics the effects induced by BCG. TNF-α, a pro-inflammatory factor, is mainly produced by activated macrophages. In this study, we found that T cells are also the producer of TNF-α (42,43). On the other hand, macrophages become a major source of IFN-γ in BCG-treated diabetic NOD mice, which might result from the apoptosis of IFN-γ-producing T cells and the increased number of activated macrophages. Similar findings have been reported in pulmonary macrophages and NK cells during mycobacterial infection and CFA immunization in NOD mice (38,44). The relationship between TNF-α and IFN-γ in the regulation of an immune response to BCG immunization remains unclear. We found that TNF-α production is prior to IFN-γ after in vivo BCG immunization or in vitro BCG stimulation, which suggests the cooperation or promotion in the production and function of these two cytokines.

The role of IFN-γ in the induction of T cell apoptosis has been investigated in IFN-γ KO mice (20), in mycobacterium and other microbe infection models (18,45). IFN-γ has also been shown to inhibit the development of diabetes in NOD mice (19). Whether CFA or BCG directly affects islets through cytokines need to be clarified. CFA reverses established diabetes by eliminating TNF-α sensitive diabetogenic T cells and promoting the regeneration of endogenous islet β cells (22). Expression of TNF-α in the islets also suppresses spontaneous diabetes by preventing the development of islet specific T cells (46). Administration of TNF-α prevents the recurrence of diabetes in NOD mice by reducing CD4 and
CD8 T cells and Th1 cytokine production in local islet grafts and in splenocytes (47). The synergistic effects of IFN-γ and TNF-α have been shown to reduce insulitis (24). We now report that IFN-γ and TNF-α may synergistically contribute to the apoptosis of diabetogenic T cells in BCG-immunized diabetic NOD mice.

In this study, the involvement of Fas-FasL and TNFR 1-TNF pathways in BCG-induced T cell apoptosis has been demonstrated by increased expression of Fas(high), FasL and TNFR on T cells and FasL/TNFR blocking assay. T cells that express high level of Fas are particularly sensitive to apoptosis. The predominant expression of FasL has been shown to mediate apoptosis in Th1 and CD8 T cells (27,28). Infection with live Mycobacterium avium induces protection against type 1 diabetes in NOD mice, which is associated with increased expression of Fas and FasL (48). Transgenic expression of soluble TNFR1 in NOD mice has been shown to prevent type 1 diabetes (49), which supports TNFR1 signalled apoptosis of diabetogenic T cell in BCG-treated mice. The relationship between Fas and TNF pathways in induction of T cell apoptosis is unclear. It has been reported that Fasl-induced apoptosis of cells in the eye is signaled by TNF through its receptor (29). We now demonstrate an in vitro synergetic effect between anti-Fas mAb and TNF-α in promoting T cell apoptosis of diabetic mice, which suggests the cooperation between Fas and TNF pathways in BCG-induced apoptosis of diabetogenic T cells.

In conclusion, BCG immunotherapy in diabetic NOD mice is mediated by the early up-regulation of TNF-α and IFN-γ production. The cooperation of TNF-α and IFN-γ triggers the apoptosis of diabetogenic T cells through both Fas–FasL and TNF–TNFR1 pathways. This study provides a rational explanation for the protection against diabetes recurrence through
BCG immunization of islet-transplanted diabetic NOD mice. These results have direct implications in preventing the recurrence of diabetes by transplanted syngeneic islets or β cells generated through stem cell technology.

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Abbreviations

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette–Guérin</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>NOG</td>
<td>non-obese diabetic</td>
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<tr>
<td>PPD</td>
<td>purified protein-derivative</td>
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<td>tumor necrosis factor receptor</td>
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<td>TUNEL</td>
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References


