Prevention of Diabetes in NOD Mice by Administration of Dendritic Cells Deficient in Nuclear Transcription Factor-κB Activity

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Abnormalities of dendritic cells (DCs) have been identified in type 1 diabetic patients and in nonobese diabetic (NOD) mice that are associated with augmented nuclear transcription factor (NF)-κB activity. An imbalance that favors development of the immunogenic DCs may predispose to the disease, and restoration of the balance by administration of DCs deficient in NF-κB activity may prevent diabetes. DCs propagated from NOD mouse bone marrow and treated with NF-κB–specific oligodeoxyribonucleotide (ODN) in vitro (NF-κB ODN DC) were assessed for efficacy in prevention of diabetes development in vivo. Gel shift assay with DC nuclear extracts confirmed specific inhibition of NF-κB DNA binding by NF-κB ODN. The costimulatory molecule expression, interleukin (IL)-12 production, and immunostimulatory capacity in presenting allo- and islet-associated antigens by NF-κB ODN DC were significantly suppressed. NF-κB ODN renders DCs resistant to lipopolysaccharide stimulation. Administration of 2×10^6 NF-κB ODN DCs into NOD mice aged 6–7 weeks effectively prevented the onset of diabetes. T-cells from pancreatic lymph nodes of NF-κB ODN DC–treated animals exhibited hyporesponsiveness to islet antigens with low production of interferon-γ and IL-2. These findings provide novel insights into the mechanisms of autoimmune diabetes and may lead to development of novel preventive strategies. *Diabetes* 52: 1976–1985, 2003

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Received for publication 16 August 2002 and accepted in revised form 16 May 2003.

APC, antigen-presenting cell; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IκB, inhibitor of κB; IL, interleukin; iNOS, inducible nitric oxide synthetase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; NF-κB, nuclear transcription factor-κB; ODN, oligodeoxyribonucleotide; TNF, tumor necrosis factor; TUNEL, transferase-mediated dUTP nick-end labeling.

Insulin-dependent diabetes (type 1 diabetes) is an autoimmune disease characterized by T-cell–mediated destruction of the pancreatic β-cells (1). The nature of immune dysregulation leading to β-cell destruction remains poorly understood, but it is clearly influenced by multiple genetic, environmental, and immunological factors. In particular, antigen-presenting cells (APCs) have a significant impact on T-cell differentiation by providing costimulatory signals and secreting the cytokine milieu at the time of T-cell priming (2). Dendritic cells (DCs) are professional APCs and control immune responses to either augment or reduce autoimmunity, which is related to their state of maturation (3,4). Mature DCs express high major histocompatibility complex (MHC) and costimulatory molecules, secrete interleukin (IL)-12, and stimulate vigorous T-helper 1 responses. In contrast, immature DCs expressing low costimulatory molecules induce antigen-specific hyporesponsiveness by triggering T-cell apoptosis (5) or differentiation of regulatory T-cells (6). Maturational and functional abnormalities of DCs have been demonstrated in humans with type 1 diabetes (7,8) and in nonobese diabetic (NOD) mice (9). Indeed, DCs are among the first cell populations detected within the islets during the onset of diabetes, and diabetes can be induced by an adoptive transfer of syngeneic DCs treated with tumor necrosis factor (TNF)-α in NOD mice (8,10).

The activation of nuclear transcription factor (NF)-κB is important in DC maturation and activation (11). Mature DCs express NF-κB, and mice lacking NF-κB complexes fail to develop myeloid-derived DCs (12,13). Many inducible genes that encode cytokines, chemokines, cell adhesion molecules, growth factors, costimulatory molecules, and immune receptors contain NF-κB binding sites in their promoters or enhancers (14,15). NF-κB is present in the cytoplasm and associated with the inhibitor of κB (IκB) as an inactive complex. Various external and internal signals, including cytokines, lipopolysaccharide (LPS), and mitogens, lead to the dissociation of the NF-κB/IκB complex by degrading IκB and allowing the nuclear translocation of free NF-κB (11). In the nucleus, NF-κB binds to a specific DNA motif and regulates transcription of target genes. The development of diabetes has been correlated with elevated levels of NF-κB activation and enhanced antigen-presentation function in DCs of NOD mice (16,17), due to

hyperactive IκB kinase (11). In addition, DCs in NOD mice show an inability to effectively elicit regulatory T-cell function and a propensity to secrete high levels of IL-12, an important proinflammatory cytokine that drives T-helper 1 responses (13). Therefore, an imbalance favoring development of immunogenic DCs, such as those with elevated NF-κB activity, might predispose to developing autoimmune diabetes (18), and restoration of the balance by administering DCs deficient in NF-κB activity might prevent the development of diabetes.

We constructed decoy double-stranded oligodeoxynucleotides (ODNs) containing a consensus of NF-κB binding sites that inhibit NF-κB activity (19). The data of this study demonstrated that NF-κB ODN specifically inhibited NF-κB DNA binding capacity and prevented DC maturation and antigen-presentation capacity. Administration of the DCs deficient in NF-κB activity prevented diabetes development in NOD mice, which was associated with T-cell hyporesponsiveness to islet antigens.

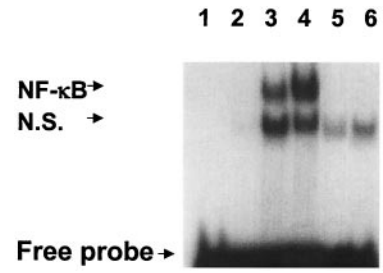


FIG. 1. NF-κB ODN inhibits NF-κB DNA binding in DCs. Nuclear proteins were extracted from DC propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (NF-κB ODN DC) for 5 days. For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. NF-κB DNA binding activity was determined by ESMA, as described in RESEARCH DESIGN AND METHODS. Medium alone, *lane 1*; DC and cold probe, *lane 2*; DC, *lane 3*; DC stimulated with LPS, *lane 4*; NF-κB ODN DC, *lane 5*; NF-κB DC stimulated with LPS, *lane 6*. NS, nonspecific band. The data are representative of three separated experiments.

RESEARCH DESIGN AND METHODS

Animals. Female NOD ($H2^{g7}$) and C3H ($H2^{k}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center and provided Purina Rodent Chow (Ralston Purina, St. Louis, MO) and water ad libitum. They were used and cared for in accordance with institutional and National Institutes of Health guidelines.

Islet lysate preparation. Islets were isolated from the pancreas by collagenase V (Sigma, St. Louis, MO) digestion as previously described (20), with slight modification (21). After separation on a Ficoll gradient (Type 400; Sigma), the islets were purified by hand picking to eliminate remaining exocrine tissues and suspended in Hank's Balanced Salt Solution (Life Technologies, Grand Island, NY). Islet lysate was prepared by repeat freezing (5 min in dry ice–ethanol bath) and thawing (10 min in 37°C warm bath) four to five times.

ODN. Double-stranded NF-κB ODN decoys were generated using equimolar amounts of single-stranded sense and antisense phosphorothioate-modified ODN containing two NF-κB binding sites (sense strand: 5′-A*GGGACTTTC C*GCTG*GGGACTTTCC*-3′; NF-κB binding sites are italicized) (19). A double-stranded ODN consisting of a random sequence (sense strand: 5′-ACCAGTC CCTAGCTACCAGTCCCTA-3′) was used as control. Sense and antisense strands of each ODN were mixed in the presence of 150 mmol/l NaCl, heated to 100°C, and cooled to room temperature to obtain double-stranded DNA.

DC culture. NOD mouse bone marrow cells were cultured in 24-well plates (2×10^6 per well) in RPMI-1640 media (Life Technologies) supplemented with antibiotics and 10% (vol/vol) FCS (hereafter referred to as "complete medium") containing both granulocyte-macrophage colony-stimulating factor (GM-CSF) (4 ng/ml) and IL-4 (1,000 units/ml) (both from Schering-Plough Research Institute, Kenilworth, NJ) for 5–7 days. The selection and purification procedures were performed as previously described (22). In vitro stimulation of DCs was achieved by exposure to LPS (2 μg/ml) for the last 18 h of culture. To incorporate ODN, 10 μmol/l ODN was added at the initiation of DC culture. For pulsing islet antigens, lysate of pancreatic islets isolated from NOD mice was added at 1:5 of DC:islet cell ratio for the last 48 h of DC culture.

Flow cytometry. Expression of cell surface molecules on DCs was determined by flow cytometric analysis, using an Epics Elite flow cytometer (Coulter Corporation, Hialeah, FL). Cells were stained with the primary hamster or rat monoclonal antibodies (mAbs) against CD40, CD80, CD86, or CD11c followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster IgG or anti-rat IgG_{2a}, as described (5). MHC antigen was detected with FITC-conjugated anti-$H2K^d$ mAb (all from B.D. PharMingen, San Diego, CA).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using a commercially available kit (Promega, Madison, WI) that was supplied with an NF-κB probe oligonucleotide (sense sequence: 5′AGTTGAGGGGACTTTCCCAGGC3′), which was end labeled with γ-^{32}P ATP (NEN, Boston, MA). A 25-fold excess of unlabeled oligonucleotide was used as cold probe. Nuclear proteins (1 μg) were loaded in each lane. The mobility shift was detected by running the mixture on a 4% acrylamide gel. Shifted bands were visualized by autoradiography.

T-cell proliferation. To examine DC allostimulatory activity, γ-irradiated (20 grays) DCs propagated from bone marrow cells of NOD mice were used as stimulators. C3H spleen T-cells (2×10^5) enriched through a nylon wool column were used as responders. For assessment of autostimulatory activity, stimulator DCs were pulsed with islet lysate, and enriched T-cells (2×10^5) isolated from NOD mesenteric lymph nodes or spleens were used as responders. Cells (200 μl/well) were cultured in complete medium in triplicate in 96-well round-bottom plates in 5% CO_2 in air at 37°C for 3–5 days. [^{3}H]TdR (1 μCi/well) was added for the final 18 h of culture. Incorporation of [^{3}H]thymidine into DNA was assessed by liquid scintillation. Results are expressed as mean counts per minute (cpm) ±1 SD.

DC administration and assessment of diabetes. Female 6- to 7-week-old NOD mice were given a single intravenous injection of 2×10^6 DCs, and blood glucose levels were monitored weekly. The first day of two consecutive readings of blood glucose >350 mg/dl was defined as the date of diabetes onset. To score insulitis, pancreata were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 mm each) were prepared and stained with hematoxylin and eosin (23). The lineage of infiltrating cells was identified by immunohistochemistry in cryostat sections using biotinylated rat anti-mouse CD4, CD8, B220, CD11b, or CD11c mAb (B.D. PharMingen) in an avidin-biotin-alkaline phosphase complex staining procedure. Isotype- and species-matched irrelevant mAbs were used as control animals.

Cytokine and nitric oxide quantitation. Levels of IL-2, IFN-γ, IL-4, IL-10, and IL-12 in supernatants of cultures were quantitated using enzyme-linked immunosorbent assay (ELISA) kits (BioSource, Camarillo, CA). Nitric oxide levels were determined by the colorimetric Griess reaction through detecting the end product nitrite (24,25).

RNase protection assay. Total RNA was extracted using the guanidinium isothiocyanate-phenol-chloroform method with TRI reagent (Sigma, St. Louis, MO) as described (24). The purity of RNA was determined from the $A_{260/280}$ absorbance ratio. Cytokine mRNA was assessed using the Ribonuclease Protection Assay Kit (RiboQuant, San Diego, CA). Briefly, probes were synthesized by T7 RNA polymerase with incorporation of α-^{32}P-UTP. Total RNA (5 μg) was treated overnight with synthesized probes (specific activity: 800 Ci · mmol/l) at 56°C, followed by treatment with RNase A (80 μg/ml) and T1 (250 units/ml) for 45 min at 30°C. The murine L32 and glyceraldehyde-3-phosphate dehydrogenase riboprobes were used as controls. Protected fragments were submitted for electrophoresis through a 7.0 mol/l urea/5% polyacrylamide gel and then exposed to Kodak X-omat film for 72 h.

Statistical analysis. Statistical significance was assessed by Student's *t* test or Kaplan-Meier log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

NF-κB ODN inhibits NF-κB DNA binding in DCs. Nuclear proteins extracted from DCs propagated from bone marrow of NOD mice in the absence or presence of NF-κB ODN was assessed for NF-κB DNA binding by EMSA. Specificity of NF-κB binding was demonstrated by utilizing the consensus NF-κB probe and unlabeled NF-κB probe (cold probe) as a competitor. As shown in Fig. 1, the NF-κB band was detected by radiolabeled NF-κB consensus sequence-specific probe in DCs without exposure to NF-κB ODN (*lane 3*). NF-κB binding capacity was en-

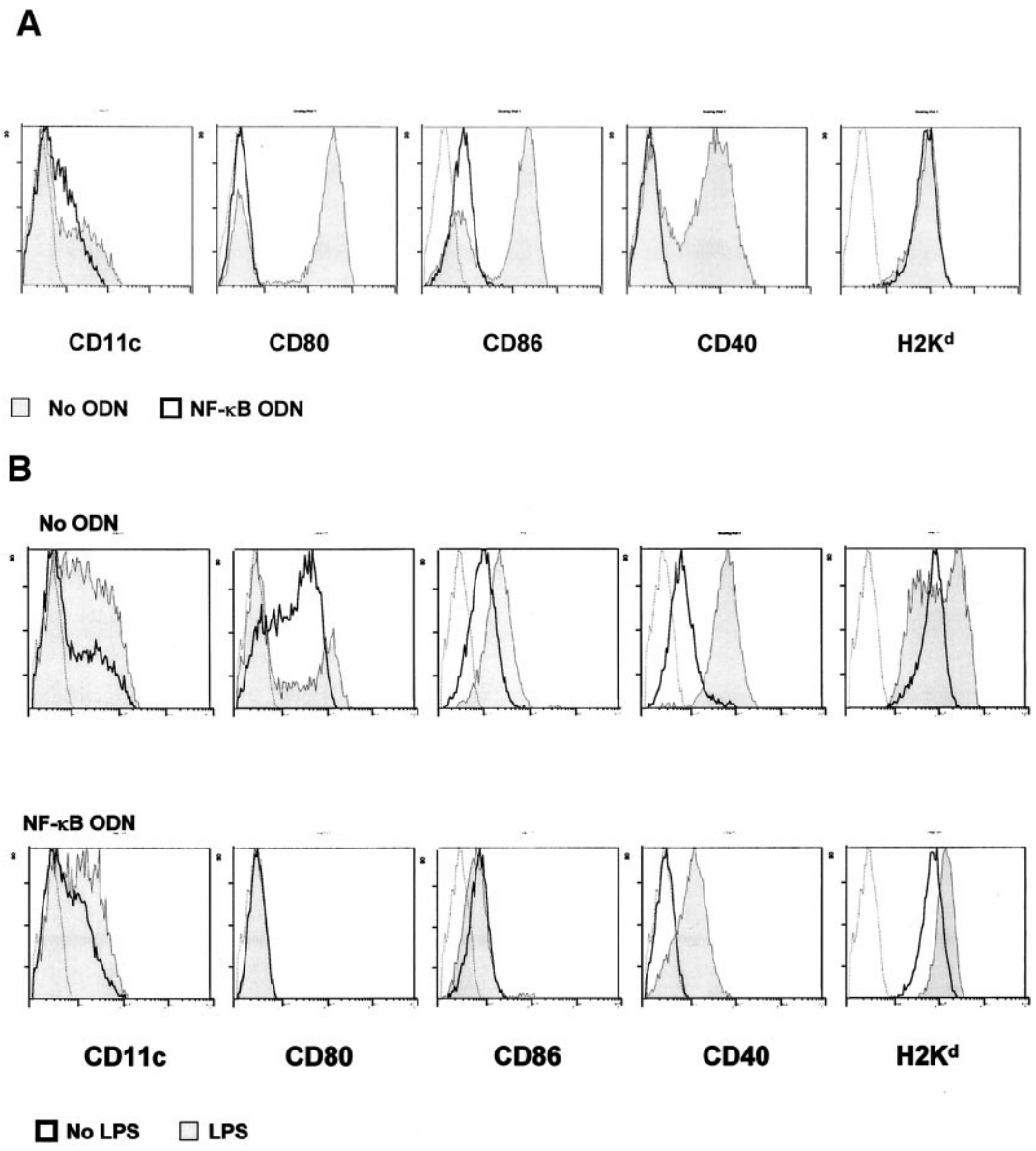


FIG. 2. Inhibition of costimulatory molecule expression on DCs by NF-κB ODN. DCs were propagated from bone marrow of NOD mice in GM-CSF and IL-4 with or without NF-κB ODN (10 μmol/l). For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. Expression of CD40, CD80, CD86, MHC, and CD11c was determined by flow cytometric analysis following mAb staining. Open profiles in dashed lines are isotype controls. *A*: NF-κB ODN markedly suppressed expression of costimulatory molecules, but not MHC and CD11c, on DCs. *B*: LPS significantly augmented CD40, CD80, and CD86 expression in normal DC, while expression of CD40, CD80, and CD86 remained at low levels on NF-κB ODN DC despite LPS stimulation. The data are representative of three separate experiments.

hanced by DC stimulation with LPS (*lane 4*). The NF-κB band disappeared when excess unlabeled probe was added as a competitor for the radiolabeled probe in the binding reaction (*lane 2*). NF-κB ODN almost totally blocked NF-κB binding activity in DCs (*lane 5*). LPS stimulation did not reverse inhibited NF-κB DNA binding capacity by NF-κB ODN (*lane 6*). These data clearly indicate that treatment with NF-κB ODN effectively and stably inhibits NF-κB DNA binding capacity in DCs derived from NOD mice.

NF-κB ODN prevents phenotypical maturation of DCs. Normal DCs propagated from the bone marrow of NOD mice in GM-CSF and IL-4 expressed high levels of MHC and costimulatory molecules, as determined by flow cytometric analysis. NF-κB ODN markedly suppressed surface expression of costimulatory molecules, including CD40, CD80, and CD86 on DCs, but the expression of MHC and DC-restricted marker CD11c was not affected (Fig. 2*A*). MHC and DC-restricted proteins are known to be required for induction of antigen-specific hyporesponsiveness (26). Since LPS-induced DC activation that results in upregulation of CD80, CD86, IL-12, and inducible nitric oxide synthase (iNOS) gene expression is NF-κB dependent (27–29), we examined whether NF-κB ODN decoys prevented LPS-stimulated DC activation. Following exposure to LPS (18 h), there was a significant upregulation in surface expression of CD40, CD80, and CD86 on DCs. Exposure to NF-κB ODN kept CD40, CD80, and CD86 expression at low levels on DCs, even in the presence of LPS stimulation (Fig. 2*B*). This indicates that competitive inhibition of NF-κB DNA binding by NF-κB ODN prevents DC phenotypic maturation, regardless of the presence of

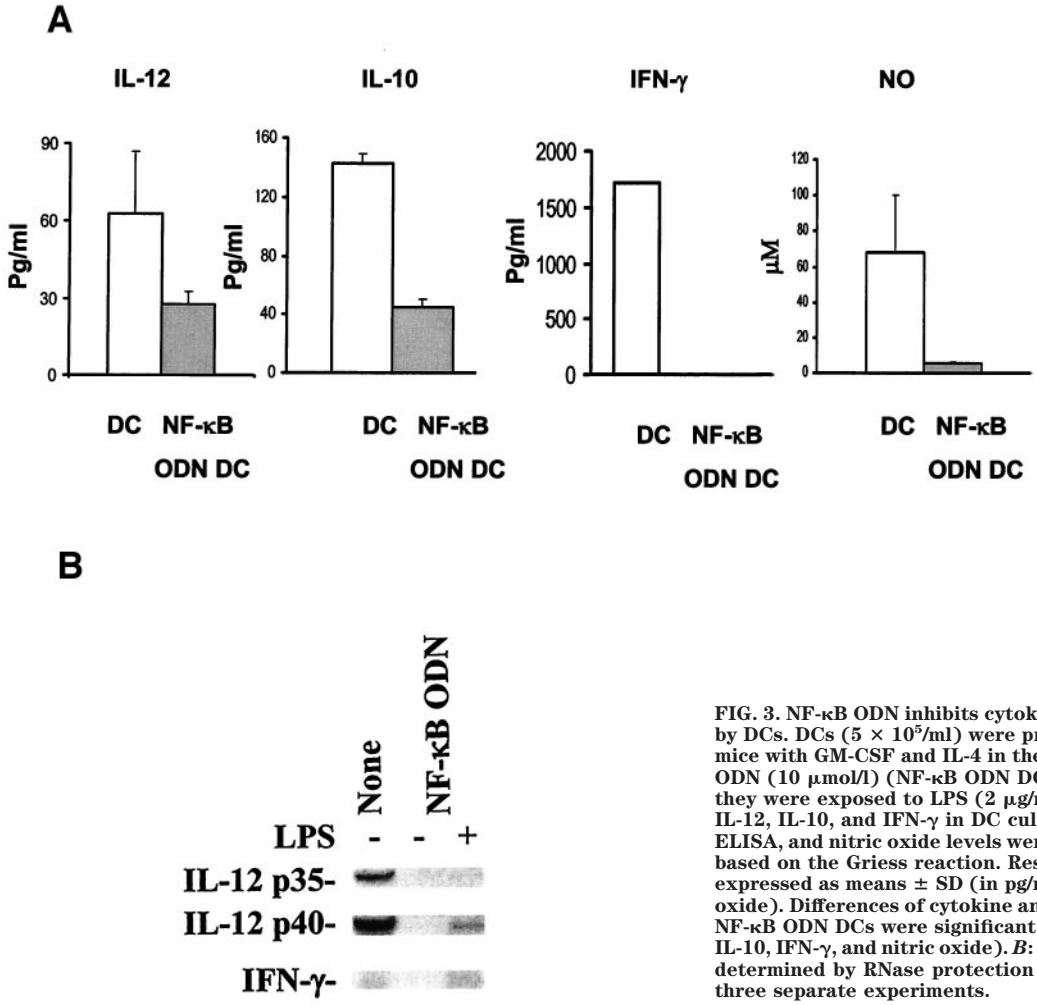


FIG. 3. NF-κB ODN inhibits cytokine and nitric oxide (NO) production by DCs. DCs (5×10^5/ml) were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (10 μmol/l) (NF-κB ODN DC) for 5 days. For further activation, they were exposed to LPS (2 μg/ml) for the last 18 h. *A*: The levels of IL-12, IL-10, and IFN-γ in DC culture supernatants were measured by ELISA, and nitric oxide levels were determined by a colorimetric assay based on the Griess reaction. Results from triplicate experiments are expressed as means ± SD (in pg/ml for cytokines and μmol/l for nitric oxide). Differences of cytokine and nitric oxide production by DCs and NF-κB ODN DCs were significant ($P < 0.05$ for IL-12 and $P < 0.01$ for IL-10, IFN-γ, and nitric oxide). *B*: mRNA expression of IL-12. IFN-γ was determined by RNase protection assay. Results are representative of three separate experiments.

the promaturation LPS signal. Therefore, this inhibition is more profound than the inhibition by suppressive cytokines, such as TGF-β, which is easily surmounted by the addition of stimuli, such as allogeneic T-cells or LPS (26).

Effect of NF-κB ODN on DC cytokine profile. NF-κB activation promotes transcription of a number of cytokines, as well as iNOS (27,30). Cytokines produced by DCs provide an important signal for T-cell proliferation, differentiation, and survival. DCs propagated from NOD mice without LPS stimulation produced very low levels of cytokines and nitric oxide (data not shown). LPS stimulation triggered increased DC production of IL-12 (p70), IFN-γ, IL-10, and nitric oxide by DC. In contrast, no IFN-γ and low levels of IL-12 (p70), IL-10, and nitric oxide were detected in the supernatant of NF-κB ODN DCs with LPS stimulation (Fig. 3*A*). Nitric oxide production is NF-κB dependent; therefore, the diminished nitric oxide production in NF-κB ODN DCs was an indication of NF-κB inhibition (29). Consistently, expression of both IL-12 p35, p40, and modest expression of IFN-γ mRNA were shown in non–ODN-treated DCs, but none of these cytokines were detected in NF-κB ODN DCs. LPS stimulation slightly restored expression of IL-12 p40 and IFN-γ in NF-κB ODN DCs, but not IL-12 p35. Expression of IL-1α or -β, the IL-1 receptor, and TNF-α or -β in DCs was not suppressed by treatment with NF-κB ODN, indicating that blocking NF-κB has a minimal effect on the expression of these monokines.

NF-κB ODN inhibits immune stimulatory activity of DCs. The effect of ODN treatment on DC allostimulatory activity was examined in a one-way mixed leukocyte reaction (MLR) assay. Figure 4*A* demonstrates that DCs propagated from NOD mice ($H2^{g7}$) stimulated strong proliferative responses in T-cells isolated from C3H ($H2^{k}$) spleen. The DC allostimulatory activity was significantly augmented by LPS stimulation. Nevertheless, the T-cell proliferative responses stimulated by NF-κB ODN DCs were markedly suppressed. This inhibition was not reversed by LPS stimulation, indicating the persistent effect of NF-κB ODN. We next assessed the effect of NF-κB ODN on the capacity of DCs to present autoantigens. T-cells from NOD mice were cultured with irradiated NOD DCs pulsed with NOD islet lysate, which provided the relevant autoantigen(s). As shown in Fig. 4*B*, without NF-κB ODN treatment, DCs pulsed with islet antigens effectively stimulated T-cell proliferation, while the autostimulatory activity of NF-κB ODN DCs was significantly suppressed. These data clearly indicate that consistent with the influence of NF-κB ODN on DC surface molecule expression, the in vitro immune stimulatory activity of NF-κB ODN DCs is also significantly suppressed.

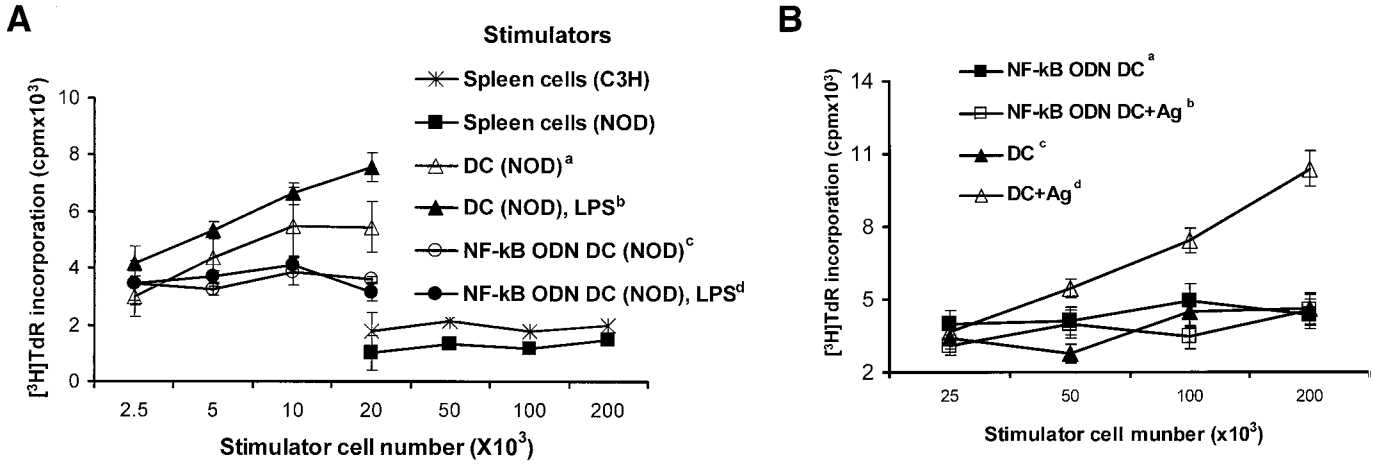


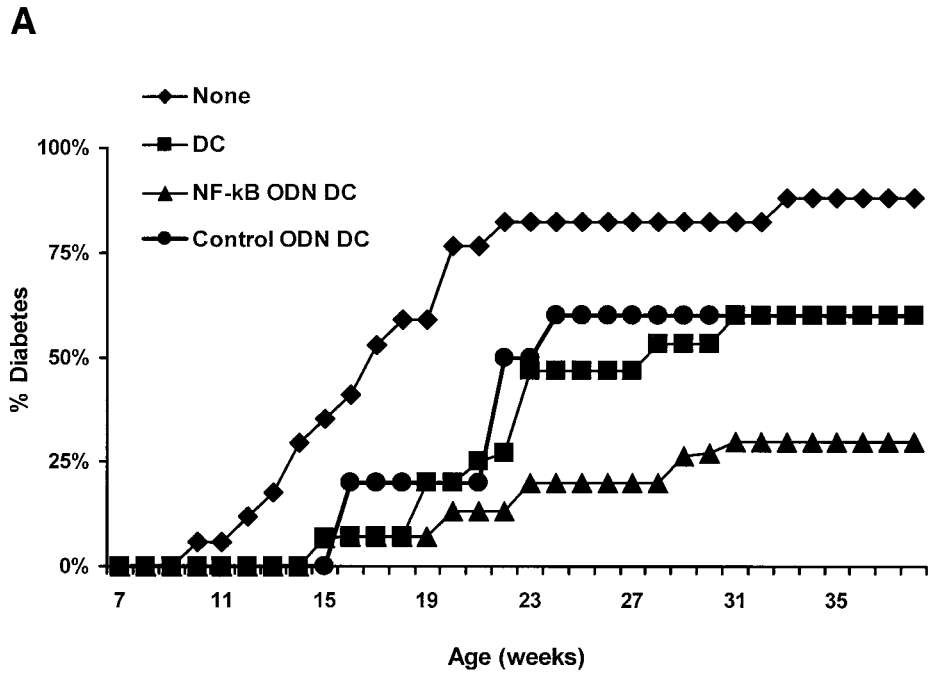
FIG. 4. Inhibition of DC immune stimulatory activity by NF-κB ODN. DCs were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF-κB ODN (10 μmol/l) (NF-κB ODN DC). For further activation, DCs were exposed to LPS (2 μg/ml) for the last 18 h. *A*: C3H ($H2^k$) splenic T-cells (2×10^5) were cultured with γ-irradiated DCs propagated from NOD mice ($H2^{g7}$) at graded concentrations for 3 days. Incorporation of [^{3}H]TdR into DNA demonstrated that LPS stimulation augmented DC allostimulatory function ($P < 0.05$, a vs. b at 2×10^4 stimulators), while NF-κB ODN inhibited DC allostimulatory activity ($P < 0.05$, a vs. c at 2×10^4 stimulators; $P < 0.01$, b vs. d at both 2×10^4 and 1×10^4 stimulators), which was irreversible by LPS ($P > 0.05$, c vs. d at all stimulator:responder ratios). Spleen cells from C3H and NOD mice were used as control stimulators. *B*: Spleen T-cells (2×10^5) of NOD mice were cultured for 5 days with γ-irradiated DCs propagated from NOD mice pulsed with or without islet lysate from NOD mice. DCs pulsed with islet antigens induced substantial T-cell proliferative responses ($P < 0.01$, c vs. d at both 1×10^3 and 2×10^5 stimulators), which was significantly reduced by treatment of DCs with NF-κB ODN ($P < 0.01$, b vs. d at both 1×10^3 and 2×10^5 stimulators). The results are expressed as counts per minute (cpm) (means ± SD) and are representative of three separate experiments.

Administration of NF-κB ODN DCs inhibits development of diabetes. Our net effort was directed toward assessing preventive effects of administration of NF-κB ODN DCs on type 1 diabetes. Female NOD mice spontaneously developed pathological insulitis, which was evident at 6–8 weeks of age with peri-islet infiltration followed by a progressive destruction of pancreatic β-cells. Histological examination revealed that the insulitis score increased from 2.18 ± 1.25 at 12 weeks of age to 4.0 ± 0 at 20 weeks of age, with 88% of the mice developing destructive insulitis and diabetes by the age of 20 weeks (Fig. 5*A* and *C*). The early inflammatory changes as a result of peri-insulitis were characterized by infiltration of macrophages ($CD11b^+$) and DCs ($CD11c^+$). After 12 weeks of age, $CD4^+$ T-cells were the predominant population of the infiltrates, accompanied by increased numbers of macrophages and DCs mixed with $CD8^+$ T-cells (Fig. 5*D*). NOD mice were treated at 6–7 weeks of age with a single intravenous injection of 2×10^6 NF-κB ODN DCs propagated from bone marrow from NOD mice. Three control groups of age-matched NOD mice included administration of normal DCs, control ODN DCs, and none. A single intravenous administration of NF-κB ODN DCs protected 70% of NOD mice from the development of diabetes up to the age of 38 weeks (Fig. 5*A*). Injection of normal DCs could protect ~30% of the mice from developing of diabetes by the age of 38 weeks, instead of accelerating the progression of disease. Similar findings reported by other investigators (31) show that normal mature DCs propagated from NOD bone marrow have a slight effect on diabetes protection in NOD mice. The diabetes incidence in the control ODN-DC treatment group is similar to that in the normal DC treatment group (Fig. 5*A*). The insulitis and islet infiltrates were histologically examined. As demonstrated in Fig. 5*B*, the severity of histological insulitis was markedly diminished with substantial reduction in peri- and intra-islet infiltration at 30 weeks of age in mice treated with NF-κB ODN DCs compared with that of non–DC-treated mice at as early as 12 weeks of age. The pancreata from three mice in each group were scored for insulitis as described in RESEARCH DESIGN AND METHODS. The insulitis score in the NF-κB ODN DC group was significantly lower than that in the non-DC and normal DC treatment groups (Fig. 5*C*). Compared with the non-DC treatment group, $CD11b^+$ macrophages and $CD8^+$ T-cells were significantly reduced in the subcapsular areas in NF-κB ODN DC–treated mice, while $CD4^+$ T-cells were markedly increased (Fig. 5*D*). Administration of NF-κB ODN DC pulsed in vitro with islet lysates did not further improve the ability of these cells to prevent disease (data not shown). This may implicate that process of in vivo existing antigens by adoptively transferred NF-κB ODN DCs is sufficient to prevent diabetes.

TABLE 1
IL-2 and IFN-γ levels in supernatants from MLR cultures.

Treatment	Ag^-		Ag^+	
	IL-2	IFN-γ	IL-2	IFN-γ
None	22.3 ± 7.6	ND	5.5 ± 7.8	9.2 ± 0.2
DC	103.8 ± 4.7	520.5 ± 28.2	97.7 ± 4.7	4,000 ± 0
NF-κB DC	0.16 ± 0.22*	ND†	ND‡	ND§

Data are mean cpm ± 1 SD and are representative of three separate experiments. Responders: T-cells (2×10^3) purified from mesenteric lymph nodes of NOD mice 4 weeks after intravenous administration of DCs (2×10^6) that were propagated from bone marrow of NOD mice in the absence or presence of NF-κB ODN (10 μmol/l). T-cells from NOD mice without DC treatment were used as controls. Stimulators: γ-irradiated DCs propagated from NOD mice bone marrow that were pulsed with islet antigens from NOD mice in vitro (Ag^+) or not pulsed with antigens (Ag^-). Responders were cultured for 5 days with stimulator at an stimulator:responder ratio of 1:10. Cytokine levels in the culture supernatants were determined by ELISA. *$P < 0.05$ vs. none, $P < 0.01$ vs. DCs; †$P > 0.05$ vs. none, $P < 0.01$ vs. DCs; ‡$P > 0.05$ vs. none, $P < 0.01$ vs. DCs; §$P < 0.01$ vs. none, $P < 0.01$ vs. DCs. ND, not detectable.



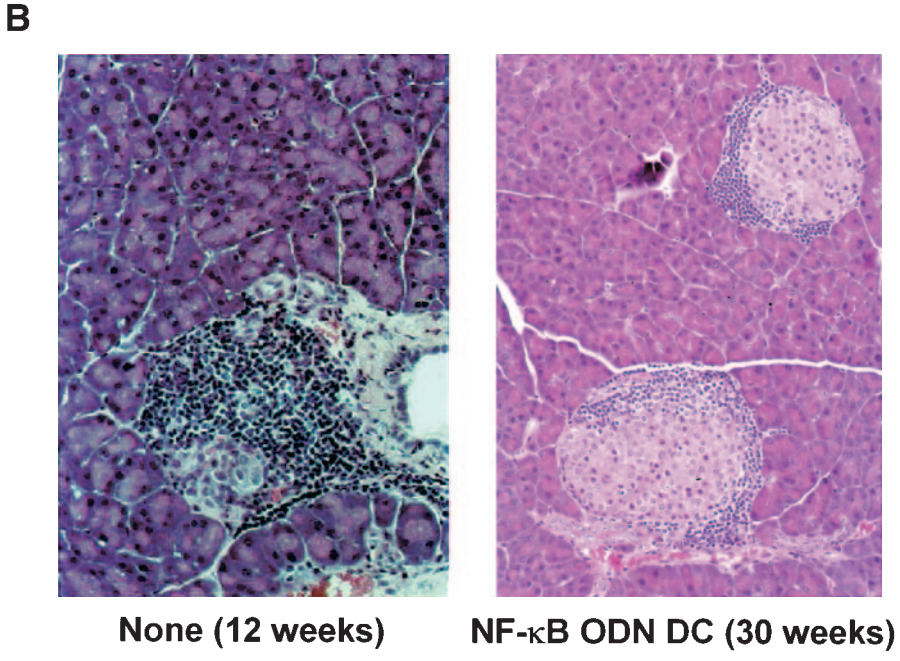


FIG. 5. Systemic administration of NF-κB ODN DCs prevents diabetes development in NOD mice. NOD mice were injected intravenously at 6–7 weeks of age with 2 × 10^6 DCs propagated from NOD bone marrow with GM-CSF and IL-4 in the presence of NF-κB ODN (NF-κB ODN DC, $n = 17$) or control ODN (control ODN DC, $n = 8$) or in the absence of ODN (DC, $n = 15$). Mice without DC treatment (none, $n = 15$) were also used as control subjects. *A*: Without DC treatment, 88% of the mice developed diabetes at the age of 33 weeks. The diabetes incidence in the control ODN-DC treatment group is similar to the non–ODN-treated DC group, showing modestly prevented diabetes development. Administration of NF-κB ODN DCs markedly reduced development of diabetes ($P < 0.01$, compared with either non–ODN DC or control ODN DC group). *B*: Histology of pancreatic islets (hematoxylin and eosin) from NOD mice treated with NF-κB ODN DC at 30 weeks of age revealed very minimal insulitis, and untreated NOD mice developed severe insulitis as early as 12 weeks of age (magnification ×400). *C*: Three mice in each group were scored (50 islets per mouse) for insulitis, as described in RESEARCH DESIGN AND METHODS, indicating that insulitis was significantly less severe in mice treated with NF-κB ODN DCs than those treated with normal DCs or without DC treatment (both $P < 0.05$). *D*: Cryostat sections of pancreata were stained with anti-CD4, -CD8, -CD11c, or -CD11b mAbs (magnification ×200). The pictures are representative of three separated experiments. For quantitation, 30 high-power fields (hpf) of islet subcapsular areas in each mouse were randomly selected and counted for staining positive cells. The data were collected from three mice in each group and are presented as positive cell numbers/hpf (±1 SD).

Administration of NF-κB ODN DCs induces T-cell hyporesponsiveness to islet antigens. To examine the effect of NF-κB ODN DC administration on T-cell responses in vivo, lymphocytes isolated from NOD spleen or mesenteric lymph nodes 4 weeks after DC treatment were assessed for T-cell proliferative responses to islet lysate in MLR where NOD DCs pulsed with pancreatic islet lysate were stimulators. T-cells from mice treated with normal DCs developed a strong proliferative response to islet antigens (Fig. 6), which was associated with increased production of IFN-γ and IL-2 (Table 1), reflecting a bias toward a T-helper 1 response. In contrast, T-cells from the NF-κB ODN DC–treated group exhibited low proliferation, along with decreased production of IFN-γ and IL-2 on in vitro restimulation (Fig. 6 and Table 1). Low levels of IL-4 and IL-10 were detectable in all groups of mice (data not shown). These results suggest that NF-κB ODN DCs elicit hyporesponsiveness of autoreactive T-cells in NOD mice, partly by suppression of T-helper 1 responses.

DISCUSSION

We demonstrate in this study that administration of syngeneic bone marrow–derived DCs, in which NF-κB activity is blocked by a designed decoy ODN, effectively prevents development of diabetes in NOD mice. At 38 weeks of age, 70% of the mice were rendered normoglycemic by one injection of NF-κB ODN DCs. This was associated with markedly inhibited autoreactive T-cell responses and T-helper 1 responses (Fig. 3). It is unlikely

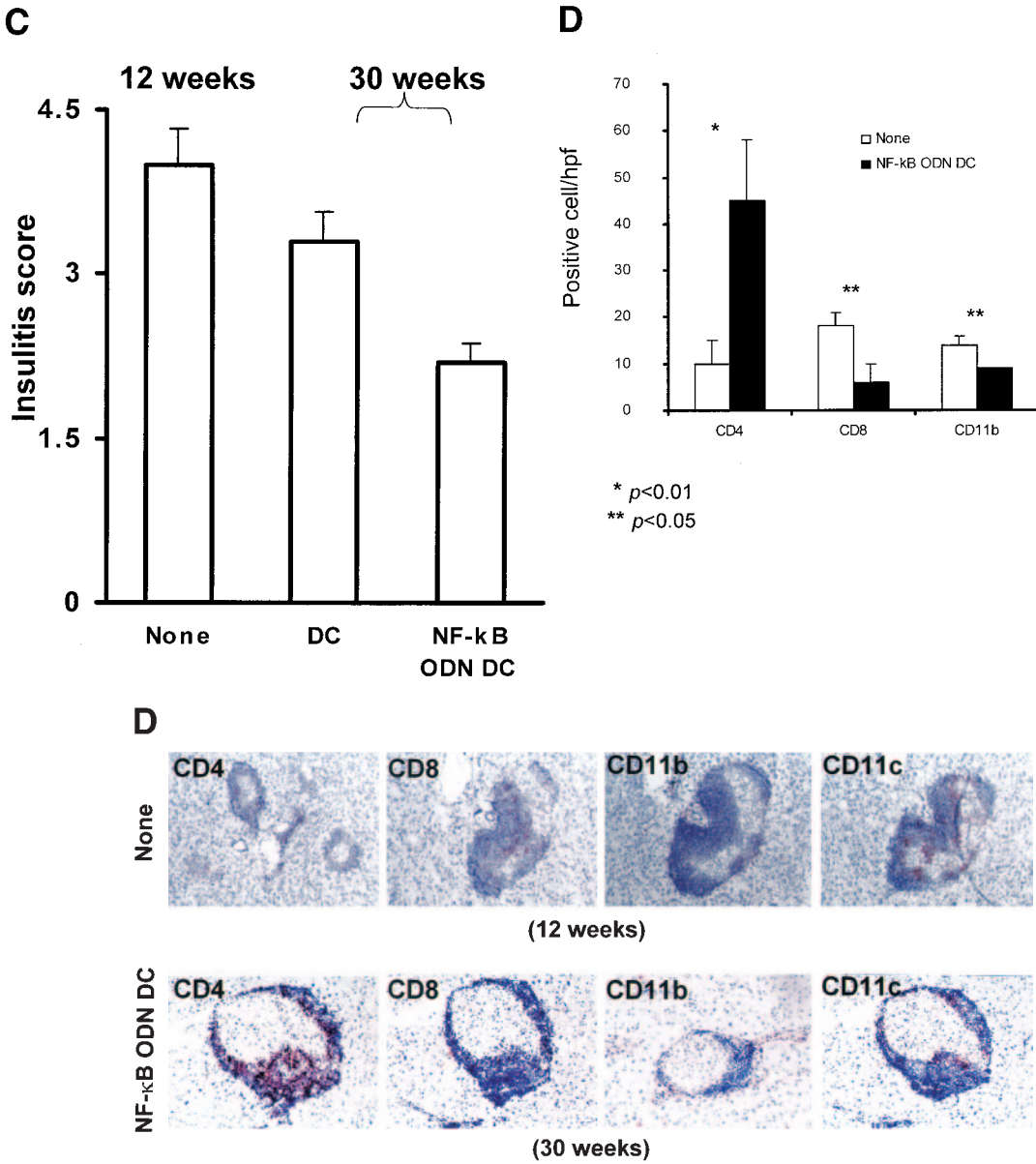


FIG. 5. Continued

that the suppressed T-cell responses by NF-κB ODN DCs are a consequence of ODN toxicity. We have previously determined the "safety window" for ODN incorporation in DCs by determining the viability of DC using dye exclusion, as well as quantitative measurement of apoptosis with transferase-mediated dUTP nick-end labeling

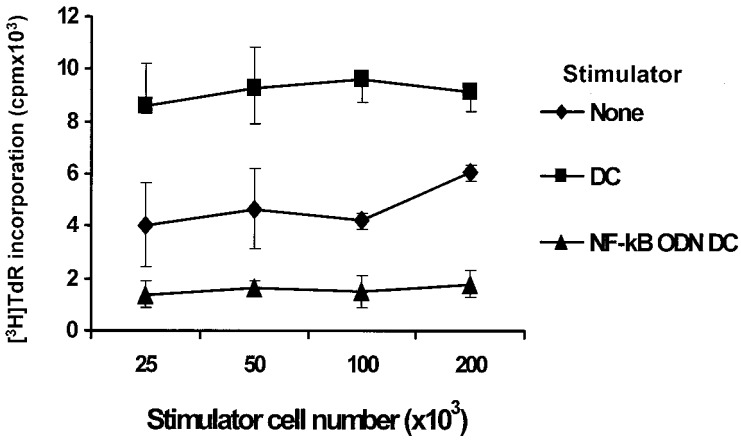


FIG. 6. Administration of NF-κB ODN DCs to NOD mice induces T-cell hyporesponsiveness to islet antigens. In a 4-day MLR assay, T-cells (2×10^3) purified from mesenteric lymph nodes of NOD mice that had received, at 6–7 weeks of age, 2×10^6 DCs intravenously were used as responders. γ-Irradiated DCs that were propagated from bone marrow of NOD mice with GM-CSF and IL-4 pulsed with islet lysates were used as stimulators. The results are expressed as counts per minute (cpm) (means ± 1 SD) and are representative of three separate experiments.

(TUNEL) staining. Using FITC-conjugated NF-κB ODN, we observed that DCs effectively incorporated ODN and survived for as long as untreated DCs. DCs treated with control ODN stimulated a strong allogeneic MLR (19). We did not observe a reduction in DC number following NF-κB ODN treatment at 10 mmol/l in the present study. These results indicate that exposure of DCs to 10 mmol/l NF-κB ODN yielded a satisfactory suppressive effect with minimal toxicity.

The results of this study coincide with the view that type 1 diabetes is initiated by autoreactive T-cell responses toward self-antigens. Indeed, T-cells from the NOD mice displayed abnormally high reactivity to self-proteins (32) and T-helper 1 responses to the autoantigens (33,34). Activation of APCs, including DCs, and subsequent migration from nonlymphoid tissues to regional lymph nodes are primary events elicited during inflammatory processes and are critical for the generation of cellular responses against self-antigens in type 1 diabetes (35). This was first suggested by the fact that adoptive transfer of DCs pulsed with a self-antigen expressed in islet β-cells, using transgene technology, activated the antigen-specific cytotoxic T-lymphocytes (CTLs) and promoted development of autoimmune diabetes, indicating that autoreactive CTL stimulation by enhanced DC antigen-presentation function is an important prerequisite for the progression of diabetes (36). DCs are also an important source of TNF-α in islet infiltrates during the early developmental stages of diabetes, which may contribute to β-cell destruction (10). The functional defects of DCs in NOD mice were correlated with significantly enhanced activity of NF-κB (11,18). Signaling by members of the TNF-α receptor family, such as CD40 and receptor activator of NF-κB (RANK), or LPS results in activation of NF-κB in DCs, leading to upregulation of costimulatory molecule expression and enhancement of IL-12 production (11). The association of immunogenic DC activity and β-cell destruction in NOD mice suggests that certain β-cell antigens are processed by the immunogenic DCs, resulting in the delivery of a signal that stimulates the autoimmune responses that lead to islet cell destruction. Therefore, it has been recently proposed (21) that an imbalance favoring development of the immunogenic DCs may predispose NOD mice to the development of autoimmune diabetes. The prevention of diabetes development in NOD mice by administration of NF-κB ODN–treated DCs at a prediabetic stage (6 weeks), as shown in this study, may reflect a consequence of the restoration of the balance between immunogenic and tolerogenic DC function, in which hyporesponsiveness of antigen-specific T-cells via the inhibition of T-helper 1 differentiation results. These results are consistent with the reports of other investigators in which immature DCs used as therapeutic vectors or adjuvants diminished or regulated autoimmune responses in type 1 diabetes (31).

Although the effect of immature DC administration on regulating immune responses has previously been demonstrated in other experimental models (31), approaches to generating DCs in a stable immature state remain to be developed. Immature DCs were initially obtained for inhibition of maturation in culture by using inhibitory cytokines, such as transforming growth factor-β. In an allograft transplantation model, administration of these cytokine-induced immature DCs induced donor-specific hyporesponsiveness and prolonged allograft survival (37). However, the immunosuppressive effect of these immature DCs is limited due to late activation following in vivo contact with inflammatory cytokines or activated T-cells (26). Genetically engineering DCs to express "designer" immunosuppressive molecules, such as IL-10, TGF-β, CTLA4Ig, or Fas ligand, in order to enhance tolerogenicity, has been explored. Adenoviral vectors can efficiently deliver the transgenes into DCs, but transgene expression is surmounted by the vigorous upregulation of costimulatory molecules on DCs by stimulation of the viral vectors themselves (38,39). Blockade of NF-κB binding to inhibit DC maturation is a new approach that is attracting extensive attention. NF-κB activation can be inhibited at each step by pharmacological agents, including glucocorticoid-dependent upregulation of IκB, Cyclosporine A–dependent calcineurin inhibition, and deoxyspergualin-dependent inhibition of nuclear translocation (40). The therapeutic use of these agents is, however, limited by weak NF-κB inhibition. The NF-κB ODN decoys used in this study effectively inhibit both phenotypic and functional maturation in DCs. NF-κB ODN not only inhibits, but also resists, the maturation of DCs in response to LPS stimulation, thus indicating a stable immature state. Moreover, NF-κB ODN selectively suppresses expression of costimulatory molecules without affecting the DC-restricted marker CD11c and MHC expression, both of which are known to be required for DCs in the induction of antigen-specific tolerance (26). Suppression of IL-12 production by NF-κB ODN DCs may contribute to the therapeutic efficacy of these modified DCs because the islet-infiltrating T-cells in NOD mice expressed high T-helper 1 (IFN-γ and IL-2) and T-helper 1–related cytokines (IL-12) (23,33,41,42) and treatment with anti–IL-12 mAb suppresses T-helper 1–dependent islet destruction (43).

The reduction in intra-islet infiltration of macrophages, DCs, and $CD8^+$ T-cells in NOD mice treated with NF-κB ODN DCs suggests that the inhibition may involve both afferent and efferent arms of the immune system. It is unlikely that the decreases in effector $CD8^+$ infiltrates are due to a lack of immune stimulation, since significant $CD4^+$ infiltrates were observed following treatment with NF-κB ODN DCs. The enhanced $CD4^+$ T-cell infiltration by NF-κB ODN DC treatment was unexpected, as earlier results demonstrated that $CD4^+$ T-cells contributed to insulitis development and transfer of $CD4^+$ islet-specific T-cells into NOD mice accelerated diabetes onset (44). The difficulty in isolating sufficient cells from islets prevented us from further characterizing the $CD4^+$ infiltrates. However, T-cells from the lymph nodes of NF-κB ODN DC–treated NOD mice secreted low IFN-γ and IL-2 when restimulated in vitro by islet antigens in vitro, suggesting the downregulation of T-helper 1 differentiation. Immature DCs have also been reported (6) to promote differentiation of T regulatory cells. The $CD4^+$ T-cells in the islets of nondiabetic mice treated with NF-κB ODN DCs may implicate the presence of a regulatory population with immunosuppressive properties (45–49).

Interestingly, despite the untreated mature phenotype and stimulatory function of mature DC propagated from NOD bone marrow in the presence of GM-CSF and IL-4 in

vitro, a single injection of them modestly reduced the incidence of diabetes in NOD mice (Fig. 5) rather than accelerated disease development. Other investigators have reported similar observations (31). These data are in contrast to those observed in our allograft transplant model, in which the administration of mature donor-derived DC exacerbates graft rejection (37). The disparate outcome may result from different antigens and the way the antigens are being presented. In NOD mice, the administered mature DCs process and indirectly present autoantigens to T-cells in vivo, thereby leading to a regulatory T-helper 2 response (50). Another possibility is that in NOD mice, the mature DCs may act like BCG or complete Freund's adjuvant to nonspecifically inhibit T-cell responses to islet antigens since the DC culture medium contains antigens, such as fetal bovine serum.

In summary, our data suggest that NF-κB ODN effectively inhibits the maturation/activation of DCs propagated from NOD mice. Compared with mature DCs, administration of DCs with suppressed NF-κB DNA binding activity can more effectively prevent diabetes development and induce diabetogenic T-cell hyporesponsiveness in NOD mice. This strategy may be utilized for selective prevention or therapy for human autoimmune diseases, including type 1 diabetes.

ACKNOWLEDGMENTS

This work was supported by a Juvenile Diabetes Foundation International Program Project Grant P1893135 and National Institutes of Health Grant DK058316.

We thank Alison Logar for flow cytometric analysis.

REFERENCES

1. Bach JF: Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15:516–542, 1994
2. Ludewig B, Odermatt B, Ochsenbein AF, Zinkernagel RM, Hengartner H: Role of dendritic cells in the induction and maintenance of autoimmune diseases. *Immunol Rev* 169:45–54, 1999
3. Lechler R, Ng WF, Steinman RM: Dendritic cell in transplantation: friend or foe? *Immunity* 14:357–368, 2001
4. Banchereau J, Briere F, Caux C, Davoust S, Lebecque S, Liu JY, PulendranB, Palika K: Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811, 2000
5. Lu L, Mccaslin D, Starzl TE, Thomson AW: Bone marrow-derived dendritic cell progenitors (NLDC 145^+, MHC classII$^+$, B7-1^{dim}, B7-2$^-$) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation* 60:1539–1545, 1995
6. Roncarolo M-G, Levings MK, Traversari C: Differentiation of T regulatory cells by immature dendritic cells (Review). *J Exp Med* 193:F5–F9, 2001
7. Takahashi K, Honeyman MC, Harrison LC: Impaired yield, phenotype, and function of monocyte-derived dendritic cells in humans at risk for insulin-dependent diabetes. *J Immunol* 161:2629–2635, 1998
8. Jansen A, van Hagen M, Drexhage HA: Defective maturation and function of antigen-presenting cells in type I diabetes. *Lancer* 345:491–492, 1995
9. Serreze DV, Gaskins HR, Leiter EH: Defects in the differentiation and function of APCs in NOD/Lt mice. *J Immunol* 150:2534–2543, 1993
10. Green EA, Flavell RA: Tumor necrosis factors and the progression of diabetes in non-obese diabetic mice. *Immunol Rev* 167:11–22, 1999
11. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P: Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med* 188:2175–2180, 1998
12. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck RP, Lira SA, Bravo R: Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-κB/Rel family. *Cell* 80:331–340, 1995
13. Wu L, D'Amico A, Winkel K, Suter M, Lo, Shortman K: RelB is essential for the development of myeloid-related CD8α$^+$ dendritic cells but not of lymphoid-related CD8α$^+$ dendritic cells. *Immunity* 9:839–847, 1998
14. Thanos D, Maniatis T: NF-κB: a lesson in family values. *Cell* 80:592–532, 1995
15. May MJ, Ghosh S: Signal transduction through NF-κB. *Immunol Today* 19:80–88, 1998
16. Weaver DJ Jr, Poligone B, Bui T, Abdel-Motal UM, Baldwin AS Jr, Tisch R: Dendritic cells from nonobese diabetic mice exhibit a defect in NF-κB regulation due to a hyperactive IκB kinase. *J Immunol* 167:1461–1468, 2001
17. Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R: Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* 168:188–196, 2002
18. Steptoe RJ, Ritchie JM, Harrison LC: Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. *J Immunol* 168:5032–5041, 2002
19. Giannoukakis N, Bonham CA, Qian, Chen Z, Peng L, Harnaha J, Li W, Thomson AW, Fung JJ, Robbins PD, Lu L: Prolongation of cardiac allograft survival using dendritic cells treated with NF-κB decoy oligodeoxyribonucleotides. *Mol Ther* 1:430–437, 2000
20. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437–438, 1985
21. Alexander AM, Crawford M, Bertera S, Rudert WA, Takikawa O, Robbins PD, Trucco M: Indoleamine 2,3-dioxygenase expression in transplanted NOD islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. *Diabetes* 51:356–365. 2002
22. Lu L, Qian S, Hershberger PA, Rudert WA, Li Y, Chambers FG, Lynch DH, Thomson AW: Fas ligand (CD95L) and B7 expression on dendritic cells provide counter-regulatory signals for T-cell survival and proliferation. *J Immunol* 158:5676–5684, 1997
23. Kim KS, Kang Y, Choi SE, Kim JH, Kim HM, Sun B, Jun HS, Yoon JW: Modulation of glucocorticoid-induced GAD expression in pancreatic β-cells by transcriptional activation of the GAD67 promoter and its possible effect on the development of diabetes. *Diabetes* 51:2764–2772, 2002
24. Lu L, Bonham CA, Liang X, Zhen Z, Li W, Wang L, Watkins S, Nalesnik MA, Schlissel MS, Demestris AJ, Fung JJ, Qian S: Liver-derived DEC205^+ B220^+ CD19^- dendritic cells regulate T-cell response. *J Immunol* 166:7042–7052, 2001
25. Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, Thomson AW: Induction of nitric oxide synthase in mouse dendritic cells by IFN-γ, endotoxin, and interaction with allogeneic T-cells: nitric oxide production is associated with dendritic cell apoptosis. *J Immunol* 157: 3577–3586, 1996
26. Lu L, Li W, Zhong C, Qian S, Fung JJ, Thomson AW, Starzl TE: Increased apoptosis of immunoreactive host cells and augmented donor leukocyte chimerism, not sustained inhibition of B7 molecule expression are associated with prolonged cardiac allograft survival in mice preconditioned with immature donor dendritic cells plus anti-CD40L mAb. *Transplantation* 68:747–757, 1999
27. Lu L, Thomson AW: Dendritic cells tolerogenicity and prospects for dendritic cell-based therapy of allograft rejection and autoimmune disease. In *Dendritic Cells: Biology and Clinical Applications*. 2nd ed. Lotze MT, Thomson AW, Eds. London, Academic Press, 2001, p. 587–607
28. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM: Regulation of interleukin-12 p40 expression through an NF-κB half-site. *Mol Cell Bio* 5:5258–5267, 1995
29. Xie QW, Kashiwabara Y, Nathan C: Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* 269:4705–4708, 1994
30. Rissoan, M-C, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu Y-J: Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283:1183–1186, 1998
31. Feili-Hariri M, Dong X, Alber SM, Watkins SC, Salter RD, Morel PA: Immunotherapy of NOD mice with bone marrow–derived dendritic cells. *Diabetes* 48:2300–2308, 1999
32. Ridgway WM, Fasso J, Lanctot A, Garvey C, Fathman CG: Breaking self-tolerance in nonobese diabetic mice. *J Exp Med* 183:1657–1662, 1996
33. Trembleau 5, Penna G, Bosi B, Mortara A, Gately MK, Adorini L: Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes. *J Exp Med* 181:817–821,1995
34. Healey D, Ozegbe P, Arden 8, Chandler P, Hutton J, Cooke A: In vivo activity and in vitro specificity of CD4^+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. *J Clin Invest* 95:2979–2985, 1995
35. Bach JF, Chatenoud L: Tolerance to islet autoantigens in type 1 diabetes. *Ann Rev Immunol* 19:131–161, 2001
36. Ludwig B, Odermatt B, Landmann S, Hengartner H, Zinkenagel: Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *J Exp Med* 188:1493–1501, 1998

37. Fu F, Li Y, Qian S, Lu L, Chambers FD, Starzl TE, Fung JJ, Thomson AW: Costimulatory molecule-deficient dendritic cell progenitors (MHC class II^+, B7-1^{dim}, B7-2^-) prolong cardiac allograft survival in non-immunosuppressed recipients. *Transplantation* 62:659–665, 1996
38. Lu L, Lee WC, Gambotto A, Zhong C, Robbin PD, Qian S, Fung JJ, Thomson AW: Transduction of dendritic cells with adenoviral vectors encoding CTLA4-lg markedly reduces their allostimulatory activity. *Transplant Proc* 31:797, 1999
39. Lee WC, Zhong C, Qian S, Wan Y, Gauldie J, Mi Z, Robbins PD, Thomson AW, Lu L: Phenotype, function, and in vivo migration and survival of allogeneic dendritic cell progenitors genetically engineered to express TGF-β. *Transplantation* 66:1810–1817, 1998
40. Lee J, Burckart GJ: Nuclear factor kappa B: important transcription factor and therapeutic target. *J Clin Pharmacol* 38:981–993, 1998
41. Chung Y-H, Jun H-S, Kang Y, Hirasawa K, Lee B-R, Rooijien NV, Yoon J-W: Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats. *J Immunol* 159:466–471, 1997
42. Alleva DG, Pavlovich RP, Grant C, Kaser SB, Beller DI: Aberrant macrophage cytokine production is a conserved feature among autoimmune-prone mouse strains: elevated interleukin (IL)-12 and an imbalance in tumor necrosis factor-α and IL-10 define a unique cytokine profile in macrophages from young nonobese diabetic mice. *Diabetes* 49:1106–1115, 2000
43. Hayashi T, Morimoto M, Iwata H, Onodera T: Possible involvement of IL-12 in reovirus type-2-induced diabetes in newborn DBA/1 mice. *Scandinavian J Immunol* 53:572–578, 2001
44. Haskins K, McDuitie M: Acceleration of diabetes in young NOD mice with a $CD4^+$ islet-specific T-cell clone. *Science* 249:1433–1436, 1990
45. Shevach EM: Regulatory T-cells in autoimmunity. *Ann Rev Immunol* 18:423–449, 2000
46. Takahashi T, Tagarni T, Yamazaki S, Uede I, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S: Immunologic self-tolerance maintained by $CD25^+$ $CD4^+$ regulatory T-cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192:303–310, 2000
47. Read S, Malmstroni V, Powrie F: Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+$ $CD4^+$ regulatory cells that control intestinal inflammation. *J Exp Med* 192:295–302, 2000
48. Groux H, O'Garra A, Bigler M, Rouleau M, Antinenko S, de Vries JE, Roncarolo M-G: A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737–742, 1997
49. Roncarolo M-G, Levings MK: The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 12:676–683, 2000
50. Feili-Hariri M, Falkner DH, Morel PA: Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *European J Immunol* 32:2021–2030, 2002