

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

Linlin Ma,¹ Shiguang Qian,¹ Xiaoyan Liang,¹ Lianfu Wang,¹ Jennifer E. Woodward,¹ Nick Giannoukakis,² Paul D. Robbins,³ Suzanne Bertera,⁴ Massimo Trucco,⁴ John J. Fung,¹ and Lina Lu¹

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)- κ B 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- κ B activity may prevent diabetes. DCs propagated from NOD mouse bone marrow and treated with NF- κ B-specific oligodeoxyribonucleotide (ODN) in vitro (NF- κ B 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- κ B DNA binding by NF- κ B ODN. The costimulatory molecule expression, interleukin (IL)-12 production, and immunostimulatory capacity in presenting allo- and islet-associated antigens by NF- κ B ODN DC were significantly suppressed. NF- κ B ODN renders DCs resistant to lipopolysaccharide stimulation. Administration of 2×10^6 NF- κ B ODN DCs into NOD mice aged 6–7 weeks effectively prevented the onset of diabetes. T-cells from pancreatic lymph nodes of NF- κ B 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

From the ¹Thomas E. Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania; the ²Diabetes Institute, Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania; the ³Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania; and the ⁴Division of Immunogenetics, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Lina Lu, Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center, W1556 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261. E-mail: lul@msx.upmc.edu.

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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; I κ B, inhibitor of κ B; IL, interleukin; iNOS, inducible nitric oxide synthetase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; NF- κ B, nuclear transcription factor- κ B; ODN, oligodeoxyribonucleotide; TNF, tumor necrosis factor; TUNEL, transferase-mediated dUTP nick-end labeling.

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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). Maturation 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)- κ B is important in DC maturation and activation (11). Mature DCs express NF- κ B, and mice lacking NF- κ B 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- κ B binding sites in their promoters or enhancers (14,15). NF- κ B is present in the cytoplasm and associated with the inhibitor of κ B (I κ B) as an inactive complex. Various external and internal signals, including cytokines, lipopolysaccharide (LPS), and mitogens, lead to the dissociation of the NF- κ B/I κ B complex by degrading I κ B and allowing the nuclear translocation of free NF- κ B (11). In the nucleus, NF- κ B binds to a specific DNA motif and regulates transcription of target genes. The development of diabetes has been correlated with elevated levels of NF- κ B activation and enhanced antigen-presentation function in DCs of NOD mice (16,17), due to

hyperactive I κ B 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- κ B activity, might predispose to developing autoimmune diabetes (18), and restoration of the balance by administering DCs deficient in NF- κ B activity might prevent the development of diabetes.

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

RESEARCH DESIGN AND METHODS

Animals. Female NOD (H2^{g7}) and C3H (H2^b) 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- κ B ODN decoys were generated using equimolar amounts of single-stranded sense and antisense phosphorothioate-modified ODN containing two NF- κ B binding sites (sense strand: 5'-AGGGACTTTC CGCTGGGGACTTTC-3'; NF- κ B 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 \times 10⁶ 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 μ g/ml) for the last 18 h of culture. To incorporate ODN, 10 μ mol/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- κ B probe oligonucleotide (sense sequence: 5' AGTTGAGGGACTTTCAGGC3'), which was end labeled with γ -³²P ATP (NEN, Boston, MA). A 25-fold excess of unlabeled oligonucleotide was used as cold probe. Nuclear proteins (1 μ g) 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

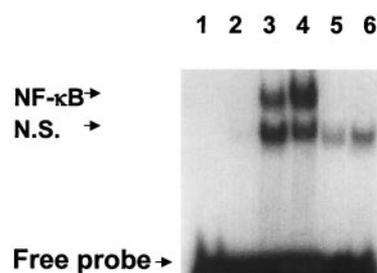


FIG. 1. NF- κ B ODN inhibits NF- κ B DNA binding in DCs. Nuclear proteins were extracted from DC propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF- κ B ODN (NF- κ B ODN DC) for 5 days. For further activation, DCs were exposed to LPS (2 μ g/ml) for the last 18 h. NF- κ B DNA binding activity was determined by EMSA, 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- κ B ODN DC, lane 5; NF- κ B DC stimulated with LPS, lane 6. NS, nonspecific band. The data are representative of three separated experiments.

stimulators. C3H spleen T-cells (2 \times 10⁵) 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 \times 10⁵) isolated from NOD mesenteric lymph nodes or spleens were used as responders. Cells (200 μ l/well) were cultured in complete medium in triplicate in 96-well round-bottom plates in 5% CO₂ in air at 37°C for 3–5 days. [³H]TdR (1 μ Ci/well) was added for the final 18 h of culture. Incorporation of [³H]thymidine into DNA was assessed by liquid scintillation. Results are expressed as mean counts per minute (cpm) \pm 1 SD.

DC administration and assessment of diabetes. Female 6- to 7-week-old NOD mice were given a single intravenous injection of 2 \times 10⁶ 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 insulinitis, 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 phosphatase 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 -³²P-UTP. Total RNA (5 μ g) was treated overnight with synthesized probes (specific activity: 800 Ci \cdot mmol/l) at 56°C, followed by treatment with RNase A (80 μ g/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- κ B ODN inhibits NF- κ B DNA binding in DCs. Nuclear proteins extracted from DCs propagated from bone marrow of NOD mice in the absence or presence of NF- κ B ODN was assessed for NF- κ B DNA binding by EMSA. Specificity of NF- κ B binding was demonstrated by utilizing the consensus NF- κ B probe and unlabeled NF- κ B probe (cold probe) as a competitor. As shown in Fig. 1, the NF- κ B band was detected by radiolabeled NF- κ B consensus sequence-specific probe in DCs without exposure to NF- κ B ODN (lane 3). NF- κ B binding capacity was en-

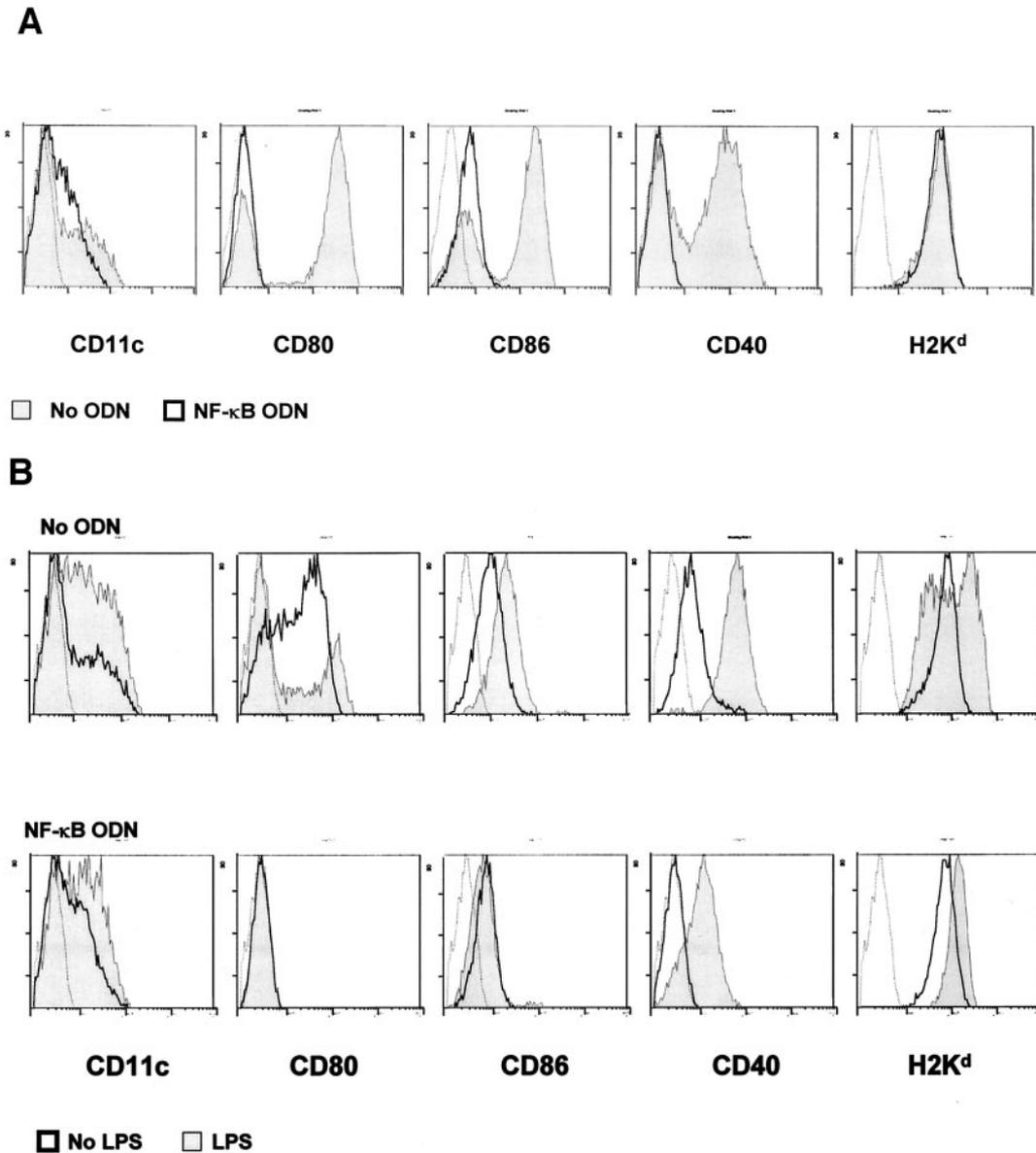


FIG. 2. Inhibition of costimulatory molecule expression on DCs by NF- κ B ODN. DCs were propagated from bone marrow of NOD mice in GM-CSF and IL-4 with or without NF- κ B ODN (10 μ mol/l). For further activation, DCs were exposed to LPS (2 μ g/ml) for the last 18 h. Expression of CD40, CD80, CD86, MHC, and CD11c was determined by flow cytometric analysis following mAb staining. Open profiles in dashed lines are isotype controls. **A:** NF- κ B 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- κ B ODN DC despite LPS stimulation. The data are representative of three separate experiments.

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

NF- κ B 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- κ B 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. 2A). 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- κ B dependent (27–29), we examined whether NF- κ B 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- κ B ODN kept CD40, CD80, and CD86 expression at low levels on DCs, even in the presence of LPS stimulation (Fig. 2B). This indicates that competitive inhibition of NF- κ B DNA binding by NF- κ B ODN prevents DC phenotypic maturation, regardless of the presence of

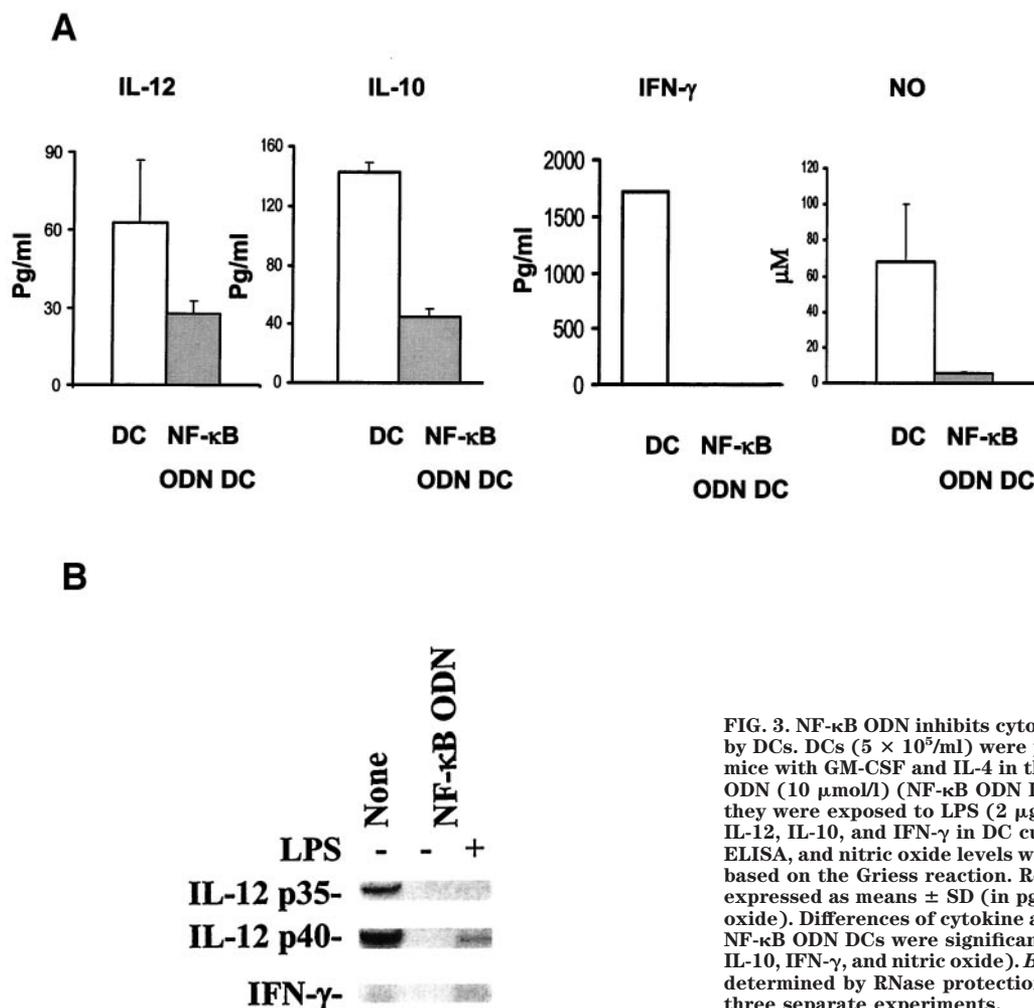


FIG. 3. NF- κ B ODN inhibits cytokine and nitric oxide (NO) production by DCs. DCs (5×10^5 /ml) were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF- κ B ODN (10 μ mol/l) (NF- κ B ODN DC) for 5 days. For further activation, they were exposed to LPS (2 μ g/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 \pm SD (in pg/ml for cytokines and μ mol/l for nitric oxide). Differences of cytokine and nitric oxide production by DCs and NF- κ B 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- κ B ODN on DC cytokine profile.** NF- κ B 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- κ B ODN DCs with LPS stimulation (Fig. 3A). Nitric oxide production is NF- κ B dependent; therefore, the diminished nitric oxide production in NF- κ B ODN DCs was an indication of NF- κ B 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- κ B ODN DCs. LPS stimulation slightly restored expression of IL-12 p40 and IFN- γ in NF- κ B ODN DCs, but not IL-12 p35. Expression of IL-1 α or - β , the IL-1 receptor, and TNF- α or - β in DCs was not suppressed by treatment with NF- κ B ODN, indicating that blocking

NF- κ B has a minimal effect on the expression of these monokines.

NF- κ B 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 4A 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- κ B ODN DCs were markedly suppressed. This inhibition was not reversed by LPS stimulation, indicating the persistent effect of NF- κ B ODN. We next assessed the effect of NF- κ B 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. 4B, without NF- κ B ODN treatment, DCs pulsed with islet antigens effectively stimulated T-cell proliferation, while the autostimulatory activity of NF- κ B ODN DCs was significantly suppressed. These data clearly indicate that consistent with the influence of NF- κ B ODN on DC surface molecule expression, the in vitro immune stimulatory activity of NF- κ B ODN DCs is also significantly suppressed.

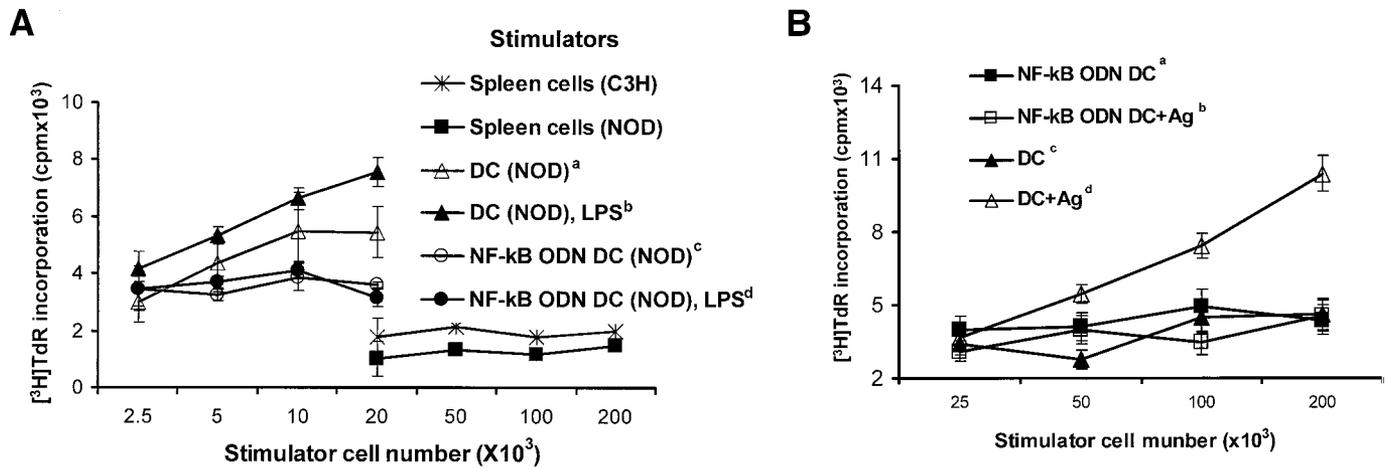


FIG. 4. Inhibition of DC immune stimulatory activity by NF- κ B ODN. DCs were propagated from bone marrow of NOD mice with GM-CSF and IL-4 in the absence (DC) or presence of NF- κ B ODN (10 μ M) (NF- κ B ODN DC). For further activation, DCs were exposed to LPS (2 μ g/ml) for the last 18 h. **A:** C3H (H2^k) splenic T-cells (2×10^5) were cultured with γ -irradiated DCs from NOD mice (H2^d) at graded concentrations for 3 days. Incorporation of [³H]TdR into DNA demonstrated that LPS stimulation augmented DC allostimulatory function ($P < 0.05$, a vs. b at 2×10^4 stimulators), while NF- κ B 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- κ B 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 \pm SD) and are representative of three separate experiments.

Administration of NF- κ B ODN DCs inhibits development of diabetes. Our net effort was directed toward assessing preventive effects of administration of NF- κ B ODN DCs on type 1 diabetes. Female NOD mice spontaneously developed pathological insulinitis, 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 insulinitis 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 insulinitis and diabetes by the age of 20 weeks (Fig. 5A and C). The early inflammatory changes as a result of peri-insulinitis 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. 5D). NOD mice were treated at 6–7 weeks of age with a single intravenous injection of 2×10^6 NF- κ B 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- κ B ODN DCs protected 70% of NOD mice from the development of diabetes up to the age of 38 weeks (Fig. 5A). 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. 5A). The insulinitis and islet infiltrates were histologically examined. As demonstrated in Fig. 5B, the severity of histological insulinitis was markedly diminished with substantial reduction in peri- and intra-islet infiltration at 30 weeks of age in mice

treated with NF- κ B 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 insulinitis as described in RESEARCH DESIGN AND METHODS. The insulinitis score in the NF- κ B ODN DC group was significantly lower than that in the non-DC and normal DC treatment groups (Fig. 5C). Compared with the non-DC treatment group, CD11b⁺ macrophages and CD8⁺ T-cells were significantly reduced in the subcapsular areas in NF- κ B ODN DC-treated mice, while CD4⁺ T-cells were markedly increased (Fig. 5D). Administration of NF- κ B 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- κ B 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 \pm 7.6	ND	5.5 \pm 7.8	9.2 \pm 0.2
DC	103.8 \pm 4.7	520.5 \pm 28.2	97.7 \pm 4.7	4,000 \pm 0
NF- κ B DC	0.16 \pm 0.22*	ND†	ND‡	ND§

Data are mean cpm \pm 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- κ B ODN (10 μ M). 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.

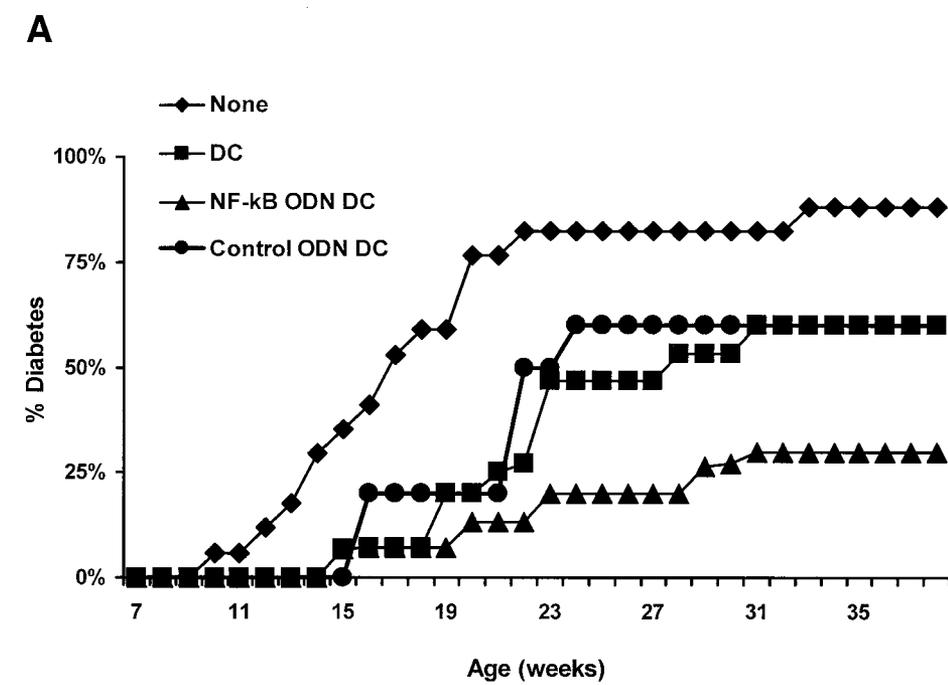
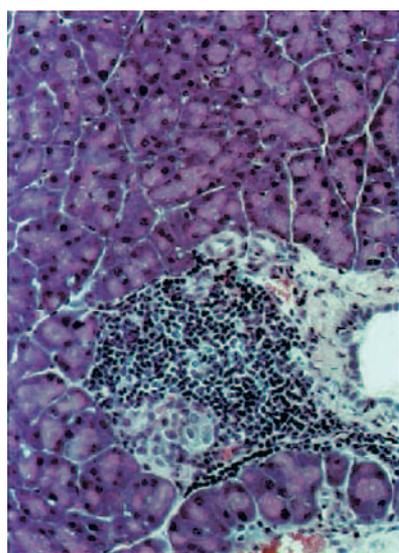
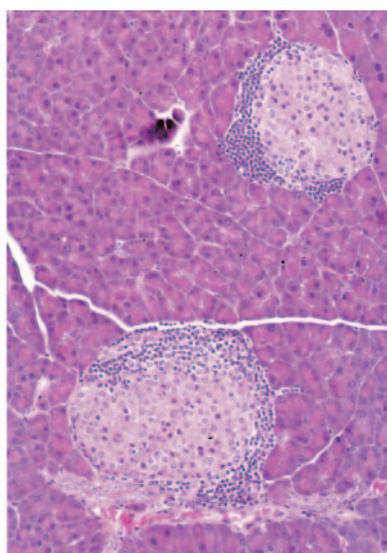


FIG. 5. Systemic administration of NF- κ B ODN DCs prevents diabetes development in NOD mice. NOD mice were injected intravenously at 6–7 weeks of age with 2×10^6 DCs propagated from NOD bone marrow with GM-CSF and IL-4 in the presence of NF- κ B ODN (NF- κ B ODN DC, $n = 17$) or control ODN (control 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- κ B 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- κ B ODN DC at 30 weeks of age revealed very minimal insulinitis, and untreated NOD mice developed severe insulinitis as early as 12 weeks of age (magnification $\times 400$). **C:** Three mice in each group were scored (50 islets per mouse) for insulinitis, as described in RESEARCH DESIGN AND METHODS, indicating that insulinitis was significantly less severe in mice treated with NF- κ B 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 $\times 200$). 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).

B



None (12 weeks)



NF- κ B ODN DC (30 weeks)

Administration of NF- κ B ODN DCs induces T-cell hyporesponsiveness to islet antigens. To examine the effect of NF- κ B 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- κ B 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- κ B 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- κ B 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- κ B ODN DCs. This was associated with markedly inhibited autoreactive T-cell responses and T-helper 1 responses (Fig. 3). It is unlikely

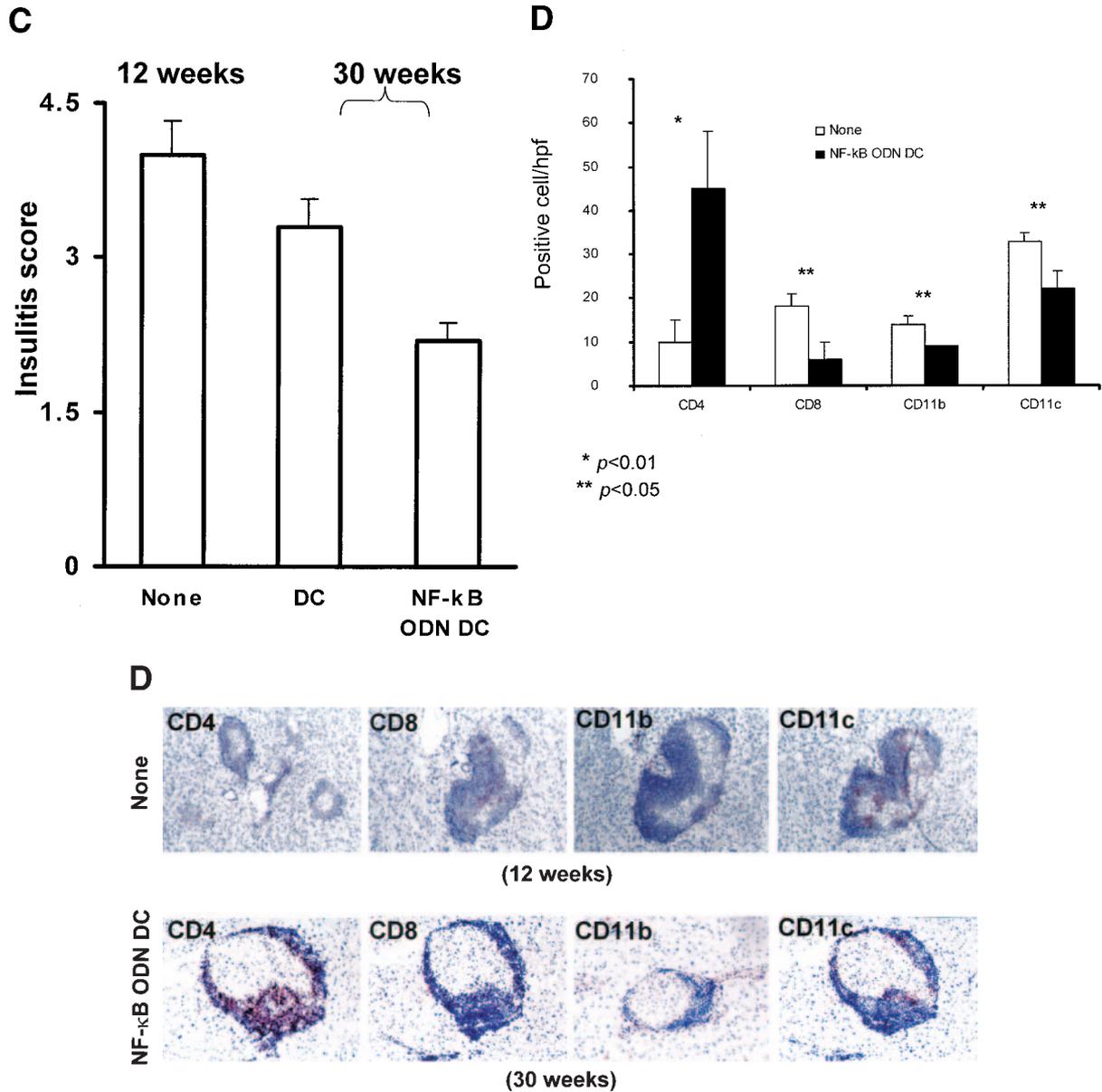


FIG. 5. Continued

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

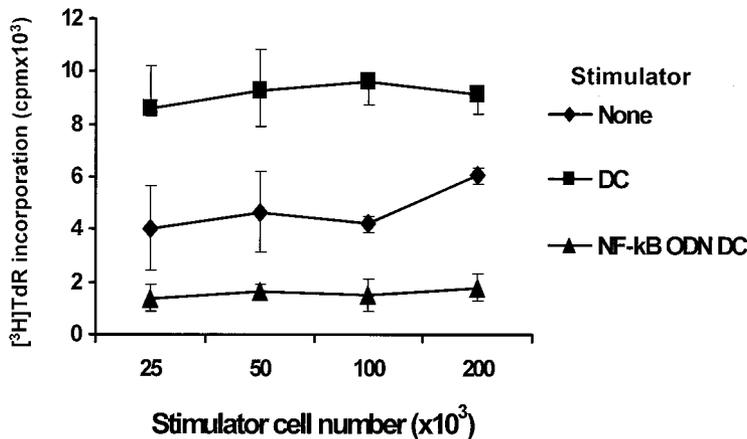


FIG. 6. Administration of NF-κB ODN DCs to NOD mice induces T-cell hyporesponsiveness to islet antigens. In a 4-day MLR assay, T-cells (2×10^3) purified from mesenteric lymph nodes of NOD mice that had received, at 6–7 weeks of age, 2×10^6 DCs intravenously were used as responders. γ -Irradiated DCs 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 \pm 1 SD) and are representative of three separate experiments.

(TUNEL) staining. Using FITC-conjugated NF- κ B 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- κ B ODN treatment at 10 mmol/l in the present study. These results indicate that exposure of DCs to 10 mmol/l NF- κ B 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- κ B (11,18). Signaling by members of the TNF- α receptor family, such as CD40 and receptor activator of NF- κ B (RANK), or LPS results in activation of NF- κ B 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- κ B 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- κ B binding to inhibit DC maturation is a new approach that is attracting extensive attention. NF- κ B activation can be inhibited at each step by pharmacological agents, including glucocorticoid-dependent upregulation of I κ B, 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- κ B inhibition. The NF- κ B ODN decoys used in this study effectively inhibit both phenotypic and functional maturation in DCs. NF- κ B ODN not only inhibits, but also resists, the maturation of DCs in response to LPS stimulation, thus indicating a stable immature state. Moreover, NF- κ B 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- κ B 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- κ B 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- κ B ODN DCs. The enhanced CD4⁺ T-cell infiltration by NF- κ B ODN DC treatment was unexpected, as earlier results demonstrated that CD4⁺ T-cells contributed to insulinitis 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- κ B 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- κ B 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- κ B ODN effectively inhibits the maturation/activation of DCs propagated from NOD mice. Compared with mature DCs, administration of DCs with suppressed NF- κ B 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.

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