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TLR9 Blockade Inhibits Activation of Diabetogenic CD8+ T Cells and Delays Autoimmune Diabetes

Yiqun Zhang,*‡ Andrew S. Lee,*‡ Afshin Shameli,‡§ Xuan Geng,¶ Diane Finegood,¶ Pere Santamaria,‡§ and Jan P. Dutz*‡

Diabetogenic CD8+ T cells are primed in the pancreatic lymph nodes (PLNs) by dendritic cells (DCs) carrying islet cell Ags. TLR signaling modifies DC function. The goal of this study was to determine the effect of TLR9 signaling on diabetogenic CD8+ T cell activation and the course of type 1 diabetes. We explored the effects of CpG oligonucleotide, TLR9 antagonists, and genetic TLR9 deficiency on the activation of diabetogenic CD8+ T cells. NOD bone marrow-derived DCs pulsed with freeze-thawed insuloma cells in the presence of TLR9 agonist CpG and CD40 agonist induced diabetogenic CD8+ T cell activation. The addition of TLR9 antagonist oligodeoxynucleotide or chloroquine inhibited bone marrow-derived DCs activation and CD8+ T cell priming in response to CpG. CpG alone or with CD40 agonist induced CTL activity that triggered diabetes development in 8.3-TCR transgenic NOD mice. Oligodeoxynucleotide treatment of 8.3-TCR transgenic NOD mice delayed spontaneous diabetes development.

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Type 1 diabetes (T1D) is the result of immune-mediated damage to the pancreatic islets (1). A crucial pathogenic role has been ascribed to CD8+ T cells (2). The activation of CD8+ T cells to tissue Ag occurs by a process termed cross-presentation. Recent data suggest that cross-presentation of apoptotic or necrotic Ag to CD8+ T cells can be tolerogenic or immunogenic and that dendritic cell (DC) activation is crucial to this distinction (3). CD40 ligation on DCs culminates in DC activation to promote immunogenic cross-presentation and the onset of T1D (4–6).

TLRs are a family of signaling molecules that bind TLR agonists released by infection or tissue injury. TLR function as a key component of the innate immune system and control multiple DC functions critically involved in the initiation of adaptive immune responses (7, 8). TLR9 is expressed in the endosome compartment of human plasmacytoid DCs (pDCs) and B cells and in a broader range of DCs in mice. TLR9 detects DNA with hypomethylated CpG motifs of microbial pathogens and their hosts (9). Ligation of TLR9 by CpG is synergistic with CD40 activation of DCs and promotes the cross-presentation of Ags by DCs (10, 11).

Cell death releases endogenous Ags and may also provide danger signals, such as endogenous DNA or RNA, which might bind to TLR9, TLR7/8, or TLR3; activate autoreactive T cells; and predispose the host to autoimmune disease (12). A self-DNA–TLR9 interaction may promote cell activation in systemic lupus erythematosus (SLE) (13). We previously observed that islet β cell death promotes diabetogenic T cell priming in situ (14). Physiological β cell death triggers the priming of self-reactive T cells by DCs in the pancreatic lymph nodes (PLNs) (15). How β cell death promotes T cell activation and diabetes development is still unclear. The clearance of dying cells is deficient in NOD mice (16). Deficient clearance in dying cells may promote the presence of secondarily necrotic cells that may then activate DCs via TLR2 (17). Because self-DNA liberated from dying cells may interact with TLR9 and promote immune responses (18), we explored the possibility that TLR9 signaling may contribute to the onset of T1D and that inhibition of this signaling could be used to delay the onset of diabetes.

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; FT, freeze-thawed; IGRP, islet-specific glucose 6 phosphatase catalytic subunit-related protein; LN, lymph node; 8.3-NOD, 8.3-TCR transgenic NOD; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cell; PLN, pancreatic lymph node; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.
complete medium (RPMI medium consisting of RPMI 1640 medium [In-vitrogen Canada, Burlington, Ontario, Canada] supplemented with 10% FBS [Sigma-Aldrich, Oakville, Ontario, Canada], penicillin [100 U/ml], and streptomycin [100 μg/ml]) for 6 d prior to use in cellular assays. An insulinoma cell line (NIT-1; ATCC, Manassas, VA), was used as the source of islet Ags. Naïve 8.3 CD8 T cells were isolated from 4–6-wk-old 8.3-NOD mice. Single-cell suspensions were prepared from peripheral lymph nodes (LNs) and spleens. CD8+ T cells were purified using CD8 microbeads and miniMac separation columns (Miltenyi Biotec, Auburn, CA).

Reagents and Abs

Phosphothioate-modified oligodeoxynucleotides (ODNs) CpG (5′-TCC ATG ACG TTC CTG AGTT-3′), ODN 2088 (5′-TCC TGG CGG GGA AGT-3′), and CpG 1982 (5′-TCC AGT ACT TCT CAGGTT-3′) were synthesized by the University of British Columbia Biotechnology Laboratory and purified by HPLC; they were endotoxin-free by Limulus assay. Recombinant mouse IL-4, IL-12, anti-CD40, biotin-anti-CD9, and biot-anti-CD25 were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Fluorescence-conjugated mAbs, including anti–IFN-γ–allophycocyanin, streptavidin-allophycocyanin, anti-CD11c-PE, anti-CD44-allophycocyanin, anti-CD122-PE, anti-CD8-FITC, and B220PerCP were from BD Pharmingen (San Diego, CA). FITC-conjugated rat anti-mouse IgM αIFN-γ was from PBL. InterferonSource (Piscataway, NJ). Anti-mouse plasmacytoid dendritic cell A-1– and CD11c-expressing cells was determined, and intracellular staining with anti–IFN-γ–FITC was performed as described (14).

In vitro CD8+ T cell priming assay

A total of 5 × 10^6 NOD BMDCs were incubated with 5 × 10^5 freeze-thawed (FT) NIT-1 cells (three cycles of freezing and thawing using alternating immersion in liquid nitrogen and 37°C water), with or without CpG (5 μg/ml), LPS (1 μg/ml; Sigma-Aldrich), or anti-CD40 mAb (clone 3/23, 15 μg/ml; BD Pharmingen) with or without ODN 2088 (12 μg/ml) or chloroquine (12 μg/ml; Sigma-Aldrich) overnight. The purity and viability of BMDCs after stimulation were confirmed by flow cytometric analysis and were independent of treatment received. A total of 2 × 10^5 CFSE-labeled 8.3 CD8+ T cells were then added to the culture. After 4 d of culture, the cells were collected, labeled with fluorescent-conjugated mAbs, and analyzed by flow cytometry.

In vivo CD8+ T cell priming assay

Female NOD TLR9+/− or NOD TLR9−/− mice were sacrificed at 11 wk of age, and PLNs were collected. The cells were stained with PE-conjugated NRP-V7/K2-tetramer on ice. After 4 h, mAbs anti–CD8-FITC and B220-PerCP were added, incubated for 30 min, and flow cytometry was performed.

Flow cytometry

Cultured CD8+ T cells, BMDCs, and peripheral blood-derived or PLN cells were harvested, washed with 0.5% BSA in PBS (Sigma-Aldrich), and stained with fluorescence-conjugated mAbs to CD25, CD69, CD40, CD44, and CD122 or with anti–IFN-γ–allophycocyanin intracellularly for the coexpression of CD25, CD69, CD40, CD44, and CD122 and IFN-γ secretion. Intracellular cytokine secretion was determined after in vitro stimulation with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 5 h. For in vitro assays, DCs were purified from PLN cells using CD11c microbeads and MiniMac separation columns (Miltenyi Biotec), prior to analysis. FACS analysis was performed on a FACSCalibur flow cytometer using CellQuest software (both from BD Biosciences, San Jose, CA). PLNs were also stained with NRP-V7/K2-tetramer, as previously described (14). For the analysis of pDCs within the PLNs, cells were gated for B220 expression, the fraction of plasmacytoid dendritic cell A1− and CD11c-expressing cells was determined, and intracellular staining with anti–IFN-γ–FITC was assessed.

Cytokine assays

ELISA. A total of 5 × 10^5 cells/ml BMDCs were cultured in complete medium with or without CpG (5 μg/ml) and LPS (1 μg/ml) alone or in combination with anti-CD40 mAb (15 μg/ml) with or without chloroquine (2 μg/ml) or ODN 2088 (12 μg/ml) in 5-ml polypropylene tubes. IL-12 p70 and IL-10 in 24-h culture supernatants were measured by standard ELISA (BD Biosciences).

IFN-γ assay. The preparation and assessment of IFN-γ production in LN homogenates were modified from the description of Li et al. (21). Briefly, LNs were extracted and homogenized into a single-cell suspension in 2% FBS/PBS. LN homogenates were lysed with radiol immunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.5% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) in the presence of a mixture of protease inhibitors (complete protease inhibitor mixture tablets; Roche Diagnostics, Indianapolis, IN). Supernatants from centrifuged lysates were removed and titrated for IFN-γ levels by ELISA (R&D Systems, Minneapolis, MN).

Cytometric bead array. Serum samples were assayed for IL-12 p70, IFN-γ, IL-6, and MCP-1 using a Mouse Inflammation Kit (BD Biosciences) and FACS.

In vivo cytotoxicity assay

8.3-NOD mice were injected i.p. with 100 μg CpG and/or 200 μg anti-CD40 mAb combined with 100 μg ODN 1982, 300 μg ODN 2088, or 200 μg rat IgG. Two days later, the mice were adoptively transferred (i.v.) with 1 μg/ml NRP-V7 peptide (KYNKAVFLF)-pulsed and 5 μM CFSE-labeled splenocytes or 1 μg/ml Tum (KYQAVTTLTEL)-pulsed and 0.5 μM CFSE-labeled splenocytes. PLN cells were collected 1 d later for analysis.

Assessment of diabetes development

Blood glucose was measured using Elite glucometers and glucometer (Bayer, Etobicoke, Ontario, Canada). Animals were considered diabetic when two consecutive measurements exceeded 14 mM.

Statistical analysis

Groups were compared using the one- or two-tailed Student t test or one- or two-way ANOVA (Prism 4, GraphPad, San Diego, CA), with post hoc significance analysis. Survival was compared using Kaplan–Meier curves and log rank tests.

Results

TLR9 agonism induces priming of diabeticogenic 8.3-NOD CD8+ T cells to self-Ag in vitro

TLR9 stimulation promotes the cross-presentation of Ags by BMDCs, resulting in the priming of CD8+ T cells (10). We investigated the effects of TLR9 signaling on diabeticogenic CD8+ T cell activation in response to a naturally occurring self-Ag. Immature BMDCs from NOD mice were pulsed with FT insulinoma cells (NIT-1) as a source of self-Ag in the presence or absence of TLR9 agonist (CpG) or TLR4 agonist (LPS), with or without agonist anti-CD40 Ab. 8.3-NOD CD8+ T cells recognize an immunodominant peptide from islet-specific glucose 6 phosphate catalytic subunit-related protein (IGRP) termed IGRP268-214, a protein expressed by pancreatic β cells; these cells are representative of a significant fraction of CD8+ T cells in pancreatic islets at the onset of inflammation (19, 22, 23). Although priming of diabeticogenic T cells may occur as early as 2 wk of age, primed T cells do not effectively home to LNs; thus, the majority of Ag-specific T cells in nonpancreatic LNs of 8.3-NOD mice remain naïve (14). Thus, naïve 8.3-NOD CD8+ T cells were purified, labeled with CFSE, and added to the Ag-pulsed cultured BMDCs. Four days later, we assessed CD8+ T cell activation using proliferation (CFSE dilution), intracellular IFN-γ production (Fig. 1A), and CD25 expression (data not shown). 8.3-NOD CD8+ T cells proliferated weakly following culture with BMDCs in the presence of FT NIT-1 cells alone. CpG, LPS, or anti-CD40 induced partial CD8+ T cell activation. CD40 agonism had a synergistic effect with CpG or LPS in inducing 8.3-NOD CD8+ T cell activation, and the combination of CpG plus anti-CD40 elicited the greatest 8.3-NOD CD8+ T cell activation.

Cell death provides a source of cellular Ags for cross-presentation, and it may release endogenous adjuvants that promote T cell responses (12, 24). To confirm that the CpG-induced 8.3-NOD CD8+ T cell activation in our model was β cell Ag specific, FT NIT-1 or FT B16 cells (melanoma cells) were added to the culture of NOD BMDCs and 8.3-NOD CD8+ T cells with CpG plus anti-CD40 (Fig. 1B). NOD BMDCs pulsed with FT NIT-1 cells induced 8.3-NOD CD8+ T cell activation, as manifested by increased CD25 expression, whereas NOD BMDCs pulsed with FT B16 cells did not (Fig. 1B; p = 0.037). Allogenic (H-2d C57B16-derived) BMDCs pulsed with NIT-1 Ag were also able to prime 8.3-NOD CD8+
T cells (data not shown), demonstrating that the NIT-1 cells provided Ag through a process termed cross-dressing (25). Thus, the assay allows an in vitro determination of CD8+ T cell activation requirements to physiologic amounts of self-Ag (derived from NIT-1 cells), but it cannot be used to assess self-Ag processing.

**ODN 2088 or chloroquine inhibits diabetogenic 8.3-NOD CD8+ T cell activation in response to β cell-derived Ags**

TLR9 activation is blocked by inhibitory oligonucleotides, such as ODN 2088 (26) or chloroquine (27). Chloroquine blocks CpG–TLR9 interaction by reducing endosomal acidification and is effective in treating B cell autoimmune diseases, such as SLE (28). In addition, chloroquine reduces CD8+ T cell activation and consequent tissue damage in murine graft-versus-host disease (27). We examined the effect of ODN 2088 and chloroquine on CpG-induced 8.3-NOD CD8+ T cell activation in response to β cell Ag. CFSE-labeled 8.3-NOD CD8+ cells were cocultured with NOD BMDCs pulsed with FT NIT-1 cells in the presence of CpG or LPS combined with anti-CD40, with or without ODN 2088 or chloroquine. The combination of CpG or LPS with anti-CD40 induced 8.3-NOD CD8+ T cell proliferation and concomitant CD25 expression (Fig. 2A). The addition of ODN 2088 or chloroquine to the culture inhibited CD8+ T cell activation induced by CpG but not by LPS. CpG-induced CD25 expression by 8.3-NOD T cells correlated with proliferation and IFN-γ secretion (Fig. 2B). CD40 stimulation enhanced CpG-induced CD8+ T cell activation. Ag-specific CD8+ T cell activation induced by CpG in the presence or absence of CD40 activation was abolished by the addition of chloroquine ($p < 0.05$; CpG+CD40 versus CpG+CD40+chloroquine).

**ODN 2088 or chloroquine inhibits CpG-induced NOD BMDC maturation**

TLR agonists promote CD8+ T cell immune responses by enhancing the processing of Ag and by the maturation and stimulation of DCs to produce inflammatory cytokines. TLR expression and function may be abnormal in NOD DCs (29). We examined the effect of ODN 2088 or chloroquine on CD40 expression on NOD BMDCs. BMDCs were cultured with or without CpG or LPS in the presence or absence of ODN 2088 or chloroquine, and consequent CD40 expression by CD11c+ DCs was assessed (Fig. 3A). CpG and LPS increased CD40 expression. ODN 2088 or chloroquine prevented CpG-induced, but not LPS-induced, CD40 expression on CD11c+ DCs.

IL-12 promotes a Th1-type immune response and accelerates the development of autoimmune diabetes (30). Conversely, the local expression of IL-10 delays diabetes onset (31). To determine the effect of ODN 2088 or chloroquine on CpG-induced cytokine production from NOD BMDCs, they were cultured with CpG or LPS with or without anti-CD40 in the absence or presence of ODN 2088 or chloroquine. CpG alone induced greater IL-12 p70 secretion than LPS. The combination of CpG and anti-CD40 mAb induced the greatest IL-12 p70 production from BMDCs, which was completely blocked by ODN 2088 or chloroquine (Fig. 3B; $p < 0.05$). In contrast, the combination of LPS and anti-CD40 induced low levels of IL-12 p70 and IL-10 secretion, an effect not modified by ODN 2088 or chloroquine. Thus, ODN 2088 and chloroquine inhibit CpG-induced proinflammatory cytokine secretion and maturation of NOD BMDCs.

**CpG triggers autoimmune diabetes, local CD8+ T cell activation, and memory T cell expansion in 8.3-NOD mice**

To determine whether TLR9 agonists can promote the spontaneous onset of diabetes, we studied the onset of diabetes in 8.3-NOD mice given CpG. Male and female 8.3-NOD mice develop spontaneous diabetes after 9 wk of age, with a more rapid rate of onset in female mice (32). We treated 8.3-NOD mice with a single dose of CpG (and control rat Ig), anti-CD40 (and control oligonucleotide ODN 1982), or both at 5 wk of age (Fig. 4) or ~1 mo prior to the spontaneous onset of disease. Half of the 8.3-NOD mice treated with CpG or anti-CD40 rapidly developed hyperglycemia ($p < 0.03$; compared with untreated). 8.3-NOD mice treated with...
a combination of CpG and anti-CD40 had the greatest prevalence of diabetes within 10 d ($p < 0.01$). Notably, coinjection of CpG and the TLR9 inhibitory ODN 2088 abrogated diabetes acceleration, confirming a TLR9-mediated effect on diabetes onset.

TLR9 agonists directly activate DCs. To confirm that consequent diabetogenic T cell activation contributed to the CpG-mediated acceleration of diabetes in 8.3-NOD mice, we examined CD8$^+$ T cells within the PLNs following treatment with CpG, with or without ODN 2088 or chloroquine, and compared this with animals treated with LPS with or without chloroquine (Fig. 5A). CpG administration induced CD69 expression on 8.3-NOD CD8$^+$ T cells; ODN 2088 ($p = 0.01$) and chloroquine (significantly at 20 mg/kg [$p = 0.02$] and less so at 10 mg/kg [$p = NS$]) diminished this expression in vivo. As expected, LPS-induced CD69 expression by CD8$^+$ T cells was not inhibited by chloroquine.

CD44/CD122$^+$ CD8$^+$ T cells represent a population of memory CD8$^+$ T cells that are responsive to IL-15 for proliferation and survival (33). Incomplete CD8$^+$ T cell activation results in abortive proliferation and absence of cytotoxicity (34). To confirm that CpG activation induces full maturation, we investigated the effect of CpG on the induction or expansion of a CD8$^+$ T cell memory phenotype. CpG plus anti-CD40 treatment of 8.3-NOD mice clearly increased the fraction of circulating CD44$^+$ hi and CD122$^+$ hi 8.3 CD8$^+$ T cells within the blood compartment (Fig. 5B). Full CD8$^+$ T cell activation is associated with the development of cytotoxic potential. To assess the cytotoxic potential of 8.3 TCR CD8$^+$ T cells in vivo following TLR9 agonist exposure, 8.3-NOD mice were treated with CpG and/or anti-CD40 combined with control ODN 1982 (which is neither stimulatory nor antagonistic) or rat IgG. These mice then received target splenocytes labeled with CFSE and loaded with the cognate L$^d$-restricted peptide Ag of 8.3 CD8$^+$ T cells (V7) or an irrelevant peptide (Tum). Target-peptide loaded splenocyte survival was diminished in CpG- or anti-CD40–treated PLNs, and it was dramatically reduced in CpG plus anti-CD40–treated PLNs (Fig. 5C; $p = 0.038$). In summary, CpG and/or CD40 stimulation induce diabetogenic 8.3-NOD CD8$^+$ T cell activation in vivo, memory phenotype, and CTL activity, culminating in diabetes development. Thus, NOD DCs process and present endogenous Ags to diabetogenic CD8$^+$ T cells following TLR9 activation.

**FIGURE 3.** Effect of ODN 2088 or chloroquine on the induction of NOD BMDC maturation. A, NOD BMDCs were cultured with CpG or LPS in the presence or absence of ODN 2088 or chloroquine overnight. CD11c$^+$ cells were analyzed for CD40 expression. Representative graphs of three independent experiments are shown. B, NOD BMDCs were cultured with or without CpG, LPS 1 µg/ml, and anti-CD40 in the presence or absence of ODN 2088 or chloroquine for 24 h. IL-12 and -10 in the culture supernatants were determined by ELISA. Bars represent the means of triplicates ± SEM. The $p$ values were determined by two-way ANOVA.

**FIGURE 4.** Effect of CpG, anti-CD40, and ODN 2088 on diabetes development in 8.3-NOD mice. Five-week-old 8.3-NOD mice were given 100 µg CpG and/or 200 µg anti-CD40 i.p., combined with 100 µg ODN 1982, or 200 µg rat IgG or 300 µg ODN 2088. Blood glucose was determined every other day for 10 d. Four female and six male mice were included in each group. The $p$ values represent log rank test results compared with untreated mice.

**FIGURE 5.** Effect of CpG alone or with anti-CD40 on 8.3-NOD CD8$^+$ T cell activation in vivo. A, 8.3-NOD mice were given 100 µg CpG or LPS alone or combined with 100 µg ODN 2088 or chloroquine at 10 or 20 mg/kg i.p. One day later, PLN cells were assayed for CD69 expression on CD8$^+$ cells. The left panels are representative of four experiments. The summary of four experiments is shown in the right panel. B, 8.3-NOD mice were given CpG plus anti-CD40 or PBS as control. After 4 d, blood-circulating CD8$^+$ T cells were assayed for CD44 and CD122 expression. The mean of triplicate experiments is shown in the right panel. C, In vivo cytotoxicity assay. Five-week-old 8.3-NOD mice were given CpG and rat IgG, anti-CD40, and ODN 1982 (control ODN), or CpG and anti-CD40. After 2 d, the mice were adoptively transferred with self-IGRP-derived V7 peptide (CFSE high) or irrelevant peptide (Tum)-pulsed (CFSE low)-labeled splenocytes. The PLN cells were assayed for CFSE profile. The mean of triplicate experiments is shown in the right panel.
Our observations indicate that TLR9 stimulation promotes the onset of diabetes. Endogenous DNA, either liberated from damaged cells or from microbial commensals, may stimulate TLR9 (12). Therefore, we explored whether the TLR9 antagonist ODN 2088 would delay the onset of spontaneous diabetes. 8.3-NOD mice at 3–4 wk of age were injected with ODN 2088 or controls (PBS or nonstimulatory and noninhibitory ODN 1982), three times weekly.

In contrast to the experimental acceleration of diabetes with CpG described previously, mice were followed for 6 wk to determine the rate of spontaneous onset of disease. Female mice treated with ODN 2088 demonstrated a delayed diabetes onset compared with the mice injected with PBS or ODN 1982 (Fig. 6A; p = 0.037).

Thus, endogenous TLR9 activation contributes to the onset of diabetes in 8.3-NOD mice. We treated 3–4-wk-old male and female 8.3-NOD mice with chloroquine (10 mg/kg i.p. daily for 5 d as a loading dose and two to three times weekly thereafter) or PBS. This dosing regimen has been shown to inhibit the progression of murine graft-versus-host disease (27). Again, the spontaneous onset of diabetes was delayed (Fig. 6B; p = 0.043).

Chloroquine prevents diabetes development in NOD mice and inhibits DC maturation

8.3-NOD mice have accelerated diabetes due to a greatly expanded population of islet-specific CD8+ T cells. High frequencies of Ag-specific T cells alter the kinetics and regulation of T cell priming (35). We sought to determine whether chloroquine would inhibit diabetes development in NOD mice, because these animals display a more physiologic frequency of self-reactive T cells and a broader repertoire of islet-specific T cells than do the transgenic mice. NOD female mice at 5–8 wk of age were treated with 10 mg/kg chloroquine, as described above. Although 19 of 29 PBS-treated mice became diabetic up to 30 wk of age, only 11 of 29 chloroquine-treated mice developed diabetes at this age (Fig. 7A; p = 0.035). Thus, chloroquine administration delays spontaneous diabetes development in NOD mice.

To explore the mechanisms by which chloroquine prevents diabetes development in NOD mice, we assessed the effect of chloroquine treatment on CD40 expression by pancreatic DCs. NOD mice at 5–7 wk of age were treated with chloroquine or PBS. The

PLNs were collected after 4 wk of treatment (at an age when insulitis is present). DCs from chloroquine-treated PLNs demonstrated diminished levels of CD40 expression by pancreatic DCs.

Repeated TLR9 stimulation with CpG was reported to delay the onset of diabetes in NOD mice (36). However, the administration of low doses of CpG in young animals was recently reported to
accelerate the onset of diabetes in NOD mice (37). To confirm a role for TLR9 in the spontaneous onset of diabetes in NOD mice, TLR9+/− NOD mice were generated by speed congenic mating of TLR9+/−-deficient mice to NOD mice. To generate control mice with TLR-signaling impairment, MyD88+/−/− NOD mice, TLR3−/−/− NOD mice, and TLR2−/−/− NOD mice were generated by speed congenic mating of TLR-deficient strains to NOD mice. The persistence of a full NOD background was confirmed in each case using 104 microsatellite markers. The spontaneous onset of diabetes in MyD88- and TLR-deficient NOD females was compared using 104 microsatellite markers. TLR9−/−/− NOD mice demonstrated a significant delay in the onset of diabetes compared with TLR9+/−/− NOD or NOD littermates (p = 0.042) (Fig. 8A). Likewise, MyD88−/−/− NOD female mice in our colony were resistant to diabetes, and TLR2−/−/− NOD, but not TLR3−/−/− NOD, mice demonstrated a delayed onset of diabetes (Supplemental Fig. 1). Thus, TLR9 signaling contributes to the spontaneous onset of diabetes in NOD mice.

To determine the mechanism of delayed onset of diabetes in TLR9−/−/− NOD mice, we examined levels of IFN-α produced within the PLNs of young mice. Type 1 IFNs promote the priming of cytotoxic T cells. It was recently demonstrated that an early and specific increase in PLN IFN-α is required for diabetogenesis (21). This increase occurs at the time of pancreatic tissue remodeling and weaning, coincident with the onset of CTL priming. The stimulus for this increase in IFN-α has been unclear. We confirmed that PLNs of young NOD mice have elevated levels of IFN-α (Fig. 8F). This increase is limited to the PLNs and is not noted in other secondary lymphoid organs or in the pancreas (data not shown). Further, this increase in IFN-α is largely absent in MyD88−/−/− NOD and TLR9−/−/− NOD mice. MyD88−/−/− NOD and TLR9−/−/− NOD mice have a decreased frequency of pDCs within the PLNs and a decreased expression of IFN-α within the pDCs (Fig. 8C) compared with NOD mice. The limited local IFN-α production correlates with a significant decrease in the frequency of NRP-V7 (IGRP206–214)-reactive CD8+ T cells within the PLNs (Fig. 8D). Thus, impaired activation and recruitment of diabetogenic CD8+ T cells in TLR9 (and MyD88)-deficient mice is associated with an impaired production of IFN-α within the PLNs, suggesting an underlying mechanism.

Discussion

We examined the role of TLR9 stimulation of DCs in the development of T1D in NOD mice. Immature NOD BMDCs pulsed with β cell Ag, in the form of necrotic insulinoma cells, and stimulated by the TLR9 agonist CpG plus anti-CD40 prime diabetogenic CD8+ T cells, resulting in proliferation, CD25 expression, and IFN-γ production (Fig. 1). Further, treatment of 8.3-NOD mice with CpG induces diabetogenic CD8+ T cell activation and CTL activity and triggers diabetes (Fig. 4). Using an in vitro assay of T cell activation, we demonstrated that a TLR9 inhibitory oligonucleotide (ODN 2088) or chloroquine inhibit CpG-induced 8.3 CD8+ T cell activation in vitro (Figs. 2 and 3). These agents inhibited CpG-induced diabetogenic CD8+ T cell activation in vivo (Fig. 5) and delayed the onset of diabetes in 8.3-NOD (Fig. 6) and NOD mice (Fig. 7). Finally, NOD mice deficient in TLR9 demonstrated a delayed onset of diabetes compared with TLR9+/− littermates (Fig. 8). Collectively, this demonstrates that TLR9 signaling, albeit dispensable for diabetogenesis, contributes to diabetogenic CD8+ T cell activation and diabetes progression. Previously, repeated CpG administration was shown to delay diabetes.

FIGURE 8. NOD TLR9-deficient mice display delayed diabetes development and decreased IFN-α secretion and NRP-V7/Kd CD8+ T cells in PLNs. A, Female NOD or NOD TLR9 knockout heterozygous (+/−) or homozygous (−/−) mice were followed for diabetes development. Differences were assessed using log rank analysis. B, Endogenous IFN-α production from PLNs in C57BL/6, NOD, NOD TLR9−/−, and NOD MyD88−/−/− mice. PLN homogenates were prepared from female 3–4-wk-old C57BL/6, NOD, and NOD TLR9−/− mice and 8-wk-old NOD MyD88−/−/− mice. IFN-α was detected by ELISA. Data were from two independent experiments. Differences were detected by ANOVA, with post hoc multiple comparison analysis. C, PLNs were collected from female NOD, NOD TLR9−/−, and NOD MyD88−/−/− mice at 4–8 wk of age. The cells were analyzed for frequencies of pDCs (B220+ CD11c+PDCA1+) (left panel) and IFN-α–producing pDCs (right panel). D, PLNs were collected from female NOD TLR9−/− or NOD TLR9−/− mice at 11 wk of age. The cells were analyzed for the frequency of NRP-V7/Kd CD8+ cells by flow cytometry. Left panel: Representative dot plots. Right panel: Mean of two experiments (five mice in each group). Differences were determined by the Student t test.
specific CD8+ T cells (42). We suggest that TLR9 stimulation may
promote the activation of diabetogenic CD8+ T cells to foreign
AgS (40) and the priming of autoreactive CD4 T cells in myo-
carditis (41). We demonstrated that TLR9 agonism combined with
CD40 ligation induced NOD DCs to produce high levels of IL-
12p70, in contrast to LPS stimulation (Fig. 3). This combined
agonism rapidly induced diabetes in 8.3-NOD mice (Fig. 4). Be-
cause 8.3-NOD CD8+ T cells respond to the islet self-Ag IGRP,
TLR9 signaling and CD40 activation induce CD8+ T cell activa-
tion to physiologic levels of this self-Ag and promote the onset of
diabetes. Recent data indicate that islet inflammation can substi-
tute for proinsulin-specific CD4+ T cell help to activate IGRP-
specific CD8+ T cells (42). We suggest that TLR9 stimulation may
induce such inflammation. TLR9 stimulation also promotes cross-
presentation of Ag. Our in vitro assay system did not permit a
quantitative assessment of this aspect of TLR9 effect.

Antimalarials have been used for decades for the treatment and
propylaxis of malaria and, more recently, for the treatment of
autoimmune diseases and inflammatory skin disease (43). The
immunologic effect of chloroquine was previously thought to relate
to inhibition of lysosomal function, resulting in decreased pre-
sentation of class II MHC Ag to CD4 T cells (44). However,
nanomolar, rather than micromolar, concentrations (required for
effects on class II MHC Ag presentation) prevent bacterial DNA-
induced IL-6 production by human PBMCs, an effect now known to
be due to inhibition of TLR9 signaling (45). Chloroquine inhibits
rheumatoid factor production by B cells induced by chromatoin-
immune complexes that signal through TLR9 (13). We demonstra-
ted that chloroquine inhibits CD8+ T cell activation and prevents the development of autoimmune diabetes. DCs activate memory cells as well as naive T cells (46). Because CTL priming in NOD mice begins as early as 2 wk of age, and we administered chloroquine after 4 wk of age, chloroquine may inhibit the activation of memory cells as well as previously naive cells. Chloroquine also delayed the onset of diabetes follow-
progression of diabetes was noted at doses of 10 mg/kg, and robust
effects on T cell activation in vivo were noted at doses of 20 mg/kg.
Antimalarials have other potentially beneficial effects. In SLE pa-
tients, these drugs lower lipid levels and are cardioprotective (47).
Antimalarials improve glucose tolerance in patients with type 2
diabetes (48). All of these observations suggest that chloroquine
therapy may be beneficial in the treatment of T1D.

Aminoquinoline antimalarials, through their effects on lysosomal
function, have multiple effects on cellular physiology. Endosomal
TLRs potentially inhibited by antimalarial therapy include TLR3,
TLR7/8, and TLR9. Suppressive ODNs have been screened and
developed to provide more specific TLR inhibition. ODN 2088 is one
such suppressive ODN (26) that blocks the stimulatory effect of
CpG on B cell activation. Such G-rich ODNs are the most effective
inhibitors of TLR9 activation (49), and they have been used to di-
rectly block the binding of CpG to TLR9 (50). We demonstrated
that TLR9 agonist-induced activation of diabetogenic CD8+ T cells
in vivo is inhibited by the systemic administration of a TLR9 ODN
antagonist (Figs. 4 and 5). This suggests that the development of
TLR9 antagonists for the prevention and treatment of T1D, as is
being pursued for other autoimmune diseases (51), is worthwhile.

The generation of specific TLR-deficient autoimmune-prone
animal models has revealed a complex role for TLR signaling in
the modulation of spontaneous and infection-associated autoimmunity.
Conventionally housed (specific pathogen-free) MyD88−/− NOD
mice do not develop diabetes (52). However, MyD88 signaling is
not obligatory for the development of diabetes, because germ-free
MyD88−/− NOD mice become diabetic (52). The protective effect
of MyD88 deficiency was ascribed to an altered commensal gut flora
that inhibited disease in a MyD88-independent fashion. A protective
effect of a single TLR deficiency (TLR2, TLR3, and TLR4 were
reported) was not noted by Wen et al. (52). However, Kim et al. (17)
reported that TLR2-deficient NOD mice have delayed onset of di-
abetes ascribed to decreased sensing of apoptotic cell-induced
particles. In our colony, MyD88−/− NOD mice were resistant to
diabetes, whereas similarly housed TLR2−/−, but not TLR3−/−,
NOD mice demonstrated delayed onset of diabetes (Supplemental
Fig. 1). Thus, we confirm that broad (MyD88−/−) and specific
inhibition of TLR signaling (TLR2 and TLR9 deficiency) can affect
the onset of diabetes. TLR9 signaling by gut flora-derived DNA
limits regulatory T cell conversion and acts as an endogenous
adjuvant to promote intestinal immune responses (53). Gut in-
flammation also promotes the activation of diabetogenic T cells
(54). Thus, our results may indicate that commensal gut flora DNA
promotes the onset of diabetes through TLR9 activation. We have
determined whether the gut flora of these strains differs in our
colony; thus, TLR-induced variations in enteric flora and the pro-
tective or deleterious immune effects thereof could contribute to
alterations in the incidence of disease.

Endogenous IFN-α was recently shown to be critical for the develop-
ment of diabetes in the NOD mouse (21). IFN-α administra-
tion can trigger autoimmune diabetes in man (55). The source of
the endogenous IFN-α promoting diabetes in the NOD mouse is not
clear. However, TLR7 or TLR9 stimulation, by viral RNA or DNA,
respectively, of pDCs results in large amounts of IFN-α production
following viral infection (56). Viral-mediated TLR9 activation
participates in the acceleration of diabetes in the biobreeding rat
(57). Self-DNA may also trigger TLR9 activation and IFN-α pro-
duction, resulting in autoimmunity (58). The therapeutic effects
of ODN2088 and chloroquine, as well as the delayed onset of diabetes
in TLR9−/− NOD mice, clearly point to TLR9 agonism as con-
tributory to diabetes progression in the pathogen-free NOD mouse.
We further demonstrate that MyD88−/− NOD and TLR9−/− NOD
mice do not exhibit the specific increase in PLN IFN-α noted in
NOD mice (Fig. 8). Further, the absence of TLR9 signaling and
consequent local IFN-α is associated with decreased diabetogenic
CD8+ T cell priming in the PLNs. This suggests that TLR9 signaling,
and more specifically, TLR9 signaling, contributes to the increase
in IFN-α that occurs in young NOD mice. The lack of modulatory
effect of TLR3 deficiency (receptor for dsRNA) noted in this study and
by other investigators (52) may point to a direct pathogenic role
for pDCs, cells that are devoid of TLR3 (59). In this regard, we further note that the absence of TLR9 or MyD88 signaling results in a decreased frequency of pDCs within the PLNs and decreased IFN-α expression within the pDCs. It needs to be determined whether the stimulus for deleterious TLR9 activation derives from endogenous tissue-derived DNA (associated with tissue remodeling) or from enteric gut bacteria (changing at the time of weaning). Paradoxically, TLR9 deficiency in mouse models of SLE accelerates disease onset (60). TLR9 deficiency results in increased TLR7-mediated activation of pDCs and IFN-α production. This may be a consequence of tonic TLR9 ligation providing a negative signal for immune responses through the induction of IDO production (61) and the subsequent induction of T regulatory cell function (60). Thus, the contrary autoimmune consequence of TLR9 signaling in the NOD mouse may be due, in part, to altered regulatory T cell function and/or immune modulation by the intestinal tract, including gut bacteria. NOD mice were recently shown to have a defect in TLR9-mediated IDO production, resulting in fewer regulatory T cells (37). Rig-1–like receptors are recently shown to have a defect in TLR9-mediated IDO production, as a consequence of tonic TLR9 ligation (177: 6018–6022).

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24. Verdaguer, J., J. W. Yoon, B. Anderson, N. Averill, T. Usugi, B. J. Park, and P. Santamaria. 1997. Spontaneous autoimmune diabetes in NOD mice suggests that the contribution of TLR7 and Rig-1 pathways to the priming of diabeticogenic CD8+ T cells may be minor. We demonstrated that TLR9 signals promote spontaneous diabetes in the NOD mouse, coincident with the upregulation of PLN IFN-α. The identification of the self versus foreign (commensal or pathogen) TLR9 agonists that drive pancreatic pDC IFN-α production and promote diabetes may allow the design of more specific interventions to prevent disease.


