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*J Immunol* 2010; 184:5645-5653; Prepublished online 14 April 2010;
doi: 10.4049/jimmunol.0901814
http://www.jimmunol.org/content/184/10/5645

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/04/14/jimmunol.0901814.DC1

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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 insulinoma 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 insulinoma


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).

TLR9, TLR7/8, or TLR3; activate autoreactive T cells; and promote immune responses (18), we explored the possibility that self-DNA liberated from dying cells may interact with TLR9 and promote 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.

Materials and Methods

Mice and cells

8.3-TCR transgenic NOD (8.3-NOD) mice expressing the rearranged TCR genes of the diabetogenic CTL clone NY 8.3 have been described (19) and were bred at the University of Calgary and at the University of British Columbia. NOD mice (The Jackson Laboratory, Bar Harbor ME) were purchased from Oriental Bio-Service (Kyoto, Japan) and were bred onto the NOD genetic background using a speed mating strategy. NOD mice had lower levels of IFN-α in PLNs and decreased frequencies of plasmacytoid DCs and diabetogenic CD8+ T cells compared with NOD mice. We propose that TLR9 activation contributes to the spontaneous onset of diabetes in NOD mice by increasing IFN-α and promoting diabetogenic CD8 T cell activation. The Journal of Immunology, 2010, 184: 5645–5653.

Received for publication June 8, 2009. Accepted for publication March 16, 2010.

This work was supported by Juvenile Diabetes Research Foundation International Grant 4-2004-783 as part of the β-Cell Apoptosis and Autoimmunity Network Program (to J.P.D.) and by the Canadian Institutes of Health Research (to P.S.). A.S. is supported by a studentship from the Alberta Heritage Foundation for Medical Research. The Julia McFarlane Diabetes Research Centre is supported by the Diabetes Association (Foothills).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: 8.3-NOD, 8.3-TCR transgenic NOD; 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; ODN, oligodeoxynucleotide; pDC, plasmacytoid dendritic cell; PLN, pancreatic lymph node; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901814

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The Journal of Immunology

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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 (LN) and spleens. CD8+ T cells were purified using CD8 microbeads and miniMACS separation columns (Miltenyi Biotec, Auburn, CA).

Reagents and Abs

Phosphoantigen-modified oligodeoxynucleotides (ODNs) CpG (5′-TCC ATG ACG TTC CTG AGGT-3′), ODN 2088 (5′-TCC TGG CGG GGA AGT-3′), and CpG 1982 (5′-TCC AGG ACT TCT CTC AGGT-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-6, anti-CD40, biotin-anti-CD69, and biotin-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 mAb IFN-γ was from PBL. InterferonSource (Piscataway, NJ). Anti-mouse plasmacytoid dendritic cell Al-PE was from Miltenyi Biotec. PE-conjugated NRP-V7/Kd-tetramer was produced as described (14).

In vitro CD8+ T cell priming assay

A total of 5 × 10^5 NOD BMDCs were incubated with 5 × 10^5 freeze-thawed (FT) NIT-1 cells (three cycles of freezing and thawing using alternate 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 (2 μg/ml; Sigma-Aldrich) overnight. The purity and viability of BMDCs after stimulation were confirmed by flow cytomteric analysis and were independent of treatment received. A total of 2 × 10^5 CFSE-labeled cells were 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/Kd-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 (50 ng/ml) and ionomycin (1 μg/ml) for 5 h. For in vitro assays, DCs were purified from PLN cells using CD11c microbeads and MiniMACS separation (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/Kd-tetramer, as previously described (14). For the analysis of pDCs within the PLNs, cells were gated for B220 expression, the fraction of plasmacytid dendritic cell A-1- 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-α assay. The preparation and assessment of IFN-α production in LN homogenates were modified from the description of Li et al. (21). Briefly, LN homogenates were lysed with radiolabeled 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-α 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 vitro cytotoxic 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 adaptively transferred (i.v.) with 1 μg/ml NRP-V7 peptide (KYNKANVFL)-pulsed and 5 μM CFSE-labeled splenocytes or 1 μg/ml Tum (KYQAVTTTL)-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’s 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 diabetogenic 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 diabetogenic 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 phosphatase catalytic subunit-related protein (IGRP) termed IGRP206-214, 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 diabetogenic T cells may occur as early as 2 wk of age, pruned 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-2b C57B16-derived) BMDCs pulsed with NIT-1 Ag were also able to prime 8.3-NOD CD8+
Histamine increases the expression of proteins involved in cell adhesion and migration; these changes are accompanied by the activation of pro-inflammatory cytokines, such as IL-1 and TNF-α, which can promote tissue damage in the disease process. The combination of these factors can contribute to the development and progression of autoimmune diabetes, particularly in the context of genetic predisposition and environmental triggers.

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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 chloroquine did not develop diabetes (p < 0.03; compared with untreated).
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 [$p = \text{NS}$]) 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$^+$ and CD122$^+$ 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.
ODN 2088 delays diabetes development in 8.3-NOD mice

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 (Fig. 7B; p = 0.029).

We next examined the effect of chloroquine on CpG-induced secretion of cytokines and chemokines in vivo. Female NOD mice were injected with CpG or LPS alone or combined with chloroquine at a dosage of 10 mg/kg. CpG treatment induced the secretion of IL-12 p70, IFN-γ, IL-6, and MCP-1. Chloroquine significantly inhibited the production of these cytokines and MCP-1. In contrast, LPS-induced secretion of cytokines and chemokines was not inhibited by chloroquine (Fig. 7C). Thus, chloroquine treatment leads to diminished pancreatic DC activation and the inhibition of cytokine release in response to TLR9 but not TLR4 agonists.

TLR9<sup>−/−</sup> NOD mice demonstrate a delayed onset of diabetes with decreased IFN-α production and decreased diabetogenic CD8<sup>+</sup> T cells in PLNs

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 with heterozygote and wild type NOD littermates. 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. 8B). 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 (IGRP 206–214)-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.
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 directly block the binding of CpG to TLR9 (50). We demonstrated that TLR9 agonist-induced activation of diabeticogenic 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 diabetes 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 inflammation also promotes the activation of diabeticogenic T cells (54). Thus, our results may indicate that commensal gut flora DNA promotes the onset of diabetes through TLR9 activation. We have not determined whether the gut flora of these strains differs in our colony; thus, TLR-induced variations in enteric flora and the protective 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 development of diabetes in the NOD mouse (21). IFN-α administration 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 production, 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 contributory 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 diabeticogenic CD8+ T cell priming in the PLNs. This suggests that TLR 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 additional cytosolic sensors of RNA and DNA that induce IFN-α production (62). The roles of TLR7-mediated stimulation of pDCs and Rig-1-like receptor stimulation of IFN-α production in diabetes are areas ripe for further investigation. However, the abrogation of the NOD-specific PLN IFN-α increase in TLR9−/− 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, coinjected 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.

Acknowledgments

We thank members of the β-Cell Apoptosis and Autoimmunity Network Program and the Dutz laboratory for comments and support.

Disclosures

P.S. is a scientist at the Alberta Heritage Foundation for Medical Research, which is supporting A.S. with a studentship.

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