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IDO Mediates TLR9-Driven Protection from Experimental Autoimmune Diabetes

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Originally predicated on the recognition of an increasing prevalence of allergy, the hygiene hypothesis was later found to accommodate the contrasting epidemiologic trends in developed countries for infectious vs autoimmune diseases. Experimentally, reduced exposure to infections will increase the risk of disease in several models of experimental autoimmunity. Although TLRs were initially considered as stimulatory molecules capable of activating early defense mechanisms against invading pathogens, emerging data suggest that they can also exert a regulatory function. In the present study, we evaluated whether TLR3 and TLR9, recognizing microbial dsDNA and CpG-containing DNA sequences, respectively, play a role in the protection from experimental autoimmune diabetes induced in C57BL/6 mice by streptozotocin. In wild-type animals, the disease was accompanied by up-regulation of IDO in pancreatic lymph nodes and would be greatly exacerbated by in vivo administration of an IDO inhibitor. Conversely, administration of a CpG-containing oligodeoxynucleotide greatly attenuated the disease in an IDO-dependent fashion. TLR9-, but not TLR3-deficient mice developed a more robust disease, an event accompanied by lack of IDO induction in pancreatic lymph nodes. Thus, our data suggest that the TLR9-IDO axis may represent a valuable target in the prevention/therapy of type 1 diabetes. The Journal of Immunology, 2009, 183: 6303–6312.

Unlike the majority of organs and tissues in the human body, the immune system requires systematic environmental pressure to develop properly. Animals raised in a germfree environment acquire poorly efficient immunoregulatory mechanisms and are at a greater risk of diseases associated with immune dysfunction (1). The incidence of major infectious diseases has significantly decreased in developed countries over the last three decades. In parallel, the incidence of most autoimmune diseases, including type 1 diabetes (T1D), has steadily been increasing in Europe and North America (2, 3). Therefore, both experimental and epidemiological data suggest that certain microorganisms and link innate with adaptive immune responses (7–9).

TLRs represent the early molecular sensors of invading microorganisms and link innate with adaptive immune responses (7–9). To date, 11 members of TLRs have been identified in humans, and 13 in mice, and a series of genetic studies revealed their respective ligands. Mammalian TLRs can be expressed on the cell membrane (i.e., TLR1, TLR2, TLR5, and TLR6), intracellularly (TLR3, TLR7, TLR8, and TLR9), or both (TLR4). The distinct localizations correlate with a different nature of stimulatory ligands—with transmembrane TLRs primarily sensing microbial membrane molecules, such as LPS and lipopeptides (TLR1, TLR2, TLR5, and TLR6), and intracellular forms recognizing microbial nucleic acids, such as CpG-containing DNA sequences (TLR9) and viral ssRNA (TLR7 and TLR8) or dsRNA (TLR3) (9), or a different signaling pathway (TLR4) (10).

The genetically diabetes-prone NOD mouse strain represents a prototypic experimental model of human T1D. In accordance with the principle of protective tolerance, decontamination of NOD mice from microbes does increase diabetes frequency. Conversely, deliberate infection of those mice with various microorganisms totally prevents diabetes onset if infection occurs early in life (5). An outstanding study has recently demonstrated that the interaction of intestinal microbes with the innate immune system is a critical epigenetic factor modifying T1D predisposition in NOD mice (11). Nonetheless, the precise mechanisms of induction of tolerance by the microbiota remain to be elucidated.

IDO is expressed in dendritic cells (DCs) in response to inflammatory stimuli, including IFN-γ and CpG oligodeoxynucleotide (ODN), and represents an important physiological mechanism capable of controlling both inflammation and autoimmunity (12–14). IDO catalyzes the first and rate-limiting step of tryptophan catabolism along the kynurenine pathway, which produces a series of tryptophan catabolites collectively known as kynurenines. We have previously demonstrated that IDO activity, hence, tryptophan catabolism, is not inducible by IFN-γ in DCs from NOD mice (15). This defect impairs tolerogenesis to a β cell-specific autoantigen also dominant in human T1D. Although maneuvers aimed at correcting the IDO defect will restore autoantigen-specific tolerogenesis (16), it is not currently known whether tryptophan catabolism plays a role in the development of the disease.

In the present study, we investigated whether TLRs and IDO are involved in the control of autoimmune diabetes in mice nongenetically prone to the disease. We found that both TLR9 and IDO are required for protection from an inflammatory/prodiabetic insult...
in healthy conditions. However, engagement of TLR9 by CpG-ODN did not efficiently induce IDO expression and activity in lymphoid tissues from NOD mice. Thus, our data: 1) confirm the protective role of TLRs in the prevention of T1D, 2) identify IDO as the critical TLR9 downstream effector in regulating inflammation/autoimmunity, and 3) suggest that successful prevention/therapy of T1D in genetically diabetes-prone subjects may require multiple and integrated approaches, capable of restoring an IDO-mediated, physiologically protective tolerance.

Materials and Methods

Mice
Four- and 8-wk-old C57BL/6 mice of either sex were obtained from Charles River Breeding Laboratories. Mice homozygous for the TLR3 (Tlr3<sup>-/-</sup>) and TLR9 (Tlr9<sup>-/-</sup>) targeted mutation raised on the C57BL/6 background were generated as described (17, 18), and bred at the breeding facilities of the University of Perugia. Female NOD/Mrk mice, 4 wk of age, were purchased from Taconic Farms. All animals were housed and fed under specific pathogen-free (SPF) conditions. All in vivo studies were in compliance with national (Italian Approved Animal Welfare Assurance A-3143-01) and Perugia University Animal Care and Use Committee guidelines.

In vivo treatments
For the induction of experimental autoimmune diabetes, wild-type (WT), Tlr3<sup>-/-</sup>, or Tlr9<sup>-/-</sup> C57BL/6 mice were injected i.p. for 5 consecutive days with 40 mg/kg body weight of freshly made streptozotocin (STZ; Sigma-Aldrich) in 0.1 mol/L citrate buffer (pH 4.5). Day 1 was that of the first STZ injection. Control mice received vehicle alone. Nonfasting glucose levels in tail vein blood samples were monitored 1–2 times/wk. Mice with a blood glucose level of ≥250 mg/dL for ≥2 consecutive days were considered diabetic. To inhibit IDO activity in vivo, groups of WT mice were treated with slow-release pellets of 1-methyl-DL-tryptophan (1-MT; Research of America) implanted on day 0 under the dorsal skin of the mice. 

Histopathology and immunohistochemistry
Paraffin-embedded sections (3–4 μm) of pancreata (five per organ) were stained with H&E and analyzed by light microscopy. Insulitis scoring was according to the following criteria: severe insulitis (score 3), 50% or higher of the islet area is infiltrated; mild insulitis (score 2), <50% of the islet area is infiltrated; peri-insulitis (score 1), infiltration is restricted to the periphery of islets; and no insulitis (score 0), absence of cell infiltration. Results are presented as the percentage of islets per mouse in each category. At least 40 islets were counted per mouse blindly by two observers. In immunostaining for insulin, 4-μm sections were cut from paraffin blocks and captured on electrically charged slides (Sigma-Aldrich). Sections were dewaxed in zylene and stained with primary pig anti-mouse insulin Abs (DakoCytomation) for 1 h, washed in PBS, and incubated with goat anti-guinea pig tetramethyl rhodamine isothiocyanate conjugate for 45 min. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole and mounted in 1,4-diazabicyclo[2.2.2]octane solution (all from Sigma-Aldrich). Slides were examined using a BX 41 apparatus in conjunction with V-View software (both from Olympus).

Cell isolation from pancreatic lymph nodes (PLNs) and peripheral lymphoid organs

Purification of CD4<sup>+</sup> T cells from pooled peripheral lymph nodes (with the exception of PLNs; see below) was conducted as described (20–22). For cytokine induction, cells (1 × 10<sup>5</sup>/ml) were cultured in the presence of plate-bound anti-CD3 (145-2C11) and anti-CD28 (PV-1) (both at the concentration of 1 μg/ml) for 48 h. Purification of PLN lymphocytes involved coculturing with irradiated T cell-depleted splenocyte samples and CD4<sup>+</sup> cells for 3 days in the presence of soluble anti-CD3 (20, 23). Proliferation was measured by incorporation of [3H]thymidine, according to standard procedures.

ELISA and TGF-β bioassay
Nonfasting blood insulin levels were measured by a mouse insulin ELISA kit (Merkodia AB). Cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, IL-23, and IFN-γ) were measured in culture supernatants by ELISA using specific kits or previously described reagents and procedures (21, 24). An ELISA-based TransAM Flexi NF-κB Family Kit (Active Motif) was used to monitor activity of NF-κB family members, as described (21, 24). Active TGF-β was measured, as described (25), using CCL-64 mink lung epithelial indicator cells (American Type Culture Collection), which do not appear to activate inactive TGF-β precursor molecules, but are extremely sensitive to growth induction initiated by the biologically active cytokine.

Real-time PCR
Real-time PCR analysis was performed, as described (26), using Iodol-specific primers (S. 5'-GAAGGATCTGGAGACAGCAC-3'; AS. 5'-GAAGCTGGATTCCACAAC-3'). For all panels, bars represent the ratio of gene to Gapdh expression, as determined by the relative quantification method (ΔΔcT cycle threshold; mean ± SD of triplicate determination).

Western blotting and IDO functional analysis
IDO expression was investigated in cells cultured overnight with complete medium either alone or in the presence of 1 μg/ml CpG-ODN by immunoblotting with a rabbit anti-IDO mAb raised in our laboratory (22). Anti-β-actin Ab (Sigma-Aldrich) was used as a normalizer. For measuring IDO functional activity, pDCs were stimulated either with 200 U/ml IFN-γ (R&D Systems) or 1 μg/ml CpG-ODN and, after 18 h, l-kyurenine, the main IDO product, was measured in culture supernatants by HPLC, as described (19). In PLN lymphocytes, treatment with IFN-γ, but not CpG-ODN, greatly reduced the cell viability, and thus, could not be used.

Nuclear extracts and EMSA

Nuclear extract preparation and EMSA were performed, as described (27). Briefly, DNA-binding reactions were conducted in 20 min at room temperature in a final volume of 20 μl. The reactions were started by adding 10 μg of nuclear protein extract to a reaction mix containing ~20,000 cpm of [γ<sup>32P</sup>]PAT-labeled NF-κB dsDNA ODN (5'-AGAGGGAGCCTTC CGAGAGGC-3') (27). Cold competitor ODN were added to the reaction mix before the radiolabeled probe (data not shown). For supershift experiments, protein extracts were incubated with anti-p65 or anti-RelB Ab (Santa Cruz Biotechnology) for 30 min at room temperature after the addition of the radiolabeled probe. Whole samples were then loaded on a 5% native polyacrylamide gel in Tris-borate-EDTA buffer.

Statistical analysis
In the in vitro experiments, glycemia data were analyzed by Kaplan-Meier plots. Paired data were evaluated by Student’s t test. All in vitro determinations are means ± SD from at least three independent experiments, unless otherwise indicated. All n values were computed by power analysis, so as to yield a power of at least 80% with an α-level of 0.05.
**Results**

**Tlr9−/− mice are highly susceptible to experimental autoimmune diabetes**

To investigate the role of TLR3 and TLR9 in the pathogenesis of chemically induced autoimmune diabetes, we injected Tlr3−/− and Tlr9−/− mice and their C57BL/6 WT counterparts with multiple low doses of STZ. From 14 days, average were significantly higher in Tlr9−/−, but not Tlr3−/− mice, as compared with WT animals, and further increased thereafter (Fig. 1A). In addition, whereas at least 40% WT and Tlr3−/− animals remained normoglycemic at day 40, all Tlr9−/− mice became diabetic as early as day 20 (Fig. 1, B and C). Although serum insulin levels were generally decreased in STZ-treated as compared with vehicle-injected counterparts, insulinemia in STZ-treated Tlr9−/− mice was significantly lower when compared with STZ-treated WT mice (Fig. 1D). Groups of mice injected with STZ were sacrificed at different times, and 5-μm-thick sections of pancreas were stained with H&E or immunostained for insulin. The majority of pancreatic islets of WT and Tlr3−/− mice were either normal or mildly infiltrated by leukocytes and clearly positive for insulin (Fig. 1, E and F). In contrast, as much as 17% (at day 14) or 50% (at day 40) of the islets in Tlr9−/− mice were characterized by intrainsulitis and low insulin. Thus, our data suggest that Tlr9−/−, but not Tlr3−/−
C57BL/6 mice develop an earlier and more severe form of experimental autoimmune diabetes than WT counterparts.

**Lack of induction of Foxp3** Treg cells and higher production of proinflammatory IL-6 and IL-17A characterize diabetes in Tlr9−/− mice

Although Th1 cells appear to play a major role in autoimmune diabetes via IFN-γ production, discordant observations have been found on NOD mice expressing targeted mutations in either IFN-γ or its receptor (28–30). More recently, Th17 cells, clearly involved in many autoimmune diseases, such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, and myocarditis, have been shown to promote pancreatic inflammation, although they induce diabetes efficiently in NOD/SCID mice only after conversion into Th1 cells (31, 32). In contrast, there is compelling evidence indicating that the development of diabetes in NOD mice is tightly controlled by Treg cells, via production of TGF-β and IL-10 (33). In experimental diabetes induced by STZ, the pattern of pathogenic vs protective cells and cytokines is less clear, although administration of IL-23, a Th17-sustaining cytokine, greatly exacerbates the disease (34).

To examine whether TLR9 expression would affect the balance of Th and Treg responses in chemically induced diabetes, we used flow cytometry to evaluate the percentage and phenotype of CD4+ T cells from lymphoid organs, i.e., spleen and lymph nodes, and PLN lymphocytes at day 21 of STZ treatment in WT, Tlr3−/−, and Tlr9−/− mice. Mice injected with vehicle alone were used as a control. Table I shows that STZ treatment did not significantly modify the basal frequency of gated CD4+CD25+ T cells coexpressing the T cell activation marker CD62L in any groups. In contrast, the percentages of CD4+CD25+ T cells coexpressing either CTLA-4 or Foxp3, the Treg cell lineage transcription factor, significantly increased upon STZ treatment in samples from WT and Tlr3−/−, but not Tlr9−/−, mice. Foxp3 staining of CD4+ T cells gated from lymph nodes further confirmed no significant modulation in CD4+Foxp3+ cells from TLR9-deficient mice treated with STZ (Fig. 2, A and B). However, although disparate in their occurrence, the suppressive activity of Treg cells purified from STZ-treated Tlr9−/− mice did not differ from that of Treg cells from diabetic WT or Tlr3−/− animals (Fig. 2C).

We next analyzed the pattern of Th1, Th2, Th17, and Treg-associated cytokine production by activated CD4+ T cells purified from peripheral lymph nodes. In addition, cytokine production by CD11c+ DCs purified from the spleen and by PLN lymphocytes, both types of cell under culture for 24 h without added stimuli, was also evaluated (Fig. 2D). In CD4+ T cell cultures, we found that in vivo treatment with STZ induced a similar trend for the majority of the tested cytokines in both WT and TLR-deficient mice, with comparable increase in IL-2, IL-10, and TGF-β and a decrease in IFN-γ production, whereas IL-4 production was not modified. In contrast, the increase in IL-17A production in response to the drug was significantly higher in CD4+ cell cultures from Tlr9−/−, but not Tlr3−/− mice as compared with WT mice. In DC cultures, IL-23 increased equally in all STZ-treated groups, although a dramatic increase in IL-6 was found in supernatants from TLR9-deficient cells. In parallel, IL-6 and IL-17A levels were also significantly higher in PLN lymphocytes from STZ-treated Tlr9−/−, but not Tlr3−/− mice, as compared with WT animals. Thus, our data suggest that lack of TLR9 expression may lead to a higher production of proinflammatory IL-6 in diabetic Tlr9−/− mice that, combined with high levels of IL-23, may impede the TGF-β-mediated expansion of Foxp3+ Treg cells and skew the balance toward the development and differentiation of pathogenic Th17 cells.

**IDO is not expressed in PLNs of diabetic Tlr9−/− mice**

IDO has a primary role in the peripheral generation of Treg cells, under physiological or pathological conditions (14). Furthermore, in experimental pathogenic inflammation, IDO helps to tame overzealous and exaggerated inflammatory response driven by IL-23 and IL-17 (22). In contrast, these cytokines can down-regulate tryptophan catabolism (35, 36). Signaling through TLR9 induces IDO expression in splenic DCs, particularly in the plasmacytoid subset (pDC), and functional tryptophan catabolism is necessary for TLR9-driven immunosuppressive effects (14, 37). We therefore became interested in ascertaining whether a defect in IDO expression could be involved in the exacerbated disease induced by STZ in Tlr9−/− mice. To this purpose, we analyzed IDO expression and activity in both splenic pDCs and PLN cells purified from mice at day 21 of STZ treatment (Fig. 3). Mice injected with vehicle alone were used as control. We found that IDO transcript and protein expression greatly increased in splenic pDCs from WT and Tlr3−/− mice, whereas in pDCs from animals lacking TLR9 the induction was barely detectable. In PLNs, IDO was expressed in samples from WT and Tlr3−/− mice and further increased following STZ treatment. In contrast, IDO expression could not be detected under basal conditions nor after in vivo treatment of Tlr9−/− mice with STZ (Fig. 3, B and C). IDO expression was accompanied by significant kynurenine production by splenic pDCs from WT and Tlr3−/− mice after in vivo treatment with STZ and further increased in response to in vitro restimulation with IFN-γ, whereas a significantly lower level of the tryptophan metabolite was present in parallel culture supernatants from Tlr9−/− pDCs, either untreated or treated with the cytokine (Fig. 3D). Thus, our data suggest that deficient TLR9 expression may determine defective IDO induction in response to pancreatic inflammation.

**In vivo treatment with CpG-ODN protects mice from STZ-induced diabetes in an IDO-dependent fashion**

To evaluate the functional role of IDO as induced in the course of diabetes development and its potential link with TLR9 activation, WT recipients were administered 1-MT, the gold standard in IDO

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### Table I. Quantitative analysis of T cell populations in gated CD4+CD25+ cells in spleens, lymph nodes, and PLNs in mice treated with STZ

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Spleen</th>
<th>Lymp Nodes</th>
<th>PLNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>27.8a</td>
<td>41.3 26.9</td>
<td>39.7 26.2</td>
</tr>
<tr>
<td>Foxp3</td>
<td>20.2</td>
<td>28.2 21.4</td>
<td>28.8 20.3</td>
</tr>
<tr>
<td>CD26L</td>
<td>ND</td>
<td>ND ND ND ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD4 CD25</td>
<td>7.5</td>
<td>8.3 6.9 8.0</td>
<td>7.6 8.9</td>
</tr>
</tbody>
</table>

*Data are mean percentages of positive cells of three experiments, each consisting of three mice per group per experiment. SD (not included in the table) never exceeded 10% of the mean value. Bold values, p < 0.01 (STZ treated vs untreated).
inhibition, in the form of slow release pellets implanted on day 0, i.e., 1 day before STZ treatment. Control mice received placebo pellets. All mice treated with STZ and 1-MT developed hyperglycemia (Fig. 4, A and B) with a kinetic pattern comparable to that manifested by Tlr9−/− mice treated with STZ alone (Fig. 1B). Conversely, administration of CpG-ODN determined a reduced frequency of hyperglycemic animals, an effect that could be reversed by cotreatment with 1-MT, but not placebo pellets (Fig. 4B). The therapeutic effect of CpG-ODN was accompanied by a significant increase in the percentage of CD4+Foxp3+ cells in lymph nodes (>50% increase in three experiments; p < 0.001, CpG-ODN vs PBS), whereas the opposite pattern could be observed in mice treated with 1-MT, either alone or combined with CpG-ODN (>50% decrease in three experiments; p < 0.001, 1-MT vs prospective placebo) (Fig. 4C). In addition, IL-17A and IL-6/IL-23 productions by lymph node CD4+ cells and by splenic DCs, respectively, further increased in mice treated with STZ and 1-MT+CpG-ODN, but decreased after in vivo treatment with STZ in combination with CpG-ODN and placebo. In PLNs, CpG-ODN treatment significantly reduced the production of both IL-6 and IL-17A in an IDO-dependent fashion (Fig. 4D). Our data therefore indicate that an intact tryptophan catabolism is required.
for mitigating the inflammatory/diabetogenic effects of STZ in C57BL/6 mice, and that activation of TLR9 in vivo can protect from the pathogenic inflammatory response at the pancreas level in an IDO-dependent fashion.

**CpG-ODN does not induce IDO expression and activity in PLNs of NOD mice**

The NOD strain of mice has become a prototypic model of autoimmune diabetes (3). Most female mice die of hyperglycemia, reflecting the onset of insulinitis at 4 wk of age and the consequent T cell-mediated destruction of pancreatic β cells. The immune dysregulation in NOD mice has been ascribed to many causes, including aberrant APC function, reduced suppressive activity of Treg cells (38), and, more recently, resistance to regulation in T cell effectors (39). In 2003, our group provided the first evidence that IFN-γ fails to induce IDO, and hence, tolerizing properties in splenic DCs from NOD female mice early in prediabetes (15). Prompted by the results obtained in the STZ diabetes model, we administered multiple doses of CpG-ODN to 4-wk-old NOD females. No protection, but rather acceleration of diabetes development could be observed in CpG-ODN-treated mice as compared with vehicle-treated counterparts (Fig. 5, A and B), despite considerable expression of Tlr9 transcripts in both splenocytes and PLN cells, which was comparable to that of C57BL/6 mice of same sex and age (data not shown). Lack of protection from diabetes was accompanied by very low induction of IDO expression in both splenic pDCs and PLN cells by in vitro incubation with CpG-ODN of cells from NOD as compared with STZ-treated C57BL/6 mice (Fig. 5, C and D). Accordingly, IDO activity was also poorly observed in the former cells (Fig. 5E). CpG-ODN, however, was capable of inducing high production of IL-6 in NOD PLNs as compared with parallel samples from C57BL/6 mice, either untreated or treated with STZ (Fig. 5F), in the face of basally higher levels of the cytokine. Because IL-6 can antagonize tryptophan catabolism via induction of protosomal degradation of IDO (40), our data suggest that an aberrant TLR9 signaling producing high levels of IL-6 may underlie the exacerbating effects of CpG-ODN in NOD mice.

We have recently demonstrated that IDO expression is contingent on the noncanonical pathway of NF-κB activation (14, 21, 26). Recent molecular dissection of NF-κB activation has shown that NF-κB can be induced by the so-called canonical (classical) and noncanonical (alternative) signaling pathways, leading to distinct patterns in the individual NF-κB subunits that are activated and the downstream genetic responses that are induced. The proinflammatory canonical pathway involves activation of the IκB kinase (IKK)-β, which leads to phosphorylation-induced proteolysis of the inhibitor IκBα and consequent nuclear translocation of the p65 subunit in the form of p50-p65 dimers. In the noncanonical pathway, activation of IκKα by NF-κB-inducing kinase results in the processing of p100 to p52 and consequent formation of p52-RelB dimers, which translocate into the nucleus and activate an anti-inflammatory gene program (13, 14). In pDCs, triggering of TLR9 can activate both IκKα- and IκKβ-mediated pathways (41). To investigate whether the inability of CpG-ODN to induce IDO in NOD mice could be due to altered signaling, PLN lymphocytes from both WT C57BL/6 and NOD mice were stimulated in vitro with CpG-ODN and, after 30–60 min, NF-κB expression was quantified by means of an ELISA kit specific for p65 and p52 (Fig. 5G). In WT C57BL/6 cells, either untreated or treated with STZ, we found that CpG-ODN activated nuclear translocation of p65 and p52 to a similar extent. In contrast, in NOD cells, the nuclear translocation of p65 was significantly higher than that of p52 as early as at 30 min, suggesting the occurrence of a dominant, proinflammatory response mediated by canonical NF-κB in NOD immune cells upon engagement of TLR9. The preferential activation of p65 in NOD PLN cells in response to TLR9 stimulation was further confirmed by EMSA (Fig. 5H).

**Discussion**

IDO is a metabolic enzyme conserved through the last 600 million years of evolution. Initially confined to the regulation of tryptophan availability in local tissue microenvironments, IDO is now considered to play a wider role, which extends to homeostasis and plasticity of the immune system, with implications for many aspects of immunopathology, including chronic inflammation and...
autoimmunity (14). Its immunoregulatory effects are mainly mediated by DCs and involve not only tryptophan deprivation, but also the production of kynurenines, which act on IDO-expressing cells, thus rendering an otherwise stimulatory DC capable of regulatory effects (42), as well as on T cells. As a result, IDO-expressing DCs mediate multiple effects on T lymphocytes, including inhibition of proliferation, apoptosis, and differentiation toward a regulatory phenotype (12–14). In addition to much evidence substantiating a critical role for tryptophan catabolism in immune regulation at the DC/T cell level, nonimmune cells such as pancreatic β cells from healthy patients as well as other islet cells will express the enzyme in response to IFN-γ (43). Furthermore, transfection of Ido1, the gene coding for mouse IDO, into β cells prolongs graft survival in NOD mice (44). Thus, in inflammatory conditions, an effective tryptophan catabolism at the pancreatic level may participate in islet protection in individuals nongenetically prone to autoimmune diabetes.

TLRs play a central role in the generation of innate and adaptive antimicrobial immune responses through recognition of conserved pathogen-associated molecular patterns. The majority of TLRs signal through the MyD88 adaptor protein, except for TLR4 and TLR3, which can or must signal by means of TIR domain-containing adaptor inducing IFN-β (7–9). After proper elimination of the invading microorganisms, anti-inflammatory signals are necessary for the restoration of the homeostatic balance, and hence, host protection from the deleterious effects of overwhelming inflammation. TLR signaling is involved not only in the primary induction of inflammation, but also in secondary regulatory mechanisms (6, 45, 46). Among these, IDO may represent the downstream effector for at least some TLRs, including the nucleic acid-binding TLR3 and TLR9. Although the TLR3 ligand poly(I:C) can induce IDO in nonimmune cells such as astrocytes (47) and gingival fibroblasts (48), CpG-ODN is known to induce the enzyme activity in immune cells, particularly pDCs, promoting strong immunoregulatory effects (37, 49–51).

Because of the crucial role of IDO in immune tolerance and the possible link between tryptophan catabolism and protective tolerance by pathogen-associated molecular patterns (6), we sought to examine whether TLR3 and TLR9 could play a role in the control of autoimmune diabetes and whether IDO would be involved in such effects. To this purpose, we examined experimental diabetes as induced by multiple low doses of STZ. Although not extensively characterized, this model is widely considered autoimmune in nature, because disease is prevented by administration of anti-CD8 mAb and can be adoptively transferred using splenocytes from diabetic animals. Furthermore, the overt disease is preceded by islet inflammation, characterized by infiltration of immune cells as early as 3–4 days after the last STZ injection (52).

We found that hyperglycemia was induced in ~60% of WT C57BL/6 mice by STZ and was accompanied by significantly reduced insulinemia, mild islet infiltration, and a cytokine pattern early as 3–4 days after the last STZ injection (52).
and IL-17A by immune cells deriving from both peripheral lymphoid tissues and PLNs was greatly increased in those mice in response to STZ, as compared with WT C57BL/6 counterparts. In PLNs, no or very low IDO protein and activity was to be detected in Tlr9/H11002/H11002 nondiabetic and diabetic mice, despite detectable basal enzyme expression and further induction in Tlr3/H11002/H11002 and WT animals by STZ in vivo treatment. In addition to substantiating and further defining the inflammatory/immunological aspects of STZ-induced diabetes, our data suggest that TLR9, but not TLR3 signaling, is necessary for the control of the disease and also for the induction of IDO at the pancreatic level.

The possible protective role of IDO was further investigated in WT mice by means of administration of either the IDO inhibitor 1-MT (which determined a more robust diabetic disease in response to STZ comparable to that of Tlr9/H11002/H11002 mice) or the TLR9 ligand CpG-ODN (which largely prevented hyperglycemia onset on condition that IDO was fully active). In the latter case, the TLR9-driven protection was accompanied by a further increase in

![FIGURE 5. Lack of IDO and high IL-6 induction by CpG-ODN in PLNs of NOD mice. Four-week-old female NOD mice (n = 10) received 25 μg of CpG-ODN or PBS twice per week for 3 wk, and average blood glucose (A) and diabetes incidence (B) were plotted over time. A and B, p < 0.05 for comparison between the CpG-ODN-treated and control group. C and D, Purified splenic pDCs and PLN lymphocytes were purified from untreated 4-wk-old female NOD and STZ-treated C57BL/6 mice (day 21) and incubated with 1 μg/ml CpG-ODN overnight. Control cultures were obtained with medium alone. Transcripts and protein cell lysates were analyzed for IDO expression by real-time PCR (C) and immunoblot analysis (D), respectively, as in Fig. 3. D. The IDO to β-actin ratio, as determined by scanning densitometry, is indicated for each sample. E, IDO activity was measured in cell supernatants, as in Fig. 3D. Cells from untreated WT mice were also included in the assay. *, Undetectable level of kynurenine. F, IL-6 production was evaluated in supernatants from PLN cells incubated overnight with different doses of CpG-ODN by ELISA. *, p < 0.001 (CpG-ODN-treated vs respective untreated control). C, E, and F, Data are means ± SD of three experiments. D, One of three experiments is shown. G, PLN cells from NOD and C57BL/6 mice, the latter being untreated or treated with STZ, as in E, were stimulated with 1 μg/ml CpG-ODN and, after 30–60 min, p65 and p52 were quantified in nuclear extracts by means of a specific ELISA. Time 0 indicates untreated cells. Relative activities (A450) are mean ± SD of three experiments, each in triplicate. *, p < 0.01. H, Nuclear extracts from PLN cells treated with CpG-ODN for 60 min were incubated with Ab against p65 or RelB. NF-κB dsDNA oligonucleotide was used as a probe. One of two experiments with the same results.}
CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells as compared with controls, whereas production of proinflammatory IL-6 and IL-17A from PLN lymphocytes was reduced. Therefore, our data further extend the protective potential of the TLR9-IDO axis from animal models of allergic disorders (14) to experimental autoimmune diabetes induced in mice nongenetically prone to the disease, and reveal an essential role of IDO expression in PLNs for the control of insulitis and hyperglycemia. Interestingly, this axis is already known to be important in the generation of human adaptive Treg cells (53), whereas either CpG-ODN (54) or kynurenines (36, 55) can control Th17-mediated inflammatory responses in murine disease models. Although TLR9 and IDO have not been directly linked yet, it is interesting to note that experimental autoimmune encephalomyelitis can be exacerbated by either lack of TLR9 expression (56) or of IDO activity (57).

However, in the NOD mouse, the situation is far more complex. Wong et al. (58) provided evidence that the incidence of diabetes in Tlr9<sup>−/−</sup> and not Tlr3<sup>−/−</sup> NOD mice maintained in SPF conditions is significantly lower when compared with their heterozygous Tlr9<sup>+/−</sup> littermates. In addition, Wen’s group (11) demonstrated that NOD mice lacking MyD88, but not TLR3, exhibit loss of diabetes development when maintained in SPF conditions, despite the development of robust diabetes in a germfree environment. However, colonization of germfree MyD88<sup>−/−</sup> NOD mice with a defined microbial consortium attenuates the disease (59). Overall, the bulk of available data suggests that, in the NOD mouse, TLR9-MyD88 signaling does not protect against, but rather sustains pathogenesis. Nonetheless, alternative microbial signals, apparently not engaging TLR3, can exert protective effects. Paradoxically, STZ treatment both prevents and reverts islet destructive autoimmunity in NOD mice (59). Considering that TLR9 can be activated also by host unmethylated CpG-containing DNA and non-CpG DNA (46) and that STZ can induce multiple DNA modifications, it might be speculated that the STZ treatment is capable of generating endogenous, regulatory TLR9 ligands. In agreement with data from knockout NOD animals, our current data indicate that multiple i.p. injections of SPF NOD mice with CpG-ODN accelerate diabetes development. However, it has been previously reported that i.m. vaccination of SPF NOD mice with CpG-ODN significantly reduces the incidence of diabetes (60). Although c.s.c. vs i.v. CpG-ODN administration has already been shown to determine opposite effects (50), it is worth mentioning that the successful i.m. administration of CpG-ODN was preceded by the injection of cardiotoxin, a molecular adjuvant particularly effective in i.m. DNA vaccinations. In this specific case, cardiotoxin pretreatment may have reset the dysregulated immune system of the NOD mouse (38), allowing CpG-ODN to exert an effective immunosuppressive effect. Thus, the inability of TLR9 to induce a regulatory pathway in NOD mice may rely on the absence of appropriate endogenous TLR9 ligands and/or the existence of signaling defects.

In evaluating the capacity of individual TLRs to promote either inflammation/autoimmunity or protective tolerance, the dominance of specific signaling pathways should be taken into account (46). TLR9 is known to signal via the MyD88 adapter, which, in pDCs, can activate two distinct NF-κB pathways, namely the classical or canonical pathway (in which IKKβ kinase plays a pivotal role) and the alternative or noncanonical pathway (which relies on IKKα activation). Although TLR9-IKKβ signaling leads to the nuclear translocation of p50-p65 dimers and production of classical proinflammatory cytokines, activation of IKKα by TLR9 triggering induces type I IFNs via nuclear translocation of p52-RelB complexes (41). We have recently found that noncanonical NF-κB activation is necessary for the induction of IDO-mediated therapeutic effects in a model of airway allergic inflammation (21). Furthermore, type I IFNs, although less potent than IFN-γ, can also act as IDO inducers (13, 14). Our current data indicate that the CpG-ODN treatment, either in vivo (data not shown) or in vitro, does not induce IDO protein and activity in NOD splenic pDCs and PLNs, despite a considerable induction in parallel samples from diabetic WT C57BL/6 mice. Lack of IDO induction is accompanied by overactivation of the proinflammatory p65 as compared with anti-inflammatory p52 subunit, whereas in cells from diabetic and nondiabetic C57BL/6 mice the amounts of p65 and p52 activated by CpG-ODN are quite similar. Although we cannot presently exclude the absence of appropriate TLR9 ligands, our data indicate that a dysfunctional TLR9 signaling may underlie the pathogenic/proinflammatory effect of this receptor in spontaneous autoimmune diabetes and may impede the effective induction of counterregulatory mechanisms mediated by IDO.

In conclusion, the identification of the molecular targets and mechanisms through which microbial ligands exert their protective effects may lead to a better understanding of the early pathogenesis processes involved in T1D, may provide new markers for an early diagnosis of the disease, and can ultimately lead to the generation of drugs mimicking protective microbial entities that can be used in either the prevention and/or treatment of T1D patients. The present study suggests that activation of TLR9, but not TLR3, can induce IDO, and thus control early inflammatory attacks to β cells. Harmonious TLR9 signaling, however, seems to be mandatory for fully protective activity of tryptophan catabolism. The latter, indeed, appears to be defective not only systemically, but also specifically in pancreas-associated lymph nodes of NOD mice. Thus, TLR9 and IDO may represent innovative and valuable molecular targets in the prevention/therapy of T1D. Although we are still currently evaluating the existence of a possible IDO defect in T1D patients, our data support the recent hypothesis that only combination therapies, capable of correcting the dysregulated immune signaling at multiple levels, will enable the permanent prevention and curing of T1D (61).

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Disclosures
The authors have no financial conflict of interest.

References


Corrections


In Fig. 1B, the color key was incorrect. The results and conclusions of the article remain unchanged. The corrected Fig. 1B is shown below. The published legend for Fig. 1 is correct, but is shown again for reference.

**FIGURE 1.** Higher susceptibility to STZ-induced diabetes in *Tlr9<sup>−/−</sup>* mice. WT (*n* = 12), *Tlr3<sup>−/−</sup>* (*n* = 10), and *Tlr9<sup>−/−</sup>* (*n* = 10) C57BL/6 mice were injected with STZ from day 1, and blood glucose was monitored over time (in A–C, one experiment is depicted of two with similar results). Diabetes was diagnosed in mice with blood glucose level >250 mg/dl. Mice were sacrificed at different times and analyzed for insulinemia (*D*) and pancreas histology (*E* and *F*) and immunohistochemistry (*E*, *F*). A, Average blood glucose in different groups is plotted over time. Data are presented as mean glucose levels ± SD. *, *p* < 0.01 (*Tlr9<sup>−/−</sup>* vs WT mice). B, Blood glucose concentrations over time in individual WT and *Tlr9<sup>−/−</sup>* mice. C, Incidence of diabetes over time in TLR-deficient and WT mice, *p* < 0.01 (*Tlr9<sup>−/−</sup>* vs WT mice). D, Blood insulin was measured by ELISA at day 30. Control, control animals treated with vehicle alone. Data are means ± SD of three experiments. *, *p* < 0.05 (STZ-treated *Tlr9<sup>−/−</sup>* vs WT mice). E and F, Pancreatic tissues were processed for H&E staining to evaluate insulitis (*E* and *F*) and immunostained for insulin (red, *E*). In fluorescent images, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Representative islet area for each group (*E*) at day 40 and the percentages of islets per mouse with different score (*F*, see Materials and Methods) of lymphocyte infiltration (days 14 and 40) are shown. Percentages represented number of islets of a given score (see Materials and Methods) over total number of islets (30–40 per pancreas). *, *p* < 0.01 and **, *p* < 0.001 (*Tlr9<sup>−/−</sup>* vs WT mice).

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