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Multiple mechanisms involved in diabetes protection by lipopolysaccharide in non-obese diabetic mice

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ABSTRACT

Toll-like receptor 4 (TLR4) activation has been proposed to be important for islet cell inflammation and eventually β cell loss in the course of type 1 diabetes (T1D) development. However, according to the "hygiene hypothesis", bacterial endotoxin lipopolysaccharide (LPS), an agonist on TLR4, inhibits T1D progression. Here we investigated possible mechanisms for the protective effect of LPS on T1D development in non-obese diabetic (NOD) mice. We found that LPS administration to NOD mice during the prediabetic state neither prevented nor reversed insulitis, but delayed the onset and decreased the incidence of diabetes, and that a multiple-injection protocol is more effective than a single LPS intervention. Further, LPS administration suppressed spleen T lymphocyte proliferation, increased the generation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), reduced the synthesis of strong Th1 proinflammatory cytokines, and downregulated TLR4 and its downstream MyD88-dependent signaling pathway. Most importantly, multiple injections of LPS induced a potential tolerogenic dendritic cell (DC) subset with low TLR4 expression without influencing the DC phenotype. Explanting DCs from repeated LPS-treated NOD mice into NOD/SCID diabetic mice conferred sustained protective effects against the progression of diabetes in the recipients. Overall, these results suggest that multiple mechanisms are involved in the protective effects of LPS against the development of diabetes in NOD diabetic mice. These include Treg induction, down-regulation of TLR4 and its downstream MyD88-dependent signaling pathway, and the emergence of a potential tolerogenic DC subset.

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Introduction

Type 1 diabetes (T1D), also known as insulin-dependent or autoimmune diabetes, is caused by the selective autoimmune destruction of insulin-secreting β cells in the pancreatic islets. The development of T1D is generally presumed to be a result of interactions between the immune system and an intricate network of genetic and environmental factors (Zoka et al., 2013). An important role of innate inflammation has been established in the insulitic process of T1D, in the form of a 'dialog' between invading immune cells and target β cells (Eizirik et al., 2009). In particular, infiltration of autoreactive $CD4^+$ and $CD8^+$ lymphocytes during immune-mediated inflammation has long been considered pathognomonic in T1D (In't Veld, 2011; Baumann et al., 2012).

Toll-like receptors (TLRs) are a family of type 1 transmembrane proteins that are important for the induction of pro-inflammatory responses (Kawai and Akira, 2010). Activation of TLRs has been linked to the pathogenesis of various autoimmune inflammatory diseases including rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease (Clanchy and Sacre, 2010). Among the TLR family members, TLR4 has been recognized as being important for islet cell inflammation and eventually β cell loss in the course of T1D development. TLR4, expressed in numerous cell types, has been identified as the integral component of the lipopolysaccharide (LPS, also known as endotoxin) transmembrane receptor complex, and a limiting factor in LPS-induced inflammatory signal transduction (Beutler, 2000). Moreover, TLR4 shares structural homology – in the intracellular domain – with the receptor for the proinflammatory cytokine interleukin (IL)-1. Activation of the TLR4 pathway may cause chronic inflammation and tissue damage (Lucas and Maes, 2013). TLR4 receptors are found in abundance on insulin-secreting β cells (Vives-Pi et al., 2003;

Abbreviations: LPS, lipopolysaccharide; NOD, non-obese diabetic; T1D, type 1 diabetes; TLRs, Toll-like receptors; Tregs, regulatory T cells; DCs, dendritic cells; APC, allophycocyanin; BMDCs, bone marrow-derived dendritic cells; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; H&E, hematoxylin–eosin; IFN, interferon; IL, interleukin; IRF-3, interferon regulatory factor-3; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetra-zolium bromide; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor; PE, phycoerythrin; MLA, multiple LPS administration; SLA, single LPS administration; TGF, transforming growth factor; TRIF, Toll/interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β.

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Garay-Malpartida et al., 2011), and TLR4 signaling pathways mediate selective damage induced by TLR4 agonists on β cells during the development of autoimmune diabetes (Li et al., 2012). TLR4-related β cell alterations thus may serve as early markers for β cell destruction. Several clinical studies (Devaraj et al., 2008, 2009, 2011a) also showed that the expression of TLR4, along with its ligands and downstream targets, was significantly elevated in patients with T1D. Furthermore, TLR4 activation and downstream cytokine production can lead to the development of T1D in the non-obese diabetic (NOD) mouse (Mohammad, 2006), while genetic deficiency of TLR4 significantly attenuates the proinflammatory state associated with T1D in a streptozotocin-induced diabetic mouse model (Devaraj et al., 2011b).

LPS, a component of gram negative bacterial outer membranes, is one of the most potent initiators of inflammation, predominantly via TLR4 receptor activation (Dobrovolskaia et al., 2003). Theoretically, LPS can cause immune-related β cell damage both directly and indirectly, by activating TLR4 receptors on both proinflammatory cytokine-releasing immune T cells and insulin-secreting β cells. Accordingly, it was once believed that LPS acts as an etiological agent, increasing the risk of autoimmune diabetes (Balasa et al., 2000). In contrast to this view, however, subsequent animal studies (Aumeunier et al., 2010; Caramalho et al., 2011) have suggested that administration of the TLR4 agonist -LPS- exerts a suppressive effect on the incidence and severity of T1D. To explain this apparently paradoxical effect, the "hygiene hypothesis" suggested that exposure to bacteria or their endotoxins during early childhood stimulates immunoregulatory mechanisms which control autoimmune reactions that might ultimately be protective in T1D (Kondrashova and Hyoty, 2014). However, the mechanisms underlying this proposed protective effect are poorly understood.

One possibility is that LPS may actually suppress the activities of cytotoxic immune components, such as inflammatory T cells, while stimulating certain immune protective mechanisms such as regulatory T cells (Tregs). The development of T1D is mediated by autoreactive T cells, which infiltrate and cause the death of pancreatic β cells. Pathogenic T cells are normally held in check by $CD4^+CD25^+$ $Foxp3^+$ Tregs, which are important components of immune suppression (Xue et al., 2012). Tregs are directly involved in limiting diabetes progression in mice, rats and humans (Caramalho et al., 2011). Dendritic cells (DCs) are also known to display a strong capacity to induce and maintain antigen-specific peripheral T cell tolerance by acting on Tregs (Hubert et al., 2007). The maturation process of DCs, marked by an increase in the surface expression of major histocompatibility complex (MHC) class II and costimulatory molecules such as CD40, CD80, CD83, and CD86, are central to DC function (Sousa, 2006). Generally, the functions of mature DCs, with immunostimulatory antigen-presenting properties, are associated with the efficient induction of primary T cell responses. In the immature developmental stages of differentiation, DCs have a higher endo-/phagocytosis capacity towards antigens, and can induce the conversion of self-reactive $CD4^+$ T cells into Tregs, thus promoting immunotolerance. They have been shown to prevent spontaneous diabetes onset in NOD mice (Morel, 2013). This bimodal concept of immature vs mature DCs has been recently challenged by studies demonstrating that mature DCs can expand Treg cells with suppressive properties (Hubert et al., 2007). Nevertheless, induction of a stable DC state with tolerogenic characteristics is believed to be potentially beneficial in the pathogenesis of T1D (Giannoukakis et al., 2011; Giannoukakis, 2013). A phase 1 clinical trial using autologous DCs stabilized into a tolerogenic immunosuppressive state has been initiated in T1D patients (Giannoukakis et al., 2011).

Accordingly, for this study we hypothesized that modulation of Treg and DC contributes to the protective actions of LPS treatment in mouse model of T1D. Here, we investigated the potential protective effects of LPS treatment in two models of diabetes: 1) NOD mice; and 2) immunocompromized NOD/SCID mice that had been made diabetic by transfer of diabetogenic splenocytes, focusing on changes in Treg and DC during the progression of diabetes.

Materials and methods

Animals. Female NOD and NOD/SCID mice were purchased from Beijing HFK Bio-Technology Co. Ltd. Experimental colonies, and maintained at the experimental animal center of Tongji Medical College (Huazhong University of Science and Technology, China) under specific pathogen-free conditions. The animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College. The animals were kept in cages at 23 ± 2 °C and fed with standard laboratory diet and tap water throughout the experiments.

LPS administration in NOD mice. Previous studies (Aumeunier et al., 2010; Caramalho et al., 2011) have proved that continuous or multiple administration of LPS had protective effects on T1D, even though the administration regimens were different. But the effect of single intervention of LPS has never been examined. In this study, LPS from *Escherichia coli* was purchased from Sigma (St. Louis, MO). Eight-week-old NOD mice were randomly divided into 3 groups of 18 animals in each group as follows: control group, single LPS administration (SLA) and multiple LPS administration (MLA) groups. LPS dissolved in normal saline (Sichuan Koren Pharmaceutical co., LTD, China) removed endotoxin according to previously reported procedures (Brenchley et al., 2006; Bell et al., 2007). The endotoxin in disposed saline was quantified with a commercially available Limulus Amebocyte assay (Cambrex), and the concentration of which was less than 0.005 EU/ml. NOD mice in the MLA group were administered 10 μg LPS intraperitoneally (i.p.) once each week for 4 weeks. In the SLA group, 8-week-old mice were given a single intraperitoneal injection of LPS at a dose of 10 μg, and then saline once each week for 3 weeks. The animals in the control group were injected once each week for 4 weeks with endotoxin-free saline only.

At the end of the 4-week treatment period, 8 mice per group (12-weeks old) were randomly selected for intraperitoneal glucose tolerance test, and for determining cytokine levels, histopathological insulitis, spleen T lymphocyte proliferation, the percentage of $CD4^{+}$-$CD25^+Foxp3^+$ Tregs, DC surface molecules, and TLR4 expression. The remaining 10 mice per group were fed until 25 weeks of age for diabetes incidence studies. The development of diabetes was monitored by measuring glycemia in a drop of blood collected from the tail vein twice weekly using the ACCU-CHEK III system (Roche Diagnostics Ltd., Shanghai, China). Mice with blood glucose levels above 16.7 mM for 2 consecutive weeks were considered diabetic.

Intraperitoneal glucose tolerance test. After overnight fasting, mice were injected i.p. with a 200 mg/ml endotoxin-free glucose solution to yield a dose of 2 g/kg. Glucose in tail vein blood was measured at baseline and 30, 60, 90, 120, and 180 min after injection (Wang et al., 2013).

Circulating cytokine analysis. The serum concentrations of cytokines including mouse interleukin(IL)-2, IL-10, transforming growth factor (TGF)-β and interferon(IFN)-γ were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using commercial kits (eBioscience, USA).

Histological analysis. Insulitis was determined histologically in at least 50 islets per group after hematoxylin–eosin (H&E) staining. Scoring was performed under double-blinded conditions. The degree of insulitis was graded according to the following: score 1: normal islet; score 2: perivascular/periductal infiltration; score 3: peri-insulitis; score 4: mild insulitis (<25% of the islet infiltrated); score 5: severe insulitis (more than 25% of the islet infiltrated).

Spleen T lymphocyte proliferation and FACS analysis of the percentage of $CD4^+CD25^+Foxp3^+$ Treg. Spleen T cell proliferation was investigated by MTT assay as described previously (Xiang et al., 2009). Regulatory T cells were isolated from spleen T cells using a Mouse Regulatory

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