Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse

Mohammad K. Mohammad1,5*, Michael Morran1*, Brandon Slotterbeck1, Douglas W. Leaman2, Yaping Sun2, Hermann von Grafenstein1, Soon-Cheol Hong3,4 and Marcia F. McInerney1

1Department of Medicinal and Biological Chemistry, College of Pharmacy, and 2Department of Biological Sciences, University of Toledo, 2801 West Bancroft Street, Toledo, OH 43606-3390, USA
3Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, USA
4Walther Oncology Center, Walther Cancer Institute, Indianapolis, IN, USA
5Present address: College of Pharmacy, University of Jordan, Amman, Jordan

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Abstract

The expression, responsiveness and regulation of mouse Toll-like receptors (TLRs) in bone marrow-derived macrophages (BM-Ø) were investigated prior to and following the development of diabetes. Expression of TLR3 and TLR5 was significantly higher in newly diabetic non-obese diabetic (NOD) mice when compared with pre-diabetic and control strains of mice. The TLR3 ligand poly(I)poly(C) triggered up-regulation of its own receptor in NOR and pre-diabetic NOD, but TLR3 was already highly expressed in diabetic NOD mice. Expression levels of TLR3 correlated with poly(I)poly(C)-triggered IFN activity. LPS triggered down-regulation of TLR4 in pre-diabetic NOD, NOR and BALB/c, while levels of TLR4 remained consistently elevated in type 1 diabetic NOD and type 2 diabetic NZL mice. Dysregulation of TLR4 expression in the diabetic state correlated with increased nuclear factor kappa B (NF-kB) activation in response to the TLR4 ligand LPS and higher expression of IL-12p40, tumor necrosis factor α (TNFα), IL-6 and inducible nitric oxide synthase but lowered expression of IL-10. Exposure of bone marrow precursor cells from NOD mice to a hyperglycemic environment during differentiation into macrophages resulted in elevated levels of TLR2 and TLR4 and the cytokine TNFα. The results indicate that macrophage precursors are influenced by systemic changes in diabetes favoring altered TLR expression and sensitivity that may influence susceptibility to macrophage-mediated diabetes complications and explain inappropriate responses to infection in diabetes.

Introduction

The Toll-like family of receptors, comprising 13 members (TLR1–TLR13), is now well recognized to play a key role in pathogen recognition by the innate immune system (1). These receptors not only initiate innate immune responses but also shape the adaptive immune response that is triggered by the initial recognition of infectious pathogens (2, 3). Besides their beneficial role in host defense, Toll-like receptors (TLRs) have been implicated in a growing number of diseases, including type 1 diabetes (T1D) (4–6), and in some of the complications of T1D and type 2 diabetes (T2D) (7, 8).

TLRs recognize molecules commonly expressed by infectious pathogens, known as pathogen-associated molecular patterns (PAMPs), that are not expressed by the mammalian host (9). The small number of innate immune receptors face a large and diverse set of pathogens and TLRs often recognize more than a single type of molecule. Indeed, TLR2 responds to peptidoglycan and lipoteichoic acid from gram-positive bacteria and lipoproteins of gram-negative bacteria. TLR4, in addition to binding LPS derived from gram-negative bacteria, recognizes a variety of molecules unrelated to LPS, including...
responses to infections (27–29). Diminished responses to inflammatory responses to external stimuli and impaired complications affect many organ systems and include altered and immune-mediated diabetes complications (26). Diabetes T1D and other autoimmune diseases but also in inflammatory upon clearance of the infection (19). Activation of macrophages and if they are not down-regulated tissue damage if they are produced upon inappropriate products are also toxic to cells of the host and may lead to prostaglandin 2 (PGE2) (12, 20–25). Most of these activation produce oxygen- and nitrogen-free radicals and inflammatory responses and by regulating their own transcription immune responses and by regulating their own transcription.

TLRs also recognize a number of molecules expressed by the mammalian host (15), particularly if these molecules are indicators of stress or disease (16) or if these molecules are modified by disease processes. Oxidation (17) and the breakdown of extracellular matrix components (18) at sites of oxidative stress or inflammation are examples of processes that convert self-molecules to agonists of TLR activation. TLR4 recognizes endogenous inflammatory products like heat shock proteins (HSP60) (16). TLR4 and TLR2 are required for recognition of endogenous HSP70 [reviewed in (1)].

The demands of host defense against a large number of potential pathogens have to be balanced by the requirement to simultaneously spare the host, despite inherent cross-reactivity and complex signaling interactions. Most likely, these conflicting requirements can only be met by a precise tuning of the combinatorial recognition of, and response to, both PAMPS and self-antigens. This balanced tuning has the potential to be easily disturbed by pathophysiological conditions during disease.

TLRs are expressed by many types of cells including dendritic cells and macrophages. Of these, macrophages play an important role in both normal host defense and the immunopathogenesis of many inflammatory and autoimmune diseases. Macrophages originate in the bone marrow, enter the circulation as monocytes and enter tissues where they undergo final differentiation (19). Given the widespread tissue distribution of macrophages, alterations in the normal program of macrophage differentiation would therefore affect many organ systems and functions, including tissue repair and remodeling, and host defense (19). Macrophages are capable of recognizing pathogens both directly through TLRs and indirectly by presenting antigens to T cells. Macrophages respond to stimulation through TLR with the production of pro-inflammatory cytokines and chemokines, induction of co-stimulatory molecules that are critical for adaptive immune responses and by regulating their own transcription [reviewed in (1)]. Once activated, inflammatory macrophages produce oxygen- and nitrogen-free radicals and inflammatory mediators such as tumor necrosis factor a (TNFα) and prostaglandin 2 (PGE2) (12, 20–25). Most of these activation products are also toxic to cells of the host and may lead to tissue damage if they are produced upon inappropriate activation of macrophages and if they are not down-regulated upon clearance of the infection (19).

Macrophages play a key role not only in the pathogenesis of T1D and other autoimmune diseases but also in inflammatory and immune-mediated diabetes complications (26). Diabetes complications affect many organ systems and include altered inflammatory responses to external stimuli and impaired responses to infections (27–29). Diminished responses to infection may underlie several complications of diabetes (27–29). These include foot infections by multiple organisms, gingivitis and periodontal disease, dyspepsia due to infection with Helicobacter pylori, an increased rate of tuberculosis and viral infections (27–29). Diabetes has been classified as a secondary immune deficiency disorder (27–29).

Neither diminished nor exaggerated signaling by Toll receptors is beneficial to the host. The inability to resolve infection by pathogenic organisms paradoxically leads to chronic stimulation of macrophages. Gingivitis and periodontal disease are examples of infections that are chronic and exacerbated in patients with diabetes (30). Products of activated macrophages, such as TNFα and PGE2, are thought to cause damage of soft tissue and, by activating osteoclasts, bone resorption (31). In addition to a defective response to infection, many other diabetes complications are thought to be immune mediated and are considered to be the consequence of chronic low-grade inflammation in which macrophages participate (32–34).

The growing evidence for a role of the innate immune system in autoimmune diabetes and diabetes complications warrants a more detailed investigation of TLR expression and signaling in macrophages. The purpose of this project was to study the expression, responsiveness and regulation of TLRs in bone marrow-derived macrophages (BM–Ø) during and after the development of diabetes. The data show that expression levels of some TLRs are significantly increased in newly diabetic non-obese diabetic (NOD) mice, indicating the in vivo up-regulation of TLRs at the onset of diabetes. Stimulation with LPS led to aberrant TLR gene expression in new onset diabetic NOD mice compared with pre-diabetic NOD and control mice. Furthermore, dysregulated TLR mRNA expression correlated with abnormally high pro-inflammatory cytokine mRNA and abnormally low IL-10 levels after LPS stimulation. Changes in the expression, responsiveness and signaling of TLRs in the diabetic state favoring a pro-inflammatory environment may impair attempts to restore beta cell mass by transplantation or stem cell grafting, may impair resolution of infection without tissue damage and may also be one of the factors that underlies susceptibility to macrophage-mediated diabetes complications (35–38).

**Methods**

**Animals**

NOD/M2² mice were bred and maintained under specific pathogen-free (SPF) conditions in laminar flow caging in the animal facility at the University of Toledo. The diabetes rate for female mice was 72% by 6 months of age (39). From the age of 12 weeks, female mice were tested every other day for diabetes development using Diastix (Bayer, Elkhart, IN, USA). Animals with blood glucose levels >250 mg dl⁻¹, as measured with a TheraSense FreeStyle monitor, were considered diabetic. Diabetic mice were used within 24–48 h of becoming diabetic. NOR/Lt breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained under the SPF conditions described above. NOR/Lt mice are genetically matched to NOD mice at the major histocompatibility antigen locus and function as a
diabetes-resistant control strain for NOD mice (40). Females were used for all experiments.

BALB/c and C57BL/6 mice were obtained from The Jackson Laboratory. NOD mice express K\(^{d}\) and D\(^{b}\) MHC class I molecules (41) and therefore share common MHC class I genes with H-2\(^{d}\) and H-2\(^{b}\) haplotype mice. BALB/c and C57BL/6 mice were housed under conventional conditions. Female mice were used as controls for experiments with NOD mice.

The New Zealand obese (NZO) mouse is an animal model of obesity-induced, insulin-resistant T2D (42). NZO mice are genetically related to the autoimmune-prone New Zealand black (NZB) and New Zealand white (NZW) mice (43). NZB × NZW F1 mice are animal models for systemic lupus erythematosus (SLE) (44). NZL is an NZO-derived mouse strain, wherein 80% of males became diabetic (blood glucose > 250 mg dl\(^{-1}\)) (45). NZL and genetic control NZW mice were gifts from Edward Leiter (The Jackson Laboratory). Animal models of T2D do not require SPF conditions for diabetes development and upon receipt, NZL as well as NZW mice, were housed under conventional conditions. NZL mice can be susceptible to urogenital tract infections, but only NZL mice clean of such infections were used. Male mice were used for experiments involving NZW and NZL strains. All animals were handled in accordance with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of Toledo.

**Isolation of murine BM-Ø**

Bone marrow myeloid progenitor cells were differentiated into macrophages following the procedure of Warren and Vogel (46) using macrophage colony-stimulating factor (Calbiochem, San Diego, CA, USA) at a concentration of 1 ng ml\(^{-1}\) in complete medium [low-glucose (100 mg dl\(^{-1}\)) DMEM (Mediatech, Herndon, VA, USA), 10% fetal bovine serum (FBS, Harlan, Indianapolis, IN, USA), 50 U ml\(^{-1}\) penicillin G, 50 \(\mu\)g ml\(^{-1}\) streptomycin (Gibco BRL, Grand Island, NY, USA), 2 mM L-glutamine (JRH Biosciences, Lenexa, KS, USA) and 5 \(\times\) 10\(^{-5}\) M 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA). To test the influence of a hyperglycemic environment on differentiation, in some experiments, BM-Ø were differentiated as described above in high-glucose (450 mg dl\(^{-1}\)) DMEM (Mediatech) medium.

**Preparation of RNA and real-time reverse transcription–PCR**

One million BM-Ø cells per well in 24-well plates were stimulated with 100 ng ml\(^{-1}\) of LPS (Escherichia coli LPS, Sigma, St Louis, MO, USA) for 2 or 4 h in a final volume of 1.0 ml of complete DMEM medium described above. In a different set of experiments, BM-Ø were exposed to poly(I)poly(C) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 250 ng ml\(^{-1}\) for 4 h before harvesting. In another set of experiments, BM-Ø were exposed to whole, viable bacteria (E. coli ATCC \#23722) at a ratio of 1:50, BM-Ø to bacteria, for 4 h. In all experiments, untreated cells were used to measure the basal level of expression. Cells were harvested and total RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA) or RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer’s protocol. Moloney murine leukemia virus reverse transcriptase (Gibco BRL) was used to make cDNA from the isolated RNA that was used in the quantitative real-time reverse transcription (RT)–PCRs.

To measure the relative amount of mRNAs, amplification of sample cDNA was monitored with the fluorescent DNA-binding dye SYBR Green in combination with the ABI 5700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) or iCycler iQ system (Bio-Rad) according to the manufacturer’s instructions. Forward and reverse primers were designed using the Primer Express 1.5 software (PE Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for an endogenous control. The PCR primers used in this study were as follows: TNF\(_x\) (F: TGGGAGTAGAAGTGAAACACCC and R: AGAGGGAAATTCGTGCCTGAC), IL-6 (F: GCCCTTCCCTACTTACAAAG and R: ATTTCACCAGATTCCCAAGAG), IL-10 (F: GGTTGCCAACGTCTATCGGA and R: ACCTGTCTCAGTCCTGCTG), inducible nitric oxide synthase (iNOS) (F: CAGGTGGGGCTTGACGAACTT and R: CGGTCGCTGGATTTA and R: TAGGCAGGCGATCAACAGGC), TLR2 (F: ATGGGGCTCGGGCAATTT and R: CTACTTTACCCAGCTCGCTCACTA), TLR3 (F: CAGAAGATTACACCTACCAACTTACA and R: TGGCTCCAGTTTGAATGGA), TLR4 (F: CAGAACATCTGCTGATT and R: AAATTTCCACGATTTCCCAGAG), IL-10 (F: GGTTGCCAACGTCTACAGGC and R: GGGTGAATGAAAGGC and R: GCCATGGACTGTGGTCATGA).

**Cytokine-specific ELISA**

BM-Ø (1 \(\times\) 10\(^6\) cells per well) were stimulated with LPS at 100 ng ml\(^{-1}\) for 24 h. Supernatants were analyzed for TNF\(_x\), IL-12p40, IL-10 and IL-6 by ELISA using cytokine kits and following the manufacturer’s instructions (BD PharMingen, San Diego, CA, USA).

**Preparation of whole-cell lysates and Western blot analysis**

BM-Ø were seeded in six-well plates and stimulated with 100 ng ml\(^{-1}\) of LPS for 15 min, washed and lysed as described previously (47). Total protein concentrations were measured using the Bradford assay, and equal amounts of protein were used for SDS-PAGE. After SDS-PAGE of the cell lysate (40 \(\mu\)g of total protein), protein bands were transferred to a polyvinylidene difluoride membrane. After blocking the membrane with 5% non-fat milk for 30 min and washing with Tris-buffered saline containing 0.2% Tween 20 (TBST), the membrane was incubated with the antibody phospho-I\(_\beta\) (Ser32) (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. After washing the membrane with TBST, HRP-conjugated ImmunoPure goat anti-rabbit IgG (H + L) ( Pierce Biotechnology, Rockford, IL, USA) was added. SuperSignal West Pico Substrate (Pierce Biotechnology) was used as an enhanced chemiluminescent substrate for the detection of HRP. CL-X Film (Pierce Biotechnology) was exposed and then developed using a Konica SRX-101 processor. The membrane was stripped
with stripping buffer (2% SDS, 100 µM 2-mercaptoethanol, 62.5 mM Tris pH 6.7) and treated sequentially as described above with anti-actin antibody (Sigma) and HRP-conjugated secondary goat anti-mouse IgG (H + L) (Jackson Immunologicals, West Grove, PA, USA). The blot was stripped a final time and treated with anti-β-actin antibody (Santa Cruz) and HRP- goat anti-rabbit secondary antibody (Pierce Biotechnology). Phospho-β-actin (Ser32)-positive control cell extract was purchased from Pierce Biotechnology, A Bio-Rad Molecular Imager FX and Quantity One Software were used to analyze band densities.

Antiviral assay to determine relative IFN production by poly(I):poly(C)-treated BM-Ø

BM-Ø (1 × 10⁶ per well), differentiated as described above and derived from pools of new onset diabetic NOD mice (aged 16–20 weeks), pre-diabetic NOD mice (aged 6 weeks) and NOR mice (aged 16–20 weeks), were seeded in 24-well plates and treated with 0, 1 or 10 µg m⁻¹ of poly(I):poly(C) (Amersham Pharmacia Biotech) for 24 h. To measure IFN production by treated macrophages, an antiviral assay was performed following the procedure of Vyas et al. (48). Briefly, conditioned medium from each well was serially diluted in complete medium (one-third log dilutions) and added to L929 mouse fibroblast indicator cells (American Tissue Culture Collection, Manassas, VA, USA) in a 96-well plate. The cells were incubated for 24 h. Cells were then exposed for 2 h to encephalomyocarditis virus (EMCV) at a multiplicity of infection of 0.5 in serum-free medium. After that time, virus was removed, complete medium was added and the L929 cells were incubated for 24 h. To determine the extent of cell lysis due to viral infection, cells were fixed (10% trichloroacetic acid) and stained with sulforhodamine B dye (0.2% w/v). Control wells contained mock-infected L929 cells (cell control), L929 cells incubated with virus (virus control) or L929 cells incubated with virus plus one-third log dilutions of IFN-α/β (Biosource International, Camarillo, CA, USA, 1000–0.45 U ml⁻¹, IFN control). IFN-protected cells were quantified by measuring the absorbance at 595 nm using a plate reader. The dilution at which 50% of the cells were protected from viral cytolysis was determined by regression analysis of absorbance values.

Lymulus amebocyte lysate test for endotoxin

Gram-negative bacterial endotoxins were detected and quantified using the pyrochrome Lymulus amebocyte lysate end-point test following the manufacturer’s instructions (Associates of Cape Cod, Falmouth, MA, USA). Liberated yellow p-nitroaniline was detected at 405 nm (curve range reading as low as 0.02 EU ml⁻¹) using a Molecular Devices SpectraMAX Plus spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

Statistical analysis

Comparison between two means was performed using a two-tailed Student’s t-test. P < 0.05 was considered significant. Whenever more than one comparison was made in a single experiment, an analysis of variance (ANOVA) test was performed to reject the null hypothesis prior to making multiple comparisons. The ANOVA test ruled out the null hypothesis in all experiments where statistical significance is mentioned.

Results

Increased level of pro-inflammatory cytokine gene expression in BM-Ø from diabetic NOD mice

Ligand binding to TLRs on macrophages leads to signal transduction, the activation of gene transcription and the production of cytokines, chemokines, co-stimulatory molecules and inflammatory markers [reviewed in (1)]. To examine the effects of signaling changes after diabetes development, the production of cytokines and the expression of the iNOS, a marker for inflammation, were analyzed after the activation of TLR4 with the specific ligand LPS (9). Even without LPS stimulation, the expression of the gene for iNOS was significantly higher in BM-Ø from newly diabetic NOD as compared with other mice (Fig. 1A). BM-Ø isolated from newly diabetic NOD mice showed increased levels of LPS-induced gene expression of iNOS and pro-inflammatory cytokines (IL-12p40 and TNFα) compared with BM-Ø obtained from either BALB/c or pre-diabetic NOD mice (Fig. 1A). Interestingly, the level of LPS-induced gene expression of the immunosuppressive cytokine IL-10 was significantly decreased in BM-Ø from diabetic mice compared with BALB/c mice and even pre-diabetic NOD mice. In another set of experiments, in which diabetic NOD mice were compared with pre-diabetic NOD mice and age-matched C57BL/6 and NOR mice, a similar pattern was observed (Fig. 1B). LPS-stimulated BM-Ø from newly diabetic NOD mice expressed significantly higher levels of TNFα, IL-12p40 and IL-6 mRNAs than BM-Ø from other mice. BM-Ø from diabetic NOD mice also expressed lower IL-10 mRNA levels than BM-Ø of pre-diabetic NOD and NOR mice.

LPS-induced changes in cytokine mRNA levels were associated with corresponding changes in cytokine production. TNFα secretion in BM-Ø from diabetic NOD mice was significantly increased compared with genetic and age-matched control NOR mice (Fig. 1C). Furthermore, IL-12 and IL-6 were significantly increased and IL-10 significantly decreased in BM-Ø from diabetic NOD mice compared with BM-Ø from BALB/c mice (Fig. 1D). Therefore, studies of both gene and protein expression of cytokines indicate an enhanced pro-inflammatory response in new onset diabetic mice along with defective regulatory control.

TLR signaling changes in diabetic NOD mice

The altered cytokine responses in diabetic NOD mice may in part be due to alterations in TLR signaling. Ligand binding to TLR triggers signal transduction and the activation of NF-κB by initiating the phosphorylation of IκB-α (1). To examine TLR signal transduction, the phosphorylation of IκB-α was measured as an indicator of TLR activation. BM-Ø from diabetic NOD mice, pre-diabetic NOD mice and C57BL/6 mice were stimulated with LPS for 15 min. BM-Ø obtained from newly diabetic NOD mice were more sensitive to LPS stimulation than those from pre-diabetic NOD or C57BL/6 mice. The amount of phosphorylated IκB-α, as measured by Western blot and scanning densitometry after LPS stimulation, showed the following changes over untreated: diabetic, 6.5-fold increase; pre-diabetic, 1.1-fold increase, and C57BL/6, 3.1-fold increase (Fig. 2A). The level of phosphorylated IκB-α in control NOR mice did not increase with LPS stimulation (Fig. 2B).
IFN-α, in particular IFN-α, leads to signal transduction and cellular production of type dsRNA (49). Treatment with dsRNA, or the mimic poly(I)poly(C), leads to signal transduction and cellular production of type 1 IFNs, in particular IFN-α and IFN-β (50–54). To investigate dsRNA responsiveness in the diabetic state, BM-Ø obtained from pre-diabetic NOD, diabetic NOD and age-matched NOR mice were stimulated with poly(I)poly(C) and type 1 IFN production was analyzed using an antiviral assay. All strains of mice produced IFNs upon stimulation with poly(I)poly(C) (Fig. 3). However, NOD mice with new onset diabetes produced 9-fold higher levels of IFNs as compared with the pre-diabetic NOD or NOR mice.

**Increased IFN production in diabetic NOD mice**

TLR3 has been identified as an endosomal or cell-surface receptor that recognizes and mediates responses to viral dsRNA (49). Treatment with dsRNA, or the mimic poly(I)poly(C), leads to signal transduction and cellular production of type 1 IFNs, in particular IFN-α and IFN-β (50–54). To investigate dsRNA responsiveness in the diabetic state, BM-Ø obtained from pre-diabetic NOD, diabetic NOD and age-matched NOR mice were stimulated with poly(I)poly(C) and type 1 IFN production was analyzed using an antiviral assay. All strains of mice produced IFNs upon stimulation with poly(I)poly(C) (Fig. 3). However, NOD mice with new onset diabetes produced 9-fold higher levels of IFNs as compared with the pre-diabetic NOD or NOR mice.

**Higher expression level of certain TLRs in new onset diabetic NOD mice**

One reason for the increased response to LPS and dsRNA stimulation could be the increased expression of TLRs. Therefore, it was determined whether diabetes development...
is associated with systemic changes of the expression of
murine TLRs on BM-Ø. The unstimulated, basal level of TLR
expression was examined by quantitative real-time RT-PCR
analysis. In the first set of experiments, BM-Ø were obtained
from diabetic NOD, pre-diabetic NOD and age-matched
BALB/c mice (Fig. 4A). The expression of mouse Toll-like
receptor 3 (mTLR3) and mTLR5 was higher in BM-Ø obtained
from diabetic NOD mice compared with pre-diabetic and
BALB/c mice. To control for age and genetics, mTLR expres-
sion was also examined in pre-diabetic NOD, diabetic NOD
and NOR mice. NOR mice had equivalent mRNA expression
levels of mTLR1, mTLR2 and mTLR9 compared with diabetic
NOD mice. NOR mice had equivalent mRNA expression
levels of mTLR1, mTLR2 and mTLR9 compared with diabetic
NOD mice (data not shown). However, mTLR3 and mTLR5
were again significantly increased in BM-Ø obtained from
diabetic NOD mice compared with pre-diabetic NOD and
NOR mice (Fig. 4B). Results with TLR4 will be discussed in
detail below. These experiments examined TLR expression
prior to stimulation with a ligand ex vivo. Therefore, TLR3 and
TLR5 must be up-regulated in vivo, resulting in increased
expression and ligand responsiveness in diabetes.

Dysregulated TLR3 expression in diabetic NOD mice

Treatment of macrophages with ligands for TLR affects
the expression of the ligand's own TLR [reviewed in (1)]. Stimu-
lation with poly(I)poly(C) resulted in significant up-regulation
of mTLR3 mRNA expression in BM-Ø obtained from control
NOR and pre-diabetic NOD mice (Fig. 5, untreated versus
poly I:C). However, in BM-Ø obtained from diabetic mice,
basal mTLR3 mRNA was already high and mRNA expression
was not increased further by poly(I)poly(C) treatment (Fig. 5,
diabetic NOD untreated versus diabetic NOD poly I:C). En-
hanced TLR3 mRNA expression in diabetes may explain the
enhanced responsiveness to dsRNA in the diabetic macro-
phages, which exhibited 9-fold more production of type 1 IFN
as compared with macrophages from the pre-diabetic and
control animals (Fig. 3).

Abnormal TLR expression in response to LPS stimulation

Down-regulation of TLR4 in response to LPS is a phenomenon
associated with endotoxin tolerance (55, 56). Thus, not only
abnormal up-regulation but also defective down-regulation
may account for the increased responsiveness to LPS ob-
served in the diabetic state. At basal untreated conditions
(Fig. 6, open bars), the expression level of TLR4 mRNA in the
NOD diabetic mice was elevated compared with BALB/c
mice, but was not greater than that observed in control NOR
mice. Stimulation TLR4 with LPS resulted in the significant
down-regulation of TLR4 mRNA in pre-diabetic NOD, BALB/c
and age-matched control NOR mice (Fig. 6A and B). In con-
trast, in diabetic NOD mice, stimulation with LPS unexpectedly
left expression levels of TLR4 mRNA unchanged. When viable
bacteria were tested, the NOR mice were again able to re-
spond with dramatic receptor mRNA down-regulation, while
TLR4 gene expression in diabetic NOD mice remained
unchanged (Fig. 6C).

TLR4 and cytokine expression after LPS stimulation in an
animal model for T2D

The development of diabetes occurs by separate pathways
in T1D and T2D. T1D is associated with the autoimmune

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**Fig. 2.** Early NF-κB activation in response to LPS stimulation. (A) BM-Ø were derived from pools of four newly diabetic NOD mice (aged 16–20 weeks), four pre-diabetic NOD mice (aged 10 weeks) and four C57BL/6 (B6) mice (aged 10 weeks) and (B) BM-Ø were derived from pools of three newly diabetic NOD mice (aged 16–20 weeks) and two NOR mice (aged 16–16 weeks). BM-Ø were either left unstimulated (–) or stimulated (+) with LPS for 15 min as indicated. Phosphorylated IκB-α (Ser32) (P-IκB-α), and actin were detected by Western blot as described in Methods. P-IκB-α cell extract (Pierce Biotechnology) was used as a positive control (control). The density of the P-IκB-α band, after LPS treatment, is greater in diabetic NOD mice compared with pre-diabetic NOD, C57BL/6 or NOR mice.

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**Fig. 3.** Relative IFN production by poly(I)poly(C)-treated BM-Ø. BM-Ø (1 × 10⁶ per well) derived from pools of four newly diabetic NOD mice (aged 16–20 weeks), four pre-diabetic NOD mice (aged 6 weeks) and four NOR mice (aged 16–20 weeks) were plated in 24-well plates and treated with 0, 1 or 10 μg ml⁻¹ of poly(I)poly(C) for 24 h (dsRNA 1, IFN Ctl). These
control wells contained mock-infected L929 cells (cell Ctl), L929 cells incubated with virus (virus Ctl) or L929 cells incubated with virus plus 10% trichloroacetic acid, and stained with sulforhodamine B dye. Control wells contained mock-infected L929 cells (cell Ctl), L929 cells incubated with virus (virus Ctl) or L929 cells incubated with virus plus one-third log dilutions of IFN-α/IFN-β (1000–0.45 U ml⁻¹), IFN Ctl). These results are representative of three experiments with equivalent numbers of mice.
destruction of insulin-secreting beta cells (57), while the hallmark of T2D is insulin resistance. However, both exhibit the clinical symptom of hyperglycemia with blood glucose levels >250 mg dl\(^{-1}\). In order to investigate responsiveness in T2D, NZL, an NZO-derived mouse strain that develops insulin-resistant T2D at a frequency of >80% (45), was studied. mTLR4 mRNA expression in the untreated state was 10-fold greater in BM-Ø from diabetic NZL mice compared with genetic, age-matched control NZW mice (Fig. 7). After stimulation with LPS, mTLR4 mRNA expression was significantly up-regulated in control NZW mice, but did not change in diabetic NZL mice. These results were similar to those observed with diabetic NOD mice. As with T1D NOD mice, T2D diabetic NZL mice exhibited enhanced TNF\(_\alpha\), IL-6, and IL-12 gene expression after stimulation with LPS, as compared with NZW mice.

**Factors affecting TLR expression**

The hyperglycemic environment in a diabetic animal may have a systemic effect on TLR expression. To test the effect of hyperglycemia, BM-Ø were differentiated in low- (normal) and high-glucose concentrations to simulate hyperglycemic conditions. Macrophages were not stimulated with LPS. As can be seen in Fig. 8, TLR2 and TLR4 mRNA expressions were significantly increased under hyperglycemic conditions in pre-diabetic (6 weeks) and diabetic NOD (16–20 weeks) mice, but not in age-matched NOR mice. In fact, TLR4 mRNA expression was down-regulated in control NOR mice comparable to reports of TLR4 expression being down-regulated by treatment with LPS (56). mTLR3 and mTLR5 mRNA expressions were unaffected by high glucose concentrations (data not shown). Concomitantly, basal expression levels of TNF\(_\alpha\) were also increased in the pre-diabetic and diabetic NOD mice under high-glucose conditions, but were unaffected in the NOR control mice. Low- and high-glucose media preparations containing FBS were negative for the presence of endotoxin. Therefore, the genetic predisposition of NOD mice and the hyperglycemic environment after diabetes development have a combined effect on some TLR and cytokine expression levels.

**Discussion**

In this study, an abnormality was uncovered at the level of myeloid progenitor cells in diabetic mice. Bone marrow cells, when differentiated into macrophages, displayed aberrant levels of TLR and iNOS expression, and upon activation, secreted an altered pattern of cytokines. It is unclear what causes these abnormalities, but they must be due to systemic changes in the immune system in the diabetic state, affecting the bone
marrow environment from which macrophage precursors were obtained. Despite the fact that macrophage precursors from diabetic and normal mice were subject to in vitro differentiation under identical conditions, differentiated macrophages obtained from diabetic NOD mice had very different characteristics as to the expression and responsiveness of TLRs. Given this long-lasting effect in vitro, it is likely that these precursors will give rise to tissue macrophages with similarly altered characteristics.

Changes in bone marrow cells occur in humans with T1D. Thus, a 28-year-old non-diabetic female patient developed T1D after bone marrow transplantation from her HLA-identical brother who had T1D. The female recipient was islet cell antibody negative before transplantation and the only circulating leukocytes after transplantation were of male origin (58). These results and the data in this paper support the hypothesis that bone marrow progenitor cells are developmentally different at the diabetic stage.

Aberrant cytokine responses were obtained upon stimulation of TLR with ligand in diabetes. LPS-activated BM-Ø isolated from diabetic NOD mice displayed significantly increased expression of pro-inflammatory molecules (IL-12p40, TLR3 expression and response to poly(I)poly(C). BM-Ø (1 x 10⁶ per well) were derived from pools of four newly diabetic NOD mice (filled bars) (aged 16–20 weeks), four pre-diabetic NOD mice (dotted bars) (aged 10 weeks) and four NOR mice (open bars) (aged 16–20 weeks). BM-Ø were left untreated or were exposed to poly(I)poly(C) (250 ng ml⁻¹) for 4 h before harvest and mRNA extraction. The data were obtained by measuring the amount of TLR3 mRNA by quantitative real-time PCR analysis using the GAPDH transcript as a reference point. The results are the mean ± SD of triplicate samples and are expressed as the percentage of GAPDH. Significant differences (P-value < 0.05) are denoted by an asterisk, indicating increases in TLR3 expression after treatment in the NOR and pre-diabetic NOD samples. Not indicated by an asterisk is the significantly higher expression of TLR3 in untreated diabetic NOD mice compared with either pre-diabetic or NOR untreated mice (P-value < 0.05).

Fig. 5. mTLR4 mRNA expression in BM-Ø after stimulation with LPS. (A) BM-Ø (1 x 10⁶ per well) were derived from pools of eight new onset diabetic NOD (aged 16–20 weeks), eight BALB/c (aged 10 weeks) and six pre-diabetic NOD (aged 10 weeks) mice and (B) BM-Ø (1 x 10⁶ per well) were derived from pools of three new onset diabetic NOD mice (aged 16–24 weeks) and three NOR mice (aged 17 weeks). (A and B) BM-Ø were either unstimulated (open bars) or stimulated with LPS at 100 ng ml⁻¹ for 4 h (filled bars). (C) BM-Ø (1 x 10⁶ per well) derived from pools of three new onset diabetic NOD mice (aged 16–24 weeks) and three NOR mice (aged 16–22 weeks) were either unstimulated (open bars) or stimulated with whole E. coli ATCC #23722 at a ratio of 1:50, BM-Ø:bacteria (filled bars). (A–C) The data were obtained by measuring the amount of mTLR4 mRNA by quantitative real-time PCR analysis using the GAPDH transcript to normalize data. The results are the mean ± SD of triplicate samples and are expressed as the percentage of GAPDH. Significant difference (P-value < 0.05) is denoted by an asterisk. TLR4 gene expression is down-regulated in pre-diabetic NOD, BALB/c and NOR mice, but remains constant in diabetic NOD mice following stimulation with either LPS or whole E. coli.
Diabetic patients with proliferative diabetic retinopathy (PDR) had significantly increased serum levels of NO, soluble IL-2R, IL-8 and TNFα compared with diabetic patients with non-PDR, diabetic patients without retinopathy and normal controls. NO and inflammatory cytokines with their endothelial implications may act together to play an important role in the pathophysiology of ocular microvascular complications in the progression of diabetic retinopathy (66).

In BM-Ø of diabetic NOD mice, relative mRNA levels and protein secretion of TNFα were significantly increased compared with those of pre-diabetic NOD and control mice after stimulation with LPS. Acute and local production of TNFα is undoubtedly beneficial in host defense against infectious pathogens, but prolonged chronic exposure to elevated or circulating TNFα may be harmful (67). Highly elevated levels of TNFα and TNFα polymorphisms have been associated with various disease states, including SLE (68), rheumatoid arthritis (69, 70), diabetes (71), multiple sclerosis (72), periodontal disease (73), myasthenia gravis (74) and Crohn’s disease (75).

Significantly higher expression of TLR 3 and TLR5 in BM-Ø of newly diabetic NOD mice indicates that these TLRs were up-regulated in vivo. It is possible that cytokines, endogenous products from damaged or stressed cells circulating in the blood stream or infection could affect the development and differentiation of bone marrow myeloid progenitor cells and TLR expression levels. HSPs, released from stressed cells, are ligands for TLRs [reviewed in (1)]. IL-7, when injected into mice, directly induces phenotypic changes in macrophages that are released from the bone marrow (76). Only TLR5 mRNA expression was dramatically increased (300-fold above untreated) when macrophages (NOD diabetic or NOR) were exposed to NOD serum that was free of endotoxin (M.F.M., unpublished observations). TLR3 expression can be up-regulated by viral infection (77) or by type 1 IFNs (52). mRNA, originating from damaged tissue, necrotic cells or endocytosed cells, is an endogenous ligand for TLR3 (78). TLR3 expression is associated with immune cells, but is also expressed on lung and brain and expression can be up-regulated by stimulation with LPS (26). Although multiple scenarios exist wherein TLR expression can be affected, the actual mechanism for the up-regulation of TLR3 or TLR5 in diabetes remains unclear.

TNFα, IL-6 and iNOS and significantly decreased expression of the immunoregulatory cytokine IL-10 compared with control mouse strains (C57BL/6 and BALB/c), pre-diabetic NOD and genetically related but not disease-prone NOR mice (40). In humans, significantly higher levels of IL-2, IFN-γ, TNFα and IL-1α circulate in the peripheral blood of recently diagnosed T1D patients (59). These cytokine results classify the diabetic NOD macrophages to be at the extreme Th1 pole. Low levels of expression of IL-10 deprive macrophages in the diabetic mice of the protective and anti-inflammatory effects of this cytokine. Furthermore, aberrantly elevated levels of IL-12 could askew the production of inflammatory Th1 cells. This hyper-sensitivity may indicate that BM-Ø from newly diabetic mice are poised to respond in a Th1 direction in the periphery even before entry into tissues such as transplanted islets, infected sites or inflamed sites. NOD mice up to 10 weeks of age, 2 weeks prior to onset of diabetes, did not have abnormal TLR or pro-inflammatory cytokine expression, although IL-10 expression, even at 6 weeks of age, was significantly decreased compared with genetically related NOR mice. The dysregulation of TLRs and the imbalance created between the over-expression of pro-inflammatory cytokines and lack of regulation clearly sets the stage for aggressive pro-inflammatory responsiveness to infection or immune insult that would result in enhanced inflammation and tissue destruction in the diabetic state.

There was significant iNOS gene expression in unstimulated and LPS-stimulated BM-Ø from newly diabetic mice that was absent in pre-diabetic and control mice. Induction of iNOS leads to internal formation of nitric oxide (NO) (60) which can be secreted from macrophages upon activation. Free radicals such as NO can penetrate cells leading to cell death if antioxidant defenses are low and free radicals persist (61). Free radical oxidative stress has been implicated in the pathogenesis of a variety of human diseases, including T1D (62–64). Patients with T1D have significant defects in antioxidant protection, increasing their vulnerability to oxidative damage and subsequent diabetic complications (62). Furthermore, high glucose concentration induces expression of inflammatory molecules such as TNFα, IL-1β and iNOS in monocytes (65). Diabetic patients with proliferative diabetic retinopathy (PDR) leads to internal formation of nitric oxide (NO) (60) which can be secreted from macrophages upon activation. Free radicals such as NO can penetrate cells leading to cell death if antioxidant defenses are low and free radicals persist (61). Free radical oxidative stress has been implicated in the pathogenesis of a variety of human diseases, including T1D (62–64). Patients with T1D have significant defects in antioxidant protection, increasing their vulnerability to oxidative damage and subsequent diabetic complications (62). Furthermore, high glucose concentration induces expression of inflammatory molecules such as TNFα, IL-1β and iNOS in monocytes (65). Diabetic patients with proliferative diabetic retinopathy (PDR) and brain and expression can be up-regulated by stimulation with LPS (26). Although multiple scenarios exist wherein TLR expression can be affected, the actual mechanism for the up-regulation of TLR3 or TLR5 in diabetes remains unclear.

Fig. 7. Basal and E. coli/LPS-stimulated cytokine expression in a T2D model. Bone marrow was isolated from diabetic NZL mice and age-matched NZW mice. All were male mice. After differentiation of macrophages in macrophage colony stimulating factor for 6 days, cells were harvested and were either left unstimulated (open bars) or stimulated with E. coli LPS (100 ng ml⁻¹) for 4 h (filled bars). RNA was isolated, converted to cDNA and quantitated by real-time PCR analysis, normalized using GAPDH. Results are the mean and SD of triplicate samples. The asterisk indicates significant differences (P-value < 0.05) as determined by the Student’s t-test. NZL diabetic mice have significantly increased gene expression of TNFα, IL-6 and IL-12 compared with NZW mice after treatment with LPS. TLR4 gene expression is high and remains elevated after LPS stimulation in diabetic NZL mice.
A contribution of the cytoplasmic dsRNA sensor RIG-I, an RNA helicase that is involved with induction of type 1 IFNs after RNA virus infection in fibroblasts and conventional dendritic cells, to the observed enhanced IFN secretion by the diabetic cells cannot be ruled out (79).

TLR3 expression can be up-regulated (13, 50, 52, 77, 78) or down-regulated by ligand binding (80). BM-Ø obtained from pre-diabetic NOD and NOR mice responded to poly(I)poly(C) stimulation with up-regulation of TLR3 expression. However, TLR3 was up-regulated in untreated BM-Ø, obtained from diabetic NOD mice, and TLR3 mRNA expression remained strongly elevated after exposure to ligand. Respiratory syncytial virus (RSV) infection of bronchoepithelial cells leads to up-regulated mRNA and protein expression of TLR3, increased expression of TLR3 on the cell surface and increased intracellular IFN-inducible, RNA-dependent protein kinase R (PKR) (77). Furthermore, RSV infection sensitizes airway epithelial cells to subsequent dsRNA exposure that results in enhanced PKR-mediated activation of NF-κB and increased production of IL-8 while maintaining TLR3 mRNA up-regulated levels. The hypersensitivity of the epithelial cells due to RSV infection extends to subsequent bacterial ligand (i.e. LPS) challenges as well (77). It is unclear what causes the initial sensitization of TLR3 in diabetes, but sensitization appears to have occurred since macrophages from diabetic mice are hypersensitive to poly(I)poly(C) stimulation, as evidenced by exaggerated IFN production.

It has been shown previously that the level of expression of TLRs changes after exposure to TLR ligands [reviewed in (1)]. Interestingly, TLR4 gene expression that was high at the onset of diabetes was not affected by treatment with LPS in either diabetic NOD or NZL mice, while pre-diabetic NOD and control mice displayed either TLR4 down-regulation as has been described previously (55, 56) or TLR4 up-regulation in the case of NZW mice. Results with TLR4 in mice correlate with findings in humans with active inflammatory bowel disease (81). TLR4 is strongly up-regulated in primary intestinal epithelial cells in active inflammatory bowel disease, facilitating the persistence of the inflammatory response. Normal controls have significantly less TLR4 expression even though E. coli is a normal resident in the bowel, indicating that in patients with active inflammatory bowel disease, alterations in the TLR expression may contribute to pathogenesis (81). The TLR4 also appears to play a role in the development of atherosclerosis, an inflammatory disease and a secondary complication of diabetes (82). TLR4 expression has been documented on macrophages in lipid-rich atherosclerotic plaques and TLR4 expression can be up-regulated by oxidized low-density lipoprotein (83, 84). Patients with hypo-responsiveness to LPS due to a TLR4 gene polymorphism from pre-diabetic NOD and NOR mice responded to poly(I)poly(C) stimulation with up-regulation of TLR3 expression. However, TLR3 was up-regulated in untreated BM-Ø, obtained from diabetic NOD mice, and TLR3 mRNA expression remained strongly elevated after exposure to ligand. Respiratory syncytial virus (RSV) infection of bronchoepithelial cells leads to up-regulated mRNA and protein expression of TLR3, increased expression of TLR3 on the cell surface and increased intracellular IFN-inducible, RNA-dependent protein kinase R (PKR) (77). Furthermore, RSV infection sensitizes airway epithelial cells to subsequent dsRNA exposure that results in enhanced PKR-mediated activation of NF-κB and increased production of IL-8 while maintaining TLR3 mRNA up-regulated levels. The hypersensitivity of the epithelial cells due to RSV infection extends to subsequent bacterial ligand (i.e. LPS) challenges as well (77). It is unclear what causes the initial sensitization of TLR3 in diabetes, but sensitization appears to have occurred since macrophages from diabetic mice are hypersensitive to poly(I)poly(C) stimulation, as evidenced by exaggerated IFN production.

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Fig. 8. Effect of high glucose on TLR mRNA expression. Bone marrow cells from pools of four pre-diabetic 6-week-old NOD, diabetic NOD (16–20 weeks) and age-matched NOR mice were differentiated in macrophage colony-stimulating factor containing either low 100 mg dl⁻¹ glucose (low-glucose DMEM, open bars) or high 450 mg dl⁻¹ glucose (high-glucose DMEM, filled bars) to mimic a hyperglycemic environment. TLR transcripts for TLR2, TLR3, TLR4 and TLR5 and TNFα were analyzed by quantitative real-time PCR analysis using the GAPDH transcript as a reference. Shown are results for TLR2 and TLR4 and TNFα mRNA for TLR3 and TLR5 did not change significantly (data not shown). Data are the mean ± SD of triplicate samples. The x-axis shows the mouse strain and the y-axis is the mRNA level of TLR expressed as a percentage of GAPDH mRNA. Statistical significance (P-value < 0.05), as indicated by an asterisk, shows that TLR2, TLR4 and TNFα mRNAs are significantly increased in pre-diabetic NOD and diabetic NOD mice, but not in NOR mice under hyperglycemic conditions.
are protected from carotid artery atherosclerosis and acute coronary problems (85–88). Single nucleotide polymorphisms (Asp299Gly and Thr399Ile) of TLR4 are associated with reduced incidence of diabetic neuropathy in T2D (8). In T1D and T2D, investigated in this paper, TLR4 expression was dysregulated, leading to hypersensitivity to LPS stimulation, as shown by significant increases in NF-κB activation and pro-inflammatory cytokine expression (TNFα, IL-12 and IL-6). Since lack of TLR4 or TLR4 polymorphisms can protect against secondary complications, hypersensitivity of TLR4 in diabetes is likely to promote inflammation, leading to secondary pathophysiological consequences.

Poor glycemic control is thought to favor the synthesis of pro-inflammatory cytokines during infection (89). Transient hyperglycemia in normal individuals results in activation of reactive oxygen species (ROS) and reduction of certain antioxidants (90). Diabetic patients in hyperglycemic crisis have elevated plasma levels of pro-inflammatory cytokines (IL-1β, TNFα, IL-6 and IL-8), ROS and cardiovascular risk factors in the absence of obvious infection or cardiovascular pathology (91). Activated monocytes that adhere to the endothelium play an important role in inflammatory and cardiovascular diseases. Human PBMCs are induced by high glucose, via oxidant stress, to express increased levels of TNFα and NF-κB transcription factor (92). Furthermore, high glucose induces multiple inflammatory cytokines and chemokines in monocytes, molecules that are involved in the pathogenesis of secondary complications of diabetes (65). In this study, hyperglycemic conditions affected macrophage differentiation with resultant increased expression of TLR 2 and TLR4 and inflammatory TNFα.

Changes in bone marrow cells in the diabetic state will influence the response to infectious pathogens. TLRs are now known to play an important role in several other diseases that occur at increased frequency in patients with diabetes. For example, vascular inflammation leading to atherosclerosis is a common complication of T1D and T2D and has been found to be a consequence of infectious load (93–95). TLR sensitivity in the diabetic state may well be one of the factors that underlies the increased risk of some types of vascular complications that are associated with diabetes. Diabetes is a major risk factor for severe periodontal disease in humans (96–98). Monocytes from patients with diabetes had significantly up-regulated secretion of pro-inflammatory cytokines, TNFα, IL-1β and PGE2, in response to stimulation with LPS from Propionibacterium gingivalis, a known periodontal pathogen, as compared with non-diabetic controls. Thus, diabetics responded in an exaggerated fashion to an equivalent oral bacterial burden and the abnormally high degree of inflammation led to severe periodontitis (97, 98). Impaired or inappropriate responses to infectious pathogens may in part be due to altered TLR responsiveness of diabetic macrophages.

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Abbreviations

ANOVA analysis of variance
BM-Ø bone marrow-derived macrophages
FBS fetal bovine serum
GAPDH glyceraldehyde-3-phosphate dehydrogenase
HSP heat shock proteins
iNOS inducible nitric oxide synthase
mTLR3 mouse Toll-like receptor
NF-κB nuclear factor kappa B
NIH National Institutes of Health
NO nitric oxide
NOD non-obese diabetic
NZB New Zealand black
NZO New Zealand obese
NZW New Zealand white
PAMP pathogen-associated molecular pattern
PDR proliferative diabetic retinopathy
PGE2 prostaglandin 2
PKR protein kinase R
ROS reactive oxygen species
RSV respiratory syncytial virus
RT reverse transcription
SLE systemic lupus erythematosus
SPF specific pathogen-free
TBST Tris-buffered saline containing 0.2% Tween 20
TLR Toll-like receptor
TNFα tumor necrosis factor α
T1D type 1 diabetes
T2D type 2 diabetes

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