

# Dysfunctional innate immune responsiveness to *Porphyromonas gingivalis* lipopolysaccharide in diabetes

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**Introduction:** Type 1 diabetes is a major risk factor for the development of severe periodontal disease. As diabetes increases in severity, so does the susceptibility to and severity of periodontitis. People with diabetes who have periodontal disease have a harder time maintaining healthy blood glucose levels. Macrophages play an important role in both diabetes and periodontitis. Previous research comparing bone-marrow-derived macrophages (BM-M $\phi$ ) from diabetic non-obese diabetic (NOD) mice and control mice illustrates that a dysregulation in cytokine, Toll-like receptor (TLR) expression, and cell signaling occurs in the diabetic state.

**Methods:** This study examines the effect of chronic hyperglycemia on BM-M $\phi$  TLR expression and activation, cell signaling, cytokine production, and phagocytic function in the diabetic state, when challenged with the periodontal stimulus *Porphyromonas gingivalis* lipopolysaccharide (LPS) to further understand how diabetes and associated hyperglycemia may contribute to the increased susceptibility of people with diabetes to periodontitis.

**Results:** When BM-M $\phi$ , obtained from diabetic NOD mice, are stimulated with *P. gingivalis* LPS under hyperglycemic conditions the following changes occur: reduced messenger RNA expression and cell surface expression of TLR2, reduced messenger RNA expression and protein production of tumor necrosis factor- $\alpha$ , reduced signal transduction, and a reduction in phagocytic function. All the activity of BM-M $\phi$  from diabetic NOD mice was restored when differentiation and stimulation occurred under normoglycemic conditions.

**Discussion:** Diabetic patients in a hyperglycemic state may be generating macrophages that are inherently immunocompromised, contributing to an environment allowing periodontal infections to flourish. As a consequence, people with diabetes who maintain proper control of blood sugar levels may experience an increased immunological benefit when challenged with a periodontal infection.

Key words: hyperglycemia; macrophage; Toll-like receptor; type 1 diabetes

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Periodontal disease is an inflammatory disease caused in part by colonization of the gingival and periodontal pockets by gram-negative bacteria. Gingivitis can lead to periodontitis and subsequently to the loss of both periodontal ligaments and

alveolar bone (36, 57). Diabetes is a major risk factor for the development of periodontal disease. Individuals with either type 1 or type 2 diabetes experience exacerbated periodontitis compared with non-diabetic subjects (11, 36, 47, 52). In

general, people with diabetes exhibit increased periodontal disease symptoms in correlation with the severity of their diabetes.

Diabetes mellitus is an endocrine disease affecting glucose tolerance and the

metabolism of carbohydrates and lipids (45). Although type 1 and type 2 diabetes have completely different etiologies, patients with either type of diabetes suffer from many of the same complications as a result of hyperglycemia and inflammation (7, 52) including: vascular disease with a higher risk of heart attack and stroke, microvascular changes leading to retinopathy, renal disease, poor wound healing, greater risk of infection, and an increased susceptibility to periodontitis (31, 32, 45, 56). However, the prevalence and severity of these complications decreases when patients maintain proper glycemic control (8), illustrating the overall negative impact of hyperglycemia on the body. Poor glycemic control leads to increased oxidative stress, advanced glycation end products, and a hyperresponsive innate immune system, all of which are contributing factors to the tissue damage observed in diabetic periodontitis (14, 19, 23, 24, 36, 52–55, 58, 67). In addition, normal immune cell functions, such as chemotaxis, cell adherence, phagocytosis, and cytokine production and secretion, are all affected by hyperglycemia (12, 34, 36). Not only does diabetes affect periodontal disease, but some research suggests that periodontal health can affect the metabolic state. Notably, treatment of periodontitis with antibiotics and dental intervention leading to improved periodontal status were accompanied by significant improvements of glycemic control (36).

The innate immune system uses sentinel cells (monocytes, neutrophils, macrophages, dendritic cells), bearing pattern recognition receptors, to recognize common molecular patterns on bacterial, viral, and fungal invaders (20). Macrophages play a pivotal role in both diabetes and periodontitis. Macrophages have been implicated in the etiology of diabetes as initiators of the autoimmune destruction of pancreatic beta cells in the non-obese diabetic (NOD) mouse, a murine model of type 1 diabetes (21, 22). Normally, a subset of macrophages aid in bacterial clearance, but in periodontal disease, macrophages have been implicated in the auto-degradation of both hard and soft connective tissue in the mouth (36). Macrophages express both Toll-like receptors (TLR) and the receptor for advanced glycation end products (RAGE), both of which are associated with diabetes disease progression and secondary complications of diabetes (27, 29, 45). Activation of specific TLRs and RAGE leads to the initiation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling, resulting in

increased gene transcription, ultimately leading to inflammatory cytokine protein production (26, 29, 52, 67).

Numerous bacteria are associated with the onset of periodontal disease among which *Porphyromonas gingivalis* is considered one of the foremost periodontal pathogens (18, 57). During a periodontal infection, the equilibrium of bacterial flora within the oral cavity becomes distinctly altered, allowing pathogenic bacterial species to flourish and survive, even when faced with an immune response. Periodontal pathogens use multiple adaptations and products to survive within the host including toxins, proteinases, and other cell-associated products (1–3, 6, 61–63). However, *P. gingivalis* lipopolysaccharide (LPS) is a known ligand recognized by TLR2 (13, 16, 17, 41). Increased TLR2 expression has been detected in the gingival tissues and periodontal pockets of patients with untreated advanced chronic periodontitis compared with weak TLR2 expression in healthy gingival tissues (50). Recognition of *P. gingivalis* LPS by TLR2 leads to gene activation, ultimately resulting in the production of cytokines and signaling molecules via host cells to mount an immune response (13, 38, 39, 41).

Previous research from our laboratory using bone-marrow-derived macrophages (BM-M $\phi$ ) generated from NOD mice demonstrated that changes in the expression, responsiveness, and signaling of TLRs occur in the diabetic state (40). An important observation was that bone marrow progenitors isolated from NOD mice and differentiated into macrophages under hyperglycemic conditions had a basal level expression of TLR and cytokine messenger RNA (mRNA) that was significantly increased from that of the same progenitors differentiated in a normal glucose environment. Hence, glucose levels have an effect on BM-M $\phi$  gene expression profiles (40). To further investigate the effect of hyperglycemia on BM-M $\phi$  gene expression, immune responsiveness, and how it may relate to the increased susceptibility of diabetics to periodontal disease, BM-M $\phi$  from diabetic NOD mice and controls were analysed to investigate BM-M $\phi$  activation, signaling, and function when differentiation into macrophages and challenge with *P. gingivalis* LPS occurred under hyperglycemic as opposed to normoglycemic conditions. Bone marrow stem cells obtained from diabetic NOD mice and differentiated and stimulated under hyperglycemic conditions displayed defective immune responsiveness,

whereas when normoglycemic conditions were present at this critical period, the subsequent immune responses were normal. These results argue for tight glucose control to allow for the generation of effective macrophages to combat periodontal infection in diabetes.

## Materials and methods

### Animals

NOD/M2 mice were bred and maintained under specific pathogen-free standard barrier conditions in laminar flow caging in the animal facility at the University of Toledo. Additional NOD mice were purchased from Taconic (Hudson, NY). Beginning at 12 weeks of age, mice were tested every other day for the presence of glucose in urine using Diastix urinalysis test strips (Bayer, Elkhart, IN). Animals with blood glucose levels equal to or exceeding 250 mg/dl, as measured with a TheraSense FreeStyle monitor (Abbott Laboratories, Chicago, IL), were considered diabetic. Diabetic mice were used within 48 h of becoming diabetic. NOR/LtJ mice, a genetically related diabetes-resistant strain (49), were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained under the specific pathogen-free conditions described above. Female 16- to 20-week-old mice were used for all experiments. As a genetic control for each experiment, NOR mice were age-matched to the diabetic NOD mice. 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-M $\phi$

Bone marrow stem cells were harvested from the femur and tibia of NOR and diabetic NOD mice following the procedure of Warren and Vogel (65). Progenitor cells were differentiated, in a humidified tissue culture incubator in 5% CO<sub>2</sub> at 37°C, into macrophages over a period of 6 days using macrophage colony-stimulating factor (M-CSF; Calbiochem, San Diego, CA) at a concentration of 1 ng/ml in either low-glucose or high-glucose Dulbecco's modified Eagle's minimum essential medium (100 mg/dl vs. 450 mg/dl; Mediatech Inc, Manassas, VA), supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN), 50 U/ml penicillin G, 50 g/ml streptomycin (Mediatech), 2 mM L-glutamine (Mediatech), and  $5 \times 10^{-5}$  M 2-mercaptoethanol (Bio-Rad,

Hercules, CA). These glucose conditions reflect approximate human normoglycemia and extreme diabetic hyperglycemia. BM-M $\phi$  were counted, replated at the desired cell concentration, and rested for an additional day without M-CSF before LPS stimulation.

#### Preparation of RNA and real-time reverse transcription–polymerase chain reaction

One million BM-M $\phi$  cells per well were stimulated in 24-well tissue culture plates with *P. gingivalis* LPS (500 ng/ml) in specific glucose-containing complete medium (described above) for 4 h. *P. gingivalis* LPS was generously provided by Dr Richard Darveau (University of Washington, Seattle, WA). In all experiments, unstimulated cells were included to measure the basal level of mRNA gene expression. Cells were harvested and total RNA was isolated by using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Moloney murine leukemia virus reverse transcriptase (Fisher Scientific, Pittsburgh, PA) was used to reverse transcribe mRNA to complementary DNA (cDNA). This cDNA was then used for quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) using ABolute QPCR SYBR green fluorescein mix (Thermo Scientific, Waltham, MA). The DNA was denatured at 95°C for 15 s, the primers were annealed at 60°C for 30 s, and the DNA was extended at 72°C for 30 s. The denaturation to extension cycle was repeated 40 times followed by a standard melt curve program. 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 iCycler iQ system (Bio-Rad, Hercules, CA). Forward and reverse primers were designed using the PRIMER EXPRESS 1.5 software (PE Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an endogenous control. Normalized results are expressed as %GAPDH. The PCR primer sequences used in this study are as follows: TLR2: forward: TTGCTGGGCTGACTTCTCTCA, reverse: GAAGAGTCAGGTGATGGATGTCG; tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ): forward: TTCTGTCTACTGAACTTCG-GGGTGATCGGTC, reverse: GTATGAGATAGCAAATCGGCTGACGGTGTGG-G; GAPDH: forward: CCAGTTGTCTCCTGCGACT; reverse: ATACCAGG-AAATGAGCTTGACAAAGT.

#### Flow cytometry

BM-M $\phi$  from diabetic NOD and age-matched NOR mice were differentiated in both low- and high-glucose complete media (described above). Cells were counted and plated to 10<sup>6</sup> cells per well in six-well tissue culture plates. Plated cells were stimulated with *P. gingivalis* LPS at 500 ng/ml for 24 h. Cells were then harvested, blocked with a 1 : 60 dilution of Fc Block (eBioscience, San Diego, CA), and stained with a 1 : 60 dilution of biotin-conjugated anti-mouse TLR2 antibody (eBioscience) on ice for 1 h. Cells were then washed three times with cold phosphate-buffered saline (PBS) and stained with a 1 : 400 dilution of streptavidin-phycoerythrin (eBioscience) on ice for 1 h. Cells were again washed three times with cold PBS and analysed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Geometric means of data histograms as calculated by BD CellQuest software (San Jose, CA) were used for comparison between conditions.

#### Enzyme-linked immunosorbent assay (ELISA)

Pools of BM-M $\phi$  were cultured and differentiated in both high- and low-glucose media from diabetic NOD and age-matched NOR mice. Supernatants from 10<sup>6</sup> unstimulated or stimulated (*P. gingivalis* LPS at 500 ng/ml for 24 h) cells were collected and analysed for protein levels of TNF- $\alpha$  using the Mouse TNF- $\alpha$  ELISA Ready-SET-Go! kit (eBioscience) following the manufacturer's protocol. Supernatants were tested in triplicate. Absorbance was read with a Molecular Devices SpectraMax Plus microplate spectrophotometer (MDS Analytical Technologies, Sunnyvale, CA) at the values of 550 nm subtracted from the read values of 450 nm following the manufacturer instructions. A standard curve was generated using included standards to convert absorbance values to a corresponding TNF- $\alpha$  concentration.

#### Preparation of cytoplasmic and nuclear extracts and Western blot analysis

Pools of BM-M $\phi$  were differentiated and matured in both high- and low-glucose media from diabetic NOD and age-matched NOR mice. BM-M $\phi$  were seeded in six-well tissue culture plates and stimulated with *P. gingivalis* LPS at 500 ng/ml for 30 min. After stimulation, cells were

washed and lysed to harvest protein as described previously (68). Total protein concentrations were measured using the Bradford assay and 40  $\mu$ g of extracts were analysed in a 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Protein bands were semi-dry electro-transferred to a poly-vinylidene difluoride (PVDF) 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 anti-phosphoI $\kappa$ B- $\alpha$  (Ser32; Cell Signaling Technology, Beverly, MA), at a dilution of 1 : 1000 overnight at 4°C. After washing the membrane with TBST, the secondary antibody horseradish peroxidase-conjugated ImmunoPure goat anti-rabbit immunoglobulin G (H + L) (Pierce Biotechnology, Rockford, IL) was added at a dilution of 1 : 10,000 and incubated for 1 h at room temperature. The membrane was again washed with TBST and incubated with SuperSignal West Pico Substrate (Pierce Biotechnology) for 5 min while protected from light. The membrane was stripped with stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris–HCl pH 6.7) and reprobed with anti- $\beta$ -actin antibody (Sigma-Aldrich, St Louis, MO) at a dilution of 1 : 10,000 as described above. For detection, CL-X Film (Pierce Biotechnology) was exposed to the membrane and developed using a Konica SRX-101 processor.

In a separate experiment, nuclear extracts were obtained from pools of BM-M $\phi$  cultured and differentiated in both high- and low-glucose media from diabetic NOD mice. BM-M $\phi$  were seeded at 10<sup>6</sup> cells per well in six-well plates and stimulated with *P. gingivalis* LPS at 500 ng/ml for 30 min. Nuclear extracts were recovered using an Active Motif Nuclear Extraction Kit according to the manufacturer's protocol (Active Motif, Carlsbad, CA). Total protein concentrations were measured using the Bradford assay and 5  $\mu$ g of nuclear extract proteins was analysed by 13% SDS–PAGE. Protein bands were semi-dry electro-transferred to a PVDF membrane. The same procedure for Western blotting was used as described above. The primary antibody used was anti-p65 antibody (a gift of Dr Brian Ashburner, University of Toledo, Toledo, OH) at a dilution of 1 : 5000. The membrane was directly visualized using a Kodak Image Station 4000R Pro. The membrane was stripped and reprobed with anti-histone H1 (Santa Cruz Biotechnologies, Santa Cruz, CA) at a dilution of 1 : 1000 as described above.

### Phagocytosis assay

Macrophage phagocytic activity was assayed using 2  $\mu\text{m}$  polystyrene fluorescent carboxylate-modified microspheres (Invitrogen, Carlsbad, CA). Pools of BM-M $\phi$  were differentiated and matured in both high- and low-glucose media. Macrophages were derived from diabetic NOD and age-matched NOR mice and were seeded at  $1 \times 10^5$  cells per well in 96-well plates. BM-M $\phi$  were either left unstimulated or stimulated for 4 h with either *P. gingivalis* LPS at 500 ng/ml or *Escherichia coli* LPS at 100 ng/ml (*E. coli* LPS 0111:B4; Sigma, St. Louis, MO). Following stimulation, BM-M $\phi$  were gently washed with PBS and incubated in the appropriate glucose media containing a concentration of 50 : 1 (fluorescent microspheres to BM-M $\phi$ ). After 4 h macrophages were gently washed twice and samples were analysed using a Packard Fusion Alpha microplate reader (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to determine the relative fluorescent units present within each given sample well. All samplings were carried out in triplicate.

Serial dilutions were generated from a fluorescent particle stock with a known concentration of microspheres. These dilutions allowed for the generation of a standard curve of particles ingested per relative fluorescent unit. Initial optimization experiments were carried out to determine proper incubation time with beads and also included incubations at 4°C to ensure that non-specific binding of beads did not occur, data not shown. All optimization experimental samples were carried out in triplicate. A Bradford assay was carried out to ensure that total protein present in each sample well was consistent (data not shown).

### Statistical analysis

Comparisons between means were performed using a two-tailed Student's *t*-test. A *P*-value <0.05 was considered significant. An analysis of variance (ANOVA) test was performed whenever more than one comparison was made in a single experiment.

## Results

### High glucose effects on BM-M $\phi$ in response to *P. gingivalis* LPS stimulation

Previous research revealed that basal level gene expression profiles of diabetic NOD BM-M $\phi$  differ in response to their glucose environment (40). To further investigate this phenomenon and the effect of hyper-

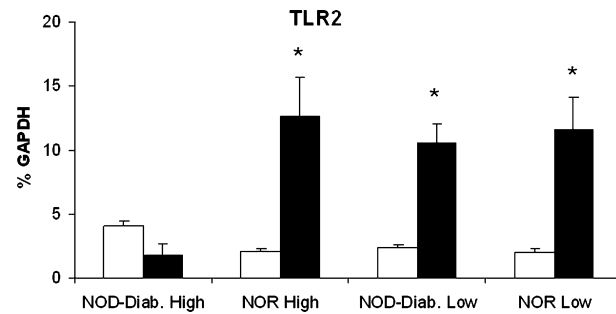


Fig. 1. High glucose effects on bone-marrow-derived macrophages (BM-M $\phi$ ) in response to *Porphyromonas gingivalis* lipopolysaccharide (LPS) stimulation. BM-M $\phi$  from pools of four diabetic female NOD mice and four age-matched female NOR mice were differentiated in either low-glucose (100 mg/dl) or high-glucose (450 mg/dl) Dulbecco's modified Eagle's medium to mimic a hyperglycemic environment. Both differentiation and stimulation occurred in low- or high-glucose media. BM-M $\phi$  were either left unstimulated (□) or were stimulated with *P. gingivalis* LPS at 500 ng/ml for 4 h (■). RNA was isolated, converted to complementary DNA, and quantified by real-time polymerase chain reaction analysis using Toll-like receptor 2 (TLR2)-specific primers. Results expressed as % glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are the mean and SD of triplicate samples for each glucose condition. These results are representative of three separate experiments. The asterisk (\*) indicates significant differences (*P* < 0.05) between high-glucose stimulated NOD diabetic BM-M $\phi$  and the three remaining conditions when stimulated.

glycemia on the ability of BM-M $\phi$  to respond to a periodontal bacterial challenge, bone marrow stem cells harvested from diabetic NOD and NOR mice were differentiated into BM-M $\phi$  using M-CSF in high-glucose or low-glucose conditions and stimulated with the TLR2 ligand *P. gingivalis* LPS. TLR2 mRNA expression detected by quantitative RT-PCR was significantly upregulated in NOR BM-M $\phi$  differentiated and stimulated in both the high- and low-glucose conditions, while only the low-glucose condition BM-M $\phi$  from diabetic NOD mice showed upregulated TLR2 expression upon stimulation (Fig. 1). BM-M $\phi$  generated from diabetic NOD mice in the high-glucose conditions showed no significant change in TLR2 expression upon stimulation with *P. gingivalis* LPS (Fig. 1, NOD-diab. High). These hyperglycemic BM-M $\phi$  (differentiated and stimulated) derived from diabetic NOD mice appear to be unable to respond via upregulation of the TLR2 receptor, as is seen in the low-glucose condition.

An examination of cell surface expression of TLR2 parallels the observation made for TLR2 mRNA expression. Hyperglycemic diabetic NOD BM-M $\phi$  stimulated with *P. gingivalis* LPS had over 20 percent less fluorescence representing cell surface TLR2 than normoglycemic NOD BM-Macrophages (Fig. 2A) compared with normoglycemic NOD BM-M $\phi$ . Control NOR mice exhibited the same level of cell surface expression of TLR2 following stimulation with *P. gingivalis* LPS in both the high- and low-glucose conditions (Fig. 2B). TLR2 cell surface expression

was equivalent for all treatment conditions except for hyperglycemic diabetic NOD BM-M $\phi$ , which expressed distinctly less TLR2 (>20% less than the average geometric means of the other conditions) on the cell surface (Fig. 2C, shown as histogram overlay of all four conditions).

TLR2 signals through the MyD88-dependent pathway, eventually activating the inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (IKK) complex to phosphorylate the I $\kappa$ B, releasing NF- $\kappa$ B dimers to translocate into the nucleus where they activate gene transcription. The low level of TLR2 expression in diabetic NOD BM-M $\phi$  generated in high-glucose conditions should correspond with a lack of TLR2 signal transduction. Therefore, BM-M $\phi$  were differentiated in both glucose conditions and cell lysates were analysed via Western blot to determine their ability to phosphorylate I $\kappa$ B- $\alpha$  in response to *P. gingivalis* LPS stimulation. Normoglycemic diabetic NOD BM-M $\phi$  and both normoglycemic and hyperglycemic NOR BM-M $\phi$  can effectively phosphorylate I $\kappa$ B (Fig. 3A). However, hyperglycemic diabetic NOD BM-M $\phi$  display diminished phosphorylated I $\kappa$ B- $\alpha$  at 30 min post-stimulation with *P. gingivalis* LPS [Fig. 3A, High glucose NOD-Diab (+) compared to NOD-Diab Low glucose (+), NOR High glucose (+), or NOR Low glucose (+) conditions]. These results suggest that diabetic NOD BM-M $\phi$  effectively phosphorylate I $\kappa$ B- $\alpha$  when differentiated and stimulated under normoglycemic conditions, but are defective when differentiated and stimulated under hyperglycemic conditions.

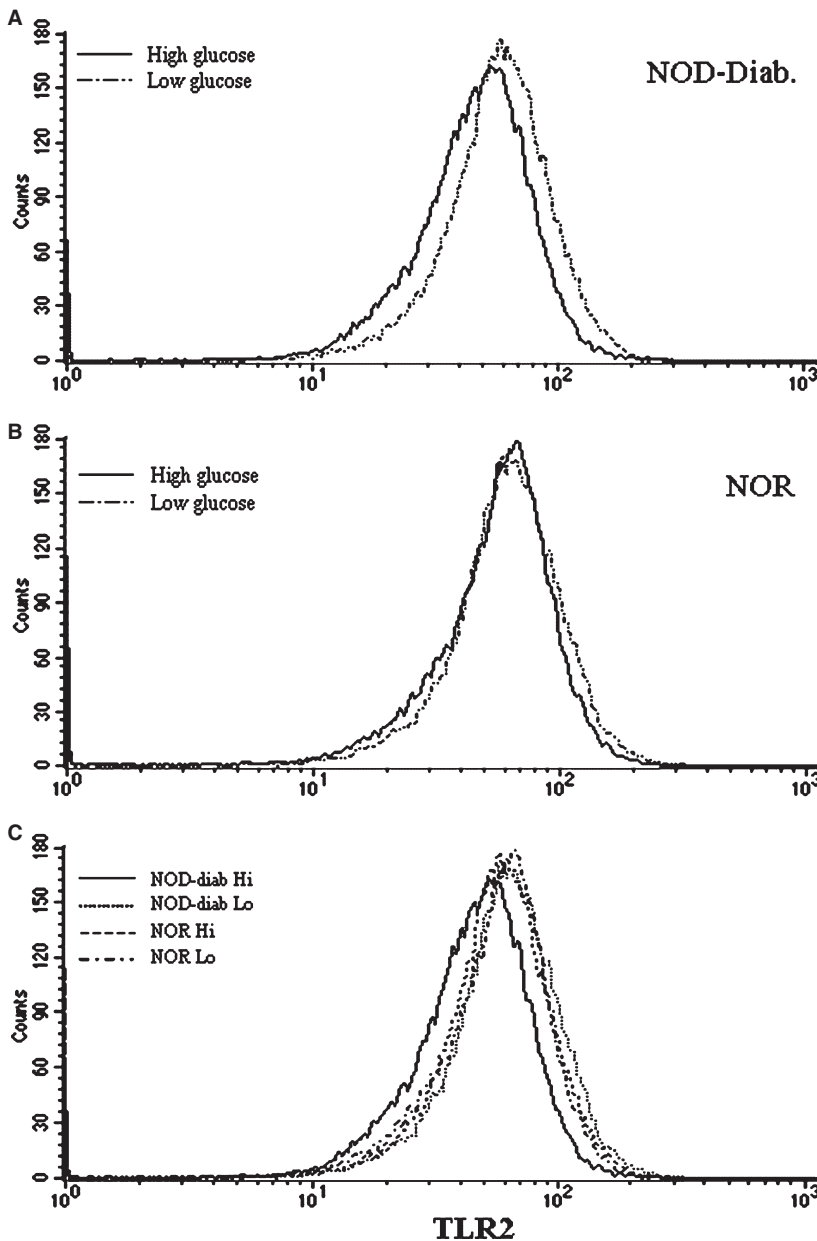


Fig. 2. Flow cytometry analysis of cell surface Toll-like receptor 2 (TLR2). Bone-marrow-derived macrophages (BM-M $\phi$ ) from pools of four diabetic female NOD mice and five age-matched female NOR mice were cultured and differentiated in either low-glucose or high-glucose media and stimulated with *Porphyromonas gingivalis* LPS at 500 ng/ml for 24 hours. Cells were analysed for surface TLR2 by flow cytometry using a biotinylated TLR2 antibody followed by secondary staining with streptavidin-phycoerythrin. (A) diabetic NOD mice and (B) NOR mice, (C) a combined overlay of all four conditions. The high-glucose NOD diabetic BM-M $\phi$  TLR2 geometric mean is >20% less than that of the average of the three other conditions.

Translocation and binding to DNA of certain NF- $\kappa$ B dimers to the nucleus induces gene transcription and expression of proinflammatory molecules such as TNF- $\alpha$ . Isolated nuclear extracts of BM-M $\phi$  derived in low- and high-glucose conditions both show the presence of the p65 (RelA) subunit of NF- $\kappa$ B in the nucleus following stimulation (Fig. 3B). However, the increase in p65 from unstimulated to post-stimulation is far more

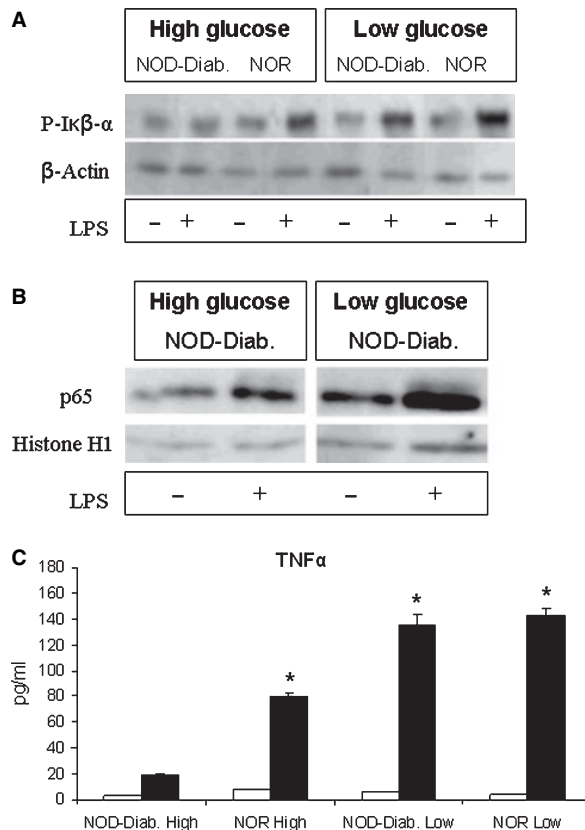
robust in the low-glucose condition. Histone H1 levels confirmed approximately equal amounts of protein in each lane. An ELISA was performed to determine the amount of TNF- $\alpha$  protein produced in response to *P. gingivalis* LPS stimulation. BM-M $\phi$  generated from both NOR and diabetic NOD mice in both high- and low-glucose conditions produce significant amounts of TNF- $\alpha$  in response to *P. gingivalis* LPS compared with untreated con-

trol cells (Fig. 3C). However, it is evident that the smallest increase in TNF- $\alpha$  production following stimulation is seen in the hyperglycemic diabetic NOD supernatants. In the hyperglycemic condition, BM-M $\phi$  generated from diabetic NOD experience a significant seven-fold reduction in the production of TNF- $\alpha$  compared with the same cells when generated in a normoglycemic condition. BM-M $\phi$  from both strains of mice in the low-glucose condition produce elevated amounts of TNF- $\alpha$  compared with those BM-M $\phi$  stimulated in the high glucose environment (Fig. 3C, intrastain low glucose compared with high glucose, closed boxes). TNF- $\alpha$  production from NOR BM-M $\phi$  was less affected by the high-glucose condition. The results indicate that BM-M $\phi$  generated from diabetic NOD mice are significantly affected by the hyperglycemic condition as evidenced by a reduction in response to *P. gingivalis* LPS-stimulated TLR2 signal transduction events and TNF- $\alpha$  protein production.

#### High-glucose effect on BM-M $\phi$ function in response to bacterial LPS stimulation

One of the most important functions of macrophages is to phagocytose the cellular debris of dead and dying cells and pathogenic material. Phagocytosis of fluorescent microspheres by BM-M $\phi$  cultured, differentiated, and stimulated in low- and high-glucose conditions was examined to test the effect of hyperglycemia on macrophage function. As expected, phagocytosis of microspheres was significantly increased following stimulation with *P. gingivalis* LPS in normoglycemic diabetic NOD BM-M $\phi$ . However, the BM-M $\phi$  generated from hyperglycemic diabetic NOD BM-M $\phi$  showed no significant increase in phagocytosis of microspheres (Fig. 4A). To confirm this effect of hyperglycemia on phagocytosis, the classical endotoxin *E. coli* LPS was used for stimulation of BM-M $\phi$ . Even upon treatment with 100 ng/ml of *E. coli* LPS, BM-M $\phi$  generated from diabetic NOD mice displayed reduced phagocytic function in the high-glucose condition compared with the low-glucose condition (Fig. 4B). In contrast, the phagocytic function in BM-M $\phi$  derived from NOR mice was unaffected by glucose state (Fig. 4B). The results suggest that the hyperglycemic effect on diabetic NOD BM-M $\phi$  is not specific to the species of the bacterial stimulus.

Further experiments using both *P. gingivalis* LPS and *E. coli* LPS as stimuli provide evidence at the mRNA level, for



**Fig. 3.** High glucose effect on phosphorylation of inhibitor of nuclear factor- $\kappa$ B- $\alpha$  (I $\kappa$ B- $\alpha$ ), nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) protein production. (A) Bone-marrow-derived macrophages (BM-M $\phi$ ) from pools of four diabetic female NOD mice and five age-matched female NOR mice were cultured and differentiated in either low-glucose or high-glucose media. (A) Cells were left unstimulated (-) or were stimulated with *Porphyromonas gingivalis* lipopolysaccharide (LPS) at 500 ng/ml for 30 min (+). Phosphorylated I $\kappa$ B- $\alpha$  (P-I $\kappa$ B- $\alpha$ ), and  $\beta$ -actin were detected by standard Western blot procedure using specific antibodies. Results are representative of two separate experiments. (B) BM-M $\phi$  either left unstimulated (-) or were stimulated with *P. gingivalis* LPS at 500 ng/ml for 30 min (+). Nuclear extract p65 and histone H1 were detected by Western blot using specific antibodies. (C) BM-M $\phi$  supernatants were analysed for TNF- $\alpha$  protein by enzyme-linked immunosorbent assay. BM-M $\phi$  were left either unstimulated (□) or were stimulated with *P. gingivalis* LPS at 500 ng/ml for 24 h (■). TNF- $\alpha$  standards were used to convert absorbance to protein concentration [pg/ml]. Results are the mean and SD of triplicate samples. The asterisk (\*) indicates significant differences ( $P < 0.05$ ) between high-glucose-stimulated NOD diabetic BM-M $\phi$  and the three remaining conditions when stimulated.

the dysfunctionality of the hyperglycemic diabetic NOD BM-M $\phi$ . As analysed by quantitative RT-PCR, significantly depressed levels of TNF- $\alpha$  mRNA were generated by hyperglycemic diabetic NOD BM-M $\phi$  in response to both LPS stimulations (Fig. 5). These results further indicate a stunting of the ability of hyperglycemic diabetic NOD BM-M $\phi$  to respond to a bacterial stimulus.

## Discussion

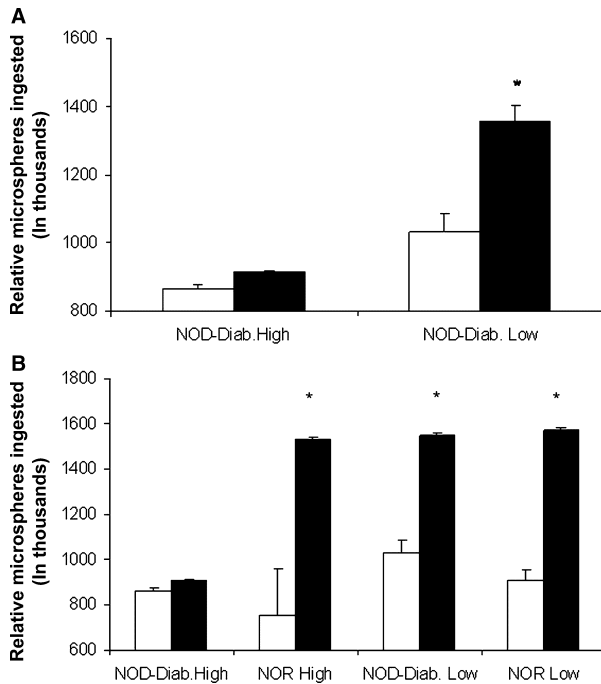
Recent insights into the progression of periodontal disease suggest that insufficient glycemic control is the most consistent risk factor associated with the severity and the extent of a periodontal infection (28). The current study examines the

effects of chronic hyperglycemia on BM-M $\phi$  derived from diabetic mice when challenged with *P. gingivalis* LPS, a common pathogenic by-product implicated in the pathogenesis of periodontal disease. It is known that hyperglycemia can alter the homeostasis of multiple body systems, including gene expression (15, 36, 44–46, 52), but it is not yet fully understood how diabetic hyperglycemia contributes to dysregulated innate immune responses that contribute to the development of a periodontal infection. Macrophages have been implicated in the autodegradation of both hard and soft connective tissue in the mouth during periodontitis (36). To investigate the effects of chronic hyperglycemia on macrophages, BM-M $\phi$  were derived from diabetic NOD mice and the NOR

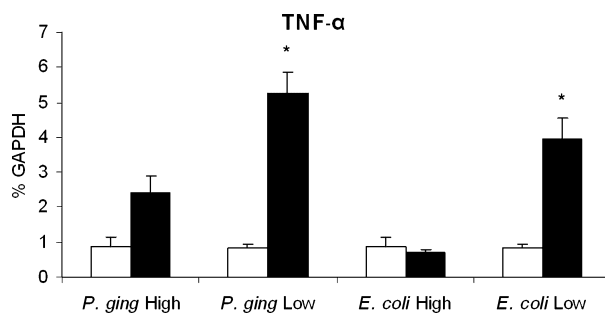
genetic control strain under normoglycemic and hyperglycemic conditions and stimulated with *P. gingivalis* LPS.

We have found that hyperglycemic conditions, comparable to those found systemically in uncontrolled diabetes, are able to stunt BM-M $\phi$  TLR2 receptor expression, NF- $\kappa$ B signal transduction, TNF- $\alpha$  cytokine production, and phagocytosis in response to *P. gingivalis* LPS. The presence of hyperglycemia does not interfere with the ability of diabetes-resistant NOR-derived BM-M $\phi$  to effectively respond to stimulation. This suggests that the diabetic NOD mouse has an inherent defect that does not allow BM-M $\phi$  compensation to hyperglycemia.

Use of primary bone marrow stem cells, differentiated into macrophages in a hyperglycemic environment, may provide insight into the *in vivo* characteristics of macrophages in individuals with long-term diabetes, as macrophages are replenished from common myeloid progenitor cells which are constantly exposed to a hyperglycemic bloodstream. Furthermore, differentiated macrophages retain the plasticity to respond to stimuli encountered in different microenvironments (reviewed in refs 35 and 42). For example, macrophages become classically activated and are capable of microbicidal activity in response to interferon- $\gamma$  and TNF- $\alpha$  whereas, the presence of interleukin-4 leads to wound-healing macrophages, and exposure to interleukin-10 leads to a regulatory macrophage phenotype (reviewed in ref. 42). Therefore, it is reasonable to assume that a hyperglycemic microenvironment could have an effect on macrophage differentiation and the ability to respond to infection. Our results on defective macrophage responsiveness in hyperglycemia are supported by a recent paper by Ma et al. (33) examining long-term (16 weeks) effects of streptozotocin-induced diabetes in C57BL/6 mice. This study showed that total F4/80<sup>+</sup> macrophage numbers were decreased and revealed immune function defects in phagocytic capability. Therefore, prolonged exposure to a diabetic milieu leading to immune defectiveness could be associated with a risk of infection. Similarly, chronic hyperglycemia (6 months) in streptozotocin-induced diabetes in BALB/c mice led to decreased B- and T-lymphocyte proliferation in primary immune responses to both T-cell-dependent and T-cell-independent antigens indicating that pathological levels of high glucose could result in the immunosuppressive state observed in diabetic mice (51).



**Fig. 4.** Effect of high glucose on macrophage phagocytosis functionality in response to bacterial lipopolysaccharide (LPS) stimulation. (A) Bone-marrow-derived macrophages (BM-M $\phi$ ) from pools of four diabetic NOD mice were differentiated in low-glucose or high-glucose media. Macrophages were either left unstimulated (□) or were stimulated with *Porphyromonas gingivalis* LPS at 500 ng/ml (■) for 4 h. (B) BM-M $\phi$  from pools of four diabetic NOD mice and two NOR mice (aged 8–10 weeks) were differentiated in low- or high-glucose media. Macrophages were either left unstimulated (□) or were stimulated with *Escherichia coli* LPS at 100 ng/ml (■) for 4 h. Following the bacterial stimulations macrophages were co-incubated in the presence of 2  $\mu$ m polystyrene fluorescent carboxylate-modified microspheres, at a ratio of 50 particles per macrophage for an additional 4 h. Plates were analysed on a fluorescent plate reader. Serial dilutions of fluorescent microspheres were utilized to generate the relative number of microspheres ingested by the BM-M $\phi$ . Results are the mean and SD of triplicates. The asterisk (\*) indicates significant differences ( $P < 0.05$ ) between high-glucose-stimulated NOD diabetic BM-M $\phi$  and the other condition(s) when stimulated. Wells were plated and analysed in triplicate and results are representative of two separate experiments.



**Fig. 5.** Effect of high glucose on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) messenger RNA in diabetic NOD bone-marrow-derived macrophages (BM-M $\phi$ ) in response to bacterial lipopolysaccharide (LPS) stimulation. BM-M $\phi$  from pools of four diabetic NOD mice were differentiated in low-glucose or high-glucose media. BM-M $\phi$  were either left unstimulated (□) or were stimulated with *Porphyromonas gingivalis* LPS at 500 ng/ml or *Escherichia coli* LPS at 100 ng/ml as indicated on the graph for 4 h (■). RNA was isolated, converted to complementary DNA, and quantified by real-time polymerase chain reaction analysis using TNF- $\alpha$ -specific primers. Results expressed as % glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are the mean and SD of triplicate samples for each glucose condition. The asterisk (\*) indicates significant differences ( $P < 0.05$ ) between low-glucose and high-glucose conditions within the same bacterial stimulation.

We have seen previously (40) that bone marrow stem cells obtained from diabetic NOD mice and differentiated in high-

glucose media had increased basal levels of TLR2, TLR4, and TNF- $\alpha$ . Similarly, Dasu et al. (5) incubated THP-1 mono-

cytic cells in high glucose and found induced expression of TLR2, TLR4, and activation of NF- $\kappa$ B with involvement of protein kinase C. Furthermore, diabetic patients in a hyperglycemic crisis have elevated plasma levels of proinflammatory cytokines in the absence of an infection (59). Even studies investigating the effects of acute hyperglycemia have found increased plasma levels of multiple proinflammatory cytokines which have metabolic effects contributing to maintaining elevated glucose levels (reviewed in ref. 64). These results suggest that a hyperglycemic environment has a stimulatory effect on the innate immune system. An important question is how these hyperglycemia-stimulated immune cells respond to further bacterial stimulation. In a study by Wasmuth et al. (66) hyperglycemia in patients admitted to the intensive care unit was associated with increased serum levels of inflammatory cytokines including TNF- $\alpha$ . However, *ex vivo* stimulation of whole blood from the same hyperglycemic patients with LPS led to significantly decreased levels of TNF- $\alpha$  compared with normoglycemic patients. These results appear to be paradoxical; the innate immune system is activated by high levels of glucose, however, secondary stimulation with a bacterial ligand leads to hyporesponsiveness. Medvedev et al. (37) have shown that pretreatment of cells with LPS causes tolerance to endotoxin (loss of responsiveness) upon restimulation with LPS. A study of patients with chronic periodontitis showed that although numbers of TLR2-positive cells increase during chronic periodontitis, there is a significant 30-fold downregulation of TLR2 mRNA in the gingival tissue compared with healthy controls (43). The same study confirmed that monocytes from healthy donors are subject to tolerance induction. Lastly, clinical studies of glycemic control in diabetic patients provide evidence that patients with adequate metabolic control, determined by fasting glucose and glycated hemoglobin, produce more TNF- $\alpha$  from stimulated mononuclear cells compared with patients with inadequate metabolic control (10). The results reported in the current study indicate that culturing BM-M $\phi$  in high-glucose media may function as a primary TLR stimulus and that subsequent restimulation with a bacterial ligand can induce tolerance and loss of responsiveness.

Previous research demonstrates that pathogen clearance by phagocytes is defective and reduced in diabetic patients (9, 12, 15, 30). Furthermore, research

utilizing polymorphonuclear cells isolated from diabetic patients with poor metabolic control demonstrated decreased phagocytic capacity compared with healthy non-diabetic patients (34). Importantly, phagocytosis improved after 36 h culture in normoglycemia, but did not return to normal levels (34). Clinically, the cause of diabetes may be less important than the detrimental disease complications of chronic exposure to hyperglycemia (25). The well-described adverse effects of hyperglycemia on insulin target tissues, and particularly pancreatic islet beta cells, have been termed 'glucotoxicity'. Chronic hyperglycemia induces multiple shortcomings in beta cells including the loss of glucose-stimulated insulin gene transcription and secretion, and is linked to beta cell apoptosis and necrosis (4, and reviewed in ref. 60). The observations in this paper may be a type of glucotoxicity of the macrophage resulting in impaired immune and phagocytic function.

Diabetes and periodontal disease is a degenerative cycle; a decline in gingival health adversely affects metabolic glucose control, which is of extreme importance for maintaining good health in a diabetic patient (48). The possibility of improving macrophage and cellular responsiveness to bacterial infections based on metabolic maintenance argues strongly for tight control of glucose levels. The current findings indicate that hyperglycemic patients with diabetes run the risk of generating macrophages that not only have compromised phagocytic function, but also possess an inability to elicit proper inflammatory mediators and cellular activation when faced with a periodontal infection.

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### References

- Baehni PC, Tsai CC, McArthur WP, Hammond BF, Shenker BJ, Taichman NS. Leukotoxic activity in different of the bacterium *Actinobacillus actinomycetem-comitans* isolated from juvenile periodontitis in man. *Arch Oral Biol* 1991; **26**: 671–676.
- Bedi GS, Williams T. Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. *J Biol Chem* 1994; **269**: 599–606.
- Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. *J Periodontal Res* 1993; **28**: 500–510.
- Brownlee M. A radical explanation for glucose-induced beta cell dysfunction. *J Clin Invest* 2003; **112**: 1788–1790.
- Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I. High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. *Diabetes* 2008; **57**: 3090–3098.
- Dennison DK, Van Dyke TE. The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol* 2000 1997; **14**: 54–78.
- The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977–986.
- The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. *N Engl J Med* 2000; **342**: 381–389.
- Djaldetti M, Salman H, Bergman M, Djaldetti R, Bessler H. Phagocytosis – the mighty weapon of the silent warriors. *Microsc Res Tech* 2002; **57**: 421–431.
- Foss-Freitas MC, Foss NT, Donadi EA, Foss MC. *In vitro* TNF-alpha and IL-6 production by adherent peripheral blood mononuclear cells obtained from type 1 and type 2 diabetic patients evaluated according to the metabolic control. *Ann N Y Acad Sci* 2006; **1079**: 177–180.
- Garcia RI, Henshaw MM, Krall EA. Relationship between periodontal disease and systemic health. *Periodontol* 2000 2001; **25**: 21–36.
- Geerlings SE, Hoepelman AI. Immune dysfunction in patients with diabetes mellitus. *FEMS Immunol Med Microbiol* 1999; **26**: 259–265.
- Gibson FC, Ukai T, Genco CA. Engagement of specific innate immune signaling pathways during *Porphyromonas gingivalis* induced chronic inflammation and atherosclerosis. *Front Biosci* 2008; **13**: 2041–2059.
- Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003; **3**: 23–35.
- Graves DT, Kayal RA. Diabetic complications and dysregulated innate immunity. *Front Biosci* 2008; **13**: 1227–1239.
- Hajishengallis G, Tapping RI, Harokopakis E et al. Differential interactions of fimbriae and lipopolysaccharide from *Porphyromonas gingivalis* with the Toll-like receptor 2 centered pattern recognition apparatus. *Cell Microbiol* 2006; **8**: 1557–1570.
- Hertz CJ, Kiertcher SM, Godowski PJ et al. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J Immunol* 2001; **166**: 2444–2450.
- Holt S, Ebersole J, Felton J, Brunsvold M, Kornman KS. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. *Science* 1988; **239**: 55–57.
- Inoguchi T, Li P, Umeda F et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 2000; **49**: 1939–1945.
- Janeway CA, Travis P, Walport M, Shlomchik M. Innate immunity. In: Janeway CA, Travis P, Walport M, Shlomchik M ed. *Immunobiology*, 6th edn. New York, NY: Garland Science Publishing, 2005: 37–102.
- Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulinitis and beta-cell destruction in NOD mice. *Diabetes* 1994; **43**: 667–675.
- Jun HS, Yoon CS, Zbytniuk L, van Rooijen N, Yoon JW. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 1999; **189**: 347–358.
- Katz J, Bhattacharyya I, Farkhondeh-Kish F, Perez FM, Caudle RM, Heft MW. Expression of the receptor of advanced glycation end products in gingival tissues of type 2 diabetes patients with chronic periodontal disease: a study utilizing immunohistochemistry and RT-PCR. *J Clin Periodontol* 2005; **32**: 40–44.
- Kitada M, Koya D, Sugimoto T et al. Translocation of glomerular p47phox and p67phox by protein kinase C-beta activation is required for oxidative stress in diabetic nephropathy. *Diabetes* 2003; **52**: 2603–2614.
- Lalla E. Periodontal infections and diabetes mellitus: when will the puzzle be complete? *J Clin Periodontol* 2007; **34**: 913–916.
- Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J Biol Chem* 1997; **272**: 17810–17814.
- Li YM, Mitsuhashi T, Wojciechowicz D et al. Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc Natl Acad Sci USA* 1996; **93**: 11047–11052.
- Lim LP, Tay FB, Sum CF, Thai AC. Relationship between markers of metabolic control and inflammation on severity of periodontal disease in patients with diabetes mellitus. *J Clin Periodontol* 2007; **34**: 118–123.
- Lin L. RAGE on the toll road? *Cell Mol Immunol* 2006; **3**: 351–358.
- Liu BF, Miyata S, Kojima H et al. Low phagocytic activity of resident peritoneal macrophages in diabetic mice: relevance to



- the formation of advanced glycation end products. *Diabetes* 1999; **48**: 2074–2082.
31. Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol* 1998; **111**: 850–857.
  32. Lusis AJ. Atherosclerosis. *Nature* 2000; **407**: 233–241.
  33. Ma H, Liu G, Ding W, Wu Y, Cai L, Zhao Y. Diabetes-induced alteration of F4/80<sup>+</sup> macrophages: a study in mice with streptozotocin-induced diabetes for a long term. *J Mol Med* 2008; **86**: 391–400.
  34. Marthofer W, Stein M, Maeser E, Federlin K. Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. *Diabetes Care* 1992; **15**: 256–260.
  35. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci* 2008; **13**: 453–461.
  36. Mealey BL. Periodontal disease and diabetes: a two-way street. *J Am Dent Assoc* 2006; **137**: 26S–31S.
  37. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J Immunol* 2000; **164**: 5564–5574.
  38. Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001; **1**: 135–145.
  39. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 1997; **91**: 295–298.
  40. Mohammad MK, Morran M, Slotterbeck B et al. Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse. *Int Immunol* 2006; **18**: 1101–1113.
  41. Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y. Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. *Oral Microbiol Immunol* 2003; **18**: 54–58.
  42. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008; **8**: 958–969.
  43. Muthukuru M, Jotwani R, Cutler CW. Oral mucosal endotoxin tolerance induction in chronic periodontitis. *Infect Immun* 2005; **73**: 687–694.
  44. Naito M. Macrophage differentiation and function in health and disease. *Pathol Int* 2008; **58**: 143–155.
  45. Nassar H, Kantarci A, van Dyke TE. Diabetic periodontitis: a model for activated innate immunity and impaired resolution of inflammation. *Periodontol 2000* 2007; **43**: 233–244.
  46. Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB. Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. *Toxicol Appl Pharmacol* 2007; **25**: 214–220.
  47. Papapanou PN. Periodontal diseases: epidemiology. *Ann Periodontol* 1996; **1**: 1–36.
  48. Perrino MA. Diabetes and periodontal disease: an example of an oral/systemic relationship. *N Y State Dent J* 2007; **73**: 38–41.
  49. Prochazka M, Serreze DV, Frankel WN, Leiter EH. NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice. *Diabetes* 1992; **41**: 98–106.
  50. Ren L, Leung WK, Darveau RP, Jin L. The expression profile of lipopolysaccharide-binding protein, membrane-bound CD14, and toll-like receptors 2 and 4 in chronic periodontitis. *J Periodontol* 2005; **76**: 1950–1959.
  51. Rubinstein R, Genaro AM, Motta A, Cremaschi G, Wald MR. Impaired immune responses in streptozotocin-induced type I diabetes in mice. Involvement of high glucose. *Clin Exp Immunol* 2008; **154**: 235–246.
  52. Ryan ME, Carnu O, Kamer A. The influence of diabetes on the periodontal tissues. *J Am Dent Assoc* 2003; **134**: 34S–40S.
  53. Salvi GE, Collins JG, Yalda B, Arnold RR, Lang NP, Offenbacher S. Monocytic TNF alpha secretion patterns in IDDM patients with periodontal diseases. *J Clin Periodontol* 1997; **24**: 8–16.
  54. Salvi GE, Yalda B, Collins JG et al. Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. *J Periodontol* 1997; **68**: 127–135.
  55. Schmidt AM, Weidman E, Lalla E et al. Advanced glycation endproducts (AGEs) induce oxidant stress in the gingiva: a potential mechanism underlying accelerated periodontal disease associated with diabetes. *J Periodontol Res* 1996; **31**: 508–515.
  56. Shlossman M, Knowler WC, Pettitt DJ, Genco RJ. Type 2 diabetes mellitus and periodontal disease. *J Am Dent Assoc* 1990; **121**: 532–536.
  57. Socransky SS, Haffajee AD. The bacterial etiology of destructive periodontal disease: current concepts. *J Periodontol* 1992; **63**: 322–331.
  58. Southerland JH, Taylor GW, Moss K, Beck JD, Offenbacher S. Commonality in chronic inflammatory diseases: periodontitis, diabetes, and coronary artery disease. *Periodontol 2000* 2006; **40**: 130–143.
  59. Stentz FB, Umpierrez GE, Cuervo R, Kitabchi AE. Proinflammatory cytokines, markers of cardiovascular risks, oxidative stress, and lipid peroxidation in patients with hyperglycemic crises. *Diabetes* 2004; **53**: 2079–2086.
  60. Stumvoll M, Goldstein BJ, van Haften TW. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 2005; **365**: 1333–1346.
  61. Sundqvist G. Pathogenicity and virulence of black-pigmented Gram-negative anaerobes. *FEMS Immunol Med Microbiol* 1993; **6**: 125–138.
  62. Tobias PS, Gegner J, Tapping R et al. Lipopolysaccharide-dependent cellular activity. *J Periodontol Res* 1997; **32**: 99–103.
  63. Travis J, Pike R, Imamura T, Potempa J. *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. *J Periodontol Res* 1997; **32**: 120–125.
  64. Turina M, Fry DE, Polk HC Jr. Acute hyperglycemia and the innate immune system: clinical, cellular, and molecular aspects. *Crit Care Med* 2005; **33**: 1624–1633.
  65. Warren MK, Vogel SN. Bone marrow-derived macrophages: development and regulation of differentiation markers by colony-stimulating factor and interferons. *J Immunol* 1985; **134**: 982–989.
  66. Wasmuth HE, Kunz D, Graf J et al. Hyperglycemia at admission to the intensive care unit is associated with elevated serum concentrations of interleukin-6 and reduced *ex vivo* secretion of tumor necrosis factor-alpha. *Crit Care Med* 2004; **32**: 1109–1114.
  67. Yan SD, Schmidt AM, Anderson GM et al. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J Biol Chem* 1994; **269**: 9889–9897.
  68. Yi AK, Krieg AM. Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J Immunol* 1998; **161**: 4493–4497.