Increased Toll-like Receptor activation and TLR ligands in Recently Diagnosed Type 2 diabetes Subjects

*Mohan R. Dasu, PhD¹, Sridevi Devaraj, PhD¹, Samuel Park, BS¹, Ishwarlal Jialal, MD PhD¹,²

¹Laboratory for Atherosclerosis and Metabolic Research
University of California Davis Medical Center, Sacramento, CA
²VA Medical Center of Northern California, Mather Field, CA

Running head: TLR2 and TLR4 expression and activation in T2D

Address for Correspondence:
Mohan R Dasu, PhD
Email: ravi.dasu@ucdmc.ucdavis.edu

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**Objective:** Individuals with Type 2 diabetes (T2DM) have a myriad of metabolic aberrations including increased inflammation that increase their cardiovascular risk. Toll-like receptors (TLRs) and their ligands play a key role in insulin resistance and atherosclerosis. However, there is a paucity of data examining the expression and activity of TLRs in T2DM. Thus, in the present study, we examined TLR2 and TLR4 mRNA and protein expression, their ligands, and signaling in monocytes of recently diagnosed T2DM patients.

**Research Design & Methods:** TLR mRNA, protein expression, TLR ligands and TLR signaling were measured in freshly isolated monocytes from healthy human controls (n=23) and T2DM subjects (n=23) using real time RT PCR, Western blot, and Flow cytometric assays.

**Results:** T2DM subjects had significantly increased TLR2, TLR4 mRNA and protein in monocytes compared to controls (P<0.05). Increased TLR2 and TLR4 expression correlated with BMI, HOMA-IR, glucose, HbA1c, CML, and FFA. Ligands of TLR2 and TLR4 namely, HSP60, HSP70, HMGB1, endotoxin, and hyaluronan levels were elevated in T2DM subjects and positively correlated with TLR2 and TLR4. T2DM subjects showed increased MyD88, phosphorylated IRAK-1, Trif, TICAM-1, IRF-3, and NF-κB p65 expression in monocytes compared to controls. Furthermore, TLR-MyD88-NF-κB signaling resulted in elevated levels of cytokines (P<0.05), but increased IL-1β, IFN-γ, and endotoxin were not significant when adjusted for BMI.

**Conclusions:** In this comprehensive study, we make the novel observation that TLR2 and TLR4 expression, their ligands, signaling, and functional activation are increased in recently diagnosed T2DM and contribute to the proinflammatory state.
Type 2 diabetes (T2DM) constitutes a group of metabolic aberrations including hyperglycemia, inflammation, and insulin resistance (IR) that increase the risk of cardiovascular disease (CVD) (1). The mechanisms by which these metabolic abnormalities cause CVD are not clear. Considerable evidence indicates that the harmful effects of elevated glucose are mediated by receptors leading to increased inflammation and oxidative stress (2,3). Recent studies demonstrating associations between elevated levels of circulating C-reactive protein (CRP), proinflammatory cytokines, and homeostasis model assessment (HOMA-IR) suggest that inflammation as an important etiological factor in the development of both IR and T2DM (4). Moreover, inflammatory markers link the pathology of IR and T2DM. Activation of innate immune system via toll-like receptors (TLRs) is implicated in the pathogenesis of IR, diabetes, and atherosclerosis (5-7). Complimentary genetic studies link TLR2 and TLR4 polymorphisms to T2DM, suggesting a causal relationship between TLR function and DM and its complications (8).

TLRs are evolutionarily preserved pattern recognition receptors (9), expressed on several cell types including monocytes, predominant cells of the innate immune system that are pivotal in diabetes and atherogenesis (10). TLRs play an important role in the activation and regulation of the innate immune system and inflammation (9). Each TLR family member recognizes a specific pathogen component, upon activation, triggers a signaling cascade leading to cytokine production and adaptive immune response (9). Among the TLRs, TLR2 and TLR4 play a critical role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis in both clinical and experimental conditions (5-7, 11). Ligands for TLR2 and TLR4 include high-mobility group B1 protein (HMGB1), heat shock protein-60 (HSP60), heat shock protein-70 (HSP70), endotoxin, hyaluronan, advanced glycation end (AGE) products, and extracellular matrix components (12). TLR2 and TLR4 bind to components of the Gram-positive and -negative bacteria, respectively (12). TLR2 has broad ligand recognition specificity due its ability to form heterodimers with TLR1, TLR6, and sometimes with CD14, CD36 (13). TLR4 does not recognize endotoxin with out the cofactor MD-2 (14). Collectively, these ligands and cofactors markedly increase TLR functionality and have not been studied in T2DM.

TLR2 and TLR4 expression have been shown to be increased in conventional IR target tissues like skeletal muscle and adipose tissue of T2DM subjects (15, 16). These important studies implicating TLRs in T2DM were derived using small sample size and the association with respective TLR2/TLR4 ligands, cofactors, down stream signaling, and functional activation remains to be properly addressed. Moreover, systemic inflammation plays a significant role in the etiology and co-morbidities of T2DM, and the role of TLR2 and TLR4 in this setting is not clear. Experimental evidence in mice demonstrates that TLR2 and TLR4 activation and downstream cytokine production lead to the development of diabetes (17, 18). More recently, TLR4 has been indicated as a molecular link between free fatty acids, inflammation, and the innate immune system (5). Also, Song et al. (19) reported increased TLR4 mRNA expression in differentiating adipose tissue of db/db mice.

While these important observations from animal and human tissue data suggest a role for TLR2 and TLR4 in T2DM, it remains unknown whether alterations in TLR2 and TLR4 pathway activation contribute to systemic inflammation in diabetic human subjects. Therefore, the purpose of this study.
was to undertake a comprehensive analysis of TLR2 and TLR4 activation, ligands, cofactors, and downstream signaling in monocytes isolated from recently diagnosed patients with T2DM and matched controls.

**RESEARCH DESIGN AND METHODS**

**Study Subjects:** Type 2 diabetic patients (T2DM; n = 23) (with mean duration of diabetes=29 months; age >35 yr) were recruited from the Diabetes and Pediatric Clinics at University of California Davis Medical Center. None of the patients were on thiazolidinediones (TZDs), angiotensin converting enzyme inhibitors (ACEs), angiotensin receptor blockers (ARBs), insulin, or statins, as they interfere with TLR expression and activation; 6 T2DM subjects were on metformin. Healthy controls (n = 23), age > 35 yr, with normal complete blood count, no family history of diabetes or other chronic diseases, normal kidney, liver, thyroid function, and fasting plasma glucose less than 100 mg/dl were included in the study. Healthy controls and T2DM patients were matched for age (within 5-10 yr), gender, and race. Subjects with mean glycated hemoglobin (HbA1c) over the last year >10%; inflammatory disorders; microvascular and macrovascular complications; abnormal liver, renal, or thyroid function; steroid therapy, anti-inflammatory, antihypertensive, or hypolipidemic drugs; antioxidant supplements in the past 3 months; pregnancy; smoking; abnormal complete blood count; alcohol consumption more than 1 oz/d; consumption of N-3 PUFA capsules (>1 g/d); and chronic high intensity exercisers were excluded. Informed consent was obtained from all the subjects and the study was approved by the UC Davis Institutional Review Board. After history and physical examination, fasting blood (30 ml) was obtained. All the T2DM patients had family history of diabetes. 60% of the subjects were Caucasians and the remaining 40% are Hispanics and African Americans.

Complete blood count, lipid and lipoprotein profile, glucose, HbA1c, C-peptide, and CRP in all the enrolled subjects, were assayed by standard laboratory techniques (11). Free fatty acid levels were assayed using reagents from Wako Chemicals (Richmond). Fasting plasma insulin concentrations were measured by using an ELISA (Linco Research). Insulin sensitivity was estimated by the HOMA index for IR (20).

**Human Monocytes:** Human monocytes were isolated by gradient density centrifugation of peripheral blood using Histopaque (Sigma) and magnetic separation (Miltenyi Biotech) as reported previously (3, 11). More than 88% of cells were identified as monocytes by CD14 staining and viability was found to be >92%. Monocytes were examined before and after activation with synthetic lipoprotein Pam3Cys-Ser-(Lys) 4 (TLR2 ligand; Pam3CSK4, 170 ng/ml; Invivogen) and LPS (TLR4 ligand; 160ng/ml; E. coli 026:B6; Sigma) overnight (12hrs), with suitable controls (3, 11). Following treatment, cells were incubated with TLR2, TLR4, or isotype matched IgG (eBioscience) depending on the cell treatment; 10,000 events were analyzed with the BD FACS Bioanalyzer as described previously (11) after gating for CD14. Intra assay and inter assay coefficient of variation for TLR2 and TLR4 expression was >8% and >12%, respectively (3, 11).

Serum and cell supernatants were used for measuring interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon-gamma (IFN-γ), Monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF-α) with a Multiplex assay (Millipore) as functional read out of TLR activation. Interferon-beta (IFN-β; Antigenix America), HSP60, HSP70 (Assay Designs), HMGB1 (Shino-Test, Japan), Hyaluronan (Echelon), Nε-(Carboxymethyl) lysine (CML; CycLex, Japan) were determined using ELISA in the serum of all
study subjects. Nuclear extracts were used to verify activation of NF-κB (Active Motif) in study subjects, indicating increased inflammation, as described previously (3,11). Intra and inter-assay CV for all the assays were determined to be between 7-12% (21). All the reagents used in the study were tested for endotoxin (<100EU/ml) using Limulus amebocyte lysate assay (Lonza), allowing precise TLR expression measurements devoid of endotoxin (3, 11).

**Real time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):** RNA was isolated from monocytes using TRI reagent (Invitrogen) reagent. c-DNA was synthesized using 1 μg total RNA and 50ng was amplified using primer probe sets for TLR1, TLR2, TLR4, TLR6, MD-2, and 18s (SA Bioscience) following manufacturer’s cycling parameters. Data are calculated using the 2^−ΔΔCT method (3, 11).

**Western blots & Coimmunoprecipitation:** Monocyte cell lysates were subjected to electrophoresis and transfer, as reported earlier (3, 11). Blots were probed with human myeloid differentiation factor-88 (MyD88), interleukin receptor associated protein kinase-1 (IRAK-1), Toll-interleukin-1 receptor domain (TIR) containing adaptor molecule (TICAM-2), interferon regulatory factor-3 (IRF-3), nuclear factor kappa B (NF-κBp65; SantaCruz Bio, CA), Toll/IL-1 receptor (TIR) domain-containing adapter-inducing interferon-β (Trif; Abcam), antibodies with respective secondary IgG antibodies and developed. In all assays, β-actin and total non-phosphorylated proteins were used as internal control. Besides, cell lysates (100µg) were immunoprecipitated with TLR2 or TLR4 antibody overnight at 4°C as reported previously (3) and blotted with TLR6, HMGB1, HSP60 (SantaCruz), and hyaluronan (Ray Biotech) antibodies as depicted in Figure 2F-G. Densitometric ratios were determined as reported earlier from four independent assays (3, 11).

**Statistical Analysis:** Statistical analyses were performed using SAS software (SAS Institute Inc. NC). Data are expressed as mean ± SD for all data. Parametric data were analyzed using paired, two-tailed t tests and nonparametric data using Wilcoxon signed rank tests. Level of significance was set at P < 0.05. Spearman’s rank correlation was computed to assess association between variables.

**RESULTS**

1. **Baseline characteristics of the study subjects:** Subject characteristics are detailed in Table 1. There were no significant differences in age and lipid profile between control and T2DM groups. Levels of glucose, HbA1c, free fatty acids, and HOMA-IR were significantly higher in T2DM compared with controls. C-peptide and advanced glycation endproduct, carboxymethyllysine (CML) levels were significantly increased in T2DM compared with controls. CRP, a prototypic atherosclerotic marker is increased in T2DM compared to controls.

2. **TLR2 and TLR4 mRNA and protein expression in T2DM monocytes:** We first determined the levels of TLR2 and TLR4 expression by flow cytometric analysis in control and T2DM subjects. Monocyte surface expression of TLR2 and TLR4 was significantly elevated in T2DM compared to control in both resting and activated state (Pam or LPS) (Fig 1A, 1D, P<0.005). Next, to determine if the increase in monocyte TLR2 and TLR4 expression in T2DM resulted from increased mRNA expression, we investigated the TLR2 and TLR4 mRNA levels by real time RT-PCR. Figure 1B and 1E depict the corresponding increased TLR2, TLR4 mRNA expression in T2DM compared to controls. We further confirmed the increased monocyte TLR2 and TLR4 protein content using Western blot assay (Figure 1C and 1F). We measured downstream functional readouts (IL-1β, IL-6, IL-8, TNF-α) of
TLR2/4 ligation in resting and activated monocytes (Figure 1G). There was a significant up-regulation of proinflammatory cytokines in T2DM patients compared with controls in both basal and activated state.

3. **TLR2 and TLR4 ligand and cofactor expression in T2DM**: Identification of TLR2 and TLR4 ligands and cofactors is a key element in TLR activation and is unclear in T2DM subjects. Beyond the established microbial ligands of TLR2 and TLR4, several molecules of endogenous origin have been suggested to act as TLR2 and TLR4 ligands notably HMGB1, HSP60, HSP70, hyaluronan fragments. Accordingly, we measured concentration of endotoxin, HMGB1, hyaluronan, HSP60, and HSP70 in the serum of T2DM and control subjects using ELISA assays. Figure 2A-E) show the significant increased levels of these ligands in T2DM subjects. In addition, coimmunoprecipitation with TLR2 and TLR4 and blotting for HMGB1, HSP60, hyaluronan (HA) showed strong association with both TLR2 and TLR4 in T2DM compared to control (Figure 2F & 2G).

It has been previously shown that TLR2 dimerizes with TLR1 or TLR6 and results in receptor activation and downstream signaling upon saturated fatty acid challenge. To determine whether TLR1 or TLR6 is required for the activation of TLR2, we measured TLR1 and TLR6 mRNA expression in monocytes of T2DM and control subjects. TLR6 mRNA levels significantly increased in T2DM (3.8±0.4 vs 1.3±0.2 mRNA/18s ratio; P<0.05) compared to controls, while TLR1 mRNA levels remained unchanged (C=1.5±0.4, T2DM: 1.9±0.5 mRNA/18s ratio), and coimmunoprecipitation showed an increased TLR2/TLR6 association in T2DM (Figure 2F). Further, MD-2 mRNA expression was also significantly increased (C=1.2±0.2 vs. 2.9±0.3 mRNA/18s ratio) in T2DM monocytes. These data are in line with our earlier observation that under hyperglycemic conditions, TLR2 heterodimerizes with TLR6 in monocytes (3).

4. **TLR2 and TLR4 activation results in MyD88 dependent and independent signaling in T2DM monocytes**: We examined the TLR mediated MyD88 dependent signaling pathway using Western blot technique. TLR2 and TLR4 both employ MyD88 and activate NF-κB, common downstream signaling components for all TLRs except TLR3 (3, 9). Therefore, activation of MyD88 dependent and independent pathways was used to interpret the activation of TLR2 and TLR4.

Phosphorylation of IRAK-1, MyD88, TRIF, TECAM-2, IRF-3 protein expression in cytoplasmic extract, and p65 protein levels in nuclear extracts of monocytes were significantly increased in T2DM compared to controls, with no change in total protein and β-actin levels (Figure 2H) suggesting activation of TLR mediated signaling cascade (both MyD88 dependent and independent) in T2DM. In addition, densitometry ratios further corroborate the data (Figure 2H).

5. **Increased TLR2 and TLR4 expression results in increased inflammation mediated by NF-κB**: To further investigate TLR2 and TLR4 mediated inflammation in T2DM, we measured NF-κB activity in the nuclear extracts of T2DM and control subjects monocytes. T2DM showed increased NF-κB p65 dependent DNA binding activity compared to control subjects (P<0.01) (Figure 2I). Increased NF-κB activity corresponded to increased systemic inflammation. We measured IL-1β, IL-6, IL-8, MCP-1, IFN-β, and TNF-α serum concentration in T2DM and control subjects as a functional readout of TLR activation. There was a significant increase in all the proinflammatory mediators in T2DM subjects compared to controls (Figure 2J, 2K).
6. Increased TLR expression correlates with BMI, Glucose, HOMA-IR, and inflammation: Additionally, there was a significant correlation between BMI and TLR2 expression (r = 0.5; P < 0.05) and TLR4 expression (r = 0.43; P < 0.01) and significant correlation between TLR expression and glucose (TLR2: r=0.7; TLR4, r=0.64, P<0.001) free fatty acid levels (TLR2: r=0.66; TLR4, r=0.66, P<0.05), HOMA-IR (TLR2: r=0.64; TLR4, r=0.54, P<0.005), and HbA1c (TLR2: r=0.66; TLR4, r=0.62, P<0.001). In addition, HSP60, HSP70, HMGB1, and endotoxin levels significantly correlated with TLR2 expression (r = 0.43; r=0.64; and r=0.48; and r=0.16; P < 0.05) and TLR4 expression (r =0.43; r=0.65; r=0.41; and r=0.21; P < 0.001), respectively. There was a significant correlation between TLR2 expression and IL-1β, TNFα (r = 0.5; r = 0.5; P < 0.001), MCP-1, and NF-κB (r = 0.42, r = 0.35; P < 0.05) and between TLR4 and IL-1β, TNFα (r = 0. 56; r = 0.55, P < 0.001), IFN-β and NF-κB (r = 0.46; r = 0.4, P < 0.05). Since BMI was significantly different between control and T2DM, additional statistical analyses using BMI as a co-variant were performed. TLR2, TLR4, with and without Pam or LPS, NF-κB, endogenous ligands, CML, and cytokines were significantly higher in T2DM compared to controls (P<0.001) while IL-1β (P=0.09), IFN-γ (P=0.16), and endotoxin (P=0.11) were no longer significant.

DISCUSSION
The interactions among inflammation, hyperglycemia, IR, and T2DM have clear implications for atherosclerosis via the innate immune system. Besides being activators of inflammation under hyperglycemia and IR (3, 5-7) both TLR2 and TLR4 are critical in atherosclerosis (7). In this study, we provided key evidence for increased monocyte TLR2 and TLR4 expression, activation, cofactor expression, ligands, and downstream signaling contributing to systemic inflammation seen in T2DM subjects. Our observations significantly add to the emerging role of TLRs in atherosclerosis and diabetes and consistent with other studies in this context (3, 7, 11). Increased TLR2 and TLR4 expression is demonstrated in atherosclerotic plaque macrophages and in animal models of atherosclerosis. TLR2/4 knockout mice on C57BL6, ApoE−/− and LDLR−/− background and MyD88 knockout mice show reduced lesion size, lipid content, and macrophage infiltration in plaques and reduced inflammation (22, 23).

In two recent studies, we showed increased TLR2 and TLR4 expression, TLR ligands, intracellular signaling, and TLR-mediated inflammation in monocytes with significant correlation to A1C levels in T1DM patients (11, 21). Moreover, TLR2/4 expression and activation is increased in human monocytes under hyperglycemia conditions (3). Reyna et al have shown abnormal TLR4 expression in skeletal muscle of a small number of insulin resistant subjects, with little information on the TLR4-MyD88 signaling, levels of TLR4 cofactors (CD14/MD-2), TLR4 ligand (endotoxin), and its correlation to TLR4 expression (15). Moreover, the invitro experiments in this paper with myotubes and FFA lacked appropriate endotoxin controls and it is not clear if the study subjects were on any cholesterol lowering medications adding complexity to the conclusions. Creely et al. (16) showed increased TLR2 expression in the adipose tissue of T2DM with strong correlates to endotoxin levels with no change in TLR4 expression. This study is inconclusive in terms of the lack of TLR4 expression even under high endotoxin levels, smaller sample size, and minimal patient medication details. Du et al in a descriptive study showed that monocytes from LADA, (latent autoimmune diabetes adults) T2DM patients on sulfonylurea therapy have
significantly higher TLR4 and CD14 expression compared to healthy controls, with no mechanistic details (24). In this context, our study fills in all the above gaps sequentially: First, we provide data on both TLR2 and TLR4 mRNA/protein levels and critical downstream signaling events that follow their ligation in T2DM; second, cofactors required for TLR2/4 activation; third, ligands in use for TLR2/4 activation; fourth, notably we and others have shown that statins, TZDs, and ARBs down regulate TLRs and our T2DM patients are not on any of these drugs; fifth, our TLR data showed significant correlation with major clinical estimates of T2DM namely adjusted BMI, glucose, HOMA-IR, FFA, ligands, cytokines; and finally, data is shown for both MyD88 dependent and independent signaling pathways, which may be acting collectively.

In addition to the well characterized microbial ligands, several molecules of the host origin (endogenous) have been proposed to act as ligands for TLR2 and TLR4 including HMGB1, HSPs, and hyaluronan (25) and were not examined in T2DM. HMGB1 is considered as a ‘late’ proinflammatory mediator in sepsis. It induces activation of intracellular signaling pathways via TLR2, TLR4, and the receptor for advanced glycation end-products (RAGE), thus acting as an extracellular alarmin. HSPs also act as endogenous ligands of TLR2 and TLR4. HSP60 and HSP70 induce the production of proinflammatory cytokines via activation of TLR2 and TLR4. The inflammatory effects of recombinant human HSP60 were shown to be TLR4 dependent, suggesting that HSP60 may be a TLR4 ligand. Low-molecular weight degradation products of hyaluronan (HA) elicit proinflammatory responses in murine alveolar macrophages in rheumatoid arthritis models and other chronic inflammatory conditions. Endotoxin is the most important ligand required for TLR4 activation (14) and its levels are significantly increased in T2DM patients. In the present study, we show that T2DM patients have high circulating levels of HMGB1, HSP60, HSP70, and HA which could trigger TLR2 and TLR4 activation, leading to a proinflammatory state by the activation of TLR2/4, synergistically with glucose, FFA, and endotoxin. Our co-IP studies further suggest an association between TLR2, TLR4, HMGB1, and HSP60.

Dimerization is a major event in the functional activation of TLRs and results in cytokine production. (3). TLR2 activity requires heterodimerization with TLR1 or TLR6 to recognize ligands. Using luciferase reporter assays and real-time RT-PCR, we showed that high glucose induces TLR2 and TLR6 heterodimerization, resulting in NF-κB activation and cytokine production (3). MD-2 is required for TLR4 ligation with endotoxin (14). Here, we demonstrate that both TLR6 and MD-2 mRNA expression is increased in T2DM patients suggesting their requirement for TLR ligation in T2DM patients.

TLRs mainly signal through the adapter protein MyD88 via activation of NF-κB, resulting in the increased transcription of inflammation-related genes, such as those encoding indicated cytokines (3,9,11). In addition, a MyD88-independent pathway involving Trif is essential to TLR3 and TLR4 signaling and induces IFN-β. In two recent studies, we showed that activation of the MyD88 pathway is increased in monocytes exposed to high glucose and in T1DM patients (3, 9, 11). In the present study, we examined MyD88 dependent and independent TLR activation, by determining IRF-3 and IFN-β levels as biological indicator of MyD88 independent activation.

Taken together, the novel findings of this comprehensive study suggest that there is significant elevation of TLR2 and TLR4 protein, mRNA, endogenous ligands, and cofactors in T2DM patients, which, in concert with hyperglycemia, contributes to the
increase in TLR2 and TLR4 signaling that results in the proinflammatory state of T2DM. In future studies, we will address the mechanism of synergistic effects of hyperglycemia, FFA, and endogenous ligands on TLR2 and TLR4 expression and signaling.

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REFERENCES


### Table 1. Clinical and Laboratory Characteristics

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<th>Control (n=23)</th>
<th>T2DM (n=23)</th>
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Values are expressed as Mean ± SD. The P values correspond to the differences between control and T2DM. BMI=Body Mass Index; FFA= Free fatty acids; HDL=high-density lipoprotein; LDL=low-density lipoprotein; CRP= C-reactive protein; CML = Nε-(Carboxymethyl) lysine; NS= not significant.
**Figure legends**

**Figure 1** TLR protein and mRNA content

A. TLR2 surface protein expression was measured in monocytes following Pam3CSK4 (Pam) challenge in control (n=23) and T2DM (n=23) subjects by Flow cytometry as described in research design and methods. Values are expressed as mean fluorescence intensity units (MFI)/10^5 cells. *P<0.005 vs Control; † P<0.05 vs C+Pam. C= Control. T2DM= Type 2 diabetes.

B. Monocyte TLR2 mRNA expression ratios in control (n=23) and T2DM (n=23) subjects by Real time RT-PCR as described in research design and methods. 18s mRNA is used as the house keeping gene. Values are expressed as mean ratio ± SD. *P<0.001 vs. Control. C= Control. T2DM= Type 2 diabetes.

C. Representative Western blot depicting the TLR2 protein expression in pooled resting monocytes from 3 controls and 3 T2DM subjects. β-actin was used as a loading control, as described in research design and methods. Each assay is repeated four times. C= Control. T2DM= Type 2 diabetes.

D. TLR4 surface protein expression was measured in monocytes following LPS challenge in control (n=23) and T2DM (n=23) subjects by Flow cytometry as described in research design and methods. Values are expressed as mean fluorescence intensity units (MFI)/10^5 cells. *P<0.005 vs Control; † P<0.05 vs C + LPS. C= Control. T2DM= Type 2 diabetes.

E. Monocyte TLR4 mRNA expression ratios in control (n=23) and T2DM (n=23) subjects by Real time RT-PCR as described in research design and methods. 18s mRNA is used as the house keeping gene. Values are expressed as mean ratio ± SD. *P<0.005 vs. Control. C= Control. T2DM= Type 2 diabetes.

F. Representative Western blot depicting the TLR4 protein expression in pooled resting monocytes from 3 controls and 3 T2DM subjects. β-actin was used as a loading control, as described in research design and methods. Each assay is repeated four times. C= Control. T2DM= Type 2 diabetes.

G. Release of Cytokines in resting and activated monocyte cell culture supernatants from controls and T2DM subjects using Multiplex assay as described in Methods. Values are expressed as pg/mg cell protein. *P<0.001 vs untreated (C); **P<0.005 vs Control (healthy control). C= untreated; LPS= Lipopolysacharide, TLR4 ligand; Pam= Pam3CSK4, synthetic TLR2 ligand.

**Figure 2**

A. Endotoxin concentration in control (n=23) and T2DM (n=23) subjects was measured using Limulus Ameobocyte lysate assay as described in research design and methods. Values are expressed as EU/ml. *P < 0.05 vs. control. C= Control. T2DM= Type 2 diabetes.

B. HMGB1 concentration in control (n=23) and T2DM (n=23) subjects was measured using ELISA assay as described in research design and methods. Values are expressed as ng/ml. *P < 0.0001 vs control. C= Control. T2DM= Type 2 diabetes.

C. Hyaluronan concentration in control (n=23) and T2DM (n=23) subjects was measured using ELISA assay as described in research design and methods. Values are expressed as ng/ml. *P < 0.0001 vs control. C= Control. T2DM= Type 2 diabetes.

D. HSP60 concentration in control (n=23) and T2DM (n=23) subjects was measured using ELISA assay as described in research design and methods. Values are expressed as ng/ml. *P < 0.0001 vs control. C= Control. T2DM= Type 2 diabetes.
E. HSP70 concentration in control (n=23) and T2DM (n=23) subjects was measured using ELISA assay as described in research design and methods. Values are expressed as ng/ml. *P < 0.0001 vs. control. C= Control. T2DM= Type 2 diabetes.

F. Association of TLR2 with its ligands and TLR6. Representative Western blot showing enhanced expression of TLR6, HMGB1, Hyaluronan (HA), and HSP60 in basal control and T2DM monocyte cell lysates immunoprecipitated with TLR2 antibody as detailed in research design and methods. β-actin was used as a loading control. Each assay is repeated four times. C= Control. T2DM= Type 2 diabetes.

G. Association of TLR4 with its ligands. Representative Western blot showing enhanced expression of HMGB1, Hyaluronan (HA), and HSP60 in basal control and T2DM monocyte cell lysates immunoprecipitated with TLR4 antibody as detailed in research design and methods. β-actin was used as a loading control. Each assay is repeated four times. C= Control. T2DM= Type 2 diabetes.

H. Monocyte TLR signaling in basal state. TLR downstream signaling proteins MyD88, pIRAK-1, Trif, IRF-3, TECAM-2, and NF-κB p65, was performed using specific antibodies to the respective (phospho) proteins as described in research design and methods using β-actin as loading and internal control for MyD88, Trif, IRF-3, TECAM-2, NF-κB p65, and IRAK for pIRAK-1. C= Control. T2DM= Type 2 diabetes. Each blot is repeated four times with pooled monocytes from 3 subjects. Densitometric ratios corroborate the data. * P<0.05 vs. Control.

I. The DNA binding activity of nuclear NF-κB p65 in control (n=23) and T2DM (n=23) monocytes was assessed by ELISA as detailed in research design and methods in the basal state. Values are normalized to mg nuclear protein and expressed as mean ± SD. * P<0.05 vs. Control. C= Control. T2DM= Type 2 diabetes.

J. Serum Concentration of Cytokines/Chemokines in study subjects were measured using Multiplex assays as described in the research design and methods. Values are expressed as pg/ml. * P<0.0001 vs. Control. C= Control. T2DM= Type 2 diabetes.

K. Serum Concentration of Cytokines/Chemokines in study subjects were measured using Multiplex assays as described in the research design and methods. Values are expressed as pg/ml. * P<0.0001 vs. Control. C= Control. T2DM= Type 2 diabetes.
Figure 1

A. TLR2

B. TLR2

C. TLR2

D. TLR4

E. TLR4

F. TLR4

G. Figure 1G
Figure 2

**A.** Endotoxin

**B.** HMGB1

**C.** Hyaluronan

**D.** HSP60

**E.** HSP70

**F.** Blot

**G.** Blot

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<td>TLR6</td>
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<td>HMGB1</td>
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<td>Hyaluronan (HA)</td>
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<td>HSP60</td>
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TLR2 and TLR4 expression and activation in T2D

**H.**

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<td>p-IRAK-1</td>
<td>1.4±0.1</td>
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<td>TRIF</td>
<td>0.4±0.1</td>
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<td>p65</td>
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<td>IRAK-1</td>
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<td>β-actin</td>
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**I.**

`NF-κB`

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<td>IFN-β</td>
<td>0.2±0.01</td>
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**J.**

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**K.**

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