INVESTIGATING TISSUE DESTRUCTION CAUSED BY ALTERATIONS IN DIABETOGENIC GINGIVAL EPITHELIAL CELLS IN THE CONTEXT OF PERIODONTAL DISEASE

By

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To my wife, Amy Tobler

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INVESTIGATING TISSUE DESTRUCTION CAUSED BY DEFECTS IN DIABETOGENIC GINGIVAL EPITHELIAL CELLS IN THE CONTEXT OF PERIODONTAL DISEASE

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Background**:** Research has established the relationship between periodontal disease and diabetes. The pathogenesis of autoimmune diabetes mellitus is mediated by activated innate immunity and inflammation. The inflammatory process, comprised of cytokines and chemokines, may be critical factors in the development of secondary diabetic complications. Periodontal disease is one secondary diabetic complication and is an inflammatory process that affects the supportive tissues around the teeth. Toll-like receptors (TLR) are receptors for microbial pathogen associated molecular patterns (PAMPs). TLR ligation result in activation of genes relevant to inflammatory and innate immune related cytokines. Advanced glycated end product receptor (RAGE) signaling has also been implicated in the pathogenesis of diabetic complication and inflammatory conditions. We ***hypothesize*** that diabetogenic gingival epithelial cells have alterations in their TLR and/or RAGE innate immune pathways.

Materials and Methods**:** Gingival epithelial cells (GECs) were harvested from C57BL6 (non-diabetogenic) and non-obese diabetic (NOD) mouse strains at 4, 8, 12, and 16 weeks of age, after which the TLR and RAGE phenotype was evaluated using qPCR and flow cytometric analysis. In addition, Human Oral Keratinocytes (HOKs) were cultured and stimulated with

TLR ligands and RAGE ligand in the presence and absence of hyperglycemic conditions. Afterwhich cytokine responses and TLR/RAGE phenotypes were determined using luminex and qPCR respectively.

Results**:** Taking into consideration the multiple comparisons made, there is no statistical significant difference in protein expression of TLR 1, 2, and 4 between NOD and C57BL6, although TLR and RAGE gene expression in C57BL6 GEC increased over time, whereas NOD GEC gene expression decreased over time. TLR and/or RAGE ligation promoted pro-inflammatory cytokine and chemokine responses, with the exception of TLR2 ligation which promoted anti-inflammatory cytokine response. Interestingly, hyperglycemic conditions contributed to increased pro-inflammatory cytokine and chemokine responses, while inhibiting TLR2 induced anti-inflammatory responses. Gene expression analysis revealed that TLR and/or RAGE ligation increased gene expression of all TLRs with the exception of TLR9. Here TLR9 expression was not induced nor did its ligation induce the expression of other innate immune receptors. In addition, while RAGE ligation induced TLR and RAGE gene expression, TLR ligation did not induce RAGE gene expression. Finally, all ligand induced gene expression was significantly up-regulated in the presence of hyperglycemic conditions.

Conclusions**:** TLR and RAGE ligation under hyperglycemic conditions results in an increase in pro-inflammatory responses and inhibits anti-inflammatory responses indicating an involvement of these receptors in the induction/progression of periodontal disease of type 1 diabetics.

CHAPTER 1
INTRODUCTION

The pathogenesis of autoimmune diabetes mellitus is mediated by activated innate immunity and inflammation. Diabetics are known to have prolonged inflammation as a result of abnormal immunological responses which can lead to tissue and organ destruction. Inflammation is an immune response in periodontal disease responsible for its severity. Periodontal disease results from an inflammatory process, which is initiated by bacterial colonization causing the production of soluble mediators, which damage the supportive tissues that surround the teeth. Periodontal disease is the foremost cause tooth loss and has been associated as a complication of diabetes. Diabetics not only have a greater risk of developing periodontal disease, but are more likely to develop a more severe form periodontal disease than non-diabetics.

Toll-like receptors (TLR) are receptors for microbial pathogen associated molecular patterns (PAMPs). TLRs result in activation of genes relevant to inflammatory and innate immune related cytokines. Advanced glycated end product (AGE) are involved in initiating, maintaining, and exacerbating inflammatory responses. Higher levels of periodontal AGE accumulation are found in diabetics than in non-diabetics. Advanced glycated end product receptor (RAGE) signaling has been implicated in the pathogenesis of diabetic complication and inflammatory conditions. Both TLRs and RAGE are expressed within gingival epithelial cells (GEC).

This study's aims were to investigate the innate immune function of gingival epithelial cells (GEC) in the context of diabetes mellitus. Specifically, we used molecular and immunohistochemical techniques to examine GEC and human oral keratinocytes (HOK) for the protein and gene expression patterns to identify altered TLR, and RAGE expression levels under

various diabetogenic conditions. In addition, we were able to decipher cytokine and chemokine responses of diabetogenic GEC to determine if type 1diabetics development of periodontal disease is due to intrinsic defects within their GEC and/or if hyperglycemic conditions are responsible.

To accomplish these aims, gingival epithelial tissue was harvested from non-obese diabetic (NOD) strain of mouse and from C57BL6 (non-diabetic) strain of mouse at 4, 8, 12, and 16 weeks of age. Real-time quantitative polymerase chain reaction (qPCR) was used to compare the expression of RAGE and TLR 1, 2, 4, 6, and 9. In addition, flow cytometric analysis (FACS) was used to determine the amount of a given protein expressed on a single cell. Here comparisons in protein expression were measured between GECs from NOD and C57BL6 mice at ages 4, 8, 12, 16 weeks.

In order to demonstrate a potential model of how RAGE, and TLR 1, 2, 4, 6, 9 can be affected by hyperglycemic condition, an *in vitro* stimulation assay using human oral keratinocyte (HOK) along with sources of TLR ligand, soluble RAGE (sRAGE), N-(carboxymethyl) lysine (CML), performed under both hyperglycemic and normoglycemic conditions. Supernatant from the *in vitro* stimulation assay was used to measure expression levels of pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines. In addition, gene expression of TLR 1, 2, 4, 6, 9 and RAGE were evaluated using qPCR.

We found that there was no difference in certain TLR protein expression between NOD and C57BL6 mice. However, our findings showed inverse TLR and/or RAGE gene expression, with C57BL6 mice increasing gene expression and NOD mice decreasing gene expression with age. Furthermore, we found that TLR and RAGE ligation under hyperglycemic conditions results in an increase in pro-inflammatory responses and inhibits anti-inflammatory responses.

Hypothesis: Diabetogenic epithelial cells have alterations in their TLR and/or RAGE innate immune pathways.

CHAPTER 2
BACKGROUND

Type 1 Diabetes

Type 1 diabetes (T1D) is characterized as a chronic cellular mediated autoimmune disease in which insulin producing pancreatic β-cells of the Islets of Langerhans are gradually destroyed by auto-reactive T cells resulting in life-long dependence on exogenous insulin (1,2). During the onset of T1D the islets of Langerhans are infiltrated with mononuclear cells and when roughly 80% of islets no longer functional, symptoms begin to occur (3).

While the cause of T1D has been linked to environmental factors that elicit the induction of an autoimmune response (4), multiple aberrant immune responses can be implicated in the gradual destruction of pancreatic β-cells resulting in the development of T1D (5). Here the mononuclear infiltrate can be identified as macrophages, T lymphocytes, CD4 and CD8 positive T cells (6, 7). In addition, auto-antibodies to insulin (IAA), glutamic acid decarboxylase (GAD), and insulinoma-associated antigen (IA-2) can be found in the peripheral blood before the onset of T1D (8). Here, the presence of IAA, GAD, and IA-2 in the peripheral blood causes a prolonged immune response that persists until the onset of T1D has been diagnosed (9).

Hyperglycemia is a consequence of diabetes resulting from abnormal fat, sugar, and protein metabolism. Hyperglycemic conditions found in diabetic patients is due to exaggerated oxidative stress caused by increased oxidation of sugars, non-saturated fats and glycated proteins leading to glucose auto-oxidation and decreased antioxidant levels (10). Hyperglycemia is a strong risk factor in the development of secondary diabetic complications including the potential to alter the periodontal environment (11). Secondary complications are consequences of indirect and direct cellular damage caused by prolonged periods of high glucose concentrations (12). Hyperglycemia affects cells indirectly due to the production of advance glycated end products

(AGE) (13), which are discussed below. Studies have shown that hyperglycemia causes impaired host defense against pathogens, prolonged inflammatory response, microvascular alterations, impaired bone formation and repair, and impaired wound healing in the periodontium (14-19). Hyperglycemia, caused by diabetes, increases glucose concentration in the gingival crevicular fluid altering the salivary environment in the periodontal pockets (20-23) leading to cellular and molecular alteration in the periodontium (24).

Innate and Adaptive Immune Response in Auto-Immunity

The adaptive immune system consists of T and B cells that display antigen specific receptors. During cell development cells somatically generated structurally unique receptors. This aspect of the adaptive immune system allows the B and T cells to response to largely diverse number of pathogens and immune insults. Interactions with self-ligands in the thymus allow for the generation of the receptors found on naïve B and T cell, resulting in the signaling of B and T cells to mature and survive. The ability of the adaptive immune system to recognize self-molecules indicates under certain inflammatory conditions, an inappropriate adaptive immune response can be elicited and therefore be responsible for the development of an autoimmune disease.

The innate immune systems response to environmental infection is a main factor in activating an aggressive adaptive immune response that can induce autoimmune disease. An innate immune response is the initial immune response resulting from any form of an environmental antigen. Microbial antigens can cause an innate immune response and under the right conditions can induce autoimmunity through molecular mimicry, polyclonal activation, the release of previously sequestered antigen, or bystander activation. For instance, self-antigens released by damaged tissues are taken up by innate immune effector cells such as macrophages and dendritic cells. If these cells have been activated by lipopolysaccharide (LPS), double

stranded RNA or activated T cell secreted factors, this bystander activation can result in dendritic cells presenting antigen and eliciting an inappropriate adaptive immune response through which autoimmune disease may develop. Thus, a cell's threshold for activation can be lowered by the up-regulation of co-stimulatory molecules on antigen-presenting cells during an infection resulting in increased chances of developing autoimmune disease if in the presence of auto-antigen (25).

Periodontal Diseases

Periodontal diseases are a group of diseases consisting of two main levels of infection: gingivitis followed by periodontitis, with periodontitis being the most severe and destructive. Gingivitis is the inflammation of the soft tissue surrounding the tooth, caused by microbial plaque that induces an immune response. Gingivitis progresses into periodontitis, resulting in the destruction of periodontal ligament, bone, and soft tissue and the supporting structure of the tooth resulting in tooth loss. Periodontitis does not affect all teeth evenly, indicating the relation to the retention plaque in areas absent of oral hygiene, leading to calculus accumulation. Interaction of genetic, environmental, host and microbial factors are correlated to the development of periodontal disease (26). The progression of periodontal disease is linked to risk factors which include genetics, age, sex, smoking, socioeconomic factors and systemic diseases.

Gingivitis is a result of inflammation in response to the accumulation of microbial plaque on the tooth surface (27-31), which results in the alteration of blood vessel network and capillary beds within the gingival tissue. An increase of inflammatory cells in the gingival tissue is caused by proteins from the blood entering through open capillary beds that under healthy circumstances would be closed. Lymphocytes, macrophages, and neutrophils are the inflammatory cells that migrate to the gingival tissue in response to microbial plaque. Bacteria are engulf and digested

by the phagocytic macrophages and neutrophils, during which an immune response against microbes is initiated by lymphocytes. Roughly ten to twenty days of accumulation of microbial plaque leads to neutrophils and the barrier of epithelial cells being overwhelmed resulting in inflammation of gingival tissue and the development of gingivitis (32). Redness, swelling, and gingival bleeding are distinctive of gingivitis; however removing plaque from the gingival crevicular area can eliminate the inflammation of the gingival tissue (33). Neutrophils are attracted to the gingival crevicular area by chemotactic peptides that are released by bacteria present in the gingival crevicular pocket (34, 35), while other leukocytes migrate to the gingival crevicular pocket in response to the release of cytokines by epithelial cells being damaged by bacteria. In addition, gingival tissue can be damaged by toxic enzymes released by neutrophils degranulating in response to being overloaded with phagocytosed bacteria.

Gingivitis can progress into periodontitis in about six or more months (34). The irreversible damage characterized as periodontitis is the result of the host immune response to the initial destruction of tissue induced by microbial plaque. In response to tissue damage caused by microbes the host immune responses by producing enzymes such as metallomatrix proteases (MMP), that are responsible for breaking down tissue. The tissue destroying enzymes are necessary in order to remove infected and non-infected yet damaged tissue from the areas of infection. The host response to remove tissue from the bacteria infected areas is a process used by the immune system to stop inflammation. This tissue destruction continues to occur and therefore periodontitis is characterized by bone loss and apical migration of the epithelial attachment eventually resulting in tooth loss. As a result of tooth loss, microbial plaque no longer has a tooth site to accumulate, and the inflammatory response subsides.

The destructive organisms involved in periodontal disease are mainly *Porphyromonas gingivalis, Actinobacilllus actinomycetemcomitans, Treponema denticola, Bacteroides forsythus, and Prevotella intermedia* (36). Microbial plaque contains periodontal pathogens that produce enzymes and toxins that initiate inflammation and damage gingival tissue (37). Host cell membranes and collagen are broken down by periodontal pathogen produced enzymes in order to produce nutrients necessary for pathogen growth. As the host immune and inflammatory processes are initiated in response to periodontal pathogens, inflammatory molecules such as proteases, cytokines, and prostaglandins are released by leukocytes and fibroblasts (38, 39). Tissues collagen structure is destroyed by proteases, creating openings for leukocyte infiltration to progress (40). Prolonged inflammation of the periodontal tissue causes connective tissue attachment to the tooth to be destroyed resulting in the epithelial cells to proliferate apically toward the root of the tooth increasing the depth of the sulcus or periodontal pocket (41). Inflammatory molecules migrating into the periodontal tissue continue to increase as the pocket depth of the sulcus increases. As the density of microbial plaque and pocket depth increase, a more destructive and chronic host response is generated until connective tissues fibers anchoring the root to gingival connective tissue are destroyed, alveolar bone loss, apical migration of the epithelial attachment, resulting tooth loss (42, 43).

Relationship between Diabetes Mellitus and Periodontal Disease

Many studies have demonstrated the close correlation between diabetes mellitus and periodontal disease (44-50). A meta-analysis using diabetic adults concluded that the majority of past studies found more severe periodontal disease in diabetic patients than in adults without diabetes, confirming a significant association between periodontal disease and diabetes (51). Also a two-year longitudinal study demonstrated that diabetic subjects had a significantly increased risk for alveolar bone loss compared to non-diabetic individuals, with an odds ratio of

4.2. Among those patients, poorly controlled diabetics had an odds ratio of 11.4 compared to 2.2 of well controlled diabetics (49). The strength of evidence on the relationship between diabetes and periodontal disease have led some to suggest that periodontal disease should be listed among the "classic" complications of diabetes (50).

Just as diabetes contributes to increased incidences and severity of periodontal disease, periodontal disease can have a significant impact on the metabolic state of diabetics (52). For instance, in a two-year longitudinal trial, diabetic subjects with severe periodontitis at baseline had a six-fold increase risk of worsening glycemic control compared to diabetic subjects without periodontitis (53). In addition eighty-two percent of diabetic patients with severe periodontitis experienced the onset of one or more diabetic complications such as major cardiovascular, cerebrovascular, or peripheral vascular events compared to only twenty-one percent of diabetic subjects without periodontitis (54). The pathology associated with chronic disease processes such as periodontitis have been implicated in the increased susceptibility to infection which is more inflammatory in nature and associated with an exaggerated secretion of innate inflammatory mediators and systemic markers of inflammation. Abnormal inflammatory responses, known as 'hyper-inflammatory trait' have been linked to diabetes (55, 56). These and other studies support the notion that the presence of periodontal disease in diabetic patients may increase insulin resistance and contribute to worsening of the diabetic state and diabetic complications (53-62), although the mechanism of how this occurs is still unclear. Importantly, studies have demonstrated that mechanical periodontal treatment can improve the level of metabolic control in patients with diabetes (63-65).

Gingival Epithelial Cells (GEC)

In the periodontal tissue gingival epithelial cells (GEC) are a central component of the barrier between oral microflora and internal tissues and exhibit innate immune cell function. Research has shown that the oral epithelium play a key role in the innate immune response by producing anti-microbial peptides and chemokines which recruit neutrophils (66, 67). In addition, GEC are capable of phagocytosing and digesting extracellular debris, erythrocytes, and microorganisms such as *Candida albicans, Mycobacterium leprae,* and *Actinobacillus actinomycetemcomitans* (68-72). Research has shown that GEC express the class II major histocompatibility complex (MHC) HLA-DR, signifying antigen presentation capability, T cell stimulation and resultant adaptive immunity (67). Co-stimulatory signals through engagement of CD28 on the T cell and B7 co-stimulatory molecules, B7-1 (CD80) and B7-2 (CD86), on the antigen presenting cell (APC) are required for antigen specific T-cell activation my MHC class II engagement. It neither has yet to be established whether GEC generally express the B7 co-stimulatory molecules (67) nor has whether they can contribute to antigen presentation.

GEC use innate immune receptors such as Toll-like receptors (TLRs), which are discussed below, to fight pathogenic infection and to prevent breaching of the epithelial barrier by pathogens (73). Indeed, GEC have demonstrated many of the same innate immune receptors for bacterial components also expressed on monocytes, macrophages, and dendritic cells (74-76). It is has been demonstrated that GEC express innate immunes Toll-like receptors (TLR) 2, 4 and 6 in healthy oral epithelial tissue allowing GEC to recognized the different range of pathogen associated molecular patterns that are encountered (75, 77). It has been found that GEC respond differently to TLR stimulation than other mucosal epithelium indicating significantly higher expression of TLR in diseased gingival epithelial tissue (75).

A ‘hyper-responsive’ trait displayed in diabetic patients has primarily been identified in monocytes and macrophages of the innate immune system and exhibits the monocytic hyper-responsiveness to bacterial antigens resulting in increased production of pro-inflammatory cytokines and mediators which induce tissue destruction, attachment loss and bone loss (40-42). Innate immune cells are known to be responsible for hyper-responsiveness in diabetic patients; however the role of GEC in the hyper-responsiveness trait has yet to be established.

Toll-like Receptor (TLR)

Toll-like receptors (TLRs) are specific recognition receptors located both intracellular and on the cell surface that responds to pathogen associated molecular patterns (PAMP). TLR signaling in response to microbial infections can directly initiate an innate immune response and can control the activation and balance of an adaptive immune response with the possibility of altering the adaptive immune response (78). Host defense responses use TLR to detect infectious microorganism and in turn elicit strong inflammatory response in order to destroy any invading pathogens. Bacterial lipopolysaccharides, peptidoglycan, lipoproteins, bacterial DNA, and double stranded RNA are all pathogen associated molecular patterns. The extracellular domains of TLR contain structural areas leucine-rich repeats, in which the consistency leucine-rich repeats responds to specific pathogen associated molecular pattern responsible for microbial infection (79, 80). The ligands recognized by TLR that have been identified are as follows: TLR 1 binds triacyl lipopeptides, TLR 2 binds peptidoglycan (81), TLR 3 binds viral double-stranded RNA (82), TLR 4 binds lipopolysaccharide (83, 84), TLR 5 binds flagellin (85), TLR 6 binds peptidoglycans and lipoteichoic acid (84), TLR 7 binds Imidazoquinoline (86), TLR 8 binds viral single-stranded RNA (87), and TLR 9 binds bacterial DNA (88). Generally, recognition of pathogen associated molecular patterns is required by a combination of TLR (79, 80). An example of combination recognition is TLR 2 forms heterophilic diamers with TLR 1 which

recognize triacyl lipopeptides and heterodiamerization of TLR 2 with TLR 6 which recognize diacyl (89). TLR 1, 2, 4, 5, and 6 are cell surface receptors and generally recognize microbial molecules whereas TLR 3, 7, 8, and 9 are intracellular receptors where TLR 3 recognizes double stranded RNA, TLR 7 and 8 recognizes single stranded RNA, and TLR 9 recognizes unmethylated CpG DNA (73).

Cytoplasmic adaptor molecules are a main component of TLR signaling pathway and interact with TLR domain at the initiation of TLR ligation. The cytoplasmic adaptor molecules identified for TLR signaling cascades are myeloid differentiation primary-response protein 88 (MyD88), Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β TRIF, TIR domain containing protein (TIRAP), TRAM, and SARM, with MyD88 being a key adapter molecule responsible in most TLR signaling pathways (90). In the MyD88-dependent pathway, TLR signaling activates MyD88 which is in turn mediates the activation of interleukin 1-receptor-associated kinase (IRAK), IRAK then activates tumor-necrosis-factor-receptor-associated factor 6 (TRAF6), which leads to the activation of two different pathways. One pathway activates a mitogen-activated protein (MAPKKK) called TAK1 and TAK1 phosphorylates and activates the IKK complex. IKK liberates NFκB from its inhibitor IκB so that it can translocate to the nucleus. The second pathway initiates the transforming growth factor β-activated kinase leading to the transforming of growth factor β-activated kinase-1-binding protein complex, the activity of the inhibitor of nuclear factor κB kinase (IkK) complex is then enhanced, which induces expression of cytokines and chemokines after translocation of NFkB into the nucleus (91). On the other hand, MyD88-independent pathway activates interferon-regulatory factor-3 which then induces type I interferon (82, 92).

Different TLR are expressed on neutrophils, monocytes, macrophages, and dendritic cells, allowing these innate immune cells to induce a large variety of immune responses on specific pathogens. Neutrophils, found predominately in the blood, are the innate immune's systems first cells to migrate to the site of infection, expressing TLR 1, 2, and 4, uses these specific receptors to recognize and bind specific microbial pathogens (93). Also involved in the innate immunes systems response are monocytes and macrophages, which recognized, engulf, and kill microorganisms by expressing TLR 1, 2,4, and 8 (94). TLR expressed on immature dendritic cells recognized pathogen associated molecular patterns from pathogens and through their signaling pathway activate the dendritic cell using co-stimulatory molecule and results in the production of cytokines and chemokines when engaging in T-cell priming and differentiation (95). TLR 2, 6, and 9 are expressed on gingival epithelial cells, allowing expressed TLR to recognize and bind pathogen associated molecular patterns that invade the oral cavity (77). Gingival epithelial cells have also been found to express TLR 4 in low levels however the level of expression was found to increase when treated by interferon-γ (96).

Advanced Glycated End Product (AGE)/Advanced Glycated End Product Receptor (RAGE)

AGEs are complex, heterogeneous molecules generated by glycation and oxidation caused by a reaction between carbohydrates and free amino groups of proteins in the environment of oxidant stress and hyperglycemia (97-99). AGEs are formed by the non-enzymatic interaction of reducing sugars with amino groups in proteins, lipids, and nucleic acids forming Schiff bases which rearrange to form a stable, and irreversible Amadori product, that further reacts with dicarbonyl intermediates (100, 101). Under conditions of hyperglycemia the concentration of intracellular glucose, fructose, and fructose-3-phosphate is increased allowing for glycation

reactions to occur by the reduction of sugars leading to the formation of AGE in the cell matrix which can migrate in the body fluids (102).

AGE chemical characteristics found in humans are pentsidine (103) and cayboxyl methyl lysine (CML) (4). AGE formation, also known as the Maillard reaction, affects long-lived proteins and therefore requires weeks to occur during which condensation between an amino group and a carboxyl group, form a Schiff base. Molecular rearrangement of the Schiff base forms an N-substituted glycosylamine which result in Amadori products. Non-oxidative rearrangement and hydrolysis of Amadori products forms 3-deoxyglucosone which reacts with free amino groups leading to cross-linked proteins (10). AGE induced formation of cross-linked proteins has been implicated in the increased stiffness of the protein matrix consequentially obstructing function and damaging the process of tissue remodeling which also occurs in advancing age and diabetes (100).

A normal physiological process of aging is the accumulation of AGEs; however, AGE accumulation is accelerated and occurs at an earlier age in individual with diabetes mellitus (103, 104-106). The severity of complications caused by diabetes has been associated to the accumulation of AGE deposits (107). Formation and accumulation of AGEs has been linked between high plasma glucose levels and tissue damage related to capillary basement membrane thickening and hypertrophy of extravascular matrix which are common diabetic microvascular complications (108). Renal failure in diabetic patients has been observed due to the increased levels of AGEs in serum and tissue causing kidneys the reduced ability of remove AGEs (109-111). Research has demonstrated irreversible cross-linking of matrix structural proteins such as collagen, laminin, and fibronectin leading to basement membrane thickening through the reduction of susceptibility to proteolytic degradation of these proteins directly correlated to the

accumulation of AGEs (112). The thickening of the basement membrane impedes the membranes functionality associated with filtration and permeability properties (113). Proteins such as albumin, hemoglobin, lens crystalline, and LDL cholesterol has been identified as AGE carriers (100). Research has shown that the activation of oxidative stress and the stimulated production and release of cytokines is due to the interaction of AGE and AGE receptor (RAGE) resulting in increased tissue damage (114).

RAGE is a multiligand receptor of the immunoglobulin superfamily and serves as a receptor not only of AGE but also for non-glycated endogenous peptide ligands, such as amyloid-β-protien, amphoterin (HMGB1), and S100/calgranulin (115, 116). RAGE recognizes and binds the multiple β-sheets and three-dimensional structure of these ligands. The extracellular ligand-bind module of RAGE contains two N-linked glycosylation sites and an N-terminal portion with three Ig domains which consist of two C-type domains and one V-type domain. In addition, RAGE is anchored to the cellular plasma membrane by a single transmembrane domain (117). Interestingly, research has demonstrated that the interaction of RAGE with AGEs and ligands leads to NF-κB activation, however the signaling pathway from RAGE to NF-κB has yet to be established. Yet it is known that the ligation of RAGE on monocytes, macrophages, smooth muscle cells, endothelial cells, and astrocytes results in prolonged inflammation caused by the production and release of pro-inflammatory cytokines and chemokines (99). In addition, activation of MAP-kinases and NF-κB mediated by AGE-RAGE interactions leads to amplified production of vascular cell adhesion molecule (VCAM-1) by which AGEs use to increase adhesion to pro-inflammatory cells at the cell surface.

Murine Model System

The non-obese diabetic (NOD) strain of mouse is a main model of autoimmune type 1 diabetes (T1D). The NOD strain of mice was developed in Japan and derived from the outbred

Jc1:ICR line of mice during selection of a cataract-prone strain of mice (118, 119). Through repetitive brother-sister mating of the cataract-prone strain, the NOD stain was developed as a strain that spontaneously developed diabetes (118-120). The NOD strains spontaneous onset of diabetes usually begins between 12 to 14 weeks of age in female mice and later in male mice with the incidence of spontaneous diabetes occurring in 60% to 80% of female mice and 20% to 30% in male mice (119, 121). Incidences of spontaneous diabetes in NOD mice occur at a higher rate when the NOD mice are housed in a germ-free facility suggesting that in a dirty environment the NOD mouse immune system is engaged in protecting against foreign proteins and protecting against autoimmunity, allergy, and other disease leading to decreased incidences of spontaneous diabetes (121, 122). The decreased incidence of spontaneous diabetes in NOD mice as result of a dirty environment is still unclear.

NOD strain possesses an immune defect leading to autoimmune destruction of the pancreas resembling the development of T1D in human. Female NOD mice develop permanent hyperglycemia at sixteen to twenty weeks of age. At three to four weeks of age NOD mice demonstrate immune cell infiltrates that surround the islet, after which these infiltrates continue to invade the islets leading to insulitis and eventual at ten weeks of age demonstrate severe insulitis (120). The mononuclear infiltrates that invade the islets consist mainly of CD4+ T cells, although CD8+ T cells, B cells, dendritic cells, natural killer (NK) cells, and macrophages are also present in the lesions (118, 119).

Research suggests that the pathogenesis of T1D in NOD mice is primarily due to both CD4+ and CD8+ T cells (121, 123, and 124). CD4+ T cells directly mediate destruction of islet cells in response to prior destruction of islet cells by CD8+ T cells (125). The pancreatic islets insulin, insulinoma-associated protein 2 (IA-2), GAD, and heat shock protein 60 (Hsp60) have

been identified as specific antigens recognized by CD+4 and CD+8 T cells in diabetogenic NOD mice (126). The combination of antigen mimicry, nonspecific inflammation, and defective tolerogenic processes may promote multiple T cell reactivities to islet expressing autoantigens in initiating the pathogenic process of T1D (120).

A number of other immune defects associated with multiple subsets of leukocytes which include defective macrophage maturation and function (127), low levels of NK cell activity (128, 129), defects in NKT cells (130, 131), deficiencies in their regulatory CD4+ CD25+ T cell population (132), and the absence of C5a and hemolytic complement (133) contribute to the development of autoimmunity in NOD mice. T1D progression in NOD mice can be contributed to intrinsic defects leading to an acquired lymphopenia that evolves with age and allows homeostatic proliferation of naïve T cells (134).

CHAPTER 3
MATERIALS AND METHODS

Harvesting Gingival Epithelial Tissue

Gingival epithelial tissue was harvest from CO2 euthanized female mice by using scissors to cut the temporalis, condyle, and masseter areas of the mandible, allowing for the mandible to be removed. Cuts were also made on the ventral surface to allow removal of the tongue and on the lower labial frenum to allow separation of skin and tissue from the outside area of the mandible. Using a dissection microscope (Zeiss), a 3.0 mm depth ROBOZ microsurgical blade was used to make incisions above and below the gingiva located around the molars of the mandible. Dissection tweezers were then used to remove the excised gingival tissue. Tissues were either placed in RLT buffer (Qiagen) or flow cytometric staining buffer for RNA purification and FACS analysis respectively.

Primary Culture of Human Oral Keratinocytes (HOK)

The vial containing frozen Human Oral Keratinocytes was placed in 37º water bath and rotated until the contents was completely thawed, after which the contents were plated onto a poly-L-lysine coated culture vessel (T75 flask) filled with 19mL of oral keratinocyte growth media (500mL Basal media, 5mL Oral keratinocyte growth supplement (OKGS), and 5mL Penicillin/Streptomycin (P/S)). The T75 flask was gently rotated to distribute the cells evenly and then allowed to incubate for 37C with 5% CO2 for sixteen hours. After which, the growth media was then removed and replaced and repeated every other day following until the cell culture reached 50% confluence. At 50% confluence the growth media was changed every day until the cell culture reached 80% confluence. At 80% confluence the cell culture was then split. Here, media was removed and cells were washed with sterile PBS and 10mL of cell stripper for 10-15 minutes at 37º 5% CO2. Cells were then removed and washed by centrifugation at 1200

rpm and 4º for 10 minutes. Resultant cells were counted and plated for the appropriate assays and frozen for later use.

HOK Stimulation Assay

HOK cells were isolated from an established primary HOK cell culture. 1x10^5 cells/mL (1mL of growth media) were added to eighteen wells of poly-L-lysine coated six-well plates. An additional 1mL of oral keratinocyte growth media (500mL Basal media, 5mL Oral keratinocyte growth supplement (OKGS), and 5mL Penicillin/Streptomycin (P/S)) was added to each of the eighteen wells. The six-well plates were gently rotated to distribute the cells evenly and allowed to incubate at 37C 5%CO2 for sixteen hours. The growth media was then change the following day and then changed every other day following until the cell culture reached 50% confluence. At 50% confluence the growth media was changed every day until the cell culture reached 80% confluence. At 80% confluence, 15mMol glucose was added to some of the wells to establish hyperglycemic environments allowed to incubate at 37C 5%CO2 for twenty-four hours. After which some cultures of both hyperglycemic and normoglycemic wells were either treated with 1µg/mL of the following stimulants: ultra-pure LPS from *P.gingivalis*, ultra-pure LPS from *E.coli*, ODN 2395 type C CpG oligonucleotide, Pam2CSK4 synthetic bacterial lipoprotein, FSL-1 synthetic diacylated lipoprotein, N-(carboxy methyl) lysine (CML), CML and soluble RAGE (sRAGE),or were left untreated. All cultures were allowed to incubate at 37C 5%CO2 for twenty-four hours. The resultant supernatants were t used in the Beadlyte® Human 22-Plex Multi-Cytokine Detection System. In addition, RNA from the cultures was also isolated using an RNeasy kit (Qiagen) as previously described. The HOK cell stimulation was performed three times for both normoglycemic and hyperglycemic conditions.

Purification of RNA

A Qiagen RNeasy Mini Kit was used for RNA purification. The excised gingival tissue or HOK cell cultures were disrupted, homogenized and/or lysed in 600µL of RLT lysis buffer in preparation for disruption and homogenization. The lysate was centrifuged for three minutes at full speed. The supernatant was carefully removed by pipetting and transferred to a new microcentrifuge tube. Only the supernatant (lysate) was used from this point. One volume of 70% ethanol was added to the clear lysate, and was immediately mixed by pipetting. The sample and any precipitate was transferred to an RNeasy spin column placed in a 2mL collection tube and then centrifuged for fifteen seconds at 8000 x g (10000 rpm). After which the flow-through was discarded. 700µL of RW1 buffer was added to the RNeasy spin column and then centrifuged for fifteen seconds at 8000 x g to wash the spin column membrane. Again the flow-through was discarded. 500µL of RPE buffer was added to the RNeasy spin column and centrifuged for fifteen seconds at 8000 x g to wash the spin column membrane after which the flow-through was discarded. Another 500µL of RPE buffer was added to the RNeasy spin column and then centrifuged for two minutes at 8000 x g to wash the spin column. The longer centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. After centrifugation the RNeasy spin column was carefully removed so that the column did not contact the flow-though and placed into a new 1.5mL collection tube. Lastly, 50µL of RNase-free water was added directly to the spin column membrane and then centrifuged for one minute at 8000 x g to elute the RNA.

Reverse Transcription

5µL/reaction of 5x buffers, 1µL/reaction of 10mM DTTs, 2µL/reaction of dNTPs, 0.25µL/reaction of reverse transcriptase, and 1µL/reaction of random oligonucleotide were added to 1.5mL eppendorf to tube to make a master mix. The tube was then mixed by vortex

and quick spin centrifugation. 9.25µL of master mix was added to the appropriate number of 0.5µL eppendorf tubes (i.e. the number of reactions being performed at a given). 15.75µL of RNA (from either GEC or HOK cells) and Rnase/Dnase free water (if less than 15.75µL of RNA was being used) were added to the appropriate 0.5µL eppendorf tube containing aliquots of master mix. The thermocycler conditions were set to the following: 40 minutes at 40º; 15 minutes at 70º; then hold at 4º.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

12.5 µL/reaction of 2x Syber Green Master Mix, 1µL/reaction of both 10µM Forward primer and 10µM Reverse primer, and 9.5µL/reaction of Rnase/Dnase free water were all add to an 1.5 eppendorf tube to make a master mix. Primers used included: Integrated DNA Technologies mRAGE (mouse), "TTG GAG AGC CAC TTG TGC TA" (forward) and "CCC TCA TCG ACA ATT CCA GT" (reverse); SA Bioscience SYBR® Green Mouse TLR 1 "TTGCCC ATCACAATCT CT"; SA Bioscience SYBR® Green Mouse TLR 2 "TGAA AAACCTGACC TCTCT"; SA Bioscience SYBR® Green Mouse TLR 4 "TCAAC TGAACTGAAC GGTTT"; SA Bioscience SYBR® Green Mouse TLR 6 "CCTGATAT CAGCTTTCTG TC"; SA Bioscience SYBR® Green Mouse TLR 9 "GCC ACACCAACAT CCTGGTT"; SA Bioscience SYBR® Green Human TLR 1 "CTTGGA TTTGTCCCAC AACA"; SA Bioscience SYBR® Green Human TLR 2 "CAGAG GTGTGTGAAC CTCCA"; SA Bioscience SYBR® Green Human TLR 4 "C TGTGTGTATT TGAAAGTGTG"; SA Bioscience SYBR® Green Human TLR 6 "TCT GCTTTCCCAA ATGGATT"; SA Bioscience SYBR® Green Human TLR 9 "CCTGAGG GTGGAAGTGT CCT"; Integrated DNA Technologies mRAGE (human), "GAC TCT TAG CTG GCA CTT GGA T" (forward) and "GGA CTT CAC AGG TCA GGG TTA C" (reverse). Glyceraldehyde 3-phosphate dehydrogenase is constitutively expressed in all cells and therefore allows for normalization of

the total DNA isolated from the PCR reaction. The primers for G3PDH were, "ACCACAGTCCATGCCATCAC" (forward) and "TCCACCACCCTGTTGCTGTA" (reverse). The tube was then mixed by vortex and quick spin centrifugation. 20µL of the master mix were then added to appropriate number of well of a 96 well thermocycler plate. 1µL of cDNA was then added to the appropriate wells of the 96 well themocycler plate containing aliquots of master mix. The 96 well plate is then place in the qPCR thermocycler set the following run cycles: 95º for 10 minutes; 95º for 15 seconds; 60º for 60 seconds for 40 cycles; then hold at 4º. Standard curves that were generated from serial dilutions of each gene used to measure mRNA transcript copy number. Each gene was detected in independent real-time PCR reactions using 10µl of a 50µl total cDNA mixture. Data are expressed as a copy number normalized GAPDH content. The normalized mRNA copy number for a gene was determined by: [raw transcript copy number derived from standard curve] X [GAPDH corrective ratio]. The GAPDH corrective ratio was calculated as [lowest GAPDH copy number within sample set]/ [GAPDH copy number for cell of interest].

Flow Cytometry (FACS)

The excised gingival tissue was ground between two frosted ends of microscope slides to create single cell suspension while in 15µL of PBS to prevent the tissue and cell from drying out. The single cell suspension/PBS was placed into a 50µL tube and centrifuged at 1200 rpm for ten minutes at 4ºC. After discarding the supernatant, the pellet was resuspended in PBS, filtered, and aliquots of 200µL of cells were added to a 96 well plate. The 96 well plate was centrifuged at 1200 rpm for ten minutes at 4ºC after which the supernatant was discarded. The cells were resuspened in 100µL of primary antibody master mix (1:200 dilution of antibody in FACS buffer (1% FBS in PBS)) and incubated in dark for thirty minutes at 4ºC. Antibodies used included:

eBioscience Biotin anti-mouse TLR 1, clone: eBioTR23; eBioscience anti-mouse TLR 2, clone: T2.5; eBioscience Biotin anti-mouse TLR 4, clone: UT41. The 96 well plate was then centrifuged at1200 rpm was ten minutes at 4ºC and the supernatant was discarded. The cells were resuspended in 200µL of FACS buffer followed by centrifugation and removal of supernatant. 100µL of the secondary antibody master mix (if needed) was added to cells followed by incubation in the dark for thirty minutes at 4ºC. The 96 well plate was then centrifuged and supernatant was removed. The cells were resuspended in 200µL FACS buffer followed by centrifugation and removal of supernatant. The cells were resuspended in 200µL of FACS buffer and transferred to FACS tubes. 200µL more of FACS buffer was added to the FACS tubes. Then BD bioscience FACS Calibur system with BD bioscience Cell quest software was used for data collection.

Luminex

Supernatant, taken after HOK cells had been stimulated for twenty-four hours, was used in the Beadlyte® Human 22-Plex Multi-Cytokine Detection System. The human 26-plex Multi-Cytokine Standard were resuspended in 1mL of tissue culture media (TCM), after which was serial diluted 1:3 to make eight standards. 50µL of the standards and sample were added to a well of a primed filter bottom 96 well plate. The Beadlyte® Human 22-plex Multi-Cytokine Beads were vortexed at high speed for fifteen seconds and then sonicated for fifteen seconds using a microbead sonicator bath after which 25µL of the bead solution was added to each well. The filter plate wells were then covered and mixed by vortex, followed by two hours of incubation in a dark room at room temperature on a plate shaker. The vacuum manifold was applied to the bottom of the filter plate and the liquid was removed, after which, 50µL of Beadlyte® Cytokine Assay Buffer was used to wash the well content two times. 75µL of Beadlyte® Cytokine Assay Buffer was used to suspend the wells contents, after which 25µL of

Beadlyte® Human 22-plex Multi-Cytokine biotin was then added to each well and the filter plate was incubated for 1.5 hours in at the dark at room temperature on a plate shaker. Beadlyte® Streptavidin-Phycoerythrin was diluted 1:12.5 in Beadlyte® Cytokine Assay Buffer. 25µL of Beadlyte® Streptavidin-Phycoerythrin dilution was added to each well. The filter plate was covered and mixed by vortex at a low speed followed by thirty minutes of incubation in a dark room at room temperature on a plate shaker. 25µL of Beadlyte® Stop solution was added after which the filter plate was vortexed gently and incubated for five minutes at room temperature in the dark. Vacuum manifold was then applied to the bottom of the filter plate and liquid was removed. 125µL of sheath fluid was then add to each well then mixed by vortex at a low speed and placed on a plate shaker for one minute. The Luminex® 100™ System was used to acquire the results and Milliplex Analyst Software (VigeneTech) was used to analyze the results.

CHAPTER 4
RESULTS

C57BL6 and NOD mice exhibit different TLR protein expression

Flow cytometry (FACS) was used in order to determine the amount of TLR2 protein expression on the surface of GECs. To accomplish this, GECs were harvested from both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age. The harvested GECs were stained with antibodies specific for TLR2 protein. TLR2 protein expression of levels of C57BL6 and NOD were than analyzed and compared using GraphPad Prism 4 analysis software. Fig. 4-1 demonstrates that TLR2 protein expression decreases in both C57BL6 and NOD mice at 16 weeks of age with a significantly different percentage of TLR2 protein expression between C57BL6 and NOD mice at 16 weeks of age ($p = 0.0058$) with the higher expression level displayed in NOD mice.

TLR4 protein expression on the surface of GECs, harvested from both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age, was evaluated using FACS. To accomplish this, GECs were stained with TLR4 specific antibodies. Fig. 4-2 demonstrates that TLR4 single positive GECs displayed a significantly different percentage of TLR4 protein expression between C57BL6 mice and NOD mice at 16 weeks of age ($p = 0.0003$). TLR4 protein expression increases in C57BL6 mice at 16 weeks of age, whereas TLR4 protein expression remains constant in NOD mice (fig. 4-2).

TLR2/4 double positive protein expression on the surface of GECs harvested from C57BL6 and NOD mice at 4, 8, 12, 16 weeks of age was determined using FACS. GECs were stained with antibodies specific for TLR2/4 protein. Fig. 4-3 demonstrates that NOD and C57BL6 mice express a significantly different percentage of TLR2/4 double positive protein expression at 16 weeks of age ($p = 0.0063$) with the higher expression level displayed in NOD

mice. TLR2/4 double positive protein expression decreases in both C57BL6 and NOD mice at 16 weeks of age.

TLR1/2 double positive protein expression on the surface of GECs harvested from NOD and C57BL6 mice at 4, 8, 12, and 16 weeks of age was evaluated using FACS. To accomplish this, GECs were stained with TLR1/2 protein specific antibodies. Fig. 4-4 demonstrates TLR1/2 double positive protein expression was significantly higher in NOD mice at both 8 and 16 weeks of age as compared to C57BL6 mice ($p = 0.0061$ 8wks; $p = 0.0055$ 16 wks). TLR1/2 double positive protein expression decreases in both C57BL6 and NOD mice at 16 weeks of age (fig. 4-4).

TLR1, 2, 1/2, and 4/2 protein expression on the surface of GECs harvested from NOD and C57BL6 mice at 4, 8, 12, and 16 weeks of age was determined using FACS. GECs were stained with TLR protein specific antibodies after which FACs was utilized in order to determine TLR protein expression. The only statistically significant finding is that both NOD and C57BL6 mice seem to decrease their innate immune TLR protein expression by 16 weeks of age, with the exception of TLR4 (all $p < 0.0058$) (fig. 4-3). When multiple comparisons were made, applying TLR protein expression at 4, 8, 12, and 16 weeks of age, there was no statistically significant difference in the protein expression of TLR1, 2, and 4 between NOD and C57BL6 mice. This demonstrates that with age TLR protein expression on the surface of GECs cannot be attributed to the different innate immune responses between NOD and C57BL6 mice. Therefore TLR protein expression cannot be employed as reasoning when comparing immune responses between type 1 diabetics and non-diabetics in regards to the triggering of prolonged inflammation resulting in tissue destruction.

Fig. 4-40 demonstrates that our data showed no difference in TLR protein expression on a single GEC between C57BL6 and NOD mice at ages 4, 8, 12, and 16 weeks.

TLR Gene Expression in C57BL6 Mice Increase over Time, Whereas TLR Gene Expression in NOD Mice Decreases over Time

Real-time quantitative polymerase chain reaction (qPCR) was used to evaluate the relative gene expression of TLR1, 2, 4, 6, and 9 on the surface of GECs harvested from C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age. DNA content of TLR1, 2, 4, 6, and 9 was normalized to G3PDH after which copy numbers of TLR1, 2, 4, 6, and 9 were determined. TLR gene expression was analyzed and compared using GraphPad Prism 4 analysis software. Figures 4-5 thru 4-8 demonstrates that the relative gene expression of TLR1, 2, 4, 6, and 9 in C57BL6 mice increase over time, whereas TLR gene expression in NOD mice decreases over time. These data demonstrates that TLR gene expression in NOD and C57BL6 is contrary to our TLR protein expression data. This suggests that a change is occurring in the signaling pathway between TLR protein and TLR gene expression at the transcriptional level.

C57BL6 and NOD mice display significantly different TLR gene expression. Figures 4-5 and 4-8 demonstrates that C57BL6 and NOD mice display significantly different TLR4 and TLR6 gene expression at 12-16 weeks of age ($p = 0.0015$ at 12 wks; $p = 0.0047$ at 16 wks). Fig. 4-7 demonstrates significantly different TLR1 gene expression between C557BL6 and NOD mice at 8-16 weeks of age ($p = 0.0208$ at 8 wks; $p = 0.0003$ at 12 wks; $p = 0.008$ at 16 wks). C57BL6 and NOD mice both display significantly different TLR2 and 9 gene expressions at 16 weeks of age ($p = 0.0048$).

TLR Ligation Promotes and/or Inhibits Cytokine/Chemokine Secretion

Luminex was used to decipher cytokine/chemokine responses of HOK when stimulated with TLR ligands. HOK cells cultures were established after which the HOK cells were

stimulated with TLR ligands for 24 hours. Following stimulation supernatant was taken from all stimulation samples. Luminex results were analyzed and compared using GraphPad Prism 4 analysis software. Fig. 4-13 demonstrates that TLR4 and TLR9 ligation promotes the secretion of IL-6 pro-inflammatory cytokine when compared to IL-6 secretion level of non-stimulated HOK cells (all $p < 0.0048$ TLR4). We also see this same trend in the following pro-inflammatory cytokines and chemokines: GM-CSF, IL-8, TNF-α, IL-1β, MIP1α, MCP-1, and IP-10. These data demonstrate that TLR ligation exhibit the basis behind the triggering of prolonged inflammation leading to tissue destruction.

Fig. 4-15 demonstrates that TLR2 ligation promotes IL-10 anti-inflammatory chemokine secretion when compared to IL-10 secretion resulting from both non-stimulated HOK cells and TLR4 ligation ($p = 0.0039$). Fig. 4-13 demonstrates this anti-inflammatory promoting trend when comparing similar levels of IL-6 pro-inflammatory cytokine secretion resulting from TLR2 ligation and non-stimulated HOK cells. We also see these affects with other pro-inflammatory cytokines and chemokines (CSF, IL-8, TNF-α, IL-1β, MIP1α, MCP-1, and IP-10). The anti-inflammatory response generated by TLR2 ligation demonstrated the innate immune systems internal control of inflammatory responses and was unknown prior to this investigation.

Fig. 4-13 demonstrates that TLR1 and/or TLR6 diamerization to TLR2 and subsequent ligation results in the promotion of IL-6 pro-inflammatory cytokine secretion when compared to IL-6 secretion from non-stimulated HOK cells (all $p < 0.0039$). This trend was also seen with other pro-inflammatory cytokines and chemokines (CSF, IL-8, TNF-α, IL-1β, MIP1α, MCP-1, and IP-10). This demonstrates that the diamerization of TLR1 and/or TLR6 to TLR2 alters TLR2 ligation from resulting in an anti-inflammatory response to a pro-inflammatory response.

This pro-inflammatory response can lead to prolonged inflammation resulting in tissue destruction.

Luminex was also used to decipher cytokine/chemokine responses of HOK when stimulated with TLR ligands in hyperglycemic conditions. HOK cells cultures were established after which half of the HOK cells were stimulated with 15mMol glucose for 24 hours to establish both hyperglycemic and normoglycemic conditions. The HOK cells under both conditions were stimulated with TLR ligands for 24 hours. Following stimulation supernatant was taken from all stimulation samples. Luminex results were analyzed and compared using GraphPad Prism 4 analysis software. TLR ligation under hyperglycemic conditions results in an increase in pro-inflammatory responses and inhibits anti-inflammatory responses. Fig. 4-13 demonstrates that under hyperglycemic conditions TLR4, 9, and 6/2 ligation result in an increased IL-6 pro-inflammatory responses when compared IL-6 secretion resulting from TLR ligation under normoglycemic conditions (all $p < 0.0363$). Fig.4-15 demonstrates that hyperglycemic conditions decrease IL-10 anti-inflammatory response resulting from TLR2 ligation when compared to IL-10 secretion resulting from TLR2 ligation under normoglycemic conditions ($p = 0.0117$).

Stimulation with TLR Ligands Increases TLR Gene Expression

Real-time quantitative polymerase chain reaction (qPCR) was used to evaluate the relative gene expression of TLR1, 2, 4, 6, and 9 on HOK cells after stimulation with TLR ligands. To accomplish this HOK cells cultures were established after which the HOK cells were stimulated with TLR ligands for 24 hours. qPCR was utilized with TLR primers. DNA content of TLR was normalized to G3PDH after which total copy numbers of TLR were determined. qPCR results were analyzed and compared using GraphPad Prism 4 analysis software. TLR ligation results in increased transcription of TLR genes. Fig. 4-28 demonstrates that HOKs stimulated with TLR4,

2, 1/2, and 6/2 ligands displayed significantly different TLR2 gene expression than HOK cells stimulated with TLR9 ligand and non-stimulated HOK cells (all $p < 0.0251$). We also saw this trend in TLR1 and TLR4 gene expression (fig. 4-29 and 4-30). Fig. 4-31 shows that HOK cells display significantly different TLR6 gene expression after stimulation with TLR2/6 ligand when compared to TLR6 gene expression by non-stimulated HOK cells ($p = 0.0018$).

Fig. 4-28 demonstrates that HOKs stimulated with TLR9 ligand displayed TLR2 gene expression levels similar to non-stimulated HOK TLR2 gene expression levels. We also saw this same trend with TLR1, 4, 6, and 9 gene expressions (fig. 4-28 thru 4-32).

RAGE Gene Expression in C57BL6 Mice Increases over Time, Whereas RAGE Gene Expression in NOD Mice Decreases over Time

In order to determine RAGE gene expression on the surface of GEC, GECs were harvested from both NOD and C57BL6 mice at 4, 8, 12, and 16 weeks of age after which qPCR were used. DNA content of RAGE was normalized to G3PDH after which total copy numbers of RAGE were determined. qPCR results were analyzed and compared using GraphPad Prism 4 analysis software. Fig. 4-9 demonstrates that within C57BL6 mice RAGE gene expression increases over time whereas in NOD mice, RAGE gene expression decreases over time. Fig. 4-9 demonstrates that C57BL6 and NOD mice display significantly different RAGE gene expression at 8-12 weeks of age ($p = 0.0026$; $p = 0.0008$ at 12 wks; $p < 0.0001$ at 16 wks). RAGE gene expression exhibits the same trend as TLR gene expression in NOD and C57BL6 mice. This data suggest that in RAGE gene expression cannot be assumed to be a primary reason behind overacting innate immune responses in type 1 diabetics resulting in prolonged inflammation leading to tissue destruction.

RAGE Ligation Promotes and/or Inhibits Cytokine/Chemokine Secretion

Luminex was used to decipher cytokine/chemokine responses of HOK when stimulated with RAGE ligands. HOK cells cultures were established after which the HOK cells were stimulated with RAGE ligands for 24 hours. Following stimulation supernatant was taken from all stimulation samples. Luminex results were analyzed and compared using GraphPad Prism 4 analysis software. Fig. 4-22 demonstrates that RAGE ligation promotes the secretion of IL-6 pro-inflammatory cytokine when compared to IL-6 secretion level of non-stimulated HOK cells ($p = 0.0045$). We also see this same trend in the following pro-inflammatory cytokines and chemokines: GM-CSF, IL-8, TNF-α, IL-1β, MIP1α, MCP-1, and IP-10. This data demonstrate that RAGE ligation exhibit the basis behind the triggering of prolonged inflammation leading to tissue destruction.

Luminex was used to decipher cytokine/chemokine responses of HOK when stimulated with RAGE ligands in hyperglycemic conditions. HOK cells cultures were established after which half of the HOK cells were stimulated with 15mMol glucose for 24 hours to establish both hyperglycemic and normoglycemic conditions. The HOK cells under both conditions were stimulated with RAGE ligands for 24 hours. Following stimulation supernatant was taken from all stimulation samples. Luminex results were analyzed and compared using GraphPad Prism 4 analysis software. RAGE ligation under hyperglycemic conditions results in an increase in pro-inflammatory responses and inhibits anti-inflammatory responses. Fig. 4-22 demonstrate that under hyperglycemic conditions RAGE ligation result in an increased IL-6 pro-inflammatory responses when compared IL-6 secretion resulting from TLR ligation under normoglycemic conditions ($p = 0.0275$). Also in hyperglycemic conditions there is an inhibiting and/or lack of IL-10 anti-inflammatory response resulting from RAGE ligation when compared to IL-10

secretion resulting from RAGE ligation under normoglycemic conditions ($p = 0.0186$) (fig. 4-24).

RAGE Gene Expression Increases after Stimulation with TLR Ligands

Real-time quantitative polymerase chain reaction (qPCR) was used to evaluate the relative gene expression of RAGE on HOK cells after stimulation with RAGE ligands. To accomplish this HOK cells cultures were established after which the HOK cells were stimulated with RAGE ligands for 24 hours. qPCR was utilized with RAGE primers. DNA content of TLR was normalized to G3PDH after which total copy numbers of TLR were determined. qPCR results were analyzed and compared using GraphPad Prism 4 analysis software. Stimulation with RAGE ligand results in increased RAGE gene transcription. Fig. 3-33 demonstrates that HOK cells stimulated with TLR 4, 2, 1/2, and 6/2 display significantly different RAGE gene expression than HOK cells stimulated with TLR 9 ligand and non-stimulated HOK cells (all $p < 0.061$).

Fig. 4-39 demonstrates that HOK cells stimulated with RAGE ligand displayed significantly different RAGE gene expression when compared to RAGE gene expression displayed by non-stimulated HOK cells ($p = 0.063$).

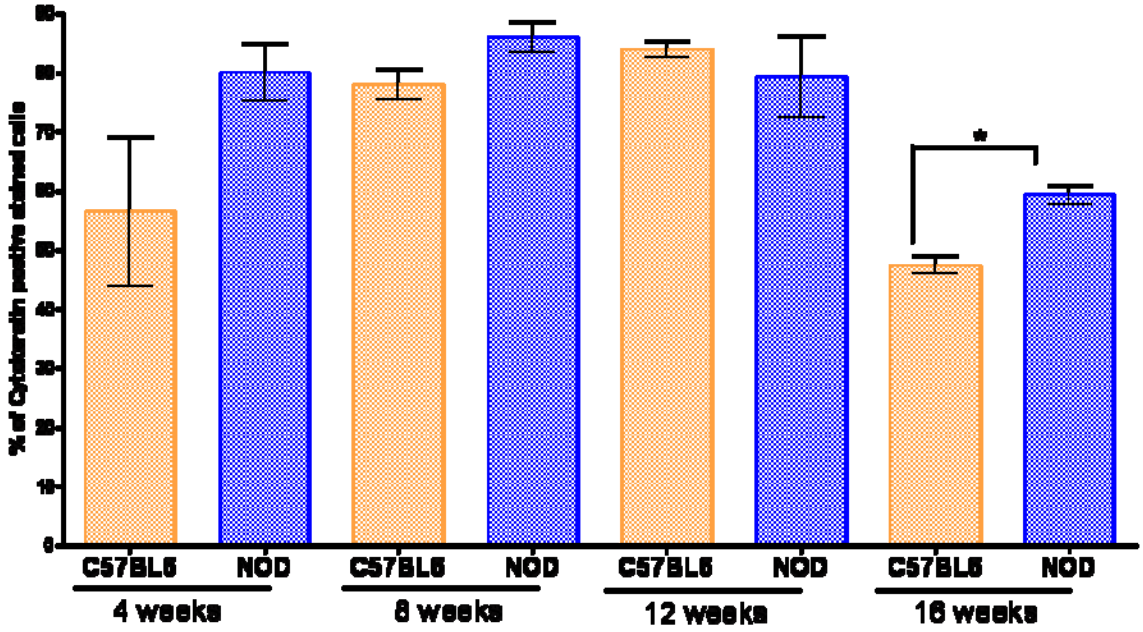

Figure 4-1. TLR2 protein expression is significantly increased in NOD mice at 16 weeks of age (*p = 0.0058). FACS analyzed relative protein expression of TLR2 single positive GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

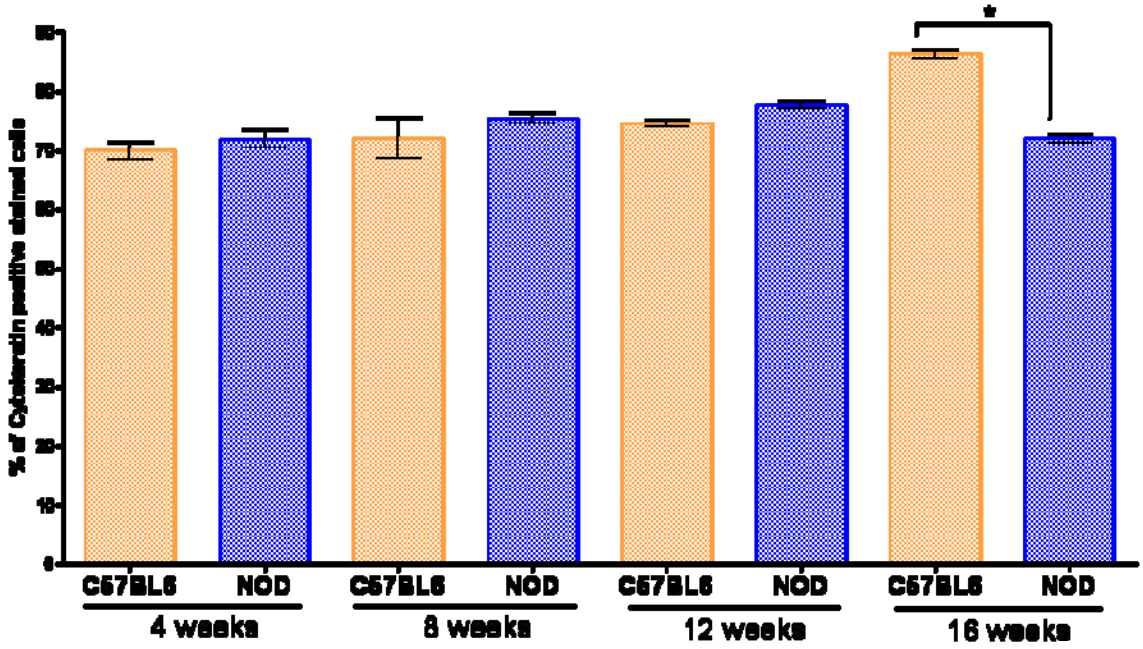

Figure 4-2. TLR4 protein expression was significantly decreased in NOD mice at 16 weeks of age (*p = 0.0003). FACS analyzed relative protein expression of TLR4 single positive GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

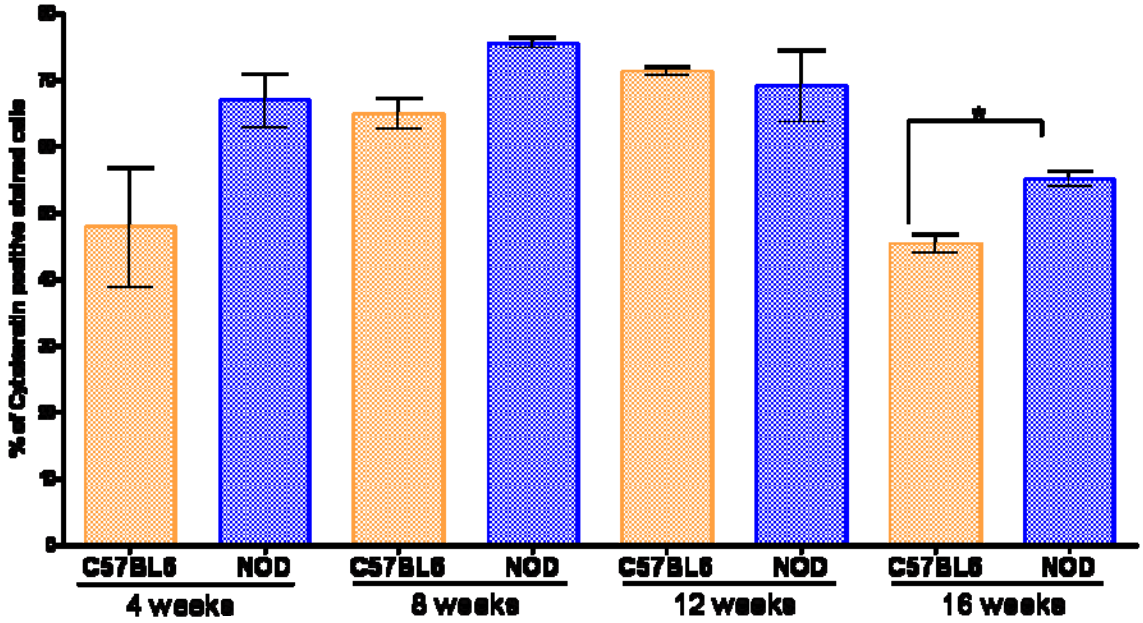

Figure 4-3. TLR2/4 double positive protein expression is significantly increased in NOD at 16 weeks of age (*p = 0.0063). FACS analyzed relative protein expression of TLR2 and TLR4 double positive GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

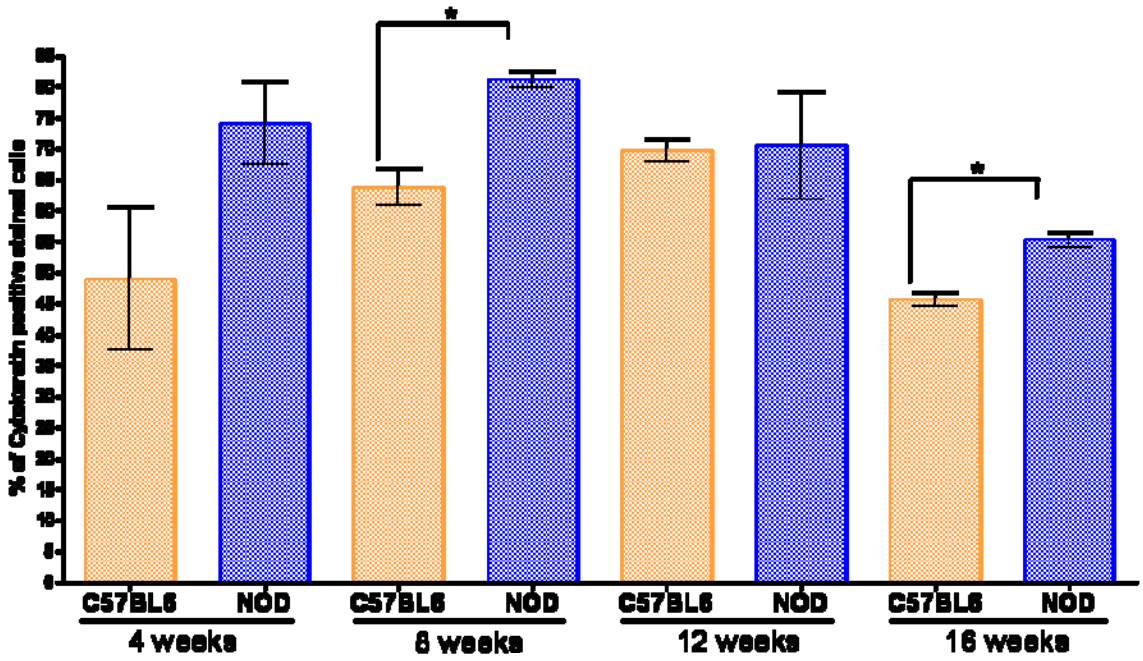

Figure 4-4. TLR1/2 double positive protein expression was significantly increased in NOD mice at both 8 and 16 weeks of age (*all $p < 0.0061$). FACS analyzed relative protein expression of both TLR1 and TLR2 double positive GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

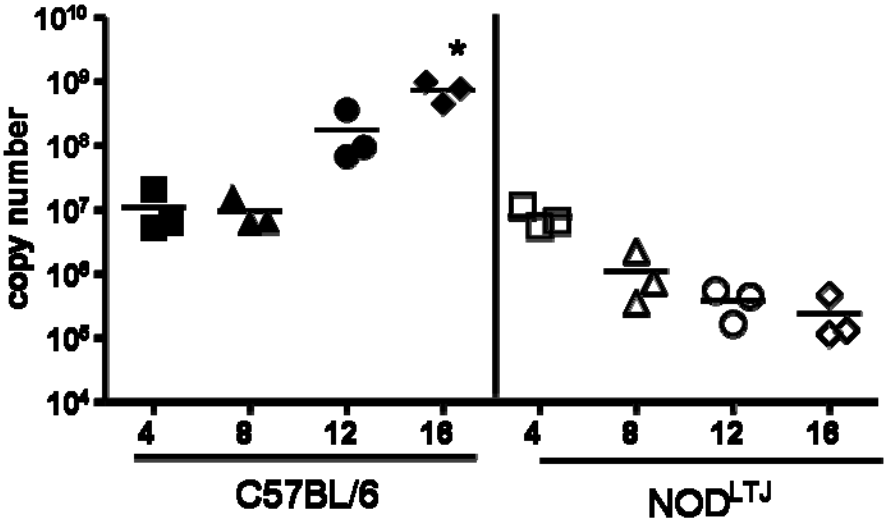

Figure 4-5. TLR2 gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (*p = 0.0093). qPCR analyzed relative gene expression of TLR2 on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

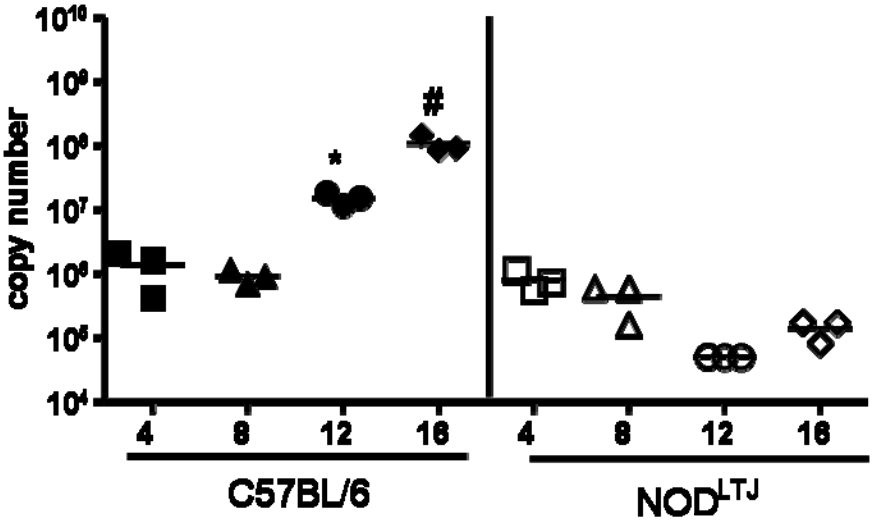

Figure 4-6. TLR4 gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (all $p < 0.0047$). qPCR analyzed relative gene expression of TLR4 on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

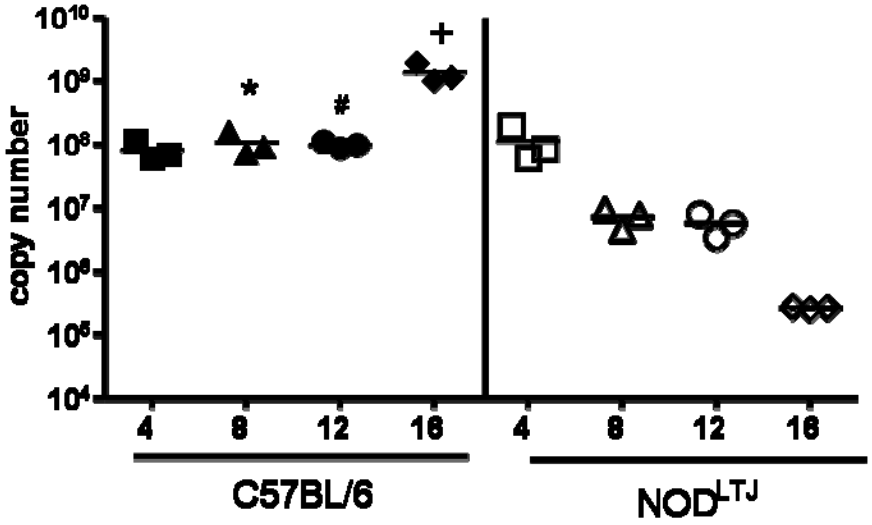

Figure 4-7. TLR1 gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (all $p < 0.00208$). qPCR analyzed relative gene expression of TLR1 on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

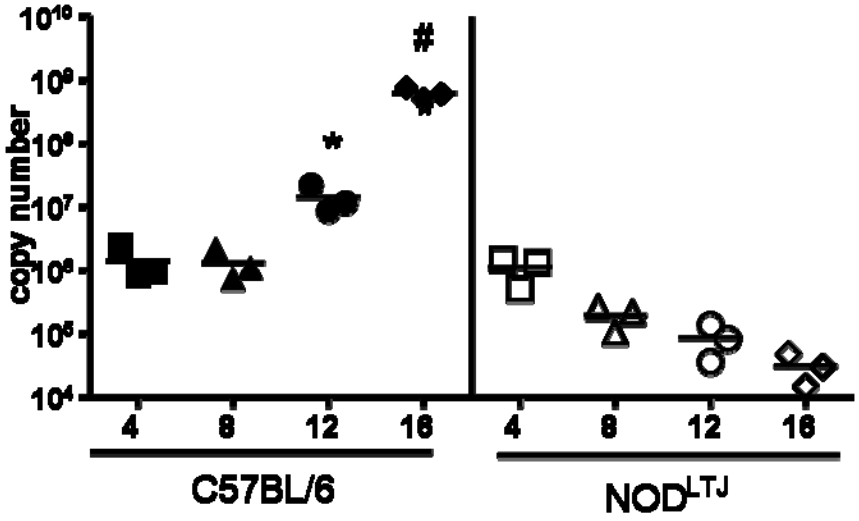

Figure 4-8. TLR6 gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (all $p < 0.0246$). qPCR analyzed relative gene expression of TLR6 on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

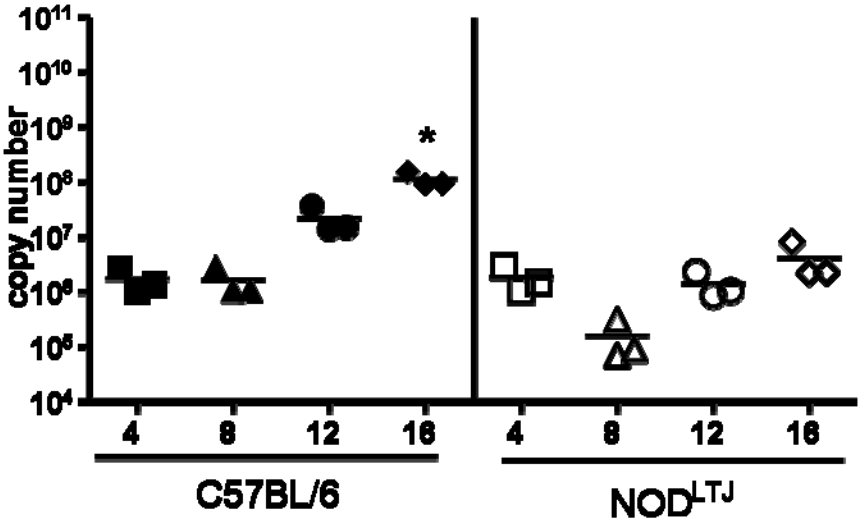

Figure 4-9. TLR9 gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (*p = 0.0048). qPCR analyzed relative gene expression of TLR9 on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

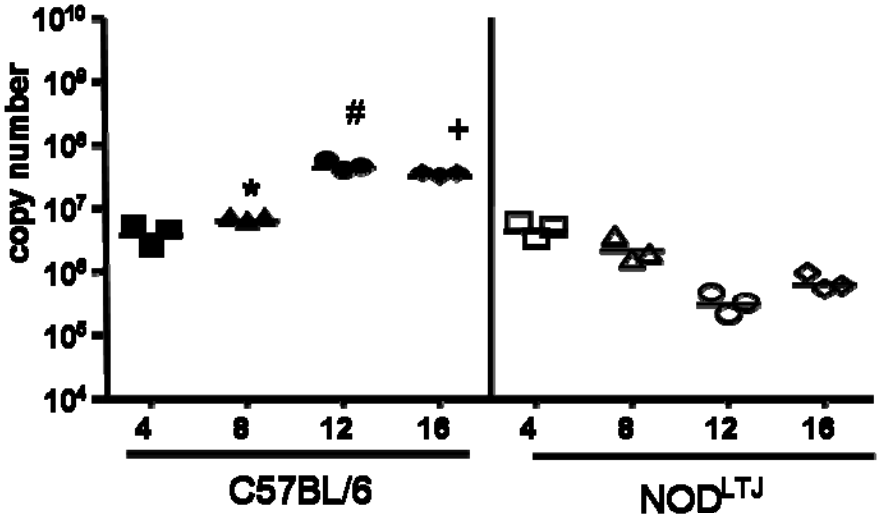

Figure 4-10. RAGE gene expression in C57BL/6 mice increases over time and decreases in NOD mice overtime (all $p = 0.0026$). qPCR analyzed relative gene expression of RAGE on GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age.

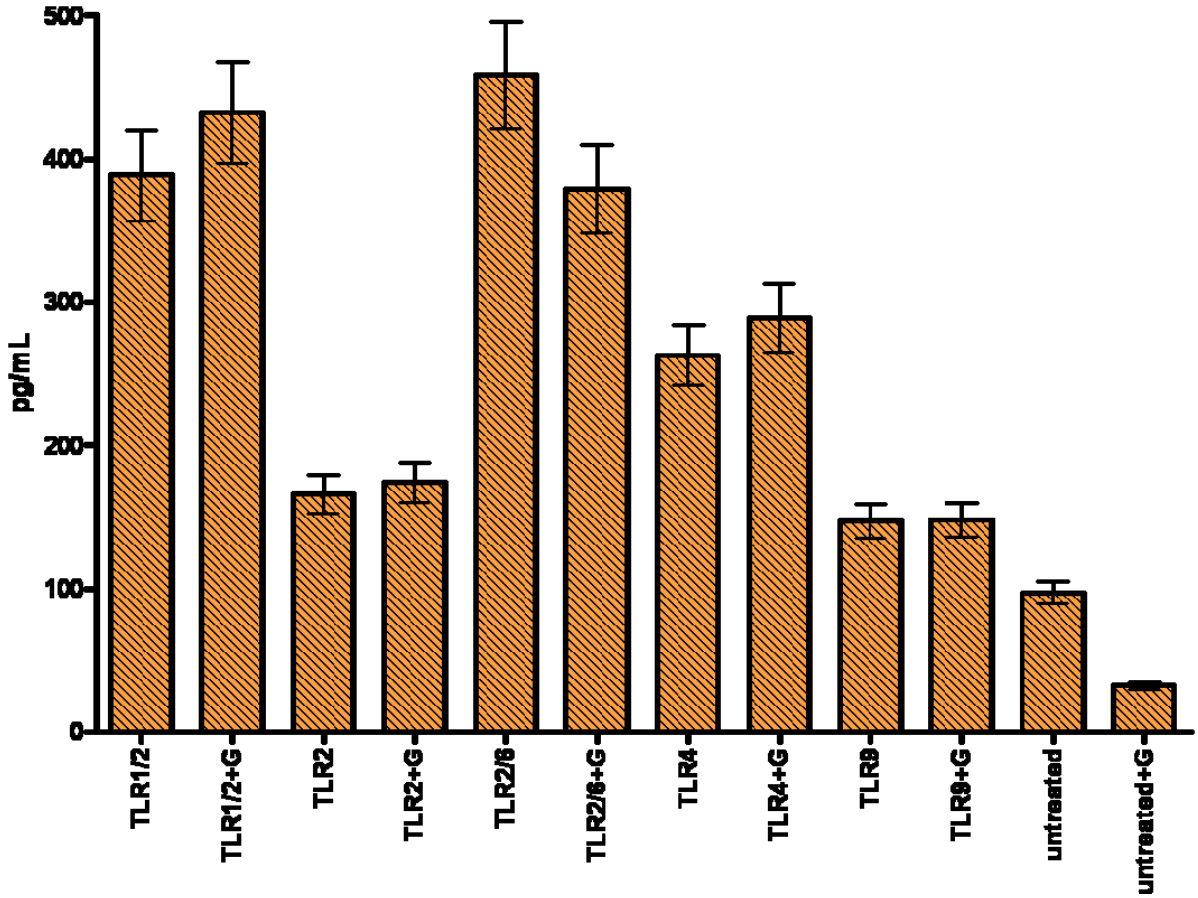

Figure 4-11. Hyperglycemic conditions induce GMCSF pro-inflammatory cytokine secretion in response to TLR ligation. Luminex® 100™ System analyzed HOK secretion levels of GMCSF pro-inflammatory cytokine in response to TLR ligation and HOK section levels of GMCSF in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

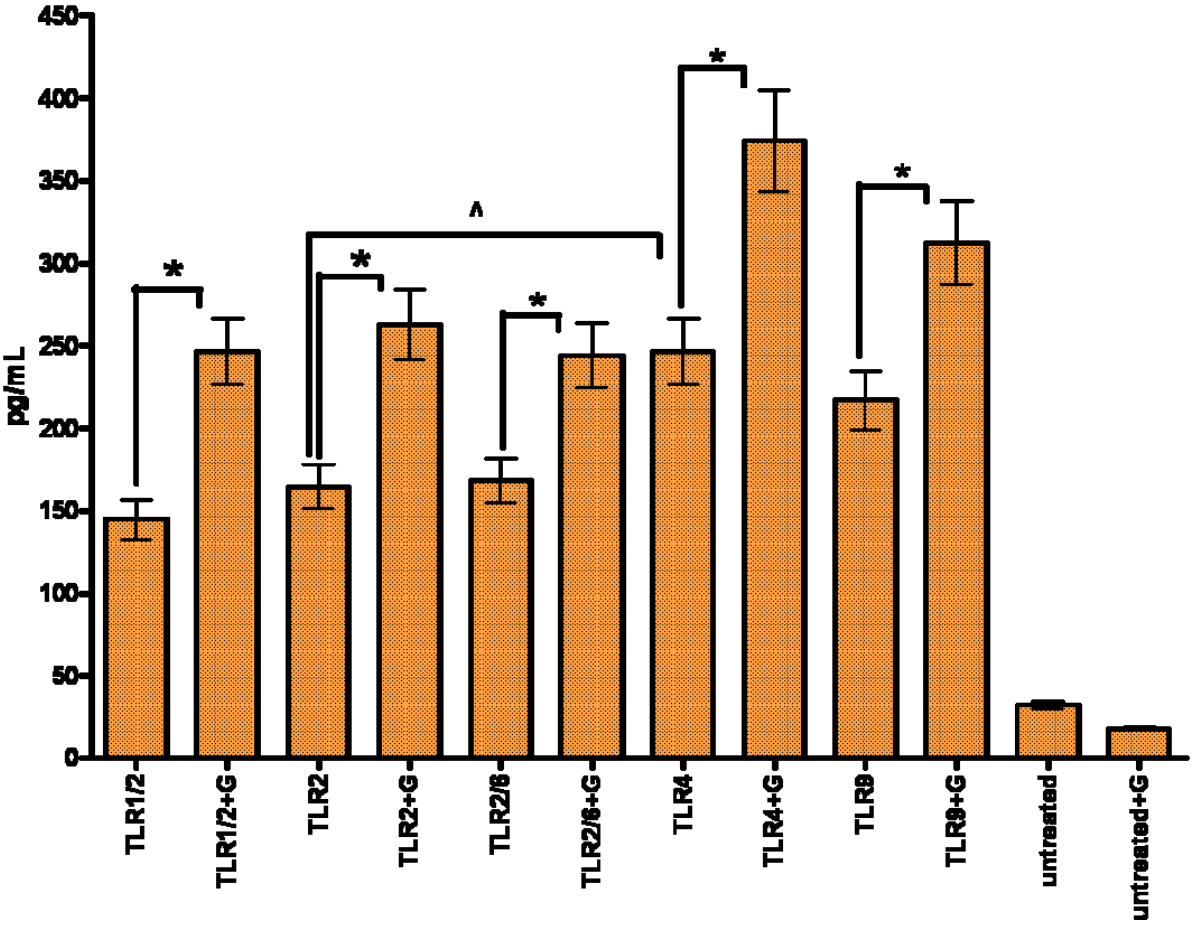

Figure 4-12. Hyperglycemic conditions induce IL-1β pro-inflammatory cytokine secretion in response to TLR ligation (*all $p<0.0274$; ^$p = 0.0218$). Luminex® 100™ System analyzed HOK secretion levels of IL-1β pro-inflammatory cytokine in response to TLR ligation and HOK section levels of IL-1β in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

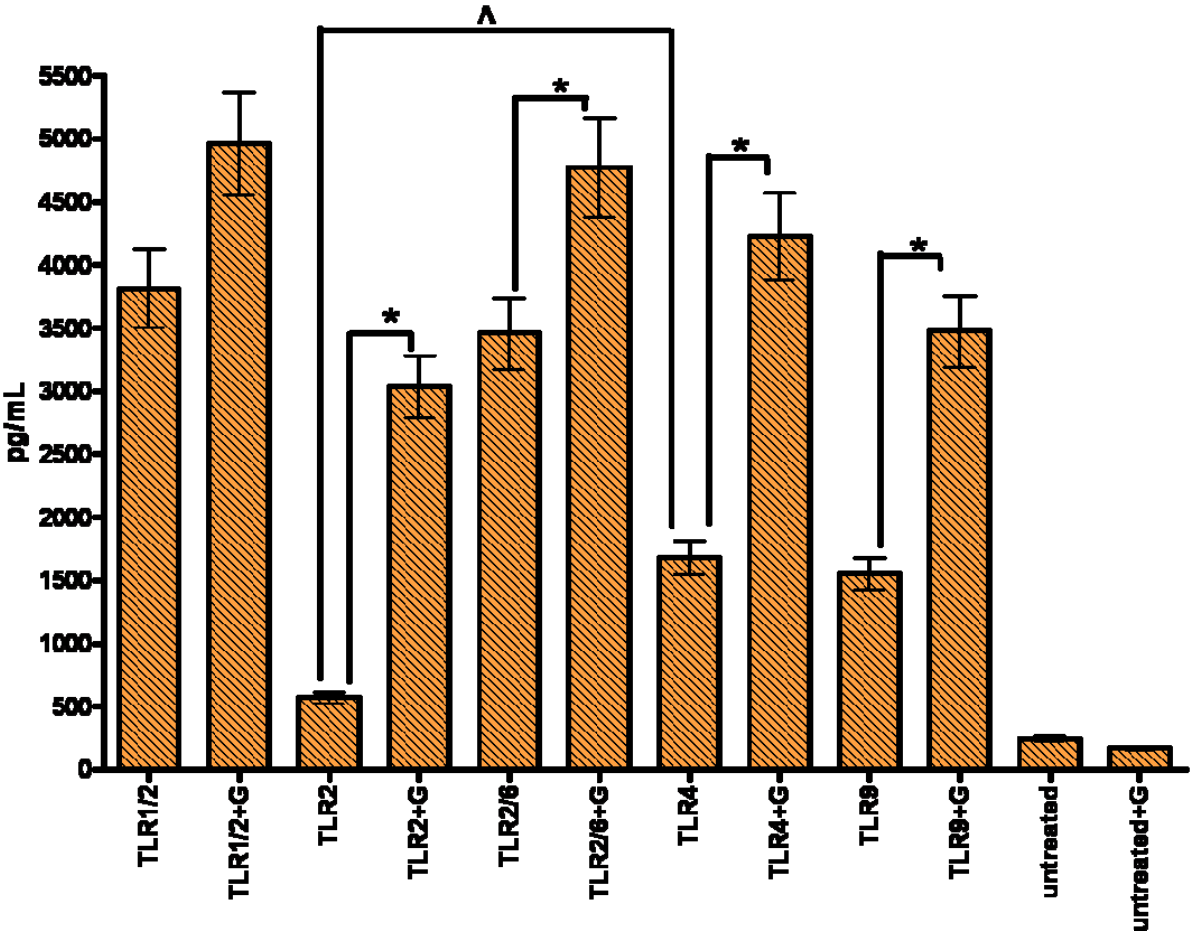

Figure 4-13. Hyperglycemic conditions induce IL-6 pro-inflammatory cytokine secretion in response to TLR ligation (*all $p < 0.363$; ^$p = 0.0045$). Luminex® 100™ System analyzed HOK secretion levels of IL-6 pro-inflammatory cytokine in response to TLR ligation and HOK section levels of IL-6 in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

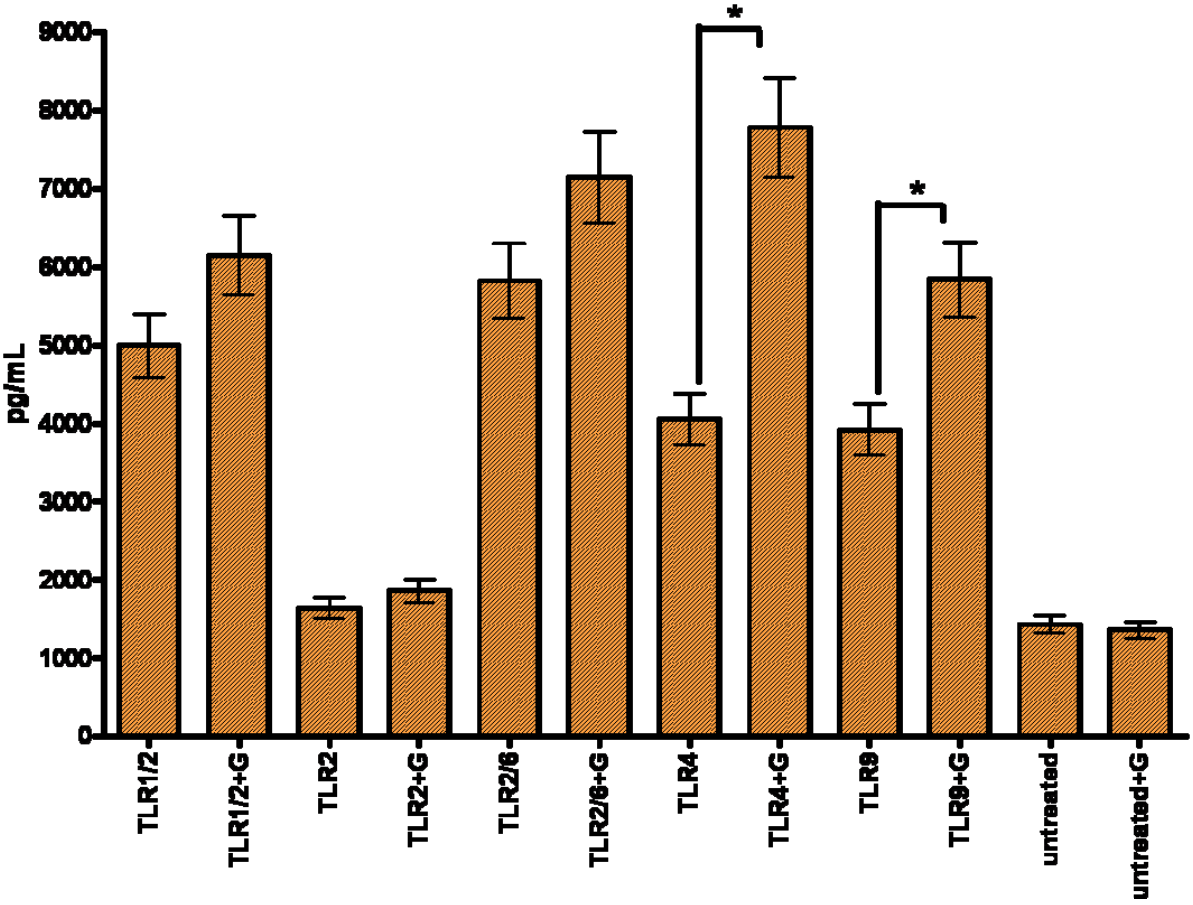

Figure 4-14. Hyperglycemic conditions induce IL-8 pro-inflammatory cytokine secretion in response to TLR ligation (*all $p < 0.0222$). Luminex® 100™ System analyzed HOK secretion levels of IL-8 pro-inflammatory cytokine in response to TLR ligation and HOK section levels of IL-8 in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

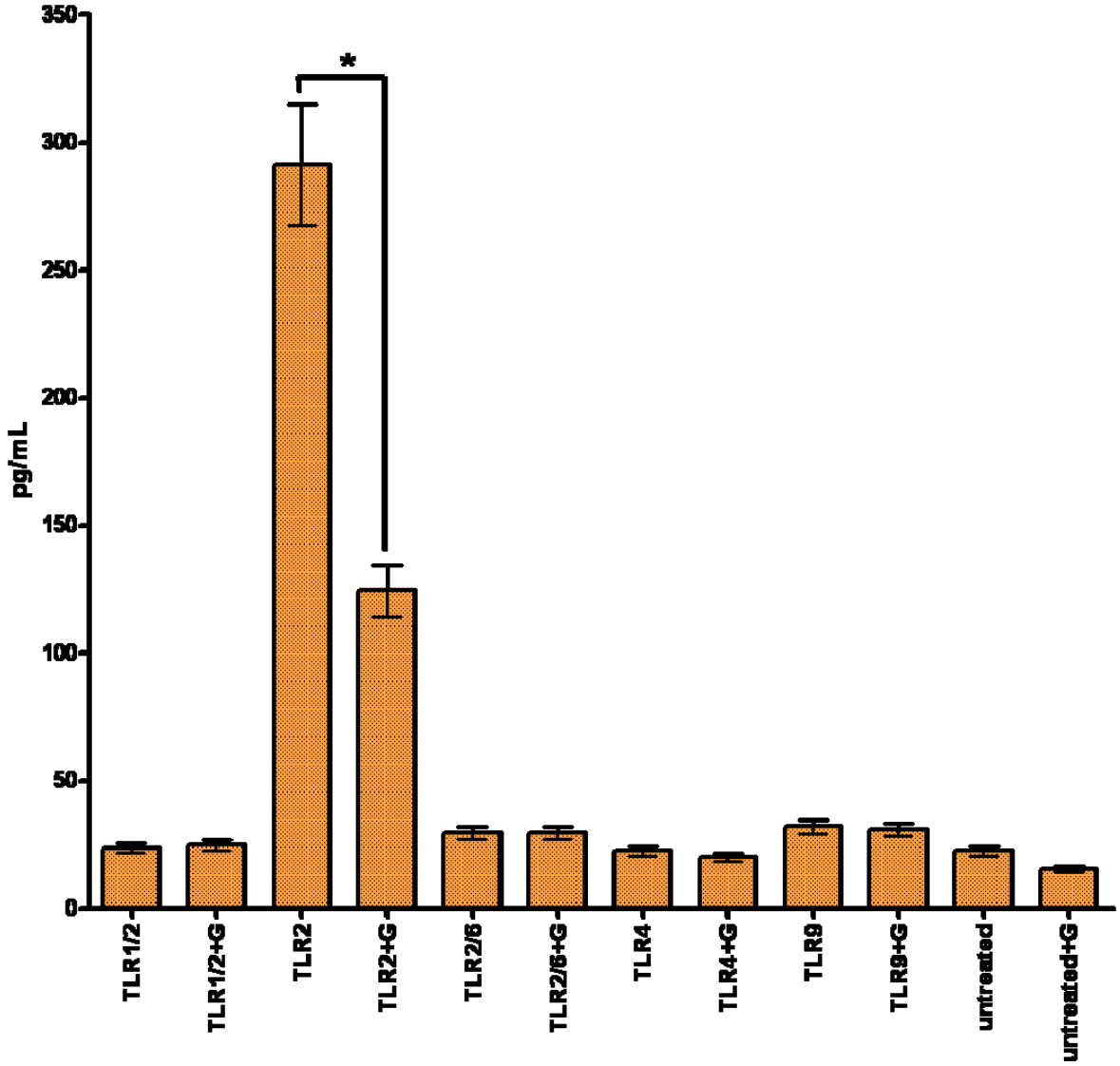

Figure 4-15. Hyperglycemic conditions inhibit IL-10 anti-inflammatory cytokine secretion in response to TLR ligation (*p = 0.0117). Luminex® 100™ System analyzed HOK secretion levels of IL-10 anti-inflammatory cytokine in response to TLR ligation and HOK section levels of IL-10 in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

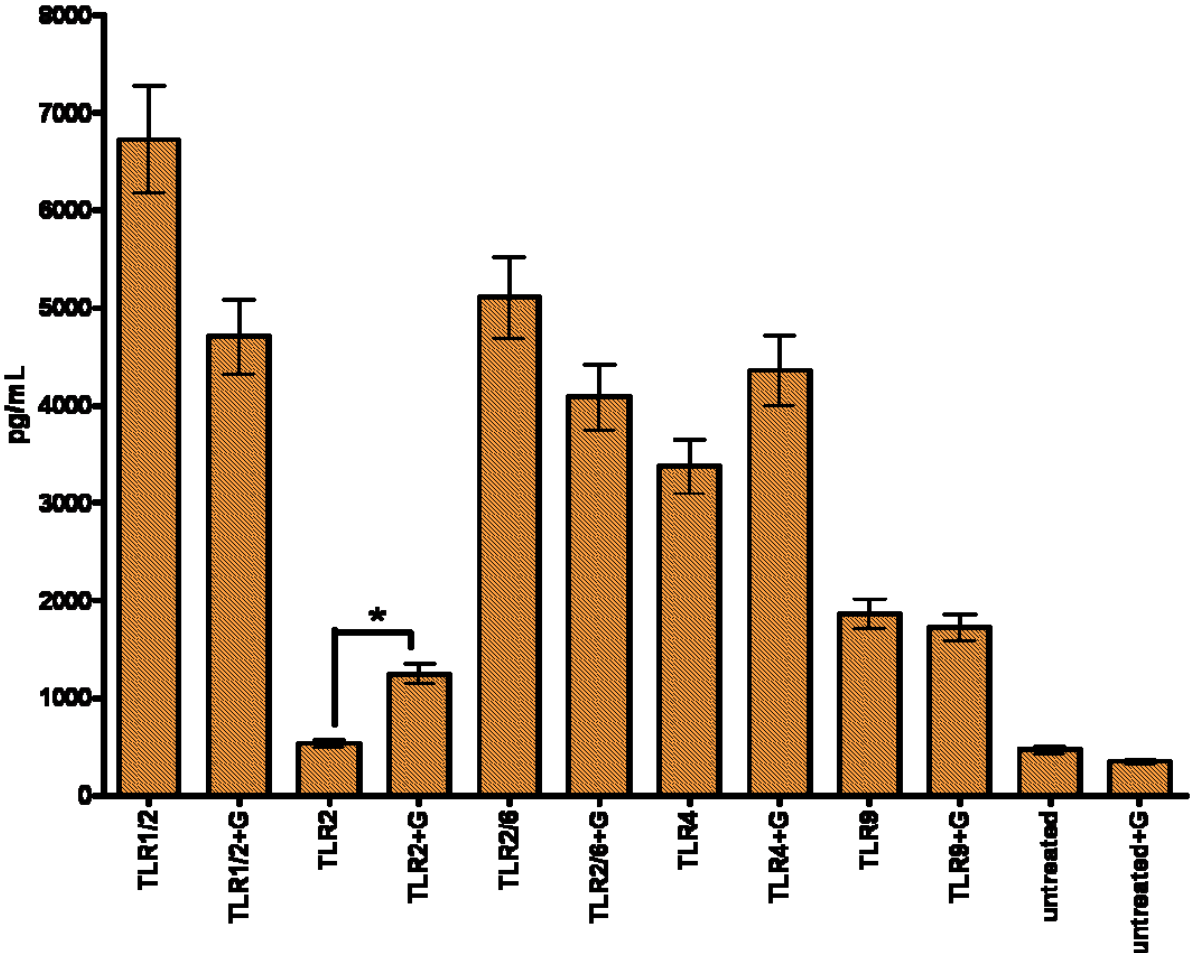

Figure 4-16. Hyperglycemic conditions induce IP-10 pro-inflammatory chemokine secretion in response to TLR ligation (*p = 0.0118). Luminex® 100™ System analyzed HOK secretion levels of IP-10 pro-inflammatory chemokine in response to TLR ligation and HOK section levels of IP-10 in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

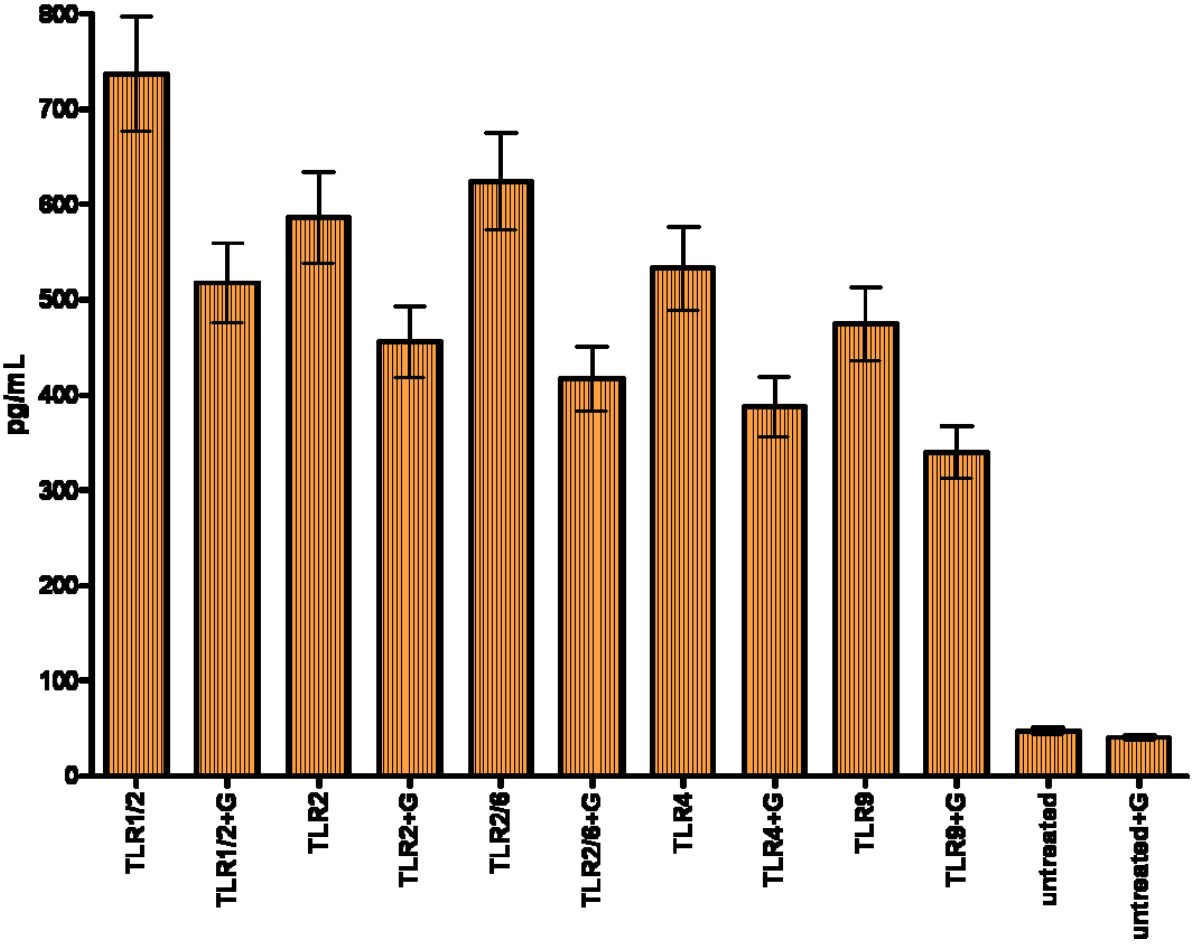

Figure 4-17. Hyperglycemic conditions did not induce MCP-1 pro-inflammatory chemokine secretion in response to TLR ligation. Luminex® 100™ System analyzed HOK secretion levels of MCP-1 pro-inflammatory chemokine in response to TLR ligation and HOK section levels of MCP-1 in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

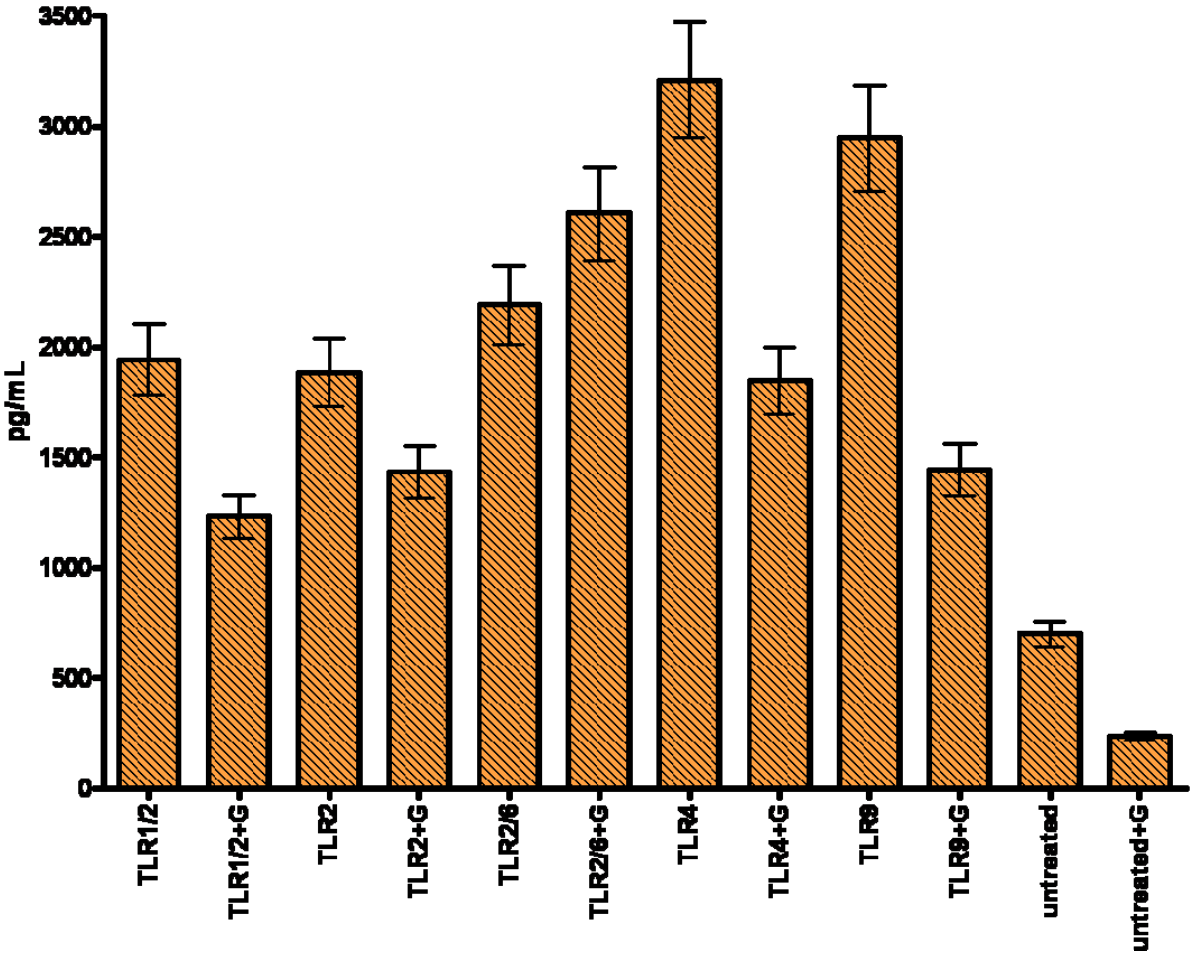

Figure 4-18. Hyperglycemic conditions did not induce MIP-1α pro-inflammatory cytokine secretion in response to TLR ligation. Luminex® 100™ System analyzed HOK secretion levels of MIP-1α pro-inflammatory cytokine in response to TLR ligation and HOK section levels of MIP-1α in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

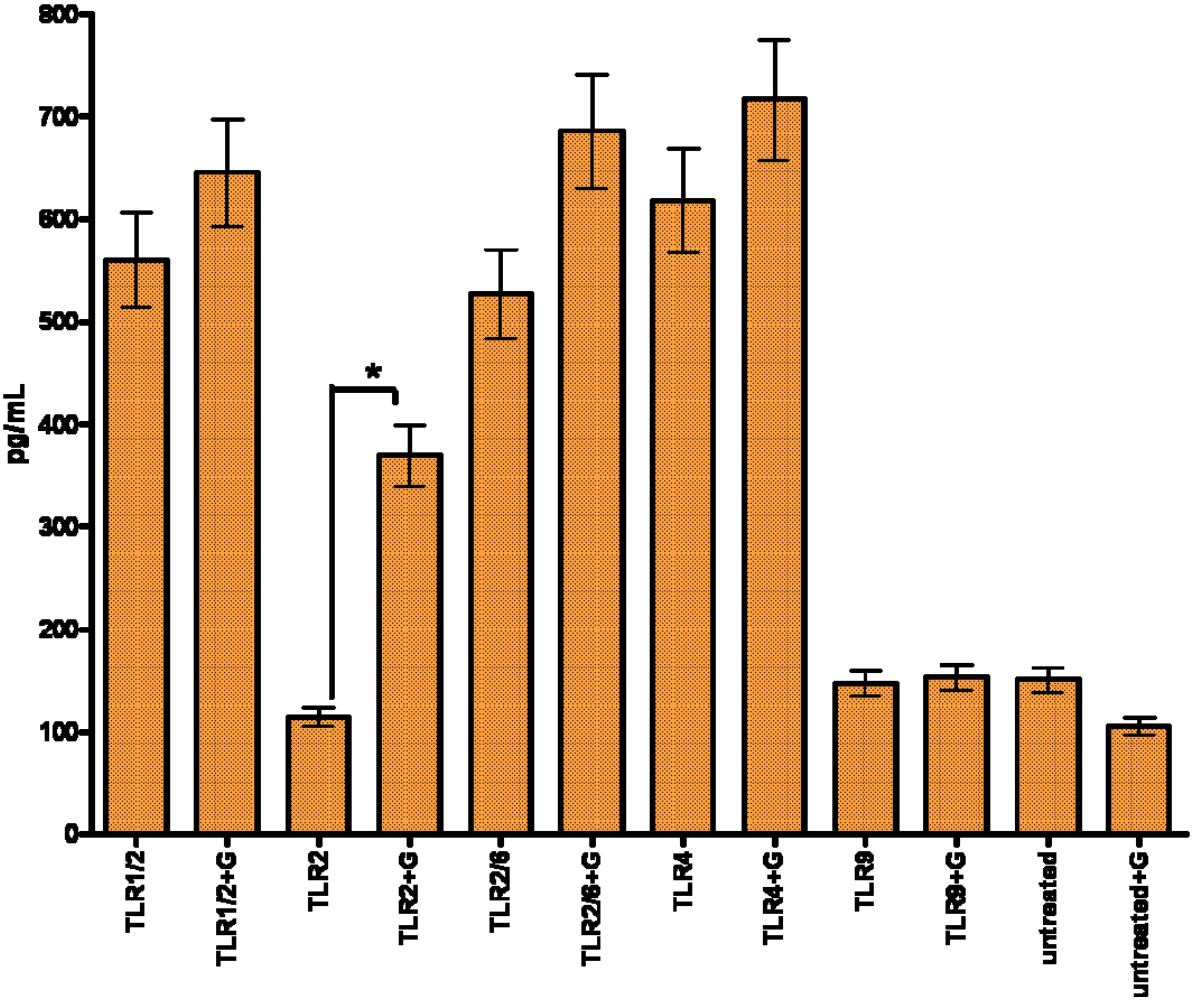

Figure 4-19. Hyperglycemic conditions induce TNF-α pro-inflammatory cytokine secretion in response to TLR ligation (*p = 0.0075). Luminex® 100™ System analyzed HOK secretion levels of TNF-α pro-inflammatory cytokine in response to TLR ligation and HOK section levels of TNF-α in response to non-stimulated TLR in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

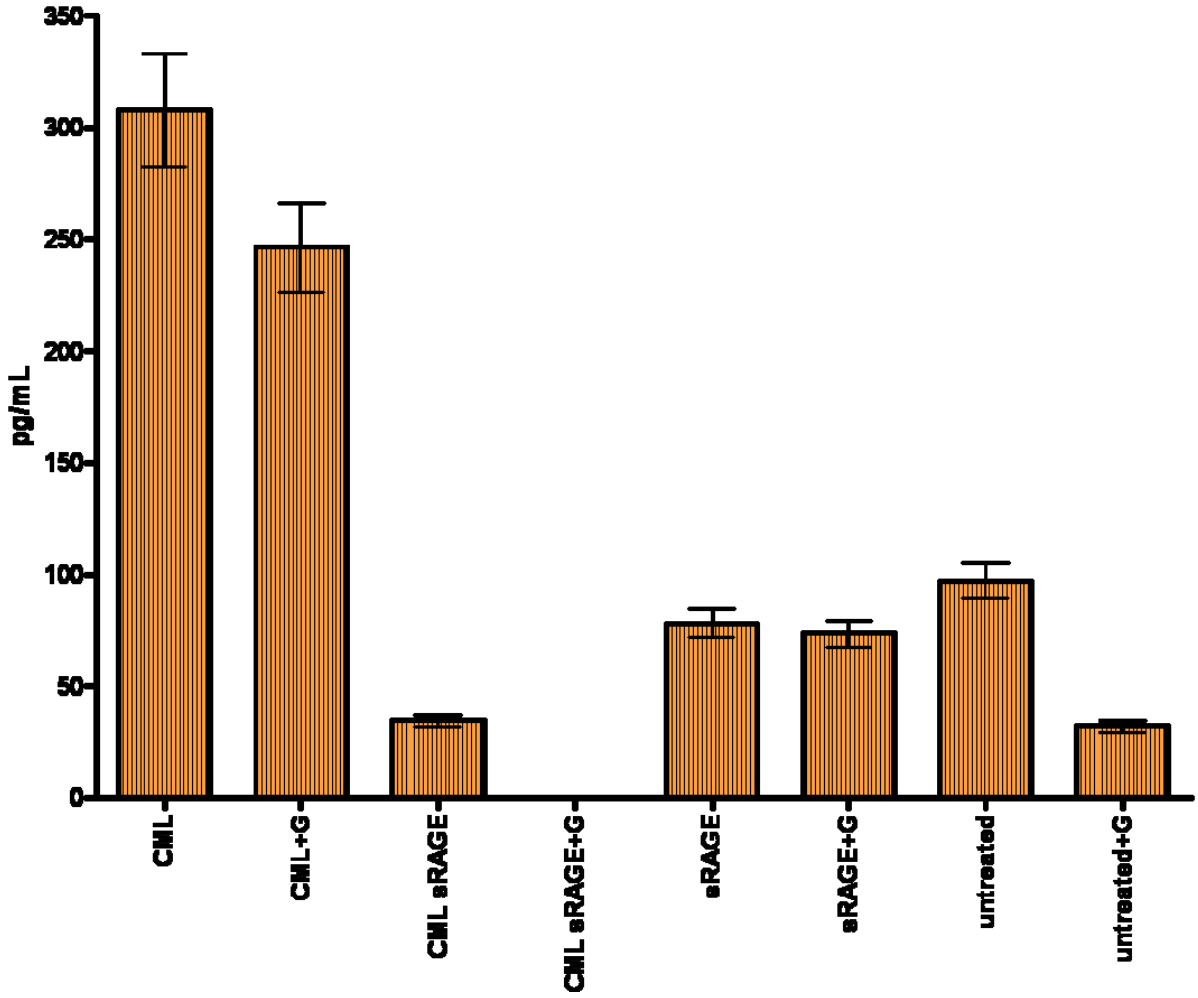

Figure 4-20. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation. Luminex® 100™ System analyzed HOK secretion levels of GMCSF pro-inflammatory cytokine in response to RAGE ligation and HOK section levels of GMCSF in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

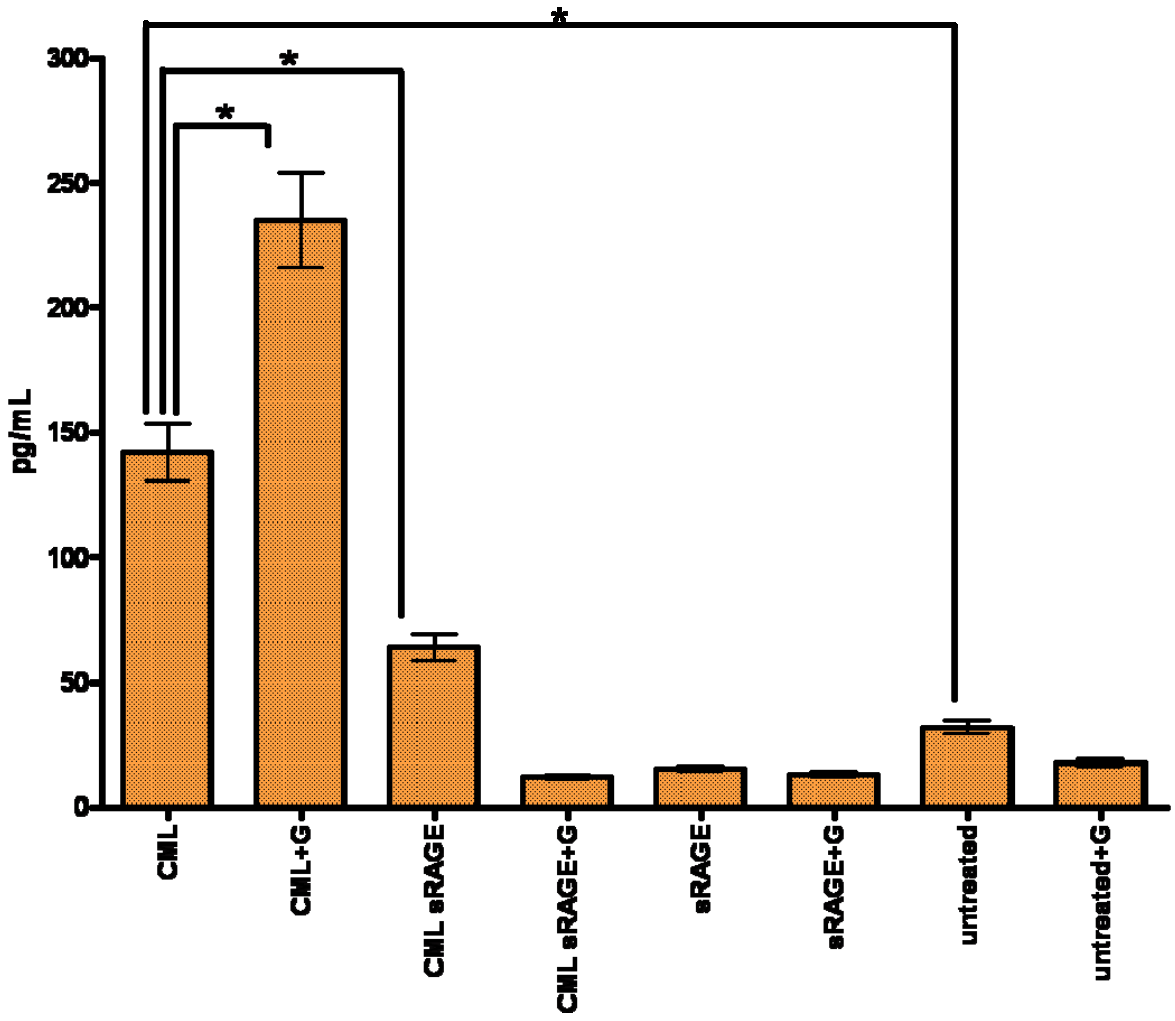

Figure 4-21. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0218$). Luminex® 100™ System analyzed HOK secretion levels of IL-1β pro-inflammatory cytokine in response to RAGE ligation and HOK section levels of IL-1β in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

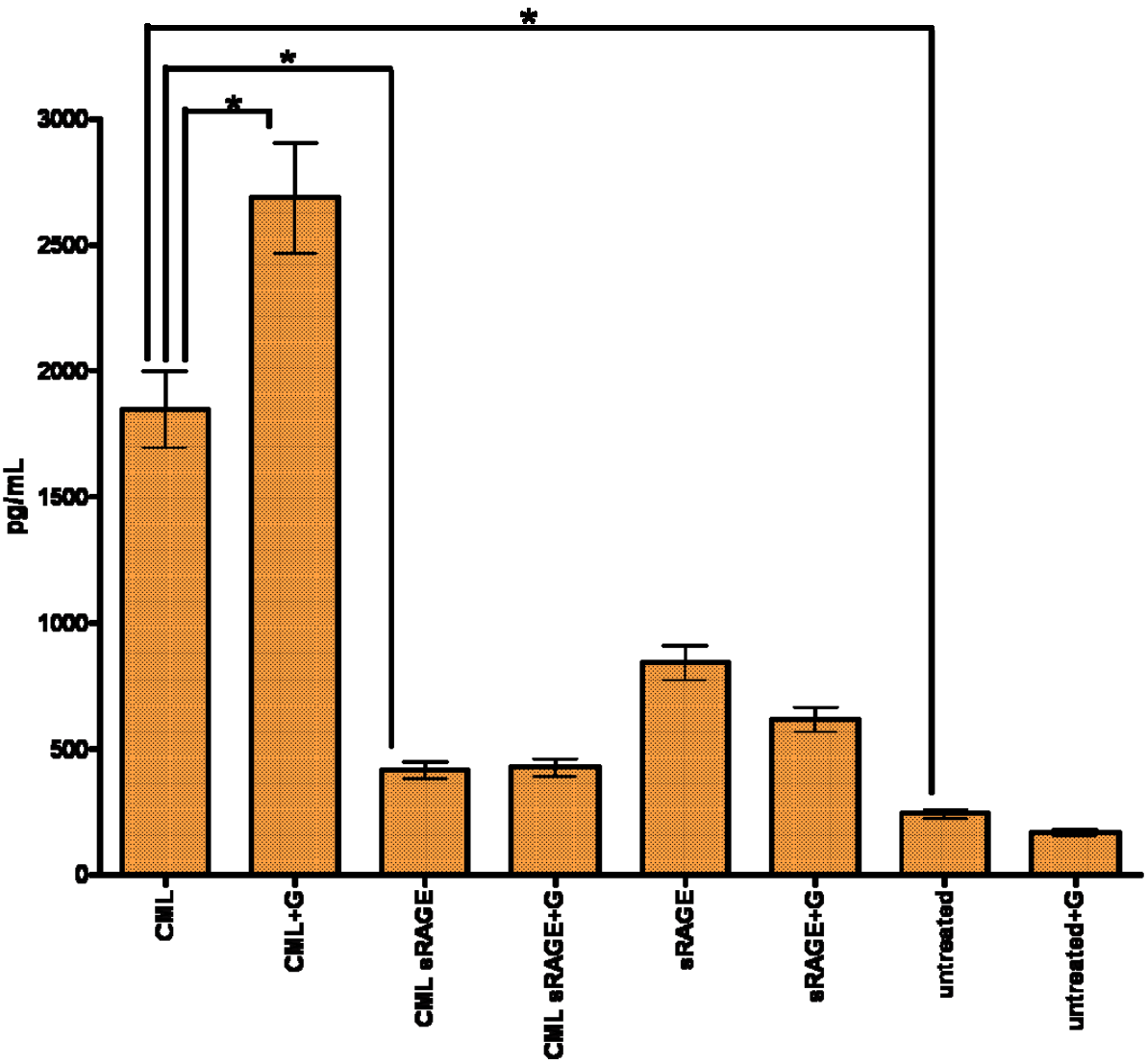

Figure 4-22. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0058$). Luminex® 100™ System analyzed HOK secretion levels of IL-6 pro-inflammatory cytokine in response to RAGE ligation and HOK section levels of IL-6 in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

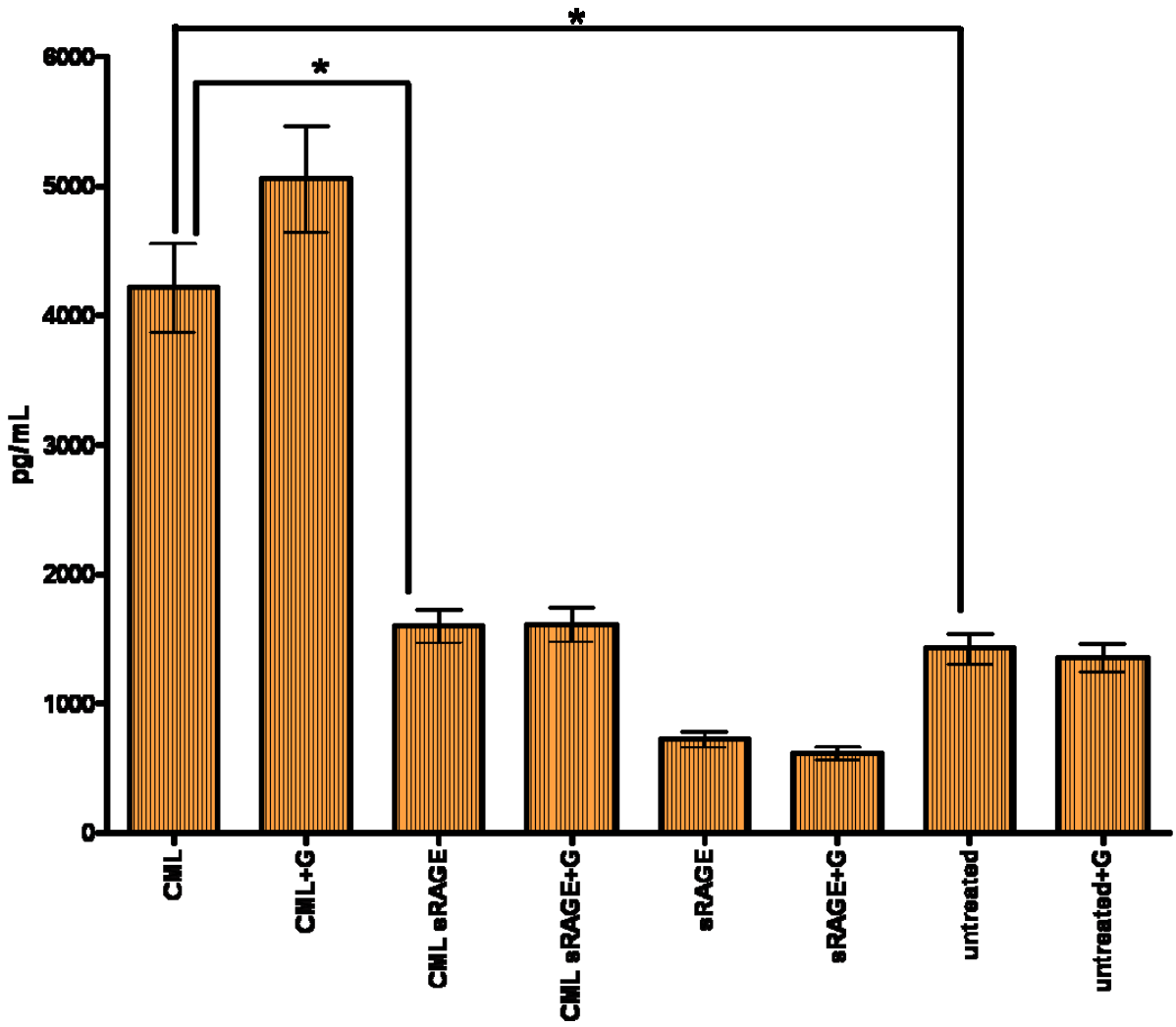

Figure 4-23. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0097$). Luminex® 100™ System analyzed HOK secretion levels of IL-8 pro-inflammatory cytokine in response to RAGE ligation and HOK section levels of IL-8 in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

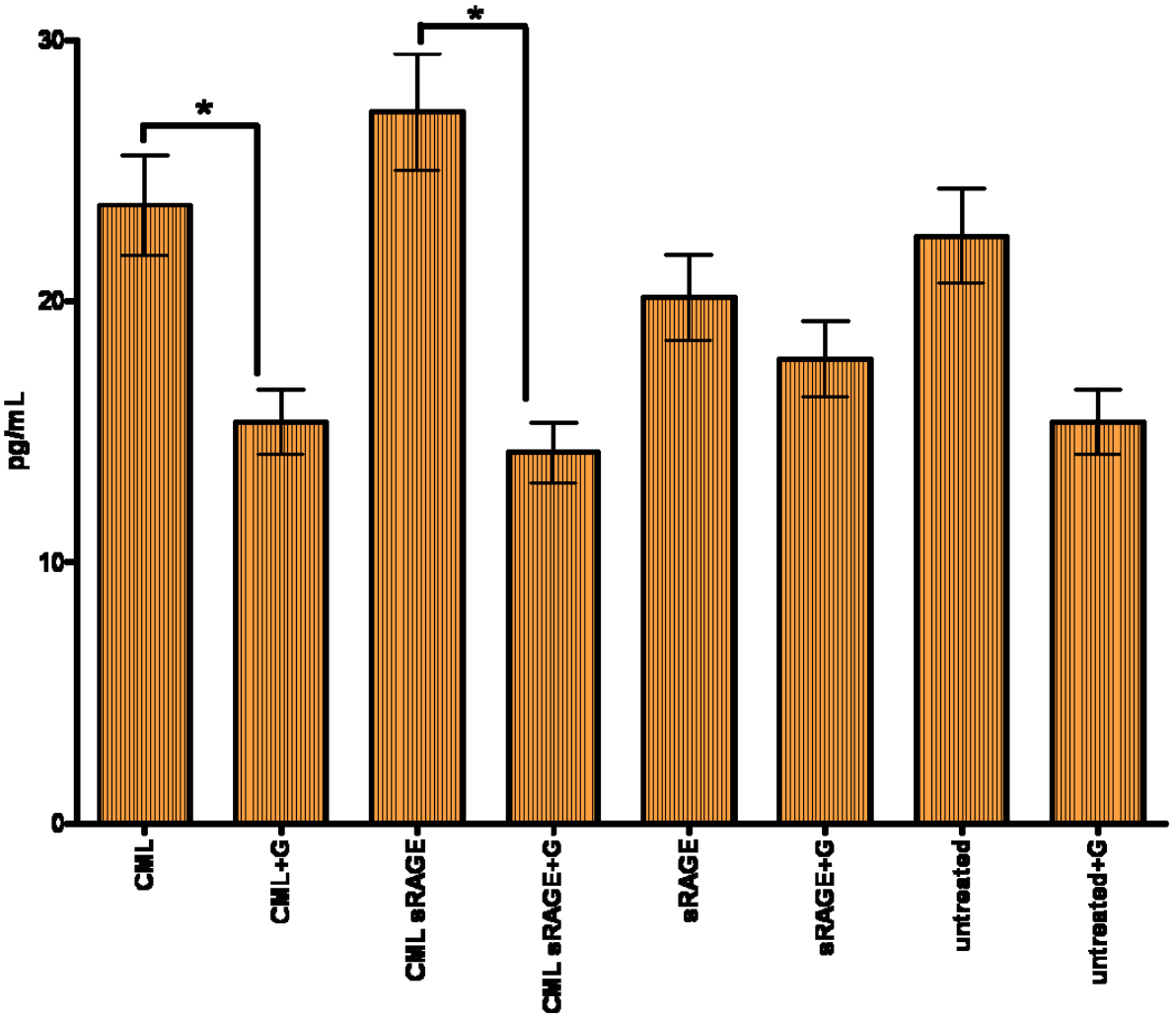

Figure 4-24. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (* all $p < 0.0186$). Luminex® 100™ System analyzed HOK secretion levels of IL-10 anti-inflammatory cytokine in response to RAGE ligation and HOK section levels of IL-10 in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

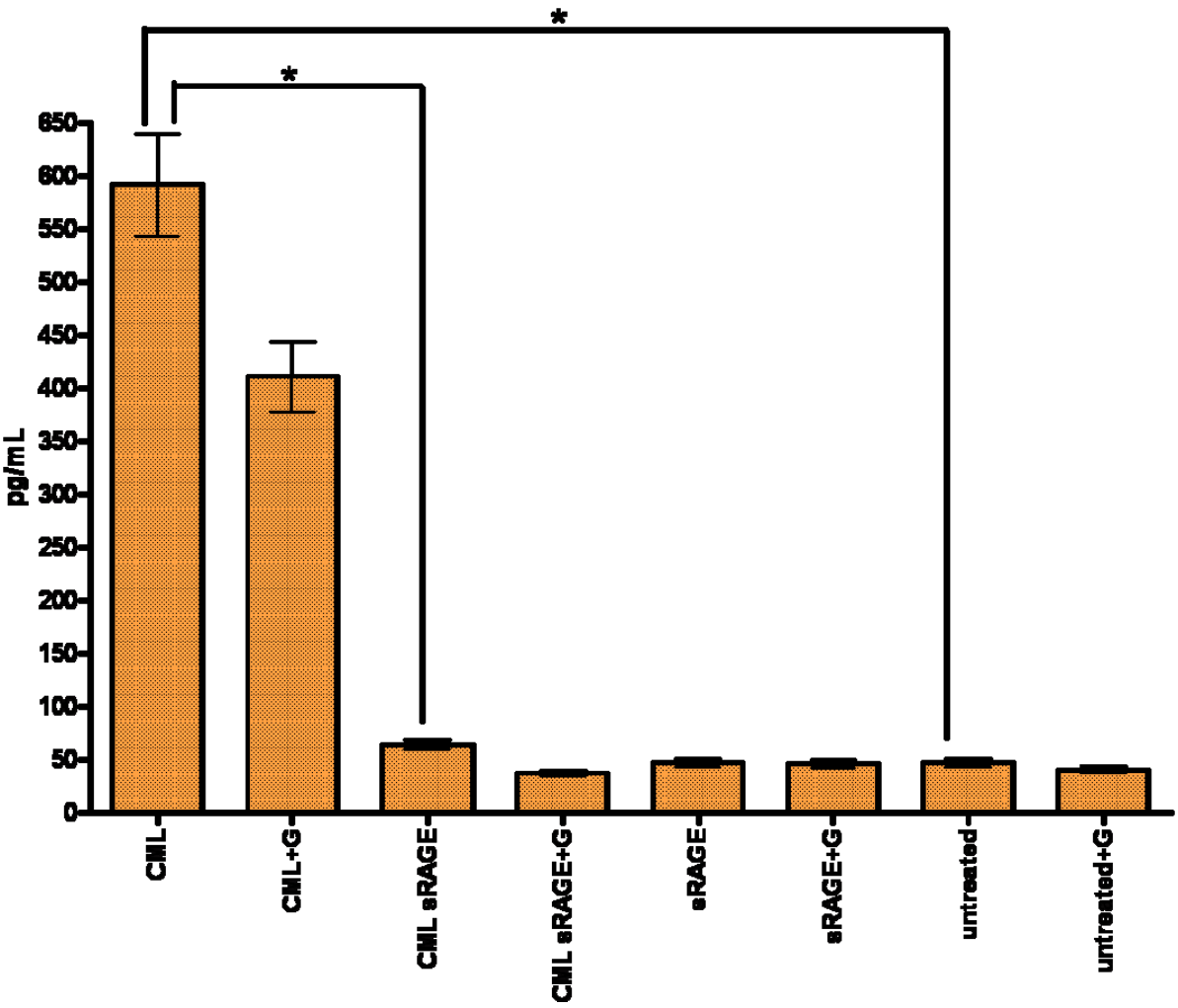

Figure 4-25. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0274$). Luminex® 100™ System analyzed HOK secretion levels of MCP-1 pro-inflammatory chemokine in response to RAGE ligation and HOK section levels of MCP-1 in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

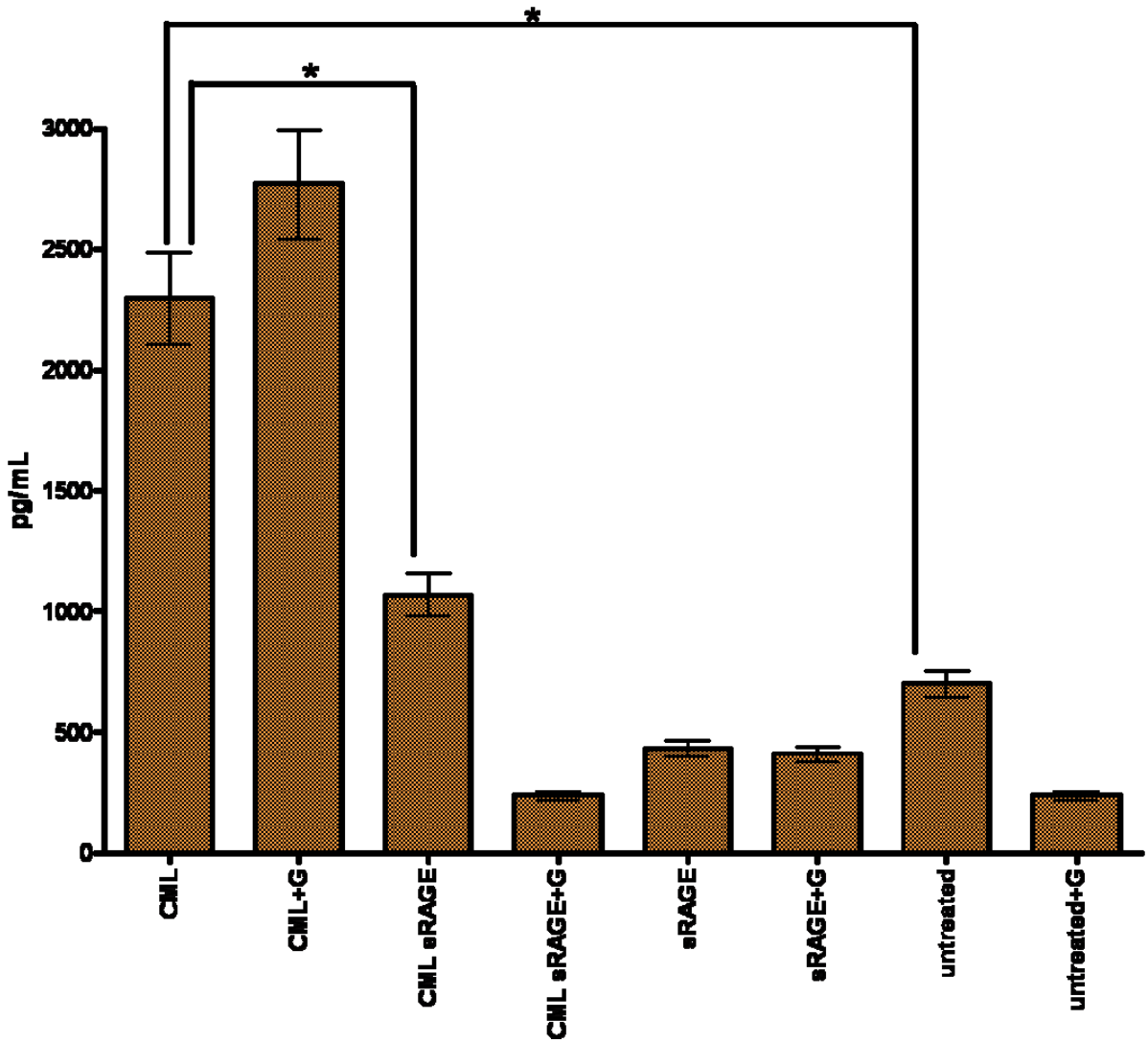

Figure 4-26. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0137$). Luminex® 100™ System analyzed HOK secretion levels of MIP-1α pro-inflammatory chemokine in response to RAGE ligation and HOK section levels of MIP-1α in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

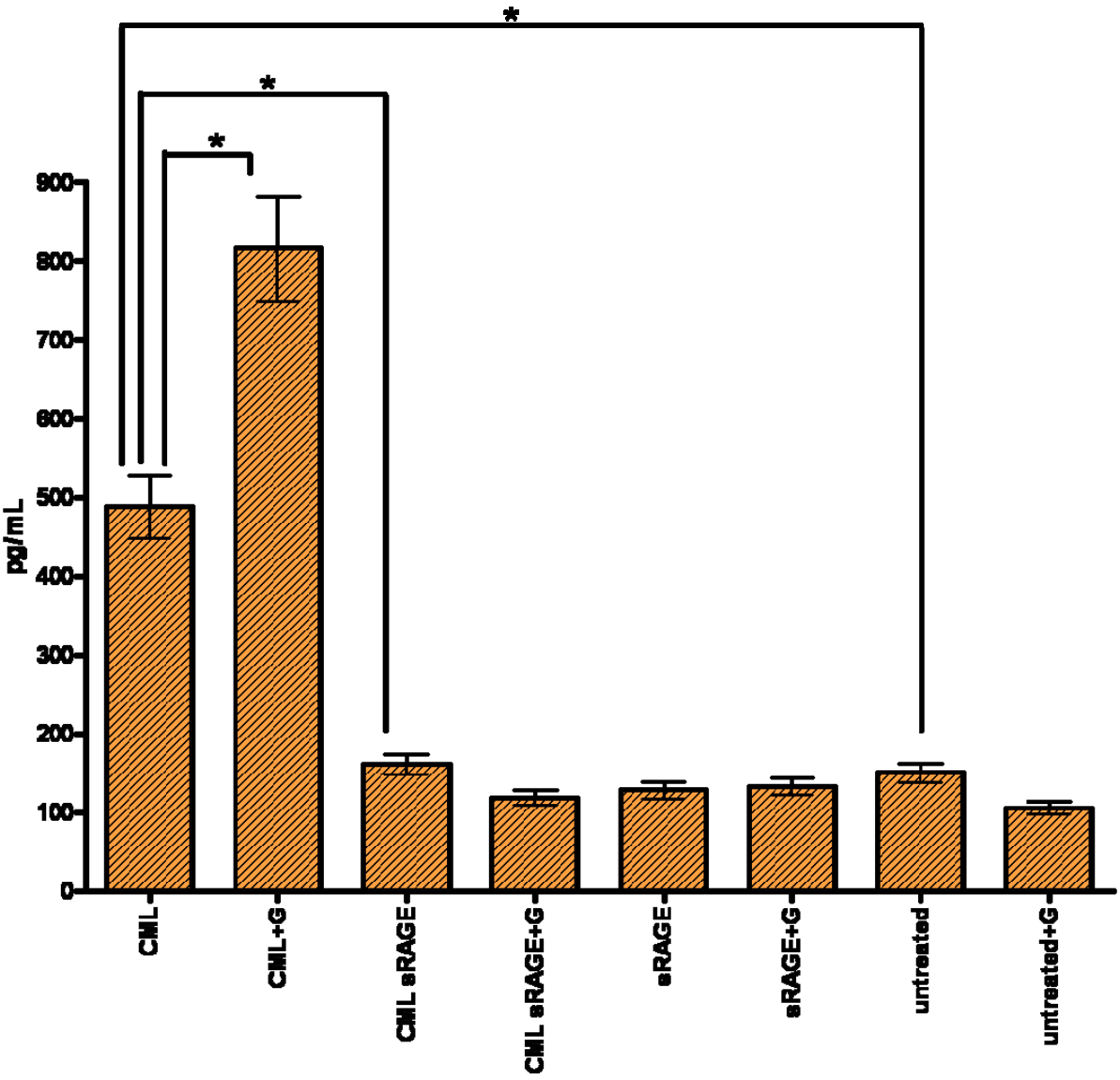

Figure 4-27. Hyperglycemic conditions induce pro-inflammatory cytokine and chemokine secretion in response to RAGE ligation (*all $p < 0.0122$). Luminex® 100™ System analyzed HOK secretion levels of TNF-α pro-inflammatory cytokine in response to RAGE ligation and HOK section levels of TNF-α in response to non-stimulated RAGE and RAGE blocking with sRAGE in both hyperglycemic and normoglycemic conditions (G = 15mM glucose).

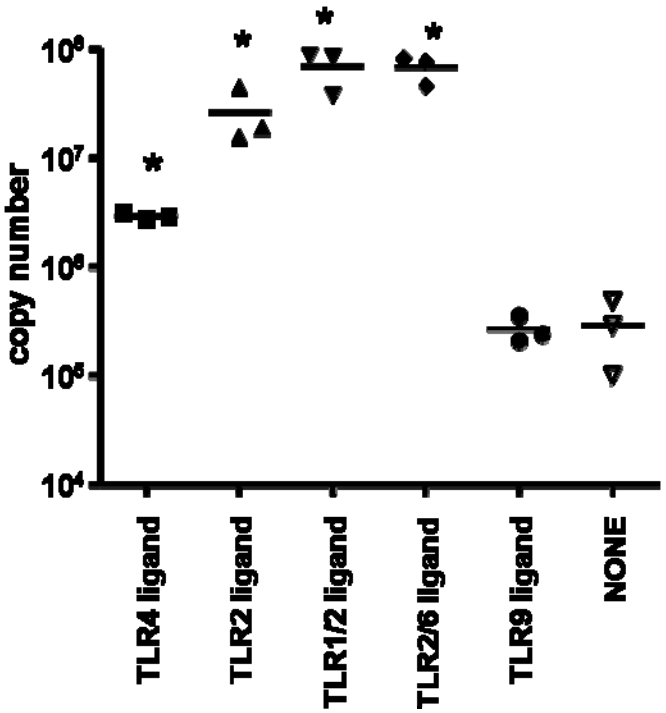

Figure 4-28. Stimulation with TLR ligands increases TLR2 gene expression (*all $p < 0.0251$). qPCR analyzed relative gene expression of TLR2 on HOK in response to TLR ligation and non-stimulation.

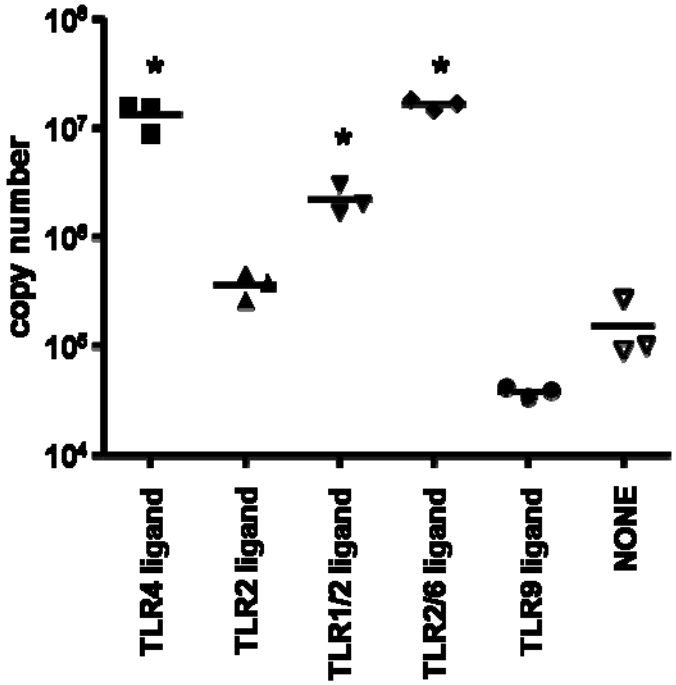

Figure 4-29. Stimulation with TLR ligands increases TLR4 gene expression (*all $p < 0.0180$). qPCR analyzed relative gene expression of TLR4 on HOK in response to TLR ligation and non-stimulation.

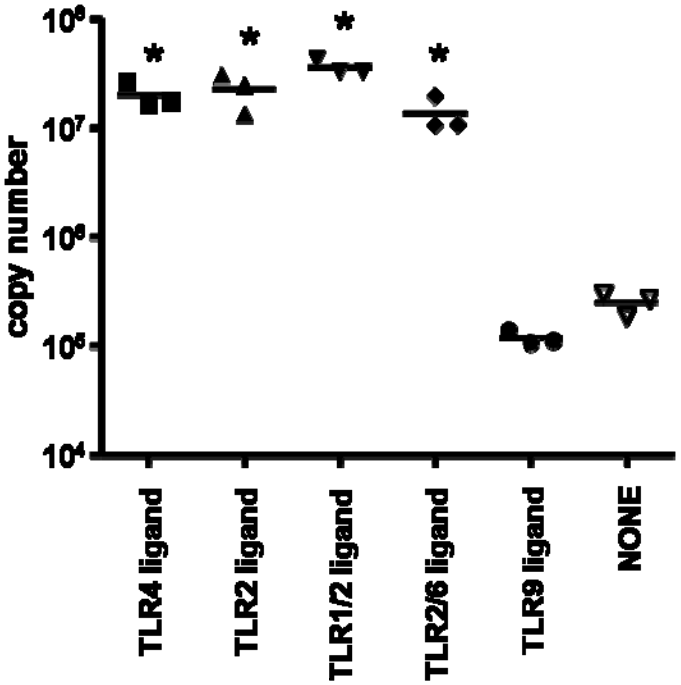

Figure 4-30. Stimulation with TLR ligands increases TLR1 gene expression (*all $p < 0.0228$). qPCR analyzed relative gene expression of TLR1 on HOK in response to TLR ligation and non-stimulation.

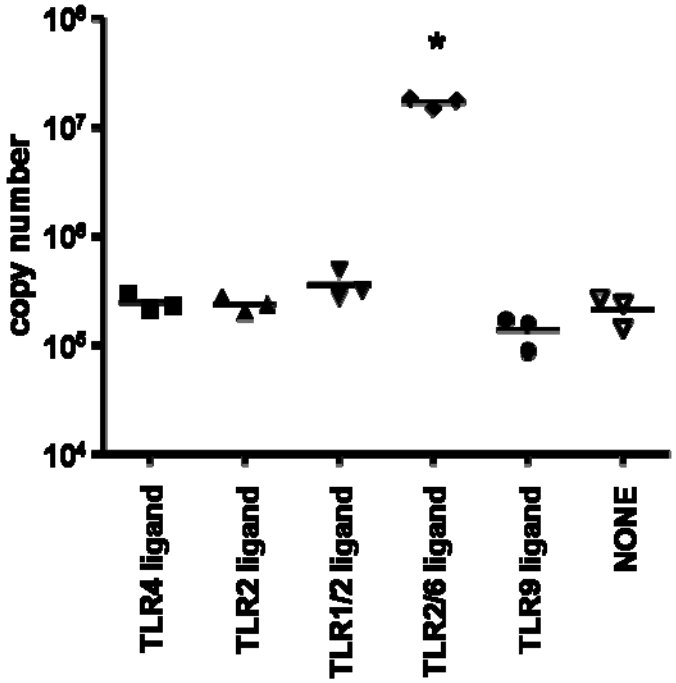

Figure 4-31. Stimulation with TLR ligands increases TLR gene expression (*p = 0.0081). qPCR analyzed relative gene expression of TLR6 on HOK in response to TLR ligation and non-stimulation.

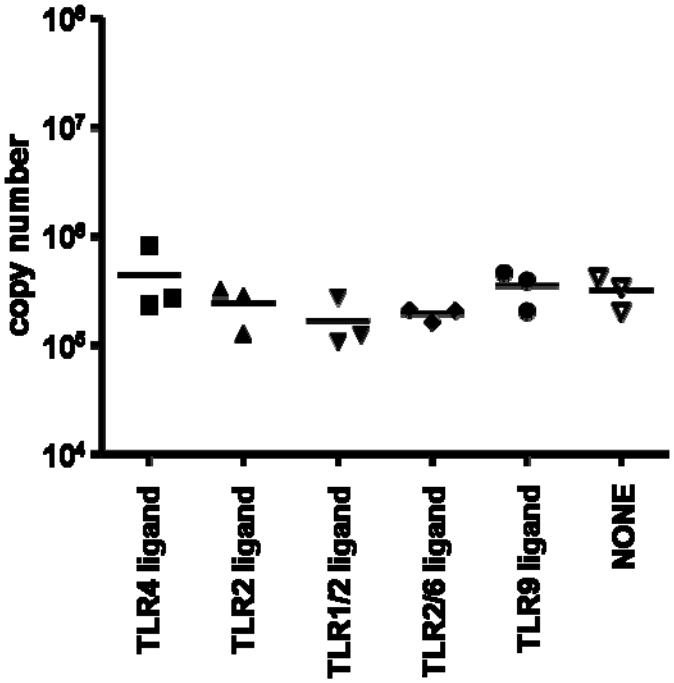

Figure 4-32. Stimulation with TLR ligands increases TLR gene expression. qPCR analyzed relative gene expression of TLR9 on HOK in response to TLR ligation and non-stimulation.

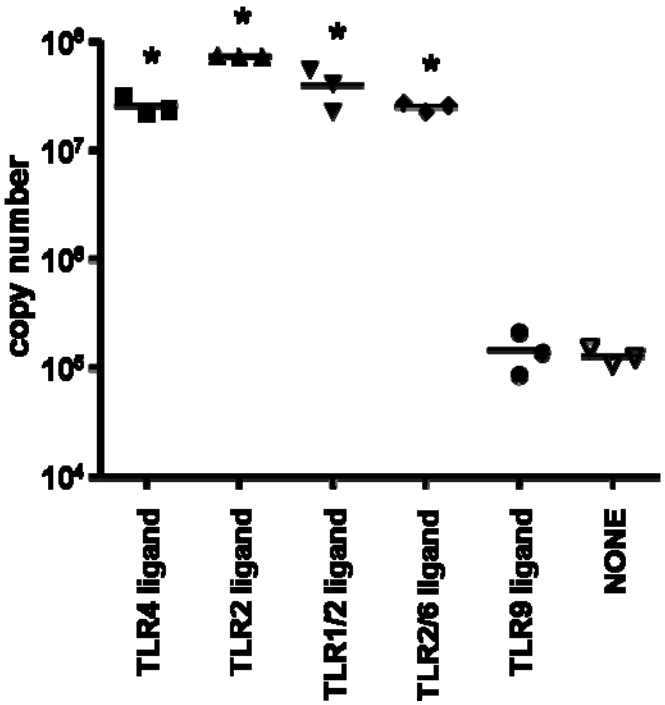

Figure 4-33. Stimulation with TLR ligands increases RAGE gene expression (*all $p < 0.0061$). qPCR analyzed relative gene expression of RAGE on HOK in response to TLR ligation and non-stimulation.

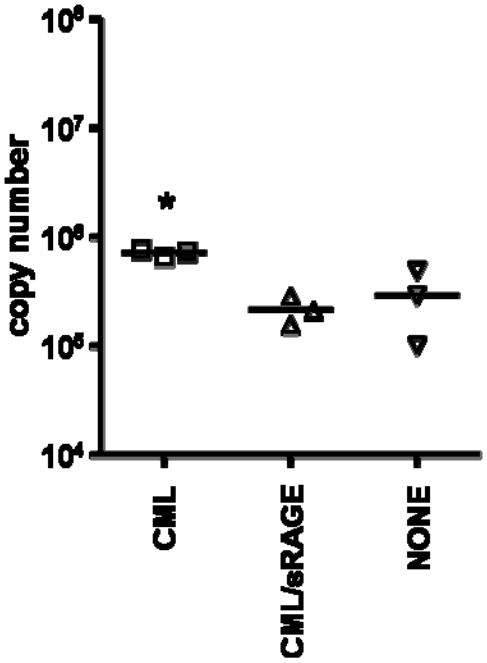

Figure 4-34. Stimulation with RAGE ligands increases TLR2 gene expression (*p = 0.034). qPCR analyzed relative gene expression of TLR2 on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

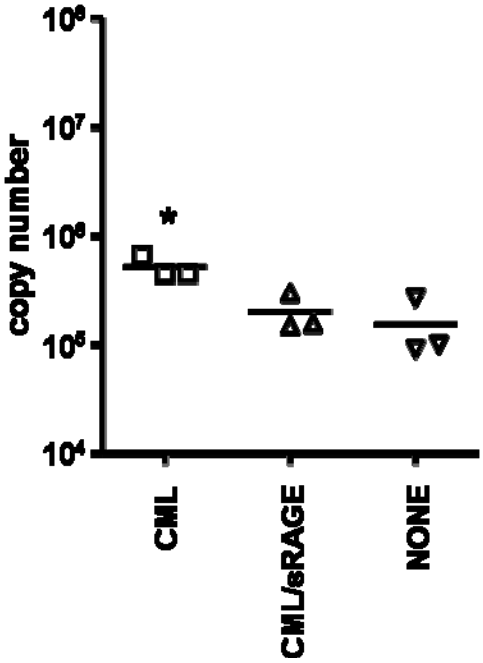

Figure 4-35. Stimulation with RAGE ligands increases TLR4 gene expression (*p = 0.0135). qPCR analyzed relative gene expression of TLR4 on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

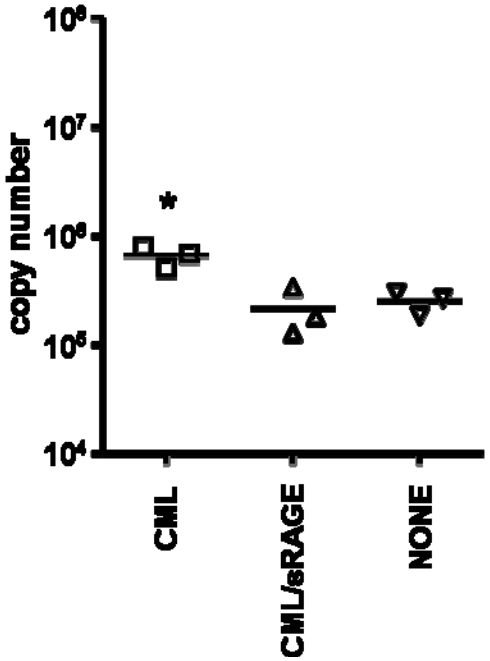

Figure 4-36. Stimulation with RAGE ligands increases TLR1 gene expression (*p = 0.0228). qPCR analyzed relative gene expression of TLR1 on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

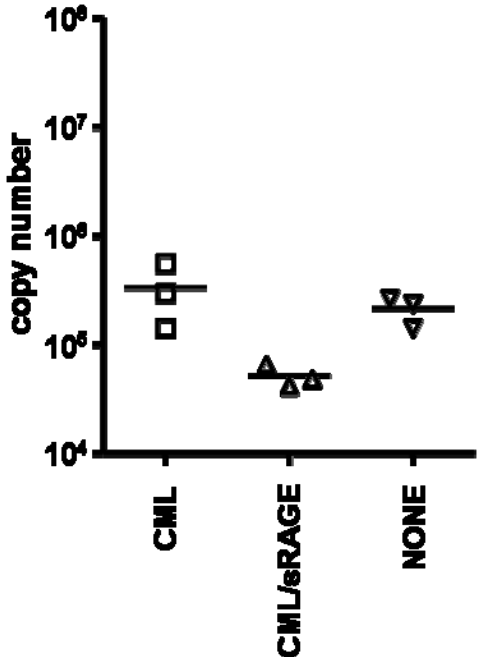

Figure 3-37. Stimulation with RAGE ligands increases TLR gene expression. qPCR analyzed relative gene expression of TLR6 on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

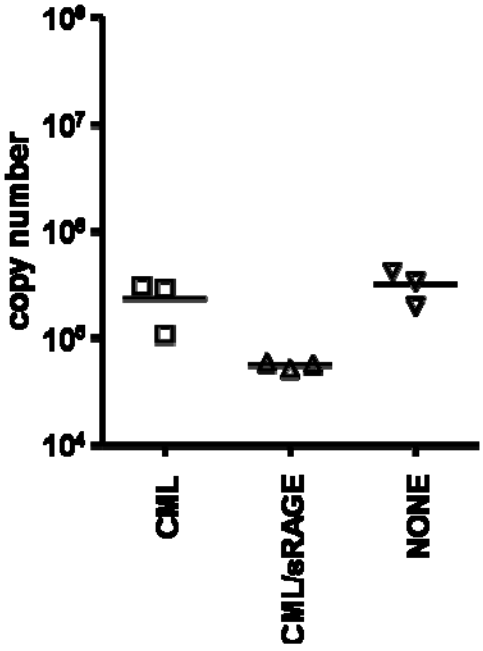

Figure 4-38. Stimulation with RAGE ligands increases TLR gene expression. qPCR analyzed relative gene expression of TLR9 on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

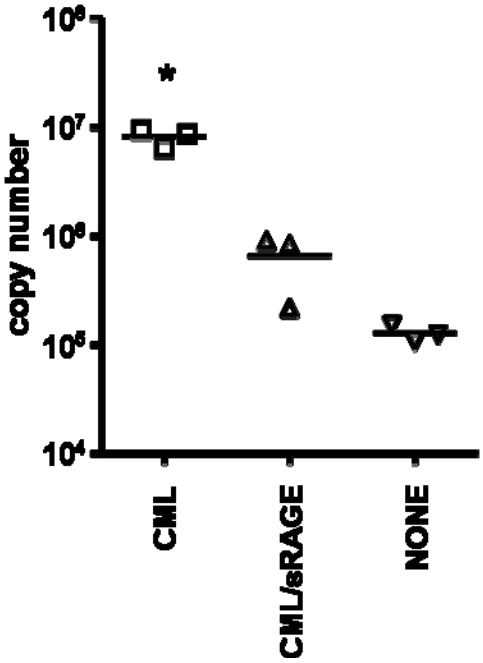

Figure 4-39. Stimulation with RAGE ligands increases RAGE gene expression (*p = 0.0063). qPCR analyzed relative gene expression of RAGE on HOK in response to RAGE ligation, blocking with sRAGE and non-stimulation.

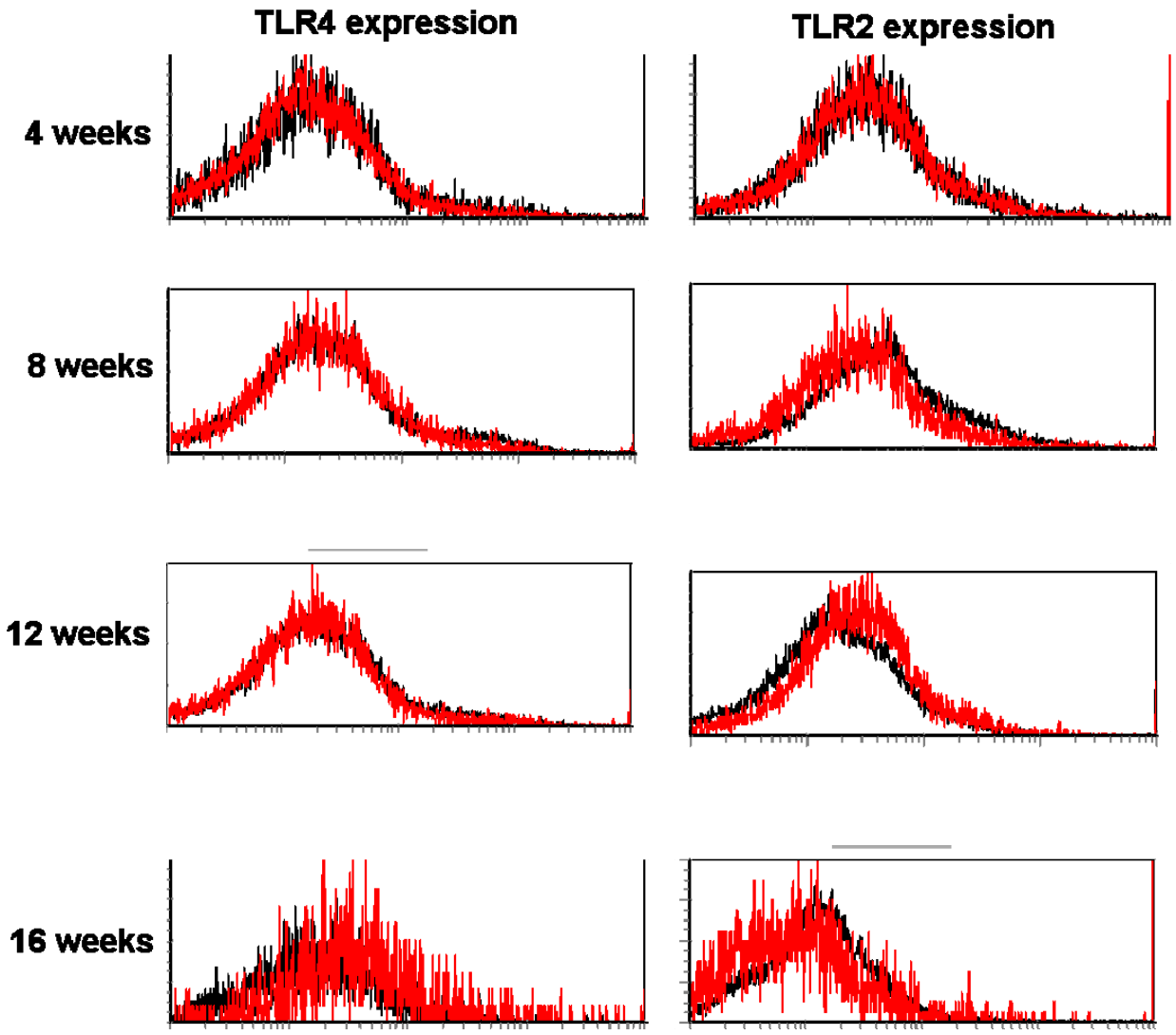

Figure 4-40. TLR2 and TLR4 single positive protein expression on a single GEC in both C57BL6 and NOD mice. FACS analyzed relative protein expression of TLR2 and TLR4 single positive GECs in both C57BL6 and NOD mice at 4, 8, 12, and 16 weeks of age

CHAPTER 5
DISCUSSION

Currently there is a lack of evidence to substantiate and clearly demonstrate the cellular and molecular mechanisms responsible for the innate immune response, protective or destructive, to microbial invasion in the periodontium (135). In this study by using immunohistochemistry, we have demonstrated the levels of expression of TLR1, 2, 4, 6, 9 and RAGE on GECs of mice with and without type 1 diabetes and on HOK cells in hyperglycemic and normoglycemic conditions. We also have demonstrated the HOK secretion levels of pro-inflammatory and anti-inflammatory cytokines and chemokines in response to TLR and RAGE stimulation in both hyperglycemic and normoglycemic conditions.

The host innate immune systems interactions with microbes are determined by TLRs differential expression, specificities, and distribution by the host's cells and tissues (135). In our immunochemistry, we did not find statistically significant differences in the protein expression of TLR between NOD and C57BL6 mice. Contrary to our TLR protein expression findings, we found that TLR gene expression in C57BL6 mice was found to increase over time, whereas TLR gene expression in NOD mice decreased over time with significant differences in TLR gene expression between C57BL6 and NOD mice at approximately 12 to 16 weeks. The down-regulation of TLR gene transcription in our NOD mice was not caused by hyperglycemic conditions associated with diabetes because the NOD mice used for this assay had failed to developed diabetes by 16 weeks of age. It is plausible that our protein and gene expression results display a critical alteration in the signaling pathway between TLR protein expression and TLR gene expression at the transcriptional level. Presently, the alternation that occurred in the signaling pathway connecting TLR protein expression to TLR gene expression is unclear. There is a need for further investigation into the mechanism that involves TLR mediated signaling

activities that signal transduction involved in the pathway between TLR gene expression at the transcriptional level and TLR protein expression.

Research shows that microbial pathogens are able to inhibit TLR mediated immune responses by impeding TLR signals or by decreasing TLR expression levels (136). Interestingly, our findings show that after stimulation with TLR ligands, HOK cells increased the transcription of TLR genes. Additionally, in the presence of hyperglycemic condition, TLR gene transcription increases were further amplified. Amplified TLR gene transcription under hyperglycemic conditions may indicate the involvement of TLR ligation in diabetic individual's susceptibility to bacterial infections leading to strong pro-inflammatory responses subsequently leading to more severe and faster progressing periodontal disease. Comparing our findings of TLR gene expression levels on both HOK and GECs, it is reasonable that diabetics may have diminished endogenous transcription of their TLR genes allowing for infection and hyperglycemic conditions to robustly up-regulate gene transcription leading to increased and prolonged inflammatory outcomes resulting in severe gingival tissue pathology.

TLR ligation triggers stimulation of a variety of cytokines, chemokines, and growth factors, mainly from innate immune cells such as neutrophils, macrophages, and monocytes, which results in the promotion of inflammation and immune cell infiltration leading to destruction of connective tissue and bone (73). In diabetics an abnormal inflammatory response to bacterial products has been shown to result in an exaggerated secretion of such mediators form innate immune cells. We have shown that TLR ligation under hyperglycemic conditions can contribute to the induction of pro-inflammatory conditions, while inhibiting the anti-inflammatory response, leading to prolonged inflammation.

In diabetics it has been demonstrated that the monocytic hyper-responsiveness to bacterial antigens results in increased production of pro-inflammatory cytokines and mediators which induce tissue destruction, attachment loss and bone loss (40-42). Interestingly, our results found that TLR4 ligation promotes pro-inflammatory cytokine and chemokine secretion, whereas TLR2 ligation promotes anti-inflammatory cytokine and chemokine secretion. Other studies have shown that *P.* gingivalis and *Leptospira* have the capability to use diverse LPS structural moieties in order to only be recognized by TLR2 and not TLR4, therefore triggering an anti-inflammatory Th2 response (137, 138) and Darveau et al. showed modified lipid-A components of LPS are used by *P. gingivalis* to undermine an aggressive TLR4 pro-inflammatory response (139). TLR2 ligation leading to an anti-inflammatory response is likely the innate immune systems adaptation to balance anti-inflammatory and pro-inflammatory mediators, avoiding overproduction of pro-inflammatory responses by TLR stimulation.

Furthermore, signaling ability of TLRs is not equally spread, implicated by potential cross-talks with between different TLRs can alter the initial innate response generated (140). In parallel, we found that TLR1 and/or TLR6 diamerization to TLR2 and subsequent ligation results in the promotion of pro-inflammatory responses and inhibits anti-inflammatory responses. It is credible that modified components of LPS that result during structural change may be recognized by TLR2/TLR1 and/or TLR2/TLR6 diamerization and consequently promoting a pro-inflammatory response instead of a TLR2 anti-inflammatory response. Moreover, we found hyperglycemic conditions amplify pro-inflammatory responses and inhibit anti-inflammatory responses. It is reasonable that the innate immune system uses TLR diameriztion to elicit stronger inflammatory responses and to purposely inhibit anti-inflammatory responses in order to eliminate microbial infections.

The amplified pro-inflammatory responses and inhibition of anti-inflammatory response in hyperglycemic conditions suggest a mechanism leading to over production of pro-inflammatory cytokines and chemokines. Abnormal pro-inflammatory responses, known as a 'hyper-inflammatory trait', have been linked to diabetes (55, 56). Our result strengthen the credibility that hyperglycemic conditions increase susceptibility to infection which is more conducive to innate inflammatory response, exhibited by exaggerated secretion of innate inflammatory cytokines and chemokines, connecting TLR ligation in hyperglycemic conditions present in diabetics to exaggerated secretion innate pro-inflammatory mediators associated with periodontal disease.

Lalla et al. (2000) demonstrated that the administration of soluble RAGE results in the halting of periodontal disease and the markers of cellular activation and tissue destruction in diabetic mice, indicating that AGEs were impeded from binding with and activating RAGE (141). Our findings revealed that RAGE gene expression in C57BL6 mice was found to increase over time, whereas RAGE gene expression in NOD mice decreased over time with significant differences in RAGE gene expression between C57BL6 and NOD mice at approximately 8 to16 weeks. We recognize that this down-regulation of RAGE gene transcription in our NOD mice was not due to hyperglycemic conditions due to the fact that our NOD mice used for this assay had not developed diabetes by 16 weeks of age and showed normal blood glucose levels at the time of gingival epithelial tissue excision.

On the contrary, Lalla et al. (1998) successfully showed that diabetic mice models exhibit increased vascular and monocyte RAGE expression along with elevated levels of AGEs in diabetic gingival compared to non-diabetic controls (142). The conflicting findings may be due to the fact that our NOD mice were still non-diabetic and therefore lacked the hyperglycemic

condition and the subsequent accumulation of AGEs that may be conducive to altering RAGE gene expression. The lack of RAGE interaction with RAGE ligands may be responsible to the down-regulation of RAGE gene transcription in GECs. It is also possible that RAGE gene expression being down-regulated in the absence of pathogens may demonstrate the innate immune systems inherent regulation of RAGE and avoiding overproduction of pro-inflammatory responses by RAGE in healthy tissue.

Studies have shown that in diabetic tissue RAGE expression increases in correlation with stimulation with RAGE ligands (143) and AGE accumulation leads to functional changes in blood proteins resulting in AGE proteins that amplify the magnitude of macrophage cytokine response leading to poor blood glucose control and more severe periodontal disease (144). We found that after stimulation with RAGE ligands, HOK cells increase the transcription of RAGE genes. Additionally, in the presence of hyperglycemic condition, these RAGE gene transcription increases are further amplified. Amplified RAGE gene transcription under hyperglycemic conditions may indicate the involvement of RAGE ligation in diabetic individual's susceptibility to bacterial infections leading to strong pro-inflammatory responses subsequently leading to more severe and faster progressing periodontal disease. The pathogenesis of diabetic complications resulting from the accumulation of AGEs has been linked to prolonged hyperglycemia (143).

The mechanism behind the increased amplification of RAGE gene transcription in hyperglycemic conditions is unclear. However, it is entirely plausible that in hyperglycemic conditions the accumulation of AGEs leads to increased AGE- RAGE interaction. Increased RAGE stimulation will lead to amplified RAGE gene expression and subsequently an increased pro-inflammatory response. We believe that diabetics may have diminished endogenous

transcription of their RAGE genes allowing for infection and hyperglycemic conditions to robustly up-regulate gene transcription leading to increased and prolonged inflammatory outcomes resulting in severe gingival tissue pathology.

RAGE is associated with pro-inflammatory response and has been shown to increase expression with aging and has been link as a fundamental receptor in the pathogenesis of periodontal disease (145). We have shown that RAGE ligation under hyperglycemic conditions results in an increase in pro-inflammatory responses and inhibits anti-inflammatory responses. We demonstrated that hyperglycemic conditions can contribute to the induction of pro-inflammatory conditions, while inhibiting the anti-inflammatory response, leading to prolonged inflammation. Lalla et al. (2000) showed that pro-inflammatory cytokine (TNF-α and IL-6) levels in gingival tissue extracts from diabetic mice were significantly higher compared to levels in non-diabetic mice. Furthermore, in diabetic mice levels of pro-inflammatory cytokines (TNF-α and IL-6) were significantly decreased when treated with sRAGE compared diabetic mice not treated with sRAGE (141). We believe that the hyperglycemic condition present in diabetic patients and the ligation of RAGE by bacteria in the oral cavity results in the overproduction of pro-inflammatory cytokines and chemokines leading to prolonged inflammation resulting in gingival tissue destruction.

One limitation of this study was that only 60% to 80% of female NOD mice turn diabetic by 16 weeks of age. This may have confounded our qPCR data and conclusions regarding relative gene expression of RAGE and TLRs in NOD mice at 16 weeks of age and subsequently any statistical analysis may have erroneous values. This limitation proved difficult to address since ordering NOD mice from breeding colonies is limited to age and not diabetic development. Additionally, Our FACs data was limited due to the fact that we were only able to staining cells

with antibodies specific for TLR 1, 2, and 4 proteins on GECs due to the limited resources available to us. Subsequently our FACS data, statistical analysis, and conclusions are limited to protein expression for only TLR 1, 2, and 4 and not for TLR 6, 9 and RAGE. This limitation has prohibited us from performing a complete investigating and determining conclusions regarding TLR and RAGE protein expression on/in GECs.

Important future research should incorporate type 2 diabetic (T2D) murine model system in order to wholly investigate the correlation between diabetes and periodontal disease. This will permit investigation of whether differences that exist between T1D and T2D correlates to differences in periodontal disease development. NOR (normoglycemic NOD) Mice should be used as normoglycemic controls allowing for clearer comparison to NOD mice because of similar genetic backgrounds. The investigation of the signaling pathway between TLR protein expression and TLR genetic expression is needed to understand the mechanism responsible for differences in protein and gene expression. Also, the investigation of the signaling pathway between TLR/RAGE and cytokine/chemokine secretion will allow for a clear understanding of the signaling pathway that occurs within the cell after TLR stimulation resulting in cytokine/chemokine secretion. Lastly, murine and human diabetogenic primary gingival epithelial cell line cultures need to be established to allow for *in vitro* investigations to be performed. This thesis provides substantial evidence linking type 1 diabetes and periodontal disease, however, additional research should incorporate the previously stated in order to further understand the association between diabetes and periodontal disease.

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BIOGRAPHICAL SKETCH

Jeffrey Wayne Tobler was born in Richland, Washington, to Karl and Jan Tobler. In 1999 he graduated from Pasco Senior High School in Pasco, Washington. In October 1999, Jeff put his academic pursuits on hold and served a two year mission in Fukuoka, Japan for the Church of Jesus Christ of Latter-Day Saints. After returning from Japan, Jeff continued his studies, receiving a bachelor's degree in East Asian languages and literature from the University of Florida in 2006. After graduating, Jeff worked for a year in the Periodontal Disease Research Center in the department of Oral Biology in the College of Dentistry at the University of Florida. Jeff began a master's degree in biomedical sciences in the College of Medicine at the University of Florida in 2007. After completing his master's degree, Jeff will continue his career goals and will pursue a D.M.D degree in the College of Dentistry at the University of Florida in the fall of 2009.