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The Role of Hyperglycemia in Mechanisms of Exacerbated Inflammatory Responses within the Oral Cavity

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Abstract

Immune modulating factors are necessary for pathogen clearance, but also contribute to host tissues damage, as those seen in periodontal diseases. Many of these responses can be exacerbated by host conditions including type 2 diabetes [T2D], where toll-like receptor 4 [TLR4] and the receptor for advanced glycosylated end products [RAGE] play a significant role. Here we investigate causality associated with the increase in inflammatory markers observed in periodontally diseased patients with T2D using multi-variant correlation analysis. Inflammation associated with periodontal diseases, characterized by elevated pro-inflammatory cytokines, innate immune receptor expression, and cellular infiltrate was exacerbated in patients with T2D. In addition, a feed forward loop regulated by poor glycemic control was associated with a loss of mucosal barrier integrity and accumulation of innate immune receptor ligands resulting in an exacerbation of ongoing inflammation, where RAGE and TLR4 cooperated to induce responses in oral epithelial cells, which were exacerbated by hyperglycemia.

Keywords

type 2 diabetes; periodontal disease; toll-like receptor 4; receptor for advanced glycosylated end products; lipopolysaccharide; *N*-carboxyl (methyl-lysine); oral epithelial cells; microbial translocation

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1. Introduction

Type 2 Diabetes [T2D] is a result of resistance to insulin combined with a failure to produce enough insulin to compensate for the resistance resulting in hyperglycemia [1; 2]. This lack of metabolic control is also implicated in the high susceptibility of these patients to infectious diseases [3]. Indeed, T2D patients have a 2- to 5- fold higher risk for periodontal diseases, where the risk for periodontal disease is reduced by effective control of hyperglycemia [2; 3; 4]. Periodontal diseases are a class of inflammatory diseases of the oral cavity which if left untreated can result in soft and hard tissue destruction. While microbial factors are the etiological agents of periodontal disease, they do not directly cause disease, but rather induce harmful inflammatory responses in a susceptible host [5; 6]. Medical and dental literature has demonstrated an association between periodontal disease and systemic chronic conditions including T2D [2; 3; 7; 8; 9; 10]. Manifestation of T2D is associated with ongoing cytokine-induced acute-phase responses which are involved in pathogenesis as well as the associated complications such as dyslipidemia and atherosclerosis [2; 3; 11]. Likewise, the mechanisms of the host-mediated response in periodontal diseases involves the activation of innate immunity, specifically through up-regulation of pro-inflammatory cytokines in the presence of gram-negative subgingival bacterial biofilms [5; 6]. Although it is clear that both of these conditions share an exacerbated inflammatory response resulting in site specific pathologies, the mechanism(s) by which these conditions are linked remains unclear.

Innate immunity is an evolutionarily conserved defense mechanism which in mammals culminates in the production and secretion of immune modulating factors [12; 13]. Here pattern recognition receptors [PRR] play a significant role where the dynamics of signaling outcomes are attributable to several factors including the nature of the ligand(s), concentration of the ligand(s), duration of stimulation by the ligand(s), and the cell type which is being engaged by the ligand(s) [14; 15]. Members of both the toll-like receptor [TLR] and immunoglobulin [Ig] super-family of receptors including TLR4 and the receptor for advanced glycosylated end-products [RAGE], respectively, have been implicated in exacerbated inflammatory responses in chronic inflammatory diseases including T2D and periodontal diseases [16; 17; 18]. Both TLR4 and RAGE have multiple ligands, allowing them to function as sensors of environmental cues and therefore play a crucial role in the regulation of both homeostasis and pathogenesis [16; 19; 20; 21]. Cells of the periodontium including epithelial cells, fibroblasts and macrophages express both TLR4 and RAGE where immune and anti-microbial factors are produced in response to stimulation [16; 17; 22; 23; 24; 25; 26; 27; 28]. Oral epithelial cells, the first line of defense within the oral cavity, serve multiple immune roles at the mucosal surface functioning as: 1. a physical barrier to microbes; 2. a source of anti-microbial peptides which inhibit microbial growth; 3. inflammatory immune cells which produce cytokines to trigger anti-microbial immunity; and 4. tolerogenic immune cells which can produce immunoregulatory cytokines or remain quiescent in response to non-pathogenic commensal microorganisms [28].

It has been suggested that hyperglycemia, a major sequela of T2D, exacerbates the inflammatory process of periodontal disease by promoting the accumulation of RAGE and TLR4 ligands within the periodontium through induction of advanced glycosylated end-product [AGE] formation and colonization of LPS-expressing gram negative bacteria [9; 18], although the direct effect of hyperglycemia on innate immune cellular responses has not been evaluated. Here regression models are used to correlate expression levels of RAGE and TLR4 in the periodontal tissues of periodontally diseased T2D patients with periodontal disease severity [clinical and state of inflammation], glycemic control and circulating levels of ligand [AGEs and LPS respectively]. In addition, cellular mechanisms for the effect of

hyperglycemia on innate immune function in the context of TLR4 and RAGE-ligation are defined using primary oral epithelial cells.

2. Materials and methods

2.1 Participant Cohorts

The Institutional Review Board [IRB] for protection of human subjects at the University of Florida approved this protocol. 20 periodontally healthy participants [PH], 20 periodontally diseased participants without diabetes [PD], and 20 periodontally diseased participants with type 2 diabetes [PDD] were recruited from the University Of Florida, College Of Dentistry, Department of Periodontology and/or Department of Orthodontics. Inclusion criteria [all groups]: 13–75yrs old, require at least one premolar tooth extraction or have a necessity for surgical treatment. Exclusion Criteria [all groups]: have clinically significant neurological, hepatic, renal, gastrointestinal, hematologic, dermatologic, metabolic, autoimmune or immune deficiency diseases, receiving any immunosuppressive medication, antibiotic or glucocorticoid therapy, smoker or oral tobacco user, pregnant or lactating, or taking medications known to affect the gingiva. Inclusion criteria [PH]: good general health according to medical history and oral examination. Exclusion criteria [PH]: diagnosed with type 1 diabetes [T1D] or T2D. Inclusion criteria [PD]: diagnosed with chronic periodontitis. Exclusion criteria [PD]: have been diagnosed with T1D or T2D. Inclusion criteria [PDD]: diagnosed with T2D without diabetic ketoacidosis, currently under physician care for diabetes control, and diagnosed with chronic periodontitis. Exclusion criteria [PDD]: have ever been diagnosed with T1D, have diabetic complications such as proliferative retinopathy, macrovascular diseases or kidney and/or liver failure.

2.2 Clinical Measurements

Periodontal clinical parameters were collected including: pocket depth [pd], clinical attachment level [cal], and bleeding on probing [BoP]. cal was calculated by $pd + \text{gingival margin position [GM]}$. GM was given a negative value when located above the cementum-enamel-junction [CEJ]. Glycated hemoglobin [HbA1c] was determined using whole blood and A1CNow (Bayer; Peoria, IL).

2.3 Tissue Collection

All tissues harvested for primary oral epithelial [HOK] cell culture was performed during third molar extractions where soft tissues [1mm waste] associated with the extraction were collected, after which epithelial cells were isolated and expanded as previously described [29]. All tissues harvested for gene expression analysis were collected in conjunction with multiple types of oral surgical procedures. PD specimens were removed as waste tissue during surgical treatment of periodontal disease, while PH specimens were removed as waste tissue during procedures not related to periodontal disease. Tissues were placed in RNeasy (Qiagen; Madison, WI) and stored at 4°C. 2.0–4.0 ml of heparinized blood was drawn from all participants, the plasma separated by centrifugation [300×g for 15mins] and stored at –80°C.

2.4 Gingival Crevicular Fluid (GCF) Collection

GCF samples were collected from a PH or PD site prior to treatment and/or tissue harvesting. Sites for GCF collection were isolated with cotton rolls, gently air-dried and supragingival plaque removed. PerioPaper GCF collection strips (OralFlow Inc, Plainview, NY) were inserted 1–2mm into the sulcus for ~30sec. Samples were eluted from the strip into 300µl of phosphate buffered saline (PBS) by centrifugation and stored at –80°C until assays were performed. Total protein content was determined by bicinchoninic acid (BCA)

assay (Pierce Thermo Fisher Scientific, Rockford, IL) according to manufacturer's instructions where the colorimetric reaction was quantified using a spectrophotometer at 562nm. A standard curve and a best fit linear trend line were used to determine ug/mL concentrations.

2.5 Plasma lipopolysaccharide [LPS]

Plasma LPS levels were quantified using an Endpoint Chromogenic LAL Assay (Lonza; Walkersville, MD). Plasma and limulus amebocyte lysate [LAL] were incubated after which a p-nitroaniline [pNA] containing chromogenic substrate was incubated and the enzymatic reaction was stopped using 25% v/v glacial acetic acid in water and the absorbance quantified using a spectrophotometer at 405nm. Endotoxin units/ml were calculated using a standard curve and best fit linear trend line.

2.6 Plasma N-carboxyl (methyl-lysine) [CML]

Plasma levels of CML were quantified using an ELISA (MBL International; Woburn, MA) according to the manufacturer's instructions where the colorimetric reaction was quantified using a spectrophotometer at 450nm with a reference of 655nm. A standard curve and a best fit linear trend line were used to determine ng/mL concentrations.

2.7 Cell culture and stimulation

1×10^6 HOK were either left untreated or stimulated for 24hrs with a TLR4 agonist [*E. coli* LPS] (InVivogen; San Diego, CA) or the advanced glycated end product [AGE] N-carboxyl (methyl-lysine) [CML] (Thermo Fisher Scientific; Waltham, MA) at indicated concentrations. In some experiments cultures were pre-treated for 1hr with soluble RAGE [sRAGE] (RayBiotech Inc.; Norcross, GA) and/or a TLR4 neutralizing antibody (InVivogen) and/or 24hrs with 5mM (100mg/dl), 12.5mM (200mg/dl), 15mM (300mg/dl), or 20mM (400mg/dl) of D-glucose. Culture supernatants were collected for soluble mediator analysis and tRNA was collected for gene expression analysis. All assays were performed using 5–7 donors.

2.8 Soluble Mediator Analysis

ELISA (Millipore; Billerica, MA) was used to detect IL1 α , IL8, and TNF α according to the manufacturer's instructions where the colorimetric reaction was quantified using a spectrophotometer at 450nm with a reference of 655nm. A standard curve and a best fit linear trend line were used to determine pg/mL concentrations. pg/ml of soluble mediators in GCF values were normalized to total protein content as follows [raw pg/ml $\times$ total protein content corrective ratio] where the corrective ratio = [lowest protein content]/[protein content for sample of interest].

2.9 Cell viability

Viability was assessed using an MTT Cell Growth Assay (Millipore) following stimulation described above according to manufacturer's instructions where absorbance was quantified with a spectrophotometer at 570nm with a reference of 630nm. Culture media alone was used as a negative control while cells lysed with 1.0% triton were used as a positive control.

2.10 real-time PCR [qPCR]

Total RNA [tRNA] from periodontal tissues or HOK cells was extracted using an RNAeasy extraction kit (Qiagen; Valencia, CA). RNA was reverse transcribed to generate cDNA using a reverse transcription master mix [5X buffer, 1mM DTTs, 2.5mM dNTPs, SuperScript III, and oligonucleotides (Invitrogen; Carlsbad, CA)]. GAPDH, RAGE (Integrated DNA Technologies; Coralville, IA), CD3 (T cell), CD19 (B cell), CD14

(macrophage), MPO (granulocyte) (Applied Biosystems, Framingham, MA), or hTLR-4 RT² qPCR primers (SABiosciences; Frederick, MD) and 2xqPCR Cyber Green Master Mix (BioRad; Hercules, CA) were used for qPCR reactions. For RAGE and TLR4, standard curves were used to determine mRNA transcript copy number, while relative expression was determined for all other genes. Each gene was detected in independent reactions. Data are expressed as copy number (or relative expression) normalized to GAPDH. Normalized mRNA copy number (or relative expression) = [raw transcript copy number (or raw *Ct* value)] × [GAPDH corrective ratio]. GAPDH corrective ratio = [lowest GAPDH *Ct* value within sample set]/[GAPDH or *Ct* value for sample of interest].

2.11 Statistics

Statistical analyses were performed using Prism Software (GraphPad Software, Inc; La Jolla, CA) where one-way ANOVA with Bonferroni's multiple comparisons test was used to determine significance when comparing groups of three or more while Student's *t* test was used when comparing two groups. For the description of clinical data, mean values and standard deviations were also calculated. Linear Regression and Pearson's correlation were used to determine *r* and *P* values for correlation analysis. *P* value <0.05 was considered significant.

3. Results

3.1 Inflammation in periodontally diseased tissues is characterized by elevated pro-inflammatory cytokines, innate immune receptor expression, and cellular infiltration

Periodontal diseases are inflammatory diseases where bacterial products induce inflammatory mediator production resulting in host tissue destruction allowing for the release of exogenous and endogenous ligands for many innate immune receptors [5]. RAGE and TLR4 respond to both exogenous and endogenous ligands and are expressed by a variety of cell types within the periodontium where ligand interactions lead to increased receptor expression and inflammation [17; 18]. Therefore, the expression levels of *rage* and *tlr4* were evaluated by qPCR in 20 periodontally diseased [PD] and 20 periodontally healthy [PH] tissue samples. The clinical demographics of the participant populations are summarized in Table I. mRNA levels of both RAGE and TLR4 were significantly higher in PD tissues when compared to those in PH tissues (Fig 1A). Expression levels of *rage* significantly correlated to expression levels of *tlr4* in these tissues ($r=0.5689$ $P=0.0089$) (Fig 1B). In addition, mRNA levels of the pro-inflammatory cytokines IL8, IL1 α , and TNF α were also quantified by qPCR. The expression levels of all three cytokines were significantly up-regulated in PD tissues when compared to PH (5.7+/-0.9, 3.9+/-0.6, 9.7+/-1.1 fold increase respectively) (Fig 1C). The protein levels of these mediators were also evaluated in the gingival crevicular fluid (GCF), where elevated levels were observed in the PD cohort (Fig 1D). The immune cellular infiltrate of the periodontal lesion has been well characterized [6; 17]. Therefore, the proportion of specific immune cells within the infiltrate was examined. As expected, elevated levels of granulocytes, macrophages, B and T cells were observed within the PD lesion (Fig 2D).

It has been suggested that prolonged expression of pro-inflammatory cytokines leads to tissue destruction resulting in translocation of bacteria and/or bacterial products into the peripheral blood [2; 30]. Therefore, the levels of LPS in the plasma of PD and PH participants were evaluated. LPS levels were significantly higher in plasma from PD participants compared to PH participants (Fig 1E), where correlation analysis in PD tissues revealed a strong association between levels of plasma LPS and expression levels of *tlr4* ($r=0.6822$ $P=0.0009$), *rage* ($r=0.6795$ $P=0.0010$), *tnfa* ($r=0.655$ $P=0.0047$), and *il8* ($r=0.7885$ $P<0.0001$), but not *il1a* ($r=0.1454$ $P=0.5408$) (Table II).

3.2 Type 2 Diabetes exacerbates inflammation in the periodontally diseased tissues

Periodontal diseases can be exacerbated by host conditions such as T2D, where conventional treatment is not always effective [31]. Although the mechanism(s) associated with these observation(s) have not been completely elucidated, it has been proposed that the condition of T2D exacerbates the inflammatory responses responsible for periodontal diseases [31]. Therefore, the expression levels of *rage* and *tlr4* as well as the pro-inflammatory cytokines *il8*, *il1a*, and *tnfa* in periodontal tissues of 20 periodontally diseased participants with T2D [PDD] were evaluated by qPCR. The clinical demographics of the participant population are summarized in Table I. Levels of *rage* and *tlr4* expression were significantly elevated in tissues from PDD participants when compared to PD participants (Fig 2A). In addition, both IL8, IL1 α , and TNF α tissue transcript and GCF protein levels were also significantly elevated in PDD tissues when compared to PD tissues (Fig 2B, C). The affect of hyperglycemia on the proportion of specific cells within the infiltrate was also examined. As expected, granulocytes, macrophages, B and T cells were all present within the PDD lesion (Fig 2D). Interestingly, while there was little change in the granulocyte and B cell compartments, there was an 8.06 $\pm$ 0.34 and 6.21 $\pm$ 0.26 fold increase in the macrophage and T cell compartments respectively in the PDD lesion when compared to that of the PD lesion (Fig 2D).

Hyperglycemia, the result of uncontrolled diabetes, leads to non-enzymatic glycation of proteins and lipids resulting in the formation of advanced glycated end products [AGEs] which can contribute to a pro-inflammatory cascade [16]. Therefore, the plasma level of one such AGE, CML, was evaluated in PH, PD, and PDD participants. While there was no significant difference in CML levels between PH and PD participants, CML levels in the plasma from PDD participants were significantly elevated (Fig 2E). Similarly, LPS levels in the plasma were significantly higher in the PDD cohort than those of the PD participants (Fig 2F).

3.3 Poor glycemic control is associated loss of mucosal barrier integrity, accumulation of ligand, and exacerbation of inflammation

In order to begin deciphering causality associated with the increase in inflammatory markers observed in periodontally diseased patients with T2D, multi-variant correlation analysis was performed. Periodontal disease severity is determined by a combination of factors including bleeding on probing, pocket depth, and clinical attachment level [32]. Within the PDD cohort, pocket depth correlated with the expression of all pro-inflammatory cytokines measured, while clinical attachment level correlated only with *tnfa* levels (Table II). RAGE and/or TLR4 ligation can lead to increased inflammation as well as induction of innate receptor expression [16]. Interestingly, while expression of *rage* and *tlr4* within the PDD cohort significantly correlated (Table II), expression of *rage* or *tlr4* correlated only with *tnfa* expression but not *il8* nor *il1a* (Table II).

Lack of glycemic control has been associated with exacerbated inflammatory responses including production of pro-inflammatory cytokines [31]. CML levels are a measurement of control over the long term while glycated haemoglobin [HbA1c] is a measurement of control over only a three month period. Within the PDD cohort, plasma CML levels correlated only with *tnfa* expression but not *il8* nor *il1a*, while HbA1c levels correlated with all of the pro-inflammatory cytokines evaluated (Table II). Lack of glycemic control has also been associated with alterations in mucosal barrier function, where plasma LPS levels can be indicative of loss of barrier integrity [33]. Interestingly, within the PDD cohort, both CML and HbA1c levels correlated with plasma LPS levels (Table II). In turn plasma LPS levels significantly correlated with the expression of *il8*, *tnfa*, *il1a* as well as *rage* and *tlr4* expression (Table II).

3.4 Cooperation of RAGE and TLR4 in the innate immune responses of oral epithelial cells

Loss of mucosal barrier integrity is regulated by multiple factors including the innate immune function of epithelial cells. To evaluate the biological outcomes of TLR4 and RAGE expression as well as ligation on epithelial cells of the mucosa, the viability and dose responsiveness of primary oral epithelial cells following exposure to LPS and CML were evaluated. While primary human oral keratinocytes [HOK] responded to all concentrations of LPS and CML tested, optimal stimulatory conditions were observed at 100ng/ml for both LPS and CML (Fig 3A, B). Because excessive inflammation can induce apoptosis and/or necrosis of cells, the viability of HOK under these stimulatory conditions was also evaluated. LPS nor CML at any concentration tested affected HOK cell viability (Fig 3C). Similarly, because the osmolarity changes associated with increase glucose concentration can induce apoptosis, the viability of HOK exposed to glucose treatment was evaluated, where epithelial cell viability was not affected by any of the D-glucose concentrations tested (Fig 3D).

It has been suggested that RAGE and TLR4 independently and cooperatively induce a wide spectrum of sustained cellular activation including induction of receptor expression and cytokine production [16]. Therefore qPCR was used to evaluate the expression of *rage* and *tlr4* following stimulation with LPS and/or CML in the presence and absence of sRAGE and/or TLR4 neutralizing antibody. Stimulation with LPS or CML increased mRNA levels of both *rage* and *tlr4*, although no additive effect was observed upon stimulation with both ligands (Fig 4A, B). In addition, interruption of RAGE ligation by the addition of sRAGE abrogated any CML-induced increases in *rage* or *tlr4* expression (Fig 4A, B). Similarly, blockade of TLR4 signalling by the addition of a TLR4-neutralizing antibody prevented LPS-induced increases in *rage* and *tlr4* expression (Fig 4 A, B). On the other hand, blockage of RAGE- or TLR4-ligation did not have an effect on *tlr4* or *rage* expression respectively (Fig 4A, B).

Increased expression of the pro-inflammatory cytokines IL8, IL1 α , and TNF α were also observed following either LPS or CML stimulation of epithelial cells (Fig 4E–G). CML-induced a more robust pro-inflammatory response than LPS, although no additive effect was seen upon simultaneous stimulation (Fig 4E–G). Interestingly, the addition of sRAGE not only abrogated CML-induced but also LPS-induced increases in pro-inflammatory cytokine expression (Fig 4E–G), although sRAGE was unable to completely inhibit responses induced by concomitant stimulation by these ligands (Fig 4E–G). Similarly, antibody mediated neutralization of TLR4 signalling prevented LPS-induced increases in cytokine expression and significantly decreased but did not completely abrogate RAGE-induced cytokine responses (Fig 4E–G).

3.5 Hyperglycemia exacerbates RAGE and TLR innate immune responses in oral epithelial cells

The affect of hyperglycemia on oral epithelial cell innate immune responses was evaluated by pre-treating primary cell cultures for 24 hours with 12.5mM glucose (200mg/dL) prior to LPS and/or CML stimulation. Although epithelial cell viability was not affected by any of the D-glucose concentrations tested, 12.5mM of D-glucose is representative of uncontrolled blood glucose levels in participants with T2D and was therefore utilized in pre-treatment of HOK (Fig 3D). Hyperglycemic conditions did not alter basal expression levels of *rage*, *tlr4*, IL8, IL1 α or TNF α levels (Figs 4). On the other hand, hyperglycemia exacerbated CML- and LPS-induced *rage* and *tlr4* expression (Fig 4A–D) as well as CML- and LPS-induced cytokine secretion (Fig 4E–I). Inhibition of both RAGE and TLR4 signalling through the use of antagonists prevented up-regulation of receptors as well as pro-inflammatory cytokine production similar to that seen under normoglycemic conditions (Fig 4).

4. Discussion

Type 2 diabetes is associated with a wide range of complications including periodontal disease where multiple underlying mechanisms are likely in play. While a majority of these complications do ensue from abnormal regulation of glucose metabolism, manifestation of T2D is associated with ongoing cytokine-induced acute-phase responses similar to those involved in the pathogenesis of periodontal disease, suggesting an additional role for the innate immune response [2; 3; 11]. Both direct and indirect consequences of hyperglycemia have been described where studies have demonstrated that even intermittent periods of hyperglycemia can result in long-term and sometimes irreversible sequela [31; 34; 35; 36; 37; 38; 39]. Hyperglycemia not only enables the accumulation of innate immune receptor ligands such as LPS and AGEs [16; 40; 41], but data presented here demonstrates that it directly affects the responsiveness of epithelial cells to these agonists. Specifically hyperglycemia induces an exacerbated TLR- and RAGE-induced pro-inflammatory cytokine response as well as increases receptor availability on the cell surface. Additional data indicates that RAGE and TLR4 cooperate to induce pro-inflammatory cytokine expression in oral epithelial cells, although this cooperatively is not involved in the induction of innate immune receptor expression.

The up-regulation of receptor expression increases the potential stimulatory capacity of the epithelial cell providing a feed-forward-loop of inflammation, which must be interrupted in order for disease to be ameliorated. Surprisingly, correlation data demonstrates that at least within the PDD cohort while disease severity is due to elevations in pro-inflammatory cytokine expression, only the expression of *mfa* is attributed to elevations in RAGE or TLR4 expression. Alternatively, the correlation data reveals that that poor glycemic control correlates with loss of mucosal barrier function, which in turn correlates with increases in innate immune receptor expression and exacerbation of pro-inflammatory responses.

These data suggest a paradigm of feed-forward-inflammation which could account for the association of T2D with other inflammatory diseases/conditions such as cardiovascular disease, adverse pregnancy outcomes and periodontal diseases[42; 43]. While it has been hypothesized that tissue destruction observed in periodontal disease can result in microbial translocation [44; 45; 46], here we propose an alternative explanation where hyperglycemia induces microbial translocation in the gastrointestinal track which in turn allows for the deposition of microbes and their products at distant sites such as arteries in the case of cardiovascular disease, the placenta in the case of adverse pregnancy outcomes, and the periodontal pocket in the case of periodontal disease. Deposition of these agonists initiates and/or exacerbates ongoing inflammation through the induction of pro-inflammatory cytokine production from multiple cell types. Indeed LPS levels in the plasma of the PDD cohort reported here correlated with all pro-inflammatory cytokines evaluated. In addition, our *in vitro* data indicate that this interaction on epithelial cells involves both TLR4 and RAGE whose expression is elevated on epithelial cells by hyperglycemic conditions. While this report only investigated the affect of hyperglycemia on the innate immune responses of oral epithelial cells, other cell types associated with these site specific pathologies such as endothelial cells, fibroblasts and traditional immune cells still need further study. Importantly, data presented here also demonstrated that the cellular infiltrate of the T2D PD lesion is altered where macrophages and T cells are significantly increased when compared to the non-diabetic PD lesion. Again, while this report only investigated the infiltrate of the periodontal lesion, the affect of hyperglycemia on the cellular infiltrate of lesions associated other inflammatory diseases/conditions such as cardiovascular disease, adverse pregnancy outcomes still need further investigation.

Based on data presented here as well as that of others, one could hypothesize that simply removing the main etiological agent of periodontal diseases, namely plaque, may not be sufficient to break this feed-forward loop in susceptible populations such as those with T2D, but rather development of supplemental therapies may be necessary not only to resolve periodontal disease progression, but prevent or dampen additional systemic sequelae. In addition, our data suggests that even transient localized hyperglycemia such as those associated with dietary intake of sugars can have adverse affects on innate immune responses of oral epithelial cells in the absence of T2D. Importantly, while hyperglycemia exacerbates the upregulation of TLR- and RAGE-induced innate immune receptor and pro-inflammatory cytokine expression, RAGE and TLR4 antagonists are effective in preventing these exaggerated inflammatory responses giving them potential as adjunctive therapies. Indeed, others have demonstrated in mouse models the effectiveness of sRAGE administration in prevention of periodontal disease induce bone loss [47]. Therefore, because oral epithelial cells are directly involved in the inflammatory process, are the first responders to insult, and are integrally involved in resolution of disease, they are an excellent target for such adjunctive therapies in the T2D population, while control of sugar intake may also provide beneficial responses in the absence and presence of T2D.

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References

1. Mealey BL, Oates TW. Diabetes mellitus and periodontal diseases. *J Periodontol.* 2006; 77:1289–303. [PubMed: 16881798]
2. Santos Tunes R, Foss-Freitas MC, Nogueira-Filho Gda R. Impact of periodontitis on the diabetes-related inflammatory status. *J Can Dent Assoc.* 2010; 76:a35. [PubMed: 20831852]
3. Loe H. Periodontal disease. The sixth complication of diabetes mellitus. *Diabetes Care.* 1993; 16:329–34. [PubMed: 8422804]
4. Taylor GW. Bidirectional interrelationships between diabetes and periodontal diseases: an epidemiologic perspective. *Ann Periodontol.* 2001; 6:99–112. [PubMed: 11887478]
5. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol.* 2005; 32(Suppl 6):57–71. [PubMed: 16128830]
6. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. *Periodontol* 2000. 1997; 14:9–11. [PubMed: 9567963]
7. Gottsegen R. Diabetes and periodontal disease. *J Periodontol.* 1991; 62:161–3. [PubMed: 2027065]
8. Khader YS, Dauod AS, El-Qaderi SS, Alkafajei A, Batayha WQ. Periodontal status of diabetics compared with nondiabetics: a meta-analysis. *J Diabetes Complications.* 2006; 20:59–68. [PubMed: 16389170]
9. Grossi SG, Genco RJ. Periodontal disease and diabetes mellitus: a two-way relationship. *Ann Periodontol.* 1998; 3:51–61. [PubMed: 9722690]
10. Taylor GW, Burt BA, Becker MP, Genco RJ, Shlossman M, Knowler WC, Pettitt DJ. Severe periodontitis and risk for poor glycemic control in patients with non-insulin-dependent diabetes mellitus. *J Periodontol.* 1996; 67:1085–93. [PubMed: 8910827]
11. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care.* 2004; 27:813–23. [PubMed: 14988310]
12. Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. *Nat Rev Genet.* 2001; 2:256–67. [PubMed: 11283698]

13. Medzhitov R, Janeway C Jr. Innate immunity. *N Engl J Med*. 2000; 343:338–44. [PubMed: 10922424]
14. Erridge C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol*. 2010; 87:989–99. [PubMed: 20179153]
15. Medzhitov R, Janeway C Jr. Innate immune recognition: mechanisms and pathways. *Immunol Rev*. 2000; 173:89–97. [PubMed: 10719670]
16. Lin L. RAGE on the Toll Road? *Cell Mol Immunol*. 2006; 3:351–8. [PubMed: 17092432]
17. Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000*. 2007; 43:41–55. [PubMed: 17214834]
18. Katz J, Wallet S, Cha S. Periodontal disease and the oral-systemic connection: “is it all the RAGE?”. *Quintessence Int*. 2010; 41:229–37. [PubMed: 20213024]
19. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis Rheum*. 2005; 52:2656–65. [PubMed: 16142732]
20. Lucas M, Stuart LM, Savill J, Lacy-Hulbert A. Apoptotic cells and innate immune stimuli combine to regulate macrophage cytokine secretion. *J Immunol*. 2003; 171:2610–5. [PubMed: 12928413]
21. Mullick AE, Tobias PS, Curtiss LK. Toll-like receptors and atherosclerosis: key contributors in disease and health? *Immunol Res*. 2006; 34:193–209. [PubMed: 16891671]
22. Sugawara Y, Uehara A, Fujimoto Y, Kusumoto S, Fukase K, Shibata K, Sugawara S, Sasano T, Takada H. Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. *J Dent Res*. 2006; 85:524–9. [PubMed: 16723649]
23. Ford PJ, Gamonal J, Seymour GJ. Immunological differences and similarities between chronic periodontitis and aggressive periodontitis. *Periodontol 2000*. 2010; 53:111–23. [PubMed: 20403108]
24. Weinberg A, Krisanaprakornkit S, Dale BA. Epithelial antimicrobial peptides: review and significance for oral applications. *Crit Rev Oral Biol Med*. 1998; 9:399–414. [PubMed: 9825219]
25. Diamond DL, Kimball JR, Krisanaprakornkit S, Ganz T, Dale BA. Detection of beta-defensins secreted by human oral epithelial cells. *J Immunol Methods*. 2001; 256:65–76. [PubMed: 11516756]
26. Dale BA, Kimball JR, Krisanaprakornkit S, Roberts F, Robinovitch M, O’Neal R, Valore EV, Ganz T, Anderson GM, Weinberg A. Localized antimicrobial peptide expression in human gingiva. *J Periodontol Res*. 2001; 36:285–94. [PubMed: 11585115]
27. Kollisch G, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, Bauer S, Jakob T, Mempel M, Ollert M. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology*. 2005; 114:531–41. [PubMed: 15804290]
28. McCormick TS, Weinberg A. Epithelial cell-derived antimicrobial peptides are multifunctional agents that bridge innate and adaptive immunity. *Periodontol 2000*. 2010; 54:195–206. [PubMed: 20712640]
29. Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun*. 1995; 63:3878–85. [PubMed: 7558295]
30. Geerts SO, Nys M, De MP, Charpentier J, Albert A, Legrand V, Rompen EH. Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. *J Periodontol*. 2002; 73:73–8. [PubMed: 11846202]
31. Lalla E, Lamster IB, Drury S, Fu C, Schmidt AM. Hyperglycemia, glycoxidation and receptor for advanced glycation endproducts: potential mechanisms underlying diabetic complications, including diabetes-associated periodontitis. *Periodontol 2000*. 2000; 23:50–62. [PubMed: 11276765]
32. *Ann Periodontol*; 1999 International International Workshop for a Classification of Periodontal Diseases and Conditions. Papers; Oak Brook, Illinois. October 30–November 2, 1999; 1999. p. 1–112.
33. Vaarala O, Atkinson MA, Neu J. The “perfect storm” for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. *Diabetes*. 2008; 57:2555–62. [PubMed: 18820210]

34. Bucciarelli LG, Wendt T, Rong L, Lalla E, Hofmann MA, Goova MT, Taguchi A, Yan SF, Yan SD, Stern DM, Schmidt AM. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. *Cell Mol Life Sci.* 2002; 59:1117–28. [PubMed: 12222959]
35. Hattori Y, Hattori S, Sato N, Kasai K. High-glucose-induced nuclear factor kappaB activation in vascular smooth muscle cells. *Cardiovasc Res.* 2000; 46:188–97. [PubMed: 10727667]
36. Inoguchi T, Xia P, Kunisaki M, Higashi S, Feener EP, King GL. Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. *Am J Physiol.* 1994; 267:E369–79. [PubMed: 7943217]
37. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science.* 1996; 272:728–31. [PubMed: 8614835]
38. Xia CQ, Peng R, Annamalai M, Clare-Salzler MJ. Dendritic cells post-maturation are reprogrammed with heightened IFN-gamma and IL-10. *Biochem Biophys Res Commun.* 2007; 352:960–5. [PubMed: 17157804]
39. Yerneni KK, Bai W, Khan BV, Medford RM, Natarajan R. Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes.* 1999; 48:855–64. [PubMed: 10102704]
40. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med.* 1988; 318:1315–21. [PubMed: 3283558]
41. Cerami A, Vlassara H, Brownlee M. Role of advanced glycosylation products in complications of diabetes. *Diabetes Care.* 1988; 11(Suppl 1):73–9. [PubMed: 3069394]
42. Scannapieco FA, Dasanayake AP, Chhun N. Does periodontal therapy reduce the risk for systemic diseases? *Dent Clin North Am.* 2010; 54:163–81. [PubMed: 20103479]
43. Mealey BL, Rose LF. Diabetes mellitus and inflammatory periodontal diseases. *Curr Opin Endocrinol Diabetes Obes.* 2008; 15:135–41. [PubMed: 18316948]
44. Wada K, Kamisaki Y. Molecular dissection of *Porphyromonas gingivalis*-related arteriosclerosis: a novel mechanism of vascular disease. *Periodontol.* 2000; 54:222–34.
45. Forner L, Larsen T, Kilian M, Holmstrup P. Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *J Clin Periodontol.* 2006; 33:401–7. [PubMed: 16677328]
46. Kinane DF, Riggio MP, Walker KF, MacKenzie D, Shearer B. Bacteraemia following periodontal procedures. *J Clin Periodontol.* 2005; 32:708–13. [PubMed: 15966875]
47. Lalla E, Lamster IB, Feit M, Huang L, Spessot A, Qu W, Kislinger T, Lu Y, Stern DM, Schmidt AM. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. *J Clin Invest.* 2000; 105:1117–24. [PubMed: 10772656]

- Increased inflammation was observed in T2D periodontally diseased tissues.
- Poor glycemic control was associated with a loss of mucosal barrier integrity.
- Poor glycemic control was associated with accumulation of innate receptor ligands.
- AGE and TLR4 cooperate in induction of oral epithelial cell immune responses.
- Hyperglycemia exacerbates RAGE/TLR4 induced oral epithelial cell immune responses.

Figure 1. Inflammation in periodontally diseased tissues is characterized by elevated pro-inflammatory cytokines and innate immune receptor expression

Periodontal tissues, GCF and plasma were collected from 20 periodontally healthy [PH] and 20 periodontally diseased [PD] participants. **(A)** Tissue mRNA copy number for RAGE and TLR4 was determined. **(B)** Linear regression and Pearson correlation analysis was performed between *tlr4* and *rage*. **(C)** Tissue mRNA levels and **(D)** GCF protein content was determined for IL8, IL1 α , and TNF α . **(E)** Plasma LPS levels were determined. **(F)** Tissue mRNA levels of MPO (neut), CD14 (mac), CD3 (T cell), and CD19 (B cell) were determined and the fold change over PH calculated. **P* value <0.05 PH vs. PD one-way ANOVA with Bonferroni's multiple comparisons correction. ^*P* value <0.0001 PH vs. PD Student's *t* test.

Figure 2. Increased inflammatory markers are present in periodontally diseased tissues of patients with Type 2 Diabetes

Periodontal tissues, GCF and plasma were collected from 20 periodontally diseased [PD] and 20 periodontally diseased participants with T2D [PDD]. (A) Tissue mRNA copy number for RAGE and TLR4 was determined. (B) Tissue mRNA levels of IL8, IL1 α , and TNF α were determined and the fold change over PD calculated. (C) GCF protein content was determined for IL8, IL1 α , and TNF α . (D) Tissue mRNA levels of MPO (neut), CD14 (mac), CD3 (T cell), and CD19 (B cell) were determined and the fold change over PD calculated. Plasma (E) CML and (F) LPS levels were determined. **P* value <0.05 PDD vs. PD or PH one-way ANOVA with Bonferroni's multiple comparisons correction. ^*P* value <0.0001 PH vs. PD Student's *t* test.

Figure 3. Responsiveness of primary oral epithelial cells to LPS, CML and glucose stimulation
 1×10^6 primary epithelial cells [HOK] from periodontally healthy participants were left unstimulated or were stimulated for 24hrs with 1ng, 10ng, 100ng or 1000ng of either (A, C) ultra-pure TLR4 agonists [*E. coli* LPS], (B, C) the advanced glycated end product [AGE] *N*-carboxyl (methyl-lysine) [CML] or (D) 5mM (100mg/dl), 10mM, (150mg/dl), 12.5mM (200mg/dl), 15mM (300mg/dl), or 20mM (250m/dl) of D-glucose. After which the (A, B) supernatants were used to quantify IL8, IL1 α , and TNF α secretion and (C, D) cell viability evaluated. All assays were performed on 5 separate donors.

Figure 4. Cooperation of RAGE and TLR4 in the innate immune responses of oral epithelial cells is exacerbated by hyperglycemia

1×10^6 primary epithelial cells [HOK] were stimulated for 24hrs with the indicated ligands in the (A–B, E–G) absence or (C–D, H–J) presence of 12.5mM glucose pre-treatment after which qPCR was used to quantify the copy number of (A, C) *rage* and (B, D) *tlr4* and ELISA was used to quantify (E, H) IL8, (F, I) IL1α, and (G, J) TNFα in the supernatants. **P* value <0.05 unstim vs. LPS, CML and CML+LPS. ^*P* value <0.05 stimulations in the absence of neutralization vs. the presence of neutralizations. † *P* value <0.01 stimulations in 5mM glucose vs. 12.5mM glucose. one-way ANOVA with Bonferroni's multiple comparisons correction.

Table 1

Participant Cohort Demographics and Clinical Parameters.

	BoP (%)	pd >4mm (%)	cal >2mm (%)	Age (yrs)	Gender (m/f)	HbA1c (%)
PH (n=20)	5.2 (+/-1.2)	0.7 (+/-0.6)	0.3 (+/-0.4)	36.1 (+/-6.89)	10/10	5.7 (+/-0.2)
PD (n=20)	47.8 (+/-10.2)	36.7 (+/-11.0)	27.2 (+/-3.0)	49.2 (+/-11.28)	9/11	5.7 (+/-0.2)
PDD (n=20)	52.4 (+/-15.2)	39.4 (+/-12.4)	30.8 (+/-3.4)	50.8 (+/-9.40)	8/12	8.2 (+/-0.3)

periodontally healthy (PH), periodontally diseased (PD), periodontally diseased with type 2 diabetes (PDD), bleeding on probing (BoP), pocket depth (pd), clinical attachment level (cal), glycated hemoglobin (HbA1c)

Table II

Correlates of Disease Severity, Inflammation and Glycemic Control.

	<i>tnfa</i>		<i>il8</i>		<i>il1a</i>		LPS	
	<i>P</i> value	<i>r</i> value	<i>P</i> value	<i>r</i> value	<i>P</i> value	<i>r</i> value	<i>P</i> value	<i>r</i> value
pd	0.0234	0.5043	0.0179	0.5232	0.0330	0.4781	0.0006	0.6686
cal	0.0371	0.4688	0.7648	0.0714	0.4176	0.1919	0.0592	0.3606
<i>rage</i>	0.0003	0.7238	0.2158	0.2894	0.4504	0.1789	0.0135	0.5425
<i>tlr4</i>	<0.0001	0.7621	0.1898	0.3058	0.7683	0.0703	0.0390	0.4029
LPS	0.0370	0.4633	0.0148	0.5375	0.0148	0.5375		
CML	0.0024	0.6395	0.0745	0.4075	0.3921	0.2024	0.0293	0.4874
HBA1c	0.0167	0.5280	0.0143	0.4188	0.0068	0.5844	0.0220	0.5085

Linear regression and Pearson's correlation analysis within the PDD cohort.

pocket depth (pd), clinical attachment level (cal)