PORPHYROMONAS GINGIVALIS LPS STIMULATES AUTOPHAGY USING A TLR MEDIATED PATHWAY

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DOCTORAL THESIS

May 2017

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A mi mujer,
por su tiempo,
dedicación y apoyo
durante todos estos años.
Porque todo este trabajo
ha sido posible gracias a ella.
Gracias
por haber cuidado de mi
con tanto esmero,
por tu comprensión y
tu cariño.

ACKNOWLEDGMENTS

ACKNOWLEDGEMENTS

Quisiera dedicara la finalización de esta tesis doctoral a todas aquellas personas que de una manera u otra me han apoyado durante estos años y la han hecho posible.

En primer lugar, quiero expresar mi agradecimiento a mis directores de tesis. A la Dra. Katheleen Boesze-Battaglia, por haberme ayudado y depositado su confianza en mi desde que empecé el proyecto en su laboratorio. Gracias por haberme enseñado todo lo que sé cómo investigador, por haberme guiado a través de estos años con tu saber y pasión por la investigación. También al Dr. Andreu Puigdollers Perez, por haber estado siempre disponible en todo momento y ofrecido su apoyo en este trabajo. Gracias por tu amistad y por tu orientación profesional mucho antes de que este proyecto empezara. Gracias a los dos por vuestra confianza depositada en mí.

Quiero mostrar un agradecimiento especial a toda mi familia, que me han ayudado con su apoyo a realizar este proyecto tan lejos de ellos. Muy especialmente a mis padres Marta y Iñaki, por su cariño, ánimo y optimismo y por suplir todo en lo que o no he llegado durante estos años. A todos mis hermanos, Alvaro, Gonzalo, Guille y Martita, cuñadas, Leti, Adriana y a Pedro, sobrinos, Alvaro y Gonzalo, que han participado directa o indirectamente en la elaboración de esta tesis.

También a mi otra parte de la familia, MªTeresa y Vicenç, a mis cuñados, Maite, Xavi, Pilar, Jordi, Carmen, Kiko, Vicenç, Toni, Sandra, Robert, Gemma, Marta, Luis, Mercè, Ramón, Francesc, y Ana, sobrinos, Roger, Anna, Martita, Aleix, Mariona, Cesc, Queralt, Xavito, Nicolas, Guadalupe, Esperanza y Bernat; por estar pendiente de todos mis proyectos y por su apoyo y cariño durante todos estos años.

Asimismo, a todo el equipo de mi laboratorio y co-autores. A Laura, Alvina, Juan, Jon, Bruce, Hassan, Anuradha, Nishat, Desiree, Edward… por haber estado trabajando a mi lado y ayudado en lo que necesitara desde los inicios de mi investigación. Algunos de los cuales ya han emprendido otros caminos y con los que he compartido los mejores momentos de estos años, debo agradecerles que han conseguido que sólo las risas acompañen nuestro trabajo diario.

También quiero expresar mi agradecimiento a todos los de la UIC que han estado pendientes de mi con mis emails. Especialmente a Dr. Javier Mareque y a Sonia Soriano, por su tiempo y por su ayuda en asesorías y dudas presentadas en la elaboración de la tesis.

Al Dr. Luís Giner, por apoyo en el desarrollo de mi formación profesional y apoyarme en todo momento.

A mis amigos, Juan, Koke, Iñigo, Slick, Dolores, Norm y Roberto porque aunque para algunos hayan sido unos años a la distancia, hemos compartido momentos muy especiales. Gracias porque de una manera u otra me habéis acompañado en este camino.

Gracias a todos por todo vuestro apoyo que ha hecho posible la elaboración de esta tesis doctoral. Gracias con todo mi cariño.

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INTRODUCTION

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Periodontitis is an oral inflammatory disease. It affects the supporting structures of the periodontium which include gingiva, alveolar bone, periodontal ligament, and cementum, causing partial or total tooth loss in the affected individuals. In United States, recent epidemiological data suggests that periodontal disease affects 46% of US adults and is the major cause of tooth loss among adults. Its prevalence is related with increasing age and is superior among males (1). There are risk factors that increase the occurrence of the disease including: smoking, stress, genetic predisposition, diabetes and rare systemic diseases. It is associated as well with various systemic conditions including colorectal and oral squamous cell carcinoma, coronary artery disease and a pre-disposition to pre-term delivery (2-5).

Periodontal disease is considered a heterogeneous disease caused by the interaction of pathogenic bacteria with various host factors. Numerous studies have been conducted trying to identify key periodontal pathogens that are thought to initiate periodontal disease. Socransky et al. (6) grouped the bacteria related to the etiology of the disease. Bacteria such as, *Porphyromonas gingivalis (P.g)*, *Tannerella forsythia (T.f)*, and *Treponema denticola (T.d)*, are characterized as the "red complex," which is very well linked with periodontal tissue destruction.

Porphyromonas gingivalis is one of the central bacterial pathogens involved in the initiation and progression of periodontitis. *P. gingivalis* is known to produce a repertoire of virulence factors that could break through the gingivae and cause tissue destruction directly or indirectly, by induction of inflammation (7). The capability of an organism to colonize and escape antibacterial host defense mechanisms, as well as the ability to produce substances that could begin tissue destruction, are basic features of a successful pathogen. Virulence factors include enzymes, capsule lipopolysaccharide, fimbriae, exopolysaccharide, outer membrane proteins, collagenase, trypsin-like protease, gelatinase and aminopeptidase.

Pg induces effects on the host ranging from inflammation mediated alveolar bone loss associated with periodontal disease to its recently ascribed role in the pathogenesis of atherosclerotic plaque (8-15). Several lines of evidence suggest that the red complex

pathogens may inhibit host defense functions thereby contributing to chronic inflammation. *Pg* has developed a number of methods to subvert innate immunity, for instance, *Pg* promotes its survival through inhibition of IL-12, and IFN-γ *in vivo* which is dependent on the pathogen's ability to hijack complement–receptor 3 (8, 16, 17). It has been proposed that using these and autophagy subversion tactics, *Pg* exploits macrophages as "Trojan Horse" for subsequent infection of gingival epithelial cells and transmission from oral cavity to systemic tissue (8).

As a vital part of the host immune response to microbial infection, macroautophagy contributes to cellular homeostasis. It is an auto-digestion method that causes a direct elimination of intracellular pathogens through lysosomes. Evolutionarily, bacteria are capable to evade or subvert host cell autophagic defense pathways, thereby promoting their survival or growth. Autophagosomes may target pathogens for degradation or, if subverted by bacteria, lead to survival. Bacteria often commandeer components of the autophagic pathway in order to survive, replicate and propagate. They employ autophagosomes which do not mature to degradative autolysosomes as a survival replicative niche. One of the earliest described examples of this evasion concept was the periodontal pathogen, *P. gingivalis.* This microorganism which traffics to double membrane autophagosomes devoid of lysosomal proteases evades the host immune system (18, 19). We have recently shown that *Pg* challenge of macrophages results in the association of LC3 with its intracellular sorting protein partner, MREG (20).

Host recognition of *P. gingivalis* as well as other gram negative bacteria is facilitated through recognition of lipopolysaccharides (LPSs), with the lipid A portion of LPS, acting as a molecular motif recognized by TLR4, the later instructs the host cell to promote an inflammatory response in an effort to eradicate the pathogen (21, 22). *Pg* modifies its lipid A structure in response to environmental stressors by synthesizing both a penta-acylated mono-phosphorylated lipid A as well as a tetra-acylated monophosphorylated species (23-25). This heterogeneity of structures is proposed to play a role in oral immune homeostasis and contributes to the microbial shift in the oral cavity characteristic of periodontitis (26-28). Recent studies have shown that lipid A modifications promote evasion of the non-canonical inflammasome by preventing caspase-1 activation (29). Thus, the heterogeneity of these structures is thought to play a role in oral immune homeostasis and contributes to the microbial shift characteristic of periodontitis (26-28).

Single nucleotide polymorphisms of pattern recognition receptors, TLRs have been linked to chronic periodontitis (30, 31). Moreover, clinical isolates of peripheral blood mononuclear cells from periodontal patients had increased levels of autophagy proteins with further enhancement of the autophagy protein microtubule–associated protein 1 light chain 3 (LC3) observed upon *Pg* LPS stimulation (32). We have recently shown that *P. gingivalis* challenge of macrophages results in association of microtubule-associated protein 1 light chain 3 (LC3) with its intracellular sorting protein partner melanoregulin, MREG, which is required for lysosome maturation in professional phagocytes (33). These clinical data coupled with recent reports suggesting that TLRs function as autophagy sensors (34) form the basis for these studies. The aim of this research was to better understand the mechanism through which *P. gingivalis* evades intracellular degradation. The experiments were designed to: (1) further assess the effect of *P. gingivalis*, specifically LPS_{1690} on autophagosome assembly, through LC3 lipidation; (2) evaluate the possibility that aberrations in LC3- and/or MREG-dependent processes are associated with susceptibility to periodontitis; and (3) determined if human gingival explants isolated from healthy and diseased individuals mediate autophagic flux in response to *Pg*LPS variants.

HYPOTHESIS

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Hypothesis 1:

- **Null hypothesis (H0):** *P. g.* sequestered into autophagosomes does not confer protection from lysosomal degradation resulting in bacterial persistence.
- **Alternative hypothesis (H1):** *P. g.* sequestered into autophagosomes confers protection from lysosomal degradation resulting in bacterial persistence.

Hypothesis 2:

- **Null hypothesis (H0):** MREG mediated lysosome maturation do not contribute to *P. g.* clearance in phagolysosome.
- **Alternative hypothesis (H1):** MREG mediated lysosome maturation contributes to *P. g.* clearance in phagolysosome.

Hypothesis 3:

- **Null hypothesis (H0):** *P. g.* mediated stimulation of TLR4 do not upregulate autophagy.
- **Alternative hypothesis (H1):** *P. g.* mediated stimulation of TLR4 upregulates autophagy.

Hypothesis 4:

- **Null hypothesis (H0):** LC3-MREG degradative pathway is not overloaded in individuals with severe periodontal disease.
- **Alternative hypothesis (H1):** LC3-MREG degradative pathway is overloaded in individuals with severe periodontal disease.

OBJECTIVES

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Main objective:

In the present studies, our objective is to determine how *P. g.* hijacks host cell responses to contribute to its long-term survival in macrophages and the role of MREG in this process.

Secondary objectives:

1. Evaluate if *P. g.* LPS upregulates autophagosome formation in a TLR dependent manner and the role of melanoregulin in *P. g.* survival.
2. Evaluate if cells isolated from chronic periodontitis patient have decreased lysosomal capacity due to diminished lysosome maturation leading to autophagy defects.

RESULTS

RESULTS

Variants of *Porphyromonas gingivalis* lipopolysaccharide alter lipidation of autophagic protein, microtubule-associated protein 1 light chain 3, LC3

Our results demonstrate that *Pg* (strain 33277) is internalized in Saos-2 cells but does not traffic to GFP-LC3 positive structures. Once GFP-LC3 expressing Saos-2 cells were stimulated with *Pg* (strain 33277), the Texas-red labeled *Pg* (TR-*Pg*) was found to be internalized, after incubation for 1h, with virtually no detectable TR-*Pg* after 24h (Fig. 1A). *Pg* incubation resulted in an increase in LC3 positive puncta suggestive of autophagosome formation (Fig. 1A & B) although no detectable TR-*Pg* was found associated with LC3 (Fig. 1A). Incubation with *Pg* stimulated the formation of MREG positive puncta by 30 min, that decreased within 1h (Fig. 1B & C).

The appearance of a 14kda LC3II band upon *Pg* challenge confirmed the increase in lipidated LC3 (Fig. 1D). MREG levels increased after incubation with *Pg* at 30 and 60 min, with a return to near baseline levels (t=0) by 3h. In contrast, *A. actinomycetemcomitans* did not stimulate LC3 lipidation (LC3II) with an overall decrease in the unlipidated form of LC3II (Fig. 1E). Furthermore, MREG levels increased only slightly upon incubation with *A. actinomycetemcomitans* (Fig. 1E).

Stimulation with *Pg* LPS_{1690} dependent increases autophagy activation where subsequently inhibited with the LPS_{1690} antagonist $LPS_{1435/1449.}$ The GFP-LC3 expressing Saos-2 cells show a time dependent increase in LC3 puncta formation when treated with any of the LPS species, with the most puncta observed upon the addition of the hexa-acylated 1,4 bis-phosphorylated *E.coli* LPS species (Fig. 2). The time course of maximal LC3 puncta formation varied amongst the LPS species, with *Pg* LPS_{1690} and *Salmonella* LPS dependent LC3 puncta formation peaking at early points (30min) and *E.coli* LPS dependent puncta formation at late time points (3h) (Fig. 2). Serum starvation of the GFP-LC3 Saos cells resulted in enhanced puncta formation, confirming predicted nutrient dependent increase in autophagy (Supplementary material Fig.S1A). To confirm that LC3 puncta formation was not due to overexpression of the GFP-LC3, Saos-2 cells (no GFP-LC3) were treated with LPS variants. LC3 and MREG puncta were enhanced upon incubation with all of the LPS

species, with LPS_{1690} dependent puncta formation maximal after 30min. By 3h MREG levels had returned almost to baseline (Fig. 2). MREG puncta also increased upon serum starvation in these cells (Supplementary material Fig.S1B). Incubation with *Pg* LPS_{1690} not only resulted in a rapid increase in LC3 puncta (within first 30min) but also the formation of large LC3-positive autophagosomal structures, these structures average in size around 3um (representative structure is 3.5um) as shown in Figure 3A and B.

When incubated with LPS_{1690} antagonist, $LPS_{1435/1449}$, such super-size autophagosomes were not observed (Fig. 4A), with the average autophagomes on size on the order of 1.5 to 2μm. Furthermore, the LPS_{1690} mediated increase in LC3 and MREG puncta diminished in the presence of $LPS_{1435/1449}$ (Fig. 4B); puncta formation decreased by at least 50% at all time points (Fig. 4C). Increased lipidated LC3 was confirmed as the appearance of a 14kda LC3II band upon stimulation with LPS_{1690} (Fig. 5A), with the highest levels of LC3II at 30min (Fig. 5B), addition of the LPS_{1690} antagonist decreased LC3 lipidation to near baseline levels (Fig. 5B). Comparably, *E.coli* LPS enhanced LC3II levels. MREG levels were increased 10 fold at 30min, with a rapid and sustained return to baseline by 1h upon LPS_{1690} stimulation, an affect that was inhibited with the simultaneous addition of the antagonist $LPS_{1435/1449}$ with a return to near baseline levels (t=0) by 3h (Fig. 5C).

Upon nutrient deprivation, LPS_{1690} was also found to augment autophagy induction; when GFP-LC3 Saos-2 cells were moderately serum starved (2.5%serum), there was a 2-fold increase in LC3II compared to *E.coli* LPS or unstimulated cells. Addition of the LPS_{1690} antagonist $LPS_{1435/1449}$ again diminished LC3 levels (Supplementary material Fig.S1C).

Samples of human gingival epithelial cells from healthy and diseased individuals, were accessed for LC3 and MREG levels. In this final series of studies, we characterized the MREG and LC3 profile in human gingival epithelial cells purified from gingival tissue isolated from patients with no, moderate or severe periodontal disease. Interestingly in this initial series of studies it appears that MREG and LC3 distributes in cells isolated from individuals with severe disease with some co-localization seen between these two proteins (Fig. 6A). In healthy tissue the proteins did not co-localize. When cells from healthy individuals and those with moderate disease were analyzed for protein levels, LC3II levels were higher in healthy individuals versus those with disease. In addition, MREG levels

appear lower in disease, with beclin levels approximately 50% lower in cells isolated from patients with disease (Fig. 6A).

Porphyromonas gingivalis LPS modulates autophagy in gingival epithelial cells.

Our results prove a LPS_{1690} stimulation of autoplagic proteins in human gingival cell line, OKF64-TERT. Our experiments demostrate a decrease in Atg5, a ubiquitin like conjugation protein necessary in the lipidation of LC3I to LC3II upon stimulation with LPS_{1690} at both 1 and 3hrs (Fig. 1A and B). $LPS_{1435/1449,}$ also showed a decrease by 3 hrs with no change at the one hour timepoint (Fig. 1A and B). Autophagic flux, defined as LC3II/LC3I is increased significantly at 3hrs both with LPS_{1690} as well as $LPS_{1435/1449}$, with little change detected at 1hr (Fig. 1A and C). After LPS_{1690} stimulation of this cell type, MREG levels were higher after 3hours, in contrast incubation with $LPS_{1435/1449}$, decreased MREG levels at this same time point.

In the evaluation of human gingival epithelial cells isolated from tissue donated by 15 subjects with no or moderate periodontitis, MREG as well as beclin levels appear to decrease. Baseline levels were approximately 25% lower in MREG and 50% lower in beclin, compared to cells isolated from disease patients (Fig.2 A and B), whereas ATG5 levels remained unchanged (Fig. 2C). We incubated the samples with LPS_{1690} for 3hours, to determine how healthy and diseased individuals react to repeated bacterial dysbiosis (Fig. 2D). Stimulation of cells with LPS_{1690} (0.1ug/mL) caused an increase in both ATG5 and MREG in all samples isolated from healthy subjects however there was no corresponding increase in the levels of these proteins in the samples isolated from patients with moderate disease (Fig.2E). Most significant is a comparison of the autophagic flux measured in individuals with moderate disease compared to healthy subjects (Table 1). There was a quantitative variability in the amount of flux (likely reflective of the heterogeneity observed between individuals in general), however, there was a consistently lower autophagic flux observed upon LPS_{1690} treatment in tissue with disease. This specifically signal as autophagic flux consistently increased by 30-50% in healthy individuals upon LPS_{1690} treatment.

DISCUSSION

DISCUSSION

Recent studies suggest that *P. gingivalis* is able to reprogram immune cells that normally protect the sub gingival crevice, into creating conditions the bacteria finds more favorable (8, 35-38). Gram-negative bacteria are recognized by host cells through the transmembrane protein TLR4 (21). Signaling through TLRs by the host's immune system initiates an innate immune response against pathogens that includes inflammatory cytokine production as well as upregulation of co-stimulatory molecules to prime the adaptive immune response (39-41). TLR receptor signaling also activates and links the processes of autophagy and phagocytosis (42). This receptor identifies lipopolysaccharide (LPS) and its activation leads to an intracellular signaling pathway NF-kappaB, an inflammatory process which activates the innate immune system (43). Several gram-negative bacteria, such as *Pg,* modify the Lipid A portion of their LPSs and have developed methods to subvert innate immunity leading to a persistent infection (8, 16, 17, 24, 44, 45). The *Pg* LPS_{1690} is a penta-acylated form with weak TLR4 agonist activity, compared to the highly immunogenic *E.coli* LPS, whereas *Pg* $LPS_{1435/1449}$ is tetra-acylated immunologically unreceptive at the TLR4 complex and acting as an LPS_{1690} antagonist.

Evasion of degradative pathways by *P.gingivalis*, provides a protective survival niche in human coronary artery endothelial cells (HCAEC), in which they traffic to LC3 positive autophagosomal structures (14, 18). Incubation of mouse macrophages with *Pg* results in the association of the autophagic protein LC3 with the cargo sorting protein MREG (20). Expanding on these observations, we pursued to determine if *Pg* enhances autophagy in general and whether it traffics to autophagosomal structures in other cell types. Autophagosome formation relies on activation of several ubiquitin-like conjugation systems that mediate the lipidation of LC3 onto membranes, thus converting LC3I to LC3II, with LC3 positive puncta representative of this lipidated form. In these studies we provide evidence that LPS_{1690} stimulation of GFP-LC3 Saos cells enhances autophagy as detected by increased LC3 puncta formation (Paper 1-Fig. 2 and Fig. 4C) as well as LC3 lipidation to LC3II (Paper 1-Fig.5A and B). The size of the LPS_{1690} GFP-LC3 containing autophagosomes (Paper 1-Fig. 3A and B) was roughly 4-times the size of the average autophagosomes, 3.5-4.0μm compared to 0.5-1.0μm respectively (46). Such large autophagomals structures where not seen upon serum starvation of the Saos2-cells (Paper 1-Supplementary Material Fig. 1A) suggesting signaling specificity in assembly of these

structures with LPS_{1690}. Similar very large autophagic compartments, termed mega-phagosomes have been observed with Coxsackie virus B3 (CVB3) infection (47), as well as *E. coli* LPS induced autophagy and subsequent infection (48). In the case of CVB3 infection, these large vacuoles are non-degradative thus providing the virus a replicative niche. A similar paradigm has been proposed for *Pg* in HCAEC cells (18, 49), although the role of *Pg* LPS_{1690} in the promotion of these large megaphagosomes in HCAEC cells has not been established. In the presence of $LPS_{1435/1449,}$ the large *Pg* LPS_{1690} induced autophagosomes were reduced in size by 50%, to 1.0 to 1.5 um (Paper 1-Fig. 4A). In addition, LC3II levels and LC3 puncta formation were reduced in the presence of the *Pg* LPS_{1690} antagonist, $LPS_{1435/1449}$ (Paper 1-Fig. 4A and B). Furthermore, Atg5 levels exhibited a decreased in OKF64-TERT cells upon LPS_{1690} and $LPS_{1435/1449}$ stimulation (Paper 2-Fig. 1A and B). Interestingly, we show evidence that LPS_{1690} and $LPS_{1435/1449}$ stimulation of OKF64-TERT cells also intensifies autophagy as detected by increased LC3II/LC3I levels with little change at a shorter time period (Paper 2-Fig. 1A and C). We further analyzed protein levels in human gingival epithelial cells extracted from individuals with no or moderate periodontitis. Gingival Epithelial cells provide an obstacle against bacterial infection and also contribute in the innate immune resistance. Once epithelial cells are infected by *P.g.*, there is subsequent downstream signaling pathways that control transcription of target genes encoding for immune response and inflammatory reactions to eradicate the pathogen (50-52). Clinical isolates from periodontal patients appear to have elevated levels of autophagy proteins with further enhancement of the autophagy protein, LC3I observed upon *Pg* LPS stimulation (32). We followed the distribution profile of the proteins in gingival epithelial cells isolated from periodontal tissue obtained from healthy and diseased individuals (Paper 1-Fig.6). Moreover, LC3II/LC3I levels was consistently lower in diseased cells following incubation with LPS_{1690} (Paper 2-Table 1). However, after LPS_{1690} stimulation consistently LC3 increased in healthy epithelial cells. ATG5 levels were equal in isolated epithelial cells from moderated diseased patients (Paper 2-Fig. 2C). These results suggest that human gingiva become refractory to LPS_{1690} upon microbial dysbiosis associated with chronic periodontitis.

LC3 in human macrophages as well as epithelial cells of the eye is associated with a small cargo sorting protein melanoregulin (MREG) (33). In retinal epithelia, MREG is required for LC3 dependent phagosome degradation. Similarly, to our LC3 observations, its binding protein, MREG, was elevated with MREG positive puncta formed by 30 min upon

Pg LPS_{1690} incubation. The addition of $LPS_{1435/1449}$ inhibited MREG upregulation. Collectively, these results suggest that the weak TLR4 agonist *Pg* LPS_{1690} activates autophagy and is necessary for the formation of large autophagosomes, in the absence of bacterial engulfment, reinforcing the role of TLR4 as autophagy sensor in pathogen clearance (34, 39). Interestingly, in contrast to our previously published studies in which engulfment of *Pg* (strain 33277) resulted in the association of LC3 with its binding partner, MREG, in these studies *Pg* LPS_{1690} induced an increase in both proteins but not their co-localization, suggesting that active phagocytosis is necessary for MREG mediated LC3 dependent degradation (20). Similarly, Wang et al. found that *E. coli* LPS induced autophagy and subsequent infection (48). Moreover, the host cell respond to bacterial pathogens up-regulating degradative processes in addition to inflammatory response stimulation (53, 54). Several studies have predicted such processes may be mediated through *Pg* LPS_{1690}. *Pg* LPS_{1690} activated the NF-κB pathway in gingival fibroblasts, in contrast its antagonist, $LPS_{1435/1449}$, showed no effect in activation of NF-κB (55). Furthermore, pro-inflammatory genes were also considerably up-regulated by *Pg* LPS_{1690}, and down-regulated by $LPS_{1435/1449}$ (55).

Pro-inflammatory genes were also significantly up-regulated by *Pg* LPS_{1690}, and down-regulated by $LPS_{1435/1449}$ (55). Local inflammatory bone loss appears to be independent of LPS Lipid A expression (15). However, Genco and colleagues (15) recently demonstrated that *P. gingivalis* expression of agonist lipid A species resulted in decreased vascular inflammation and atherosclerosis progression in *ApoE*$^{-/-}$ mice. Whereas, *P. gingivalis* expression of antagonist lipid A species resulted in vascular inflammation and atherosclerosis progression in *ApoE*$^{-/-}$ mice. In addition, Frost et al. suggested that active phagocytosis is necessary for MREG mediated LC3 dependent degradation (33). Similar to LC3 response, we show data that MREG levels were elevated after OKF64-TERT cells stimulation with LPS_{1690}. However, incubation with $LPS_{1435/1449}$, decreased MREG, suggesting that autophagy process could be inadequate (Paper 2-Fig. 1A and D). The exact molecular mechanism of *P. gingivalis* LPS action which contributes to bacterial subsistence, innate immune detection and its ability to induce local and systemic inflammatory process require further investigation. In gingival epithelial cells MREG and beclin levels did not follow the same pattern as ATG5 and were found to be decreased half and ¼ respectively, suggesting that the autophagy axis may be compromised in diseased patients (Paper 2-Fig.2 A and B). Other authors, such as Bullon et al have found a dysregulation of autophagy in

periodontitis (32). We treated both group of cells with LPS_{1690} to observe the difference of autophagy responses between healthy and disease epithelial samples. In the presence of $LPS_{1690,}$ there was an increased level of ATG5 and MREG in healthy tissue cells, however such increase was not observed in cell isolated from moderate disease subjects (Paper 2-Fig. 2E). Therefore, these consistent results may indicate that the autophagic pathway is compromised in patients with periodontitis disease.

The use of Saos-2 cells to study *Pg* mediated enhancement of autophagy was described by Reyes, et al.(14); in those studies inoculations of GFP-LC3 Saos-2 cells with *Pg* strain W83 did not lead to internalization of bacteria but the formation of large LC3 positive puncta (56). In our studies, *Pg* strain (33277), a less virulent strain than W83 (57), was found to internalize however was not detectable in LC3 positive structures (Paper 1-Fig. 1). Differences in internalization and intracellular trafficking patterns and autophagy induction may be due to a truncation in the PG07171 gene (lipoprotein gene) in *Pg* (33277) that is present in its full-length form in *Pg*W83 (58). Furthermore, we observed, in isolated in human gingival epithelial cells, a co-localization between LC3 and MREG in severely affected individuals with no co-localization between these proteins in healthy or moderately affected individuals. MREG-mediated LC3-dependent degradation is necessary for the clearance of lipid debris (20) and we propose for clearance of bacteria. The observation that LC3 and MREG- colocalize only in cells from severely affected patients suggest that this clearance system may be overloaded. Collectively, these results suggest that mutations in MREG and /or LC3 may contribute to a predisposition to chronic periodontitis.

CONCLUSION

CONCLUSION

1. **Evaluate if *P. g.* LPS upregulates autophagosome formation in a TLR dependent manner and the role of melanoregulin in *P. g.* survival.**

In conclusion, it has been demonstrated that LPS upregulates autophagosome formation in a TLR dependent manner.

LPS_{1690} cell stimulation enhances autophagy as detected by increased LC3-positive puncta as well as LC3 lipidation to LC3II. An increase in the formation of large LC3-positive autophagosomal structures was also observed, leading to bacterial persistence (Hypothesis 1). Similarly, detection of the LC3 binding protein, MREG, was elevated with MREG-positive puncta; demonstrating that MREG contributes to *P. g.* clearance (Hypothesis 2).

Furthermore, LPS_{1690} activates autophagy and is necessary for the formation of large (super-size) autophagosomes in the absence of bacterial engulfment, reinforcing the role of TLR4 as autophagy sensor in pathogen clearance (Hypothesis 3).

2. **Evaluate if cells isolated from chronic periodontitis patient have decreased lysosomal capacity due to diminished lysosome maturation leading to autophagy defects.**

Additionally, the observation that LC3 and MREG co-localize only in cells from severely affected patients suggest that this clearance system may be overloaded. We suggested that mutations in MREG and /or LC3 may contribute to a predisposition to chronic periodontitis (Hypothesis 4).

Our results provide an explanation to better understand the mechanism through which *P. gingivalis* evades intra-cellular degradation leading to bacterial survival.

ARTICLES

molecular oral microbiology

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Variants of *Porphyromonas gingivalis* lipopolysaccharide alter lipidation of autophagic protein, microtubule-associated protein 1 light chain 3, LC3

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Keywords: GFP-LC3; human gingival epithelia; periodontal pathogens; *Porphyromonas gingivalis* LPS_{1690}; Saos-2 cells; toll-like receptor agonist
Accepted 5 October 2015
DOI: 10.1111/omi.12141

SUMMARY

Porphyromonas gingivalis **often subverts host cell autophagic processes for its own survival. Our previous studies document the association of the cargo sorting protein, melanoregulin (MREG), with its binding partner, the autophagic protein, microtubule-associated protein 1 light chain 3 (LC3) in macrophages incubated with *P. gingivalis* (strain 33277). Differences in the lipid A moiety of lipopolysaccharide (LPS) affect the virulence of *P. gingivalis*; penta-acylated LPS_{1690} is a weak Toll-like receptor 4 agonist compared with *Escherichia coli* LPS, whereas tetra-acylated $LPS_{1435/1449}$ acts as an LPS_{1690} antagonist. To determine how *P. gingivalis* LPS_{1690} affects autophagy we assessed LC3-dependent and MREG-dependent processes in green fluorescent protein (GFP)-LC3-expressing Saos-2 cells. LPS_{1690} stimulated the formation of very large LC3-positive vacuoles and MREG puncta. This LPS_{1690}-mediated LC3 lipidation decreased in the presence of $LPS_{1435/1449}$. When Saos-2 cells were incubated with *P. gingivalis* the bacteria internalized but did not traffic to GFP-LC3-positive structures. Nevertheless, increases in LC3 lipidation and MREG puncta were observed. Collectively, these results suggest that *P. gingivalis* internalization is not necessary for LC3 lipidation. Primary human gingival epithelial cells isolated from patients with periodontitis showed both LC3II and MREG puncta whereas cells from disease-free individuals exhibited little co-localization of these two proteins. These results suggest that the prevalence of a particular LPS moiety may modulate the degradative capacity of host cells, so influencing bacterial survival.**

INTRODUCTION

Periodontitis is a chronic inflammatory disease that is driven by a polymicrobial infection including the red complex of periodontal pathogens, the best

characterized of which is *Porphyromonas gingivalis.* This organism causes dysbiosis within dental plaque biofilms resulting in the complex microflora that induces the inflammation-mediated alveolear bone loss characteristic of periodontitis (Liang *et al.*, 2010). Recently, *P. gingivalis* has also been ascribed a role in the pathogenesis of atherosclerotic plaque formation (Gibson *et al.*, 2006; Hayashi *et al.*, 2010; Reyes *et al.*, 2013b; Slocum *et al.*, 2014). Several lines of evidence suggest that evasion of the host immune system facilitates persistence of red-complex pathogens within the gingival microenvironment and in so doing contributes to a state of chronic inflammation. *Porphyromonas gingivalis* has developed a number of strategies to avoid mechanisms of innate immunity, including blockade of inflammasome activation and inhibition of intracellular killing in macrophages (Hajishengallis *et al.*, 2008; Hajishengallis, 2009, 2010). *Porphyromonas gingivalis* promotes its survival through inhibition of interleukin-12, and interferon-γ *in vivo*, which is dependent on the pathogen's ability to hijack complement-receptor 3 (Hajishengallis *et al.*, 2008; Hajishengallis, 2009, 2010). It has been proposed that *P. gingivalis* uses these evasion maneuvers in concert with tactics to subvert autophagy to exploit macrophages as 'Trojan Horses' for subsequent infection of other cell types and potentially transmission from the oral cavity to systemic tissues (Hajishengallis, 2009).

Macroautophagy (hereto referred to as autophagy) contributes to cellular homeostasis as an autodigestion process that adjusts cellular biomass in response to bacteria, among other infectious agents. It is a vital part of the host immune response to microbial infection through direct elimination of intracellular pathogens by mediating their delivery to lysosomes. Evolutionarily, some bacteria have evolved mechanisms to evade or subvert host cell autophagic defense pathways, thereby promoting their survival or growth. Autophagosomes may target pathogens for degradation or, if subverted by bacteria, lead to survival. For example, *Streptococcus pyogenes* is degraded within autolysosomes (Deretic, 2005; Paludan *et al.*, 2005; Munz, 2006), whereas, other microbes have developed strategies to avoid autolysosomal degradation (Meresse *et al.*, 1999; Cossart, 2004; Cossart & Sansonetti, 2004). These strategies include avoidance of capture (*Shigella*) (Kanno *et al.*, 2005), survival in endothelial cells (*P. gingivalis*) (Dorn *et al.*, 2002), replication (*Listeria monocytogenes*) (Otto *et al.*, 2004) or cell egress (*Francisella)* (Checroun *et al.*, 2006). Microbes often commandeer components of the autophagic pathway to promote their survival, replication and dissemination, often by using autophagosomes, which do not mature to form degradative autolysosomes as a survival replicative niche. One of the earliest described examples of this evasion concept was *P. gingivalis*, which traffics to double membrane autophagosomes devoid of lysosomal proteases (Dorn *et al.*, 2001, 2002).

Host recognition of *P. gingivalis* as well as other Gram-negative bacteria is facilitated through recognition of lipopolysaccharides (LPS). The lipid A portion of LPS serves as a molecular motif that is recognized by Toll-like receptor 4 (TLR4), a so-called pattern recognition receptor, found on the surface of a variety of human cell types. The interaction between the two molecules induces a proinflammatory response by the host cell that takes part in the eradication of the pathogen (Akira *et al.*, 2001; Munford & Varley, 2006). *Porphyromonas gingivalis* modifies its lipid A structure in response to environmental stressors by synthesizing both a penta-acylated lipid A as well as a tetra-acylated species (Coats *et al.*, 2003, 2009, 2011). Recent studies have shown that lipid A modifications promote evasion of the non-canonical inflammasome by preventing caspase-1 activation (Kayagaki *et al.*, 2013). Hence, the heterogeneity of these structures is thought to play a role in oral immune homeostasis and contributes to the microbial shift characteristic of periodontitis (Jain & Darveau, 2010; Berezow & Darveau, 2011; Darveau *et al.*, 2012).

Single nucleotide polymorphisms of TLRs have been linked to chronic periodontitis (Sahingur *et al.*, 2011; Kim *et al.*, 2015). Furthermore, peripheral blood mononuclear cells from patients with periodontal disease had increased levels of autophagy-related gene expression with further enhancement of the autophagy protein microtubule-associated protein 1 light chain 3 (LC3) observed upon *P. gingivalis* LPS stimulation (Bullon *et al.*, 2012). We have recently shown that *P. gingivalis* challenge of macrophages results in association of microtubule-associated protein 1 light chain 3 (LC3) with its intracellular sorting protein partner melanoregulin, MREG, which is required for lysosome maturation in professional phagocytes (Frost

et al., 2015). These clinical and *in vitro* data coupled with recent reports suggesting that TLRs function as autophagy sensors (Sanjuan *et al.*, 2007) form the basis for our current work. To better understand the mechanism through which *P. gingivalis* evades intracellular degradation the experiments in this study were designed to: (1) further assess the effect of *P. gingivalis*, specifically LPS_{1690} on autophagosome assembly, through LC3 lipidation; and (2) evaluate the possibility that aberrations in LC3-dependent and/or MREG-dependent processes are associated with susceptibility to periodontitis.

METHODS

Materials

Commercially available antibodies and LPS were purchased as follows. For immunoblotting: goat anti-Actin (Santa Cruz Biotechnology Santa Cruz, CA), rabbit anti-MREG (Abnova, Taipei City, Taiwan), rabbit anti-LC3 (Abcam, Cambridge, MA), rabbit anti-beclin and rabbit anti-claudin 1 (Novus, Littleton, CO), rabbit anti-goat and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Thermo Scientific, Rockford, IL). For immunohistochemistry: mouse anti-MREG monoclonal antibody (Novus Biologics, Littleton, CO), rabbit anti-LC3 (Cell Signaling, Boston, MA), Alexa Fluor 594 donkey anti-mouse IgG and Alexa Fluor 647 donkey anti-rabbit (Life Technologies, Grand Island, NY) and Hoechst 33,258 (Ana Spec Inc. Fremont, CA). Lipopolysaccharides: *P. gingivalis* LPS_{1690}, *P. gingivalis* $LPS_{1435/1449}$ (Astarte Biologics, Redmond, WA), *Salmonella enterica* LPS and *Escherichia coli* LPS (Sigma, St Louis, MO). Analysis of LPS_{1690} and $LPS_{1435/1449}$ is summarized online at: http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/ASB_/7000.20131008.pdf. In brief, the LPS_{1690} and $LPS_{1435/1449}$ are purified from *P. gingivalis* strain 33277 as described previously (Darveau *et al.*, 2004). It is important to note that matrix-assisted laser desorption/ionization time-of-flight analysis detected a minor structure at negative m/z 1440 in the LPS. This fraction does not appear to have biological activity based on assessment of E-selectin and tumor necrosis factor-α stimulation; in contrast, the LPS_{1690} biological activity is predominantly associated with the m/z 1690 penta-acyl lipid A moiety. These analyses are consistent with previously published work, in which the 1690 lipid A species is a major component of total lipid A with a second minor lipid A centered around m/z 1448 bearing a 4′-phosphate that was undetectable by thin-layer chromatography (Coats *et al.*, 2009; Jain *et al.*, 2013). The use of such commercial LPS preparations is well documented (Yee *et al.*, 2012; Angosto *et al.*, 2014; Zhu *et al.*, 2014).

Patient samples

Human gingival tissue was obtained during routine periodontal surgery performed at the University of Pennsylvania – School of Dental Medicine. In all cases, the donated tissue represented the discarded secondary gingival flaps. Healthy tissue was procured from patients presenting for clinical crown-lengthening surgery. Diseased tissue was obtained from individuals diagnosed with chronic periodontitis and who presented for pocket reduction surgery. Disease severity was based upon mean clinical attachment loss (CAL) for the teeth being treated: mild (CAL $\leq$ 2 mm), moderate (CAL 3–4 mm) and severe (CAL $>$ 4 mm). Clinical attachment loss was determined at each patient's initial comprehensive periodontal examination. Upon removal, the tissue was immediately placed in chilled F12 medium supplemented with 1% antibiotic–antimycotic solution and transported to the laboratory on ice for processing. The protocol for tissue procurement received Institutional Review Board approval, and all donors provided informed consent.

Cell culture

GFP-LC3 Saos-2 cells

Saos-2 cells stably expressing green fluorescent protein (GFP-) LC3 [a generous gift from Dr W. Dunn, University of Florida, Gainesville (Reyes *et al.*, 2013a)] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with G418 Sulfate (Mediatech, Inc., Manassas, VA) and 10% fetal bovine serum (FBS). For immunoblot studies, Saos-2 cells were plated at a density of 5×10^5 in a 35-mm culture dish. After 24 h, in some experiments, the cells were serum starved using DMEM supplemented with 2.5% FBS and G418 Sulfate. For imaging studies, Saos-2 cells were plated on glass coverslips at 1×10^5 per well in a 24-well dish. After 24 h, the cells were either maintained in medium as described

or serum starved for 24 h using DMEM supplemented with 2.5% FBS and G418 Sulfate.

ScCr cells

These cells are a commercially available (23 ScCr [10ScNCr/23] ATCC® CRL-2751™; American Type Culture Collection, Manassas, VA) bone-marrow-derived macrophage cell line developed from a mouse strain with a deletion of the TLR4 locus. Cells were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g l^{-1} sodium bicarbonate and 4.5 g l^{-1} glucose, 70%; 10% FBS and 20% LADMAC Conditioned Media [produced from the LADMAC cell line (CRL-2420)]. For immunoblot studies, 23 ScCr cells were plated at a density of 5×10^5 in 35-mm culture dish and treated with LPS as indicated in the figure legends.

Human gingival epithelial cells

Human gingival explants were obtained as described above. Clinically healthy or diseased periodontal tissue were used to isolate primary human gingival epithelial cells as described (Oda & Watson, 1990; Ogawa *et al.*, 2005; Damek-Poprawa *et al.*, 2011). Cells were grown in serum-free keratinocyte medium and 10% FBS and were phenotyped for epithelial markers by assessing claudin expression. Cells were maintained in culture from 7 to 14 days and analyzed when semi-confluent. In the case of cells from severely diseased individuals, cells were analyzed when patches of morphologically distinct (hexagonal architecture) epithelial cells were present. Viability was assessed using Trypan blue before experiments to be $\geq$ 90% viable.

BACTERIA

Porphyromonas gingivalis strain ATCC 33277 was grown as described (Kinane *et al.*, 2006). Briefly, *P. gingivalis* were grown to mid-log phase in GAM media (Nissui Pharmaceutical, Tokyo, Japan) under anaerobic conditions (85% N_2, 10% CO_2 and 10% H_2; Coy Laboratory anaerobic chamber) and harvested by centrifugation at 3000 rpm for 15 min, washed in phosphate-buffered saline (PBS) and used immediately at the multiplicities of infection indicated in individual figures (Galicia *et al.*, 2009). In some experiments the *P. gingivalis* was labeled with Texas Red (TR) as described for outer segments (Frost *et al.*, 2015). In brief, $\sim 2 \times 10^9$ per ml *P. gingivalis* was incubated with an equal volume of 25 μg ml^{-1} TR at 37°C, washed three times to remove unbound TR and resuspended in PBS, and the optical density was determined.

For *Aggregatibacter actinomycetemcomitans*, the JP2 strain was grown overnight in AAGM broth (Fine *et al.*, 1999) supplemented with antibiotics, 12.5 μg ml^{-1} vancomycin and 75 μg ml^{-1} bacitracin as described previously (Brown *et al.*, 2013).

IMMUNOBLOTTING

Cleared Saos-2 and human gingival cell lysates were prepared in RIPA buffer as described elsewhere (Frost *et al.*, 2015). Samples (30 μg) were separated on 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Invitrogen, Grand Island, NY) under reducing conditions, transferred to nitrocellulose (iBlot system; Invitrogen), blocked with BLOTTO and probed with anti-LC3 (1 : 1000), anti-MREG (1 : 500), or anti-Claudin (1 : 500) and anti-Actin (1 : 5000) antibodies for 18 h at 4°C. Membranes were washed and incubated with goat anti-rabbit (1 : 3000) or rabbit anti-goat (1 : 5000) horseradish peroxidase-conjugated secondary antibodies. The Western blots were developed using enhanced chemiluminescence (Thermo Scientific) and analyzed by digital densitometry (Kodak Image System) as previously described (Shenker *et al.*, 2015).

MICROSCOPY

GFP-LC3-Saos-2 or Saos-2 cells were grown on glass coverslips for 48 h, rinsed in PBS, and fixed. Cells were permeabilized in 4% buffered paraformaldehyde, with 0.2% Triton X100 for 10 min at room temperature, washed in PBS with 0.05% Triton X100 (PBST) (three times, for 10 min each) and blocked in PBST containing 4% bovine serum albumin for 60 min at 37°C. After blocking, the cells were incubated at 37°C for 60 min with primary antibodies: mouse anti-MREG mAb (1 : 300) and rabbit anti-LC3 (1 : 200), washed three times in PBST and then incubated with secondary antibodies: Alexa Fluor 594 donkey anti-mouse IgG (1 : 1000), Alexa Fluor 647 donkey anti-rabbit (1 : 1000) and Hoechst 33258 (1 : 10 000) at 37°C for 60 min, washed in PBST and mounted in Cytoseal (Electron Microscopy Sciences,

Hatfield, PA). Images were captured on a Nikon A1R laser scanning confocal microscope (60 × water or 100 × oil objective) and processed using Nikon's ELEMENTS ANALYSIS software 4.0.0, with binary mask setting as described in the figure legends.

STATISTICAL ANALYSES

Data were analyzed using SIGMASTAT version 3.1 and are reported as mean ± SEM. Statistical analysis used a one-way analysis of variance Dunn's test; results with $P < 0.05$ were considered significant.

RESULTS

Porphyromonas gingivalis is internalized by GFP-LC3 containing cells

Evasion of degradative pathways by *P. gingivalis*, provides a protective survival niche in human coronary artery endothelial cells (HCAEC), in which they traffic to LC3-positive autophagosomal structures (Dorn *et al.*, 2001; Reyes *et al.*, 2013a). Incubation of mouse macrophages with *P. gingivalis* results in the association of the autophagic protein LC3 with the cargo sorting protein MREG (Frost *et al.*, 2015). To extend these observations, we sought to determine if *P. gingivalis* enhances autophagy in general and whether it traffics to autophagosomal structures in other cell types. When GFP-LC3-expressing Saos-2 cells were challenged with *P. gingivalis*, TR-labeled *P. gingivalis* (TR-*P. gingivalis*) were internalized after incubation for 1 h, with virtually no detectable TR-*P. gingivalis* after 24 h (Fig. 1A). Incubation with *P. gingivalis* resulted in an increase in LC3-positive puncta, indicative of autophagosome formation (Fig. 1A, B) although no detectable TR-*P. gingivalis* was found associated with LC3 (Fig. 1A) at any of the time points studied. Incubation with *P. gingivalis* stimulated the formation of MREG positive puncta by 30 min, which decreased within 1 h (Fig. 1B, C).

Autophagosome formation relies on the activation of several ubiquitin-like conjugation systems that mediate the lipidation of LC3 onto membranes, thus converting LC3I to LC3II, with LC3-positive puncta representative of this lipidated form. In these next studies we compared the effect of two different pathogens, *A. actinomycetemcomitans* and *P. gingivalis*, on LC3 lipidation. The increase in lipidated LC3 was confirmed by the appearance of a 14-kDa LC3II band upon *P. gingivalis* challenge (Fig. 1D). An average of three independent experiments showed a 200 ± 34% increase in LC3II after 30 min compared with No Addition (NA). Incubation with *P. gingivalis* increased MREG levels at 30 and 60 min, with a return to near baseline levels ($t = 0$) by 3 h (Fig. 1C, D). In contrast, *A. actinomycetemcomitans*, a periodontal pathogen associated with localized aggressive periodontitis (Armitage, 1999) did not induce LC3 lipidation (LC3II), instead an overall decrease in LC3II was observed (Fig. 1E): when three independent experiments were averaged we observed a 25 ± 5% decrease in LC3II/actin at 30 min compared with no addition (NA). Furthermore, MREG levels increased only slightly upon incubation with *A. actinomycetemcomitans* (Fig. 1E). Levels of MREG in most cell types are often low with increases observed upon challenge with stress-inducing toxins or pathogens (unpublished observations).

Porphyromonas gingivalis LPS_{1690} stimulates autophagy

Toll-like receptors serve as host cell autophagic sensors to mediate the upregulation of autophagic processes in an effort to clear ingested bacteria (Sanjuan *et al.*, 2007). In this series of experiments, cells were incubated with LPS from *E. coli*, *P. gingivalis* and *Salmonella enterica* with the autophagic response measured as the formation of LC3-positive vacuoles. Furthermore, we sought to determine if the level of MREG, the LC3 binding partner, was affected by LPS. The GFP-LC3-expressing Saos-2 cells show a time-dependent increase in LC3-positive puncta when treated with any of the LPS species; most puncta were observed upon the addition of the hexa-acylated 1,4 bis-phosphorylated *E. coli* LPS species (Fig. 2). The time course of maximal LC3-positive puncta formation varied among the LPS species, with *P. gingivalis* LPS_{1690} and *Salmonella enterica* LPS-dependent LC3-positive puncta formation peaking at early points (30 min) and *E. coli* LPS-dependent puncta formation peaking at late time points (3 h) (Fig. 2). Serum starvation of the GFP-LC3 Saos-2 cells resulted in enhanced puncta formation, confirming a predicted nutrient-dependent increase in autophagy (see Supplementary material, Fig. S1A). To confirm that LC3 puncta formation was not the result of overexpression of the GFP-LC3, the Saos-2 cells

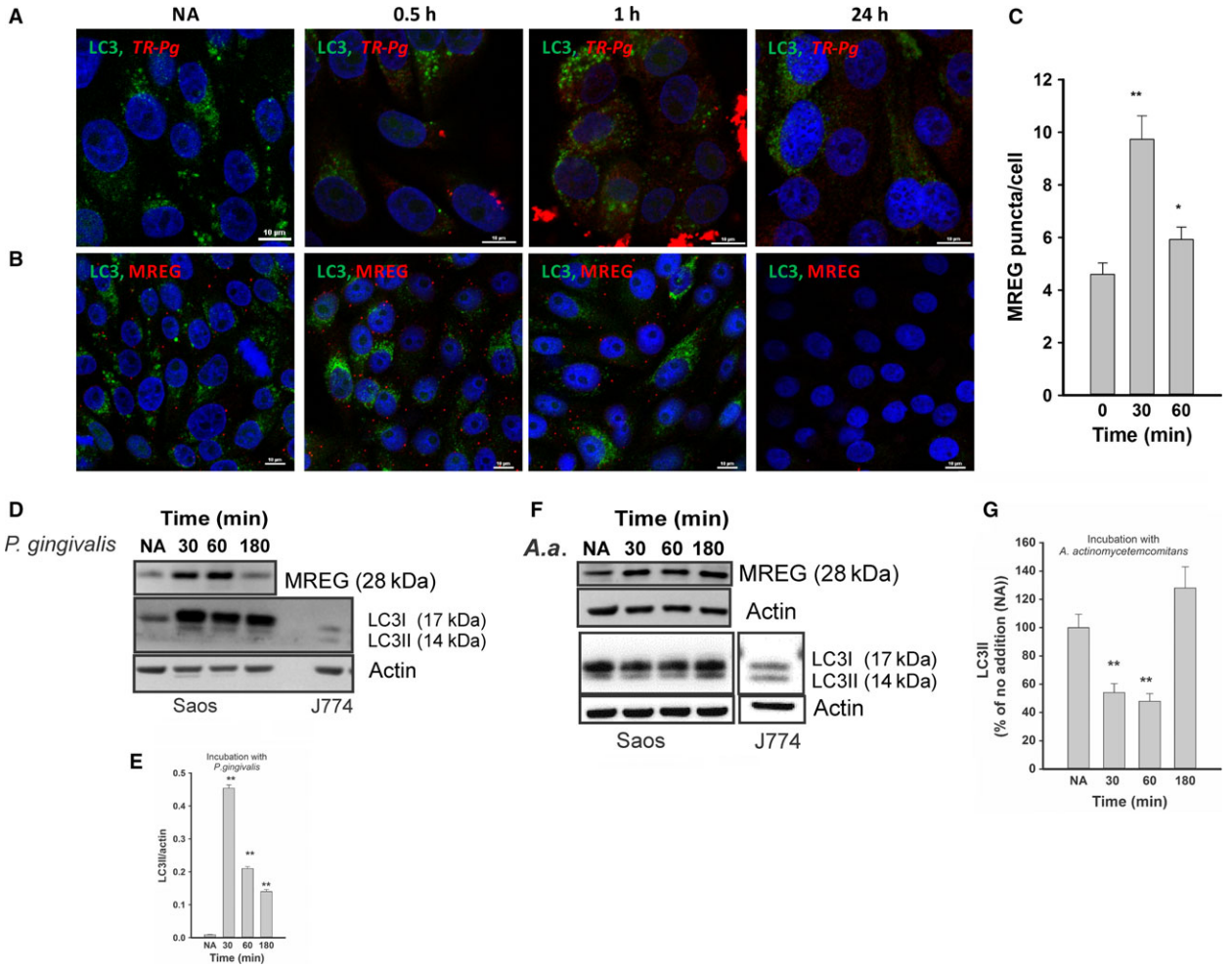

Figure 1 *Porphyromonas gingivalis* incubation with green fluorescent protein-labeled microtubule-associated protein 1 light chain 3 (GFP-LC3) Saos-2 cells. (A) *P. gingivalis* is internalized by Saos-2 cells but does not co-distribute with GFP-LC3. GFP-LC3-expressing Saos-2 cells were incubated with Texas red-labeled (TR-) *P. gingivalis* [multiplicity of infection (MOI) 100] for the indicated times, cells fixed and imaged as described in the Methods. Scale bars are all 10 ums (B) *P. gingivalis* enhances LC3 and melanoregulin (MREG) puncta. GFP-LC3-expressing Saos-2 cells were incubated with unlabeled *P. gingivalis* (MOI 100) for the indicated times, cells were fixed, stained for MREG and imaged as described in the Methods. (C) Incubation with *P. gingivalis* increases the number of MREG-positive puncta per cell within 30 min. The image quantification data are an average of 40 cells per field, with five fields analyzed in two independent experiments. Error bars represent ± SEM (**$P < 0.005$, *$P < 0.010$). (D) *P. gingivalis* enhances LC3II and MREG protein levels. Saos-2 cells were treated with unlabeled *P. gingivalis* (MOI 100) for the times indicated. Cell extracts were assessed for MREG and LC3 levels as described in the Methods. J774 cells (positive control to confirm position of LC3II and LC3I bands). NA refers to No Addition. (E) Quantification of LC3II/actin levels over time upon incubation with *P. gingivalis*. Results are the means ± SEM for three experiments, asterisks indicate statistical significance compared with no addition (NA) ($P \leq 0.05$). (F) *Aggregatibacter actinomycetemcomitans* decreases LC3. Saos-2 cells were treated with *A. actinomycetemcomitans* (MOI 100) for the times indicated. Cell extracts were assessed for MREG and LC3 levels as described in the Methods. J774 cells (positive control to confirm position of LC3II and LC3I bands). NA refers to No Addition. (G) Quantification of per cent change in LC3II relative to NA over time upon incubation with *A. actinomycetemcomitans*. Results are the means ± SEM for three experiments, asterisks indicate statistical significance compared with no addition (NA) ($P \leq 0.05$).

were treated with LPS variants. Both LC3 and MREG puncta were enhanced upon incubation with all of the LPS species, with LPS$_{1690}$-dependent puncta formation maximal after 30 min. By 3 h the MREG levels had returned almost to baseline (Fig. 2). MREG puncta also increased upon serum starvation in these cells (see Supplementary material, Fig. S1B). Incubation with *P. gingivalis* LPS$_{1690}$ not only resulted in a rapid increase in LC3-positive puncta (within the first 30 min) but also in the formation of large LC3-positive

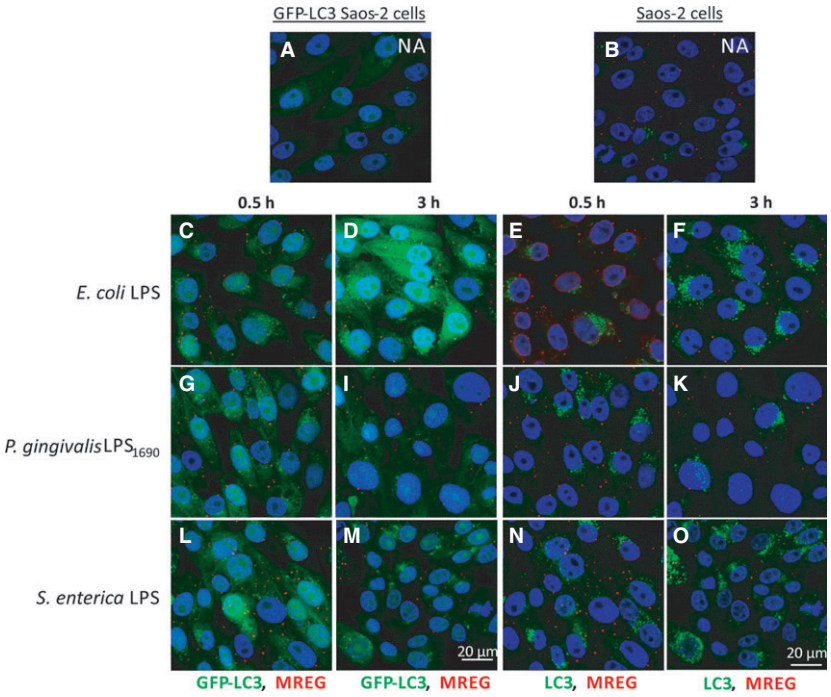

Figure 2 Time course of microtubule-associated protein 1 light chain 3 (LC3) puncta formation in response to lipopolysaccharides (LPS). (A, B) Saos-2 cells with or without stably transfected green fluorescent protein-labeled LC3 (GFP-LC3) before LPS stimulation. Time course of LC3- and melanoregulin (MREG) -positive puncta formation upon stimulation with *Escherichia coli* LPS at 0.1 μg ml^{-1} (C–F), *Porphyromonas gingivalis* LPS_{1690} at 0.1 μg ml^{-1} (G–K), or *Salmonella* LPS at 0.1 μg ml^{-1} (L–O). In (B, E, F, J, K, N and O) cells were immunostained for MREG and endogenous levels of LC3. Cells were fixed and stained for MREG and LC3 as described in the Methods.

autophagosomal structures, the average size of the structures was determined to be approximately 3 μm (representative structure is 3.5 μm) as shown in Fig. 3(A, B). *Salmonella enterica* LPS stimulation produced similar size super autophagosomes with fewer structures > 3 μm per cell; we found on average 3 ± 0.5 super-autophagosomes upon LPS_{1690} stimulation and 1.5 ± 1.0 super-autophagosomes upon incubation with *S. enterica* LPS within 30 min (data not shown).

In the presence of the LPS_{1690} antagonist, $LPS_{1435/1449}$, such super-sized autophagosomes were not observed (Fig. 4A). The average size of the autophagosomes under these conditions was on the order of 1.5–2 μm. Furthermore, the LPS_{1690} mediated increase in LC3- and MREG-positive puncta diminished in the presence of $LPS_{1435/1449}$ (Fig. 4B); puncta formation decreased by at least 50% at all time points (Fig. 4C). Increased lipidated LC3 was confirmed as the appearance of a 14-kDa LC3II band upon stimulation with LPS_{1690} (Fig. 5A), with the highest levels of LC3II at 30 min (Fig. 5B). In the presence of both LPS_{1690} and $LPS_{1435/1449}$ LC3 lipidation was decreased to below baseline levels (Fig. 5B). Similarly, *E. coli* LPS enhanced LC3II levels. MREG levels were increased 10-fold at 30 min, with a rapid and sustained return to baseline by 1 h upon LPS_{1690} stimulation, an affect that was inhibited with the simultaneous addition of the antagonist $LPS_{1435/1449}$ with a return to near baseline levels ($t = 0$) by 3 h (Fig. 5C).

The LPS_{1690} was also found to augment autophagy induction upon nutrient deprivation; when GFP-LC3 Saos-2 cells were moderately serum starved (2.5% serum), there was a two-fold increase in LC3II upon treatment with *P. gingivalis* LPS_{1690} compared with *E. coli* LPS or unstimulated cells. Addition of the LPS_{1690} antagonist $LPS_{1435/1449}$ again diminished LC3 levels (see Supplementary material, Fig. S1C). LPS_{1690} is a weak TLR4 agonist, so to further define the mode of MREG upregulation, 23 ScCr cells, a bone-marrow-derived macrophage cell line developed from a mouse strain with a deletion of the TLR4 locus were tested. As shown in the Supplementary material (Fig. S2), LPS_{1690}-mediated increases in MREG were not observed in the absence of TLR4.

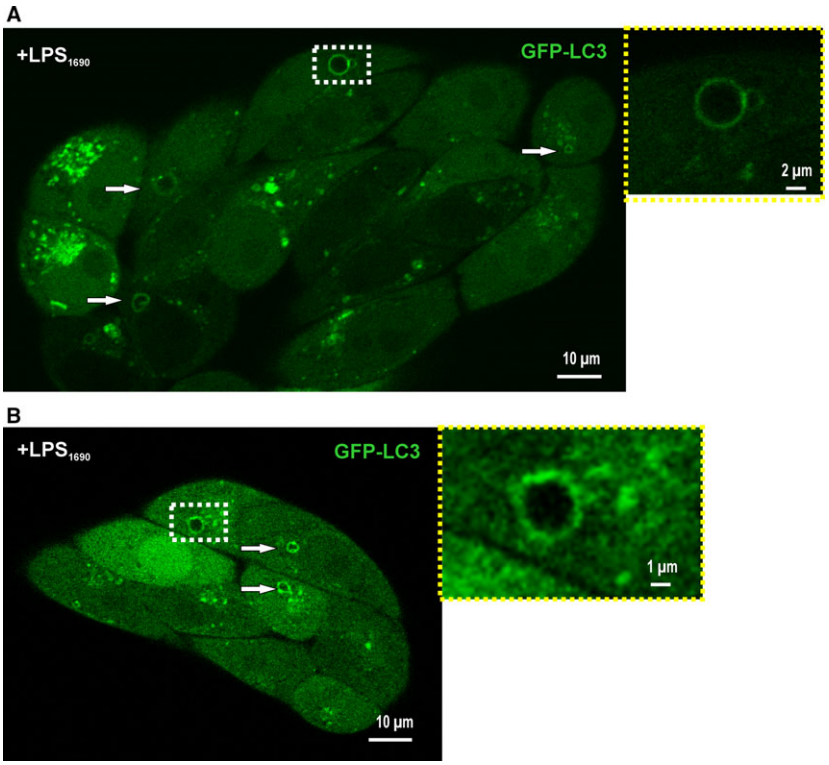

Figure 3 LPS_{1690} stimulates microtubule-associated protein 1 light chain 3 (LC3) -positive super-sized autophagosome formation in green fluorescent protein-labeled LC3 (GFP-LC3) -expressing cells. (A) GFP-LC3-Saos-2 cells were incubated with 0.1 μg ml^{-1} *Porphyromonas gingivalis* LPS_{1690} for 30 min as described in the Methods. The area designated by the white square is shown at right at a higher magnification in the yellow box. The size of the autophagosome is 3.5 μm. (B) Second representative example of GFP-LC3-Saos-2 cells incubated with 0.1 μg ml^{-1} LPS_{1690} for 30 min as described in the Methods. The area designated by the white square is shown at right at a higher magnification in the yellow box.

Expression and distribution of LC3 and MREG in human gingival epithelial cells

In this final series of experiments we characterized the unstimulated LC3 and MREG profile in human gingival epithelial cells isolated from tissue donated by subjects with no, moderate or severe periodontitis. Interestingly, in this initial series of studies it appears that MREG and LC3 redistributed in cells isolated from individuals with severe disease, with some co-localization seen between these two proteins (Fig. 6A). In contrast, when cells from healthy individuals and those with moderate disease were analyzed, the two proteins did not co-localize. From a quantitative perspective, LC3II levels were higher in healthy individuals compared with those with disease. MREG as well as beclin levels appear to decrease, with baseline levels approximately 50% lower in cells isolated from patients with disease (Fig. 6B). Collectively, these results indicate that in subjects with periodontitis the autophagy axis may be compromised.

DISCUSSION

Recognition of Gram-negative bacteria by host cells relies on the detection of LPS moieties by cell surface TLR4 (Akira *et al.*, 2001). Subsequent downstream signaling pathways instruct the host to promote an inflammatory response thereby eradicating the pathogen. *Porphyromonas gingivalis*, like several other Gram-negative bacteria, modify the lipid A portion of their LPS, thereby modulating the host response, in some cases leading to evasion of host detection and persistent infection (Al-Qutub *et al.*, 2006; Coats *et al.*, 2009; Maeshima & Fernandez, 2013). *Porphyromonas gingivalis* expresses a heterogeneous population of LPS, with a divergence in structural moieties that is dependent on temperature, growth phase and

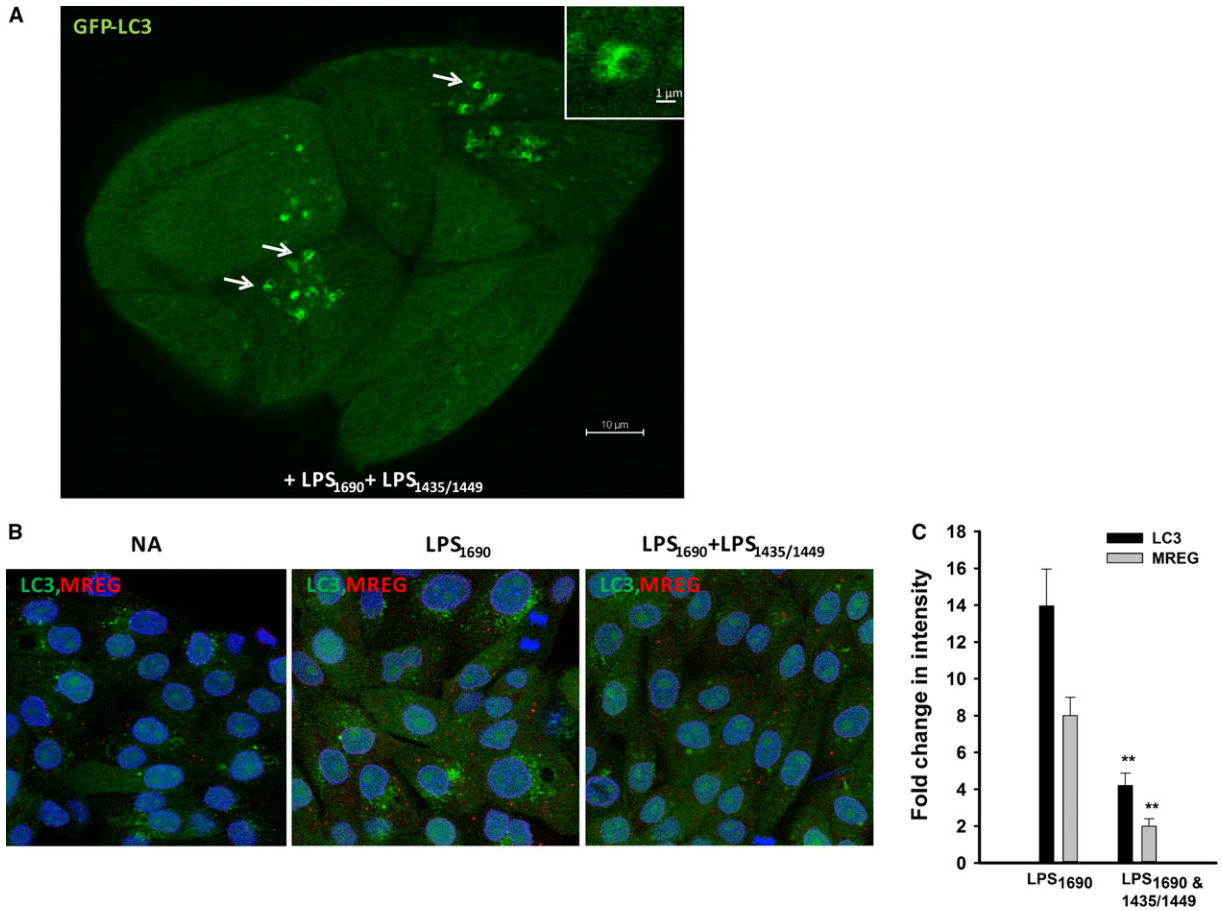

Figure 4 $LPS_{1435/1449}$ diminishes microtubule-associated protein 1 light chain 3 (LC3) -positive super-sized autophagosome formation induced by LPS_{1690}. (A) Green fluorescent protein-labeled (GFP-) LC3-Saos-2 cells were incubated with 0.1 μg ml^{-1} LPS_{1690} + $LPS_{1435/1449}$ for 60 min, fixed and imaged as described in the Methods. Inset: shows GFP-LC3-postive autophagosome. (B) GFP-LC3-Saos-2 cells were incubated with 0.1 μg ml^{-1} LPS_{1690} or LPS_{1690} + $LPS_{1435/1449}$ at 0.1 μg ml^{-1} for 30 min, fixed, stained for melanoregulin (MREG) and imaged as described in the Methods. (C) The image quantification data are an average of 20 cells per field, with five fields analyzed in two independent experiments. Data are fold change in intensity/cell relative to NA. Error bars represent ± SEM (**$P < 0.005$).

hemin levels (Curtis *et al.*, 2011). Two LPS forms identified in *P. gingivalis* are a non-phosphorylated, tetra-acylated lipid A as well as a mono-phosphorylated, penta-acylated lipid A designated $LPS_{1435/1449}$ and LPS_{1690}, respectively. LPS_{1690} exhibits weak TLR4 agonist activity compared with the highly immunogenic *E. coli* LPS, whereas $LPS_{1435/1449}$ is essentially biologically inert relative to TLR4 activation. In these studies we provide evidence that LPS_{1690} stimulation of GFP-LC3-expressing Saos-2 cells enhances autophagy as detected by increased LC3-positive puncta (Fig. 2 and Fig. 4C) as well as LC3 lipidation to LC3II (Fig. 5A, B). The size of the LPS_{1690}-induced GFP-LC3-containing autophagosomes (Fig. 3A, B) was roughly four times the size of typical autophagosomes, 3.5 to 4.0 μm compared with 0.5–1.0 μm, respectively (Mizushima *et al.*, 2002). Such large autophagosomal structures were not seen upon serum starvation of the Saos-2 cells suggesting signaling specificity in assembly of these structures with LPS_{1690}. Similarly, very large autophagic structures, termed mega-phagosomes, have been observed with coxsackievirus B3 infection (Kemball *et al.*, 2010), as well as *E. coli* LPS-induced autophagy and subsequent infection (Wang *et al.*, 2013). In the case of coxsackievirus B3 infection, these large vacuoles are non-degradative so providing the virus with a replicative niche. A similar paradigm has been proposed for *P. gingivalis* in HCAEC (Dorn *et al.*, 1999, 2001), although the role of LPS_{1690} in the promotion of these large mega-phagosomes in HCAEC has not been established. In the presence of

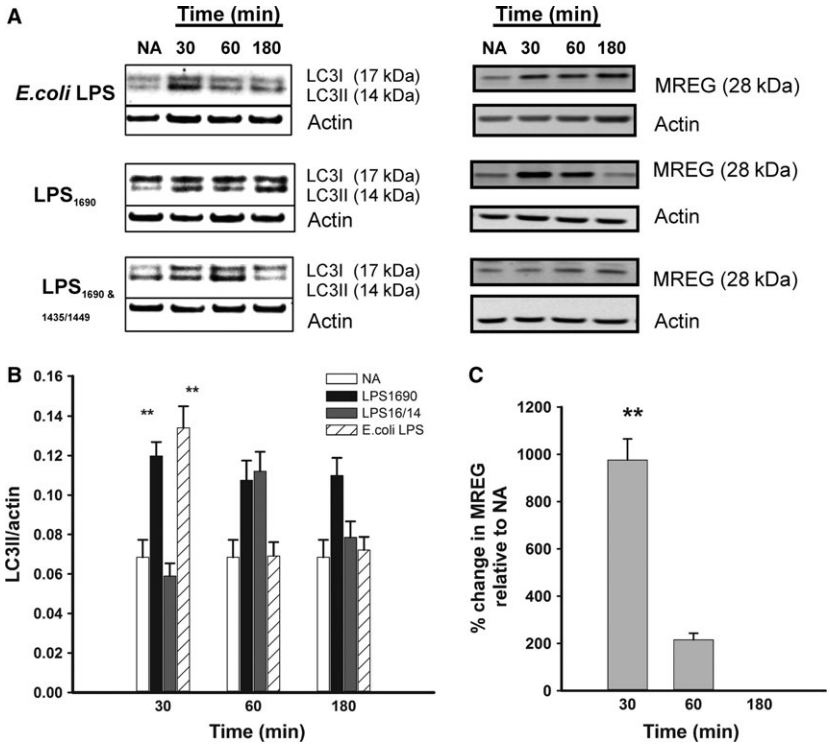

Figure 5 The LPS_{1690} antagonist, $LPS_{1435/1449}$ inhibits microtubule-associated protein 1 light chain 3 (LC3) lipidation. (A) Time course of melanoregulin (MREG), LC3I and LC3II protein expression in response to LPS_{1690}, LPS_{1690} + $LPS_{1435/1449}$ or *Escherichia coli* lipopolysaccharide (LPS) by Western blot. Green fluorescent protein-labeled (GFP-) LC3-Saos-2 cells were incubated with 0.1 μg ml^{-1} of LPS_{1690} or LPS_{1690} + $LPS_{1435/1449}$ or *E. coli* LPS, at 0.1 μg ml^{-1} for the time points indicated, NA represents no addition. Cell lysates prepared and proteins separated and subsequently identified by Western blot as described in the Methods. These blots are a representative example of four independent experiments. (B) Quantification of LC3II levels over time upon stimulation with various LPS species. Results are the means ± SEM for four experiments, each performed in duplicate; asterisks indicate statistical significance compared with control (*$P \leq 0.05$). NA represents no addition. (C) Quantification of MREG levels over time upon stimulation with LPS_{1690} or LPS_{1690} + $LPS_{1435/1449}$. Results are the means ± SEM for four experiments, each performed in duplicate; asterisks indicate statistical significance compared with control (*$P \leq 0.05$). At 180 minutes MREG levels were below those observed at NA.

$LPS_{1435/1449}$ the large LPS_{1690}-induced autophagosomes were reduced in size by 50%, to 1.0–1.5 μm in diameter (Fig. 4A). In addition, LC3II levels and LC3-positive puncta were reduced in the presence of the LPS_{1690} antagonist, $LPS_{1435/1449}$ (Fig. 4A, B). Similarly, detection of the LC3 binding protein, MREG, was elevated with MREG-positive puncta formed by 30 min upon *P. gingivalis* LPS_{1690} incubation. The addition of $LPS_{1435/1449}$ inhibited MREG upregulation. Collectively, these results suggest that the weak TLR4 agonist LPS_{1690} activates autophagy and is necessary for the formation of large autophagosomes, in the absence of bacterial engulfment, reinforcing the role of TLR4 as autophagy sensor in pathogen clearance (Sanjuan *et al.*, 2007; Xu *et al.*, 2007). Interestingly, in contrast to our previously published studies in which engulfment of *P. gingivalis* resulted in the association of LC3 with its binding partner, MREG, in these studies LPS_{1690} induced an increase in both proteins but not their co-localization, suggesting that active phagocytosis is necessary for MREG-mediated LC3-dependent degradation (Frost *et al.*, 2015). Furthermore, these studies suggest that *P. gingivalis* internalization, although it may occur, is not a prerequisite for LC3 lipidation.

In addition to promoting an inflammatory response, host cells respond to bacterial pathogens through an upregulation of degradative processes, including macroautophagy (Deretic, 2005; Deretic & Levine, 2009). The studies herein predict that such enhancement is probably mediated through LPS_{1690}. However, the role of LPS_{1690}-mediated enhancement of autophagy in local versus systemic inflammation is unknown. In gingival fibroblasts, LPS_{1690} was shown

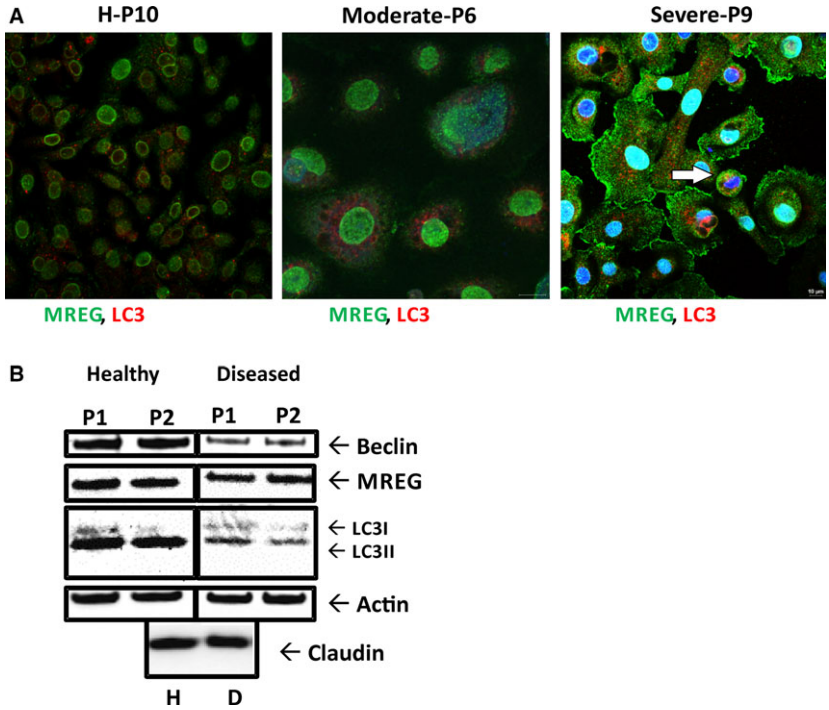

Figure 6 Microtubule-associated protein 1 light chain 3 (LC3) and its binding partner melanoregulin (MREG) are detected in human gingival epithelial cells from patients with periodontitis. (A) Confocal images of gingival cells from normal (P10), and diseased individuals (moderate: P6, severe: P9) immunostained for MREG and LC3. Human gingival epithelial cells isolated from tissue donated by subjects with no, moderate or severe periodontitis were fixed and stained for MREG and LC3 as described in the Methods. Images shown are representative of three individual samples for healthy patients and two individual samples from individuals with moderate and severe disease. Arrows indicate areas of LC3 and MREG co-localization with a Pearson's coefficient > 0.60. (B) Western blotting showing expression levels of beclin, MREG, LC3I and II as well as claudin in gingival epithelial cells from healthy patients designated P1 and P2 and individuals with moderate disease designated P1 and P2.

to activate the nuclear factor-κB pathway, with $LPS_{1435/1449}$ having virtually no effect (Herath *et al.*, 2013). Pro-inflammatory genes were also significantly upregulated by LPS_{1690}, and downregulated by $LPS_{1435/1449}$ (Herath *et al.*, 2013). Local inflammatory bone loss appears to be independent of LPS lipid A expression (Slocum *et al.*, 2014). However, Slocum *et al.* (2014) recently demonstrated that *P. gingivalis* expression of agonist lipid A species resulted in decreased vascular inflammation and atherosclerosis progression in *ApoE*$^{-/-}$ mice. Whereas *P. gingivalis* expression of antagonist lipid A species resulted in vascular inflammation and atherosclerosis progression in *ApoE*$^{-/-}$ mice. The molecular mechanism by which modulation of *P. gingivalis* lipid A expression contributes to innate immune recognition, survival and the ability of the pathogen to induce local and systemic inflammation is probably cell-type specific and the focus of further investigation.

The use of Saos-2 cells to study *P. gingivalis*-mediated enhancement of autophagy was recently described by Reyes *et al.*; in those studies inoculation of GFP-LC3-Saos-2 cells with *P. gingivalis* strain W83 did not lead to internalization of bacteria but rather to the formation of large LC3-positive puncta (Reyes *et al.*, 2013a). In our studies, *P. gingivalis* (Lin *et al.*, 2006) was found to internalize, but it was not detectable in LC3-positive structures, although LPS_{1690} appeared to stimulate LC3 lipidation and its antagonist $LPS_{1435/1449}$ inhibited lipidation. Unexpectedly, in contrast to *E. coli* LPS, LPS_{1690}-mediated LC3II lipidation was prolonged, remaining elevated over the 3-h time course, possibly as a result of the degradation of the mega-autophagosomes. Furthermore, incubation with LPS_{1690} and $LPS_{1435/1449}$ together did not lead to diminished LC3II at the 60-min time point. We predict that this may be the result of an off-target effect of $LPS_{1435/1449}$ that regulates

the turnover of LC3II via Atg 4. Whether Atg 4 is regulated by $LPS_{1435/1449}$ through a TLR2-mediated pathway remains to be determined. Differences in internalization and intracellular trafficking patterns and autophagy induction may be due to a truncation in the lipoprotein gene in *P. gingivalis* strain 33277 that is present in its full-length form in *P. gingivalis* W83 (Chen *et al.*, 2004). In this regard, *P. gingivalis* persistence is proposed to be independent of lipid A structure but is rather the result of a component that co-purifies with LPS. It is proposed that this component is part of a novel class of TLR2 agonists composed of lipoproteins (Jain *et al.*, 2013).

When cells were treated with *A. actinomycetemcomitans* versus *P. gingivalis* no increase in LC3 lipidation was observed, on the contrary incubation with *A. actinomycetemcomitans* resulted in a decrease in LC3II (Fig. 1D, E). Both *A. actinomycetemcomitans* and *P. gingivalis* can trigger immune responses and production of pro-inflammatory and anti-inflammatory cytokines in the host response, which is mediated by TLR pathways (Ando-Suguimoto *et al.*, 2014; Shaik-Dasthagirisaheb *et al.*, 2015). Specifically, the immune responses to *P. gingivalis* are mediated by extracellular signal-related kinase (ERK), c-Jun N-terminal protein kinase (JNK) in HL-60 and JY (B cells) whereas immune responses in macrophages challenged by *A. actinomycetemcomitans* involve p38 pathways other than the ERK and JNK pathways (Korostoff *et al.*, 1998, 2000; Hajishengallis *et al.*, 2004). In *A. actinomycetemcomitan*s-challenged cells, the levels of interferon-γ, interleukin-1β, interleukin-12, tumor necrosis factor-α and tumor necrosis factor-β are decreased compared with those in *P. gingivalis*-stimulated cells. Differences in host response between these two bacteria may be partially the result of LC3-mediated non-canonical inflammasome activation; with *A. actinomycetemcomitans* leading to decreased interleukin-1β secretion through an LC-II-mediated pathway (Dupont *et al.*, 2011). A major virulence factor of *A. actinomycetemcomitans* is the secretion of leukotoxin A, which induces apoptosis in white blood cells (Taichman *et al.*, 1980, 1991). Leukotoxin A-mediated apoptosis occurs via different pathways such as a mitochondrial signaling pathway that results in collapse of the mitochondrial membrane potential and arrest of oxidative phosphorylation as well as by activation of caspase 1 (Korostoff *et al.*, 1998, 2000; Yamaguchi *et al.*, 2001).

Lastly, we evaluated the distribution profile of LC3 and MREG in gingival epithelial cells isolated from tissue obtained from healthy and diseased individuals (Fig. 6). We observed co-localization between LC3 and MREG in severely affected individuals with no co-localization seen between these proteins in healthy or moderately affected individuals. MREG-mediated LC3-dependent degradation is necessary for the clearance of lipid debris (Frost *et al.*, 2015) and we propose for clearance of bacteria. The observation that LC3 and MREG co-localize only in cells from severely affected patients suggest that this clearance system may be overloaded. In a manner analogous to that proposed for dendritic cells (El-Awady *et al.*, 2015a,b). Collectively, these results suggest that mutations in MREG and /or LC3 may contribute to a predisposition to chronic periodontitis.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Manju Benakanakere for directly supplying the *P. gingivalis* 33277 used in these studies as well as Dr A. Kazi for his initial observations. This work was supported by funds from PHS; DE022465 (KBB), DE023071 (BJS) and DE09517 (ETL) as well as an SDM-Rabinowitz Award (KBB and JK) and Schoenleber Pilot Grant (JK).

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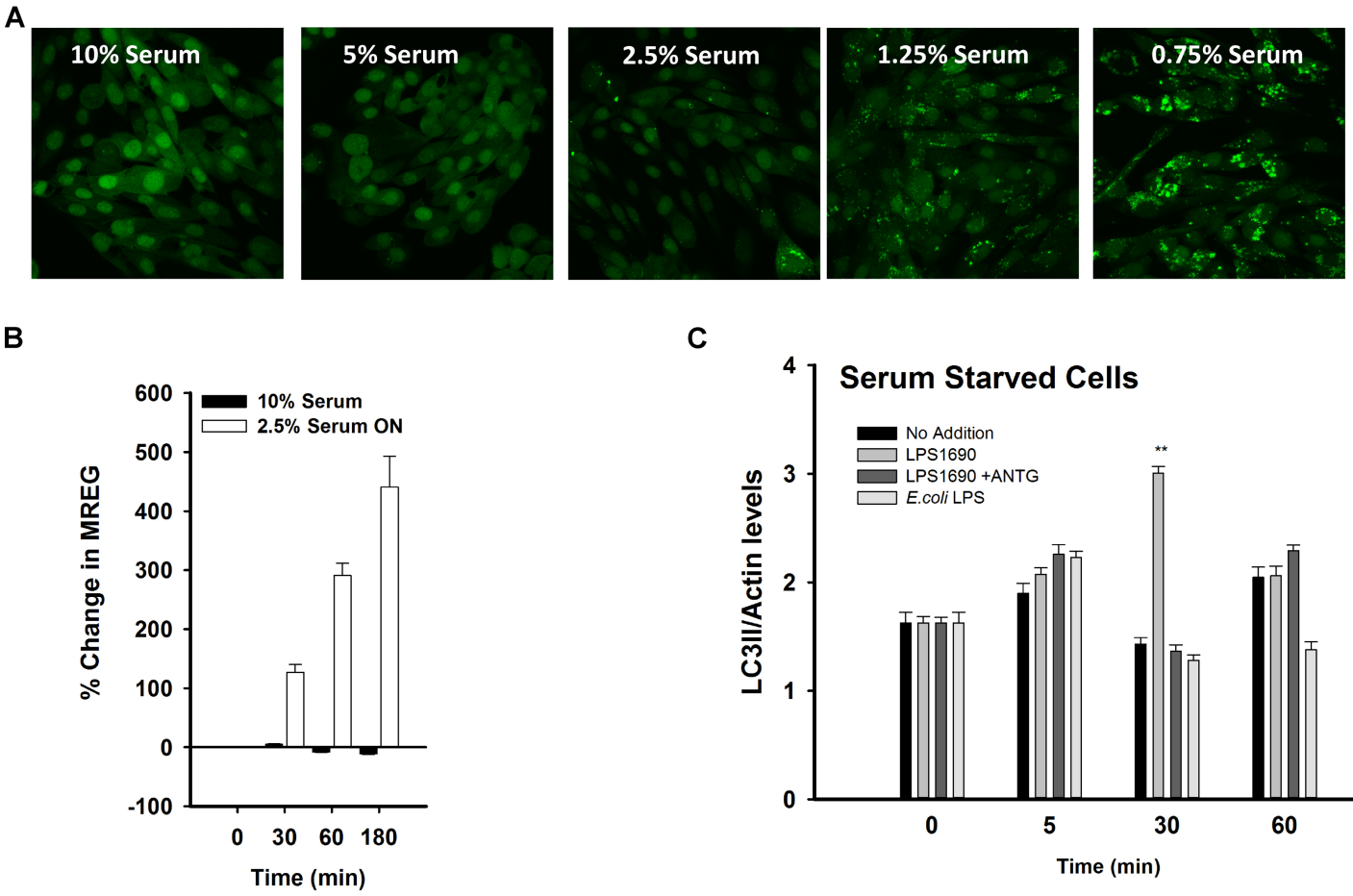
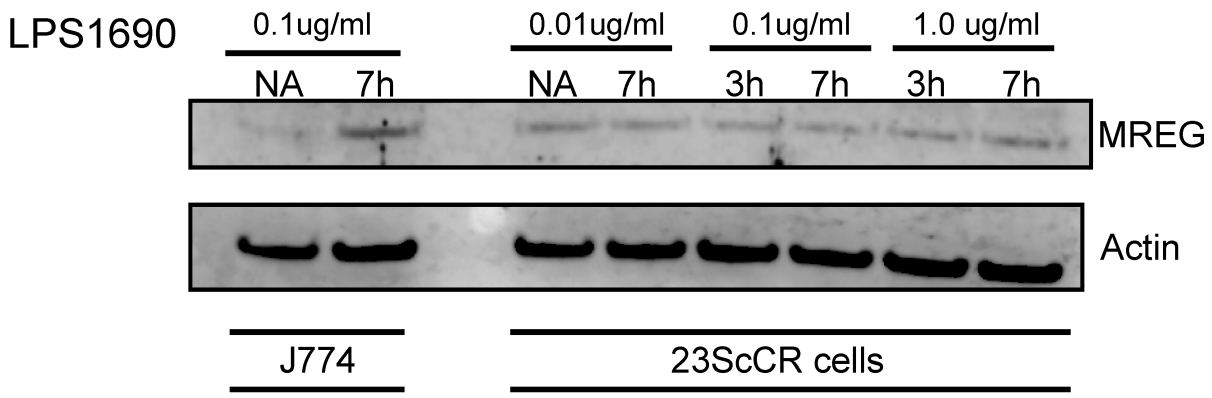
S. Figure 1


A
10% Serum
5% Serum
2.5% Serum
1.25% Serum
0.75% Serum
B
10% Serum
2.5% Serum ON
% Change in MREG
600
500
400
300
200
100
0
-100
0
30
60
180
Time (min)
C
Serum Starved Cells
No Addition
LPS1690
LPS1690 +ANTG
E.coli LPS
**
LC3II/Actin levels
4
3
2
1
0
0
5
30
60
Time (min)


LPS1690
0.1ug/ml
0.01ug/ml
0.1ug/ml
1.0 ug/ml
NA
7h
NA
7h
3h
7h
3h
7h
MREG
Actin
J774
23ScCR cells

S. Figure 2

Porphyromonas gingivalis LPS modulates autophagy in gingival epithelial cells.

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Running Title: LPS_{1690} stimulates LC3

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Keywords: periodontal pathogens, human gingival epithelia, Toll like receptor agonist

Summary (250) words

Porphyromonasgingivalis (*P. gingivalis*) is a keystone pathogen that contributes to periodontal pathogenesis by disrupting host-microbe homeostasis and promoting dysbiosis. *Porphyromonas gingivalis* often subverts host-cell response including degradative pathways to facilitate its survival. Virulence of *P.gingivalis* likely reflects an alteration in the lipid A composition of its lipopolysaccharide (LPS) from the penta-acylated (*Pg*LPS_{1690}) to the tetra-acylated (*Pg*$LPS_{1435/1449}$) form. In the human gingival epithalia, Pg is not readily degraded but survives, whether survival is mediated through the hijacking of autophagic degradative pathways is unknown. Our previous studies document enhanced autophagic flux upon incubation with Pg LPS, without internalization of bacteria. Moreover, individuals with severe periodontal disease were shown to have decreased levels of LC3 and its binding partner melanoregulin (MREG), with the bulk of these proteins forming intracellular complexes. In contrast, cells from disease-free individuals showed little colocalization of LC3 with MREG. Based on those studies we predicted that the LC3-MREG degradative pathway may be overloaded in diseased individuals. To further explore the molecular mechanism of this overload, in these studies we asked if Pg LPS variants, enhance autophagy in vitro, using human gingival cell line. In addition, we treated gingival cells from diseased and disease free patients with LPS to define their responsiveness. Our results suggest that mutations in MREG and /or LC3 may contribute to a predisposition to chronic periodontitis.

Introduction

Periodontal infection and bacteria associated with this condition are linked to various systemic conditions including colorectal and oral squamous cell carcinoma, coronary artery disease and a pre-disposition to pre-term delivery (1-4). Periodontitis is a chronic inflammatory disease that develops due to dysbiosis of the bacterial community colonizing the hard and soft tissue of the periodontium (5, 6). A critical component of this poly-microbial flora is *Porphyromonas gingivalis* (*P.gingivalis*) a Gram- negative anaerobe, designated a keystone pathogen due to its effect on disruption of tissue homeostasis as well as enhancing the virulence of the polymicrobial community as a whole (6-11).

The gingival epithelium is an early line of defense against microbial assault that when damaged allows invasive oral bacteria and their products to enter the connective tissue. These cells line the gingival crevice thus providing a physical barrier and a signaling platform to communicate the presence of bacteria to the underlying immune cells. *P. gingivalis* actively invades gingival epithelial cells, establishing a protective niche for survival (12, 13) and intracellular redistribution with a transfer of *P. gingivalis* 24 hours after infection (14). Essential for *P. gingivalis* survival is the hijacking of host-cell intracellular degradative pathways designed eliminate the bacteria. A common subversion of degradation by numerous bacteria is modulation of the autophay pathway.

Macroautophagy (hereto referred to as autophagy) contributes to cellular homeostasis as an auto- digestion process that adjusts cellular biomass in response to bacteria, among other

infectious agents. It is a vital part of the host immune response to microbial infection through direct elimination of intra-cellular pathogens. Autophagosomes target pathogens for degradation or, if subverted by bacteria, lead to survival. For example, *Streptococcus pyogenes* is degraded within autolysosomes (15-17), whereas, other microbes commandeer components of the autophagic pathway to promote their survival, replication and dissemination, often by using autophagosomes, which do not mature to form degradative autolysosomes as a survival replicative niche (18-20). One of the earliest described examples of this evasion concept was *P. gingivalis,* which traffics to double membrane autophagosomes devoid of lysosomal proteases (21, 22). We have recently shown that *P. gingivalis* challenge of macrophages results in association of microtubule-associated protein 1 light chain 3 (LC3) with its intracellular sorting protein part-ner melanoregulin, MREG, which is required for lysosome maturation in professional phagocytes (23).

P. gingivalis lipopolysaccharide (PgLPS) is one of the key virulence factors that contribute to periodontitis (8-10). Lipid A is the most biologically active component of LPS and its structure differs greatly among Gram-negative bacterial species; the canonical *E. coli* LPS possesses a hexa-acylated lipid A structure and promotes a strong inflammatory response via the activation of TLR4. Conversely, PgLPS is heterogeneous consisting of penta- (LPS_{1690}) and tetra- ($LPS_{1435/1449}$) acylated lipid structures that generally have opposing effects on innate immune responses, thereby playing a key role in modulating host immune-inflammatory reactions (9, 11, 12). *P. gingivalis* strains expressing penta-acylated lipid A activate TLR4 and are more susceptible to killing by macrophages compared to strains that predominantly generate tetra-acylated lipid A (8, 9, 13, 14). The lipid A structure of *P. gingivalis* is modified in response

to environmental stressors by synthesizing both a penta-acylated lipid A as well as a tetra-acylated species (24-26). Lipid A modifications promote evasion of the non-canonical inflammasome by preventing caspase-1 activation (27). Hence, the heterogeneity of these structures is thought to play a role in oral immune homeostasis and contributes to the microbial shift characteristic of periodontitis (28-30).

Susceptibility to chronic periodontitis (CP) is determined by both environmental and genetic factors [13, 14]. To date, genetic studies have failed to identify an association between CP and major disease genes. Rather, it appears that the interaction between multiple polymorphic genes, each with relatively small but significant associations with disease risk, contribute to overall susceptibility [14]. Numerous studies have suggested that unique inherited defects in phagocyte function render individuals more susceptible to specific forms of periodontitis including CP [14]. Single nucleotide polymorphisms (SNPs) of TLRs have been linked to chronic periodontitis (31, 32). Furthermore, peripheral blood mononuclear cells from patients with periodontal disease had increased levels of autophagy-related gene expression with further enhancement of the autophagy protein microtubule-associated protein 1 light chain 3 (LC3) observed upon *P. gingivalis* LPS stimulation (33). Our previous studies evaluating the distribution profile of LC3 and MREG in gingival epithelial cells isolated from patients suggest that mutations in MREG and /or LC3 may contribute to a predisposition to chronic periodontitis. The observation that LC3 and MREG co-localize only in cells from severely affected patients suggest that this clearance system may be overloaded. In a manner analogous to that proposed for dendritic cells (34, 35). In the current studies, we tested the effect of PgLPS variants *in vitro* using an immortalized gingival cell line, OKF61 TERT-1 cells and determined if human gingival

explants isolated from healthy and diseased individuals mediate autophagic flux in response to PgLPS variants.

Methods

Materials. Commercially available antibodies were purchased as follows: mouse anti-Actin (A2228; Sigma-Aldrich, St. Louis, MO), rabbit anti-MREG from Abnova (Taipei City, Taiwan), rabbit anti-LC3 from Abcam (Cambridge, MA), rabbit anti-goat and goat anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody from Thermo Scientific (Rockford, IL). LPS: *Porphyromonas gingivalis* (*P.g.*) LPS_{1690}, *P.g.* $LPS_{1435/1449}$ from Astarte Biologics (Redmond, WA) and *Escherichia coli* (*E. coli*) LPS from Sigma (St. Louis, MO). Analysis of LPS_{1690} and $LPS_{1435/1449}$ is summarized online at: http://search.cosmobio.co.jp/cosmo_search_ p/search_gate2/docs/ASB_/7000.20131008.pdf. In brief, the LPS_{1690} and $LPS_{1435/1449}$ are purified from *P. gingivalis* strain 33277 as described previously (36). It is important to note that matrix-assisted laser desorption/ionization time-of-flight analysis detected a minor structure at negative m/z 1440 in the LPS. This fraction does not appear to have biological activity based on assessment of E-selectin and tumor necrosis factor-α stimulation; in contrast, the LPS1690 biological activity is predominantly associated with the m/z 1690 penta-acyl lipid A moiety. These analyses are consistent with previously published work, in which the 1690 lipid A species is a major component of total lipid A with a second minor lipid A centered around m/z 1448 bearing a 40-phosphate that was undetectable by thin-layer chromatography (25, 37). The use of such commercial LPS preparations is well documented (38-40).

Patient Samples Human gingival tissue was obtained during routine periodontal surgery done at the University of Pennsylvania School of Dental Medicine. In all cases, the donated tissue represented the discarded secondary gingival flaps. Healthy tissue was procured from patients presenting for clinical crown lengthening surgery. Diseased tissue was obtained from individuals diagnosed with chronic periodontitis and presenting for pocket reduction surgery. Disease severity was based upon mean clinical attachment loss (CAL) for the teeth being treated: mild (CAL $\leq$ 2 mm), moderate (CAL of 3 to 4 mm) and severe (CAL > 4 mm). Clinical attachment loss was determined at each patient's initial comprehensive

periodontal examination. Upon removal, the tissue was immediately placed in chilled F12 medium supplemented with 1% Antibiotic-Antimycotic solution and transported to the laboratory on ice for processing. The protocol for tissue procurement received Institutional Review Board approval, and all donors provided informed consent.

Cell Culture

OKF61 TERT-1 cells, a generous gift from the Rheinwald labs are keratinocytes immortalized by transfection to express TERT (41, 42). Cells were initially cultured in modified "GIBCO Keratinocyte serum-free medium (K-sfm)" (Life Technologies catalog #17005-042) as described (43). Upon reaching 30% confluence in K-sfm the cells were sub-cultured and switched to a 1:1 mixture of K-sfm and DF-K medium. DF-K medium is prepared by mixing calcium-free, glutamine-free DMEM (Life Technologies catalog #21068-028) with Ham's F-12 medium (Life Technologies catalog #11765-054) 1:1 (vol/vol) and supplementing with 0.2 ng/ml EGF, 25 μg/ml BPE (half of the tube of BPE supplied by Invitrogen for K-sfm per 500 ml medium, the same as for making Ksfm), 2 mM L-glutamine, 0.25 mM CaCl2, and pen/strep. We routinely carry stocks by plating 10^4, $3X10^4$, and 10^5 cells per T75 flask or p100 dish (or 1/3 these numbers per T25 flask or p60 dish). Cultures are refed every two days and used as needed for experiments.

Human gingival epithelial cells. Human gingival explants were obtained as described above. Clinically healthy or diseased periodontal tis-sue were used to isolate primary human gingival epithelial cells as described (44-46). Cells were grown in serum-free keratinocyte medium and 10% FBS and were phenotyped for epithelial markers by assessing claudin expression. Cells were maintained in culture from 7 to 14 days and analyzed when semi-confluent. In the case of cells from severely diseased individuals, cells were analyzed when patches of

morphologically distinct (hexagonal architecture) epithelial cells were present. Viability was assessed using Trypan blue before experiments to be ≥ 90% viable.

Immunoblotting Cells were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin (Cell Signaling, Boston, MA) and protease inhibitor cocktail (Roche) and centrifuged at 13,500rpm for 15 min at 4°C. Proteins in the cleared lysates were separated on 4-12% SDS–PAGE (Invitrogen, Grand Island, NY) under reducing conditions, transferred to nitrocellulose, blocked and probed with anti-LC3 (1:1000), or anti-MREG (1:500) and anti-Actin (1:500) antibody. Appropriate horseradish peroxidase-conjugated secondary antibodies were subsequently used for detection. Blots were developed using enhanced chemiluminescence (ECL) (SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) and captured on ImageQuant™ LAS 400 image reader (GE Healthcare, Mickleton, NJ) and quantified as described (47).

Statistical Analyses. Data were analyzed using SigmaStat version 3.1. Data are reported as mean ± SEM. Statistical analysis used a student's t-test, results with $p<0.05$ were considered significant and are indicated in the figures.

Results

P. gingivalis LPS_{1690} stimulates autophagic proteins *in vitro,* in human gingival cells.

Recently, TLRs were proposed to act as host cell autophagy sensors to mediate the upregulation of autophagic processes to clear ingested bacteria. *E.coli* LPS for example a hexa-acylated 1,4 bis-phosphorylated species is a potent stimulator of autophagy through TLR4. Whether *P.gingivalis,* LPS_{1690}, also a TLR4 agonist, albeit less potent, stimulates autophagy in the human gingival cell line, OKF64-TERT is the focus of our first experiment. OKF64-TERT cells show a decrease in Atg5, a ubiquitin like conjugation protein necessary in the lipidation of LC3I to LC3II upon stimulation with LPS_{1690} at both 1 and 3hrs (Fig. 1A and B). Interestingly, $LPS_{1435/1449,}$ also showed a decrease by 3 hrs with no change at the one hour timepoint (Fig. 1A and B). Autophagic flux, defined as LC3II/LC3I is increased at significantly at 3hrs both with LPS_{1690} as well as $LPS_{1435/1449}$, little change was detected at 1hr (Fig. 1A and C). LC3 in human macrophages as well as epithelial cells of the eye is associated with a small cargo sorting protein melanoregulin (MREG) (23). In retinal epithelia, MREG is required for LC3 dependent phagosome degradation. When OKF64-TERT cells were stimulated with $LPS_{1690,}$ MREG levels were elevated after 3hours, in contrast incubation with $LPS_{1435/1449}$, decreased MREG levels at this same time point.

Gingival cells from patients with moderate periodontal disease respond less robustly to *P. gingivalis* LPS_{1690}.

Our previous studies showed an increase in the extent of co-localization between MREG and LC3 in samples isolated from healthy patients and individuals with moderate or severe periodontal disease. Blasi et al. suggested that in subjects with periodontitis the autophagy axis

202 may be compromised (48). Here we extend those studies with the analysis of 15 patients,
203 characterizing the profile of MREG, ATG5 and beclin as well as assessing autophagic flux in
204 human gingival epithelial cells isolated from tissue donated by subjects with no or moderate
205 periodontitis. MREG as well as beclin levels appear to decrease, with baseline levels
206 approximately 25 and 50% lower, respectively in cells isolated from patients with disease (Fig.2
207 A and B), while ATG5 levels remained unchanged (Fig. 2C). To determine how healthy and
208 diseased individuals may react to repeated bacterial dysbiosis, we treated the cells with
209 $PgLPS_{1690}$, for 3hours (Fig. 2D). Incubation of cells with LPS_{1690} (0.1ug/mL) resulted in an
210 increase in both ATG5 and MREG in all samples isolated from healthy subjects however there
211 was no corresponding increase in the levels of these proteins in the samples isolated from
212 patients with moderate disease (Fig.2E). These results are an average of two technical replicates
213 from three different patients. Most telling is a comparison of the autophagic flux measured in
214 individuals with moderate disease compared to healthy subjects (Table 1). While there was a
215 quantitative variability in the amount of flux, likely reflective of the heterogeneity observed
216 between individuals in general, there was a consistently lower autophagic flux observed upon
217 LPS_{1690} treatment in individuals with disease. This is particularly striking as autophagic flux
218 consistently increased by 30-50% in healthy individuals upon LPS_{1690} treatment.

Table 1.- Autophagic Flux LC3II/LC3I in Patient isolates

SAMPLE	Autophagic flux (LC3II/LC3I)	
	No Addition	+LPS_{1690} (0.1µg/ml)
P2-Healthy	0.498	0.7040
P3-Healthy	2.85	11.90
P7-Healthy	0.55	0.70
P10-Healthy	0.89	1.28
P4-Moderate Disease	2.23	1.34
P6-Moderate Disease	3.08	1.71
P12-Moderate Disease	0.80	0.64
P14-Moderate Disease	0.75	0.55

Discussion

Gram-negative bacteria are recognized by host cells through the transmembrane protein TLR4 (49). This receptor identifies lipopolysaccharide (LPS) and its activation leads to an intracellular signaling pathway NF-kappaB, an inflammatory process which activates the innate immune system(50). Several gram-negative bacteria, such as *Pg,* modify the Lipid A portion of their LPSs and have developed methods to subvert innate immunity leading to a persistent infection (25, 51-55). The *Pg* LPS_{1690} is a penta-acylated form with weak TLR4 agonist activity, compared to the highly immunogenic *E.coli* LPS, whereas *Pg* $LPS_{1435/1449}$ is tetra-acylated immunologically unreceptive at the TLR4 complex and acting as an LPS_{1690} antagonist. Our previous studies demonstrated an enhanced autophagy after LPS_{1690} stimulation of GFP-LC3 Saos cells detected through the increased LC3 puncta formation(48). However, when induced with $LPS_{1435/1449}$, LC3 puncta formation decreased. In these present studies, Atg5 levels exhibited a decreased on OKF64-TERT cells upon LPS_{1690} and $LPS_{1435/1449}$ stimulation (Fig. 1A and B). Interestingly, we show evidence that LPS_{1690} and $LPS_{1435/1449}$ stimulation of OKF64-TERT cells also intensifies autophagy as detected by increased LC3II/LC3I levels with little change at a shorter time period (Fig. 1A and C). These results suggest that Atg5 might not be the only molecular key component that play a major role in LC3 conjugation. Similarly, Wang et al. found that *E. coli* LPS induced autophagy and subsequent infection (56). Moreover, the host cell respond to bacterial pathogens up-regulating degradative processes in addition to inflammatory response stimulation (15, 57). Several studies have predicted such processes may be mediated through *Pg* LPS_{1690}. *Pg* LPS_{1690} activated the NF-κB pathway in gingival fibroblasts, in contrast its antagonist, $LPS_{1435/1449}$, showed no effect in activation of NF-κB (58). Furthermore, pro-

inflammatory genes were also considerably up-regulated by *Pg* LPS_{1690}, and down-regulated by $LPS_{1435/1449}$ (58).

In addition, Frost et al. suggested that active phagocytosis is necessary for MREG mediated LC3 dependent degradation (23). Similar to LC3 response, we show data that MREG levels were elevated after OKF64-TERT cells stimulation with LPS_{1690}. However, incubation with $LPS_{1435/1449}$, decreased MREG, suggesting that autophagy process could be inadequate (Fig. 1A and D). The exact molecular mechanism of *P. gingivalis* LPS action which contributes to bacterial subsistence, innate immune detection and its ability to induce local and systemic inflammatory process require further investigation.

Lastly, we analyzed levels of MREG, ATG5 and beclin as well as levels of auphagic flux in human gingival epithelial cells extracted from individuals with no or moderate periodontitis. Gingival Epithelial cells provide an obstacle against bacterial infection and also contribute in the innate immune resistance. Once epithelial cells are infected by *P.g.*, there is subsequent downstream signaling pathways that control transcription of target genes encoding for immune response and inflammatory reactions to eradicate the pathogen (59-61). The stimulation of gingival epithelial cells with *P.g.* has been investigated by several authors (12-14, 60, 61). In this present study ATG5 levels were equal in isolated epithelial cells from moderated diseased patients (Fig. 2C). However, MREG and beclin levels did not follow the same pattern as ATG5 and were found to be decreased half and ¼ respectively, suggesting that the autophagy process may be jeopardized in diseased patients (Fig.2 A and B). Other authors, such as Bullon et al have found a dysregulation of autophagy in periodontitis (33). We treated both group of cells with

271 LPS_{1690} to observe the difference of autophagy responses between healthy and disease epithelial
272 samples. In the presence of $LPS_{1690,}$ there was an increased level of ATG5 and MREG in healthy
273 tissue cells, however such increase was not observed in cell isolated from moderate disease
274 subjects (Fig. 2E). Therefore, these last results are consistent with our previous studies and
275 indicate that the autophagic pathway is compromised in patients with periodontitis disease.
276 Moreover, the autophagic flux was consistently lower in diseased cells following incubation with
277 LPS_{1690} (Table 1). However, autophagic flux after LPS_{1690} stimulation consistently increased in
278 healthy epithelial cells. Collectively, these results suggest that human gingiva become refractory
279 to LPS_{1690} upon microbial dysbiosis associated with chronic periodontitis.

Figure Legends

Figure 1- LPS_{1690} and the LPS_{1690} antagonist, $LPS_{1435/1449}$, stimulation of OKF64-TERT cells. (A) Time course of ATG5, melanoregulin (MREG), LC3I and LC3II protein expression in response to LPS_{1690}, $LPS_{1435/1449}$ or Escherichia coli lipopolysaccharide (LPS) by Western blot. OKF61 TERT-1 cells, keratinocytes immortalized by transfection to express TERT, were incubated with 0.1ug/mL^{-1} of LPS_{1690} or $LPS_{1435/1449}$ or E. coli LPS, at 0.1ug/mL^{-1} for the time points indicated, No Addition represents Control. Cell lysates prepared and proteins separated and subsequently identified by Western blot as described in the Methods. (B) Quantification of AGT5 levels over time upon incubation with LPS_{1690} or $LPS_{1435/1449}$ or E. coli LPS. Control represents no addition. (C) Quantification of level of expression of LC3I/LC3II over time upon stimulation with various LPS species. (G) Quantification of per cent change in MREG over time upon incubation with LPS_{1690} or $LPS_{1435/1449}$ or E. coli LPS.

Figure 2- Gingival epithelial cells from healthy patients and subjects with moderate periodontal disease. (A) Quantification of Beclin/Actin levels on epithelial cells isolated from healthy subjects and subjects with moderate periodontitis. P indicates statistical significance compared with healthy group ($*P \leq 0.05$). (B) Level of MREG/Actin expression quantified in healthy cells and in moderated diseased cells. P indicates statistical significance compared with healthy group ($*P \leq 0.05$). (C) ATG5/Actin protein levels in healthy and moderate periodontal disease patient. ATG5 levels remain unchanged. (D) Incubation of gingival cells with LPS_{1690}. Western blotting showing expression levels of beclin, MREG, LC3I and II in gingival epithelial cells from healthy individuals and patients with moderate disease. Gingival cells cells were incubated with 0.1ug/mL^{-1} of LPS_{1690} or LPS_{1690} + LPS1435/1449 for 3 hours. NA represents no addition. Cell extracts were assessed for Beclin, MREG and LC3 levels and recognized by

Western blot as described in the Methods. (E) Quantification of ATG5 and MREG levels upon stimulation with 0.01ug/mL^{-1} of LPS_{1690} or 0.1ug/mL^{-1} of LPS_{1690} of healthy and moderated disease gingival cells for 3 hours. These results are the means of two technical replicates from three different patients, asterisk indicates statistical significance compared with 0.01ug/mL^{-1} of LPS_{1690} ($*P \leq 0.05$).

Acknowledgements;

This work was supported by grants from PHS; DE006014 (BJS) and DE022465-02 (KBB).

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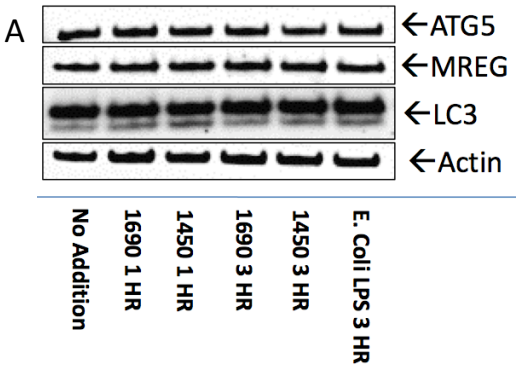
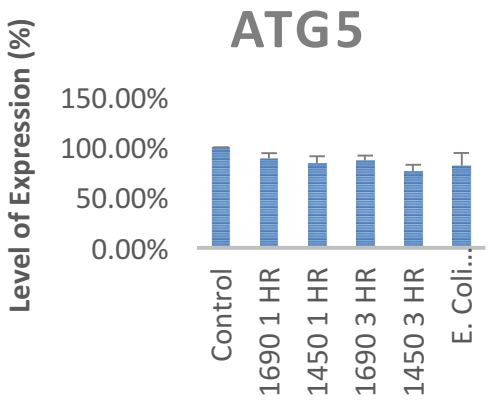
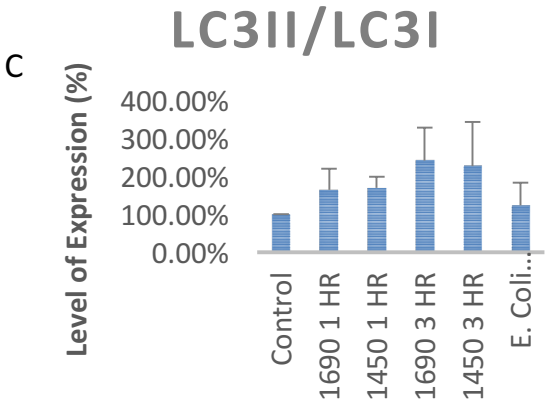

A
←ATG5
←MREG
←LC3
←Actin
No Addition
1690 1 HR
1450 1 HR
1690 3 HR
1450 3 HR
E. Coli LPS 3 HR


B
ATG5
Level of Expression (%)
150.00%
100.00%
50.00%
0.00%
Control
1690 1 HR
1450 1 HR
1690 3 HR
1450 3 HR
E. Coli...


C
LC3II/LC3I
Level of Expression (%)
400.00%
300.00%
200.00%
100.00%
0.00%
Control
1690 1 HR
1450 1 HR
1690 3 HR
1450 3 HR
E. Coli...

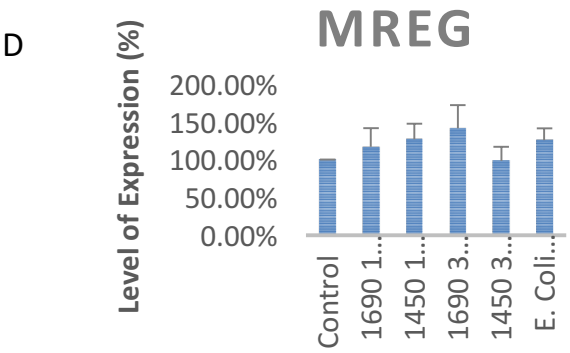

D
MREG
Level of Expression (%)
200.00%
150.00%
100.00%
50.00%
0.00%
Control
1690 1...
1450 1...
1690 3...
1450 3...
E. Coli...

Figure 1

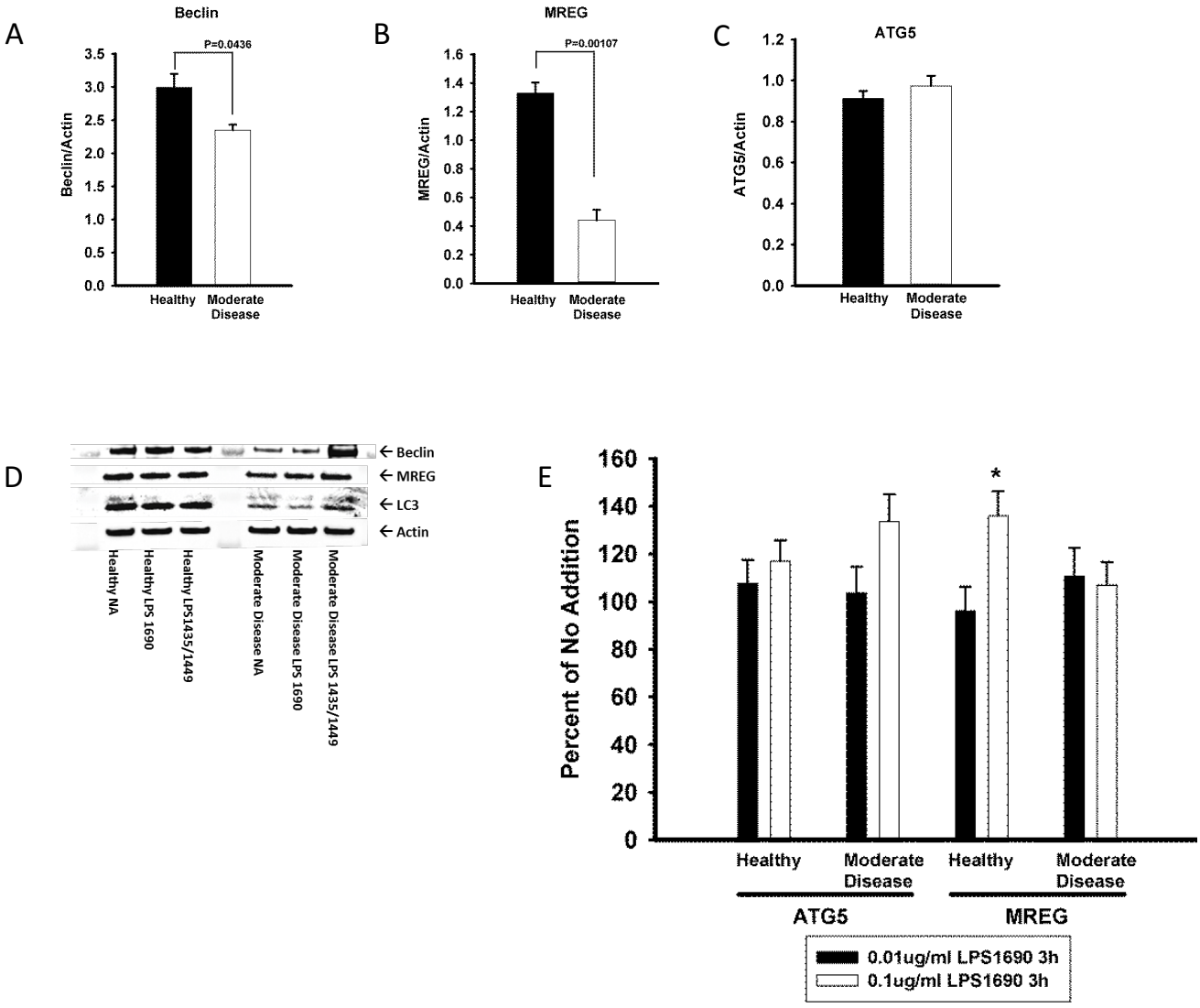

A
Beclin
P=0.0436
Beclin/Actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Healthy
Moderate Disease
B
MREG
P=0.00107
MREG/Actin
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Healthy
Moderate Disease
C
ATG5
ATG5/Actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Healthy
Moderate Disease
D
Beclin
MREG
LC3
Actin
Healthy NA
Healthy LPS 1690
Healthy LPS1435/1449
Moderate Disease NA
Moderate Disease LPS 1690
Moderate Disease LPS 1435/1449
E
Percent of No Addition
160
140
120
100
80
60
40
20
0
*
Healthy
Moderate Disease
Healthy
Moderate Disease
ATG5
MREG
0.01ug/ml LPS1690 3h
0.1ug/ml LPS1690 3h

Figure 2

FUTURE DIRECTIONS

FUTURE DIRECTIONS

Dhingra, A., Reyes-Reveles, J., Shenker, B.J., **Blasi, I**., Alexander, D., Walker, L. and Boesze-Battaglia, K., *"P. gingivalis traffics to LC3 positive structures in phagocytes in a TLR4 dependent manner."* In preparation for *J. Biol Chem*. 2017

1. **Further testing on MREG role and *P. g.* systemic consequences in its absence.**

We provide evidence that *P.g.* traffics to autophagosomes resulting in enhanced microbial persistence.

Our future studies will focus on determining the systemic consequences of *P. g.* persistence in the absence of MREG by assessing dissemination of *P. g.* in the *Mreg* $^{-/-}$ mice. MREG knockdown experiments will provide direct evidence that MREG mediated lysosome maturation is required for autophagosome formation. In addition, we will determine if other gram negative bacteria upregulate MREG mediated lysosome maturation; and if persistence contributes to bone loss in this model.

2. **Evaluate if CR3 receptors targets *P.g.* to autophagosomes.**

Trafficking to autophagosomes resulted in persistence/survival.

In future studies, we will determine if binding to the raft associated CR3 receptors targets *P.g.* to autophagosomes using macrophages isolated from *Cd11b* $^{-/-}$ mice.

3. **Determine if there is a preferential TLR agonist for optimal MREG upregulation.**

Our experiments determined that TLR is required for regulation of lysosome maturation and autophagy as well as *P.g.* LPS is critical in the upregulation of these processes. We showed that *P.g.* LPS_{1690} upregulates lysosome maturation required for autophagosome formation.

In future studies, we will assess the contribution of *P.g.* lipid A configuration to determine if there is a preferential TLR agonist for optimal MREG upregulation and trafficking of *P.g.* to autophagosomes to aid in targeting TLR agonists as therapeutics.

TRANSLATION

HIPÓTESIS

HIPÓTESIS

Hipótesis 1:

- **Hipótesis nula (H0):** *P. g.* secuestrada en autofagosomas no confiere protección contra la degradación lisosomal que da como resultado la persistencia bacteriana.
- **Hipótesis alternativa (H1):** *P. g.* secuestrada en autofagosomas confiere protección contra la degradación lisosomal que da lugar a la persistencia bacteriana.

Hipótesis 2:

- **Hipótesis nula (H0):** la maduración del lisosoma mediada por MREG no contribuye a la eliminación de *P. g.* en el fagolisosoma.
- **Hipótesis alternativa (H1):** La maduración de lisosomas mediada por MREG contribuye a la eliminación de *P. g.* en el fagolisosoma.

Hipótesis 3:

- **Hipótesis nula (H0):** La estimulación de TLR4 mediada por *P. g.* no regula positivamente la autofagia.
- **Hipótesis alternativa (H1):** La estimulación de TLR4 mediada por *P. g.* regula positivamente la autofagia.

Hipótesis 4:

- **Hipótesis nula (H0):** La vía degradación de LC3-MREG no está sobrecargada en individuos con enfermedad periodontal severa.
- **Hipótesis alternativa (H1):** La vía de degradación LC3-MREG está sobrecargada en individuos con enfermedad periodontal severa.

OBJETIVOS

OBJETIVOS

Objetivo principal:

En los estudios presentes, nuestro objetivo es determinar como *P.g.* intercepta las respuestas de las células huésped para contribuir a su supervivencia a largo plazo en los macrófagos y el papel de MREG en este proceso.

Objetivos secundarios:

1. Evaluar si *P.g*. LPS regula positivamente la formación de autofagosomas de una manera dependiente de TLR y el papel de la melanoregulina en la supervivencia de *P. g*.
2. Evaluar si las células aisladas de pacientes con periodontitis crónica han disminuido la capacidad lisosómica debido a la disminución de la maduración de los lisosomas que conduce a defectos de autofagia.

CONCLUSIÓN

CONCLUSIÓN

1. **Evaluar si *P.g*. LPS regula positivamente la formación de autofagosomas de una manera dependiente de TLR y el papel de la melanoregulina en la supervivencia de *P. g*.**

En conclusión, se ha demostrado que el LPS regula positivamente la formación de autofagosomas de una manera dependiente de TLR.

La estimulación de células con LPS_{1690} intensifica la autofagia según se detecta por el aumento de punta-positiva de LC3 y de la lipidación de LC3 a LC3II. También se observó un aumento en la formación de grandes estructuras autofagosómicas de LC3-positivas, lo que conduce a la persistencia bacteriana (Hipótesis 1). Del mismo modo, la detección de la proteína de unión a LC3, MREG, se elevó con punta-positiva; demostrando que MREG contribuye a la eliminación de *P. g.* (Hipótesis 2).

Además, el LPS_{1690} activa la autofagia y es necesario para la formación de grandes (super-size) autofagosomas en ausencia de absorción bacteriana, reforzando el papel de TLR4 como sensor de autofagia en la eliminación de patógenos (Hipótesis 3).

2. **Evaluar si las células aisladas de pacientes con periodontitis crónica han disminuido la capacidad lisosómica debido a la disminución de la maduración del lisosoma conduciendo a defectos de autofagia.**

Además, la observación de que LC3 y MREG se co-localizan sólo en las células de los pacientes gravemente afectados sugieren que este sistema de eliminación bacteriana puede estar sobrecargado. Sugerimos que las mutaciones en MREG y / o LC3 puede contribuir a una predisposición a la periodontitis crónica (Hipótesis 4).

Nuestros resultados proporcionan una explicación para entender mejor el mecanismo a través del cual *P. gingivalis* evade la degradación intracelular que conduce a la supervivencia bacteriana.

RESUMEN

RESUMEN

La periodontitis es una enfermedad inflamatoria crónica asociada con los patógenos del complejo rojo. El patógeno más característico del complejo es *Phorphyromonas gingivalis (P. g.)* (59). *P. g.* induce efectos sobre el huésped que van desde la pérdida de hueso alveolar mediada por inflamación hasta su recientemente atribuido papel en la patogénesis de la placa aterosclerótica (8-12).

Los patógenos, entre ellos *P. gingivalis*, han ideado una serie de mecanismos mediante los cuales evaden o subvierten los mecanismos de defensa del huésped para persistir y sobrevivir, contribuyendo así a la infección crónica (35, 60). Uno de estos mecanismos es evitar la degradación mediada por los lisosomas. Los macrófagos son un componente crítico de la defensa del huésped y degradan los patógenos bacterianos en fagolisosomas y / o autolisosomas (61).

Estas vías de degradación también están íntimamente unidas a la superficie celular por TLR. Los agonistas de TLR aceleran la maduración del fagosoma. También estimulan la regulación positiva de la autofagia como un medio por el cual la célula elimina patógenos que podrían haber escapado a la degradación. Con frecuencia los patógenos subvierten el proceso de degradación autofágica y continúan persistiendo y sobreviviendo (62).

La autofagia en los macrófagos se estimula a través del TLR4 induciendo interferón-β (39). El lipopolisacárido (LPS) de *P. gingivalis* es capaz de actuar como un agonista de TLR2 y / o TLR4 para proceder con la maduración lisosomal del autofagosoma temprano al tardío (27, 63). *P. gingivalis* sintetiza dos tipos diferentes de LPS; Pg_{1690} y $Pg_{1435\,/\,1449}$. Pg_{1690} es un lípido A de penta-acilo que es agonista de TLR4 en monocitos humanos y HUVEC (células endoteliales de venas umbilicales humanas) (27, 64). $Pg_{1435\,/\,1449}$ es una especie de lípido A en forma tetra-acilada. Es un antagonista de Pg_{1690}.

La autofagia y la fagocitosis comparten los intermediantes lisosómicos comunes, cuya formación está regulada por una proteína, la melanoregulina (MREG) (65). Recientemente se ha reportado de que el producto del gen mamífero $Mreg^{dsu}$ es necesario para la maduración de los lisosomas en fagocitos. La disminución de MREG da lugar a la acumulación e inactivación de enzimas lisosómicas debido a la alcalinización de los

lisosomas. Esto conduce a la acumulación de restos fagocíticos no degradados que contribuyen a la acumulación de lipofuscina (65). La pérdida de MREG también da como resultado la secreción de Catepsina D debido a la separación de proteínas (66). Tales defectos en la función de los lisosomas se correlacionan con una mayor persistencia bacteriana que contribuye a la enfermedad crónica como la periodontitis (67). Sobre la base de trabajos previos en los que se demostró que *P. gingivalis* sobrevive durante 72 h (68) y nuestra observación innovadora que *P. gingivalis* regula positivamente intermediarios lisosómicos y que trafica a autofagosomas en macrófagos; propusimos que *P. gingivalis* regula positivamente procesos de autofagia. Específicamente, propusimos que la regulación positiva de la formación de autofagosomas es crítica para la supervivencia de *P. gingivalis* en macrófagos y requiere maduración de lisosomas mediada por MREG.

En estos estudios, proporcionamos evidencia de que la estimulación con LPS_{1690} de células Saos de GFP-LC3 aumenta la autofagia tal y como se detecta mediante una mayor formación de punta LC3 (Artículo 1-Fig. 2 y Fig. 4C) así como la lipidación de LC3 a LC3II (Artículo 1-Fig. 5A y B). El tamaño de los autofagosomas que contenían LPS_{1690} GFP-LC3 (Artículo 1-Figuras 3A y B) era aproximadamente 4 veces el tamaño de los autofagosomas medios, 3,5-4,0 μm en comparación con 0,5-1,0 μm respectivamente (46). Tales estructuras autofagomales de gran tamaño que no se vieron en la inanición de las células Saos2 (Artículo 1-Material Suplementario Figura 1A), sugiere la especificidad de señalización en la disposición de estas estructuras con LPS_{1690}. En presencia de $LPS_{1435\ /\ 1449}$, los grandes autofagosomas inducidos por *Pg* LPS_{1690} se redujeron en tamaño en un 50%, hasta 1,0 a 1,5 μm (Artículo 1-Figura 4A). Además, los niveles de LC3II y la formación de puncta LC3 se redujeron en presencia del antagonista *Pg* LPS_{1690}, $LPS_{1435\ /\ 1449}$ (Artículo 1-Fig. 4A y B). Además, Atg5 niveles mostraron una disminución en células OKF64-TERT tras la estimulación de LPS_{1690} y $LPS_{1435\ /\ 1449}$ (Artículo 2-Fig. 1A y B). Curiosamente, se demuestra que la estimulación con LPS_{1690} y $LPS_{1435\ /\ 1449}$ de células OKF64-TERT también intensifica la autofagia tal y como se detectó en el aumento de LC3II / LC3I niveles con pocos cambios en un período de tiempo más corto (Artículo 2-Fig. 1A y C).

Además, seguimos el perfil de distribución de las proteínas en células epiteliales gingivales aisladas de tejido periodontal obtenido de individuos sanos y enfermos (Artículo 1-Fig.6). Los niveles de LC3II / LC3I fueron consistentemente más bajos en las células enfermas después de la incubación con LPS_{1690} (Artículo 2-Tabla 1). Sin embargo, después

de la estimulación de LPS_{1690}, LC3 consistentemente aumentó en células epiteliales sanas. Los niveles de ATG5 fueron iguales en células epiteliales aisladas de pacientes enfermos moderados (Artículo 2-Fig. 2C). Estos resultados sugieren que la encía humana se convierte en refractaria a LPS_{1690} sobre disbiosis microbiana asociada con periodontitis crónica.

De manera similar, a nuestras observaciones de LC3, su proteína de unión, MREG, se elevó con puntas positivas de MREG formadas tras 30 minutos de incubación con *Pg* LPS_{1690}. La adición de $LPS_{1435\,/\,1449}$ inhibió la regulación positiva de MREG. Curiosamente, en contraste con nuestros estudios publicados anteriormente en los que la absorción de *Pg* (33277) dio lugar a la asociación de LC3 con su pareja vinculante, MREG, en estos estudios *Pg* LPS_{1690} indujo un aumento en ambas proteínas, pero no su co-localización, lo que sugiere que la fagocitosis activa es necesaria para la degradación dependiente de LC3 mediada por MREG (20). Colectivamente, estos resultados sugieren que el agonista débil TLR4 *Pg* LPS_{1690} activa la autofagia y es necesaria para la formación de grandes autofagosomas, en ausencia de absorción bacteriano, reforzando el papel de TLR4 como sensor autofágico en la eliminación del patógeno (34, 39).

Similar a la respuesta de LC3, se muestra que los niveles MREG fueron elevados después de la estimulación de las células OKF64-TERT con LPS_{1690}. Sin embargo, la incubación con $LPS_{1435\,/\,1449}$, disminuyó MREG, lo que sugiere que el proceso de autofagia podría ser inadecuado (Artículo 2-Fig. 1A y D). En las células epiteliales gingivales, los niveles de MREG y beclin no siguieron el mismo patrón que ATG5 y se encontró que disminuyeron la mitad y ¼ respectivamente, lo que sugiere que el eje de la autofagia puede estar comprometido en pacientes enfermos (Artículo 2-Fig.2 A y B). Tratamos ambos grupos de células con LPS_{1690} para observar la diferencia de respuestas de autofagia entre las muestras sanas y epiteliales de la enfermedad. En presencia de LPS_{1690}, hubo un mayor nivel de ATG5 y MREG en células de tejido sano, sin embargo, dicho aumento no se observó en células aisladas de sujetos con enfermedad moderada (Artículo 2 - Fig. 2E). Por lo tanto, estos resultados consistentes pueden indicar que la vía autofágica está comprometida en pacientes con enfermedad periodontal.

En nuestros estudios, *Pg* (33277) se encontró que interiorizó, sin embargo, no era detectable en estructuras positivas de LC3 (Artículo 1-Fig. 1). Las diferencias en la internalización y los patrones de tráfico intracelular y la inducción de autofagia puede

deberse a un truncamiento en el gen PG07171 (gen de la lipoproteína) en *Pg* (33277) que está presente en su forma de longitud completa en *Pg*W83 (58). Además, se observó, en células epiteliales gingivales humanas aisladas, una co-localización entre LC3 y MREG en individuos gravemente afectados sin ninguna co-localización entre estas proteínas en individuos sanos o moderadamente afectados. La degradación dependiente de LC3 mediada por MREG es necesaria para la eliminación de los desechos lipídicos (20) y se propone la eliminación de bacterias. La observación de que LC3 y MREG-colocalizan sólo en las células de los pacientes gravemente afectados sugieren que este sistema de eliminación puede estar sobrecargado. Colectivamente, estos resultados sugieren que las mutaciones en MREG y / o LC3 pueden contribuir a una predisposición a la periodontitis crónica.

En conclusión, se ha demostrado que el LPS regula positivamente la formación de autofagosomas de una manera dependiente de TLR. Además, proponemos que las mutaciones en MREG y / o LC3 puede favorecer a una susceptibilidad a la enfermedad periodontital crónica. A pesar de que más investigación es necesaria en este ámbito, nuestros resultados sirven para entender mejor el mecanismo a través del cual *P. gingivalis* evade la degradación intracelular que conduce a la supervivencia bacteriana.

ABSTRACT

ABSTRACTS

Variantes del lipopolisacárido de *Porphyromonas gingivalis* alteran la lipidación de la proteína autofágica, la proteína 1 asociada a los microtúbulos de cadena ligera 3, la LC3.

Porphyromonas gingivalis suele subvertir los procesos autofágicos de la célula huésped para su propia supervivencia. Nuestros estudios previos documentan la asociación de la proteína melanoregulina (MREG), con su socio de unión, la proteína autofágica, proteína 1 ligada a microtúbulos de cadena ligera 3 (LC3) en macrófagos incubados con *P. gingivalis* (strain 33277). Las diferencias en la parte del lípido A del lipopolisacárido (LPS) afectan a la virulencia de *P. gingivalis*; penta-acilado LPS_{1690} es un débil agonista del receptor TLR4 comparado con el LPS de *Escherichia coli*, mientras que el $LPS_{1435/1449}$ tetra-acilado actúa como un antagonista de LPS_{1690}. Para determinar cómo el LPS_{1690} del *P. gingivalis* afecta a la autofagia, evaluamos procesos dependientes de LC3 y MREG en células Saos-2 que expresan proteína de fluorescencia verde (GFP)-LC3. LPS_{1690} estimuló la formación de grandes vacuolas positivas de LC3 y puncta de MREG. Esta lipidación de LC3 mediada por LPS_{1690} disminuyó en presencia de $LPS_{1435/1449}$. Cuando las células Saos-2 se incubaron con *P. gingivalis*, las bacterias se internalizaron, pero no se propagaron a estructuras positivas de GFP-LC3. Sin embargo, se observaron incrementos en la lipidación de LC3 y puncta de MREG. Colectivamente, estos resultados sugieren que la internalización de *P. gingivalis* no es necesaria para la lipidación de LC3. Las células epiteliales gingivales humanas primarias aisladas de pacientes con periodontitis mostraron punta de LC3II y MREG mientras que las células de individuos sanos sin enfermedad periodontal mostraron poca co-localización de estas dos proteínas. Estos resultados sugieren que la prevalencia de una porción particular de LPS puede modular la capacidad degradativa de las células huésped, influyendo así en la supervivencia bacteriana.

LPS de *Porphyromonas gingivalis* modula la autofagia en células epiteliales gingivales.

Porphyromonas gingivalis (P. gingivalis) es un patógeno clave que contribuye a la patogénesis periodontal interrumpiendo la homeostasis del microorganismo huésped y promoviendo la disbiosis. *Porphyromonas gingivalis* suele subvertir la respuesta de la célula huésped incluyendo las vías de degradación para facilitar su supervivencia. La virulencia de *P. gingivalis* probablemente refleja una alteración en la composición del lípido A de su lipopolisacárido (LPS) desde la forma penta-acilada (*Pg*LPS_{1690}) a la forma tetra-acilada (*Pg*$LPS_{1435/1449}$). En el epitelio gingival humano, *Pg* no se degrada fácilmente, pero sobrevive, si la supervivencia es a través de la intercepción de las vías de degradación autofágica o no, es desconocido. Nuestros estudios previos documentan la intensificación del flujo autofágico tras la incubación con *Pg LPS*, sin internalización de bacterias. Además, se demostró que los individuos con enfermedad periodontal severa presentaban niveles disminuidos de LC3 y su pareja de unión melanoregulina (MREG), con la mayor parte de estas proteínas formando complejos intracelulares. En contraste, las células de individuos sanos mostraron poca co-localización de LC3 con MREG. Basándonos en esos estudios, predijimos que la vía degradativa de LC3-MREG puede estar sobrecargada en individuos con enfermedad periodontal. Para explorar aún más el mecanismo molecular de esta sobrecarga, en estos estudios nos preguntamos si las variantes del LPS de *Pg*, intensifican la autofagia in-vitro, utilizando la línea de células gingivales humanas. Además, tratamos las células gingivales de pacientes con enfermedad y sin enfermedad con LPS para definir su capacidad de respuesta. Nuestros resultados sugieren que las mutaciones en MREG y / o LC3 pueden contribuir a una predisposición a la periodontitis crónica.

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APPENDIX

APPENDIX

Appendix 1. Presentations/Abstracts/Posters

- Dhingra, A., Reyes-Reveles, J., Shenker, B.J., **Blasi, I**., Alexander, D., Walker, L. and Boesze-Battaglia, K., *"P. gingivalis traffics to LC3 positive structures in phagocytes in a TLR4 dependent manner."* In preparation for *J. Biol Chem*. 2017
- Boesze-Battaglia K, **Blasi I**, Stefano FP, Shenker BJ. *"Melanoregulin (MREG), an Intracellular Sorting Protein in Critical for P. gingivalis Degradation"*. May 31st, 2013 SDM retreat. University of Pennsylvania. Philadelphia, PA.
- Boesze-Battaglia K, **Blasi I**, Stefano FP, Shenker BJ. *"Melanoregulin (MREG), an Intracellular Sorting Protein in Critical for P. gingivalis Degradation"*. June 2013 Aegean Conference on Adaptive and Innate Immunity. Greece.
- Boesze-Battaglia K, Shenker BJ, Stefano FP, Brancato J, **Blasi I**. *"LPS Mediated Induction of the Intracellular Sorting Protein MREG is Critical for P. gingivalis Processing and Degradation"*. February 2013. Mark Wilson Conference, San Juan, PR.
- **Blasi I**, Stefano FP, Boesze-Battaglia K. *"Porphyromonas gingivalis LPS stimulates autophagy using a TLR mediated pathway"*. Abstract. June 8th, 2012 SDM retreat. University of Pennsylvania. Philadelphia, PA.
- **Blasi I**, Stefano FP, Boesze-Battaglia K. *"Porphyromonas gingivalis LPS stimulates autophagy using a TLR mediated pathway"*. Poster. June 8th, 2012 SDM retreat. University of Pennsylvania. Philadelphia, PA
- **Blasi I**, Boesze-Battaglia K. "*Porphyromonas gingivalis LPS stimulates autophagy using a TLR mediated pathway"*. Presentation. June 14th, 2012. University of Pennsylvania. Philadelphia, PA.

Appendix 2. Other publications during PhD period.

- **Blasi I Jr.,** Pavlin D. *"Minimally Invasive Approach to Accelerate Tooth Movement."* Chapter 31. In: Graber LW, Vanarsdall RL, Vig KWL, Huang GJ, eds. Orthodontics- Current Principles and Techniques. 6th ed. Louis: Elsevier; 2017; 913-25.
- Vanarsdall RL Jr., **Blasi I Jr.,** Secchi AG. *"Periodontal-Orthodontic Interrelationships."* Chapter 22. In: Graber LW, Vanarsdall RL, Vig KWL, Huang GJ, eds. Orthodontics- Current Principles and Techniques. 6th ed. Louis: Elsevier; 2017; 621-68.
- Vanarsdall RL Jr, **Blasi I Jr.** *"Applying new knowledge to the correction of the transverse dimension."* In: Kapila SD, Vig KWL, Huang GJ, eds. *Anecdote, Expertise and Evidence: Applying New Knowledge to Everyday Orthodontics.* Craniofacial Growth Series, Center for Human Growth and Development, The University of Michigan, Ann Arbor, MI 2017;53:167-193.
- DeGeorge A, **Blasi I**, Boucher NJ, Katz SH, Vanarsdall RL. *"Phase I Treatment Effect of Lip Bumper Therapy in the Transverse Dimension as Measured on 3-D Models."* Submitted.
- DeGeorge A, **Blasi I**, Boucher NJ. *"The Effect of Mandibular Lip Bumper Therapy on Early Treatment Using Digitized Models and Ct Scans."* Thesis 2015. University of Pennsylvania. Philadelphia, PA.
- DeGeorge A, Boucher NS, **Blasi I**. The Effect of Mandibular Lip Bumper Therapy on the WALA Ridge Using Digitized Models and CT Scans. MASO Annual Meeting. Williamsburg, VA. October 17-20th, 2014.
- Vanarsdall RL, **Blasi I**, Evans M, Kocian P. *"Rapid maxillary expansion with skeletal anchorage vs. bonded tooth/tissue born expanders: A case report comparison utilizing CBCT."* RMO Clinical Review. 1(1): p. 18-22, 2012.