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***Porphyromonas gingivalis* RagB is a pro-inflammatory STAT4 agonist**

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Summary

Periodontal diseases are semi-ubiquitous and caused by chronic, plaque-induced inflammation. The 55kDa immunodominant RagB outer membrane protein of *Porphyromonas gingivalis*, a keystone periodontal pathogen, has been proposed to facilitate nutrient transport. However, potential interactions between RagB and the innate response have not been examined. We determined that RagB exposure led to the differential and dose-related expression of multiple genes encoding pro-inflammatory mediators (IL-1 α , IL-1 β , IL-6, IL-8 and CCL2; all, $p < 0.05$) in primary human monocytes and to the secretion of TNF and IL-8, but not IFN- γ or IL-12. RagB was shown to be a TLR2 and TLR4 agonist that activated STAT4 and NF κ B signaling. In keeping, a *ragB* mutant similarly exhibited reduced inflammatory capacity which was rescued by *ragB* complementation. These results suggest that RagB elicits a major pro-inflammatory response in primary human monocytes and, thus, could play an important role in the etiology of periodontitis and systemic sequelae.

Keywords

inflammation; monocyte; periodontitis; *Porphyromonas gingivalis*; STAT4; TLR

Introduction

Chronic periodontitis, characterized by the destruction of the hard and soft tissues surrounding the teeth, is predominantly caused by the immune response to pathogenic bacteria present in dental plaque. *Porphyromonas gingivalis*, a Gram negative,

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Conflict of Interest

The authors have no conflict of interest to declare.

asaccharolytic, anaerobe, is a causative agent of chronic periodontitis and a keystone pathogen of the sub-gingival biofilm (Hajishengallis *et al.*, 2012).

The *rag* locus of *P. gingivalis* is comprised of receptor antigen gene A (*ragA*, PG0185), encoding a 115 kDa TonB-dependent receptor, and *ragB* (PG0186), encoding a 55 kDa putative lipoprotein, related to the SusD starch utilization protein of gut Bacteroidetes (Martens *et al.*, 2009, Hanley *et al.*, 1999), although it has been hypothesized to function in iron acquisition in *P. gingivalis* (Curtis *et al.*, 1999). The *rag* genes are co-transcribed, flanked by insertion sequences, and consist of a lower G+C ratio than the rest of the genome, suggesting the Rag locus is a pathogenicity island that arose from horizontal transfer (Hanley *et al.*, 1999). While host-RagB interactions are poorly understood, RagB is the most immunodominant *P. gingivalis* antigen, and so is clearly recognized by the immune system (Curtis *et al.*, 1999, Nagano *et al.*, 2007, Imai *et al.*, 2005). Recent data suggest that the *rag* operon may be essential to *P. gingivalis* (Klein *et al.*, 2012).

Comparative genomics have identified RagB as potentially important in epithelial cell invasion (Dolgilevich *et al.*, 2011) while *ragB* mutants exhibit reduced virulence in murine soft tissue infection models (Shi *et al.*, 2007, Nagano *et al.*, 2007). Furthermore, RagB has been proposed to aid *P. gingivalis* survival in severe forms of periodontitis (Imai *et al.*, 2005). Thus, RagB is under investigation as a potential periodontitis vaccine (Zheng *et al.*, 2013). Rag locus homologs found in gut *Bacteroides* *sp.* have also been shown to be associated with inflammatory bowel disease (Wei *et al.*, 2001b).

We set out to determine the inflammatory potential of RagB and underlying mechanisms. Herein, we show, for the first time, that the RagB protein of *P. gingivalis* is an unusual TLR agonist that is recognized both by human monocytic TLR2 and -4, induces STAT4, activates NF κ B and promotes multiple mediators of inflammation at the transcriptional and protein levels.

Results

RagB induces multiple pro-inflammatory cytokines in a dose-related manner

Recombinant RagB induces the secretion of multiple pro-inflammatory cytokines (IL-8, TNF, IL-6, all $p < 0.05$) from primary human monocytes in a dose-related manner, as shown in Figure 1 and Supplemental Figure 1. A RagB-induced IFN- γ or IL-12 p70 signal was not observed, while IL-10 was minimal (*data not shown*). Heat-treated (boiled) or denatured (SDS-treated) RagB did not induce a cytokine signal, indicating that the recombinant protein was free from immunologically significant contamination with TLR agonists including LPS, or other pyrogens, from the *E. coli* expression system (*data not shown*).

RagB induces multiple pro-inflammatory genes

Five genes were upregulated in monocytes by RagB, as determined using TLR Signaling Pathway RT² Profiler PCR arrays, as presented in Table 1. As canonical TLR-signaling was not strongly suggested by these data, whole genome arrays were performed, with key target molecules confirmed by RT-PCR. The top inflammation-related genes induced by RagB [(i) 1.0 μ g/ml $>$ 0.1 μ g/ml $>$ 0.0 μ g/ml; (ii) $>$ 2x induction; (iii) $p < 0.05$; and (iv) confirmed by

RT-PCR) are presented in Table 2. The full array data set is provided in Supplemental Table 2. There was considerable overlap between RagB differential monocyte genes identified in the focused TLR and genome-wide arrays (IL-1 β , IL-8, IL-6). IL-8, TNF and IL-6 regulation by RagB was also seen at the protein level (Figure 1 and Supplemental Figure 1).

RagB activates TLR2 and -4

RagB induction of cytokines in monocytes was initiated by engagement of TLR2 or TLR4, as indicated by the inhibition of IL-8 release by anti-TLR antibodies and also by TLR gene silencing, as presented in Figures 2 and 3.

Inflammatory activity of RagB is STAT4-related

As array data suggested STAT4 may be an important regulator of RagB-induced inflammation, we examined the influence of the pharmaceutical STAT4 inhibitor, LSF, and *stat4* gene silencing on RagB-induced cytokine production. Both strategies were highly effective in suppressing RagB, but not LPS-stimulated, IL-8 production (Figure 4), indicating potentially divergent signaling pathways between RagB and a classical TLR-agonist.

Inflammatory activity of RagB is p65-related

A similar approach was taken to determine the importance of the primary inflammatory gene activity regulator, p65, in RagB-induced cytokine production. Both pharmaceutical p65 inhibition, using a p65 decoy peptide, and *stat4* gene silencing were potent suppressors of RagB- and, as expected, LPS-induced cytokine production (Figure 5).

RagB stimulates phosphorylation of p65 and STAT4

As shown in Figure 6, RagB induces the rapid phosphorylation of p65 (within 15 minutes) followed by the transient phosphorylation of STAT4 (within 60 – 120 minutes).

Cytokine induction by whole *P. gingivalis* is RagB-dependent

As shown in Figure 7, a *ragB* strain *P. of gingivalis* induces a significantly reduced IL-8 response from primary human monocytes, compared to WT ($p < 0.001$). The same phenomenon was apparent for IL-6 and TNF; $p < 0.001$ and $p < 0.01$, respectively - **data not shown**). The introduction of *ragB*-expressing plasmid rescued cytokine release. These data, therefore, suggest that RagB is key to the induction of the innate response, in the context of the whole bacterium.

Discussion

RagB is a predominant, highly antigenic, environmentally regulated, surface-exposed outer membrane protein of *P. gingivalis* (Bonass *et al.*, 2000, Zeller *et al.*, 2013, Zheng *et al.*, 2013, Bagaitkar *et al.*, 2009). RagB expression is temperature regulated, with increased temperature a hallmark of plaque-induced periodontal inflammation. Tobacco smoke, a major risk factor for periodontitis, promotes *P. gingivalis* infection (Zeller *et al.*, 2013, Bagaitkar *et al.*, 2008) and alters the inflammatory capacity of *P. gingivalis* through suppression of capsule generation, enhancement of fimbrial protein expression and

augmentation of biofilm formation (Bergstrom, 2003, Bagaitkar *et al.*, 2010, Bagaitkar *et al.*, 2009). Tobacco smoke is also a potent inducer of RagB expression (Bagaitkar *et al.*, 2009). RagB is homologous to the SusD group of polysaccharide uptake proteins of other Bacteroidetes species, including *Bacteroides fragilis* NanU (Hall *et al.*, 2005, Phansopa *et al.*, 2013, Bolam & Koropatkin, 2012). A Rag homologue in *Bacteroides caccae* (OmpW) has been associated with inflammatory bowel disease (Wei *et al.*, 2001a, Iltanen *et al.*, 2006). Subcutaneous inoculation of RagB mutant *P. gingivalis* into mice resulted in attenuated virulence, assessed as soft tissue lesion size and survival (Shi *et al.*, 2007). Furthermore, the ability of RagB mutants to invade transformed epithelial-like cells is curtailed (Dolgilevich *et al.*, 2011). Despite such data implicating RagB as an important immunomodulatory molecule, RagB-innate interactions have not, to the best of our knowledge, been previously examined mechanistically.

We show RagB to be a strong but unusual TLR agonist. As has been reported for *P. gingivalis*-derived LPS (Kocgozlu *et al.*, 2009), RagB can engage both TLR2 and -4, resulting in the induction of multiple pro-inflammatory cytokines. This innate activation occurs in a STAT4-dependent manner, as determined using the pharmaceutical inhibitor, lisofylline, and gene silencing. STAT4 is best known as an important transcription factor in non-antigenic induction of cytokines in T-cells, type I interferon signaling, and IL-12-mediated development of Th₁ cells from naive CD4⁺ T cells (Remoli *et al.*, 2007, Ronnblom, 2011, Guo *et al.*, 2009). More recently, STAT4 has been shown to be expressed in other immune cells, particularly activated monocytic cells (Remoli *et al.*, 2007). TLR2, -3 and -4 agonists have been shown or suggested to activate STAT4 in innate immune cells, with STAT4 suggested as potential therapeutic target for various inflammatory diseases, including shock (Matsukawa *et al.*, 2001, Kim & Chung, 2012, Kim *et al.*, 2007, Shirota *et al.*, 2005, Kamezaki *et al.*, 2004, Frucht *et al.*, 2000, Remoli *et al.*, 2007). Whole microbial induction of STAT4 expression in innate cells has been established for *Mycobacterium tuberculosis* and *Candida albicans* (Remoli *et al.*, 2007). STAT4 has been reported to bind to MyD88, the TLR-associated signal transducer, and also to the TNF promoter (Good *et al.*, 2009). Indeed, microbial STAT4 activation has been shown to result in the induction of classic NFκB-regulated cytokines, but not IFNγ, in dendritic cells (Remoli *et al.*, 2007). Similarly, we could not detect an IFN-γ signal in RagB-exposed cells, despite STAT4 upregulation. It has been reported that the activation of STAT4 by IL-12, well-defined in T cells, does not occur in innate cells of monocytic lineage (Frucht *et al.*, 2000), indeed monocytic cell expression of IL-12 receptors is contentious (Remoli *et al.*, 2007, Grohmann *et al.*, 2001), and that the STAT4 promoter contains an NF-κB binding site -969/-959 bp upstream from the transcriptional start site that binds p65/p50 and p50/p50 dimers (Remoli *et al.*, 2007). In addition to STAT4-dependence, RagB induction of pro-inflammatory cytokines requires NFκB (p65), as determined using both pharmaceutical and siRNA approaches. Thus, RagB activation of the innate response in human monocytes exhibits both classical and atypical elements of TLR signaling pathways.

Clearly, recombinant RagB may differ from the natural protein, particularly as it is not anchored in the cell membrane. With this in mind, we also examined IL6 and IL-8 production in monocytes exposed to WT, *ragB* mutant and complemented *P. gingivalis*

cells. These data support a pro-inflammatory role for RagB in the context of the whole bacterium, similar to the recombinant protein.

RagB, then, seems to have two roles in *P. gingivalis* that could promote sub-gingival colonization. Firstly, it is assumed that the RagB, along with RagA, facilitates polysaccharide, or other nutrient, transport. As *P. gingivalis* is considered asaccharolytic, however, the physiological relevance of sugar uptake is not immediately transparent. Secondly, RagB, as a promoter of inflammation, is likely to contribute to the availability of protein, required by *P. gingivalis* as a carbon source.

It must be remembered that there is considerable antigenic variation at the *rag* locus, with at least four non-cross reactive RagB proteins identified (Hall *et al.*, 2005). It is possible, then, that RagB produced by different *P. gingivalis* strains may have altered inflammatory activities and, thus, exhibit differing virulence potential. Indeed, the highly virulent *P. gingivalis* lab strains W83 (used herein) and W50 each carry *rag1*, which has been clinically associated with deep periodontal pockets, along with 26% of 168 isolates. The less pathogenic strain, ATCC 33277, carries *rag4*, along with 14% of isolates, which cross reacts poorly with W83-specific RagB antibodies (Hall *et al.*, 2005, Imai *et al.*, 2005, Hanley *et al.*, 1999). This may be important in the drive to develop RagB-based vaccines for periodontal disease prevention (Zheng *et al.*, 2013).

Established immunomodulatory virulence factors of *P. gingivalis* include LPS, fimbrial proteins (FimA and Mfa1), capsular polysaccharides and gingipains (Bagaitkar *et al.*, 2010, Bostanci & Belibasakis, 2012, Guo *et al.*, 2010, Curtis *et al.*, 1999). It appears that RagB, which induces multiple pro-inflammatory cytokines in a dose-related, TLR-, STAT4- and p65-dependent manner, may now be added to this list.

Experimental Procedures

Materials

P. gingivalis W83 was purchased from the American Type Culture Collection (Manassas, VA); Gifu Anaerobe Medium (GAM) from Nissui Pharmaceutical (Tokyo, Japan); *ragB* and pTCBex2:*ragBW83*-carrying complemented *P. gingivalis* strains were kindly provided by Dr. Fuminobu Yoshimura (Aichi-Gakuin University, Nagoya, Japan) and have been described previously (Nagano *et al.*, 2007) and; lymphocyte separation medium (LSM; Fischer Scientific, Pittsburgh, PA); CD14 microbeads from Miltenyi Biotec (Auburn, CA); *Escherichia coli* TOP10, TOPO expression kits and 5-Prime RNA kits from 5-Prime (Gaithersburg, MD); cytokine ELISAs came from R&D systems (Minneapolis, MN) and eBioscience (San Diego, CA); Human Toll-Like Receptor (TLR) Signaling Pathway RT² Profiler PCR array and RT² SYBR Green ROX qPCR Mastermix came from Qiagen (Valencia, CA); whole genome microarrays were from Affymetrix (Santa Clara, CA); siRNA (TLR4, sc-40260; TLR2, sc-40256; p52, sc-29409; p65, sc-29410; STAT4, sc-36568; control, sc-37007) from Santa Cruz Biotechnology (Santa Cruz, CA); ultrapure *E. coli* K12 LPS, anti-TLR2 and -4 antibodies (clones B4H2 and W7C11, respectively), qScript cDNA SuperMix from Quanta BioSciences Inc. (Gaithersburg, MD); THP1-XBlue NF-κB/AP-1 SEAP Reporter cells and TLR-transfected HEK293 cells came from InvivoGen (Santa

Clara, CA); the STAT4 inhibitor, lysofylline (1-[(5*R*)-5-Hydroxyhexyl]-3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione; LSF), was from Cayman Chemical Co (Ann Arbor, MI); the p65 (Ser276) inhibitory peptide set came from Novus Biologicals (Littleton, CO); while LB agar, kanamycin, arabinose, imidazole, RPMI 1640, fetal bovine serum (FBS), penicillin G, streptomycin, proteinase K, polymyxin B and agarose came from Sigma-Aldrich (St. Louis, MO).

P. gingivalis culture—*P. gingivalis* was maintained as frozen stock culture, grown in GAM under anaerobic conditions (80% N₂, 10% H₂, 10% CO₂) at 37°C in a Coy Laboratories (Grass Lake Charter Township, MI) anaerobic chamber and harvested at an OD₆₀₀ of 1 (1 × 10⁹ bacteria/ml). Purity of the cultures was routinely screened by Gram stain.

Isolation and maintenance of primary blood mononuclear cells (PBMCs) and monocytes—Whole blood was isolated from anonymized consenting, healthy donors (18 years; 49.9 kg; free of any chronic health problems; medication free; hemoglobin 12.5 gr/dl; not pregnant). Buffy coat was collected from an LSM gradient, washed in PBS (10mM, pH 7.2) and monocytes isolated from PBMCs using CD14 microbeads. Purified monocytes were maintained in RPMI 1640 containing 10% FBS, 100 µg/ml penicillin G, 100 µg/ml streptomycin, 37°C, 5% CO₂. Monocyte preparations were routinely > 95% pure and > 96% viable. For siRNA transfection, monocytes were isolated by negative selection. Blood collection was approved by the Institutional Research Board of the University of Louisville.

Production and purification of RagB—Genomic DNA was isolated from *P. gingivalis* W83 and the *ragB* gene was amplified by PCR (Fwd: CACCGAGCTTGACCGCGACCCC; Rev: TATCGGCCAGTTCTTTATTAAGTGGG). The *ragB* gene product was purified by electrophoresis 1% agarose gel and transformed into *E.coli* TOP10 using the pBAD202D-TOPO vector and the TOPO expression kit, according to the manufacturer's instructions. Transformants were selected by screening using LB agar plates containing 50 µg/ml kanamycin and PCR confirmation of *ragB* uptake. RagB expression was induced by arabinose and the full length, his-tagged protein collected by column purification (300mM imidazole fraction; HiTrap Chelating HP column) and dialyzed in PBS. Purity confirmed by SDS-PAGE and sequencing by mass spectrometry. Sequence comparison, using UNIPROTKB (Consortium, 2014), and structural prediction, using the Phyre² server at Imperial College London, UK (Kelley & Sternberg, 2009), were employed to confirm RagB identity, both with 100% confidence (*P. gingivalis* W83 RagB and Bacteroides SusD-like protein, respectively; Supplemental Figure 2).

Quantification of RagB-induced cytokines—PBMCs and primary monocytes were rested overnight in 96 (300,000 cells/well) or 6 well (3 × 10⁶ cells/well) plates, 37°C, 5% CO₂. Innate cells were incubated with RagB and various agonists and inhibitors, under the conditions described in the figure legends. Cytokine release was determined by ELISA. Lack of biologically meaningful recombinant protein contamination with vector TLR agonists was confirmed by analysis of cytokine release from monocytes exposed to

denatured (SDS-treated), heat-treated (boiled) and polymyxin B-treated RagB preparations (*data not shown*).

Quantification of RagB-induced genes—RNA was harvested from primary human monocytes 4 hr after stimulation with RagB. RagB-induced genes were determined by (a) TLR Signaling Pathway RT² Profiler PCR arrays (0 vs 1 µg/ml RagB) and (b) whole genome microarrays (0 vs 1 vs 10 µg/ml RagB), with (c) activation of promising candidate genes confirmed by qPCR. (a) Human TLR Signaling Pathway RT² Profiler PCR arrays (100ng of total RNA per array) were performed, according to the manufacturer's instructions, with RT-PCR performed using the ABI 7500 system (Life Technologies, Grand Island, NY). PBS control and RagB stimulations were compared using the C_T method. (b) GeneChip® 3' IVT Express kits were used to prepare RNA for microarray analyses. Human PrimeView arrays (100 ng of total RNA per array) were scanned using Command Console 3.2 (Affymetrix, St. Clara CA), according to the manufacturer's instructions, and files imported into Genomics Suite 6.6 (Partek Inc., Saint Louis, MO) for summarization and background correction using RNA. One way ANOVA was conducted to compare the individual treatment conditions. FDR was conducted along with the Bonferroni corrected p-value test. Ingenuity IPA software (Redwood City, CA) was used to select for genes: ± 2 fold change, $p < 0.001$, RagB 10 µl treatment group $>$ fold change than 1 µl treatment group when compared with negative control. (c) For qPCR of selected RagB regulated monocyte genes, RNA concentration was measured using Nanodrop Spectrophotometer ND-1000 (Wilmington, DE) and cDNA reverse transcribed using qScript cDNA SuperMix, according to manufacturer's protocol. RT² SYBR Green ROX qPCR Mastermix along with 1/10th of cDNA synthesis reaction was added to PCR tubes with total volume of 25 µl. The primers used are listed in Supplementary Table 1 and were employed at a final concentration of 25 pM per tube. RT-PCR was conducted using ABI 7500 system with the following cycle conditions: 2 mins at 50°C; 10 mins at 95°C; 15 sec at 95°C, 1 min at 55°C for 40 cycles.

Gene silencing—TLR2, TLR4, p65 and STAT4 genes were individually silenced in human monocytes by siRNA transfection using the 4D-Nucleofactor system (Lonza Group, Allendale, NJ), according to the manufacturer's P3 primary cell protocol. Briefly, ~4 million cells were suspended in 100µl transfection reagent in single nucleocuvettes and 3µL (30 pM) of siRNA added. Transfected monocytes were rested in RPMI 1640 containing 10% FBS for 48 h at 37°C prior to stimulation with RagB and other agonists, as described in the Figure legends.

Induction of cytokines by whole *P. gingivalis*—*P. gingivalis* (WT, ragB and ragB complemented strain) was fixed in 1% paraformaldehyde for one hour and washed with PBS. Monocytes were stimulated with MOI 1:10 fixed *P. gingivalis* strains for 18 h and cytokines (IL-6, IL-8) measured in cell free supernatants by ELISA.

Quantitative Data and Statistical Analyses—All experiments were performed in triplicate. All results are presented as mean ($\pm$ s.d.), unless otherwise stated. Comparisons between groups were analyzed by t-test or one-way ANOVA using Prism 5 software (GraphPad, La Jolla, CA). Significance was accepted at $p = 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. RagB induces pro-inflammatory cytokines

Primary human monocytes were untreated or treated with *E. coli* LPS (0.1, or 1 $\mu\text{g/ml}$) or with RagB (0.1, 1, or 10 $\mu\text{g/ml}$). IL-8 concentrations in 20 hr cell-free supernatants were determined by ELISA. Similar results were found for IL-6 and TNF (Supplementary Figure 2).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. RagB signals through TLR2

Primary human monocytes were untreated or exposed to combinations of RagB (1 $\mu\text{g/ml}$); the TLR2 agonist, Pam3CSK (1 $\mu\text{g/ml}$), anti-TLR2 (1 $\mu\text{g/ml}$, 1 hr pre-treatment) and control (IgA₂, 1 $\mu\text{g/ml}$, 1 hr pre-treatment) antibodies. In an alternative strategy, TLR2 gene silencing was performed, as described in the text.

(A) IL-8 concentrations in 20 hr cell-free supernatants were determined by ELISA. TLR2 knockdown was confirmed by (B) Western blot and (C) densitometry. (D) Inhibition of RagB-induced IL-8 on TLR2 gene silencing was determined by ELISA.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) and (C) represent typical data.

Figure 3. RagB signals through TLR4

Primary human monocytes were untreated or exposed to combinations of RagB (1 μ g/ml); the TLR2 agonist, *E. coli* LPS (1 μ g/ml), anti-TLR4 (1 μ g/ml), 1 hr pre-treatment) and control (IgG₁, 1 μ g/ml), 1 hr pre-treatment) antibodies. In an alternative strategy, TLR4 gene silencing was performed, as described in the text.

(A) IL-8 concentrations in 20 hr cell-free supernatants were determined by ELISA. TLR4 knockdown was confirmed by (B) Western blot and (C) densitometry. (D) Inhibition of RagB-induced IL-8 on TLR4 gene silencing was determined by ELISA.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) and (C) represent typical data.

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Figure 4. RagB-induction of IL-8 is STAT-dependent

Primary human monocytes were untreated or exposed to combinations of RagB (1 μ g/ml) and the STAT4 inhibitor, LSF (25 and 100 μ M, 1 hr pre- treatment). STAT4 gene silencing was performed, as described in the text.

(A) IL-8 concentrations in 20 hr cell-free supernatants were determined by ELISA. STAT4 knockdown was confirmed by (B) Western blot and (C) densitometry. (D) Inhibition of RagB-induced IL-8 on STAT4 gene silencing was determined by ELISA.

*** $p < 0.001$. (B) and (C) represent typical data.

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Figure 5. RagB-induction of IL-8 is Nf κ B (p65)-dependent

Primary human monocytes were untreated or exposed to combinations of RagB (1 μ g/ml) and a p65 peptide inhibitor set (100 μ M, 1 hr pre-treatment). p65 gene silencing was performed, as described in the text.

(A) IL-8 concentrations in 20 hr cell-free supernatants were determined by ELISA. p65 knockdown was confirmed by (B) Western blot and (C) densitometry. (D) Inhibition of RagB-induced IL-8 on p65 gene silencing was determined by ELISA.

*** $p < 0.001$. (B) and (C) represent typical data.

Figure 6. RagB induces the phosphorylation of STAT4 and p65

Monocytes were treated with 1 µg/ml rRagB. Western blot of proteins harvested at 0, 15, 30, 60, 90, 120, and 180 mins.

Results represent typical data.

Figure 7. Mutation of *ragB* reduces *P. gingivalis*-induction of IL-8 production in monocytes Monocytes were incubated with fixed WT, *ragB* and pTCBex2: *ragBW83*-complemented *P. gingivalis* cells (MOI 1:10) for 18 h and IL-8 monitored in cell free supernatants by ELISA.

***p<0.001 compared to WT (W83).

Table 1

RagB induces multiple TLR-related genes in primary human monocytes.

Gene	Description	Fold change
IL-1 β	Interleukin 1, beta	83.4
IL-8	Interleukin 8	35.3
IL-1A	Interleukin 1, alpha	18.7
CCL2	Chemokine (C-C motif) ligand 2	7.1
IL-6	Interleukin 6	5.8

RNA was harvested 4 hr post treatment and reverse transcribed for hTLR RT-PCR array. The $2^{-\text{Ct}}$ method was used to determine significant differences from the control (unstimulated) group. 5 of the 84 genes in the TLR Signaling Pathway RT² Profiler PCR array were upregulated, as noted above.

Table 2

RagB induces multiple pro-inflammatory genes in dose-related manner.

Gene	Fold Change		
	Array		RT-PCR
	1vs0	10vs0	1vs0
IL-6	612	800	153
CCL20	380	449	34
IL-1 β	295	311	113
IL-12 β	146	765	2.8
STAT4	35	51	6
TNF	24	32	9
IL-8	7.3	7.5	2.7

Primary human monocytes were stimulated with 0, 1 or 10 μ g/ml of rRagB. RNA was harvested 4 hr post-stimulation and used for microarray analysis. 243 genes were differentially regulated, as determined by Bonferroni correct p-value, and also exhibited a dose-related response (10 > 1 > 0 μ g/ml RagB). Selected genes were verified by RT-PCR. The complete array data set is presented in Supplemental Table 1 (**.xls file*). Primers used for PCR-verification of array data are presented in Supplemental Table 2.