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High-throughput sequencing reveals key genes and immune homeostatic pathways activated in myeloid dendritic cells by *Porphyromonas gingivalis* 381 and its fimbrial mutants

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SUMMARY

The human microbiome consists of highly diverse microbial communities that colonize our skin and mucosal surfaces, aiding in maintenance of immune homeostasis. The keystone pathogen *Porphyromonas gingivalis* induces a dysbiosis and disrupts immune homeostasis through as yet unclear mechanisms. The fimbrial adhesins of *P. gingivalis* facilitate biofilm formation, invasion of and dissemination by blood dendritic cells; hence, fimbriae may be key factors in disruption of immune homeostasis. In this study we employed RNA-sequencing transcriptome profiling to identify differentially expressed genes (DEGs) in human monocyte-derived dendritic cells (MoDCs) in response to *in vitro* infection/exposure by *Pg*381 or its isogenic mutant strains that solely express minor-Mfa1 fimbriae (DPG3), major-FimA fimbriae (MFI) or are deficient in both fimbriae (MFB) relative to uninfected control. Our results yielded a total of 479 DEGs that were at least twofold upregulated and downregulated in MoDCs significantly ($P \leq 0.05$) by all four strains and certain DEGs that were strain-specific. Interestingly, the gene ontology biological and functional analysis shows that the upregulated genes in DPG3-induced MoDCs were more significant than other strains and associated with inflammation, immune response, anti-apoptosis, cell proliferation, and other homeostatic functions. Both transcriptome and quantitative polymerase chain reaction results show that DPG3, which solely expresses Mfa1, increased ZNF366, CD209, LOX1, IDO1, IL-10, CCL2, SOCS3, STAT3 and FOXO1 gene expression. In conclusion, we have identified key DC-mediated immune homeostatic pathways that could contribute to dysbiosis in periodontal infection with *P. gingivalis*.

Keywords

dendritic cells; dysbiosis; immune homeostasis; microbiome; *Porphyromonas gingivalis*; RNA-sequencing

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INTRODUCTION

Periodontitis is a chronic inflammatory disease that entails progressive destruction of the supporting tissues of the dentition. Chronic periodontitis, which affects 47% of the US population (Eke *et al.*, 2015), has been identified as a risk factor for multiple inflammatory diseases (Zhang *et al.*, 2013). The intensive accumulation of microorganisms in the oral biofilm or dental plaque subjects the teeth and gingival tissues to high concentrations of bacterial metabolites, leading to a progressive inflammation. An aggregate of microorganisms, which comprise the human microbiota, resides on the surface and in deep layers of skin, oral mucosa and saliva, conjunctiva and in the gastrointestinal tracts. The presence of a symbiotic relationship between the host and microbiota significantly impacts on the immune system and is essential to maintenance of health. We recently described a diverse microbiome contained within the blood dendritic cells (DCs) of patients with chronic periodontitis, which could have a profound influence on systemic health (Carrion *et al.*, 2012).

Porphyromonas gingivalis, a gram-negative anaerobe and oral amphibiont, is one of the most prominent microbes disseminated by blood DCs and has been recognized as a key causative microorganism in the pathogenesis of destructive chronic periodontitis (Griffen *et al.*, 1998). *Porphyromonas gingivalis* effectively colonizes the oral tissues, multiplies rapidly with invasion and survives for protracted periods in the gingival epithelial cells (Yilmaz *et al.*, 2006). It is postulated that the pathogenicity of *P. gingivalis* is multi-factorial and it possess a number of properties to enhance growth and survival, including those that regulate host inflammatory responses (Palm *et al.*, 2013). The virulence factors of *P. gingivalis* can be characterized based on their involvement in colonization and attachment, evading host responses, and in damaging host tissues and dissemination. There is considerable diversity in strain and virulence of *P. gingivalis* depending upon the changes in either the pathogen itself or the host (Zenobia & Hajishengallis, 2015). *Porphyromonas gingivalis* also invades DCs, fibroblasts, and heart or aortic endothelial cells, brain, placenta, and other organs. In the oral cavity, *P. gingivalis* colonization changes the composition of the oral commensal microbiota accelerating microbiota-mediated bone-destructive periodontitis, signifying that this low-abundance species is a keystone in periodontal disease (Hajishengallis & Lambris, 2011). It can accumulate into complex multi-species consortia with other organisms (Kuboniwa & Lamont, 2010) resulting in the differential expression of a large percentage of its genome and proteome (Kuboniwa *et al.*, 2009; Redanz *et al.*, 2011).

Dendritic cells are highly migratory antigen-presenting cells that ‘patrol’ the blood, skin, mucosa, and all the major organ systems (El-Awady *et al.*, 2015). These DCs, upon capture of microbes, undergo a tightly regulated series of events, including directed migration towards the secondary lymphoid organs, where processed antigens are ostensibly presented to T cells. This stimulates the adaptive immune response, generating distinct CD4 effector T helper responses (Miles *et al.*, 2014). We have recently documented that *P. gingivalis* is able to subvert autophagic destruction within DCs, where autophagy is an integral component of microbial clearance, antigen processing and presentation by DCs (El-Awady *et al.*, 2015). This occurs through its glycoprotein minor fimbriae called Mfa-1, which targets the C-type

lectin DC-SIGN on DCs. It is shown that Mfa1 are immunosuppressive, compared with FimA (Zeituni *et al.*, 2009); moreover, Mfa1 are essential for cell–cell auto-aggregation and recruitment for microcolony formation. The FimA have a chief role in initial attachment and organization of biofilms, as they act as adhesins that mediate invasion and colonization of host cells. The minor Mfa1 fimbriae interact with other dental commensals, co-adhere and develop biofilm in conjunction with *Streptococcus gordonii* (Lamont *et al.*, 2002).

High-throughput RNA sequencing (RNA-seq) is recognized as a major quantitative transcriptome-profiling platform that aids in relating functional genomics to basic biology (Hirano *et al.*, 2012). In the case of bacteria, small non-coding regulatory RNA mediated post-transcriptional regulation influences pathogenic bacteria to modulate their virulence and survival (Bardill & Hammer, 2012; Mann *et al.*, 2012; Ortega *et al.*, 2012), iron homeostasis and quorum sensing (Lenz *et al.*, 2005). RNA sequencing generates millions of reads in a relatively short time and analyzes the transcriptome of eukaryotic genomes (Wang *et al.*, 2009). The available literature states that a number of bacteria have been analyzed at the transcriptional level by deep sequencing approaches (Oliver *et al.*, 2009; Perkins *et al.*, 2009; Yoder-Himes *et al.*, 2009; Beck *et al.*, 2011; Isabella & Clark, 2011), which has provided novel insights into pathogenic regulons, as well as identifying sRNAs. As put forth by Hirano *et al.* (2012), with respect to the periodontopathogenic bacterium *P. gingivalis*, the transcriptome profiles generated genome-wide identification of transcription patterns (Hovik *et al.*, 2012) using a targeted array approach has identified many putative novel small non-coding RNAs and the expression of few hypothetical genes validated. Recent studies have also used RNA-seq data to examine expression profiles of multiple immune cells (Hutchins *et al.*, 2015).

We have shown in our previous studies that engagement of DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) by Mfa1 fimbriae of *P. gingivalis* yields weak DC maturation and an immunosuppressive cytokine profile. Furthermore, DC-SIGN–Toll-like receptor 2 (TLR2) crosstalk determines the intracellular fate of this pathogen within DCs, and may have implications for the management of many low-grade infections (El-Awady *et al.*, 2015). We hypothesize that bacteremic oral pathogens taken up by blood DCs activate specific genes and pathways responsible for disruption of immune homeostasis in chronic periodontitis. In our recent review, we also speculated that the frequency and phenotype of blood mDCs along with an assessment of the transcriptome and microbiome may provide a diagnostic tool for assessing patient risk for chronic inflammation (Miles *et al.*, 2014). Given these facts, in order to establish the immune homeostatic pathways disrupted in chronic periodontitis and to identify the genes in human myeloid DCs, as a first report, we have documented here two novel approaches using *P. gingivalis* 381 and bacterial mutants that solely express minor-Mfa1 fimbriae (DPG3), major-FimA fimbriae (MFI) or are deficient in both fimbriae (MFB) relative to uninfected control. We performed RNA sequencing of human monocyte-derived DCs (MoDCs) subjected to *in vitro* infection with *Pg381* or its defined isogenic mutants. This transcriptome analysis demonstrates the important and distinctive roles that the fimbriae play in disruption of DC-mediated immune homeostatic pathways that could contribute to dysbiosis in periodontitis. Overall, our investigation advances our understanding of the molecular basis of gene regulation during *P. gingivalis*-

mediated disease progression and also sheds light on how microbes interact with human DCs.

METHODS

Cultivation and phenotypic characterization of MoDCs

The Human Assurance Committee (HAC) at Georgia Regents University approved this protocol involving human cells. All human subject studies conducted conform to the principles of the Declaration of Helsinki. This study was human subject exempt as all monocytes were obtained from healthy anonymous donors. The conventional MoDCs were generated *in vitro* as described elsewhere (Sallusto & Lanzavecchia, 1994), with slight modifications. Briefly, human monocytes were isolated from mononuclear fractions of peripheral human blood by a human monocyte enrichment technique. Ethylenediamine tetraacetic acid (EDTA) was added to the whole blood (1 mM) and samples were incubated with the RosetteSep™ Human Monocyte Enrichment Cocktail kit (StemCell Technologies Inc., Vancouver, BC, Canada) for 20 min. Samples were diluted with an equal volume of phosphate-buffered saline (PBS) + 2% fetal bovine serum and 1 mM EDTA and layers of diluted samples were placed on the top of medium-density Ficoll. Monocyte separation was carried out using medium density Ficoll (GE Health-care Bio-Sciences, Piscataway, NJ). Samples were centrifuged at 1200 *g* for 20 min at room temperature. The layer of enriched cells was removed and washed twice with PBS and re-suspended in medium for cell culture. Cells were seeded in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF)/ interleukin-4 (IL-4) (1000 unit ml⁻¹) (Gemini Bio-Product, GA, Broderick, CA) at a concentration of 3 × 10⁵ to 4 × 10⁵ cells ml⁻¹ for 5–6 days. Fresh medium treated with GM-CSF and IL-4 was used every other day for cultured cells. Flow cytometry analyses were carried out to verify the immature DC phenotype (CD1a⁺ CD83⁻ CD14⁻ DC-SIGN⁺) (Carrion *et al.*, 2012; Miles *et al.*, 2014; El-Awady *et al.*, 2015) Cell surface markers of DCs were evaluated by four-color immunofluorescence staining with the following antibodies: phycoerythrin-conjugated CD1a, fluorescein isothiocyanate-conjugated DC-SIGN, Peridinin chlorophyll protein-conjugated CD14, and allophycocyanin-conjugated CD83 (Miltenyi Biotec, Bergisch Gladbach, Germany). After 30 min at 4°C and washing with staining buffer (PBS pH 7.2, 2 mM EDTA, and 2% fetal bovine serum), cells were fixed in 1% paraformaldehyde. Positive marker expression was calculated as a percentage of total DCs by forward scatter and side scatter characteristics (Zeituni *et al.*, 2009, 2010a).

Porphyromonas gingivalis strains and mutants

Four *P. gingivalis* strains were used in this study and were obtained courtesy of C.A. Genco (Tufts University School of Medicine, Boston, MA): (i) Pg381, which expresses both minor (Mfa1) and major (FimA) fimbriae; (ii) isogenic minor-Mfa1 fimbria-deficient mutant (MFI), which expresses only the major-FimA fimbriae; (iii) isogenic major-FimA fimbria-deficient mutant (DPG3), which expresses only the minor-Mfa1 fimbriae; and (iv) the double fimbriae mutant (MFB) (Takahashi *et al.*, 2006) (Table 1). The *P. gingivalis* strains were maintained anaerobically (10% H₂, 10% CO₂ and 80% N₂) in a Forma Scientific anaerobic system glove box model 1025/ 1029 at 37°C in Difco anaerobe broth MIC (Gibson *et al.*, 2006). Mutant strains were maintained using erythromycin (5 µg ml⁻¹) for

mutant DPG3, tetracycline ($2 \mu\text{g ml}^{-1}$) for mutant MFI and both erythromycin and tetracycline for the double fimbriae mutant MFB.

DC infection with *P. gingivalis*

Bacterial suspensions were washed five times in PBS and re-suspended for spectrophotometer reading of 0.11 for optical density at 660 nm, which was previously determined to be equal to 5×10^7 colony-forming units (Cutler *et al.*, 1991). Corresponding bacterial counts were calculated and dilutions were prepared to infect MoDCs at a multiplicity of infection (MOI) of 10 : 1. Bacterial suspensions were washed three times with PBS before MoDC infection. The MoDCs were co-incubated with *Pg381*, DPG3, MFI, and MFB and incubated for 12 h. Four biological replicates were prepared for each strain.

RNA isolation and purification

After 12 h of infection, direct lysis of the infected MoDC suspensions was achieved by adding 350 μl of Qiagen's buffer RLT (Qiagen, Hilden, Germany). The lysate was collected and pipetted directly into the Qia-shredder spin column. Ethanol (70%) was added to the lysate, which was mixed by pipetting. Samples were then transferred to RNeasy spin columns placed in 2-ml collection tubes. The samples were centrifuged at 11,963 g for 15 s. The flow-through was discarded and 700 μl of buffer RW1 was added to the spin column. The samples were centrifuged at 11,963 g for 15 s. The flow-through was discarded and 500 μl of buffer RBE was added to the spin column. The buffer RBE step was repeated; then the spin column was placed in a 1.5-ml collection tube. RNA-free water (20 μl) was added directly to the spin column membrane, and the samples were centrifuged at 11,963 g for 1 min. All steps of the procedure were performed at room temperature, and all centrifugation was performed at 20–25°C in a standard microcentrifuge. RNA quantity and integrity were tested and only ratios of absorbance at 260 and 280 nm of 1.8–2.0, were included in the study. Samples were then stored at -80°C for further RNA-seq studies.

RNA-seq library preparation and sequencing

High-molecular-weight (> 200 nucleotides) RNA (300 ng) was pooled from three individual wells within each strain phenotype: *Pg381*, DPG3, MFI, MFB and uninfected control, for a total of five samples. For each strain, two pools of 900 ng of RNA were created and libraries were prepared using Illumina regular approach. The rationale for pooling the RNA from individual samples was to obtain sufficient quantities of RNA for library preparation using samples. Illumina TruSeq RNA Sample Prep Kit (Illumina Inc., San Diego, CA) was used with 800 ng of total RNA for the construction of sequencing libraries. RNA libraries were prepared for sequencing using standard Illumina protocols. In brief, RNA was purified using Sera-Mag Magnetic Oligo(dT) beads and fragmented, followed by cDNA synthesis with random hexamers. This product then underwent end repair, adapter ligation, and gel purification (2% TAE) to isolate 300-nucleotide fragments. DNA from the gel bands was purified using the QIAquick Gel Extraction Kit, polymerase chain reaction (PCR) amplified (15 cycles), and libraries were quantified using an Agilent bioanalyzer (DNA-1000 kit). Each library was sequenced using Illumina GAIIx sequencer on one lane of the flow cell, generating 50-nucleotide paired-end reads.

Data processing

The raw reads were processed using cutadapt (<http://journal.embnet.org/index.php/embnetjournal/article/view/200/458>) to filter for low-quality bases (QV < 20) and trimming of adapter sequences. After trimming, any reads with < 32 bp were also filtered out. High-quality reads were then aligned to the ribosomal RNA database to remove rRNA reads using in-house perl scripts.

Principal component analysis

For quantifying gene expression data from the signal-processing viewpoint, samples correlation and detection of outliers, the raw data (all transcripts) were subjected to a principal component analysis (PCA) before cluster exploration using Partek® Software as previously described (Koh *et al.*, 2012). Samples were plotted in three-dimensional plots across the first three principal components and the five conditions (*Pg381*, DPG3, MFI, MFB and uninfected control) were studied. The plot of PC1 (*x*-axis), PC2 (*y*-axis), and PC3 (*z*-axis) gave rise to ~95% of the variability, revealing distinct clusters corresponding to the conditions.

Differential gene expression, functional, and pathway analysis

Filtered high-quality RNA-seq data sets (see Table S1) were aligned against the hg38/GRCh38 human genome build (see Fig. S1) using the splice-aware aligner tophat (Trapnell *et al.*, 2012). Normalized gene counts were obtained with cufflinks/2.2.1 with default parameters and differential gene expression between the untreated and treated samples was investigated using cuffdiff with default parameters (Trapnell *et al.*, 2012). Genes were defined as expressed using an average minimum fragments per kilo base per million reads (FPKM) value of 2 across all samples and transcript length ≥ 300 bp. In each comparison, a gene is defined as differentially expressed using a fold change of ≥ 2, average FPKM ≥ 5 and *P* ≤ 0.05. R scripts were written using the packages gplot, heatmap.2, Venn diagram, and topGO. Clustering and visualization of differentially expressed transcripts as heatmaps were generated using heatmap.2. Overlaps between differentially expressed genes were performed using Venn diagram tools and biological processes were assessed with topGO. A classic Fisher test was used to define enriched Gene Ontology (GO) terms compared with the background of expressed transcripts with *P* ≤ 0.01. Genes involved in different steps of a common pathway tend to overlap in their expression profiles. As such, pathway-based analysis can provide insights into the biological functions of genes. Functional and pathway analysis was performed using GO terms/biological process and annotated genes. All the raw FASTQ data generated in this study using the Illumina protocols have been deposited in GEO Series GSE67141.

TaqMan PCR array of MoDCs

Analysis of gene expression in *Pg381*, DPG3, MFI, and MFB infected MoDCs was performed by real-time quantitative PCR (RT-qPCR) using the aliquots of the same total RNA isolated as described above. Briefly, cDNA was synthesized using 1 µg RNA through a reverse-transcription reaction (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed on TaqMan array fast plates using TaqMan fast universal PCR master mix

(2 $\times$). Three experimental (technical) replicates were analyzed for each biological sample. The calculations for fold regulation used the 2^{-C_t} method. The fold regulation of the gene in each group was calculated relative to its expression in the controlled samples for that gene, and normalized with 18S rRNA as an internal control.

RESULTS

Purity of mDCs and RNA-seq profiling quality analysis

Isolated immature DCs were over 96% pure, as determined by phenotypic markers as described (El-Awady *et al.*, 2015). To understand the transcriptional events in MoDCs associated with pathogen *P. gingivalis* and its fimbriae mutants, we performed high-throughput RNA-seq of poly-A purified RNA samples using an Illumina Genome Analyzer. Figure S1 shows that approximately 50 million high-quality reads were obtained per sample after filtering for base quality, adapter trimming, and rRNA reads. Of these high-quality reads, 48 million (~94%) reads were aligned and properly paired to the human mRNA reference sequences (see Fig. S1B) and 20,900 transcripts were measured in DCs. Fragments per kilobase of exon per FPKM was used to represent the expression level of each transcript as shown in the (Fig. S1C–E) (Mortazavi *et al.*, 2008). Among the transcripts obtained, 20,900 transcripts and their variants with stringent filter criteria of $P < 0.05$ and the absolute $\log_2$ -fold change ≥ 2 were annotated to 479 differentially expressed genes (see Table S1).

Principal component analysis

To identify possible correlations between genes altered by different strains of *P. gingivalis* and their fimbrial phenotypes and genotypes (Table 1) we performed a PCA (Fig. 1A). The PCA shows interesting and not-unexpected results, demonstrating the scattered samples among the three PCA components, which clearly indicate a strain-specific gene expression pattern. The first component result shows that *Pg381*, DPG3, and MFI are distinctly separated from uninfected control and MFB (double mutant). The second components show that genes induced in MoDCs by *Pg381* and MFI (both FimA+) and DPG3 (FimA–) are strongly related to the strain's phenotype (Table 1), as the strains that might have more or less similar virulence tend to cluster together. As expected, when the third PCA component is taken into account, the genes of MoDCs infected with DPG3 are further separated from the genes of MoDCs infected with *Pg381* and MFI, according to the virulence level of the mutant, although it is clustered in the same PCA pattern. Environmental cues are particularly influential in fimbriae expression patterns of *P. gingivalis* (Xie *et al.*, 1997). These data further suggest the important role that the local environment can play in host immune cell gene regulation by *P. gingivalis* (Xie *et al.*, 1997).

Transcriptome profiling of *in vitro* MoDCs

RNA-seq was employed to profile the global transcriptome changes induced in DCs by infection with different fimbriae expressing *P. gingivalis* strains (DPG3, MFI, and MFB) in three experimental replicate cultures. To achieve our goal, first unsupervised hierarchical clustering was performed on 479 differentially expressed genes of MoDCs infected by MOI of 10 for 12 h of *Pg381*, DPG3, MFI, and MFB relative to uninfected control. The conditional and hierarchical cluster-grams clearly indicate the clustered samples (per strain)

in the column and the highly upregulated (red) and downregulated (blue) genes in the rows (Fig. 1B). The differential expression patterns demonstrate clustering of most of the up-regulated and down-regulated genes are related to the *Pg381*, DPG3, and MFI strain's virulent dependent manner. Expression patterns of *Pg381*, DPG3, and MFI infected groups are similar, whereas a variation in expression patterns is evident in the double mutant MFB. This cluster trend is further confirmation of the PCA as shown in Fig. 1A.

Next, we used the Venn diagram analysis for shared and unique genes induced by *P. gingivalis* strains compared with uninfected control (Fig. 1C). Results of our analysis yielded a total of 479 differentially expressed genes in MoDCs that passed all stringent filters including 2-fold upregulated and downregulated in all four strains (*Pg381*-338, DPG3-350, MFI-318, and MFB-42), significantly ($P \leq 0.05$), compared with the uninfected control, using cuffdiff. Among these, 243 genes were commonly altered by three strains, *Pg381*, DPG3, and MFI, whereas none of the genes were altered commonly between these and MFB double mutants (except for two genes shared with DPG3), which were mostly comparable with the uninfected control. Remarkably, 70 genes in MoDCs were uniquely differentially expressed in response to DPG3, 24 in *Pg381*, 20 in MFI, and 40 in MFB. In addition, 25 genes were shared in response to *Pg381* and DPG3, 10 between DPG3 and MFI, and 48 between *Pg381* and MFI. To visualize the differentially expressed genes, we clustered each strain individually compared with the uninfected control (Fig. 1D). These tightly clustered and functionally congregated upregulated and downregulated genes were selected and are listed in the (Tables S2–S5).

Further analyses revealed the distribution of differentially upregulated and downregulated genes using MA (Fig. 2) and scatter (inset) plots based on $\log_2$ -fold change compared with average expression 5 FPKM. The MA plot shows, respectively, 200 and 138 genes upregulated and downregulated in MoDCs in response to *Pg381* (Fig. 2A), 238 and 118 increased and decreased in response to DPG3 (Fig. 2B), 188 and 130 increased and decreased in response to MFI (Fig. 2C), but only 40 altered (18 upregulated and 24 downregulated) in response to MFB (Fig. 2D), relative to the uninfected control. This indicates that *P. gingivalis* MFB, which is not internalized by MoDCs, nonetheless induces 40 differentially expressed genes. In contrast, DPG3 and MFI, which were both taken up by MoDCs, induced distinct changes in genes and homeostatic pathways in MoDCs likely reflective of distinct pattern recognition receptor crosstalk by Mfa1 and FimA. The expression and distribution of genes shown in scatter plots (Inset) indicate the similar trend and the reliability of gene expressions induced by *P. gingivalis* and its mutant strains.

Functional categories and enriched pathways

After analysis of the differentially expressed genes in MoDCs by *Pg381* and its mutants, our interest turned towards the genes that were uniquely altered by each strain in comparison with the uninfected control (Fig. 3A–D). These unique genes are also listed in the (Tables S2–S5). Among the 479 identified genes in total, 154 were found to correspond to unique genes shown as clustergram in Fig. 3(B–D), respectively. These include 24 genes induced by *Pg381*-, 70 by DPG3-, 20 by MFI-, and 40 by MFB-infected cells. To characterize these genes, we initially categorized them based on biological process terms of GO and conducted

an enrichment analysis. The most significant categories are shown in the functional pie-charts (Fig. 4). These processes include apoptosis in response to *Pg381* (16%) and MFI (18%), anti-apoptotic processes in response to DPG3 (20%), and immune effector process in response to MFB (15%) -infected cells. Further interesting gene-mediated functions affected by *Pg381* are angiogenesis (7%), immune response (16%), immune effectors (10%), IL-17 production (1%) and regulation of chemokine production (2%), bone resorption (2%), and CD40 signaling/TLR2/4 signaling pathways (7%) (Fig. 4A). In contrast, processes influenced most by DPG3 (Fig. 4B) include immune-homeostasis (10%), dendritic/cell migration/differentiation (17%), proliferation (20%), negative regulation of apoptotic-signaling pathways (4%), and others listed. Strain MFI (Fig. 4C) induced immune/inflammatory response (27%), programmed cell death/positive regulation of apoptosis (27%), bone remodeling/resorption (5%), phagolysosome assembly/phagocytosis (2%), cytokine-mediated and TLR2 signaling pathways (7%); while MFB (Fig. 4D) influenced activated T-cell proliferation (5%), innate immune response (15%), regulation of IL-2 production (2%) and TLR signaling pathways (10). Furthermore, the predicted molecular function and subcellular localization of the identified genes were also retrieved from GO and enrichment analysis (see Tables S2–S3). All 154 genes were then classified into five major groups upon which we focused according to their molecular function, as follows: immune-inflammatory response, apoptotic activities, immune-homeostatic regulation, differentiation, and migration.

Validation of differentially expressed genes data by RT-qPCR array

In a separate series of experiments, MoDCs were infected with *P. gingivalis* and its mutants for 12 h. An array of 17 genes (Table 2) related to DC phenotype, maturation, inflammation and migration profiles of DCs were investigated by our in-house TaqMan qPCR array. The results are as follows.

DC phenotype and maturation state—*Pg381* infection significantly downregulated the immaturity marker DC-SIGN in MoDCs (−4.0-fold, $P = 0.0001$) whereas DPG-3 upregulated DC-SIGN (CD209) mRNA (+6.9-fold, $P = 0.0188$). Both *Pg381* and DPG-3 significantly downregulated the expression of CD1a, CD1c, and CD36. In addition, both strains upregulated CD80 mRNA (+4.8-fold, $P = 0.023$) (+5.5-fold, $P = 0.023$), respectively. MFI infection significantly downregulated the expression of CD1a, CD1c, and CD36 (−3.4, −3.6, and −3.6-fold), respectively. In contrast strain MFB, which is not taken up by MoDCs, downregulated CD1c and CD36 mRNA (−4.2-fold and −4.1-fold). Moreover, MFB infection downregulated DC-SIGN (CD209) mRNA (−4.1-fold, $P = 0.027$). No significant changes were detected in the expression of CD14, CD40, CD83, and CD86 after infection with all strains (Table 2).

Immunoregulatory and inflammatory/atherogenesis markers—These markers showed a distinct pattern of expression when comparing *Pg381* to DPG-3 infections. Indoleamine 2,3-dioxygenase 1 (IDO1) mRNA was significantly upregulated in MoDCs after infection with DPG-3 (+151-fold, $P = 0.015$), *Pg381* (+76.5-fold, $P = 0.004$) and MFI (+54.4-fold, $P = 0.006$), whereas the lowest upregulation of IDO1 was in MFB-infected cells (+32.4-fold, $P = 0.006$). In addition, Lox1/OLR1 mRNA was significantly upregulated

in cells infected with DPG-3 (+8.6-fold, $P = 0.014$). The change in Lox1 expression in MoDCs infected with *Pg381* was < 2-fold (+1.8, $P = 0.026$). No significant change was detected in Lox1 expression after MFI infection, whereas slight upregulation of the gene was recorded in MFB-infected cells (+3.7-fold, $P = 0.029$). No significant change was detected in the expression of matrix metalloproteases (1, 8 and 9) among cells infected with all *P. gingivalis* strains, although the tissue inhibitor of matrix metalloproteases, TIMP1, was upregulated by all strains. Fimbriated strains (*Pg381*, DPG3, and MFI) significantly upregulated TLR2 (~ +4-fold), whereas MFB did not change the gene expression within MoDCs. Although all strains downregulated TLR4 expression, the statistical significance was only achieved in MFI- and MFB-infected cells (−3.7, $P = 0.041$) (−4.1, $P = 0.029$), respectively (Table 2).

Inflammatory cell recruitment and homing—*Pg381* infection significantly upregulated CCL19, CCL5, and CCR7 mRNA 2.3-, 4.2-, and 8.4-fold, respectively, in MoDCs while *Pg381* downregulated CCL21, CCR2, and CXCL16 mRNA (−7.1, −5.4, and −3.4-fold). In contrast, DPG3 significantly downregulated CCL21, CCR2, and CX3CR1 (−3.1, −2.4, and −2.3-fold) in MoDCs. Those MoDCs infected with MFI strains showed upregulation of CCL5 (+4.5-fold, $P = 0.015$) and downregulation of CCL21 (−4.2-fold, $P = 0.001$). In addition, cells infected with MFB exhibited significant downregulation in CCL19, CCL21, CCR2, and CX3CR1 mRNA (−1.5, −9.1, −5.8 and −3.2-fold) (Table 2).

We also confirmed several key annotated genes activated in DCs by DPG3 (Fig. 3B, E), which are mainly involved in regulation of DC differentiation (ZNF366) programmed cell death (apoptosis) [Fork-head box protein O1 (FOXO1)], DC recruitment (CCL2, CCR7) intracellular signaling [suppressor of cytokine signaling 3 (SOCS3), signal transducer and activator of transcription 3 (STAT3)] uptake of low-density lipoproteins (LOX1) and immunoregulation (DC-SIGN, IL-10, IDO1) compared with uninfected control. Among the upregulated genes examined by qPCR, IDO1 was the gene most significantly ($P < 0.001$) upregulated more than 50-fold by DPG3 (Mfa1) relative to uninfected control. Overall, the expression changes of nine selected genes validated by TaqMan qPCR data show the similar trend from RNA-seq analysis (Fig. 3E).

DISCUSSION

A major determinant of *P. gingivalis* pathogenicity in chronic periodontitis and its impact on immune homeostasis has been attributed to fimbriae expression. The fimbriae mediate initial interactions with the host that are imperative for the initiation and progression of periodontal diseases (Lamont & Jenkinson, 1998). *Porphyromonas gingivalis* plays a particularly influential role in periodontal pathogenesis by disrupting the local microbiome (Hajishengallis & Lambris, 2011), invading the host epithelial barrier (Njoroge *et al.*, 1997) and evading autophagy in DCs (El-Awady *et al.*, 2015). Here we used RNA-seq to analyze globally the transcripts induced in DCs by infection with different fimbriae-expressing *P. gingivalis* strains. The RNA-seq method is regarded as a powerful digital gene expression measurement with many advantages over previous microarray-based assays; in particular, its ability to sensitively analyze transcriptomes in an unbiased and comprehensive manner (‘t Hoen *et al.*, 2008; Wilhelm *et al.*, 2008). Through this approach we were able to identify

certain key immune homeostatic pathways that are disrupted by *P. gingivalis* infection; moreover, many pathways are induced in a fimbriae-specific manner. We will focus our discussion on several key annotated genes activated in our *in vitro* DC infection model that were dependent on the fimbrial expression pattern of the infecting *P. gingivalis* strain.

DC-SIGN has emerged as a key player in the induction of immune responses against numerous pathogens via modulation of TLR-induced immune activation (Sukhithasri *et al.*, 2013). We have previously observed that the native minor Mfa1 fimbriae of *P. gingivalis* are glycosylated proteins with mannose and fucose residues (Zeituni *et al.*, 2010b) required for binding to the endocytic receptor DC-SIGN, leading to internalization in DC-SIGN-rich compartments. This uncouples cytokine secretion from the maturation of DCs and elicits a T helper type 2-biased effector T-cell response (Zeituni *et al.*, 2009). Dendritic cells are regarded as immune ‘sentinels’ that determine the fate of infection through activation of immunity or tolerance. This dichotomous response is regulated by pattern recognition receptor crosstalk (Hajishengallis & Lambris, 2011), which triggers distinctive intracellular signaling pathways (e.g. STAT3) and transcription factors (e.g. FOXO1). Pattern recognition receptors on DCs include TLRs, Nod-like receptors and the endocytic C-type lectins such as DC-SIGN (CD209) and mannose receptor (Hammer & Ma, 2013). As DC-SIGN and TLR2 on DCs are targeted by *P. gingivalis* Mfa1 and FimA fimbriae, respectively (El-Awady *et al.*, 2015) the analysis of the unique genes activated in DCs by these fimbriae-deficient strains becomes a powerful model system to examine how microbes disrupt immune homeostasis. In this study, DPG3-infected MoDCs upregulated STAT3 and DC-SIGN mRNA. This is consistent with the reported role for DC-SIGN ligation (i.e. by Kaposi’s sarcoma-associated herpesvirus) in induction of STAT3 activation, leading to DC dysfunction (Santarelli *et al.*, 2014). STAT3 induction in DCs also correlates with block of autophagy, leading to persistence of Kaposi’s sarcoma-associated herpesvirus (Santarelli *et al.*, 2014) and DPG3 in DCs (El-Awady *et al.*, 2015). Moreover, engagement of TLR2 on DCs by FimA of strain MFI (El-Awady *et al.*, 2015) activates DC maturation (Zeituni *et al.*, 2009) and as shown here, downregulation of DC-SIGN and upregulation of TLR2.

ZNF366 or DC-SCRIPT (cDNA-encoding DC-specific transcript) is a novel protein member of the zinc finger family of transcription factors and is expressed in all DC subsets (Triantis *et al.*, 2006). DC-SCRIPT plays an important role in DC differentiation and maturation. DC-SCRIPT-silenced DCs are less capable of inducing T-cell proliferation (Hontelez *et al.*, 2012). DC-SCRIPT was prominent among the genes upregulated in *Pg381*-infected DCs. *In vitro* studies from our group have validated the ability of *Pg381* and DPG3 at a low MOI to induce DC differentiation from human monocytes in the absence of GM-CSF/IL-4, the canonical growth factor/cytokine normally used to generate MoDCs from monocytes (Miles *et al.*, 2013).

Among other activities, FOXO1 is involved in the regulation of programmed cell death (apoptosis), necessary for clearance of dysfunctional immune cells and homeostasis (Hou & Van Parijs, 2004). We show here high levels of FOXO1 in DCs infected *in vitro* with *Pg381* and DPG3, but not uninfected control. Moreover, pro-apoptotic Bcl-2 member Bim was not induced by DPG3-infected DCs, explaining the high resistance of these non-canonical MoDCs to apoptosis (Miles *et al.*, 2013). Moreover, the pro-apoptosis-related genes,

especially BH3-only proteins (BAD, BAX), are upregulated by *Pg381* and MFI but downregulated by DPG3. Efforts in the laboratory are directed towards validating whether or not FOXO1 is active or inactivated by phosphorylation in DCs infected with *P. gingivalis* DPG3.

Interestingly, mRNA for the immunoregulatory enzyme IDO1 shows significant upregulation after infection with DPG3, and upregulation was also detected in *Pg381*-infected MoDCs. MFI similarly upregulated IDO1 mRNA whereas, MFB-infected cells showed the lowest upregulation of IDO1. IDO catalyzes the breakdown of tryptophan needed for T-cell proliferation and in chronically inflamed sites, IDO blocks T helper type 1 and type 17 effector differentiation, promoting T-cell apoptosis and FOXP3⁺ regulatory T-cell formation (Huang *et al.*, 2010). Efforts in our laboratory are directed to determining whether IL-23 α , IL-1 β , IL-10 and IDO1 proteins are selectively induced in DCs by the different *P. gingivalis* strains. We are particularly interested in whether DPG3-infected DCs will produce low levels of IL-23, IL-1 β and IL-17 relative to MFI and *Pg381*, as we expect. These inflammatory mediators have previously been reported to be elevated in patients with periodontitis (Davanian *et al.*, 2012). In support of our current study, these cytokines, notably tumor necrosis factor- α , CCL2 (MCP-1), IL-8, IL-6, and IL-1 β were elevated by *Pg381*-infected MoDCs. Moreover, IL-6 and CCL2 also increased by DPG3 that recruits DCs to the sites of inflammation.

It has been shown that *P. gingivalis* inhibits the production of the chemokine MCP-1 by *P. gingivalis*-specific T cells, monocytes and B cells (Gemmell *et al.*, 2001). *Porphyromonas gingivalis* also inhibits neutrophil chemotaxis (Madianos *et al.*, 1997) as well as influx and activation of monocytes/macrophages (Hajishengallis *et al.*, 2008). In the present report we find differential induction of chemokines and chemokine receptors depending on fimbrial expression patterns of the *P. gingivalis* strain. In general, upregulation of CCL19, CCL2, CCR7, and CCL5 mRNA are dependent on fimbrial expression (or internalization as all the fimbriated strains are taken up by DCs (Zeituni *et al.*, 2009). It is notable that although CCR7 mRNA is upregulated in DCs infected with *P. gingivalis* the protein is not expressed, resulting in disruption of secondary lymphoid organ homing (Miles *et al.*, 2013). It is also important to note that *Pg381* downregulated CCL21, CCR2 and CXCL16 mRNA, whereas DPG3 downregulated CCL21, CCR2, and CX3CR1 and MFI downregulated CCL21 only. Likewise, MFB downregulated CCL19, CCL21, CCR2, and CXCR1 mRNA. Efforts in our laboratory are directed to understanding how these differential chemokine responses translate to DC and T-cell recruitment to the gingival tissues during different stages of the disease, as they are potential targets for therapy (Gemmell *et al.*, 2001).

We further showed that lectin-like oxidized low-density lipoprotein receptor-1 (LOX1) mRNA was significantly upregulated in DPG3-infected cells compared with LOX1 expression in cells infected with *Pg381*. LOX-1 is a pattern recognition receptor for a variety of endogenous and exogenous ligands. LOX-1 expressed on B cells and DC cells has complementary functions to promote humoral immune responses (Joo *et al.*, 2014). The TLRs play a critical role in the early innate immune response to invading pathogens by sensing microorganisms, and are involved in detecting endogenous danger signals. Signaling by TLRs results in a variety of cellular responses including the production of interferons,

proinflammatory cytokines, and effector cytokines that direct the adaptive immune response (Rakoff-Nahoum & Medzhitov, 2009). TLR2 is essential for the recognition of a variety of pathogen-associated microbial patterns from gram-positive bacteria, including bacterial lipoproteins. Our results show that TLR2 has been noticeably upregulated in comparison with MFB-infected cells, which did not change the gene expression within MoDCs. There is statistical significance only in MFI-and MFB-infected cells even though all strains down-regulated the expression of TLR4, the receptor for gram-negative enterobacterial lipopolysaccharide.

Collectively, our results highlight the consequence for genes involved in immune homeostasis, of selective DC-SIGN and TLR2 engagement by *P. gingivalis* fimbriae (El-Awady *et al.*, 2015). We propose that this DC-SIGN-TLR2 crosstalk represents a microbial ‘switch’ that regulates immune homeostasis and the T-cell effector response (see Fig. S2). These findings will further advance the understanding of the pathogenesis of periodontal diseases; eventually leading to the identification of novel candidate molecules for pathogen-specific therapeutic approaches to chronic inflammatory diseases. Further investigation is warranted to validate these genes at the level of protein, function and in controlled human studies, to eventually provide important clues for therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Principal component, hierarchical clustergram and Venn analyses. (A) Principal component analysis shows good agreement with sample correlation: *Pg381*, *DPG3*, and *MFI* are similar and distinctly separated from uninfected control (Ui. Ctrl) monocyte-derived dendritic cells (MoDCs) and *MFB* (the double mutant), which are also similar. The *DPG3* is clearly separated from *Pg381* and *MFI* as well. (B) Hierarchical cluster analysis performed on the profiles of 479 differentially expressed transcripts. The heatmap of $\log_2$ (fold change) of samples compared with uninfected control shows correlation in differential expression levels (rows) over *DPG3*, *Pg381*, and *MFI* with subtle changes in *MFB* (columns). Heatmap for fold changes over uninfected control shown on the left. Deep red and blue are genes 10-fold upregulated and 10-fold downregulated, respectively. Note that the *DPG3* and *MFB* are deviated from *Pg381* and *MFI*, as shown in the principal components analysis. (C) Four-way Venn diagram comparison of the differentially expressed genes shows that *Pg381*, *DPG3*, and *MFI* share most of the genes in common (243) from uninfected control. The *MFB* genes do not overlap with other strains as well as uninfected control, though only a few of the genes are altered. (D) Comparison of fragments per kilo base per million reads (FPKM) value differences by individual strain. Subcluster-gram shows the differential expression of genes in genetic mutants of *Porphyromonas gingivalis* strains in DCs compared with the uninfected control (Tables S2 and S3). The pseudo colored representation of transcript expression is shown according to the scale at the top of each cluster, respectively.

Figure 2.

MA and scatter plots for transcripts distribution analyses. MA plots for the average expression and fold changes in log scale to visualize the change in gene expression and distribution against uninfected control for all 20,900 transcripts (A–D). Note: some values cannot be displayed in log space, a majority of the genes defined as significant have a 0 fragments per kilo base per million reads (FPKM) in one sample (bottom right plot). Scatter plots for comparison of FPKM for coding sequences (inset). For all four strains, each locus has one point with the $\log_2$ FPKM in one strain plotted against the $\log_2$ FPKM of the uninfected control. The straight line shows identity (A) *Pg381* vs. Uninfected Control, (B) *DPG3* vs. Uninfected Control, (C) *MFI* vs. Uninfected Control, and (D) *MFB* vs. Uninfected Control. Red, blue, and black colored data points indicate upregulated, downregulated and unaltered, respectively, as well as demonstrating the significant relative abundance differences between uninfected control monocyte-derived dendritic cells and infected with *Porphyromonas gingivalis* plus its mutant strains.

Figure 3.

Unique and functional cluster analysis. (A–E) The Venn and cluster-gram shows the uniqueness of each genetic mutant of *Porphyromonas gingivalis* based on the available annotated genes, fold (± 2) change, CuffDiff defined *P*-value comparison between observed and expected differentially expressed genes in a gene ontology term (see the gene list in the Tables S2–S5). Analyzed results demonstrate the set of genes that are uniquely and differentially expressed in dendritic cells (DCs) based on the virulence level of each mutant strain (B–D). (E) TaqMan quantitative polymerase chain reaction results shows the key annotated genes are strongly influenced by DPG3-infected DCs. Note: both, RNA-sequencing and quantitative polymerase chain reaction demonstrate the similar trend in expression level at fold changes. Genes were declared to be differentially expressed by comparing four types of *P. gingivalis* strains requiring the filter criteria as mentioned above.

Figure 4.

Functional categorization. Pie chart shows the classification of differentially expressed genes based on relevant biological processes (Gene Ontology terms) of four strains, (A) *Pg381*; (B) *DPG3*, (C) *MFI*, and (D) *MFB* relative to uninfected control. The pie chart depicts the major function of *Pg381* (Apoptosis/inflammatory response) and its fimbriae mutants, *DPG3* (cell proliferation/survival/dissemination), *MFI* (bone resorption/programmed cell death), *MFB* (feeble pattern recognition receptor signaling/immune effector process).

Table 1*Porphyromonas gingivalis* wild type and isogenic fimbriae-deficient mutant strains

Strain	Phenotype	Genotype	Predominant PRRs targeted
Pg381	mfa1+/fimA+	381	DC-SIGN/TLR2
Pg-DPG3	mfa1+/fimA–	381 fimA	DC-SIGN
Pg-MFI	mfa1–/fimA+	381 mfa1	TLR2
Pg-MFB	mfa1–/fimA–	381 fimA mfa1	TLRs

PRR, pattern recognition receptor; TLR, Toll-like receptor.

Table 2

Quantitative TaqMan-PCR array of monocyte-derived dendritic cells infected with Pg381, DPG3, MFI and MFB strains

Functional group	Gene name	Pg381		DPG3		MFI		MFB	
		Fold	P-value	Fold	P-value	Fold	P-value	Fold	P-value
Phenotype/maturation	CD1A	-3.9	0.0270*	-1.7	0.0814	-3.4	0.0170**	-2.3	0.0534*
	CD1C	-4.0	0.0280*	-3.7	0.0258*	-3.6	0.0441*	-4.2	0.0212*
	CD209	-4.0	0.0001***	6.9	0.0188**	-0.5	0.2196	-4.1	0.0002***
	CD36	-3.9	0.0228*	-3.7	0.0197**	-3.6	0.0176**	-4.1	0.0277*
	CD80	4.8	0.0238*	5.5	0.0237*	2.6	0.1034	2.2	0.1160
Inflammation	IDO1	76.5	0.0041***	151.4	0.0159**	54.4	0.0068**	32.4	0.0062**
	IL6	52.0	0.0210*	34.0	0.0361*	50.0	0.0232*	-3.5	0.0253*
	LOX1	1.8	0.0262*	8.6	0.0141**	0.3	0.4542	3.7	0.0260*
	TIMP1	4.1	0.0305*	9.2	0.0060**	4.6	0.0094**	2.0	0.1523
	TLR2	4.1	0.0307*	4.3	0.0271*	4.0	0.0133**	2.0	0.1293
Migration	TLR4	-2.0	0.0974	-1.9	0.0889	-3.7	0.0410*	-4.1	0.0293*
	CCL19	2.3	0.0204*	2.9	0.0944	1.4	0.2986	-1.5	0.0046**
	CCL21	-7.1	0.0084**	-3.1	0.0155**	-4.2	0.0015***	-9.1	0.0020**
	CCL5	4.2	0.0291*	9.2	0.0095**	4.5	0.0156**	2.0	0.1572
	CCR2	-5.4	0.0162**	-2.4	0.0414*	-1.9	0.1652	-5.8	0.0045**
	CCR7	8.4	0.0017***	17.9	0.0157**	0.1	0.4449	4.0	0.0008***
	CX3CR1	-3.4	0.0190**	-2.3	0.0051**	-0.7	0.3121	-3.2	0.0107**

ND, not detectable.

The statistical analysis of fold regulations (2^{-C_T}) was performed using the *t*-test with uninfected controls within three different experiments (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).