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Research Article

RESEARCH ARTICLE

Macrophage polarization in response to oral commensals and pathogens

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One sentence summary: Plasticity of macrophage populations related to microbial stimulation and ability of commensal bacteria to help regulate maturation towards inflammatory and tissue destructive cells via oral pathogens.

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ABSTRACT

Macrophages have been identified in the periodontium. Data have phenotypically described these cells, demonstrated changes with progressing periodontal disease, and identified their ability to function in antigen-presentation critical for adaptive immune responses to individual oral bacterium. Recent evidence has emphasized an important role for the plasticity of macrophage phenotypes, not only in the resulting function of these cells in various tissues, but also clear differences in the stimulatory signals that result in M1 (classical activation, inflammatory) and M2 (alternative activation/deactivated, immunomodulatory) cells. This investigation hypothesized that the oral pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* induce M1-type cells, while oral commensal bacteria primarily elicit macrophage functions consistent with an M2 phenotype. However, we observed that the M1 output from *P. gingivalis* challenge, showed exaggerated levels of pro-inflammatory cytokines, with a much lower production of chemokines related to T-cell recruitment. This contrasted with *A. actinomycetemcomitans* infection that increased both the pro-inflammatory cytokines and T-cell chemokines. Thus, it appears that *P. gingivalis*, as an oral pathogen, may have a unique capacity to alter the programming of the M1 macrophage resulting in a hyperinflammatory environment and minimizing the ability for T-cell immunomodulatory influx into the lesions.

Keywords: macrophage; oral bacteria; pathogens; commensals; cytokines

INTRODUCTION

Mucosal tissues are colonized by an extremely dense and diverse microbiota of commensal bacteria, are often the first site of interaction with pathogenic microorganisms (Socransky *et al.* 1998; D'Aiuto *et al.* 2004; Dye *et al.* 2005; Tatakis and Kumar 2005; Pedron and Sansonetti 2008). These sites continuously sample foreign material via various cells types, including macrophages (MΦ), which are innate immune cells at epithelial surfaces that respond rapidly to infection, carrying crucial information about the infection to lymph nodes to trigger an immune response (Nestle *et al.* 1994; Jotwani *et al.* 2001; Makino *et al.* 2001; Kopitar, Ihan Hren and Ihan 2006). Historically, the MΦ were identified to effectively engage microbes using a repertoire of pattern recognition receptors (PRRs) (Hemmi and Akira 2005; Benko *et al.* 2008)' which recognize distinct classes of microorganism-associated molecular patterns (MAMPs), including a range of bacterial, viral and fungal pathogens, through engagement of LPS, LTA and nucleic acid (e.g. CpG, DNA, dsRNA) ligands (Wollenberg *et al.* 2002; Blach-Olszewska 2005; Kawai and Akira 2006; Kumar, Kawai and Akira 2009). This enables an avid uptake of these foreign materials by the macrophages (Lauvau and Glaichenhaus 2004).

Macrophages are effective as antigen presenting cells (APCs) and are particularly adept at stimulating T cells for controlling

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the quality of the T effector cells (Girardi 2006; Gray and Cyster 2012). These cells also play a critical role in innate immunity, responding to microbial challenge and producing elevated levels of various cytokines that contribute to host innate defenses. These cells recognize and respond to microbial structures using the PRRs, which decorate the surface of the mΦ enabling uptake of antigenic components (Allavena *et al.* 2004; Blach-Olszewska 2005; den Haan and Kraal 2012; Zanoni and Granucci 2012; Locati, Mantovani and Sica 2013; Striz *et al.* 2014) and triggering activation and phenotype plasticity after engagement of microbial or viral pathogens as ligands (Kawai and Akira 2006; Schaible, Schaffer and Taylor 2010; Ferrante and Leibovich 2012; Salomao *et al.* 2012). This recognition of microbial components by the APCs triggers the production of selected cytokines, e.g. IL-1ß and TNFα that enhance cellular differentiation and maturation and are also linked to upregulation of a repertoire of cytokines and chemokines, enabling communication with both B and T cells as major effector cells in adaptive immunity (Guiney, Hasegawa and Cole 2003; Gervassi *et al.* 2004; Karlsson *et al.* 2004; Kranzer *et al.* 2004; Hart *et al.* 2005; Hu *et al.* 2006).

Recent evidence has emphasized an important role for the plasticity of MΦ phenotypes, not only in the resulting function of these cells in various tissues, but also clear differences in the stimulatory signals that result in M1 (classical activation, inflammatory) and M2 (alternative activation/deactivated, immunomodulatory) cells (Gratchev *et al.* 2001; Martinez *et al.* 2008; Sica and Mantovani 2012; Mantovani *et al.* 2013; Rodriguez *et al.* 2014). These different MΦ phenotypes perform distinct and crucial functions in innate and adaptive immunity in local tissues (Benoit, Desnues and Mege 2008; Labonte, Tosello-Trampont and Hahn 2014). It is now clear that the innate and adaptive immune response outcomes of antigen recognition depends upon these functions of subpopulations of MΦ (Ivashkiv 2013; Locati, Mantovani and Sica 2013; Mantovani *et al.* 2013; Labonte, Tosello-Trampont and Hahn 2014; Zhou *et al.* 2014). These variations are regulated by the types of microorganisms providing the stimulus and the local host factor microenvironment (Banchereau *et al.* 2000). The resulting signaling pathways activated through these receptors and processes lead to different immune cell response patterns. Through 'classical activation', the MΦ expresses an inflammatory function that leads to cytotoxicity, tissue injury and fibrosis (Locati, Mantovani and Sica 2013). This differentiation is related to host derived IFNγ as either an autocrine or paracrine factor and LPS (Labonte, Tosello-Trampont and Hahn 2014). The 'alternative activation' process is driven by host factors, IL-4 and IL-13, that can also be autocrine or paracrine derived and is an immunomodulatory cell type that controls the response, aids in tissue repair and cellular regeneration (Mantovani *et al.* 2013). Finally, the 'deactivated' macrophage phenotype is triggered by IL-10 and is highly phagocytic contributing to tissue remodeling, and parasite encapsulation (Zhou *et al.* 2014).

Periodontitis is a chronic immunoinflammatory lesion of mucosal surfaces triggered by a polymicrobial challenge derived from subgingival biofilms in local host tissues, that undermines soft tissue integrity and progresses to resorption of alveolar bone (Tatakis and Kumar 2005). The lesion is a result of a complex host response comprising inflammatory cells, cytokines, chemokines and mediators produced by resident gingival cells and inflammatory cells that infiltrate into the infected tissues (Kornman, Page and Tonetti 1997; Kantarci and Van Dyke 2005; Salvi and Lang 2005; Tatakis and Kumar 2005; Trombelli *et al.* 2006). A range of APCs, including MΦ, have been identified in the periodontium, with data providing phenotypic descriptions of these cells, detecting changes in these cell populations with progressing periodontal disease, and demonstrating *in vitro* that these APCs can function in antigen-presentation critical in controlling the adaptive antibody response patterns in periodontal disease to individual bacteria (Cutler *et al.* 1999; Cohen, Morisset and Emilie 2004; Tanaka *et al.* 2006; Cutler and Teng 2007; Zelkha, Freilich and Amar 2010; Nanbara *et al.* 2012; Papadopoulos *et al.* 2013). However, the regulatory role of these cell types is of particular importance at mucosal surfaces as they are in constant association with external antigenic stimuli. While much emphasis has been expended examining host responses to members of the oral ecology purported to contribute to the pathogenic potential of the biofilm, little information is available examining the characteristics of host responses to commensal bacteria that represent 'early colonizers' and how this horde of bacteria compete, co-exist and/or synergize with opportunistic pathogens to initiate this chronic disease process (Paster *et al.* 2001). Numerous biomarkers of innate immunity are observed in gingival tissues, e.g. LBP, CD14, TLRs, irrespective of the health of the tissues, although changes in TLR2/TLR4 appear in diseased gingiva (Ren *et al.* 2005). Combined these results support the likely role of MΦ in diseased tissues, and suggest that the development and function of these APCs, may actually differ between the forms of periodontal disease (Cutler and Jotwani 2004; Gonzalez *et al.* 2014, 2015). The mechanisms of action at mucosal surfaces include ignoring commensal MAMPs, compartmentalized TLR expression and commensal-driven attenuation of proinflammatory signaling (Kelly *et al.* 2004; Ramos, Rumbo and Sirard 2004). In this regard, intestinal epithelial cells have been shown to be 'tolerant' of commensal MAMPs through additional mechanisms that regulate MAMP binding and PRR signaling (Sirard, Bayardo and Didierlaurent 2006). However, how commensal bacteria and pathogens are distinguished by these cells in the periodontium is not completely understood, and some new concepts are beginning to emerge. Gaps exist in our knowledge of how the host discriminates among these microorganisms, specifically as related to the interaction of MΦ with the polybacterial challenge that can occur in the subgingival sulci of the oral cavity. Although, while both types of macrophages would be expected to be functioning in the mucosal gingival tissues, there is little information on the distribution in health and disease, and more importantly, how pathogens like *Porphyromonas gingivalis* versus commensal bacteria might be critical in molecularly programming the tissue macrophages towards differing phenotypes.

This report focuses on our findings of the capacity of the major periodontal pathogen, *P. gingivalis*, to alter the functional/phenotypic characteristics of macrophages that would help to create a hyperinflammatory environment in the gingival tissues and, as such, affect the characteristics of the microbial ecology (e.g. dysbiosis), as well as undermine normal host response maturation that would enhance resolution of the chronic inflammatory tissue damage.

METHODS

Cell line and culturing conditions

The THP-1 cell line was used to generate M1 macrophages. For M1 polarization, THP-1 cells were cultured in medium (RPMI 1640 + 10% FBS; Gibco). The cells were expanded prior to an experiment for 24 hr, harvested by centrifugation and resuspended to the appropriate concentration to be evaluated in 12-well culture plates. One ml of media containing 1×10^6 cells was added to wells coated with PEI (polyethyleneimine) and allowed to incubate overnight to allow attachment and phenotype

change reflecting a macrophage-like cell (Finlin *et al.* 2013). For polarization to an M1 phenotype the cells are treated for 16 hr with 50 ng/ml of *Escherichia coli* LPS (Sigma) and 1000 U/ml of recombinant IFNγ (R&D Systems, Minneapolis, Minn.). The treated macrophages were harvested in 3 ml of PBS, lysed using 100 μl lysis buffer, and the lysates stored frozen at −80°C until analyzed.

Microorganisms and cell culture

The bacterial strains used in this study were *Porphyromonas gingivalis* ATCC 33277, *Aggregatibacter actinomycetemcomitans* JP2, *Fusobacterium nucleatum* ATCC 25586, *Prevotella intermedia* ATCC 25611, *Streptococcus mutans* ATCC 33535, *S. gordonii* ATCC 10558 and *S. sanguis* ATCC 10556. All bacteria were grown, harvested, washed sonicates prepared and protein levels determined by BCA assay (Pierce, Rockford, IL, USA) as we have described previously (Huang *et al.* 2011b). Macrophages (10^5 cells/well) were stimulated in duplicate with sonicates or live bacteria (*P. gingvalis, S. gordonii*) at various MOI for 16 hrs.

Analysis of mRNA

Total RNA was isolated from THP-1, untreated and treated M1 macrophages using pure Link RNA Mini Kit (Ambion), and reverse transcription reaction was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche). Conditions were optimized using 2 μg of total RNA and 2.5 μM oligo(dT). Real-time PCR primers and probes were designed using Universal Probe Library for humans (Roche). A primer and probe for GAPDH was designed to use it as an internal control. The real-time PCR experiment was performed in 96-well plates using Light Cycler 480 (Roche) at conditions which were all uniformed for each probe and primers MonoColor Hydrolysis probe-UPL probe 96. QPCR was performed with pre-incubation for 30 s at 95°C, amplification: denaturation 95° 10 sec, annealing 60° for 30 sec and elongation 72°-1 s for 45 cycles. A LightCycler 480 (Roche) software was used to perform the analysis. Each reaction was performed in triplicate and the average number of cycles required to detect DNA (Cp) was plotted and used to calculate the quantitative value of real-time RT-PCR (Gonzalez *et al.* 2013, 2016). Table 1 provides a summary of the primers and conditions for each of the mRNA expression assessments.

Analysis of chemokines/cytokines

Culture supernatants were evaluated for IL-8, TNFα, IL-6, IL-12 heterodimer (p70) or IL-10 by standard sandwich ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions as we have described previously (Huang *et al.* 2011b). All samples were tested in triplicate.

Statistical analysis

Statistical analyses were performed using a Mann–Whitney U or Kruskal–Wallis analysis of variance on ranks with a post hoc Dunn's test for multiple testing (SigmaStat 3.5, Point Richmond, CA, USA). An alpha value of $p < 0.05$ was accepted as statistically significant when comparing the mediator levels under test conditions to media derived from untreated cells.

Table 1. Primers for qPCR for gene expression analysis.

Target gene	Primer	Amplicon Size (bp)
CCL5	Forward – TGCCCACATCAAGGAGTATTT	72
	Reverse – TTTCGGGTGACAAAGACGA	
CXCL10	Forward – GAAAGCAGTTAGCAAGGAAAGGT	132
	Reverse – GACATATACTCCATGTAGGGAAGTGA	
CD86	Forward – CAGAAGCAGCCAAAATGGAT	97
	Reverse – TCAGGTTGACTGAAGTTAGCAGA	
IL1ß	Forward – TACCTGTCCTGCGTGTTGAA	76
	Reverse – TCTTTGGGTAATTTTTGGGATCT	
IL6	Forward – GATGAGTACAAAAGTCCTGATCCA	130
	Reverse – CTGCAGCCACTGGTTCTGT	
IL8	Forward – GAGCACTCCATAAGGCACAAA	90
	Reverse – ATGGTTCCTTCCGGTGGT	
IL12	Forward – CACTCCCAAAACCTGCTGAG	91
	Reverse – CAATCTCTTCAGAAGTGCAAG	
IL23	Forward – AGCTTCATGCCTCCCTACTG	71
	Reverse – CTGCTGAGTCTCCCAGTGGT	
TNFα	Forward – CAGCCTCTTCTCCTTCCTGAT	123
	Reverse – GCCAGAGGGCTGATTAGAGA	
GAPDH	Forward – GGTGTGAACCATGAGAAGTATGA	112
	Reverse – GAGTCCTTCCACGATACCAAAG	

RESULTS

We examined the outcomes of stimulation of the THP-1 myelomonocytic human cell line (M0 type of cell), with various oral bacteria. Figure 1 summarizes the variations in responses related to the types of bacteria [e.g. Gram(+) vs. Gram(−); pathogen vs. commensal]. The data were derived from experiments that challenged THP-1 cells with 50 μg/ml of sonicates of each bacteria. Supernatants were harvested after 24 hr and assayed. While these analytes provide only a rough snapshot of the macrophage responses, the data are consistent with the oral streptococci stimulating primarily an M2 response, and both *A. actinomycetemcomitans* and *P. gingivalis* eliciting responses consistent with M1 macrophages. Interestingly, both *P. intermedia* and *F. nucleatum* sonicates stimulated a mixed response with markers for both M1 and M2-type cells.

We then evaluated in greater detail macrophage responses following challenge with *P. gingivalis* and *S. gordonii*, as a prototype commensal microorganism (Fig. 2). The data focused on the phenotype of the M1 type cells following challenge with live bacteria of these two species. The results demonstrated high levels of IL-6, TNFα and IL-1ß following *P. gingivalis* stimulation, all markers of TLR engagement and NFκB activation of proinflammatory responses. Of interest was the decrease in IL-6 mRNA levels with the highest dose of *P. gingivalis*. Since this pathogen has the capacity to interact with various cell surface receptors, beyond TLRs, for example Protease Activated Receptors (PARs), and macrophages tend to display enhanced expression of PAR1, PAR2 and PAR3, this outcome could be a composite of the complex *P. gingivalis* bacterium engaging multiple receptors leading to complementary or competing signaling events in the cells (Chung *et al.* 2004; Holzhausen, Spolidorio and Vergnolle 2005; Holzhausen *et al.* 2006; Hajishengallis and Sahingur 2014). *Streptococcus gordonii* challenge did stimulate a response of these cytokines, but at much lower levels. In contrast, IL-12 and IL-23 products of the M1 phenotype, that are response markers of interferon-gamma receptor (IFNGR) engagement and activation through interferon regulatory factor (IRF)5 were not increased, and appeared even below the normal M1 cell levels following

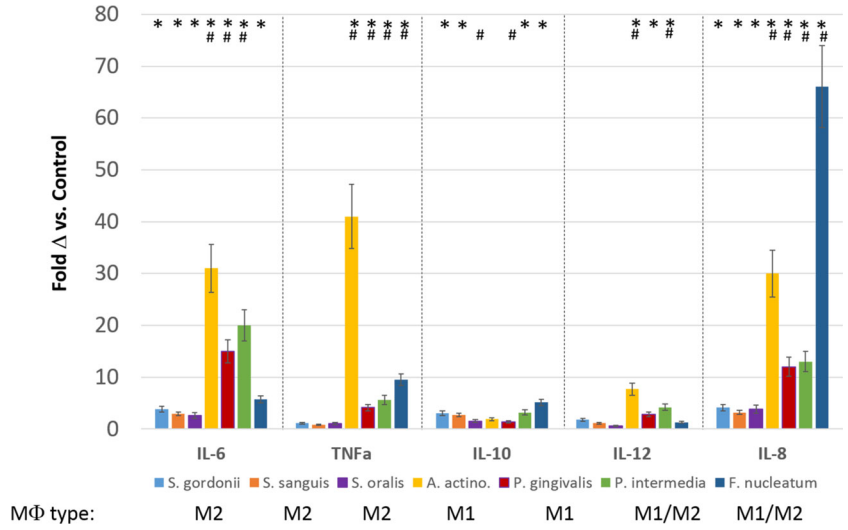

Figure 1. Cytokine/chemokine profiles from THP-1 macrophages induced by challenge with oral bacterial sonicates. Values denote mean fold difference (Δ) from control unstimulated THP-1 cells and vertical brackets enclose 1 SD from triplicate determinations. * denotes significantly different from control levels at least at $p < 0.05$. # denotes significantly different from levels with *S. gordonii* challenge at least at $p < 0.05$. Patterns of cytokines that were used to identify the MΦ type identified for each bacterial species in the figure, indicated that the Gram-positive commensal oral bacteria primarily elicit an M2 macrophage phenotype, while the oral pathogens (*A. actinomycetemcomitans*, *P. gingivalis*) induced an M1 type of macrophage. The oral bacteria (*P. intermedia*, *F. nucleatum*) associated with gingival inflammation and development of a pathogenic biofilm elicited a patterns of cytokines/chemokines reflective of both M1 and M2 phenotypes.

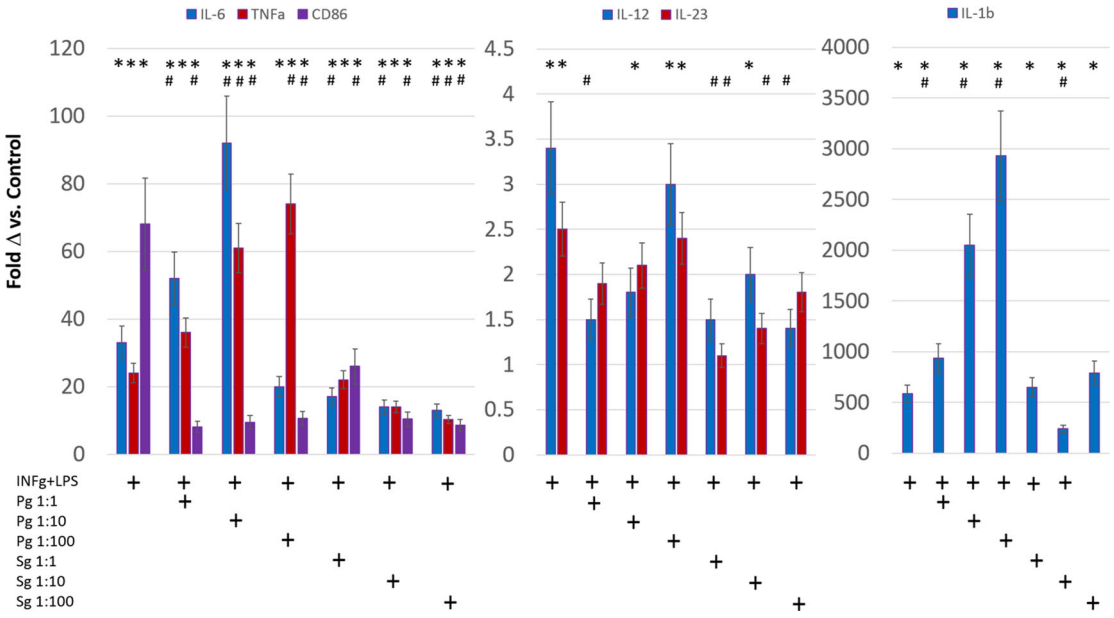

Figure 2. Message levels of cytokines/chemokines/receptors following challenge of M1 macrophages (i.e. IFNγ+LPS) with different oral bacteria at different MOI (MΦ:bacteria). Values denote mean fold difference (Δ) from control unstimulated THP-1 cells and vertical brackets enclose 1 SD from triplicate determinations. * denotes significantly different from control levels at least at $p < 0.05$. # denotes significantly different from levels with IFNγ+LPS along at least at $p < 0.05$.

P. gingivalis challenge. CD86 a more pan-marker of macrophage activation was decreased somewhat by both types of bacteria, with rather consistent observations that higher concentrations of the commensal bacteria appeared to decrease this expression to a greater degree. It is clear that these types of co-stimulatory molecules can be elicited during innate immune responses to activate adaptive immune responses (Kobayashi and Flavell 2004). However, the literature also supports that various bacteria including pathogens and commensals can regulate the expression of CD86 (Shklovskaya *et al.* 2011; Xin *et al.* 2014; Christoffersen *et al.* 2015; Gaikwad and Agrawal-Rajput 2015; Li *et al.* 2015a) with numerous reports of commensal bacteria down-regulating these surface receptors to modify the characteristics of T-cell activation and create a more tolerigenic environment for the commensal bacteria to permanently colonize (Kobayashi and Flavell 2004; Shklovskaya *et al.* 2011; Xin *et al.* 2014; Li *et al.* 2015a) similar to the results with these oral bacteria. Our data are consistent with these previous concepts about regulation by commensals, and as *P. gingivalis* also appeared to decrease the CD86 levels, it may suggest another mechanisms for virulence and dysbiotic regulation by this primary oral pathogen.

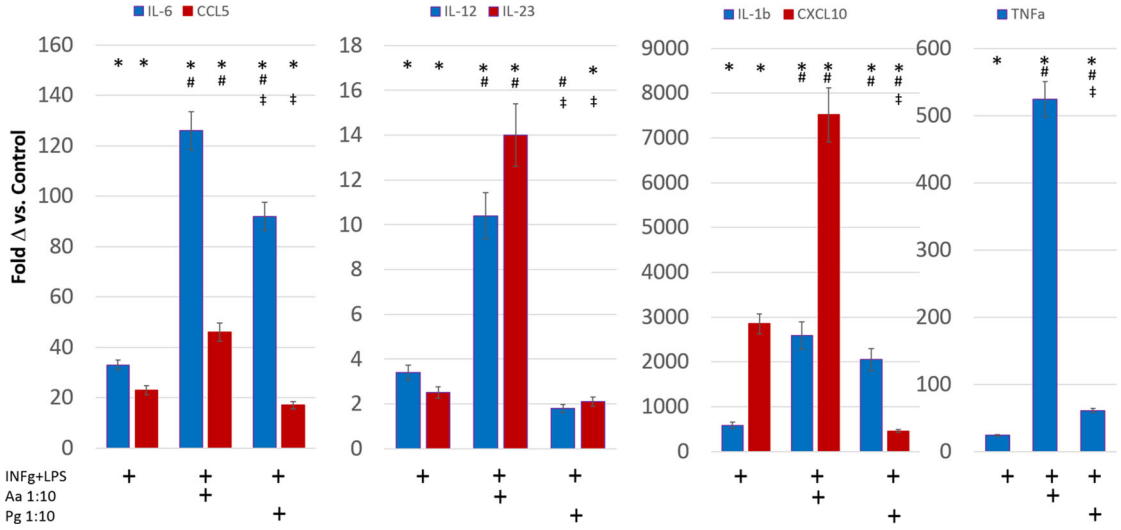

Figure 3. Message levels of cytokines/chemokines from M1 macrophages (i.e. INFγ+LPS) challenged with oral pathogens at MOI (MΦ:bacteria). Values denote mean fold difference (Δ) from control unstimulated THP-1 cells and vertical brackets enclose 1 SD from triplicate determinations. * denotes significantly different from control levels at least at $p < 0.05$. # denotes significantly different from levels with IFNγ+LPS alone at least at $p < 0.05$. ‡ denotes significantly different from *A. actinomycetemcomitans* levels at least at $p < 0.05$.

We extended these studies to more specifically address pathogenic features of the *P. gingivalis* interaction with the macrophages. As shown in Fig. 3, the oral pathogen *A. actinomycetemcomitans* elicited a pattern of substantially elevated gene expression representing both the pro-inflammatory (e.g. IL-1β, IL-23 etc.) and T-cell engagement activities [e.g. CCL5 (regulated on activation, normal T cell expressed and secreted; RANTES), CXCL10 (interferon-gamma induced protein 10; IP-10)] of M1 macrophages. This contrasted with the *P. gingivalis* challenge that appeared to elicit a 'hyperinflammatory' type of response with decreased levels of chemokines related to T-cell activation and immunoregulation.

DISCUSSION

A summary of the literature demonstrates that MΦ are present in the periodontium, respond to the environment at diseased sites, and likely contribute crucial functions to maintaining or re-establishing homeostasis of these oral tissues. The differential induction of host cell responses by commensals and pathogens, and the ability of the host to differentiate and respond to trigger adaptive immunity remains unclear (Lu, Kurago and Brogden 2006). The characteristics of gingival MΦ interactions with the complex microbial biofilms in the oral cavity, triggering innate and adaptive immune response specificity to members of the biofilms must be presumed to play a role in the maintenance of homeostasis, formation of chronic destructive inflammatory lesions, and the adaptive immune response contribution to correcting the dysregulated inflammatory response of disease.

The results of this study demonstrate that commensal bacteria primarily elicit an M2 phenotype macrophage. This might be predicted based upon the existing literature describing the type of immune cell activities that occur within the periodontium (Lappin *et al.* 1999; Hillmann *et al.* 2001). However, of interest are reports suggesting a limited production of IL-4, albeit these reports focused on changes with periodontitis (Yamazaki *et al.* 1997; Duarte *et al.* 2012). Additionally, elevated levels of IL-13 in gingival tissues with periodontal lesions could contribute to the molecular environment to drive M2 formation (Roberts, McCaffery and Michalek 1997; Johnson and Serio 2007). Additionally, challenge of macrophages that have been driven towards an M1 phenotype with INFγ and LPS, with commensal bacteria appears to provide some modulation of the inflammatory nature of the M1 cells. There have been rather limited reports on the modulation of M1 macrophage maturation/functions related to specific bacterial infections. Recently, Christoffersen *et al.* (2014) demonstrated that Gram-negative gastrointestinal pathogens tended to elicit M1 macrophages compared with Gram-positive probiotic bacteria identified by targeted gene expression (e.g. iNOS, ARG), as well as cytokine secretion. *Escherichia faecalis* was found to polarize colon macrophages to the M1 phenotype and contribute to a 'bystander effect' mediating DNA damage to neighboring cells (Yang *et al.* 2013). Using a THP-1 monocyte cell line-derived model, Habil *et al.* (2011) examined the immunomodulatory effects of probiotic bacteria and their secreted proteins on a macrophage subset-specific inflammatory marker profile. The cells were stimulated by enteropathic lipopolysaccharides in the presence or absence of the probiotic bacteria. The probiotics differentially regulated M1 and M2 production of TNFα, whereas M2 IL-6 production was suppressed.

An interesting observation was the identification of the apparent capacity of both *F. nucleatum* and *P. intermedia* to induce a profile of functions in the macrophages that exhibited characteristics of both M1 and M2 types. These bacteria are often identified as representative of the Socransky and colleagues 'orange complex' (Socransky *et al.* 1998). As such, they are considered to represent the change in microbial ecology that occurs with biofilm accumulation leading to inflammation, clinically described as gingivitis. Additionally, they have been identified by Kolenbrander and co-workers (Kolenbrander, Palmer and Rickard 2006) to display an array of surface biomolecules that enable cognate interactions with putative pathogens, enabling co-aggregation, accretion and emergence of the pathogens at sites that progress to periodontitis. We and others have found that *F. nucleatum*, while thought of as a less pathogenic member of the subgingival ecology, has the *in vitro* capability to elicit very high levels of an array of cytokines and chemokines from multiple cell types (Gonzalez, Ebersole and Huang 2011; Huang *et al.*

2011a,b; Peyyala *et al*. 2012). Thus, it may actively contribute to changing the local nutritional milieu through this induced inflammation that allows the opportunistic pathogens to emerge and create a climax microbial community that is periodontopathic (Uzel *et al*. 2011; Teles *et al*. 2012). Also of interest is periodontal microbiological data that indicated a somewhat inverse relationship between levels of *P. intermedia* and *P. gingivalis* in subgingival plaque samples from health through periodontitis (Zambon 1996; Haffajee *et al*. 2004; Sakamoto, Umeda and Benno 2005). This was interpreted as *P. intermedia* providing a resource for enhancing *P. gingivalis* binding and growth in the transition from gingivitis to periodontitis; however, *P. gingivalis* could outcompete *P. intermedia* for a similar ecological niche and thus emerge in the disease ecology. Consequently, the ability of both of these transition or 'bridging' microorganisms to activate the macrophage population more broadly, would in theory provide some ability of the host to mount an antimicrobial effort through M1 cells, contributing to local inflammation although inducing chemokines that would help engage the adaptive immune response cells. This combined with the induction of M2-like activities could then enable the local tissue environment to enhance the armamentarium of host immune responses to more effectively manage the noxious bacterial challenge. A caveat to these interpretations is that the commensal bacteria tested were Gram-positive, with the pathogens being Gram-negative reflecting the predominate distribution of these morphotypes of bacteria in periodontal health and disease. We have also developed some additional preliminary information suggesting that selected Gram-negative commensal bacteria also induce a more M2-like response profile (unpublished data). Thus, this polarization may reflect more general characteristics of the oral bacteria, not simply the cell wall/membrane structure of the microorganisms.

Based upon the existing literature we had predicted that *P. gingivalis*, as an oral pathogen contributing to the chronic inflammatory lesions of periodontitis, would primarily induce an M1 polarized macrophage population. This type of macrophage is primarily associated with inflammatory responses to bacterial infections, and is a primary cell type for combatting these infections (Zhou *et al*. 2014). However, a collateral aspect of the induction of M1 cells, particularly through engagement of TLRs, is signaling through the NF-κB pathway and production of an array of proinflammatory mediators (Martinez *et al*. 2008). While it is clear that inflammation is required as a presage to the development of adaptive immunity, chronic elevated levels of these biomolecules in the local tissues is associated with undermining epithelium integrity, enzymatic degradation of connective tissue matrix and loss of fibroblast function/viability, and activation of osteoclastogenesis leading to alveolar bone resorption (Souza and Lerner 2013; Hajishengallis 2014). Our data demonstrate that *P. gingivalis* clearly has the ability to trigger this pathway in macrophages and synergizes with host factors, i.e. IFNγ and extrinsic LPS to induce significant elevations in M1-produced inflammatory mediators. The finding that was rather unexpected was the apparent capacity of *P. gingivalis* to down-regulate/block M1 production of an array of cytokines and chemokines (e.g. IL-12, CCL5, CXCL10) that would help engage immunoregulatory cells and adaptive immunity (Gemmell, Carter and Seymour 2001). Recent results from Foey and Crean (Foey and Crean 2013) examined the impact on MΦ subsets of challenge with heat-killed *P. gingivalis* or LPS prior to stimulation by bacterial PRRs (e.g. lipoteichoic acid, peptidoglycan). Both *P. gingivalis* pretreatments suppressed PRR-induced TNFα, IL-6 and IL-10, but not IL-1β expression in both M1 and M2 MΦs. In addition, suppressed NFκB activation in M2 MΦs, but not in pro-inflammatory M1 MΦs, was noted. Thus, the authors suggested that this oral pathogen appeared to selectively tolerize MΦ subsets that could facilitate immunopathology and marginalize immunity. Recent results have supported the importance of macrophages in alveolar bone resorption elicited by *P. gingivalis* infection of mice, which also emphasized the profile of periodontal infiltrating macrophages to be dominantly an M1 type cell (Lam *et al*. 2014). This same group also demonstrated that *P. gingivalis* LPS only weakly activated macrophage polarization, while still inducing pro-inflammatory mediators via TLR2 engagement (Holden *et al*. 2014). These findings are generally consistent with our observations regarding the capacity of *P. gingivalis* to induce a specific response profile in macrophages with some predilection for polarizing towards an M1 phenotype.

In order to assess if our findings were a specific characteristic of *P. gingivalis* or a more general characteristic of oral pathogenic bacteria, we performed similar assessments examining *A. actinomycetemcomitans*, as the likely etiologic agent in many cases of aggressive periodontitis (Kononen and Muller 2014). The results showed that *A. actinomycetemcomitans* appeared to synergize with the M1 cell maturation and polarization process to induce a macrophage that has inflammation regulatory mechanisms intact, as well as the capacity to actively interface with the adaptive immune responses that would be predicted to help reestablish tissue homeostasis.

These results suggested that the *P. gingivalis* challenge was stimulating M1 cells towards a somewhat different functional phenotype that could have ramifications on the local environment in diseased gingival tissues, while commensal bacteria tend to polarize these cells towards an M2 phenotype. Multiple mechanisms could be contributing to inducing this plasticity in the periodontium, with potential targets including that *P. gingivalis* has the capacity to regulate the level and function of STAT1 (Signal Transducer and Activator of Transcription-1) (Matsukawa 2007) as a crucial molecule for generation of an array of host responses to external stimuli during inflammation that includes numerous autocrine/paracrine factors. This critical cellular outcome could occur via *P. gingivalis* effects on multiple molecular controls. ShP-1/2 can negatively regulate the Jak/STAT pathway in the nucleus, as well as by interacting with cytosolic STAT1 and preventing the recruitment of STAT1 to IFNγR, thus specifically inhibiting STAT1 signaling (Christophi *et al*. 2009; Wu *et al*. 2012). PIAS proteins sumoylate various transcription factors to modulate their function with PIAS1 as a transcriptional corepressor of STAT1 (Liu *et al*. 2004, 2013). Suppressors of cytokine signaling (SOCS) negatively regulate cytokine signaling, and form part of a negative feedback loop for inflammation via STATs and NFκB (Baetz, Zimmermann and Dalpke 2007; Delgado-Ortega *et al*. 2013; Carow and Rottenberg 2014). Several phosphatases have also been implicated in negative regulation of cytokine signaling via STAT1 (PTP1B; TCPTP). These enzymes can dephosphorylate JAK and TYK kinases that are crucial for STAT phosphorylation and cellular responses to IFN (Heinonen *et al*. 2009; Ma *et al*. 2011). Finally, an additional mechanism for these varied cellular responses to *P. gingivais* is through epigenetic actions of histone deacetylases (HDACs) on targeted gene transcription related to inflammatory phenotypes and various diseases, ie. septic shock, rheumatoid arthritis, asthma and colitis (Hawtree, Muthana and Wilson 2013; Royce and Karagiannis 2014; Cantley *et al*. 2015; Li *et al*. 2015a,b; Wendling *et al*. 2015). Histone modifications have also been shown to govern multiple aspects of inflammation and immunity, including impacts on the functions and polarization of macrophages (Halili *et al*. 2010; Sweet *et al*. 2012; Turgeon *et al*.

2013), and *P. gingivalis* has been shown to alter HDAC levels (Cantley *et al.* 2011; Imai, Ochiai and Okamoto 2009; Imai and Ochiai 2011).

These findings support the importance of understanding the molecular events that are triggered by various oral bacteria resulting in a polarization of macrophages in the gingival tissues that would proscribe an environment exacerbating destructive inflammation or one oriented towards a 'wound healing' and resolution of the chronic inflammatory response. Detailed mechanisms of this polarization modulation within the context of a polymicrobial challenge need to be evaluated to better understand how the host reacts in the complex microbial milieu.

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