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Immunologic Environment Influences Macrophage Response to *Porphyromonas gingivalis*

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SUMMARY

Macrophages adapt both phenotypically and functionally to the cytokine balance in host tissue microenvironments. Recent studies established that macrophages contribute an important yet poorly understood role in the development of infection-elicited oral bone loss. We hypothesized that macrophage adaptation to inflammatory signals encountered prior to pathogen interaction would significantly influence the subsequent immune response of these cells to the keystone oral pathobiont *Porphyromonas gingivalis*. Employing classically activated (M1) and alternatively activated (M2) murine bone marrow-derived macrophage (BMDMØ), we observed that immunologic activation of macrophages prior to *P. gingivalis* challenge dictated phenotype-specific changes in the expression of inflammation-associated molecules important to sensing and tuning host response to bacterial infection including Toll-like receptors (TLR) 2 and 4, CD14, CD18 and CD11b (together comprising CR3), MHCII, CD80, and CD86. M2 cells responded to *P. gingivalis* with higher expression of TNF- α , IL-6, MCP-1, MIP-1 α , RANTES, and KC than M1 cells. M1 BMDMØ expressed higher levels of IL-10 to *P. gingivalis* than M2 BMDMØ. Functionally, we observed that M2 BMDMØ bound *P. gingivalis* more robustly than M1 BMDMØ. These data describe an important contribution of macrophage skewing in the subsequent development of the cellular immune response to *P. gingivalis*.

Keywords

Periodontal disease; cytokines; chemokines; cell receptors; bacterial attachment; polarization

INTRODUCTION

Periodontal diseases are chronic inflammatory diseases of the periodontium that in severe cases lead to destruction of the hard and soft tissues supporting the teeth, and bacteria-elicited inflammation serves as the nidus of this destructive host response. *Porphyromonas*

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gingivalis is an organism associated with chronic periodontitis in part through its ability to stimulate microbial dysbiosis in subgingival plaque, and locally modulate expression of host immune factors (Darveau *et al.* 2012; Hajishengallis *et al.* 2016; Hajishengallis *et al.* 2015). Individuals with periodontal disease present with a complex array of expressed inflammatory and anti-inflammatory mediators (Gemmell *et al.* 2001a; Seymour & Gemmell 2001), as well as a complex cellular infiltration with early neutrophilic responses followed by lymphocytes, dendritic cells, and monocytes/macrophages (Charon *et al.* 1981; Kinane 2000; Pihlstrom *et al.* 2005; Schenkein 2006). This array of tissue specific cues serves as the driver of cellular recruitment, and promotes the shifts in cell populations present in PD tissues. Lymphocytes contribute to *P. gingivalis*-elicited oral bone loss (Baker *et al.* 1994; Wang *et al.* 2014); however, recent studies point to a critical role for macrophages in the host response to, and pathogen-elicited oral bone loss by *P. gingivalis* (Lam *et al.* 2014; Papadopoulos *et al.* 2013).

Macrophages develop from a common myeloid progenitor cell that also gives rise to dendritic cells, polymorphonuclear leukocytes, and mast cells (Epelman *et al.* 2014; Gordon & Taylor 2005). In addition, tissue resident macrophage arise from local progenitor cell populations seeded at sites early in ontogeny (Dey *et al.* 2014). Macrophages perform a myriad of functions in the host including phagocytic uptake of foreign material, innate immune sensing and antigen presentation to T cells, secretion of inflammatory and anti-inflammatory mediators, as well as assist with tissue homeostasis through clearance of necrotic and apoptotic cells (Geppert & Lipsky 1989; Gordon 2003; Sica & Mantovani 2012).

Upon tissue localization, macrophages respond to inflammatory, anti-inflammatory, and regulatory cues in the cellular microenvironment that further drive phenotypic change in these cells, a process of immunologic skewing termed polarization (Biswas & Mantovani 2010). In mice, two principal macrophage activation phenotypes have been characterized and are designated classically activated (M1), and alternatively activated (M2) cells (Mantovani *et al.* 2005). These activation states represent the antitheses of a polarization continuum (Mosser & Edwards 2008). *In vitro* M1 cells develop in response to interferon (IFN) γ and LPS, or tumor necrosis factor (TNF)- α treatment (Cassetta *et al.* 2011). M1 macrophages produce copious nitric oxide (Mulder *et al.* 2014), and are associated with protection during infection (Mosser 2003). M2 macrophages were originally described based on the atypical activation observed in response to interleukin (IL)-4 (Stein *et al.* 1992); however, M2 cells also arise from stimuli including IL-10, and immune complexes (Martinez & Gordon 2014). Broadly M2 macrophages are associated with tissue homeostasis, resolution of inflammation, tissue repair, and chronic infections (Benoit *et al.* 2008; Sica & Mantovani 2012; Snyder *et al.* 2016).

Differing levels of key mediators of macrophage skewing including IFN- γ and IL-4 are reported between healthy individuals and those with periodontitis (Navarrete *et al.* 2014). Thus, although macrophages comprise 5–30% of cells identified in the cellular infiltrate of human periodontal disease lesions (Okada & Murakami 1998), little is known regarding how macrophage skewing contributes to periodontal disease, and more specifically in the downstream development of a macrophage immune response to periodontal disease associated

bacteria. By modeling the effect of different inflammatory cues that drive macrophage activation prior to pathogen encounter, we report that skewed BMDMØ interact with, and respond to, live *P. gingivalis* challenge with unique signatures that may have important implications in the pathogenesis of periodontal disease.

METHODS

Mice, macrophage collection, and immunologic skewing

Male 6–10 week old C57BL-6 mice (Jackson Laboratories, Bar Harbor, ME) were used as a source of BMDMØ, and all animals were cared for and used in accordance with Boston University Institutional Animal Care and Use Committee approvals. Following sacrifice, BMDMØ were generated from bone marrow cells as previously described (Shaik-Dasthagirisahab *et al.* 2010). After 7 days, BMDMØ were collected (~95% pure by F4/80+ staining), placed into 12-well tissue culture plates and M1 BMDMØ were generated by 3-day treatment with recombinant murine IFN γ (100 U ml⁻¹; R&D Systems Minneapolis, MN) and ultrapure *E. coli* 0111:B4 LPS (10 ng ml⁻¹; Invivogen, San Diego, CA), while M2 BMDMØ were generated by 3-day treatment with recombinant murine IL-4 (20 U ml⁻¹; R&D Systems). Reference BMDMØ (M0) were maintained in differentiation medium. Following skewing, cells were washed and cultured in antibiotic-free complete cell culture medium. BMDMØ skewing was confirmed by flow cytometry targeting MHCII and CD86 surface protein expression, RT-qPCR measuring *nos2* (M1 marker) and *arg1* (M2 marker) gene expression, Griess assay measuring nitrite production (Shaik-Dasthagirisahab *et al.* 2010), and ELISA measuring TNF- α and IL-6.

RNA harvest and reverse transcription quantitative-PCR (RT-qPCR)

Levels of *nos2* and *arg1* gene expression were determined by RT-qPCR using Taqman murine primer sets (Life Technologies, Carlsbad, CA). RNA isolated from cells was converted to cDNA using High-Capacity RNA-to-cDNA kit (Life Technologies), which served as template in qPCR assays. Target gene expression was calculated using CT method, and β -actin served as the housekeeping gene.

Bacterial culture, FITC-labeling, and infection assays

P. gingivalis strain 381 was cultured anaerobically on blood agar plates, and after 3–5 days, plate growth was used to seed Brain-Heart Infusion broth supplemented with yeast extract, hemin, and menadione (Gibson & Genco 2001). After 24 h, bacteria were harvested by centrifugation, washed, adjusted to OD₆₆₀ of 1 (1 $\times$ 10⁹ colony forming units ml⁻¹), and either placed in antibiotic-free complete RPMI-1640, or labeled with fluorescein isothiocyanate (FITC; 0.15 mg ml⁻¹; Molecular Probes) under anaerobic conditions for 30 min (Pathirana *et al.* 2007). Gram staining confirmed purity of all bacterial cultures. Unlabeled, or FITC-labeled *P. gingivalis* were added to M1, M2, and M0 BMDMØ at multiplicity of infection (MOI) 100 in antibiotic-free RPMI-1640 medium for up to 24 h. For assays to define BMDMØ immune response to *P. gingivalis*, bacteria were added to M1, M2, and M0 BMDMØ for 24 h, at which time BMDMØ viability was determined by Trypan blue staining, in addition, culture supernatant fluids were collected and stored frozen at -80°C until multiplex-based immunoassays were performed. The remaining BMDMØ

were washed, and surface molecule expression was assessed by flow cytometry. For binding and internalization assays, unbound bacteria were removed by washing, BMDMØ were harvested, divided into two aliquots for flow cytometric measurement of mean fluorescent intensity (MFI) of total bound bacteria, and to define internalized bacteria following Trypan blue quenching of extracellular FITC (Liang *et al.* 2009).

Griess assay

In brief, the presence of total nitrite was measured in collected cell culture supernatant fluids by incubating individual 50 µl samples with 50 µl Griess reagent A and 50 µl Griess reagent B for 10 minutes in wells of 96-well plates, and absorbance 540 nm for each sample was recorded. A standard curve using sodium nitrite was run in parallel with experimental samples, and was used to convert absorbance values to µM units.

Multiplex immunoassays

An 8-plex immunoassay kit (Invitrogen, Carlsbad, CA) was used to measure murine TNF-α, IL-1β, IL-6, IL-10, KC (CXCL1), monocyte chemotactic protein-1 (MCP-1; CCL2), macrophage inflammatory protein 1 alpha (MIP-1α; CCL3), and regulated on activation normal T cell expressed and secreted (RANTES; CCL5). Briefly, 50 µl samples were applied to the beads and the samples manipulated following manufacturer's instructions. Levels of each cytokine and chemokine were measured on a Luminex 100, and expressed as pg ml⁻¹ determined from standards run in parallel with the test samples.

Flow cytometry

BMDMØ were washed, treated with Fc block (eBiosciences, San Diego, CA), then were incubated with fluorescently labeled antibodies against mouse F4/80 (eBiosciences), MHCII (eBiosciences), TLR2 (eBiosciences), TLR4 (eBiosciences), CD11b (MAC-1; BD Pharmingen, San Jose, CA), CD14 (eBiosciences), CD18 (BD Pharmingen), CD80 (Santa Cruz Biotechnology, Santa Cruz, CA), and CD86 (BD Pharmingen), or isotype control antibodies, per manufacturer's instructions. The cells were then fixed with 2% buffered paraformaldehyde, 10,000 gated events were collected for each sample and MFI, and percentage of positive staining cells were determined.

Statistical analyses

All experiments were performed 2–3 times and data from individual experiments were combined, imported in Prism statistical analysis software (Graphpad Software, La Jolla, CA), and the mean ± standard error of the mean for each group was determined. One-way ANOVA and Tukey post-tests, or Two-way ANOVA with Bonferroni post-test were performed as indicated. A $P < 0.05$ was considered significant.

RESULTS

Establishment of activated BMDMØ subsets

Prior to defining the skewed BMDMØ response to *P. gingivalis*, we confirmed that our cytokine treatments generated phenotypically distinct macrophage populations. Employing

flow cytometry to measure surface expression of MHCII and CD86 we observed as anticipated that M1 BMDMØ expressed the highest levels of these markers as compared with either M0 or M2 skewed cells (Fig 1A–H). RT-qPCR revealed that groups of BMDMØ treated for 72h with IFN γ +LPS or treated with IL-4 presented with high *nos2* (M1 marker), or *arg1* (M2 marker) gene expression, respectively. M0 expressed low levels of each gene (Fig. 1I). Nitrite measurements revealed that only the M1 population of BMDMØ expressed this marker (Fig. 1J), while ELISA assays supported that TNF- α (Fig. 1K) and IL-6 (Fig. 1L) were expressed by M1 BMDMØ to a greater extent than either M0 or M2 BMDMØ. Taken together, these data confirmed reproducible generation of M1 and M2 skewed BMDMØ subsets.

M1 and M2 skewed BMDMØ display different surface receptor profiles in response to *P. gingivalis*

It is known that macrophage display an array of surface receptors to *P. gingivalis* (Hajishengallis *et al.* 2006b); however, the influence of immunologic skewing prior to bacterial interaction in the evolution of surface receptor responses is essentially unknown. Prior to measuring expression of surface marker expression, we investigated whether immunologic skewing of BMDMØ influenced cell viability after 24h culture with *P. gingivalis*. We observed that there were no significant changes in viability between subsets of BMDMØ cultured with *P. gingivalis* (data not shown). Employing flow cytometry we observed that all BMDMØ subsets cultured with *P. gingivalis* significantly increased F4/80 expression (Fig. 2A). Complement receptor (CR)3 is a β 2-integrin heterodimer of CD11b and CD18 that plays an important role in host recognition of *P. gingivalis* (Hajishengallis *et al.* 2007). We observed that M0 BMDMØ responded to *P. gingivalis* with increased CD11b expression, while no effect on CD11b was observed with challenged M1 or M2 cells (Fig. 2B). *P. gingivalis* challenge elicited increased CD18 expression on M0 BMDMØ, while M1 and M2 skewed BMDMØ responded with decreased CD18 staining, with M2 possessing greater reduction in CD18 staining than observed with M1 cells (Fig. 2C).

TLRs are pattern recognition receptors used by macrophages to recognize and process foreign material and participate in various aspects of the host response to *P. gingivalis*. TLR2 is linked to development of inflammatory responses, and oral bone loss to *P. gingivalis* (Burns *et al.* 2006; Ukai *et al.* 2008; Yumoto *et al.* 2005). M0 and M2 BMDMØ responded to *P. gingivalis* with significant increases in TLR2 expression, while M1 cells did not (Fig. 2D). A slight increase in TLR4 expression by M0 cells cultured with *P. gingivalis* was observed as compared with unchallenged BMDMØ; however, this did not reach the level of significance ($P > 0.05$; Fig. 2E). M2 BMDMØ responded to *P. gingivalis* with a slight increase in TLR4 expression. Interestingly, M1 BMDMØ responded to *P. gingivalis* challenge by significantly enhancing TLR4 expression (Fig. 2E). CD14 is a co-receptor for TLRs (Hajishengallis *et al.* 2006a). A profile of enhanced CD14 expression in response to *P. gingivalis* challenge was similar between all BMDMØ subsets (Fig. 2F).

Macrophage present antigen to T cells via major histocompatibility complex (MHC)II and aided by co-receptor engagement including CD80 (B7-1), and CD86 (B7-2) (Banchereau & Steinman 1998; Neeffjes *et al.* 2011; Underhill *et al.* 1999). Therefore, we were interested in

understanding if immunologically skewed macrophages responded to *P. gingivalis* challenge by modifying MHCII or co-receptor expression. M2 and M0 BMDMØ responded to *P. gingivalis* challenge with a significant increase in MHCII (Fig. 3A), while MHCII expression did not change in M1 cells (Fig. 3A). In the M2 and M0 BMDMØ populations, CD80 expression changed modestly following *P. gingivalis* challenge, while M1 BMDMØ responded with a strong decrease in CD80 expression (Fig. 3B). *P. gingivalis* exposure did not shift expression of CD86 in M0 BMDMØ, whereas M1 responded to *P. gingivalis* challenge with a significant decrease in CD86 expression, and M2 cells increased CD86 expression following exposure (Fig. 3C).

M1 and M2 skewing affects BMDMØ cytokine and chemokine response to *P. gingivalis*

As chronic inflammation is a hallmark of periodontal disease (Pihlstrom *et al.* 2005; Seymour & Gemmell 2001; Silva *et al.* 2007), we wanted to determine whether immunologically skewed BMDMØ respond to *P. gingivalis* by producing different cytokine and chemokine profiles. Skewed and reference M0 BMDMØ cultured with *P. gingivalis* possessed significantly higher levels of TNF- α and IL-6 than the unchallenged cells with TNF- α expression profiles M2>M1>M0, and IL-6 expression M2>M0>M1 (Fig. 4). Little change in IL-1 β production was observed between unchallenged and *P. gingivalis* challenged BMDMØ (Fig. 4). *P. gingivalis* challenge elicited IL-10 from all BMDMØ with M0>M1>M2 (Fig. 4), a profile inverse of that observed for TNF- α . M2 and M0 responded to *P. gingivalis* with robust KC production M2>M0, while M1 cells had little change in KC production relative to unchallenged BMDMØ (Fig. 4). Profiles of MCP-1, and RANTES were similar to that observed for KC (Fig. 4), although no difference in MCP-1 was observed between M0 and M1 cells cultured with *P. gingivalis*. MIP-1 α production in response to *P. gingivalis* challenge was different between macrophage subsets with M2>M1>M0 (Fig. 4).

M2 macrophages bind *P. gingivalis* more robustly than M1 macrophages

As macrophages play key roles in handling bacterial pathogens (Boisvert *et al.* 2014), and *P. gingivalis* adheres to, and is internalized within macrophages (Liang *et al.* 2009), we investigated whether skewed macrophages differ in their capacity to bind and internalize *P. gingivalis*. Flow cytometry revealed that BMDMØ subsets bound FITC-*P. gingivalis* in a time dependent manner (Fig. 5A). M2 cells bound *P. gingivalis* more robustly than M1 cells, and M0 BMDMØ bound *P. gingivalis* least robustly. To determine whether skewing influenced the capacity of BMDMØ to internalize *P. gingivalis*, FITC-*P. gingivalis* infected BMDMØ subsets were treated with trypan blue to quench extracellular FITC (Liang *et al.* 2009). All cultures treated with trypan blue showed a decrease in detectable fluorescence as compared with untreated aliquots. M1 and M2 cells possessed similarly enhanced levels of internalized *P. gingivalis* as compared with M0 (Fig. 5B), suggesting that the numbers of *P. gingivalis* internalized by phenotypically distinct BMDMØ were similar.

DISCUSSION

Despite the development of robust immune responses, the host is unable to resolve periodontal infections and disease progression. The reasons for this are unclear; however,

several factors including microbial dysbiosis, immune dysfunction, and apparent failure of antibodies to clear key organisms likely contribute (Darveau *et al.* 2012; Lamster *et al.* 1990; Nakagawa *et al.* 1990; Wang 2015). Macrophages comprise an important fraction of the total inflammatory cell population present in periodontal disease lesions (Okada & Murakami 1998); however their role in disease particularly in the context of responsivity to immunologic cues guiding cell function prior to pathogen interaction is poorly understood. Recently it was reported that injection of naïve animals with *P. gingivalis* drives recruitment of differing sets of macrophages, including M1 and M2 cell populations, with M1 > M2 (Lam *et al.* 2014), and are broadly in agreement with the clinical environment in periodontitis which possesses molecules favorable for simultaneous M1 and M2 macrophage skewing (Navarrete *et al.* 2014). Thus, macrophage skewing as a consequence of the environment encountered by infiltrating cells during periodontal disease prior to bacterial exposure is a poorly defined attribute that likely plays an important, and functionally relevant role in host recognition and subsequent immune response of these cells to *P. gingivalis* challenge.

TLRs, and in particular TLR2, is implicated in the host response to *P. gingivalis* (Burns *et al.* 2010; Costalonga *et al.* 2009; Gibson & Genco 2007; Hajishengallis *et al.* 2006b). BMDMØ respond to *P. gingivalis* with enhanced TLR2 (Liang *et al.* 2009; Zhou & Amar 2007), yet little change in TLR4 expression (Liang *et al.* 2009). Unexpectedly, we observed that macrophages skewed in M1 orientation prior to *P. gingivalis* challenge failed to change TLR2 expression; however, TLR4 expression was augmented on both M1 and M2 skewed macrophages. This finding suggested that skewed macrophages adapt different pattern recognition receptor repertoires in responses to *P. gingivalis* than observed by naïve macrophages typically used to study cell response to challenge, and that microenvironments favoring M1 skewing switch macrophage from a predominating TLR2, to a predominating TLR4, response. Our findings build on those of Burns *et al.* (2006) regarding TLR4, where wild type and TLR4^{-/-} mice, but not TLR2^{-/-} mice, developed a potent cytokine response to *P. gingivalis*. Indeed, we observed that M1 skewed BMDMØ do not produce a cytokine and chemokine response to *P. gingivalis* that is as robust as M2 activated cells, and M2 cells bind *P. gingivalis* more robustly than M1 cells. CD14, a co-receptor for TLR4 that is important in recognition of bacterial LPS (Fenton & Golenbock 1998), expression was significantly increased in all macrophage populations in response to *P. gingivalis* challenge, suggesting that other elements of TLR4 sensing of bacterial LPS were not affected. *P. gingivalis* signaling via TLR2 induces conformational changes in CR3 (a heterodimer of CD11b and CD18) to a high affinity receptor, that is important for attachment and internal survival of *P. gingivalis* in macrophages (Hajishengallis 2011). We observed that immunologic activation of macrophages prior to *P. gingivalis* infection led to a similar and modest baseline reduction in CD11b as compared with M0 cells; however, in response to *P. gingivalis* exposure only M0 cells responded with increased CD11b. Interestingly, both M1 and M2 BMDMØ responded to *P. gingivalis* challenge with reduced CD18 expression. One explanation for the observed reduction in CD18 by skewed macrophage to *P. gingivalis* reflects its utilization in attachment/internalization of *P. gingivalis* (such as through CR3); however, the absence of change in CD11b does not fully substantiate attachment-mediated

utilization of CD18 via intact CD3. Our data thus point to possible utilization of multiple receptors by macrophages to interact with *P. gingivalis*.

As macrophages participate in antigen presentation to T cells to engage adaptive immunity (Hume 2008; Unanue 1984), and the host is unable to resolve periodontal disease, the immunologic environment encountered by macrophages prior to infection may critically influence expression key receptors in this process (Balbo *et al.* 2001). Enhanced MHCII expression was observed on M0 and M2 cells challenged with *P. gingivalis*, while MHCII levels did not change on M1 cells, thus suggesting that immunologic skewing may impact key elements of macrophage capacity to properly present antigen. These findings extend knowledge of macrophage types encountered in the oral cavity where two distinct populations based on MHCII and iNOS expression were observed to co-exist, MHCII⁻/iNOS⁺, and MHCII⁺/iNOS⁻ (Suzuki *et al.* 1999). It was suggested that MHCII⁻/iNOS⁺ macrophage populations may have phagocytic/antimicrobial function, while MHCII⁺/iNOS⁻ macrophages perform antigen presentation activities. Our data suggest that MHCII⁻/iNOS⁺ macrophages may be skewed M1, while MHCII⁺/iNOS⁻ macrophages may display markers consistent with M2 cells. Although, in our hands, M1 cells bound *P. gingivalis* more robustly than M0 cells, M2 macrophages possess greater capacity to bind *P. gingivalis*. Antigen presentation requires co-stimulatory molecule engagement; interestingly we observed strong *P. gingivalis* dependent reduction in the expression of CD80 and CD86 by M1 macrophages. M2 cells did not shift expression of CD80 in response to *P. gingivalis* challenge, these cells responded with robust increase in CD86. In culture naïve non-skewed macrophages that encounter *P. gingivalis* LPS or its fimbriae express MHCII and CD80, (Cohen *et al.* 2004; Hajishengallis *et al.* 2004). CD80 and CD86 expression has been reported on macrophages in clinical samples, but levels did not reflect clinical status (Gemmell *et al.* 2001b). Employing mice deficient in CD80 or CD86 and *P. gingivalis* HagB protein, Zhang *et al.* (2004) revealed important roles for CD86 (B7-2) in antibody production, and cytokine response to *P. gingivalis*. Our observed reduction in co-stimulatory molecule expression by M1 cells, when compared with the findings of Zhang *et al.* (Zhang *et al.* 2004) suggest that immunologic skewing to M1 prior to *P. gingivalis* exposure may impair the development of a properly coordinated innate immune response to *P. gingivalis*, thus dysregulate integration of T cell help during *P. gingivalis* infection, and may impact downstream coordination of host antibody production.

The periodontium during periodontal disease presents with a dynamic immune response (Gemmell *et al.* 2007), thus it is plausible that local shifts in inflammatory mediator expression during disease contributes to progression of periodontitis (Ellis *et al.* 1998). We observed an unexpected overall profile where M2 cells generated a more robust response to *P. gingivalis* challenge than either M1 or M0 cells. The notable exception was IL-10 that was secreted most robustly by reference M0 cells followed by M1, then M2 cells. IL-10 is detected at high levels in periodontal disease (Lappin *et al.* 2001), and has been linked to oral bone loss (Sasaki *et al.* 2004). The profile of IL-10 production to *P. gingivalis* was opposite to that observed for TNF- α , which was produced in greater abundance in M2 BMDM ϕ than M1 or M0 cells. This was unanticipated as M2 cells are associated with higher IL-10 production than M1 cells (Weisser *et al.* 2013), wound healing, and are considered not inflammatory (Sica & Mantovani 2012). Although differences in data could

reflect differences in systems employed, we speculate that this profile reflects a cellular response to pathogen encountered at a site where infection is not anticipated by the host, such as a site of wound healing. To this point, our observations into chemokine production by immunologically skewed macrophages reflected an overall enhanced responsivity of M2 over that of M1 macrophages.

We generated M1 cells using a cocktail of IFN γ with enteric LPS. Thus the reduced response of M1 BMDM ϕ as compared with M2 cells at several points may suggest an anergic profile (Foey & Crean 2013). In our hands, anergy as a consequence of our macrophage activation approach is not supported as we observed that *P. gingivalis* elicited more TNF- α , and MIP-1 α in activated BMDM ϕ than from non-activated macrophages. Furthermore, we previously reported that *E. coli* LPS treatment of macrophages prior to *P. gingivalis* exposure did not lead to tolerization, rather partial sensitization of these cells to subsequent *P. gingivalis* exposure was observed (Papadopoulos *et al.* 2013). Our results agree and extend from recent findings regarding reduction in chemokine production from M1 skewed macrophages to *P. gingivalis* challenge (Huang *et al.* 2016). On the contrary, a recent study investigating skewed macrophage immune responses to *P. gingivalis* LPS suggested a less robust response from M2 macrophages than M1 cells (Holden *et al.* 2014). We do not understand why our data differ from this study in that we observed differences between activation types and cytokine, chemokine, and cell molecule expression; however the reported differences may reflect systems employed, as we used live *P. gingivalis* to define macrophage response to live intact bacteria, rather than a purified ligand.

Our findings identify that the inflammatory milieu encountered by macrophages prior to pathogen interaction dysregulates the responsivity of these cells to *P. gingivalis* challenge and the observed phenotypes may impact significantly in initiation, progression, and complexity of inflammation observed in periodontal disease. Based on the complex inflammatory milieu described in human periodontitis, it is plausible that both M1 and M2 macrophage subsets exist in periodontal lesions and thus would be in position to respond to bacterial infection or microbial insult simultaneously. Alternatively, there may be tissue-level microenvironments in periodontal lesions that could favor M1 and M2 subset activation. Suzuki *et al.* (Suzuki *et al.* 1999) employed a histological approach to demonstrate the presence of different macrophage types in rat periapical lesion tissue, thus supporting the notion that phenotypically distinct macrophages may exist in periodontal lesions. In the context of atherosclerosis, presence of phenotypically distinct macrophages in diseased vascular tissue has been identified, and there is a degree of spatial partitioning of these phenotypic subsets in these compartments (Chinetti-Gbaguidi *et al.* 2011). Future investigations are needed to define the relative numbers and phenotypic characteristics of the macrophage cell infiltrate in periodontal tissues, as well as to determine the spatial and functional distribution of macrophage subsets within tissues obtained from individuals with periodontal disease.

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Fig. 1. Phenotypic description of M1 and M2 activated BMDMØ subsets prior to *P. gingivalis* exposure

Bone marrow cells from C57BL-6 mice were cultured in M-CSF for 7 days, harvested, and cultured with fresh medium (M0; control), recombinant murine IFN γ (100 U ml⁻¹)+ultrapure *E. coli* LPS 0111:B4 (10 ng ml⁻¹) (M1), or recombinant murine IL-4 (20 U ml⁻¹) (M2). Flow cytometry was used to measure surface expression of MHCII (A–C = representative histograms where bold trace is specific staining and shaded trace is isotype control, D = MFI, n=6 $\pm$ SEM for each), and CD86 (E–G = representative histograms where bold trace is specific staining and shaded trace is isotype control, H = MFI, n=6 $\pm$ SEM for each); RT-QPCR was used to define *nos2* and *arg1* gene expression by macrophage subsets normalized to β -actin (I, n=2 $\pm$ SEM for each); culture supernatant fluid levels of nitrite (μ M; J), TNF- α (pg ml⁻¹; K), and IL-6 (pg ml⁻¹; L) expressed by polarized macrophages (n=6 $\pm$ SEM for each).

Fig. 2. Surface receptor profile of skewed BMDMØ cultured with *P. gingivalis*
Control M0 and skewed M1 and M2 BMDM were cultured in medium alone (open bars) or with *P. gingivalis* 381 MOI 100 (filled bars) for 24h and the mean fluorescence intensity (MFI) expression of (A) F4/80, (B) CD11b, (C) CD18, (D) TLR2, (E) TLR4, (F) CD14 was determined by flow cytometry. Bars = mean $\pm$ SEM; n = 6 mice per group; *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ by Two-way ANOVA with Bonferroni post test.

Fig. 3. MHCII, CD80, and CD86 surface expression by skewed BMDMØ to *P. gingivalis* Control M0 and skewed M1, and M2 BMDMØ were cultured in medium alone (open bars) or with *P. gingivalis* 381 MOI 100 (filled bars) for 24h and the mean fluorescence intensity (MFI) expression of (A.) MHCII, (B.) CD80, and (C.) CD86 was determined by flow cytometry. Bars = mean $\pm$ SEM; n = 6 mice per group; *** = $P < 0.001$ by Two-way ANOVA with Bonferroni post test.

Fig. 4. Cytokine and chemokine expression by skewed BMDMØ cultured with *P. gingivalis* Control M0 and skewed M1, and M2 BMDMØ were cultured in medium alone (open bars) or with *P. gingivalis* 381 MOI 100 (filled bars) for 24h and culture supernatant fluid levels of (A.) TNF- α , (B.) IL-1 β , (C.) IL-6, (D.) IL-10, (E.) KC, (F.) MCP-1, (G.) MIP-1 α , and (H.) RANTES were determined by multiplex immunoassay. Bars = mean $\pm$ SEM; n = 6 mice per group; *** = P < 0.001, ** = P < 0.01, or NS = not significantly different by One-way ANOVA with Tukey post test.

Fig. 5. Binding and internalization of *P. gingivalis* by skewed BMDMØ

Control M0 (circles) or skewed into M1 (squares), and M2 (triangles) BMDMØ were cultured with FITC-labeled *P. gingivalis* 381 MOI 100 from 15 to 120 minutes, and mean fluorescence intensity (MFI) was determined for each sample by flow cytometry. (A.) Total BMDMØ cell-associated MFI, (B.) BMDMØ MFI following treatment with trypan blue to quench extracellular FITC activity. Bars = mean $\pm$ SEM; n = 6 mice per group.