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Role of MyD88-dependent and -independent signaling in *Porphyromonas gingivalis*-elicited macrophage foam cell formation

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SUMMARY

Clinical studies and experimental modeling identify a potential link between periodontal disease and periodontal pathogens such as *Porphyromonas gingivalis* with atherosclerosis and formation of macrophage foam cells. Toll-like receptors and molecules governing their intracellular signaling pathways such as MyD88 play roles in atherosclerosis, as well as host response to *P. gingivalis*. The aim of this study was to define roles of MyD88 and Lps2 during macrophage foam cell formation in response to *P. gingivalis*. In the presence of human low-density lipoprotein (LDL) mouse bone marrow derived macrophages (BMØ) cultured with *P. gingivalis* responded with significant reduction in TNF- α and IL-6. BMØ stained strongly with oil red O, regardless whether bacterial challenge occurred concurrent with or prior to LDL treatment. Heat-killed *P. gingivalis* stimulated foam cell formation similar to live organisms. BMØ from MyD88-knockout (KO) and Lps2 mice revealed a significant role for MyD88, and a mild role for Lps2 in *P. gingivalis*-elicited foam cell formation. *P. gingivalis*-elicited TNF- α and IL-6 was affected by MyD88 ablation and to a lesser extent by Lps2 status. These data indicate that LDL affects TNF- α and IL-6 response of macrophage to *P. gingivalis* challenge and that MyD88 and Lps2 play important roles in *P. gingivalis*-elicited foam cell formation.

Keywords

Innate immunity; *Porphyromonas gingivalis*; macrophage; foam cell; cytokine

INTRODUCTION

Periodontal diseases are a complex group of chronic oral inflammatory diseases. *Porphyromonas gingivalis* is a Gram negative anaerobic bacterium implicated as a primary periodontal pathogen (Socransky & Haffajee, 1992). Host response to this bacterium is thought to contribute significantly to loss of soft and hard tissues supporting the teeth (Baker, 2000). Various host expressed inflammatory mediators including cytokines and chemokines are implicated in periodontal disease pathogenesis (Page, 1991, Kornman, *et al.*, 1997), and experimental modeling has confirmed many of these clinical findings (Genco, *et al.*, 1998). *P. gingivalis* and its purified antigens stimulate cytokine and chemokine production from macrophages *in vitro* (Zhou, *et al.*, 2005, Shaik-Dasthagirisahab, *et al.*,

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2009). Clinical and experimental data support roles for toll-like receptors (TLR) in periodontal disease and *P. gingivalis*-elicited oral bone loss (Folwaczny, *et al.*, 2004, Schroder, *et al.*, 2005, Burns, *et al.*, 2006). Furthermore, TLRs participate in host recognition and inflammatory response to *P. gingivalis* and several of its antigens (Tabeta, *et al.*, 2000, Darveau, *et al.*, 2004, Ukai, *et al.*, 2008). The TLR family is comprised of 11 members (Takeda & Akira, 2005). These molecules provide innate immune recognition of conserved pathogen-associated molecular patterns, or PAMPS, including lipopeptides (TLR2), lipopolysaccharide (TLR4), CpG DNA (TLR9), and others (Takeda & Akira, 2005), as well as modified endogenous host ligands (Wagner, 2006). On binding these receptors, intracellular adaptor molecules including myeloid differentiation factor (MyD) 88, TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN- β ; the protein product of the *Ips2* gene), TIRAP/MAL (TIR domain containing-adaptor protein), and TRAM (TRIF-related adaptor molecules) are recruited to the TLR TIR domain, to initiate intracellular signaling cascades that culminate in NF- κ B activation and cytokine gene expression (Takeda & Akira, 2004). Recent *in vivo* studies have reported that MyD88-dependent and -independent pathways play important roles in development of inflammation and clearance of *P. gingivalis* (Burns, *et al.*, 2010).

Emerging studies suggest a connection between periodontal disease and systemic diseases such as atherosclerosis (Haraszthy, *et al.*, 2000, Padilla, *et al.*, 2006). Cardiovascular disease with atherosclerosis is the leading cause of death in the United States (Heron MP, 2009). Clinical and experimental findings identify innate immunity as a key factor in most aspects of atherosclerosis from endothelial cell activation to vascular plaque rupture (Libby & Aikawa, 2002). Elevated TLR expression in human atherosclerotic plaques has been reported (Mullick AE, 2006). Experimental models support this clinical finding and implicate TLRs and members of TLR signaling pathways in the development of atherosclerosis (Bjorkbacka, *et al.*, 2004, Michelsen, *et al.*, 2004). Macrophages are present in early and late atherosclerotic lesions, typically found in a lipid laden state, termed foam cells (Kruth, 2001). Little is known regarding the specific mechanisms that stimulate macrophage foam cell formation, although innate immune mechanisms play important roles in this process (Cao, *et al.*, 2007). Several pathogens are known to stimulate macrophage foam cell formation (Kalayoglu & Byrne, 1998, Qi, *et al.*, 2003, Wang, *et al.*, 2007, Okahashi, *et al.*, 2011). Roles for TLRs and TLR signaling pathways have been implicated in macrophage foam cell formation in response to *Chlamydia pneumoniae* (Kalayoglu & Byrne, 1998, Chen, *et al.*, 2008). *P. gingivalis* accelerates atherosclerosis in mouse models of atherosclerosis (Li, *et al.*, 2002, Lalla, *et al.*, 2003, Gibson, *et al.*, 2006), a phenomenon which is in part dependent on TLR2 (Hayashi, *et al.*, 2010). *P. gingivalis* can stimulate macrophage foam cell formation (Qi, *et al.*, 2003, Giacona, *et al.*, 2004); however the host mechanisms underlying *P. gingivalis*-elicited macrophage foam cell formation are poorly defined. We speculated that TLR signaling pathways participate in *P. gingivalis*-elicited macrophage foam cell formation. In the present study we define roles for MyD88 and Lps2 in macrophage foam cell formation and cytokine response to *P. gingivalis*.

METHODS

Mouse strains and macrophage culture

Wild type C57BL-6 (Wt) mice were purchased from Jackson Laboratories (Bar Harbor, ME). MyD88-knockout (KO) mice were obtained from Dr. S. Akira (Osaka University, Osaka, Japan; (Kawai, *et al.*, 1999), and Lps2 mice (point mutation in *Ips2* gene rendering its protein product TRIF non-functional) were provided by Dr. B. Beutler (The Scripps Institute, La Jolla, CA; (Hoebe, *et al.*, 2003). Boston University Medical Center Institutional IACUC approvals governed handling and use of all laboratory animals in these studies. Bone marrow cells were harvested from femurs of mice and were cultured in RPMI-1640 +

10% FBS supplemented with penicillin-streptomycin (50 IU/mL, 50 µg/mL, respectively) and 20% conditioned medium from L929 cells (source of M-CSF, kind gift from Dr. Robin Ingalls, Boston University Medical Center, Boston, MA). After 7 days, adherent bone marrow derived macrophage (BMØ) cells were collected, placed into fresh medium without penicillin-streptomycin and L929 conditioned medium, and added to wells of tissue culture plates or 8-well chamber slides (Thermo Fisher Scientific-Nunc, Rochester, NY). In some experiments we employed thioglycolate-elicited peritoneal macrophages from Wt mice (Baer, *et al.*, 2009).

Growth of *P. gingivalis*

Porphyromonas gingivalis strain 381 was plated on anaerobic blood agar plates and cultivated anaerobically for 3-5 days at 37°C. Plate grown *P. gingivalis* were harvested and used to seed BHI-yeast extract broth cultures (Baer, *et al.*, 2009). After 24h growth, bacteria were harvested, washed, and suspended in antibiotic-free BMØ cell culture medium. Heat-killed *P. gingivalis* were generated by 30 min. incubation at 60°C (Baer, *et al.*, 2009).

Macrophage challenge

To examine the effect of LDL on *P. gingivalis* elicited TNF-α and IL-6, BMØ from Wt mice were challenged with *P. gingivalis* at a multiplicity of infection (MOI) of 100 in medium alone or medium containing human low-density lipoprotein (LDL; 200 µg/mL, Intracell, Fredrick, MD) for 24h and cell culture supernatant fluids were harvested and used for ELISA. For BMØ foam cell formation, two challenge methods were employed. In the first method, *P. gingivalis* and LDL (200 µg/mL) were added simultaneously to BMØ cultures (concurrent method), and after 24h incubation, culture supernatant fluids were harvested and BMØ stained for foam cells. In the second method *P. gingivalis* was added to BMØ for 24h in the absence of LDL, culture supernatant fluids were harvested, cells were then washed, and LDL (200 µg/mL) was added for an additional 24h. These final culture supernatant fluids were also collected and BMØ stained for foam cells (uncoupled method). Dose-dependent stimulation of *P. gingivalis*-elicited BMØ foam cell formation was performed using MOI of 1, 10 and 100, or equivalent numbers of heat-killed *P. gingivalis*. In several experiments, BMØ from MyD88-KO and Lps2 mice were cultured with *P. gingivalis* using both concurrent and uncoupled method to define the roles of these molecules in cytokine production and foam cell response.

Lipid staining and foam cell counts

Oil red O staining was used to observe lipid accumulation in BMØ (Cao, *et al.*, 2007). In brief, BMØ were paraformaldehyde fixed, washed and stained with oil red O solution (100 µg/mL in 70% isopropanol, 30% water) for 15 minutes. Following washing with DPBS, the slides were processed for microscopic evaluation. Five digital images were taken of representative microscopic fields for each condition for each experiment, and the percentage of oil red O stained cells was determined from total cell counts of at least 450 cells/field by a person blinded to sample identity using Image J (Rasband, 1997-2011).

ELISA

Following manufacturers instructions, murine-specific tumor necrosis factor (TNF)-α and interleukin (IL)-6 Duo-set ELISA kits (R&D Systems Inc, Minneapolis, MN) were used to measure these molecules in BMØ culture supernatant fluids.

Statistical Analysis

All experiments were performed at least two times with BMØ from individual mice. Data were imported into PRISM statistical analysis software for comparative analysis by one-way

ANOVA for one variable or two-way ANOVA for two or more variables with Bonferroni post-test. A $p < 0.05$ was considered significant.

RESULTS

LDL affects pro-inflammatory cytokine production in response to *P. gingivalis*

As high levels of circulating LDL represent an important risk factor for atherosclerosis, we were interested to investigate if LDL affected pro-inflammatory cytokine production by BMØ challenged with *P. gingivalis*. To accomplish this, Wt BMØ were cultured for 24h in medium alone, medium with LDL (200 µg/mL), medium with *P. gingivalis* (MOI 100), or medium with LDL and *P. gingivalis* together and TNF-α and IL-6 levels measured in culture supernatant fluids by ELISA. Low **or non-detectable** levels of TNF-α or IL-6 were measured in response to medium alone or medium with LDL (Fig. 1). *P. gingivalis* readily stimulated significant levels of TNF-α and IL-6 from Wt BMØ as compared to medium alone or medium with LDL ($p < 0.05$ for both; Fig. 1A and B). When LDL was present at the time of *P. gingivalis* challenge, Wt BMØ produced significantly less TNF-α and IL-6 as compared with *P. gingivalis* challenge in the absence of LDL ($p < 0.05$ for each; Fig. 1A and B). These results indicate that high levels of LDL significantly reduce pro-inflammatory cytokine production by BMØ cultured with *P. gingivalis*.

P. gingivalis stimulates macrophage foam cell formation

Previous studies have identified that *P. gingivalis* stimulates macrophage foam cell formation (Qi, *et al.*, 2003, Giacona, *et al.*, 2004). We observed that BMØ from Wt mice cultured with LDL alone did not readily stain with oil red O (Fig. 2 panel I and Fig. 2B), a dye, which stain lipid droplets thus indicates foam cell formation. Wt BMØ cultured for 24h with *P. gingivalis* at MOI 100 in the presence of LDL (200 µg/mL) elicited foam cell formation, as indicated by oil red O staining (concurrent method; Fig. 2A, panel II and Fig. 2B). As we observed that in the presence of added LDL there was a profound effect on *P. gingivalis*-elicited TNF-α and IL-6 (Fig. 1), we explored an uncoupled LDL treatment method where BMØ were cultured with medium alone or medium with *P. gingivalis* for 24h in the absence of LDL, followed by supernatant collection, cell washing to remove extracellular *P. gingivalis*, then incubation for 24h in medium with LDL. This strategy allowed us to measure the effect of LDL treatment on inflammatory response of BMØ to *P. gingivalis*, and to investigate whether BMØ challenged with *P. gingivalis* in the absence of LDL retained the ability to form foam cells if high-levels of LDL were later encountered. In the absence of an initial 24h *P. gingivalis* challenge, addition of LDL to Wt BMØ failed to elicit foam cell formation (Fig. 2A panel III and Fig. 2B). Wt BMØ challenged with *P. gingivalis* at MOI 100 prior to LDL treatment readily took on foam cell phenotype (Fig. 2A panel IV and Fig. 2B) similar to that observed with concurrent LDL challenge ($p > 0.05$; Fig. 2B). To understand if the ability of Wt BMØ cultured with *P. gingivalis* followed by LDL treatment was specific to BMØ, we obtained thioglycolate-elicited peritoneal macrophages from Wt mice performed uncoupled *P. gingivalis* challenge and LDL treatment. We observed that thioglycolate-elicited peritoneal macrophages also readily took on a foam cell phenotype when challenged in this manner (data not shown).

Next we were interested to know if the BMØ foam cell response was dependent on dose of *P. gingivalis* encountered. We observed that with increasing MOI of *P. gingivalis* (MOI 1, 10 and 100) regardless of the concurrent or uncoupled LDL treatment elicited a greater percentage of foam cells (Fig. 2B). Moreover, foam cell formation occurred readily at low MOI (Fig. 2B). To determine if killed *P. gingivalis* retained the ability to evoke BMØ foam cell formation live or heat-killed *P. gingivalis* (MOI 100, or equivalent number of killed organisms) were added to BMØ and following oil red O staining we observed that heat-

killed *P. gingivalis* stimulated foam cell formation similar to live *P. gingivalis* regardless of LDL presence at the time of challenge ($p>0.05$; Table 1). These data support that *P. gingivalis* can stimulate macrophage foam cell formation in a dose-dependent manner, and that viability of *P. gingivalis* is not required to elicit this response.

MyD88 plays a role in BMØ foam cell formation, lesser role observed for Lps2

Animal modeling using hyperlipidemic mice has implicated TLR-signaling pathways governed by MyD88 and TRIF with atherosclerosis (Michelsen, *et al.*, 2004, Cole, *et al.*, 2010). Based on this we performed studies using BMØ from MyD88-KO and Lps2 mice to determine roles for these molecules in *P. gingivalis*-elicited foam cell formation. In relationship to Wt, BMØ from MyD88-KO mice exhibited significant reduction in number of foam cells when challenged with *P. gingivalis* at MOI 100 using both concurrent and uncoupled methods ($p<0.05$ for each; Fig. 3A and B). Employing LDL treatment concurrent with *P. gingivalis* challenge we observed that BMØ from Lps2 mice responded to *P. gingivalis* and formed foam cells similar to Wt ($p>0.05$; Fig. 3A). Unexpectedly, using the uncoupled method where LDL addition followed after 24h *P. gingivalis* challenge, BMØ from Lps2 mice presented with reduced percentage of foam cells as compared with Wt ($p<0.05$; Fig. 3B). These data support that MyD88 has a role in *P. gingivalis* elicited BMØ foam cell formation, while a limited role for Lps2 appears to exist.

P. gingivalis stimulates pro-inflammatory cytokine production in BMØ in response to addition of LDL

Next we were interested to define the expression of the pro-inflammatory cytokines TNF- α and IL-6 in the context of *P. gingivalis*-elicited macrophage foam cell formation. To accomplish this we measured cytokine levels in the culture supernatant fluids from Wt BMØ cultured with *P. gingivalis* and LDL (concurrent method), as well as levels from cells cultured with *P. gingivalis* or medium alone for 24h, and following washing, and at 24h after LDL treatment (uncoupled method). In all instances, regardless of model system, culture supernatant fluids from BMØ cultured in medium alone or medium with LDL had non-detectable, or low level TNF- α and IL-6 (Fig. 4A-F). As anticipated from our results in Fig. 1, a strong TNF- α (638 ± 47 pg/mL) and IL-6 (450 ± 22 pg/mL) response followed 24h *P. gingivalis* challenge in the absence of LDL (Fig. 4C and D). When LDL was present at the time of *P. gingivalis* challenge, Wt BMØ produced significantly less TNF- α (327 ± 56 pg/mL) and IL-6 (310 ± 31 pg/mL), than when LDL was absent from the challenge ($p<0.05$ for both; Fig. 4A and B). When LDL was added following *P. gingivalis* challenge in the uncoupled method, BMØ continued to produce TNF- α and IL-6; however, a drop in residual levels of TNF- α (170 ± 32 pg/mL; Fig. 4E) was observed. Interestingly, residual expression of IL-6 following 24h LDL treatment resembled levels following 24h *P. gingivalis* challenged (Fig. 4F). These results confirm that addition of LDL reduces *P. gingivalis* induced cytokine response of BMØ in foam cell formation, and identify that BMØ following removal of *P. gingivalis* followed by the addition of LDL retain the ability to produce TNF- α and IL-6.

Effect of MyD88, and Lps2 on BMØ TNF- α and IL-6 expression in response to *P. gingivalis*

As we observed that in the presence of LDL Wt BMØ TNF- α and IL-6 response to *P. gingivalis* challenge was significantly reduced compared to when LDL was absent, and also that MyD88 and Lps2 participated, albeit at differing levels, in BMØ foam cell formation, we were interested to understand the contribution of MyD88 and Lps2 to the inflammatory response of BMØ during foam cell formation. To accomplish this, culture supernatant fluids were collected during both the concurrent and uncoupled LDL treatment foam cell assays from Wt, MyD88-KO, and Lps2 BMØ and levels of TNF- α and IL-6 were measured. In all cases, Wt, MyD88-KO and Lps2 BMØ cultured in medium alone or medium with LDL

produced non-detectable or very low levels of TNF- α and IL-6 (Fig. 4). When *P. gingivalis* and LDL were added concurrently to BM ϕ from MyD88-KO mice we observed significant reduction in TNF- α and IL-6 as compared with Wt ($p < 0.05$ for each; Fig. 4A and B). Supernatant fluid levels of TNF- α and IL-6 from BM ϕ of Lps2 mice challenged with *P. gingivalis* in the presence of LDL were also significantly reduced as compared to Wt BM ϕ ($p < 0.05$ for both; Fig. 4A and B); however, levels of TNF- α and IL-6 between MyD88-KO and Lps2 BM ϕ revealed significant differences for TNF- α ($p < 0.05$; Fig. 4A), but not IL-6 ($p > 0.05$; Fig. 4B). In the uncoupled model, supernatant fluids harvested from MyD88-KO BM ϕ challenged with *P. gingivalis* prior to LDL addition, showed reduction in both TNF- α and IL-6 as a consequence of MyD88 ablation vs. Wt ($p < 0.05$ for both; Fig. 4C and D). Modest yet significant TNF- α reduction was observed with Lps2 BM ϕ as compared to Wt ($p < 0.05$; Fig. 4C), while IL-6 levels were unchanged from those of Wt ($p > 0.05$; Fig. 4D). Lastly we measured the levels of TNF- α and IL-6 in culture supernatants of *P. gingivalis*-challenged BM ϕ following LDL treatment in uncoupled method (Fig. 4E and F). Residual levels of produced TNF- α were not detected from either MyD88-KO or Lps2 mouse BM ϕ following LDL addition, and was significantly different than Wt ($p < 0.05$ for both genotypes, Fig. 4E). IL-6 levels from MyD88-KO were significantly reduced from that of Wt ($p < 0.05$; Fig. 4F) and were significantly different than that produced by Lps2 BM ϕ ($p < 0.05$; Fig. 4F); although significantly reduced from Wt, IL-6 in culture supernatant fluids from Lps2 BM ϕ following LDL treatment revealed an intermediate level different from both Wt and MyD88-KO ($p < 0.05$ for both; Fig. 4F).

Discussion

Atherosclerosis is a complex multi-factorial chronic inflammatory disease (Ross, 1999). It is speculated that periodontal disease may serve as a risk factor for atherosclerosis (Scannapieco & Genco, 1999). Presence of cholesterol-engorged macrophage foam cells in vascular wall tissues is hallmark of atherosclerosis (Greenspan, *et al.*, 1997, Lusis, 2000). *In vitro* studies have reported that in the presence of LDL *P. gingivalis* stimulates macrophages to take on a foam cell-like phenotype (Qi, *et al.*, 2003, Giacona, *et al.*, 2004). Little is known regarding potential host mechanisms underlying *P. gingivalis*-elicited foam cell formation, although animal modeling supports that TLRs are implicated in atherosclerosis accelerated by this organism (Hayashi, *et al.*, 2010). In this study we were interested to define the role of TLR-signaling adaptor molecules MyD88 and Lps2 on BM ϕ foam cell formation and to understand whether high lipid environments affected host immune response to *P. gingivalis*. Our *in vitro* studies using mouse macrophages identified foam cell formation by BM ϕ in response to *P. gingivalis* challenge. This response involved MyD88, and by employing BM ϕ from Lps2 mice our data suggest partial involvement of functional TRIF protein. In the absence of MyD88, *P. gingivalis* also failed to stimulate TNF- α or IL-6 production, which are pro-inflammatory cytokines with suspected roles in oral bone loss and atherosclerosis that are observed following *P. gingivalis* challenge (Baker, *et al.*, 1999, Gibson, *et al.*, 2006, Miyauchi, *et al.*, 2011). We observed that the presence of LDL at the time of *P. gingivalis* challenge led to reduced BM ϕ TNF- α and IL-6 expression. This latter point led us to **adopt** a dual approach to study *P. gingivalis*-elicited foam cell formation. The majority of investigations focusing on infection-elicited foam cell formation utilize LDL treatment at the time of challenge. Indeed, this is in keeping with convention as elevated LDL is a common feature of individuals with or at risk for atherosclerosis (Schaefer, *et al.*, 1994). Employing our dual challenge strategy we observed that presence of LDL lowered BM ϕ TNF- α and IL-6 response to *P. gingivalis* challenge, and that *P. gingivalis* challenge in the absence of LDL **predisposed** BM ϕ to become foam cells when LDL was provided after challenge. We do not know what mechanisms underlay this reduced pro-inflammatory cytokine phenotype as a consequence of high-level LDL. Recently it was reported using human monocytes incubated with enteric LPS that the presence of oxidized-LDL, but not

unmodified LDL, decreased TNF- α and IL-6 levels as compared with LPS treatment alone (Kannan, *et al.*, 2012). Unexpectedly, in the same study no effect was observed with either LDL or oxidized LDL treatment on LPS-elicited cytokine levels when human monocyte-derived macrophages were employed (Kannan, *et al.*, 2012). These findings identify cellular level complexity between monocytes and macrophages with regards to effect of LDL species influencing host cytokine responses. In our studies we employed murine BM ϕ , and a live bacterium with complex LPS structure rather than purified enteric LPS. Our findings differ from that seen with either human monocytes or human monocyte-derived macrophages as elevated levels of unmodified LDL reduced macrophage TNF- α and IL-6 production to *P. gingivalis* challenge. Similar to our findings, enhanced foam cell formation was observed with human monocytes challenged with *Chlamydomphila* (*Chlamydia*) *pneumoniae* in the presence of unmodified LDL (Kalayoglu & Byrne, 1998). Further analysis revealed oxidative modification to LDL by human monocytes infected with *C. pneumoniae* (Kalayoglu, *et al.*, 1999). Neither of these reports investigated effects of LDL on *C. pneumoniae*-elicited cytokine production; however, a separate study identified that *Chlamydia*-infected foam cells secrete pro-inflammatory cytokines when in the absence or presence of LDL or oxidized LDL (Blessing, *et al.*, 2002). In the context of *P. gingivalis*, addition of this organism to whole human blood resulted in increase detection of reactive oxygen species (Bengtsson, *et al.*, 2008), thus suggesting the potential for oxidative change to lipids.

Our findings of reduced host inflammatory response to *P. gingivalis* in an elevated LDL environment may also suggest activation of lipid signaling pathways. Liver X receptors (LXRs) are nuclear hormone receptors with identified roles in lipid signaling. LXRs control expression of genes involved in removal of excess cholesterol from cells (Laffitte, *et al.*, 2001). In addition to their role in lipid signaling, LXRs influences host immunity (Joseph, *et al.*, 2003), and response to bacterial infection (Joseph, *et al.*, 2004, Korf, *et al.*, 2009). Cross-talk between LXR and TLR pathways has also been identified, with evidence for interactions through TLR2 and TLR4 (Cao, *et al.*, 2007). At the level of the macrophage, LXR agonist treatment reduces expression of the NF- κ B dependent genes iNOS and COX2 to LPS treatment. Interestingly, the promoters of iNOS and COX-2 do not possess LXR binding sites (Joseph, *et al.*, 2003). Further studies are needed to determine what molecules are involved in the reduced host response to *P. gingivalis* in high LDL environments.

Prior studies have identified that *P. gingivalis* elicits foam cell formation (Qi, *et al.*, 2003, Giacona, *et al.*, 2004, Miyakawa, *et al.*, 2004); however, role of specific host pathways such as TLR signaling is unclear. Our study employing BM ϕ from Wt mice shows that *P. gingivalis* drives foam cell formation, and is in agreement with others (Qi, *et al.*, 2003, Giacona, *et al.*, 2004, Miyakawa, *et al.*, 2004). Moreover, we report that heat-killed *P. gingivalis* elicited foam cell formation similar to live organism. These data support that viability is not required for triggering foam cell response, and suggest important roles for specific antigens in this process. *P. gingivalis* possesses several factors including LPS, fimbriae, gingipains, and others that are thought important to the virulence of this organism (Hamada, *et al.*, 1998, Lamont & Jenkinson, 1998, Potempa, *et al.*, 2000). Studies employing the major fimbriae-deficient *P. gingivalis* mutant strain DPG3, identified an important role for major fimbriae in human macrophages foam cell formation (Giacona, *et al.*, 2004). Studies focusing on gingipains, more specifically arginine-specific gingipains, have identified that these enzymes are also important in *P. gingivalis*-elicited foam cell formation. Human LDL was directly modified by gingipain, and the foam cell phenotype resulted following addition of gingipain-treated LDL to cells (Miyakawa, *et al.*, 2004). This modification could have important implications in uptake of modified LDL as scavenger receptors readily take-up modified LDL (Moore & Freeman, 2006). Indeed, scavenger receptor-A and CD36 have been shown to play important roles in aspects of *P. gingivalis*

challenge (Triantafilou, *et al.*, 2007, Baer, *et al.*, 2009), albeit not directly in foam cell formation elicited by this organism. *P. gingivalis* and its gingipains have also been shown to degrade ApoB-100, and affects both foam cell formation (Miyakawa, *et al.*, 2004) and atherosclerosis (Hashimoto, *et al.*, 2006). Other groups have shown that bacteria and infectious agents associated with diseases linked to atherosclerosis stimulate macrophage foam cell formation. Indeed, *in vitro* challenge with *C. pneumoniae*, *Mycobacterium tuberculosis*, HIV are examples of organisms reported to elicit a foam cell response (Kalayoglu & Byrne, 1998, Mujawar, *et al.*, 2006, Peyron, *et al.*, 2008). Recently, this capacity of bacteria to elicit foam cell formation has been expanded to include killed organisms and specific TLR ligands in macrophage foam cell formation (Nicolaou, *et al.*, 2012). As the focus of our present study was identification of host cell pathways involved, and not the bacterial antigens driving foam cell formation, we did not follow-up our findings to investigate what *P. gingivalis* antigens may be driving foam cell formation.

TLRs perform immune recognition and ability to distinguish conserved pathogen-associated molecular patterns, as well as modified endogenous ligands (Takeda & Akira, 2005, Wagner, 2006). Most TLRs signal through the adaptor protein MyD88, except TLR3, which uses TRIF, while TLR4 utilizes both MyD88 and TRIF (Hoebe, *et al.*, 2003, Yamamoto, *et al.*, 2003, Takeda & Akira, 2004). Several groups have identified that host response to *P. gingivalis* and its antigens involves TLRs (Bainbridge, *et al.*, 2002, Davey, *et al.*, 2008, Gaddis, *et al.*, 2009, Hajishengallis, *et al.*, 2009). In this study we focused on roles for MyD88 and Lps2 to cover the majority of TLR signaling in BMØ to define their roles in foam cell formation and cytokine expression in response to *P. gingivalis*. We observed significant reduction in BMØ foam cell formation due to MyD88 ablation regardless of challenge method employed (Fig. 3A and B). Similarly, reduction in cytokine production in response to *P. gingivalis* challenge was observed with BMØ from MyD88-KO mice compared to Wt (Fig. 4A-D). Importance of MyD88 in infection-elicited foam cell formation has previously been identified for the obligate intracellular pathogen *C. pneumoniae* (Chen, *et al.*, 2008). Our studies are in agreement with this prior study, supporting an important role for MyD88 in BMØ foam cell formation to *P. gingivalis* exposure. Clearly MyD88 has been established in the development of inflammatory response to *P. gingivalis* (Wang, *et al.*, 2000), as well as in *P. gingivalis* clearance from host (Burns, *et al.*, 2010). As TLR2 appears to play an important role in *P. gingivalis*-elicited cytokine production (Burns, *et al.*, 2006, Ukai, *et al.*, 2008), we anticipated an important role for MyD88 in the development of pro-inflammatory cytokine production by BMØ to *P. gingivalis* challenge. In addition we observed a subtle change in TNF- α or IL-6 expression in BMØ from Lps2 mice compared to WT (Fig. 4A-D). From our studies, a more consistent role for MyD88 in immune response and foam cell formation is suggested; however, subtle involvement of the TRIF arm may play a role in BMØ foam cell formation and innate immune response elicited by *P. gingivalis*. Further investigations in this area are needed to confirm our findings in the context of *P. gingivalis*-accelerated atherosclerosis *in vivo*.

Our data employing macrophages from MyD88-deficient and Lps2 mice supports a connection to TLR signaling in foam cell formation. From these studies it is clear that *P. gingivalis* stimulates BMØ foam cell formation and MyD88 signaling plays a role in foam cell formation as well as induction of cytokine expression in the context of immune response to *P. gingivalis*.

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Figure 1. BMØ cytokine levels in response to *P. gingivalis* and added LDL

BMØ from C57BL-6 mice were cultured for 24h in medium alone (Control), medium with 200 µg/mL human LDL (LDL), *P. gingivalis* strain 381 MOI 100 (*Pg*), or *P. gingivalis* and LDL (*Pg* + LDL). Culture supernatant fluids were collected and levels of (A) TNF-α, and (B) IL-6 were determined by ELISA. N = 6 separate experiments; * = $p < 0.05$ as determined by one-way ANOVA with Bonferroni post-test. **ND = none detected.**

Figure 2. BMØ foam cell formation in response to *P. gingivalis*

BMØ from C57BL-6 mice were cultured in 8-well chamber slides for foam cell determinations using concurrent or uncoupled method. BMØ were washed, fixed, then stained with oil red O. Micrographs (A) of BMØ cells cultured for 24h in 200 µg/mL human LDL (panel I), *P. gingivalis* 381 (*Pg*) MOI 100 + LDL (panel II), 24h medium followed by 24h LDL (panel III), or 24h *P. gingivalis* 381 MOI 100 followed by 24h LDL (panel IV). (B) Percent positive BMØ foam cells elicited by dose of *P. gingivalis* strain 381 MOI 1, 10, and 100 using either concurrent (open bars) and uncoupled (filled bars) methods. On-screen counts were performed by person blinded to sample identify. N = 3 **separate experiments**; * = p<0.05 compared to matched LDL alone (Cont) as determined by one-way ANOVA with Bonferroni post-test. No significant differences were observed between concurrent and uncoupled methods in the ability of *P. gingivalis* to elicit foam cell formation (p>0.05).

Figure 3. Role of MyD88-dependent and -independent signaling on BMØ foam cell formation in response to *P. gingivalis*

BMØ from wild type C57BL-6 (Wt), MyD88-KO, and Lps2 mice were cultured in 8-well chamber slides and following fixation were stained with oil red O. (A) Concurrent method and (B) Uncoupled method. Medium with LDL (open bars), medium with *P. gingivalis* strain 381 MOI 100 and LDL (filled bars). N = 3 **separate experiments**; * = p<0.05 as determined by one-way ANOVA with Bonferroni post-test, NS = not significant.

Figure 4. MyD88-dependent and -independent signaling on TNF-α and IL6 levels in BMØ foam cell formation in response to *P. gingivalis*

Culture supernatant fluid levels of TNF-α (A, C, and E) and IL-6 (B, D, and F) from wild type C57BL-6 (Wt), MyD88-KO, Lps2 BMØ were determined by ELISA during Concurrent method (A and B), and Uncoupled method (C-F) foam cell experiments. In concurrent method cytokine levels were measured in supernatant fluids harvested from BMØ 24h after medium and LDL (open bars), or medium and LDL with *P. gingivalis* MOI 100 (filled bars) treatments. For uncoupled method cytokine levels were determined after 24h medium alone (open bars) or medium with *P. gingivalis* (filled bars) challenge (C and D), as well as 24h following BMØ wash and addition of medium (open bars) or medium with LDL (filled bars) to define residual cytokine production (E and F). Cytokine measurements presented as mean pg/mL ± standard error of the mean. N = 3 **separate experiments**; * = p < 0.05 as determined by two-way ANOVA with Bonferroni post-test, NS = not significant, **ND = none detected**.

Table 1Effect of *P. gingivalis* viability on BMØ foam cell formation.

Treatment	<u>Foam cells (%)</u>	
	Concurrent method	Uncoupled method
LDL alone	0.92 ± 0.69	2.21 ± 2.29
LDL and Pg live	48.9 ± 10.3 *	47.9 ± 5.4 *
LDL and Pg HK	48.6 ± 14.0 *#	42.5 ± 6.8 *#

Foam cells presented as percent oil red O stained cells **determined from 3 separate experiments**. Comparisons between groups were performed using one-way ANOVA with Bonferroni post-test.

* = p<0.05 compared to the treatment with LDL alone;

= Not significant compared to *Pg* live challenge (**p>0.05**). *Pg* = *P. gingivalis*; HK=heat killed.