molecular oral microbiology

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Role of MyD88-dependent and MyD88-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* and 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 TRIF during macrophage foam cell formation in response to *P. gingivalis*. In the presence of human low-density lipoprotein (LDL) mouse bone-marrow-derived macrophages (BMϕ) cultured with *P. gingivalis* responded with significant reduction in tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The BMϕ stained strongly with oil red O, regardless of whether bacterial challenge occurred concurrent with or before LDL treatment. Heat-killed *P. gingivalis* stimulated foam cell formation in a similar way to live bacteria. The BMϕ from MyD88-knockout and Lps2 mice revealed a significant role for MyD88, and a minor role for TRIF in *P. gingivalis*-elicited foam cell formation. *Porphyromonas gingivalis*-elicited TNF-α and IL-6 were affected by MyD88 ablation and to a lesser extent by TRIF status. These data indicate that LDL affects the TNF-α and IL-6 response of macrophages to *P. gingivalis* challenge and that MyD88 and TRIF play important roles in *P. gingivalis*-elicited foam cell formation.

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). Both *P. gingivalis* and its purified antigens stimulate cytokine and chemokine production from macrophages *in vitro* (Zhou *et al.*, 2005; Shaik-Dasthagirisaheb *et al.*, 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 comprises 11 members (Takeda & Akira, 2005). These molecules provide innate immune recognition of conserved pathogen-associated molecular patterns, including lipopeptides (TLR2), lipopolysaccharide (TLR4), CpG DNA (TLR9) (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/interleukin-1 receptor domain-containing adaptor inducing interferon-β; the protein product of the *lps2* gene), TIRAP/MAL (Toll/interleukin-1 receptor 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 nuclear factor-κB activation and cytokine gene expression (Takeda & Akira, 2004). Recent *in vivo* studies have reported that MyD88-dependent and MyD88-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 USA (Heron *et al.*, 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 *et al.*, 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.*, 2004a). 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 *Chamydiophila* (*Chlamydia*) *pneumoniae* (Kalayoglu & Byrne, 1998; Chen *et al.*, 2008). *Porphyromonas 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). *Porphyromonas 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 TRIF 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 *lps2* 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% fetal bovine serum supplemented with penicillin–streptomycin (50 IU ml^{-1}, 50 μg ml^{-1}, respectively) and 20% conditioned medium from L929 cells (source of macrophage colony-stimulating factor, a kind gift from Dr. Robin Ingalls, Boston University Medical Center, Boston, MA). After 7 days, adherent bone marrow-derived macrophages (BMϕ) were collected, placed into fresh medium without penicillin–streptomycin and L929-conditioned medium, and added to wells of tissue culture plates or eight-well chamber slides (Thermo Fisher Scientific-Nunc, Rochester, NY). In some experiments we employed thioglycollate-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 brain–heart infusion –yeast extract broth cultures (Baer *et al.*, 2009). After 24 h of growth, bacteria were harvested, washed and suspended in antibiotic-free BMϕ 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 tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), BMϕ 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^{-1}, Intracell, Fredrick, MD) for 24 h and cell culture supernatant fluids were harvested and used for enzyme-linked immunosorbent assay (ELISA). For BMϕ foam cell formation, two challenge methods were employed. In the first method, *P. gingivalis* and LDL (200 μg ml^{-1}) were added simultaneously to BMϕ cultures (concurrent method), and after 24 h of incubation, culture supernatant fluids were harvested and BMϕ were stained for foam cells. In the second method, *P. gingivalis* were added to BMϕ for 24 h in the absence of LDL, culture supernatant fluids were harvested, cells were then washed, and LDL (200 μg ml^{-1}) was added for an additional 24 h. These final culture supernatant fluids were also collected and BMϕ were stained for foam cells (uncoupled method). Dose-dependent stimulation of *P. gingivalis*-elicited BMϕ foam cell formation was performed using MOI of 1, 10 and 100, or equivalent numbers of heat-killed *P. gingivalis*. In several experiments, BMϕ from MyD88-KO and Lps2 mice were cultured with *P. gingivalis* using both concurrent and uncoupled methods 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 BMϕ (Cao *et al.*, 2007). In brief, BMϕ were paraformaldehyde fixed, washed and stained with oil red O solution (100 μg ml^{-1} in 70% isopropanol, 30% water) for 15 min. Following washing with Dulbecco's phosphate-buffered saline, 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, 1997–2012).

ELISA

Following manufacturer's instructions, murine-specific TNF-α and IL-6 Duo-set ELISA kits (R&D Systems Inc., Minneapolis, MN) were used to measure these molecules in BMϕ culture supernatant fluids.

Statistical analysis

All experiments were performed at least twice with BMϕ from individual mice. Data were imported into PRISM statistical analysis software for comparative analysis by one-way analysis of variance for one variable or two-way analysis of variance 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 BMϕ challenged with *P. gingivalis*. To accomplish this, Wt BMϕ were cultured for 24 h in medium alone, medium with LDL (200 μg ml^{-1}), medium with *P. gingivalis* (MOI 100), or medium with LDL and *P. gingivalis* together and TNF-α and IL-6 levels were 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). The *P. gingivalis* readily stimulated significant levels of TNF-α and IL-6 from Wt BMϕ compared with medium alone or medium with LDL ($P < 0.05$ for both; Fig. 1A,B). When LDL was present at the time of *P. gingivalis* challenge, Wt

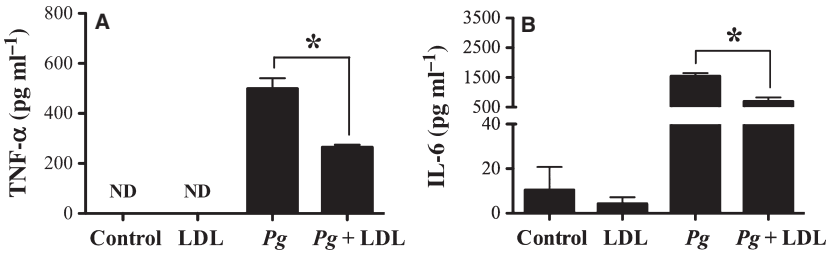

Figure 1 Bone-marrow-derived macrophage (BMϕ) cytokine levels in response to *Porphyromonas gingivalis* and added low-density lipoprotein (LDL). BMϕ from C57BL/6 mice were cultured for 24 h in medium alone (Control), medium with 200 μg ml^{-1} human LDL (LDL), *P. gingivalis* strain 381 multiplicity of infection (MOI) 100 (*Pg*), or *P. gingivalis* and LDL (Pg + LDL). Culture supernatant fluids were collected and levels of (A) tumor necrosis factor-α (TNF-α), and (B) interleukin-6 (IL-6) were determined by enzyme-linked immunosorbent assay. $n = 6$ separate experiments; $^*P < 0.05$ as determined by one-way analysis of variance with Bonferroni post-test. ND, none detected.

BMϕ produced significantly less TNF-α and IL-6 than *P. gingivalis* challenge in the absence of LDL ($P < 0.05$ for each; Fig. 1A,B). These results indicate that high levels of LDL significantly reduce pro-inflammatory cytokine production by BMϕ cultured with *P. gingivalis*.

Porphyromonas gingivalis stimulates macrophage foam cell formation

Previous studies have shown that *P. gingivalis* stimulates macrophage foam cell formation (Qi *et al.*, 2003; Giacona *et al.*, 2004). We observed that BMϕ from Wt mice cultured with LDL alone did not readily stain with oil red O (Fig. 2A, panel I and Fig. 2B), a dye that stains lipid droplets and so indicates foam cell formation. Wild-type BMϕ cultured for 24 h with *P. gingivalis* at an MOI of 100 in the presence of LDL (200 μg ml^{-1}) 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 BMϕ were cultured with medium alone or medium with *P. gingivalis* for 24 h in the absence of LDL, followed by supernatant collection, cell washing to remove extracellular *P. gingivalis*, then incubation for 24 h in medium with LDL. This strategy allowed us to measure the effect of LDL treatment on the

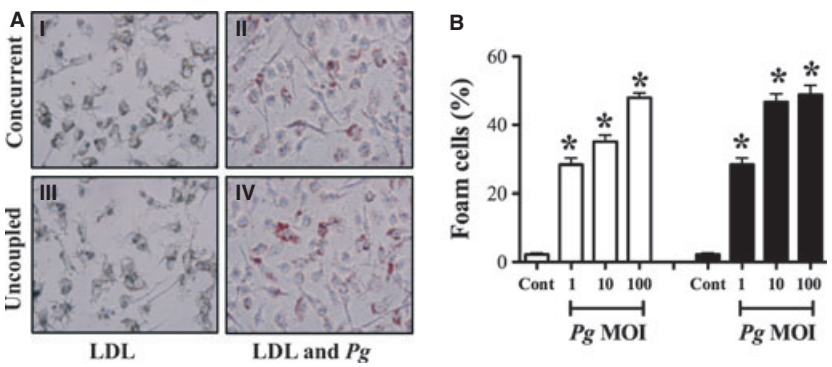

Figure 2 Bone-marrow-derived macrophage (BMϕ) foam cell formation in response to *Porphyromonas gingivalis*. BMϕ from C57BL/6 mice were cultured in eight-well chamber slides for foam cell determinations using a concurrent or an uncoupled method. BMϕ were washed, fixed, then stained with oil red O. (A) Micrographs of BMϕ cells cultured for 24 h in 200 μg ml^{-1} human low-density lipoprotein (LDL) (panel I), *P. gingivalis* 381 (*Pg*) multiplicity of infection (MOI) 100 + LDL (panel II), 24 h medium followed by 24 h LDL (panel III), or 24 h *P. gingivalis* 381 MOI 100 followed by 24 h LDL (panel IV). (B) Percent positive BMϕ foam cells elicited by dose of *P. gingivalis* strain 381 MOI 1, 10 and 100 using either concurrent (open bars) or uncoupled (filled bars) methods. On-screen counts were performed by a person blinded to sample identity. $n = 3$ separate experiments; $^*P < 0.05$ compared with matched LDL alone (Cont) as determined by one-way analysis of variance 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 ($P > 0.05$).

inflammatory response of BMϕ to *P. gingivalis*, and to investigate whether BMϕ 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 24-h *P. gingivalis* challenge, addition of LDL to Wt BMϕ failed to elicit foam cell formation (Fig. 2A, panel III and Fig. 2B). Wild-type BMϕ challenged with *P. gingivalis* at an MOI of 100 before LDL treatment readily took on a 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 BMϕ cultured with *P. gingivalis* followed by LDL treatment was specific to BMϕ, we obtained thioglycollate-elicited peritoneal macrophages from Wt mice performed uncoupled *P. gingivalis* challenge and LDL treatment. We observed that thioglycollate-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 BMϕ foam cell response was dependent on dose of *P. gingivalis* encountered. We observed that 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 BMϕ foam cell formation live or heat-killed *P. gingivalis* (MOI 100, or equivalent number of killed organisms) were added to BMϕ 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 the theories 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.

Table 1 Effect of *Porphyromonas gingivalis* viability on BMϕ foam cell formation

Treatment	Foam cells (%)	
	Concurrent method	Uncoupled method
LDL alone	0.92 ± 0.69	2.21 ± 2.29
LDL and live *P. gingivalis*	48.9 ± 10.3*	47.9 ± 5.4*
LDL and heat-killed *P. gingivalis*	48.6 ± 14.0*,[1]	42.5 ± 6.8*,[1]

*$P < 0.05$ compared with the treatment with LDL alone.
[1]Not significant compared with *P. ginigvalis* live challenge ($P > 0.05$).
LDL, low-density lipoprotein. Foam cells presented as % oil red O stained cells determined from three separate experiments. Comparisons between groups were performed using one-way analysis of variance with Bonferroni post-test.

MyD88 plays a role in BMϕ foam cell formation, a lesser role is observed for TRIF

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

Porphyromonas gingivalis stimulates pro-inflammatory cytokine production in BMϕ in response to the 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 BMϕ cultured with *P. gingivalis* and LDL (concurrent method), as well as levels from cells cultured with *P. gingivalis* or medium alone for 24 h, and following washing, and at 24 h after LDL treatment (uncoupled method). In all instances, regardless of model system, culture supernatant fluids from BMϕ cultured in medium alone or in medium with LDL had non-detectable,

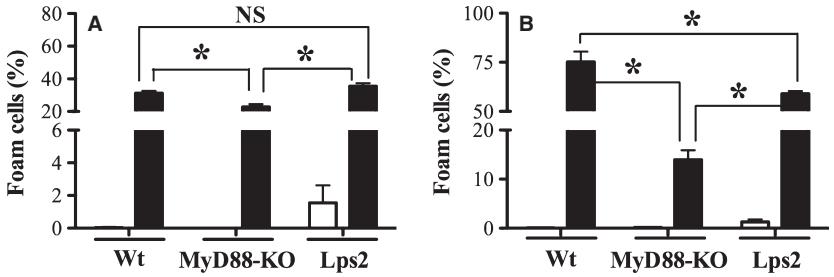

Figure 3 Role of MyD88-dependent and MyD88-independent signaling on bone-marrow-derived macrophage (BMϕ) foam cell formation in response to *Porphyromonas gingivalis*. The BMϕ from wild-type C57BL/6 (Wt) mice, MyD88 knockout (KO) mice and Lps2 mice were cultured in eight-well chamber slides and following fixation were stained with oil red O. (A) Concurrent method and (B) Uncoupled method. Medium with low-density lipoprotein (LDL) (open bars), medium with *P. gingivalis* strain 381 multiplicity of infection 100 and LDL (filled bars). $n = 3$ separate experiments; $^*P < 0.05$ as determined by one-way analysis of variance with Bonferroni post-test; NS, not significant.

or low-level TNF-α and IL-6 (Fig. 4A–F). As anticipated from the results shown in Fig. 1, a strong TNF-α (638 ± 47 pg ml^{-1}) and IL-6 (450 ± 22 pg ml^{-1}) response followed 24 h of *P. gingivalis* challenge in the absence of LDL (Fig. 4C,D). When LDL was present at the time of *P. gingivalis* challenge, Wt BMϕ produced significantly less TNF-α (327 ± 56 pg ml^{-1}) and IL-6 (310 ± 31 pg ml^{-1}), than when LDL was absent from the challenge ($P < 0.05$ for both; Fig. 4A, B). When LDL was added following *P. gingivalis* challenge in the uncoupled method, BMϕ continued to produce TNF-α and IL-6; however, a drop in residual levels of TNF-α (170 ± 32 pg ml^{-1}; Fig. 4E) was observed. Interestingly, residual expression of IL-6 following 24 h of LDL treatment resembled levels following 24 h of *P. gingivalis* challenge (Fig. 4F). These results confirm that addition of LDL reduces *P. gingivalis*-induced cytokine response of BMϕ in foam cell formation, and indicate that BMϕ 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 TRIF on BMϕ TNF-α and IL-6 expression in response to *P. gingivalis*

As we observed that in the presence of LDL the Wt BMϕ TNF-α and IL-6 response to *P. gingivalis* challenge was significantly reduced compared with when LDL was absent, and also that MyD88 and TRIF participated, albeit at differing levels, in BMϕ foam cell formation, we were interested to understand the contribution of MyD88 and TRIF to the inflammatory response of BMϕ 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 BMϕ and levels of TNF-α and IL-6 were measured. In all cases, Wt, MyD88-KO and Lps2 BMϕ 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 BMϕ from MyD88-KO mice we observed significant reductions in TNF-α and IL-6 compared with Wt ($P < 0.05$ for each; Fig. 4A,B). Supernatant fluid levels of TNF-α and IL-6 from BMϕ of Lps2 mice challenged with *P. gingivalis* in the presence of LDL were also significantly reduced compared with Wt BMϕ ($P < 0.05$ for both; Fig. 4A,B); however, levels of TNF-α and IL-6 between MyD88-KO and Lps2 BMϕ 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 BMϕ challenged with *P. gingivalis* before LDL addition, showed a reduction in both TNF-α and IL-6 as a consequence of MyD88 ablation vs. Wt ($P < 0.05$ for both; Fig. 4C,D). Modest yet significant TNF-α reduction was observed with Lps2 BMϕ compared with Wt ($P < 0.05$; Fig. 4C), whereas 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 BMϕ following LDL treatment in the uncoupled method (Fig. 4E,F). Residual levels of produced TNF-α were not detected from either MyD88-KO or Lps2 mouse BMϕ following LDL addition, and were significantly different from Wt ($P < 0.05$ for both genotypes, Fig. 4E). Interleukin-6 levels from MyD88-KO were significantly reduced compared with those from Wt ($P < 0.05$; Fig. 4F) and were significantly different from that produced by

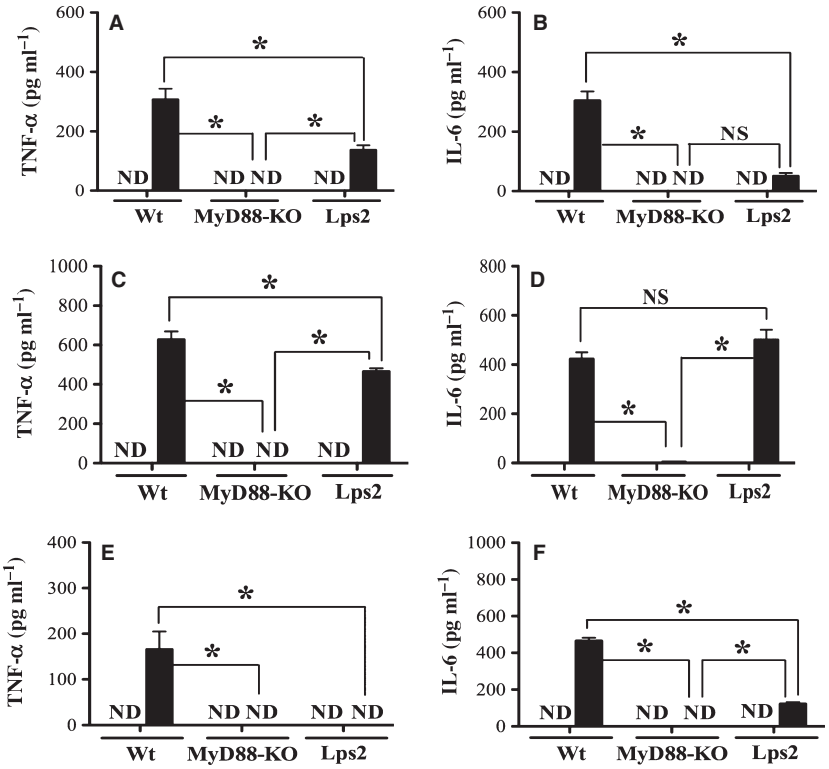

Figure 4 MyD88-dependent and MyD88-independent signaling on tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in bone-marrow-derived macrophage (BMϕ) foam cell formation in response to *Porphyromonas gingivalis*. Culture supernatant fluid levels of TNF-α (A, C and E) and IL-6 (B, D and F) from wild-type C57BL/6 (Wt), MyD88 knockout (KO) and Lps2 BMϕ were determined by enzyme-linked immunosorbent assay during the Concurrent method (A, B), or the Uncoupled method (C–F) foam cell experiments. In the concurrent method cytokine levels were measured in supernatant fluids harvested from BMϕ 24 h after medium and low-density lipoprotein (LDL) (open bars), or medium and LDL with *P. gingivalis* multiplicity of infection (MOI) 100 (filled bars) treatments. For the uncoupled method cytokine levels were determined after 24 h medium alone (open bars) or medium with *P. gingivalis* (filled bars) challenge (C, D), as well as 24 h following BMϕ wash and addition of medium (open bars) or medium with LDL (filled bars) to define residual cytokine production (E, F). Cytokine measurements presented as mean pg ml^{-1} ± standard error of the mean. $n = 3$ separate experiments; $*P < 0.05$ as determined by two-way analysis of variance with Bonferroni post-test, NS, not significant, ND, none detected.

Lps2 BMϕ ($P < 0.05$; Fig. 4F); although significantly reduced from Wt, IL-6 in culture supernatant fluids from Lps2 BMϕ 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 a 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 the idea 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 the TLR-signaling adaptor molecules MyD88 and TRIF on BMϕ foam cell formation and to understand whether high lipid environments affected the host immune response to *P. gingivalis*. Our *in vitro* studies using mouse macrophages identified foam cell formation by BMϕ in response to *P. gingivalis* challenge. This response involved MyD88, and by employing BMϕ from Lps2 mice our data suggest partial involvement of the functional TRIF protein. In the absence of MyD88, *P. gingivalis* also failed to stimulate produc-

tion of TNF-α or IL-6, 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.*, 2012). We observed that the presence of LDL at the time of *P. gingivalis* challenge led to reduced BMϕ 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 use 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 the BMϕ TNF-α and IL-6 response to *P. gingivalis* challenge, and that *P. gingivalis* challenge in the absence of LDL predisposed BMϕ to become foam cells when LDL was provided after challenge. We do not know what mechanisms underlie this reduced pro-inflammatory cytokine phenotype as a consequence of high-level LDL. Recently it was reported using human monocytes incubated with enteric lipopolysaccharide (LPS) that the presence of oxidized-LDL, but not unmodified LDL, decreased TNF-α and IL-6 levels 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 BMϕ, and a live bacterium with complex LPS structure rather than purified enteric LPS. Our findings differ from those 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 *Chlamydophila* (*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 increased detection of reactive oxygen species (Bengtsson *et al.*, 2008), 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. The 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 influence 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-κb-dependent genes for inducible nitrous oxide synthase (iNOS) and cyclo-oxygenase 2 (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.

Previous studies have identified that *P. gingivalis* elicits foam cell formation (Qi *et al.*, 2003; Giacona *et al.*, 2004; Miyakawa *et al.*, 2004); however, the role of specific host pathways such as TLR signaling is unclear. Our study employing BMϕ from Wt mice shows that *P. gingivalis* drives foam cell formation, and is in agreement with other studies (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 responses, and suggest important roles for specific antigens in this process. *Porphyromonas gingivalis* possesses several factors including LPS, fimbriae, gingipains and others that are thought to be important to the virulence of this organism (Hamada *et al.*, 1998; Lamont & Jenkinson, 1998; Potempa *et al.*, 2000). Studies employing the major

fimbria-deficient *P. gingivalis* mutant strain DPG3, identified an important role for major fimbriae in human macrophage 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 after the addition of gingipain-treated LDL to cells (Miyakawa *et al.*, 2004). This modification could have important implications in the 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. *Porphyromonas gingivalis* and its gingipains have also been shown to degrade ApoB-100, and affect 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*, and 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 the host cell pathways involved, and not the bacterial antigens driving foam cell formation, we did not follow-up our findings to investigate which *P. gingivalis* antigens may be driving foam cell formation.

Toll-like receptors 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, and TLR4, which uses 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 the roles of MyD88 and TRIF to cover the majority of TLR signaling in BMϕ, to define their roles in foam cell formation and cytokine expression in response to *P. gingivalis*. We observed a significant reduction in BMϕ foam cell formation due to MyD88 ablation regardless of challenge method employed (Fig. 3A,B). Similarly, reduction in cytokine production in response to *P. gingivalis* challenge was observed with BMϕ from MyD88-KO mice compared with 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 previous study, supporting an important role for MyD88 in BMϕ foam cell formation to *P. gingivalis* exposure. Clearly MyD88 has been established in the development of an 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 BMϕ to *P. gingivalis* challenge. In addition, we observed a subtle change in TNF-α or IL-6 expression in BMϕ from Lps2 mice compared with 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 BMϕ 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 support a connection to TLR signaling in foam cell formation. From these studies it is clear that *P. gingivalis* stimulates BMϕ 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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