Lipid raft-dependent uptake, signalling and intracellular fate of *Porphyromonas gingivalis* in mouse macrophages

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Summary

Lipid rafts are cholesterol-enriched microdomains involved in cellular trafficking and implicated as portals for certain pathogens. We sought to determine whether the oral pathogen *Porphyromonas gingivalis* enters macrophages via lipid rafts, and if so, to examine the impact of raft entry on its intracellular fate. Using J774A.1 mouse macrophages, we found that *P. gingivalis* colocalizes with lipid rafts in a cholesterol-dependent way. Depletion of cellular cholesterol using methyl-β-cyclodextrin resulted in about 50% inhibition of *P. gingivalis* uptake, although this effect was reversed by cholesterol reconstitution. The intracellular survival of *P. gingivalis* was dramatically inhibited in cholesterol-depleted cells relative to untreated or cholesterol-reconstituted cells, even when infections were adjusted to allow equilibration of the initial intracellular bacterial load. *P. gingivalis* thus appeared to exploit raft-mediated uptake for promoting its survival. Consistent with this, lipid raft disruption enhanced the colocalization of internalized *P. gingivalis* with lysosomes. In contrast, raft disruption did not affect the expression of host receptors interacting with *P. gingivalis*, although it significantly inhibited signal transduction. In summary, *P. gingivalis* uses macrophage lipid rafts as signalling and entry platforms, which determine its intracellular fate to the pathogen’s own advantage.

Introduction

Host-pathogen cross-talk involves microbial interactions with the signalling apparatus of the infected cell, and in this regard lipid rafts serve as a major interface (Simons and Toomre, 2000). Lipid rafts are membrane microdomains rich in cholesterol, sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins, which partition receptors for various cellular signalling and trafficking processes (Simons and Ikonen, 1997). The formation of lipid rafts is attributed to the property of sphingolipids and cholesterol to preferentially interact with each other resulting in their spontaneous separation from other phospholipids in the cell membrane. Moreover, cholesterol is believed to stabilize lipid rafts by filling the voids between the relatively bulky glycosphingolipids (Riethmuller et al., 2006).

Microbe-detecting molecules of the immune system such as Toll-like receptors (TLRs) as well as T-cell and B-cell receptors are recruited to lipid rafts of activated cells, although both their recruitment and ability to signal are inhibited by cholesterol-depleting drugs (Pierce, 2002; Triantafilou et al., 2002a; Pizzo and Viola, 2003). This indicates that cholesterol-enriched membrane microdomains participate in the induction of both innate and adaptive immunity. Lipid rafts play additional roles in host–pathogen interactions as they are implicated as the sites of action of several bacterial toxins (Nagahama et al., 2003; Badizadegan et al., 2004; Boesze-Battaglia et al., 2006; Fong et al., 2006) and as portals of entry for certain intracellular pathogens (reviewed by Manes et al., 2003). For example, lipid raft integrity is essential for the uptake of mycobacteria by macrophages, whereas the cholesterol-depleting drug methyl-β-cyclodextrin (MCD) prevents this activity (Gatfield and Pieters, 2000).

Given the importance of lipid rafts in host defence signalling, it might seem paradoxical that intracellular pathogens, such as *Salmonella typhimurium*, *Shigella flexneri* and *Mycobacterium* spp. have ‘selected’ these microdomains for cell entry (Manes et al., 2003). Ironically, however, it could be the very significance of lipid rafts in host defence that may have rendered them targets for immune subversion by pathogens. Although there are only a few examples of lipid raft exploitation by microbes as a means to enhance their adaptive fitness, it has been suggested that the lipid-raft route may afford protection from the intracellular degradative lysosomal pathway (reviewed by Manes et al., 2003). In this respect, it has been proposed that internalized lipid rafts do not readily
fuse with late endosomes and lysosomes (Simons and Gruenberg, 2000). Moreover, at least certain pathogens proactively attempt to prevent post-phagocytosis killing. For example, mycobacteria induce recruitment of a coat protein known as TACO (tryptophane aspartate-containing coat protein) which associates in a cholesterol-dependent way with the phagosomal membrane and prevents its fusion with lysosomes (Gatfield and Pieters, 2000). In stark contrast to lipid raft-mediated phagocytosis, opsonic phagocytosis through Fc receptors is not affected by cholesterol depletion and normally leads to pathogen degradation (Joiner et al., 1990; Shin et al., 2003; Naroeni and Porte, 2002; Manes et al., 2003).

The main objective of this study was to determine whether the oral pathogen Porphyromonas gingivalis enters macrophages through lipid rafts, and if so, to examine the impact of lipid raft entry on the intracellular fate of this bacterial organism. *P. gingivalis* is associated with chronic periodontitis and implicated as a contributory factor in systemic conditions such as atherosclerotic heart disease, on the basis of human epidemiological findings and experimental evidence in mouse models (reviewed by Gibson et al., 2006). Our focus on macrophages in this paper is based on the significance of this cell type in the innate host response in periodontitis and other chronic infections including atherosclerosis (Linton and Fazio, 2003; Teng, 2006). The rationale for investigating macrophage lipid rafts as a possible portal of entry for *P. gingivalis* was provided by recent findings from our group.

Specifically, we have previously compared the phagocytic activities of normal and pattern-recognition receptor (PRR)-deficient mouse macrophages and found that CD14, CD11b and TLR2 are important for the uptake of *P. gingivalis* (Hajishengallis et al., 2006a). Although TLR2 is not known to be a bona fide phagocytic receptor, its involvement in *P. gingivalis* uptake is attributed to TLR2 inside-out signalling which activates the ligand-binding capacity of complement receptor-3 (CR3; CD11b/CD18) (Harokopakis and Hajishengallis, 2005; Harokopakis et al., 2006), TLR2 and CR3 are recruited to lipid rafts following activation with appropriate stimuli including *P. gingivalis* fimA-encoded fimbriae (Triantafilou et al., 2004; 2007; Hajishengallis et al., 2006b), whereas CD14 constitutively resides in lipid rafts by virtue of being a GPI-anchored molecule (Triantafilou et al., 2002b). We have thus hypothesized that the macrophage uptake of *P. gingivalis* is mediated, at least partly, via lipid rafts. Previous reports provided suggestive evidence for *P. gingivalis* interactions with lipid rafts, but these studies did not involve professional phagocytic cells. Specifically, polystyrene beads coated with *P. gingivalis* outer membrane vesicles are taken up by HeLa epithelial cells via lipid rafts, whereas MCD inhibits uptake (Tsuda et al., 2005). Moreover, MCD inhibits *P. gingivalis* invasion of KB epithelial cells (Tamai et al., 2005). However, control experiments with cholesterol-reconstituted MCD-treated cells were not performed to rule out potential pleiotropic MCD effects and, furthermore, the biological significance of lipid raft-mediated entry was not addressed in these pioneering studies (Tamai et al., 2005; Tsuda et al., 2005). Our current findings from functional and imaging studies underscore the importance of macrophage lipid rafts as uptake and signalling platforms for *P. gingivalis* in a way that favours its survival.

**Results**

Disruption of lipid rafts suppresses the phagocytosis of *P. gingivalis*

To investigate a possible role of lipid rafts in *P. gingivalis* virulence, we used J774A.1 mouse macrophages which have been widely employed as a model for lipid raft-dependent host–microbial interactions, such as bacterial entry or toxin action (Gatfield and Pieters, 2000; Norkin et al., 2001; Naroeni and Porte, 2002; Maldonado-Garcia et al., 2004; Pucadyil et al., 2004; Bavdek et al., 2007). Although we have previously shown that J774A.1 macrophages readily take up *P. gingivalis*, the role of lipid rafts in this phagocytic activity was not addressed (Wang et al., 2007). We have thus examined the effect of the raft-disrupting agent MCD (Riethmuller et al., 2006) on the ability of J774A.1 macrophages to interact with and internalize *P. gingivalis*. This was determined using FITC-labelled *P. gingivalis* and a flow cytometric uptake assay (Wang et al., 2007), which can determine the levels of macrophage-associated bacteria (extracellularly attached or intracellularly located) or of phagocytosed bacteria (upon quenching extracellular fluorescence with trypan blue).

Methyl-β-cyclodextrin (10 mM) significantly inhibited both the association of *P. gingivalis* with macrophages and its phagocytosis (*P < 0.05; Fig. 1A). Interestingly, MCD displayed a stronger inhibitory effect on phagocytosis than on association (53% versus 28% respectively; Fig. 1A). This finding indirectly suggested that the attachment of *P. gingivalis* to macrophages may not have been affected to the same extent as its phagocytosis. Indeed, when cytochalasin D was used to block phagocytosis (in order to specifically address bacterial adherence), *P. gingivalis* displayed almost comparable binding to untreated and MCD-treated macrophages (Fig. 1B). Specifically, attachment to MCD-treated cells was 87 ± 5% of that to untreated cells, and the difference did not reach statistical significance (Fig. 1B).

The observed inhibitory effects of MCD on association and phagocytosis (Fig. 1A) were strictly dependent on...
cholesterol depletion. First, we confirmed the ability of MCD to extract cholesterol. Indeed, MCD extracted 65% of cellular cholesterol, although the cholesterol content was readily restored by adding exogenous cholesterol to MCD-treated cells (Fig. 1C). Moreover, cholesterol reconstitution of MCD-treated macrophages effectively reversed the inhibitory effects of MCD (Fig. 1A), thus indicating that the MCD effects are mediated through cholesterol extraction and cannot be attributed to non-specific toxic effects.

To determine whether the observed levels of cholesterol depletion by MCD were sufficient to disrupt lipid rafts (and thus to attribute the inhibited phagocytosis of P. gingivalis to disruption of lipid raft organization), we used a confocal microscopy-based approach. In untreated cells, lipid rafts were readily visualized after staining of the lipid raft marker GM1 ganglioside with Alexa Fluor 594-labelled cholera toxin subunit B (CTB), followed by anti-CTB antibody to cross-link them into distinct patches (Fig. 1D, left). However, lipid rafts were hardly detectable in MCD-treated cells indicating that they were disrupted (Fig. 1D, middle). On the other hand, cholesterol reconstitution restored the appearance of lipid rafts (Fig. 1D, right). In summary, these data show that cholesterol depletion diminishes the functionality of lipid rafts and inhibits macrophage phagocytosis of P. gingivalis, although its cell attachment is not significantly influenced.

**Cholesterol-dependent colocalization of P. gingivalis with lipid rafts**

In view of the above findings implicating lipid rafts in the phagocytosis of P. gingivalis, we sought to demonstrate that P. gingivalis interacts directly with lipid rafts. Specifically, we examined whether this pathogen colocalizes with GM1 ganglioside, an established lipid raft marker (Tian-taflou et al., 2002b), stained with Alexa Fluor 594-CTB. Confocal microscopy confirmed that FITC-labelled...
P. gingivalis bacteria (green) attached to or entered macrophages, and revealed evident colocalization between GM1-containing membrane microdomains (red) and bacteria (appearing yellow) (Fig. 2A1 and A2). However, some bacteria (remaining green) did not show colocalization with GM1, suggesting that macrophage–P. gingivalis interactions may also involve non-raft membrane regions.

In Fig. 2A2 for example, neighbouring bacteria stained differently (yellow versus green) indicating, respectively, the presence or lack of GM1 colocalization. In contrast to the above, P. gingivalis did not colocalize with GM1 in cholesterol-depleted cells (Fig. 2B); therefore, under these conditions, its observed uptake may involve a lipid raft-independent route. However, cholesterol reconstitu-
significantly (*P < 0.05) reduced recovery of viable cfu from MCD-treated compared with MCD-untreated or cholesterol-reconstituted cells.

We have previously shown that *P. gingivalis* resists killing by human monocytes or mouse macrophages, wherein it can persist for at least 72 h (Wang et al., 2007). We have now determined whether lipid rafts play a role in the ability of *P. gingivalis* for intracellular persistence in J774A.1 macrophages. For this purpose, MCD was used to deplete the cells of cholesterol and disrupt lipid rafts. MCD-treated, MCD-untreated, as well as cholesterol-reconstituted MCD-treated cells were then infected with *P. gingivalis* (moi = 10:1) and viable internalized bacteria (colony forming units, cfu) were enumerated using an antibiotic protection-based survival assay. MCD treatment resulted in about 50% reduction in recovered *P. gingivalis* cfu after 1.5 h incubation (compared with untreated cells; *P < 0.05, Fig. 3A). The inhibitory effect of MCD was reversed in cholesterol-reconstituted cells, confirming that MCD acts in a cholesterol-specific way. Interestingly, following overnight incubation (15 h), viable *P. gingivalis* bacteria were hardly recoverable from MCD-treated macrophages which displayed 20 times lower cfu levels compared with untreated or cholesterol-reconstituted cells (*P < 0.05; Fig. 3A). Although cholesterol depletion appeared to accelerate the intracellular clearance of *P. gingivalis*, we modified the assay in a way that would allow us to conclusively determine whether lipid rafts influence *P. gingivalis* intracellular survival. Specifically, we ensured that MCD-treated and untreated cells would contain comparable numbers of starting intracellular bacteria. In preliminary flow cytometric uptake experiments, using twofold serial increases in the numbers of bacteria added to MCD-treated cells, we found that at an moi of 80:1, MCD-treated cells display comparable

**Table 1.** Quantification of *P. gingivalis* colocalization with lipid rafts in J774A.1 macrophages.*

| Cell condition                  | % Colocalization |
|--------------------------------|------------------|
| Untreated                      | 76.7 ± 13.3      |
| Cholesterol depletedb          | 13.7 ± 6.2       |
| Cholesterol reconstitutedc     | 67.3 ± 13.1      |
| Impaired phagocytosisd         | 70.1 ± 19.9      |

a. The colocalization of FITC-*P. gingivalis* 33 277 with Alexa Fluor 594-CTB stained GM1 (lipid raft marker) was quantified using ImageJ intensity correlation analysis. See Fig. 2 legend for experimental details.

*P < 0.05 compared with all other conditions.

**Fig. 3.** Cholesterol depletion suppresses the ability of *P. gingivalis* for intracellular persistence in mouse macrophages. A. J774A.1 macrophages were pretreated for 30 min with 10 mM MCD to deplete cholesterol, or were pretreated for 30 min with 10 mM MCD followed by addition of 1 mM cholesterol for an additional 30 min. The MCD- and MCD/cholesterol-pretreated macrophages, as well as cells pretreated with medium only, were subsequently incubated with *P. gingivalis* (moi = 10:1) for the indicated time points.

B. Similar intracellular survival assay, which was modified to ensure comparable numbers of starting intracellular bacteria in MCD-treated and control cells, by using an moi of 80:1 for MCD-treated cells (the inset confirms similar uptake at 30 min post infection, determined by the flow cytometric uptake assay). The persistence of viable internalized bacteria was determined using an antibiotic protection-based survival assay. Data are mean ± SD (*n* = 3) from typical experiments performed three (A) or two (B) times yielding similar findings. Asterisks indicate significantly (*P < 0.05) reduced recovery of viable cfu from MCD-treated compared with MCD-untreated or cholesterol-reconstituted cells.

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phagocytosis to that seen in untreated cells incubated with bacteria at an moi of 10:1 (Fig. 3B, inset). We next performed an intracellular survival assay (Fig. 3B) using differential moi values as specified above. Although there were no significant differences between MCD-treated and control groups at 1.5 h regarding the numbers of intracellular viable bacteria (Fig. 3B), there was a dramatic and statistically significant reduction of viable counts in MCD-treated cells at 15 h relative to untreated cells or cholesterol-reconstituted MCD-treated controls (Fig. 3B). Moreover, MCD-treated cells did not harbour any viable bacteria 24 h later (39 h time point), in stark contrast to the control groups (Fig. 3B). These data clearly indicate that cholesterol depletion promotes the intracellular clearance of \textit{P. gingivalis}. Taken together with the earlier findings of this study (Figs 1 and 2), it may be concluded that the lipid raft route of entry influences the intracellular fate of \textit{P. gingivalis} to the pathogen’s own advantage.

**Cholesterol depletion promotes the colocalization of \textit{P. gingivalis} with lysosomes**

As a possible mechanism whereby certain pathogens may exploit lipid rafts, it has been proposed that lipid rafts mediate an uptake pathway which does not readily fuse with lysosomes (Nagahama \textit{et al}., 2003). If this mechanism applies to \textit{P. gingivalis}, we would expect to see increased trafficking of this pathogen to lysosomes upon cholesterol depletion. We have thus examined the colocalization of FITC-labelled \textit{P. gingivalis} with LysoTracker Red-labelled lysosomes in MCD-treated and untreated J774A.1 macrophages. As would be expected from the hypothesis, confocal microscopy revealed increased colocalization of green fluorescent \textit{P. gingivalis} and red fluorescent lysosomes (manifested as yellow spots) in MCD-treated compared with untreated control macrophages (Fig. 4A–C). Whereas green fluorescent bacteria could readily be found intracellularly in untreated control cells (along with yellow spots indicating fusion with lysosomes; Fig. 4A), ‘green’ bacteria were either absent or rarely found within MCD-treated cells which contained mostly yellow spots (Fig. 4B and C). However, ‘green’ bacteria could be found attached extracellularly in MCD-treated (Fig. 4C) as well as in untreated control cells (Fig. 4A). Cholesterol-reconstituted MCD-treated cells resembled untreated control cells in that both ‘green’ intracellular bacteria (viable) and yellow spots (probably representing non-viable bacteria) could readily be found within the same cell (Fig. 4D). Quantification of the degree of colocalization of \textit{P. gingivalis} with lysosomes revealed that an overwhelming majority (about 90%) of internalized bacteria traffic to lysosomes in cholesterol-depleted cells, in contrast to untreated or cholesterol-reconstituted cells which roughly contain comparable numbers of lysosome-associated and non-associated bacteria (Table 2). In contrast to \textit{P. gingivalis}, another periodontal pathogen, \textit{Aggregatibacter actinomycetemcomitans}, cannot be recovered in a viable state from macrophages as it is readily killed intracellularly (Wang \textit{et al}., 2007). \textit{A. actinomycetemcomitans} was thus used as a control and was found exclusively associated with lysosomes (Fig. 4E, yellow spots). In conclusion, cholesterol depletion enhances the trafficking of \textit{P. gingivalis} to the lysosomes, suggesting that intact lipid rafts allow a relatively safe entry of this pathogen into macrophages.

**Cholesterol depletion inhibits \textit{P. gingivalis}-induced cytokine production without affecting cell surface interactions or expression of signalling receptors**

We next determined whether intact lipid raft function and/or phagocytosis are required for \textit{P. gingivalis}-induced signalling. MCD pretreatment of J774A.1 macrophages resulted in significant (\(P \leq 0.05\)) inhibition of \textit{P. gingivalis}-induced TNF-\(\alpha\) and IL-6 production (>70% inhibition compared with untreated cells; Fig. 5A and B). Moreover, MCD significantly (\(P \leq 0.05\)) inhibited TLR2/1-dependent NF-\(\kappa\)B activation by \textit{P. gingivalis} in transfected human embryonic kidney (HEK)-293 cells (83% inhibition; Fig. 5C). However, cholesterol reconstitution of MCD-treated cells reversed the MCD inhibitory effects (Fig. 5). On the other hand, the inhibitory effects of MCD could not be attributed to its ability to suppress \textit{P. gingivalis} phagocytosis. Indeed, pretreatment with cytochalasin D, which prevents phagocytosis by blocking actin polymerization (Deshpande \textit{et al}., 1998), had no significant influence on \textit{P. gingivalis}-induced cell activation (Fig. 5). That cytochalasin D inhibits the internalization of \textit{P. gingivalis} was verified by confocal microscopy (e.g. see Fig. 2D). These results show that cellular cholesterol is important for maximal NF-\(\kappa\)B activation and cytokine induction by \textit{P. gingivalis}, although its phagocytosis is

\*\(\frac{\text{d}}{\text{d}}\) refers to cholesterol depletion, \(\text{r}\) to cholesterol reconstitution.
not essential in this respect. It can thus be concluded that *P. gingivalis*-induced signalling for cell activation is initiated at the cell surface in cholesterol-enriched lipid rafts.

The inhibitory effect of MCD on *P. gingivalis*-induced cytokine production may be attributed to disruption of lipid rafts, which partition TLRs and other PRRs required for initiation of signal transduction (Simons and Ikonen, 1997; Triantafilou *et al.*, 2002a). However, it was essential to rule out the possibility that MCD may cause loss or shedding of receptors involved in *P. gingivalis*-induced signalling, such as TLR1, TLR2, TLR6, CD11b and CD14 (Hajishengallis *et al.*, 2006b). We have thus examined the expression of these PRRs in MCD-treated or untreated cells. Flow cytometric analysis showed that MCD does not alter the expression of these receptors (Fig. 6), thus suggesting the notion that cholesterol depletion affects the receptor capacity for signalling rather than the receptors per se.

That MCD predominantly affects signalling rather than cell surface interactions was also supported by additional, independent experiments. As MCD appeared...
to affect the adherence of \textit{P. gingivalis} to J774A.1 macrophages only slightly, we decided to determine the effect of MCD on the binding of purified fimbriae, a major adhesin of \textit{P. gingivalis} encoded by the \textit{fimA} gene (Lamont and Jenkinson, 1998; Hajishengallis, 2007). By comparing MCD-treated to untreated J774A.1 macrophages, we found that MCD had no significant influence on the binding of fimbriae (Fig. 7A) although, by great contrast, dramatically inhibited cytokine induction by fimbriae in a cholesterol-specific way [results shown for Fig. 5. Lipid raft-dependent but phagocytosis-independent cytokine induction by \textit{P. gingivalis}. J774A.1 macrophages (A, B) or HEK293 cells transfected as indicated (C) were pretreated or not with MCD (10 mM) or cytochalasin D (5 \( \mu \text{g ml}^{-1} \)). Half of the MCD-treated groups were subsequently reconstituted with cholesterol (1 mM). The cells were stimulated or not with \textit{P. gingivalis} (moi = 10:1) and assayed for TNF-\( \alpha \) (A) or IL-6 (B) by ELISA, or for NF-\( \kappa \)B activation (C) reported as relative luciferase activity (RLA). Data are mean \pm SD (n = 3) from one of two sets of experiments with similar results. Asterisks show statistically significant (\( P < 0.05 \)) inhibition of cell activation.

**Fig. 6.** Effect of cholesterol depletion on PRR expression. J774A.1 macrophages were treated or not with MCD (10 mM) for 30 min. Flow cytometry was then used to analyse expression of surface PRRs (TLR1, TLR2, TLR6, CD11b and CD14) using appropriate FITC-labelled mAbs. The histograms shown are from one of three independent experiments that yielded similar findings.
TNF-α in Fig. 7B; similar findings obtained for IL-6 (not shown). The findings from Figs 5–7, taken together with the rest of the study, underscore the importance of cholesterol-enriched lipid rafts as signalling and entry platforms for P. gingivalis.

Discussion

To persist in a hostile host environment, pathogens have developed mechanisms to evade or subvert immune defences aiming to control or eliminate them. Our findings implicate lipid rafts in the macrophage uptake of P. gingivalis, and show for the first time that this entry route impacts on the intracellular fate of the pathogen in a way that may promote its adaptive fitness. Our results are also consistent with the notion that cholesterol is crucial for the integrity and signalling capacity of lipid rafts (Goluszko and Nowicki, 2005), as in cholesterol-depleted cells P. gingivalis does not colocalize with lipid rafts and loses significant cytokine-inducing ability. The latter effect (inhibition of intracellular signalling) is not attributed to suppressed uptake of P. gingivalis. Indeed, cytochalasin D, which inhibits phagocytosis without affecting P. gingivalis-lipid rafts interactions, does not influence TLR2-dependent NF-κB activation and cytokine induction by this organism. Although inhibition of signalling by lipid raft disruption is not unique to P. gingivalis challenge and has been seen with other bacterial agonists (Triantafillou et al., 2002b; Liang et al., 2007a), it is of interest to note that rafts have also been implicated in negative regulation of signalling, as seen in response to Pseudomonas aeruginosa (Grassme et al. 2003).

Interestingly, cholesterol depletion had a much greater inhibitory effect on the phagocytosis of P. gingivalis than on its binding to macrophages. This preferential effect on phagocytosis may be related to observations that cholesterol depletion inhibits cytoskeletal rearrangements (Riff et al., 2005), which are necessary for phagocytic function (Niedergang and Chavrier, 2004). The inhibition of cytoskeletal rearrangements may in turn be related to diminished capacity for signal transduction in cholesterol-depleted cells. For example, cholesterol depletion results in reduced TLR activation (Triantafillou et al., 2002b) as seen in this study with reduced P. gingivalis-induced TLR2/1 signalling. Strikingly, TLR signalling plays an important role in the induction of cytoskeletal rearrangements promoting endocytosis (West et al., 2004). In cholesterol-depleted cells therefore inhibition of lipid raft-dependent TLR signalling may not simply suppress cytokine induction but may account, at least partly, for impaired phagocytosis.

At least for epithelial cells, it could be argued that microbes tend to invade through lipid rafts simply because these microdomains are concentrated on the apical side of polarized epithelial cells (Simons and Toomre, 2000), i.e. on the side which directly faces the microbial challenge. However, even in macrophages where rafts and non-raft regions are similarly available for interactions, certain pathogens still prefer to enter via lipid rafts as shown in this and previous studies (reviewed by Manes et al., 2003). This strongly suggests that the lipid-raft route of entry may provide an advantage to at least some pathogenic organisms. Indeed, we found that cholesterol depletion promotes the intracellular killing of P. gingivalis, even when moi values are adjusted to allow equilibration of the initial intracellular bacterial load in cholesterol-depleted and control macrophages. These findings support similar claims about lipid raft exploitation by certain other pathogens (Gatfield and Pieters, 2000; Duncan et al., 2002;
Watarai et al., 2002; Manes et al., 2003). It could thus be speculated that P. gingivalis-containing phagosomes originating from lipid rafts may follow a different intracellular fate than phagosomes emanating from non-raft regions of the cell membrane. In this regard, it is thought that internalized lipid rafts may not readily fuse with lysosomes (Simons and Gruenberg, 2000). This concept is readily supported by our findings that cholesterol depletion results in increased colocalization of P. gingivalis with lysosomes.

The specific molecular events underlying the capacity of P. gingivalis to avoid targeting to lysosomes following lipid raft-mediated uptake by macrophages are currently uncertain. At least in human coronary artery endothelial cells, P. gingivalis was shown to traffic from early phagosomes to autophagosomes which, however, do not appear to acquire cathepsins that would indicate formation of autolysosomes (Dorn et al., 2001; Dorn et al., 2002). Consistent with this observation, P. gingivalis appeared to replicate within autophagosomes which also contained degraded cytoplasmic material (Dorn et al., 2001; Dorn et al., 2002). Whether P. gingivalis localizes within autophagosomes in macrophages and, if so, what signals are used to manipulate autophagy remain intriguing questions. However, subversion of autophagy in macrophages has actually been demonstrated for another organism. Indeed, Legionella pneumophila modifies the autophagosomal compartment to establish a niche that is permissive for intracellular replication (Swanson and Isberg, 1996; Dubuisson and Swanson, 2006). Interestingly, recent evidence suggests that pathogens which enter macrophages through lipid rafts, tend to localize in autophagosomes (Amer et al., 2005).

The capacity of P. gingivalis for lipid raft-mediated uptake and increased intracellular persistence is intriguing in the context of the epidemiological and mechanistic link between periodontitis and systemic diseases such as atherosclerosis (Gibson et al., 2004; Desvarieux et al., 2005; Pussinen et al., 2007). Remarkably, viable P. gingivalis has been demonstrated in atherosclerotic plaques (Kozarov et al., 2005). Conceivably, the persistence of P. gingivalis within macrophages may be sufficient to allow this organism to co-opt the migration potential of macrophages, facilitating relocation to systemic tissues and infection of more permissive cells (e.g., endothelial cells). The notion that macrophages might be exploited as ‘Trojan horses’ for P. gingivalis systemic dissemination is an intriguing hypothesis that warrants further investigation. However, the capacity of P. gingivalis to exit initially infected host cells and then to enter and multiply within new host epithelial or vascular cells has been documented (Yilmaz et al., 2006; Li et al., 2008).

On the basis of published literature and our recent work investigating molecular interactions between P. gingivalis and PRRs, it is possible that CR3 may be at least one of potential receptors exploited by P. gingivalis in lipid rafts. First, CR3 is recruitable to lipid rafts upon activation with appropriate microbial stimuli, including P. gingivalis fimbriae (Peyron et al., 2000; Pfeiffer et al., 2001; Hajishengallis et al., 2006b; Nakayama et al., 2007). Second, we have shown that CR3 blockade or deficiency impairs macrophage phagocytosis of P. gingivalis and exerts an even more dramatic inhibitory effect on its intracellular survival (Wang et al., 2007). Mechanistically, this could be explained by findings that CR3 is not linked to vigorous microbicidal mechanisms (Wright and Silverstein, 1983; Caron and Hall, 1998; Rosenberger and Finlay, 2003; Lowell, 2006). It is tempting to speculate that this may in turn be attributed, at least partly, to the functioning of CR3 in lipid rafts, wherefrom derived phagosomes are thought to not readily lead to lysosomal degradation (Simons and Gruenberg, 2000; Watarai et al., 2001; Manes et al., 2003).

In summary, lipid rafts serve as signalling platforms and portals of entry for P. gingivalis into macrophages. Importantly, this route of entry appears to promote the pathogen’s capacity for survival and virulence. Our results and those of others (Gatfield and Pieters, 2000; Watarai et al., 2001; Naroeni and Porte, 2002; Manes et al., 2003) implicating lipid rafts in microbial virulence raise the possibility for lipid raft-oriented strategies as a means to counteract immune evasion and control bacterial infection.

**Experimental procedures**

**Reagents, cells and bacteria**

Cytochalasin D, MCD and cholesterol were purchased from Sigma-Aldrich. Murine-specific mAbs to TLR1 (clone TR23), TLR2 (6C2), CD14 (Sa2–8) and CD11b (M1/70), and their isotype controls were obtained from e Bioscience. Anti-TLR6 mAb (clone 418 601) was from the R&D Systems. Alexa Fluor 594-labelled CTB and LysoTracker Red DND-99 were from Molecular Probes/Invitrogen. The reagents were used at effective concentrations determined in preliminary experiments or in previous publications (Hajishengallis et al., 2006a; Wang et al., 2007). The mouse macrophage cell line J774A.1 (ATCC TIB-67) was cultured at 37°C and 5% CO₂ atmosphere, in RPMI 1640 (Invitrogen/Gibco) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM Hepes, 100 U ml⁻¹ penicillin G, 100 μg ml⁻¹ streptomycin and 0.05 mM 2-mercaptoethanol. HEK-293 cells (ATCC CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated FBS, 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Invitrogen/Gibco). Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega). None of the experimental treatments affected cell viability with medium-only control treatments. P. gingivalis ATCC 33 277 was grown anaerobically at 37°C in modified GAM medium (contains 5 μg ml⁻¹ hemin and 1 μg ml⁻¹ menadione) (Nissui Pharmaceutical) and fimA-encoded fimbriae were purified as previously described (Harokopakis and Hajishengallis, 2005). The purified fimbriae
preparations were free of any contaminating substances on silver-stained SDS-PAGE, and tested negative for endotoxin (<6 EU mg⁻¹ protein) as indicated by the quantitative Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Fimbria binding assay

The binding of purified fimbriae to macrophages was performed using a fluorescent cell-based assay in 96-well plates as we previously described (Harokopakis and Hajishengallis, 2005). Briefly, biotinylated fimbriae (1 μg ml⁻¹) were allowed to bind for 30 min at 37°C, and bound protein was probed with FITC-labelled streptavidin. Upon washing, binding was determined by measuring cell-associated fluorescence on a microplate fluorescence reader (FL600, Bio-Tek Instruments, Winooski, VT) with excitation/emission wavelength settings of 485/530 nm.

Flow cytometry

Flow cytometric analysis was performed to assess the uptake of fluorescently labelled P. gingivalis (Wang et al., 2007). Briefly, J774A.1 macrophages were incubated at 37°C with FITC-labelled P. gingivalis at an moi of 10:1 for 30 min. Phagocytosis was stopped by cooling the incubation tubes on ice. After cell washing to remove non-adherent bacteria, in some groups extracellular fluorescence (representing attached but not internalized bacteria) was quenched with 0.2% trypan blue. The cells were washed again, fixed with 1% paraformaldehyde and analysed by flow cytometry (% positive cells for FITC-P. gingivalis and mean fluorescence intensity (MFI)) using the FACSCalibur and the CellQuest software (Becton–Dickinson). Association (i.e. representing both adherence and phagocytosis) or phagocytic indices were calculated using the formula (% positive cells × MFI)/100. Control experiments indicated that cytochalasin D-pretreated macrophages incubated with FITC-P. gingivalis and subsequently exposed to trypan blue did not show significant fluorescence, thus confirming that cytochalasin D blocks phagocytosis and that trypan blue effectively quenches extracellular fluorescence. Flow cytometry was also used to analyse the effect of MCD pretreatment on J774A.1 macrophage expression of surface receptors (TLR1, TLR2, TLR6, CD11b and CD14) using appropriate FITC-labelled mAbs (see above).

Cell activation assays

NF-xB-dependent transcription of a luciferase reporter gene was determined as previously described in detail (Hajishengallis et al., 2006b; Liang et al., 2007b). Briefly, HEK-293 cells were transiently co-transfected with CD14 (pUNO-hCD14; Invivogen) and TLR1/TLR2 (pDUO-hTLR1/TLR2; Invivogen) and with a NF-xB reporter system, comprising a NF-xB-dependent firefly luciferase plasmid (pNF-xB-Luc; Stratagene) and a Renilla luciferase transfection control (pRLnull; Promega). Two days post transfection, the cells were stimulated for 6 h with P. gingivalis (moi = 10:1) and relative luciferase activity was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity, to correct for transfection efficiency. The results were then normalized to those of unstimulated control cells transfected with reporter and empty vectors, the value of which was taken as 1. Induction of cytokine (TNF-α and IL-6) production in stimulated cell culture supernatants was measured as previously described (Hajishengallis et al., 2006b) using specific ELISA kits (eBioscience).

Antibiotic protection-based intracellular survival assay

The capacity of phagocytosed P. gingivalis for intracellular persistence in macrophages was determined by an antibiotic protection-based survival assay, as we previously described (Wang et al., 2007). Briefly, viable counts (cfu) of internalized P. gingivalis were determined by plating serial dilutions of macrophage lysates on blood agar plates subjected to anaerobic culture. Prior to macrophage lysis, extracellular non-adherent bacteria were removed by washing, while extracellular adherent bacteria were killed by addition of gentamicin (300 μg ml⁻¹) and metronidazole (200 μg ml⁻¹).

Confocal microscopy

Confocal laser scanning microscopy was used to determine colocalization of P. gingivalis with lysosomes. For this purpose, J774A.1 macrophages, cultured on glass coverslips, were infected with FITC-labelled P. gingivalis (moi = 10:1) for 30 min, washed and incubation was allowed to proceed for an additional 90 min at 37°C. Subsequently, the cells were stained for 15 min with LysoTraker Red DND-99, a freely permeant and vital dye that stains acidified late endosomes and lysosomes (Basyuk et al., 2003). The cells were then fixed and imaged on an Olympus FV500 confocal microscope. Confocal microscopy was also used to demonstrate colocalization of P. gingivalis with the lipid raft marker, GM1 ganglioside. In this case, J774A.1 macrophages were exposed to FITC-labelled P. gingivalis (moi = 10:1), and infection was allowed to proceed for 15 min at 37°C. Unattached bacteria were removed by washing. After fixation and staining for GM1 with Alexa Fluor 594-labelled CTB (1 μg ml⁻¹; 15 min), the cells were imaged as above. Shown in Figs 2 and 4 are representative single optical sections as well as two-colour overlay (merge) confocal images. Where appropriate (Fig. 4), additional overlay with differential interference contrast (DIC) images is shown. The degree of colocalization of P. gingivalis with lipid rafts or lysosomes was quantified using the public domain Image J software with the Intensity Correlation Analysis plugin (NIH; http://rsb.info.nih.gov/ij).

MCD treatment and cholesterol reconstitution

To deplete macrophages of cholesterol using MCD and reconstituting cellular cholesterol in MCD-treated cells, we used a modification of previously published methodology (Christian et al., 1997; Lawrence et al., 2003). Briefly, J774A.1 macrophages were incubated in the presence of 10 mM MCD for 30 min at 37°C to deplete the cells of cholesterol. The cells were washed and incubated for an additional 30 min with medium only or with 1 mM cholesterol. Subsequently, the cells (MCD-treated, MCD-treated plus cholesterol-reconstituted, and cells treated with medium only) were used in functional assays. To confirm depletion of cholesterol by MCD, cellular cholesterol was quantified using the
fluorometric Amplex Red cholesterol assay kit (Molecular Probes/Invitrogen) and normalized to total protein concentration determined by the BCA Protein Assay (Pierce Biotechnology). The MCD concentration used in the study (10 mM) was decided based on preliminary experiments. These showed that the use of MCD at increasing concentrations (1–25 mM) reduced cellular cholesterol content in a dose-dependent manner, although concentrations > 10 mM did not extract significantly more cholesterol than the 10 mM dose.

**Statistical analysis**

Data were evaluated by analysis of variance and the Dunnett multiple-comparison test using the InStat program (GraphPad Software). Where appropriate (comparison of two groups only), two-tailed t-tests were also performed. Statistical differences were considered significant at the level of $P < 0.05$.

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