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Mechanism and implications of CXCR4-mediated integrin activation by *Porphyromonas gingivalis*

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SUMMARY

In monocytes and macrophages, the interaction of *Porphyromonas gingivalis* with Toll-like receptor 2 (TLR2) leads to the activation of a MyD88-dependent antimicrobial pathway and a phosphatidylinositol-3 kinase (PI3K) -dependent pro-adhesive pathway, which activates the β_2-integrin complement receptor 3 (CR3). By means of its fimbriae, *P. gingivalis* binds CXC-chemokine receptor 4 (CXCR4) and induces crosstalk with TLR2 that inhibits the MyD88-dependent antimicrobial pathway. In this paper, we investigated the impact of the *P. gingivalis*-CXCR4 interaction on the pro-adhesive pathway. Using human monocytes, mouse macrophages, or receptor-transfected cell lines, we showed that the binding of *P. gingivalis* fimbriae to CXCR4 induces CR3 activation via PI3K, albeit in a TLR2-independent manner. An isogenic strain of *P. gingivalis* expressing mutant fimbriae that do not interact with CXCR4 failed to efficiently activate CR3, leading to enhanced susceptibility to killing *in vivo* compared with the wild-type organism. This *in vivo* observation is consistent with previous findings that activated CR3 mediates safe entry of *P. gingivalis* into macrophages. Taken together with our previous work, these results indicate that the interaction of *P. gingivalis* with CXCR4 leads to inhibition of antimicrobial responses and enhancement of pro-adhesive responses, thereby maximizing its adaptive fitness in the mammalian host.

INTRODUCTION

Pattern recognition receptors expressed on sentinel immune cells detect microbial pathogens and initiate a complex set of signaling pathways (Kawai & Akira, 2010). The host-cell response represents the integration of activated signaling pathways that may result in synergistic or antagonistic effects (Natarajan *et al.*, 2006; Hajishengallis & Lambris, 2010). Therefore, signaling crosstalk may potentiate the host response or regulate it to prevent unwarranted inflammation. However, pathogens may also instigate crosstalk signaling for subverting or skewing the host response in ways that promote their virulence and survival (Hajishengallis & Lambris, 2011).

We have previously shown that *Porphyromonas gingivalis*, a keystone pathogen in periodontal disease (Hajishengallis *et al.*, 2011; Hajishengallis & Lamont, 2012), induces crosstalk between the CXC-chemokine receptor 4 (CXCR4) and Toll-like receptor 2 (TLR2) that undermines the killing function of human monocytes or mouse macrophages

(Hajishengallis *et al.*, 2008). Specifically, *P. gingivalis* induces co-association and activation of CXCR4 and TLR2 in membrane lipid rafts resulting in enhanced cAMP-dependent protein kinase A (PKA) signaling, which in turn inhibits the production of nitric oxide, a potent antimicrobial molecule (Hajishengallis *et al.*, 2008). Consistent with this, treatment of mice with a CXCR4 antagonist, the bicyclam drug AMD3100 (Donzella *et al.*, 1998), confers protection against periodontal tissue colonization by *P. gingivalis* and development of periodontitis (McIntosh & Hajishengallis, 2012).

The *P. gingivalis* virulence factor responsible for CXCR4 exploitation is its surface fimbriae, which comprise polymerized fimbrilin (FimA) associated with a complex of accessory proteins (FimCDE) (Nishiyama *et al.*, 2007; Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009). Specifically, the binding of *P. gingivalis* fimbriae to CXCR4 is mediated by the FimCDE complex, whereas mutant fimbriae **d**evoid of these **a**ccessory **p**roteins (dubbed DAP fimbriae) fail to interact with CXCR4 (Pierce *et al.*, 2009).

In monocytes and macrophages, TLR2 activation by *P. gingivalis* induces two distinct signaling pathways, a MyD88-dependent antimicrobial pathway and phosphatidylinositol-3 kinase (PI3K) -dependent pro-adhesive pathway (Hajishengallis *et al.*, 2009). The pro-adhesive pathway involves TLR2 inside-out signaling via Rac1, PI3K and cytohesin-1, leading to activation of complement receptor 3 (CR3; a β_2-integrin consisting of CD11b and CD18 subunits), which thereby assumes its high-affinity binding state (Harokopakis *et al.*, 2006; Hajishengallis *et al.*, 2009). Although the *P. gingivalis*-induced CXCR4–TLR2 crosstalk inhibits the TLR2/MyD88-dependent pathway (Hajishengallis *et al.*, 2008), its effect on the TLR2/CR3 pro-adhesive pathway is yet to be determined.

CR3 is not linked to strong microbicidal mechanisms (Caron & Hall, 1998; Lowell, 2006; Ricklin *et al.*, 2010) and is exploited by *P. gingivalis* and other pathogens as a safe portal of entry that permits enhanced intracellular survival (Wang *et al.*, 2007; Oliva *et al.*, 2009; Hajishengallis & Lambris, 2011). The objective of this study was to determine whether the interaction of *P. gingivalis* with CXCR4 exerts an impact on the pro-adhesive pathway. If so, this could constitute a second mechanism by which *P. gingivalis* exploits CXCR4 to promote its adaptive fitness.

METHODS

Reagents

Monoclonal antibodies (mAbs) to human CD11b [clone CBRM1/5, fluorescein isothiocyanate (FITC) -labeled; IgG1], to human/mouse CD11b (clone M1/70; IgG2b), or to mouse CXCR4 (clone 247506; IgG2b) and isotype controls were from R&D Systems (Minneapolis, MN). Immunoglobulin isotype controls were purchased from eBioscience (San Diego, CA). AMD3100 (CXCR4 inhibitor), phorbol myristate acetate (PMA), wortmannin (irreversible inhibitor of PI3K), LY294002 (reversible inhibitor of PI3K), LY30351 (inactive analog of LY294002), H89 (PKA inhibitor; blocks the ATP site of the enzyme), and GF109203X (inhibitor of protein kinase C; PKC) were from Sigma-Aldrich (St Louis, MO), and PKI 6-22 (PKA inhibitor; blocks the substrate site) was from Calbiochem-Millipore (Billerica, MA). Recombinant human or mouse intercellular adhesion molecule-1 (ICAM-1) was purchased from the R&D Systems. The small-molecule inhibitor XVA143 (molecular weight 585.35), which antagonizes CR3 (Shimaoka *et al.*, 2002; Harokopakis *et al.*, 2006), was kindly provided by N. Fotouhi (Roche, Nutley, NJ). *Porphyromonas gingivalis* ATCC 33277 and its isogenic mutant OZ5001C were grown anaerobically at 37°C in hemin-containing and menadione-containing Gifu anaerobic medium (GAM) medium (Nissui Pharmaceutical, Tokyo, Japan) (Wang *et al.*, 2007) and their fimbriae were extracted and chromatographically purified as previously described (Yoshimura *et al.*, 1984). The final fimbrial preparations were free of any contaminating substances on silver-stained sodium dodecyl sulfate—polyacrylamide gel electrophoresis, and tested negative for endotoxin (< 0.7 ng mg^{-1} protein) according to quantitative *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). Moreover, the purity of the fimbriae was confirmed using an Applied Biosystems 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Carlsbad, CA). All reagents were used at effective concentrations determined in preliminary experiments or in previous publications (Harokopakis *et al.*, 2006; Hajishengallis *et al.*, 2008; Pierce *et al.*, 2009).

Cell culture

Monocytes were purified from human peripheral blood upon centrifugation over NycoPrep™1.068 (Axis-Shield, Oslo, Norway) as previously described (Harokopakis & Hajishengallis, 2005). Incidental non-monocytes were removed by magnetic depletion using a cocktail of biotin-conjugated mAbs and magnetic microbeads coupled to anti-biotin mAb (Monocyte isolation kit II; Miltenyi Biotec, Auburn, CA). Purified monocytes were cultured at 37°C and 5% CO_2 atmosphere, in RPMI-1640 (Invitrogen/Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 0.05 mM 2-mercaptoethanol (complete RPMI). Human blood collections were conducted under institutional review board approval and in compliance with established federal guidelines. Chinese hamster ovary (CHO) cells stably transfected with human CR3 (CHO-CR3 cells) were kindly provided by Dr. D.T. Golenbock (University of Massachusetts Medical School, Worcester, MA) (Levitz *et al.*, 1997). These cells were cultured in Ham's F-12 nutrient mixture (Invitrogen/Gibco) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The CHO-CR3 cells were transiently transfected with TLR2 and/or CXCR4 using the PolyFect transfection reagent (Qiagen, Valencia, CA), as we previously described (Hajishengallis *et al.*, 2006, 2008; Pierce *et al.*, 2009). Briefly, CHO-CR3 cells were transfected with human TLR2 and CD14, using a single plasmid (pDUO-hCD14/TLR2; Invivogen), with or without co-transfection with human CXCR4 (pORF-hCXCR4; Invivogen). The total amount of plasmid DNA per well was kept constant by supplementing with empty control vector. The cells were used in functional assays 48 h after transfection. Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), as we previously described (Hajishengallis *et al.*, 2005b). Briefly, mice were intraperitoneally injected with 1 ml sterile 3% thioglycollate, and cells were harvested 5 days later by flushing the peritoneal cavity with 10 ml ice-cold phosphate-buffered saline (PBS) three times. Isolated cells were then subjected to density gradient centrifugation (Histopaque 1.083) to remove dead cells and red blood cell contamination. The purity of macrophage preparations (> 90%) was confirmed by flow cytometry using phycoerythrin-labeled anti-F4/80 (clone BM8; eBioscience). The macrophages were rested overnight (at 37°C and 5% CO_2 atmosphere in complete RPMI) before use in experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee, in compliance with established Federal and State policies. Cell viability was monitored using the CellTiter-Blue™ assay kit (Promega, Madison, WI). None of the experimental treatments affected cell viability compared with medium-only control treatments.

Binding assay for *P. gingivalis* fimbriae

Biotinylated wild-type or DAP fimbriae (1 μg ml^{-1}) were allowed to bind to human monocytes or human cell lines for 30 min at 37°C, as previously described (Harokopakis & Hajishengallis, 2005). Subsequently, the cells were washed and incubated on ice with FITC-labeled streptavidin. After washing, binding was determined by measuring cell-associated fluorescence (in relative fluorescence units) on a microplate fluorescence reader (FL600, Bio-Tek Instruments, Winoosski, VT) with excitation/emission wavelength settings of 485/530 nm. Background fluorescence was determined in cells treated with medium only and FITC-streptavidin.

CR3 activation assays

The CBRM1/5 epitope induction assay was used to monitor the activation state of human CR3 (CD11b/CD18), as we have previously described (Harokopakis & Hajishengallis, 2005). The assay is based on the property of the CBRM1/5 mAb to detect a conformational change on human CD11b that signifies the high-affinity binding state of CR3 (Diamond & Springer, 1993). Activation of mouse CR3 was assessed by monitoring its binding activity for soluble ICAM-1 (sICAM-1), a ligand that binds activated but not resting CR3 (Diamond *et al.*, 1993; Harokopakis *et al.*, 2006). Specifically, biotinylated sICAM-1 was allowed to bind to mouse macrophages for 30 min at 37°C. Subsequently, the cells were washed and incubated on ice with FITC-labeled streptavidin. After washing, binding was determined by measuring cell-associated fluorescence (in relative fluorescence

units) on a microplate fluorescence reader (FL600, Bio-Tek) with excitation/emission wavelength settings of 485/530 nm. Background fluorescence was determined in cells treated with medium only and FITC-streptavidin.

Intraperitoneal infection model

Specific pathogen-free BALB/cByJ mice (8–10 weeks old; The Jackson Laboratory) were pretreated with AMD3100 (intraperitoneally, 25 μg in 0.1 ml PBS) or PBS alone. After 1 h, the mice were infected intraperitoneally with *P. gingivalis* 33277 or OZ5001C (5×10^7 colony-forming units). Peritoneal lavage was performed 24 h after infection. Serial 10-fold dilutions of peritoneal fluid were plated on blood agar plates and cultured anaerobically at 37°C for enumerating recovered peritoneal colony-forming units. All animal procedures were approved by the Institutional Animal Care and Use Committee and performed in compliance with established Federal and State policies.

Statistical analysis

Data were evaluated by analysis of variance and the Dunnett multiple comparison test using the InStat program (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed *t*-tests were also performed. $P < 0.05$ was taken as the level of significance. All experiments were performed at least twice for verification.

RESULTS

The interaction of *P. gingivalis* fimbriae with CXCR4 induces CR3 activation

To determine the role of CXCR4 in *P. gingivalis* fimbria-induced activation of CR3 in human monocytes, we examined whether the CXCR4 antagonist AMD3100 inhibits the ability of fimbriae to induce an activation-specific epitope (CBRM1/5) (Diamond & Springer, 1993; Harokopakis & Hajishengallis, 2005). AMD3100 was used at 1 μg ml^{-1}, which completely inhibits the interaction of fimbriae with CXCR4 (Hajishengallis *et al.*, 2008). Induction of the CBRM1/5 epitope was evident at 10 min after stimulation with fimbriae, peaked at 30 min, and slowly declined thereafter, consistent with the transient nature of CR3 activation (Shimaoka *et al.*, 2002; Harokopakis & Hajishengallis, 2005). However, the induction of the CBRM1/5 epitope was suppressed in the presence of AMD3100 ($P < 0.01$; Fig. 1A), suggesting that CXCR4 contributes to pro-adhesive signaling for CR3 activation.

The notion that CXCR4 activates CR3 was further substantiated by experiments in mouse macrophages:

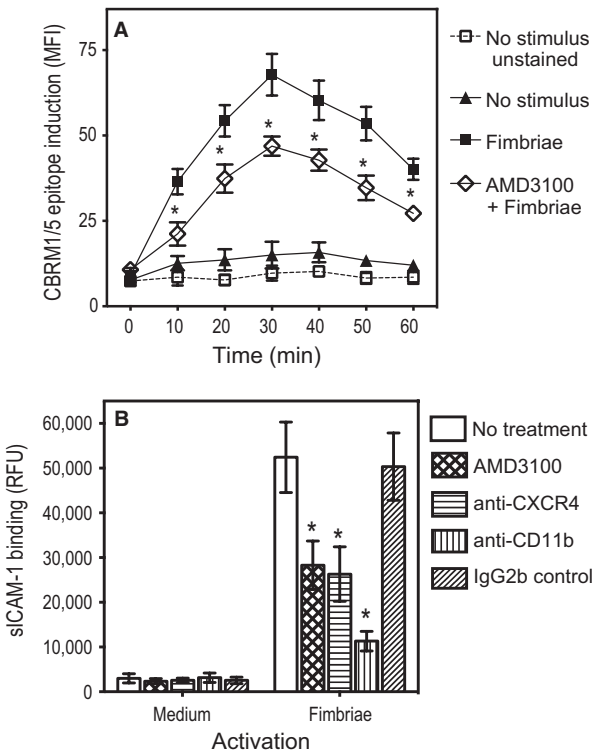

Figure 1 CXCR4 is involved in *Porphyromonas gingivalis* fimbria-induced CR3 activation. (A) Human monocytes were stimulated or not with fimbriae (1 μg ml^{-1}) for the indicated times, with or without AMD3100 (1 μg ml^{-1}), and assayed for CBRM1/5 epitope induction using fluorescein isothiocyanate (FITC) -labeled CBRM1/5 monoclonal antibody (mAb) and flow cytometry. FITC-labeled IgG1 control was used to assess background fluorescence ("unstained" group). CBRM1/5 induction is reported in mean fluorescence intensity (MFI) values. (B) Mouse peritoneal macrophages were activated with fimbriae (1 μg ml^{-1}), with or without AMD3100 (1 μg ml^{-1}) or anti-CXCR4 (5 μg ml^{-1}), and assayed for CR3-dependent binding of FITC-labeled soluble intercellular adhesion molecule-1 (sICAM-1) at 30 min following activation. CR3 dependence was confirmed by including groups treated with anti-CR3 (anti-CD11b) mAb or isotype control. The sICAM-1 binding is reported in relative fluorescent units (RFU). Data are means ± SD ($n = 3$). *Statistically significant ($P < 0.01$) inhibition of CBRM1/5 epitope induction (A) or of sICAM-1 binding (B).

CXCR4 blockade with AMD3100 or a blocking anti-CXCR4 mAb suppressed the ability of *P. gingivalis* fimbria-activated mouse macrophages to bind sICAM-1 (Fig. 1B), a ligand that binds activated but not resting CR3 (Diamond *et al.*, 1993; Hajishengallis *et al.*, 2009). Indeed, as expected, the binding of sICAM-1 to medium-treated macrophages was negligible (Fig. 1B). Moreover, a mAb to the CD11b subunit of CR3 inhibited the binding of sICAM-1 to fimbria-activated macrophages confirming the CR3 dependence of sICAM-1 binding (Fig. 1B). Taken together, these data show that *P. gingivalis* fimbriae induce CXCR4-dependent CR3 activation.

PI3K, but not TLR2, is involved in CXCR4-dependent CR3 activation by *P. gingivalis* fimbriae

We next examined whether the observed activating effect of CXCR4 on CR3 involved crosstalk with TLR2, as is the case for the inhibitory effect of CXCR4 on the MyD88-dependent antimicrobial response (Hajishengallis *et al.*, 2008, 2009). For this purpose, we used a recombinant TLR2 inside-out pro-adhesive signaling system developed in CHO cells stably transfected with human CR3 (CHO-CR3 cells) (Hajishengallis *et al.*, 2006). CHO cells lack functional endogenous TLR2 but express TLR1 and TLR6, either of which is capable of cooperative signaling with TLR2 (Heine *et al.*, 1999; Ozinsky *et al.*, 2000; Henneke *et al.*, 2001). Therefore, transfection of exogenous TLR2 in CHO-CR3 cells permits reconstitution of the TLR2 pro-adhesive signaling for CR3 activation (Hajishengallis *et al.*, 2006). Similarly, in these experiments, CHO-CR3 cells transfected with human TLR2 and its CD14 co-receptor acquired the capacity for CR3-dependent binding of sICAM-1, upon stimulation with purified fimbriae or whole cells of *P. gingivalis* (Fig. 2A and B, respectively). Consistent with the involvement of CXCR4 in CR3 activation (Fig. 1), co-transfection of CD14/TLR2 with CXCR4 further enhanced the ability of CHO-CR3 cells to bind sICAM-1 (Fig. 2). As expected, the CXCR4 effect was reversed by AMD3100 (Fig. 2). However, significant CR3-dependent binding of sICAM-1 was also seen in CHO-CR3 cells transfected with CXCR4 alone, i.e. without CD14/TLR2 co-transfection (Fig. 2). It therefore appeared that CXCR4 exerted an additive effect on CR3 activation independent of TLR2, ruling out the possibility that CXCR4 activates CR3 by potentiating TLR2 inside-out signaling.

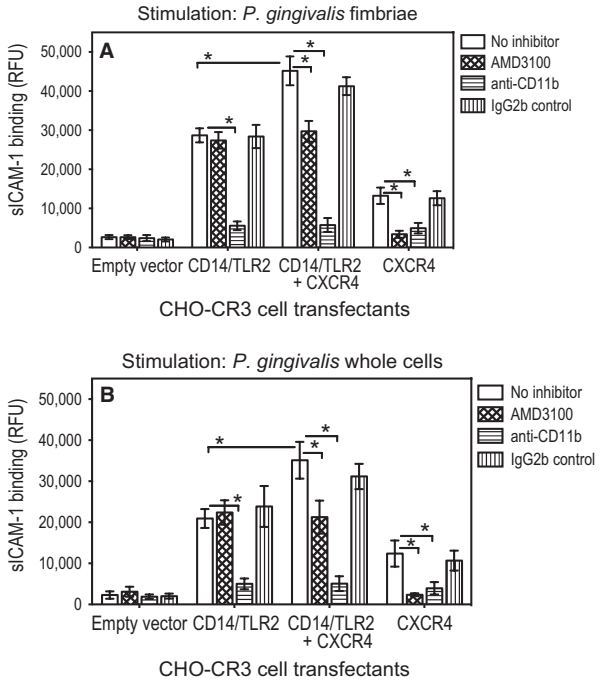

Figure 2 CR3-dependent binding activities of CHO-CR3 cells transfected with Toll-like receptor 2 (TLR2) and/or CXCR4. CHO-CR3 cells, transiently transfected with the indicated receptors or empty vector control, were activated with *Porphyromonas gingivalis* fimbriae (1 μg ml^{-1}) (A) or whole cells of *P. gingivalis* (multiplicity of infection = 10 : 1) (B) and assayed for CR3-dependent binding of fluorescein isothiocyanate-labeled soluble intercellular adhesion molecule-1 (sICAM-1) at 30 min following activation. CR3 dependence was confirmed by including groups treated with an anti-CR3 (anti-CD11b) monoclonal antibody or isotype control. The sICAM-1 binding is reported in relative fluorescent units (RFU). Data are means ± SD ($n = 3$). *Statistically significant ($P < 0.01$) differences between the indicated groups.

Several kinases, such as PI3K, PKA and PKC, have been implicated in integrin activation (Nagel *et al.*, 1998; Luo *et al.*, 2007). The ability of *P. gingivalis* fimbriae to induce CXCR4-dependent activation of CR3 was not reversed by inhibitors of PKA (H89 and PKI 6-22) or PKC (GF109203X), but was significantly ($P < 0.01$) inhibited by inhibitors of PI3K, specifically wortmannin and LY294002 (but not by the inactive analog LY303511) (Fig. 3A). The inhibitory effects of wortmannin and LY294002 could not be attributed to non-specific toxic effects, because both compounds failed to affect PMA-induced CR3 activation, which – as expected – was inhibited by the PKC inhibitor GF109203X ($P < 0.01$; Fig. 3B). In summary,

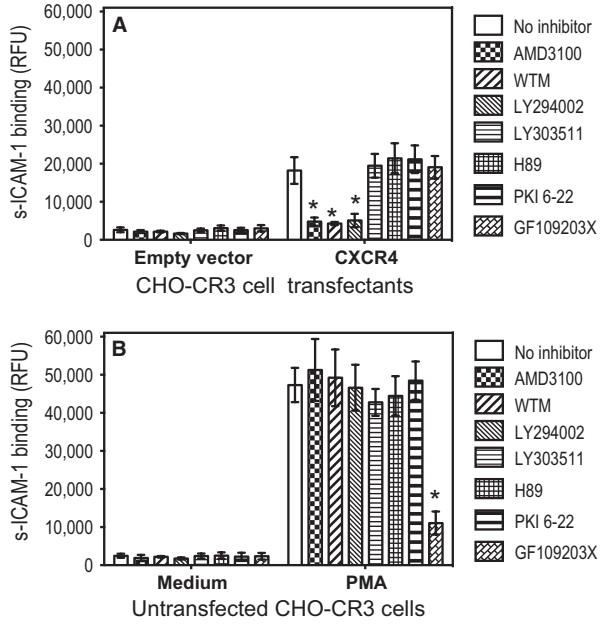

Figure 3 CXCR4-dependent activation of CR3 is mediated by phosphatidylinositol-3 kinase (PI3K). (A) CHO-CR3 cells, transiently transfected with CXCR4 or empty vector control, were stimulated with *Porphyromonas gingivalis* fimbriae (1 μg ml^{-1}) and assayed for binding fluorescein isothiocyanate-labeled soluble intercellular adhesion molecule-1 (sICAM-1) at 30 min following activation. Before stimulation, the cells were pretreated for 30 min with AMD3100 (1 μg ml^{-1}), wortmannin (WTM; 50 nM), LY294002 (20 μM), LY30351 (20 μM), H89 (5 μM), PKI 6-22 (1 μM), or GF109203X (10 μM). (B) Similar experiment in untransfected CHO-CR3 cells which were stimulated with phorbol myristate acetate (PMA; 0.1 μg ml^{-1}) or medium-only control. The sICAM-1 binding is reported in relative fluorescent units (RFU). Data are means ± SD ($n = 3$). *Statistically significant ($P < 0.01$) inhibition of of sICAM-1 binding.

the data from Figs 1–3 show that *P. gingivalis* fimbriae induce CXCR4-dependent and PI3K-mediated activation of CR3.

Comparative interactions of wild-type and DAP fimbriae with CXCR4 and CR3

We previously showed that a mutant form of *P. gingivalis* fimbriae that lacks FimCDE (DAP fimbriae) fails to interact with CXCR4 (Pierce *et al.*, 2009). Moreover, unlike wild-type fimbriae, DAP fimbriae interacted relatively poorly with CR3, although the molecular basis of this observation was not addressed (Wang *et al.*, 2007). In view of the role of CXCR4 in fimbria-induced CR3 activation (Figs. 1 and 2), we hypothesized that the poor interaction of DAP fimbriae with CR3 could be attributed to their inability to bind CXCR4, rather than to an inherent defect preventing CR3 binding. To address this hypothesis, we first compared the abilities of wild-type and DAP fimbriae to bind PMA-treated CHO-CR3 cells. PMA activates CR3 independently of receptor interactions and inside-out signaling (not functional in untransfected CHO-CR3 cells) (Harokopakis & Hajishengallis, 2005; Hajishengallis *et al.*, 2006). Wild-type and DAP fimbriae bound comparably to PMA-activated CHO-CR3 cells in a CR3-dependent way (Fig. 4A), indicating that DAP fimbriae do not have any defect that prevents their binding to CR3.

This finding lent support to the hypothesis that the poor interaction of DAP with CR3 in normal cells (which have not been treated with PMA and CR3 activation would depend on inside-out signaling) reflects their inability to optimally activate CR3. Indeed, in human monocytes, DAP fimbriae showed significantly reduced ability to activate CR3 compared with wild-type fimbriae ($P < 0.01$; Fig. 4B). In the same cells, AMD3100 significantly ($P < 0.01$) suppressed CR3 activation by wild-type fimbriae but not by DAP fimbriae (Fig. 4B), further confirming that wild-type but not DAP fimbriae interact with CXCR4 to activate CR3. Moreover, in contrast to wild-type fimbriae, DAP fimbriae failed to induce CR3 activation in CXCR4-transfected CHO-CR3 cells (Fig. 4C). Therefore, the differential abilities of wild-type and DAP fimbriae to activate CR3 can be attributed, at least in large part, to the inability of DAP fimbriae to bind CXCR4, which contributes to CR3 activation.

Combined inhibition of CXCR4 or CR3 *in vivo* further promotes the killing of *P. gingivalis*

Porphyromonas gingivalis exploits both CXCR4 (Hajishengallis *et al.*, 2008) and CR3 (Hajishengallis *et al.*, 2007) to resist killing *in vivo*, and therefore blockade of either CXCR4 or CR3 with specific receptor antagonists [AMD3100 (Hajishengallis *et al.*, 2008) or XVA143 (Hajishengallis *et al.*, 2007), respectively] promotes the ability of the mouse host to clear intraperitoneal infection with *P. gingivalis*. However, whether combined treatment with AMD3100 and XVA143 achieves a greater protective effect against *P. gingivalis* in this model was not previously examined. This question was addressed in parallel with experiments using an isogenic mutant (strain

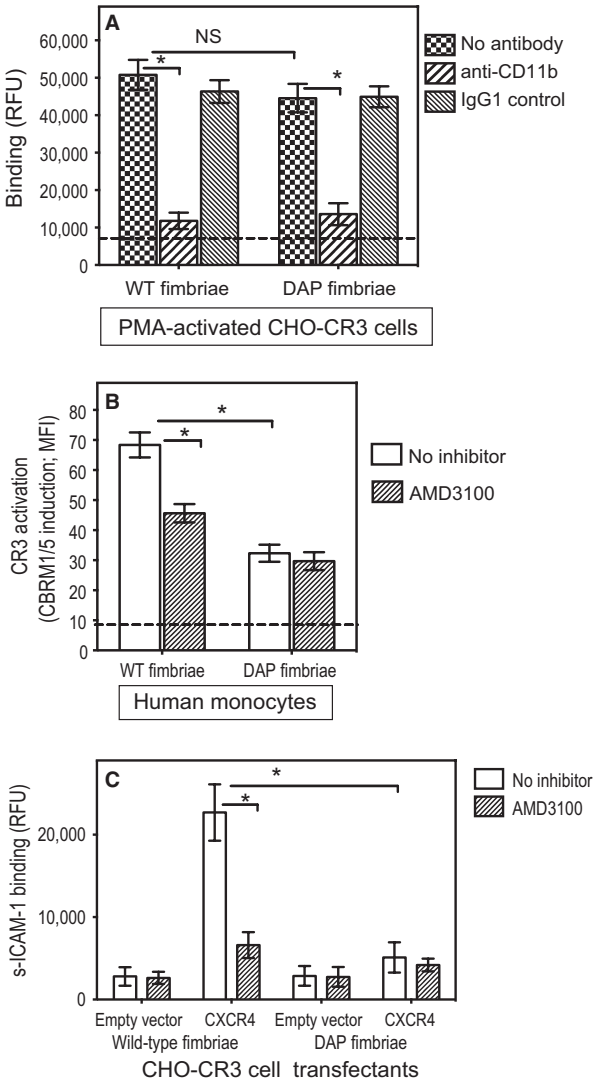

Figure 4 DAP fimbriae bind pre-activated CR3 but do not efficiently activate CR3. (A) CHO-CR3 cells were pre-activated with phorbol myristate acetate (PMA; 0.1 μg ml^{-1}) and assayed for binding wild-type (WT) or DAP fimbriae (both at 1 μg ml^{-1}) after a 30-min incubation, in the absence or presence of anti-CD11b monoclonal antibody or IgG1 isotype control. (B) Human monocytes were stimulated with WT or DAP fimbriae (both at 1 μg ml^{-1}) with or without AMD3100 (1 μg ml^{-1}) and assayed for CBRM1/5 epitope induction at 30 min following activation. CBRM1/5 induction is reported in mean fluorescence intensity (MFI) values. The horizontal dashed lines indicate background binding (6943 ± 892 RFU) to empty vector-transfected cells (A) or baseline CBRM1/5 induction (8.8 ± 1.7 MFI) in unstimulated cells (B). (C) CHO-CR3 cells, transiently transfected with CXCR4 or empty vector control, were stimulated with WT or DAP fimbriae (both at 1 μg ml^{-1}) with or without AMD3100 (1 μg ml^{-1}) and after 30 min were assayed for binding of fluorescein isothiocyanate-labeled soluble intercellular adhesion molecule-1 (sICAM-1). Data are means ± SD (n = 3). *Statistically significant ($P < 0.01$) differences between the indicated groups. NS, not significant.

OZ5001C) expressing DAP fimbriae, which – as alluded to above – do not mediate efficient exploitation of the receptors under investigation.

At 24 h after infection, the recovery of the OZ5001C mutant (viable colony-forming unit counts) from the peritoneal cavity of PBS-pretreated mice was significantly ($P < 0.01$) lower compared with wild-type *P. gingivalis* (Fig. 5), suggesting that OZ5001C is not as capable as the wild-type strain in resisting killing. As expected, pretreatment of mice with either AMD3100 or XVA143 promoted the killing of wild-type *P. gingivalis* (Fig. 5). Importantly, combined pretreatment with AMD3100 and XVA143 resulted in significantly ($P < 0.01$) enhanced killing of *P. gingivalis* compared with either antagonist alone (Fig. 5). None of these antagonist treatments could significantly affect the killing of the OZ5001C mutant (Fig. 5), reflecting the limited interactions of this strain with the receptors involved. In summary, the expression of fully functional (i.e. wild-type) fimbriae is important for the ability of *P. gingivalis* to productively exploit both CXCR4 and CR3 and thereby effectively promote its survival in the mammalian host.

DISCUSSION

An imbalance either in the composition of the periodontal microbiota (dysbiosis) or in local regulatory

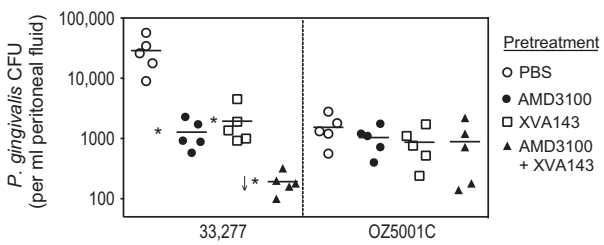

Figure 5 *Porphyromonas gingivalis* expressing DAP fimbriae does not exploit CXCR4 or CR3. BALB/cByJ mice were intraperitoneally pretreated with AMD3100 [25 μg in 0.1 ml phosphate-buffered saline (PBS)], XVA143 (15 μg in 0.1 ml PBS), both AMD3100 and XVA143, or PBS alone. After 1 h, the mice were intraperitoneally infected with 5×10^7 colony-forming units (CFU) *P. gingivalis* 33277 or OZ5001C (FimCDE-deficient isogenic mutant). Peritoneal lavage was performed 24 h after infection. Serial 10-fold dilutions of peritoneal fluid were plated for anaerobic growth and enumeration of recovered CFU. Horizontal lines show mean CFU counts. Asterisks indicate significant ($P < 0.01$) differences in *P. gingivalis* peritoneal CFU between PBS-treated mice and mice treated with receptor antagonists. The arrow sign shows significant ($P < 0.01$) difference between dual and single antagonist treatments. The difference between 33277 and OZ5001C CFU in PBS-pretreated mice is statistically significant ($P < 0.01$).

mechanisms that control inflammatory cell recruitment can precipitate pathological periodontal inflammation (Eskan *et al.*, 2012; Hajishengallis *et al.*, 2012). *Porphyromonas gingivalis* appears to be one of the causes of periodontal dysbiosis through its ability to subvert the host response in ways that may benefit also bystander bacterial species (Hajishengallis & Lamont, 2012). One of the receptors exploited by *P. gingivalis* to inhibit macrophage killing is CXCR4 (Hajishengallis *et al.*, 2008), the pharmacological inhibition of which blocks experimental periodontitis in a mouse model (McIntosh & Hajishengallis, 2012). In this paper we demonstrated a second CXCR4-dependent mechanism by which *P. gingivalis* undermines the host response to its own benefit. Specifically, in addition to hijacking CXCR4 to interfere with the TLR2 antimicrobial response (Hajishengallis *et al.*, 2008), *P. gingivalis* was now shown to exploit CXCR4 to activate CR3, which mediates safe internalization of this pathogen by macrophages (Wang *et al.*, 2007) (Fig. 6). Combined inhibition of CXCR4 and CR3 resulted in enhanced killing of *P. gingivalis in vivo* compared with single inhibition of either receptor.

Rac1 is a key signaling component of the TLR2 pro-adhesive pathway leading to CR3 activation (Harokopakis *et al.*, 2006). Since the signaling activity of Rac1 may be enhanced by PKA (O'Connor & Mercurio, 2001), the ability of *P. gingivalis* fimbriae to activate PKA through CXCR4 (Hajishengallis *et al.*, 2008) could potentially lead to enhanced Rac1 signaling and hence to potentiation of TLR2-induced CR3 activation. However, our results showed that the ability of CXCR4 to contribute to CR3 activation in cells exposed to purified fimbriae or whole cells of *P. gingivalis*, does not involve a crosstalk between CXCR4 and TLR2. Rather, CXCR4 independently activates CR3 and additively contributes to TLR2-induced CR3 activation (Fig. 6).

Stimulation of CXCR4 by its physiological ligand CXCL12 (also known as stromal cell-derived factor-1) can activate integrins and initiate firm adhesion of rolling leukocytes (Campbell *et al.*, 1998; Constantin *et al.*, 2000). Therefore, by activating the CR3 integrin through interaction with CXCR4, *P. gingivalis* actually mimics a physiological process, which thereby comes under the control of the pathogen.

Optimal CXCR4 and CR3 exploitation by *P. gingivalis* requires wild-type fimbriae, comprising FimA and the FimCDE accessory proteins. The relative inability of DAP fimbriae to interact with CR3 (Wang

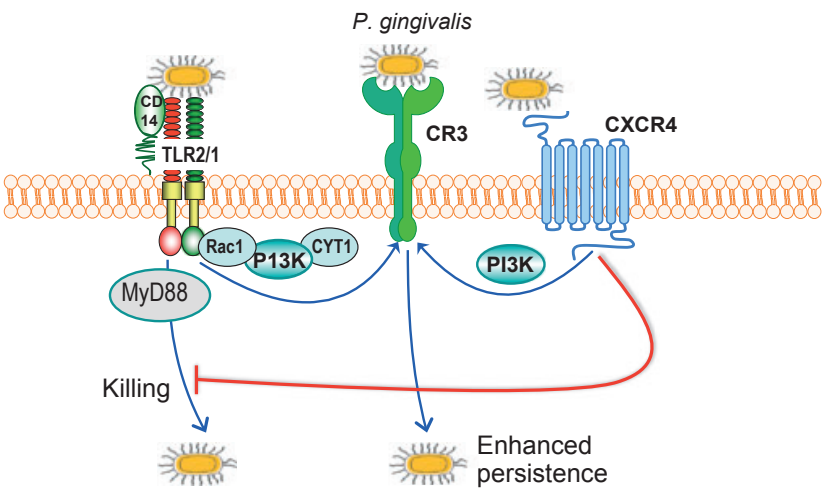

Figure 6 Exploitation of CXCR4 by *Porphyromonas gingivalis.* In macrophages, *P. gingivalis* interacts with CD14 and the Toll-like receptor 2 (TLR2)/TLR1 signaling complex resulting in inside-out signaling for activating and binding CR3, which leads to a relatively 'safe' uptake of these organisms by macrophages (Hajishengallis *et al.*, 2006, 2007; Wang *et al.*, 2007). The signaling pathway that activates the high-affinity state of CR3 is mediated by Rac1, PI3K and cytohesin 1 (Cyt1) (Harokopakis & Hajishengallis, 2005; Harokopakis *et al.*, 2006; Hajishengallis *et al.*, 2009). The *P. gingivalis*-activated TLR2/TLR1 also induces a MyD88-dependent pathway that can potentially promote the killing of this bacterium (Hajishengallis *et al.*, 2008, 2009). However, by means of its fimbriae, *P. gingivalis* instigates a crosstalk between CXCR4 and TLR2 which interferes with this antimicrobial mechanism (Hajishengallis *et al.*, 2008). In this study, *P. gingivalis* was shown to also use CXCR4 to induce phosphatidylinositol-3 kinase (PI3K) -dependent activation of CR3, independently of TLR2, which further contributes to its capacity to evade killing. CXCR4 exploitation requires fully functional fimbriae, i.e. containing both the FimA and FimCDE components, which can directly bind CXCR4 (Pierce *et al.*, 2009).

et al., 2007) could not be attributed to intrinsic defects of DAP fimbriae for CR3 binding, but rather to lack of interaction with CXCR4. Indeed, DAP fimbriae could bind pre-activated CR3, consistent with our previous identification of CR3-binding epitopes in the FimA peptide sequence (Hajishengallis *et al*., 2005a).

The inability of DAP fimbriae to interact with CXCR4 and thereby efficiently activate CR3 resulted in poor interactions with this integrin. Consequently, *P. gingivalis* expressing DAP fimbriae (OZ5001C) was more susceptible to *in vivo* killing, being unable to exploit either CXCR4 or CR3, as shown by the experiments using antagonists of these receptors (AMD3100 and XVA143, respectively) alone or in combination. In contrast, wild-type *P. gingivalis* used both receptors to promote its *in vivo* survival. We previously showed that CXCR4 crosstalks with complement C5a receptor (C5aR) in *P. gingivalis*-challenged macrophages and *P. gingivalis* exploits this crosstalk to evade killing (Wang *et al*., 2010). Therefore, CXCR4 blockade would likely affect the ability of *P. gingivalis* to crosstalk with both CR3 and C5aR. On the other hand, OZ5001C still expresses gingipains that are required for C5aR exploitation (Wang *et al*., 2010) and this could provide a degree of protection, consistent with the relatively modest persistence of this strain in the intraperitoneal infection model. Apparently, the ability of wild-type *P. gingivalis* to integrate subversive crosstalk signaling involving several receptors maximizes its adaptive fitness.

Taken together with our previous results (Hajishengallis *et al*., 2008), our current findings support the concept that *P. gingivalis* exploits CXCR4 for inhibiting antimicrobial responses and promoting pro-adhesive activities, both of which lead to impaired host defense against this keystone pathogen (Darveau *et al*., 2012) (Fig. 6). These CXCR4-dependent evasive tactics can potentiate the impact of *P. gingivalis* on periodontal disease, a notion that is consistent with our recent report that CXCR4 blockade inhibits *P. gingivalis*-induced periodontitis in a mouse model (McIntosh & Hajishengallis, 2012).

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