Complement Receptor 3 Blockade Promotes IL-12-Mediated Clearance of *Porphyromonas gingivalis* and Negates Its Virulence In Vivo

George Hajishengallis, Muhamad-Ali K. Shakhatreh, Min Wang and Shuang Liang

*J Immunol* 2007; 179:2359-2367; doi: 10.4049/jimmunol.179.4.2359

http://www.jimmunol.org/content/179/4/2359

References
This article cites 61 articles, 31 of which you can access for free at:
http://www.jimmunol.org/content/179/4/2359.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Complement Receptor 3 Blockade Promotes IL-12-Mediated Clearance of Porphyromonas gingivalis and Negates Its Virulence In Vivo

George Hajishengallis, Muhamad-Ali K. Shakhatreh, Min Wang, and Shuang Liang

The ability of certain pathogens to exploit innate immune function allows them to undermine immune clearance and thereby increase their persistence and capacity to cause disease. Porphyromonas gingivalis is a major pathogen in periodontal disease and is associated with increased risk of systemic conditions. We have previously shown that the fimbriae of P. gingivalis interact with complement receptor 3 (CR3; CD11b/CD18) in monocytes/macrophages, resulting in inhibition of IL-12p70 production in vitro. The in vivo biological implications of this observation were investigated in this study using a CR3 antagonist (XVA143). XVA143 was shown to block CR3 binding of P. gingivalis fimbriae and reverse IL-12p70 inhibition; specifically, CR3 blockade resulted in inhibition of ERK1/2 phosphorylation and up-regulation of IL-12 p35 and p40 mRNA expression. Importantly, mice pretreated with XVA143 elicited higher IL-12p70 and IFN-γ levels in response to P. gingivalis i.p. infection and displayed enhanced pathogen clearance, compared with similarly infected controls. The notion that CR3 is associated with reduced IL-12p70 induction and impaired P. gingivalis clearance was confirmed using i.p. infected wild-type and CR3-deficient mice. Moreover, XVA143 dramatically attenuated the persistence and virulence of P. gingivalis in experimental mouse periodontitis, as evidenced by reduced induction of periodontal bone loss. Therefore, CR3 blockade may represent a promising immunomodulatory approach for controlling human periodontitis and possibly associated systemic diseases. The Journal of Immunology, 2007, 179: 2359–2367.

The β2 integrins are heterodimeric receptors consisting of a common β subunit (CD18) associated noncovalently with a unique α subunit (CD11a, b, c, or d) (1). These receptors orchestrate crucial processes involved in immunity and inflammation and, in this context, mediate cell-cell, cell-extracellular matrix, and cell-pathogen interactions (2, 3). To integrate the intracellular and extracellular environments, β2 integrins use inside-out and outside-in bidirectional signaling. Inside-out signaling refers to dynamic regulation of their adhesive activity from within the cell via signals generated by other receptors, whereas outside-in signaling refers to the ability of the activated integrin to respond to ligands by inducing downstream intracellular signaling (1). CD11b/CD18 is also known as complement receptor 3 (CR3) and is abundantly expressed by phagocytic leukocytes (2). CR3 has the capacity to interact with a wide variety of structurally unrelated molecules derived from either the host (e.g., ICAM-1, fibrinogen, and the complement fragment iC3b) or pathogens (e.g., Bordetella pertussis filamentous hemagglutinin and Leishmania gp63) (4–6). The ligand-binding promiscuity of CR3 suggests that it may function as a pattern-recognition receptor (PRR) and, in this regard, CR3 clusters with other PRRs (e.g., CD14 and TLRs) in lipid rafts of activated cells (7, 8).

We have recently described a novel inside-out signaling pathway in human monocytes or mouse macrophages activated by Porphyromonas gingivalis (9, 10), an oral pathogen that is strongly associated with periodontal disease and implicated in atherosclerosis (11, 12). This proadhesive pathway is initiated when P. gingivalis fimbriae interact with the CD14/TLR2 recognition/signaling complex, leading to PI3K-mediated induction of the high-affinity conformation of CR3 (9, 10, 13). Activation of this TLR2 inside-out signaling pathway by P. gingivalis or purified fimbriae leads to enhanced CR3-dependent monocyte adhesion and transendothelial migration (10). However, additional work by our group has shown that P. gingivalis has co-opted this proadhesive pathway for CR3 binding, intracellular entry, and inhibition of IL-12p70 (13–15) (Fig. 1A), a heterodimeric cytokine composed of disulfide-linked p35 and p40 subunits (16). We have thus speculated that CR3 may be exploited by P. gingivalis for undermining IL-12-mediated bacterial clearance, thereby promoting its virulence and capacity to cause disease. Interestingly, several pathogens have developed distinct mechanisms for IL-12 suppression (5, 17–20). This may represent an effective microbial strategy for immune evasion because production of IL-12p70 by macrophages is a key event in host defense against infection (16). Indeed, IL-12p70 activates T and NK cells to produce IFN-γ, which in turn activates the bactericidal function of macrophages (16).

Because macrophages play important roles at the front line of host defense while at the same time constituting targets of pathogens aiming to subvert immune surveillance (21), the elucidation of macrophage-pathogen interactions is essential to understanding the pathogenesis of many infection-driven diseases, including periodontitis. The notion that macrophage CR3 function may be exploited by P. gingivalis for undermining IL-12-mediated bacterial clearance seems paradoxical at first, given that CR3 can potentially

*Division of Oral Health and Systemic Disease/Department of Periodontics, and "Department of Microbiology and Immunology, University of Louisville Health Sciences Center, Louisville, KY 40292
Received for publication May 17, 2007. Accepted for publication June 13, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by U.S. Public Health Service Grants DE015254 and DE018292 (to G.H.) from the National Institutes of Health.
2 Address correspondence and reprint requests to Dr. George Hajishengallis, University of Louisville Health Sciences Center, 501 South Preston Street, Room 206, Louisville, KY 40292. E-mail address: ghaj01@louisville.edu
3 Abbreviations used in this paper: CR3, complement receptor 3; ABC, alveolar bone crest; CEJ, cementoenamel junction; CHO, Chinese hamster ovary; PRR, pattern-recognition receptor.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
not be activated by fimbria-induced TLR2 inside-out signaling (13). PMA (0.1 μg/ml) was used to activate CR3 in the binding assay.

**Cell activation assays**

Human monocytes or mouse macrophages were stimulated with *P. gingivalis* fimbriae or *E. coli* LPS, as outlined in the figure legends, and induction of cytokine release (TNF-α, IL-1β, IL-6, IL-8, and IL-12p70) in culture supernatants was measured by ELISA using kits from eBioscience or Cell Sciences. Total and phosphorylated ERK1/2 was determined using total ERK1/2 and Phospho Detect ERK1/2 (pTyr185/187) ELISA kits from Calvinbo.

**Real-time PCR**

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen Life Technologies) and quantified by spectrometry at 260 and 280 nm. First strand cDNA synthesis was performed using the High-Capacity cDNA Archive kit (Applied Biosystems). Real-time PCR with cDNA was performed with an ABI 7500 system (Applied Biosystems). TaqMan probes, sense primers, and antisense primers for gene expression of human IL-12p35 and IL-12p40 or a housekeeping gene (GAPDH) were purchased from Applied Biosystems. The reactions were performed, according to the manufacturer’s protocol. Relative expression level was determined by normalization to the housekeeping gene.

**Confocal microscopy**

To demonstrate colocalization of *P. gingivalis* with TLR2 and CR3, mouse macrophages were grown on chamber slides (Lab-Tek) and were exposed to FITC-labeled *P. gingivalis* for 20 min. Immediately afterward, the cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were then stained with Texas Red-labeled anti-CD11b mAb (3A3; Abcam) and allophycocyanin-labeled anti-TLR2 mAb (6C2; eBioscience) and mounted with cover slips before imaging on an Olympus FV500 confocal microscope.

**In vivo mouse studies**

**Peritonitis model.** Specific pathogen-free BALB/cByJ mice (8–10 wk old; The Jackson Laboratory) were pretreated by i.p. injection of XVA143 (0.5 ml of 50 μM) or PBS alone. After 1 h, the mice were infected i.p. with *P. gingivalis* 33277 (5 × 10⁶ CFU). Peritoneal lavage was performed 20 h postinfection. Serial 10-fold dilutions of peritoneal fluid were plated onto blood agar plates supplemented with hemin/menadione and cultured anaerobically for enumerating recovered peritoneal CFU. The peritoneal fluid was also used to measure IL-12p70 and IFN-γ production by ELISA. In a separate, modified experiment, mice were previously given i.p. polyclonal anti-mouse IL-12 IgG (0.1 mg/mouse) or equal amount of nonimmune IgG. Similar procedures were followed to determine *P. gingivalis* clearance from the peritoneal cavity of infected wild-type or CR3-deficient (CD11b−/−) C57BL/6 mice (The Jackson Laboratory).

**Periodontitis model.** Ten-week-old BALB/cByJ mice were orally infected with *P. gingivalis* American Type Culture Collection 33277 for induction of periodontal bone loss, according to the Baker et al. model (34). To determine the role of CR3 blockade on *P. gingivalis*-induced bone loss, the mice were administered XVA143 or PBS control through microinjection. Specifically, XVA143 was injected using a 28.5-gauge MicroFine needle (BD Biosciences) into the palatal gingivae, on the mesial of the first molar (1 ml of 1 mM solution per site; total of six sites). XVA143 was administered five times at 2-day intervals (days 1, 3, 5, 7, and 9). Each administration of XVA143 or PBS preceded, by 1 day, oral infection with 10⁶ CFU *P. gingivalis* 33277 in 2% carboxymethylcellulose or with carboxymethylcellulose vehicle alone (sham infection), also given five times at 2-day intervals (i.e., days 2, 4, 6, 8, and 10). Therefore, four groups were used (PBS pretreated/sham infected; PBS pretreated/*P. gingivalis* infected; XVA143 pretreated/sham infected; and XVA143 pretreated/*P. gingivalis* infected). In each comprising the final five mice. Six weeks after the final infection, mice were euthanized with CO₂ inhalation. Assessment of periodontal bone loss in defleshed maxillae was performed under a dissecting microscope (×40) fitted with a video image marker measurement system (model VIA-170K; Fryer) standardized for measurements in millimeters (mm). Specifically, the distance from the cementoenamel junction (CEJ) to the marginal bone crest (ABC) was measured on 14 predetermined points along the palatal surfaces of the maxillary molars (34). To calculate periodontal bone loss, the 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance from the group of sham-infected
FIGURE 1. *P. gingivalis* colocalizes with TLR2 and CR3 and exploits these interactions to undermine innate immunity. *A. P. gingivalis* exploits its fimbrial-mediated interaction with macrophage TLR2 to activate the high-affinity conformation of CR3 within minutes. This pathway proceeds through Rac1/PI3K-mediated inside-out signaling and requires CD14 for facilitating the fimbria-TLR2 interaction (9, 10). TLR2 stimulation by *P. gingivalis* also induces NF-kB activation and cytokine production (8, 14). Upon CR3 activation, fimbriated *P. gingivalis* can readily interact with CR3, leading to outside-in signaling, involving ERK1/2, which down-regulates IL-12 p35 and p40 and consequently inhibits IL-12p70 production (this study). In vivo evidence presented in this study indicates that exploitation of CR3 function by *P. gingivalis* undermines IL-12-mediated immune clearance, leading to persistence of the pathogen and enhanced virulence in the mouse periodontitis model. *B. Mouse macrophages were exposed to FITC-labeled *P. gingivalis* for 20 min. After cell fixation and staining with allophycocyanin-labeled anti-TLR2 mAb and Texas Red-labeled anti-CD11b mAb, the cells were examined by confocal microscopy. The merge images demonstrate colocalization of *P. gingivalis* with both TLR2 and CR3.*

Results

**Inhibition of *P. gingivalis* fimbria-CR3 interaction and up-regulation of IL-12p70 by a CR3 antagonist**

*P. gingivalis* fimbriae stimulate TLR2 inside-out signaling for activation and binding of CR3 (CD11b/CD18) (9, 13), which is consistent with the observation that this pathogen colocalizes with both PRRs in macrophages (Fig. 1B). To determine the biological implications of CR3 binding by *P. gingivalis* fimbriae, we used XVA143 (m.w. 585.35), an allosteric antagonist that binds CD18 and blocks transmission of activation signals to the ligand-binding domain of CD11b (35). Although we have previously shown that XVA143 blocks the binding activity of human or mouse CR3 for ICAM-1 or fibrinogen (10), it was essential to determine whether XVA143 can similarly inhibit CR3 binding of *P. gingivalis* fimbriae. The fimbriae could readily bind CR3-expressing, but not CR1-expressing CHO cells; however, XVA143 inhibited binding by >80% (*p < 0.05*) and its effect was comparable to a blocking anti-CD11b mAb (Fig. 2). We next examined the ability of XVA143 to antagonize CR3-dependent biological activities of *P. gingivalis* fimbriae. Because *P. gingivalis* fimbriae inhibit production of IL-12p70 by interacting with CR3, we have investigated the ability of XVA143 to reverse this potential immune evasion effect. For this purpose, human macrocytes were stimulated with fimbriae (1 μg/ml) in the absence or presence of XVA143 (1 μM). XVA143 partially blocked fimbria-induced TNF-α, IL-1β, IL-6, or IL-8 (*p < 0.05*; Fig. 3, A–D), but exerted an up-regulating effect on IL-12p70 production (Fig. 3E). In a separate experiment in which monocytes were previously primed with IFN-γ (0.1 μg/ml) to enhance IL-12p70 induction, XVA143 maintained the ability to further up-regulate fimbria-induced IL-12p70 production by >5-fold (*p < 0.05*; Fig. 3F). Similar results were obtained using mouse macrophages (data not shown). These findings suggest that XVA143 reverses the inhibitory effect of the *P. gingivalis* fimbria-CR3 interaction on IL-12p70 induction.

**The *P. gingivalis* fimbria-CR3 interaction down-regulates IL-12 p35 and p40 through ERK1/2 signaling**

We next investigated the effect of XVA143 on expression of the individual IL-12 p35 and p40 subunits at the mRNA level. Using real-time PCR and XVA143-pretreated monocytes exposed to *P. gingivalis* fimbriae, we found that XVA143 enhanced induction of both IL-12 p35 and p40 subunits compared with XVA143-non-pretreated (PBS) controls (*p < 0.05*; Fig. 4, A and B). Because IL-12 is controlled by ERK1/2 signaling in several experimental systems (36, 37), we next determined the IL-12 regulatory effect of
PD98059 (20 μM), which inhibits the activation (phosphorylation) of ERK1/2 (36). Similarly to XVA143, PD98059 exerted a potent up-regulating effect on the expression of both IL-12 p35 and p40 mRNA and on the production of IL-12p70 protein (p < 0.05 compared with 0.1% DMSO vehicle control; Fig. 4, C–E). In contrast, pretreatment with a neutralizing dose of anti-IL-10 mAb resulted in modest up-regulation of IL-12p70, at levels 30% of those seen with PD98059 (Fig. 4 F). This suggests that IL-12 down-regulation by the P. gingivalis fimbria-CR3 interaction is only partially attributable to endogenously produced IL-10. We next showed that ERK1/2 is activated by P. gingivalis fimbriae in a CR3-dependent way. Indeed, fimbriae induced significant phosphorylation of ERK1/2, which was reversible by CR3 blockade using XVA143 (p < 0.05; Fig. 4 G). None of the treatments influenced total ERK1/2 levels (Fig. 4 G). The Fig. 4 data collectively suggest that the interaction of P. gingivalis fimbriae with CR3 induces ERK1/2 phosphorylation leading to down-regulation of IL-12 p35 and p40 subunits, and suppression of bioactive (p70) IL-12 production.

FIGURE 3. CR3 blockade in human monocytes inhibits release of several cytokines, but enhances IL-12p70 in response to P. gingivalis fimbriae. Human monocytes were pretreated with or without XVA143 (1 μM) for 30 min and then incubated with medium only or fimbriae (1 μg/ml). After 16 h, supernatants were collected for cytokine analysis by ELISA (A–E). F, Shows a separate experiment for determining IL-12p70 induction using IFN-γ-primed monocytes. Results are shown as means ± SD (n = 3) from one of two independent sets of experiments that yielded similar findings. Asterisks indicate significant (p < 0.05) differences in cytokine induction due to treatment with XVA143.

FIGURE 4. The interaction of P. gingivalis fimbriae with CR3 inhibits IL-12 expression through ERK1/2. Human monocytes were pretreated or not for 45 min with 1 μM XVA143 (A and B) or with 20 μM PD98059 (C and D), and subsequently incubated for 5 h with medium only or with P. gingivalis fimbriae. Real-time PCR was used to determine IL-12 p35 and p40 mRNA levels, normalized against GAPDH mRNA levels. Data are shown as fold induction relative to medium-only treated control. E and F, PD98059-pretreated monocytes were incubated with fimbriae for 16 h, with or without IFN-γ (0.1 μg/ml), and culture supernatants were assayed for IL-12p70. F, The IL-12-up-regulatory effect of a neutralizing dose of anti-IL-10 mAb (200 μg/ml) or IgG2b isotype control (IC) was compared with that of PD98059. G, Cells were pretreated with XVA143, and fimbria-induced phosphorylation of ERK1/2 was determined using PhosphoDetect ERK1/2 (pTpY185/187) ELISA. Total ERK1/2 content was quantitated using Total ERK1/2 ELISA kit. Results are means ± SD (n = 3) and were confirmed in repeated experiments. Asterisks indicate significant (p < 0.05) differences between experimental and control treatments.
Enhanced in vivo clearance of \textit{P. gingivalis} by CR3 antagonist

IL-12p70 production by macrophages activates T cells and NK cells to produce IFN-\(\gamma\), which in turn activates the bactericidal function of macrophages (16). We have thus investigated the effect of IL-12p70 induction on the in vivo clearance of \textit{P. gingivalis}. The CR3 antagonist XVA143, which up-regulates IL-12 in vitro (Fig. 3, E and F, and Fig. 4, A and B), was used as the immunomodulatory agent. Initially, we tested the ability of XVA143 to up-regulate IL-12p70 in vivo. Mice were administered XVA143 or PBS by the i.p. route, followed 1 h later with i.v. challenge with \textit{P. gingivalis} 33277. XVA143 pretreatment of \textit{P. gingivalis}-challenged mice resulted in significantly higher serum levels of IL-12p70 and IFN-\(\gamma\) compared with pretreatment with PBS vehicle control (\(p < 0.05\); Fig. 5). No IL-12p70 or IFN-\(\gamma\) was detectable in mice not challenged with \textit{P. gingivalis} regardless of whether they had previously received XVA143 or not (Fig. 5).

We then determined whether XVA143 helps the mice clear an i.p. infection with \textit{P. gingivalis}. We found that 20 h postinfection, the peritoneal lavage fluid from XVA143-pretreated mice contained \(\sim 40\) times less \textit{P. gingivalis} CFU compared with mice pre-treated with PBS instead (\(p < 0.05\); Fig. 6A). Analysis of peritoneal fluid for IL-12p70 and IFN-\(\gamma\) confirmed the enhancing effect of XVA143 on the production of these cytokines (Fig. 6B). Therefore, positive regulation of IL-12p70 and IFN-\(\gamma\) by XVA143 correlates with enhanced in vivo clearance of \textit{P. gingivalis}. To conclusively implicate IL-12p70 in protection, mice were i.p. given goat polyclonal anti-mouse IL-12 IgG (0.1 mg/mouse; R&D Systems) or an equal amount of nonimmune IgG. Both groups were then administered XVA143 and 1 h later were challenged with \textit{P. gingivalis}. Mice treated with anti-IL-12 yielded significantly higher levels of \textit{P. gingivalis} CFU from the peritoneal cavity 20 h postinfection, compared with mice receiving nonimmune IgG (\(p < 0.05\); Fig. 6C). The anti-IL-12 Ab used reacts with both IL-12 p35 and p40, which is shared by IL-23, a heterodimeric cytokine containing a shared p40 and a unique p19 subunit (38). It was thus possible that the observed \textit{P. gingivalis} clearance was mediated, at least in part, by IL-23. However, the use of goat anti-mouse IL-23 p19-neutralizing Ab had no significant effect on \textit{P. gingivalis} clearance (Fig. 6C), suggesting that clearance is attributable to IL-12p70.

\textit{P. gingivalis fimbriae inhibit LPS-induced IL-12p70 induction in vitro and in vivo}

The ability of \textit{P. gingivalis} fimbriae to suppress IL-12p70 induction through CR3 interaction has prompted us to investigate whether fimbriae can exert a similar inhibitory effect in cells activated with potent inducers of IL-12p70. For this purpose, we used IFN-\(\gamma\)-primed and \textit{E. coli} LPS-activated human monocytes. We found that the ability of LPS to induce IL-12p70 in monocytes was significantly inhibited in the presence of fimbriae, although an enhancing additive effect was observed for four other proinflammatory cytokines (\(p < 0.05\); Fig. 7). Similar results were obtained using LPS purified from the oral pathogen \textit{A. actinomycetemcomitans} (data not shown). Moreover, mice systemically challenged with \textit{E. coli} LPS elicited significantly less serum IL-12p70 or IFN-\(\gamma\) if they were previously administered \textit{P. gingivalis} fimbriae rather than PBS control (\(p < 0.05\); Fig. 8). Therefore, \textit{P. gingivalis} fimbriae interfere with LPS-induced IL-12p70 production in vivo as seen in vitro.
FIGURE 8. *P. gingivalis* fimbriae inhibit *E. coli* LPS-induced IL-12p70 and IFN-γ production in vivo. BALB/cByJ mice were pretreated with *P. gingivalis* fimbriae (i.p.; 10 µg) or PBS alone. After 1 h, the mice were i.v. injected with *E. coli* LPS (1 µg). Six hours later, blood was collected to analyze serum levels of IL-12p70 and IFN-γ. Data are means ± SD (n = 4) from one of two experiments yielding similar results. Asterisks indicate significant (p < 0.05) differences from one of two experiments yielding similar results. 

**Enhanced in vivo clearance of *P. gingivalis* in CR3-deficient mice**

The findings that XVA143 promotes *P. gingivalis* clearance (Fig. 6) are attributable to CR3 blockade by XVA143 and the consequent inability of *P. gingivalis* to exploit CR3 for enhancing its survival. This is a significant finding because *X. vulgaris* may be a useful immunomodulatory agent for controlling *P. gingivalis* infection. However, to conclusively implicate CR3 as an exploited receptor, we examined the ability of *P. gingivalis* 33277 to survive in i.p. infected wild-type or CR3-deficient mice. We found that 20 h postinfection, the peritoneal lavage fluid from CR3-deficient mice contained significantly lower *P. gingivalis* CFU, but significantly higher IL-12p70 and IFN-γ levels, compared with wild-type mice (p < 0.05; Fig. 9, A and B). Therefore, the presence of CR3 correlates with reduced induction of IL-12p70 and IFN-γ and impaired pathogen clearance. In a similar experiment using a non-fimbriated isogenic mutant (JI-1), we did not observe significant differences in the number of recovered CFU or the levels of IL-12p70 and IFN-γ between PBS-treated/periodontal bone loss by CR3 antagonist.

Our in vitro and in vivo findings suggest that CR3 may be exploited by *P. gingivalis* in ways that promote its virulence. In the context of periodontal disease, the virulence of this pathogen is determined by its capacity to induce periodontal bone resorption in animal models (39). We thus used a validated model of mouse periodontitis (34) and hypothesized that the CR3 antagonist XVA143 suppresses *P. gingivalis*-induced periodontal bone loss. For this purpose, groups of five BALB/cByJ mice were microinjected into the palatal gingivae with XVA143 or PBS control and orally infected with *P. gingivalis* (P.g.) or 2% carboxymethylcellulose vehicle only (Sham), as described in Materials and Methods. A. The mm distance from the CEJ to the ABC was measured at 14 predetermined sites in defleshed maxillae and was totaled for each mouse. Data are shown for each mouse, and horizontal lines indicate the mean value. B. The data from A were transformed to indicate bone loss, as outlined in Materials and Methods. Data are shown as means ± SD (n = 5), and negative values indicate bone loss. Asterisks denote significant (p < 0.05) differences between PBS-treated/*P. gingivalis*-infected mice and the rest of the groups, among which no significant differences were found.

**FIGURE 9.** Effect of CR3 deficiency on in vivo clearance of *P. gingivalis* and induction of IL-12 and IFN-γ. Wild-type or CR3-deficient (CD11b−/−) C57BL/6 mice were infected i.p. with wild-type *P. gingivalis* 33277 (A and B) or with the isogenic nonfimbriated mutant JI-1 (C and D) (both strains used at 5 × 10^7 CFU). Peritoneal lavage was performed 20 h postinfection. Serial 10-fold dilutions of peritoneal fluid were plated onto blood agar plates with hemin/ menadione and cultured anaerobically for enumerating recovered peritoneal CFU (A and C). The peritoneal fluid was also used to measure IL-12p70 and IFN-γ production by ELISA (B and D). Data are shown for each mouse, and horizontal lines indicate the mean value (A) or are shown as means ± SD (n = 5) (B), from one of two experiments that yielded similar findings. Asterisks indicate significant (p < 0.05) differences in *P. gingivalis* peritoneal CFU (A) or in cytokine production (B).

**FIGURE 10.** The CR3 antagonist, XVA143, prevents *P. gingivalis*-induced periodontal bone loss. BALB/cByJ mice were pretreated with XVA143 or PBS control and orally infected with *P. gingivalis* (P.g.) or 2% carboxymethylcellulose vehicle only (Sham), as described in Materials and Methods. A. The mm distance from the CEJ to the ABC was measured at 14 predetermined sites in defleshed maxillae and was totaled for each mouse. Data are shown for each mouse, and horizontal lines indicate the mean value. B. The data from A were transformed to indicate bone loss, as outlined in Materials and Methods. Data are shown as means ± SD (n = 5), and negative values indicate bone loss. Asterisks denote significant (p < 0.05) differences between PBS-treated/*P. gingivalis*-infected mice and the rest of the groups, among which no significant differences were found.

Inhibition of *P. gingivalis*-induced periodontal bone loss by CR3 antagonist.

Our in vitro and in vivo findings suggest that CR3 may be exploited by *P. gingivalis* in ways that promote its virulence. In the context of periodontal disease, the virulence of this pathogen is determined by its capacity to induce periodontal bone resorption in animal models (39). We thus used a validated model of mouse periodontitis (34) and hypothesized that the CR3 antagonist XVA143 suppresses *P. gingivalis*-induced periodontal bone loss. For this purpose, groups of five BALB/cByJ mice were microinjected into the palatal gingivae with XVA143 or PBS control and were orally infected with *P. gingivalis* or vehicle control, as detailed in Materials and Methods. In the groups microinjected with PBS, *P. gingivalis* infection resulted in significant periodontal...
bone loss compared with sham infection (p < 0.05; Fig. 10). However, the virulence of \textit{P. gingivalis} in causing bone loss was significantly attenuated in mice microinjected with XVA143 (p < 0.05; Fig. 10). No significant differences were observed in sham-infected mice regardless of whether they were treated with XVA143 or PBS (Fig. 10). At termination of the experiment, viable \textit{P. gingivalis} was recoverable by paper point sampling of the oral cavity of PBS-treated/infected, but not XVA143-treated/infected mice (viable cell counts = 3480 ± 1843 CFU, following anaerobic culturing on blood agar). This indicates that XVA143 inhibits the ability of \textit{P. gingivalis} to persist in the mouse host. In conclusion, the CR3 antagonist XVA143 counteracts \textit{P. gingivalis} virulence in the mouse periodontitis model.

**Discussion**

Our findings implicate CR3 in immune evasion by \textit{P. gingivalis}. This pathogen exploits its fimbrial-mediated interaction with CR3 and proactively down-regulates IL-12 production via ERK1/2 signaling. The ability of \textit{P. gingivalis} to escape immunosurveillance and promote its survival is counteracted by CR3 deficiency or a CR3 antagonist, or by lack of fimbria expression. The antagonist approach is of particular interest because it could be harnessed to negate immune evasion by \textit{P. gingivalis} and thereby to attenuate its virulence in human periodontitis and possibly associated systemic conditions such as atherosclerosis. In a proof-of-concept study using the mouse periodontitis model, we showed that XVA143, an antagonist of both mouse and human CR3 (10, 31), inhibits the ability of \textit{P. gingivalis} to persist in the mouse host and cause periodontal bone loss. Whether the attenuating effect of XVA143 on \textit{P. gingivalis} virulence involves additional mechanisms cannot be formally ruled out. However, the fact that XVA143 is a potent CR3 antagonist that exerts similar effects on IL-12/IFN-γ production and \textit{P. gingivalis} clearance as CR3 deficiency does, strongly supports that the XVA143 effects are attributable, at least partly, to actions on CR3.

Suppression of IL-12p70 is most likely a major bacterial evasion strategy, because this cytokine induces the production of IFN-γ and plays a key role in mediating bacterial clearance (16). The observation that \textit{P. gingivalis} fimbria-exposed macrophages display diminished induction of IL-12p70 and IFN-γ in response to other independent bacterial stimuli suggests that this evasion mechanism may impact on the survival of both \textit{P. gingivalis} and other periodontal pathogens that cohabit its niche in mixed species biofilm. Fimbriae could act in cell-associated form, as free fimbriae shed from the cell surface, or as components of outer membrane vesicles released from the bacterial cell surface (40). Interestingly, an inverse relationship exists between IL-12p70 and severity of periodontal disease (41, 42). Moreover, removal of \textit{P. gingivalis} by periodontal therapy results in selective augmentation of monocyte production of IL-12p70 (43), perhaps attributable in part to diminished \textit{P. gingivalis} fimbria-CR3 interactions. It is thus possible that proactive inhibition of IL-12 induction may lead to \textit{P. gingivalis} persistence, explaining, at least partly, the chronicity of periodontal disease. Although IL-23 did not seem to play a significant role in \textit{P. gingivalis} clearance from systemically infected mice (Fig. 6C), this cytokine stimulates production of IL-17 by the Th17 T cell subset, and its involvement in periodontal host defense cannot be ruled out. In this context, a recent study using IL-17R knockout mice indicated an important role for innate responses and the Th17 axis of immunity in the control of \textit{P. gingivalis}-induced bone loss (44).

Although CR3 exploitation by \textit{P. gingivalis} appears to undermine effective innate immunity, it is unlikely to suppress inflammation altogether because the fimbria-CR3 interaction specifically down-regulates IL-12 (Fig. 3). In fact, inflammation could benefit \textit{P. gingivalis} through acquisition of serum exudate-derived nutrients that are absolutely required for its growth (40). Inflammation that fails to clear the pathogen could be characterized as nonproductive and may underlie inflammatory periodontal bone resorption when infection becomes chronic.

From the host viewpoint, CR3-dependent inhibition of IL-12 appears to serve a physiological role. Indeed, the phagocytosis of apoptotic cells by macrophages is heavily dependent on CR3 and is associated with inhibition of IL-12p70, because apoptotic cells are not normally recognized as danger (45, 46). These findings in conjunction with our current data suggest that \textit{P. gingivalis} has co-opted a natural anti-inflammatory CR3-dependent mechanism to evade innate immunity. This mechanism may be exploited also by other pathogens. For example, the interaction of \textit{B. pertussis} filamentous hemagglutinin with CR3 similarly leads to inhibition of IL-12p70 (5), and, interestingly, the in vivo phagocytic uptake of \textit{B. pertussis} via CR3 fails to promote its clearance (25). \textit{P. gingivalis} fimbriae and other microbial virulence proteins are not sensu stricto pathogen-associated molecular patterns, because they are responsible for adaptive fitness and thus do not represent invariant structures. This is in stark contrast to conserved molecular patterns that were selected by evolution as targets of pattern recognition (47). We and others have speculated that microbial virulence proteins have evolved to interact with and possibly exploit TLRs and the pattern recognition system in general, in ways that increase the potential of the pathogens for survival (8, 13, 14, 48–51). In this regard, the interaction of \textit{P. gingivalis} fimbriae with CR3 depends strictly upon the ability of fimbriae to exploit TLR2 inside-out signaling for activating the ligand-binding capacity of CR3 (9, 10) (Fig. 1A), and the pathogen colocalizes with both PRRs in macrophages (Fig. 1B). However, in addition to stimulating proadhesive inside-out signaling through Rac1/PI3K (9, 10), TLR2 also activates NF-κB through MyD88 (52). In fact, TLR2 deficiency abrogates IL-12p70, whereas CR3 deficiency augments IL-12p70 induction by \textit{P. gingivalis} fimbriae (14), implying that CR3 engages in a cross-talk with TLR2 for IL-12 down-regulation. These findings suggest that under certain conditions TLR2 has the inherent potential for inducing protective immunity. Indeed, both in vitro and in vivo studies have shown that TLR2 mediates protection against various pathogens through induction of IL-12 and production of IL-12-dependent IFN-γ (53–57). By contrast, TLR2 signaling has been implicated in immune evasion by \textit{Yersinia enterocolitica}. Specifically, \textit{Y. enterocolitica} expresses the virulence Ag LcrV, which induces immunosuppression through TLR2-dependent IL-10 release, resulting in enhanced pathogen survival (48). Moreover, a recent report has shown that TLR2 deficiency attenuates \textit{P. gingivalis}-induced periodontal bone loss (58). The mechanism(s) whereby \textit{P. gingivalis} exploits TLR2 to promote its virulence is largely uncertain at the moment. However, it is intriguing to suggest that TLR2 deficiency limits efficient activation of CR3, which in turn cannot be readily exploited by \textit{P. gingivalis} in the way described in our study. This could explain, at least partly, why TLR2 deficiency attenuates \textit{P. gingivalis} virulence in experimental periodontitis.

In our investigation of the role of CR3 in \textit{P. gingivalis}-induced periodontal bone loss, we used a CR3 antagonist rather than a comparison of CR3-deficient mice with wild-type controls. The CR3 (CD11b \(^{-/-}\)) deficiency is available on a C57BL/6 genetic background (The Jackson Laboratory), which is quite resistant to induction of periodontal bone loss, in contrast to the relatively susceptible BALB/c background (34). C57BL/6 mice are thus quite useful for examining receptor deficiencies that would predispose to susceptibility to periodontitis, rather than to resistance,
which applied to our hypothesis with CR3. In the context of host receptor exploitation in periodontitis, therefore, BALB/c mice constitute a more useful model. There is an additional advantage in using the CR3 antagonist approach; if effective, as shown in our study, the antagonist can further be considered as a potential immunomodulatory agent for controlling human periodontitis. Interestingly, Baker et al. (59) used the relatively resistant C57BL/6 mice to determine the effect of adhesion molecule deficiencies on susceptibility to *P. gingivalis*-induced periodontal bone loss. Although deficiencies in P-selectin or ICAM-1 rendered the mutant mice susceptible to significant bone loss, a hypomorphic mutation in CD18—deficient mice displayed a trend for increased resistance to experimental periodontitis, although this could not be clearly shown in an already resistant genotype. Although CD18 deficiency affects more $\beta_2$ integrins (LFA-1 and $p150, 95$) than just CR3, these data are consistent with the notion that CR3 is associated with increased *P. gingivalis* virulence in the periodontal bone loss model. It is moreover intriguing to suggest that the increased resistance of C57BL/6 mice to *P. gingivalis*-induced periodontitis, relative to BALB/c mice, could be attributable to the capacity of C57BL/6 mice to elicit higher levels of IL-12 and to exert enhanced bacterial clearance (61).

In conclusion, our findings indicate that CR3 is exploited by *P. gingivalis* for proactive suppression of the innate response in ways that promote its persistence and virulence. The ability of a CR3 antagonist (XVA143) to reverse suppression of IL-12p70, and that promote its persistence and virulence. The ability of a CR3 antagonist approach; if effective, as shown in our study, the antagonist can further be considered as a potential immunomodulatory agent for controlling human periodontitis. Interestingly, Baker et al. (59) used the relatively resistant C57BL/6 mice to determine the effect of adhesion molecule deficiencies on susceptibility to *P. gingivalis*-induced periodontal bone loss. Although deficiencies in P-selectin or ICAM-1 rendered the mutant mice susceptible to significant bone loss, a hypomorphic mutation in CD18—deficient mice displayed a trend for increased resistance to experimental periodontitis, although this could not be clearly shown in an already resistant genotype. Although CD18 deficiency affects more $\beta_2$ integrins (LFA-1 and $p150, 95$) than just CR3, these data are consistent with the notion that CR3 is associated with increased *P. gingivalis* virulence in the periodontal bone loss model. It is moreover intriguing to suggest that the increased resistance of C57BL/6 mice to *P. gingivalis*-induced periodontitis, relative to BALB/c mice, could be attributable to the capacity of C57BL/6 mice to elicit higher levels of IL-12 and to exert enhanced bacterial clearance (61).


