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Cytokine Profiling of Macrophages Exposed to *Porphyromonas gingivalis*, Its Lipopolysaccharide, or Its FimA Protein

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To characterize the roles of *Porphyromonas gingivalis* and its components in the disease processes, we investigated the cytokine profile induced by live *P. gingivalis*, its lipopolysaccharides (LPS), and its major fimbrial protein, fimbrillin (FimA). Using cytokine antibody arrays, we found that *P. gingivalis* LPS and FimA induced a similar profile of cytokine expression when exposed to mouse peritoneal macrophages but that this profile differed significantly in response to live *P. gingivalis*. In vitro, mouse peritoneal macrophages were stimulated to produce interleukin-6 (IL-6), granulocyte colony-stimulating factor, and lymphotactin by live *P. gingivalis*, but not by *P. gingivalis* LPS or FimA, while RANTES, gamma interferon, IL-17, vascular cell adhesion molecule 1 (VCAM-1), and vascular endothelial growth factor were induced by *P. gingivalis* LPS or FimA, but not by live *P. gingivalis*. In vivo, IL-6 mRNA was strongly induced only by live *P. gingivalis* while monocyte chemoattractant protein 1 mRNA was strongly induced only by *P. gingivalis* LPS and FimA in mouse calvarial scalp, further confirming the differences of cytokine profile induced in vitro. Cytokine antibody arrays using toll-like receptor 2 (TLR2)- and TLR4-deficient macrophages revealed that most of the cytokines induced by *P. gingivalis* LPS or FimA signal through TLR2, while most of cytokines induced by live *P. gingivalis* signal through both TLR2 and TLR4. Interestingly, the activation of TLR2 by live *P. gingivalis* inhibited the release of RANTES, VCAM-1, and IL-1α from mouse peritoneal macrophages. A tumor necrosis factor alpha enzyme-linked immunosorbent assay further confirmed that *P. gingivalis* LPS and FimA activate mouse peritoneal macrophages via TLR2. These results indicate that host immune cells sense live *P. gingivalis* and its components differently, which translates into the expression of different inflammatory cytokine profiles.

P. gingivalis, a black-pigmented anaerobic gram-negative bacterium, has been implicated as a major pathogen in the development and progression of periodontal diseases (5, 22). It is known to possess structures on its surface, such as lipopolysaccharides (LPS) and fimbriae, which are well-characterized pattern recognition receptor ligands. LPS are a major component of the outer membrane of gram-negative bacteria capable of host activation, while fimbriae, peritrichous filamentous appendages, mediate the adherence of bacteria to host cells and to a variety of oral substrates and molecules (34). Numerous studies have shown that the host uses these molecules to detect both microbial colonization and infection. They play important roles in the induction of immune responses, including recruitment of peripheral leukocytes (8), production of cytokines (36), and activation of inflammation-related signaling pathways (29).

A dense infiltration of inflammatory cells, including monocytes/macrophages, occurs in the periodontal tissues of adult patients with periodontitis. Mononuclear phagocytes play an important role in the regulation of inflammatory host responses, in part through their ability to secrete mediators, particularly cytokines, in response to microorganisms and microbial products. Macrophages constitute a substantial proportion of the cells recovered from the gingival tissues, particularly the inflammatory tissues, of patients with periodontitis (42). These macrophages play an essential role in the development and progression of periodontal diseases. It is likely that the high expression of the molecular mediators of inflammation facilitates the performance of monocytes/macrophages in protecting the host from bacterial challenge (46).

Macrophages also play diverse roles in atherogenesis and lipoprotein metabolism (27). They function as scavenger cells, immune mediator cells, and as sources of chemotactic molecules and cytokines (43). Certain chemokines have been implicated in promoting the migration of monocytes into the gingiva (50). The host response to *P. gingivalis* and other oral pathogens is thought to be responsible for the local tissue destruction seen with periodontitis (2). In addition, the response to oral pathogens has systemic consequences. For example, infection and chronic inflammatory conditions, such as periodontitis, may influence the atherogenic process (14, 25, 39).

Toll-like receptors (TLRs), a pattern-recognition receptor family, have been identified in mammals based on their homology to the *Drosophila* protein Toll (19). Mammalian TLRs comprise a large family with extracellular leucine-rich repeats and a cytoplasmic Toll/interleukin-1 (IL-1) receptor homology domain and have been implicated in the recognition of bacterial components (31). Two members of the Toll-like receptor family, TLR2 and TLR4, have been identified as signaling receptors for bacterial cell wall components. For example, the expression of TLR2 in Chinese hamster ovary (CHO) fibroblasts or human embryonic kidney (HEK293) cells, which are TLR2 deficient, confers responsiveness to various bacterial components, such as peptidoglycan, lipoprotein, and lipoarabinomannan (26, 30). TLR4 cloned from naturally LPS-resistant

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C3H/HeJ mice was found to harbor a point mutation that renders it nonfunctional (38). While TLR4-deficient mice are unresponsive to LPS from *Escherichia coli*, macrophages from TLR2-deficient mice lack the ability to respond to gram-positive bacterial cell walls, strengthening the hypothesis that TLR4 is the principal signal transducer for most types of LPS and that TLR2 is a signal transducer for other bacterial components, such as peptidoglycan and lipoprotein (44). Although highly conserved, LPS from different bacterial species displays important structural differences that can significantly alter host responses (15). LPS obtained from *P. gingivalis* is noteworthy in that it causes a highly unusual innate host response. *P. gingivalis* LPS is both an agonist for human TLR2 and an antagonist for human TLR4 (12). In addition, *P. gingivalis* LPS is also an effective inhibitor of *E. coli* LPS-induced p38 phosphorylation (6).

In the present study, we investigated the cytokine expression profile of mouse peritoneal macrophages exposed to live *P. gingivalis*, *P. gingivalis* LPS, and FimA. Our results demonstrated that *P. gingivalis* LPS and FimA induce, mainly through TLR2, similar patterns of macrophage cytokine expression. However, this pattern was found to be significantly different when the cells were challenged with live *P. gingivalis*, which was found to signal through both TLR2 and TLR4.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. gingivalis* was grown in brain heart infusion broth (*P. gingivalis* 381) or Schaedler broth (*P. gingivalis* A7436) enriched with hemin (5 μg/ml) and menadione (1 μg/ml) in an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2) for 24 h at 37°C. For infection experiments, *P. gingivalis* 381 was grown until the culture reached an optical density at 660 nm of 0.8 and was then pelleted by centrifugation and resuspended in sterile 20% glycerol to make frozen stocks that were thawed for the experiments.

Purification of FimA. FimA was purified to homogeneity by size exclusion chromatography of sonicated extracts of *P. gingivalis* 381 or *P. gingivalis* A7436 (23). Briefly, *P. gingivalis* cells were harvested by centrifugation, resuspended in 25 mM Tris-Cl (pH 8.0) containing 10 mM $MgCl_2$ and 0.15 M NaCl, and sonicated for 5 min. A supernatant fluid was obtained by centrifugation at 10,000 × *g* for 15 min. Proteins were precipitated from the supernatant by adding solid ammonium sulfate in a stepwise manner to achieve 40% saturation, after which they were recovered by centrifugation at 10,000 × *g* for 25 min. The pellet was resuspended in a minimal volume of 5 mM Tris-Cl (pH 8.0) and dialyzed against the same buffer. The dialyzed sample was subjected to further purification on a Sepharose CL-6B column equilibrated and eluted with 6 M guanidine-HCl (pH 4.75). The fimbria-rich fractions detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver stain were pooled, dialyzed, lyophilized, and processed repeatedly through the Sepharose CL-6B column by using 6 M guanidine-HCl (pH 4.75) as the elution buffer until purified proteins were obtained. Fractions that were negative for any contaminating substances on silver-stained SDS-PAGE were selected, dialyzed, and lyophilized. The 41-kDa FimA was confirmed by N-terminal amino acid sequencing, and its molecular weight was further determined by matrix-assisted laser desorption ionization–time-of flight mass spectrometry. The total fimbrial protein was quantified by using a NanoOrange protein quantification kit (Molecular Probes, Eugene, Oreg.).

Preparation of LPS. LPS from *P. gingivalis* 381 and *P. gingivalis* A7436 were purified by phenol-water extraction and subsequent treatment with DNase I, RNase A, and proteinase K, followed by cesium chloride isopyknic density gradient centrifugation (32). The fractions with a density of 1.42 to 1.52 g/cm^3, which contained peak endotoxin activity as determined by the *Limulus* amebocyte clotting assay, were pooled, dialyzed, lyophilized, and repurified by the method of Manthey and Vogel (28). The purity of the preparation was confirmed by amino acid analysis and by SDS-PAGE with silver staining. The molecular weight of LPS was determined by gas chromatography to determine the number of moles of hexadecanoic acid (16:0) in a given weight of LPS as previously described (49).

Mouse peritoneal macrophage isolation and culture. C3H/OuJ (wild-type), C3H/HeJ (TLR4-deficient), and TLR2 knockout (TLR2$^{-/-}$) mice (Jackson Laboratory, Bar Harbor, Maine) were injected with 3 ml of sterile thioglycolate medium (Remel, Lenexa, Kans.) into the peritoneal cavity. Four days thereafter, thioglycolate-elicited peritoneal exudate macrophages were harvested by peritoneal lavage with 9 ml of RPMI 1640 medium. Macrophages were pooled and centrifuged at a speed of 1,500 × *g* at 4°C for 5 min. If the pellets contained red blood cells, the pellets were resuspended in 3 ml of ice-cold Tris-buffered ammonium chloride lysis buffer (Sigma, St. Louis, Mo.), incubated at 4°C for 2 to 3 min, and then washed with 10 ml of RPMI 1640 medium. Macrophages were resuspended in 10 ml of RPMI 1640 medium and counted by a hemacytometer. Cells were placed into 24- or 6-well plates at a concentration of 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and standard penicillin-streptomycin. After a 2-h incubation at 37°C in an atmosphere containing 5% CO_2, nonadherent cells were washed out with warm phosphate-buffered saline (PBS). Adherent macrophages were cultured for 3 days before exposure to stimulants. Media were changed 1 h before experiments were begun.

Macrophage exposure to live *P. gingivalis*. Live *P. gingivalis* 381 frozen stocks containing 10^8 to 10^9 bacteria were thawed and diluted in media to a final volume of 50 μl, which was added to cultures containing 2×10^6 mouse peritoneal macrophages, resulting in a final multiplicity of infection (MOI) of 50:1. Dilutions were plated on brain heart infusion agar plates for anaerobic culture, and colonies were counted to confirm the accuracy of dilution and viability of bacteria. Cell culture media were collected after 24 h of infection.

Macrophage exposure to *P. gingivalis* components. Mouse peritoneal macrophages were stimulated with isolated and purified *P. gingivalis* LPS and FimA in media containing 10% fetal bovine serum for 24 h. Both LPS and FimA were added to a final concentration of 10 μg/ml or in a dose-dependent manner. Cell culture media were collected after 24 h of stimulation.

Inoculation of *P. gingivalis*, *P. gingivalis* LPS, or FimA in vivo. The calvarial scalps of C3H/OuJ mice were inoculated with 1×10^8 or 5×10^8 live *P. gingivalis* or 50 or 250 μg of LPS or FimA from *P. gingivalis* 381 in 50 μl of sterile PBS as we have previously described (4). Control animals were inoculated with PBS alone. The scalps were harvested 20 h later and immediately frozen in liquid nitrogen. Total RNA was extracted from pulverized tissue by using TRIzol reagent according to the manufacturer's instructions and was quantified spectrophotometrically. The expression of selected genes was measured by an RNase protection assay as described below.

RNase protection assay. Total RNA was extracted from pulverized tissue as described above. Detection of cytokine mRNA was performed with a multiprobe RNase-protection assay system (Pharmingen, San Diego, Calif.). Briefly, a mixture of [^{32}P]UTP-labeled antisense riboprobes was generated from a multiprobe template set (Pharmingen). The cytokine templates included IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and inducible nitric oxide synthase (iNOS). The housekeeping gene product, glyceraldehyde-3-phosphate dehydrogenase (GADPH), was also included in the multiprobe templates to ensure equal loading of total RNA onto the gels. A total of 10 μg of RNA was hybridized overnight at 56°C with 10^6 cpm of the ^{32}P-labeled antisense riboprobe mixture. After hybridization, the unprotected RNA was digested with a mixture of RNases A and T1. Nuclease-protected RNA fragments were resolved on a 6% polyacrylamide sequencing gel. After exposure to the Imaging-Screen K (Bio-Rad, Hercules, Calif.), the specific chemokine bands were identified on the basis of their individual mobilities compared with those of labeled standard probes.

Cytokine antibody array. Culture samples were analyzed with cytokine antibody array by using either RayBio Human Cytokine Antibody Array V or RayBio Mouse Cytokine Antibody Array III (RayBiotech, Inc., Norcross, Ga.) according to the manufacturer's instructions. Briefly, cytokine array membranes were blocked in 2 ml of 1× blocking buffer for 30 min and then incubated with 1 ml of samples at room temperature for 1 to 2 h. Samples were then decanted from each container, and the membranes were washed three times with 2 ml of 1× wash buffer I, followed by two washes with 2 ml of 1× wash buffer II at room temperature with shaking. Membranes were then incubated in 1:250-diluted biotin-conjugated primary antibodies at room temperature for 1 to 2 h and washed as described above before incubation in 1:1,000-diluted horseradish peroxidase-conjugated streptavidin. After incubation in horseradish peroxidase-conjugated streptavidin for 30 to 60 min, membranes were washed thoroughly and exposed to a peroxidase substrate (detection buffers C and D; RayBiotech, Inc.) for 5 min in the dark before imaging. Membranes were exposed to X-ray film (Kodak X-OMAT AR film) within 30 min of exposure to the substrate. Signal intensities were quantified with a Bio-Rad VersaDoc Imaging System 3000 and analyzed with Quantity One software (Bio-Rad). Biotin-conjugated immunoglobulin G served as a positive control at six spots, where it was used to identify membrane orientation and to normalize the results from different membranes that were being compared. For each spot, the net optical density level was

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS	POS	POS	POS	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
2	NEG	NEG	NEG	NEG	Blank	Axl	BLC	CD30L	CD30T	CD40	CRG-2	CTACK	CXCL16	Eotaxin
3	Eotaxin-2	Fas Ligand	Fractakine	GCSF	GM-CSF	IFN-r	IGFBP-3	IGFBP-5	IGFBP-6	IL-1a	IL-1b	IL-2	IL-3	IL-3Rb
4	Eotaxin-2	Fas Ligand	Fractakine	GCSF	GM-CSF	IFN-r	IGFBP-3	IGFBP-5	IGFBP-6	IL-1a	IL-1b	IL-2	IL-3	IL-3Rb
5	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	KC	Leptin R	LEPTIN(OB)	LIX	L-Selectin
6	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	KC	Leptin R	LEPTIN(OB)	LIX	L-Selectin
7	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1a	MIP-1r	MIP-2	MIP-3b	MIP-3a	PF-4	P-Selectin	RANTES	SCF
8	Lymphotactin	MCP-1	MCP-5	M-CSF	MIG	MIP-1a	MIP-1r	MIP-2	MIP-3b	MIP-3a	PF-4	P-Selectin	RANTES	SCF
9	SDF-1a	TARC	TCA-3	TECK	TIMP-1	TNF-a	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	Blank	Blank
10	SDF-1a	TARC	TCA-3	TECK	TIMP-1	TNF-a	sTNF RI	sTNF RII	TPO	VCAM-1	VEGF	Blank	POS	POS

A

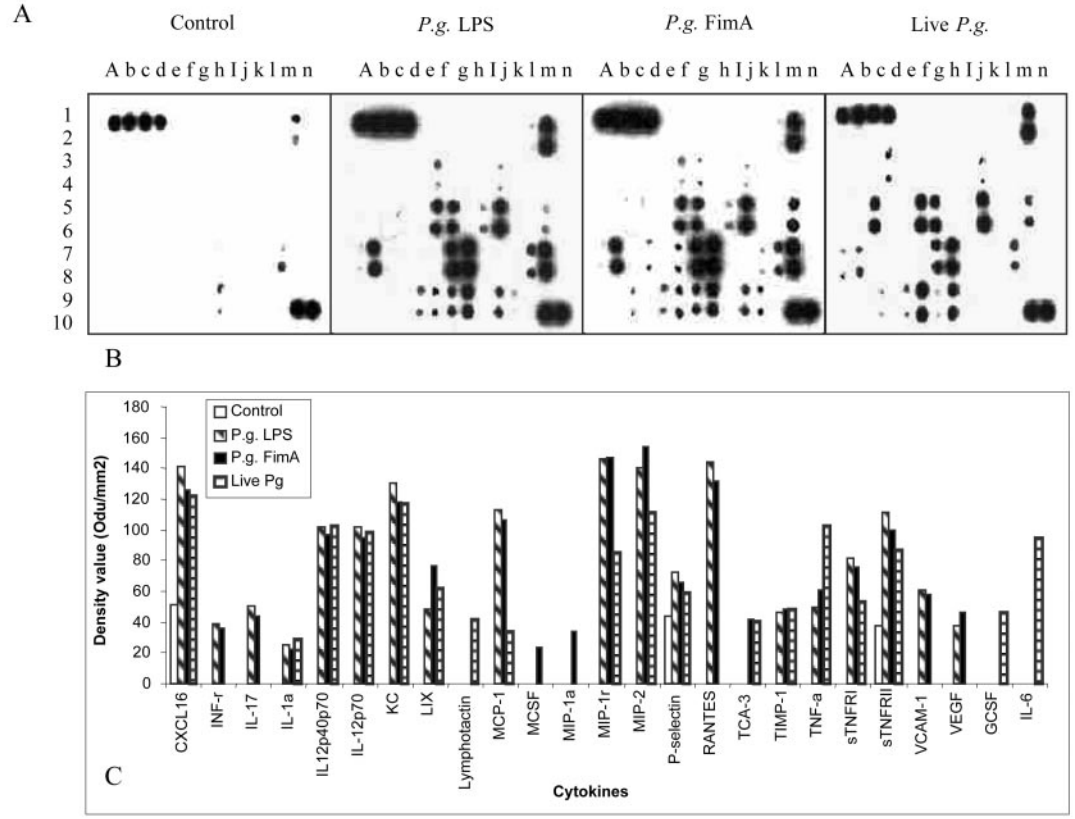

FIG. 1. Cytokine profile of murine peritoneal macrophages treated with live *P. gingivalis*, *P. gingivalis* LPS, or FimA. Peritoneal macrophages from C3H/OuJ mice were exposed to live *P. gingivalis* to a MOI of 50:1 or to 10 μg of either *P. gingivalis* LPS or FimA/ml for 24 h. Untreated cell cultures were used as a control. Cell culture supernatants were subject to cytokine antibody array. (A) Each cytokine is represented by duplicate spots in the locations shown. (B) The cytokine array image represents results of one of two independent experiments that found similar patterns of expression. (C) The average net optical intensity for each pair of cytokine spots is shown *P.g.*, *P. gingivalis*; POS, positive; NEG, negative; BLC, B-lymphocyte chemoattractant; CRG-2, cytokine-responsive gene 2; IGFBP-3, insulin-like growth factor binding protein 3; MIG, monokine induced by IFN-γ; TARC, thymus- and activation-regulated chemokine; TECK, thymus-expressed chemokine; CTACK, cutaneous T-cell-attracting chemokine; PF-4, platelet factor 4; TPO, thrombopoietin.

determined by subtracting the background optical level from the total raw optical density level.

TNF-α ELISA. The supernatants from LPS- or FimA-stimulated mouse peritoneal macrophages were analyzed for TNF-α by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minn.) performed according to the manufacturer's instructions.

Statistical analysis. Analysis was performed by two-tailed Student's *t* test for comparison between two groups. A P value of <0.05 was considered statistically significant.

RESULTS

Cytokine profile induced in mouse peritoneal macrophages by *P. gingivalis* LPS, FimA, or live *P. gingivalis*. To determine differences in cytokine induction between *P. gingivalis* and its two important inflammatory components, FimA and LPS, we examined the cytokine expression in C3H/OuJ mouse peritoneal macrophages by using a cytokine antibody array. Twenty-five cytokines were detected and semiquantified on membrane arrays containing 62 different cytokine antibodies (Fig. 1A to C). Untreated peritoneal macrophage cultures (control) were found constitutively to express low levels of CXC chemokine ligand 16 (CXCL16), P-selectin, and soluble TNF receptor II (sTNFRII), while all of the other cytokines were undetectable. The presence of these cytokines in the control was not an

TABLE 1. Major differences of cytokine expression in mouse peritoneal macrophage cultures treated with live *P. gingivalis*, *P. gingivalis* LPS, or Fim A[a]

Cytokine	Relative levels in culture supernatants at 24 h			
	Control	Live *P. gingivalis*	*P. gingivalis* LPS	Fim A
RANTES	−	−	++++	++++
VCAM-1	−	−	+++	+++
VEGF	−	−	+	+
IL-17	−	−	+	+
INF-γ	−	−	+	+
IL-6	−	++++	−	−
G-CSF	−	++	−	−
Lymphotactin	−	+	−	−
MCP-1	−	++	++++	++++

[a] Relative levels: −, undetectable; +, low; ++, medium; +++, high; ++++, very high.

artifact related to the inclusion of fetal bovine serum in the RPMI medium, since incubation with the medium alone did not result in reactivity with any of the antibody spots (data not shown). We did not observe release of IL-6, granulocyte colony-stimulating factor (G-CSF), or lymphotactin in mouse peritoneal macrophage cultures incubated with *P. gingivalis* LPS or FimA, while live *P. gingivalis* strongly stimulated the expression of those cytokines. It is interesting that *P. gingivalis* LPS and FimA stimulated RANTES, vascular cell adhesion molecule 1 (VCAM-1), vascular endothelial growth factor (VEGF), gamma interferon (IFN-γ), and IL-17 expression in mouse peritoneal macrophages, but live *P. gingivalis* did not stimulate expression of those cytokines at all, even though it possesses cell-associated LPS and FimA. *P. gingivalis* LPS and FimA also induced large amounts of MCP-1 and MIP-1γ, while live *P. gingivalis* induced much smaller amounts of them. MIP-1α and macrophage CSF (M-CSF) were present in FimA-treated cultures at only low levels. TNF-α, CXCL16, IL-12p40, IL-12p40p70, keratinocyte-derived chemokine (KC), LPS-induced CXC chemokine (LIX), MIP-2, sTNFRI, and sTNFRII were the most predominant cytokines detected in response to all three stimuli. Overall, stimulation with live *P. gingivalis* induced a pattern of cytokine responses that was different from that induced by *P. gingivalis* LPS or FimA. However, *P. gingivalis* LPS and FimA induced similar patterns of cytokine production (Table 1).

In vivo cytokine mRNA expression in murine calvarial scalp samples inoculated with live *P. gingivalis*, *P. gingivalis* LPS, or FimA. To confirm the patterns of cytokine expression induced by live *P. gingivalis*, *P. gingivalis* LPS, and FimA in vivo, murine calvarial scalps were inoculated with live *P. gingivalis*, *P. gingivalis* LPS, or FimA. Twenty hours later, total RNA was extracted from scalp tissue, and mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, MIP-2, and iNOS were detected by RNase protection assay (Fig. 2). We observed strong induction of IL-6 mRNA by live *P. gingivalis*; however, little induction of IL-6 mRNA by *P. gingivalis* LPS or FimA was observed with either low- or high-dose stimulation. In contrast, MCP-1 mRNA was strongly stimulated by *P. gingivalis* LPS or FimA, but little was induced by live *P. gingivalis*. The expression of IL-1β and MIP-2 mRNA was strongly induced by all of those stimuli. With a low dose (50 μg/mouse) of either *P. gingivalis* LPS or FimA, FimA induced much more IL-1β, TNF-α, MCP-1, MIP-2, and iNOS transcript in murine scalp tissue than did *P. gingivalis* LPS. When a high dose (250 μg/mouse) was used, the levels of induction of IL-1β, MCP-1, and MIP-2 mRNA by *P. gingivalis* LPS and FimA were quite comparable. But induction of TNF-α and iNOS mRNA by FimA was less than that stimulated by *P. gingivalis* LPS. Induction of iNOS mRNA by live *P. gingivalis* is much stronger at a high dose (5×10^8) than at a low dose (1×10^8). The expression of untreated mice as well as the injection control (PBS) showed baseline expression of the cytokine genes. This result indicates that live *P. gingivalis* also induced a pattern of cytokine mRNA expression that was different from that induced by *P. gingivalis* LPS and FimA, while *P. gingivalis* LPS and FimA induced the same general pattern of cytokine mRNA expression in vivo, although the mRNA levels differed somewhat, depending on the dose.

In order to compare the potency of *P. gingivalis* LPS and FimA in cytokine induction, we determined their molecular weight as described in Materials and Methods (data not shown). The molecular weights (in thousands) of *P. gingivalis* LPS and FimA were 7.04 and 36.4, respectively. Therefore, the low-dose (50 μg/50 μl) concentrations of LPS and FimA were 142 and 28 μM, respectively, and the high-dose (250 μg/50 μl) concentrations of LPS and FimA were 710 and 140 μM, respectively. We could then conclude that at the lower dose, FimA is much more potent than LPS in inducing MCP-1, MIP-2, IL-1β, and iNOS expression in vivo, but at a fivefold higher dose, LPS is more potent than FimA in inducing TNF-α and iNOS expression.

Loss of TLR2 or TLR4 function alters the cytokine profile induced by live *P. gingivalis*, *P. gingivalis* LPS, or FimA. TLR2 and TLR4 are two important signaling receptors for bacteria and their cell wall components. To test the roles of TLR2 and TLR4 in cytokine induction, a cytokine protein array was performed to detect cytokine expression in C3H/OuJ, C3H/HeJ, or $TLR2^{-/-}$ mouse peritoneal macrophages treated with live *P. gingivalis*, *P. gingivalis* LPS, or FimA.

After *P. gingivalis* LPS treatment, cytokines induced by C3H/HeJ peritoneal macrophages matched those produced by C3H/

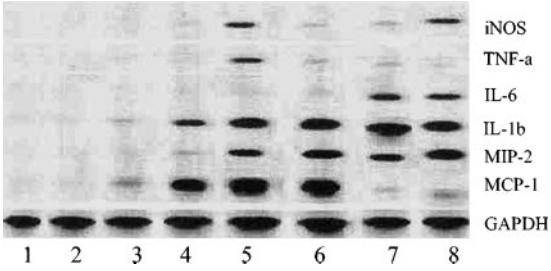

FIG. 2. In vivo cytokine mRNA expression induced by live *P. gingivalis*, *P. gingivalis* LPS, or FimA. Total RNA from calvarial scalps in each group ($n = 6$) was harvested and pooled after local inoculation with live *P. gingivalis*, *P. gingivalis* LPS, or FimA. Cytokine mRNA was analyzed by an RNase protection assay as described in the text. GAPDH was used as the constitutive expression control. Similar results were obtained in two independent experiments. Lane 1, no treatment control; lane 2, PBS (50 μl); lane 3, LPS (50 μg/50 μl); lane 4, FimA (50 μg/50 μl); lane 5, LPS (250 μg/50 μl); lane 6, FimA (250 μg/50 μl); lane 7, live *P. gingivalis* (1×10^8); and lane 8, live *P. gingivalis* (5×10^8).

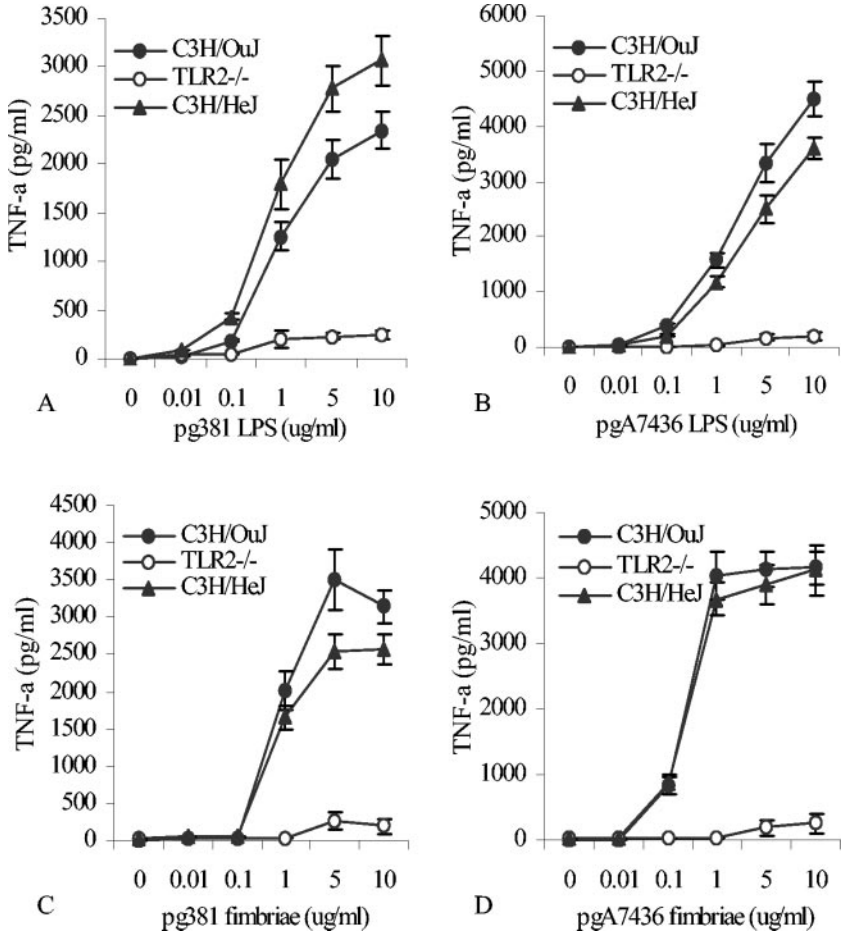

FIG. 3. *P. gingivalis* LPS and FimA activate mouse peritoneal macrophages via TLR2. Peritoneal macrophages from C3H/OuJ, C3H/HeJ, and TLR2$^{-/-}$ mice were stimulated with LPS or FimA from *P. gingivalis* 381 and *P. gingivalis* A7436 at the indicated concentrations. The macrophage culture supernatants were collected after 24 h. The TNF-α concentrations were measured by ELISA. The data are the means ± standard errors from three independent experiments. *P.g.*, *P. gingivalis*.

OuJ peritoneal macrophages, but TNF-α, IL-12p40, IL-12p40/p70, LIX, IL-1α, IL-17, IFN-γ, lymphotactin, VCAM-1, and VEGF were totally absent in TLR2$^{-/-}$ peritoneal macrophages (Fig. 3A and B). Production of MCP-1, MIP-1γ, sTNFRI, and sTNFRII was also significantly ($P < 0.05$) reduced in TLR2$^{-/-}$ peritoneal macrophages, while production of RANTES, MIP-2, KC, CXCL16, P-selectin, and tissue inhibitor of metalloproteinase 1 (TIMP-1) remained unchanged compared to C3H/OuJ macrophages (Fig. 3A and B).

With FimA treatment, the amounts of RANTES, CXCL16, MIP-1γ, MIP-2, P-selectin TCA-3, MIP-1, and sTNFRII induced by C3H/HeJ and TLR2$^{-/-}$ macrophages were quite comparable ($P > 0.05$) to those induced by C3H/OuJ macrophages. However, TNF-α, MCP-1, LIX, KC, IL-12p40, IL-12p40p70, IL-1α, IL-17, and IFN-γ were absent or much less induced ($P < 0.05$) in TLR2$^{-/-}$ macrophages. IFN-γ, IL-17, sTNFRI, VCAM-1, and VEGF were also much less induced ($P < 0.05$) in C3H/HeJ macrophages (Fig. 3A and C).

When C3H/HeJ macrophages were treated with live *P. gingivalis*, G-CSF, IL-1α, and MIP-1γ were not observed; IL-6, KC, LIX, MCP-1, MIP-1γ, T-cell activation gene 3 (TCA-3), TIMP-1, and TNF-α were not observed with TLR2$^{-/-}$ macrophages compared to C3H/OuJ macrophages (Fig. 3A and D). The production of IL-6 and MCP-1 was diminished ($P < 0.05$) in C3H/HeJ macrophages, and the production of MIP-2 was diminished ($P < 0.05$) in TLR2$^{-/-}$ macrophages. In contrast, the production of IL-1α, RANTES, and VCAM-1 was greatly increased ($P < 0.05$) in TLR2$^{-/-}$ macrophages (Fig. 3A and D).

Overall, the data showed that TLR2 is a major macrophage receptor for whole *P. gingivalis*, LPS, and FimA, while TLR4 also participates in the response to live *P. gingivalis*. However, induction of some cytokines, such as RANTES, MIP-2, TIMP-1, P-selectin, and CXCL, by *P. gingivalis* LPS or FimA and induction of sTNFRII by live *P. gingivalis* or FimA appear to be independent of both TLR2 and TLR4. The activation of TLR2 by live *P. gingivalis* inhibited the induction of RANTES, IL-1α, and VCAM-1.

Both *P. gingivalis* LPS and FimA stimulate TNF-α expression via TLR2. To confirm whether *P. gingivalis* LPS and FimA signal through TLR2 or TLR4, we compared TNF-α responses to *P. gingivalis* LPS and FimA among mouse peritoneal macrophages from C3H/OuJ, TLR2$^{-/-}$, and C3H/HeJ mice. Macrophages from C3H/OuJ and C3H/HeJ mice responded in a dose-dependent manner to *P. gingivalis* 381LPS (Fig. 4A), *P. gingivalis* A7436LPS (Fig. 4B), *P. gingivalis* 381FimA (Fig.

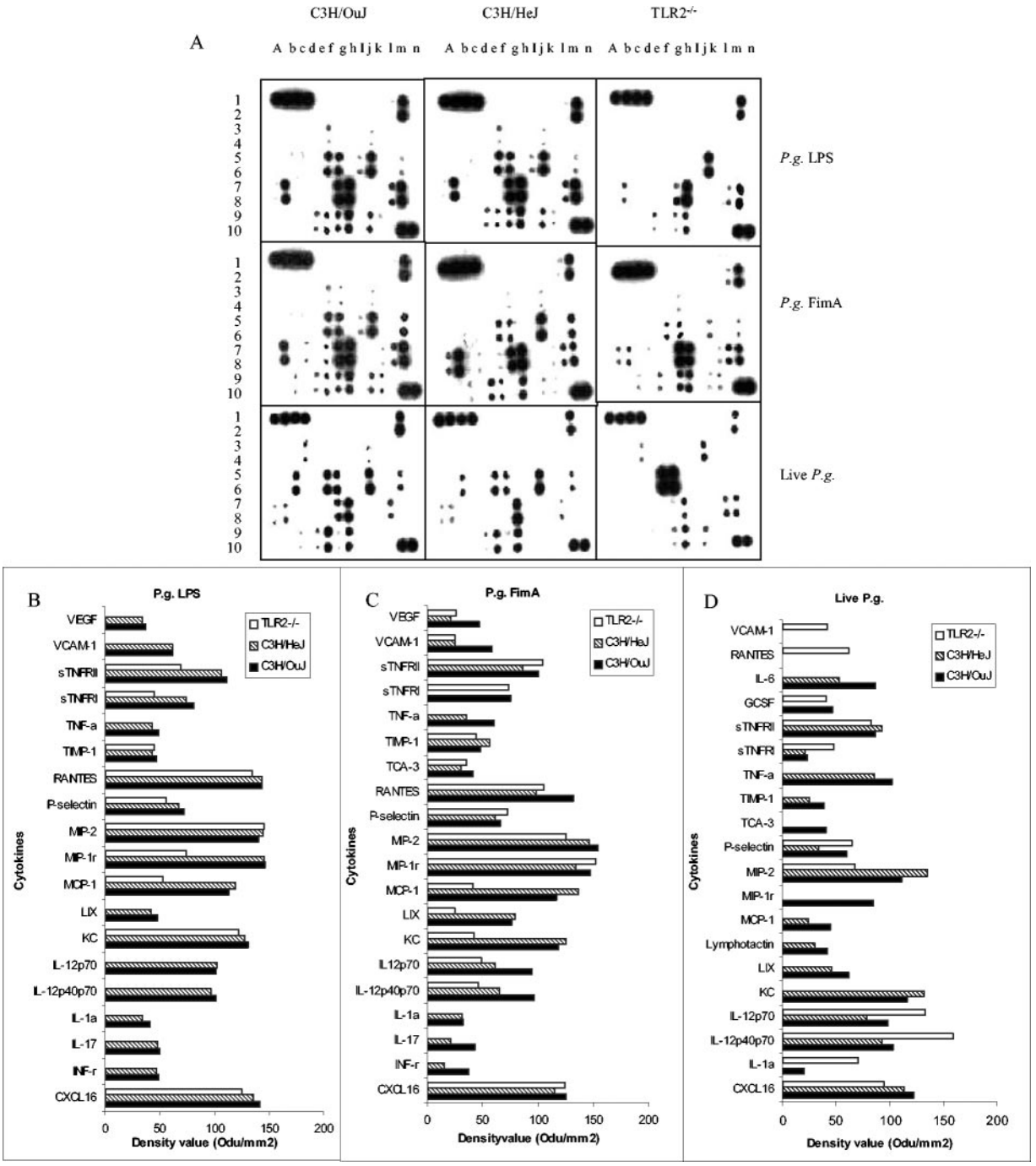

FIG. 4. Effect of TLR2 and TLR4 on cytokine production in mouse peritoneal macrophages. Peritoneal macrophages from C3H/OuJ, C3H/HeJ, and TLR2$^{-/-}$ mice were treated with live *P. gingivalis*, *P. gingivalis* LPS, or FimA for 24 h, and the cell culture supernatants were subjected to cytokine antibody arrays. (A) The cytokine array image represents one of two independent experiments obtained by a 15-min exposure of membranes to X-ray film. The spot orientation is as described in the legend to Fig. 1A. (B to D) Average net optical intensities for each pair of cytokine spots induced by *P. gingivalis* LPS, FimA, and live *P. gingivalis*, respectively. *P.g.*, *P. gingivalis*.

4C), and *P. gingivalis* A7436FimA (Fig. 4D) as assayed by TNF-α production. In contrast, *P. gingivalis* LPS (Fig. 4A and B) and FimA (Fig. 4C and D) did not elicit TNF-α production in response to doses of up to 10 μg/ml in TLR2$^{-/-}$ macrophages. In order to eliminate the possibility that the TLR2$^{-/-}$ macrophages also did not respond to any other stimuli, we incubated macrophages with a TLR4 agonist, *E. coli* LPS. In fact, *E. coli* LPS induced TNF-α expression in TLR2$^{-/-}$ macrophages in a dose-dependent manner, while C3H/HeJ macrophages did not respond to *E. coli* LPS at all (data not shown). This result strongly supports the conclusion that *P. gingivalis* LPS and FimA each activate mouse peritoneal macrophages via TLR2.

DISCUSSION

P. gingivalis is an important etiological agent of chronic adult periodontitis and may also be associated with systemic diseases, including atherosclerosis (3, 11). Part of its virulence is thought to derive from structures on its bacterial cell wall and appendage, such as LPS and fimbriae. These components play important roles in the induction of innate and acquired immune responses, such as the induction of proinflammatory cytokines and of Th1 or Th2 responses in vitro and in vivo in individuals or animals previously exposed to *P. gingivalis* (17, 18, 48). Although LPS and FimA are generally considered to be major pathogenic factors, the host defense system may sense live bacteria differently than individual bacteria components, thus launching a different immune response. Our results showed that the cytokine profile of mouse peritoneal macrophages induced by live *P. gingivalis* is different from that induced by isolated LPS and FimA, whereas LPS and FimA exhibited similar cytokine profiles. This differential cytokine profile was further confirmed by similar findings in vivo. As observed in vitro, the expression of MCP-1 and IL-6 mRNA was regulated differently by live *P. gingivalis* than by its components (LPS or FimA).

The differential cytokine response to live *P. gingivalis* and to its components LPS or FimA suggests that live *P. gingivalis* and its components play different roles in *P. gingivalis* infection and subsequent breakdown of its cell walls by host immune cells. The observation that live *P. gingivalis* is a weak inducer of RANTES, MCP-1, and IFN-γ may lead to relatively small inflammatory infiltrate and less intensive antigen-specific immune responses (16, 47). These reduced immune responses can result in an exaggerated growth of previously constrained subgingival bacteria in the early infection stages. Meanwhile, the overgrowth of bacteria in subgingival tissue could stimulate IL-1β, IL-6, and TNF-α expression, which leads to local tissue destruction (4, 20). In the late stages of infection, LPS and FimA released via bacterial breakdown might penetrate the gingival tissues and move into blood circulation, from which they might contribute to systemic inflammatory responses, such as those believed to be involved in atherosclerosis. *P. gingivalis* LPS and FimA have been demonstrated to have the ability to induce the production of IL-8 and MCP-1 in human vascular endothelial cells (35), which is closely related to atherosclerosis (3, 11). Our data substantiate these observations and further demonstrate that *P. gingivalis* LPS and FimA, but not live *P. gingivalis*, strongly stimulate macrophage to produce cytokines involved in atherosclerosis, such as MCP-1, RANTES, VCAM-1, and VEGF. These results suggest that *P. gingivalis* LPS and FimA may play more important roles in atherosclerosis than live *P. gingivalis* and could explain the lack of substantive results of antibiotics trials for the treatment of coronary artery disease (10, 33). Therefore, further studies are needed to explore the molecular mechanisms of atherosclerosis triggered by *P. gingivalis* and its components.

The differential cytokine induction by *P. gingivalis*, LPS, and FimA also indicates that the bacteria and their components can activate different receptors to mediate intracellular signaling. Our data demonstrated that TLR2 is a common receptor for *P. gingivalis* LPS and FimA, a finding consistent with previous studies (1, 15); however, FimA was also reported to activate TLR4 (13). *P. gingivalis* LPS and FimA activation of the same receptor, TLR2, may account for the fact that they induce similar cytokine profiles in macrophages. We also found that live *P. gingivalis* utilizes both TLR4 and (especially) TLR2 for stimulation of most cytokine responses, which could explain why IL-6, a preferentially TLR4-inducible cytokine (41), was induced by live *P. gingivalis* but not by LPS or FimA. Some cytokines induced by live *P. gingivalis*, *P. gingivalis* LPS, or FimA were not found to signal though TLR2 or TLR4, suggesting that other receptors may also be involved in the recognition of these ligands. Furthermore, some cytokines induced by isolated LPS or FimA were not seen in response to whole *P. gingivalis*, even though it would have contained cell-associated LPS and FimA. There are several possible explanations for this phenomenon. First, the activity of cell-associated LPS or FimA may be tempered by capsular polysaccharide that can physically mask LPS or FimA on the cell surface (40). Second, cytokines produced locally could subsequently be degraded by live *P. gingivalis* (9). Third, live *P. gingivalis* might antagonize some of the cytokine production induced by LPS or FimA (7). Our data showed that live *P. gingivalis* inhibited RANTES, VCAM-1, and IL-1α, via TLR2. Finally, the amount of LPS and FimA present in live *P. gingivalis* cultures used in the in vitro and in vivo assays is much lower than the amount used with purified LPS or FimA. As determined by gas chromatography, LPS and FimA typically represent about 7 to 10% and 3 to 5% of the dry weight of bacteria, respectively. So a culture of *P. gingivalis* (optical density, 2) yields about 100 μg (dry weight) of bacteria per ml, corresponding to 2×10^9 bacteria. Assuming no losses, this amount of bacteria harbors approximately 7 μg of LPS and 3 μg of FimA. Since macrophages were plated at a density of 1×10^6 cells per ml, 5×10^7 of live bacteria were added to the culture to give a MOI of 50:1. Therefore, we estimate that 0.18 μg of LPS/ml and 0.075 μg of FimA/ml were present in our live *P. gingivalis* cultures, amounts substantially lower than the LPS and FimA amounts used in our experiments.

Although *P. gingivalis* LPS and FimA presented similar cytokine profiles in wild-type macrophages and both of them activated TLR2, they induced different cytokine profiles with $TLR2^{-/-}$ macrophages. These differences may not be surprising, considering that TLR2 is thought to associate with other TLRs to form heterodimers, such as TLR1-TLR2 (37), TLR4-TLR2 (21), and TLR6-TLR2 (45). The formation of heterodimers between TLR2 and another TLR dictates the specificity of ligand recognition, thereby diversifying the possible outcomes of TLR2 activation. Our data indicate that FimA activates the TLR2-TLR4 heterodimer. In vivo results also showed that at a relatively low concentration, FimA was more potent than LPS in stimulating IL-1β, TNF-α, MCP-1, MIP-2, and iNOS mRNA expression, while at a fivefold higher concentration, LPS was more potent than FimA in the induction of TNF-α and iNOS mRNA expression. This difference suggests that different TLR2 agonists may induce differential cell activation (12, 24), and it is possible that the use of different coreceptors or different TLR interfaces involved in pathogen recognition may influence the intensity or quality of the induced signals.

Recent studies demonstrated that the apparent TLR2 dependence of many LPS preparations can be eliminated by

phenol re-extraction, while TLR4 dependence is retained (28). In contrast, our *P. gingivalis* LPS preparation, which was confirmed to be protein free by amino acid analysis, did not appear to stimulate cytokine expression through TLR4. Moreover, macrophages from C3H/OuJ and C3H/HeJ mice produced identical cytokine profiles in response to *P. gingivalis* LPS or FimA treatment, while the TLR4-inducible IL-6 (41) was not induced by *P. gingivalis* LPS or FimA in vitro or in vivo. These results confirmed that TLR4-mediated signaling is independent of the *P. gingivalis* LPS and FimA preparations and that those preparations were free of any contaminating environmental LPS.

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