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# *Porphyromonas gingivalis* Outer Membrane Vesicles Induce Selective Tumor Necrosis Factor Tolerance in a Toll-Like Receptor 4- and mTOR-Dependent Manner

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***Porphyromonas gingivalis* is an important member of the anaerobic oral flora. Its presence fosters growth of periodontal biofilm and development of periodontitis. In this study, we demonstrated that lipophilic outer membrane vesicles (OMV) shed from *P. gingivalis* promote monocyte unresponsiveness to live *P. gingivalis* but retain reactivity to stimulation with bacterial DNA isolated from *P. gingivalis* or AIM2 ligand poly(dA·dT). OMV-mediated tolerance of *P. gingivalis* is characterized by selective abrogation of tumor necrosis factor (TNF). Neutralization of interleukin-10 (IL-10) during OMV challenge partially restores monocyte responsiveness to *P. gingivalis*; full reactivity to *P. gingivalis* can be restored by inhibition of mTOR signaling, which we previously identified as the major signaling pathway promoting Toll-like receptor 2 and Toll-like receptor 4 (TLR2/4)-mediated tolerance in monocytes. However, despite previous reports emphasizing a central role of TLR2 in innate immune recognition of *P. gingivalis*, our current findings highlight a selective role of TLR4 in the promotion of OMV-mediated TNF tolerance: only blockade of TLR4—and not of TLR2—restores responsiveness to *P. gingivalis*. Of further note, OMV-mediated tolerance is preserved in the presence of cytochalasin B and chloroquine, indicating that triggering of surface TLR4 is sufficient for this effect. Taking the results together, we propose that *P. gingivalis* OMV contribute to local immune evasion of *P. gingivalis* by hampering the host response.**

Periodontitis (PD) is probably the most frequent chronic inflammatory disorder associated with an alteration of the local microbiota. Clinically, release of inflammatory mediators induces collagen degradation and bone resorption, which ultimately result in tooth loss. The carbohydrate-deficient subgingival environment fosters the growth of *Porphyromonas gingivalis* (1). Tissue degradation caused by enzymes released from bacteria and neutrophils provokes an inflammatory response and supplies bacteria with additional nutrients. This specific milieu permits growth of *P. gingivalis*, whose sophisticated mechanisms for immune evasion enhance growth of the periodontal biofilm, thus leading to bacterial overgrowth and excessive immune stimulation (2, 3).

*P. gingivalis* releases outer membrane lipophilic microvesicles, which contain lipopolysaccharide (LPS) as a major structural component (4–6). These outer membrane vesicles (OMV) overcome the epithelial barrier, thus transporting LPS and other virulence factors into the host tissue (7). Subsequently, OMV elicit a robust mucosal immune response (8), an effect exploited in OMV-based vaccines (8, 9) and mainly attributed to their LPS content (6). However, unlike LPS from *Escherichia coli* or *Salmonella* spp., which are Toll-like receptor 4 (TLR4) agonists, *P. gingivalis* LPS contains a mixture of chemically diverse lipid A species with distinct immune stimulatory properties (10). Albeit *P. gingivalis* LPS was formerly thought to act as a TLR2 ligand (11, 12), recent studies described the opposing effects of *P. gingivalis* LPS-derived penta- and tetra-acylated lipid A forms on TLR4 (13, 14).

Numerous studies have noted that LPS recognition by TLR4 plays a central role in sepsis caused by Gram-negative bacteria where LPS triggers inflammation and lethality (endotoxin shock) and, with a short delay, promotes compensatory immune suppression (15–17). On a cellular level, this counterregulatory action results in a specific form of immunological anergy called "endotoxin tolerance." This phenomenon is associated with cellular unresponsiveness to repeated stimulation with microbes or their components and a cytokine response characterized by low concentrations of proinflammatory cytokines and high levels of anti-inflammatory mediators. Although those studies were carried out using LPS derived from enteric bacteria, LPS from *P. gingivalis* also induces proinflammatory cytokine responses and endotoxin tolerance (2).

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Based on these findings, changes in host cell susceptibility to *P. gingivalis* or its LPS might contribute to the establishment of periodontitis. In the present study, we hypothesized that OMV might represent regulatory factors in the gingival immune response. Our experimental findings demonstrate their pro- and anti-inflammatory effects.

## MATERIALS AND METHODS

**Bacterial strains and OMV preparation.** *P. gingivalis* (DSMZ no. 20709; ATCC no. 33277) was purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and ATCC BAA-308 (= W83) from LGC Standards GmbH, Wesel, Germany). Bacteria were grown under anaerobic conditions on blood agar at 37°C (BD Bioscience, Heidelberg, Germany). For stimulation of human monocytes, bacteria were washed in phosphate-buffered saline (PBS), resuspended in 0.9% saline solution at a McFarland standard of 1 (corresponding to $3 \times 10^8$ CFU/ml), and diluted in RPMI 1640 (Gibco/Life Science, Darmstadt, Germany) to achieve a final densitometric equivalent of a multiplicity of infection (MOI) of 20 ($2 \times 10^6$ bacteria and $10^5$ monocytes/well). For OMV preparation, *P. gingivalis* was grown in 250 ml tryptic soy broth (TSB) supplemented with hemin and menadione for 48 h under anaerobic conditions. Preparation of OMV was performed as described in reference 18 with modifications: the culture supernatant was harvested by centrifugation at 8,000 × *g* at 4°C for 15 min, filtered through a 0.45-μm-pore-size polyvinylidene difluoride (PVDF) membrane (Nalgene rapid-flow; Thermo Scientific, Darmstadt, Germany), and ultracentrifuged at 100,000 × *g* for 3 h at 4°C. The OMV pellets were resuspended in 20 mM Tris-HCl (pH 8.0), pooled, and, after additional sterile filtration (0.45-μm-pore-size Nalgene syringe filters; Thermo Scientific, Darmstadt, Germany), frozen at −20°C. For the protease experiments, pretreatment of OMV with 100 μM leupeptin (Sigma-Aldrich, Munich, Germany) was performed for 60 min on ice as previously described in reference 19. Protein concentrations were determined by the use of a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Bonn, Germany). LPS concentrations were measured by *Limulus* amebocyte lysate (LAL) assay (Pyroquant; Diagnostik GmbH, Moerfelden, Germany). For stimulation of monocytes, *P. gingivalis* OMV were diluted to a final concentration of 25 μg/ml (5 μg/well), unless otherwise indicated.

**Transmission electron (trans-electron) microscopy.** A negative staining protocol was adapted from reference 20: carbon-coated 300-mesh copper grids (Quantifoil, Jena, Germany) were coated with poly-L-lysine solution (Sigma-Aldrich, Munich, Germany) and washed with ultrapure water (Milli-Q; Merck Millipore, Darmstadt, Germany). A 20-μl volume of OMV suspension at 500 μg/ml was applied to the grid and incubated for 1 min, excess liquid was removed, 50 μl 2% methylamine tungstate (Nano-W9; Nanoprobes, Yaphank, NY, USA) was applied for 30 s, excess liquid was removed, and the grid was dried for 5 min before analysis was performed on a Zeiss TEM-900 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) at 70 kV.

**SDS-PAGE and Coomassie staining.** For analysis of protein lysates, bacterial cells were grown to the exponential phase in 15 ml TSB (24 h), harvested by centrifugation (10 min and 4,000 rpm), and washed with cold PBS. The bacterial and OMV pellets were resuspended in radioimmunoprecipitation assay (RIPA) lysis buffer containing aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), NaF, and $Na_3VO_4$ (all from Sigma-Aldrich, Munich, Germany). After mechanical disruption performed with 0.1-mm-diameter glass beads in a Precellys 24 homogenizer (Peqlab, Erlangen, Germany) and incubation on ice for 30 min, lysates were centrifuged at 13,000 rpm for 15 min and supernatants stored at −20°C. Protein concentrations were determined with a Pierce BCA protein assay kit (Thermo Scientific). SDS-PAGE (12%) was performed with 15 μg protein per lane (equivalent to approximately $3 \times 10^7$ bacterial cells). Proteins were stained with 0.2% Coomassie brilliant blue R-250 (Bio-Rad, Munich, Germany).

**Purification of nucleic acids and detection of bacterial DNA and RNA.** DNA and RNA isolation from *P. gingivalis* and *P. gingivalis* OMV was performed using DNeasy and RNeasy blood and tissue kits (Qiagen, Hilden, Germany) and TRIzol (Invitrogen, Karlsruhe, Germany); concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Peqlab). Bacterial DNA was amplified with a 16S rRNA gene PCR performed with consensus primers (21).

**Analysis of TLR2 activity.** Human embryonic kidney (HEK) 293 cells (DSMZ no. ACC305) were transiently transfected with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) with or without plasmid bearing TLR2 cDNA as described in reference 22. After lipofection, cells were washed, resuspended in RPMI 1640–10% fetal calf serum (FCS) (Invitrogen)–1% penicillin-streptomycin (PAA Laboratories), and stimulated with *P. gingivalis* (MOI = 20), OMV (25 μg/ml), or $Pam_3CSK_4$ (100 ng/ml) (EMC Microcollections, Tuebingen, Germany) at 37°C and 5% $CO_2$ for 24 h. Interleukin-8 (IL-8) was quantified by enzyme-linked immunosorbent assay (ELISA) (BD OptEIA; BD Biosciences).

**Monocyte isolation, stimulation, and viability.** The use of human peripheral blood leukocytes was approved by the ethics committee of the Medical Faculty of the University of Bonn (approval no. 36/12). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from buffy coats. Human monocytes were isolated by the use of anti-CD14 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). The purity was analyzed using a FACSCanto I system and anti-human CD14 fluorescein isothiocyanate (FITC) (both from BD Biosciences) and ranged from 85% to 99%. Isolated cells were resuspended in RPMI 1640 supplemented with 2% heat-inactivated autologous human AB serum (Lonza, Cologne, Germany), 100 IU/ml of penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 1% HEPES buffer (all from PAA Laboratories). If not otherwise indicated, stimulation of monocytes was conducted in a two-step approach as follows. (i) Prestimulation was performed with *P. gingivalis* OMV with $4 \times 10^6$ monocytes/ml in a 12-well plate for 24 h or was not performed. (ii) After washes were performed, cells were counted, resuspended in cell culture medium, plated in 96-well flat-bottom plates at $10^5$ monocytes/well, and (re)stimulated for 24 h at 37°C and 5% $CO_2$. Viability of monocytes was assessed by trypan blue exclusion (Applichem Panreac, Darmstadt, Germany), WST-1 assay (Roche, Mannheim, Germany), or propidium iodide staining (eBioscience, Frankfurt am Main, Germany).

**Stimulatory reagents.** The following stimulatory reagents and inhibitors were used at the indicated concentrations, unless otherwise noted: highly purified LPS from *Salmonella* (gift from U. Zaehringer, Research Center Borstel, Germany) (5 ng/ml); $Pam_3CSK_4$ (EMC Microcollections) (100 ng/ml). DNA from the *Staphylococcus aureus* SA113 Δ*lgt* strain (devoid of TLR2 activity) (23) or *P. gingivalis* and poly(dA·dT) (Sigma-Aldrich) (100 ng/well) were transfected with Lipofectamine (Invitrogen) (100 ng DNA per well in Opti-MEM [Gibco/Life Science]). The following neutralizing antibodies (Ab) (R&D Systems, Minneapolis, MN, USA) were used at 10 μg/ml: goat anti-human IL-10 polyclonal Ab (catalog no. AF-217-NA), mouse anti-human TLR2 $IgG_{2B}$ monoclonal Ab (MAb) (catalog no. MAB2616), goat anti-human TLR4 polyclonal Ab (catalog no. AF1478), goat IgG isotype control (catalog no. AB-108-C), and mouse $IgG_{2B}$ isotype control (clone 20116; catalog no. MAB004). Rapamycin (Sigma-Aldrich) (10 ng/ml) and cytochalasin D (2 μM) and chloroquine (2 μM) (both from Enzo Life Sciences) were dissolved in dimethyl sulfoxide (DMSO).

**Cytokine ELISA.** Supernatants from monocytes were collected after 24 h and 48 h as indicated. Levels of cytokines (IL-8, tumor necrosis factor [TNF], IL-10, IL-12p40, and IL-1β) were quantified using BDOptEIA kits (BD Biosciences).

**Fluorescence microscopy.** For fluorescence microscopy, the OMV pellet was labeled with Vybrant DIO cell-labeling solution (Life Technologies, Darmstadt, Germany) at 37°C for 20 min. After washes were performed, the pellet was resuspended in PBS. $CD14^+$ monocytes ($6 \times 10^5$) were incubated with OMV suspension (100 μg/ml) at 37°C and 5% $CO_2$

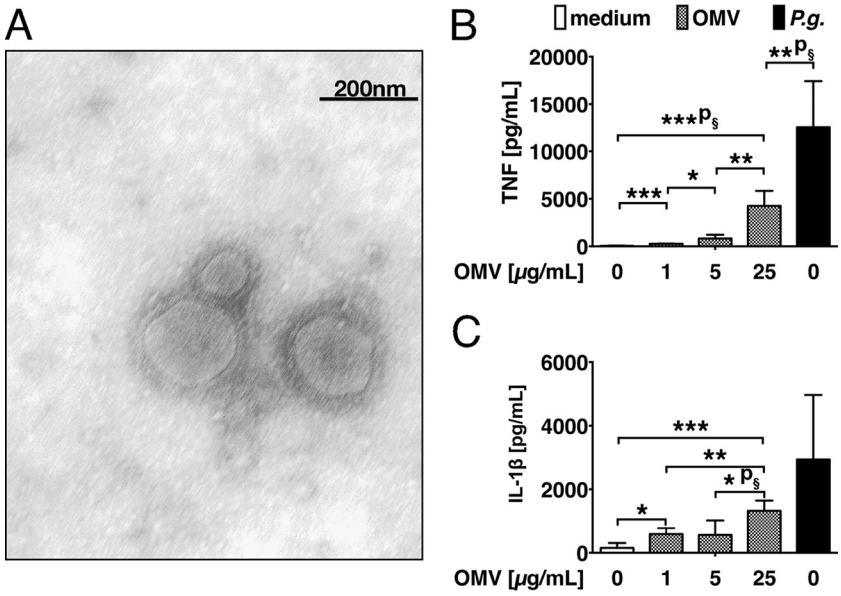


**FIG 1** Secretion of proinflammatory cytokines in response to *P. gingivalis* OMV. (A) Trans-electron microscopy of the purified OMV. The image shows a negative stain with 2% methylamine tungstate and is representative of the results of $n = 6$ experiments. (B and C) Human $CD14^+$ monocytes were stimulated with OMV at the indicated concentrations or with live *P. gingivalis* (*P.g.*). Cellular supernatants were harvested after 24 h and cytokines quantified by ELISA. (B) TNF. (C) IL-1β. The graphs summarize the results obtained from $n = 6$ independent donors ($n = 3$ independent experiments). ***, $P = 0.0002$; ***$p_§ = 0.0008$; *, $P = 0.0281$; **, $P = 0.0031$; **$p_§ = 0.0027$ (B); *, $P = 0.0119$; ***, $P = 0.0003$; **, $P = 0.0051$; *$p_§ = 0.0217$ (C).

for 5 h. To eliminate excess OMV, monocytes were washed before incubation on slides (Marienfeld, Lauda Königshofen, Germany) (37°C and 5% $CO_2$ for 30 min.), fixed with PBS–4% paraformaldehyde (PFA) for 10 min, washed, stained with DAPI (4′,6-diamidino-2-phenylindole) (Life Science, Darmstadt, Germany) for 15 min, washed, and covered with mounting medium before analysis on an Olympus IX81 microscope (Olympus, Hamburg, Germany).

**Statistical analysis.** Statistical analysis of results was carried out using GraphPad Prism 6.0 (Graphpad Software Inc., San Diego, CA, USA). A two-sided Student's *t* test was used to calculate significance for two groups. Results were considered statistically relevant at *P* values of ≤0.05 and are presented as means ± standard deviations. *P* values were labeled as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

## RESULTS

***Porphyromonas gingivalis* sheds OMV with immune stimulatory activity.** *P. gingivalis* releases round vesicles of various sizes between 50 and 200 nm in diameter into the extracellular space (Fig. 1A) (24). These OMV are composed of a limited spectrum of bacterial proteins compared to the whole bacterium (see Fig. S1A in the supplemental material) and LPS, with levels ranging from 0.0027 to 0.033 μg/μg protein. IL-8 induction in TLR2-transfected HEK293 cells further confirmed TLR2 activity (see Fig. S1B). Notably, DNA and RNA levels remained below the detection limit; the rRNA gene was also undetectable by 16S rRNA gene PCR (see Fig. S1C), thus confirming earlier reports that claimed that *P. gingivalis* OMV lack nucleic acids (25).

Measurement of immune stimulatory activity in human monocytes exposed to OMV induced dose-dependent secretion of the TNF and IL-1β proinflammatory cytokines (Fig. 1B and C) but not IL-12 (data not shown). Notably, the OMV concentration of 25 μg/ml (5 μg protein/well) was used in all subsequent experiments. Furthermore, despite the higher immune stimulatory capacity, $2 \times 10^6$ *P. gingivalis* cells per well corresponded to a protein equivalent of approximately 20% of the OMV protein mass, e.g., 1.2 μg protein/well.

In addition, we costimulated monocytes with bacterial DNA or AIM2 ligand poly(dA·dT) and OMV to assess whether complementation of the missing bacterial DNA in OMV potentiates their immune stimulatory activity to the level achieved by stimulation with whole bacteria. Notably, the addition of DNA resulted in an increase in OMV-induced release of IL-1β from monocytes whereas TNF levels remained unaffected (see Fig. S1D in the supplemental material).

**OMV block TNF secretion in response to *P. gingivalis*.** Reasoning that OMV might serve as sentinels that come into earlier and closer contact with innate immune cells than the bacterium itself, we investigated the monocyte response to *P. gingivalis* subsequently to exposure to OMV. Thus, we prestimulated monocytes with OMV for 24 h, washed the cells, and restimulated the monocytes with live *P. gingivalis*. The results obtained revealed that monocytes prestimulated with OMV failed to secrete TNF in response to restimulation with *P. gingivalis* (Fig. 2A, left panel). Moreover, monocytes retained responsiveness to stimulation with bacterial DNA and poly(dA·dT) (Fig. 2A, right panel), the non-TLR stimuli selected for viability control to avoid TLR cross-tolerance. Accordingly, monocyte viability was preserved after stimulation with OMV (Fig. 2B).

**OMV-induced TNF tolerance is mediated by IL-10.** Further analysis revealed that prestimulation of monocytes with OMV induces IL-10 (Fig. 3A). Neutralization of IL-10 restored the TNF response of OMV-stimulated monocytes to live *P. gingivalis* (Fig. 3B), which was not seen using the isotype control. We deducted that OMV-induced tolerance is regulated by IL-10. Notably, IL-1β release was not subject to regulation by IL-10 (Fig. 3B). Of note, failure to detect IL-10 under conditions of IL-10 blocking

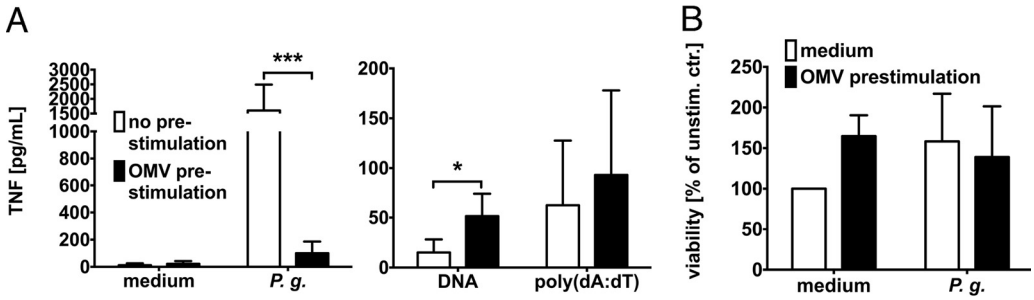


**FIG 2** Effects of OMV preexposure on TNF secretion in response to secondary stimulation with live *P. gingivalis*. (A) Human monocytes were prestimulated or not prestimulated with OMV for 24 h; after washing, cells were left unstimulated (medium) or restimulated with live *P. gingivalis*, *P. gingivalis* DNA, or poly(dA·dT) for another 24 h. TNF secretion was quantified in the monocyte supernatants. Results are shown as mean values ± standard deviations (SD) of data from 12 donors ($n = 6$ independent experiments). ***, $P < 0.0001$; *, $P = 0.0329$. (B) WST-1 was used to determine viability of OMV-stimulated monocytes after 48 h. Results from $n = 12$ donors ($n = 6$ independent experiments) were normalized to the unstimulated control (unstim. ctr.) results, and data are given in percentages (mean values ± SD).

and the presence of IL-10 in the presence of the isotype control proves both the specificity and the efficacy of the neutralizing antibody (Fig. 3A).

**Inhibition of mTOR restores the TNF response of OMV-stimulated monocytes.** Previous work indicated that TLR2- and TLR4-dependent induction of IL-10 release in monocytes is mediated via phosphatidylinositol 3-kinase (PI3K)/PKB/Akt/mTOR-dependent signaling associated with a tolerogenic response (26). In view of the strong TLR2 activity in OMV (see Fig. S1B in the supplemental material), we hypothesized that similar mechanisms underlie OMV-mediated inhibition of TNF secretion. We therefore targeted this pathway using the mTOR inhibitor rapamycin. The results revealed that, although the initial OMV-induced TNF levels were not altered by mTOR inhibition (Fig. 4A), the defective TNF response to *P. gingivalis* could be restored after treatment with rapamycin during OMV pretreatment (Fig. 4B). However, IL-10 secretion levels were not reduced upon rapamycin treatment (Fig. 4B). Interestingly, after treatment with rapamycin, the levels of OMV- and *P. gingivalis*-induced IL-1β secretion (Fig. 4) and *P. gingivalis*-induced IL-12p40 (Fig. 4B) trended upwards, despite not reaching statistical significance. Similar findings were obtained using the VIII Akt inhibitor (data not shown). We thus concluded that PKB/Akt/mTOR signaling is involved in the regulation of OMV-mediated suppression of the TNF response.

**OMV-mediated interference with the TNF response depends on TLR4.** Multiple studies on *P. gingivalis* have highlighted the central role of TLR2 activity in innate immune recognition as well

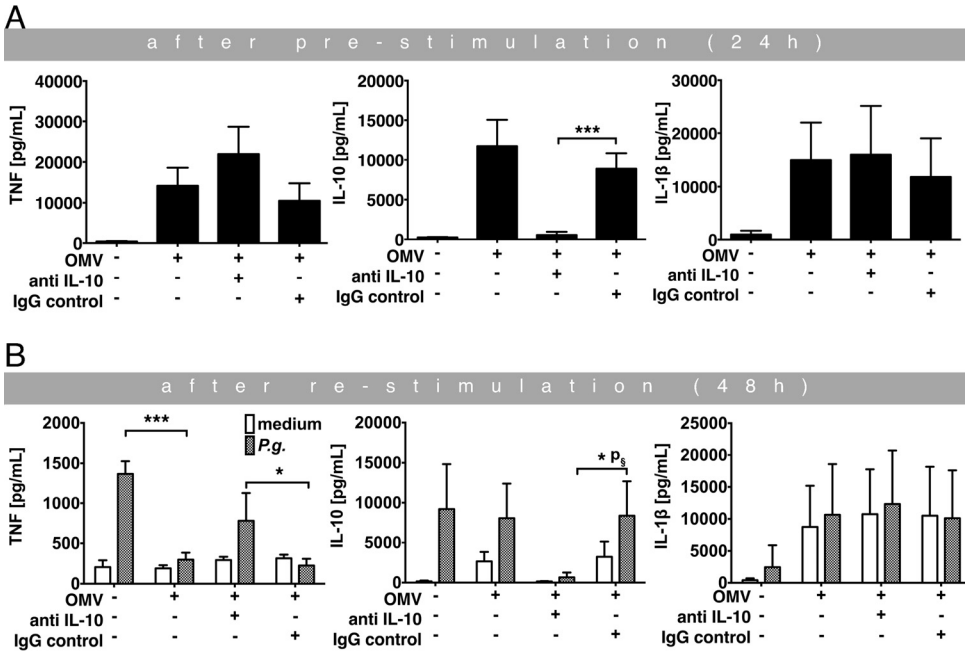


**FIG 3** Role of IL-10 in OMV-induced monocyte tolerance. (A and B) Monocytes were left unstimulated or prestimulated with OMV in the presence or absence of anti-IL-10 or the IgG isotype control for 24 h. Cells were subsequently washed and restimulated with or without live *P. gingivalis* for an additional 24 h. TNF, IL-10, and IL-1β levels were measured in the cellular supernatants obtained after prestimulation (24 h) (A) and after secondary challenge (48 h) (B). The results are shown as mean values ± SD of data from $n = 4$ donors ($n = 2$ independent experiments). ***, $P = 0.0002$ (A); *, $P = 0.02083$; *$p_{§} = 0.0121$; ***, $P < 0.0001$ (B).

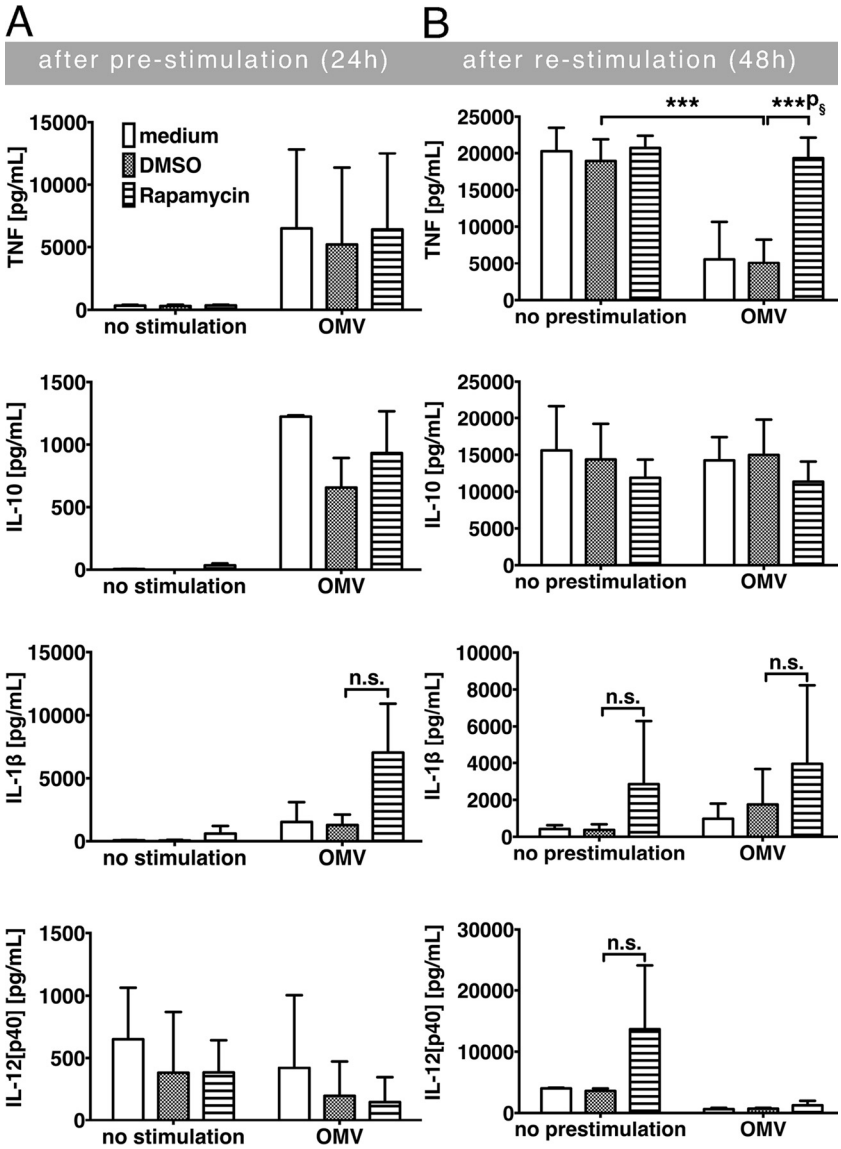


**FIG 4** Role of mTOR in OMV-induced TNF tolerance. (A and B) $CD14^+$ monocytes were preincubated with or without OMV in the presence or absence of rapamycin DMSO as a solvent control for 24 h. Restimulation after washing of cells was performed with or without live *P. gingivalis*. Only data from conditions that included *P. gingivalis* are shown in the diagrams. Concentrations of TNF, IL-10, IL-1β, and IL-12p40 were quantified in the supernatants collected after prestimulation (24 h) (A) and restimulation (48 h) (B). Data are provided as mean values ± SD of results from $n = 6$ donors ($n = 3$ independent experiments). ***, $P = 0.00071$; $^{***}p_{§} = 0.00019$; n.s. = not significant.

as immune subversion by *P. gingivalis* (27). To assess the contribution of TLR2, we prestimulated monocytes in the presence of a TLR2-blocking MAb or the isotype control. Despite complete blockage of cytokine release induced by OMV within the first 24 h (Fig. 5A), TNF secretion could not be restored in the presence of TLR2 neutralization (Fig. 5B). Therefore, the possibility that TLR2 is a major regulator of OMV-induced TNF deficiency was excluded. Further experiments, however, revealed that blockade of TLR4 not only abolished OMV-mediated cytokine secretion (measured after 24 h of stimulation with OMV) (Fig. 5C) but also restored TNF secretion in OMV-stimulated monocytes subsequently challenged with *P. gingivalis* (Fig. 5D). Notably, a reduction in TNF secretion was also observed when TLR4 ligand *Salmonella enterica* serovar Minnesota LPS was neutralized with anti-TLR4 antibody but—in contrast to the results seen with OMV—cellular responsiveness could not be reversed by TLR4 neutralization during secondary challenge (see Fig. S1E in the supplemental material).

Taken together, these data provided proof of differential roles of TLR2 and TLR4 in *P. gingivalis* OMV-mediated immune stimulation and tolerance.

**Role of bacterial fimbriae and proteases in abrogation of the TNF response.** Induction of tolerance was further independent of fimbriae, as it was also induced by OMV derived from the nonfimbriated *P. gingivalis* W83 strain (Fig. 6A). Moreover, pretreatment of OMV with leupeptin, an inhibitor of *P. gingivalis* gingipain proteases (28), did not affect TNF tolerance or IL-10 secretion levels (Fig. 6B).

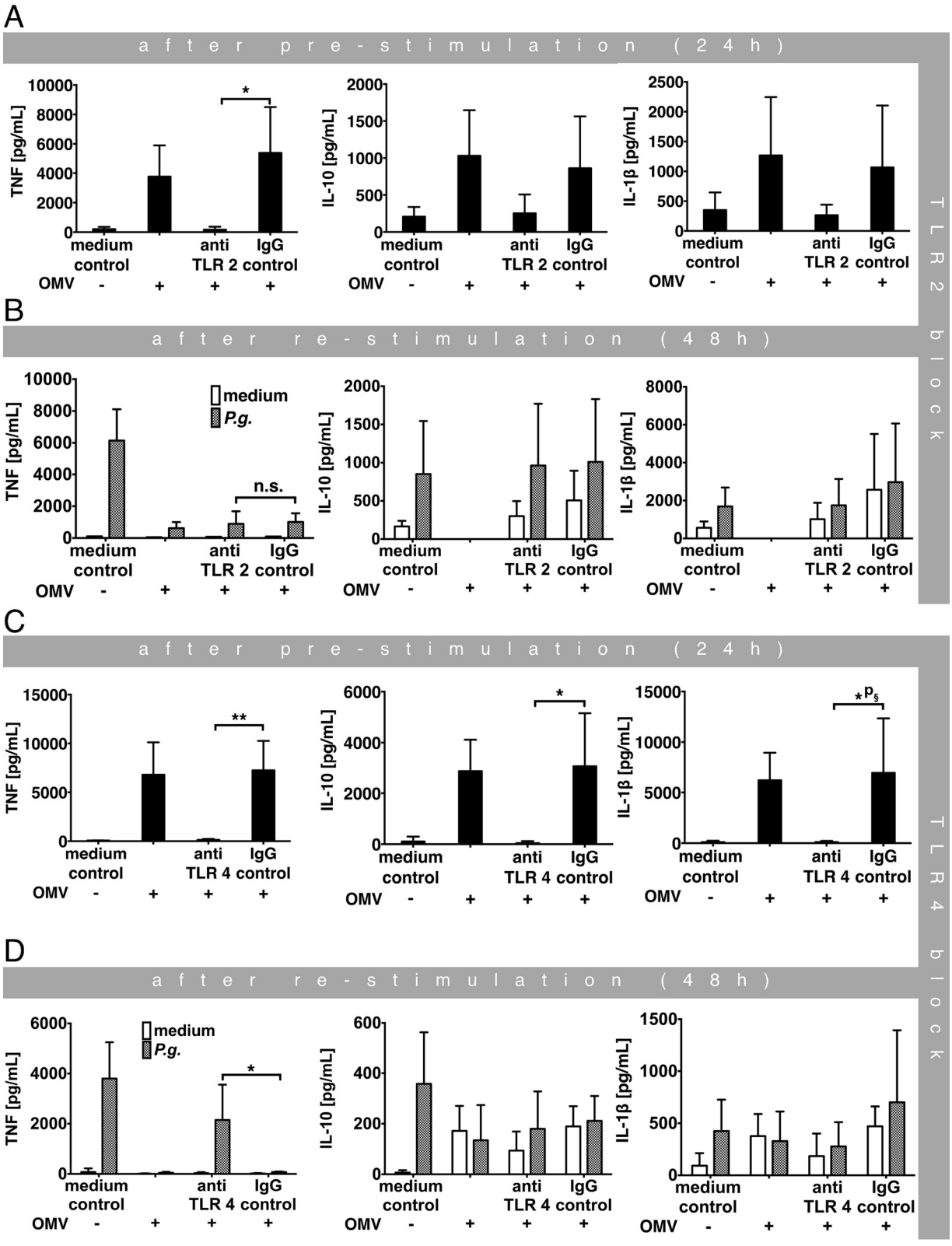


**FIG 5** Role of TLR2 and TLR4 in OMV-mediated abrogation of the TNF response. (A to D) Human monocytes were prestimulated with OMV or not prestimulated; live *P. gingivalis* was used for secondary challenge after washing. The experiments were performed in the presence of neutralizing anti-TLR2 (A and B) and anti-TLR4 (C and D) antibodies or of the respective isotype controls. TNF secretion was measured in the supernatants harvested after 24 (A and C) and 48 h (B and D). The diagrams summarize the data obtained as mean values ± SD of the results from $n = 4$ donors ($n = 4$ independent experiments). *, $P = 0.0154$ (A); n.s. = not significant (B); *, $P = 0.0281$; *$p_{\S} = 0.0452$; **, $P = 0.0032$ (C); *, $P = 0.0261$ (D).

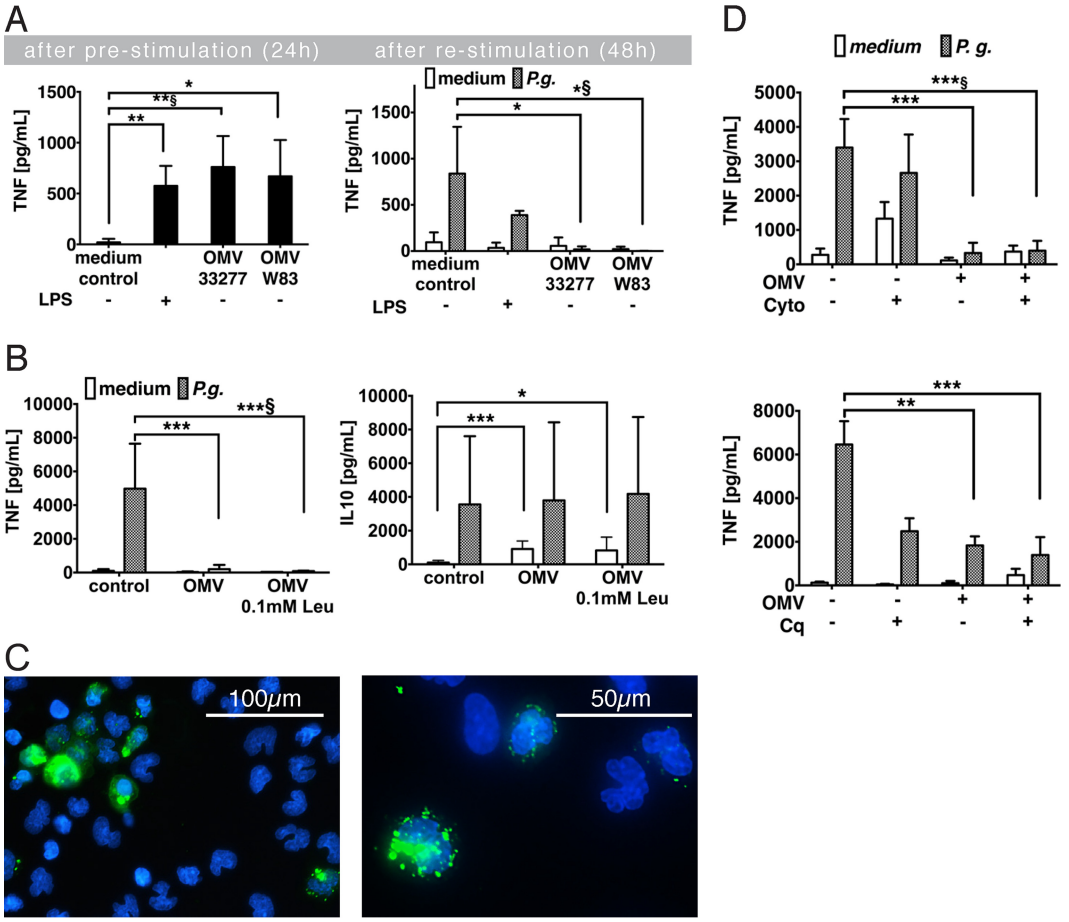


**FIG 6** Role of fimbriae, gingipains, and endocytosis in TNF tolerance. (A) Monocytes were left unstimulated or prestimulated with *Salmonella* Minnesota LPS, *P. gingivalis* (ATCC 33277) OMV, or OMV of the nonfimbriated *P. gingivalis* W83 strain for 24 h. Cells were subsequently washed and restimulated with live *P. gingivalis* (ATCC 33277) for an additional 24 h or not restimulated. TNF, IL-10, and IL-1β levels were measured in the cellular supernatants obtained after prestimulation (24 h) (A) and after secondary challenge (48 h) (B). The results are shown as mean values ± SD of data from $n = 4$ donors (summary of 2 independent experiments). Left panel: **, $P = 0.0023$; **$p_{§} = 0.0053$; *, $P = 0.0143$. Right panel: *, $P = 0.0487$; *$p_{§} = 0.0187$. (B) Monocytes were left unstimulated or prestimulated with OMV that had been pretreated with 100 μM leupeptin (Leu). Cells were subsequently washed and restimulated with live *P. gingivalis* (ATCC 33277) for an additional 24 h or not restimulated. TNF and IL-10 concentrations in the supernatants were measured after secondary challenge (48 h). Results are given as mean values ± SD of data from $n = 6$ donors ($n = 3$ independent experiments). Left panel: ***, $P = 0.0002$; ***$p_{§} = 0.0004$. Right panel: ***, $P = 0.00042$; *, $P = 0.02513$. (C) Immunofluorescence microscopy was used to visualize cellular binding and internalization of OMV labeled with Vybrant-DIO (green) coincubated with monocytes for 5 h. Nuclei were counterstained with DAPI (blue). The images were acquired with 40-fold magnification (upper panel) and 2× zoom (lower panel) and are representative of the results of $n = 2$ experiments. Both images were cropped for better visualization. (D) Human monocytes were prestimulated with OMV or not prestimulated in the presence or absence of cytochalasin B (Cyto) (upper panel), chloroquine (Cq) (lower panel), or the DMSO solvent control. Restimulation after washing was performed with or without live *P. gingivalis*, but only data from conditions that included *P. gingivalis* are depicted in the graphs. TNF concentrations in the supernatants were measured 24 h after restimulation (48 h). Results are given as mean values ± SD of data from $n = 4$ independent donors ($n = 2$ independent experiments). Upper panel: ***, $P = 0.00044$; ***$p_{§} = 0.00049$. Lower panel: **, $P = 0.00222$; ***, $P = 0.00083$.

**OMV-mediated suppression of the TNF response is not affected by blockade of endocytosis.** Next, we asked whether uptake of OMV is required for OMV-induced TNF tolerance. Immunofluorescence staining of OMV and monocytes revealed a spatial association of OMV with monocytes, i.e., a punctuate pattern of surface-bound OMV and, to a lesser degree, intracellular uptake (Fig. 6C). In view of these findings, we investigated whether OMV-mediated interference with the TNF response requires endocytosis of OMV or receptor internalization. To test this, we performed the experiments in the presence of an inhibitor of actin polymerization (cytochalasin D) (Fig. 6D), which interferes with both endocytosis and receptor internalization, and of an inhibitor of endosomal maturation and acidification (chloroquine) (Fig. 6D), typically used to demonstrate involvement of nucleic acid-sensing TLRs. However, chloroquine affected *P. gingivalis*-induced TNF secretion in the absence of OMV, thus limiting the significance of the data. Nevertheless, the experiments showed that treatment of monocytes with cytochalasin D had no effect on OMV-mediated suppression of the TNF response. We concluded that triggering of expression of surface TLR4 by *P. gingivalis* LPS in OMV is sufficient to prevent the release of TNF after secondary exposure of monocytes to *P. gingivalis*.

## DISCUSSION

In a previous study on human monocytes, we demonstrated that TLR2 and -4 induce MyD88-dependent signaling, leading to

IL-10 production, which suppresses IL-12 expression and the T cell response to alloantigen (26). This tolerogenic response is mediated in a PI3K/PKB/Akt/mTOR-dependent pathway and counterregulated by IRAK4 (26). Similarly, in the study described in reference 29), the PI3K/Akt pathway reciprocally regulated *P. gingivalis* LPS-induced IL-10 and IL-12 synthesis, notably, in a TLR2-dependent manner. Our present data demonstrate a different form of monocyte tolerance: in accordance with our earlier findings, OMV-induced monocyte unresponsiveness is sensitive to the presence of rapamycin (Fig. 4), thus confirming the regulatory role of PKB/Akt/mTOR in tolerogenic TLR responses (30–32); however, in the present context, tolerance was limited to TNF responsiveness (Fig. 2).

In a previous study on murine macrophages, the authors proposed that *P. gingivalis* LPS suppresses TNF expression but concomitantly induces IL-10 production (33). In our study, IL-10 levels remained unchanged by prior exposure to OMV (Fig. 3) but neutralization of IL-10 almost completely restored *P. gingivalis*-induced TNF secretion in OMV-pretreated cells (Fig. 3). Thus, similarly to our previous study, our data identify IL-10 as an important mediator of tolerance (26). It has further been suggested that increased TNF levels in monocytes and macrophages after rapamycin treatment reflect loss of IL-10 (32, 34). Here, however, OMV-induced IL-10 was not sensitive to rapamycin (Fig. 4).

Earlier studies limited the definition of endotoxin tolerance to the failure to secrete TNF upon reexposure to LPS (35). Taking into consideration that one of the most important effects of TNF is the priming of neutrophils to enhance chemotaxis, phagocytosis, and respiratory burst reactions (36–38), continuous exposure to OMV may prevent TNF-induced influx of neutrophils and neutrophil-mediated clearance of *P. gingivalis*. However, neutrophil responsiveness to TNF priming in PD patients was equivalent to that in healthy controls (39). Nevertheless, use of peripheral blood neutrophils in the cited study must be critically viewed because only peripheral (i.e., oral) neutrophils are potentially exposed to *P. gingivalis* OMV.

In contrast to studies using purified *P. gingivalis* LPS, monocytes stimulated with OMV did not secrete IL-12 (Fig. 4). Secretion of IL-12 is, however, induced by stimulation with live *P. gingivalis* and repressed by prior OMV exposure (Fig. 4). Notably, abrogated IL-12 production desensitizes T cells to alloantigen and prevents the development of protective Th1 and Th17 responses in the mucosa (26, 40).

Our data show that costimulation of OMV with bacterial DNA or AIM2 ligand increases IL-1β secretion but does not affect TNF levels (see Fig. S1D in the supplemental material). This difference most likely arises from the key role of cytosolic DNA recognition and inflammasome activation in the induction and processing of IL-1β, while triggering of surface TLR expression is sufficient for TNF induction. In line with this argument, we further show that cytosolic DNA recognition and IL-1β production are preserved in the presence of OMV-mediated suppression (Fig. 2 and 3B). Thus, OMV-mediated immune modulation should not be confounded with cellular anergy.

Earlier studies, however, delivered conflicting results: prestimulation with purified *P. gingivalis* LPS inhibited IL-1β release after restimulation in THP-1 cells and human PBMC (41–43). These differences most likely reflect that the microbial stimulus provided by OMV differs from that provided by a single TLR2 or TLR4 agonist. Furthermore, PI3K dependency of *P. gingivalis*-induced IL-1β secretion was observed in a murine PD model (27), albeit secretion of OMV-induced IL-1β in our study increased even in the presence of rapamycin (Fig. 4), a finding supported by a report noting that mTOR negatively regulates IL-1β production (31).

In contrast to our previous study, where levels of TLR2- and TLR4-dependent monocyte tolerance were indistinguishable (26), our present data highlight differential roles of TLR2 and TLR4 in OMV-mediated induction of TNF tolerance of *P. gingivalis*. While selective blockade of either receptor nearly abrogates the TNF response upon OMV stimulation, only anti-TLR4 treatment prevents OMV-mediated tolerance (Fig. 5). The finding that inhibition of either TLR2 or TLR4 reduces OMV-induced cytokine secretion to the unstimulated baseline is unexpected. Previous reports described a reduction in TNF secretion of only 70% after blockade of either TLR2 or TLR4, indicating that these receptors were partially redundant (44). We can only speculate that synergistic activation of these receptors might be necessary for OMV-induced release of cytokines (45).

Albeit we observed selective reversal of TNF tolerance by neutralization of TLR4 (Fig. 5), *P. gingivalis* has long been viewed as a Gram-negative pathogen bearing an atypical LPS with TLR2 activity but lacking TLR4 binding (11, 46). In contradiction of our results, *P. gingivalis* LPS-mediated induction of TNF in THP-1 cells and phosphorylation of Akt in human monocytes were previously reported to be strictly dependent on TLR2 and could not be inhibited by anti-TLR4 monoclonal antibodies (29, 41). Furthermore, in an *in vivo* pharmacodynamics (PD) model, inhibition of both PI3K and TLR2 interfered with *P. gingivalis* expansion, but TLR4 blockade had no effect (27). Thus, in contrast to our results obtained with *P. gingivalis* OMV, earlier studies failed to demonstrate a role for *P. gingivalis* LPS in TLR4 activation.

Notably, previous reports investigating whether *P. gingivalis* LPS interferes with the activity of *E. coli* LPS provide different molecular explanations: they support the hypothesis that the inhibitory potential was attributable either to tolerance mediated by loss of the ability to upregulate TLR2 or TLR4 expression upon restimulation with *E. coli* or *P. gingivalis* LPS (33, 47) or, alternatively, to TLR4 antagonism mediated via direct interaction of *P. gingivalis* LPS with TLR4 (42, 43). In addition, dephosphorylation of lipid A by *P. gingivalis* phosphatases prevents TLR4 activation and promotes resistance to cationic antimicrobial peptides (48). Other reports, however, describe *P. gingivalis* LPS preparations as a highly heterogeneous mixture of different types of lipid A species with differential levels of stimulatory potency in their effects on TLR2 and TLR4 (10, 14, 49). In addition, a few studies demonstrated that TLR2 activity can be attributed to *P. gingivalis* lipoproteins (50–52). However, since methods that are more refined than the SDS-PAGE performed in this study (see Fig. S1A in the supplemental material) are required to visualize the full protein spectrum, including the lipoproteins (5, 6), we can only speculate that *P. gingivalis* OMV contain lipoproteins that account for TLR2 activity (Fig. 5; see also Fig. S1B in the supplemental material). Nevertheless, one study showed that *P. gingivalis* lipid A is a specific ligand for murine TLR4 (53), thus providing a molecular basis for the prominent role of TLR4 in OMV-mediated immune modulation demonstrated in the present study.

One major difference between TLR4 signaling and TLR2 signaling is the recruitment of TIR-domain-containing adapter-inducing beta interferon (IFN-β) (TRIF) by TLR4, which occurs in

a MyD88-independent manner (54). It was recently reported that TLR4 is superior to TLR2 in regard to IL-10 production because signaling via TRIF prevented degradation of IL-10 mRNA in macrophages (55). Furthermore, TRIF-dependent release of IFN-β has been implicated in endotoxin tolerance (56). However, the level of IFN-β induction by *P. gingivalis* is low and was previously suggested to depend on the presence of TLR7 (57). Also, recruitment of TRIF and activation of nucleic-acid-sensing TLRs require internalization of TLR4 or the respective ligands. Similarly to Klein et al., who described punctuate structures upon LPS binding to its receptor in the plasma membrane (58), we detected punctuate membrane-bound OMV and only limited amounts of OMV within the cells (Fig. 6C). Nevertheless, our data suggest that internalization is not a prerequisite for OMV-induced TNF suppression because blockade of actin polymerization and endocytosis with cytochalasin D had no effect (Fig. 6D).

Recent work by Slocum et al. provides evidence for agonistic and antagonistic effects of *P. gingivalis* LPS on TLR4 that depend on its chemical modification in the form of, e.g., tetra- and pentaacylation, respectively (13). The heterogeneity in affinity, binding, and immunological potency of different *P. gingivalis* LPS forms might also explain the reversibility of TLR4-mediated TNF tolerance in the presence of anti-TLR4 neutralizing antibodies, which was not achieved when monocytes were stimulated with classical TLR4 agonist LPS from *Salmonella* Minnesota (see Fig. S1E in the supplemental material). Furthermore, previous reports associated OMV-mediated TNF tolerance with proteolytic degradation of surface LPS receptor CD14 by *P. gingivalis* proteases such as gingipains (59–61). At this point, we can only speculate that the resultant lack of CD14 could be responsible for the reversibility of *P. gingivalis* OMV-mediated TNF tolerance of anti-TLR4. When surface CD14 is degraded by gingipains, tolerance can become solely dependent on TLR4 signaling and, thus, can be reversed by anti-TLR4. In contrast, endotoxin tolerance induced by *Salmonella*-derived LPS is additionally mediated by TLR4-independent CD14 signaling and therefore might not be inhibited solely by TLR4 antagonism (62, 63).

In control experiments, however, the presence of protease inhibitor leupeptin did not affect TNF tolerance (Fig. 6B). Nevertheless, further work will have to clarify the relative contributions of gingipains and TLR4 to TNF tolerance. Recent data additionally suggested that *P. gingivalis* induces C5aR-dependent proteasomal degradation of MyD88, which strengthens MyD88-independent TLR2-Mal-PI3K signaling (27). Since OMV might contain gingipains (8), the enzymes acting as C5 convertases (59), it is plausible that TLR4 ligands and gingipains in OMV might synergistically drive the switch of TLR signaling to the PI3K/PKB/Akt/mTOR-dependent pathway. Notably, gingipain and C5aR engagement inhibited secretion of IL-12p70 (59), a finding fully compatible with the lack of IL-12 secretion seen upon monocyte stimulation with OMV (Fig. 4).

Taken together, our results demonstrate that exposure of monocytes to *P. gingivalis* OMV exerts pro- and anti-inflammatory effects. We can only speculate that continuous exposure to *P. gingivalis* OMV in periodontal disease induces selective TNF deficiency that hampers microbial recognition and hence represents a possible immune evasion strategy of *P. gingivalis*. Changes in the proportional representation of *P. gingivalis* and/or OMV release could promote inflammation and periodontitis.

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