Activation of the TREM-1 pathway in human monocytes by periodontal pathogens and oral commensal bacteria

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SUMMARY

Periodontitis is a highly prevalent disease caused in part by an aberrant host response to the oral multi-species biofilm. A balance between the oral bacteria and host immunity is essential for oral health. Imbalances in the oral microbiome lead to an uncontrolled host inflammatory response and subsequent periodontal disease (i.e. gingivitis and periodontitis). TREM-1 is a signaling receptor present on myeloid cells capable of acting synergistically with other pattern recognition receptors leading to amplification of inflammatory responses. The aim of this study was to investigate the activation of the TREM-1 pathway in the human monocyte-like cell line THP-1 exposed to both oral pathogens and commensals. The relative expression of the genes encoding TREM-1 and its adapter protein DAP12 were determined by quantitative real-time polymerase chain reaction. The surface expression of TREM-1 was determined by flow cytometry. Soluble TREM-1 and cytokines were measured by enzyme-linked immunosorbent assay. The results demonstrate that both commensal and pathogenic oral bacteria activate the TREM-1 pathway, resulting in a proinflammatory TREM-1 activity-dependent increase in proinflammatory cytokine production.

INTRODUCTION

Periodontitis is a common oral inflammatory disease leading to destruction of tooth supporting structures and alveolar bone, and, when left untreated, ultimately causes tooth loss. Periodontitis affects a substantial proportion of the population, with the prevalence of severe periodontitis estimated to be 8.5% (Eke *et al.*, 2012). Bacteria play a critical role in initiating gingivitis and periodontitis. Several species of oral bacteria, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* have been associated with aggressive and chronic forms of periodontitis (Haubek *et al.*, 2008; Oliveira *et al.*, 2016). These bacteria stimulate host innate immune responses that play a crucial role in the pathogenesis of periodontal infection

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(Yucel-Lindberg & Bage, 2013). Uncontrolled host inflammatory responses can contribute to the progression of periodontitis, including effects such as severe tissue damage and loss of alveolar bone.

Triggering Receptor Expressed on Myeloid cells (TREM)-1, a member of the immunoglobulin superfamily, is a receptor expressed on monocytes and neutrophils that plays an important signaling role in the innate immune response against pathogenic bacteria, viruses and fungi (Sharif & Knapp, 2008; Buckland *et al.*, 2011; Ivan *et al.*, 2012). Activation of TREM-1 leads to a cascade of intracellular signaling events resulting in activation and degranulation of neutrophils, enhanced phagocytosis and cytokine production (Arts *et al.*, 2013). TREM-1 consists of an extracellular domain, a transmembrane region, as well as a short cytoplasmic domain that lacks signaling motifs (Ramanathan *et al.*, 2005). Consequently, the association of TREM-1 with the 12-kDa adaptor protein DNAX activation protein (DAP12) is required for induction of downstream signaling (Bouchon *et al.*, 2000). Although TREM-1 ligands are yet to be definitively identified, putative ligands include high-mobility group box 1 (HMGB1), 70-kDa heat-shock protein (hsp 70), a ligand expressed on platelets, peptidoglycan recognition protein 1 (PGLYRP1) of neutrophils, as well as bacterial peptidoglycans and lipopolysaccharides (Bouchon *et al.*, 2001; El Mezayen *et al.*, 2007; Haselmayer *et al.*, 2007; Read *et al.*, 2015).

TREM-1 plays a major role in bacterial infection and sepsis by acting as an amplifier of inflammation and is necessary for a successful antibacterial response. In a polymicrobial sepsis mouse model, a moderate amount of TREM-1 silencing by small interfering RNA (siRNA) enhanced mouse survival, likely by reducing the systemic inflammatory response, whereas high doses of siRNA resulted in impaired neutrophil function and increased mouse mortality (Gibot *et al.*, 2007) suggesting that optimized levels of TREM-1 are required for mounting an effective immune response and clearance pathogens. TREM-1 can activate the nuclear factor-$\kappa\beta$ (NF-κB) pathway leading to a proinflammatory response characterized by increased production of cytokines tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-12p40 and IL-1β (Gibot *et al.*, 2004; Sharif *et al.*, 2007). TREM-1 also modulates the signaling pathways of pattern recognition receptors, including Toll-like receptors (TLRs) and Nod-like receptors (Arts *et al.*, 2013). For example, TREM-1 can act synergistically with TLR-2 in inducing cytokine production (Radsak *et al.*, 2004), whereas TLR-2 concomitantly upregulates TREM-1 via the MyD88-dependent pathway (Buckland *et al.*, 2011). Molecular cross-talk between TREM-1 and TLR-2 may also occur at the level of NF-κB transcription factor as it is involved in both pathways (Arts *et al.*, 2013). TREM-1 additionally modulates the TLR-4 pathway (Arts *et al.*, 2011), by regulating the expression of MyD88 and CD14, through regulation of NF-κB (Ornatowska *et al.*, 2007), and by downregulating negative regulators of the TLR pathway (e.g. IRAK-M) (Lagler *et al.*, 2009).

A soluble form of TREM-1 (sTREM-1) has been detected in the plasma of endotoxin-treated mice, bronchoalveolar lavage fluids from patients with pneumonia, serum of patients with septic shock and gingival crevicular fluid of patients with periodontitis (Gibot *et al.*, 2004; Bisson *et al.*, 2012; Palazzo *et al.*, 2012), raising the possibility that sTREM-1 may be useful as a diagnostic marker for sepsis and inflammation. The mechanism of formation of sTREM-1 is not yet understood, although alternate splicing of TREM-1 mRNA (Gingras *et*

al., 2002; Baruah *et al.*, 2015) and proteolytic cleavage of membrane-bound TREM-1 by matrix metalloproteinases (Gomez-Pina *et al.*, 2007) have been proposed as possibilities. The function of sTREM is also not clear, but when released it may competitively bind TREM-1 ligands, thereby acting as a negative regulator of the TREM-1 pathway; sTREM-1 has been shown to have a protective effect on murine septic peritonitis (Bouchon *et al.*, 2001).

Fine-tuning of the immune response is critical in preventing excessive inflammation and tissue damage. TREM-1 receptors can act as amplifiers of the inflammatory response and cytokine production by monocytes and neutrophils. Therefore, modulating TREM-1 expression may help to dampen immune responses to benefit the host. Little is known regarding the regulation of TREM-1 expression in response to periodontal pathogens, and it is not clear how different periodontal bacteria, including Gram-negative pathogens, as well as Gram-positive commensals, activate the TREM-1 pathway in the context of periodontitis. The objective of this study was to compare the induction of TREM-1 expression and proinflammatory cytokine production in THP-1 monocytes following exposure to several species of periodontal bacteria.

MATERIALS AND METHODS

Host cells

The human monocyte-like cell line THP-1 was obtained from Sigma Aldrich (St Louis, MO). The cells were maintained in RPMI Glutamax medium (Life Technologies, Grand island, NY) supplemented with 10% fetal bovine serum (FBS; Life Technologies) at a density of 1×10^6 to 2×10^6 cells ml^{-1}. The cells were split every 3 to 4 days. Cells were maintained in a 5% CO_2 incubator at 37°C. For experiments, 1×10^6 cells per well were seeded in 12-well tissue-culture coated plates (Falcon).

Bacteria

The bacterial species *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Streptococcus gordonii*, *Streptococcus cristatus* and *Streptococcus pneumoniae* were tested. Both the Gram-positive respiratory pathogen *S. pneumoniae* and the Gram-negative periodontal pathogen *P. gingivalis* are known positive activators of the TREM-1 pathway and were used as positive experimental controls. *S. gordonii* and *S. cristatus* were chosen to represent oral commensal members of the mitis group of streptococci that also includes *S. pneumoniae*. The bacterial strains obtained from our culture collection and the growth conditions used are listed in Table 1. THP-1 cells were routinely infected with bacteria at multiplicities of infection (MOI) of 10 and 100 for 4 h and 24 h. Uninfected cells were used as controls.

Survival of bacteria in co-culture with THP-1 cells

To determine the survival of each bacterial strain co-cultured with the cell line, 1×10^8 bacteria were added to 1×10^6 THP-1 cells in 1 ml of cell culture medium and incubated in 5% CO_2 at 37°C for 4 and 24 h. At the end of each time period, the bacterial suspension was serially diluted and spotted onto duplicate agar plates and incubated under conditions appropriate for the bacterial strain. The number of bacteria recovered was enumerated by

counting colony-forming units (CFU) using the spotting–spreading method as described elsewhere (Thomas *et al.*, 2012). Uninfected THP-1 cells were included as a control for sterility.

Cytotoxicity of bacteria on THP-1 cells

Cytotoxicity was determined using the Pierce LDH cytotoxicity assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Briefly, THP-1 cells were seeded in RPMI medium with 10% FBS at a density of 1×10^4 cells per well in 96-well tissue culture-coated plates (Falcon). Cells were co-incubated with bacteria at MOIs of 10 and 100, in triplicate, and incubated in 5% CO_2 at 37°C for 4 and 24 h. At the end of the incubation period, the 96-well plate was centrifuged at 250 **g** for 3 min. The cell culture medium (50 μl) was transferred to appropriate wells of a new 96-well flat-bottomed plate (Falcon). The reaction mixture (50 μl) was added to each well and mixed by gentle tapping. The plate was incubated at room temperature for 30 min protected from light. Stop solution (50 μl well^{-1}) was added to each sample well and mixed by gentle tapping. Absorbance was measured at 490 nm and 680 nm using the Beckman AD340 microplate reader (Beckman Coulter, Brea, CA). To determine LDH activity, absorbance value at 680 nm (background signal from the instrument) was subtracted from the 490 nm absorbance. Cytotoxicity was expressed as a percentage of the maximum LDH activity.

RNA extraction

RNA extraction was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). After 4 and 24 h of infection, THP-1 cells were centrifuged, and the supernatant was removed and stored at −80°C for cytokine analysis. RNA was extracted from the cell pellet following the manufacturer's instructions. RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer. RNA was used immediately for cDNA synthesis or stored at −80°C until further use.

Reverse transcription (cDNA synthesis)

Reverse transcription was performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. An equal amount of RNA (1 μg) was used for all the samples. Reverse transcription was performed in a BioRad MyCycler thermocycler (BioRad, Hercules, CA) at 37°C for 60 min followed by 95°C for 5 min. Samples without reverse transcriptase (no RT) were used as negative controls. The cDNA was stored at −20°C until further use.

Quantitative real-time polymerase chain reaction

TaqMan Gene Expression Assays and the TaqMan Gene Expression Master Mix (Life Technologies) were used according to the manufacturer's instructions. β-Actin was used as an endogenous control (TaqMan Assay ID: Hs 01060665-g1). The relative expression of TREM-1 (TaqMan Assay ID: Hs 00218624-m1) and DAP12 (TaqMan assay ID: Hs00182426-m1) genes was determined. The cDNA from uninfected cells was used as a control. Polymerase chain reaction (PCR) conditions used were 50°C for 2 min, 95°C for 5 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Quantitative PCR was

carried out in a 7500 Real-time PCR System (Applied Biosystems). Data were analyzed by the ⊿CT method using the ABI 7500 system software.

Flow cytometry

Surface expression of TREM-1 was determined by flow cytometric analysis. THP-1 cells were co-incubated with the bacteria at an MOI of 10 and 100 for 4 and 24 h. After incubation, the cells were washed with phosphate-buffered saline. Fc receptors were blocked using human FcR blocking reagent (Miltenyi Biotec, San Diego, CA). Cells were stained using mouse anti-human TREM-1 antibody (Abcam, Cambridge, MA) followed by goat anti-mouse IgG Alexa Fluor 488 secondary antibody (Abcam). Mouse IgG1 monoclonal antibody (Abcam) was used as an isotype control. After washing, the cells were resuspended in phosphate-buffered saline with 0.5% bovine serum albumin. Cells were fixed in 2% formaldehyde in FACS buffer and analyzed using a BD Fortessa flow cytometer (Becton Dickinson). The results were analyzed using FLOWJO (version 8.8.7).

Cytokine analysis and measurement of sTREM-1

Levels of sTREM-1, IL-1β and TNF-α in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN).

Effect of TREM-1 inhibitor on cytokine release

Involvement of the TREM-1 pathway in the secretion of proinflammatory cytokines was tested using the synthetic LP17 peptide (LQVTDSGLYRCVIYHPP) (Pepscan Presto B.V, Lelystad, the Netherlands) that mimics the highly conserved extracellular domain of TREM-1 believed to be involved in the binding of TREM-1 to its ligand (Radaev *et al.*, 2003; Gibot *et al.*, 2004). A second peptide with the same amino acids but in a different order was used as a control (LP 17 control peptide; TDSRCVIGLYHPPLQVY) (Pepscan Presto B.V). THP-1 cells were infected with the bacteria at an MOI of 100. Some wells were also treated with either LP17 or LP17 control peptide at a concentration of 100 ng ml^{-1}. After incubating the cells for 24 h, IL-1β and TNF-α levels in the supernatant were quantified using ELISA.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Tukey's honest significant difference post hoc test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Cytotoxicity

A general measure of bacterial toxicity against monocytes was initially performed, with cytotoxicity determined by measuring LDH released from the THP-1 cells treated with different oral bacterial species (Table 2). *Streptococcus pneumoniae* induced the highest cytotoxicity and *A. actinomycetemcomitans* induced the least cytotoxicity.

Bacterial survival in cell culture conditions

We next examined the ability of the bacteria to survive in co-culture with THP-1 cells in RPMI medium with 10% FBS in 5% CO_2 at 37°C. This test was performed to make sure that the strict anaerobes (*P. gingivalis* and *T. forsythia*) would be able to survive under cell culture conditions. Aerobic and microaerophilic bacteria (*S. gordonii*, *S. cristatus*, *S. pneumoniae* and *A. actinomycetemcomitans*) survived well under cell culture conditions and increased in numbers during the 24 h period. However, as was expected, the strict anaerobes (*P. gingivalis* and *T. forsythia*) showed significant decreases in CFUs recovered after 4 and 24 h of incubation under THP-1 cell culture conditions (Fig. 1). There was no difference between the CFUs recovered when the bacteria were cultured alone (data not shown) or in co-culture with the THP-1 cells for all the bacteria tested.

TREM-1 and DAP12 gene expression

Patterns of TREM-1 and DAP12 expression were next determined by qPCR in THP-1 cells co-incubated with different oral bacteria at an MOI of 10 and 100 for 4 or 24 h (Fig. 2). At 4 h exposure, *P. gingivalis* (MOI 10 and 100) and *S. pneumoniae* (MOI 100), two well-known pathogens, induced significantly higher expression of TREM-1 mRNA (Fig. 2A). Following 24 h of exposure, all tested bacterial species induced increased TREM-1 gene expression (Fig. 2B). In the case of *S. pneumoniae* at 24 h exposure, the bacteria caused very significant monocyte cytotoxicity. Hence, the RNA yield and quality from these samples was low, resulting in failure of qPCR at this time-point. Gram-positive organisms generally induced higher TREM-1 expression in THP-1 cells compared with the Gram-negative organisms at this time-point. Similar to TREM-1 gene expression, *S. pneumoniae* (MOI 100) induced significantly higher DAP12 expression following 4 h exposure (Fig. 2C). No other bacterial strain tested induced increased DAP12 expression at this time-point. However, after 24 h exposure, all bacterial strains tested, with the exception of *T. forsythia*, induced increased DAP12 expression in THP-1 cells (Fig. 2D).

Surface expression of TREM-1

Changes in surface expression of TREM-1 were additionally examined (Fig. 3A,B). Exposure of THP-1 cells to bacteria for 24 h resulted in significantly increased surface expression of TREM-1 in all cases, with the exception of *P. gingivalis*. No differences in THP-1 surface expression of TREM-1 were seen after 4 h exposure to any of the tested bacteria (data not shown).

sTREM-1 release

The release of sTREM-1 in cell culture supernatants was determined by ELISA. *Porphyromonas gingivalis* (MOI 100) was the only bacterial strain to induce significant levels of sTREM-1 at 4 h (Fig. 4A), and at 24 h it induced the highest levels of sTREM-1 (Fig. 4B). Moderate levels of sTREM-1 were also induced by *S. cristatus* and *S. pneumoniae* at 24 h (MOI 10). sTREM-1 release was not induced by *A. actinomycetemcomitans, T. forsythia* or *S. gordonii*.

Cytokine production and effect of TREM-1 inhibitor

Exposure of THP-1 cells to oral bacteria resulted in proinflammatory cytokine production in all cases, as determined by measuring IL-1β and TNF-α release into the cell culture supernatant by ELISA (Fig. 5). We found the release of these inflammatory cytokines was significantly reduced when the THP-1 cells were incubated with the TREM-1 inhibitor LP-17 peptide simultaneously with the bacteria (Fig. 6). TNF-α was reduced 26–57% and IL-1β was reduced 26.4–53.2% by LP-17 treatment compared with control cells incubated with bacteria alone or with bacteria and a control peptide (LP-17 control peptide in Fig. 6).

DISCUSSION

Periodontitis is the most prevalent disease of the oral cavity. The multispecies oral biofilm, along with the host immune response, are responsible for the onset and progression of this disease. The present study investigated TREM-1 production, as well as its importance in signaling for the production of proinflammatory cytokines, by human monocytes exposed to a variety of oral bacteria. It has been reported that *P. gingivalis* infection of the MonoMac-6 cell line resulted in the upregulation of TREM-1 and DAP12 genes, and was associated with increased sTREM levels in culture supernatants (Bostanci *et al.*, 2011). This study confirms this finding, but additionally shows that TREM-1 is important for the immune reaction to Gram-positive commensal as well as Gram-negative pathogenic species.

Commensal bacteria play an important role in immunomodulation of tissues that support polymicrobial communities, such as the gut and oral cavity. Any imbalance in resident microbiota may lead to disease conditions such as periodontitis (Darveau, 2010) and inflammatory bowel disease (Serban, 2015). Commensal bacteria can also help to prime the immune system, enabling a rapid and efficient response to pathogens (Ivanov & Honda, 2012). Activation of the TREM-1 pathway by the commensals may help to sensitize the immune system to other oral pathogens. The normal healthy host regulates the inflammatory response to commensals through the mucosal immune mechanisms (Tlaskalova-Hogenova *et al.*, 2004). Epithelial cells are an important component of the mucosal immune response, and depending upon the location they may be unresponsive to the common pathogen-associated molecular patterns, as shown by the reduced expression of TLR-4 in intestinal epithelial cells (Abreu *et al.*, 2001). Also, it has been shown that intestinal macrophages do not express CD14 and CD89 (receptor for IgA) in healthy individuals and may contribute to the low level of inflammation in healthy intestinal mucosa (Smith *et al.*, 2001). When the mucosal barrier is breached, however, the commensal bacteria may induce a proinflammatory response. Nakanishi and co-workers showed that breakdown of the intestinal epithelial barrier can result in an improper immune response to the Gram-positive intestinal commensal organisms leading to the development of colitis (Nakanishi *et al.*, 2015). This may apply to the oral cavity, as expression of TLR-4 and CD14 vary within the oral cavity (Li *et al.*, 2014). Although only Gram-positive oral commensals were included in the current study, we expect that both Gram-positive and Gram-negative bacteria once exposed to monocytes will induce a proinflammatory response.

TREM-1 is an amplifier of inflammation that depends on the association with the adaptor protein DAP12 for downstream signal transduction (Bouchon *et al.*, 2000), which culminates

in increased proinflammatory cytokine production. DAP12 is a transmembrane adapter protein that partners with a number of different cell surface receptors including TREM-1, TREM-2 and MDL-1 (Hamerman *et al.*, 2009), leading to either activation or inhibition of cellular responses depending on the affinity/avidity of ligand binding (Hamerman *et al.*, 2009). In the present study, as early as 4 h, *P. gingivalis*- and *S. pneumoniae*-exposed monocytes showed increased TREM-1 expression, and *S. pneumoniae* co-incubation resulted in significantly higher monocyte DAP12 expression as well. At 24 h post-exposure, both TREM-1 and DAP12 gene expression were upregulated in THP-1 monocytes by co-incubation with nearly all bacteria tested.

There are very few studies on the involvement of DAP12 in host response to bacterial pathogens. MonoMac-6 cells co-incubated with *P. gingivalis* W50 showed increased expression of DAP12 at MOIs of 1 and 10 after 24 h of infection, whereas there was no significant increase in DAP12 expression at an MOI of 100 (Bostanci *et al.*, 2011). We found increased DAP12 expression both with MOI 10 and 100. Additionally, DAP12-deficient mice infected with *S. pneumoniae* have increased survival and reduced lung inflammation, concomitant with increased TNF-α production and phagocytic activation of alveolar macrophages (Hommes *et al.*, 2014b). These results indicate that upregulation of DAP12 could be beneficial for the pathogens. In addition to TREM-1, DAP12 is associated with various other inhibitory and activating receptors including TREM-2, myeloid DAP12-associated lectin-1 (MDL-1) and signal-regulatory protein β1 (SIRPβ1) (Lanier, 2009). Whether interactions of pathogenic bacteria with TREM-1, or other interaction partners of DAP12, are more important for modifying the immune response to favor their survival remains unclear, though we did find inhibition of TREM-1 reduced the activation of THP-1 cells, suggesting the latter.

TREM-1 exists in two forms; as a surface receptor and a soluble peptide (sTREM-1) (Bouchon *et al.*, 2001). sTREM-1 is believed to be generated either as a result of proteolytic cleavage of the surface receptor (Gomez-Pina *et al.*, 2007) or by alternate splicing (Gingras *et al.*, 2002; Baruah *et al.*, 2015). Although sTREM cannot induce signal transduction, it likely competes with the surface receptor for available ligand, thereby acting as an antagonist to TREM-1 signaling. *Porphyromonas gingivalis* increases sTREM-1 through cleavage of the surface receptor by the bacterial protease enzymes called gingipains (Darveau, 2010). So while we did not find a decrease in the surface levels of TREM-1 below that seen in the uninfected controls, when combined with increased gene expression and significantly elevated sTREM-1 levels it suggests the possibility that cleavage of the surface TREM-1 is an additional mechanism by which *P. gingivalis* can suppress the local immune response (Darveau *et al.*, 1998; Hajishengallis & Lamont, 2014). However, our data do not definitely rule out the possibility of alternate splicing. We also observed moderate amounts of sTREM-1 in supernatants from cells treated with *S. pneumoniae* and *S. cristatus*, consistent with reports of increased sTREM-1 levels in the bronchoalveolar lavage and lung homogenates of mice infected with *S. pneumoniae* (Hommes *et al.*, 2014a). Further studies are required to determine if proteases produced by early colonizers such as *S. cristatus* (Wang *et al.*, 2011) are capable of cleaving TREM-1 to actively modify the immune response.

Activation of TREM-1 also resulted in increased production of proinflammatory cytokines by the monocytic cells, as they were significantly reduced when the cells were treated with TREM-1 inhibitor. The inhibitory effect of the LP-17 peptide supports the notion that it competes with TREM-1 for the binding of the available ligand thereby inhibiting the TREM-1 pathway. The increase in cytokines is consistent with earlier reports that stimulation of TREM-1 can result in synergistic upregulation of signaling initiated by other pattern recognition receptors such as the TLRs (Arts *et al.*, 2011, 2013; Prufer *et al.*, 2014). *Streptococcus pneumoniae* induced lesser amounts of cytokines at MOI 100 when compared with the MOI 10. This may be because *S. pneumoniae* induced a very significant amount of cytotoxicity of THP-1 cells at MOI of 100. We also noticed a reduction in the cytokine production at MOI of 100 for *P. gingivalis*, known previously to cause degradation of released cytokines locally (Fletcher *et al.*, 1997). Additionally, we found that treatment with the TREM-1 inhibitor did not abrogate cytokine production completely, as previously noted for other non-oral bacterial infections (Bostanci *et al.*, 2011; Hu *et al.*, 2014).

In summary, this study compared the activation of the TREM-1/DAP12 pathway by both commensal and pathogenic oral bacteria and found that both activate the pathway. The oral cavity supports a multispecies microbial community that stimulates inflammation as a default condition, similar to the situation seen in the gut (Devine *et al.*, 2015), in which commensal bacteria play an important role regulating innate immune processes to maintain health. Future studies may investigate strategies using this information to diminish imbalance in the innate immune response to prevent or diminish inflammation and hence periodontal disease activity.

Acknowledgments

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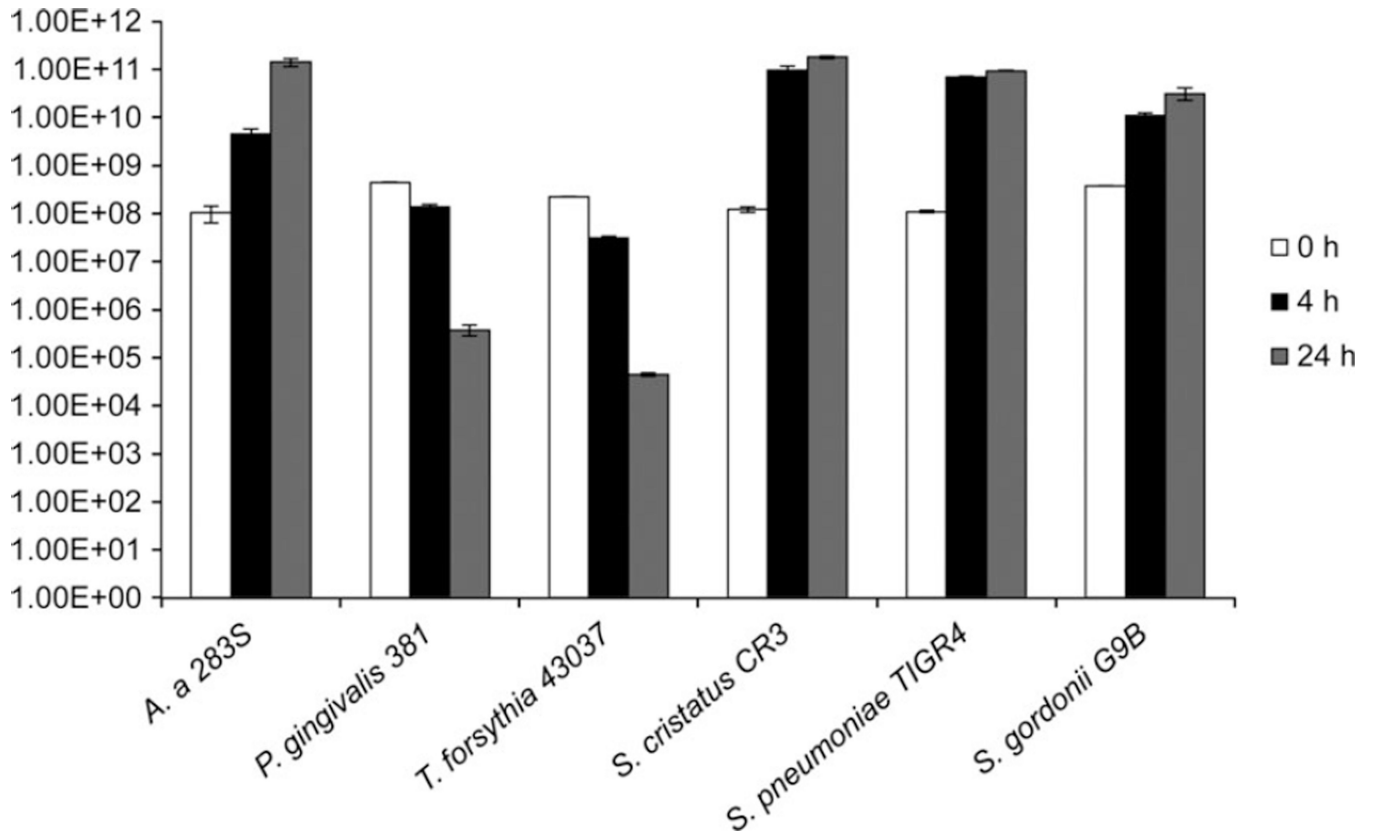
Figure 1.
Survival of bacteria under co-incubation with THP-1 cell culture conditions. THP-1 cells were exposed to bacteria at multiplicity of infection (MOI) of 100. After 4 and 24 h post exposure the CFUs were determined. Experiments were repeated three times. Data shown are mean ± SD of triplicate cultures from a representative experiment. *A. a.*, *Aggregatibacter actinomycetemcomitans*.
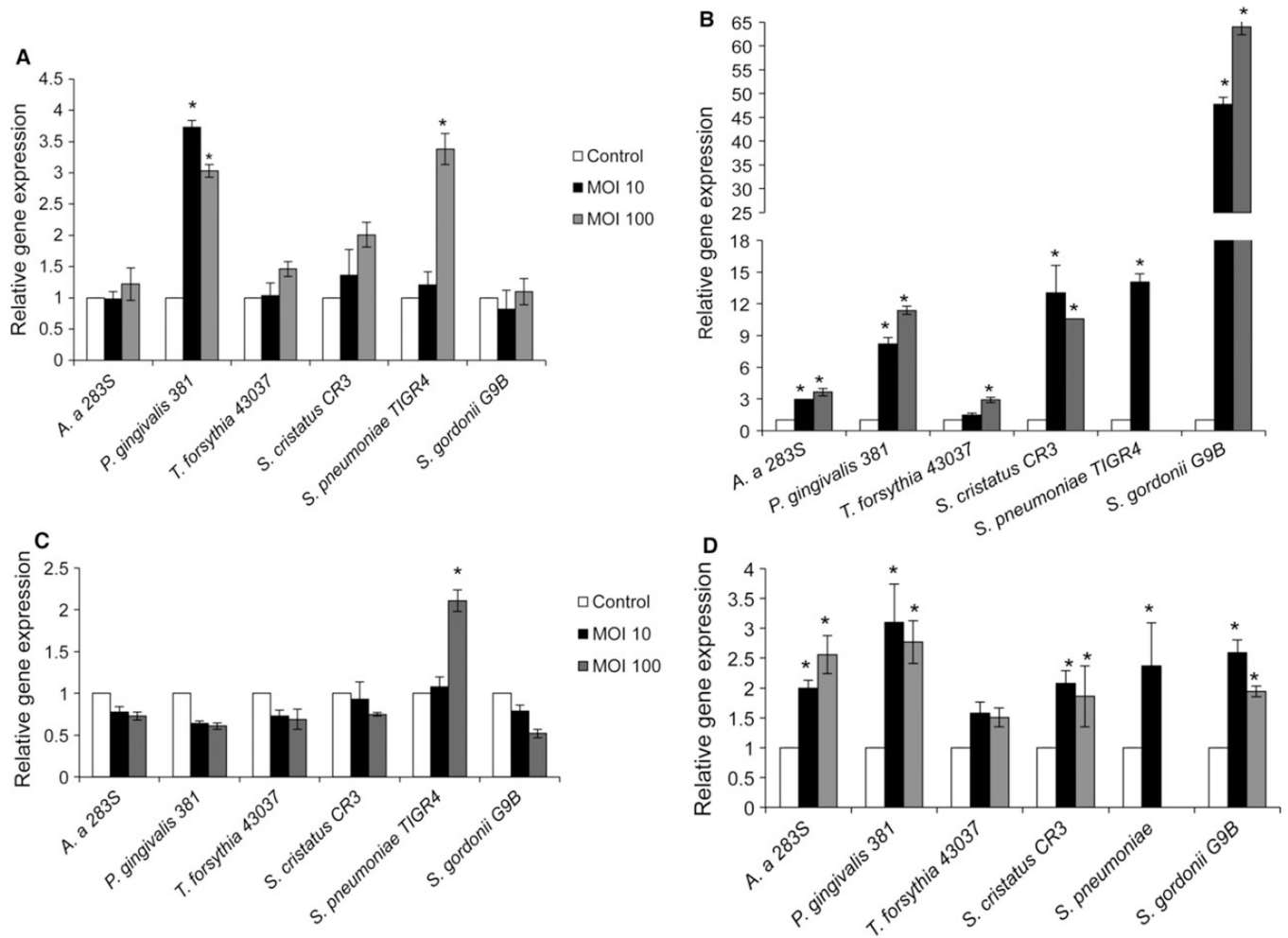
Figure 2.
Relative expression of TREM-1 and DAP12 genes. THP-1 cells were exposed to bacteria at multiplicity of infection (MOI) of 10 or 100. After 4 and 24 h post exposure RNA was extracted, cDNA synthesized and the relative gene expression was determined by quantitative polymerase chain reaction, as described. (A) TREM-1 at 4 h; (B) TREM-1 at 24 h, (C) DAP12 at 4 h, (D) DAP12 at 24 h. Experiments were repeated three times. Data shown are mean ± SD of triplicates from a representative experiment. * P 0.05 compared with uninfected control.
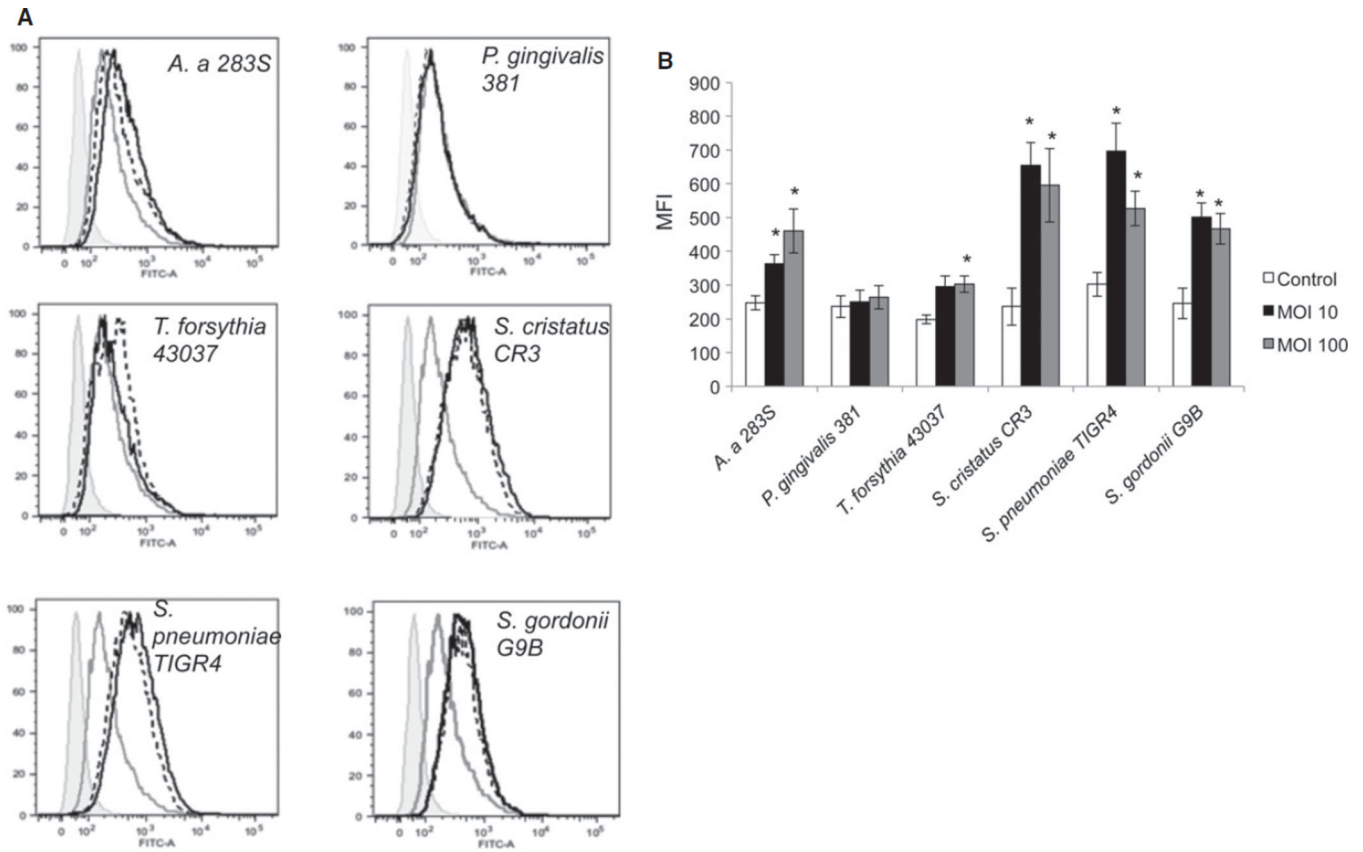
Figure 3.
Surface expression of TREM-1 receptors. THP-1 cells were exposed to the indicated oral bacteria at multiplicity of infection (MOI) of 10 and 100 for 24 h. Surface expression of TREM-1 receptor was determined by flow cytometry. (A) Histograms from a representative experiment, (grey filled – isotype control, grey line- uninfected controls, black dotted lines – MOI 10, black solid lines – MOI 100). (B) Mean fluorescence intensity (MFI) from two independent experiments for *Tannerella forsythia* and three independent experiments for all the other bacteria * *P*   0.05 compared with uninfected control.
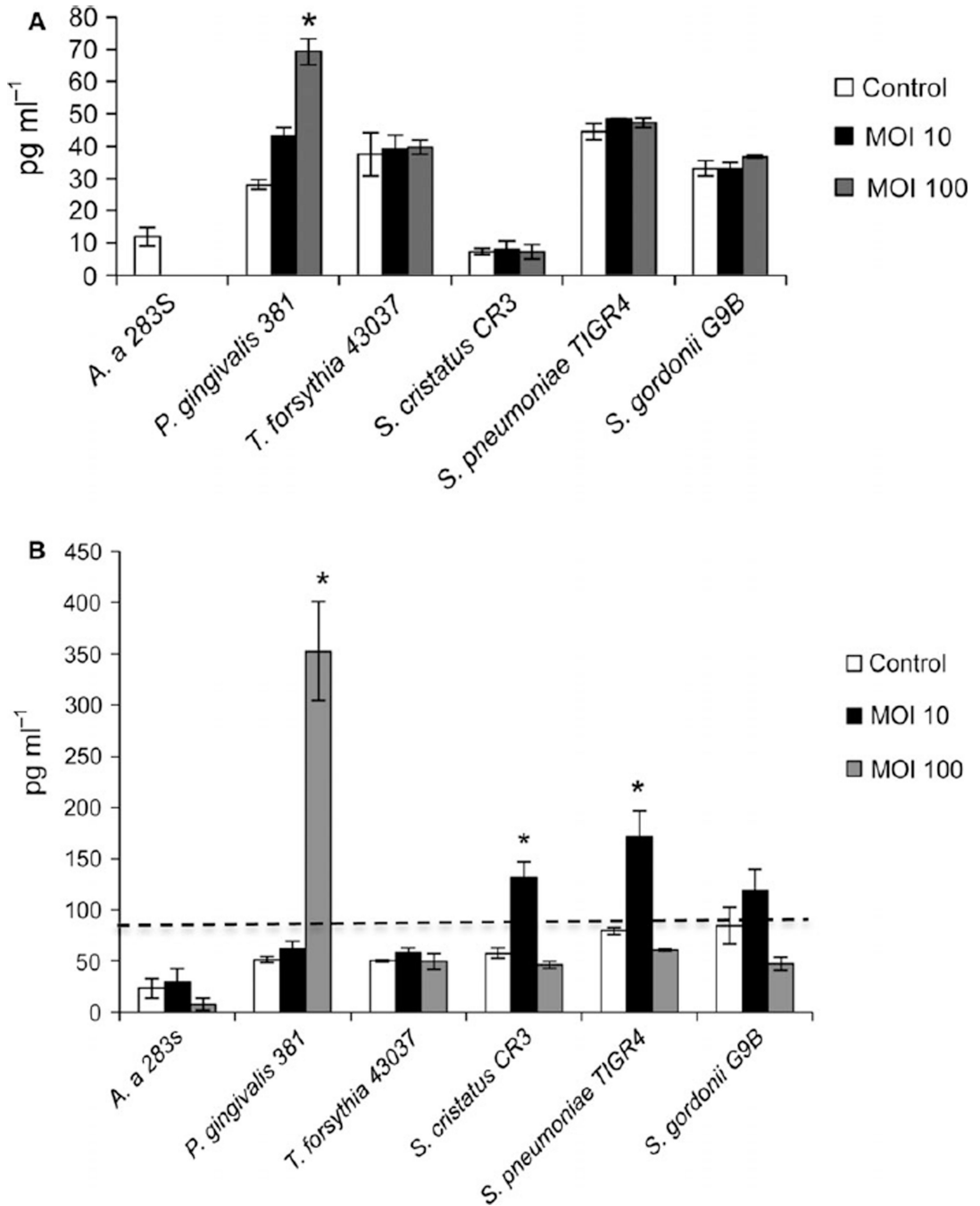
Figure 4.
Soluble TREM-1 (sTREM-1) release from THP-1 cells exposed to oral bacteria. THP-1 cells were exposed to bacteria at an MOI of 10 or 100. After 4 h (A) and 24 h (B) exposure, supernatants were collected and sTREM-1 levels were measured by enzyme-linked immunosorbent assay. Data shown are mean ± SD from three replicates. Line (- - -) indicates the start of the linear range of the assay (93.8 to 6000 pg ml^{-1}). * P 0.05 compared with uninfected control.
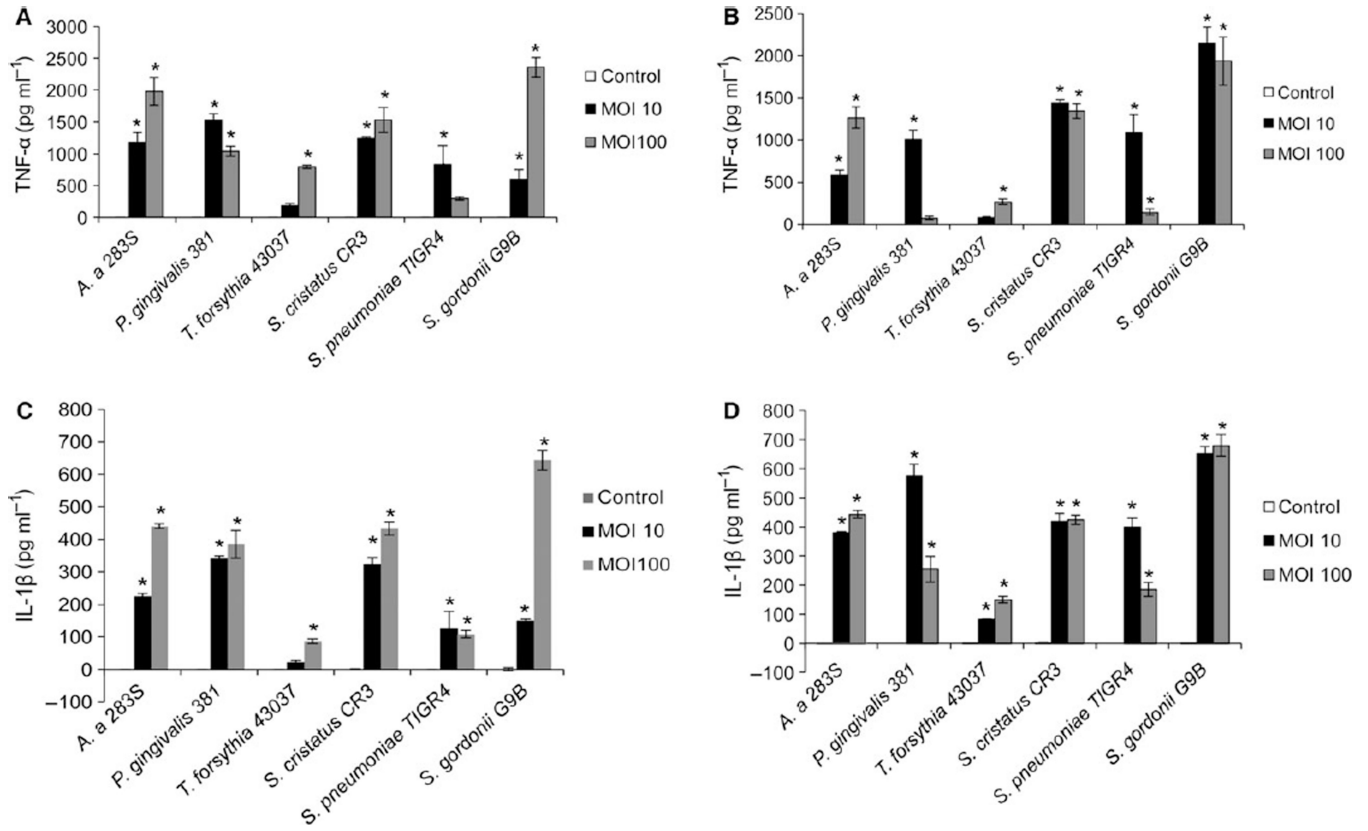
Figure 5.
Pro-inflammatory cytokine release from THP-1 cells exposed to oral bacteria. THP-1 cells were exposed to the indicated oral bacteria at multiplicity of infection (MOI) of 10 and 100. After 4 and 24 h exposure supernatants were collected. Tumour necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β) levels were estimated by enzyme-linked immunosorbent assay. (A) TNF-α at 4 h, (B) TNF-α at 24 h, (C) IL-1β at 4 h, (D) IL-1β at 24 h. Control is THP-1 cells only. Shown are means ± SD from three independent experiments. * P 0.05 compared with uninfected controls.
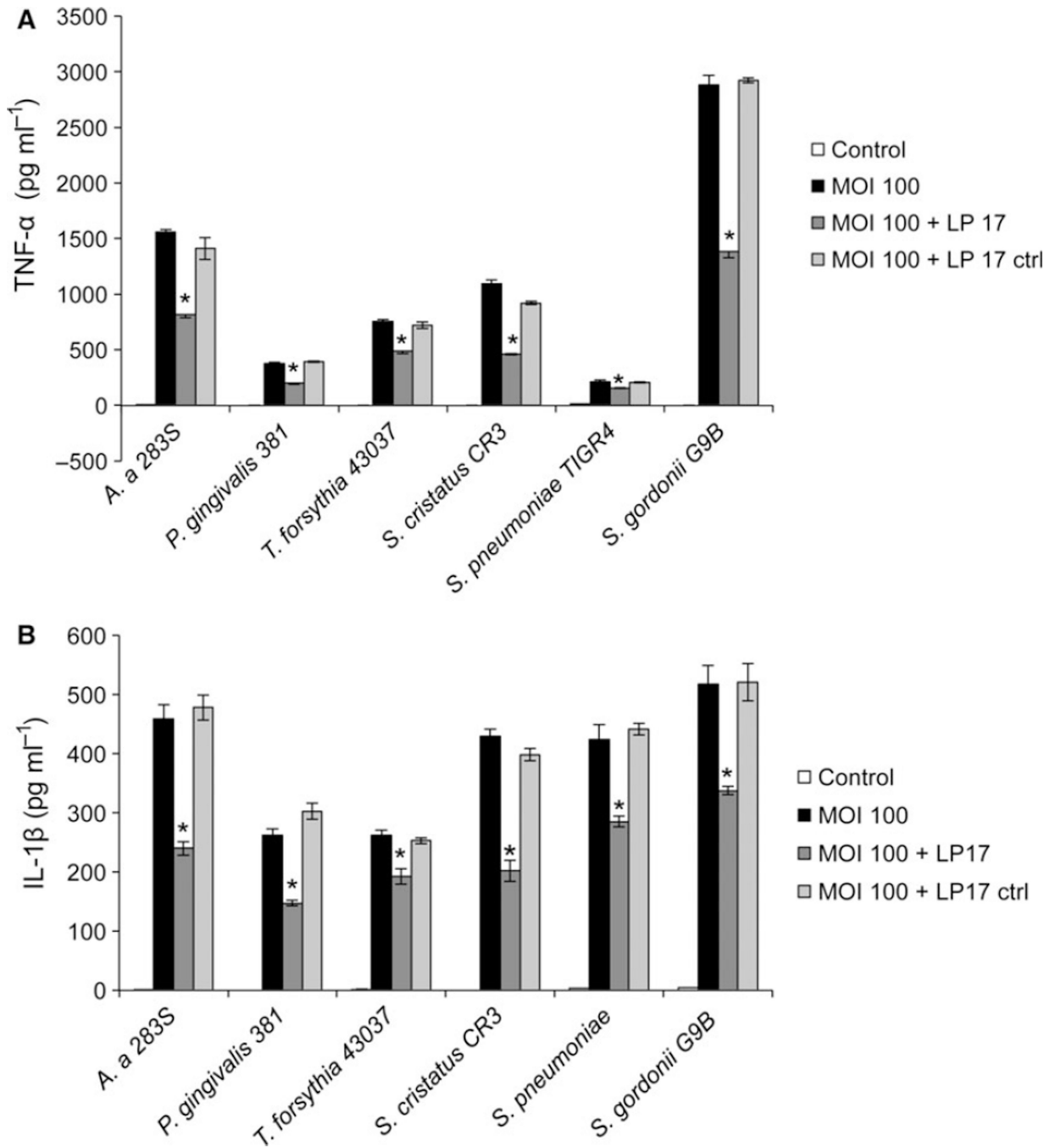
Figure 6.
Effect of TREM-1 inhibitor on cytokine production. THP-1 cells were exposed to indicated oral bacteria at a multiplicity of infection (MOI) of 100 for 24 h. Some of the wells were also treated with the LP17 or LP17 control peptide at a concentration of 100 ng ml^{-1}. After 24 h the supernatant was collected and tumour necrosis factor-α (TNF-α) (A) and interleukin-1β (IL-1β) (B) levels were determined by enzyme-linked immunosorbent assay.

Data shown are mean ± SD from three replicates. * P 0.05 compared with samples treated with bacteria only. Control is THP-1 cells only.

Table 1

Bacterial strains and growth conditions

Bacteria	Medium	Condition
Aggregatibacter actinomycetemcomitans 283S (smooth phenotype, serotype A)	TSBY (0.6% yeast extract and 0.04% sodium bicarbonate)	Candle jar
Porphyromonas gingivalis 381	Sheep blood agar, TSBY with hemin and vitamin K	Anaerobic chamber
Streptococcus gordonii G9B	TSBY (0.5% yeast extract)	Candle jar
Streptococcus cristatus CR3	Todd–Hewitt agar/broth	Candle jar
Streptococcus pneumoniae TIGR4	Blood agar, Todd-Hewitt broth	Aerobic
Tannerella forsythia 43037	TF agar/broth (with *N*-acetyl muramic acid)	Anaerobic chamber

TSBY, tryptic soy broth supplemented with yeast extract as indicated. TF, *Tannerella forsythia*.

Table 2

Cytotoxicity induced by bacteria

Bacteria	MOI 10	MOI 100
Aggrega tibacter actinomycetemcomitans 283S	1.8 ± 0.2	4.2 ± 0.5
Porphyromonas gingivalis 381	3.2 ± 1.0	7.1 ± 0.2
Streptococcus gordonii G9B	17.0 ± 2.8	21.0 ± 1.9
Streptococcus cristatus CR3	14.8 ± 1.6	19.0 ± 2.4
Streptococcus pneumoniae TIGR4	26.0 ± 4.8	59.0 ± 2.9
Tannerella forsythia 43037	7.9 ± 0.9	11.5 ± 0.4

THP-1 cells were exposed to indicated bacteria at multiplicities of infection (MOIs) of 10 and 100 for 24 h. Cytotoxicity was detected by measuring the LDH release from the cells and is expressed as percentage of the total LDH activity upon lysis of uninfected cells. Data shown are mean ± SD from triplicate cultures of a representative experiment.