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MMP-9/TIMP-1 Imbalance Induced in Human Dendritic Cells by *Porphyromonas gingivalis*

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Abstract

Matrix metalloproteinase-9 (MMP-9) cleaves collagen, allowing leukocytes to traffic towards the vasculature and the lymphatics. When MMP-9 is unregulated by tissue inhibitor of metalloproteinase 1 (TIMP-1), this can lead to tissue destruction. Dendritic cells (DCs) infiltrate the oral mucosa increasingly in chronic periodontitis (CP), characterized by infection with several pathogens including *Porphyromonas gingivalis*. In this study, human monocyte-derived DCs (MoDCs) were pulsed with different doses of LPS of *P.gingivalis* 381 and of *E. coli* type strain 25922, as well as whole live isogenic fimbriae deficient mutant strains of *P.gingivalis 381*. Levels of induction of MMP-9 and TIMP-1, as well as IL-10, which reportedly inhibits MMP-9 induction, were measured by several approaches. Our results reveal that LPS of *P.gingivalis*, compared to LPS from *E.coli* type strain 25922, is a relatively potent inducer of MMP-9, but a weak inducer of TIMP-1, contributing to a high MMP-9/TIMP-1 ratio. Whole live *P. gingivalis* strain 381, major fimbriae mutant DPG-3 and double mutant MFB were potent inducers of MMP-9, but minor fimbriae mutant MFI was not. MMP-9 induction was inversely proportional to IL-10 induction. These results suggest that LPS and the minor and major fimbriae of *P. gingivalis* may play distinct roles in induction by DCs of MMP-9, a potent mediator of local tissue destruction and leukocyte trafficking

Keywords

MMP-9; TIMP-1; dendritic cells; *Porphyromonas gingivalis*; LPS; fimbriae; periodontitis

INTRODUCTION

Matrix metalloproteinase-9 (MMP-9) is a member of a family of proteolytic enzymes that regulate cell-matrix composition by requiring zinc for their proteolytic activities. MMP-9 cleaves denatured collagen (gelatin), in particular, type IV collagen, which constitutes the major component of the basement membranes (Opdenakker, *et al.*, 2001, Matache, *et al.*, 2003, Ram, *et al.*, 2006). This cleavage helps lymphocytes and other leukocytes like dendritic cells (DCs) to enter and leave the blood and lymph circulations. MMP-9 also cleaves myelin compounds such as myelin basic protein (MBP) and type 2 gelatins, which leads to remnant epitopes that can generate autoimmunity (Opdenakker, *et al.*, 2001, Matache, *et al.*, 2003,

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Ram, *et al.*, 2006). Expression and secretion of MMP-9 by activated lymphocytes and monocytes is tightly regulated by cytokines, chemokines, eicosanoids and peptidoglycans (Matache, *et al.*, 2003). In most cell types, gene transcription of MMP-9 is inducible by cytokines and cellular interactions. MMP-9 is secreted as a zymogen (proenzyme), which remains inactive unless it is activated by the removal of the propeptide domain by proteolytic enzymes like stromelysin-1, MMP-2 and other MMPs. MMP-9 is usually secreted together with variable amounts of its specific inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1) which controls its proteolytic activity. Disturbances in the balance between MMPs and TIMPs may result in excessive degradation of tissue, a condition often associated with chronic inflammatory diseases like chronic periodontitis (CP), atherosclerosis, systemic lupus erythematosus, Sjogren's syndrome, systemic sclerosis, rheumatoid arthritis, multiple sclerosis and polymyositis (Reynolds, 1996, Ejeil, *et al.*, 2003, Ram, *et al.*, 2006).

It is now recognized that in CP, MMPs play an important role in the degradation of collagen, the major component of the extracellular matrix of the gingival tissue (Ingman T, 1994). CP is initiated by a biofilm of bacteria on the teeth and below the gum-line, which often contains *Porphyromonas gingivalis*, a pathogenic organism associated with CP (Colombo, *et al.*, 2009). The virulence factors of *P. gingivalis*, e.g. LPS, play a role in triggering inflammatory responses in host tissues and can lead to bone loss (Nishida E, 2001). During CP, pathologically elevated levels of active MMP-9 have been found in the saliva, gingival crevicular fluid, and gingival tissue (Teng, *et al.*, 1992, Makela, *et al.*, 1994, Ingman, *et al.*, 1996, Ejeil, *et al.*, 2003, Sorsa, *et al.*, 2004). In addition, active forms of MMP-9 have been associated with irreversible tissue destruction and the progression of periodontitis (Sorsa, *et al.*, 2004). Several studies have documented that *P. gingivalis* can also induce epithelial cells and polymorphonuclear leukocytes (PMN) to secrete bioactive MMP-9. Human whole blood stimulated with *P.gingivalis* LPS releases IL-1β, IL-8, MMP-8 and MMP-9 (Cazalis, *et al.*, 2009). The fimbriae of *P. gingivalis* are also important virulence factors, particularly in invasion of epithelial cells (Yilmaz, 2002), dendritic cells (Zeituni, 2009) and in induction of bone loss (Umemoto & Hamada, 2003). However, there is no information on the induction and activation of MMP-9 by *P. gingivalis* in dendritic cells (DCs). DCs are the most potent antigen-presenting cells (APC), possessing unique capacity to recognize and acquire microbial antigens. Antigen acquisition can lead to DC maturation and expression of chemokine receptor CCR7. Mature DCs disengage from the extracellular matrix, cross basement membranes, and travel to draining lymph nodes to activate T cells (Dieu, *et al.*, 1998, Yanagihara, *et al.*, 1998). We have previously shown that human gingiva contains at least two sub-populations of DCs: Langerhans cells that populate the epidermis and dermal dendritic cells in the connective tissues or lamina propria (Jotwani & Cutler, 2003). We and others have reported that the number of DCs varies in human gingiva during health and disease, suggestive of active trafficking of these cells (Jotwani, *et al.*, 2004). Recent studies in mice have highlighted the role of MMP-9 in DC migration *in vitro* and *in vivo* and have shown that DCs matured within inflammatory sites require both CCR7 and PGE_2-induced MMP-9 for their directional migration to draining lymph nodes (Yen, *et al.*, 2008). It has been shown that secreted MMP-9 can associate itself to CD44 and CD11b expressed by murine and human DCs (Ratzinger, *et al.*, 2002) which would allow activated DCs to use MMP-9 activity in a targeted manner for directional migration to lymph nodes.

In the present study we hypothesized that DCs would be a significant source of MMP-9 and TIMP-1 when challenged with the LPS of *P. gingivalis* or the whole live *P. gingivalis*; furthermore, that fimbrial expression on *P. gingivalis* would alter MMP-9 induction. We therefore analyzed MMP-9 and TIMP-1 levels from *in vitro* cultured monocyte-derived DCs (MoDCs), when pulsed with LPS of *P.gingivalis* or whole live *P. gingivalis* strain 381 and its isogenic fimbriae deficient mutant strains. We show that *P. gingivalis* LPS induces in DCs elevated levels of bioactive MMP-9, but low TIMP-1 levels when compared to LPS from

Escherichia coli type strain 25922. Interestingly, all strains with the exception of minor fimbriae deficient mutant *P. gingivalis* strain MFI induced relatively high levels of MMP-9. Overall, MMP-9 induction was inversely proportional to IL-10 induction by the different strains. We surmise that a high MMP-9/TIMP-1 ratio induced by the periodontal pathogen *P gingivalis* in part due to induction of low levels of IL-10 may contribute to DC responses in CP.

MATERIALS AND METHODS

DC culture and isolation

MoDCs were generated by the procedure of Palucka et al. (Palucka, *et al.*, 1998), as we have previously described (Jotwani, *et al.*, 2001). Briefly, monocytes were isolated from mononuclear fractions of peripheral blood of healthy donors and seeded in the presence of granulocyte-monocyte colony stimulating factor (GM-CSF) and IL-4 (1–2 × 10^5 cells/ml) for 6–8 days, after which flow cytometry was performed to confirm the immature DC phenotype ($CD14^-CD83^-CD1a^+$). Cell surface markers of DCs were evaluated by four-color immunofluorescence staining with the following mAbs, at concentrations according to the manufacturers' recommendations (from 1–3 μg/ml, depending on the fluorochrome): CD1a-FITC (BioSource International, Camarillo, CA); CD80-PE (BD Biosciences, Mountain View, CA); CD83-PE (Immunotech); CD86-PE (BD PharMingen, San Diego, CA); HLA-DR-PerCP (BD Biosciences); and CD14 APC (Caltag Laboratories, Burlingame, CA). After30 min at 4° C and washing with staining buffer (phosphate-bufferedsaline [pH 7.2], 2 mM EDTA, 2% fetal bovine serum), cells were fixed in 1% paraformaldehyde. Analysis was performed with FACSCalibur (BD Biosciences). Marker expression was analyzed as the percentage of positive cells in the relevant population defined by forward scatter and side scatter characteristics. Expression levels were evaluated by assessing mean fluorescence intensity indices calculated by relating mean fluorescence intensity noted with the relevant mAb to that with the isotype control mAb for samples labeled in parallel and acquired by using the same setting.

LPS isolation

The methodology for isolation and purification of LPS from *P. gingivalis* 381 and *E. coli* American Type Culture Collection (ATCC, Manassas, VA) type strain 25922 was as described previously in our laboratory (Cutler, *et al.*, 1996). Briefly, whole cell pellets were subjected to hot-phenol water extraction; the aqueous phase was subjected to extensive dialysis against distilled water, followed by lyophilization and then isopycnic density gradient centrifugation. The LPS-containing fractions were dialyzed extensively against distilled water, lyophilized, and subjected to biochemical analysis for purity. LPS was analyzed for protein content by the bicinchoninic-acid protein assay (Pierce, Rockford, IL, USA). LPS samples were also separated by SDS-PAGE and stained for protein with Coomassie blue and were uniformly negative. Selected samples were also subjected to proteinase K digestion and nuclease treatment, and reanalyzed by SDS-PAGE to confirm the purity of the LPS moieties (data not shown).

Western blot analysis of MMP-9 secretion

MoDCs (5 × 10^5 cells/ml) were stimulated with indicated doses of *P. gingivalis* LPS and *E. coli* LPS for 24 hours. The total protein concentration in each culture supernatant was quantitated using protein assay kit (Bio-Rad Laboratories, Hercules, CA). Culture supernatants were then analyzed for MMP-9 secretion. Aliquots of cell culture supernatants containing equal concentrations of total protein [includes FCS in the medium] were suspended in equal volumes of Laemmle sample buffer (Bio-Rad) and subjected to SDS-PAGE (8% gel) at 120V, transferred to PVDF membrane at 25 V overnight and blocked with 5% blocking agent (ECL blocking agent, Amersham Biosciences, Pittsburgh, PA) in PBS and 0.1% Tween 20. Primary

anti-human MMP-9 [mouse anti-human immunoglobulin IgG1, clone GE-213, from Research Diagnostics, Inc, NJ, USA] or was used at 1:15000, followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences) at 1:50000 dilutions. The proteins were detected using an enhanced chemiluminescence detection system (Amersham Biosciences).

Zymography

MoDC supernatants were mixed with Tris-Glycine SDS sample buffer (2x) (Millipore) and allow to stand for 10 min at room temperature. Samples normalized by volume according to protein concentration were loaded on a 10% zymogram (gelatin) Gel. Samples were run with 1x Tris-Glycine SDS running buffer at 125V for approximately 90 min, or when the Bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, gel was removed and incubated in 1X Zymogram Renaturing Buffer (Triton × –100, 2.5% (v/v) in water) (Millipore) for 30 minutes at room temperature with gentle agitation. After decanting the Zymogram Renaturing Buffer, 1X Zymogram Developing Buffer (50mM Tris base, 0.2M Nacl, 5mM $CaCl_2$, 0.02% Brij 35) (Millipore) was added. Gel was equilibrated for 30 minutes at room temperature with gentle agitation, buffer was decanted and fresh 1X zymogram developing buffer was added. Gel was incubated at 37°C for at least 4 hours or overnight for maximum sensitivity. The optimal result was determined empirically by varying the sample load or incubation time. Gels were stained with Coomassie Blue R-250 for 30 minutes, then destained with an appropriate Coomassie R-250 destaining solution (Methanol: Acetic acid: Water (50: 10: 40). Areas of protease activity appeared as clear bands against a dark blue background, where the protease has digested the substrate.

ELISA assay for MMP-9, TIMP-1 and IL-10

Supernatants of MoDCs pulsed with 100 ng *P. gingivalis*, *E. coli* LPS or *P. gingivalis* strains at a 1:25 MOI for 24 hrs were analyzed in triplicate for MMP-1, TIMP-1 and IL-10 levels in pg/ml, by quantitative sandwich enzyme immunoassay technique (ELISA), as described by the manufacturer (Quantikine®, R&D systems, Minneapolis, MN).

Statistical analysis

All assays were performed in triplicate and results expressed as mean ± S.D., unless otherwise indicated in figure legends. Data with parametric distribution were analyzed by Student's t-test, while non-parametric data were analyzed by Kruskal–Wallis test (Minitab®.ver. 15, State College PA). Statistical significance was assessed at $p<0.05$.

RESULTS

P.gingivalis LPS is a highly potent MMP-9 agonist

We analyzed the ability of *P. gingivalis* LPS, at physiologically relevant doses of 10 ng and 100 ng (Copeland, *et al.*, 2005) and a higher dose of 1000 ng, to induce secretion of MMP-9 by MoDCs, as determined by semi-quantitative Western blotting analysis (Fig. 1). *P. gingivalis* LPS at 10, 100 and 1000 ng induced a dose-dependent increase in MMP-9 in relative densitometry units (DU). The MMP-9 levels secreted by MoDCs in response to 100 ng and 1000 ng of *P. gingivalis* LPS was greater than that induced by equivalent dose of *E.coli* LPS (Fig 1A, Fig. 1B).

Enzymatically active of MMP-9 is secreted by DCs in response to *P. gingivalis* LPS

To determine the gelatinolytic activity of the MMP-9 secreted by MoDCs, we performed gelatin zymography (Fig. 2), and titered the LPS doses from 100 ng to 800 ng. We show that

P. gingivalis LPS indeed induces higher levels of enzymatically active MMP-9 than *E.coli* LPS (Fig. 2A, B).

High MMP-9/TIMP-1 ratio induced by *P. gingivalis* LPS as compared to *E coli* LPS

MMP-9 levels in cell supernatants were quantitated by ELISA (Quantikine ®, R&D systems, Minneapolis, MN). The results in pg/ml (Fig. 2C) confirm Western blotting analysis and zymography. *P.gingivalis* LPS induces significantly higher levels of MMP-9 (17.1 ± 0.02 ng/ml) than *E. coli* LPS (4.8 ± 0.03 ng/ml) (Students t-test, $p<0.05$). We then determined the levels of tissue inhibitor of metalloproteinase -1 (TIMP-1) in the MoDC supernatants. We show that induction of TIMP-1 by *P.gingivalis* LPS was equivalent to *E.coli* LPS (Fig. 2D). Thus the ratio of MMP-9/TIMP-1 induced by *P. gingivalis* LPS (= 3.25) was nearly three-fold greater than that induced by *E. coli* LPS (=1.2).

Whole live *P. gingivalis* strains also induce MMP-9 production

Due to distinct roles for minor and major fimbriae in binding to and invasion of MoDCs (Zeituni, 2009), we analyzed the ability of *P. gingivalis* strains to induce MMP-9 and TIMP-1 production. MoDCs were pulsed with type Pg 381 and its isogenic major- (DPG-3), minor- (MFI) and double- (MFB) fimbriae deficient mutant strains {Takahashi, et al. 2006). We observed that, with the exception of minor fimbriae mutant MFI, all the strains of *P. gingivalis* induced high levels of MMP-9, ranging from 5.8–6.3 ng/ml (Fig 3A). This was reflected in elevated MMP-9/TIMP-1 ratios with these strains (Fig. 3B). Minor fimbriae deficient strain MFI, which expresses major fimbriae, induced ~6-fold lower MMP-9 levels and 10–15 fold lower MMP-9/TIMP-1 ratio than the other strains (Fig. 3B). This correlates with higher levels of IL-10 induced by MFI (Fig. 3C).

DISCUSSION

The pathogenic potential of *P. gingivalis*, the predominant pathogen associated with CP, has been attributed to several virulence factors, including its fimbriae, a unique LPS and cysteine proteinases {Lamont, 1998 #75}. Several lines of evidence suggest that in order to survive in the hostile host environment, *P. gingivalis* must evade or subvert the host immune system (Domon, *et al.*, 2008, Hajishengallis, *et al.*, 2008) by targeting innate immunity via these virulence factors. *P. gingivalis* has also been called a ‘keystone” species due to its ability to influence the entire oral microbial community by modulation of innate responses (Darveau, 2009). In the present study we show a role for *P. gingivalis* LPS in modulation of MMP-9 and TIMP-1 by DCs. Our results show that *P. gingivalis* LPS is more potent in inducing bioactive MMP-9 than *E.coli* LPS (Figs 1–3). Moreover while both LPS moieties increase TIMP-1 levels compared to untreated control, the ratio of MMP9/TIMP1 is higher when MoDCs are stimulated with *P. gingivalis* LPS. TIMP-1 functions as an inhibitor of MMPs by forming non-covalent complexes with MMPs, thus blocking access of substrates to the MMP catalytic site (Gomez, *et al.*, 1997, Bode, *et al.*, 1999). A high MMP/TIMP-1 ratio, therefore, implies an imbalance or loss of MMP-9 regulation, as reported in inflammatory diseases such as lupus nephritis (Jiang Z, 2009), encephalitis (Ichiyama T, 2009), appendicitis (Solberg A, 2008) and periodontitis (Kubota T, 2008). While the present study was carried out in vitro, we used the potent antigen presenting cells DCs, which infiltrate lamina propria in CP (Jotwani, *et al.*, 2001) and have been shown to contact *P. gingivalis* in situ.(Cutler, *et al.*, 1999)

The reported structure of *P. gingivalis* LPS, particularly its diverse lipid A species, is associated with its unorthodox targeting of TLR2, compared to TLR4 by *E. coli* LPS, used as a control here (Fig. 1, 2). *P. gingivalis* LPS can also weakly stimulate TLR4, but potently antagonize TLR4 activation by other stronger agonists (Darveau, *et al.*, 2004). Our previous published studies on the immuno-biological functions of *P. gingivalis* LPS with MoDCs have

demonstrated that its LPS is also a weak inducer of MoDC maturation and induces a Th2 effector response, versus a Th1 effector response by *E.coli* LPS (Jotwani, *et al.*, 2001,Jotwani & Cutler, 2003). Differential TLR targeting, as well as our recent in press studies may offer clues as to the mechanisms involved in the functional differences in *P. gingivalis* LPS, including the MMP-9 observations here. We recently reported that *P. gingivalis* LPS induces in DCs increased translocation of NFκB p50 subunits and of p50 homodimer formation into the nucleus. Moreover, this same pattern of increased p50 subunits and of p50 homodimer formation is found in tissues from CP patients (Jotwani, 2009). Induction of MMP-9 and TIMP-1 has been reported to be transcriptionally regulated via the MEK/ERK pathway (Maddahi, *et al.*, 2009). The MEK-ERK pathway acts upstream of NFκB p50 homodimer activity (Kurland, *et al.*, 2003). Our results therefore imply a mechanistic link between the unusual immuno-biological activities of *P. gingivalis* LPS as reported here, with the intracellular signaling pathways and transcription factors that it induces.

Pulsing MoDCs with whole live *P. gingivalis* 381, as well as its major fimbriae deficient mutant DPG-3 and double-fimbriae mutant MFB resulted in equivalent MMP-9 levels. This was not the case however, with strain MFI, which has no minor fimbriae, but only expresses major fimbriae (Takahashi, *et al.*, 2006, Zeituni, 2009) and produces lower levels of MMP-9 (Fig. 3). This is interesting in that strain MFI induces in MoDCs much higher levels of inflammatory cytokines, costimulatory molecules (Zeituni, 2009) and IL-10 (Fig 3C) than the other strains. Low MMP-9 induction by major fimbriae seems to be due to the important role that IL-10 plays in inhibition of MMP-9 production (Lacraz, *et al.*, 1995). The major fimbriae is reported to target TLR2 (Davey, 2008)), as does *P. gingvalis* LPS, but this is apparently insufficient to overcome the inhibitory activity of IL-10. TLR2 appears to be particularly important in IL-10-mediated mucosal immune homeostasis in response to intestinal commensals (Cario, 2008).

In conclusion, in the present study we demonstrate by several techniques that *P. gingivalis* LPS is a more potent inducer of MMP-9 in DCs than *E. coli* LPS. Combined with induction of equivalent levels of TIMP-1 by both LPS moieties, this suggests a MMP-9/TIMP-1 imbalance by *P. gingivalis* LPS that could contribute to chronic inflammation (Kubota T, 2008, Solberg A, 2008, Jiang Z, 2009, Ichiyama T, 2009).. Elevated MMP-9/TIMP-1 was also induced by the whole bacterium, except the minor fimbriae deficient mutant MFI, due to high levels of MMP-inhibitory IL-10.

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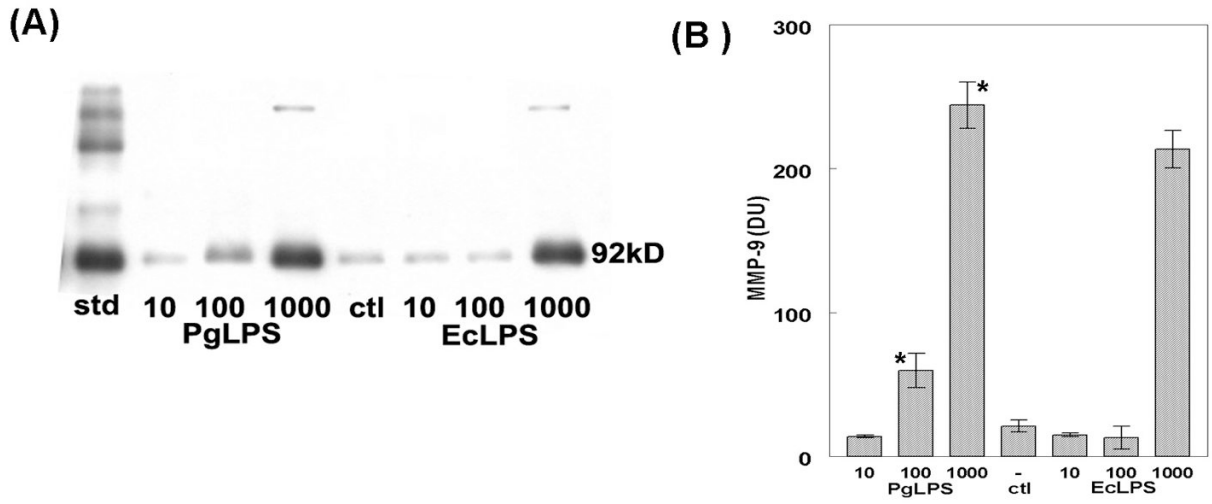

Fig. 1. Semi-quantitative analysis of MMP-9 production by MoDCs in response to *P. gingivalis* LPS and *E. coli* LPS

(A) Western blotting analysis of supernatants from MoDCs stimulated with 10, 100 and 1000 ng/ml of *P. gingivalis* (Pg) LPS and *E.coli* LPS or no LPS (control [ctl]) for 24 hrs. Equal amounts of protein were loaded onto SDS-PAGE and separated by electrophoresis and transferred to nitrocellulose membranes as described in the Materials and Methods. Representative bands corresponding to 92 kDa (MMP-9). (B) Western blot bands corresponding to 92 kDa (MMP-9) were quantified by optical densitometry (GelPro Analyzer, Media Cybernetics, Silver Spring, MD) and results expressed as densitometric units (DU). Results represent means ± S.D. of densitometric units (DU). * Significant difference between *P. gingivalis* LPS and *E.coli* LPS at the same dose ($p < 0.01$, Kruskal–Wallis test).

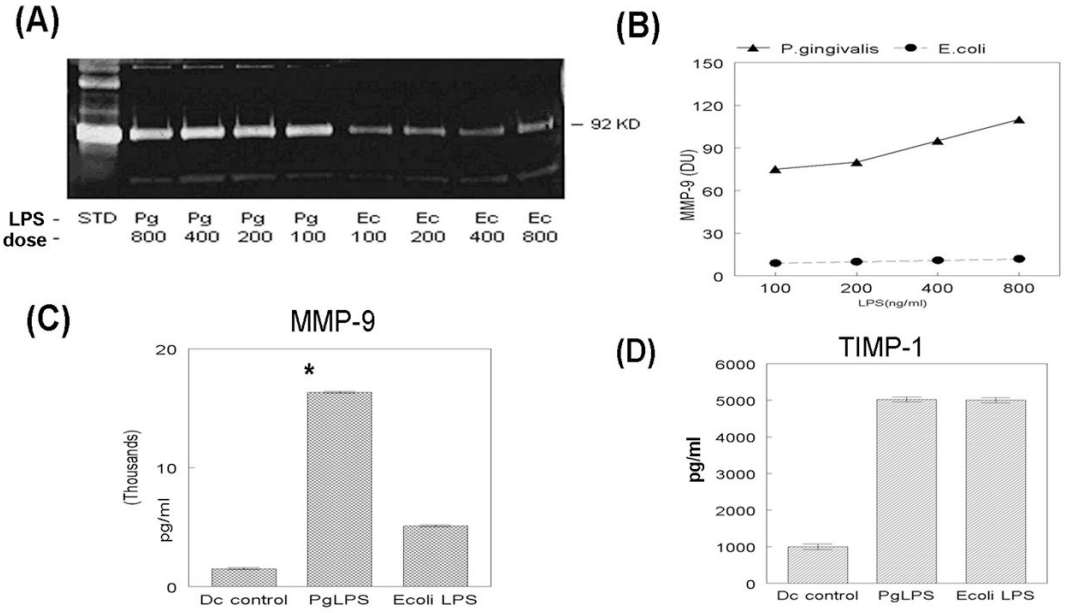

Fig. 2. Enzymatic and quantitative analysis of MMP-9 and of the MMP-9/TIMP-1 ratio in MoDCs exposed to *P. gingivalis* LPS

(A) MoDCs were stimulated with 100, 200, 400 and 800 ng/ml of *P. gingivalis* LPS and *E.coli* LPS for 24 hrs. MMP-9 in cell supernatants was analyzed by gelatin zymography. Equal amounts of protein were loaded and separated by electrophoresis as described in the Materials and Methods. (B) Gelatin zymogram bands corresponding to 92 kDa (MMP-9) were quantified by optical densitometry (GelPro Analyzer, Media Cybernetics, Silver Spring, MD). Results represent means ± S.D. of densitometric units (DU). (C) ELISA analysis of MMP-9 production in pg/ml by MoDCs in response to 100 ng/ml of either LPS or no LPS (control). Shown are the means ± S.E. of assay performed in triplicate. * Significant increase in MMP-9 induction by *P. gingivalis* LPS compared to *E. coli* LPS and DC control ($p<0.05$, Student's t-test (D) ELISA analysis of TIMP-1 production in pg/ml by MoDCs in response to 100 ng/ml of either LPS or no LPS (control). Shown are the means ± S.E. of assay performed in triplicate.

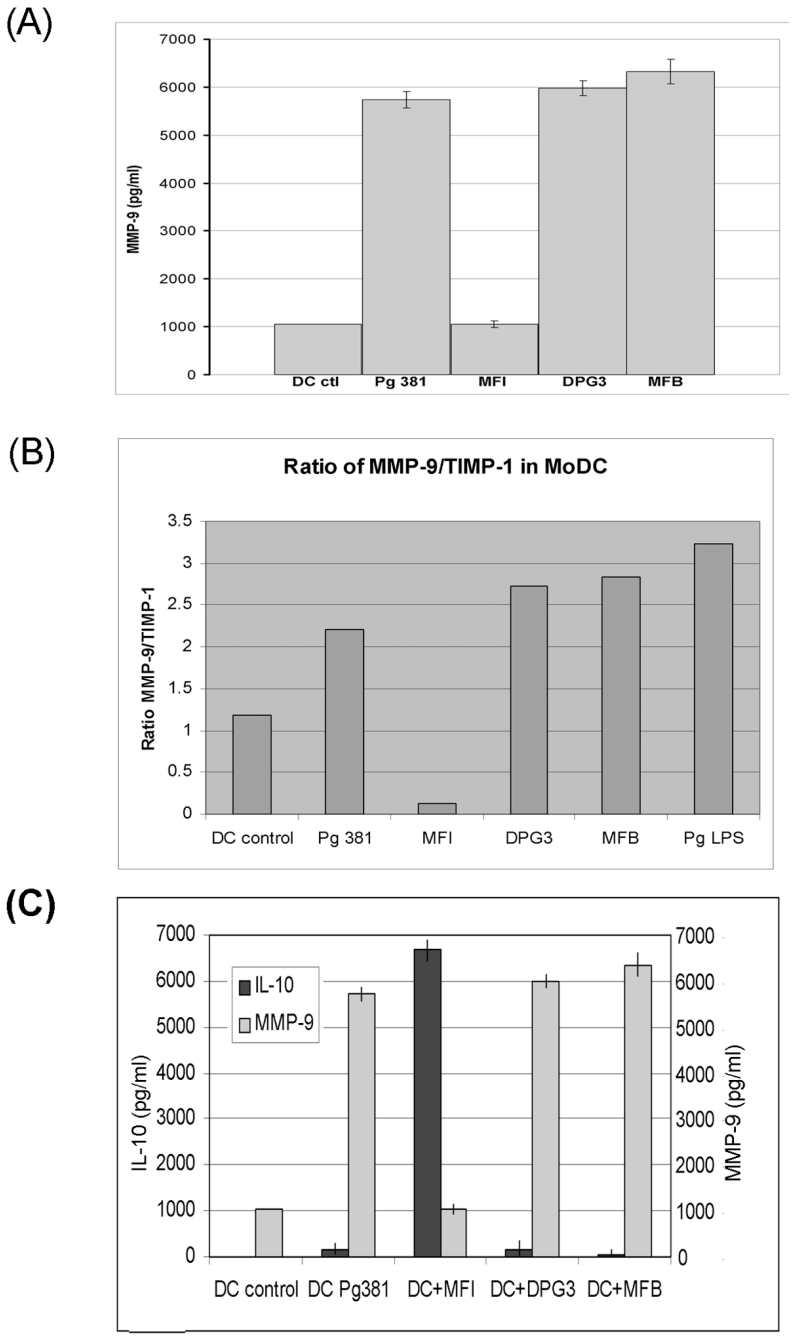

Fig. 3. Induction of MMP-9, TIMP-1 and IL-10 by *P.gingivalis* 381, its fimbriae deficient mutants

MoDCs were pulsed with whole cells of wild type Pg 381, its minor fimbriae deficient strain (MFI), major fimbriae deficient strain (DPG-3) and double-fimbriae deficient mutant strain (MFB) for 18 h at a MOI of 1:25. Secretion of MMP-9, TIMP-1 and IL-10 in pg/ml were assessed by ELISA, as described in Materials and Methods. The data are the mean ± S.D. of triplicate assays. (A) MMP-9 production in response to different fimbriae deficient strains. (B) Ratio of MMP-9/TIMP-1. (C) Comparison of MMP-9 and IL-10 produced by MoDCs in response to different fimbriae deficient strains.