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Porphyromonas gingivalis infection and prothrombotic effects in human aortic smooth muscle cells

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Abstract

Introduction—Accumulating evidence has demonstrated an association between periodontal infectious agents, such as *Porphyromonas gingivalis*, and vascular disease. Tissue factor (TF) and its specific tissue factor pathway inhibitor (TFPI) are produced by vascular smooth cells and are important regulators of the coagulation cascade.

Materials and Methods—To assess the role of *P. gingivalis* in atherothrombosis, we infected primary human aortic smooth cells (HASMC) with either *P. gingivalis 381*, its non-invasive mutant DPG3, or heat-killed *P. gingivalis 381*. Levels and activity of TF and TFPI were measured 8 and 24 hours after infection in cell extracts and cell culture supernatants.

Results—*P. gingivalis* 381 did not affect total TF antigen or TF activity in HASMC, but it significantly suppressed TFPI levels and activity compared to uninfected control cells, and those infected with the non-invasive mutant strain or the heat-killed bacteria. Further, *P. gingivalis'* LPS (up to a concentration of 5 μg/ml) failed to induce prothrombotic effects in HASMC, suggesting a significant role for the ability of whole viable bacteria to invade this cell type.

Conclusion—These data demonstrate for the first time that infection with a periodontal pathogen induces a prothrombotic response in HASMC.

Keywords

infection; periodontitis; *P. gingivalis*; thrombosis; atherosclerosis; smooth muscle cell

Vascular smooth muscle cells (VSMC) are the major cellular component of the vessel wall and play a pivotal role in vascular function. They contribute to the short-term regulation of the blood pressure by altering the luminal vessel diameter, but also contribute to long-term adaptation, by structural remodeling. They also constitute a significant portion of the

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atherosclerotic lesion and proliferation, migration and apoptosis of VSMC are essential to atherogenesis, plaque progression and rupture [1].

Accumulating evidence suggests that inflammation and coagulation are essential in the pathogenesis of vascular disease: inflammation leads to activation of coagulation, and coagulation considerably affects inflammatory activity [2]. In addition, multiple studies have shown that certain infections are implicated in atherogenesis and may serve as inflammatory stimuli that also contribute to acute events via plaque destabilization [3]. Periodontal diseases, chronic oral infections leading to destruction of tooth supporting structures and eventually tooth loss, are very common. Mild to moderate forms affect the majority of adults and severe forms have a prevalence of 10% to 15% [4]. Several studies have demonstrated a link between periodontal infections and atherosclerosis in human subjects [5-8]. It has also been postulated that since several infectious agents are likely involved in atherogenesis, the risk relates to the aggregate pathogen load [9]. As periodontal diseases are largely preventable and treatable, periodontal therapy with resultant reduction of the oral infection burden may contribute to the efforts to reduce the risk for vascular disease [10].

Porphyromonas gingivalis, a principal etiologic agent of chronic periodontitis in humans, can gain access into the bloodstream and has been identified in human atherosclerotic plaques [11,12]. Frequent low-level bacteremias, such as those resulting from chewing, brushing and flossing in periodontitis patients [13], may provide a chronic insult to the vasculature that could initiate and/or exacerbate atherogenesis. *P. gingivalis* has been shown to enhance foam cell formation in human macrophages [14] and invade vascular cells, including endothelial and smooth muscle cells [15,16]. Further, *P. gingivalis* has been shown to accelerate atherosclerosis in animal model studies [17-19]. We recently demonstrated that infection with *P. gingivalis* induces increased leukocyte adhesion to human aortic endothelial cells (HAEC), enhances procoagulant responses in HAEC and promotes up-regulation of adhesion molecule expression and pro-inflammatory cytokine production by this cell type [20,21].

Although *P. gingivalis* has been shown to invade VSMC, its effects on VSMC phenotype and function have not been previously explored. Therefore, we sought to investigate *P. gingivalis'* ability to modulate human aortic smooth muscle cell (HASMC) expression of tissue factor (TF), a principal initiator of the coagulation cascade. In addition, we aimed to assess *P. gingivalis*' modulation of tissue factor pathway inhibitor (TFPI), the primary physiological inhibitor that regulates TF-dependent blood coagulation.

Materials and methods

Cells, bacterial strains, and culture conditions

Clonetics® HASMC were purchased and maintained in smooth muscle cell medium (SmGM-2, Lonza Walkersville Inc, Walkersville, MD) with supplements provided by the supplier. Confluent 4^{th}-6^{th} passage cells were used in all experiments. *P. gingivalis* FDC381 was grown on blood agar plates (Anaerobe Systems, Morgan Hill, CA) in anaerobic chambers at 37°C. DPG3, its fimbriae-deficient mutant constructed by insertional inactivation of the fimA gene [22], was grown on blood agar plates supplemented with erythromycin (Anaerobe Systems) in anaerobic chambers at 37°C. Bacterial suspensions were prepared in phosphate buffered saline without Mg^{2+}/Ca^{2+} (PBS) using established growth curves and spectrophotometric analysis. In some experiments, heat-killed *P. gingivalis* 381 and ultrapure *P. gingivalis* lipopolysacharide (LPS) (Invivogen, San Diego, CA) were used as controls. The endotoxin activity of the *P. gingivalis* LPS preparation was 17.3 ± 3.96 EU/ng, as determined by the *Limulus Amebocyte* lysate method (Lonza Walkersville Inc, Walkersville, MD).

Co-incubation of HASMC with P. gingivalis and its LPS

HASMC were seeded (10^5/well) in 24-well plates (Corning, Acton, MA) for 24 hours and were then infected with viable, or heat-killed, *P. gingivalis* 381 or DPG3 at a multiplicity of infection (MOI) of 1:100 (i.e. 100 bacteria per cell) for 90 minutes in a 37°C, 5% CO_2 environment. In order to approximate *in vivo* conditions, the bacteria were not centrifuged onto the HASMC to promote intimate contact. For all experiments MOI was calculated based on the number of cells per well when seeded. After the incubation period, cells were washed with PBS, and maintained in SmGM-2 medium without antibiotics for 8 or 24 hours. The cell culture supernatant was collected and stored at -70°C until analyzed. In additional experiments, HASMC were co-incubated with various concentrations of *P. gingivalis* LPS for either 8 or 24 hours. After the incubation period, cell culture supernatants were harvested and kept frozen until further analyses.

Antibiotic protection assay and assessment of HASMC necrosis

In order to confirm the potential of *P. gingivalis* 381 to invade HASMC in our experiments, recovery of viable microorganisms from antibiotic-treated cells was assessed. Confluent HASMC on a 96-well plate (Corning) were infected with *P. gingivalis* 381 or DPG3 (MOI 1:100) for 90 min at 37°C, 5% CO_2. After washing, metronidazole (200 mg/ml, Sigma) was added to each well for 1 hour. Control experiments confirmed that extracellular bacteria were killed at this antibiotic concentration. Following metronidazole treatment, cells were washed, lysed with sterile deionized water, and lysates were plated on blood agar plates. Colonies were counted after a three-day incubation under standard anaerobic conditions. To assess non-specific necrosis, smooth muscle cell membrane integrity was measured using a lactate dehydrogenase (LDH) assay (Roche, Mannheim, Germany). Data are given as percent specific LDH release.

Determination of TF levels, TF activity, TFPI levels, and TFPI activity

After the cell culture supernatants were removed, cells were lysed by 3 freeze-thaw cycles. TF was extracted for 30 minutes in TRIS buffered saline containing 0.1% Triton X-100 at 37°, samples were collected and stored at -70°C until analyzed. Expression of total TF in cell extracts was assessed by ELISA (American Diagnostica, Stamford, CT). TF procoagulant activity in cell extracts was determined using a two-stage chromogenic activity assay (American Diagnostica). In the first stage of this assay, the TF in the sample is allowed to complex with factor (F) VIIa to generate TF/FVIIa complexes and convert FX into FXa. In the second stage, FXa cleaves Spectrozyme® FXa, a highly specific FXa chromogenic substrate and a chromophore is released. Absorbance is read and compared to values from a standard curve generated using known amounts of active TF. Levels of total TFPI and TFPI activity were quantified in culture supernatants, using a commercially available ELISA kit and a three-stage chromogenic activity assay, respectively (American Diagnostica). The latter measures the ability of TFPI to inhibit the catalytic activity of the TF/FVIIa complex to activate Fx to FXa. After incubation of samples with TF/FVIIa and FX, the residual activity of the TF/FVIIa complex is measured using Spectrozyme® FXa. The TFPI activity present in the sample is interpolated from a standard curve constructed using known TFPI activity levels.

Data and statistical analysis

All experiments were performed in duplicate wells for each condition and repeated at least three times. Data are presented as mean ± SD and n represents the number of experiments. Statistical comparisons were performed using Student's t-tests and the resultant p values are reported. Under the Bonferroni adjustment for multiple comparisons, an individual $p<0.0125$ is necessary to achieve statistical significance at the 5% level.

Results

P. gingivalis interaction with HASMC

We first addressed the potential of *P. gingivalis* to adhere to and/or invade HASMC in our experimental setting using antibiotic protection assays. *P. gingivalis* 381 had the ability to adhere to/invade HASMC, which was significantly higher compared to its fimbriae-deficient mutant, DPG3. Mean counts of invading bacteria were $3.22 \pm 0.35 \times 10^3$ for strain 381 vs $0.50 \pm 0.18 \times 10^3$ for DPG3 (p=0.0013, n=3).

Since infection of eukaryotic cells with an intracellular pathogen may lead to cell death, we carefully observed our infected cells under phase-contrast microscopy. At no time did the infected HASMC show any signs of cell death, such as morphologic shrinking, rounding or detachment at the MOI used in our experiments. To further assess cell death, we employed an LDH release assay. As depicted in Fig. 1A, infection of HASMC with *P. gingivalis 381* at MOI 1:100 did not increase LDH release after 8h (19.22 ± 0.90 %) compared to non-infected control (16.77 ± 2.09 %), DPG3 (18.10 ± 3.87 %), or heat-killed *P. gingivalis 381* (17.73 ± 1.64 %). This was also true for the 24h time point (Fig. 1B; non-infected 21.01 ± 3.10 %, *P. gingivalis* 381 20.62 ± 2.91 %, DPG3 22.23 ± 6.37 %, and heat-killed *P. gingivalis 381* 18.98 ± 1.47 %).

P. gingivalis and TF levels and activity in HASMC

We next determined if *P. gingivalis*-infected HASMC demonstrated increased TF expression. As shown in Fig. 2, there was no significant upregulation in TF antigen in HASMC extracts at 8h (non-infected 73.14 ±5.19 pg/ml, *P. gingivalis* 381 75.70 ± 6.70 pg/ml, DPG3 69.46 ± 0.72 pg/ml, and heat-killed *P. gingivalis 381* 75.01 ± 4.30 pg/ml), or 24h after infection (non-infected 117.81 ± 4.48 pg/ml, *P. gingivalis* 381 127.45 ± 9.18 pg/ml, DPG3 121.93 ± 6.76 pg/ml, and heat-killed *P. gingivalis 381* 120.52 ± 6.70 pg/ml). Further, we assessed TF activity, as the extent of TF protein induction in vascular cells does not always correlate well with its activity [23,24]. *P. gingivalis* 381-infected HASMC also failed to demonstrate increased TF activity at both times points tested (data not shown).

P. gingivalis and TFPI antigen and activity in HASMC

Since TFPI, the endogenous inhibitor of TF, is also an important regulator of coagulation, we assessed its expression in HASMC in response to *P. gingivalis* infection. As shown in Fig. 3A, at 8 hours after infection with *P. gingivalis* 381 we found a statistically significant 40% decrease in levels of TFPI in HASMC supernatants (0.35 ± 0.05 ng/ml) as compared to the non-infected control group (0.58 ± 0.07 ng/ml; n=4 for both, p=0.0029). Similarly, the levels of TFPI in *P. gingivalis* 381 infected cells were significantly suppressed compared to both those in DPG3-infceted cells (0.55 ± 0.1 ng/ml; p=0.0107) and in the heat-killed *P. gingivalis* 381 group (0.68 ± 0.01 ng/ml, p=0.0003; n=4 for all). Further, as shown in Fig. 3B, at 24 hours post-infection *P. gingivalis* 381 significantly reduced TFPI levels by approximately 70% in HASMC (1.41 ± 0.22 ng/ml), as compared to 5.25 ± 0.44 ng/ml in the non-infected control group (p<0.001), 4.45 ± 0.79 ng/ml in the DPG3-infected group (p=0.0036) and 4.77 ± 0.47 ng/ml in the heat-killed *P. gingivalis* 381 group (p=0.0001; n=4 for all). At both time points tested, levels of TFPI antigen were similar in control, DPG3- and heat killed *P. gingivalis* 381-infected cells.

We then assessed TFPI activity, which was below detection at the early time point, but could be quantified at the 24h timepoint. *P. gingivalis* 381 infection completely suppressed TFPI activity in HASMC (0.001 ± 0.0005 units/ml) compared to the non-infected control group (0.055 ± 0.009 units/ml; p=0.0011), the DPG3-infected group (0.013 ± 0.004 units/ml; p=0.0099) and the heat-killed *P. gingivalis* 381-treated group (0.047 ± 0.011 units/ml; p=0.0039; n=4 for all groups). TFPI activity in DPG3-infected cells was also significantly

suppressed compared to control cells (p=0.0006), but it was still significantly higher than in *P. gingivalis* 381-infected cells (p=0.0099).

As *P. gingivalis* is a Gram (-) pathogen, in additional experiments we tried to elucidate the effect of its LPS (in various concentrations) on TFPI production. As seen in Fig. 4A, *P. gingivalis* 381 LPS did not induce any effect on HASMC TFPI levels at 8h (0.25 μg/ml: 0.59 ± 0.08 ng/ml, 1 μg/ml: 0.55 ± 0.03 ng/ml, 5 μg/ml: 0.50 ± 0.01 ng/ml) as compared to control untreated cells (0.48 ± 0.08 ng/ml; n=3 for all). Similarly at 24h (Fig. 4B), TFPI levels in LPS-treated cells were not significantly affected (0.25 μg/ml: 5.55 ± 0.72 ng/ml, 1 μg/ml: 4.31 ± 0.24 ng/ml, 5 μg/ml: 4.30 ± 0.38 ng/ml, control: 4.80 ± 0.33 ng/ml; n=3 for all). These data suggest that the observed *P. gingivalis* 381 procoagulant effect cannot be attributed to its LPS, but requires the invasion of HASMC by viable bacterial cells.

Discussion

Our data demonstrate that *P. gingivalis* 381 has the ability to adhere to and invade HASMC and does not have a deleterious effect on smooth muscle cell survival within 24 hours following infection at MOI 1:100. Moreover, infection of HASMC with live whole *P. gingivalis* 381 does not affect levels or activity of TF, but leads to a significant decrease in TFPI levels and activity. Interestingly, this cannot be attributed to a sole LPS effect, but requires the invasion of smooth muscle cells by viable bacteria.

Viability of bacteria is necessary for invasion and heat-killed bacteria cannot invade. Indeed, in this study, heat killed *P. gingivalis* 381 had no effect on HASMC. In an analogous fashion, *P. gingivalis* 381 LPS had no effect on TFPI production by HASMC. In agreement with our present findings, prior evidence indicates that LPS of *P. gingivalis* exhibits unique properties distinct from that of other bacterial species [21,25].

The exact mechanisms by which *P. gingivalis* may enter human cells are still elusive, but the use of lipid rafts as a portal of entry has been suggested [26]. The precise intracellular fate of internalized bacteria and the exact nature of the processes by which they modulate cell functions are also not completely clarified. However, in coronary endothelial cells as well as epithelial cells, internalized *P. gingivalis* cells have been shown to multiply and persist *in vivo* [27,28]. This feature is common in bacteria that cause mucosal infections and could enable *P. gingivalis* to avoid host defense mechanisms.

Intracellular prokaryotic pathogens are well known to be able to induce cell death, by either apoptosis or necrosis, in the infected host cells. To ensure that any observed effects were not due to cell death, we addressed the question of smooth muscle cell survival following *P. gingivalis* infection in our study. At a MOI of 1:100 *P. gingivalis* 381 did not lead to increased smooth muscle cell death, as confirmed by phase contrast microscopy and LDH release, which is in line with our recent findings in human aortic endothelial cells [29].

Tissue factor is a key initiator of the coagulation cascade. TF-mediated activation can lead to occlusive thrombosis after plaque disruption, which is the immediate cause of most acute coronary symptoms. TF is also involved in promoting atherosclerotic plaque growth, by inducing migration and proliferation of vascular smooth muscle cells [30,31]. In addition, it plays an important role at the crossroad of coagulation and inflammation. Previous studies have reported that mediators like TNF-α, CD40 ligand or endotoxin can induce TF expression on vascular endothelial cells and smooth muscle cells and thereby commence pathways that lead to thrombin generation [31]. We have previously demonstrated that endothelial cell infection with *P. gingivalis* 381 lead to a significant upregulation of TF levels and activity [20]. Interestingly, it appears that *P. gingivalis* infection effects are cell-specific, as in our current

studies, we could not demonstrate an influence on levels or activity of TF in HASMC, although *P. gingivalis* 381 diminished TFPI production and activity in this cell type.

TFPI is a Kunitz-type serine protease inhibitor, which inhibits the initial reactions of the blood coagulation cascade, by specifically blocking TF. TFPI inhibits the FVIIa-TF complex and FXa with its first and second Kunitz domains, respectively [32,33]. TFPI expression in vascular endothelial and smooth muscle cells is comparable, and its expression and co-localization with TF in atherosclerotic plaques suggests a significant role for TFPI in the regulation of TF activity [34,35]. Moreover, in animal models reduced endogenous TFPI levels lead to enhanced thrombus formation [36], whereas overexpression was protective after vascular injury and resulted in reduced atherosclerosis [37,38]. In addition to the anticoagulant effects, recent findings indicate that TFPI has an inhibitory effect on vascular cells [39] and it is able to attenuate the inflammatory response of the vascular wall to TF [32,40]. We recently demonstrated that live *P. gingivalis* 381 can adhere to and invade HAEC, and that it is its invasive capacity that is involved in modulating properties of endothelial cells linked to prothrombotic pathways, including suppression of TFPI production [20,21]. Based on the present findings, it appears that *P. gingivalis* 381 may also exert a prothrombotic effect in HASMC, by suppressing TFPI levels, and its ability to invade these cells appears to play a vital role in this. Although the *in vivo* significance of TFPI activity is not as well understood, we found that *P. gingivalis* 381 also significantly reduced TFPI activity, compared to control or DPG3-infected cells. Interestingly, despite the fact that the non-invasive mutant strain failed to affect TF levels and activity or TFPI production by HASMC, it did reduce TFPI activity. The latter effect was significant when compared to control cells, but was also significantly smaller that the effect of *P. gingivalis* 381. This suggests that bacterial properties beyond the pathogen's invasive capacity may also contribute in this setting. Previous studies have also shown that DPG3 is unable to invade vascular cells [16] or to accelerate atherosclerosis in hypercholesterolemic apoE-null mice [18], although its LPS is not distinguishable from that of the wild type strain [22].

Taken together, the present study makes the novel observation that *P. gingivalis* elicits a prothrombotic response in HASMC, by modulating TFPI production. Our findings add to the understanding of this pathogen's role in pathways linked to plaque progression and instability, and further help to explain the link between periodontal infections and atherosclerosis-related vascular disease.

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Abbreviations

VSMC
: vascular smooth muscle cells

HAEC
: human aortic endothelial cells

HASMC
: human aortic smooth muscle cells

TF
: tissue factor

TFPI
: tissue factor pathway inhibitor

LPS
: lipopolysacharide

LDH
: lactate dehydrogenase

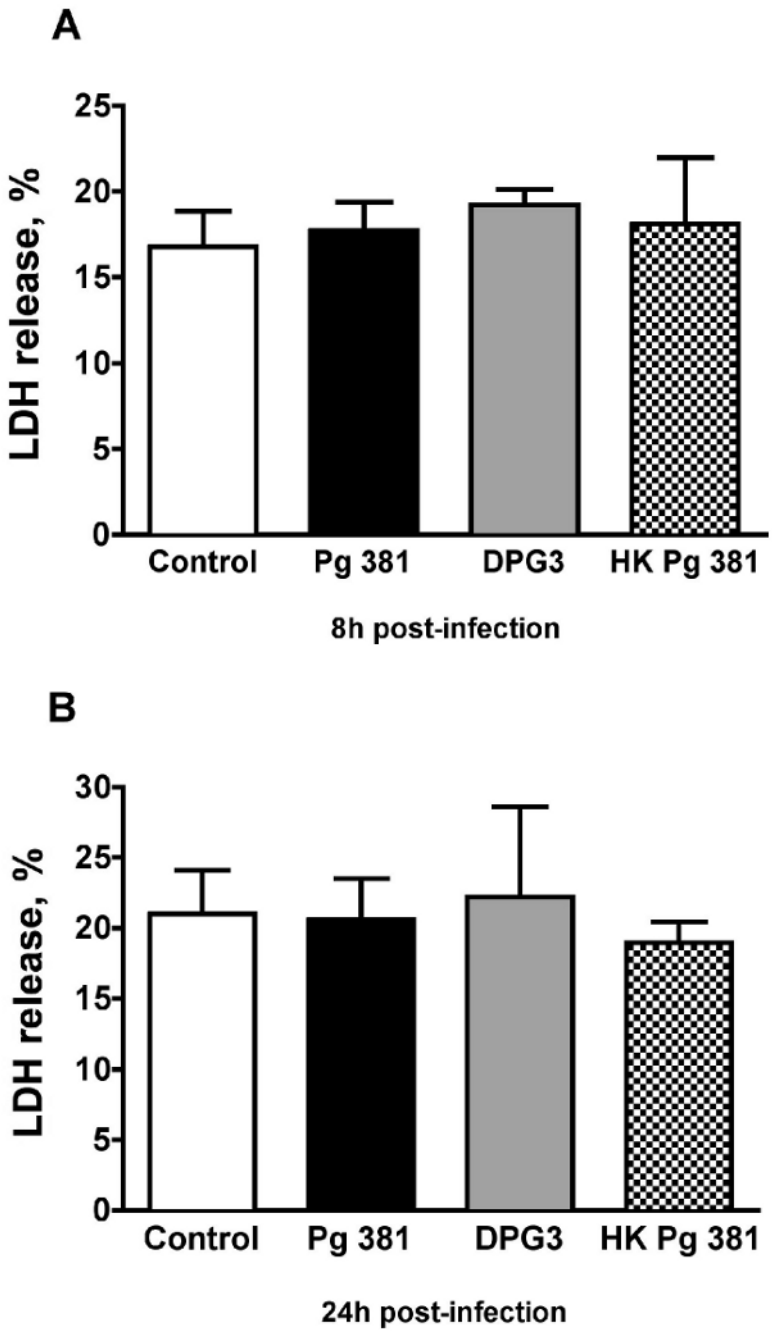

Figure 1. HASMC necrosis assessed by lactate dehydrogenase (LDH) release

Infection of HASMC with *P. gingivalis 381* (Pg 381) at MOI 1:100 did not increase LDH release after 8h (**A**) compared to non-infected control, non-invasive mutant DPG3, or heat-killed *P. gingivalis 381* (HK Pg 381). This was also the case at the 24h time point (**B**); n=4 for all groups. Mean values are shown and error bars denote standard deviations.

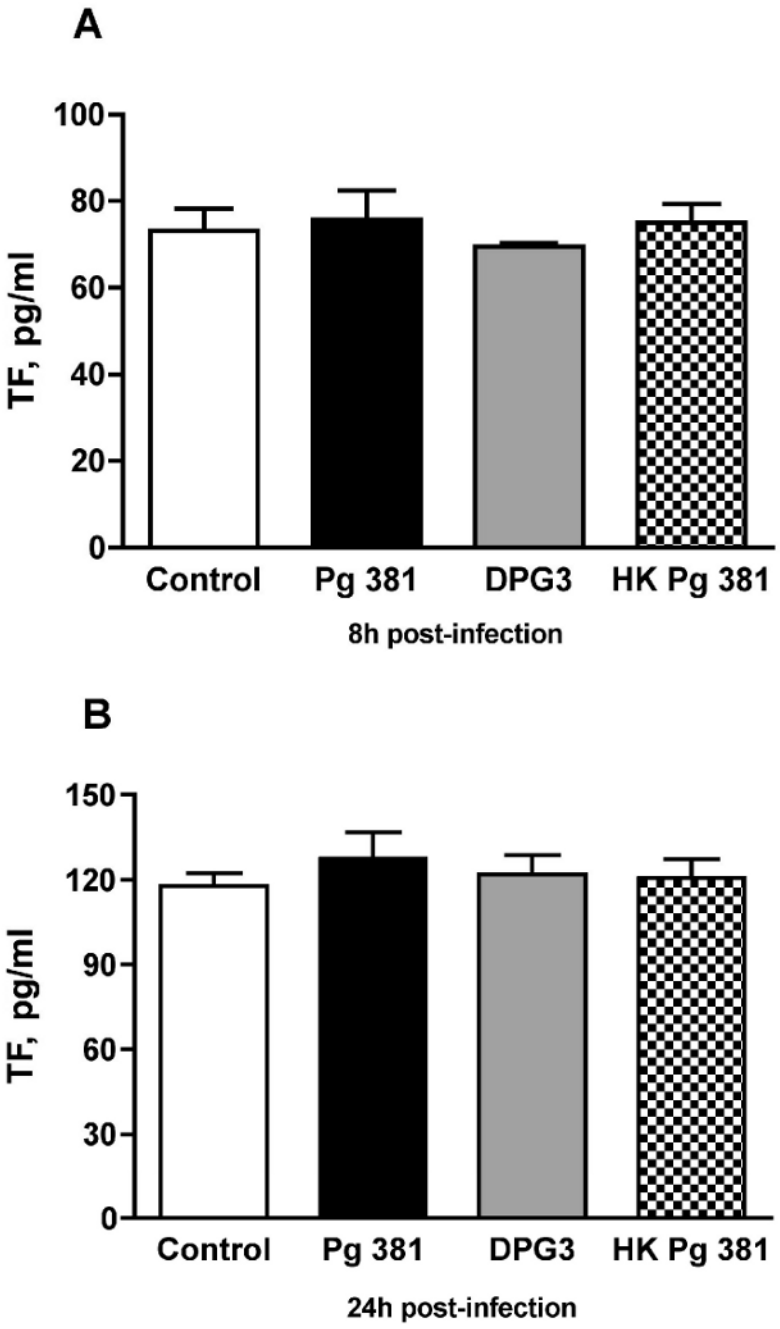

Figure 2. P. gingivalis and TF antigen levels in HASMC extracts

There was no significant upregulation in TF levels in HASMC extracts at 8h (**A**) or 24h (**B**) after *P. gingivalis* 381 infection (Pg 381), compared to non-infected control, DPG3 infection, or heat-killed *P. gingivalis* 381 (HK Pg 381); n=5 for Pg 381 group and 4 for all other groups. Mean values are shown and error bars denote standard deviations.

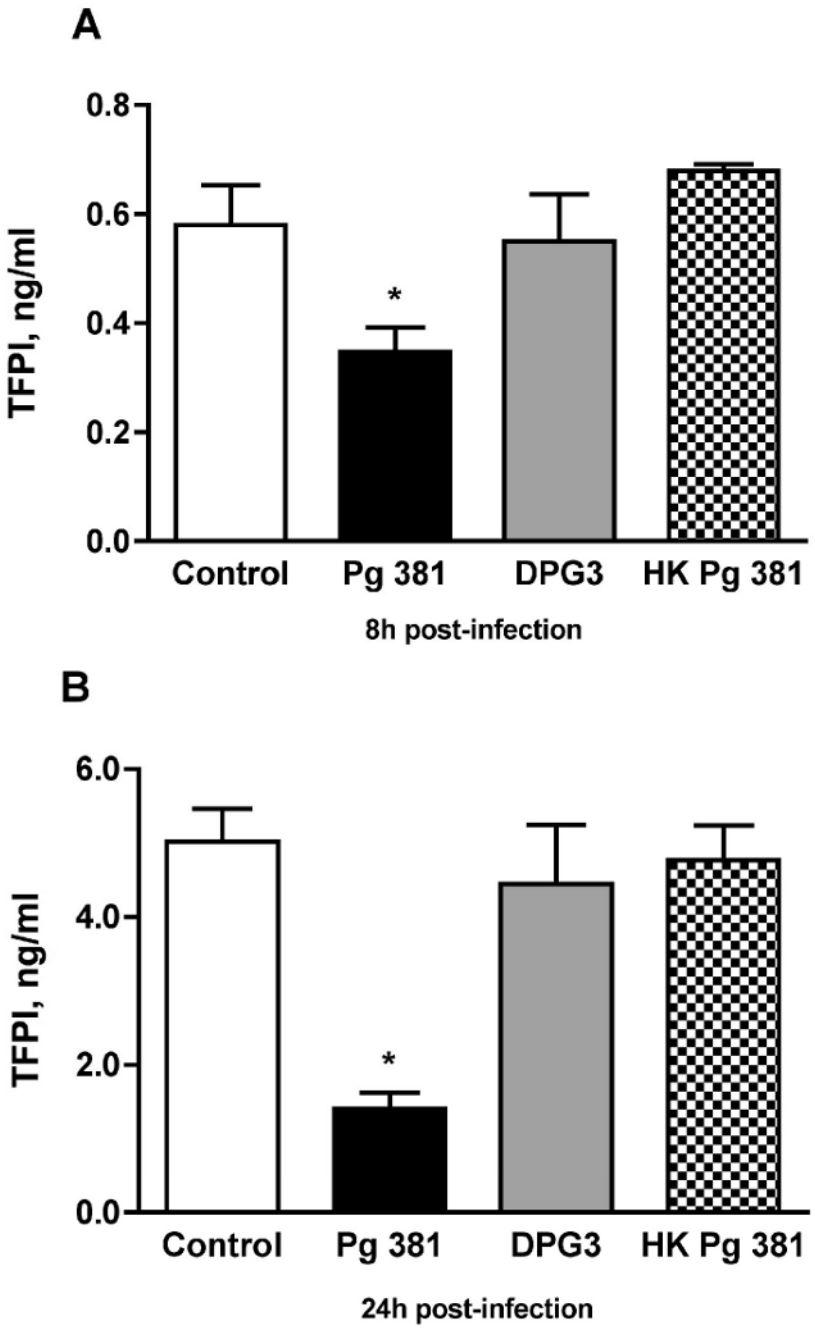

Figure 3. P. gingivalis and TFPI antigen levels in HASMC

(**A**) At 8 hours after infection with P. gingivalis 381 (Pg 381), levels of TFPI in HASMC supernatants were significantly decreased as compared to the non-infected control group (n=4 for both, p=0.0029). Similarly, the levels of TFPI in P. gingivalis 381 infected cells were significantly suppressed compared to those in DPG3-treated cells (p=0.0107) and the heat-killed P. gingivalis 381 (HK Pg 381) group (p=0.0003; n=4 for all groups). (**B**) At 24 hours after infection P. gingivalis 381 significantly reduced TFPI in HASMC, as compared to the non-infected group (p<0.001), the DPG3-treated group (p=0.0036) and the heat killed P. gingivalis 381 group (p=0.0001; n=4 for all). At both time points tested, levels of TFPI antigen were similar in control, DPG3- and heat killed P. gingivalis 381-infected cells. Mean values

are shown and error bars denote standard deviations. *indicates statistically significant differences (i.e., $p < 0.0125$ which under the Bonferroni adjustment for multiple comparisons is necessary for significance at the 5% level) compared to control, DPG3 and HK Pg 381 groups (individual p values for each comparison shown above)

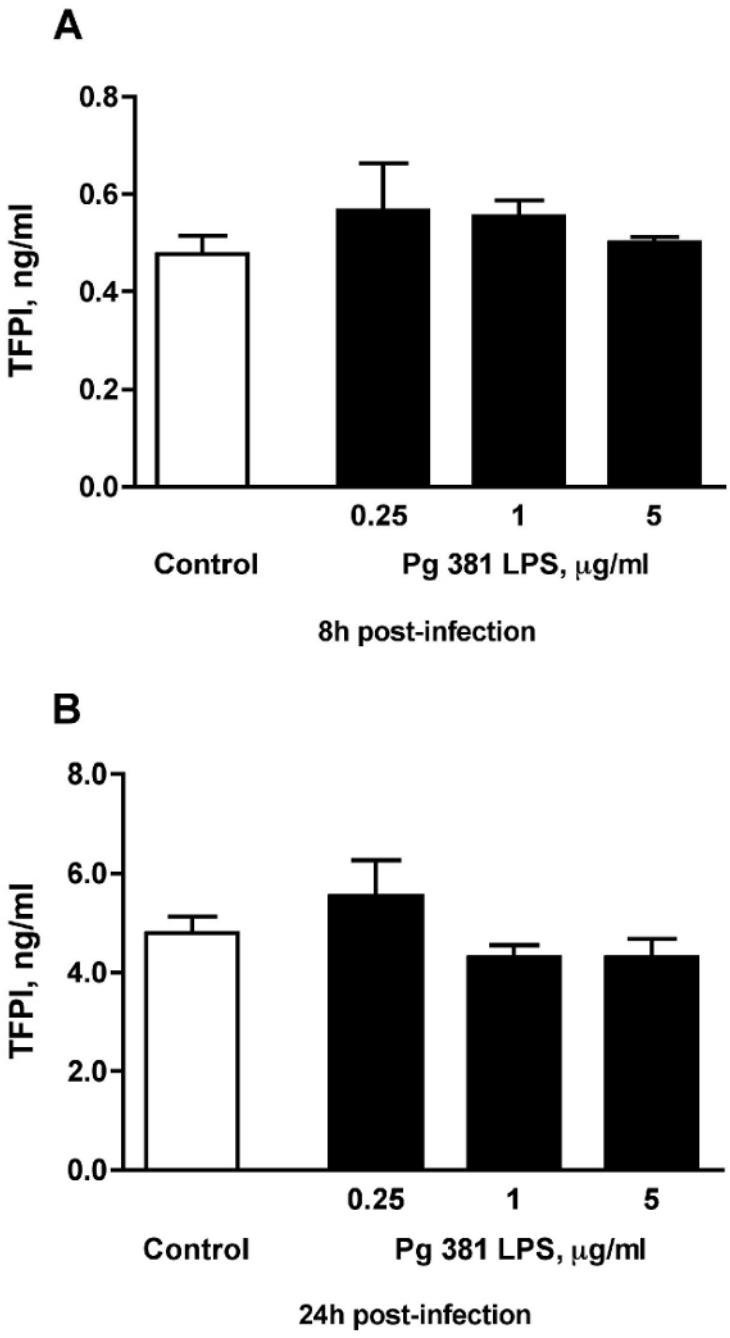

Figure 4. Effect of P. gingivalis 381 LPS on HASMC TFPI production

(**A**) *P. gingivalis* 381 LPS at 3 different concentrations (0.25 μg/ml, 1 μg/ml, or 5 μg/ml) did not induce any effect on HASMC TFPI levels at 8h as compared to control untreated cells. Similarly at 24h (**B**), TFPI levels in LPS-treated cells were not significantly affected; n=3 for all groups. Mean values are shown and error bars denote standard deviations.