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Porphyromonas gingivalis ***differentially modulates apoptosome APAF-1 in epithelial cells and fibroblasts***

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Abstract

Porphyromonas gingivalis is able to invade and modulate host-immune response to promote its survival. This bacterium modulates the cell cycle and programmed cell death, contributing to periodontal lesion worsening. Several molecular pathways have been identified as key triggers of apoptosis including apoptosome APAF-1. In this study, Apaf-1 and X-linked inhibitor of apoptosis protein (Xiap) mRNA were differentially expressed between gingival samples harvested from human healthy and chronic periodontitis tissues (Apaf-1: 19.2-fold; caspase-9: 14.5-fold; caspase-3: 6.8-fold; Xiap: 2.5-fold in chronic periodontitis) ($P < 0.05$)), highlighting their potential role in periodontitis. An increased proteic expression of APAF-1 was also observed in a murine experimental periodontitis model induced by *P.gingivalis*–soaked ligatures. *In vitro,* it was observed that *P.gingivalis* targets apoptosome-related pathways, including APAF-1, XIAP, caspase-3, and caspase-9, to inhibit epithelial cell death at both mRNA and protein levels. Opposite effect was observed in fibroblasts where *P.gingivalis* increased cell mortality and apoptosis. To assess if the observed effects were associated to APAF-1, epithelial cells and fibroblasts were transfected with siRNA targeting Apaf-1. Herein, we confirmed that APAF-1 is targeted by *P.gingivalis* in both cell types. Taken together, this study identified APAF-1 apoptosome and XIAP as intra-cellular targets of *P.gingivalis*, contributing to the deterioration of periodontal lesion through an increased persistence of the bacteria within tissues and the subversion of host-response.

Introduction

Periodontitis is a group of common chronic inflammatory diseases characterized by the destruction of tooth-supporting tissues and affecting over 30% to 50% of the adult population worldwide [1]. The pathogenesis of periodontitis is related to the disruption of homeostasis between host-immune response and dysbiotic microbial flora at the lesion site [2]. Periodontopathogenic bacteria, such as *Porphyromonas gingivalis (P.gingivalis), Tannerella forsythia,* and *Treponema denticola* interact with the host cells, including epithelial cells and fibroblasts, aiming to colonize and bypass this barrier for invading the profound periodontal tissues [3]. *P.gingivalis*, a Gram-negative assacharolytic bacteria, is considered as one of the keystone pathogens involved in the transition from periodontal health to disease [4]. This bacterium exhibits a wide array of virulence factors including lipopolysaccharide (*Pg*-LPS), extracellular proteases, and several factors that contribute to its capability of cellular invasion such as fimbriae [5]. Intracellular persistence is one strategy developed by the bacteria, to escape the host-immune response, contributing to the pathology of periodontal diseases [6]. This bacterium is also able to modulate cell cycle and programmed cell death in an attempt to avoid the host-cell response and to promote its spread to distant sites [7,8]. For instance, it has been observed that epithelial invasion by *P.gingivalis* subdues apoptosis and stimulates cell proliferation through modulation of several molecular triggers such as p53 and Jak/Stat [9,10]. However, the mechanisms involved remain under investigation and several contradictory results have been observed regarding the influence of *P.gingivalis* on apoptosis and cell viability with some studies endorsing induction of apoptosis by infection [11,12], whereas others describing its ability to inhibit such a process as a mechanism leading to induction of apoptosis [10,13].

Apoptosis has been described as a potential mechanism involved in the extensive tissue destruction associated to infectious and inflammatory diseases such as gastric ulcer related to

Helicobacter pylori [14], atherosclerosis [15] and also chronic periodontitis (CP) [12]. Dysregulation of apoptosis-related genes, such as *Bax*, *Bcl2*, *Nlrp3*, or *Smad2* has been observed in the gingival tissues of patients with periodontitis [16]. Apoptosis is involved in homeostasis and is characterized by cellular morphological changes including cell shrinkage, DNA fragmentation, and engulfment by macrophages or neighboring cells. It is under the influence of regulators including caspases that could be stated as key executioners of the cell-death machinery [17]. Apoptosis is induced by both extrinsic and intrinsic pathways. The intrinsic apoptosis pathway requires the release of pro-apoptotic factors from mitochondria, especially cytochrome c to the cytosol, which activates apoptosome-related components [18]. The apoptosome complex is a cytosolic wheel-shaped signaling platform, constituted by the oligomerization of APAF-1 [19] induced by cytochrome c release that leads to the activation of several caspases, including caspase-9 and -3, and ultimately to apoptosis [20]. APAF-1 apoptosome activity is controlled by several proteins such as X-linked inhibitor of apoptosis protein (XIAP) [21]. XIAP is a member of inhibitors of apoptosis proteins (IAPs) that is able to interact both directly with the activated caspases and under the control of activator of caspases such as Smac/Diablo [22]. XIAP acts as a guard against accidental induction of the cell death program [23] and may also be targeted by pathogens [24]. Interestingly, it was already demonstrated that apoptosome expression is modulated by *P.gingivalis* in an endothelial cell model [25] with potential influence on atherosclerosis worsening [26]. However, the effect of such infection at the periodontal level has not been investigated so far.

Therefore, our aim was to evaluate the expression of APAF-1 in CP tissues *in vivo*. Simultaneously, we examined the expression and enzymatic activities of key molecules related to apoptosome in response to *P.gingivalis* infection in epithelial cells and fibroblasts *in vitro*, in an attempt to identify new molecular mechanisms associated to periodontitis onset.

Materials and methods

Ethics Statement

A total of 20 patients accepted to participate in this study that received approval from Ethic Committee (French Ministry of Research, Bioethic department authorization DC-2014-2220). All patients gave written and informed consent prior to enrollment. Complete medical and dental histories were taken from all the subjects.

Study population and clinical examination

None of the patients had systemic diseases or cigarette smoking habit, and had not taken any prior medications such as antibiotics in the last three months. Clinical periodontal examination included measurement of pocket probing depth and bleeding on probing at six sites around each tooth with a manual periodontal probe. The healthy group consisted of nine patients (five males and four females, mean age 37.8 ± 17.3) and the CP group of 11 patients (four males and seven females, mean age 62.4 ± 7.3). Periodontal diagnosis of CP was based on the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions [27].

Collection of gingival tissue samples

Gingival samples were obtained during periodontal surgeries (open access flap) or dental extractions. Samples were inserted immediately in a sterile tube and stored at -80 °C until RNA extraction.

Bacterial culture

Porphyromonas gingivalis ATCC 33277 (ATCC, Manassas, VA) was cultured under strict anaerobic conditions at 37 °C in brain-heart infusion medium (Sigma-Aldrich, St-Louis,

MO) supplemented with 5 mg/mL hemin and 1 mg/mL menadione. On the day of infection, bacteria were collected and counted as previously described [25]. To inactivate *P.gingivalis* (H*Pg*), bacteria were heated at 85 °C for 10 minutes before the beginning of the experiment. Commercial ultrapure *Pg*-LPS was purchased from InvivoGen (San Diego, CA).

Experimental periodontitis

After anesthesia, *P.gingivalis*–infected ligature was placed at the cervical palatal site of first and second maxillary molars of mice (C57/BL6, Charles River, Larbresle, France). Ligatures were replaced twice a week during 30 days as described previously [28]. All procedures were approved by the local ethical committee and performed according to the regulations for animal experimentation. Mice were examined to evaluate pain and stress and their weight was monitored daily.

Immunohistochemistry

Immunohistochemistry for APAF-1 has been performed on sections from *P.gingivalis*–infected mice and control. Slides were treated as described in Saadi-Thiers et al [28]. Briefly, after preliminary treatment, slides were incubated with primary antibody against APAF-1 (rabbit, PA5-19894, ThermoFischer, Illkirch, France) (1:200) at 4 °C for 24 hours and then with peroxidase Dog anti-Rabbit IgG (1/200) for 30 minutes (ImmunoCruzTM rabbit ABC Staining System, Santa Cruz Biotechnology, Heidelberg, Germany). As a negative control, sections were treated with phosphate-buffered saline (PBS) or with IgG as the primary antibody to rule out non-specific binding. Finally, the sections were treated with diaminobenzadine (DAB) as a substrate chromogen and counterstained with hematoxylin. The slides were then mounted and observed under a light microscope. Image analysis software was used to evaluate the relative protein expression of APAF-1 on both healthy and

diseased tissue.

Cell culture

Human oral epithelial cells (GEC) used in this study derived from the TERT-2/OKF-6 cell line (BWH Cell Culture and Microscopy Core, Boston, MA). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in Defined Keratinocyte-SFM basal medium (KSFM) supplemented with a cocktail of growth supplements (Invitrogen, Carlsbad, CA). Human oral fibroblasts (FB) were isolated from gingival biopsy and cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Saint-Aubin, France), 2 mM glutamine, 250 U/mL fungizone, and 10 U/mL antibiotics (10 U/mL penicillin and 100 µg/mL streptomycin).

Infection of GECs with P.gingivalis and stimulation by its LPS

Twenty-four–hours before the experiment, $2x10^5$ cells were plated in each well of a 24-wells plate. At the day of the experiment, cells were washed twice with PBS and infected for 24 to 48h with *P.gingivalis* at a multiplicity of infection (MOI) of 100 and 10 bacteria/cell or stimulated by 1µg/mL *Pg*- LPS and 1µg/mL *E.Coli*-LPS for 24 to 48h.

Cells' metabolic activity

Metabolic activity/cell viability was measured using colorimetric AlamarBlue test (resazurin reduction test) [29] (ThermoFischer, Illkirch, France). After 24h of infection, stimulation and/or transfection, 300µL of incubation media were transferred to 96-well plates and measured at 570 and 600 nm to determine the percentage of AlamarBlue reduction.

Flow-cytometry analysis

GEC and FB were treated according to experimental design (infection, stimulation) as

described. To measure apoptotic and necrotic cells, Annexin V- propidium iodide staining and flow-cytometry analyses were performed. Briefly, after removal of the supernatant, cells were centrifuged and re-suspended. Cells were stained with Annexin-V-FLUOS Staining Kit (Roche Applied Science, Meylan, France) according to the manufacturer's instructions. For each condition, a total of 10,000 isolated cells were considered, using a wavelength of 488 nm for fluorescein and a wavelength of 617 nm for propidium iodide. BD LSR II was used for fluorimetric analysis. The percentage of Annexin V-positive cells was calculated from the quadrants as follows: percent of Q1: Positive Cells for IP^{PE} (Necrosis), Q2: Positive Cells for Annexin V^{FITC} and IP^{PE} (late apoptosis), Q3: Annexin Positive Cells V^{FITC} (apoptosis), Q4: Unlabeled (Viable Cells).

RNA isolation and reverse transcription

Total RNA from gingival samples and cells was extracted using Tri reagent (Sigma-Aldrich) according to the manufacturer's instructions. The Total RNA concentration was quantified using NanoDrop 1000 (ThermoFischer). Reverse transcription was performed with the iScript Reverse Transcription Supermix (Biorad, Miltry-Mory, France) according to the manufacturer's instructions.

Quantitative Real-time PCR analysis

To quantify mRNA expression, qPCR was performed on the cDNA samples. PCR amplification and analysis were performed with CFX Connect™ Real-Time PCR Detection System (Biorad). Amplification reactions have been performed using iTAq Universal SYBR Green Supermix (Biorad). Beta-actin was used as endogenous RNA control (housekeeping gene) in all samples. Primer sequences related to *Bcl2*, Bax1, Caspase-3, Caspase-9 were purchased from Qiagen (Les Ulis, France) and sequence for *Apaf*1 (3'-GTCTGCTGATGGTGCAAGGA-5'; 5'-GATGGCCCGTGTGGATTTC-3'), Keratin-10 (3'-

CATGAGTGTCCCCCGGTATC-5' ; 5'- CAGTATCAGCCGCTTTCAGA-3'), and *Xiap* (3'-TGGGACATGGATATACTCAGTTAACAA-5' ; 5'- GTTAGCCCTCCTCCACAGTGAA-3') were synthesized (ThermoFischer). The specificity of reaction was controlled using melting curve analysis. The expression level was calculated after normalization to the housekeeping gene. All PCR assays were performed in triplicate and results were represented by the mean values.

Caspase activity fluorogenic assays

Caspase-3 and -9 enzymatic activities were determined using specific fluorogenic enzymatic assays. Cells were sonicated and lysates were incubated with 200 µL of substrate solution (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 0.75 µm of caspase substrate) for one h at 37 °C as previously described [30,31]. The activity of each caspase was calculated from the cleavage of the respective specific fluorigenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9) (Bachem, Bobendorf, Switzerland). Substrate cleavage was measured with a fluorescence spectrophotometer with excitation wavelength of 360 nm and emission at 460 nm. Results were expressed as fluorescence units/mg of total protein.

Western-blotting

Proteins were separated by SDS/Page and transferred to a nitrocellulose membrane (Hybond ECL, Fischer Scientific, Illkirch, France). Primary antibodies were directed against APAF-1 (PA5-19894, ThermoFischer), XIAP (SC-11426, Santa Cruz), and β-actin (SC-130301, Santa Cruz). Protein were visualized using secondary antibody conjugated to alkaline phosphatase (anti-mouse REF: A120-101AP; anti-rabbit REF: A90- 116-AP; Bethyl

Laboratories, Montgomery, TX). All antibodies were used at the dilutions recommended by the manufacturer.

TUNEL

Direct terminal deoxynucleotidyl transferase fluorescein-dUTP nick end labeling (TUNEL) assay was performed using a commercially-available kit (TACS 2 TdTDAB In situ apoptosis detection kit, Trevigen Inc, Gaithersburg, MD), according to the manufacturer's instructions.

Small Interfering RNA Transfection and XIAP inhibition

One day prior to the transfection, $5x10^6$ cells were seeded in a 6-wells plate. Transfection with 5 nM of small interfering RNA (siRNA) targeted against APAF-1 (SI00022792) or with negative control (Qiagen) was performed using a HiPerfect Transfection reagent, according to the manufacturer's protocol.

To inhibit XIAP, cells were pre-treated with 50mM of Embelin (S7025, Selleckchem, Munich, Germany) diluted in 0.3% DMSO 48 hours before infection. Cells were then infected according to the experimental design.

Statistical analysis

Statistical analysis was performed using pair-wise Anova test and post-hoc Tukey's test. Statistical significance level was considered for $P < 0.05$. Data were analyzed using PRISM 6.0 (GraphPad, La Jolla, CA). All experiments have been performed in triplicate and in at least three sets of experiments.

Results

Expression levels of apoptosome-related components are modulated in chronic periodontitis

The expression of mRNAs related to Apaf-1, Xiap, caspase-3, and -9 was evaluated in gingival biopsies from healthy and CP patients to assess their potential relationship with periodontal lesion. Analysis showed that the expression of Apaf-1, caspase-3, and -9 mRNAs was significantly increased in CP group (Apaf-1: 19.2-fold; caspase-9: 14.4-fold; caspase-3: 6.8-fold) ($P < 0.05$) (Figure 1A, 1C, 1D). At contrary, the expression of the apoptosome inhibitor Xiap was decreased in CP group (2.5-fold) (Figure 1B). The decrease of Xiap expression correlates with the increased expression of Apaf-1 as estimated with Spearman's correlation test (R= -0,5; $P < 0.05$).

These results were also observed *in vivo*, in a murine model of experimental periodontitis associated to *P.gingivalis* infection (Figure 1E to 1L). In control group, Apaf-1 was detected mainly in soft tissues, especially within the epithelium (Figure 1E to 1H). At diseased site, Apaf-1 was still observed within the sulcular epithelium and also in the underlying connective tissue (Figure 1I to 1L). No significant staining was observed in alveolar bone suggesting a role for apoptosome mainly in the soft tissue compartment during CP pathogenesis.

Infection with* P.gingivalis *modulates metabolic activity in a dose- and cell-dependent manner

To evaluate the impact of *P.gingivalis* infection on gingival cells, assessment of cell's metabolic activity was evaluated in GECs and FBs (Figure 2). A dose-related effect has been observed in GECs regarding cells' metabolic activity. At a high MOI (100), *P.gingivalis* increased significantly the metabolic activity of the GECs whereas such an effect could not be observed with a low MOI (10) and heat-killed *P.gingivalis* (H*Pg*) after 24 and 48h (Figure 2A). A cell-specific response has been observed. In FBs, infection with *P.gingivalis* reduced significantly the metabolic activity for each tested MOI (Figure 2B). In both cell types, *Pg*-LPS displayed toxic effects at 48h (Figure 2A, 2B).

Increased epithelial cell viability is correlated with decreased apoptotic rate

To evaluate the qualitative impact of *P.gingivalis* infection on cell death, apoptotic or necrotic state of infected cells was evaluated by flow cytometry. After 24h of infection, at high MOI (100), the increase of metabolic activity induced by *P.gingivalis* infection was correlated with a decrease in the apoptotic rate in GECs. No differences were observed between infection at MOI 10 and uninfected cells (Figure 2C, 2E). A cell-specific response has been observed while, unlike GECs, same conditions of infection and *Pg*-LPS stimulation significantly increased apoptosis in FBs (Figure 2D, 2F).

Modulation of apoptosome-related mRNAexpression by* P.gingivalis *infection

To evaluate if intrinsic apoptosis-related pathways, especially associated to APAF-1 apoptosome, were modulated by *P.gingivalis* infection, mRNA expression was analyzed in infected GECs and FBs at 24h (Figure 3).

In GECs, *P.gingivalis* infection (MOI 100) decreased significantly Apaf-1 and caspase-3 mRNA expression ($P < 0.05$), and a descending trend was observed regarding caspase-9 mRNA expression as well (Figure 3A, 3E). Interestingly, the anti-apoptotic Bcl-2 and Xiap mRNAs were concomitantly increased and pro-apoptotic Bax-1 was decreased (Figure 3A). An increase in Keratin-10 expression has also been recorded, confirming the increase of GECs' proliferation. When GECs were infected with a lower MOI (MOI=10), opposite effects compared to those obtained with MOI 100 have been observed, especially regarding Apaf-1 and caspase-3 expression (Figure 3A, 3C). *Pg*-LPS may be implicated in the observed effect as it induced a significant increase in Apaf-1 and caspase-3 mRNAs expression (Figure 3C and 3E).

In FBs, *P.gingivalis* infection, at each tested MOI (10 and 100), increased significantly *Apaf1*, caspase-3, and -9 gene expression (Figure 3B to 3F). Same pro-apoptotic effects have been observed regarding Bcl-2/Bax-1 mRNA expression as infection leads to a decrease in Bcl-2 and an increase in Bax-1 mRNA expression. Interestingly, a toxic effect of *Pg*-LPS has also been highlighted (Figure 3D).

Infection with* P.gingivalis *modulates APAF-1 and XIAP protein levels and caspases' enzymatic activity

To corroborate the results obtained at the gene level, protein concentrations of APAF-1, XIAP, and caspases 3 and 9 enzymatic activities have been measured. In GECs, *P.gingivalis* (MOI 100) decreased significantly APAF-1 and increased XIAP concentrations, whereas *P.gingivalis* infection at MOI 10 and H*Pg* did not induce any modification (Figure 4A, 4C, 4E). Similar to the trend observed for mRNA level, opposite results were obtained for APAF-1 in FBs as infection at both tested MOIs increased APAF-1 concentration (Figure 4B, 4D, 4F). *Pg*-LPS may be involved as XIAP concentration was decreased following *Pg*-LPS stimulation in GECs.

Caspases are major components of apoptosome-related pathways as they are key activators of apoptosis. In our model, infection modulates caspase-9 and -3 enzymatic activities in a dose-dependent manner. In GECs, for MOI 100 condition, the enzymatic activity of both caspases was decreased whereas at MOI 10, an increase was measured. Intriguingly, *Pg*-LPS did not modify caspase-3 enzymatic activity; however, it seems to be implicated in the activation of caspase-9. In FBs, *P.gingivalis* infection increased caspase-3 significantly. Interestingly, an increase in caspase-9 activity was also observed after *Pg*-LPS stimulation (Figure 5).

P.gingivalis *modulates cell survival and cell death in infected GECs and FBs through APAF-1 and XIAP modulation*

To evaluate if APAF-1 is a key effector of cell-death associated to *P.gingivalis* infection, RNA silencing was performed using siRNA targeting APAF-1 in GECs and FBs**.** Metabolic activity, ratio of apoptosis and necrosis, and caspases' enzymatic activity were measured after Apaf-1 RNA silencing in GECs and FBs.

In transfected GECs, infection at high MOI did not increase metabolic activity as observed previously or in control. Furthermore, the inhibition of APAF-1 was associated to an increase in cell's metabolic activity at low MOI, highlighting the role of apoptosome in cell's response to *P.gingivalis* (Figure 6A). In FBs, an opposite effect was observed confirming the differential mechanisms involved in relation to cell type (Figure 6B). A qualitative change has also been observed regarding the type of cell death induced. When transfected cells were infected with *P.gingivalis*, an increase in apoptosis was observed in comparison with the negative control and H*Pg* in GECs (Figure 6C). Interestingly, such result was correlated with an increase in caspase-3 activity (Figure 6E). As observed for metabolic activity, contradictory effects were observed in FBs compared to that in GECs (Figure 6B, 6D, 6F).

To determine the implication of XIAP, cells were treated with Embelin, an XIAP inhibitor, before infection. In GECs, the increase of metabolic activity induced by *P.gingivalis* (MOI 100) infection was inhibited (Figure 7A) and was correlated with an increased apoptosis and caspases activity (Figure 7C, 7E). In FBs, pre-treatment with Embelin did not modulate cellular metabolic activity (Figure 7B) in infected cells. However, a qualitative change related to the type of cell death induced was observed (Figure 7D).

Discussion

This study aimed at determining the ability of *P.gingivalis* to modulate APAF-1 apoptosome activity and programmed cell death in the periodontal soft tissue cells. First, in gingival samples from periodontitis' patients, an increase of Apaf-1 expression and a decrease of its inhibitor, Xiap was demonstrated, showing that APAF-1 apoptosome may be involved in the periodontal lesion. However, this difference in terms of gene expression may also be related to changes of the cellular composition of the analyzed samples, CP samples exhibiting more inflammatory cells than healthy ones. In an *in vivo* model of experimental periodontitis, an increased expression of APAF-1 was observed at diseased site especially at connective tissue level consolidating our hypothesis.

In vitro, *P.gingivalis* acted differentially on this specific pathway according to the MOI and cell type. Herein, we observed an augmentation in epithelial cell viability, correlated with a decrease in apoptosis triggers (APAF-1, caspases) and increase of apoptosis inhibitors (XIAP), when cells were infected at a high MOI. In addition, when XIAP was inhibited, the increased proliferation of GEC was counteracted. Such effect was not observed in FBs.

Due to their specific location at the interface between subgingival biofilms and gingival sulcus, epithelial cells constitute the first line of host-defense. Integrity of the epithelial barrier is a key factor for periodontal health as epithelial cells play a key role in the overall tissue homeostasis and initiation of innate immune response [5]. Not only do these cells release soluble mediators such as cytokines [32] that sustain the inflammatory process but also antimicrobials peptides such as β-defensins [33]. *P.gingivalis* is considered as one of the keystone pathogens and is able to modulate cell cycle and programmed cell death to favor its survival [7,34]. Cellular invasion by *P.gingivalis* is followed by intracellular persistence, bacterial multiplication, and leads to bacterial spread to adjacent tissues including connective tissue and bone [35]. Moreover, several studies showed that this *bacterium* uses a panel of

strategy to hijack host mechanisms such as autophagy [36]. In this study, it was confirmed that *P.gingivalis* infection inhibits apoptosis and increases the proliferation rate of epithelial cells. This result is in accordance with the previous studies [7,9,10,37] that implicated the dysregulation of cell cycle by bacteria during pathogenic process [8,25]. Recently, the importance of cell cycle in the invasion process of *P.gingivalis* has been demonstrated, as the bacteria preferentially target cells in S phase [38]. This increase, in terms of proliferation rate, has prominent clinical consequences as it was suggested that fast turnover of epithelial cells may reduce immune response at the sulcular level [9]. Moreover, this may also have influence on cellular senescence that has been associated to impaired innate immune response, increased oxidative stress and that reduce bacterial clearance [39-41]. Contradictory results could also be found and some studies described an increased epithelial cell death induced by *P.gingivalis* infection. Such discrepancies were already observed by Stathopoulou et al and might be explained by the different protocols used (MOI, bacterial strain, length of exposure, cell type) [11,12].

In FBs, the effects observed were similar to those in endothelial cells [25] whereas opposite outcomes in comparison with GECs were noticed, highlighting the differential effect of the infection according to cell type. This result is in accordance with previous studies that showed the cytotoxic effect of *P.gingivalis* on FBs [42] but differs from those observed in periodontal ligament fibroblasts [43]. This reduction of proliferation rate diminishes the capability of the host to regenerate or to repair the injured tissues and contributes to periodontal lesion worsening [44].

Apoptosis is a well-described cellular process and plays a key role in homeostasis and its dysregulation has been associated to periodontitis [16]. Modulation of apoptosis or programmed cell death by periodontal pathogens is an important feature of the disease especially at the epithelial level where anti-apoptotic effects and therefore pro-survival cellular phenotype will be a key mechanism of bacterial colonization [10] and to sustained

inflammation. For instance, reduced apoptosis within periodontal tissues correlates with chronic persistence of inflammatory cells [45]. It has been described that *P.gingivalis*–induced effects on apoptosis regulation are cell-type–dependent and associated with the modulation of several pathways such as phosphatidylinositol 3-kinase/Akt or Jak/Stat pathways [10,34]. In this study, we focused on APAF-1–related pathway that has been described as an important component of the mitochondria-mediated intrinsic pathway [46]. It was observed that *P.gingivalis* modulates significantly the expression of APAF-1 in a cell- and dose-dependent manner. In epithelial cells, at high-dose, *P.gingivalis* blocked cell death through a decrease in APAF-1 expression, reduction in the enzymatic activity of caspases and an increase in XIAP expression. APAF-1 has already been described as a target of several pathogens including bacteria such as *Chlamydia pneumoniae* [47] or viruses such as *Influenza A* virus [48]. The decrease of APAF-1, at both mRNA and protein levels, may explain the increased metabolic activity observed. Therefore, it can be hypothesized that such dysregulation may be a specific bacterial strategy to avoid clearance from the organism with potential consequences regarding induction of inflammatory cell death through necrosis or necroptosis as suggested in a mouse model [49]. The regulatory role of XIAP has also been investigated by using an inhibitor. In our model of epithelial cells, inhibition of XIAP inhibited the *P.gingivalis*–induced increase of cell viability. This effect was associated with an elevated caspase-3 and -9 enzymatic activity, as already demonstrated in other cellular models [22,50]. IAP, such as cIAP1, cIAP2, and XIAP are key elements of homeostasis as their loss or inhibition sensitizes the cell to death associated to inducers such as TNF [51]. A better understanding of mechanisms involved between IAP and regulation of cell death will be instrumental in developing therapeutic strategies. In FBs, inhibition of XIAP did not affect *P.gingivalis*–associated cell death as hypothesized. However, it is important to consider the multitude of pathways that control apoptosis process and crosstalk between them especially in the context of *P.gingivalis*

infection. For instance, *P.gingivalis* is able to modulate NLRP3 inflammasome expression [52,53]. NLRP3 inflammasome is implicated in the release of mature IL-1β and IL-18 and to pyroptosis but also in the control of apoptosis as demonstrated in osteoblast infected by *Aggregatibacter actinomycetemcomitans* [54]. Interestingly, inflammasome and apoptosome are similar in their structure and are subject to crosstalk mediated by cytochrome c [55]. Future studies need to focus on the effect induced by apoptosome modulation by *P.gingivalis* on inflammasome activity and especially on its impact on sustained periodontal inflammation.

A differential cellular effect was also observed between high and low dose of infection. To date no MOI has been regarded as the most relevant to *in vivo* situation, even if MOI 100 corresponds to the optimal dose for *P.gingivalis* invasion [6]; however, the use of a low and high MOI may give some data relevant to low-grade versus acute infection. We could hypothesize that in case of high bacterial load, as observed in deep pockets that occur during severe forms of periodontitis, host defense is submerged by the bacterial aggression, leading to an unregulated cellular proliferation. In case of low bacterial charge, host defense is able to control bacterial spreading and eliminate infected cells more efficiently. Hence, it may be considered that, apoptosome may be positive in the case of mild infection but detrimental in case of severe one. Such effect has already been observed with regard to another molecular platform, the inflammasome [56], in an infectious context. Here, we showed that APAF-1 was increased in periodontitis based on clinical biopsies' and ligature-induced experimental periodontitis analyses. However, even if we can suppose that high bacterial load was associated to the analyzed tissues, no bacterial sampling was available restraining the possibility to directly assess the correlation between *P.gingivalis* presence and APAF-1 expression. Furthermore, even if relevant to study periodontal pathogenesis [57], the local trauma that occurs while using ligature-induced periodontitis should be considered as observed modulatory effects could not be solely associated to *P.gingivalis* in this model.

However, to date, no data are available regarding the specific effect induced by chronic mechanical trauma on programmed cell-death at periodontal lesion site.

To better understand the bacterial mechanisms involved, the effects induced by *P.gingivalis* LPS were investigated. *Pg*-LPS is a well-described virulence factor that activates host inflammatory response. It has already been shown that *Pg*-LPS induces secretion of several pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α or IL-1 [58] or extra-cellular matrix proteases such as cathepsins [59]. In this study, *Pg*-LPS exhibits cytotoxic effects in both cell types as observed in endothelial cells [25]. As for the bacteria, cytotoxicity of LPS has been demonstrated to be dependent on the strain and structure, such as the presence of O-antigen [60], and also the cell type [61]. Appealingly, LPS was found to modulate apoptosome-related pathway by increasing APAF-1 expression and caspases activity as observed in endothelial cells [25]. *Pg*-LPS acts through toll-like receptors (TLRs) such as TLR-2 [58] and TLR-4 [60,62]. Besides, TLRs have already been linked to apoptosis in several infectious models such as in macrophages simulated with *Mycobacterium* proteins [63,64]. However, specific mechanisms associated with *Pg*-LPS need to be elucidated.

Finally, this study demonstrated the putative role of APAF-1/XIAP in periodontal pathogenesis within soft tissues. Infection with *P.gingivalis* modulates APAF-1 apoptosome and XIAP activities in a cell-type–dependent manner (Figure 8). These mechanisms may be one of those explaining the subversion of innate immune response by *P.gingivalis* that occurs during periodontitis. However, further experiments such as knock-in of *Apaf-1* should be conducted to evaluate its potential impact on the prevention or management of *P.gingivalis*–associated diseases. Targeting programmed cell death in a time-controlled manner may be a promising tool, as suggested for autophagy; however, specific drugs should be developed with limited risk of side effect.

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Figures legends

Figure 1: Apaf-1, caspase-3, caspase-9, and Xiap mRNA expression in chronic periodontitis. The mRNA expression levels were evaluated in healthy (H) (n=10) and CP (n=9) group. mRNA expression was normalized against the expression level of β-actin ($P < 0.05$). Relative expression of Apaf-1 (**A**), Xiap (**B**), caspase-3 (**C**), and caspase-9 (**D**). *differences between H and CP group ($P < 0.05$). Apaf-1 expression in healthy periodontal tissue (left 1st molar) (**E, F, G, H**) and in experimental periodontitis induction (right 1st molar) (**I, J, K, L**), in wild-type mouse (Arrows indicate the zones of Apaf-1 expression; EP: Epithelium, CT: Connective tissue; AB: Alveolar Bone), (Bars size = 100µm).

Figure 2: Effects of *P.gingivalis* infection on cell metabolic activity and cell death. A: Metabolic activity of human oral epithelial cells (GECs) infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS at 24 and 48h. **B:** Metabolic activity of FBs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24 and 48h. **C:** Analysis of cell death of GECs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS at 24h, by Annexin V^{FITC} and IP^{PE} labelling measured by flow cytometry showing apoptotic, late apoptotic, and necrotic cell populations. **D:** Analysis of cell death of FBs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS at 24h, by Annexin V^{FITC} and IP^{PE} labelling measured by flow cytometry showing apoptotic, late apoptotic, and necrotic cell populations. Data are expressed as mean ± SD. *differences between infected or stimulated cells with control ($P < 0.05$). **E, F:** Histogram of Annexin V^{FITC} and IP^{PE} labelling on GECs and FBs (Q1: IP^{PE} positive cells; Q2: Annexin $V^{FITC/}$ IP^{PE} positive cells; Q3: Annexin V^{FITC} positive cells; Q4: no labelled / viable cells).

Figure 3: *P.gingivalis* and its lipopolysaccharide (LPS) differentially modulates

apoptosis-related gene expression in human oral epithelial cells (GECs) and human oral fibroblasts (FBs). A: Gene expression of Bax-1, Bcl-2, Apaf-1, Xiap, and Ker-10 in GECs infected with *P.gingivalis* MOI 100 and MOI 10 and H*Pg* at 24h. **B:** Gene expression of Bax-1, Bcl-2, APAF-1, and Xiap in FBs infected with *P.gingivalis* MOI 100 and MOI 10 and H*Pg* at 24h. **C:** Gene expression of Bax-1, Bcl-2, Apaf-1, Xiap, and Ker-10 in GECs stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. **D:** Gene expression of Bax-1, Bcl-2, Apaf-1, and Xiap in FBs stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. **E:** Gene expression of caspase-1, -3, and -9 in GECs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. **F:** Gene expression of caspase-1, -3, and -9 in FBs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. Data are expressed as mean ± SD. *differences between infected or stimulated cells with control ($P < 0.05$).

Figure 4: *P.gingivalis* modulates APAF-1 and XIAP protein concentration in human oral epithelial cells (GECs) and human oral fibroblasts (FBs). A, C, E: Western blot analysis for APAF-1 and XIAP in GECs infected with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. **B, D, F:** Western blot analysis for APAF-1 and XIAP proteins in FBs infected with *Pg* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. *differences between infected or stimulated cells with control ($P < 0.05$).

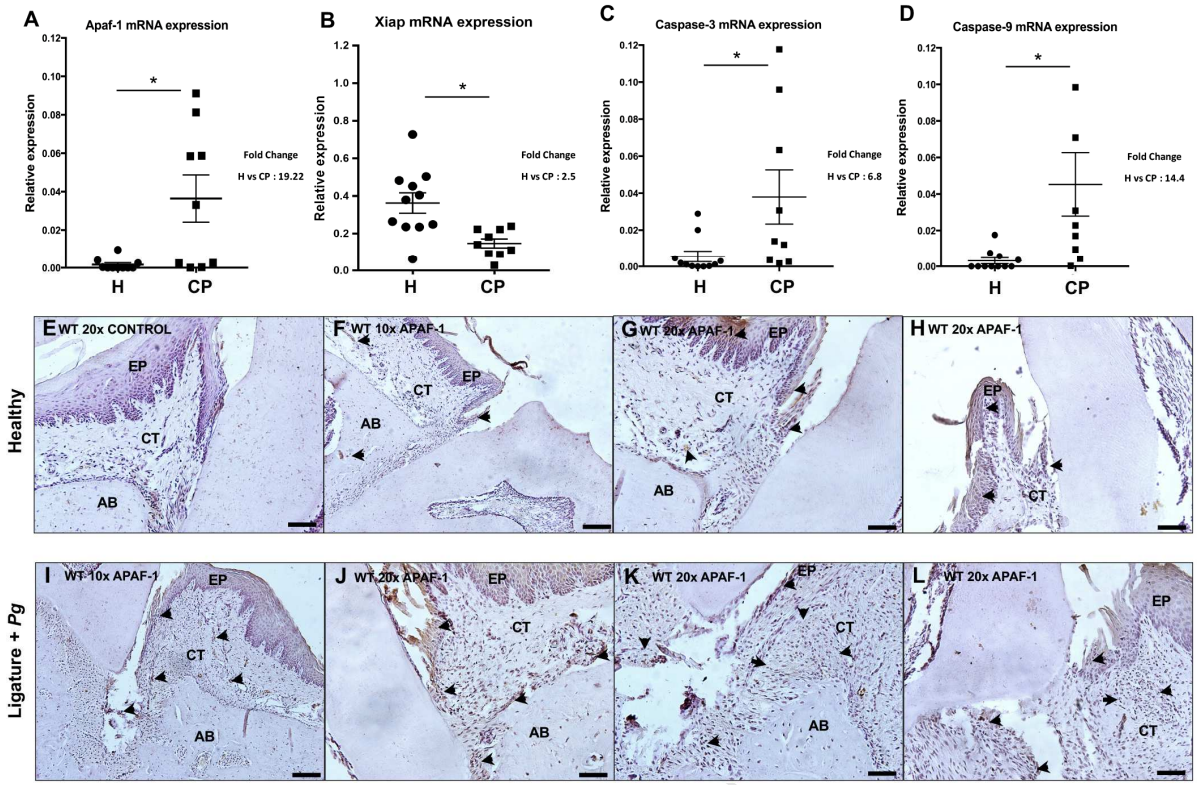
Figure 5: *P.gingivalis* and its lipopolysaccharide (LPS) modulate caspases' enzymatic activity in a dose- and cell-dependent manner. Enzymatic activity of caspase-3 (**A, C**) and caspase-9 (**B, D**) in human oral epithelial cells (GECs) and human oral fibroblasts (FBs)infected with with *P.gingivalis* MOI 100 and MOI 10, H*Pg* and stimulated by *Pg*-LPS and *E.Coli*-LPS (1µg/mL) at 24h. Data are expressed as mean ± SD. *differences between infected or stimulated cells with control ($P < 0.05$).

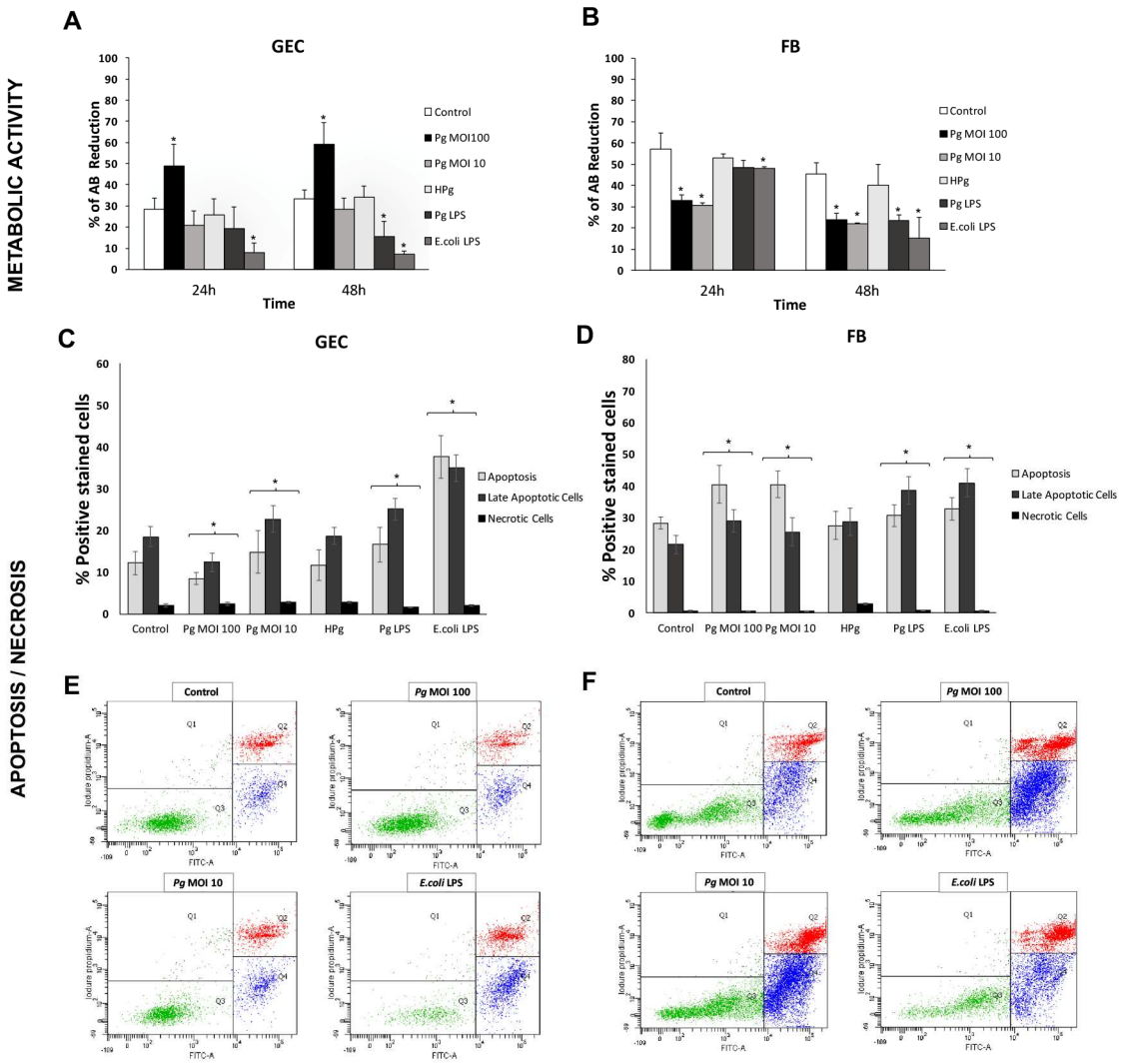
Figure 6: *P.gingivalis* modulates cell survival and apoptosis rate in infected human oral epithelial cells (GECs) and human oral fibroblasts (FBs) through apoptosome-related proteins. A, B: Metabolic activity of GECs and FBs transfected with siRNA targeting APAF-1 and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24h. **C, D:** Apoptosis/necrosis ratio, in transfected GECs and FBs and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24h. **E, F:** Enzymatic activity of caspase-3 (c3) and caspase-9 (c9) in transfected GECs and FBs and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24h. Data are expressed as mean ± SD. *differences between infected or stimulated cells with control ($P < 0.05$). †differences between transfected and non-transfected cells ($P < 0.05$). ‡differences between infected or stimulated transfected cells with transfected control ($P < 0.05$).

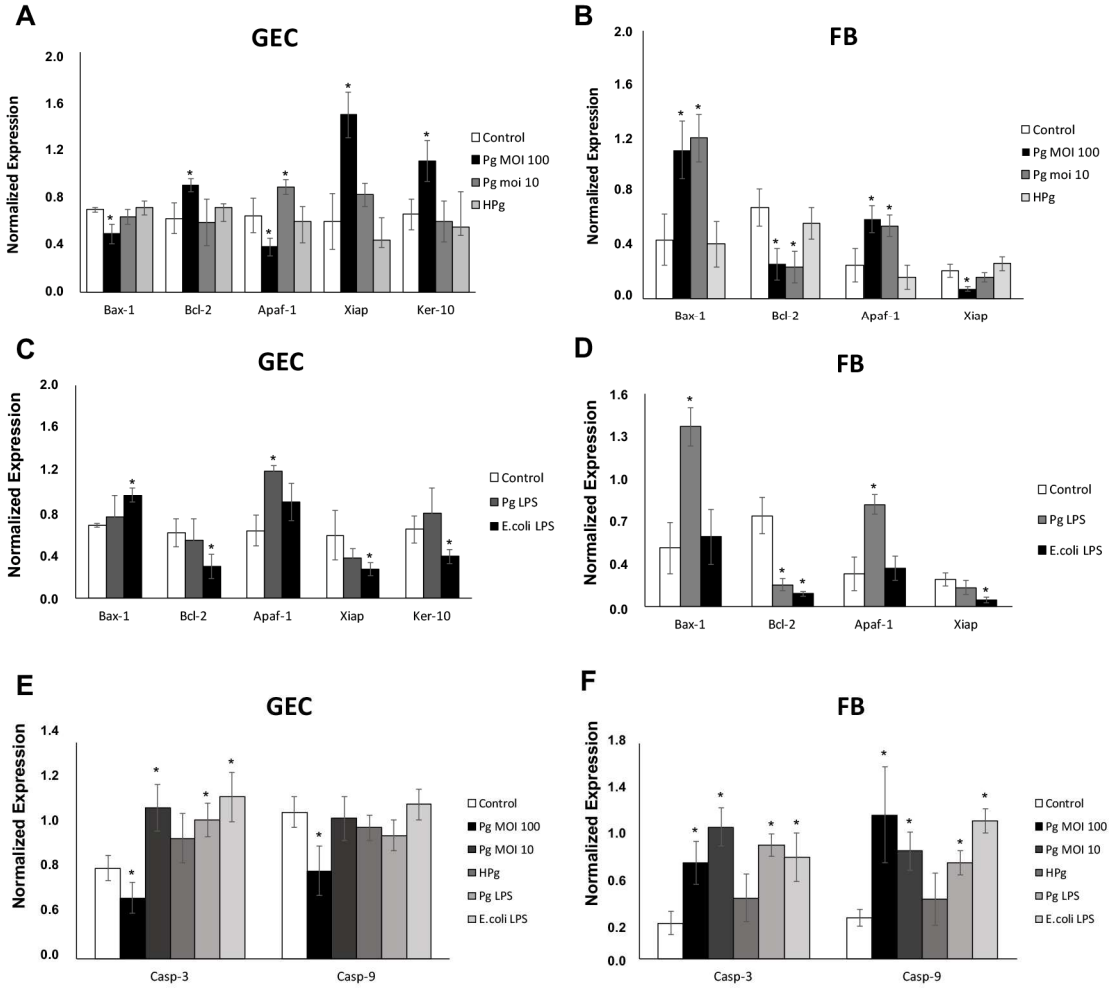
Figure 7: *P. gingivalis* modulates cell survival and apoptotic rate in human oral epithelial cells (GECs) and human oral fibroblasts (FBs)through apoptosome-related proteins. A, B: Metabolic activity of GECs and FBs treated with Embelin during 48h and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24h. **C, D:** Apoptosis evaluation by Tunel assay in transfected GECs and FBs and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24. **E, F:** Enzymatic activity of caspase-3 (c3) and caspase-9 (c9) in transfected GECs and FBs and infected with *P.gingivalis* MOI 100, MOI 10 and H*Pg* at 24h. Data are expressed as mean ± SD. *differences between infected or stimulated cells with control ($P < 0.05$). †differences between pre-treated and non-treated cells ($P < 0.05$). ‡differences between infected or stimulated cells with pre-treated control ($P < 0.05$).

Figure 8*:* Cell death and activation of APAF-1–related pathway in response to *P.gingivalis* infection in epithelial cells and fibroblasts. Cell-specific modulation of apoptosis by *P.gingivalis* in epithelial cells and fibroblasts. In epithelial cells, *P.gingivalis*

decreases APAF-1 and increases XIAP expression leading to an augmented cell survival. At contrary, in fibroblasts, bacteria stimulate APAF-1 pathway and reduce XIAP expression inducing apoptosis.



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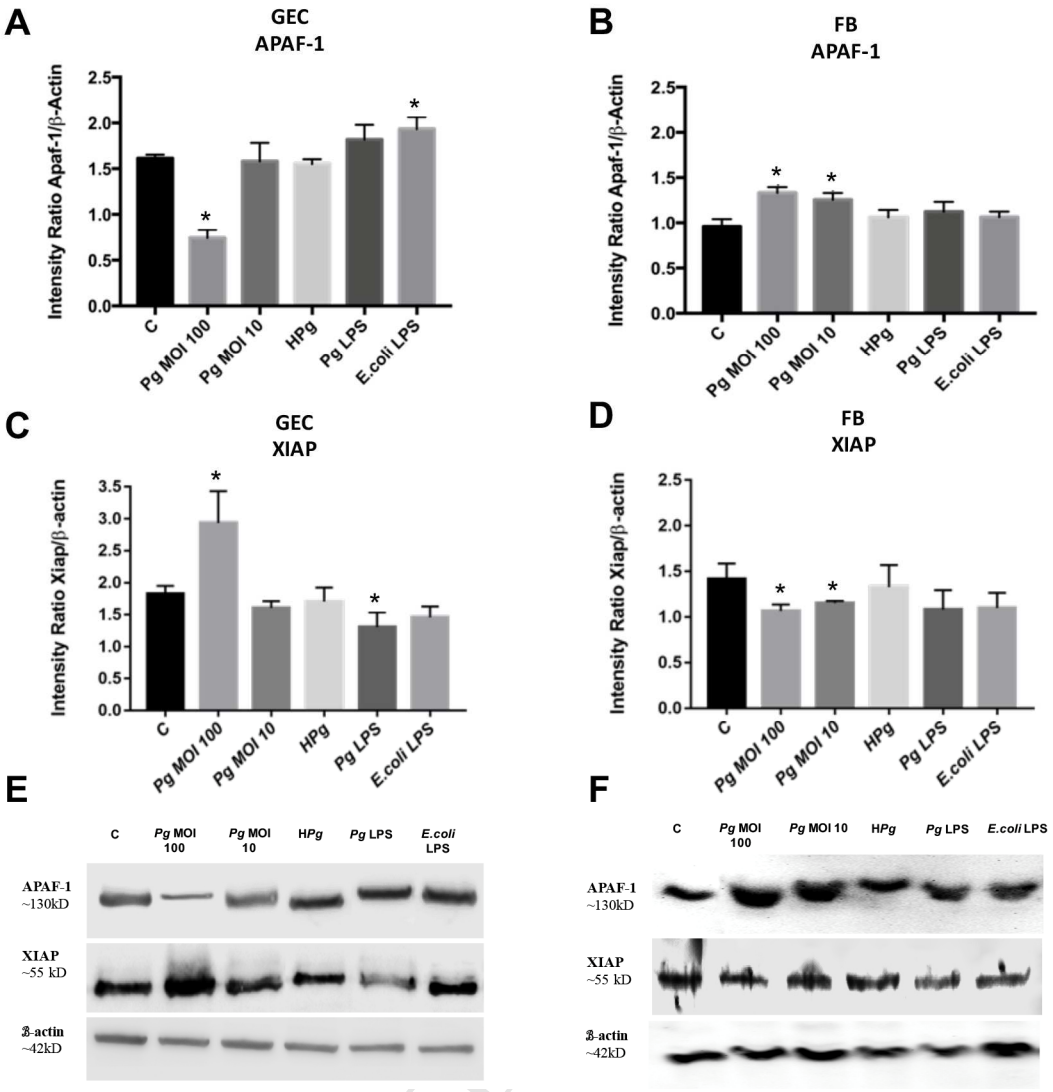
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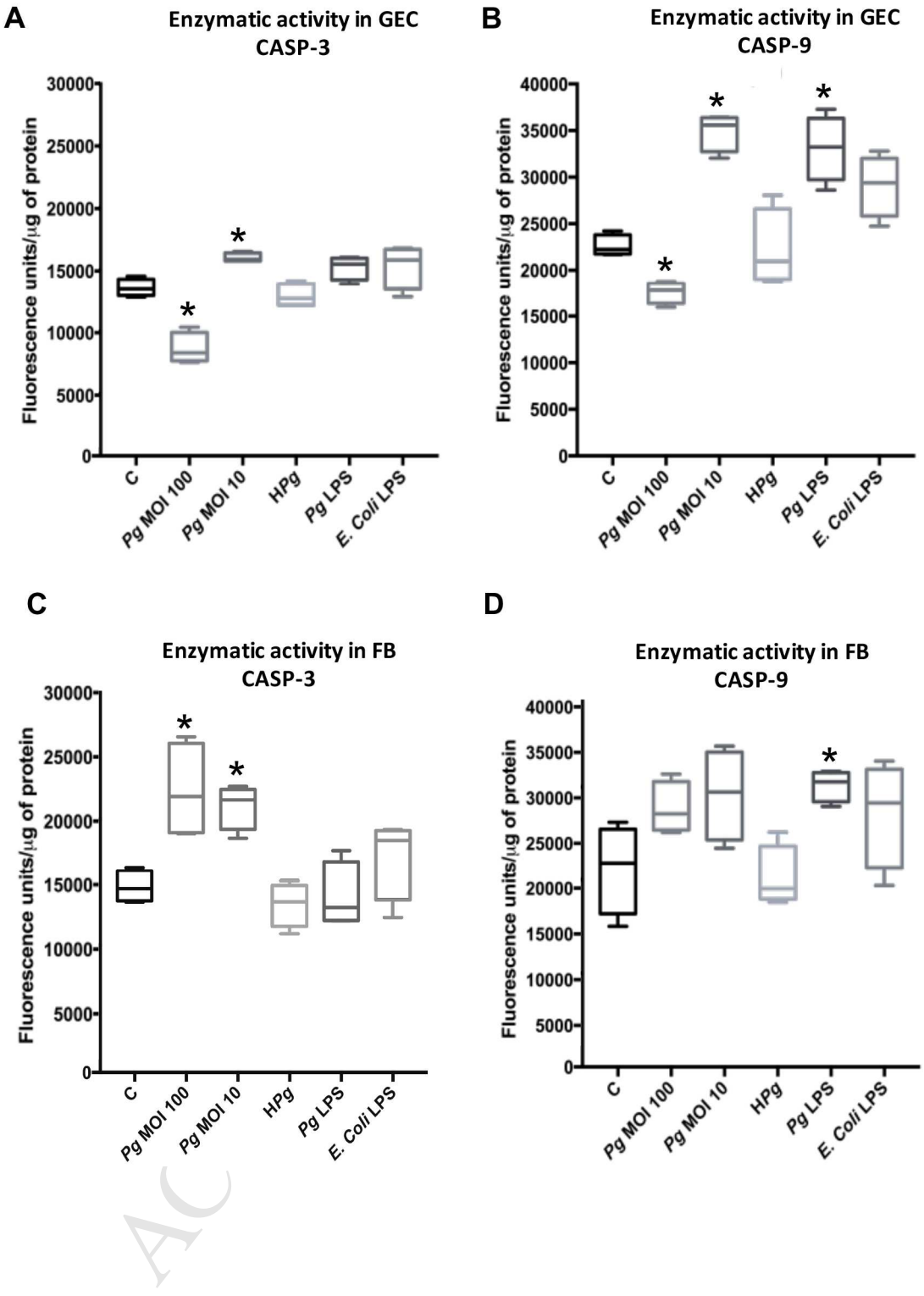
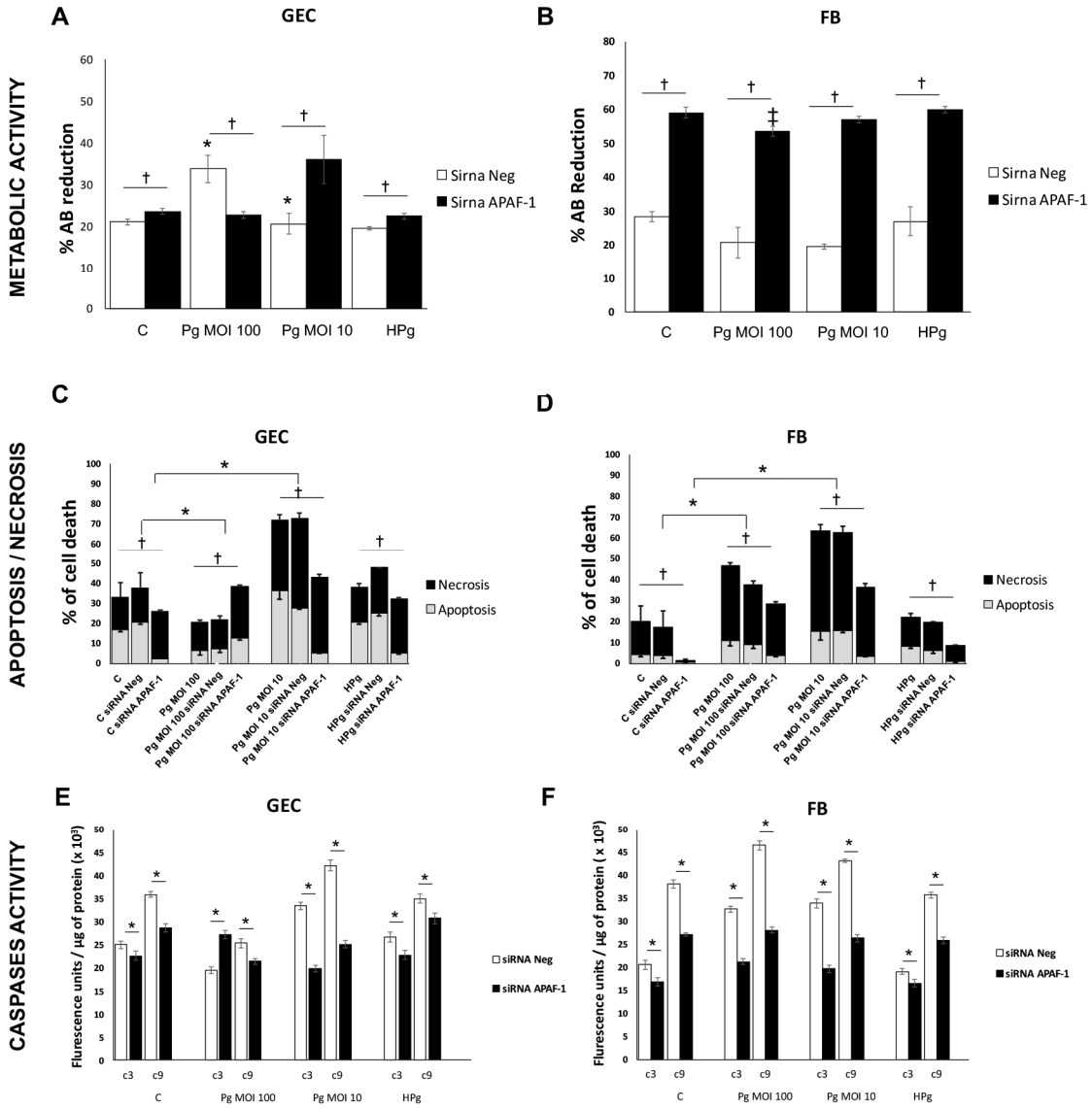
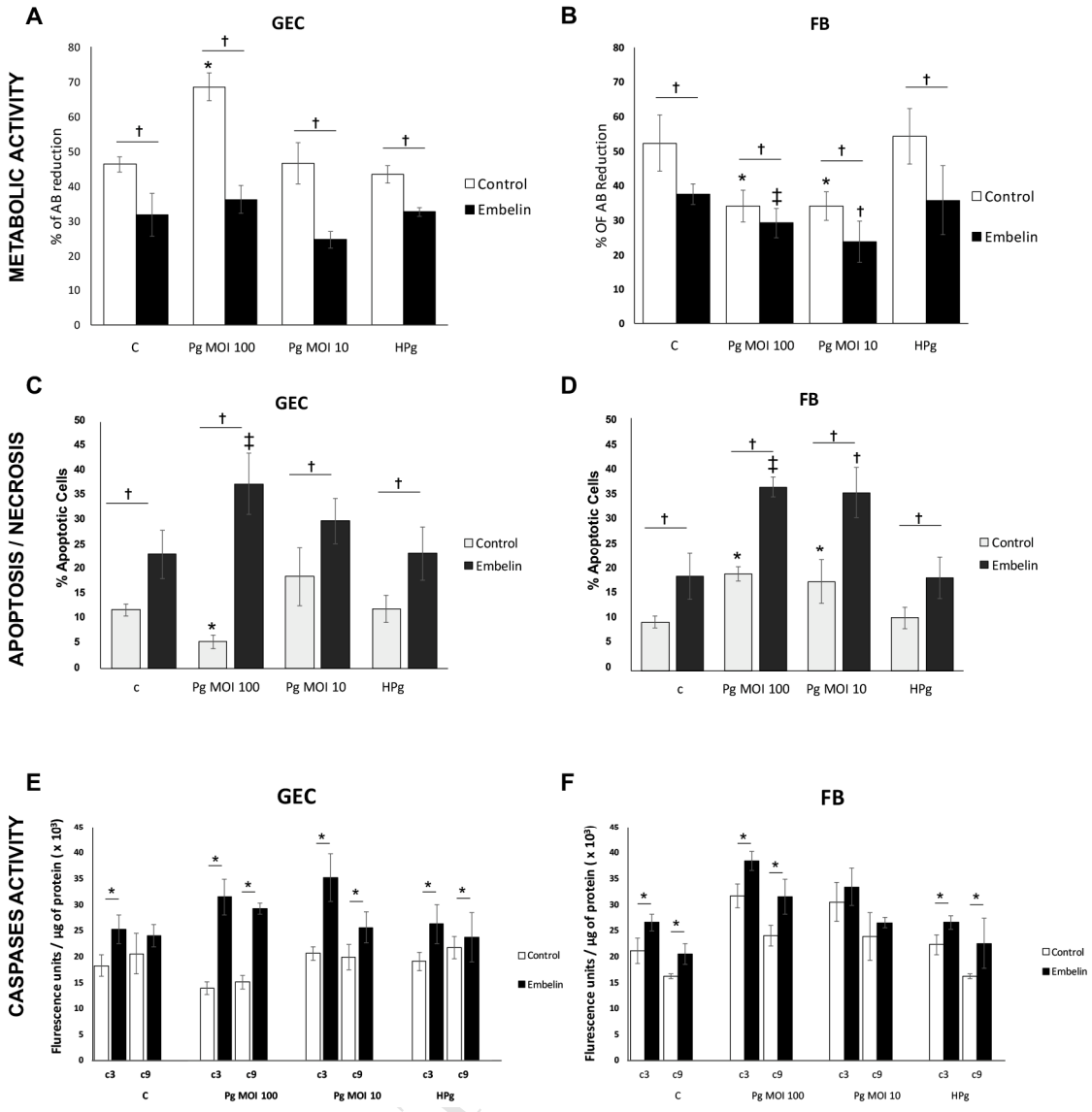
FB
XIAP
Intensity Ratio Xiap/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E.coli LPS
E
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E.coli LPS
APAF-1
~130kD
XIAP
~55 kD
β-actin
~42kD
F
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E.coli LPS
APAF-1
~130kD
XIAP
~55 kD
β-actin
~42kD


A
Enzymatic activity in GEC
CASP-3
Fluorescence units/µg of protein
30000
25000
20000
15000
10000
5000
0
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E. Coli LPS
B
Enzymatic activity in GEC
CASP-9
Fluorescence units/µg of protein
40000
35000
30000
25000
20000
15000
10000
5000
0
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E. Coli LPS
C
Enzymatic activity in FB
CASP-3
Fluorescence units/µg of protein
30000
25000
20000
15000
10000
5000
0
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E. Coli LPS
D
Enzymatic activity in FB
CASP-9
Fluorescence units/µg of protein
40000
35000
30000
25000
20000
15000
10000
5000
0
C
Pg MOI 100
Pg MOI 10
HPg
Pg LPS
E. Coli LPS




A
GEC
METABOLIC ACTIVITY
% of AB reduction
Control
Embelin
C
Pg MOI 100
Pg MOI 10
HPg
B
FB
% OF AB Reduction
C
Pg MOI 100
Pg MOI 10
HPg
C
GEC
APOPTOSIS / NECROSIS
% Apoptotic Cells
c
Pg MOI 100
Pg MOI 10
HPg
D
FB
% Apoptotic Cells
E
GEC
CASPASES ACTIVITY
Flurescence units / µg of protein (x 10³)
c3
c9
F
FB