Periodontal Commensals and Pathogens Differentially Modulate Immuno-Inflammatory Response in Human Oral Keratinocytes

Hua Jing LI[1,2], Chaminda Jayampath SENEVIRATNE[3], Cun Yu WANG[4], Li Jian JIN[1]

Objective: *To investigate the immunoinflammatory response in the crosstalk of human oral keratinocytes (HOKs) with selected periodontal commensals and pathogens.*
Methods: *Four representative viable oral bacteria, including periodontal commensals (Streptococcus mutans, Sm; and Actinomyces israelii, Ai) and pathogens (Aggregatibacter actinomycetemcomitans, Aa; and Porphyromonas gingivalis, Pg), were selected. A viable bacteria-HOKs interactive model was tested under various conditions of oxygen, antibiotics, duration and multiplicity of infection (MOI). The expression of IL-6 and IL-8 in HOKs was assessed by real-time qPCR and ELISA.*
Results: *An MOI of 1 was determined to be the appropriate ratio of bacteria and HOKs with substantial amounts of viable bacterial cells and HOKs in an antibiotic-free medium under aerobic conditions for 2 h. Sm and Pg significantly upregulated the expression of IL-6 and IL-8 ($P < 0.05$), while Ai and Aa could not induce significant levels of these cytokines with reference to the control.*
Conclusion: *Within the limitations of this study, the current findings suggest that periodontal commensals and pathogens may differentially modulate immunoinflammatory response in human oral keratinocytes.*
Key words: *commensals, periodontopathogens, human oral keratinocytes, bacteria-host interaction, cytokines*
Chin J Dent Res 2019;22(2):105–112; doi: 10.3290/j.cjdr.a42514

Periodontal disease is a highly prevalent infection and inflammation and remains one of the major global oral health issues with marked socioeconomic impacts and healthcare costs[1-3]. Severe periodontitis results in substantial destruction of tooth-supporting tissues and predominantly accounts for severe tooth loss and edentulism in the adult population worldwide[4]. Current evidence also shows that periodontal disease is closely linked with an array of systemic diseases and disorders such as diabetes mellitus and cardiovascular disease[5]. It is therefore crucial to further promote periodontal health and disease prevention for optimal oral and general health[1,3,6].

It is currently recognised that a symbiosis between oral commensal bacteria and the host defence system is crucial for periodontal health, while the microbial shift of oral biofilms may account for microbe-host dysbiosis and the initiation of periodontal disease, and subsequently contribute to periodontal destruction[7]. A dysregulated and aberrant immunoinflammatory response to an uncontrolled bacterial challenge in susceptible individuals is indeed the major contributing factor influencing the severity and progression of the disease[8,9]. Oral commensals and pathogens could evoke different immunoinflammatory responses when interacting with various host cells, and identification of these bacteria with different characteristics may crucially determine the resultant immunoinflammatory response[10-13], whereas what remains to be further defined are the detailed profiles

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This study was supported by the General Research Fund (GRF) of Hong Kong Research Grants Council (GRF No. 768713) and the Modern Dental Laboratory/HKU Endowment Fund to LJJ.

and underlying mechanisms of the crosstalk of host cells with periodontal commensals and pathogens.

Epithelial cells act at the frontline in dealing with complex microbial challenges, and gingival epithelial cells play a pivotal role in the integrity of innate defence systems and periodontal health[14,15]. Human oral keratinocytes (HOKs) have often been used as an *in vitro* model to study microbe-host interactions[16-18]. Standardised protocols with well-defined laboratory parameters for *in vitro* studies on variable oral microbe-host interactions are urgently required to generate meaningful and comparable data.

The present study investigated the immunoinflammatory response in the crosstalk of HOKs with selected periodontal commensals and pathogens by using a defined viable bacteria-HOKs interactive model.

Materials and methods

Selection and culture of oral bacteria

The selected Gram-positive bacteria including *Streptococcus mutans (Sm)* (ATCC 35668) and *Actinomyces israelii (Ai)* (ATCC 10048) and Gram-negative bacteria including *Aggregatibacter actinomycetemcomitans (Aa)* (ATCC 29523) and *Porphyromonas gingivalis (Pg)* (ATCC 33277) were from the archival microbial collection at the Oral Biosciences, Faculty of Dentistry, The University of Hong Kong. *Sm*, *Ai* and *Aa* were subcultured 1 day prior to the experiment and *Pg* was subcultured 1 week early on blood agar at 37°C in an anaerobic chamber. These species were then collected by washing with sterile phosphate-buffered saline (PBS) to prepare bacterial suspensions for the subsequent experiments.

Cell culture

The primary HOKs (ScienCell Research Laboratories, Carlsbad, CA, USA) were subcultured in serum-free oral keratinocyte medium (OKM) (ScienCell) with 1% growth supplement and 1% penicillin/streptomycin solution (ScienCell). Preliminary studies revealed that the third passage cells were optimal for the experiments without any signs of senescence. The cells at 5×10^3 cells/cm^2 were then seeded in 6-well plates or 25T flasks, and properly grown at 37°C in a humidified incubator of 5% CO_2. Medium changes were made on the first day after seeding, and then every other day until the cell confluence was reached.

Bacteria-HOKs interactions

The HOKs were challenged with the viable bacteria under different circumstances, and the most appropriate conditions were determined for the bacteria-HOKs interactive model. Briefly, the selected viable bacteria were adjusted to 10^5 cells/ml using the OKM, then the HOKs were treated with these bacteria under various conditions of oxygen (aerobic or anaerobic), with or without antibiotics, for a duration of 2 to 24 h, and different multiplicity of infection (MOI) with 1 and 10. The viability of both bacteria and HOKs during their interactions was assessed under different circumstances.

Colony forming units (CFUs)

In order to examine the survival of bacteria in the culture medium without interference of the HOKs, the selected bacteria alone were adjusted to 10^5 cells/ml using OKM, with or without antibiotics, and incubated at 37°C in an anaerobic or aerobic chamber for 2, 6 and 24 h. A serial dilution of the bacterial suspension was made, and the aliquots were spirally plated in blood agar in duplicates. The plates were incubated under anaerobic conditions and the resultant CFU was counted.

Quantitative real-time polymerase chain reaction (qPCR)

As an alternative approach to examine the multiplication of the species, the selected bacteria were adjusted to 10^5 cells/ml using OKM without antibiotics and incubated at 37°C in an aerobic chamber for 2 h. Following the incubation period, the bacteria were recollected and the

Table 1 Nucleotide sequence of primers for real-time qPCR.

Genes	Forward	Reverse
Sm	GCCTACAGCTCAGAGATGCTATTCT	GCCATACACCACTCATGAATTGA
Ai	GGCCACATTGGGACTGAGAT	CGCCCATTGTGCAATATTCC
Aa	CGTAAGGGCCATGATGACTTG	ACCAACCAGCGATGGGG
Pg	TACCCATCGTCGCCTTGGT	CGGACTAAAACCGCATACACTTG

Sm: Streptococcus mutans; Ai: Actinomyces israelii; Aa: Aggregatibacter actinomycetemcomitans; and *Pg: Porphyromonas gingivalis*.

deoxyribonucleic acid (DNA) was extracted from each sample. qPCR was conducted with validated species-specific primers (Table 1).

MTT assay

The HOKs were cultured in OKM in a 96-well plate at 37°C in a humidified incubator of 5% CO_2 to confluence, according to the manufacturer's instructions. Bacteria were added with an MOI of 1 or 10 into the confluent HOKs and incubated for 2 h. Supernatants were discarded and 200 µl of MTT solution was added to each well. The plate was then incubated in 37°C for 3 h. Subsequently, the MTT solution was discarded and the cells were washed with 200 µl of dimethyl sulfoxide (DMSO) and incubated in 37°C for 30 min. The supernatants were transferred into wells in a new plate. Absorbance of the converted dye was measured by a microplate reader (Victor, Vienna, VA, USA) at 570 nm with background subtraction at 650 nm.

Assay of IL-6 and IL-8

The levels of IL-6 and IL-8 in the culture supernatants were measured using a standard ELISA (R&D systems, Minneapolis, MN, USA)[19]. The absorbance was recorded by a microplate reader (Victor) at 450 nm with subtraction at 540 nm. The concentrations of IL-6 and IL-8 were then calculated with reference to a standard curve.

RNA extraction, cDNA synthesis and qPCR

Cell pellets were collected after centrifuge at 1000 rpm for 10 min. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Five micrograms of total RNA were reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Thereafter, qPCR was performed by using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification reactions were undertaken in 20 µl of reaction mixture containing 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 50 µg of cDNA template and 0.5 µM of each pair of primers for the targeting genes (Sigma, St. Louis, MO, USA). β-actin was used as the internal control for each experiment as previously optimised by our group[19].

Statistical analysis

The data were presented as mean ± standard deviation (SD) following three separate assays. The significant difference between the groups was analysed with the one-way analysis of variance (ANOVA) for the normalised dataset, whereas the non-normalised data were tested with non-parametric methods. A P value < 0.05 was considered to be a significant difference by using SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA).

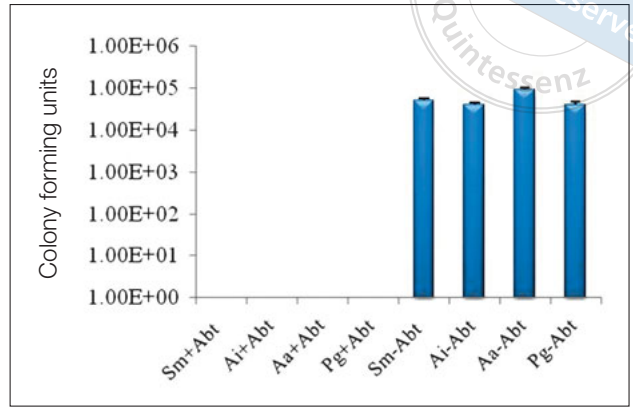


Fig 1 The colony forming units (CFUs) of *Sm*, *Ai*, *Aa* and *Pg* in OKM. The CFUs of each species (10^4 at baseline) were determined after incubation in the OKM with or without antibiotics for 2 h under aerobic conditions (Abt: antibiotics; +/-: with or without).

Results

The pilot study showed that the HOKs were unable to grow in anaerobic conditions. The interaction of the HOKs with *Sm*, *Ai*, *Aa* and *Pg* was then undertaken and analysed in aerobic conditions with 5% CO_2. None of these bacteria could survive for over 2 h in the OKM containing 1% penicillin/streptomycin, while they could grow and proliferate in the serum-free OKM with growth supplement but without penicillin/streptomycin for at least 2 h (Fig 1).

In a time-dependent assay, *Sm* and *Pg* were screened for determination of bacterial viability by spiral plating of the bacteria in OKM without penicillin/streptomycin solution at baseline and after an incubation period of 2 h, 6 h and 24 h, and the CFU was then counted after 5 days. *Sm* multiplied well with the time course up to 24 h, while *Pg* showed the highest and similar proliferative ability at 2 h but failed to survive in OKM at 24 h (Fig 2). After the medium content and the bacteria-HOKs interactive time were examined, *Sm*, *Ai*, *Aa* and *Pg* were tested with this interactive model under such circumstances. These selected species showed similar proliferative ability after 2 h bacteria-HOKs interaction with reference to the baseline (approximately 10 times)

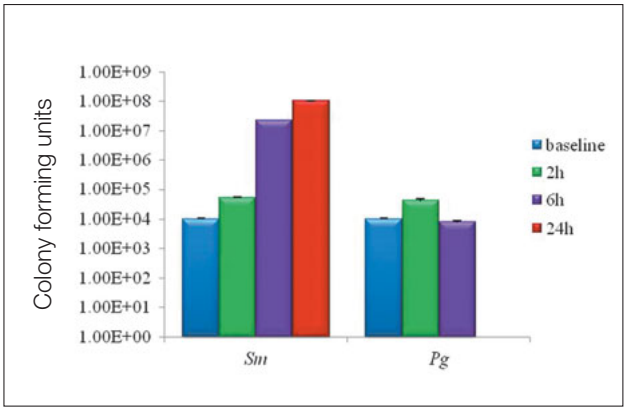
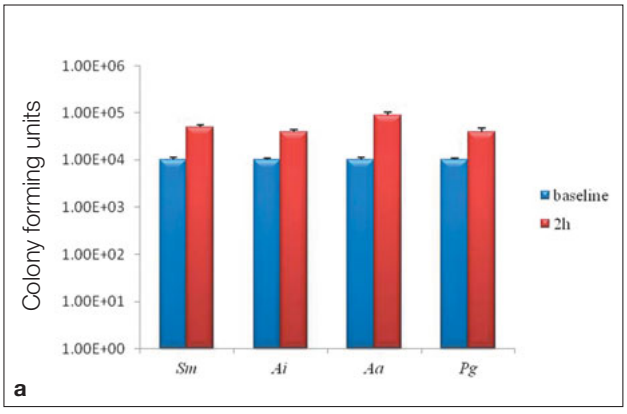


Fig 2 The CFU of *Sm* and *Pg* in a time-dependent assay. The CFUs of *Sm* and *Pg* were determined after incubation in the OKM without antibiotics for 2 h, 6 h and 24 h under aerobic conditions.



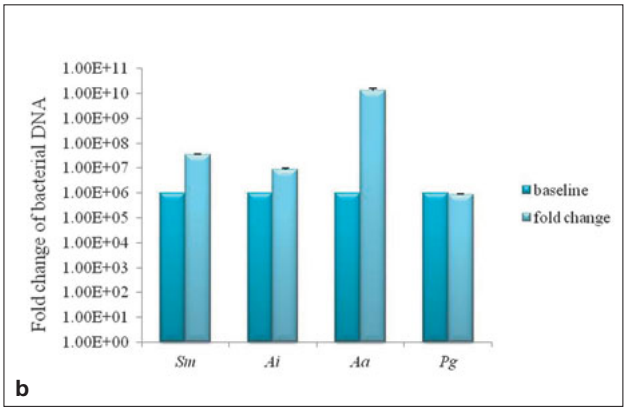


Fig 3 The CFU of *Sm*, *Ai*, *Aa* and *Pg* in their interactions with HOKs. The bacterial levels of these species were determined after incubation in the OKM without antibiotics for 2 h under aerobic conditions. Both the CFU (a) and the fold change of the bacterial DNAs (b) are presented.

in respect of their viability and DNA levels (Fig 3). It should be noted that the level of DNA did not necessarily represent the level of live bacteria in the *in vitro* model. Moreover, the HOKs challenged with different bacteria demonstrated similar viability with the MOI value equal to 1. On the contrary, an MOI of 10 seemed to cause an imbalanced growth of the HOKs in their interactions with *Sm* and *Aa* (Fig 4).

These experiments showed that an MOI of 1 was the appropriate ratio of bacteria and HOKs with the presence of substantial amounts of viable bacteria and HOKs in an antibiotic-free OKM under aerobic conditions for 2 h.

Overall, the expression of IL-6 and IL-8 proteins in the interactions of HOKs with the variable *Sm*, *Ai*, *Aa* and *Pg* increased from 30 min to 2 h in a time-dependent manner (Fig 5a and b). Notably, the IL-6 and IL-8 levels at 2 h were significantly upregulated by *Pg* ($P < 0.01$) and *Sm* ($P < 0.05$), compared with the controls (Fig 5c and d). However, no significant difference was found in the interactive groups of HOKs with *Ai* and *Aa*. Furthermore, the expression of IL-6 and IL-8 mRNAs at 2 h significantly increased in the HOKs challenged by *Pg* ($P < 0.01$), compared with the control and all the other interactive groups ($P < 0.01$) (Fig 6).

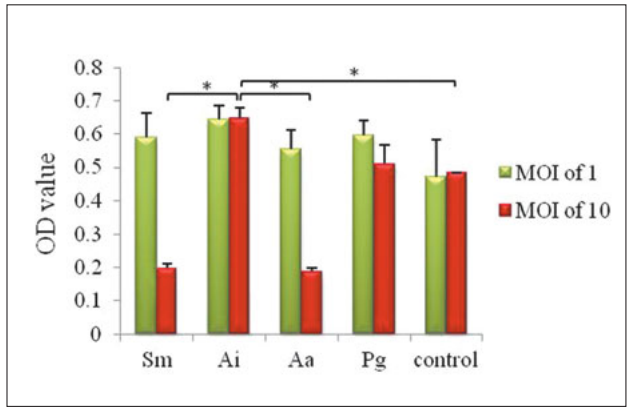


Fig 4 The viability of the HOKs at different MOI. The HOKs were treated with *Sm*, *Ai*, *Aa* and *Pg* at an MOI of 1 and 10 for 2 h under aerobic conditions. The viability of the HOKs was assessed by MTT assay (*$P < 0.05$).

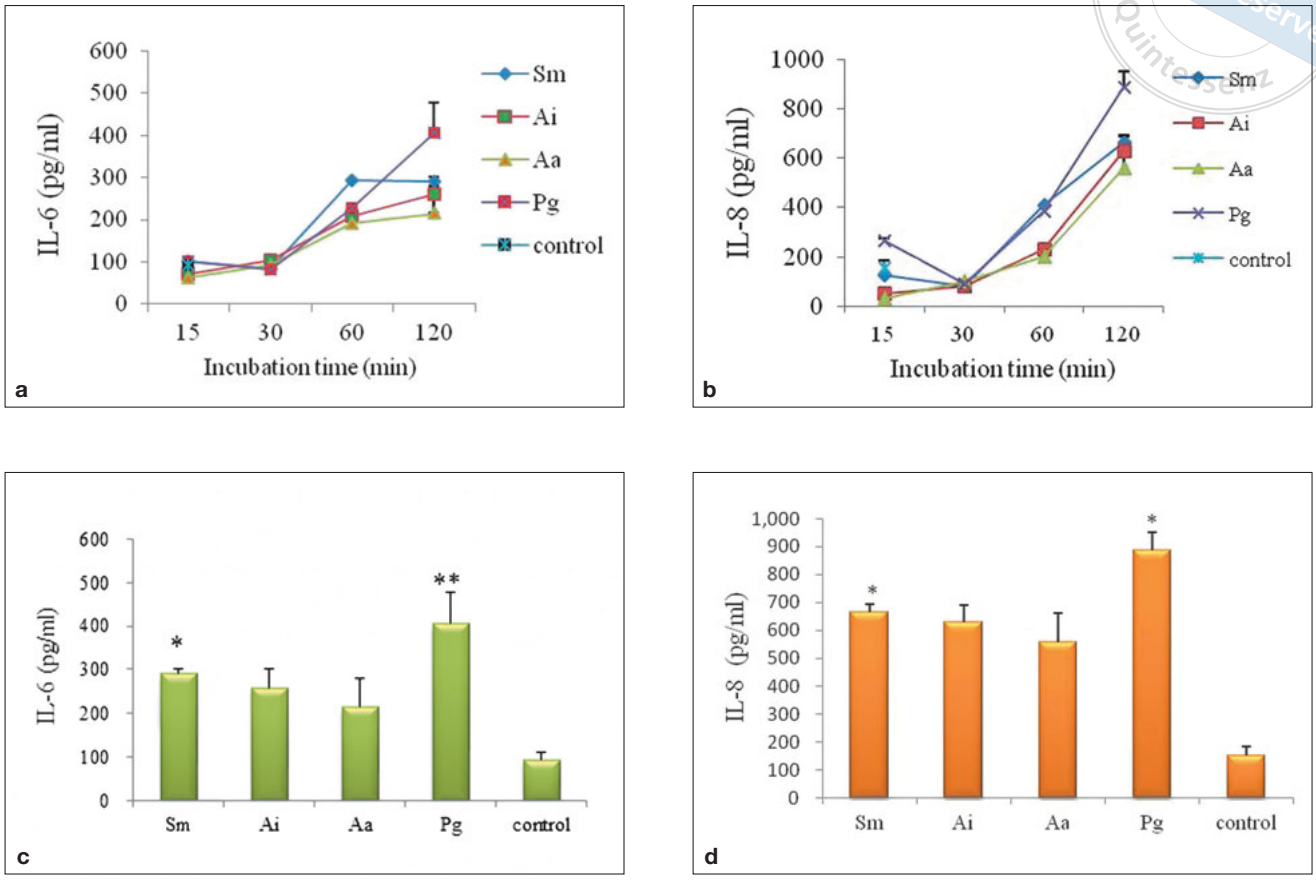


Fig 5 The levels of IL-6 and IL-8 proteins in the interactions of the HOKs with *Sm*, *Ai*, *Aa* and *Pg* in a time-dependent assay. The concentrations (pg/ml) of IL-6 (a) and IL-8 (b) were analysed by ELISA after the interactions of the HOKs with these species (with an MOI of 1) at 15 min, 30 min, 60 min and 120 min under aerobic conditions. The levels of IL-6 (c) and IL-8 (d) at 120 min were significantly higher in HOKs treated by *Sm* and *Pg* than the controls (*$P < 0.05$ and **$P < 0.01$).

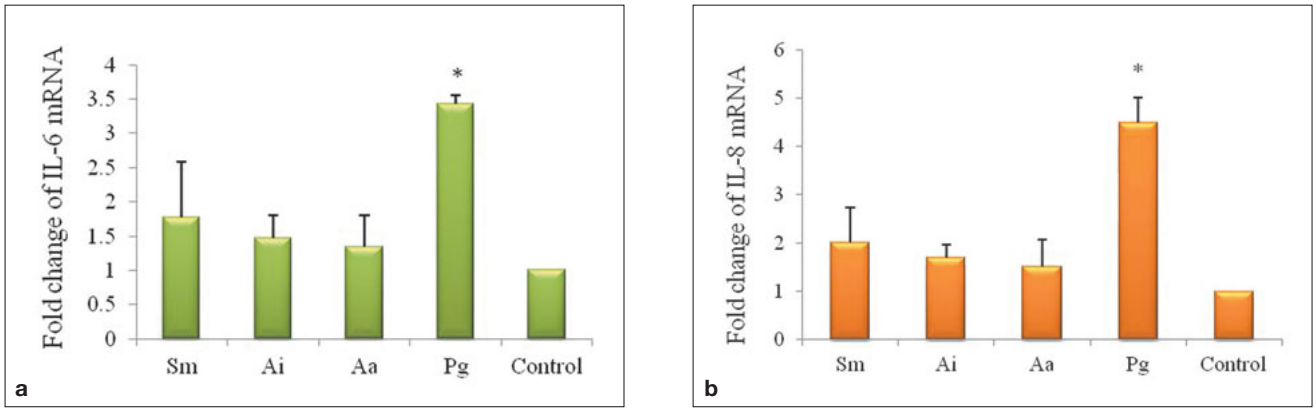


Fig 6 The levels of IL-6 and IL-8 mRNAs in the interactions of HOKs with *Sm*, *Ai*, *Aa* and *Pg*. The fold change of IL-6 (a) and IL-8 (b) mRNAs in the HOKs treated with *Sm*, *Ai*, *Aa* and *Pg* at an MOI of 1 for 2 h under aerobic conditions. There was a significant difference compared with the control and all the other interactive groups (*$P < 0.01$).

Discussion

It is currently well appreciated that bacteria-host symbiosis critically accounts for periodontal homeostasis and health, while the interruption of the balance may result in the initiation and development of periodontal disease[7-9,20]. Establishment of an appropriate interactive model of bacteria-host cells like HOKs is an essential approach to better understand the interactive profiles of live commensal/pathogenic bacteria with host cells and the underlying biological implications. There are limited studies on well-defined interactive models of bacteria and host cells in periodontal science. The present study has tested a bacteria-HOKs interactive model with validated parameters *in vitro*, which could facilitate further study on the crosstalk of bacteria-host cells in periodontal health and disease.

A crucial step in establishing a bacteria-cells interactive model is to determine the appropriate aerobic or anaerobic environment. Our initial experiments suggest that HOKs are unable to survive under anaerobic conditions, whereas it is noteworthy that both selected bacteria and HOKs at an MOI of 1 were substantially viable under aerobic conditions for 2 h. Therefore, this environmental condition was used for subsequent experiments on host-bacteria interactions. Meanwhile, we also found that penicillin/streptomycin-free culture medium could support the growth of both selected bacteria and HOKs, and hence it was selected as the appropriate medium for the experiments. Next, *Sm* and *Pg* were selected to work out the appropriate time duration of the bacteria-HOKs interactions. The viability of *Pg* dropped in a time-dependent manner after 2 h due to its preference for an anaerobic environment, i.e., a mix of H_2 and nitrogen (N_2) (5/95%) or N_2/carbon dioxide (CO_2)/H_2 (85/10/5%) without oxygen. On the other hand, *Sm* as a facultative anaerobe was able to grow and multiply from 2 to 24 h. As *Pg* is proposed to be a keystone periodontal pathogen[21] and a major focus of the current study, a well-fitted time course (2 h) of the interactions of HOKs with the selected bacteria including *Pg* was determined. Afterwards, all of the four selected bacteria were tested in their interactions with HOKs. As a similar proliferative ability was confirmed among these species, the significant differences in the cytokine expression levels of HOKs may reflect the profiles of bacteria-host cells crosstalk. On the other hand, an important target of establishing the current model is to maintain the viability of HOKs during the experiment. Most studies on host-microbe interaction have used an MOI of 50 to 150[22-24], while in the present study various efforts were made to mimic the *in vivo* interaction of bacteria with host cells. In order to achieve a balanced viability of HOKs among the interactive groups of different species with HOKs, an MOI of 1 was tested and then selected as the appropriate one for this model.

In the present study, the time-dependent assay showed that at the first 30 min, the levels of IL-6 and IL-8 expression in all bacteria-HOKs interactive groups were similar to the control group, which may suggest the tolerance of the mammalian cells to these commensal and pathogenic bacteria within such a relatively short timeline. All these species were able to induce the expression of IL-6 and IL-8 in HOKs from 30 min to 2 h in a time-dependent manner. Interestingly, *Pg* was able to markedly increase the expression of both IL-6 and IL-8 proteins and mRNAs; this finding has been supported by our previous studies of those and other groups[19,25-27]. On the other hand, with reference to *Pg*, *Sm* exhibited a comparatively weaker role in the activation of innate host response during bacteria-host interaction. However, other periodontal commensals (e.g., *Ai*) and pathogens (e.g., *Aa*) showed a considerable mute reaction to HOKs.

Sm frequently transits from the coccal phase to the coccobacillary phase. It is a Gram-positive bacterium and presents with three serotypes c, e and f that have been detected from human isolates[28]. Its role in periodontal pathogenesis has been controversial. *Sm* was previously thought to be related to periodontal lesion and bone resorption[29,30]. However, this bacterium could be associated with periodontal health[31]. Unlike the 'red complex' species that are frequently found in deep periodontal pockets, *Sm* is a member of the 'yellow complex' species, and they usually precede the presence of 'red complex' pathogens[32]. Recently, *Sm* has been shown to possess a quorum-sensing-dependent character and may therefore antagonise periodontal pathogens[33]. Moreover, an interesting study demonstrates that *Sm* may potentially serve as a beneficial species applied in periodontal pockets to reverse dysbiosis via probiotics for the adjunctive treatment of periodontitis[34].

On the other hand, *Pg* is a well-recognised pathogen for periodontal disease. Its unique role in the pathogenesis of periodontitis has been elucidated in a number of *in vitro* experiments, animal models and clinical studies, regardless of whole live/dead bacteria or its critical components such as lipopolysaccharide[17-19,22,25-27,35-39]. Lately, the role of *Pg* as a 'keystone' pathogen has been proposed by the pioneer researchers in oral microbiology, which has illustrated that the existence of *Pg* may critically remodel commensal plaque biofilms into pathogenic ones in animal

models and significantly contribute to disease development[21,40].

In our study, the viability and reactivity of these selected bacteria and HOKs during 2 h interactions, amidst a relatively shorter time period, have provided useful data for statistical analysis among different bacterial-HOKs interactive groups. The *in vitro* observations on a similar trend of IL-6 and IL-8 expression among the groups suggest that the immunoinflammatory response may not necessarily depend on the classical 'commensal' or 'pathogenic' nomenclature of the bacterial species concerned. Further work is required to clarify this point.

The current study tested a well-defined variable bacteria-HOKs interactive model for periodontal research. However, there are some limitations to be addressed. It is very challenging to maintain the viability of both bacteria and HOKs in an interactive model, and a 2 h duration is found to be the appropriate timeframe, while this short duration could not reflect the *in vivo* situation. Moreover, the present interactive model is bound to planktonic bacteria and monolayer HOKs. The current findings should therefore be interpreted with caution. Future research is required to refine the experimental protocols and further investigate the interactive models of tissue block with single and/or multiple species biofilms. As such, the critical effects of microenvironmental factors such as hemin concentration and temperature on host-bacteria crosstalk should be further investigated[41-43] and the relevant clinical implication should be explored.

Within the limitations of the present study, the current findings suggest that periodontal commensals (e.g., *Sm* and *Ai*) and pathogens (e.g., *Aa* and *Pg*) may differentially modulate immunoinflammatory response in a defined bacteria-HOKs interactive model. This study gives insight into the complex innate host response to a bacterial challenge *in vitro*, which may not be simply yet necessarily determined by the commonly defined categories of 'commensal' and 'pathogenic' species. This study could enhance our understanding of the complex and dynamic crosstalk of variable bacteria and host cells in periodontal health and disease.

Acknowledgements

The authors are grateful to the late Prof A.S. LAU from Li Ka Shing Faculty of Medicine, The University of Hong Kong, for his suggestion to undertake this work.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Hua Jing LI contributed to the experiments, data analyses and manuscript writing; Dr Chaminda Jayampath SENEVIRATNE contributed to the study design, data analyses and manuscript writing; Prof Cun Yu WANG contributed to the interpretation of the data and manuscript writing; Prof Li Jian JIN initiated the project and contributed to the study design, interpretation of the data, manuscript writing and revision. All authors approved the manuscript.

(Received Sep 27, 2018; accepted Nov 19, 2018)

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