Archives of Oral Biology 174 (2025) 106240

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Akkermansia muciniphila enhances mucosal immunity against porphyromonas gingivalis

Qin Hu [a], Wai Keung Leung [a], Aneesha Acharya [a,b], George Pelekos [a,*]

[a] Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Sai Ying Pun, Hong Kong
[b] Dr D Y Patil Dental College and Hospital, Pune, India

ARTICLE INFO

Keywords:
Akkermansia muciniphila
Porphyromonas gingivalis
Periodontal disease
Mucosal immunity

ABSTRACT

Objective: This study aims to investigate the immunomodulatory potential of *Akkermansia muciniphila* in restoring *Porphyromonas gingivalis*-induced immune dysfunction.
Design: The immune response was assessed by measuring the expression of pro-inflammatory cytokines and antibacterial peptides in human gingival epithelial cells and human gingival fibroblasts treated with *A. muciniphila, P. gingivalis*, or a combination of both. Activation of the NF-κB pathway was analyzed using immunofluorescent staining and western blot. *In vivo* validation was performed using a mouse model, where *A. muciniphila* and *P. gingivalis* were administered alongside a MyD88-specific inhibitor to confirm the immunomodulatory mechanisms.
Results: A. muciniphila significantly enhanced the defensive immune response through TLR-MYD88-NF-κB pathway. *In vitro* and *in vivo*, *A. muciniphila* upregulated chemokine expression to recruit immune cells. *A. muciniphila* also reduced the adhesion and internalization of *P. gingivalis* and increased the expression of genes encoding antimicrobial peptides (*DEFB103B* and *CAMP*).
Conclusions: A. muciniphila demonstrates potential in combating *P. gingivalis* infection highlighting its role as a promising immune modulator for periodontal disease management.

1. Introduction

Periodontitis is an inflammatory condition affecting the supporting structures of teeth, characterized by the irreversible loss of attachment between the teeth and the surrounding bone. This condition can lead to tooth loss and is associated with systemic health issues such as cardiovascular disease and diabetes (Preshaw et al., 2020; Ramseier et al., 2017). The primary driver of periodontitis is a dysbiotic biofilm. The oral cavity hosts the second-largest bacterial community after the gut microbiome and ranks first in bacterial diversity within the human body (Caselli et al., 2020). Maintaining a balanced microbial community is crucial for both oral and systemic health.

P. gingivalis, although low in abundance in the oral cavity, is a keystone pathogen in periodontitis due to its ability to cause microbial dysbiosis in the periodontium (Hajishengallis et al., 2012). Immune paralysis is a crucial strategy employed by *P. gingivalis* to evade immune surveillance and facilitate the overgrowth of other pathogens. Firstly, *P. gingivalis* produces proteases like gingipain, which can digest chemokines and paralyze the localized immune response (Mikolajczyk-Pawlinska et al., 1998). Additionally, SerB, a serine phosphatase secreted by *P. gingivalis*, can dephosphorylate S536 of p65 in gingival epithelial cells which inhibits the nuclear translocation of p65, thereby suppressing the transcription of target genes involved in the immune response (Takeuchi et al., 2013). Hence, it can repress the innate immunity at mucosal surfaces against microbial dysbiosis. Moreover, *P. gingivalis* also interacts with Toll-like receptors and manipulates complement systems to evade bacterial clearance (Maekawa et al., 2014). Traditional treatments often focus on the mechanical removal of *P. gingivalis* and biofilm. However, these mechanical approaches may not reach all areas where *P. gingivalis* and biofilm are present, particularly in deep periodontal pockets or hard-to-reach regions and may cause tooth sensitivity. Furthermore, even after mechanical removal, *P. gingivalis* can rapidly recolonize the treated areas if the underlying conditions that promote its growth are not addressed. Mobilizing the host immune response to tackle *P. gingivalis*-induced periodontal disease presents a promising alternative.

Mucosal immunity plays a crucial role in preserving tolerance towards beneficial commensal microorganisms while defending against

* Correspondence to: The Prince Philip Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong.
E-mail address: george74@hku.hk (G. Pelekos).

https://doi.org/10.1016/j.archoralbio.2025.106240
Received 5 February 2025; Received in revised form 18 March 2025; Accepted 19 March 2025
Available online 23 March 2025

harmful pathogens (Moutsopoulos & Konkel, 2018). Recent research has highlighted the potential of leveraging beneficial microbes to modulate host immune responses and combat periodontal pathogens from ecological perspective (Maier et al., 2024). *A. muciniphila*, a commensal bacterium residing in the gut, has garnered attention for its immunomodulatory properties (Ansaldo et al., 2019; Bae et al., 2022). Studies have shown that a reduction or absence of *A. muciniphila* is linked to various diseases, including cancers, metabolic disorders, and inflammatory diseases (Dao et al., 2016; Wang et al., 2020). Supplementing with *A. muciniphila* has been found to benefit human health in multiple ways (Depommier et al., 2019; Fan et al., 2021). *A. muciniphila* enhances gut barrier function and modulates immune responses via TLR signaling, suggesting its potential application in other mucosal sites, including the oral cavity (Fan et al., 2021; Wang et al., 2020).

This study aims to explore the therapeutic potential of *A. muciniphila* in mitigating *P. gingivalis*-induced periodontal disease. Specifically, we investigate how *A. muciniphila* influences the host immune response through the TLR-MYD88-NFκB signaling pathway, a critical pathway in innate immunity. By examining the effects of *A. muciniphila* on human gingival epithelial cells (HGECs) and human gingival fibroblasts (HGFs), both *in vitro* and *in vivo*, we aim to elucidate the mechanisms by which *A. muciniphila* modulates immune responses and enhances host defense against *P. gingivalis*.

2. Materials and methods

2.1. Cell culture

Primary human gingival epithelial cells (HGECs) and their culture media, CnT-PRIME, were ordered from CELLnTEC (Switzerland). Primary human gingival fibroblasts (HGFs, American Type Culture Collection, ATCC, USA) were grown in fibroblast basal medium (ATCC, USA) supplemented with fibroblast growth kit-low serum (ATCC, USA). THP-1 cells (ATCC, USA) were maintained in RPMI-1640 medium (ATCC, USA) containing 10 % fetal bovine serum, 100 μg mL^{-1} normocin (InvivoGen, USA) and 50 μM β-mercaptoethanol. All cells were cultured at 37°C in a 5 % CO_2 atmosphere.

2.2. Bacterial strains and culture conditions

A. muciniphila (BAA-835) and *P. gingivalis* (W83) were purchased from ATCC (USA). *A. muciniphila* was maintained on the brain infusion agar plates (Difco, USA) while *P. gingivalis* was grown on the blood agar plates (39 g L^{-1} Columbia agar base from Difco, 5 % defibrinated horse blood from Hemostat, and 1 % hemin and vitamin K1 solution) anaerobically at 37°C. *P. gingivalis* was cultured in tryptic soy broth (TSB; 30 g/L tryptic soy broth from Difco, 5.0 g L^{-1} yeast extract from Difco, and 1 % hemin and vitamin K1 solution), and *A. muciniphila* in brain heart infusion (BHI, Difco) broth medium with 0.3 % mucin.

2.3. Cytocompatibility and bacterial administration protocol for In Vitro assays

HGECs and HGFs were seeded in 6-well plates and cultured overnight. The cells were then exposed to *A. muciniphila* at a varying multiplicity of infection (MOI). After the bacterial infection, the metabolic activity of the cells was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan), while plasma membrane integrity was assessed using CyQUANT LDH cytotoxicity assay kit (Thermo Fisher Scientific, USA). Meanwhile, cell viability was determined using the LIVE/DEAD viability/cytotoxicity kit (Invitrogen, USA).

Referring to following assays, the treatment groups were established as follows: Ctrl (medium alone), *A. muciniphila* (MOI: 100), *P. gingivalis* (MOI: 10), and Mixture (mixture of *A. muciniphila* at MOI of 100 and *P. gingivalis* at MOI of 10).

2.4. Enzyme-linked immunosorbent assay (ELISA)

The supernatants of cells challenged by different bacteria were collected, and debris was removed by centrifugation at 100 × g for 5 min. The selected cytokine or chemokine levels were quantified using ELISA kits (R&D Systems, USA).

2.5. Real-time quantitative transcription PCR (qRT-PCR)

mRNA samples were collected after a six-hour co-culture of different bacteria and cells. Total RNA was extracted with RNAfast200 kit (Fastagen, China). A total of 2 μg of RNA was reverse transcribed into cDNA with the TB Green® Premix DimerEraser™ Kit (Takara, Japan). Quantitative PCR (qPCR) was performed on the ABI Prism 7700 (Applied Biosystems, USA). The primer sequences used were listed in Table 1. Relative gene expression levels were normalized to endogenous reference genes *ACTB* with the comparative threshold cycle ($2^{-\triangle\triangle ct}$) method.

2.6. Transwell cell migration assay

24-well transwell plates containing inserts with a 5 μm pore size, separating the upper and lower compartments, were purchased from Corning Costar Inc. (Corning, USA). Before the experiments, the cell culture supernatants collected after the challenges by different bacteria were filtered through 0.4 μm μm membrane filters to obtain the cell-free culture supernatant. In the lower chamber, 600 μL of each cell-free culture supernatant was added, followed by the addition of 5 × 10^5 THP-1 cells in 100 μL RPMI medium to the top insert. After 24 hour of incubation, the inserts were removed from the plates and rinsed twice with PBS prior to the removal of non-migrated cells using a swab. Then, the insert membrane was fixed with 4 % paraformaldehyde (PFA) for 15 min and stained with crystal violet at room temperature. After incubating for 15 min, the staining solution was carefully removed, and the inserts were thoroughly washed three times with PBS before counting the cells under an optical microscope.

2.7. Animal model and experimental procedures

All animal experiments were conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and were approved by the Ethics Committee of Lanzhou University School of Stomatology (LZUKQ-2024–052).

Animals and Grouping:

A total of 20 C57BL/6 mice were used in this study. The mice were randomly divided into 4 groups (n = 5 per group):

Control group: Received oral gavage of 1 % carboxymethylcellulose (CMC) solution every 3 days for a total of 7 days.

P. gingivalis group: Orally administered 100 μL of *P. gingivalis* suspension (1 × 10^9 CFU/mL) prepared in 1 % CMC solution using a syringe.

Table 1
Primers and probes for quantitative real-time RT-PCR.

Gene	Primer (5'-3')
ACTB	CACCATTGGCAATGAGCGGTTC
	AGGTCTTTGCGGATGTCCACGT
TLR2	CTTCACTCAGGAGCAGCAAGCA
	ACACCAGTGCTGTCCTGTGACA
TLR4	CCCTGAGGCATTTAGGCAGCTA
	AGGTAGAGAGGTGGCTTAGGCT
MYD88	GAGGCTGAGAAGCCTTTACAGG
	GCAGATGAAGGCATCGAAACGC
DEFB103B	TTATTGCAGAGTCAGAGGCGGC
	CTTTCTTCGGCAGCATTTTCGGC
LL−37	GACACAGCAGTCACCAGAGGAT
	TCACAACTGATGTCAAAGGAGCC

Mixture group: Received 100 μL of a mixture containing *A. muciniphila* (1×10^9 CFU/mL) and *P. gingivalis* (1×10^9 CFU/mL) prepared in 1 % CMC solution, administered orally using a syringe.

Inhibitor group: Administered the same mixture as the mixture group. On day 7, mice in this group were anesthetized with isoflurane (induction at 4 % and maintenance at 2 % in oxygen) and injected with 100 μL of MyD88 inhibitor (10 mM) at the buccal site of the upper jaw.

On day 8, all mice were euthanized via cervical dislocation without prior anesthesia. Gingival tissues were immediately dissected for RNA extraction and subsequent qRT-PCR analysis.

2.8. Immunofluorescence Staining

HGECs or HGFs were seeded in μ-Slide 8-well chambers (ibidi, Germany) and treated as described in Section 2.3 for one hour. After treatment, the medium was removed, and the cells were washed twice with PBS. The cells were then fixed with 4 % paraformaldehyde for 15 minutes at room temperature. Following fixation, the cells were washed three times with PBS to remove the paraformaldehyde. Permeabilization was performed using 0.1 % Triton X-100 in PBS for 10 minutes, after which the cells were washed three times with PBS. To block non-specific binding, the cells were incubated with goat serum for one hour at room temperature. The primary antibody against pP65 (Cell Signaling Technology, #8242, dilution rate: 1:500) was diluted and applied to the cells, which were then incubated at 4°C overnight. Subsequently, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology). Finally, the cell nuclei were stained with DRAQ5 (Invitrogen, USA).

2.9. Western blotting

In general, cells were lysed on ice in M-PER mammalian protein extraction reagent (Thermo Fisher, USA) containing Halt protease and phosphatase inhibitor cocktail (Sigma Aldrich, USA) for 15 min. The lysate was centrifuged at 4°C for 15 minutes at 15,000 × g to collect the soluble proteins in the supernatant. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher, USA). The proteins were denatured using Pierce lane marker reducing sample buffer (Thermo Fisher, USA) and boiled for 10 min. Equivalent amounts of the protein aliquots were loaded on and separated using 10 % SDS polyacrylamide gels (EpiZyme, China). The separated proteins were transferred onto Amersham Hybond P western blotting polyvinylidene fluoride membranes (GE Healthcare, USA). Membranes were then blocked with 5 % BSA for one hour and then incubated with the diluted (1:1000) rabbit monoclonal primary antibodies (Cell Signaling Technology, USA) overnight at 4°C. After the incubation, the membranes were washed with tris-buffered saline (pH 7.0) containing 0.1 % Tween 20 followed by the incubation of the respective diluted (1:3000) secondary antibodies with conjugation of horse radish peroxidase for one hour at room temperature. The blots were detected using WesternBright Sirius chemiluminescent detection kit (Advansta, San Jose, USA), and imaged by ChemiDoct XRS + System (Thermo Fisher, USA).

2.10. Effect of Am on the adhesion and cellular internalization of *P. gingivalis*

HGECs and HGFs were seeded in 6-well plates at a density 4×10^5 cells/well and pre-treated with/without *A. muciniphila* for three hours at an MOI of 100. To eliminate the interference of *A. muciniphila* in the colony-forming unit counting, gentamicin (GTM, Sigma-Aldrich, USA) and ampicillin (AMP, Sigma-Aldrich, USA) were administrated to the cells for one hour. After the treatment of mixed antibiotics, the antibiotics were removed, and the cells were washed twice with PBS. The cells were then infected by *P. gingivalis* at an MOI of 100 for another hour followed by extensive washing in PBS to remove the loosely attached bacteria. To assess the number of adherent and internalized *P. gingivalis* in THP-1-derived macrophages, GTM and metronidazole (MTZ, Sigma Aldrich, USA) were selectively added to the cells for one hour. Then all cells were washed twice with PBS, lysed with sterile water, and serially diluted, and the cell lysates were plated for counting CFUs.

2.11. Statistical analysis

All experiments were repeated at least three times independently. The one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used for statistical evaluation of all results. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Cytocompatibility of *A. muciniphila* with host cells

The CCK-8 assay demonstrated that HGECs and HGFs maintained good viability with *A. muciniphila* up to an infection ratio of 1000:1 (Fig. 1A). At this infection ratio, no significant difference was observed for released LDH amount compared with the control group, suggesting intact cell structures after *A. muciniphila* infection (Fig. 1B). After co-culturing for 24 hours, the viability of cells was measured using Live/Dead staining. It was found that *A. muciniphila*-infected cells presented good viability under different MOI, which was consistent with CCK-8 and LDH results (Fig. 1C).

3.2. *A. muciniphila* stimulated and restored the immune response suppressed by *P. gingivalis*

The expression of three representative pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein 1 (MCP-1), was determined to evaluate the effects of *A. muciniphila* on host cells. Fig. 2A and B showed that *A. muciniphila* significantly increased the transcriptional levels of IL-6 ($p = 0.0041$) and IL-8 ($p = 0.0037$), as well as the protein levels of IL-6, IL-8, and MCP-1 ($p < 0.0001$) in HGECs. However, *P. gingivalis* did not alter the expression of these three cytokines in HGECs. The mixture of *A. muciniphila* and *P. gingivalis* increased the mRNA levels of IL-6 ($p = 0.0015$), and IL-8 ($p = 0.253$) compared to *P. gingivalis* alone group, but the protein levels of the three cytokines were not increased. For HGF (Fig. 2C, D), similarly, *A. muciniphila* enhanced both the transcriptional and protein expressions of the three cytokines. *P. gingivalis* showed a tendency to increase the mRNA levels of the three cytokines but failed to elevate their protein levels. The combined bacterial challenge of *A. muciniphila* and *P. gingivalis* to HGFs resulted in elevated mRNA levels of the three cytokines and a significantly increased MCP-1 protein level ($p = 0.0461$) compared to the *P. gingivalis* group. The decreased protein levels in *P. gingivalis* group could be attributed to the gingipains that the cysteine-based proteases produced by *P. gingivalis*, which can digest various proteins including cytokines and chemokines (Darveau et al., 1998).

3.3. *A. muciniphila* stimulated the TLR-MYD88-NF-κB pathway

To further explore the underlying mechanisms behind the manipulation of inflammatory responses by various bacteria, the relative expression and activation of key proteins involved in the NF-κB pathway were investigated. Fig. 3A showed that in HGECs, the combination of *A. muciniphila* and *P. gingivalis* promoted the phosphorylation and nuclear translocation of NF-κB (P65) compared with *P. gingivalis* alone group ($p = 0.0334$). In HGFs, nuclear translocation of NF-κB was significantly facilitated ($p < 0.0001$) when given *A. muciniphila* or the bacterial mixture (Fig. 3B, C). Meanwhile, western blot results further verified the activation of the NF-κB pathway by looking at the levels of IκBα and phosphorylated P65 (p-P65) after the treatments of different bacteria and combinations (Fig. 3D). The data showed no statistically

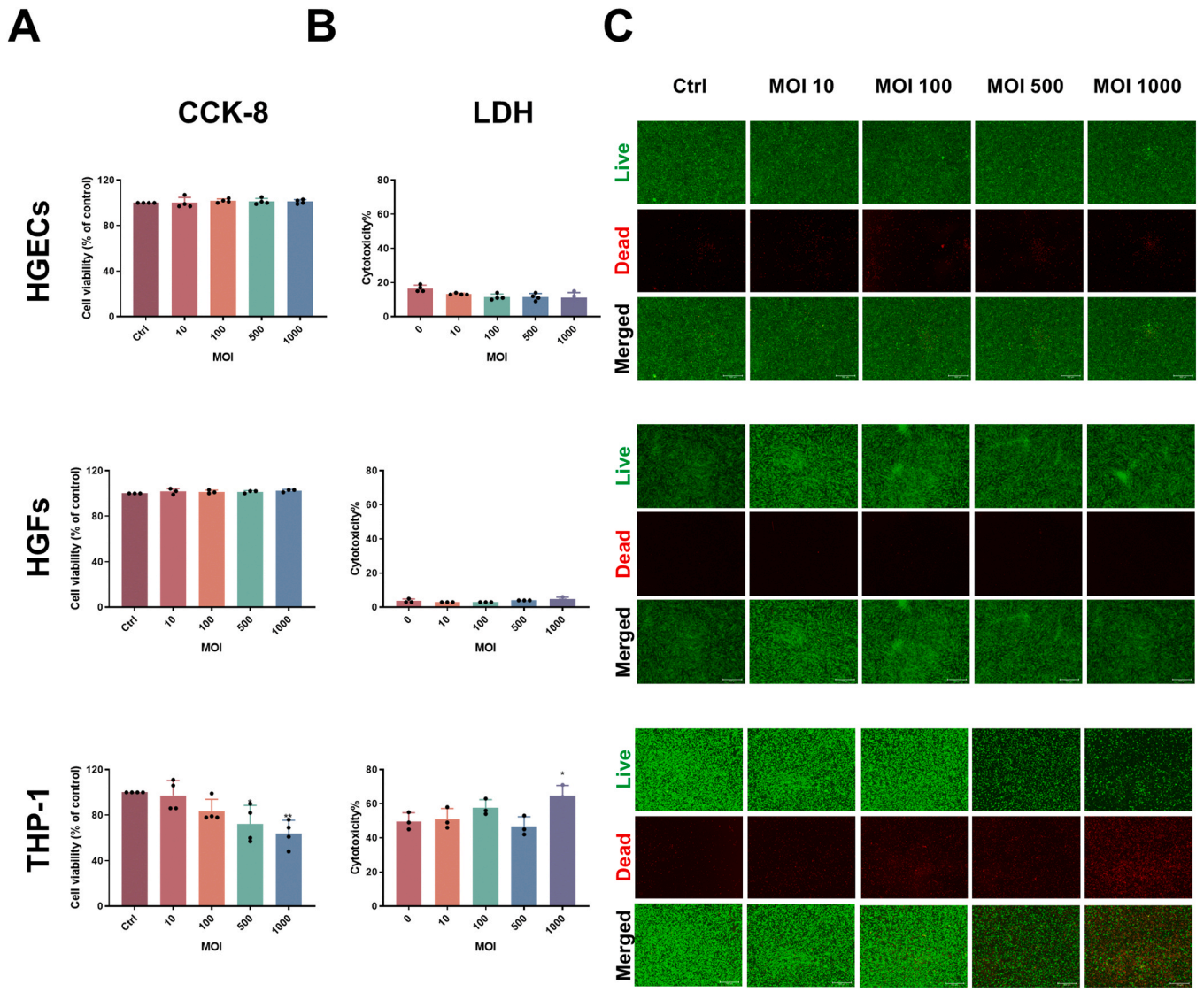

Fig. 1. Effects of *A. muciniphila* on the viability and cytotoxicity on HGECs and HGFs. (A) Cell viability and (B) relative LDH release in periodontal cells treated with *A. muciniphila* at the infection ratio of 0,10:1, 100:1, 500:1, and 1000:1 for 24 h. (C) Live/Dead cell staining of cells after culturing with *A. muciniphila* for 24 hour in culture medium. Living cells were detected as green and dead cells were detected as red fluorescence (scale bar, 500 μm). Statistical significance in comparison to the control were analysed using one-way ANOVA followed by Tukey's post hoc test (n = 3). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ *versus* the control groups.

significant differences among the four groups for the four proteins, due to the large number of comparisons (Fig. 3E). In HGECs, the levels of the four proteins remained consistent across the four groups, showing no significant trends of increase or decrease. However, *A. muciniphila* and the bacterial mixture tended to elevate the phosphorylated P65 (pP65) in HGFs. TLR, MyD88 and NF-κB are critical components of the innate immune signaling pathway, mediating the recognition of microbial pathogens and subsequent inflammatory responses. Potential proteins interaction with the MyD88 protein was searched using the STRING database (Fig. 3F). It provided the evidence that *A. muciniphila* could modulate immune response through TLR2/4-MyD88-NFκB pathway.

Therefore, RT-PCR was performed to assess gene expression related to the NF-κB pathway, including TLR2, TLR4, and MYD88. As shown in Fig. 3G and H, administration of *A. muciniphila*, *P. gingivalis*, or their combination significantly upregulated TLR2/4 gene expression. Specifically, *A. muciniphila* significantly increased MYD88 expression in both HGECs ($p = 0.0023$) and HGFs ($p = 0.0179$), whereas *P. gingivalis* did not. Moreover, the addition of *P. gingivalis* partially impaired the MYD88 expression upregulated by *A. muciniphila*. These results suggested that *P. gingivalis* might block the NF-κB pathway via MYD88.

3.4. *A. muciniphila suppressed the adherence and internalization of P. gingivalis*

The effect of *A. muciniphila* on affecting *P. gingivalis*'s invasion in periodontal cells was investigated (Fig. 4A). The findings revealed a significant reduction in the number of *P. gingivalis* that adhered to and invaded the cells in the groups receiving *A. muciniphila* (Fig. 4C, D). Moreover, even after a one-hour disinfection process using MTZ/GTM to remove *P. gingivalis* attached to the cell surface, the pre-treatment with *A. muciniphila* continued to significantly lower the amount of viable intracellular *P. gingivalis*. Beta-defensin 3 and cathelicidin are essential components of the innate immune system, providing a first line of defense against pathogens in the oral cavity. Cathelicidin also modulates the immune response by influencing various immune cells, including neutrophils, monocytes, and T cells. The expression of *DEFB103B* (encoding beta-defensin 3) and *CAMP* (encoding cathelicidin) was evaluated under different treatments. Both *DEFB103B* and *CAMP* were

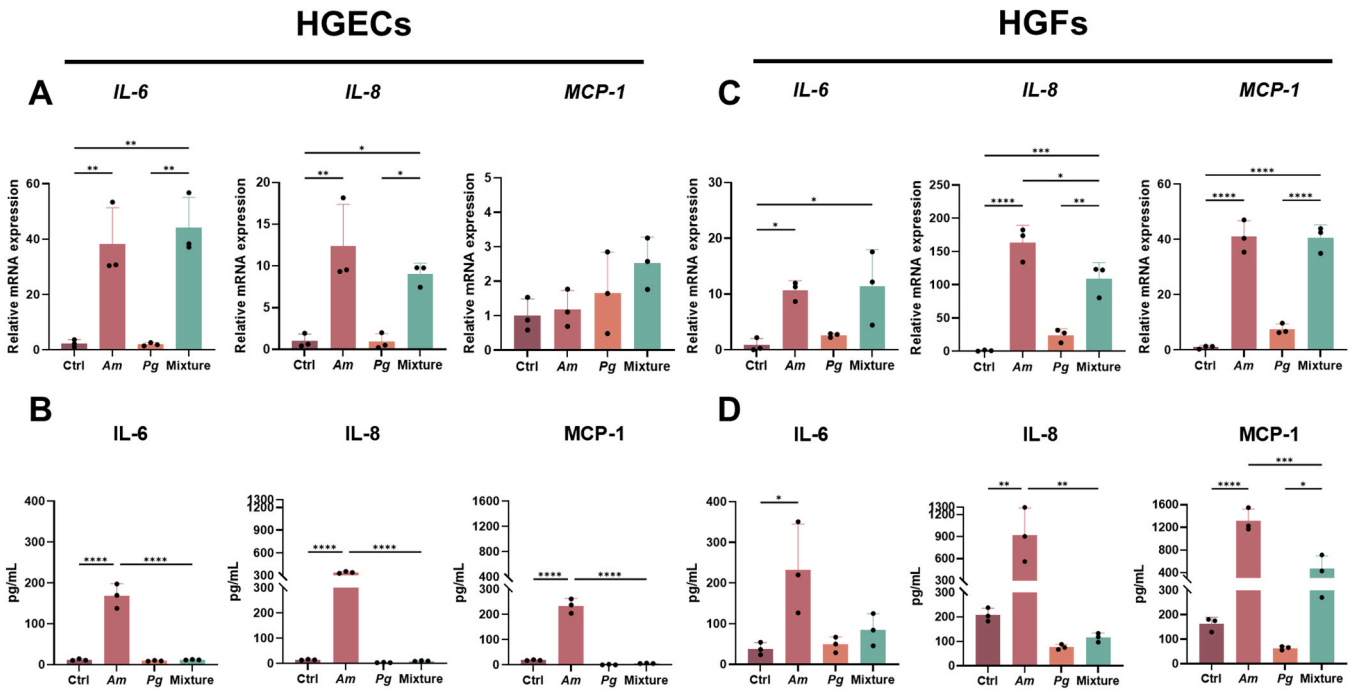

Fig. 2. *A. muciniphila* restored the inflammatory response subverted by *P. gingivalis* in HGECs and HGFs. (A) HGECs and (C) HGFs were treated with *A. muciniphila* and/or *P. gingivalis* for 6 h. Relative mRNA expression was evaluated to evaluate the expression of IL6, IL-8, and MCP-1. (B) HGECs and (D) HGFs were treated with *A. muciniphila* and/or *P. gingivalis* for 24 h. Supernatants were collected for the ELISA assays. Statistical significance in comparison to the control were analysed using one-way ANOVA followed by Tukey's post hoc test (n = 3). Data are presented as mean ± SD. *$P < 0.05$, * *$P < 0.01$, * **$P < 0.001$, and * ** *$P < 0.0001$ *versus* the control and matched groups. *Am*, *A. muciniphila*. *Pg*, *P. gingivalis*.

significantly upregulated, which may explain the reduced viable internalized *P. gingivalis* in *A. muciniphila*-primed HGECs and HGFs.

3.5. *A. muciniphila activated the chemotatic activity of immune cells*

Recruitment and accumulation of monocytes are critical for host defense against pathogenic bacteria. Hence, we evaluated how the supernatants from infected HGECs (Fig. 5A) or HGFs (Fig. 5B) could function on the migration of THP-1 cells, and it was found that they demonstrated similar effects on the monocyte/macrophage chemotaxis. In the control group, only a minimal number of THP-1 cells were found on the insert membranes, while supernatants from *P. gingivalis*-infected cells also failed to recruit THP-1 cells. In accordance with the ELISA results, the cell-free supernatants collected from HGECs or HGFs treated with *A. muciniphila* showed a capacity in attracting the migration of THP-1 cells. And the addition of *A. muciniphila* in the Mixture group could reverse *P. gingivalis*'s effect with a higher capacity in recruiting THP-1 cells.

To confirm the immune modulation capacity of *A. muciniphila in vivo*, mice were inoculated with bacteria every 3 days for 7 days (Fig. 5C). On day 7, the inhibitor group received a MyD88 inhibitor treatment. The results demonstrated that the short-term administration of *P. gingivalis* tended to downregulate the expressions of CXCL1 and CXCL10. However, the addition of *A. muciniphila* effectively reversed this impairment, with a particularly significant elevation in CXCL1 levels ($p < 0.0001$, Fig. 5D). Blocking MyD88 with a specific inhibitor weakened the activation effect of *A. muciniphila*, suggesting that *A. muciniphila* induces a protective response through the MyD88 pathway.

4. Discussion

This study presents a novel therapeutic approach using *A. muciniphila* to manage *P. gingivalis*, emphasizing its immunomodulatory potential. The data reveal a dual role of *A. muciniphila* in combating *P. gingivalis* infection: it not only mobilizes intracellular protective mechanisms to defend against the adherence and invasion of *P. gingivalis* but also recruits effective immune cells to the local site to eradicate the pathogens.

Previous studies have reported that *P. gingivalis*, as the key pathogen in periodontal disease, develops multiple strategies to evade the host immune system. It can suppress the NF-κB pathway, degrade immune mediators like chemokines with proteases, and invade and replicate inside gingival epithelial cells to escape immune surveillance. These strategies help establish chronic infections and contribute to periodontal disease (Darveau et al., 1998; Hasegawa et al., 2008; Takeuchi et al., 2013; Yilmaz et al., 2002). In line with these findings, our study revealed that *P. gingivalis* failed to elicit a robust host defensive immune response. This was evidenced by the inactivation of the NF-κB pathway, reduced expression of key cytokines and chemokines, and minimal effects on the chemotaxis of THP-1 cells. Such a suppressed immune response in the periodontal mucosa is detrimental to the maintenance of microbial homeostasis, potentially exacerbating dysbiosis and compromising periodontal health.

Numerous studies have revealed that commensals maintain microbial and immune balance by mildly activating host protective mechanisms against infection, primarily controlled by continuous priming of the host via key receptors known as toll-like receptors (TLRs) (Khan et al., 2019; Murdoch & Rawls, 2019). It can help and enhance the recognition of exogenous pathogens by the epithelial tissues, the first barrier of the host, to mediate the immune-inflammatory responses for controlling infections and improving oral/general health (van Essche et al., 2013). A study revealed that oral keratinocytes treated with periodontal commensal, *Streptococcus sanguinis*, exhibited stronger chemotactic activity towards THP-1 cells compared to those infected with *P. gingivalis* (Li et al., 2022). These studies suggest that beneficial microorganisms can be adopted to modulate the immune system and modify the periodontal microbiota for improved health.

A. muciniphila as the one of the next generation probiotics has presented its potential in immune modulation. Extensive research has

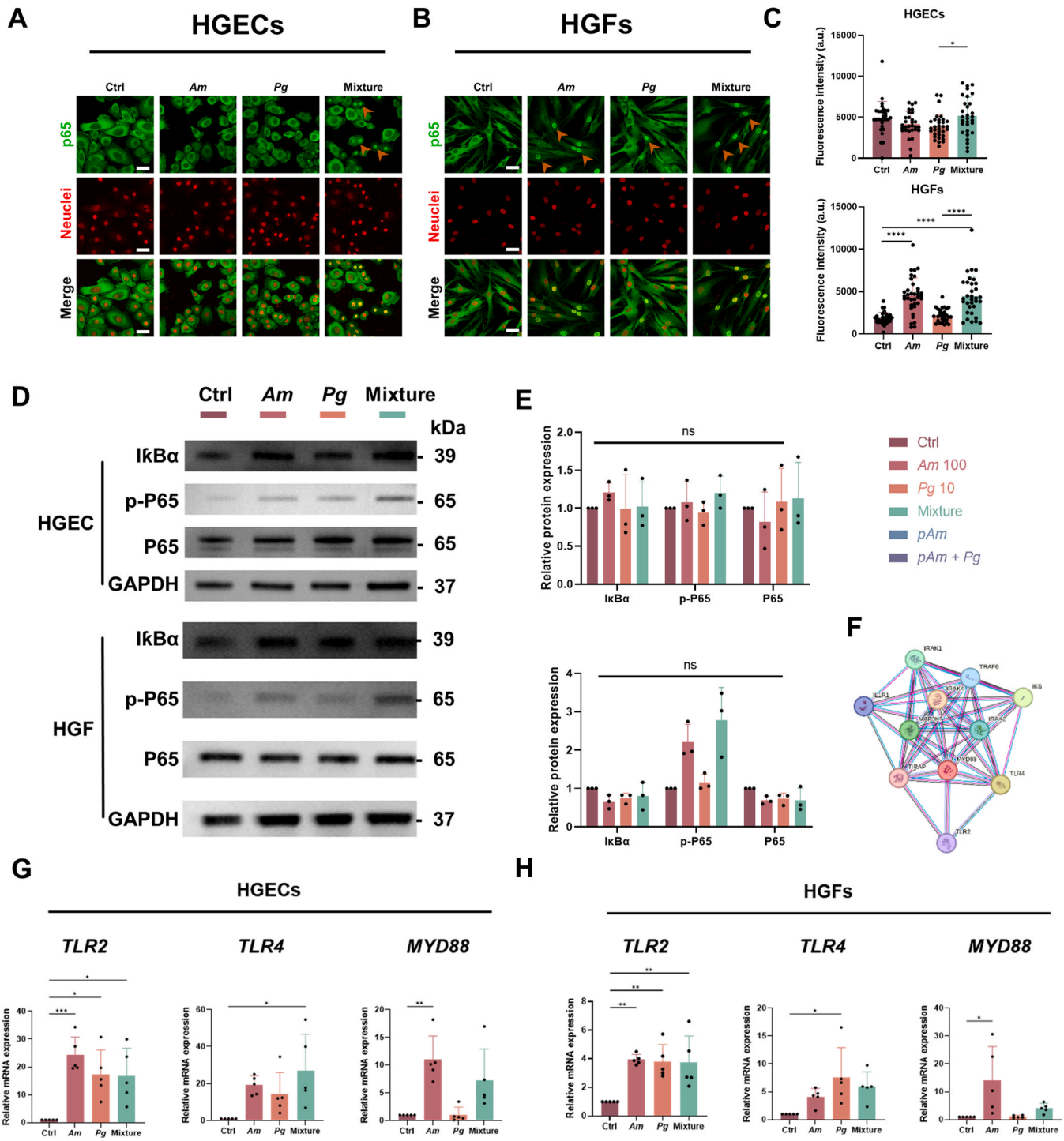

Fig. 3. *A. muciniphila* restored the inflammatory response via TLR-MyD88-NF-κB signaling pathway. The immunofluorescent staining of P65 in (A) HGECs and (B) HGFs after one-hour exposure to different bacteria or their combinations (scale bar, 20 μm). (C) The fluorescent intensity of P65 in the cell nuclei was analyzed from randomly selected cell nuclei (n ≥ 30). (D) The immunoblotting analysis of IκBα, p-P65, P65, and GAPDH in HGECs or HGFs after the same bacterial stimulations for one hour. (E) Their relative expression was quantified from three independent experiments (n = 3). (F) MyD88, TLR2/4 and NF-κB pathway related proteins interaction according to the STRING program. (G) HGECs and (H) HGFs were treated with *A. muciniphila* with or without *P. gingivalis* for 6 h. Relative mRNA expression was evaluated to evaluate the gene expression of TLR2, TLR4, and MYD88. Statistical significance in comparison to the control were analysed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as mean ± SD. *$P < 0.05$, * *$P < 0.01$, * **$P < 0.001$, and * ** *$P < 0.0001$ *versus* the control and matched groups. Ns, not significant ($P > 0.05$).

shown that *A. muciniphila* or its components can activate TLR receptors, subsequently stimulating the NF-κB pathway and NLRP3 inflammasome. These findings indicate that *A. muciniphila* has promising anti-tumor and anti-infection properties. Our results showed that *A. muciniphila* increased the expression of TLR, activated the NF-κB pathway, targeted MyD88 protein, and induced downstream cascades. Its ability to attract immune effectors was also confirmed *in vivo*. CXCL1 and CXCL10 are important peptides in inflammatory responses, guiding immune cells, primarily neutrophils, to sites of infection. The oral administration of *A. muciniphila* in mice significantly upregulated the

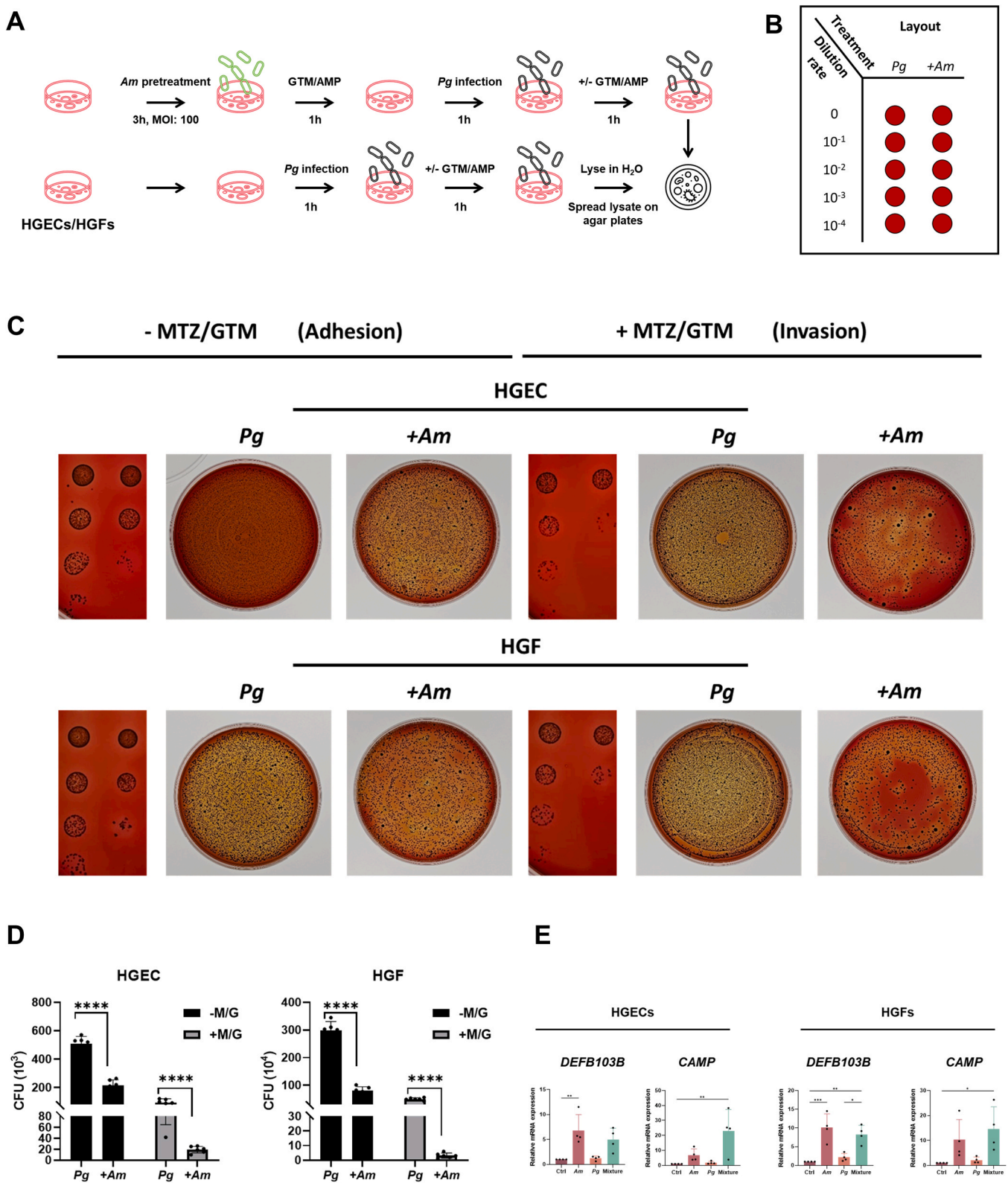

Fig. 4. *A. muciniphila*-primed HGECs and HGFs were resistant to the adherence and internalization of *P. gingivalis*. (A) Infection procedures. HGECs and HGFs were pre-treated with *A. muciniphila* before being infected with *P. gingivalis*. After infection, the cells were rinsed, with or without the addition of MTZ and GTM, to remove any *P. gingivalis* that had adhered to the cell surfaces. After each antibiotic treatment, the cells were rinsed with PBS twice to remove residual antibiotics. The cells were then lysed in water. The resulting lysates were plated on blood agar plates and incubated under anaerobic conditions for seven days to determine the number of colony-forming units (CFU) of *P. gingivalis*. (B) Layout of the serial dilution. (C, D) The CFU results of *P. gingivalis*. (E) HGECs and HGFs were treated with *A. muciniphila* with or without *P. gingivalis* for 6 h. Relative mRNA expression was evaluated to evaluate the gene expression of genes encoding antimicrobial peptide, including *DEFB103B* and *CAMP*. Statistical significance in comparison to the control were analysed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as mean ± SD. *$P < 0.05$, * *$P < 0.01$, * **$P < 0.001$, and * ** *$P < 0.0001$ *versus* the matched groups.

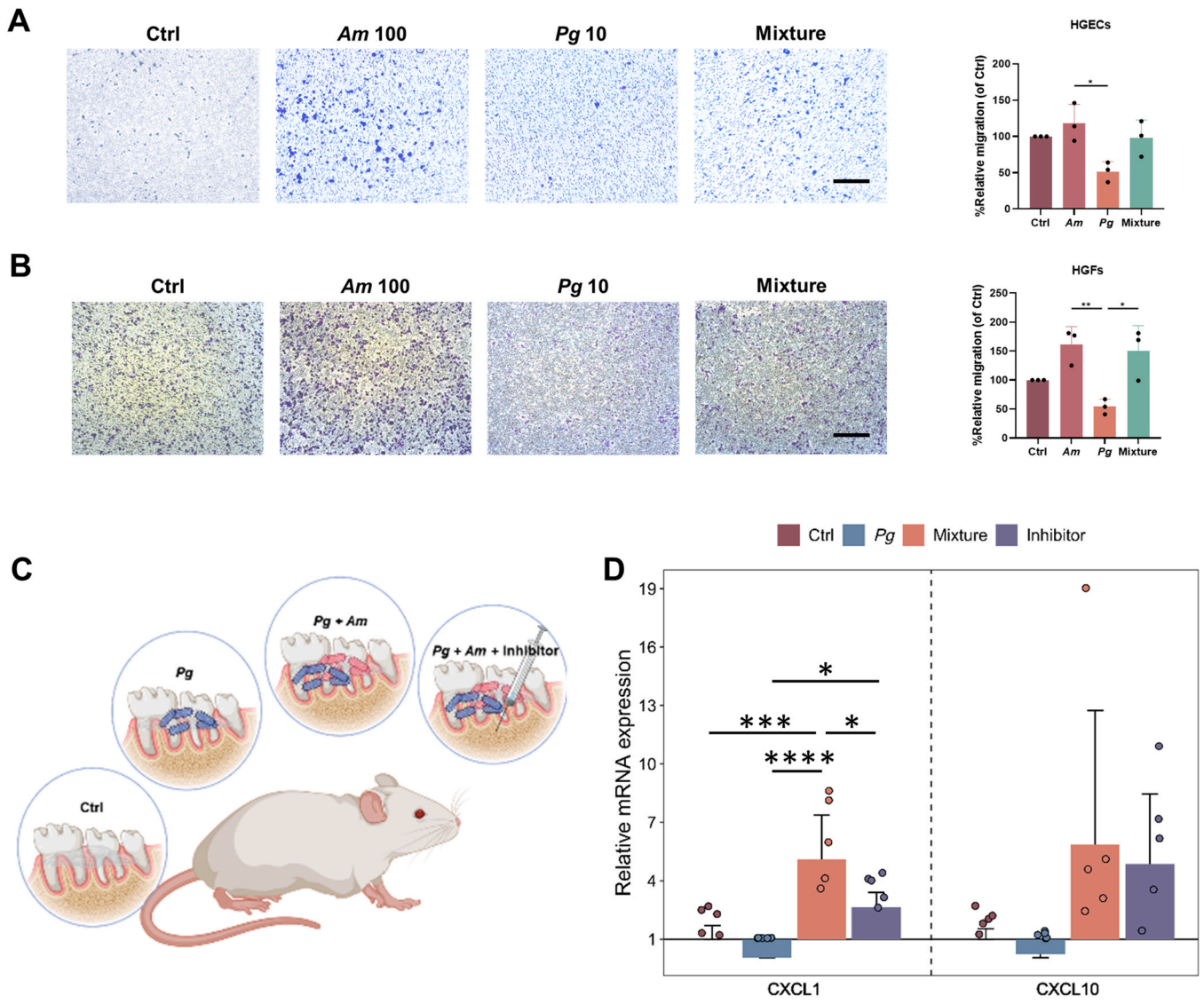

Fig. 5. *A. muciniphila* enhanced the chemotactic activity of immune cells. (A, B) Supernatants collected from bacteria-treated HGECs or HGFs were administered in the bottom of the transwell plate (scale bar, 250 μm). THP-1 cells recruited from the top chambers were stained with crystal violet and quantified (n = 3). (c) Experiment design for short-term bacterial inoculation: specific-pathogen-free mice received bacteria suspended in the carboxymethylcellulose (CMC) solution every 3 days for a total of 7 days. The groups include Ctrl (n = 5, receiving germ-free CMC), *P. gingivalis* (n = 5, receiving *P. gingivalis*), *P. gingivalis* + *A. muciniphila* (n = 5, receiving a mixture solution of *P. gingivalis* and *A. muciniphila*), and *P. gingivalis* + *A. muciniphila* + inhibitor (n = 5, receiving the mixture solution followed by MyD88 inhibitor injection on the final day). (d) Gene expression levels of CXCL1 and CXCL10 in gingival tissues from the short-term inoculated mice across different groups (n = 5). Statistical significance in comparison to the control were analysed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as mean ± SD. *$P < 0.05$, * *$P < 0.01$, and * **$P < 0.001$ *versus* the matched groups. Ns, not significant ($P > 0.05$).

localized expression of these peptides which was mediated by MyD88. Additionally, *A. muciniphila* increased the expression of *DEFB103B* and *CAMP*, which encode antibacterial peptides essential for mucosal immunity against microbial dysbiosis and pathogenic infection. This could explain the reduction of viable adherent and intracellular *P. gingivalis* in HGECs and HGFs (Inoue et al., 2024). On the other hand, there are other previous studies that have explored *A. muciniphila*'s capacity in alleviating periodontal diseases (Mulhall et al., 2021, 2022). The other two studies indicated that *A. muciniphila* could suppress the virulence of periodontal pathogens *P. gingivalis* and *F. nucleatum* and enhance epithelial integrity (Huck et al., 2020; Song et al., 2023). All these studies revealed that the *A. muciniphila* could reduce inflammation in experimental periodontitis. At first glance, our results may seem contradictory to these preclinical studies. However, this apparent conflict can be understood through *A. muciniphila*'s ability to elicit a moderate immune response, which helps control further bacterial dysbiosis-induced inflammation. Additionally, Song et al. reported that a 24-hour co-culture with *A. muciniphila* inhibited the TLR/MyD88/NF-κB pathway at the transcriptional level in gingival epithelial cells, thereby reducing inflammation. However, the mechanism by which TLR was inhibited by the bacteria remains unclear. This finding contrasts with our results, and the difference might arise from the use of different cells. Our results indicated that epithelial cells were less sensitive to the bacterial challenge compared to fibroblasts. Moreover, previous studies have shown that the activation of the NF-κB pathway is dynamic and that signal transduction through MyD88 with activated TLRs is transient (Fisch et al., 2024). Therefore, observations made after a 24-hour treatment period may not capture the signaling cascade. Furthermore, Song et al. demonstrated that *A. muciniphila* could inhibit the expression of *F. nucleatum* virulence which could indirectly diminish pathway activation. Given *A. muciniphila* is a sophisticated bacteria with its components and metabolites that can function, to confirm the functional

parts of *A. muciniphila* and their underlying mechanisms, further studies should be conducted.

Although *A. muciniphila* activated the host's ability to recognize bacteria and enhanced the immune response of the host, which was beneficial for controlling periodontal plaque, we must remain cautious regarding the immunogenicity exhibited by it. Previous studies have reported that the metabolic activity of *A. muciniphila* could deglycosylate IgA1, contributing to autoimmune kidney disease (Gleeson et al., 2024). It is particularly noteworthy that *A. muciniphila* originates from the gut and is not a commensal organism of the oral cavity. Therefore, supplementation with *A. muciniphila* may trigger undesired immune responses and potentially lead to ectopic colonization, necessitating further research. Considering these aspects, exploring the use of pasteurized forms or extracted functional components of *A. muciniphila* as the immune modulators for clinical treatment of periodontal diseases is warranted.

In summary, we demonstrated that *A. muciniphila* has the inherent capacity to activate the innate immune system, providing a promising therapeutic avenue for combating *P. gingivalis*-induced immune subversion. These results show the potential of *A. muciniphila* as an immune modulator for periodontal disease.

CRediT authorship contribution statement

Hu Qin: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Leung Wai Keung:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization. **Acharya Aneesha:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Pelekos George:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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