Cytokine 119 (2019) 24–31

Contents lists available at ScienceDirect

Cytokine

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

IL-36γ regulates mediators of tissue homeostasis in epithelial cells

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ARTICLE INFO

Keywords:
Epithelial cells
IL-36
Innate immunity
MMP9
NGAL
Porphyromonas gingivalis

ABSTRACT

IL-36 cytokines are critical regulators of mucosal inflammation and homeostasis. IL-36γ regulates the expression of inflammatory cytokines and antimicrobial proteins by gingival epithelial cells (e.g. TIGK cells). Here, we show that IL-36γ also regulates the expression of matrix metalloproteinase 9 (MMP9) and neutrophil gelatinase-associated lipocalin (NGAL), important mediators of antimicrobial immunity and tissue homeostasis in mucosal epithelia. MMP9 and NGAL were not similarly induced by IL-17 or IL-22, thus indicating the importance of IL-36γ in the regulation of MMP9 and NGAL. Mechanistically, MMP9 and NGAL expression was demonstrated to be induced in an IRAK1- and NF-κB-dependent manner. Furthermore, signaling by p38 MAP kinase may enable their expression to be independently regulated by IL-36γ. The stronger IL-36γ-inducible expression of MMP9 and NGAL in terminally differentiating TIGK cells suggests that control of their expression is associated with the maturation of the gingival epithelium. Although MMP9 and NGAL expression in epithelial cells can also be induced by bacteria, their expression in TIGK cells was not induced by the periodontal pathogen *Porphyromonas gingivalis*, most likely due to antagonism by the gingipain proteinase virulence factors. This study advances our understanding of how IL-36γ may promote oral mucosal immunity and tissue homeostasis, and how this may be dysregulated by bacterial pathogens.

1. Introduction

Mucosal surfaces interact directly with the external environment, continuously encountering commensal microbes as well as potential pathogens. Therefore, mucosal epithelial cells are critical mediators of host defence against infection. They maintain this essential barrier by undergoing coordinated cycles of cell proliferation and differentiation [1]. Epithelial cells also actively participate in host defence through the expression of pattern recognition receptors (PRRs), including members of the Toll-like receptor (TLR) family [2]. The recognition of microbial ligands by TLRs initiates downstream signaling that induces the expression of inflammatory cytokines and chemokines to activate the immune response [3].

Epithelial-derived antimicrobial proteins (AMPs) also have a significant role in the protection of mucosa from infection and maintenance of host-microbe homeostasis. AMPs produced in response to microbes are commonly regulated by TLR signaling; however, they are also regulated by signaling initiated by innate and adaptive immune cytokines (e.g. IL-1β and IL-17) [4,5]. AMPs can have varied modes of action, including cationic antimicrobial peptides (e.g. defensins) that disrupt microbial membranes, causing osmotic lysis and cell death [4]. Another group of AMPs includes proteins that inhibit microbial iron metabolism, either through direct sequestration of iron (e.g. lactoferrin) [6] or binding bacterial siderophores (e.g. neutrophil gelatinase-associated lipocalin; NGAL) [7]. Some AMPs can also regulate the immune response by modulating cytokine production or functioning as chemokines [8,9].

Mucosal homeostasis requires constant remodelling of the extracellular matrix (ECM) [10]. This is a tightly controlled process involving the cleavage of ECM proteins to regulate the ECM composition and structure as well as the release of bioactive molecules (e.g. epidermal growth factor). Matrix metalloproteinases (MMPs) are the major enzymes responsible for ECM degradation, with diverse substrate and tissue specificities. MMPs also play a direct role in immune regulation. For example, pro-TNF can be converted to mature TNF by MMP3 [11], and N-terminal processing of IL-8 by MMP9 can greatly enhance its chemotactic activity [12]. Conversely, N-terminal processing of CCL2 by MMP1 produces an antagonistic factor [13].

Abbreviations: ActD, actinomycin D; AMP, antimicrobial protein; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; IL, interleukin; IL-36R, IL-36 receptor; IRAK1, IL-1 receptor-associated kinase 1; LFQ, MaxQuant label-free quantitation metric; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; PRR, pattern recognition receptors; TIGK, telomerase immortalised gingival keratinocyte; TIMP, tissue inhibitor of metalloproteinase; TLR, toll-like receptor

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https://doi.org/10.1016/j.cyto.2019.02.012
Received 17 December 2018; Received in revised form 31 January 2019; Accepted 12 February 2019
Available online 08 March 2019

IL-36 family cytokines (IL-36α, IL-36β and IL-36γ), members of the IL-1 superfamily, have recently been demonstrated to be important mediators of mucosal inflammation and homeostasis [14–18]. IL-36 receptor (IL-36R) signaling conferred protection against bacterial infection in a mouse model of colitis [16]. Moreover, IL-36γ was critical for mucosal protection in mouse models of bacterial pneumonia [18]. The IL-36 cytokines are expressed in response to TLR signaling by a range of cell types, including mucosal epithelial cells [19,20]. IL-36 cytokines are potent stimulators of the expression of cytokines and chemokines, such as IL-8 and CXCL1, by mucosal epithelial cells and innate immune cells (e.g. dendritic cells) [20–22]. IL-36 cytokines are also important regulators of adaptive immunity, shown to regulate T helper cells [16,17,23]

We recently established that IL-36γ plays a role in the inflammatory response of gingival epithelial cells to the bacterial pathogen *Porphyromonas gingivalis*, regulating the expression of inflammatory cytokines and antimicrobial proteins [20,24,25]. Using an unbiased proteomics approach, we have now shown that IL-36γ also stimulates the secretion of MMP9 and NGAL by gingival epithelial cells (i.e. TIGK cells). IL-36γ was demonstrated to also regulate MMP9 and NGAL at the level of gene transcription, which was mediated by IRAK1-dependent signaling and enhanced by terminal differentiation. Notably, MMP9 and NGAL expression was not induced by *P. gingivalis*, most likely due to antagonism by proteinase virulence factors. Together, our current findings indicate that IL-36γ may mediate immune homeostasis of the oral mucosa through its regulation of the tissue remodelling and antimicrobial effectors MMP9 and NGAL.

2. Materials and methods

2.1. Reagents

DermaLife keratinocyte growth medium and supplements (TGFα, insulin, epinephrine, apo-transferrin, hydrocortisone, bovine pituitary extract, and glutamine) were from Lifeline Cell Technology. The human MMP9 ELISA kit, human IL-17A (Gly24-Ala155), IL-22 (Ala34-Ile179), IL-36α (Lys6-Phe158), and IL-36γ (Ser18-Asp169) were from R&D Systems. The human NGAL ELISA kit was from ELISAkit.com. Opti-MEM I reduced serum medium, Lipofectamine RNAiMAX transfection reagent, Zymogram buffers, and precast 10% NuPAGE and 10% Zymogram gels were from Life Technologies. The ON-TARGETplus IRAK1 and non-targeting control siRNA were from GE Healthcare. BAY11-7082, SB203580, and U0126 were from Merck Millipore, while actinomycin D was from Sigma-Aldrich.

2.2. Mammalian cell culture

Human Telomerase Immortalised Gingival Keratinocytes (TIGK) [26] were cultured in DermaLife keratinocyte growth medium supplemented with 0.5 ng/ml TGFα, 5 μg/ml insulin, 1 μM epinephrine, 5 μg/ml apo-transferrin, 100 ng/ml hydrocortisone, 0.4% bovine pituitary extract, and 6 mM glutamine. For proteomic analysis, TIGK cells were stimulated in keratinocyte growth medium without supplements. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Bacterial cell culture

P. gingivalis (ATCC 33277) were obtained from the culture collection of the Melbourne Dental School (University of Melbourne). The isogenic Kgp and RgpA/B deficient *P. gingivalis* mutant KDP136 (Δ*kgp*Δ*rgpA*Δ*rgpB*) has been previously described [27]. The bacteria were maintained on horse blood agar plates at 37 °C in an anaerobic atmosphere of 5% H_2, 80% N_2, and 15% CO_2. Bacterial colonies were used to inoculate Brain Heart Infusion medium supplemented with 5 μg/ml hemin plus 0.5 mg/ml cysteine, and 5 μg/ml menadione [28].

2.4. Challenging of gingival epithelial cells with P. gingivalis

Late logarithmic growth phase bacteria were harvested by centrifugation at 7000*g* for 20 min at 4 °C, and then suspended in antibiotic-free keratinocyte growth medium. TIGK cell monolayers were incubated with bacteria at a multiplicity of infection (MOI) of 100:1 [28].

2.5. Proteomic analysis

Proteins in cell culture supernatants were precipitated with 15% v/v trichloroacetic acid and washed with acetone. Whole samples were solubilised with NuPAGE LDS sample buffer containing 50 mM dithiothreitol and subjected to SDS-PAGE run at 120 V for 15 min. Each sample was excised as a single band and underwent in-gel trypsin digestion, LC-MS/MS analysis on an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA), and MaxQuant label-free quantitation, as previously described [29]. Three biological replicates of IL-36γ-stimulated and unstimulated control samples were analysed. Each of the six samples was injected three times (technical replicates) for a total of 18 raw MS files. The files were analysed together by MaxQuant software (v1.5) [30] and included database searching against the human proteome, containing a total of 20,156 proteins from UniProt (www.uniprot.org). The technical replicates within each biological replicate were averaged by MaxQuant and then for each paired biological replicate the MaxQuant label-free quantitation metric (LFQ) was employed to calculate the ratio of IL-36γ-stimulated TIGK cells/unstimulated control. For the secretion of a protein to be defined as being up-regulated by IL-36γ-stimulation, at least two peptides from the protein had to be identified in all three IL-36γ-stimulated replicates and the average ratio (geometric mean) had to be at least 2.0. In total, 1781 proteins were identified and 1167 quantifiable. The quantifiable proteins were centred around a ratio of 1.0, thus demonstrating successful normalisation of the data.

2.6. RNA purification, reverse transcription, and quantitative real-time PCR

Total RNA was purified using the ReliaPrep RNA Cell miniprep system (Promega), which included an on-column DNase-treatment step. RNA was reverse-transcribed using random primers and GoScript Reverse Transcriptase (Promega), per the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in duplicate using GoTaq qPCR Master Mix (Promega) and pre-developed TaqMan assays (Life Technologies) for the following genes: *IVL* (Hs00902520_m1), *KRT1* (Hs01549614_g1), *MMP9* (Hs00957562_m1), *NGAL* (Hs01008571_m1), *TIMP1* (Hs01092512_g1), *TIMP2* (Hs00234278_m1), *TIMP3* (Hs00165949_m1). PCR was performed on a QuantStudio 7 Flex Real-Time PCR system (Life Technologies). The data were normalised against the hypoxanthine guanine phosphoribosyl transferase (HPRT) or TATA-box binding protein (TBP) genes.

2.7. RNA interference-mediated gene-silencing

A reverse transfection protocol was used for siRNA transfection of TIGK cells [20]. Briefly, the siRNAs were diluted to 120 nM with 100 μl Opti-MEM I reduced serum medium, mixed with 100 μl Opti-MEM medium containing 1 μl Lipofectamine RNAiMAX transfection reagent, and incubated at room-temperature for 20 min. TIGK cells (2×10^5 cells in 1 ml keratinocyte growth medium) were seeded in 12-well plates and cultured with the transfection cocktail for 24 h. Thereafter, the medium was refreshed, and the cells cultured for an additional 24 h.

2.8. Enzyme-linked immunosorbent assays

NGAL ELISA was performed by incubating diluted culture supernatants and standards in capture antibody-coated 96-well microplates

at room temperature for 2 h, per the manufacturer's instructions (ELISAkit.com). The wells were washed prior to the addition of biotinylated anti-NGAL antibody and incubated at room temperature for 2 h. The plates were washed, and then incubated with a streptavidin-HRP conjugate at room temperature for 45 min. MMP9 ELISA was performed by incubating diluted culture supernatants and standards in capture antibody-coated 96-well microplates at room temperature for 2 h, per the manufacturer's instructions (R&D Systems). The wells were washed prior to the addition of HRP-conjugated anti-MMP9 antibody and incubated at room temperature for 1 h. Following washing, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and the plates incubated at room temperature. Colour development was subsequently stopped and measured at 450 nm with wavelength correction at 560 nm using a microplate reader (Victor^3, PerkinElmer).

2.9. Zymography

Culture supernatants were mixed with Tris-glycine SDS sample buffer and then subjected to electrophoresis on a 10% Zymogram (Gelatin) gel using Tris-glycine SDS running buffer. The proteins in the gel were renatured with Zymogram Renaturing Buffer and developed overnight with Zymogram Developing Buffer. The gel was stained with 0.1% w/v colloidal Coomassie Blue G-250. Areas of proteinase activity appeared as clear bands against the dark stained background. The gel was imaged with a Fujifilm Las-3000 Imager (Fujifilm, Japan), and relative proteinase activity was quantitated by densitometric analysis with ImageJ [31].

3. Statistical analysis

Unless otherwise stated, data combined from three independent biological-replicate experiments are presented as the mean ± SEM. Proteomic data are presented as an average ratio (geometric mean). Statistical analyses were performed using GraphPad Prism 7. Differences between two groups were evaluated using the Student's *t*-test. For multiple comparisons, statistical analysis was performed by ANOVA with Dunnett's or Sidak's post-hoc test. A p value < 0.05 was considered statistically significant.

4. Results

4.1. IL-36γ up-regulates secretion of immune mediators by gingival epithelial cells

We have recently shown that IL-36γ regulates the expression of various inflammatory cytokines and AMPs by gingival epithelial cells [20,24,25]. Thus, we sought to establish whether IL-36γ also regulates the expression of other mediators of innate immunity. Using a label-free quantitative proteomics technique, we investigated the proteins secreted by human gingival epithelial cells (i.e. TIGK cells) in response to IL-36γ stimulation. This analysis revealed that IL-36γ stimulates increased secretion of numerous proteins important for host immunity and tissue homeostasis (Table 1). For example, IL-8 secretion was strongly up-regulated by IL-36γ. The IL-36γ-inducible secretion of this chemokine as well as CXCL1 and CXCL5 has been previously reported in gingival epithelial cells, macrophages and dendritic cells [20]. These results, taken together support IL-36γ as a potent stimulator of chemokine responses in gingival epithelial cells. Interestingly, the secretion of proteins involved in the complement pathway, including complement factor B and complement C3, were also up-regulated by IL-36γ. Similarly, IL-36γ stimulated the secretion of proteins with roles in protein turnover, tissue remodelling and/or homeostasis (e.g. MMP9 and carboxypeptidase A2). Furthermore, increased secretion of pentraxin 3 and tissue plasminogen activator, with known functions in both the complement pathway and tissue remodelling, were identified. PGLYRP2 was also identified, having previously been shown to be regulated at the gene expression level by IL-36γ in gingival epithelial cells [25]. Also of notable interest was the identification of NGAL as an IL-36γ-regulated protein. Together, these data suggest that IL-36γ produced by the gingival epithelium may promote host defence and tissue homeostasis via the stimulation of chemokines, proteins with antimicrobial function, and proteinases with roles in tissue remodelling.

4.2. IL-36γ stimulates MMP9 secretion and expression in gingival epithelial cells

Our proteomics data (Table 1) indicated that IL-36γ stimulates MMP9 secretion by gingival epithelial cells, which we confirmed by ELISA (Fig. 1A). MMP9 is secreted as a pro-domain-containing precursor (pro-MMP9), which is subsequently processed to its enzymatically active form. Gelatin zymography was used to confirm the secretion of enzymatically active MMP9. As shown in Fig. 1B, IL-36γ stimulated a 10-fold increase in MMP9 activity in the cell culture supernatants. Quantitative PCR (qPCR) revealed that IL-36γ also strongly stimulates MMP9 mRNA expression (Fig. 1C). To confirm that the increased MMP9 mRNA levels were due to increased transcription, and not mRNA stabilisation, TIGK cells were treated with actinomycin D (ActD) prior to IL-36γ stimulation. Accordingly, ActD inhibited the increase in MMP9 mRNA levels (Fig. 1D). IL-36α was also found to stimulate MMP9 mRNA expression with comparable kinetics (Fig. 1E). Interestingly, IL-36γ did not stimulate the expression of tissue inhibitor of metalloproteinase-1 (TIMP1), TIMP2 or TIMP3 (data not shown), which can function to inhibit MMP9 activity [32]. The adaptive immune cytokines IL-17 and IL-22 mediate host defence in part by stimulating the expression of inflammatory cytokines and AMPs by epithelial cells, both independently and in synergy [33]. Therefore, we tested the ability of IL-17A and IL-22 to stimulate MMP9 expression. Neither IL-17A nor IL-22 stimulated MMP9 expression by TIGK cells (Fig. 1F). Collectively, these data indicate that IL-36R signaling regulates MMP9 production by gingival epithelial cells.

4.3. IL-36γ stimulates NGAL secretion and expression in gingival epithelial cells

The ability of IL-36γ to stimulate NGAL secretion by gingival epithelial cells (Table 1) was confirmed by ELISA (Fig. 2A). We also established that IL-36γ stimulates increased levels of NGAL mRNA (Fig. 2B), which was inhibited by ActD (Fig. 2C). IL-36α was also demonstrated to stimulate NGAL mRNA expression (Fig. 2D). As shown in Fig. 2E, IL-17A stimulated NGAL expression only weakly (< 3-fold), while its expression was not stimulated by IL-22. Furthermore, IL-17A and IL-22 did not synergise to induce NGAL expression (Fig. 2E). In addition to antimicrobial activity [7], NGAL can form a disulphide-linked heterodimer with MMP9, which might protect MMP9 from proteolytic degradation [34]. However, we did not detect the presence of MMP9/NGAL complexes in tissue culture supernatants of IL-36γ-stimulated TIGK cells (data not shown). Taken together, these data suggest that IL-36γ specifically controls the expression of NGAL by the gingival epithelium.

4.4. IL-36γ stimulates MMP9 and NGAL expression via IRAK1 and NF-κB signaling

Given the above data suggest that IL-36γ is an important regulator of MMP9 and NGAL in gingival epithelial cells, we investigated the signaling pathways that regulate their expression. We have previously shown that IRAK1 mediates the IL-36γ-inducible stimulation of inflammatory cytokine and AMP expression in gingival epithelial cells [20,25]. Consistently, siRNA-mediated gene silencing of IRAK1 inhibited IL-36γ-inducible MMP9 (Fig. 3A) and NGAL (Fig. 3B) expression in TIGK cells. We have also recently shown that IL-36γ activates ERK1/2 and p38 MAP kinases, and the NF-κB transcription factor in TIGK cells

Table 1
IL-36γ-inducible protein secretion by gingival epithelial cells. TIGK cells were stimulated with IL-36γ (50 ng/ml) for 24 h, and the protein composition of the cell culture medium then analysed by label-free quantitation proteomics ($n = 3$).

UniProt Accession	Protein Name	LFQ Ratio (IL-36γ-stimulated/unstimulated)				p	Function/Pathway
		N1	N2	N3	Average[a]		
P10145	IL-8	41.3	67.5	35.2	46.1	0.0178*	Neutrophil chemoattractant
P09341	CXCL1	36.8	8.6	14.0	16.4	0.1357	Neutrophil chemoattractant
P13501	CCL5	9.3	8.0	4.0	6.7	0.0995	T cell chemoattractant
P02778	CXCL10	137.2	449.3	10.7	87.1	0.1562	T cell chemoattractant
P26022	Pentraxin-related protein	70.7	62.8	70.5	67.9	0.0220*	Antimicrobial activity, immune modulation, tissue remodelling
P00751	Complement factor B	18.3	22.4	22.2	20.9	0.0001*	Component of the alternative complement pathway
P01024	Complement C3	5.0	7.0	7.1	6.3	0.0008*	Component of the alternative complement pathway
P14780	MMP9	27.1	52.7	39.8	38.5	0.0249*	Proteinase (collagenase) involved in tissue remodelling and immune modulation
P45452	MMP13	5.5	3.3	4.8	4.4	0.0750	Proteinase (collagenase) involved in bone formation/ remodelling and inflammation
P03956	MMP1	2.1	1.8	2.9	2.2	0.1823	Proteinase (collagenase) involved in tissue remodelling and immune modulation
Q96PD5	PGLYRP2	5.7	[b]	3.5	4.5	0.2014	Antimicrobial activity, immune modulation
P80188	NGAL	20.0	16.6	18.3	18.2	0.0282*	Antimicrobial activity, immune modulation
P48052	Carboxypeptidase A2	5.1	7.1	7.9	6.6	0.0158*	Protein degradation
P00750	Tissue-type plasminogen activator	4.1	2.5	3.6	3.3	0.0009*	Activation of plasmin (involved in tissue remodelling, wound healing, coagulation cascade, complement)
P49769	Vascular endothelial growth factor C	2.6	1.9	2.0	2.2	0.1302	Angiogenesis, tissue remodelling, immune modulation

[a] Average indicates the geometric mean.
[b] Identified in cell culture medium from IL-36γ-stimulated TIGK cells but not from unstimulated cells.
* Indicates significance ($p < 0.05$).

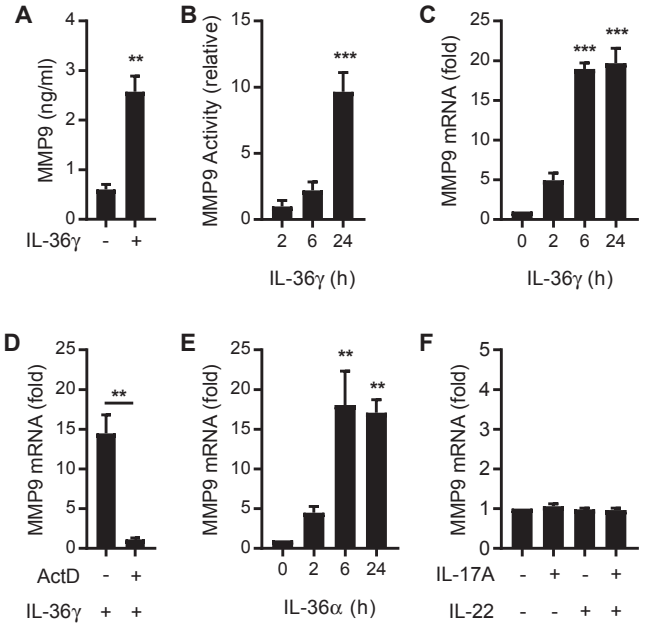

Fig. 1. Stimulation of MMP9 expression in gingival epithelial cells by IL-36γ. (A) TIGK cells were stimulated with IL-36γ (100 ng/ml) for 24 h, and the concentrations of MMP9 in the cell culture supernatants were then measured by ELISA ($n = 3$). (B) TIGK cells were stimulated with IL-36γ (100 ng/ml) for the time indicated. The enzymatic activity of MMP9 in the cell culture supernatants was then assessed by gelatin zymography and quantitated ($n = 3$). (C) TIGK cells were stimulated with IL-36γ (100 ng/ml) for the time indicated. MMP9 mRNA expression levels were then measured by qPCR ($n = 3$). (D) TIGK cells were treated with actinomycin D (ActD; 1 μg/ml) for 30 min, and then stimulated with IL-36γ (100 ng/ml) for 6 h. MMP9 mRNA expression levels were then measured by qPCR ($n = 3$). (E) TIGK cells were stimulated with IL-36α (100 ng/ml) for the time indicated. MMP9 mRNA expression levels were then measured by qPCR ($n = 3$). (F) TIGK cells were stimulated with IL-17A (100 ng/ml) or IL-22 (50 ng/ml) for 24 h. MMP9 mRNA expression levels were then measured by qPCR ($n = 3$). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

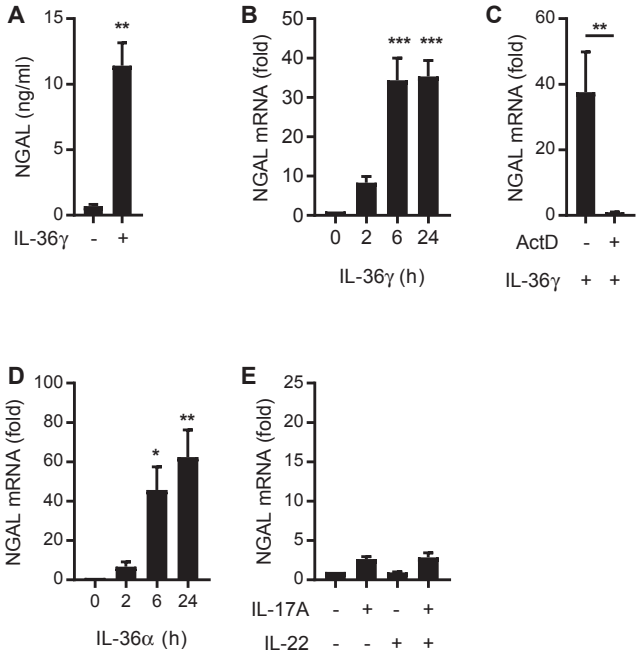

Fig. 2. Stimulation of NGAL expression in gingival epithelial cells by IL-36γ. (A) TIGK cells were stimulated with IL-36γ (100 ng/ml) for 24 h, and the concentrations of NGAL in the cell culture supernatants were then measured by ELISA ($n = 3$). (B) TIGK cells were stimulated with IL-36γ (100 ng/ml) for the time indicated. NGAL mRNA expression levels were then measured by qPCR ($n = 3$). (C) TIGK cells were treated with actinomycin D (ActD; 1 μg/ml) for 30 min, and then stimulated with IL-36γ (100 ng/ml) for 6 h. NGAL mRNA expression levels were then measured by qPCR ($n = 3$). (D) TIGK cells were stimulated with IL-36α (100 ng/ml) for the time indicated. NGAL mRNA expression levels were then measured by qPCR ($n = 3$). (E) TIGK cells were stimulated with IL-17A (100 ng/ml) or IL-22 (50 ng/ml) for 24 h. NGAL mRNA expression levels were then measured by qPCR ($n = 3$). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

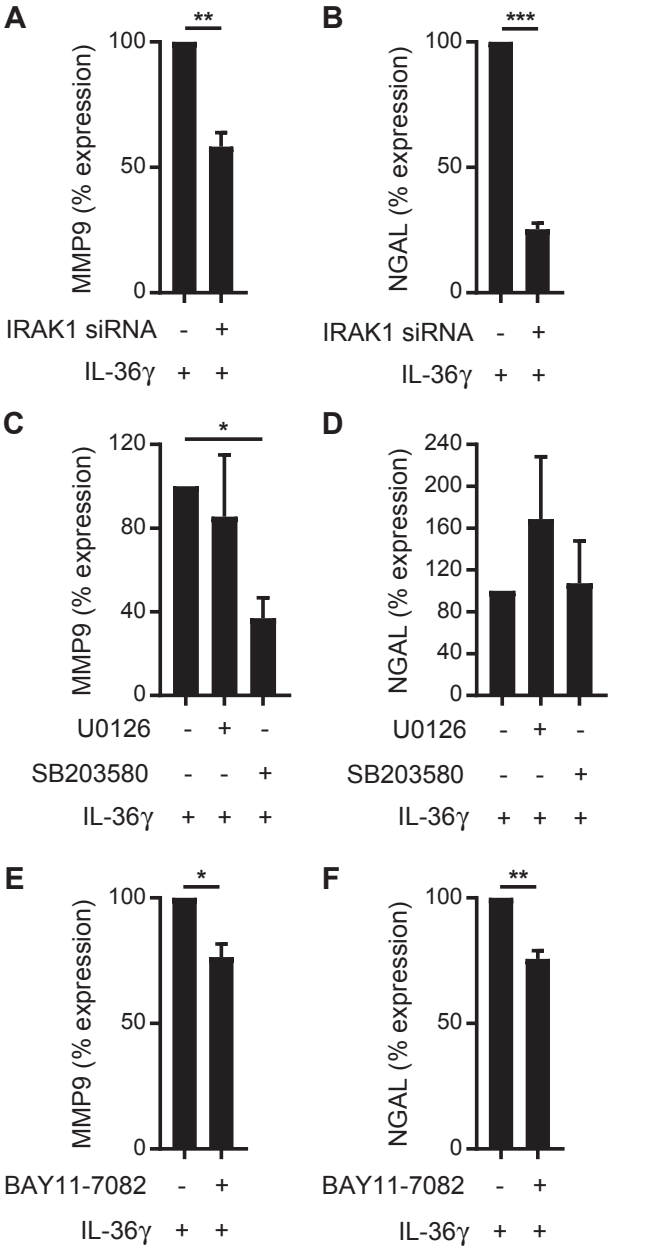

Fig. 3. Signaling pathways regulating the IL-36γ-inducible expression of MMP9 and NGAL in gingival epithelial cells. (A and B) TIGK cells were transfected with an IRAK1 (+) or control (–) siRNA, and subsequently stimulated with IL-36γ (100 ng/ml) for 24 h. (A) MMP9 and (B) NGAL mRNA expression levels were then measured by qPCR ($n = 3$). (C–F) TIGK cells were treated with 10 μM U0126 or 5 μM SB203580 (C and D) or 10 μM BAY11-7082 (E and F) for 30 min, and then stimulated with IL-36γ (100 ng/ml) for 24 h. (C and E) MMP9 and (D and F) NGAL mRNA expression levels were then measured by qPCR ($n = 3$). $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

[24]. Therefore, we used pharmacologic inhibitors to establish the importance of these signaling proteins for the stimulation of MMP9 and NGAL expression. Neither the IL-36γ-inducible expression of MMP9 (Fig. 3C) nor NGAL (Fig. 3D) were affected by the MEK (ERK1/2) inhibitor, U0126. Furthermore, the p38 MAP kinase inhibitor, SB203580, did not affect IL-36γ stimulation of NGAL expression (Fig. 3D), whereas stimulation of MMP9 expression was reduced by more than 50% (Fig. 3C). The NF-κB inhibitor, BAY11-7082, inhibited only weakly the stimulation of MMP9 (Fig. 3E) and NGAL (Fig. 3F) expression by IL-36γ. These data indicate that IL-36γ induces the expression of MMP9 and NGAL in an IRAK1- and NF-κB-dependent manner. Furthermore, p38

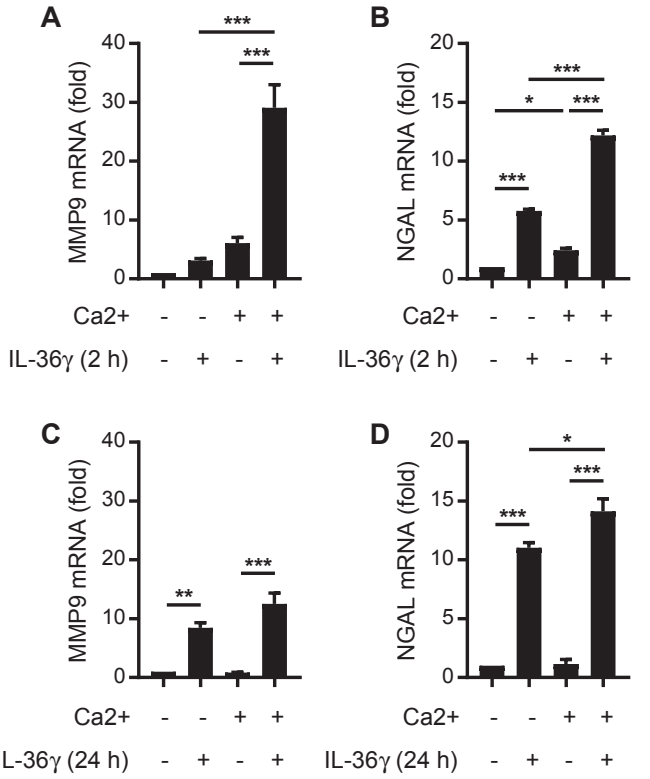

Fig. 4. Terminal differentiation modulates IL-36γ-inducible MMP9 and NGAL expression in gingival epithelial cells. TIGK cells were cultured in the presence of 0.09 mM (−) or 1.8 mM (+) Ca^{2+} for 48 h, and then stimulated with IL 36γ (100 ng/ml) for 2 h (A an B) or 24 h (C and D). (A and C) MMP9 and (B and D) NGAL mRNA expression levels were then measured by qPCR ($n = 3$). $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

MAP kinase signaling downstream of IRAK1 may enable the IL-36γ-inducible expression of MMP9 to be regulated independently of NGAL.

4.5. Effect of epithelia differentiation state on IL-36γ-inducible MMP9 and NGAL expression

The terminal differentiation of basal cells as they migrate towards the epithelium surface is important for maintaining the integrity and barrier function of the gingival epithelium [1]. Therefore, we investigated the effects of differentiation on the regulation of MMP9 and NGAL expression by IL-36γ. Terminal differentiation was induced by culturing TIGK cells in the presence of 1.8 mM Ca^{2+} for 48 h, and differentiation confirmed by demonstrating the up-regulation of involucrin and keratin 1 mRNA expression (data not shown). Given we had found that IL-36γ induced maximal MMP9 (Fig. 1C) and NGAL (Fig. 2B) expression in undifferentiated TIGK cells in a somewhat delayed manner, we examined the effect of differentiation on IL-36γ-inducible MMP9 and NGAL expression at early (e.g. 2 h) and late (e.g. 24 h) time-points post-stimulation. As shown in Fig. 4A, differentiation dramatically enhanced the stimulation of MMP9 by IL-36γ at the 2 h time-point. By contrast, differentiation did not significantly enhance IL-36γ-inducible MMP9 expression at the 24 h time-point (Fig. 4C). IL-36γ-inducible NGAL expression was also found to be strongly enhanced by differentiation at the 2 h time-point (Fig. 4B), but only weakly at 24 h post-stimulation (Fig. 4D). These data suggest that epithelial differentiation state plays an important role in modulating the regulation of MMP9 and NGAL expression by IL-36γ.

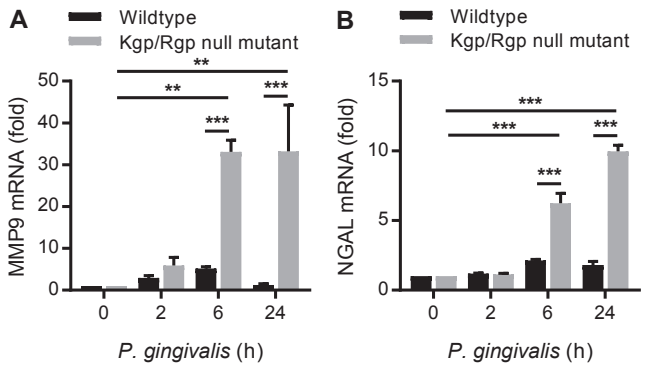

Fig. 5. *P. gingivalis* gingipain proteinases antagonise MMP9 and NGAL expression in gingival epithelial cells. TIGK cells were challenged with wildtype *P. gingivalis* or *P. gingivalis* KDP136 (Kgp/Rgp-null mutant) at a multiplicity of infection (MOI) of 100:1 for the time indicated. (A) MMP9 and (B) NGAL mRNA levels were then measured by qPCR ($n = 3$) $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

4.6. MMP9 and NGAL expression in response to P. gingivalis is antagonised by the gingipain proteinases

MMP9 and NGAL expression in epithelial cells can also be induced by bacteria [35–37]. Therefore, we tested the ability of the bacterial pathogen *P. gingivalis* to stimulate their expression by gingival epithelial cells. *P. gingivalis* challenge of TIGK cells did not induce significant MMP9 (Fig. 5A) or NGAL (Fig. 5B) expression. The extracellular gingipain proteinases (Kgp and RgpA/B) produced by *P. gingivalis* are key virulence factors and can proteolytically degrade immune mediators as well as dysregulate host signaling [38]. Accordingly, we investigated whether the gingipain proteinases antagonise the stimulation of MMP9 or NGAL expression in response to *P. gingivalis*. Both MMP9 (30-fold) and NGAL (10-fold) expression were strongly stimulated when TIGK cells were challenged with a *P. gingivalis* gingipain proteinase-null mutant (Fig. 5A and B). This indicates that the *P. gingivalis* gingipain proteinases antagonise the stimulation of MMP9 and NGAL expression by TIGK cells.

5. Discussion

IL-36γ has recently been established to be an important mediator of mucosal immunity [14,15,17,18]. For instance, IL-36γ has been shown to regulate the expression of inflammatory cytokines and AMPs by epithelial cells [19,20,24]. In this study, IL-36γ was shown to also regulate the expression of MMP9 and NGAL by epithelial cells. MMP9 and NGAL have established roles in immunity [39,40]. Thus, IL-36γ might promote mucosal immunity through its capacity to also regulate MMP9 and NGAL expression.

MMP9 has a primary role in ECM remodelling [10,41]. MMP9 preferentially degrades ECM proteins, including collagen, elastin, and fibronectin. ECM remodelling by MMP9 is involved in normal physiologic processes, such as wound healing, development and angiogenesis. Significantly, MMP9 is also involved in regulating immune cell recruitment and function. N-terminal processing of IL-8 by MMP9 was shown to enhance its inflammatory activity by potentiating neutrophil chemotaxis [12]. Similarly, MMP9 increased neutrophil recruitment in a mouse model of peritonitis through N-terminal processing of CXCL5 [42]. The ability of IL-36γ to stimulate the expression of neutrophil chemokines (e.g. IL-8 and CXCL1) by various cell types, including gingival epithelial cells, positions it as a key regulator of neutrophil responses [20]. The regulation of MMP9 by IL-36γ may therefore represent an additional mechanism for the control of neutrophil responses.

We found that IL-36γ also regulates the expression of NGAL by gingival epithelial cells. NGAL has a multifaceted role in mediating host defence against infection. It can exert direct antimicrobial effects by sequestering bacterial catecholate-type ferric siderophores (e.g. enterochelin), thereby preventing the capture of host iron and thus impairing bacterial metabolism [7,43]. NGAL may also play a role in modulating neutrophil function. NGAL was shown *in vitro* to be a potent paracrine chemoattractant for human and murine neutrophils [44]. Consistently, in NGAL-deficient mice neutrophils failed to extravasate to sites of bacterial infection due to impaired chemotaxis and adhesion [45]. The importance of NGAL in host defence is further evident from the finding that NGAL-deficient mice were more susceptible to infection by bacterial pathogens (e.g. *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*) [43,45–47]. Therefore, the regulation of NGAL by IL-36γ might promote mucosal immunity by not only sequestering bacterial siderophores, but also by modulating neutrophil responses.

The Th17 cell cytokines IL-17 and IL-22 are also important regulators of the host defence functions of epithelial cells [4,5]. We have previously shown that IL-17 and IL-22 can synergistically stimulate AMP expression (e.g. β-defensin 2) by TIGK cells [25]. In this study, IL-17 and IL-22 did not similarly stimulate the expression of MMP9 or NGAL. The expression of MMP9 and NGAL in the gingival epithelium is therefore likely to be largely regulated by epithelial-derived IL-36γ. Importantly, this would allow the rapid expression of MMP9 and NGAL in response to infection. Notably, we found that IL-36γ can potentially regulate MMP9 and NGAL expression independently via p38 MAP kinase signaling. This might also be important for mucosal homeostasis as it could allow appropriate tailoring of the inflammatory response.

The superficial layer of the gingival epithelium, which is comprised of terminally differentiating epithelial cells, is constantly exposed to bacteria, including potential pathogens. The terminal differentiation of epithelial cells in this layer is essential to barrier function [1]. Consistently, we recently established that terminal differentiation of TIGK cells enhanced their IL-36γ-inducible expression of AMPs (e.g. β-defensin 2) [25]. In this current study, we found that terminal differentiation also enhanced their IL-36γ-inducible expression of MMP9 and NGAL. Taken together, these findings suggest that epithelial cell differentiation may mediate spatial modulation of IL-36γ-mediated responses to promote mucosal homeostasis.

The expression of MMP9 and NGAL by epithelial cells can also be directly induced by bacteria [35–37]. Notably though, their expression in TIGK cells was not significantly induced by the periodontal pathogen *P. gingivalis*. This is consistent with other studies, which demonstrated that MMP9 and NGAL expression in primary gingival epithelial cells was not induced by *P. gingivalis* [48,49]. The extracellular gingipain proteinases produced by *P. gingivalis* are critical virulence factors [38]. Using a gingipain proteinase-deficient *P. gingivalis* mutant, we were able to establish that the proteinases antagonise the induction of MMP9 and NGAL expression in TIGK cells. Notably, we recently established that TIGK cells respond to *P. gingivalis* by expressing IL-36γ [20]. The expression of IL-36γ by the gingival epithelium in response to *P. gingivalis* is therefore likely to be important in mediating MMP9 and NGAL expression.

Several studies point to important roles for MMP9 and NGAL in oral mucosal immunity. MMP9 and NGAL levels in gingival crevicular fluid were shown to be increased in chronic periodontitis [50,51], and MMP9 was shown in a mouse model of apical periodontitis to be an important regulator of oral inflammation [52]. Recent studies indicate that oral mucosal immunity is likely to also be important for maintaining health at other sites in the body. For example, the oral cavity may be a reservoir for pathobionts that can exacerbate inflammatory bowel disease [53] and that have been linked to colorectal cancer [54,55]. Thus, further studies to establish the importance of the regulation of MMP9 and NGAL by IL-36γ for oral mucosal immunity are warranted.

In summary, IL-36γ strongly stimulates MMP9 and NGAL expression in gingival epithelial cells. Although MMP9 and NGAL have several functions in various tissues and pathologies, they play important roles

in promoting a normal microbiome and tissue homeostasis through antimicrobial activity and modulation of neutrophil function [12,35,42–45,56]. Therefore, this study extends our understanding of the role IL-36γ may play in promoting homeostasis of the oral mucosa.

Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Jacqueline E. Heath: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Glen M. Scholz:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision. **Paul D. Veith:** Methodology, Formal analysis, Writing - review & editing. **Eric C. Reynolds:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - review & editing, Supervision.

Acknowledgments

We thank Richard Lamont (University of Louisville) and Koji Nakayama (Nagasaki University) for generously providing the human TIGK cell line and the *Porphyromonas gingivalis* KDP136 mutant, respectively. We thank Shuai Nie, Ching-Seng Ang, and Nicholas Williamson for the acquisition of Orbitrap LC-MS/MS data within the Melbourne Mass Spectrometry and Proteomics Facility of the Bio21 Molecular Science and Biotechnology Institute at The University of Melbourne.

Funding

This research was supported by the Australian Government, Department of Industry, Innovation and Science Grant ID 20080108.

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