



# Regulation of the Peptidoglycan Amidase PGLYRP2 in Epithelial Cells by Interleukin-36 $\gamma$

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**ABSTRACT** Interleukin-36 (IL-36) cytokines are important regulators of mucosal homeostasis and inflammation. We have previously established that oral epithelial cells upregulate IL-36 $\gamma$  expression in response to the bacterial pathogen *Porphyromonas gingivalis*. Here, we have established that IL-36 $\gamma$  can stimulate the gene expression of mechanistically distinct antimicrobial proteins, including the peptidoglycan amidase PGLYRP2, in oral epithelial cells (e.g., TIGK cells). PGLYRP2 gene expression was not stimulated by either IL-17 or IL-22, thus demonstrating selectivity in the regulation of PGLYRP2 by IL-36 $\gamma$ . The IL-36 $\gamma$ -inducible expression of PGLYRP2 was shown to be mediated by IRAK1- and p38 mitogen-activated protein (MAP) kinase-dependent signaling. Furthermore, our finding that IL-36 $\gamma$ -inducible PGLYRP2 expression was reduced in proliferating TIGK cells but increased in terminally differentiating cells suggests that control of PGLYRP2 expression is associated with the maturation of the oral epithelium. PGLYRP2 expression in TIGK cells can also be directly stimulated by oral bacteria. However, the extracellular gingipain proteases (Kgp and RgpA/B) produced by *P. gingivalis*, which are critical virulence factors, can antagonize PGLYRP2 expression. Thus, the expression of IL-36 $\gamma$  by oral epithelial cells in response to *P. gingivalis* might enable the subsequent autocrine stimulation of PGLYRP2 expression. In summary, our data identify how IL-36 $\gamma$  may promote oral mucosal homeostasis by regulating PGLYRP2 expression.

**KEYWORDS** EGF, IL-36, *Porphyromonas gingivalis*, antimicrobial, epithelial cells, peptidoglycan recognition protein

The oral epithelium is a critical barrier to infection by bacterial pathogens. The integrity of the epithelium is maintained by cycles of cell proliferation and differentiation, whereby epithelial cells in the basal layer terminally differentiate as they migrate toward the epithelium surface (1). The expression of proteins with antimicrobial activity by the oral epithelium also provides protection against infection (2, 3). The  $\beta$ -defensins cause bacterial cell membrane permeation, resulting in cell lysis (4), while calprotectin, a heterodimeric complex of S100A8 and S100A9, exerts bacteriostatic activity by chelating essential divalent metal ions (5). Pattern recognition receptors, including Toll-like receptors (TLRs), are important regulators of the inducible expression of antimicrobial proteins in response to microorganisms (6). Importantly, antimicrobial protein expression is also induced by inflammatory cytokines. For example, interleukin-17 (IL-17) and IL-22 are important regulators of antimicrobial protein expression by epithelial cells in mucosal tissues and epidermis (7, 8).

Peptidoglycan recognition proteins (PGLYRP1 to 4) are a unique family of antimicrobial proteins (9). Peptidoglycan is a major component of the Gram-positive bacterial cell wall, while in Gram-negative bacteria it forms a thin layer in the periplasmic space between the inner and outer cell membranes. PGLYRP1, PGLYRP3, and PGLYRP4 can directly kill bacteria by inducing stress responses (10, 11). In contrast, PGLYRP2 is an *N*-acetyl-muramoyl-L-alanine amidase and while not directly bactericidal may function

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together with other host factors to cause bacterial cell death (12). PGLYRP2 can also potentially digest proinflammatory peptidoglycan into biologically inactive fragments (12, 13), which might be important for preventing inappropriate inflammation. Indeed, in an experimental model of colitis, PGLYRP2 was shown to protect mice from inflammation and loss of epithelial barrier function by promoting a normal gut microbiome (14). PGLYRP2 has also been reported to protect mice from psoriasis-like skin inflammation (15). Intriguingly, PGLYRP2 was shown to be important for the induction of inflammation by peptidoglycan in a mouse model of arthritis (16). Thus, the role of PGLYRP2 in host inflammation might be context dependent.

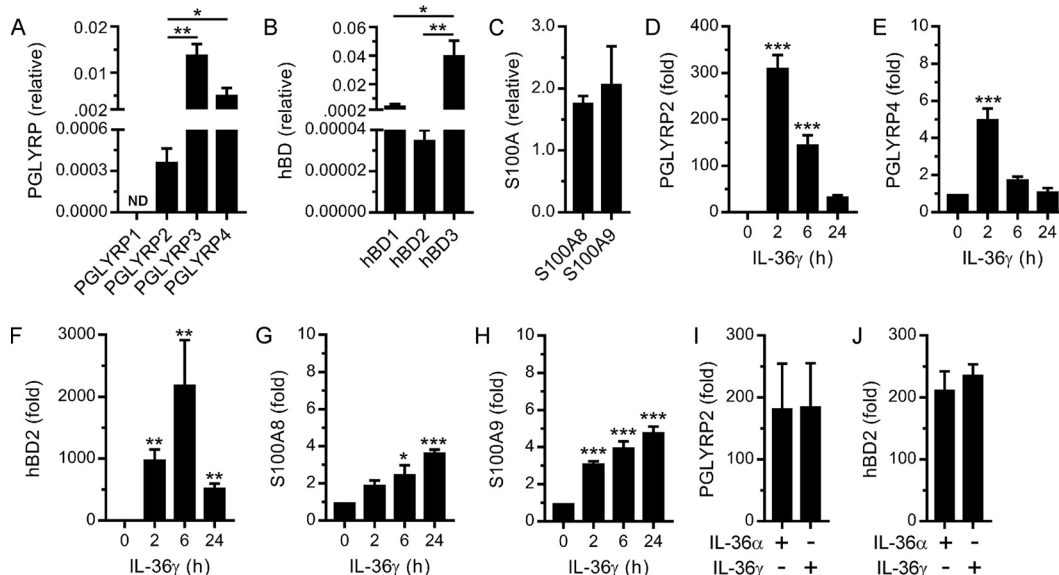
IL-36 cytokines (IL-36 $\alpha$ , IL-36 $\beta$ , and IL-36 $\gamma$ ) have recently emerged as key regulators of mucosal homeostasis and inflammation (17–22). Signaling by the IL-36 receptor (IL-36R) was shown to provide protection against bacterial infection in a mouse model of colitis (18), while IL-36 $\gamma$  was essential for protective mucosal immunity in mouse models of bacterial pneumonia (17). The expression of IL-36 cytokines by different cell types, including epithelial cells, has been shown to be induced by TLR signaling (23, 24). IL-36 cytokines can induce inflammation by stimulating the expression of cytokines and chemokines by epithelial cells and innate immune cells (e.g., dendritic cells) (17–26). IL-36 cytokines have also been shown to regulate T-helper cells (20, 25).

We recently established that oral epithelial cells upregulate IL-36 $\gamma$  expression in response to *Porphyromonas gingivalis* (24). *P. gingivalis* is a keystone pathogen in chronic periodontitis, an inflammatory disease that results from the breakdown of homeostasis between the host and plaque bacteria (27–29). In this study, we investigated the ability of IL-36 $\gamma$  to regulate the expression of antimicrobial proteins in oral epithelial cells (e.g., TIGK cells). We show that PGLYRP2 gene transcription in TIGK cells is robustly stimulated by IL-36 $\gamma$ . Furthermore, we show that IL-36 $\gamma$ -inducible PGLYRP2 expression in TIGK cells is reduced by epidermal growth factor receptor (EGFR) signaling but enhanced by terminal differentiation. Although PGLYRP2 expression can also be directly stimulated by oral bacteria, its expression in response to *P. gingivalis* is antagonized by the extracellular gingipain proteases produced by the pathogen. Collectively, our findings suggest that IL-36 $\gamma$  may play an important role in promoting tissue homeostasis in the oral mucosa through its regulation of PGLYRP2.

## RESULTS

### IL-36 $\gamma$ stimulates expression of antimicrobial proteins in oral epithelial cells.

Antimicrobial proteins and cytokines are important mediators of mucosal homeostasis and inflammation. We have previously shown that IL-36 $\gamma$  regulates the expression of various inflammatory cytokines in oral epithelial cells (24, 26). Thus, we sought to establish whether it also regulates the expression of antimicrobial proteins. First, we assessed the basal gene expression levels of antimicrobial proteins. Human oral epithelial cells (i.e., TIGK cells) were found to express PGLYRP2, PGLYRP3, and PGLYRP4 but not PGLYRP1 (Fig. 1A). Notably, the basal expression levels of PGLYRP3 and PGLYRP4 were significantly higher than that of PGLYRP2. TIGK cells also express the human  $\beta$ -defensins, hBD1, hBD2, and hBD3 (Fig. 1B), with hBD3 most highly expressed. The cells were also found to express S100A8 and S100A9 (Fig. 1C). Next, we tested the ability of IL-36 $\gamma$  to stimulate their expression. As shown in Fig. 1D, IL-36 $\gamma$  strongly stimulated the expression of PGLYRP2. In contrast, PGLYRP3 expression was not stimulated (data not shown), and PGLYRP4 expression was stimulated only weakly (Fig. 1E). IL-36 $\gamma$  strongly stimulated the expression of hBD2 (Fig. 1F) but not hBD1 and hBD3 (data not shown). IL-36 $\gamma$  also stimulated the expression of S100A8 and S100A9 (Fig. 1G and H), albeit weakly in comparison to PGLYRP2 and hBD2. Accordingly, we subsequently focused on the regulation of PGLYRP2 and hBD2 by IL-36 $\gamma$ . By treating TIGK cells with actinomycin D prior to IL-36 $\gamma$  stimulation, we could confirm that the upregulation of PGLYRP2 and hBD2 mRNA levels was due to increased gene transcription (data not shown). In addition to shared functions, *in vivo* studies indicate that IL-36 cytokines may also have unique functions (18, 22, 30). Therefore, we tested the ability of IL-36 $\alpha$  to stimulate PGLYRP2 and hBD2 expression. The levels of stimulation of PGLYRP2 (Fig. 1I) and



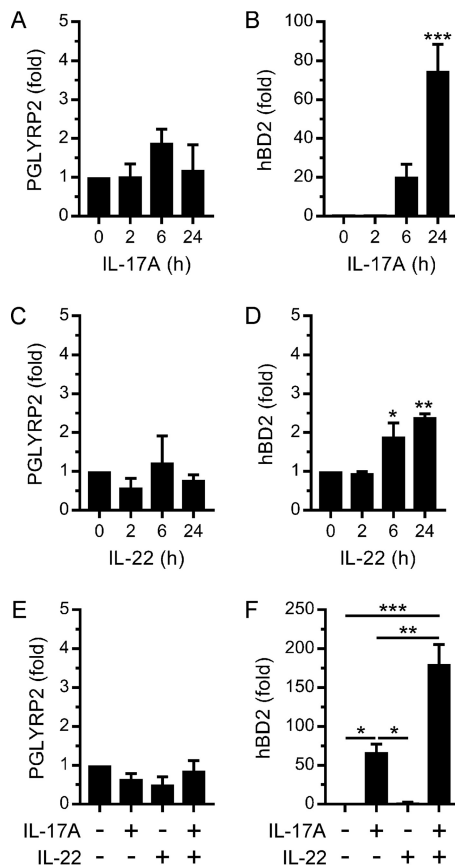
**FIG 1** Stimulation of antimicrobial protein gene expression in oral epithelial cells by IL-36 $\gamma$ . (A to C) Basal mRNA expression levels of the indicated PGLYRP (A), hBD (B), and S100A (C) proteins were measured ( $n = 3$ ). (D to H) TIGK cells were stimulated with IL-36 $\gamma$  (100 ng/ml) for the time indicated. PGLYRP2 (D), PGLYRP4 (E), hBD2 (F), S100A8 (G), and S100A9 (H) mRNA levels were then measured ( $n = 3$ ). (I and J) TIGK cells were stimulated with IL-36 $\alpha$  (100 ng/ml) or IL-36 $\gamma$  (100 ng/ml) for 2 h. PGLYRP2 (I) and hBD2 (J) mRNA levels were then measured ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

hBD2 (Fig. 1J) expression by IL-36 $\alpha$  and IL-36 $\gamma$  were highly comparable. These data suggest that IL-36 $\gamma$  produced by the oral epithelium might promote host defense and mucosal homeostasis by stimulating the expression of mechanistically diverse antimicrobial proteins, including the peptidoglycan amidase PGLYRP2.

#### PGLYRP2 expression in oral epithelial cells is not stimulated by IL-17 or IL-22.

The adaptive immune cytokines IL-17 and IL-22 are also important mediators of host defense at mucosal surfaces, where they stimulate the expression of antimicrobial proteins in epithelial cells (7, 8). Therefore, we tested the ability of IL-17 and IL-22 to stimulate the expression of PGLYRP2 and hBD2 in TIGK cells. As shown in Fig. 2A, IL-17A did not stimulate PGLYRP2 expression. In contrast, IL-17A stimulated hBD2 expression (Fig. 2B), although the magnitude of the response was smaller than when TIGK cells were stimulated with IL-36 $\gamma$  (Fig. 1F). Similarly, IL-22 did not stimulate PGLYRP2 expression (Fig. 2C) and stimulated hBD2 expression only weakly (Fig. 2D). IL-17 and IL-22 have been shown to synergistically stimulate antimicrobial protein (e.g., hBD2) expression in epidermal keratinocytes (31). Therefore, we investigated their ability to synergistically stimulate PGLYRP2 and hBD2 expression in TIGK cells. Notably, PGLYRP2 expression was not stimulated when the cells were treated concurrently with IL-17A and IL-22 (Fig. 2E). In contrast, hBD2 expression was synergistically stimulated (Fig. 2F). Collectively, the above data identify important differences in the regulation of antimicrobial protein gene expression in oral epithelial cells by IL-17/IL-22 and IL-36 $\gamma$ .

**IL-36 $\gamma$  stimulates PGLYRP2 expression via IRAK1 and p38 MAP kinase.** Our findings position IL-36 $\gamma$  as an important regulator of PGLYRP2 in oral epithelial cells. Therefore, we investigated the signaling pathways that regulate its expression downstream of the IL-36R. We have previously shown that IRAK1 mediates the stimulation of inflammatory cytokines and chemokines by IL-36 $\gamma$  in oral epithelial cells (e.g., TIGK cells) (24). Consistently, small interfering RNA (siRNA)-mediated gene silencing of IRAK1 in TIGK cells inhibited the stimulation of PGLYRP2 expression by IL-36 $\gamma$  (Fig. 3A); it also inhibited the stimulation of hBD2 expression (Fig. 3B). In addition to IRAK1, IL-36 $\gamma$  activates the mitogen-activated protein (MAP) kinases p38 MAP kinase and ERK1/2 and the NF- $\kappa$ B transcription factor in TIGK cells (26). Thus, we used pharmacologic inhibitors to establish their importance for the stimulation of PGLYRP2 by IL-36 $\gamma$ . The p38 MAP

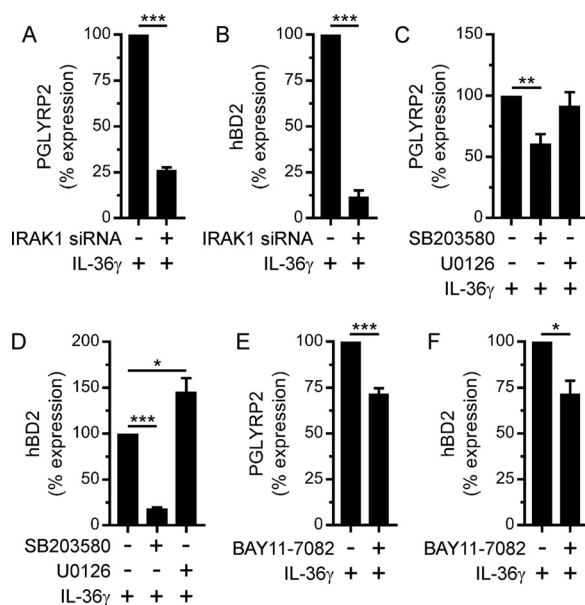


**FIG 2** Regulation of PGLYRP2 and hBD2 expression in oral epithelial cells by IL-17 and IL-22. (A to D) TIGK cells were stimulated with IL-17A (100 ng/ml) (A and B) or IL-22 (50 ng/ml) (C and D) for the times indicated. PGLYRP2 (A and C) and hBD2 (B and D) mRNA levels were then measured ( $n = 3$ ). (E and F) TIGK cells were stimulated with IL-17A (100 ng/ml) and IL-22 (50 ng/ml) for 24 h. PGLYRP2 (E) and hBD2 (F) mRNA levels were then measured ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

kinase inhibitor SB203580 reduced the IL-36 $\gamma$  stimulation of PGLYRP2 expression by 40% (Fig. 3C). In contrast, the stimulation of PGLYRP2 expression was not affected by the MEK (ERK1/2) inhibitor U0126 (Fig. 3C). For comparison, the p38 MAP kinase inhibitor significantly reduced the IL-36 $\gamma$ -inducible stimulation of hBD2 expression (Fig. 3D), whereas the MEK (ERK1/2) inhibitor potentiated the stimulation of hBD2 expression (Fig. 3D). The NF- $\kappa$ B inhibitor BAY11-7082 partially inhibited the stimulation of PGLYRP2 (Fig. 3E) and hBD2 (Fig. 3F) expression by IL-36 $\gamma$ . These data indicate that IL-36 $\gamma$  induces the transcription of PGLYRP2, as well as hBD2, by activating IRAK1 and p38 MAP kinase signaling. Interestingly, activation of MEK/ERK signaling by IL-36 $\gamma$  may limit the upregulation of hBD2 expression without affecting PGLYRP2.

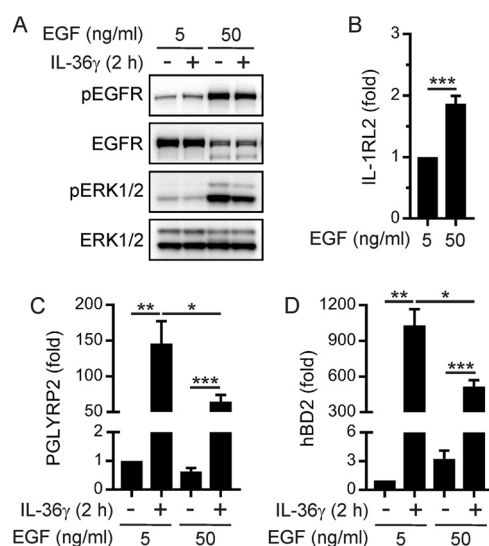
#### EGFR signaling antagonizes the stimulation of PGLYRP2 expression by IL-36 $\gamma$ .

The proliferation of epithelial cells is important for the ongoing functional integrity of the oral epithelium and repair following injury. Therefore, we investigated the effect of EGF-induced proliferation on the stimulation of PGLYRP2 expression by IL-36 $\gamma$ . Specifically, TIGK cells were cultured in the presence of either 5 ng/ml EGF or 50 ng/ml EGF to induce different levels of EGFR signaling; thereafter, the cells were stimulated with IL-36 $\gamma$ . Western blotting of cell lysates with phospho-specific antibodies confirmed that the culturing of TIGK cells in the presence of 50 ng/ml EGF resulted in greatly increased EGFR and ERK1/2 phosphorylation (Fig. 4A). Consistent with ligand-induced internalization and degradation of receptor tyrosine kinases, total EGFR levels were reduced when TIGK cells were cultured in the presence of 50 ng/ml EGF (Fig. 4A). The expression levels of the IL-36R subunit IL-1RL2 were weakly increased (Fig. 4B) and IL-1RAcP levels unchanged (data not shown) under the same conditions. We then determined whether

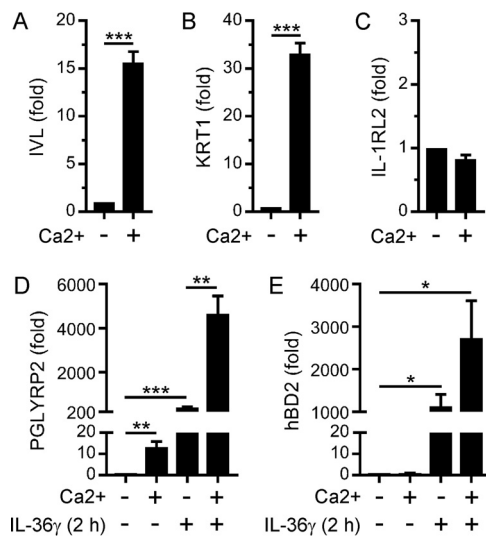


**FIG 3** IRAK1 and p38 MAP kinase signaling regulates IL-36 $\gamma$ -inducible PGLYRP2 and hBD2 expression in oral epithelial cells. (A and B) TIGK cells were transfected with an IRAK1 (+) or control (-) siRNA and then subsequently stimulated with IL-36 $\gamma$  (100 ng/ml) for 2 h. PGLYRP2 (A) and hBD2 (B) mRNA levels were then measured ( $n = 3$ ). (C to F) TIGK cells were treated with 5  $\mu$ M SB203580 or 10  $\mu$ M U0126 (C and D) or 10  $\mu$ M BAY11-7082 (E and F) for 30 min and then stimulated with IL-36 $\gamma$  for 2 h. PGLYRP2 (C and E) and hBD2 (D and F) mRNA levels were then measured ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

increased EGFR signaling affects the stimulation of PGLYRP2 expression by IL-36 $\gamma$ . Notably, the stimulation of PGLYRP2 expression was significantly reduced (Fig. 4C). Increased EGFR signaling also reduced IL-36 $\gamma$ -inducible hBD2 expression (Fig. 4D). Collectively, these data indicate that EGFR signaling in oral epithelial cells dampens the stimulation of PGLYRP2 and hBD2 expression by IL-36 $\gamma$ .



**FIG 4** EGFR signaling dampens IL-36 $\gamma$ -inducible PGLYRP2 and hBD2 expression in oral epithelial cells. TIGK cells were cultured with 5 ng/ml or 50 ng/ml EGF for 24 h and then stimulated with IL-36 $\gamma$  (100 ng/ml) for 2 h. (A) The cells were lysed, and then aliquots of the lysates were subjected to Western blotting with the indicated antibodies. The data are representative of two independent experiments. (B to D) IL-1RL2 (B), PGLYRP2 (C), and hBD2 (D) mRNA levels were then measured ( $n \geq 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

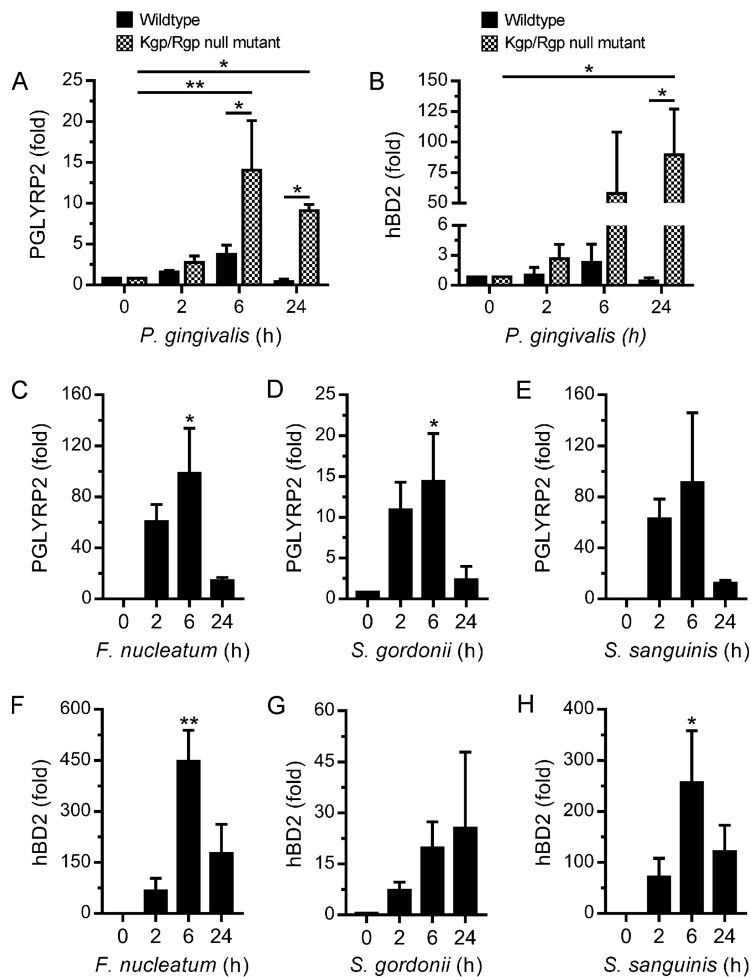


**FIG 5** Terminal differentiation enhances IL-36 $\gamma$ -inducible PGLYRP2 and hBD2 expression in oral epithelial cells. TIGK cells were cultured in the presence of 0.09 mM (–) or 1.8 mM (+) calcium for 48 h (A to C) and then stimulated with IL-36 $\gamma$  (100 ng/ml) for 2 h (D and E). IVL (A), KRT1 (B), IL-1RL2 (C), PGLYRP2 (D), and hBD2 (E) mRNA levels were then measured ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

### Epithelial differentiation potentiates the stimulation of PGLYRP2 expression by IL-36 $\gamma$ .

The differentiation of oral epithelial cells, whereby cells in the basal layer terminally differentiate as they migrate toward the epithelium surface, is important for maintaining the integrity and barrier function of the oral epithelium. Therefore, we also investigated the effect of differentiation on the regulation of PGLYRP2 expression by IL-36 $\gamma$ . TIGK cells were cultured in the presence of 1.8 mM calcium to induce their terminal differentiation, which was confirmed by measuring the expression levels of the differentiation markers involucrin (IVL) (Fig. 5A) and keratin 1 (KRT1) (Fig. 5B). Differentiation did not affect the expression levels of IL-1RL2 (Fig. 5C) or IL-1RAcP (data not shown). Remarkably, though, whereas IL-36 $\gamma$  stimulated a 400-fold increase in PGLYRP2 expression in undifferentiated TIGK cells, PGLYRP2 expression in terminally differentiated cells was stimulated more than 4,000-fold (Fig. 5D). The basal expression levels of PGLYRP2 were also significantly higher in differentiating cells (Fig. 5D). Differentiation likewise potentiated the stimulation of hBD2 expression by IL-36 $\gamma$  (Fig. 5E), albeit not to the same extent as for PGLYRP2. In contrast to PGLYRP2, differentiation did not affect the basal expression levels of hBD2 (Fig. 5E). These data suggest that IL-36 $\gamma$  likely stimulates stronger homeostatic antimicrobial responses in differentiating oral epithelial cells, in line with the superficial cells of the oral mucosa being directly exposed to bacteria.

**Stimulation of PGLYRP2 expression by oral bacteria.** PGLYRP2 gene expression in epidermal keratinocytes has previously been shown to be stimulated by bacteria (32). This raised the possibility that *P. gingivalis* might also directly stimulate PGLYRP2 gene expression by oral epithelial cells, independently of its stimulation of IL-36 $\gamma$ . As shown in Fig. 6A, however, PGLYRP2 gene expression was not significantly induced (<4-fold) when TIGK cells were challenged with *P. gingivalis*, thus suggesting that *P. gingivalis* does not directly stimulate PGLYRP2 expression in oral epithelial cells. The extracellular gingipain proteases (Kgp and RgpA/B) produced by *P. gingivalis* are critical virulence factors (28, 29), and therefore we investigated whether they might antagonize the stimulation of PGLYRP2 expression. Notably, PGLYRP2 expression was significantly stimulated (10- to 15-fold) when TIGK cells were challenged with an isogenic *P. gingivalis* gingipain protease (Kgp/Rgp)-null mutant (Fig. 6A). Similar observations were made for hBD2, whereby its expression was significantly stimulated by a *P. gingivalis* gingipain protease-null mutant (Fig. 6B). Given these findings, we investigated the ability of other oral bacteria to stimulate PGLYRP2 expression. In contrast to the case for



**FIG 6** Stimulation of PGLYRP2 and hBD2 expression in oral epithelial cells by bacteria. (A and B) TIGK cells were cultured with wild-type *P. gingivalis* or *P. gingivalis* KDP136 (Kgp/Rgp-null mutant) at an MOI of 100:1 for the times indicated. PGLYRP2 (A) and hBD2 (B) mRNA levels were then measured ( $n = 3$ ). (C to H) TIGK cells were cultured with *F. nucleatum* (C and F), *S. gordonii* (D and G), and *S. sanguinis* (E and H) at an MOI of 100:1 for the times indicated. PGLYRP2 (C to E) and hBD2 (F to H) mRNA levels were then measured ( $n = 3$ ). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

*P. gingivalis*, PGLYRP2 expression was strongly stimulated by *Fusobacterium nucleatum*, *Streptococcus gordonii*, and *Streptococcus sanguinis* (Fig. 6C to E). These bacterial species also strongly stimulated hBD2 expression (Fig. 6F to H). Collectively, these results suggest that oral epithelial cells can directly upregulate the expression of PGLYRP2 and hBD2 in response to some bacterial species. In the case of *P. gingivalis*, the gingipain proteases can antagonize the stimulation of PGLYRP2 and hBD2 expression.

## DISCUSSION

IL-36 cytokines are important regulators of mucosal homeostasis and inflammation. We recently established that oral epithelial cells selectively upregulate IL-36 $\gamma$  in response to the bacterial pathogen *P. gingivalis* (24) and that IL-36 $\gamma$  can stimulate oral epithelial cells to express inflammatory cytokines (24, 26). Here, we have established that IL-36 $\gamma$  also regulates the expression of antimicrobial proteins by oral epithelial cells, including the peptidoglycan amidase PGLYRP2. PGLYRP2 has previously been demonstrated to be important for intestinal homeostasis (14). By analogy, the regulation of PGLYRP2 by IL-36 $\gamma$  might be important for homeostasis in the oral mucosa.

PGLYRP2 gene expression levels in oral epithelial cells (e.g., TIGK cells) were found to be 20- to 40-fold lower than those for PGLYRP3 and PGLYRP4. However, PGLYRP2 expression was strongly induced by IL-36 $\gamma$ , such that the expression levels of PGLYRP2

are likely to be higher than those of PGLYRP3 and PGLYRP4 following IL-36 $\gamma$  stimulation. Significantly, PGLYRP2 expression was not induced by either IL-17A or IL-22. These adaptive immune cytokines are important mediators of host defense at mucosal sites where they stimulate epithelial cells to express antimicrobial proteins (7, 8). Thus, our findings identify IL-36 $\gamma$  as a key regulator of PGLYRP2 expression in epithelial cells.

In contrast to PGLYRP3 and PGLYRP4, which can kill bacteria by inducing stress responses (10, 11), PGLYRP2 is not directly bacteriolytic and may function together with other host factors to cause bacterial cell death (12). In this regard, we found that IL-36 $\gamma$  also strongly stimulated oral epithelial cells to express hBD2. The  $\beta$ -defensins kill bacteria by forming multimeric pores in the bacterial cell membrane, resulting in cell lysis (4). Although not demonstrated, PGLYRP2 could potentially cooperate with hBD2 to mediate bacterial killing, for instance, increasing the accessibility of hBD2 to the cell membranes of Gram-positive bacteria by hydrolyzing peptidoglycan.

The epithelial cells of the oral mucosa would potentially be exposed to bacterial cell wall fragments with proinflammatory properties (e.g., peptidoglycan fragments) when bacterial killing is mediated by  $\beta$ -defensins, thus possibly stimulating inappropriate inflammation. PGLYRP2 has been reported to digest inflammatory peptidoglycan into biologically inactive fragments (12, 13). Moreover, PGLYRP2 was shown in mice to promote the maintenance of a normal gut microbiome and thereby protected the mice from experimentally induced colitis (14). Consequently, the coexpression of PGLYRP2 with hBD2 might be important for preventing inappropriate inflammation in the oral mucosa.

The IL-36 $\gamma$ -inducible expression of PGLYRP2 in TIGK cells was dependent on signaling by IRAK1 and p38 MAP kinase. This is consistent with an earlier study which demonstrated that the inducible expression of PGLYRP2 in epidermal keratinocytes was likewise regulated by p38 MAP kinase (32). Interestingly, we found that EGFR signaling, which promotes epithelial cell proliferation, dampened the stimulation of PGLYRP2 expression by IL-36 $\gamma$ . Conversely, calcium-induced terminal differentiation greatly potentiated the IL-36 $\gamma$ -inducible expression of PGLYRP2. Differentiation also enhanced the IL-36 $\gamma$ -inducible expression of hBD2, albeit not to the same extent. The augmenting effects of cell differentiation on PGLYRP2 and hBD2 expression are consistent with the superficial cells of the oral mucosa being directly exposed to bacteria. Most of the bacteria that colonize the oral mucosa are commensal species, and therefore overt host inflammation is normally not required to maintain host-microbiota homeostasis; in fact, inappropriate inflammation can promote dysbiosis, as occurs in chronic periodontitis (27, 28). Given the ability of PGLYRP2 to inactivate inflammatory peptidoglycan (12, 13), higher levels of PGLYRP2 expression by terminally differentiating oral epithelial cells might serve to limit the stimulation of host inflammation by commensal bacteria.

We have shown that *P. gingivalis* induces IL-36 $\gamma$  expression in TIGK cells (24). However, we did not detect significant upregulation of PGLYRP2 gene expression when TIGK cells were challenged with the pathogen. Although this might suggest that IL-36 $\gamma$  may not act in an autocrine manner to induce PGLYRP2 expression, following its stimulation by *P. gingivalis*, IL-36 $\gamma$  is secreted as pro-IL-36 $\gamma$ , which is up to 1,000 times less active than mature IL-36 $\gamma$  (33). Hence, proteolytic processing of pro-IL-36 $\gamma$  to IL-36 $\gamma$  by neutrophil proteases is required for significant activation of IL-36R signaling (34). The absence of neutrophils in the cell culture system used in this study would thus preclude the stimulation of PGLYRP2 expression by autocrine IL-36 $\gamma$ .

PGLYRP2 gene expression was strongly stimulated when TIGK cells were challenged with other oral bacterial species, such as *F. nucleatum* and *S. sanguinis*. This suggests that PGLYRP2 expression in oral epithelial cells can also be directly stimulated by bacteria. Indeed, an earlier report demonstrated the ability of bacteria (e.g., *Bacillus subtilis*) to stimulate PGLYRP2 expression in epidermal keratinocytes (32). The extracellular gingipain proteases (Kgp and RgpA/B) expressed by *P. gingivalis* are central to the ability of the pathogen to subvert the host immune response and promote the breakdown of tissue homeostasis (28, 29). Notably, PGLYRP2 expression was significantly induced when TIGK cells were challenged with an isogenic *P. gingivalis* Kgp/



Rgp-null mutant. The gingipain proteases may therefore subvert the tissue-homeostatic functions of PGLYRP2 by antagonizing its expression by oral epithelial cells. Consequently, the stimulation of PGLYRP2 expression in response to *P. gingivalis* might be dependent on the production of IL-36 $\gamma$  by the oral epithelium.

In summary, IL-36 $\gamma$  robustly stimulates PGLYRP2 gene expression in oral epithelial cells. Although the functions of PGLYRP2 are still to be completely defined, in the intestines PGLYRP2 appears to play an important role in promoting a normal microbiome and mucosal homeostasis (14). Thus, our study provides new insight into how IL-36 $\gamma$  may promote oral mucosal homeostasis by regulating PGLYRP2 expression.

## MATERIALS AND METHODS

**Reagents.** DermaLife keratinocyte growth medium and supplements (transforming growth factor  $\alpha$  [TGF- $\alpha$ ], insulin, epinephrine, apo-transferrin, hydrocortisone, bovine pituitary extract, and glutamine) were from Lifeline Cell Technology. Human IL-17A (Gly24 to Ala155), IL-22 (Ala34 to Ile179), IL-36 $\alpha$  (Lys6 to Phe158), and IL-36 $\gamma$  (Ser18 to Asp169) were from R&D Systems. Recombinant human EGF, Opti-MEM I reduced-serum medium, Lipofectamine RNAiMAX transfection reagent, and precast 10% NuPAGE gels were from Life Technologies. The ON-TARGETplus IRAK1 and nontargeting control siRNAs were from GE Healthcare. The anti-phospho-EGFR (pY1068), anti-EGFR, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies were from Cell Signaling Technology. BAY11-7082, SB203580, and U0126 were from Merck Millipore, while actinomycin D was from Sigma-Aldrich.

**Mammalian cell culture.** Human TIGK gingival epithelial cells (35) were cultured in DermaLife keratinocyte growth medium supplemented with 0.5 ng/ml TGF $\alpha$ , 5  $\mu$ g/ml insulin, 1  $\mu$ M epinephrine, 5  $\mu$ g/ml apo-transferrin, 100 ng/ml hydrocortisone, 0.4% bovine pituitary extract, and 6 mM glutamine. The cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Bacterial cell culture.** *P. gingivalis* (ATCC 33277), *F. nucleatum* (ATCC 10953), *S. gordonii* (ATCC 35105), and *S. sanguinis* (ATCC 10556) 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 ( $\Delta$ kgp  $\Delta$ rgpA  $\Delta$ rgpB) was cultured as previously described (36). The bacteria were maintained on horse blood agar plates at 37°C in an anaerobic atmosphere of 5% H<sub>2</sub>, 80% N<sub>2</sub>, and 15% CO<sub>2</sub> (*P. gingivalis*, *F. nucleatum*, and *S. sanguinis*) or an aerobic atmosphere of 100% air (*S. gordonii*). Bacterial colonies were used to inoculate brain heart infusion medium supplemented with 5  $\mu$ g/ml hemin plus 0.5 mg/ml cysteine (anaerobes) and 5  $\mu$ g/ml menadione (*P. gingivalis*) (37).

**Infection of oral epithelial cells with bacteria.** Logarithmic-growth-phase bacteria were harvested by centrifugation at 7,000  $\times$  g for 20 min at 4°C and suspended in keratinocyte growth medium. TIGK cell monolayers were incubated with bacteria at a multiplicity of infection (MOI) of 100:1 (37).

**RNA purification, reverse transcription, and qPCR.** 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 predeveloped TaqMan assays (Life Technologies) for the following genes: hBD1 (Hs00608345\_m1), hBD2 (Hs00175474\_m1), hBD3 (Hs04194486\_g1), IL-1RAcP (Hs00895050\_m1), IL-1RL2 (Hs00909276\_m1), IRAK1 (Hs01018347\_m1), IVL (Hs00902520\_m1), KRT1 (Hs01549614\_g1), PGLYRP1 (Hs00175475\_m1), PGLYRP2 (Hs00994650\_m1), PGLYRP3 (Hs00364657\_m1), PGLYRP4 (Hs01120180\_m1), S100A8 (Hs00374264\_g1), and S100A9 (Hs00610058\_m1). PCR was performed on a QuantStudio 7 Flex real-time PCR system (Life Technologies). The data were normalized against the hypoxanthine guanine phosphoribosyl transferase (HPRT) or TATA box binding protein (TBP) gene.

**RNA interference-mediated gene silencing.** A reverse transfection protocol was used for siRNA transfection of TIGK cells (38). Briefly, the IRAK1 and nontargeting control siRNAs were diluted to 120 nM with 100  $\mu$ l Opti-MEM I reduced-serum medium, mixed with 100  $\mu$ l Opti-MEM medium containing 1.0  $\mu$ l Lipofectamine RNAiMAX transfection reagent, and incubated at room temperature for 20 min. TIGK cells ( $2 \times 10^5$  cells in 1 ml keratinocyte complete growth medium) were seeded in 12-well plates and cultured with the transfection cocktail for 24 h. Thereafter, the medium was replaced and the cells cultured for a further 24 h.

**Cell lysis and Western blotting.** TIGK cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with IGEPAL lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 10% glycerol, 20 mM  $\beta$ -glycerol phosphate, 1 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors) on ice for 60 min. Thereafter, the lysates were clarified by centrifugation (13,000  $\times$  g for 10 min at 4°C), and the protein concentrations were measured using a protein assay kit (Bio-Rad). Cell lysates were incubated with NuPAGE lithium dodecyl sulfate (LDS) sample buffer for 10 min at 70°C and then subjected to electrophoresis on a 10% NuPAGE gel using MOPS (morpholinepropanesulfonic acid) buffer (Life Technologies). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which was then blocked with 3% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.02% Tween 20) for 60 min at room temperature. The membrane was incubated with the primary antibody (diluted in 1% BSA in TBST) overnight at 4°C. The membrane was washed with TBST, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in 1% BSA in TBST) for 60 min at room temperature. Following washing with TBST, immunoreactive proteins were visualized using ECL reagents (Millipore) and a Fujifilm Las-3000 Imager (Fujifilm, Japan).

**Statistical analysis.** Data combined from three or more independent biological replicate experiments are presented as the mean  $\pm$  standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 7. Differences between two groups were evaluated using the Student *t* test. For multiple comparisons, statistical analysis was performed by analysis of variance (ANOVA) with Dunnett's *post hoc* test. A *P* value of  $<0.05$  was considered statistically significant.

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