**MEK-ERK signaling diametrically controls the stimulation of IL-23p19 and EBI3
expression in epithelial cells by IL-36 γ**

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ABSTRACT (250 word limit – currently 240)

Interleukin (IL)-36 (IL-36) cytokines are important regulators of mucosal homeostasis and inflammation. We previously established that oral epithelial cells strongly upregulate IL-36 γ expression in response to the bacterial pathogen *Porphyromonas gingivalis*. Here, we have established that IL-36 γ stimulates the expression of the IL-12 cytokine family members, *IL-23p19* and *Epstein-Barr Virus-Induced Gene 3 (EBI3)*, by oral epithelial cells; their expression was also selectively stimulated by IL-36 α . Notably, *IL-23p19* and *EBI3* expression was not stimulated by *P. gingivalis*, thus suggesting that their expression by the oral epithelium in response to *P. gingivalis* is likely to be mediated in an autocrine manner by IL-36 γ . The IL-36 γ -inducible expression of *IL-23p19* and *EBI3* was found to be diametrically regulated by the mitogen-activated protein kinase/extracellular signal regulated kinase (MEK)-extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, whereby the activation of MEK-ERK signaling likely functions as a negative feedback mechanism to limit *EBI3* expression. Furthermore, epidermal growth factor (EGF) receptor (EGFR) signaling, which is important for mucosal homeostasis, was demonstrated to modulate, in a MEK-ERK-dependent manner, the stimulation of *IL-23p19* and *EBI3* expression by IL-36 γ . *IL-23p19* and *EBI3* have recently been shown to heterodimerize to form the novel cytokine IL-39 and promote neutrophil expansion. *EBI3* has been shown to also have IL-12 cytokine family-independent functions (e.g. mediating IL-6 *trans*-signaling). Thus, this study not only advances our understanding of how IL-36 cytokines may control mucosal inflammation, but also establishes EGFR signaling as a potentially important modulator of IL-36 cytokine function.

INTRODUCTION

Mucosal epithelial cells are critical mediators of host defense against infection by microbial pathogens. In addition to barrier function, they express pattern recognition receptors, including members of the Toll-like receptor (TLR) family, which enable them to also actively participate in host defense.¹⁻⁴ Specifically, the engagement of TLRs by microbial ligands triggers downstream signaling pathways that activate the host immune response. For example, the TLR adapter protein myeloid differentiation primary response 88 can initiate downstream signaling via Interleukin (IL)-1 receptor associated kinase 1 (IRAK1), resulting in the activation of mitogen-activated protein (MAP) kinases (e.g. extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinase) and transcription factors, including nuclear factor- κ B (NF- κ B). This ultimately culminates in the expression of inflammatory cytokines and chemokines that activate the immune response.⁵

IL-36 family cytokines (IL-36 α , IL-36 β , and IL-36 γ), which belong to the IL-1 superfamily, have recently emerged as key regulators of immune responses at mucosal sites.⁶⁻¹¹ They are expressed by a range of cell types, including mucosal epithelial cells, macrophages, and dendritic cells, and induced in response to TLR signaling.¹²⁻¹⁴ IL-36 cytokines are potent stimulators of the expression of neutrophil chemokines, such as *IL-8* and *chemokine (C-X-C motif) ligand 1 (CXCL1)*, by mucosal epithelial cells and innate immune cells,^{12, 13, 15} and accordingly are important regulators of neutrophil responses during infection.⁷⁻¹¹ IL-36 cytokines can also regulate immune responses by stimulating the expression of T cell chemokines, including *CXCL10* and *chemokine (C-C motif) ligand 20 (CCL20)*.¹²⁻¹⁵ They can also directly regulate T cell responses by stimulating the proliferation of CD4⁺ T helper (Th) cells.^{15, 16}

IL-12 family cytokines are likewise important regulators of immune responses.^{17, 18} These heterodimeric cytokines are formed by the chain-pairing of an alpha subunit

(IL-12p35, IL-23p19, and IL-27p28) with a soluble receptor-like beta subunit (IL-12p40 and Epstein-Barr Virus-Induced Gene 3 (EBI3)). The family includes: IL-12 (IL-12p35/IL-12p40), IL-23 (IL-23p19/IL-12p40), IL-27 (IL-27p28/EBI3), IL-35 (IL-12p35/EBI3), and IL-39 (IL-23p19/EBI3).^{17, 18} IL-12 and IL-23, which are largely produced by macrophages and dendritic cells, are pro-inflammatory and regulate the development and effector functions of Th cells, namely Th1 and Th17 cells, respectively.¹⁸ IL-27 exerts mainly anti-inflammatory effects, such as inducing T regulatory cells and inhibiting the development of Th17 cells.¹⁹ IL-35 is an anti-inflammatory cytokine, and primarily produced by T regulatory cells.²⁰ IL-39 was identified only very recently, and has been shown to be expressed by keratinocytes and B cells.^{21, 22} Although a role for IL-39 in the regulation of T cells has not been reported, it induces inflammation and promotes the expansion of neutrophils in lupus-prone mice.^{22, 23} EBI3 also has IL-12 family cytokine-independent functions, including mediating IL-6 *trans*-signaling in CD4⁺ T cells,²⁴ and inhibiting collagen expression in dermal fibroblasts.²⁵ Significantly, IL-36 cytokines can synergize with IL-12 to promote the Th1 polarization of naïve T cells,^{7, 16} and conversely inhibit the development of T regulatory cells.⁹

We recently established that oral epithelial cells specifically up-regulate the expression of IL-36 γ in response to the bacterial pathogen *Porphyromonas gingivalis*.¹³ *P. gingivalis* is a keystone pathogen in chronic periodontitis, an inflammatory disease in which the breakdown of periodontal tissues can result in tooth loss as well as compromise systemic health.^{26, 27} In addition to neutrophil chemokines, IL-36 γ also potently stimulates the expression of T cell chemokines (e.g. *CCL5* and *CCL20*) by oral epithelial cells.¹³ Here, we investigated whether IL-36 cytokines may regulate immune responses by also stimulating the expression of IL-12 family cytokines. We show that IL-36 γ selectively stimulated the expression of *IL-23p19* and *EBI3* by oral epithelial cells. Mechanistically, *IL-23p19* and *EBI3*

91 expression were diametrically regulated by the MEK-ERK pathway, whereby the activation
92 of MEK-ERK signaling likely functions as a negative feedback mechanism to limit *EBI3*
93 expression. Significantly, the IL-36 γ -inducible expression of *IL-23p19* and *EBI3* were
94 modulated, in a MEK-ERK-dependent manner, by epidermal growth factor (EGF) receptor
95 (EGFR) signaling. Together, these findings extend our understanding of how IL-36 cytokines
96 regulate immune responses, as well as establish how EGFR signaling may modulate IL-36
97 cytokine function.

RESULTS

IL-36 γ selectively stimulates *IL-23p19* and *EBI3* expression in oral epithelial cells

The inflammatory cytokine response of mucosal epithelia to microbial pathogens is important for activating the immune response. We recently established that IL-36 γ plays a role in the inflammatory response of oral epithelial cells to the bacterial pathogen *P. gingivalis*.¹³ For instance, IL-36 γ was shown to strongly stimulate the expression of T cell chemokines (e.g. *CCL5* and *CCL20*) by not only dendritic cells and macrophages, but also by oral epithelial cells¹³. This indicates that oral epithelium-derived IL-36 γ likely activates the immune response to microbial pathogens by also functioning in an autocrine manner. IL-12 family cytokines are important regulators of T cell responses,^{17, 18} and therefore we investigated the ability of IL-36 γ to stimulate their expression in oral epithelial cells (e.g. TIGK cells). We have previously shown that IL-36 γ can stimulate strong cytokine responses in TIGK cells.¹³ Although IL-36 γ did not stimulate *IL-12p35* expression in TIGK cells (Figure 1a), it strongly stimulated *IL-23p19* expression (Figure 1b), and weakly stimulated *IL-27p28* expression (Figure 1c). Interestingly, although *IL-12p40* expression was not stimulated (Fig. 1d), *EBI3* expression was robustly stimulated by IL-36 γ (Figure 1e). To confirm that the increased *IL-23p19* and *EBI3* mRNA levels were due to increased transcription, TIGK cells were treated with actinomycin D (ActD) prior to IL-36 γ stimulation. Consistently, ActD inhibited the increase in *IL-23p19* and *EBI3* mRNA levels (Figures 1f and g). IL-23p19 was recently shown to heterodimerize with EBI3 to form IL-39.^{21, 22} Additionally, EBI3 can also function independently of IL-23p19 (and IL-12p35 and IL-27p28).^{24, 25} Thus, the levels of IL-23p19 and EBI3 in the cell culture supernatants were measured. Although IL-23p19 was not detected in the supernatants (data not shown), EBI3 levels were greatly increased in culture supernatants from IL-36 γ -stimulated cells (Figure 1h). Oral epithelium-derived IL-36 γ is likely to also function in a paracrine manner to

stimulate inflammation. Therefore, we tested the ability of IL-36 γ to stimulate the expression of *IL-23p19* and *EBI3* in human monocyte-derived dendritic cells and monocyte-derived macrophages. Notably, IL-36 γ did not stimulate significant increases in the expression levels of *IL-23p19* and *EBI3* in dendritic cells (Figure 1i and j) and macrophages (Figure 1k and l). Taken together, these data suggest that oral epithelium-derived IL-36 γ may regulate the immune response to pathogen infection, in part, by stimulating in an autocrine manner the expression of *IL-23p19* and *EBI3*.

***IL-23p19* and *EBI3* expression is also selectively stimulated by IL-36 α and IL-1 β**

In addition to shared functions, *in vivo* studies indicate that IL-36 cytokines (IL-36 α , IL-36 β , and IL-36 γ) also have unique functions.^{7, 11, 28, 29} Therefore, we tested the ability of IL-36 α to stimulate the expression of IL-12 cytokines by oral epithelial cells. We also tested their stimulation by IL-1 β for comparison. As with IL-36 γ , IL-36 α and IL-1 β did not stimulate the expression of *IL-12p35* in TIGK cells (Figure 2a). In contrast, they strongly stimulated *IL-23p19* expression (Figure 2b) and weakly stimulated *IL-27p28* expression (Figure 2c). Although IL-36 α and IL-1 β did not stimulate significant *IL-12p40* expression (Figure 2d), they both robustly stimulated *EBI3* expression (Figure 2e). The secretion of *EBI3* was also strongly stimulated by IL-36 α and IL-1 β (Figure 2f). These data indicate that IL-36 cytokines (and IL-1 β) can stimulate oral epithelial cells to selectively express the IL-12 family members *IL-23p19* and *EBI3*.

***P. gingivalis* does not stimulate *IL-23p19* and *EBI3* expression in oral epithelial cells**

Given our previous report demonstrating the stimulation of IL-36 γ expression in oral epithelial cells in response to *P. gingivalis*,¹³ we sought to establish whether *P. gingivalis* may therefore also stimulate *IL-23p19* and *EBI3* expression. Consistent with our earlier

report, *IL-36γ* expression in TIGK cells was strongly stimulated in response to *P. gingivalis* (Figure 3a). However, *IL-23p19* expression was not stimulated (Figure 3b), and *EBI3* expression was stimulated only weakly (Figure 3c). Although this might suggest that the stimulation of *IL-36γ* expression by *P. gingivalis* does not subsequently result in the stimulation of *IL-23p19* and *EBI3* by autocrine IL-36γ signaling, IL-36γ is secreted as pro-IL-36γ, which is at least 1,000-fold less active than mature IL-36γ.³⁰ Pro-IL-36γ needs to be proteolytically processed to IL-36γ by neutrophil proteases to activate significant IL-36 receptor (IL-36R) signaling.³¹ The absence of neutrophils in our *in vitro* culture system precludes such processing of secreted pro-IL-36γ, thereby explaining, at least in part, the lack of *IL-23p19/EBI3* stimulation in response to *P. gingivalis*.

Diametric regulation of the IL-36γ-inducible expression of *IL-23p19* and *EBI3* by MEK-ERK signaling

In view of the above findings, we next sought to identify the signaling pathways that mediate the stimulation of *IL-23p19* and *EBI3* expression by IL-36γ. We have previously established that the stimulation of neutrophil chemokine expression (e.g. *IL-8* and *CXCL1*) in oral epithelial cells (e.g. TIGK cells) by IL-36γ is regulated by IRAK1-dependent signaling.¹³ The transfection of TIGK cells with IRAK1-targeting siRNA, which reduced IRAK1 expression levels by >90% (Figure 4a), significantly inhibited the stimulation of *IL-23p19* and *EBI3* expression by IL-36γ (Figure 4b and c). Oral epithelial cells express IRAK2, and thus the incomplete inhibition of IL-36γ-inducible *EBI3* expression might be due to partial compensation by IRAK2. IRAK1 can activate several signaling proteins downstream of the IL-36R, including NF-κB and MAP kinases (e.g. p38 MAP kinase and ERK1/2). To further elucidate the signaling pathways controlling the IL-36γ-inducible expression of *IL-23p19* and *EBI3*, we tested the ability of IL-36γ to activate NF-κB, p38 MAP kinase, and ERK1/2 in

TIGK cells. Western blotting of cell lysates with phospho-specific antibodies revealed the rapid activation of NF- κ B (Figure 4d). p38 MAP kinase and ERK1/2 were likewise shown to also be rapidly activated (Figure 4d). Next, we used pharmacologic inhibitors to establish the importance of their activation for *IL-23p19* and *EBI3* expression. The NF- κ B inhibitor, BAY11-7082, reduced the stimulation of both *IL-23p19* and *EBI3* expression by IL-36 γ (Figure 4e and f). IL-36 γ -inducible *IL-23p19* expression was also significantly inhibited when the cells were pretreated with either the p38 MAP kinase inhibitor, SB203580, or the mitogen-activated protein kinase/extracellular signal regulated kinase kinase (MEK) inhibitor, U0126 (Figure 4g). In contrast, the stimulation of *EBI3* expression was not inhibited by the p38 MAP kinase inhibitor (Figure 4h). Moreover, its IL-36 γ -inducible expression was potentiated by the MEK inhibitor (Figure 4h). The basal expression level of *EBI3* in unstimulated TIGK cells was not affected by the MEK inhibitor (data not shown). Collectively, these data suggest that the p38 MAP kinase pathway enables the expression of *IL-23p19* to be regulated independently of *EBI3*, and that the activation of the MEK-ERK pathway may function as a feedback mechanism to limit *EBI3* expression.

MEK-ERK signaling represses the stimulation of *EBI3* transcription by IL-36 γ

The MEK-ERK pathway can transcriptionally as well as post-transcriptionally (e.g. mRNA stability) regulate the expression of inflammatory genes.^{32, 33} Therefore, to determine the mechanism underlying the potentiation of the IL-36 γ -inducible expression of *EBI3* by the MEK inhibitor, TIGK cells were treated concurrently with ActD and U0126, and then stimulated with IL-36 γ . Significantly, ActD inhibited the ability of U0126 to potentiate the upregulation of *EBI3* mRNA levels by IL-36 γ (Figure 5a), suggesting that MEK-ERK signaling negatively regulates *EBI3* at the level of gene transcription. However, because the U0126-independent stimulation of *EBI3* mRNA levels by IL-36 γ was also inhibited, this

experimental approach did not enable us to establish whether MEK-ERK signaling may also regulate the stability of *EBI3* mRNA transcripts. Therefore, TIGK cells were stimulated with IL-36 γ for 2 h to induce *EBI3* transcription; thereafter, the cells were treated with ActD in the absence and presence of U0126, and *EBI3* mRNA levels monitored over the next 8 h. As shown in Figure 5b, *EBI3* mRNA levels were unchanged over the 8 h time-course, even when MEK-ERK signaling was inhibited with U0126. Collectively, these data indicate that MEK-ERK signaling negatively-regulates the IL-36 γ -inducible expression of *EBI3* at the level of gene transcription. We also examined the effects of U0126 on the stability of *IL-23p19* mRNA transcripts for comparison. *IL-23p19* mRNA levels declined slowly over the 8 h time-course, and were not affected by U0126 (Figure 5b).

EGFR signaling can differentially modulate the IL-36 γ -inducible expression of *IL-23p19* and *EBI3*

Signaling by the EGFR, which activates the MEK-ERK pathway, has been shown to differentially regulate the expression of inflammatory cytokines (e.g. *IL-8* and *CCL2*) in epidermal keratinocytes.³⁴ Therefore, we investigated the effect of EGFR signaling on the stimulation of *IL-23p19* and *EBI3* expression in oral epithelial cells by IL-36 γ . Specifically, TIGK cells were cultured for 24 h in the presence of 5 ng mL⁻¹ EGF and 50 ng mL⁻¹ EGF to induce different levels of EGFR and ERK1/2 activity, which was confirmed by Western blotting with phospho-specific antibodies (Figure 6a). Consistent with ligand-induced receptor internalization and degradation, EGFR expression levels were reduced when TIGK cells were cultured in the presence of 50 ng mL⁻¹ EGF (Figure 6a). Culturing the cells under those conditions enhanced the IL-36 γ -inducible expression of *IL-23p19*, and this effect was abolished when the cells were treated with U0126 prior to IL-36 γ stimulation (Figure 6b). In contrast, the IL-36 γ -inducible expression of *EBI3* was significantly reduced in a MEK-ERK-

223 dependent manner (Figure 6c). To determine whether the inhibitory effect of EGFR signaling
224 on the IL-36 γ -inducible expression of *EBI3* might be due to decreased IL-36R expression, we
225 also measured the expression levels of the IL-36R subunits, *IL-1RL2* and *IL-1RAcP*. *IL-1RL2*
226 expression was found to be increased around 2-fold when TIGK cells were cultured in the
227 presence of 50 ng mL⁻¹ EGF (Figure 6d), while *IL-1RAcP* expression was unchanged (Figure
228 6e). Taken together, these data indicate that the EGFR can differentially modulate the IL-36 γ -
229 inducible expression of *IL-23p19* and *EBI3* by activating MEK-ERK signaling.

DISCUSSION

IL-36 cytokines are important regulators of mucosal homeostasis and inflammation.⁶⁻
¹¹ In this study, we discovered that IL-36 γ stimulates oral epithelial cells to selectively express the IL-12 cytokine family members, *IL-23p19* and *EBI3*. IL-23p19 and EBI3 have recently been shown to form the novel cytokine IL-39 and regulate neutrophil responses.²¹⁻²³ EBI3 has been shown to also have IL-12 cytokine-independent functions.^{24, 25} The selective stimulation of *IL-23p19* and *EBI3* expression is therefore likely to play an important role in the control of mucosal immunity by IL-36 cytokines.

We previously established that oral epithelial cells up-regulate *IL-36 γ* expression in response to the bacterial pathogen *P. gingivalis*, and that IL-36 γ likely regulates immune responses in the oral mucosa in part by stimulating in both a paracrine and autocrine manner the expression of neutrophil and T cell chemokines.¹³ We have now found that IL-36 γ also stimulates oral epithelial cells (e.g. TIGK cells) to express *IL-23p19* and *EBI3*; their expression was also selectively stimulated by IL-36 α . In contrast, *IL-12p35* and *IL-12p40* expression was not stimulated. Although TIGK cells exhibit characteristics of primary oral epithelial cells, the possibility that subtle differences exist needs to be considered. Regardless, our data indicate that *IL-23p19* and *EBI3* are likely to be conserved targets of IL-36 cytokines in human oral epithelial cells.

IL-36 γ did not stimulate *IL-23p19* and *EBI3* expression in human monocyte-derived dendritic cells and macrophages. A recent study found that *IL-23p19* expression in human monocyte-derived dendritic cells was also not stimulated by IL-36 β .³⁵ Another study, however, reported that *IL-23p19* expression in human monocyte-derived dendritic cells was stimulated by IL-36 cytokines.¹⁵ Interestingly, although the authors found that *IL-12p35* expression in human dendritic cells was only weakly stimulated, its expression was strongly stimulated in mouse bone marrow-derived dendritic cells.¹⁵ IL-36 γ has been reported to

stimulate the expression of *IL-23p19* in mouse bone marrow-derived macrophages.⁶ Therefore, stimulation of IL-12 family cytokine subunit expression in dendritic cells and macrophages by IL-36 cytokines may be species-dependent and/or depend on *in vitro* culture conditions.

IL-23p19 and EBI3 have recently been shown to heterodimerize to form IL-39.^{21, 22} Although we did not detect IL-23p19 secretion by IL-36 γ -stimulated TIGK cells, it remains possible that *in vivo* IL-36 γ , potentially in concert with other inflammatory cytokines, stimulates the secretion of IL-23p19 and results in the formation of IL-39 (IL-23p19/EBI3). Unlike other IL-12 family cytokines, IL-39 does not appear to regulate T cells; instead, IL-39 has been shown in lupus-prone mice to promote the expansion of neutrophils.²³ This raises the possibility that IL-36 cytokines might also drive the neutrophil-mediated host response to microbial pathogens (e.g. *P. gingivalis*) by stimulating IL-23p19/EBI3 (IL-39) production.

EBI3 is also secreted independent of a pairing alpha subunit (e.g. IL-12p35, IL-23p19, and IL-27p28).^{21, 36} This suggests that EBI3 also has IL-12 family cytokine-independent functions. Indeed, EBI3 was recently shown to mediate IL-6 *trans*-signaling in endothelial cells and CD4⁺ T cells.²⁴ IL-6 *trans*-signaling differs from “classical” signaling in that IL-6 binds to a soluble form of the IL-6 receptor, which then activates intracellular signaling via transmembrane gp130.³⁷ IL-6 signaling plays a critical role in promoting the development of Th17 cells.³⁸ Moreover, the maintenance of IL-17-secreting Th17 cells is mediated by IL-6 *trans*-signaling.³⁹ IL-6 *trans*-signaling also activates inflammatory responses (e.g. *CCL2* expression) in other cell types, including endothelial cells, fibroblasts, and epithelial cells.³⁷ IL-36 γ has previously been shown to stimulate IL-6 expression, including in oral epithelial cells.^{12, 13, 15} The ability of IL-36 γ to coordinately stimulate both IL-6 and EBI3 expression by epithelial cells may therefore be important for the differential regulation of the responses that are activated by “classical” and “*trans*” IL-6 signaling in the oral mucosa.

The infection of TIGK cells by *P. gingivalis* did not stimulate *IL-23p19* expression, and *EBI3* was only weakly stimulated, despite the strong stimulation of *IL-36γ* expression. *IL-36γ* is secreted as pro-*IL-36γ*, which is around 1,000-fold less active than mature *IL-36γ*.⁴⁰ *In vivo*, pro-*IL-36γ* is thought to be proteolytically processed to *IL-36γ* by neutrophil proteases (e.g. cathepsin G and elastase).³¹ Such processing of pro-*IL-36γ* would not have occurred in our TIGK cell-*P. gingivalis* co-culture system, thus precluding autocrine stimulation of *IL-23p19* and *EBI3* expression. Furthermore, the major virulence factors of *P. gingivalis* (Kgp and RgpA/B) are lysine-specific and arginine-specific proteases. Cytokines that are secreted into the cell culture medium in response to *P. gingivalis* have previously been shown to be degraded by these proteases, which prevents autocrine cytokine responses.⁴¹⁻⁴³ Indeed, the presence of nine lysine and five arginine residues in *IL-36γ* would likely make the cytokine highly susceptible to degradation by *P. gingivalis* proteases. This raises the possibility that *P. gingivalis* might be able to dysregulate the stimulation of *IL-23p19* and *EBI3* expression by autocrine *IL-36γ* signaling.

Remarkably, signaling by the MEK-ERK pathway was found to diametrically regulate the stimulation of *IL-23p19* and *EBI3* expression by *IL-36γ*; i.e. *IL-23p19* expression was stimulated and *EBI3* expression inhibited by MEK-ERK signaling. The activation of MEK-ERK signaling by *IL-36γ* is therefore likely to function as a negative feedback mechanism to limit *EBI3* expression (Figure 7). Although this would likely restrain *IL-36γ*-mediated responses (e.g. neutrophil expansion), the inhibitory effects on *EBI3*-mediated responses (e.g. *IL-6 trans*-signaling) would arguably be greater. Interestingly, the expression of *IL-23p19*, but not *EBI3*, was also dependent on signaling by the p38 MAP kinase pathway. Further studies will be required to understand the importance of the diametric regulation of *IL-23p19* and *EBI3* by the MEK-ERK pathway, and the selective regulation of *IL-23p19* by the p38 MAP kinase pathway.

Importantly, the MEK-ERK pathway might enable the modulation of the IL-36 γ -inducible expression of *IL-23p19* and *EBI3* by other host factors (e.g. growth factors). We demonstrated that EGF, which activates MEK-ERK signaling in epithelial cells, enhanced the stimulation of *IL-23p19* expression and inhibited that of *EBI3*. Consequently, the local growth factor milieu might play a role in modulating the expression of IL-23p19/EBI3 by the oral epithelium in response to inflammatory stimuli (e.g. IL-36 cytokines). This may be important during mucosal wound healing, when the levels of EGFR ligands are upregulated and promote wound healing by stimulating the proliferation of epithelial cells.⁴⁴ It is worth noting that *P. gingivalis* has been proposed to interfere with wound healing by inactivating EGF.⁴⁵ The dysregulation of *IL-23p19* and *EBI3* expression in the oral mucosa might be one consequence of the inactivation of EGF by *P. gingivalis*.

In summary, IL-36 cytokines stimulate oral epithelial cells to selectively express IL-23p19 and EBI3. Although the immunoregulatory functions of IL-36 (IL-23p19/EBI3) are still to be clearly defined, the cytokine appears to play a role in the control of neutrophil responses. Furthermore, the stimulation of EBI3 expression may enable IL-36 cytokines to also indirectly control inflammatory responses in other cell types, for example, by facilitating IL-6 *trans*-signaling. Finally, the diametric regulation of *IL-23p19/EBI3* expression by MEK-ERK signaling may be important for the resolution of mucosal inflammation. Thus, this study not only extends our understanding of how IL-36 cytokines regulate immune responses, but also establishes a likely role for EGFR signaling in the modulation of IL-36 cytokine function.

METHODS

Reagents

DermaLife keratinocyte growth medium and supplements (TGF α , insulin, epinephrine, apo-transferrin, hydrocortisone, bovine pituitary extract, and glutamine) were from Lifeline Cell Technology. Human IL-1 β (Ala117-Ser269), IL-36 α (Lys6-Phe158), and IL-36 γ (Ser18-Asp169) were from R&D Systems. The human IL-23p19 and EBI3 ELISA kits were from Aviva Systems Biology. Opti-MEM I reduced serum medium, Lipofectamine RNAiMAX transfection reagent, and precast 10% NuPAGE gels were from Life Technologies. The ON-TARGETplus IRAK1 and non-targeting control siRNA were from GE Healthcare. The anti-phospho-EGFR (pY1068), anti-EGFR, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38 MAP kinase, anti-phospho-p65 NF- κ B, and anti-p65 NF- κ B 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 cells⁴⁶ were cultured in DermaLife keratinocyte growth medium supplemented with 0.5 ng mL⁻¹ TGF α , 5 μ g mL⁻¹ insulin, 1 μ M epinephrine, 5 μ g mL⁻¹ apo-transferrin, 100 ng mL⁻¹ hydrocortisone, 0.4% bovine pituitary extract, and 6 mM glutamine. Human monocyte-derived dendritic cells and monocyte-derived macrophages were cultured as previously described.¹³ All cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

Bacterial cell culture

P. gingivalis (ATCC 33277) was maintained on horse-blood agar plates at 37 °C in an anaerobic atmosphere of 5% H₂, 80% N₂, and 15% CO₂. Bacterial colonies were used to

inoculate Brain Heart Infusion medium supplemented with 0.5 mg mL⁻¹ cysteine, 5 µg mL⁻¹ hemin, and 5 µg mL⁻¹ menadione.^{13, 47}

Infection human oral epithelial cells with *P. gingivalis*

P. gingivalis cell concentrations were determined spectrophotometrically and confirmed retrospectively by counting viable cell colonies on horse-blood agar plates. Late logarithmic growth phase bacteria were harvested by centrifugation at 7,000 x g for 20 min at 4 °C, and suspended in antibiotic-free keratinocyte growth medium. TIGK cells were infected with *P. gingivalis* at a multiplicity-of-infection (MOI) of 100:1.¹³

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: *EBI3* (Hs01057148_m1), *IL-1RAcP* (Hs00895050_m1), *IL-1RL2* (Hs00909276_m1), *IL-12p35* (Hs01073447_m1), *IL-12p40* (Hs01011518_m1), *IL-23p19* (Hs00372324_m1), *IL-27p28* (Hs00377366_m1), *IRAK1* (Hs01018347_m1), and *IRAK2* (Hs00176394_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* and *TATA-box binding protein* genes.

RNA interference-mediated gene-silencing

A reverse transfection protocol was used for siRNA transfection of TIGK cells.¹³ Briefly, the

siRNA were diluted to 120 nM with 100 μ L Opti-MEM I reduced serum medium, mixed with 100 μ L Opti-MEM medium containing 1 μ L 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 replaced and the cells cultured for an additional 24 h.

Enzyme-linked immunosorbent assays

ELISA assays were performed by incubating diluted culture supernatants and standards in capture antibody-coated 96-well microplates at 37 °C for 2 h, per the manufacturer's instructions (Aviva Systems Biology). The wells were washed prior to the addition of biotinylated anti-IL-23p19 and anti-EBI3 antibodies, and incubated at 37 °C for 1 h. The plates were washed, and then incubated with an avidin-horse radish peroxidase conjugate at 37 °C for 1 h. Following washing, 3,3',5,5'-tetramethylbenzidine substrate was added and the plates incubated at room temperature. Color development was subsequently stopped and measured at 450 nm with wavelength correction at 560 nm using a microplate reader (Victor³, PerkinElmer).

Cell lysis and Western blotting

TIGK cells were washed with ice-cold PBS, and then lysed on ice with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 10% glycerol, 20 mM β -glycerol phosphate, 1 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors) for 30 min. The lysates were clarified by centrifugation (13,000 $\times$ g for 10 min at 4 °C) and the protein concentrations measured using a protein assay kit (Bio-Rad). Cell lysates were incubated with NuPAGE LDS sample buffer for 10 min at 70 °C, and then

subjected to electrophoresis on a 10% NuPAGE gel using MOPS buffer (Life Technologies). The proteins were transferred to a PVDF membrane, which was then blocked with 3% 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 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 enhanced chemiluminescence reagents (Millipore) and a Fujifilm Las-3000 Imager (Fujifilm, Japan).

Statistical analysis

Data combined from n independent replicate experiments are presented as the mean $\pm$ SEM. 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 post-hoc test. A P value <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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545

FIGURE LEGENDS

Figure 1. Stimulation of IL-12 family cytokine expression in oral epithelial cells and innate immune cells by IL-36 γ . **(a-e)** TIGK cells were stimulated with IL-36 γ (100 ng mL⁻¹) for the time indicated. **(a)** *IL-12p35*, **(b)** *IL-23p19*, **(c)** *IL-27p28*, **(d)** *IL-12p40*, and **(e)** *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. **(f and g)** TIGK cells were treated with actinomycin D (1 μ g mL⁻¹) for 30 min, and then stimulated with IL-36 γ for 2 h. **(f)** *IL-23p19* and **(g)** *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. **(h)** TIGK cells were stimulated with IL-36 γ (100 ng mL⁻¹) for the time indicated, and the concentrations of EBI3 in the cell culture supernatants were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. **(i and j)** Human monocyte-derived dendritic cells, and **(k and l)** human monocyte-derived macrophages were stimulated with IL-36 γ (100 ng mL⁻¹) for 2 h. **(i and k)** *IL-23p19*, and **(j and l)** *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ separate donors are presented as the mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Figure 2. Stimulation of IL-12 family cytokine expression in oral epithelial cells by IL-36 cytokines and IL-1 β . **(a-e)** TIGK cells were stimulated with IL-36 γ (100 ng mL⁻¹), IL-36 α (100 ng mL⁻¹), or IL-1 β (20 ng mL⁻¹) for 2 h. **(a)** *IL-12p35*, **(b)** *IL-23p19*, **(c)** *IL-27p28*, **(d)** *IL-12p40*, and **(e)** *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. **(f)** TIGK cells were stimulated with IL-36 γ (100 ng mL⁻¹), IL-36 α (100 ng mL⁻¹), or IL-1 β (20 ng mL⁻¹) for 24 h. The concentrations of EBI3 in the cell culture supernatants were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

0.01, * $P < 0.05$.

Figure 3. Effects of *P. gingivalis* on *IL-23p19* and *EBI3* expression in oral epithelial cells. TIGK cells were cultured with *P. gingivalis* (MOI 100:1) for the time indicated. (a) *IL-36γ*, (b) *IL-23p19*, and (c) *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. *** $P < 0.001$, ** = $p < 0.01$.

Figure 4. Signaling pathways that regulate the IL-36 γ -inducible expression of *IL-23p19* and *EBI3* in oral epithelial cells. (a-c) TIGK cells were transfected with an IRAK1 (+) or control (–) siRNA, and subsequently stimulated with IL-36 γ (100 ng mL^{–1}) for 2 h. (a) IRAK1, (b) *IL-23p19*, and (c) *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. (d) TIGK cells were stimulated with IL-36 γ (100 ng mL^{–1}) for the indicated time. Thereafter, the cells were lysed and aliquots subjected to Western blotting with the indicated antibodies. The data are representative of three independent experiments. (e-h) TIGK cells were treated with (e and f) 10 μ M BAY11-7082 or (g and h) 5 μ M SB203580 or 10 μ M U0126 for 30 min, and then stimulated with IL-36 γ (100 ng mL^{–1}) for 2 h. (e and g) *IL-23p19* and (f and h) *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

Figure 5. Negative-regulation of IL-36 γ -inducible *EBI3* transcription by the MEK-ERK pathway. (a) TIGK cells were treated with 1 μ g mL^{–1} actinomycin D (ActD) and 10 μ M U0126 for 30 min, and then stimulated with IL-36 γ (100 ng mL^{–1}) for 2 h. *EBI3* mRNA expression levels were then measured. Data combined from $n = 3$ replicate experiments are

presented as the mean $\pm$ SEM. **(b)** TIGK cells were stimulated with IL-36 γ (100 ng mL⁻¹) for 2 h, and then treated with 1 μ g mL⁻¹ actinomycin D (ActD) and 10 μ M U0126 for the time indicated. *IL-23p19* and *EBI3* mRNA expression levels were then measured. Data combined from $n = 2$ replicate experiments are presented as the mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

Figure 6. Regulation of the IL-36 γ -inducible expression of *IL-23p19* and *EBI3* by EGF signaling. TIGK cells were cultured in the presence of 5 ng mL⁻¹ and 50 ng mL⁻¹ EGF for 24 h. Thereafter, the cells were treated with 10 μ M U0126 for 30 min, and then stimulated with IL-36 γ (100 ng mL⁻¹) for 2 h. **(a)** The cells were lysed and aliquots subjected to Western blotting with the indicated antibodies. The data are representative of two independent experiments. **(b)** *IL-23p19*, **(c)** *EBI3*, **(d)** *IL-1RL2*, and **(e)** *IL-1RAcP* mRNA expression levels were measured. Data combined from $n = 3$ replicate experiments are presented as the mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

Figure 7. A proposed model for the regulation of *IL-23p19* and *EBI3* expression in epithelial cells by IL-36 cytokines. The activation of p38 MAP kinase and MEK-ERK signaling by the IL-36 receptor (IL-36R) stimulates the transcriptional upregulation of *IL-23p19*. In contrast, the IL-36R stimulates the transcriptional upregulation of *EBI3* independently of p38 MAP kinase signaling, while its activation of MEK-ERK signaling represses *EBI3* expression. The EGF receptor (EGFR) positively- and negatively-regulates *IL-23p19* and *EBI3* expression, respectively, by activating MEK-ERK signaling.