

Chapter 25

Possible Roles of IL-33 in Periodontal Diseases: *Porphyromonas gingivalis* Induced IL-33 in Human Gingival Epithelial Cells

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Abstract In the oral mucosa, epithelial cells work not only as a physical barrier to pathogens, but also play a pivotal role in initiating immune responses to microbes. Interleukin (IL)-33, a member of the IL-1 family, is constitutively expressed in epithelial cells and amplifies Th2-type inflammatory immune responses. We found that IL-33 was detected in the inflamed gingival epithelium from chronic periodontitis patients, and periodontopathic *Porphyromonas gingivalis* strongly increased expressions of IL-33 mRNA and molecules in human gingival epithelial cells. In contrast, fimbriae, a lipopeptide and lipopolysaccharide derived from *P. gingivalis* were not active in this respect. Protease inhibitors specific for gingipains efficiently inhibited the induction of IL-33 mRNA by stimulation with *P. gingivalis*. Furthermore, *P. gingivalis* KDP136, a gingipains-null mutant, did not increase IL-33 mRNA expression. We also demonstrated that *P. gingivalis* upregulated IL-33 mRNA expression through protease-activated receptor-2, phospholipase C, mitogen-activated protein kinase p38 and NF- κ B. IL-33 is suggested to negatively regulate antimicrobial peptide LL-37, resulting in attenuation of innate immune responses of gingival epithelial cells in chronic periodontitis. Possible roles of IL-33 in inflammation in the oral mucosa are discussed.

Keywords Gingipains • Gingival epithelial cells • Interleukin-33 • LL-37 • *Porphyromonas gingivalis*

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25.1 Introduction

In mucosal immune systems, including the oral mucosa, epithelial cells work not only as a physical barrier to bacterial pathogens, but also play a pivotal role in initiating and amplifying Th2-type immune responses in response to bacterial components [1, 2]. Epithelial cells produce interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin, which may be involved in the development and regulation of Th2-type inflammatory responses. IL-33 is a member of the IL-1 cytokine family, and is constitutively expressed in epithelial cells, endothelial cells and fibroblasts [3]. IL-33 consists of two domains: a non-classical homeodomain-like helix-turn-helix DNA-binding domain, which consists of a chromatin-binding motif (CBM) and a nuclear localization sequence (NLS), and an IL-1-like domain [4] (Fig. 25.1a). IL-33 localizes in the nuclei of resting epithelial cells and acts as an alarmin when released from necrotic cells (Fig. 25.1b). IL-33 has a protective role in inflammatory bowel disease [7] and in the initiation of *Toxoplasma* infection that polarizes adaptive responses towards a Th2-biased response, which is protective in this disease [8]. In contrast, a lot of evidence suggests that IL-33 is also involved in the development of chronic inflammatory diseases such as arthritis [9]. IL-33 signals through the IL-33 receptor (IL-33R), which consists of heterodimers of ST2 and IL-1 receptor accessory protein (IL-1RAcP) [5, 6] (Fig. 25.2). IL-33 upon binding to ST2 induces the recruitment of IL-1RAcP and myeloid differentiation primary-response protein 88 (MyD88) to the Toll/IL-1R (TIR) domain in the

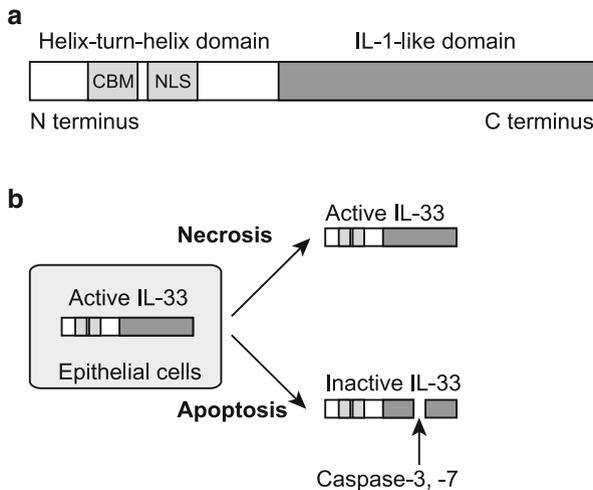


Fig. 25.1 (a) Structure of IL-33 protein. IL-33 is a 30-kDa protein that consists of 270 amino acids. IL-33 consists of two domains: a helix-turn-helix domain and an IL-1-like domain. The helix-turn-helix domain contains a chromatin-binding motif (CBM) and a nuclear-binding signal (NLS). (b) Release of IL-33. Active form of IL-33 is thought to be released by necrosis. IL-33 is inactivated by cleaving with caspase-3 or caspase-7 during apoptosis. Based on Martin, Oboki et al. and Palmer and Gabay [4–6]

Fig. 25.2 IL-33 signaling pathways. IL-33 signaling through the IL-33 receptor (IL-33R), which consists ST2 and IL-1 receptor accessory protein (IL-1RAcP) dimers and myeloid differentiation primary-response protein 88 (MyD88) to the Toll/IL-1R (TIR) domain in the cytoplasmic region of ST2. The MyD88 and TRAF6 complex activates NF- κ B- and MAP kinase-mediated signaling pathways. Based on Martin, Oboki et al. and Palmer and Gabay [4–6]

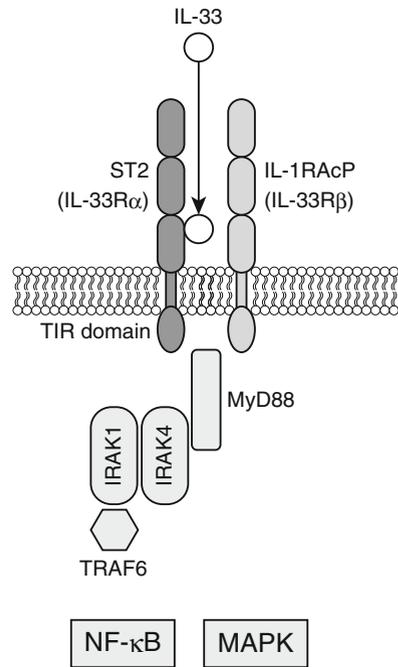


Table 25.1 Articles on IL-33 in oral/dental science

Finding	Reference
IL-33 mRNA and protein expressions are enhanced by <i>P. gingivalis</i> LPS in human monocytes	Nile et al. 2010 [10]
IL-33 protein expressions are not differentiated from human gingival crevicular fluid, saliva, or plasma in chronic periodontitis	Buduneli et al. 2012 [11]
IL-33 protein expression is enhanced by TNF- α in human gingival fibroblasts	Beklen et al. 2013 [12]
IL-33 protein expression is not detected from human gingival crevicular fluid in gingivitis or periodontitis	Papathanasiou et al. 2014 [13]

cytoplasmic region of ST2. The MyD88 and TRAF6 complex activates NF- κ B- and MAP kinase-mediated signaling pathways [5, 6].

Porphyromonas gingivalis is a periodontopathic pathogen in chronic periodontitis and has a variety of virulence factors that induce proinflammatory cytokines leading to chronic inflammation, resulting in destruction of periodontal tissues. In the dental/oral field, only limited information on IL-33 is available (see Table 25.1). TNF- α induces IL-33 expression in human gingival fibroblasts [12], although whether IL-33 expression is increased in the periodontal tissues in chronic periodontitis patients and the functions of IL-33 in the modulation of chronic periodontitis remain unelucidated. This review article shows possible regulation of IL-33

expression in human gingival epithelial cells in response to *P. gingivalis* based on our recent studies, and discusses possible roles of IL-33 expressed in gingival epithelial cells in relation to the pathogenesis of chronic periodontitis.

25.2 Expression of IL-33 in Periodontal Tissues from Chronic Periodontitis Patients

We first examined whether inflamed gingival tissues from chronic periodontitis patients expressed IL-33 by immunohistochemical studies using an anti-human IL-33 monoclonal antibody (mAb). As expected, IL-33 was strongly expressed in the cytoplasm of the inflamed gingival epithelium from chronic periodontitis patients, but only weakly detected in the normal gingival epithelium from healthy individuals. Unlike the expression of IL-33 in the gingival epithelium, IL-33 expression in the lamina propria of gingival tissues was only weakly observed. IL-33 expression is possibly upregulated in epithelial, mesenchymal, and myeloid cells in response to proinflammatory stimuli, pathogen-associated molecular patterns, and pathogens [5]. These findings suggest that gingival epithelial cells are capable of inducing IL-33 expression upon infection with periodontal pathogens.

25.3 Possible Induction of IL-33 by Gingipains from *P. gingivalis* in Gingival Epithelial Cells

25.3.1 *P. gingivalis* Induces IL-33 mRNA Expression in Human Gingival Epithelial Cells in Culture

As *P. gingivalis* is implicated as a major pathogenic bacteria for chronic periodontitis [14], we examined the effect of *P. gingivalis* infection on IL-33 mRNA expression in human gingival epithelial cells in culture. The IL-33 mRNA expression was increased 20-fold at 48 h after stimulation with *P. gingivalis* W83 in Ca9-22 cells, which is a human gingival epithelial cell line established from squamous cell carcinoma. Pretreatment of the cells with cycloheximide, a protein synthesis inhibitor, blocked the induction of IL-33 mRNA levels, suggesting that *de novo* protein synthesis was required for *P. gingivalis*-mediated IL-33 mRNA induction. Nile et al. [10] reported that *P. gingivalis* LPS induced IL-33 expression in human monocytes. Therefore, we examined the possible IL-33 mRNA-inducing capacity of *P. gingivalis*-related specimens; fimbriae (Toll-like receptor (TLR) 2 ligand [15]) and LPS (TLR2 and 4 ligand [16]) prepared from *P. gingivalis*, and synthetic *P. gingivalis*-type lipopeptide PGTP2-RL (TLR2/1 ligand [17]). Unlike *P. gingivalis* whole cells, fimbriae and lipopeptide were completely

inactive, and LPS specimens were only weakly active in this respect. These results suggest that gingival epithelial cells respond to *P. gingivalis* rather than TLR ligands for induction of IL-33 mRNA.

25.3.2 Involvement of Gingipains in the Induction of IL-33 mRNA Expression in Human Gingival Epithelial Cells by *P. gingivalis*

P. gingivalis produces two types of arginine-specific cysteine proteinases (Arg-gingipains, RgpA and RgpB) and a lysine-specific cysteine proteinase (Lys-gingipain, Kgp) [18]. Gingipains are localized to a cell-associated form, a soluble form, and released as outer membrane blebs [18]. We next examined whether enzymatic activities of gingipains are involved in the IL-33-inducing capacity in Ca9-22 cells and primary oral epithelial cells. The induction of IL-33 mRNA expression by *P. gingivalis* W83 in the two types was completely and significantly inhibited by FPR-cmk (Rgp inhibitor) and KYT-36 (Kgp inhibitor), respectively. Furthermore, *P. gingivalis* KDP136, a gingipains-null mutant, unlike *P. gingivalis* ATCC 33277, a wild-type parent strain of KDP136, did not induce IL-33 mRNA expression in either cell type. *P. gingivalis*-induced IL-33 mRNA expression was abolished by heat treatment (70 °C, 1 h) to inactivate enzymatic activities of *P. gingivalis*. We confirmed intact proteolytic activities for Rgps and Kgp in the whole cells of *P. gingivalis* W83 and ATCC 33277, but not in those of *P. gingivalis* KDP136. These observations suggest that the proteolytic activity of gingipains is essential for the induction of IL-33 mRNA expression by *P. gingivalis* in human gingival epithelial cells.

25.3.3 Induction of the IL-33 Molecule by *P. gingivalis* in Human Gingival Epithelial Cells

Next, we examined whether *P. gingivalis* induced IL-33 expression in human gingival epithelial cells using an immunoblot analysis. IL-33 expression in Ca9-22 cells was increased tenfold with a peak at 4 days by stimulation with whole cell preparations of *P. gingivalis*. Epithelial cells constitutively express IL-33 in their nuclei under resting conditions [19]. IL-33 is a nuclear protein that is also released into the extracellular environment. To further determine whether *P. gingivalis*-induced IL-33 was accumulated in the nuclei or the cytoplasm, we analyzed the location of IL-33 protein in Ca9-22 cells after stimulation with *P. gingivalis* using immunocytochemical analysis. Although IL-33 was constitutively expressed in the resting cells, IL-33 molecules accumulated in the cytoplasm of the cells when they were stimulated with *P. gingivalis* for 4 days. However, the

released IL-33 level was quite low (approximately 30 pg/mL), even after stimulation with *P. gingivalis* whole cells. IL-33 is not detected in viable human monocytic cells, even upon stimulation with *P. gingivalis* LPS [10]. Furthermore, IL-33 levels in gingival crevicular fluid (GCF) are not different between chronic periodontitis patients and healthy controls [11]. In fact, IL-33 is not detected in the GCF of inflamed regions from chronic periodontitis patients [13]. Further studies are required to elucidate the fate of IL-33 induced by *P. gingivalis* in gingival epithelial cells, which may modulate the innate immune functions of the cells in infected periodontal lesions.

25.4 PAR-2-p38/NF- κ B-Mediated Signals in IL-33 Induction

25.4.1 Role of PAR-2 in the Induction of IL-33 by Gingipains

Proteinase-activated receptor-2 (PAR-2) is a seven-transmembrane domain receptor family which couples to G-proteins [20]. Rgps are capable of activating PAR-2 expressed on human gingival epithelial cells, which produce proinflammatory cytokines [21]. We examined the possible involvement of PAR-2 in *P. gingivalis*-induced IL-33 mRNA expression. Ca9-22 cells constitutively expressed PAR-1, -2, -3, and -4 mRNA. PAR-2 mRNA expression was increased upon stimulation with *P. gingivalis* W38 in Ca9-22 cells. Next, we performed inhibition of PAR-2 mRNA expression by RNA interference using a PAR-2-specific small interference RNA (siRNA). The induction of IL-33 mRNA by *P. gingivalis* W83 was partially inhibited in PAR-2 siRNA-transfected cells. PAR-2 is activated by a tethered ligand when cleaved by protease. A PAR-2 agonist peptide based on the tethered ligand sequences can activate PAR-2 in a proteolysis-independent manner. However, the PAR-2 agonist peptide was not able to induce IL-33 mRNA in Ca9-22 cells. Pretreatment of the cells with cytochalasin D, a particle internalization inhibitor, inhibited the IL-33 mRNA induction by *P. gingivalis*. These findings suggested that both proteolytic activation of PAR-2 by gingipains and the endocytosis of *P. gingivalis* are required for the up-regulation of IL-33 expression induced by *P. gingivalis* in oral epithelial cells. *P. gingivalis* enters gingival epithelial cells by endocytosis, which mediates binding of Rgp to the cells [22]. It must be noted that gingipains are required for maturation of *P. gingivalis* fimbriae [23], which is essential for internalization of the bacterium into epithelial cells [24]. However, endocytosis of both PAR-2 and *P. gingivalis* are probably required for the up-regulation of IL-33 in gingival epithelial cells.

25.4.2 Involvement of PLC in the Induction of IL-33 by Gingipains

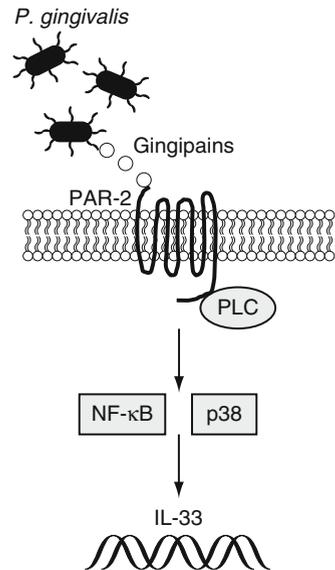
Because PAR-2 has been reported to be coupled to G protein, leading to activation of phospholipase C (PLC) [25, 26], we examined whether or not the PLC pathway is involved in *P. gingivalis*-induced IL-33 mRNA expression in Ca9-22 cells. The cells were pretreated with U-73122, a PLC inhibitor, and then stimulated *P. gingivalis* W83 for 48 h. The pretreatment significantly inhibited the *P. gingivalis*-induced IL-33 mRNA expression. In contrast, a protein kinase C (PKC) inhibitor, GF-109203X, did not inhibit either the basal level of IL-33 or the *P. gingivalis*-induced increase in IL-33. These findings suggest that *P. gingivalis*-induced up-regulation of IL-33 expression is mediated via a PAR-2-PLC-signaling pathway.

25.4.3 Involvement of p38 and NF- κ B in the Induction of IL-33 by Gingipains

As p38 and NF- κ B are implicated in PAR-2 signaling [27], we first confirmed p38 phosphorylation in Ca9-22 cells stimulated with *P. gingivalis* W83. Then, we demonstrated that p38 signaling is involved in *P. gingivalis*-induced IL-33 mRNA expression using SB203580, a p38 inhibitor. In contrast, the *P. gingivalis* IL-33 mRNA expression was not inhibited by PD98059, an ERK1/2 inhibitor, or SP600125, a JNK inhibitor. Further, we demonstrated that gingipains are responsible for p38 activation caused by whole cells of *P. gingivalis* because p38 phosphorylation induced by *P. gingivalis* W83 was completely inhibited when *P. gingivalis* was treated with Rgp inhibitor FPR-cmk plus Kgp inhibitor KYT-36. In addition, p38 phosphorylation was not observed in the cells stimulated with *P. gingivalis* KDP136, a gingipains-null mutant.

Next, we demonstrated NF- κ B activation in Ca9-22 cells stimulated with *P. gingivalis* W83 using a luciferase reporter assay. We demonstrated that NF- κ B signaling is involved in *P. gingivalis*-induced IL-33 mRNA expression because pretreatment of cells with PDTC, an NF- κ B inhibitor, significantly inhibited *P. gingivalis*-induced IL-33 mRNA expression. The NF- κ B activities induced by *P. gingivalis* W83 were markedly diminished by FPR-cmk plus KYT-36, and the NF- κ B activation was attenuated in the cells stimulated with *P. gingivalis* KDP136, indicating that gingipains are responsible for NF- κ B activation caused by whole cells of *P. gingivalis*. Taken together, we demonstrated that the *P. gingivalis*-derived gingipain-mediated IL-33 increase was dependent on PAR-2-PLC-p38/NF- κ B signaling (Fig. 25.3).

Fig. 25.3 Role of gingipains in the induction of IL-33 via PAR-2-p38/NF- κ B-mediated signals



25.5 Future Prospects: Possible Roles of IL-33 in Gingival Epithelial Cells in Chronic Periodontitis

Gingival epithelial cells play integral roles in innate immune defense by sensing periodontal pathogens, maintaining a physical barrier, and expressing antimicrobial peptides (AMPs) [28]. AMPs constitute an important component in the innate immune response. In humans, two main classes of cationic AMPs, the cathelicidins and the defensins, are expressed in a various type of cells, such as epithelial cells, neutrophils, and macrophages. LL-37, a 37-residue mature antimicrobial peptide with two leucine residues, is a 37 amino acid peptide derived from human cathelicidin, a cationic antimicrobial peptide of 18-kDa (CAP18). The peptide is mainly produced by epithelial cells and neutrophils [29] and detected in GCF [30]. Human β -defensin-2 (hBD-2), an antimicrobial peptide of the β -defensin family, is induced by gingipains through PAR-2 in gingival epithelial cells [31]. It has been reported that hBD-2 production is down-regulated by pretreatment with IL-33 in human foreskin keratinocytes [32]. Endogenous expression of LL-37 plays an important role in intracellular killing of mycobacteria in macrophages [33].

To address whether development of chronic periodontitis is attributable to IL-33, we examined the influence of *P. gingivalis*-mediated IL-33 production on the induction of LL-37 in human gingival epithelial cells. Indeed, we found that *P. gingivalis*-induced CAMP (LL-37 gene) mRNA expression was up-regulated in IL-33 siRNA-transfected Ca9-22 cells. LL-37 is capable of proteolytically degradation by gingipains secreted by *P. gingivalis*; however, the antibacterial activity of LL-37 is still intact in the presence of *P. gingivalis* proteases [34].

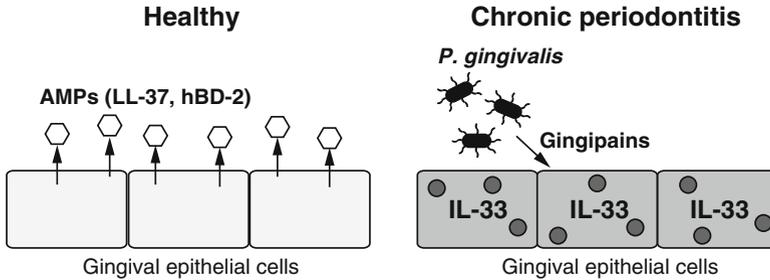


Fig. 25.4 A hypothetical model for the role of IL-33 induced by *P. gingivalis* in the down-regulation of LL-37 expression in human gingival epithelial cells

These findings indicate that IL-33 may attenuate antimicrobial immune responses by epithelial cells against bacterial mucosal infections.

In this study, we revealed that *P. gingivalis* induced IL-33 via PAR-2-PLC-p38/NF- κ B signaling pathways and that the IL-33 down-regulated LL-37 expression in human gingival epithelial cells. These findings suggest evasion of innate immune responses by *P. gingivalis* is due to inhibition of antimicrobial peptide expression (Fig. 25.4). Further studies are necessary to elucidate the role of intracellular IL-33 in maintaining host defense mechanisms in gingival epithelial cells against periodontal diseases.

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