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Expression and function of resolvin $RvD1_{n\text{-}3\ DPA}$ receptors in oral epithelial cells

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Abstract

Chronic inflammatory responses can inflict permanent damage to host tissues. Specialized pro-resolving mediators downregulate inflammation but also can have other functions. The aim of this study was to examine whether oral epithelial cells express the receptors FPR2/ALX and DRV1/GPR32, which bind $RvD1_{n\text{-}3\ DPA}$, a recently described pro-resolving mediator derived from omega-3 docosapentaenoic acid (DPA), and whether $RvD1_{n\text{-}3\ DPA}$ exposure induced significant responses in these cells. Gingival biopsies were stained using antibodies to FPR2/ALX and DRV1/GPR32. Expression of FPR2/ALX and DRV1/GPR32 was examined in primary oral epithelial cells by qRT-PCR, flow cytometry, and immunofluorescence. The effect of $RvD1_{n\text{-}3\ DPA}$ on intracellular calcium mobilization and transcription of beta-defensins 1 and 2, and cathelicidin was evaluated by qRT-PCR. FPR2/ALX and DRV1/GPR32 were expressed by gingival keratinocytes in situ. In cultured oral epithelial cells, FPR2/ALX was detected on the cell surface, whereas FPR2/ALX and DRV1/GPR32 were detected intracellularly. Exposure to $RvD1_{n\text{-}3\ DPA}$ induced intracellular calcium mobilization, FPR2/ALX internalization, DRV1/GPR32 translocation to the nucleus, and significantly increased expression of genes coding for beta-defensin 1, beta-defensin 2, and cathelicidin. This shows that the signal constituted by $RvD1_{n\text{-}3\ DPA}$ is recognized by oral keratinocytes and that this can strengthen the antimicrobial and regulatory potential of the oral epithelium.

KEYWORDS
beta defensin, cathelicidin, DRV1/GPR32, FPR2/ALX, oral epithelium

INTRODUCTION

Pro-resolving molecules (lipoxins, resolvins, maresins, and protectins) are described by the umbrella term 'specialized pro-resolving mediators' [1]. These mediators are important for the inflammatory resolution phase, which is temporally activated to allow the return of the affected tissue to its normal homeostatic state and function [1]. Resolution is essential because non-resolving chronic inflammation is recognized as a key component of many commonly occurring inflammatory diseases [1]. In the oral cavity for example, the inability of the host to successfully control and resolve inflammation

within the gingival tissues may contribute to the development of chronic periodontitis [2, 3]. Hence, understanding and controlling the activities of pro-resolving pathways can have impact on designing novel host-modulatory therapies. Other than their role in resolution, specialized pro-resolving mediators can also enhance the generation of anti-microbial peptides and regulate macrophage phagocytosis, hemostasis, cell migration and proliferation, all critical prerequisites for angiogenesis, wound healing and regeneration of damaged tissues [4–7].

Specialized pro-resolving mediators are generated enzymatically from essential polyunsaturated fatty acids, often via cell-cell interactions involving the transfer of biosynthetic intermediates (transcellular biosynthesis) after the initial stages of the inflammatory process [2]. Both leukocytes and stromal cells (e.g., endothelial cells) and undifferentiated and differentiated stem cells (e.g., embryonic, hematopoietic, and periodontal stem cells) can produce specialized pro-resolving mediators [8–10]. Binding of specialized pro-resolving mediators to their receptors, which are expressed on a variety of host cells, activates a wide spectrum of cell type-specific responses that collectively inhibit the inflammatory process and promote tissue healing [11]. For example, the resolvin RvE1 directs osteoclasts and osteoblasts towards bone regeneration in animal models of ligature-induced periodontitis [12, 13]. It also increases human periodontal ligament stem cell viability, accelerating wound healing and up-regulating periodontal ligament markers and cementogenic-osteogenic differentiation [14]. In mammals, alpha-linolenic acid obtained via dietary intake is converted via elongation and desaturation to eicosapentaenoic acid and subsequently to docosahexaenoic acid. An intermediate product in this conversion is n-3 docosapentaenoic acid (n-3 DPA) (Figure S1). Recently, novel omega-3 DPA-derived resolvins, such as resolvin $D1_{n\text{-}3\ DPA}$ ($RvD1_{n\text{-}3\ DPA}$), have been identified and shown to exhibit potent anti-inflammatory effects on human neutrophils and endothelial cells [15–18]. Polymorphonuclear neutrophils and monocyte/ macrophages have been shown to produce $RvD1_{n\text{-}3\ DPA}$ from n-3 DPA [16]. To date, two G-protein coupled receptors for $RvD1_{n\text{-}3\ DPA}$ have been described: formyl peptide receptor 2 (FPR2/ALX) and G protein-coupled receptor 32 (DRV1/GPR32) [15]. Several endogenous and exogenous lipids, peptides, and proteins may serve as ligands for FPR2/ALX, stimulating both pro- and anti-inflammatory functions. In addition to $RvD1_{n\text{-}3\ DPA}$, DRV1/GPR32 also binds other specialized pro-resolving mediators, including RvD1, AT- RvD1, RvD3, AT-RvD3, and RvD5 [1].

The epithelial lining of the oral cavity represents not only a passive physical barrier, but also actively participates in the epithelial-immune crosstalk through the generation and secretion of cytokines, chemokines, and anti-microbial peptides [19–20]. Epithelia may also recognize signals from specialized pro-resolving mediators as was demonstrated in epithelia from the skin and the intestine [5, 7, 21]. This study aims to identify whether the receptors FPR2/ALX and DRV1/GPR32 are expressed by primary oral epithelial cells and whether activation by $RvD1_{n\text{-}3\ DPA}$ induces responses in these cells.

MATERIAL AND METHODS

Study design and biopsy materials

In situ protein expression of the receptors FPR2/ALX and DRV1/GPR32, both binding the resolvin $RvD1_{n\text{-}3\ DPA}$, was examined using immunohistochemistry in gingival biopsies that were obtained from the buccal gingiva of six volunteers with clinically healthy gingiva (mean age ± SD = 29.6 ± 11.6 years, four females and two males). Probing depth at the biopsy sites was <5 mm, clinical attachment loss was ≤2 mm, and there was no bleeding on probing.

Ex vivo mRNA and protein expression of the receptors FPR2/ALX and DRV1/GPR32 were studied on primary oral epithelial cell lines by flow cytometry and qRT-PCR. The cell lines were also used to investigate responses of oral epithelial cells to the addition of $RvD1_{n\text{-}3\ DPA}$, including examination of FPR2/ALX receptor internalization, DRV1/GPR32 nuclear translocation, Ca^{2+} mobilization, and transcription of the genes coding for antimicrobial peptides (beta-defensin 1, beta-defensin 2, and cathelicidin). Biopsies for these cellular investigations were obtained during tooth extractions from another seven healthy volunteers (mean age ± SD = 36.7 ± 14.2 years, four females and three males). Both tissue collection processes were approved by the Regional Committee for Medical Research Ethics in South-East Norway (nr. 2017/2196), and the study was carried out according to the Declaration of Helsinki's principles for biomedical research.

Isolation of primary oral epithelial cells

Oral epithelial cells were isolated from biopsies as described previously [22]. Briefly, biopsies were transferred to Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 1.25 mg/ml dispase (Gibco) and incubated over night at 4°C. The epithelial sheets were peeled off, cut into small pieces, and incubated in 10× trypsin EDTA (Sigma-Aldrich) for 7 min at 37°C. A Pasteur pipette was used to loosen the cells and the enzymatic treatment was stopped by addition of fetal calf serum. The cells were then cultured in keratinocyte serum-free medium (Gibco), supplemented with 25 µg/ml bovine pituitary extract (Gibco), 1 µg/ml epidermal growth factor (Gibco), and 1% antibiotic-antimycotic (Gibco), in a humidified atmosphere of 5% CO_2 in air at

37˚C. In the experiments, 2nd–6th passaged cells were used. For all experiments, 500,000 cells per well were seeded, incubated overnight, and then grown in keratinocyte serum-free medium without addition of bovine pituitary extract and epidermal growth factor 24 h before stimulation. For analysis of FPR2/ALX, oral epithelial cells were harvested by trypsinization (Trypsin (1×) with EDTA (Sigma-Aldrich) for 5 min, 37˚C with subsequent blocking with fetal calf serum) or by scraping. For flow cytometry of DRV1/GPR32, attached cells were lifted by scraping, as treatment with trypsin was found to affect the presence of the receptor in the cells' surface (see Results).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded sections were immunostained according to procedures described previously [22]. Briefly, heat induced epitope retrieval was performed using 0.05% citraconic anhydride (Sigma-Aldrich) for 15 min at 100˚C using a decloaking chamber (Biocare Medical). Endogenous peroxidase was blocked by 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min, before blocking with 1 mg/ml human IgG (Gammanorm; Octapharma) and 5% serum matching the species of the secondary antibody. Sections were incubated overnight with 1 μg/ml unlabeled mouse anti-FPR2/ALX (RRID AB 10952146; Aldevron) and 1 μg/ml rabbit anti-DRV1/GPR32 (RRID AB 1950440; GeneTex) antibodies or isotype- and concentration-matched mouse IgG1 and rabbit IgG (Dako). Bound antibodies were amplified by incubation with the matched biotinylated secondary antibodies horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories) for 1 h at 21˚C, and finally with peroxidase-conjugated ABC reagent (Vector Laboratories). The antibody label was visualized using 3,3′-diaminobenzidine, and nuclei were counterstained with hematoxylin.

Flow cytometry

The oral epithelial cells were stained with eBioscience fixable viability dye eFluor780 (Life Technologies) in PBS to exclude dead cells. After washing in FC buffer (PBS containing 10% fetal bovine serum, cells suspended in 100 μl FC buffer were incubated for 30 min on ice with 0.5 μl of primary anti-FPR2/ALX (Aldevron) and 1 μl anti-DRV1/GPR32 antibodies (GeneTex) or isotype- and concentration-matched rabbit IgG (Peprotech) and mouse IgG1 (Sigma-Aldrich) followed by secondary PE-Cy7-conjugated anti-mouse IgG1 and BV510-conjugated anti-rabbit IgG (Biolegend). For intracellular staining, primary oral epithelial cells were fixed and permeabilized prior to staining with primary antibodies (Cytofix/Cytoperm Kit; Becton Dickinson). Data acquisition was performed on BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo v10.6.1 software (Three Star).

For internalization studies, oral epithelial cells were exposed to $RvD1_{n\text{-}3\ DPA}$ (10^{-11}, 10^{-10} and 10^{-9} M) or to vehicle for 15 min and analyzed by flow cytometry as described above. Percentage internalization was calculated as $100 - ((MFI_{RvD1n\text{-}3\ DPA}/MFI_{vehicle}) \times 100)$, with MFI denoting the mean fluorescent intensity.

Resolvin

$RvD1_{n\text{-}3\ DPA}$ was prepared by total organic synthesis [15]. The structural integrity of $RvD1_{n\text{-}3\ DPA}$ was monitored using UV tandem liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matched against authentic material of $RvD1_{n\text{-}3\ DPA}$ [15]. Before use, $RvD1_{n\text{-}3\ DPA}$ was diluted in phosphate-buffered saline with 1% v/v ethanol.

Intracellular Ca^{2+} mobilization

Primary oral epithelial cells (10^6 cells) were passively loaded with the calcium indicator Fluo-3 AM (3 μM) (ThermoFisher Scientific) in keratinocyte culture medium (45 min, 5% CO_2, 37˚C), washed twice with Ca^{2+} free PBS to remove excess dye, and then incubated for 30 min to allow complete de-esterification of intracellular acetoxymethyl (AM). Increase in intracellular Ca^{2+} mobilization was quantified by flow cytometry directly after stimulation with $RvD1_{n\text{-}3\ DPA}$ (10^{-12}–10^{-8} nM). Flow cytometric analysis was performed as described above.

Immunofluorescence

Primary oral epithelial cells were seeded on glass cover slips at a density of 250,000 cells per well in a 24-well culture plate.

For staining of cells without further stimulation, cover slips were washed twice with PBS, fixed with 4% paraformaldehyde (10 min, 37˚C), washed again and blocked overnight with 1% bovine serum albumin in PBS. Some cover slips were treated with 0.1% Triton-X100 in 0.1% sodium citrate (w/v) before staining to permeabilize the attached cells. Then, cover slips were immersed in 5% normal goat serum and incubated overnight with 1 μg/ml unlabeled mouse anti-FPR2/ALX (Aldevron) and 1 μg/ml rabbit anti-DRV1/GPR32 (GeneTex) antibodies at 4˚C. Bound antibody was visualized by 1 μg/ml cyanine-(Cy)3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Europe) and/or 7.5 μg/ml biotinylated horse

anti mouse IgG (Vector Laboratories) followed by 1 μg/ml Cy2-labeled streptavidin (GE LifeSciences). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific). Images were taken using an Olympus Fluoview FV1000 confocal system mounted on a IX81 microscope and image overlays mounted using Adobe Photoshop CS5. Cells on cover slips were also exposed to $RvD1_{n\text{-}3\ DPA}$ (0.1 nM) for 15 min or to vehicle only (< 0.1% ethanol), after which cells were fixed, stained, and examined as described above.

RNA extraction and qPCR

Cells seeded at a density of 500,000 cells per well in 6-well plates were stimulated with 0.1 nM $RvD1_{n\text{-}3\ DPA}$ or vehicle (keratinocyte serum-free medium containing < 0.1% ethanol) for 24 h. Cells were washed twice with cold PBS before lysis in RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). Total RNA was extracted using the QIAcube and the QIAcube standard RNeasy mini kit (Qiagen) using the DNase digestion protocol. Total RNA (1 μg) was transcribed into cDNA using a mixture of reverse transcriptase and random nonamers (Eurogentec). cDNA synthesis was performed in a GeneAmp PCR System 9700 (Applied Biosystems). Primers were designed using NIH primer blast (*DEFB1*: forward: 5′-GTCGCCATGAGAACTTCCTAC-3′ and reverse 5′-TCGGGCAGGCAGAATAGAGA-3′; *DEFB4A*: forward 5′-TGAGCATTGCACCCAATACCAG-3′ and reverse 5′-GGACATCAAGCCTTCCACCTT-3′; *CAMP*: forward 5′-ATCATTGCCCAGGTCCTCAG-3′ and reverse 5′-GTCCCCATACACCGCTTCAC-3′). Detection of mRNA was performed using a SYBR Green qPCR Master Mix (Bimake). Each real-time PCR reaction consisted of 2.5 μl of RNase-free water, 12.5 μl of Master Mix, 1 μl each of forward and reverse primers, and 8 μl of template. Reactions were carried out in duplicates on a AriaMx Real-Time PCR system G8830A (Agilent Technologies). *GAPDH* was used as a reference gene. Data were expressed as fold changes.

Statistical analysis

The data were normally distributed based on the Shapiro-Wilk test. To evaluate the effect of $RvD1_{n\text{-}3\ DPA}$ on receptor internalization, paired two-sided *t*-tests comparing vehicle- and $RvD1_{n\text{-}3\ DPA}$-treated cells were carried out with Bonferroni adjustments for multiple hypothesis testing. The effect of $RvD1_{n\text{-}3\ DPA}$ addition on the transcription of anti-microbial peptides was analyzed with paired *t*-tests comparing vehicle- and $RvD1_{n\text{-}3\ DPA}$-treated cells. Differences associated with *p* values less than 0.05 were considered to be statistically significant. Statistical analyses were carried out using GraphPad Prism V8.0.1 (GraphPad Software).

RESULTS

FPR2/ALX and DRV1/GPR32 expression in oral epithelium and in primary oral epithelial cells

Immunohistochemical staining for FPR2/ALX and DRV1/GPR32 on healthy gingival biopsies showed that both receptors were expressed in the epithelium and in cells in the connective tissue (Figure 1). FPR2/ALX presented a uniform moderate epithelial staining with a strong intensity across the superficial epithelial layers (Figure 1A–D). DRV1/GPR32 generally presented low to moderate epithelial staining with a stronger expression at the basal and suprabasal layers of the epithelium (Figure 1E–H). Staining with isotype control antibodies was negative (Figure 1I). The same staining pattern was observed after staining of oral organotypic co-cultures of oral keratinocytes and fibroblasts (Figure S2).

In cultured primary oral epithelial cells, qRT-PCR showed that the cells produced mRNA specific for *FPR2/ALX* and *DRV1/GPR32* ($n = 7$; mean $\Delta Ct_{FPR2/ALX} = -11.14$ and $\Delta Ct_{DRV1/GPR32} = -8.63$, respectively) (Figure 2A).

To establish a protocol for flow cytometric staining of oral epithelial cells, monocytes isolated from fresh blood of healthy donors served as positive controls for FPR2/ALX and DRV1/GPR32 staining (Figure S3). This showed that the majority of the monocytes expressed high levels of FPR2/ALX, whereas DRV1/GPR32 expression was confined to a smaller cell subset (Figure S3A). Moreover, we observed that surface staining for FPR2/ALX on monocytes was reduced by trypsin treatment (Figure S3), indicating trypsin-induced cleavage of the antigen epitope recognized by the detecting antibody. Cell scraping was therefore used to collect the cultured oral epithelial cells when DRV1/GPR32 was analyzed. On primary oral epithelial cells, flow cytometric analysis showed strong and uniform surface staining for FPR2/ALX on non-permeabilized cells (Figure 2B). Conversely, surface labeling with the DRV1/GPR32 antibody on non-permeabilized oral epithelial cells revealed nearly no staining. Staining of fixed and permeabilized cells revealed staining of most cells for both FPR2/ALX and DRV1/GPR32.

Immunofluorescence analysis also showed that both $RvD1_{n\text{-}3\ DPA}$ receptors were expressed in the cytoplasm of cultured primary oral epithelial cells (Figure 2C). FPR2/ALX staining was also observed along the cell plasma membrane. In some cells, DRV1/GPR32 showed a tendency to localize closer to the nuclear compartment, whereas FPR2/ALX presented a more even distribution in the cytoplasm.

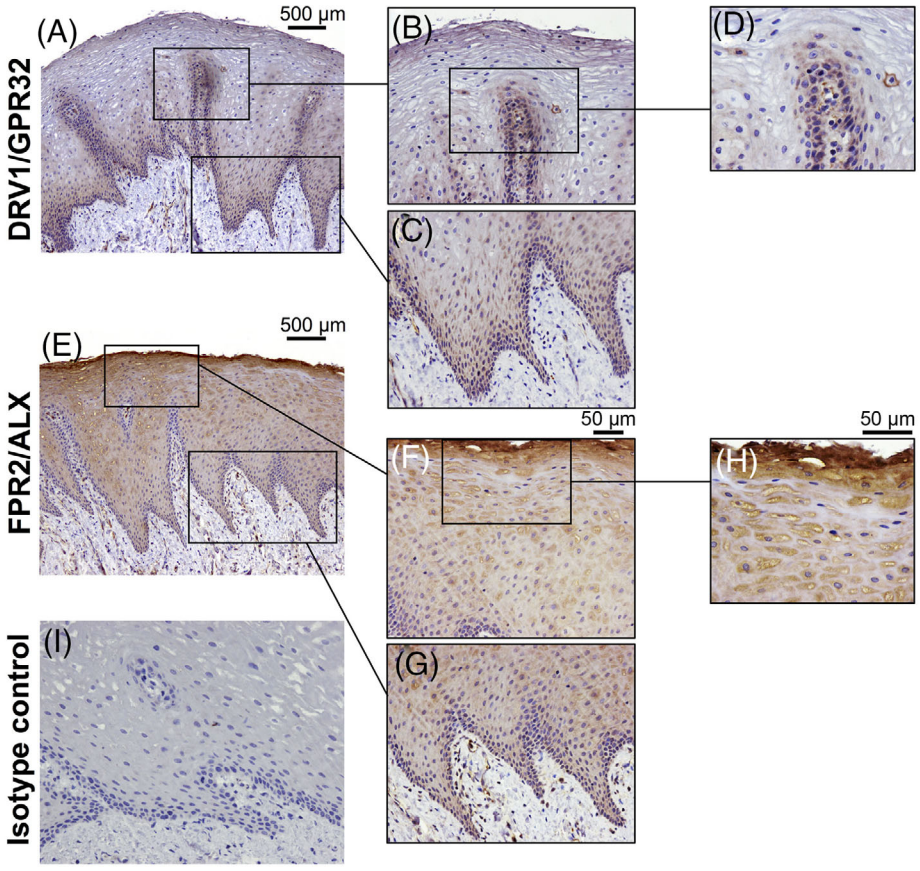

FIGURE 1 Expression of FPR2/ALX and DRV1/GPR32 in oral gingival epithelium. Immunohistochemical staining of sections of healthy human gingival epithelium. Staining was performed on formalin-fixed and paraffin-embedded tissues with antibodies reactive for the receptors FPR2/ALX (A–D) and DRV1/GPR32 (E–H) or the isotype- and concentration-matched controls (I). Figures A, E, and I present 10× original magnification. Inserts show higher magnification of the marked areas. Results are representative of six separate human donors

Effect of RvD1$_{n-3\ DPA}$ on FPR2/ALX and DRV1/GPR32 expression in oral epithelial cells

Membrane trafficking of cell surface G-protein-coupled receptors regulates receptor signaling. As flow cytometry revealed no staining for DRV1/GPR32 on intact oral epithelial cells, we focused on FPR2/ALX internalization. After exposure for 15 min to varying concentrations of RvD1$_{n-3\ DPA}$, FPR2/ALX showed significant internalization within the range of ligand concentrations tested (Figure 3A).

Confocal microscopy analysis showed that treatment with RvD1$_{n-3DPA}$ (0.1 nM) for 20 min resulted in translocation of DRV1/GPR32 from the cytoplasm to the nucleus, a phenomenon that was not seen for FPR2/ALX (Figure 3B).

Intracellular calcium mobilization is important for G-protein-coupled receptor signaling, including that through FPR2/ALX and DRV1/GPR32. Oral epithelial cells in culture responded to RvD1$_{n-3\ DPA}$ exposure by an increase in intracellular calcium mobilization, even at a dose of 10^{-11} M (Figure 3C). This indicates activation of G-protein-coupled receptors even at low concentrations of RvD1$_{n-3\ DPA}$.

Induction by RvD1$_{n-3\ DPA}$ of mRNA expression coding for antimicrobial proteins in primary oral epithelial cells

The regulatory effect of RvD1$_{n-3\ DPA}$ on the transcription of the genes coding for the antimicrobial peptides beta-defensin 1, beta-defensin 2 and cathelicidin (*DEFB1*, *DEFB4A* and *CAMP*, respectively) was analyzed by qRT-PCR. A 0.1 nM concentration of RvD1$_{n-3\ DPA}$ significantly up-regulated RNA expression in oral epithelial cells at 24 h, with *DEFB4A* mRNA showing the highest fold change after adjustment to the house keeping gene (Figure 4).

DISCUSSION

The n-3 docosapentaenoic acid has recently emerged as a potential precursor in the biological generation of specialized pro-resolving mediators, including resolvins such as RvD1$_{n-3\ DPA}$ [16, 23, 24]. RvD1$_{n-3\ DPA}$, first described to be produced by activated leukocytes in a murine ischemia

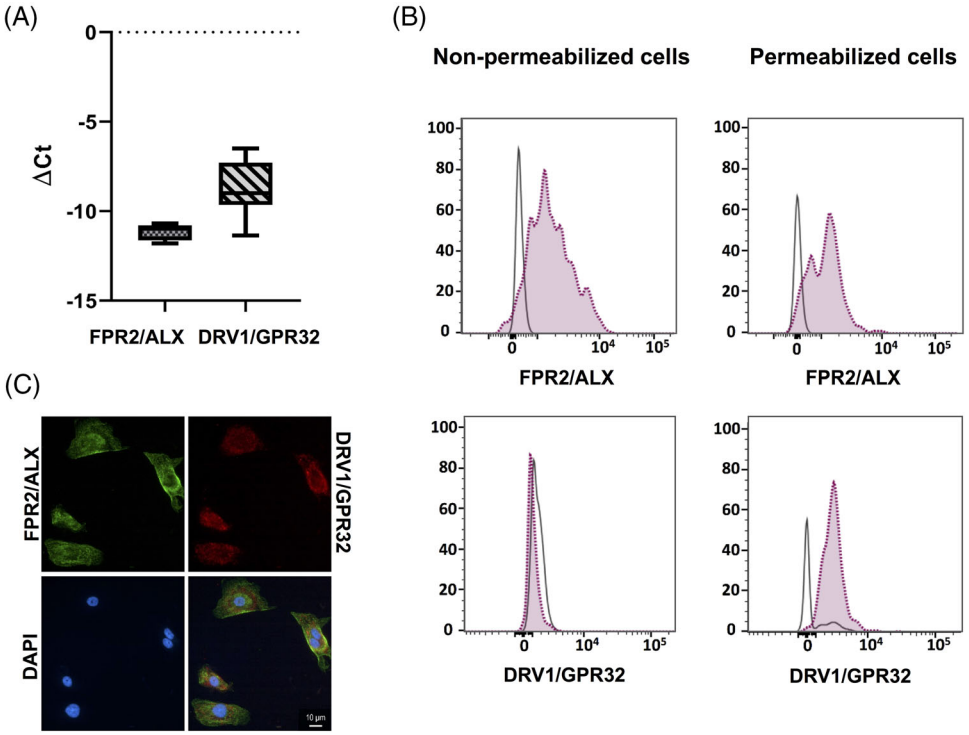

FIGURE 2 Expression of FPR2/ALX and DRV1/GPR32 in cultured primary oral epithelial cells. (A) Box (25-75 percentiles) and whiskers (10-90 percentiles) plot with median (horizontal line) of ΔCt values for *FPR2/ALX* and *DRV1/GPR32* mRNA expression, $n = 7$ donors. (B) Representative histograms from flow cytometric analyses of FPR2/ALX and DRV1/GPR32 staining on non-permeabilized (left) and permeabilized (right) oral keratinocytes. Pink dotted lines represent primary oral epithelial cells stained for FPR2/ALX and DRV1/GPR32. The corresponding isotypes are shown in black solid lines. The *Y*-axis shows percent normalized to mode. (C) Representative immunofluorescence microscopy images of FPR2/ALX (green) and DRV1/GPR32 (red) expression in quiescent primary oral epithelial cells. Nuclei were stained with DAPI (blue). Results are representative of three separate human donors

reperfusion model, inhibits polymorphonuclear neutrophil recruitment and chemotaxis [16]. Here, we found that the two known receptors for $RvD1_{n\text{-}3\ DPA}$, FPR2/ALX and DRV1/GPR32, are constitutively expressed in primary oral epithelial cells. Addition of $RvD1_{n\text{-}3\ DPA}$ to cultured primary oral epithelial cells led to intracellular calcium release, internalization of FPR2/ALX, nuclear translocation of DRV1/GPR32 and increased transcription of genes coding for antimicrobial peptides. This indicates that oral keratinocytes can react to $RvD1_{n\text{-}3\ DPA}$ and that resolvins therefore may participate in the modulation of inflammatory and antimicrobial responses in oral epithelial cells.

FPR2/ALX and DRV1/GPR32 have been reported to be expressed on different types of leukocytes, such as monocytes and polymorphonuclear neutrophils [25], but only few reports are available on their expression on epithelial cells. In situ, FPR2/ALX was expressed in all layers of the gingival epithelium. This accords with the finding of FPR2/ALX expression in human dermal epithelium [7]. Other epithelial cells expressing FPR2/ALX include ductal and acinar cells in human minor salivary glands [26–28], as well as cultured human submandibular gland cells [26]. In cultured oral epithelial cells, qRT-PCR showed transcription of *FPR2/ALX* and flow cytometry showed that the receptor was present on both the cell surface and within the cytoplasm. This was confirmed by immunofluorescence. In situ, DRV1/GPR32 expression differed from that of FPR2/ALX with staining mostly in the deeper gingival epithelial layers. Although flow cytometry showed DRV1/GPR32 on the cell surface of a subset of blood monocytes, we failed to detect it on the plasma membrane of cultured oral epithelial cells. We found, however, ample cytoplasmic DRV1/GPR32 expression by flow cytometry and immunofluorescence. Lack of cell surface DRV1/GPR32 on the oral epithelial cells is in contrast to what is seen in human small airway epithelial cells where it also is detected on the surface of the cells [29]. The presently observed absence of surface DRV1/GPR32 expression in quiescent oral epithelial cells could be due to low levels of the receptor, below the level of detection. Spatiotemporal regulation of localization of G protein receptors [30] could also be the cause: receptors can recirculate from the intracellular compartment to the plasma membrane and in the resting phase DRV1/GPR32 could be absent from the cell surface. Receptor recirculation has, however, thus far not been described for DRV1/GPR32, in contrast to FPR2/ALX [25, 31]. Together, the present findings indicate that FPR2/ALX is

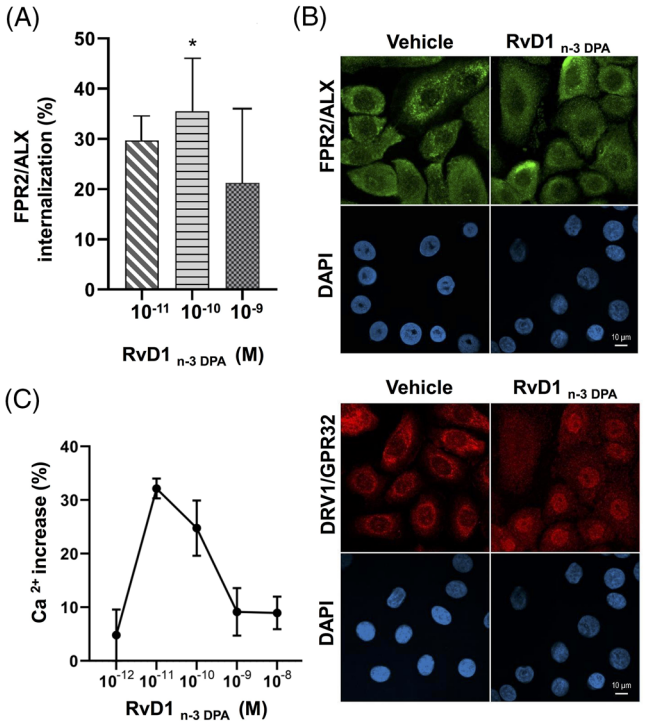

FIGURE 3 FPR2/ALX internalization, nuclear translocation of DRV1/GPR32 and intracellular Ca^{2+} mobilization after addition of RvD1$_{n\text{-}3\ DPA}$ to primary oral epithelial cells. (A) Primary oral epithelial cells were incubated with vehicle (<0.1% ethanol) or RvD1$_{n\text{-}3\ DPA}$ (10^{-11}–10^{-9} M) for 15 min at 37°C. Percent internalization was calculated (see Material and Methods) and shown as mean ± SD ($n = 3$; *$p < 0.05$; paired *t*-tests comparing RvD1$_{n\text{-}3\ DPA}$ vs vehicle after Bonferroni adjustment for multiple comparisons). (B) Representative confocal microscopy images of expression of FPR2/ALX (green) and DRV1/GPR32 (red) in vehicle-treated primary oral epithelial cells (left column) and after 20 min incubation with RvD1$_{n\text{-}3\ DPA}$ (0.1 nM; right column). Nuclei were stained with DAPI (blue). Results are representative of three separate human donors. (C) Primary oral epithelial cells were loaded with Fluo-3 AM (3 μM in keratinocyte serum-free medium) for 45 min, washed twice with Ca^{2+}-free PBS, incubated for 30 min to allow complete de-esterification of intracellular Fluo-3 AM ester and then stimulated with RvD1$_{n\text{-}3\ DPA}$ (10^{-12}–10^{-8} M). Increase in intracellular Ca^{2+} mobilization was quantified by flow cytometry. Results are expressed as mean% ± SD, $n = 3$ donors

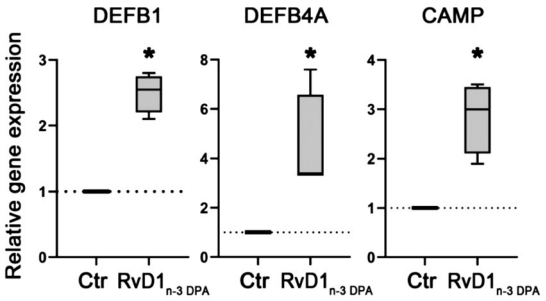

FIGURE 4 Effect of RvD1$_{n\text{-}3\ DPA}$ addition on mRNA levels of antimicrobial peptides in oral epithelial cells. *DEFB1*, *DEFB4A*, and *CAMP* mRNA expression patterns after incubation with RvD1$_{n\text{-}3DPA}$ (0.1 nM) in primary oral epithelial cells were evaluated by qRT-PCR and compared with vehicle-treated (<0.1% ethanol) control oral epithelial cells (Ctr). Box (25–75 percentiles) and whiskers (10-90 percentiles) plot with median (horizontal line) of fold change after adjustment to the house keeping gene ($n = 4$–5 donors). * $p < 0.05$, two-sided paired *t*-test

the main RvD1$_{n\text{-}3\ DPA}$-binding surface receptor expressed by primary oral epithelial cells, whereas DRV1/GPR32 appears to be largely confined to the intracellular compartment. Incidentally, it is conceivable that DRV1/GPR32 can be activated by RvD1$_{n\text{-}3\ DPA}$ intracellularly, as described for other GPCR/ligand systems [32]. Indeed, RvD1$_{n\text{-}3\ DPA}$ is a lipid compound, and may pass through the cell membrane and induce intracellular DRV1/GPR32 signaling.

Trafficking of the receptor from the cell surface can regulate cell surface signaling mediated by G-protein-coupled receptors. Once internalized, G-protein-coupled receptors can follow distinct trafficking routes that further shape the signaling outcome [31]. We examined alterations in surface expression of FPR2/ALX on primary oral epithelial cells following incubation with RvD1$_{n\text{-}3DPA}$ and found that FPR2/ALX was internalized within the range of the tested concentrations. This is in agreement with observations in polymorphonuclear neutrophils [25, 31]. DRV1/GPR32 can also be internalized [25], but as we did not observe cell surface DRV1/GPR32 expression on oral epithelial cells, this was not examined.

Several G-protein-coupled receptors can be detected in cell nuclei and many of them contain putative nuclear localization signals, even though the mechanisms of their translocation to the nucleus remain poorly understood [33]. FPR2/ALX contains a nuclear localization signal and has been recently described to be localized and functional in the nuclei of lung and gastric cancer cell lines [34]. In oral epithelial cells, however, FPR2/ALX did not translocate to the nucleus of the cell, unlike DRV1/GPR32, shortly after addition of the ligand to the cultures. We detected a potential nuclear localization signal sequence in DRV1/GPR32 (^{21}R…I^{54}) as analyzed by cNLS Mapper [35], but whether this sequence is functional in targeting the receptor to the nucleus is as yet unknown. Nuclear translocation of DRV1/GPR32 indicates new, previously unrevealed molecular functions of this receptor within the nuclear compartment.

Signaling through G-protein-coupled receptors is coupled to intracellular calcium (Ca^{2+}) mobilization. We found that addition of even low concentrations of RvD1$_{n\text{-}3\ DPA}$ (0.01–0.1 nM) induced calcium mobilization in primary oral epithelial cells. This can be the result from signaling through FPR2/ALX and/or DRV1/GPR32 but activation of other signaling pathways cannot be excluded. The low RvD1n-3 DPA doses used in the calcium mobilization assay point to a

high potency of this agonist compared with other specialized pro-resolving mediators [36].

Specialized pro-resolving mediators can induce antimicrobial peptides which interfere directly with microbes [37–39]. As cathelicidin (LL-37) and beta-defensins 1 and 2 are widely expressed in oral epithelium [40, 41], we chose those antimicrobial peptides to explore whether $RvD1_{n\text{-}3\ DPA}$ acts on their transcription in oral epithelial cells. Cathelicidin and beta-defensins act by disintegration of cell membranes of sensitive microorganisms [42]. Beta-defensins can also attenuate lipopolysaccharide-stimulated inflammatory responses and suppress the expression of pro-inflammatory cytokines [43]. Addition of $RvD1_{n\text{-}3\ DPA}$ to the present cultured oral keratinocytes induced transcription of *CAMP*, *DEFB1*, and *DEFB4A*, the genes coding for cathelicidin and beta-defensins 1 and 2, respectively. Increased transcription of *CAMP* after exposure of oral epithelial cells to $RvD1_{n\text{-}3\ DPA}$ is in keeping with that seen with other specialized pro-resolving mediators [39]. The activity of antimicrobial peptides in the moist mucosal tract, densely populated by microbes, is essential to keep a healthy balance between the host and the microbiota, amply shown in experiments in mice [44] and by clinical observations in patients with chronic inflammatory bowel diseases [45]. Our findings indicate that $RvD1_{n\text{-}3\ DPA}$ may prime oral epithelial cells to strengthen their defense against microbial infections.

Taken together, the present findings show that oral epithelial cells have the ability to respond to $RvD1_{n\text{-}3\ DPA}$. When released during the inflammatory process, $RvD1_{n\text{-}3\ DPA}$ may prime oral keratinocytes to participate in defense against oral infection (present study) and contribute to the resolution of inflammation as described earlier [16–18]. $RvD1_{n\text{-}3\ DPA}$ may therefore be a promising therapeutic candidate in the prevention and treatment of chronic inflammatory diseases, such as periodontitis.

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CONFLICTS OF INTERESTS

The authors declare no potential conflicts of interest with respect to the authorship and publication of this article.

AUTHOR CONTRIBUTIONS

Conceptualization: M. Balta, I. J. Blix, K. Schenck, E. Baekkevold; **Methodology**: M. Balta, O. Schreurs; **Formal analysis**: M. Balta, E. Glaab; **Investigation**: M. Balta, O. Schreurs, T. Küntziger; **Resources**: T. V. Hansen, J. E. Tungen, A. Vik, I. J. Blix, K. Schenck, E. Baekkevold; **Writing**: M. Balta, K. Schenck, I. J. Blix; **Writing – review and editing**: O. Schreurs, T. V. Hansen, J. Tungen, A. Vik, E. Glaab, T. Küntziger, E. Baekkevold; **Supervision**: I. J. Blix, K. Schenck, E. Baekkevold; **Project administration**: M. Balta, I. J. Blix, K. Schenck; **Funding acquisition**: M. Balta, I. J. Blix.

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SUPPORTING INFORMATION

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