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Short communication

IFN-β secretion is through TLR3 but not TLR4 in human gingival epithelial cells

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ABSTRACT

The oral cavity is home for a plethora of bacteria and viruses. Epithelial barriers encounter these micro-organisms and recognize them via pathogen recognition receptors (PRRs) that instigate antibacterial and antiviral responses. We and others have shown that human gingival epithelial cells (HGECs) express PRRs to defend invading pathogens. Among these PRRs, TLR2, TLR3 and TLR4 are highly expressed in HGECs and appear to be important based on our previous findings. IFN-β is one of the major type 1 interferons induced to defend viral attack. In this report, we sought to dissect TLR3 and TLR4 mediated secretion of IFN-β in HGECs. We stimulated HGECs with ultrapure LPS (TLR4 ligand) and Poly I:C (TLR3 ligand) for 24 h and supernatant was used to determine IFN-β secretion. We show that cells treated with Poly I:C induced IFN-β secretion but not cells treated with LPS. In addition, silencing of TLR3 prior to Poly I:C stimulation significantly downregulated IFN-β secretion. On the contrary, overexpression of MD2 and TLR4 in HGECs restored IFN-β secretion. Upon further evaluation, we found that TLR3 stimulation but not TLR4 induced the phosphorylation of interferon regulatory factor 3 (IRF3), which is critical for IFN-β secretion. We conclude that IFN-β secretion is through TLR3 and not via TLR4 in HGECs.

1. Introduction

Oral epithelium forms the first line of defense against bacteria and viruses (Kinane et al., 2008; Benakanakere et al., 2019, 2015; Benakanakere et al., 2009; Zhao et al., 2010). Recognition of these microorganisms is achieved by pattern recognition receptors that recognize highly conserved microbial-associated molecular patterns (Akira et al., 2006) and initiate signal transduction pathways leading to the activation of immune defense genes (Medzhitov and Horng, 2009). TLR4 was the first toll-like receptor in humans to be characterized (Medzhitov et al., 1997) that recognizes bacterial lipopolysaccharide (LPS) (Poltorak et al., 1998). TLR4 is a an important receptor in gingival epithelial cells sensing Gram -ve bacteria that are known to cause the chronic inflammatory condition termed 'periodontitis' (Kinane et al., 2008; Socransky and Haffajee, 2002). Polymorphisms of the TLR4 gene manifest hypo-responsiveness to bacteria in human gingival epithelial cells (HGECs) and may account for periodontal disease susceptibility (Kinane et al., 2006). Apart from bacterial infections, viruses of the herpes family, especially Epstein-Barr virus (EBV) may also account for the severity of periodontitis (Cappuyns et al., 2005; Slots et al., 2006, 2003; Sabeti et al., 2003; Sunde et al., 2008). Recently, RNA released from EBV infected cells has been shown to activate TLR3 and induce type I interferons and proinflammatory cytokines (Iwakiri et al., 2009).

Type 1 interferons are classical antiviral and immune defense molecules (Garcia-Sastre and Biron, 2006). IFN-α and IFN-β are primarily induced by nucleic acids (dsRNA) (van Boxel-Dezaire et al., 2006). IFN-β in particular can be induced by a non-viral source such as bacterial LPS (Toshchakov et al., 2002). Our laboratory has shown IFN-β production by gingival epithelial cells by TLR4 in cooperation with S1P1 receptor (Eskan et al., 2008a). However, it is still not clear why the HGECs do not induce IFN-β upon LPS challenge. In the present report we aimed to characterize IFN-β production triggered by both TLR3 and TLR4 perturbation to understand and determine the receptor for IFN-β production in HGECs.

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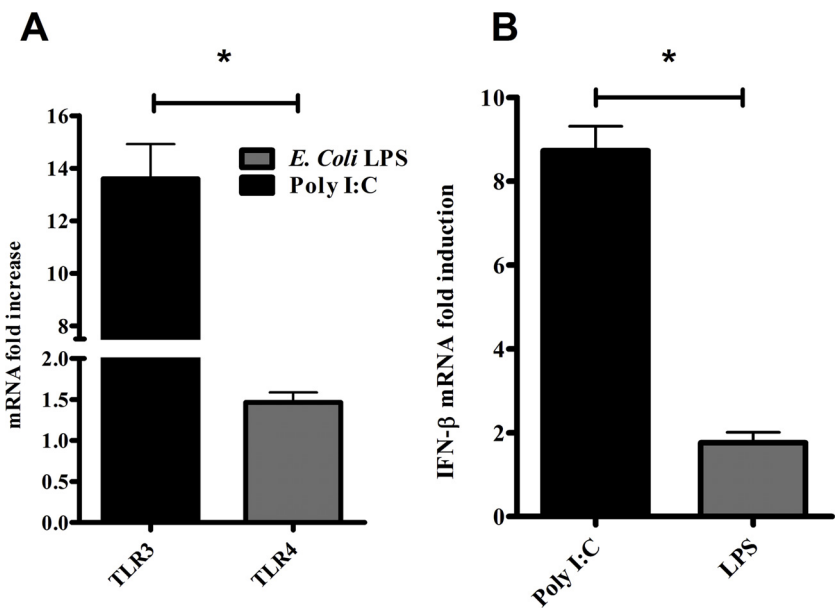

Fig. 1. TLR3 mediated IFN-β gene expression: HGECs were treated poly I:C (5 μg/ml) and LPS (1 μg/ml) for 24 h. Quantitative real-time PCR was performed on cDNA as stated above. Poly I:C induced higher IFN-β gene expression at 24 h post stimulation, but TLR4 activation failed to induce IFN-β gene expression. Statistical test: One-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). Results are mean ± SE.

2. Material & methods

2.1. Cell isolation, culture and challenge assay

Human gingival epithelial cells (HGECs) were isolated as described previously (Shiba et al., 2005, 2005; Kinane et al., 2012; Benakanakere et al., 2010). The isolated HGECs were seeded in 60-mm type-I collagen coated tissue culture plates coated (BD Biocoat) and incubated in 5% CO_2 at 37 °C using KSFM complete medium (Invitrogen, Carlsbad, CA) as described in our previous reports (Zhao et al., 2010; Eskan et al., 2008a; Benakanakere et al., 2010). When the cells reached 90% confluence, they were harvested and cultured in 6 well plates for stimulation assays. The cells were stimulated with 5 μg/ml of Poly I:C (Invivogen) and 1 μg/ml *E. Coli* LPS (Invivogen, CA) for 24 h. The control cells received DMSO or PBS depending on solubility of agonists. The supernatant was collected and subjected to IFN-β ELISA using human IFN-β ELISA kit (PBL Interferonsource, NJ) according to the manufacturer's instructions.

2.2. Quantitative real-time PCR

RNA was extracted from cultured cells using TRIzol (Invitrogen) and quantified. 10 μg total RNA was converted to first-strand complementary DNA (cDNA) synthesis using the cDNA kit (Thermofisher Scientific) in a total volume of 100 μl. Real-time PCR was performed using 50 ng of cDNA on ABI 7500 system (Applied Biosystems). *Taq*-Man probes and primers for gene expression of human IFN-β and GAPDH as an endogenous control were all purchased from Thermofisher Scientific.

2.3. Western blotting

The Western blots were performed using 50 μg total proteins per lane (Benakanakere et al., 2009; Zhao et al., 2010). After electrophoresis is complete, the membranes were blocked, washed and incubated with ser32/36 phospho specific IkBα antibody, Ser396 phospho specific IRF-3 antibody and β-actin as loading controls. The membranes were developed using ECL plus™ western blotting detection reagent (GE Healthcare) and exposed X-ray films which were developed using Konica Medical Film Processor (Konica Corp., Taiwan). The membranes were washed and stripped with Restore plus Western blot stripping buffer (Thermofisher Scientific) and re-probed accordingly.

2.4. Transfection

HGECs at fourth passage were seeded at a density of 0.5 × 10 (Zhao et al., 2010) cells/well in 6 well plates, and maintained in 2 ml of medium until the cells reached 50–60% confluency. HGECs were transfected with 100 pmol of siTLR3 or non-target siRNA pool as control (Dharmacon). 100 pmol of siRNA was mixed with siPORT NeoFX Transfection Agent (Thermofisher Scientific) and incubated at room temperature for 15 min. The transfection mixture was then added drop wise to the wells and the reaction was continued overnight. After overnight incubation, cells were replaced with fresh media. Following transfection, the cells were stimulated as mentioned above. For TLR4 and MD2 overexpression, 1 μg each of pUNO-TLR4 (Invivogen), pFlag-CMV1-hMD2 (Addgene Inc, MA) and empty vector control (Invivogen, CA) plasmids were mixed separately with Fugene 6 transfection reagent (Roche) and incubated for 15 min according to the manufacturer's instructions. The mixture was then added drop wise on to the cells and the transfection reaction was carried out for 24 h. After 24 h post transfection, the cells were stimulated as mentioned above.

2.5. Statistical analysis

The gene expression was calculated according ΔΔCT method (Livak and Schmittgen, 2001). Statistical analysis (analysis of variance and Tukey multiple comparison test) was done using GraphPad Pism 5.0 and GraphPad Instat 3.0. The difference between treatment and controls were considered significant at $p < 0.05$ level. Statistically significant data are indicated by an asterisk ($p < 0.05$ (*).

3. Results

3.1. TLR3 stimulation drives IFN-β expression in HGECs

TLR4 in gingival epithelial cells is a weak receptor in stimulating inflammatory cytokines upon LPS challenge (Eskan et al., 2008a, b). To address this issue, we tested the level of TLR3 and TLR4 receptor gene expression in HGECs. Further, we sought to determine if either TLR4 or TLR3 contribute to IFN-β induction. The cells were stimulated with 1 μg/ml *E. coli* LPS and 5 μg Poly I:C for 24 h. The concentrations of ligands were chosen based on our previous publications (Zhao et al., 2010; Benakanakere et al., 2010). After stimulation, the total RNA was extracted and subjected to real time PCR to determine the level of TLR3

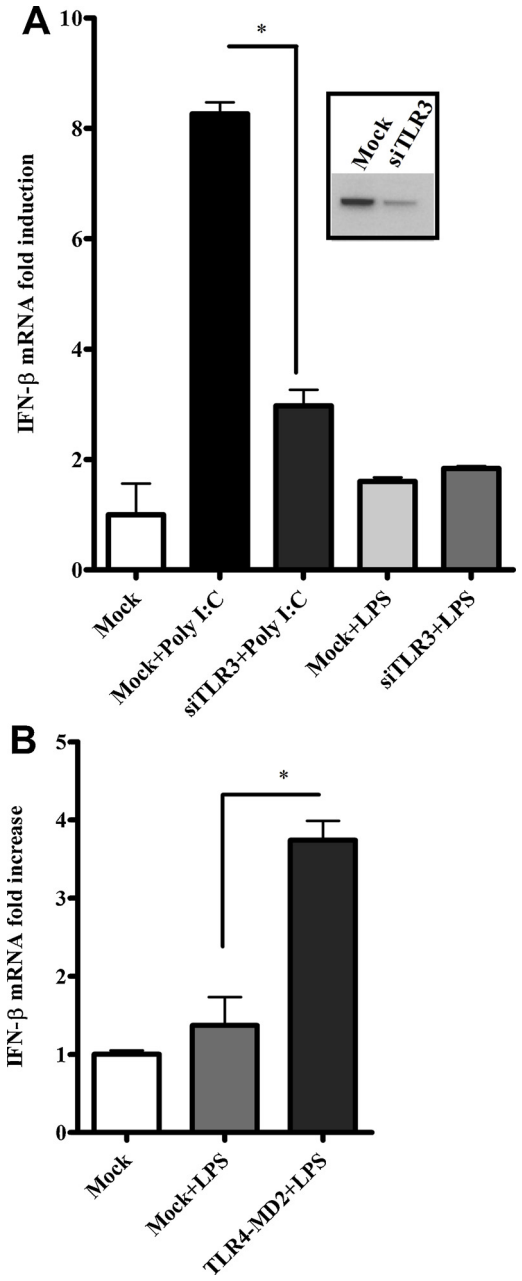

Fig. 2. Silencing TLR3 reduced IFNβ gene expression in HGECs: When TLR3 is silenced, the expression of IFN-β significantly downregulated after stimulation with poly I:C (inlet shows TLR3 knockdown efficiency). On the other hand, LPS had no effect on IFN-β gene expression **(A)**. TLR4 and MD2 over-expression in HGECs restored IFN-β gene expression **(B)**. Statistical test: One-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). Results are mean ± SE.

and TLR4 gene expression. The relative mRNA expression of TLR3 stimulated with Poly I:C was ˜13.4 fold increase compared to the control cells. On the other hand, TLR4 expression was only ˜1.4 folds compared to its control (Fig. 1A). This data clearly show that TLR4 gene expression is minimal compared to that of TLR3 in HGECs. Furthermore, we tested the expression of IFN-β after stimulation with LPS and Poly I:C in HGECs. IFN-β was up-regulated ˜9 fold upon Poly I:C treatment whereas LPS induced ˜1.7 fold increase in IFN-β mRNA expression. This data demonstrates the importance of receptor expression in inducing IFN-β in gingival epithelial cells and confirms that TLR3 predominantly drives IFN-β expression.

3.2. IFN-β secretion is mediated by TLR3 in gingival epithelial cells

To address the receptors involved in IFN-β secretion in gingival epithelial cells, we utilized RNA silencing in HGECs. Cells were grown to 60–70% confluence and transfected with siRNA against TLR3 with scrambled siRNA pool as control. After 24 h post transfection, the cells were stimulated with Poly I:C or LPS. The efficiency of knockdown was determined by immunoblotting against human TLR3 antibody (Fig.2**A inlet**). The real time PCR data on IFN-β showed marked decrease in cells treated with siTLR3 compared to the mock transfected cells (Fig. 2A). IFN-β production by LPS in siTLR3 treated cells did not induce IFN-β compared to control cells. This data clearly indicates that IFN-β induction in HGECs is through TLR3 but not TLR4 as shown by receptor expression. Furthermore, we overexpressed TLR4 in HGECs by transfecting the overexpression plasmid to check if the receptor expression or its signaling was responsible for paralysis of IFN-β in LPS stimulation. When TLR4 is overexpressed in HGECs, the IFN-β mRNA expression was restored to ˜3.7 fold (Fig.2B). This data shows that the receptor expression itself is crucial in inducing IFN-β expression in HGECs.

3.3. TLR3 activates IRF3 to induce IFN-β in gingival epithelial cells

Interferon Regulatory Factor 3 (IRF3) is a crucial transcriptional regulatory element of antiviral response genes (Doyle et al., 2002). There are several reports claiming the role of IRF3 in TLR4 mediated signaling (Doyle et al., 2002; Kawai et al., 2001; Navarro and David, 1999). IRF3 phosphorylation can either can be induced by TLR3 or by TLR4 to mount IFN-β secretion (Doyle et al., 2002). We wanted to test IRF3 phosphorylation levels after stimulation of HGECs with LPS and Poly I:C. The cells were grown to confluence and challenged with either LPS or Poly I:C for 0, 15, 30, 60, 90 min, 2 h, 4 h, 6 h and 24 h. The total protein was collected and subjected to immunoblot against Phospho-IRF3 (Ser396) antibody. Interestingly, Poly I:C induced phosphorylation of IRF3 on Ser396 at 60 min and reached maximum at the 2 h time point. However, LPS failed to induce phosphorylation of IRF3 (Fig. 3A). Interestingly, it has already been shown that TLR3 recognizes dsRNA and activates NF-kB to induce IFN-β (Alexopoulou et al., 2001). Hence, we wanted to examine the activation of NF-kB by measuring the phosphorylation state of IkBα, an inhibitor of NF-kB to determine its level of phosphorylation with LPS and Poly I:C stimulation in HGECs. Poly I:C treated cells dramatically induced IkBα Ser32/36 phosphorylation at 60 min and along the time points tested. However, LPS treated cells induced weak phosphorylation of IkBα Ser32/36 when compared to Poly I:C (Fig. 3A). We next investigated IFN-β induction by both TLR3 and TLR4 agonists. Poly I:C challenged cells induced IFN-β significantly compared to LPS stimulated cells (Fig. 3B). When TLR4 and MD2 were overexpressed in HGECs prior to stimulation with LPS, the IFN-β induction was restored (Fig. 3B). Transient expression of TLR4 and MD2 confer LPS responsiveness to IFN-β induction demonstrating both TLR4 and MD2 is required for epithelial IFN-β secretion. This data shows that the level of TLR4 receptor and MD2 protein expression are crucial in inducing IFN-β in HGECs and also demonstrates that the IFN-β secretion is mainly through TLR3 but not TLR4 in HGECs.

4. Discussion

The major aim of this study was to determine how IFN-β is secreted in human gingival epithelial cells (HGECs). Our laboratory demonstrated that extracellular addition of S1P stimulated S1P1 receptor in cooperation with TLR4 to induce IFN-β secretion in gingival epithelial cells (Eskan et al., 2008a). In this study, we explored why TLR4 by itself cannot induce IFN-β in HGECs upon *E. coli* LPS stimulation. We demonstrate that Poly I:C activated TLR3 to induce IFN-β at both transcriptional and at protein levels. Our results demonstrate that activation

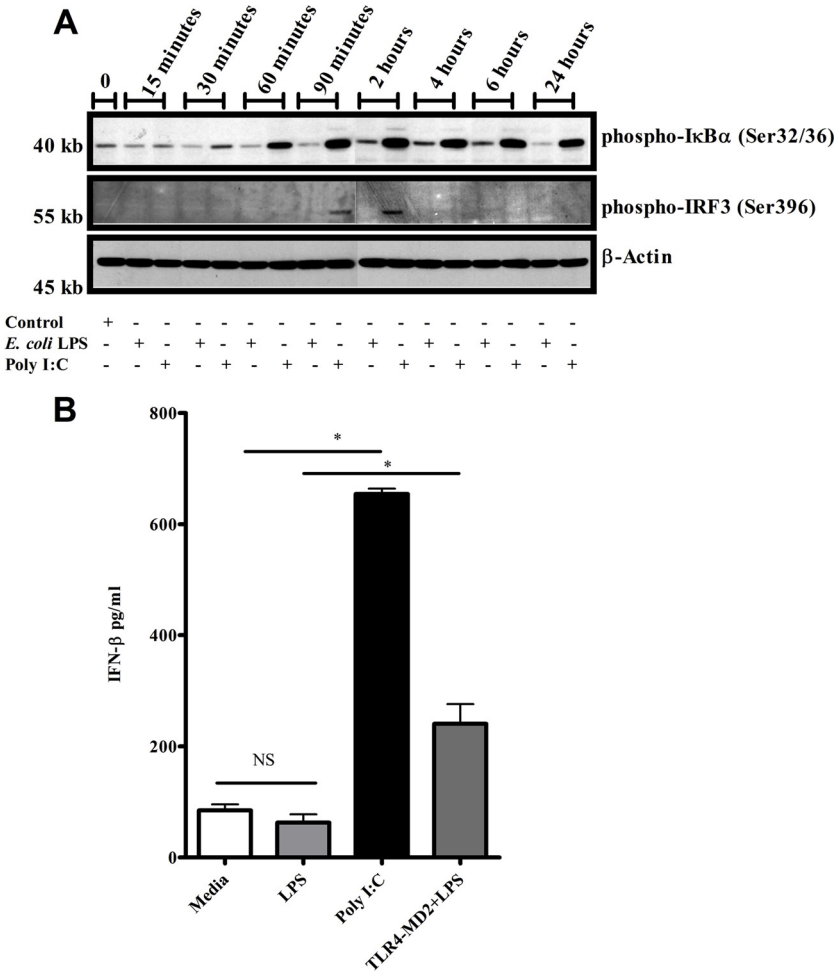

Fig. 3. TLR3 induces phosphorylation if IRF3 in HGECs: Immunoblot showing the phosphorylation of IRF3 and IRF3 (ser396) and IκBα (Ser32/36). TLR3 stimulation in HGECs with Poly I:C leads to the phosphorylation of IRF3 (ser396) and IκBα (Ser32/36) but not with LPS **(A).** IFN-β expression was measured using ELISA in cell supernatants. HGECs treated with Poly I:C significantly upregulated IFN-β secretion but not LPS. However, when HGECs were co-transfected with TLR4 and MD2 overexpression plasmids, IFN-β secretion was restored **(B)**. Statistical test: One-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$). Results are mean ± SE.

of IRF-3 by TLR3 induces IFN-β but not by TLR4. RNA mediated silencing of TLR3 down-regulated IFN-β induction by Poly I:C. This shows TLR3 is unarguably the IFN-β inducing receptor in HGECs.

The association of subgingival EBV and higher levels of gram –ve bacteria, such as *P. gingivalis* has been shown in adult periodontitis and indeed both micro-organisms may synergistically worsen periodontitis (Contreras et al., 1999; Sugano et al., 2004; Chalabi et al., 2008). It has also been shown that EBV is sensed through TLR3 in humans (Iwakiri et al., 2009). In terms of future potential therapeutic approaches it is imperative to know the receptor that mounts the antiviral response in HGECs. This is the first report demonstrating that TLR3 as an IFN-β inducing receptor in HGECs that can contribute in mounting a robust immune response and signaling non-myeloid cells against EBV infection in periodontitis.

Reports in the past have shown that Poly I:C ligands do not activate IRF-3 to induce IFN-β in myeloid cells especially macrophages (Reimer et al., 2008) and dendritic cells (Lundberg et al., 2007). However, non-myeloid cells like endothelial cells and synovial fibroblasts activated IRF-3 (Lundberg et al., 2007). Here we show that non-myeloid HGECs phosphorylated IRF-3 upon Poly I:C treatment. However, LPS failed to activate IRF-3 in contrast to myeloid lineage cells. We have previously shown that activation of IRF3 is important in the induction of IFN-β by HGECs (Eskan et al., 2008a). Hence, the failure of IRF3 activation by *E.coli* LPS in epithelial cells is because of the diminished expression of TLR4 and MD2 as evidenced by the induction of IFN-β in transiently transfected cells. Our results with HGECs corroborated previous observation by Abreu et al. [Abreu et al., 2001] in intestinal epithelial cells where the decreased expression of TLR4 and MD-2 accounted for dysregulated pro-inflammatory gene expression to LPS challenge (Abreu et al., 2001). Interestingly, Franchi et al., identified a new signaling pathway that mediated the sensing of dsRNA in cytosol activating nucleotide-binding oligomerization domain, leucine rich repeat and pyrin 3 (NLRP3) inflammasome (Franchi et al., 2014). In this study, the authors showed that poly I:C induced NLRP3 inflammasome activation is independent of TLR signaling, but dependent on the retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) with a common adaptor mitochondrial antiviral signaling protein (MAVS) in the activation of NLRP3 inflammasome (Franchi et al., 2014). However, we previously showed that Poly I:C do not activate RIG-I or MDA5 in HGECs to induce type-1 interferon secretion (Zhao et al., 2010). Based on our previous observations, we believe that Poly I:C may not have a role in activating NLPR3 inflammasome in HGECs. In summary, we conclude that IFN-β secretion is through TLR3 but not via the TLR4 in HGECs.

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