Utilization of Protease-Activated Receptor signaling pathways in oral innate immune regulation

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Abstract

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Protease Activated Receptors (PARs) are G-protein coupled receptors and they play an active role in host defense. PAR1 and PAR2 are the most highly expressed members of the PAR family in gingival epithelial cells (GECs). This thesis is focused on the role of these two receptors.

Objective: This study investigated how PAR receptors regulate downstream signaling pathways and subsequent cytokines secretion in response to specific bacteria in gingival epithelial innate immunity.

Methods: Human GECs were transfected with small interfering RNA (siRNA) specific for PAR1 or PAR2. The cells were then stimulated with perio-pathogenic *Porphyromonas gingivalis (PG)*, *Aggregatibacter actinomycetemcomitans (AA)* or non-pathogenic bacteria *Streptococcus gordonii (SG)*. We used quantitative real-time polymerase chain reaction (QRT-PCR) to detect the messenger RNA (mRNA) expression level of the different innate immune markers; in addition, we used Multi-ELISA ARRAY to measure the protein level of select cytokines and ELISA to analyze intercellular proteins involved in two signal transduction pathways.

Results: PAR1 and PAR2 knock-down affected the signaling pathways of both PI3K and IKBkB. When PAR1 was knocked down, we observed up-regulation of IKBKB ($p \leq 0.05$) following either AA+SG or PG+SG stimulation, and PI3K up-regulation ($p \leq 0.001$) following AA+SG stimulation. When PAR2 was knocked down, we observed up-regulation of PI3K ($P \leq 0.05$) and IKBKB ($p \leq 0.05$) following SG stimulation. Also our results showed that the secretion levels of innate immune markers varied. IL-8 was down-regulated ($p \leq 0.05$) with AA+SG, PG+SG, or SG stimulation when PAR1 was knocked down. When PAR2 was knocked down, IL-8 secretion decreased after PG+SG stimulation, and increased with SG stimulation ($p \leq 0.05$) compared to bacteria-only controls. TNF-α protein secretion level increased with AA+SG, PG+SG, or SG stimulation ($p \leq 0.05$) and PAR1 knock-down, while with PAR2 knock-down and PG+SG stimulation the protein level of TNF-α decreased compared to controls with the same conditions and no receptors knocked down. IL-1β was down-regulated with PAR2 knock-down with AA+SG or AA+SG stimulation ($p \leq 0.05$). IL-6 was up-regulated in the un-stimulated group and with AA+SG stimulation when PAR2 was knocked down ($p \leq 0.05$), while when PAR1 was knocked down, IL-6 down-regulated following AA+SG stimulation ($p \leq 0.001$). IL-1α was down-regulated when PAR2 was knocked down following AA+SG or SG stimulation ($p \leq 0.05$).

Conclusion: These results suggest that PAR1 and PAR2 receptors conduct their signal through IKBKB and PI3K. Moreover, IL-8, TNF-α, IL-6, IL-1α and IL-1β secretion are modulated by PARs in response to bacterial stimulations. We foresee the information gained from this study will be a stage to further study how different PAR receptors induce appropriate innate immune responses, and also how PAR receptors might work through different signaling pathways to achieve appropriate innate immune response.

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ACKNOWLEDGEMENTS

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I also would like to thank my many friends and whoever has supported me throughout the process. I will always appreciate all they have done.

DEDICATION

I dedicate my thesis work to my family, and my country. A special feeling of gratitude to my loving parents, Abdalla and Fathia Borgeia whose words of encouragement and push for tenacity ring in my ears. My sisters and brothers have never left my side and are very special. I finally dedicate this work to Libya, my country, and I appreciate the scholarship they gave to achieve my advanced education.

Chapter One: Background

1. Introduction

Periodontitis is an inflammatory condition leading to destruction of connective tissues and migration of the gingival attachment to create a pocket (1). Periodontal disease begins with bacterial growth in the mouth which leads to tooth loss if it is not treated properly. In the early stage of this disease, bacteria grow in plaque, causing a swelling or inflammation in gums that can damage the tissues which surround the teeth. These changes are induced by the diffusion of bacterial products through the affected epithelium (2, 6). An inappropriate host immune response also has a role in tissue destruction in periodontitis (9).

The part of the gingiva that is affected in periodontitis is the attached gingiva around the teeth, which consists of gingival epithelial cells (GECs). The gingival epithelia comprise the epithelial tissue that covers the external surface of the gingiva as well as the epithelium lining, the gingival sulcus and the junctional epithelium. The gingival epithelia act as a physical barrier and also have an important value in innate immunity by expressing antimicrobial peptides (3, 4, and 5). The non-keratinized sulcular and junctional epithelia are the main barriers that prevent the microbial products from penetrating through the periodontal tissue. In the case of periodontitis, the epithelial lining loses the tight junction with itself and becomes easily permeable (11, 12).

Oral epithelia are exposed to various pathogenic and non-pathogenic bacteria in both those that are healthy or have periodontitis. Oral epithelia maintain homeostasis by carefully setting up and delegating the immune responses which rely on cellular receptors and on the expression of innate immune markers. These markers of immunity include

antimicrobial peptides, chemokines and cytokines (8). Cytokines like IL-1α, IL-1β, IL-8, IL-6 and TNF-α have a significant role in inflammation and host response to infection. They play a role in both innate and adaptive immunity by activating neutrophils and enhancing T and natural killer cell maturation (27). However, overproduction of cytokines by epithelial cells, such as occurring during periodontitis, increases local blood flow, neutrophil infiltration, and stimulation of Matrix Metalloproteinase (MMP) secretion from osteoclasts, fibroblasts and neutrophils, leading to bone resorption and pocket formation (27).

The oral cavity harbors millions of bacteria during both health and disease. *Porphyromonas gingivalis* (PG) and *Aggregatibacter actinomycetemcomitans* (AA) are Gram negative bacteria strongly connected with periodontitis (13, 14). *Streptococcus gordonii* (SG) are considered as a part of the normal oral microbiota (13, 14). Pathogenic bacteria, particularly PG within the microbial biofilm that start accumulating around the gum, release products that deregulate the host immune response (10). Recent analysis of bacterial population in subgingival plaque biofilms showed pathogens, including PG, were relatively abundant in microbiota from subjects with periodontitis, whereas *streptococci*, including SG, were major components of microbiota from periodontally healthy subjects (15). Diffusion of bacterial products through the affected epithelium leads to periodontal disease (7).

During periodontitis the first colonizer on the dental plaque is SG, then PG and/or AA will colonize later in the biofilm. The availability of SG allows and provides a platform to other colonies to attach to the dental plaque and like PG, has an important role in initiation and the progression of chronic periodontitis. AA is also a good example of a pathogen which acts as an etiologic agent of locally aggressive periodontitis (39).

Protease-activated receptors (PARs) are G-protein coupled receptors with a unique mechanism of activation. These receptors have sequences which can result in tethered ligands when activated by proteolytic activity of serine proteases (16, 23). Among the four

members of the PAR family, PAR1 and PAR2 are highly expressed in human gingival epithelial cells (20, 25). Gingival epithelial cells express protease activated receptors as well as other receptors like TLR and NOD in order to sense their environment. Presence of pathogenic and commensal bacterial products leads to activation of these receptors, subsequently resulting in up-regulation or down-regulation of cytokines. These cytokines initiate and regulate the innate immune responses (16, 17, 21, 22 and 35). PAR1 is activated by thrombin, while PAR2 is activated by trypsin (16). Activation of PARs by proteases of pathogens, PG and AA, suggests an active participation for PARs, and in particular PAR2, as an assumed mediator of periodontitis (16, 18, 19, and 20). However, there is not sufficient information about the signaling downstream of PAR receptors when PARs are activated by bacteria. The PI3K/Akt signaling pathway plays a role in coordinating defense mechanisms in innate immunity. In a previous study by Rohani and colleagues (3) it was suggested that PI3K down-regulates PAR signaling in gingival epithelial cells. This effect was observed at the mRNA level and also for CXCL5 at the protein level (3). This study also suggested that PI3K acts as a compensatory mechanism which suppresses inflammatory responses. A similar inhibitory role for PI3K signaling in response to TLR2 and TLR5 activation has been reported in dendritic cells and epithelial cells (33, 34), suggesting that PI3K may act as a balancing regulator to prevent excessive innate immune responses. However, little information is available about PAR-mediated PI3K signaling in normal human gingival epithelial cells in response to bacteria.

The NF-κB signaling pathway is important in inflammatory, immune, proliferative, differentiation, and survival responses of cells. In the majority of cell types, NF-κB complexes are preserved in the cytoplasm by a family of IkB inhibitory proteins (IkBs). Upon activation of the pathway, the cytoplasmic IkB kinase (IKK) complex is activated and phosphorylates the IkB molecules (26). This leads to its proteasomal degradation and release of the NF-κB complex, allowing this complex to translocate to the nucleus to start

activating its DNA targets (26). Thus, signaling through the NF-κB pathways involves the release of NF-κB from IkB in the cytosol; followed by translocation into the nucleus. This, in turn, could lead to regulate the secretion of a group of cytokines, like IL-8, IL-6, IL-1α, TNF-α, and IL-1β, that are important in innate immune defense during the periodontal inflammation. These markers are essential for the innate immune response by immune cells like macrophages, B-cell, and T-cell in general and also they are important for the innate host response by epithelial cells in periodontal diseases. TNF-α is an important immune marker and its increase is one of the signs for acute inflammation (28). Also this cytokine is involved in the periodontal disease in which the secretion of this cytokine increases the blood flow in the adjacent connective tissue, attracts other adaptive immune cells, and stimulates more gingival epithelial cell in the area of infection (32). The primary role of TNF-α is in the regulation of immune cells especially in the acute phase. Interleukin-8 (IL-8) is a chemoattractant cytokine produced by various tissues. Unlike many other cytokines, it has specificity for the neutrophil, without any significant effects on other blood cells. IL-8 attracts and activates neutrophils at inflammation sites. The activation of neutrophil is important for fighting the pathogenic bacterial invasion in periodontitis (38). These cytokines could be released in response to invaders such as pathogenic bacteria, PG and AA. Significance of NF-κB in inflammatory signaling has made it a natural focus of research about periodontal inflammation (24). But little has been studied about whether this signaling pathway is utilized in the PAR-mediated response to periodontal pathogens. Our GECs could be utilizing these markers to initiate the process of defense against microbes, and also these markers need the intracellular signaling component in order to be secreted.

Discerning the signaling pathway downstream of PAR activation will lead to better understanding of how innate immune responses are regulated in maintaining oral health. The aim of this research was to explore the function of these receptors and to understand the role of the receptors in immune responses to bacteria.

2. Specific Aims

- **The first specific aim** was to investigate if PIK3, a regulator of innate immune markers, and IKBKB, a component of NF-κB signaling pathway, are regulated by PARs in epithelial responses to oral bacteria.
- **The second specific aim** was to investigate the role of PARs in the secretion of identified cytokines in response to oral bacteria stimulation.

Chapter Two: Materials and Methods

1. Human gingival epithelial cells and growth conditions

Gingival tissue in healthy condition was collected from patients undergoing surgery to remove their third molars. Gingival epithelia cells (GECs) were isolated from these tissues and cultured. For experiments, GECs were cultured in T75 flasks until 80% confluency. GECs were then removed with Detachin (Genlantis San Diego, CA), a non-trypsin-based cell release agent, and counted using The Countess® cell counter (ProductWiki Inc. Palo Alto, CA). Finally, the appropriate cell number was used according to the seeding density of the experiment.

For these specific experiments GECs were plated in a 12-well plate and incubated for one hour to attach to the bottom of the wells. GECs were then transfected with specifically selected small interfering RNA (siRNA) for PAR1 or PAR2. This siRNA was custom-synthesized by Qiagen (Valencia, CA) and added at a 5nM concentration. After 48 hours, GECs were stimulated with a combination of AA+SG, PG+SG or SG alone for 16 hours. Supernatant, total RNA and the whole cell lysates were then extracted.

2. Bacterial culture

The three different bacteria used for the experiments are: *Porphyromonas gingivalis* (PG) (ATCC33277), *Aggregatibacter actinomycetemcomitans* (AA) (ATCC43718), and *Streptococcus gordonii (*SG) (ATCC49818). AA was grown in Todd-Hewitt Broth media (Fluka T1438 Sigma-Aldrich, St. Louis, MO) supplemented with yeast extract at 1g/1L of media. PG was grown in Bacto Trypticase Soybroth (Becton, Dickinson and Company, Sparks,MD) supplemented with yeast extract at 1g/1L, Hemin at 5mg/1L, and Menadione (Vitamin K) at 1mg/1L of media. For SG, Brain Heart infusion broth (Becton, Dickinson and Company) was used for culturing. Culture media were pre-reduced in anaerobic jars, with a catalytic pillow and an anaerobic indicator strip, in 37°C incubators overnight before adding

the PG or AA from the frozen stock, while SG was grown in aerobic condition. AA and PG took 2-3 days to grow and SG took only overnight to grow. CFU/OD estimates established in our lab were used to calculate the multiplicity of infection (MOI). For our stimulation conditions, we used an MOI of 50:1 (50 SG cells to 1 GEC), followed by an MOI of 50:1 for PG or AA. For stimulation with SG alone, we used an MOI of 100:1. We chose this MOI because we tested different MOI concentrations and we found that this MOI is suitable to initiate innate immune response in GECs without causing cellular stress. We used the combination of PG+SG or AA+SG to simulate the oral environment.

3. RNA isolation

RNA was extracted from GEC lysates using Qiagen RNeasy kit (Valencia, CA). Total RNA was quantified using a Nanodrop® (Thermo Scientific, Wilmington, DE). Reverse transcription was performed with 250 ng/µl of the total available RNA using the high capacity kit (Applied Biosystems®, Foster city, CA). Following the manufacturer's protocol, we synthesized cDNA using iScript (Bio-Rad, Hercules, CA) and a Master cycler® (Eppendorf, Germany).

4. QRT-PCR

Quantitative analysis of the cDNA was performed using CFX (Bio-Rad) and SYBR green PCR kit (Bio-Rad) according to the manufacturer's instructions. PCRs were performed in 96-well plates in a total volume of 20 µl, including 2 µl of cDNA and 250 nM primers. At the end of every real-time PCR, melting curve analysis was performed to confirm that the amplified product was specific. Standard curve analysis was conducted. All reactions were carried out in duplicate and average threshold cycle values were calculated. Sample values were normalized to the expression values of the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Negative PCR controls were performed using water without cDNA.

5. Western Blot

We performed Western blots to check the efficiency and the specificity of siRNA transfection at the protein level. GECs cell lysates after siRNA transfection were run on NuPAGE® Novex 4-12% Bis Tris Gel (Life Technologies, NY). The gel was then transferred to PVDF membrane for Western blot. The PAR1 primary antibody (goat: R&D, Carlsbad, CA) was used at a concentration of 1:1000. Donkey-anti-goat (R&D) secondary antibody was used at a 1:20,000 concentration. The signal on the membrane was imaged by Gel Doc (Bio-Rad).

6. IKBKB ELISA

PathScan® (s177/181) Sandwich ELISA Kit (Cell Signaling, Danvers, MA) was used to determine IKBKB protein level. We used undiluted cell lysates. From each undiluted cell lysate, 100 µl was added to the appropriate well. The plate was incubated overnight at 4°C, then 100 µl of detection antibody was added to each well, and the plate was incubated at 37°C for 1 hr. Secondary antibody was added to each well and incubated for 30 min at 37°C. Optical density was measured at 450 and 570 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnydale, CA). To analyze the data, the reading at 570 was subtracted from the 450 reading to remove background noise due to plate imperfection. Then the data for each condition were averaged and normalized to the un-stimulated control.

7. PI3K ELISA

We used PI3 Kinase reaction in the Glutathione-coated strips/plate (EMD Millipore Corporation, Billerica, MA) to perform this experiment. The kinase and inhibitor were pre-incubated for 10 minutes prior to adding Phosphatidylinositol 4, 5-bisphosphate (PIP2) substrate. Five µL/well of 5X Kinase reaction buffer and 5 µL/well of PIP2 substrate were added. Samples were set up in triplicate wells according to the manufacturer's

recommendation. Twenty-five µL/well of Phosphatidylinositol (3, 4, 5) - triphosphate (PIP3) working solution was added to all wells excluding the buffer control wells. The wells were monitored for the appearance of a blue color after adding the Stop Solution to avoid over-development, and then the plate was read at 450nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnydale, CA). All the sample values were divided with Biotinylated-PIP3 average, and then the data were averaged. Finally, the data were multiplied by 100 to show the relative percentage to the positive signal.

8. Multi-Analyte ELISA ARRAY

A Multi-Analyte ELISA ARRAY kit (Qiagen) contains 6 different pre-coated capture antibodies for IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF for detection of all markers simultaneously. Of these, we were only interested in the first 5 cytokines because of their relevance in gingival innate immunity. The kits are composed of detection antibodies, antigen standards, detection reagents, and a positive and a negative control. To account for optical imperfection in the ELISA plates, a correction is made by subtracting the 450nm wavelength readouts by the corresponding 570 nm wavelength readouts. To account for the fold differences in protein levels, all conditions were compared to the control (un-stimulated GECs) in order to obtain a relative measurement of expression, and then we averaged our data.

9. Statistical Analysis

All experiments were performed twice and each experiment was set up in duplicate, except for IKBKB and PI3K ELISA which were done in triplicate. The samples were pooled per experiment for both ELISA and QRT-PCR experiments. Our statistical analyses involved a two-tailed t-test to determine statistical significance, and significance was determined based on a P value< 0.05. The data are presented as mean $\pm$SD.

Chapter Three: Results

1. Efficient and specific knock-down of PAR1 and PAR2 receptors

Analysis of PAR1 and PAR2 mRNA expression after transfection with siRNA is important to determine whether PAR1 and PAR2 receptors were silenced successfully before we continue our experiments. The result of our transfection for both PAR1 and PAR2 was determined by QRT-PCR. We achieved 85% knock-down for PAR2 and 95% for PAR1 (Figure 1). We also included a negative control when we performed siRNA experiments to eliminate the possibility of non-specific silencing effects. Non-silencing (NS) siRNA has a sequence that has been shown to have no effect on gene silencing. In addition, PAR1 did not cross react with PAR2 and vice versa (Figure 1).

The efficient knock-down for PAR1 was also determined at the protein level using Western-Blot method. Western blots with PAR2 antibody showed too much background after multiple attempts at optimization, thus we were unable to verify the specificity of PAR2 knock-down at the protein level. The PAR1 receptor protein band should appear at 66-70 KD range (Figure 2). The knock-down of the PAR1 receptor is indicated by the circle where there is an absence of the band where PAR1 receptor expected to appear (Figure 2).

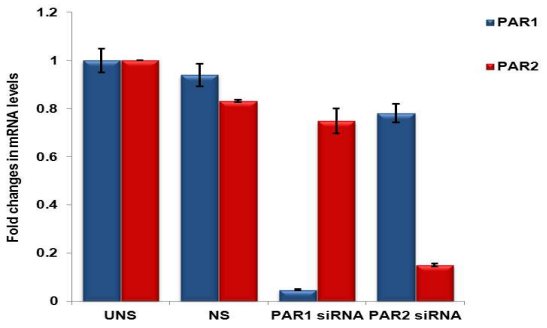

Figure 1: Specific and efficient knock-down of PAR1 and PAR2 was determined with QRT-PCR. **NS:** negative control, siRNA sequence that has been shown to have no effect on gene silencing.

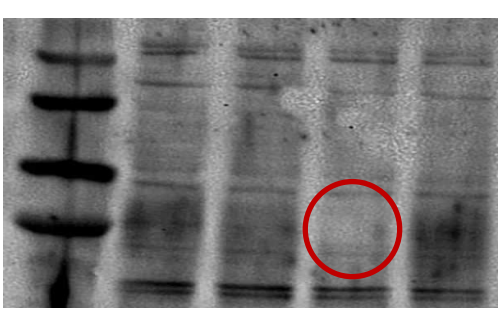

Figure 2*:* Successful knock-down of PAR1 receptor at the protein level. The red circle represents a region where the PAR1 protein band is expected.

2. Result from the first aim

2-1. PAR1 changes the gene expression level of IKBKB as determined by QRT- PCR

To determine if IKBKB is utilized in the signaling of PAR1 and PAR2 receptors, we knocked-down PAR1 and PAR2 by siRNA , and stimulated the GECs with AA+SG, PG+SG, or SG, and then tested the changes in IKBKB expression. The level of expression of mRNA for IKBKB was increased with PAR1 knock-down in the case of both AA+SG and PG+SG stimulation ($p \leq 0.05$) (in comparison to the control which is the GECs that were stimulated only with the specific bacterial combination and no receptor knock-down) (Figure 3). PAR2 knock-down did not change the IKBKB gene expression significantly with both bacterial stimulations.

2-2. PAR1 and PAR2 change the gene expression level of PI3K as determined by QRT-PCR

The level of expression of mRNA for PI3K was increased ($p \leq 0.001$) with PAR1 knock-down in the case of AA+SG stimulation compared to the control (GECs stimulated only with the specific bacterial combination). The PI3K expression was not changed significantly with PG+SG stimulation and either PAR1 or PAR2 knock-down. The expression of PI3K was increased ($p \leq 0.05$) with PAR2 knock-down and SG stimulation (Figure 4).

2-3. Protein level for IKBKB detected by ELISA

SG stimulation resulted in increased protein expression of IKBKB with PAR2 knock-down ($p \leq 0.05$) in comparison to our control (GECs stimulated with only the specific bacterial combination) (Figure 5). Since the NS control in this condition was higher than bacteria-stimulated GECs control, the protein expression level for IKBKB was compared with NS and the difference was not significant.

2-4. Protein level for PI3K detected by ELISA

When PI3K is activated, it converts PIP2 to PIP3, which is the active form of this lipid kinase. We utilized PI3K ELISA to measure the activity of PI3K in our samples after the stimulation conditions as listed above. No significant changes in the protein level of PI3K are observed with PAR1 or PAR2 knock-down followed by stimulation with AA+SG, PG+SG, or SG (Figure 6).

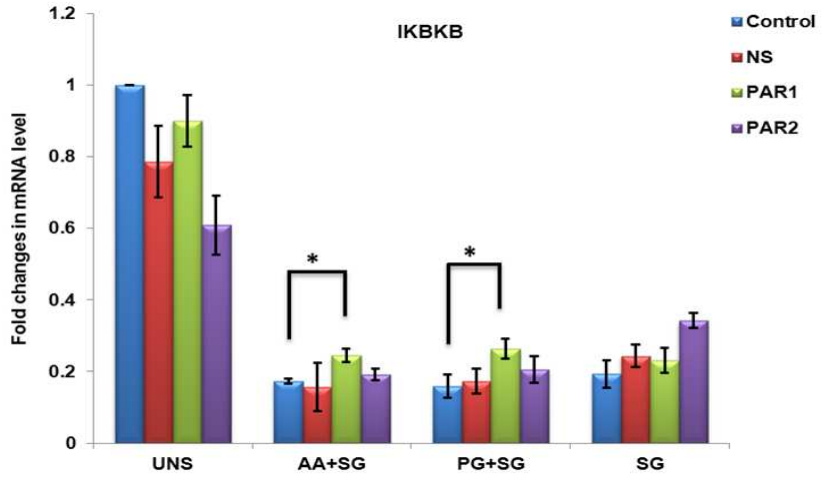

Figure 3: Changes in gene expression determined with QRT-PCR with SYBR green show PAR1 knock-down resulted in a significant increase in the mRNA expression level of IKBKB with AA+SG and PG+SG stimulation compared to control. ***** ($p \leq 0.05$)

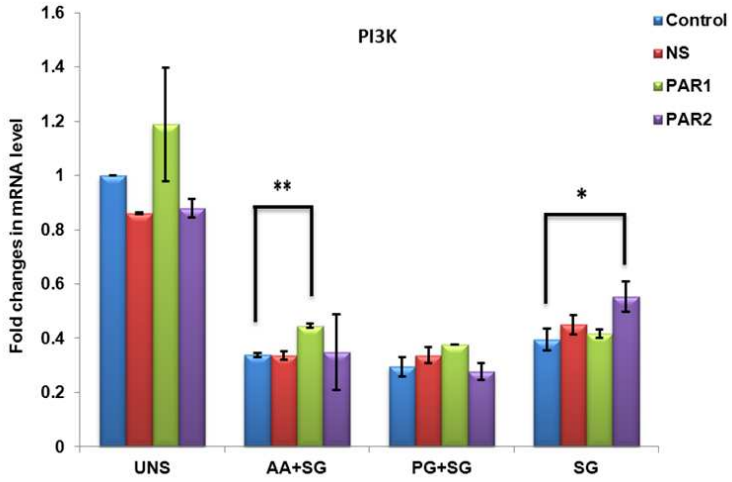

Figure 4: Changes in gene expression determined with QRT-PCR with SYBR green show PAR1 knock-down resulted in a significant increase of the expression of PI3K with AA+SG stimulation, while PAR2 knock-down resulted in a significant up-regulation of the expression of PI3K with SG stimulation compared to control.***** ($p \leq 0.05$) ****** ($p \leq 0.001$)

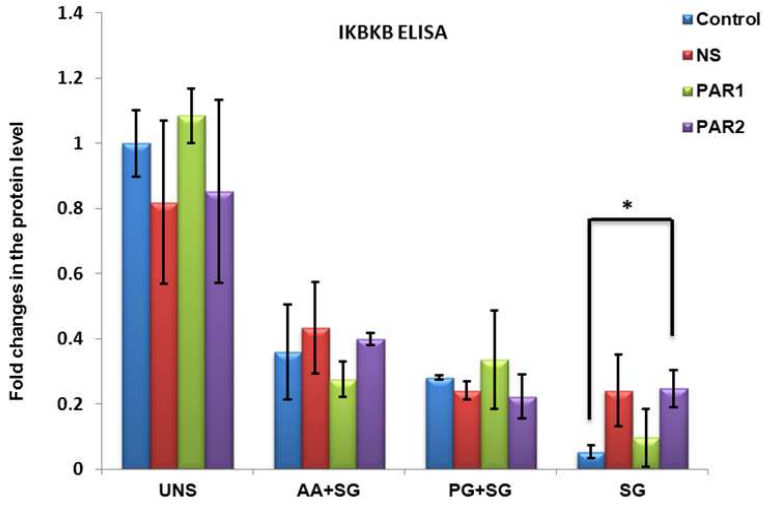

Figure 5: Changes in protein level determined with ELISA show that PAR2 knock-down resulted in a significant increase in IKBKB protein level with SG stimulation compared to control ***** ($p \leq 0.05$)

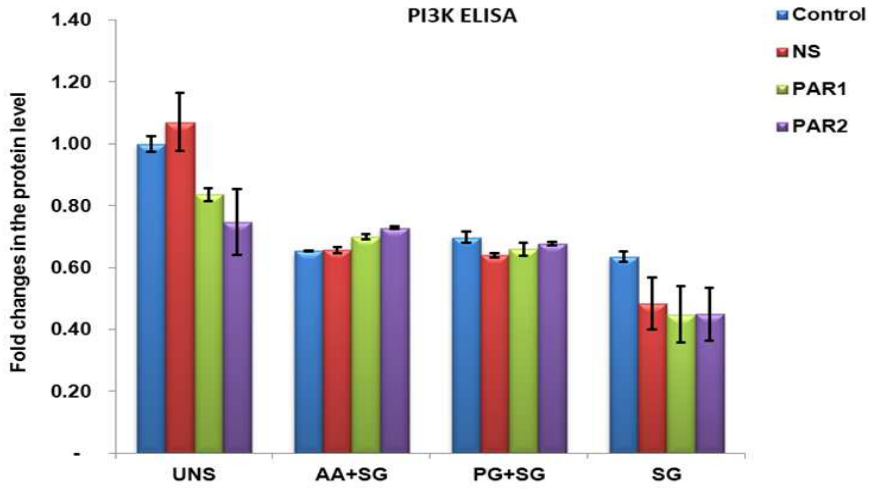

Figure 6: Protein level determined with ELISA shows that PAR1 and PAR2 knock-down did not change the protein level of PI3K.

Result from the second aim

For the second part of the study, we investigated how some of the important inflammatory markers are regulated by PAR receptors

3-1. Cytokine secretion levels detected by Multi-ELISA ARRAY with AA+SG, PG, and SG stimulation

Down-regulation of IL-8 protein secretion level was observed with PAR1 and PAR2 knock-down in all the groups except with AA+SG, in which the IL-8 secretion was decreased only when PAR1 receptor was knocked down (all $p \leq 0.05$) (Figures 7,8,9).

TNF-α protein secretion level was significantly up-regulated with AA+SG stimulation when PAR1 was knocked down, while TNF-α protein secretion level down-regulated with PAR2 knock-down following AA+SG stimulation ($p \leq 0.05$) (Figure 7)

IL-1β protein expression level was significantly down-regulated with AA+SG or PG+SG bacterial stimulation when PAR2 was knocked down ($p \leq 0.05$) (Figures 7, 8).

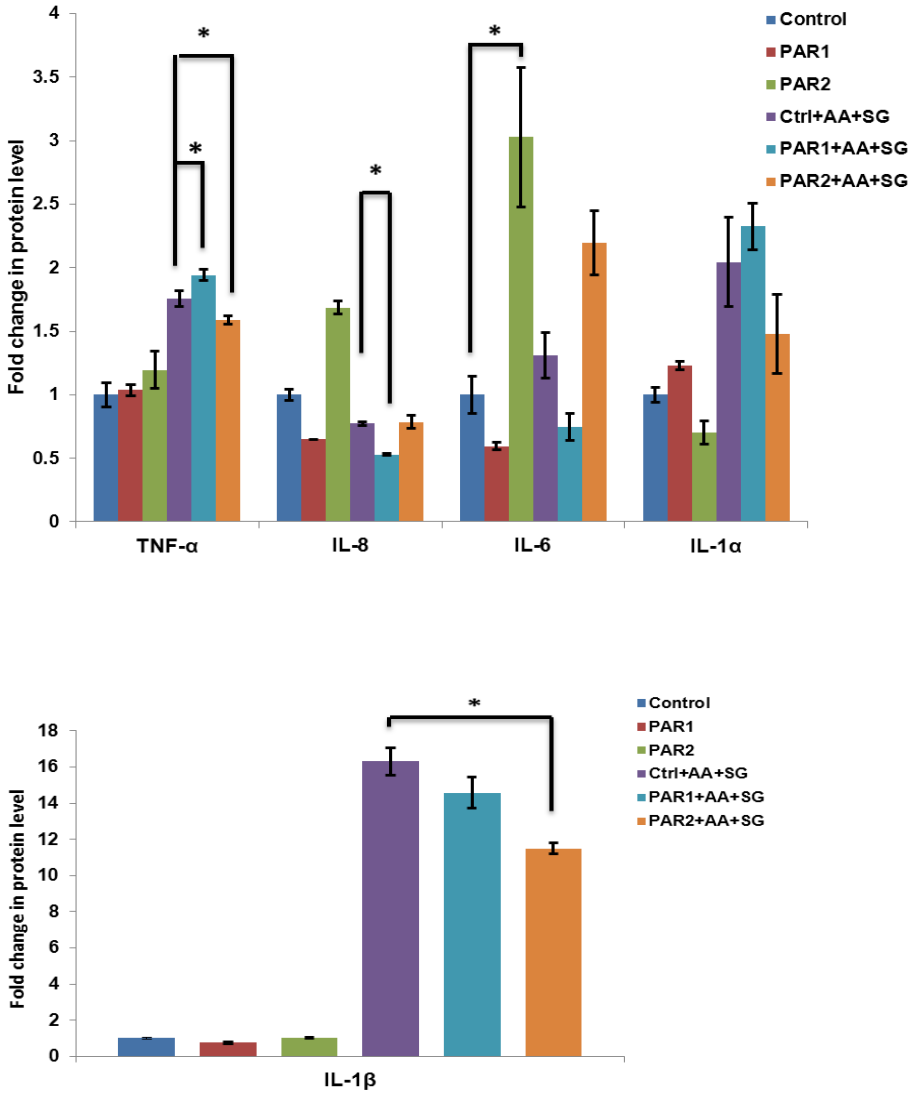

Figure 7: Cytokine secretion levels detected by Multi-Analyte ELISA ARRAY show TNF-α protein secretion level increased significantly as a synergistic effect from multi-bacterial stimulation with AA+SG with PAR1 knock-down, while PAR2 knock-down down-regulated the protein secretion level of TNF-α. IL-1β secretion also was significantly increased with PAR2 knock-down followed by multi-bacterial stimulation by AA+SG. IL-6 protein secretion level increased with PAR2 knock-down with absence of any bacterial stimulation. A significant decrease was seen in IL-8 secretion with PAR1 knock-down. *(p 0.05)

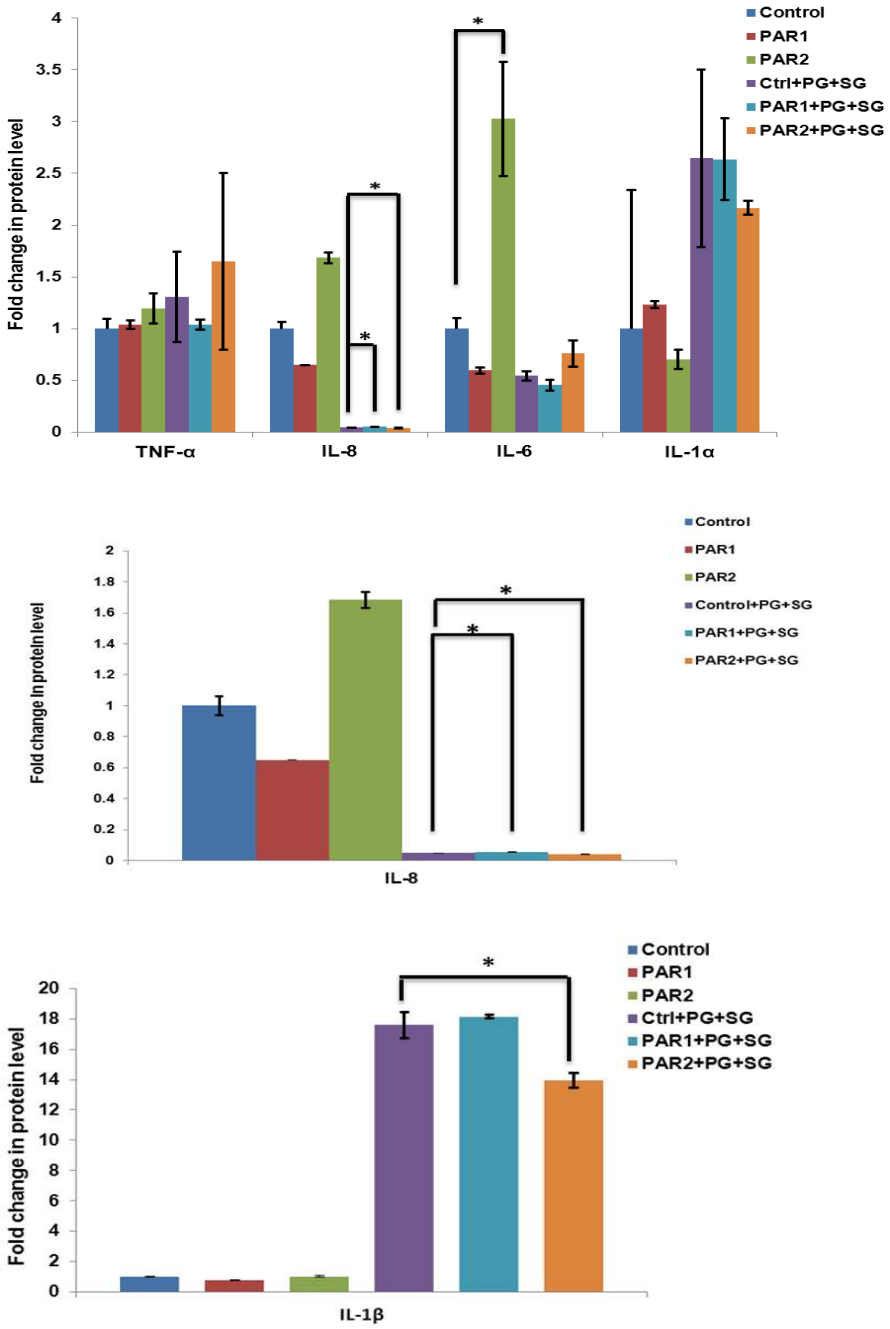

Figure 8: Cytokine secretion levels detected by Multi-Analyte ELISA ARRAY show IL-8 secretion is significantly decreased with PAR1 and PAR2 knock-down in comparison to the control which is un-stimulated GECs. IL-6 protein secretion level increased with PAR2 knock-down with absence of any bacterial stimulation in comparison to the control. IL-1β protein secretion level decreased with PAR2 knock-down. *($p \leq 0.05$)

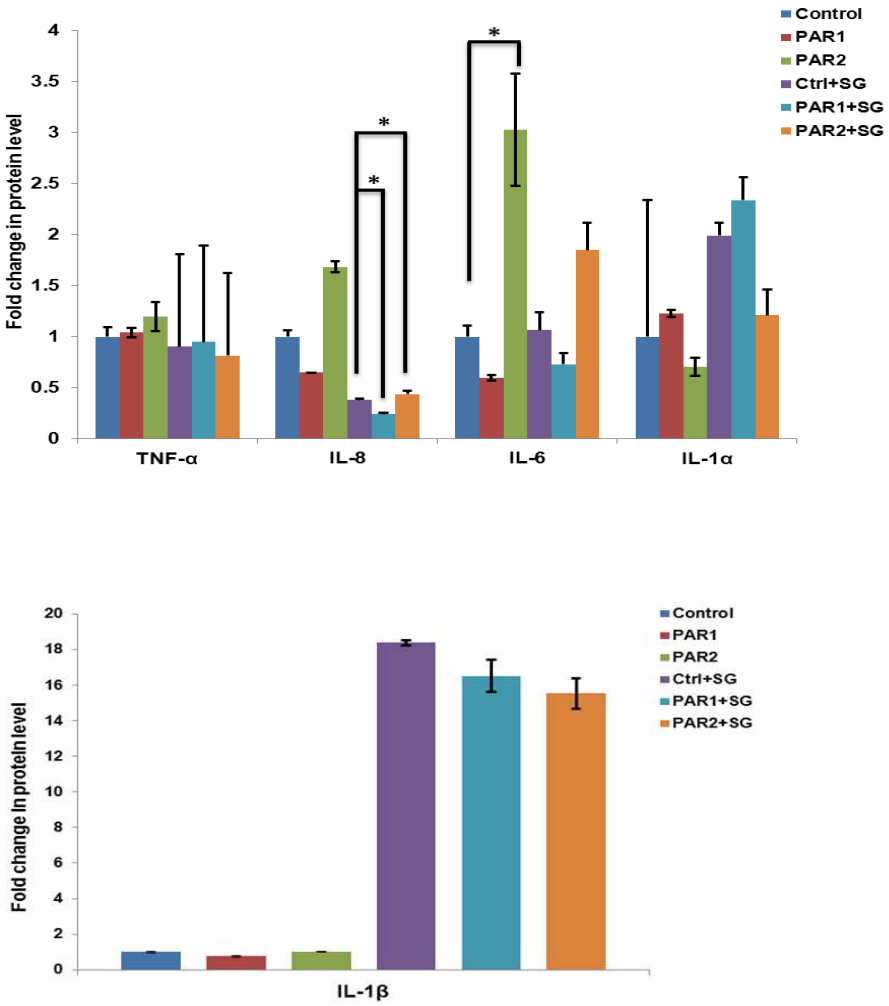

Figure 9: Cytokine secretion levels detected by Multi-Analyte ELISA ARRAY showed IL-8 protein secretion level decreased significantly with PAR1 knock-down in comparison to the control which is un-stimulated GECs, while IL-8 protein secretion level increased with PAR2 knock-down following SG stimulation. IL-6 protein secretion level increased with PAR2 knock-down with absence of any bacterial stimulation in comparison to the control ***** ($p \leq 0.05$)

3-2. Cytokine mRNA expression levels detected by QRT-PCR with AA+SG, PG+SG, and SG stimulation

We performed QRT-PCR to determine the gene expression level for the same cytokines that were tested for their protein secretion level to compare the results at the protein and mRNA levels.

PAR1 knock-down decreased the mRNA expression level of IL-8 significantly ($p \leq 0.05$) with PG+SG stimulation in comparison to our control which is the GECs stimulated with PG+SG without transfection (Figure11). The mRNA expression level for IL-8 increased significantly with PAR1 knock-down with AA+SG stimulation ($p \leq 0.001$) (Figure 10), while, the mRNA expression level of IL-8 increased significantly with PAR2 knock-down and PG+SG stimulation (Figure 11).

In comparison to TNF-α protein secretion level, we measured the mRNA expression level for TNF-α which was significantly up-regulated with PAR1 knock-down in all of the bacterial conditions in comparison to our control ($p \leq 0.05$). The mRNA expression for TNF-α decreased significantly with PAR2 knock-down and PG+SG stimulation ($p \leq 0.001$) (Figure 11).

The mRNA level for IL-6 increased significantly with PAR2 knock-down without any bacterial stimulation (Figures 10, 11), and with PAR2 knock-down following AA+SG stimulation ($p \leq 0.05$) (Figure 11). The IL-6 mRNA level decreased significantly when PAR1 was knocked down following AA+SG stimulation ($p \leq 0.001$).

We ran the QRT-PCR for IL-1α and observed that the mRNA expression level for IL-1α is significantly down-regulated with PAR2 knock-down followed by AA+SG or SG stimulation ($p \leq 0.05$)(Figures 10, 12). QRT-PCR for IL-1β showed that the knock-down of PAR receptors did not change the mRNA expression level for this cytokine (Figures 10, 11, 12).

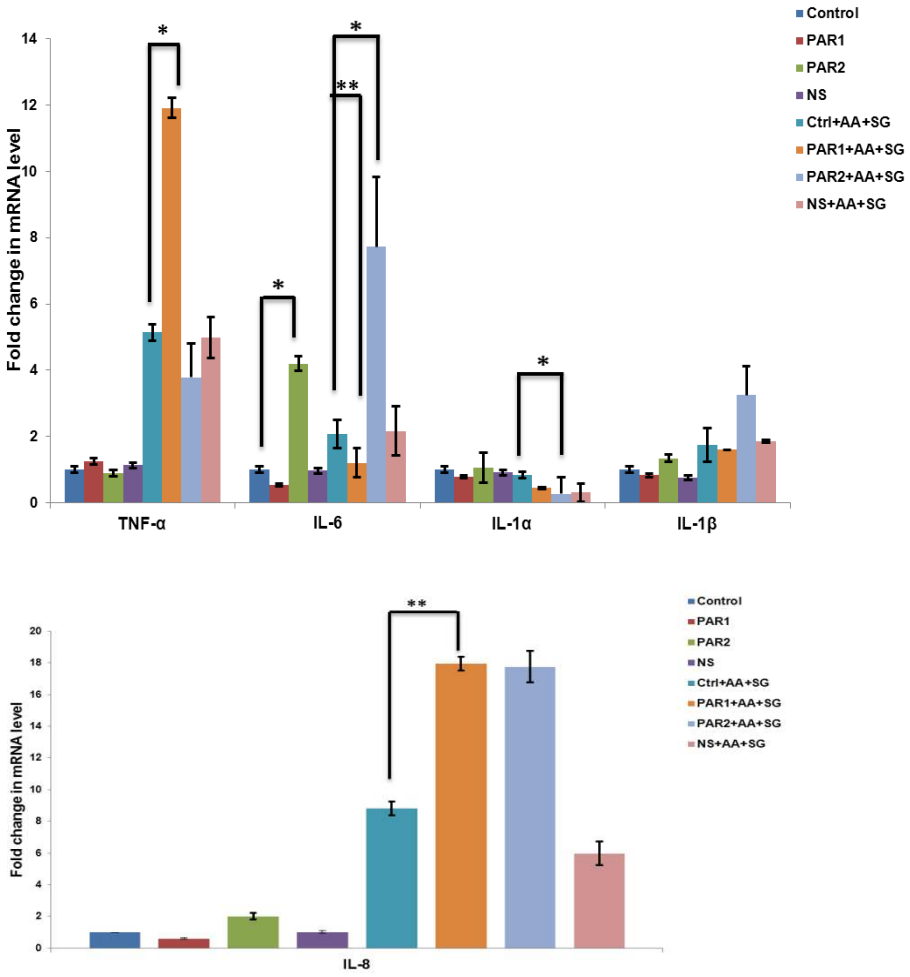

Figure 10: Changes in gene expression determined with QRT-PCR using SYBR green showed PAR1 knock-down significantly up-regulated the mRNA expression level of TNF-α following AA+SG stimulation. PAR1 knock-down significantly down-regulated the mRNA expression level of IL-6 with AA+SG stimulation. PAR1 knock-down also significantly up-regulated the mRNA expression level of IL-8 with AA+SG stimulation. PAR2 knock-down without bacterial stimulation up-regulated IL-6 mRNA expression level. PAR2 up-regulated the mRNA expression level of IL-6 with AA+SG stimulation. PAR2 knock-down down-regulated the mRNA expression level of IL-1α. All of these conditions are compared to the bacterial control *($p \leq 0.05$) ** ($p \leq 0.001$)

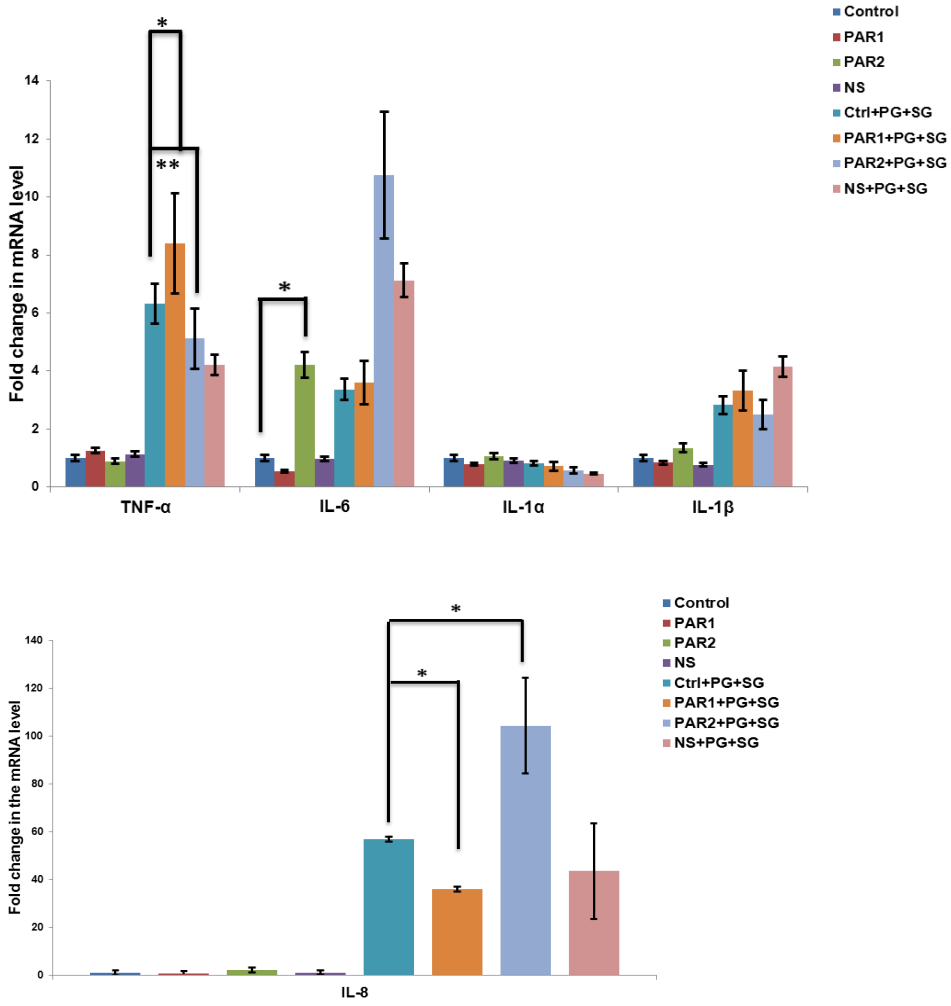

Figure 11: Changes in gene expression determined with QRT-PCR using SYBR green show PAR1 knock-down significantly up-regulated the mRNA expression level of TNF-α with PG+SG stimulation, and PAR1 knock-down significantly down-regulated the mRNA expression level of IL-8 with PG+SG stimulation. PAR2 knock-down significantly down-regulated the mRNA expression level of TNF-α with PG+SG stimulation, while PAR2 knock-down significantly up-regulated the mRNA expression level of IL-8 with PG+SG stimulation. The gene expression level of IL-6 was affected by PAR2 knock-down resulting in an increased expression in the un-stimulated group. *($p \leq 0.05$) ** ($p \leq 0.001$)

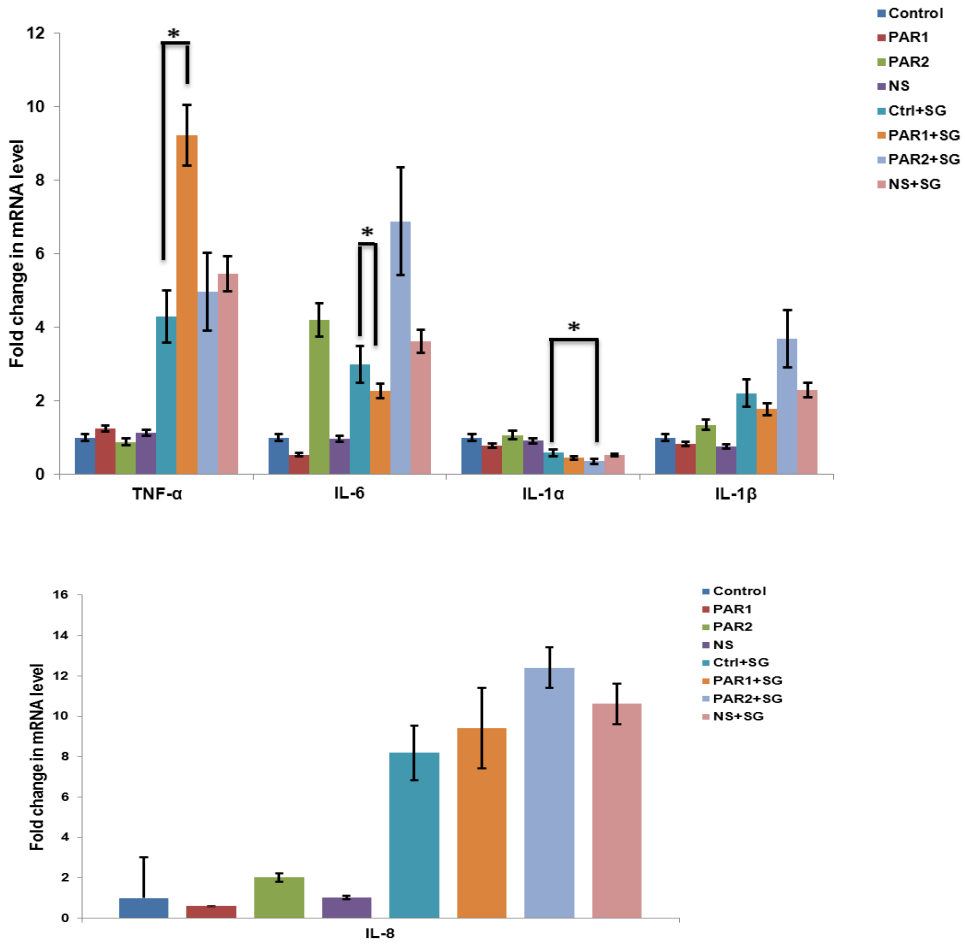

Figure 12: Changes in gene expression determined with QRT-PCR using SYBR green show PAR1 knock-down significantly up-regulated TNF-α mRNA expression level with SG stimulation, while PAR1 knock-down significantly down-regulated the mRNA expression level of IL-6 with SG stimulation. PAR2 knock-down down-regulated the mRNA expression level of IL-1α with SG stimulation. All of these condition are compared to the bacterial control *($p \leq 0.05$).

Chapter Four: Discussion

Periodontal diseases are characterized by alveolar bone resorption and destruction of gingival tissues, which may eventually lead to tooth loss. The disease starts at the epithelial surface of the sub-gingival compartment, and the gingival epithelial cells have a significant role in responding to microbial infection and coordinating immune responses. Gingival epithelial inflammatory responses include PAR receptors, downstream signaling, and expression of cytokines and chemokines (3, 20).

IKBKB is one of the signaling components of NF-κB signaling. In order for the transcriptional factor NF-κB to be liberated and translocate to the nucleus, IKBKB should be phosphorylated and degraded. During this process, IKBKB must separate from the IkB kinase complex in the cytoplasm. IKBk-β and IKBk-α are the two proteins that form the IkB kinase complex. Upon interaction between these two proteins, IkB phosphorylates and in turn activates NF-κB (26). Multiple studies reported that NF-κB can also be activated in an IKBKB-independent way, where the absence of IkB degradation does not interfere with the nuclear translocation (36).

The unique result that we showed in this study was the connection between IKBKB and PAR receptor. Many studies showed that IKBKB is important for the NF-κB to translocate to the nucleus (26) but there were no studies investigating if PAR receptors utilize IKBKB to conduct the signal from the cell surface to the nucleus. We investigated the possibility that PAR receptors may be controlling the down-stream signal of IKBKB and its effect on NF-κB. Our data showed that the expression level of IKBKB modulated with different bacterial combination stimulation. In both cases of our experiments using QRT-PCR and ELISA, the change is significant with AA+SG and PG+SG stimulation with PAR1 knock-down in comparison to the control condition. In addition, our data showed that the protein level of IKBKB measured with ELISA is up-regulated with PAR2 knock-down followed by SG

stimulation in comparison to the control. Based on our data, we suggest that the PAR1 receptor regulates the signaling pathway of IKBKB and in turn, PAR1 is also involved in NF-κB signaling down-stream. Taken together, these data demonstrate the novel finding that NF-κB in gingival epithelial cells is regulated by PAR through IKBkB-dependent mechanism.

From our result, we suggest also that PAR receptor regulates gene expression level of PI3K. We showed in this study that PI3K mRNA and protein expression levels are up-regulated with PAR1 knock-down with AA+SG stimulation. PI3K was also up-regulated with SG stimulation and PAR2 knock-down; suggesting PAR receptors exert control over the signaling pathway of PI3K. A previous study suggested that PAR receptors act as inhibitors for PI3K signaling and also suggested that this inhibition might down-regulate the innate immune response (3). This study used the two enzymes trypsin and thrombin to activate PAR2 and PAR1, respectively (3), while in our study we stimulated the receptors by using specific bacterial combination after silencing PAR receptors.

For the second part of our study, we investigated the expression level for five key cytokines that are thought to be important in epithelial cell response and specifically in periodontitis. Our data showed that TNF-α protein secretion level was up-regulated with PAR1 and PAR2 knock-down and AA+SG stimulation. Likewise, TNF-α mRNA expression level was up-regulated with PAR1 knock-down and AA+SG, PG+SG, or SG in comparison to control. All of these data suggest that PARs play a role in the modulation of TNF- α.

We observed a significant up-regulation in IL-1β cytokines with all of our bacterial stimulation conditions and both PAR1 and PAR2 knock-down. Our data suggest that PAR receptors regulate the secretion level of IL-1β, since we detected high level of this cytokine upon the deletion of PAR receptor signal.

IL-6 plays a role in fighting infection (33). IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae* (33). Also it has been

shown that over-expression of PAR2 was positively associated with clinical inflammatory signs (35). From our data it is relevant that IL-6 protein secretion level increased with PAR2 knock-down with AA+SG stimulation and even in the absence of any bacterial stimulation. We also saw a change in the IL-6 mRNA expression level that was down-regulated with PAR1 knock-down and AA+SG or SG stimulation. This suggested that IL-6 could be secreted to initiate the innate immunity response through utilizing PAR1 and PAR2 receptors in response to single and multiple bacterial stimulations.

We observed from our data that IL-8 protein secretion level was down-regulated with PAR1 and PAR2 knock-down and PG+SG or SG stimulation, while AA+SG stimulation only affected IL-8 with PAR1 knock-down. However, IL-8 mRNA regulation differed for PG+SG stimulation. A previous study suggested that IL-8 responses can be triggered at the mRNA level by PG through PAR1 (22, 40). These studies also suggested that the fluctuation in the level of IL-8 among mRNA and protein secretion in the same epithelial cells that have been stimulated with PG may be due to degradation of IL-8 by virulence factors of PG (22, 40). In our present study we stimulated our cells with combination of the pathogenic bacteria, AA, PG, and the commensal bacteria SG. We observe here that IL-8 secretion at the mRNA level and at the protein level are different after PAR knock-down. This might be due to the fact that bacterial stimulation is triggering the release of this cytokine in normal status when PAR receptors are activated; however, the virulence factors of these pathogenic bacteria may be degrading IL-8 once it is secreted outside the cells.

The decreased mRNA level of inflammatory cytokine IL-1α under AA+SG, and SG stimulation with PAR2 receptor knock-down, suggests the link between PAR2 receptor activation and IL-1α secretion. PAR2 receptors may regulate the mRNA expression level of IL-1α with AA+SG, and SG stimulation.

Our data showed that PARs utilize PI3K and IKBKB in gingival inflammatory responses to periodontal bacteria. PAR receptors utilize IKBKB to conduct the signal from

the cell surface down to the cell nucleus through the activation of NF-κB signaling component. We conclude from our data that PAR receptors alter the PI3K molecule signaling, and this is an important mechanism for these receptors in order to control the innate immune response during periodontitis.

These findings indicate that the epithelial cells elicit cytokine responses to a bacterial challenge via PAR receptors. This information will provide a better understanding of the development of periodontal disease and inform the strategy for identification of therapeutic approaches for this disease. If we understand the mechanism of down-stream signaling pathway that could be activated through PAR1 and PAR2 receptors, and how PAR receptors utilize them, we could invent a therapeutic to induce or suppress these signaling pathways through PAR receptors activation or inhibition. This would decrease the over- secretion of cytokines, which could be harmful to the supporting structures of the periodontal tissues, especially bone in the case of periodontitis.

Conclusion

- PARs utilize PI3K and IKBKB in gingival inflammatory responses to Perio-pathogenic bacteria.
- Gingival epithelial cells elicit cytokine responses to a bacterial challenge via PAR receptors.

Future plans

- Further studies will need to be conducted to test other cytokines of interest and to understand the mechanisms of how PAR activation induces or suppresses the release of additional cytokines.
- We have used only one subject (cell line) for this project and testing additional donor cell lines will provide better insight into the possibility of individual variations in PAR-mediated signaling.

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