이학석사 학위논문

# The Role of Toll-like Receptors in Oral Bacteria-Induced Regulation of Major Histocompatibility Complex I in Human Submandibular Gland Cells

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이 재 원

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이재원

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# Abstract

## Role of Toll-like Receptors in Oral Bacteria-Induced Regulation of Major Histocompatibility Complex Ⅰ in Human Submandibular Gland Cells

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**Background**

Bacteria that invade into host epithelial cells may be presented to $CD8^+$ and $CD4^+$ T cells through major histocompatibility complex (MHC) class Ⅰ and Ⅱ, respectively. Previously, it was found that Sjogren's syndrome-associated oral bacteria *Prevotella melaninogenica* (Pm) and *Rothia mucilaginosa* (Rm) efficiently invade into human submandibular

gland tumor (HSG) cells and regulate MHC Ⅰ expression differently. The aim of this study was to investigate molecular mechanisms for the regulation of MHC Ⅰ expression in HSG cells.

**Methods**

HSG cells were infected with Pm KCTC 5457 or Rm KCTC 19862 stained with pHrodo red. After staining with PerCP-conjugated anti-MHC Ⅰ antibodies, Sytox green, Annexin V, and Propidium Iodide (PI), bacterial invasion, the viability of HSG cells, cell death stages and the expression of MHC Ⅰ were simultaneously analyzed by flow cytometry. The expressions of Toll-like receptor (TLR) 2, 4, and 9 in HSG cells were examined by flow cytometry. After HSG cells were stimulated with Pam3CSK4, Lipopolysaccharide (LPS), or CpG-ODN the expression of MHC Ⅰ was measured. The abilities of Pm and Rm to activate TLR2, TLR4, and TLR9 were evaluated using reporter cells.

**Results**

Pm did not affect the viability of HSG cells in various MOI, and the levels of MHC Ⅰ expression had a weak positive correlation with the degree of bacterial invasion ($r_s$ = 0.273, $p$ = 0.023). Rm induced a substantial decrease in host cell viability in a dose-dependent and an invasion dependent manner. Rm significantly induced necrosis and late apoptosis in HSG cells in 24 h. The levels of MHC Ⅰ expression in the

Rm-infected cells had a positive correlation with the cell viability ($r_s$ = 0.496, $p$ < 0.001) and a negative correlation with the bacterial invasion ($r_s$ = -0.358, $p$ = 0.003) in 48 h. Not only TLR9 but also TLR2 and TLR4 were intracellularly expressed but not on the surface of HSG cells. The ligands for all three TLRs up-regulated MHC Ⅰ expression in concentration-dependent manners. Whereas Pm activated TLR2 and TLR9, Rm activated only TLR2. Interestingly, Rm DNA regulated TLR9 activation by its agonist.

**Conclusion**

TLR9 may mediate the Pm-induced up-regulation of MHC Ⅰ in HSG cells. The down-regulation of MHC Ⅰ by Rm seems to be associated with cell death induced by infection. However, the mechanisms of which need to be clarified.

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**Keywords:** Sjogren's syndrome, Human salivary gland cell, *Prevotella melaninogenica*, *Rothia mucilaginosa*, MHC Ⅰ

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# Contents

## Ⅰ. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease in which the immune system mistakenly attacks the body's own cells and tissues, especially the excretory glands such as the salivary and lacrimal glands. Its primary symptoms are dry mouth and dry eyes, and affected patients are at an increased risk of lymphoma [1]. SS generally occurs in females ten times more than males and commonly begins in middle ages [2]. One of the diagnostic criteria for SS is focal lymphocytic sialadenitis (FLS) with a focus score ≥ 1 [3]. The lymphocytic infiltrates consist of T cells and B cells and are found initially around the ductal epithelia [4]. Other studies report that lymphocytic infiltrates isolated from the lacrimal and salivary glands of humans and NOD mice are composed largely of $CD4^+$ T cells, yet $CD8^+$ T cells are consistently present [5, 6]. Minor salivary gland biopsies from SS patients revealed the expression of apoptosis-related molecules and substances released by CD8+ cytotoxic T cells [7]. In several autoimmune diseases, $CD8^+$ T cells are involved in the initiation of diseases and progression and the regulation of diseases [8].

SS is also described as autoimmune epithelitis, suggesting a central role for epithelial cells in the pathogenesis of SS [9]. Salivary glandular epithelial cells (SGECs) in SS patients often present the phenotype of antigen presenting cells (APCs) by expressing MHC Ⅰ and Ⅱ molecules, costimulatory molecules CD80 and CD86, and adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule

(VCAM), and E-selectin in ducts. Therefore, SGECs in SS patients are suitable to interact with T cells and to form immune synapses between T cells and epithelial cells [10]. In addition, SGECs with upregulated MHC class II expression activate $CD4^+$ T cells and develop glandular inflammation [11]. In the activation of T cells, MHC molecules are the most crucial in the immune system. MHC molecules are cell surface proteins that are essential for T cell-mediated acquired immunity. The main function of MHC molecules is to bind to antigens derived from pathogens and display them on the cell surface to present to specific T cells. These interactions are referred as signal 1 for the activation of T cells. MHC Ⅰ interacts with $CD8^+$ T cells and MHC Ⅱ interacts with $CD4^+$ T cells, respectively. Thus, the role of the phenotypical change of SGECs on APC markers may be crucial in the induction of the inflammatory process in SS. However, the exact factors that induce the phenotypical change of SGECs in SS have not been well elucidated.

SGECs in SS patients express functional Toll-like Receptor (TLR) molecules: TLR1, TLR2, TLR3, TLR 4, and TLR9 [12, 13]. TLRs are one of the well-identified pattern recognition receptors (PRRs). TLRs recognize molecules that are broadly shared by microbes referred to as microbe-associated molecular patterns (MAMPs), have a key role in the innate immune system, and also have a role as a bridge between the innate and adaptive immune systems [14]. Generally, TLR1, TLR2, and TLR4 are expressed on the cytoplasmic membranes of cells, whereas TLR3 and TLR9

are usually located in endosomes. TLR1, TLR2, and TLR4 recognize bacterial cell wall components such as bacterial lipoproteins, peptidoglycans, lipoteichoic acid, and lipopolysaccharides. TLR3 recognizes double-stranded RNA, and TLR9 is activated by un-methylated CpG oligodeoxynucleotide DNA. The signaling through these TLRs leads to the up-regulation of APC-related molecules, adhesion molecules, and the production of pro-inflammatory cytokines and chemokines [12]. Especially, TLR signaling induces phagosomal MHC Ⅰ delivery into endosomes and augments the subsequent cross-presentation of microbial antigens [15]. Overall, TLR signaling may have a role in the autoimmune epithelitis phenotype of SS.

The importance of dysbiosis in autoimmune diseases is emerging. In SS, several studies have reported dysbiosis in the intestinal and oral microbiota, suggesting a potential role in SS pathogenesis [16~21]. However, the changes in the microbiota of SS patients have never directly been shown to trigger SS, and its connection to the etiopathogenesis of SS remains unclear.

Recently, our research group analyzed the relative abundance of selected oral bacteria at the phylum and genus levels among healthy controls, sicca patient controls, and SS patients by pyrosequencing the 16S bacterial RNA gene. The pyrosequencing results indicated that the change in proportion of the bacterial community may be related to the pathogenesis of SS. In a previous study, six of selected oral bacteria *Streptococcus salivarius* (Ss), *Streptococcus oralis* (So), *Rothia mucilaginosa*

(Rm), *Fusobacterium nucleatum* (Fn), *Prevotella melaninogenica* (Pm), and *Prevotella histicola* (Ph) were tested for the regulation of APC molecules, secretion of cytokines by HSG cells, and invasion ability. Pm is a gram-negative anaerobic bacterium, and Rm is a gram-positive aerobic bacterium. Interestingly, Pm and Rm efficiently invaded into HSG cells and regulated the expressions of MHC Ⅰ molecules in the HSG cells differently. It has been hypothesized that specific oral bacteria may be responsible for the induction of the dysregulation of SGECs, which manifests as APC-like phenotypes. The aim of this study was to elucidate the molecular mechanisms for the regulation of MHC Ⅰ molecule expression in HSG cells by Pm and Rm infection.

## Ⅱ. Materials & Methods

### 2.1 Cell culture

HSG cell line was obtained from Korean cell line bank (KCBL, Seoul, Korea). HSG cells were cultured at 37℃ in a humidified atmosphere of 95% air and 5% $CO_2$. Cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) High glucose (Hyclone, Uppsala, Sweden) with 10% fetal bovine serum (Hyclone) and 100 unit/ml of penicillin and 100 μg/ml of streptomycin.

Chinese hamster ovary (CHO)/CD14/TLR2 and TLR4 cell lines were obtained from Dr Douglas Golenbock (Boston Medical Center, Boston, MA). CHO/CD14/TLR2 and TLR4 cells were cultured at 37℃ in a humidified atmosphere of 95% air and 5% $CO_2$. Cells were maintained in Ham's F-12 Nutrient Mix (Gibco, CA, USA) medium with 10% fetal bovine serum and 100 unit/ml of penicillin and 100 μg/ml of streptomycin in the presence of 1 mg/ml of G418 and 0.4 mg/ml of Hygromycin B.

Human embryonic kidney (HEK)-Blue™ human TLR9 cell line was purchased from Invivogen (Toulouse, France). HEK-Blue hTLR9 cells were cultured at 37℃ in a humidified atmosphere of 95% air and 5% CO2. Cells were maintained in DMEM with 10% fetal bovine serum, 100 unit/ml of penicillin and 100 μg/ml of streptomycin and 100 μg/ml of Normocin™ in the presence of 10 μg/ml of Blasticidin and 100 μg/ml of Zeocin™.

HSG cells and CHO/CD14/TLR2 and TLR4 cells were detached by treatment with 0.05% Trypsin-EDTA at 37℃ cell culture incubator.

HEK-Blue hTLR9 cells were detached by phosphate buffered saline (PBS) in same condition of HSG and CHO/CD14/TLR cells. After detaching, cells were seeded in 24-well or 96-well plates, for flow cytometry or HEK-Blue assay respectively.

2.2 Bacteria culture

All used bacteria for this study were from ATCC (American type culture collection) and KCTC (Korean collection for type cultures, Jeong-eup, Korea). Pm KCTC 5457 and Fn ATCC 25586 were cultured in recommended KCTC-5457 medium and brain heart infusion (BHI) medium, respectively, supplemented with 5 μg/ml of hemin (Sigma, St Louis, MO, USA) and 5 μg/ml of vitamin K under anaerobic condition (5% $H_2$, 10% $CO_2$, and 85% $N_2$) at 37℃. Rm KCTC 19862 were grown in BHI medium under aerobic condition with shaking (180 rpm) at 37℃. All bacteria were harvested in log phase and washed more than twice with PBS for further experiments. Heat-killed bacteria were prepared by 1 h incubation in 95℃ heat block. After heat-killing, bacteria were washed with PBS more than 2 times for further experiments.

2.3 Bacterial infection

HSG cells, CHO/CD14/TLR2 or TLR4 cells were seeded into the 24 well plates at $6\times10^4$ cells/well. After O/N incubation, cells were washed by PBS and changed with antibiotics-free medium. Then, cells were infected with Pm, Fn or Rm at multiplicity of infection (MOI) 10, 50, 100 and 500 for 24, 48 and 72 h. To prevent the overgrowth of bacteria, gentamicin was added to the infected cells simultaneously.

2.4 Analysis of bacterial invasion of HSG cells

For the analysis of bacterial invasion of HSG cells, Pm and Rm were stained with pHrodo™ Red succinimidyl ester (Thermo Fisher Scientific, Illinois, USA) to investigate whether bacteria are localized to endosomes or not. After harvesting the bacteria, bacteria were stained 1μM of pHrodo red dye and incubated for 1 h at room temperature with avoiding light. After bacterial staining, bacteria were washed more than 3 times with PBS and infected into HSG cells.

2.5 Stimulation of cells with TLR ligands

HSG cells were seeded at $6\times10^4$ cells/well in 24 well plates one day before use. While changing the culture medium, HSG cells were treated with 0.1, 1, 10 μg/ml of Pam3CSK4, LPS derived from *Escherichia coli* K12 (Invivogen) or 0.77, 7.7, 38.5 μg/ml of ODN 2006 (Invivogen)

for 24, 48 and 72 h. In reporter cell assay, the HEK-Blue TLR9 cells were treated with 10 μg/ml of ODN 2006 and the CHO/CD14/TLR2 and TLR4 cells were treated with 0.1 μg/ml of Pam3CSK4 and 0.1 μg/ml of LPS for 16 h.

2.6 Flow cytometry

Suspended cells were incubated with PerCP-conjugated anti-Human HLA-A, B, C mAb clone W6/32 (BioLegend, Sandiego, CA, USA) for 15 min on ice. Then cells were stained with 5 μM of SYTOX™ Green Nucleic Acid Stain (Invitrogen) for analyze cell viability and incubated for 15 min on ice. HSG cells were stained with FITC-conjugated Human TLR2 clone TL2.1 (BioLegend, Sandiego, CA, USA), PE-conjugated Human TLR4 clone TF901 (BD Biosciences Korea, Seoul, Korea) or PE-conjugated anti-Human TLR9 clone eB72-1665 (BD Biosciences). For intracellular staining, HSG cells were prepared with fixation/permeabilization solution kit (BD Biosciences). Also, FITC-conjugated mouse anti-Human CD25 (BD Biosciences) were stained to CHO/CD14/TLR2 and TLR4 cells. After that, stained cells were washed with FACS buffer (5% FBS in PBS) more than 2 times and analyzed by using FACS Calibur (BD Biosciences) equipped with CellQuest software and FlowJo (ver. 10) for data analysis.

2.7 Preparation of bacterial DNA and endotoxin removal

During the log phase of bacterial growth, Pm and Rm were harvested centrifuge by 11,000 g in 15 min at 4℃. After harvest of bacteria, bacterial DNA was extracted using Blood & Cell culture DNA maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In case of Rm, Garnet beads 0.15 mm (Qiagen) were first applied to disrupt peptidoglycan cell wall before DNA extraction. Isolated bacterial DNA was treated with TritonX-114 (Sigma, St.Louis, USA) to remove endotoxin. TritonX-114 was added to DNA samples at a 1 % of total volume, then mixtures were incubated at 4℃ for 20 min in shaking incubator. After incubation, the mixtures were incubated at 37℃ for 10 min, next, centrifugation for 20,000 g for 30 min, the upper phase that contains the DNA was carefully extracted and subjected to isopropanol precipitation. Finally, endotoxin quantification test was performed using a LAL chromogenic endotoxin quantitation kit (Thermo Fisher Scientific) according to manufacture instructions.

2.8 HEK-blue detection assay

First, addition of 2 μg of ODN 2006 (~up to total 20 μl) as a positive control, bacterial DNA with lipofectamine (Invitrogen) by using according to manufacture instructions or negative control (endotoxin-free water) in each well of 96 well plate was done. After detaching the

HEK-Blue hTLR9 cells, cells were resuspended into HEK-Blue Detection medium (Invivogen). Resuspended cells were added into each wells up to ~$8\times10^4$ cells per well. Then, cells were incubated at 37℃ in 5% $CO_2$ for 6~24 h. After incubation, 96 well plates were analyzed with spectrophotometer at 620 nm.

2.9 Statistical analysis

All data are presented as the mean ± standard error of the mean of at least three times repeated experiments. The difference between control and experimental groups was analyze by t-test. Significance was set at $P<0.05$. Correlation analysis was done by Spearman correlation analysis in Statistical Package for the Social Sciences (SPSS) 23.

## Ⅲ. Results

### 3.1 Effects of TLR stimulation on MHC Ⅰ expression in HSG cells.

In epithelial cells, PRRs including TLRs recognize and mediate responses to bacteria. Because TLR2, TLR4, and TLR9 recognize bacterial components, it was investigated whether their ligands can up-regulate MHC Ⅰ in HSG cells. All tested ligands up-regulated MHC Ⅰ expression in a dose-dependent manner 24 and 48 h after treatment but went back to basal level 72 h after treatment (Fig. 1).

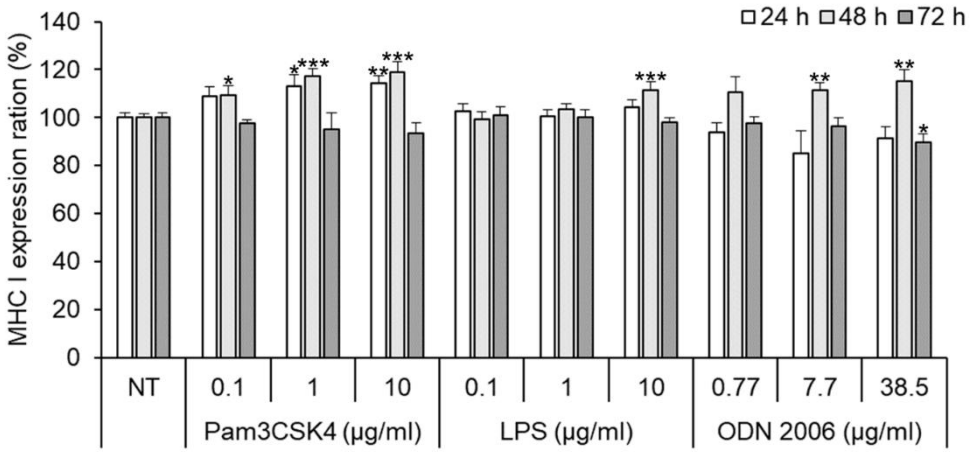


**Fig 1. Activation of TLR2, TLR4 or TLR9 by TLR ligands upregulated MHC Ⅰ expressions.**

HSG cells ($6 \times 10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, TLR ligands (Pam3CSK4; TLR2, LPS; TLR4 and CpG-ODN; TLR9) were treated into HSG cells and analyzed MHC Ⅰ expressions in various time points. After stimulation, cells were analyzed by flow cytometry. Pam3CSK4 induced MHC Ⅰ expressions in 24 and 48 h with dose-dependent manner. LPS also induced MHC Ⅰ expressions but, only in 48 h with 10 ug/ml. ODN 2006 up-regulated MHC Ⅰ expressions in 48 h. Each value represents the mean ± standard error of the mean of five independent experiments in duplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control.

### 3.2 HSG cells express bacteria-sensing TLRs.

It has been shown that SGECs from SS patients constitutively express high levels of TLRs [12, 13]. In the case of HSG cells, there are no expressions of TLR2 and TLR 4; however, only TLR9 is expressed in endosomes. For further accurate analysis of TLR expression in HSG cells, both surface and intracellular staining was performed. HSG cells expressed no TLR2, TLR4, or TLR9 on the surface of the cells (Fig. 2). However, intracellular staining revealed constitutive expression of TLR2, TLR4, and TLR9 (Fig. 2). Therefore, HSG cells can sense Pm and Rm via TLR2, TLR4, and TLR9.

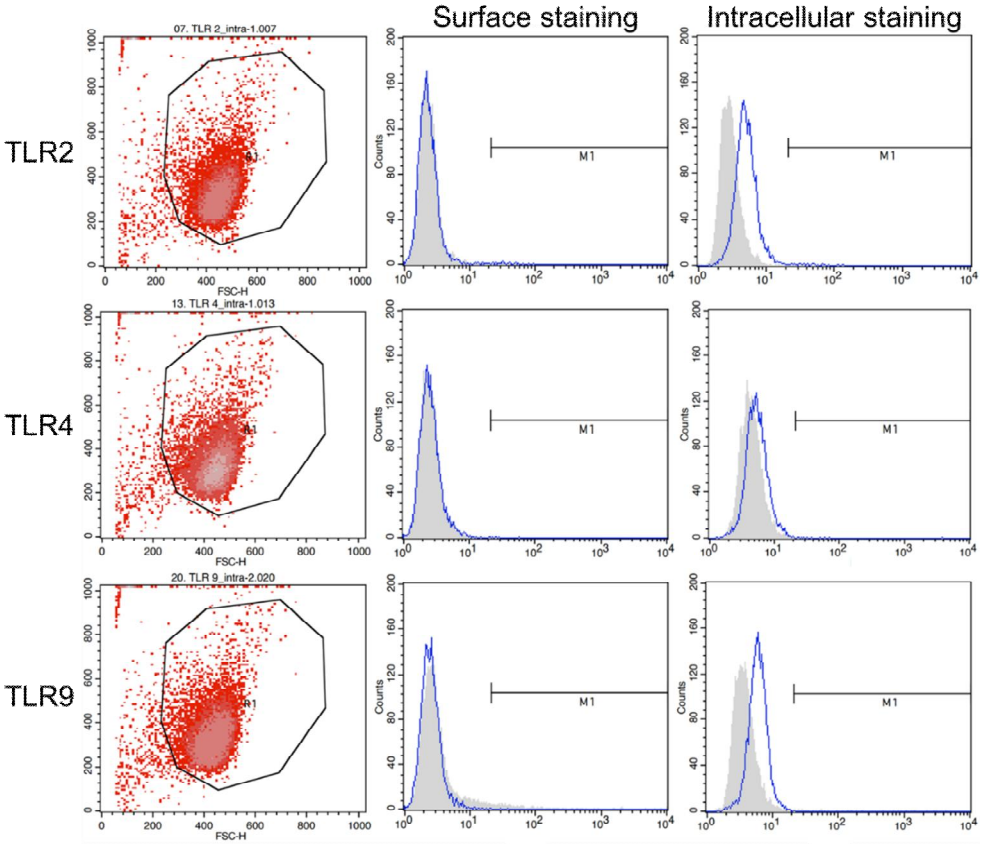


**Fig 2. Expressions of intracellular TLR2, TLR4, and TLR9 in HSG cells.**

HSG cells ($6 \times 10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, HSG cells were stained with anti- TLR2, 4, or 9 antibodies in both surface and intracellular staining. HSG cells expressed TLR 2, 4 and 9 in intracellular space but, not in the surface layer on HSG cells. TLR2, and 9 were expressed higher than TLR4. Gray line indicated isotype control and blue line indicated experimental groups.

### 3.3 Invasion ability and effects of Pm and Rm on cell viability and MHC Ⅰ expression of HSG cells.

Because not only TLR9 but also TLR2 and TLR4 were intracellularly expressed in HSG cells (Fig. 2), the degree of bacterial invasion and MHC Ⅰ expression were examined simultaneously. In addition, the cell viability of HSG cells were also analyzed with flow cytometry after sytox green staining. Pm and Rm efficiently invaded into the HSG cells, and Rm was the more potent invading species compared to Pm (Fig. 3A). Pm did not induce any cell death compared with the non-infection control. However, Rm induced cell death in the HSG cells (Fig. 3B). In addition, Pm up-regulated MHC Ⅰ expression, but Rm down-regulated MHC Ⅰ expression (Fig. 3C). Taken together, HSG cells infected with Pm and Rm have their expression of MHC Ⅰ modulated, which is characteristic of dysregulated SGECs.

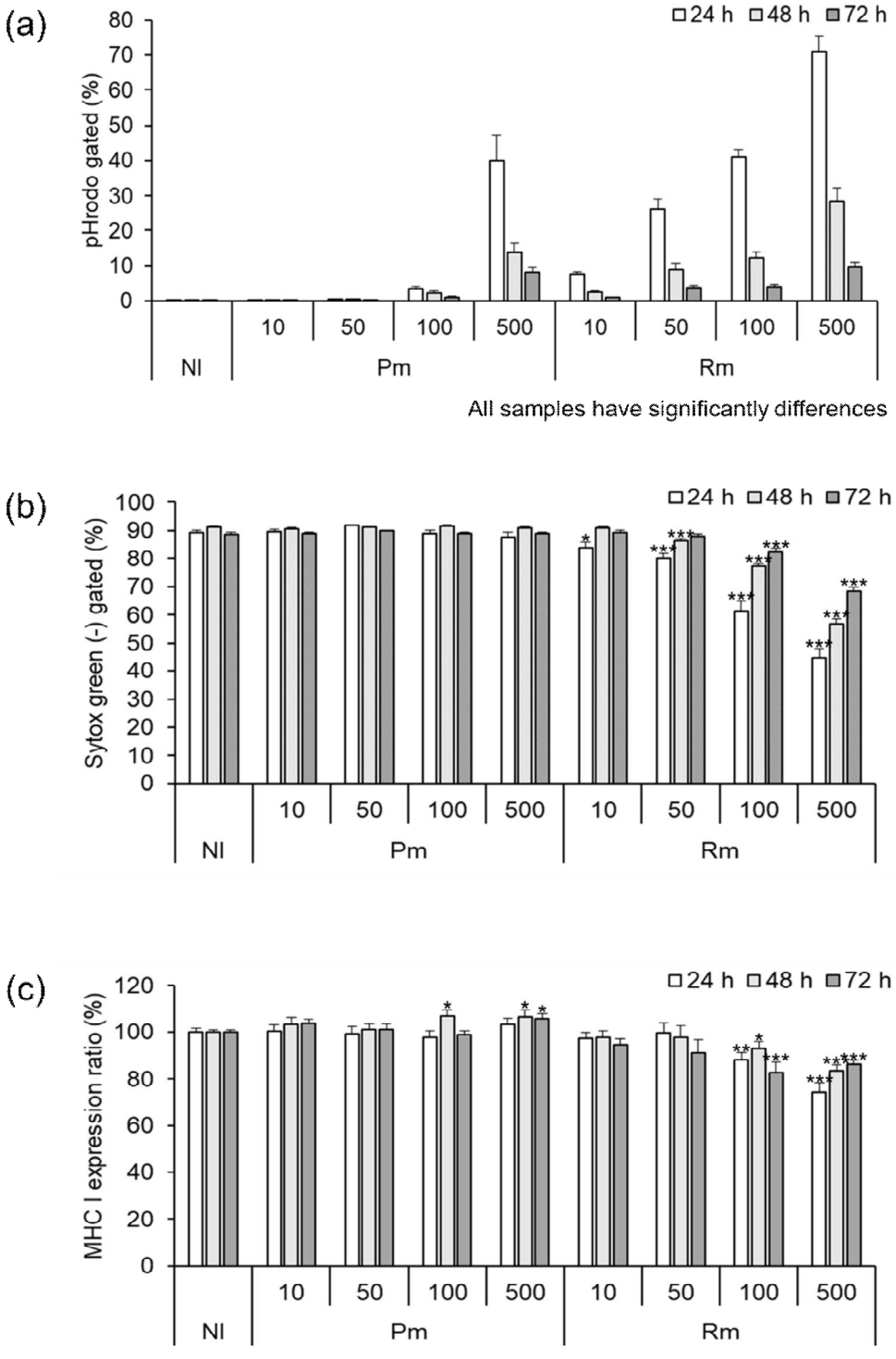
(a)
24 h
48 h
72 h
pHrodo gated (%)
80
70
60
50
40
30
20
10
0
10
50
100
500
10
50
100
500
NI
Pm
Rm
All samples have significantly differences
(b)
24 h
48 h
72 h
Sytox green (-) gated (%)
100
90
80
70
60
50
40
30
20
10
0
10
50
100
500
10
50
100
500
NI
Pm
Rm
(c)
24 h
48 h
72 h
MHC I expression ratio (%)
120
100
80
60
40
20
0
10
50
100
500
10
50
100
500
NI
Pm
Rm

**Fig 3. Whereas Pm up-regulated MHC Ⅰ without affecting the viability, Rm down-regualted MHC Ⅰ accompanied with cell death.**

HSG cells ($6 \times 10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, pHrodo red labeled Pm and Rm were infected into HSG cells with various MOI. After infection, infected cells were analyzed by flow cytometry. (a) Pm and Rm invaded into HSG cells efficiently, also they located in endosomes. (b) Cell viability of HSG cells were stained with sytox green staining and analyzed by flow cytometry. Pm did not induce cell death however, Rm induced cell death with various MOI in several time points. (c) Pm induced MHC Ⅰ expressions of HSG cells in 48, and 72 h at MOI 100 and 500. Rm suppressed MHC Ⅰ expressions of HSG cells in all time points at MOI 100 and 500. Each value represents the mean ± standard error of the mean of five independent experiments in triplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control.

### 3.4 Correlation between bacterial invasion, cell viability, and MHC Ⅰ expression.

Fig. 3C shows the Pm up-regulated and Rm down-regulated MHC Ⅰ expression. Additionally, Rm induced the cell death of the HSG cells while Pm did not. To analyze the correlation of the bacterial invasion, cell viability, and MHC Ⅰ expression, a Spearman correlation analysis was done. There was a weak positive correlation between invasion and MHC Ⅰ expression at 48 hours post infection (hpi) of Pm ($r_s$=0.273, $p$=0.023) (Fig. 4A). There was a negative correlation between Rm invasion and MHC Ⅰ expression at 48 hpi of Rm ($r_s$=-0.358, $p$=0.003) (Fig. 4B). Moreover, Rm had a positive correlation between MHC Ⅰ expression and cell viability at 48 hpi ($r_s$=0.496, $p$<0.001) (Fig. 4C). The same patterns were observed at 72 hpi for the Rm infection (Invasion vs MHC Ⅰ expression: $r_s$=-0.249, $p$=0.039; MHC Ⅰ expression vs Cell viability: $r_s$=0.496, $p$<0.001) (Fig. 4E~F). The results indicate that the HSG cells infected with Pm do not affect the viability of cells, and the levels of MHC Ⅰ expression have a weak positive correlation with the degree of bacterial invasion. However, Rm infected HSG cells have a substantial decrease in host cell viability in a dose-dependent and in an invasion-dependent manner. Furthermore, Rm reduces the MHC Ⅰ expression in HSG cells dependent on the invasion index.

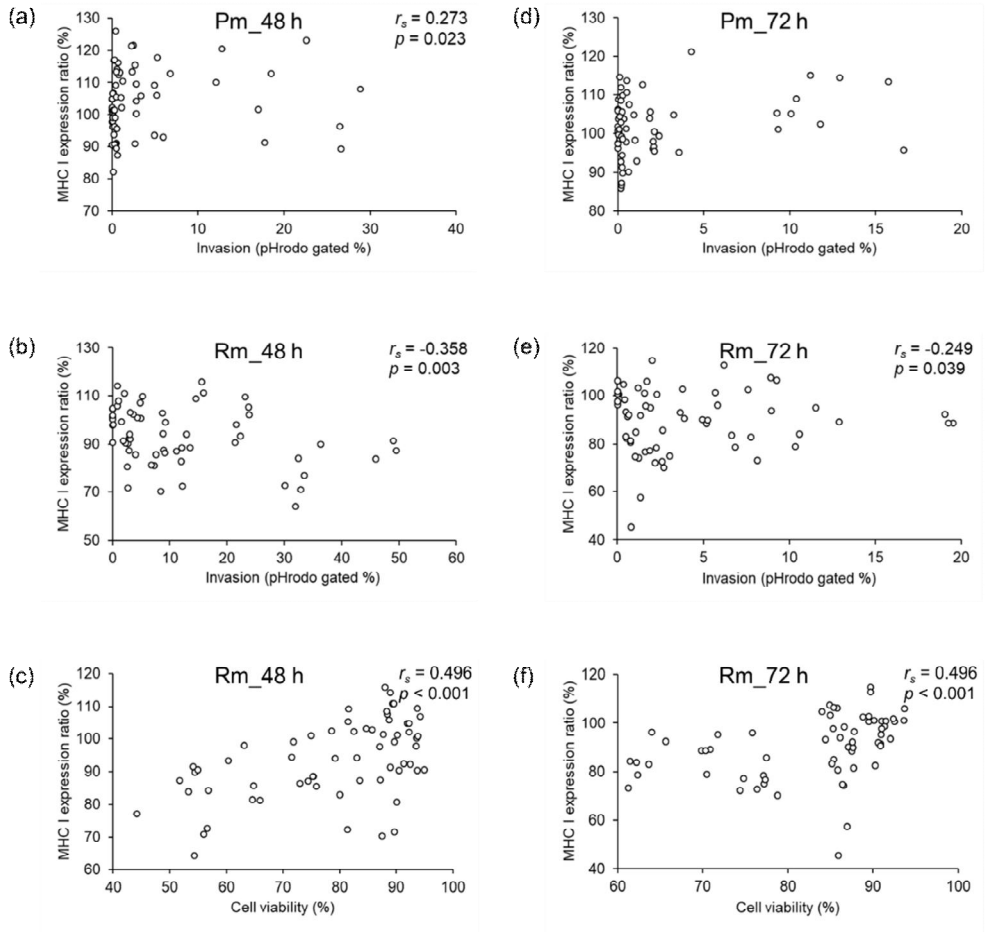


**Fig 4. Correlation between bacterial invasion and MHC Ⅰ expression.**

(a) and (d) Pm had a weak positive correlation between invasion and MHC Ⅰ expressions in 48 h. (b) and (e) Rm had a weak negative correlation between invasion and MHC Ⅰ expressions in 48 and 72 h. (c) and (f) Rm had a positive correlation between MHC Ⅰ expressions and cell viability in 48 and 72 h. (Statistical analysis was done by SPSS 23 software in Spearman Correlation analysis)

### 3.5 Analysis of the Rm-induced cell death stage in HSG cells.

Fig. 3B shows the Rm induced cell death in HSG cells by analysis of sytox green staining. To analyze which cell death stages were induced by Rm, Annexin V and PI staining was done. In addition, to confirm that the Rm-induced cell death was specific only in HSG cells, human oral keratinocyte (HOK-16B) cells were used as a control. Rm increased necrosis (defined as Annexin $V^-$, $PI^+$) and late apoptosis (defined as Annexin $V^+$, $PI^+$) in the HSG cells. Interestingly, Rm did not induce cell death in the HOK-16B cells. Taken together, Rm-induced cell death was specific to the HSG cells, and it may affect and lead to the dysfunction and death of salivary gland cells or tissues.

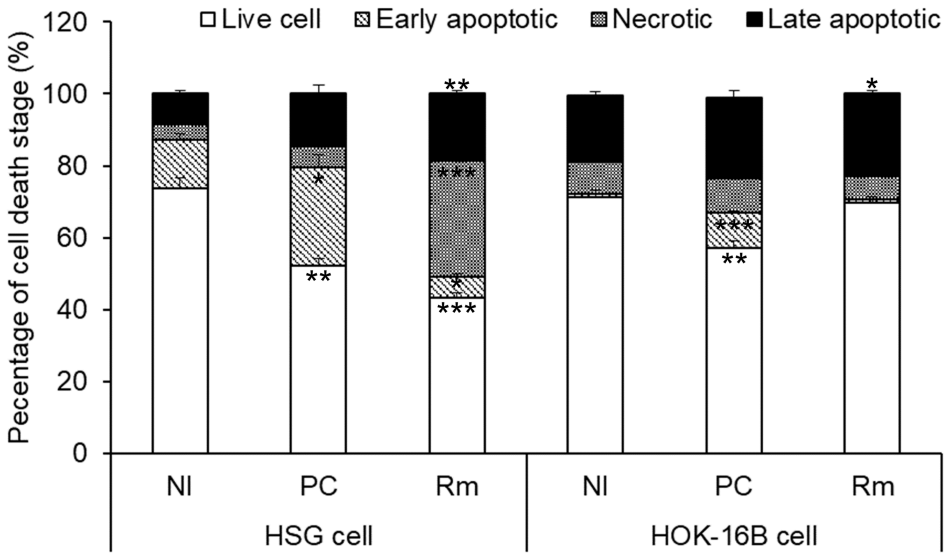


**Fig 5. Rm induced necrosis and late apoptotic stage in HSG cells whereas, not in HOK-16B cells.**

HSG cells and HOK-16B cells ($6 \times 10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, Rm was infected into HSG cells and HOK-16B cells with MOI 500. After 24-hour incubation, cells were harvested and stained with annexin V and PI and analyzed by flow cytometry. Rm-infected HSG cells significantly increased necrotic and late apoptotic cells compare to control. However, Rm did not induce cell death in HOK-16B cells. Each value represents the mean ± standard error of the mean of one independent experiments in triplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control. PC; 0.5 μg/ml of staurosporine for positive control.

### 3.6 Abilities of Pm and Rm to activate TLR2 and TLR4.

Because the ligands for TLR2 and TLR4 up-regulate MHC Ⅰ expression in HSG cells, the abilities of Pm and Rm to activate these TLRs were investigated using CHO/CD14/TLR2 and TLR4 reporter cells. Both Pm and Rm activated TLR2, but not TLR4 (Fig. 6A). As a gram-negative bacterium, Pm not activating TLR4 was an unexpected result. A periodontal pathogen *Treponema denticola* inhibits the activation of TLR2 although it contains TLR2 ligands [22]. To test the presence of inhibitory machinery, CHO/CD14/TLR2 and TLR4 cells were treated with heat-killed bacteria. Heat-killed Pm activated TLR2 more compared to the live Pm but still did not activate TLR4. Such an increase in TLR2 activation was not observed by the heat-killed Rm (Fig. 6B). The results indicate that Pm has a unique mechanism, which modulates TLR2 activation. Additionally, the factors that modulated TLR2 activation are susceptible to heat. Conclusively, both Pm and Rm activate TLR2.

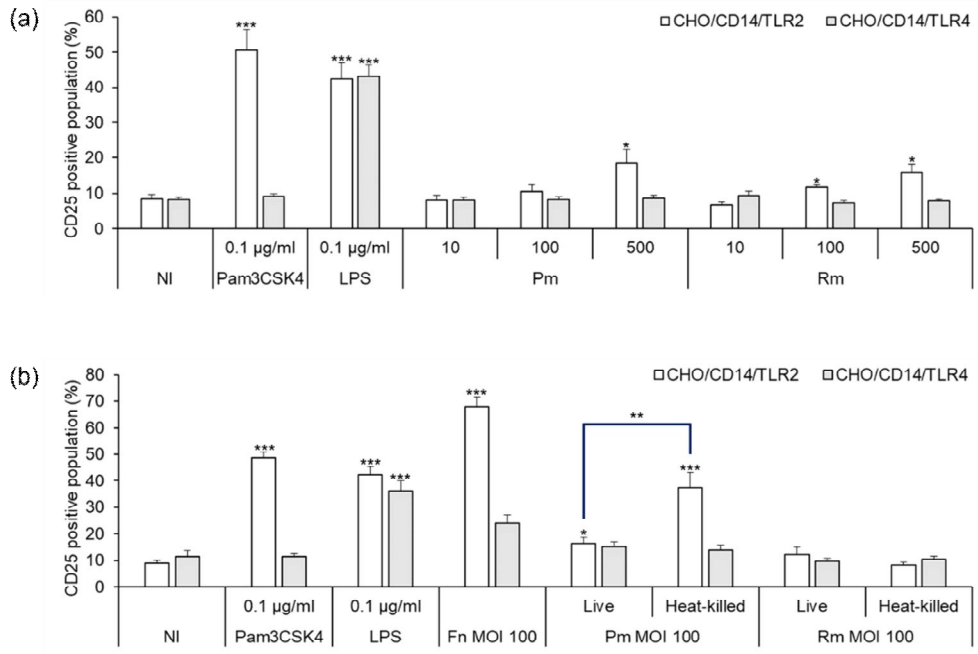


**Fig 6. Both Pm and Rm activated TLR2.**

CHO/CD14/TLR2 and TLR4 cells ($6 \times 10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, Pm, Rm, Fn, heat-killed Pm and Rm were added to CHO/CD14/TLR2 and TLR4 cells. (a) Pm and Rm activated CHO/CD14/TLR2 cells in MOI-dependent manners. However, Pm and Rm did not activate CHO/CD14/TLR4 cells. (b) Heat-killed Pm activated CHO/CD14/TLR2 cells higher than live Pm. However, heat-killed Rm did not have a difference compare to live Rm. Each value represents the mean ± standard error of the mean of five independent experiments in duplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control.

### 3.7 TLR9 activation by Pm DNA in HEK-blue_hTLR9 cells.

TLR9 recognizes un-methylated CpG DNA like bacterial or viral DNA. Therefore, Pm and Rm may activate TLR9 by their DNA and transduce signaling. In contrast, mammalian DNA, which is methylated, acts as an antagonist for TLR9 activation [23]. In the assay using HEK-blue_hTLR9 cells, Pm DNA activated TLR9 in a dose dependent manner. However, the Rm and HSG DNA did not activate TLR9 (Fig. 7A). This different bacterial activation may lead to different regulation of MHC Ⅰ expression in HSG cells. Whether Rm and HSG DNA can act as TLR9 antagonist was further tested by co-treatment with ODN 2006. Both Rm and HSG DNA down-regulated the ODN 2006-induced activation of TLR9 in a dose-dependent manner (Fig. 7B).

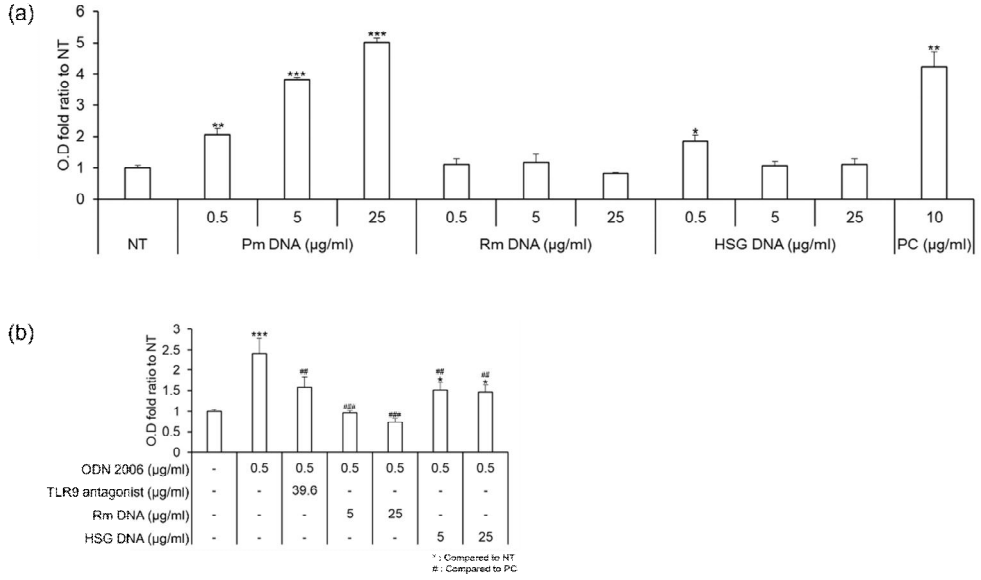


**Fig 7. Whereas Pm DNA activated TLR9 in TLR9 reporter cells, Rm DNA and HSG cells DNA inhibited TLR9 activation.**

HEK-blue_hTLR9 cells ($8 \times 10^4$ cells/well) were seeded into 96-well plates. After O/N incubation, cells were transfected Pm, Rm, and HSG DNA with lipofectamine 3000 as follow manufactures protocol. After transfected with DNA in O/N, HEK-blue detection medium was treated in each well and for 24 h incubation, optical density was analyzed by spectrometer. (a) Pm DNA activated TLR9 signaling in dose-dependent manners. However, Rm and HSG DNA did not activate TLR9. (b) In Fig 7a, Rm and HSG DNA results indicated that, they have a role of regulation in TLR9 activation. For further analysis of this result, Rm and HSG DNA were treated with ODN 2006 as a ligand for TLR9 activation. Interestingly, Rm and HSG

DNA blocked TLR9 activation by ODN 2006. Each value represents the mean ± standard error of the mean of two independent experiments in triplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control. #, $P < 0.05$, ##, $P < 0.005$, and ###, $P < 0.001$ versus to ODN 2006; positive control (PC).

### 3.8 Antagonistic regulation between TLR2 and TLR9.

Pathogens express ligands for more than just one TLR or innate immune receptor, and innate immune recognition by multiple receptors may produce a distinct response pattern. As seen in Figs. 4 and 5, the results indicate that Pm activated TLR2 and TLR9; however, Rm only activated TLR2. Therefore, in Pm infection, Pm may activate both TLR2 and TLR9 in SGECs. To mimic the infection condition, HSG cells were co-treated with TLR2 and TLR9 ligands, and their expression of MHC Ⅰ was analyzed. Interestingly, TLR2 and TLR9 had an antagonistic effect on the regulation of MHC Ⅰ expression.

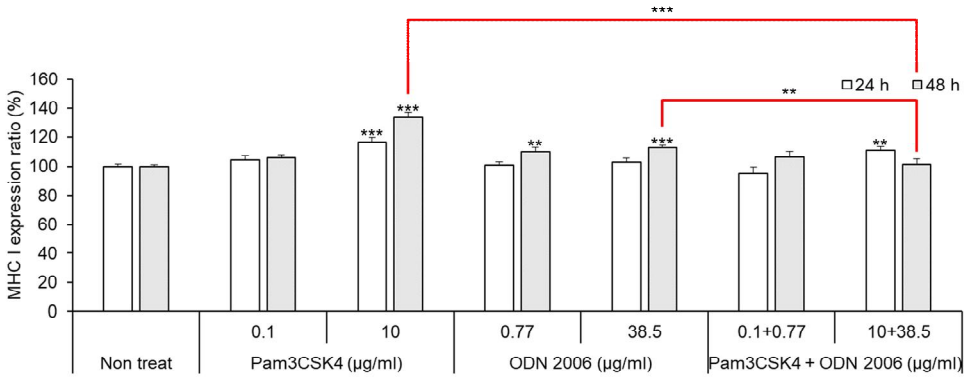


**Fig 8. Antagonistic effect between TLR2 and TLR9 activation.**

HSG cells (6 × $10^4$ cells/well) were seeded into 24-well plates. After O/N incubation, to mimic the Pm infection situation, Pam3CSK4 and ODN 2006 were co-treated into HSG cells with various concentration. Each of single ligand up-regulated the expression of MHC Ⅰ molecules. However, co-treat of TLR2 and TLR9 ligands down-regulated the expression of MHC Ⅰ molecules compare to each of single ligand treated samples. This results indicate that TLR2 and TLR9 have an antagonistic effect in HSG cells. Each value represents the mean ± standard error of the mean of four independent experiments in duplicates. *, $P < 0.05$, **, $P < 0.005$, and ***, $P < 0.001$ versus to control.

## Ⅳ. Discussion

In this study, I investigated the molecular mechanisms for the regulation of MHC Ⅰ expression in HSG cells induced by SS-associated bacteria. The dysregulated APC molecules of SGECs in SS are not only the target of the immune system but also seem to be potent triggers of immune responses [10]. In this study Pm and Rm efficiently invaded into the HSG cells and localized to the endosomes. Pm up-regulated the expression of MHC Ⅰ molecules, whereas Rm down-regulated the expression of MHC Ⅰ molecules. Recent findings indicate that SGECs in SS patients express functional TLR molecules [12, 13]. The signaling of these TLRs leads to not only the up-regulation of APC-related molecules in SGECs [12] but also phagosomal MHC Ⅰ delivery into endosomes and an increase in cross-presentation [15]. In addition, when the bacteria infect the cells, the bacteria are recognized by several TLRs such as TLR1, TLR2, TLR4, TLR5, and TLR9 (Table1). In the case of Pm and Rm, there are no flagellin. Therefore, TLR5 can be excluded from the experimental group. Each of the TLR ligands (TLR2; Pam3CSK4, TLR4; LPS, TLR9; ODN 2006) up-regulated the expression of MHC Ⅰ in the HSG cells at various time points.

**Table 1. Ligands and subcellular location of TLRs.**

| TLRs | Ligands | Location | Agonist |
|---|---|---|---|
| TLR1 | Triacetylated lipopeptides | Cell surface | Pam3CSK4 |
| TLR2 | Lipoproteins, Lipopeptides, Glycolipids, Lipoteichoic acid | Cell surface | Pam3CSK4 |
| TLR3 | Double-stranded RNA | Endosome | Poly(I:C) |
| TLR4 | Lipopolysaccharide(LPS), Heat shock proteins | Cell surface | LPS |
| TLR5 | Flagellin | Cell surface | Flagellin |
| TLR6 | Diacyl lipopeptides | Cell surface | FSL-1, Pam2CSK4 |
| TLR7 | Single-stranded RNA | Endosome | Gardiquimod |
| TLR8 | Single-stranded RNA | Endosome | CPD14b |
| TLR9 | Unmethylated CpG DNA | Endosome | ODN 2006 |

Pm is a gram negative bacterium; therefore, Pm is expected to activate TLR2, TLR4, and TLR9. In reporter cell assays, Pm activated TLR2 and TLR9; however, Pm did not activate TLR4. In the case of *Porphyromonas gingivalis* (Pg) which is also a gram negative bacterium, their LPS activate TLR2, but not TLR4 [24]. Likewise, the LPS of Pm may activate TLR2, but not TLR4, which needs to be confirmed using isolated LPS. In contrast, Rm is a gram positive bacterium; therefore, Rm is expected to activate both TLR2 and TLR9. However, Rm only activated TLR2. Furthermore, the Rm DNA inhibited rather than activated TLR9.

Compared to Rm, the specific feature of the Pm infection is TLR9 activation. Thus, their differential tendencies in MHC Ⅰ expression of HSG cells may be related to TLR9 activation. One of the possible mechanisms

can be explained by TLR9 signaling cascades. When TLR9 signaling is activated, TLR9 interacts with Myd88, the primary adaptor protein in its signaling pathway [25]. After that, interferon-1 receptor-associated kinase (IRAK) 4, 1, and 2 are assembled. Then, I kappa B kinase (IKK) alpha is activated by IRAK and induces a transcription factor, which is interferon regulatory factor (IRF) 7. Finally, IRF 7 induces Type Ⅰ interferons (IFNs), and Type Ⅰ IFNs induce MHC Ⅰ expression in the host cells [26~28]. Thus, this mechanism may be interpreted as the result of MHC Ⅰ induction following Pm infection. Moreover, there were interesting results in TLR2 activation by Pm. Based on the results of the CHO/CD14/TLR2 reporter cells, Pm has a unique mechanism to modulate TLR2 activation. Pathogenic bacteria have evolved elaborate strategies to evade or modulate the intracellular signaling pathways of host cells that activate the host immune response [29]. One of the immune evasion strategies is to inhibit TLR-dependent signaling through MAPK and NF-kB signaling. For example, in *Brucella melitensis*, they mimic TIRAP to block TLR2/TLR4 signaling targets for proteasomal degradation [30]. Additionally, *enteropathogenic Escherichia coli* (EPEC) and *enterohemorrhagic Escherichia coli* (EHEC) cleave the JNK and p39 phosphorylation motif [31] or the amino-terminus of p65 NF-kB targeting it for proteasomal degradation [32]. Similarly, Pm may have mechanisms such as proteases to degrade TLR2 pathway molecules or block TLR2 signaling. However, one thing to note about the un-known factors of Pm that block TLR2 signaling

is that they are susceptible to heat. Therefore, the unknown factors may be a protein. For further investigation, observing TLR2 and TLR9 expression after HSG cells are infected by Pm and Rm may indicate and explain these results.

On the other hand, Rm decreased MHC Ⅰ expression in the HSG cells. This result can be explained by the cytotoxicity induced by the bacterial challenge. Several viruses such as adenovirus type2 and herpes simplex virus 1 and 2 have evolved strategies to reduce MHC Ⅰ expression in host cells [33, 34]. However, regulation of MHC Ⅰ expression in cells by bacterial infection has not been well elucidated. In the case of entero-bacterial infection, bacteria regulate the expression of MHC Ⅰ in peripheral blood mononuclear cells (PBMCs) [35]. Another possible explanation is from the unique function of Rm DNA. TLR9 is activated by un-methylated DNA such as virus and bacterial DNA. Surprisingly, Rm DNA down-regulated the activation of TLR9 by its agonist ODN 2006. This indicates that Rm DNA has antagonistic activity for TLR9. One of the possible explanations is that Rm DNA has an inhibitory motif for TLR9 in its genome. There are many reports about a TLR9 activation sequence motif [36~39]. If Rm DNA lacks the TLR9 activation motif, it can explain our result. In addition, CpG suppression, methylation, and a saturable amount of DNA uptake can affect TLR9 suppression [40, 41]. Therefore, in SGECs, their TLR9 signaling cascade may be diminished by Rm challenge. Conclusively, Type Ⅰ IFNs may decrease, and the expression

of MHC Ⅰ molecules may decrease. However, the role of TLR9 or TLR2 in the bacteria-induced regulation of MHC Ⅰ expression should be confirmed using HSG cells with TLR2 or TLR9 knocked-down.

Interestingly, there was an antagonistic effect between TLR2 and TLR9 signaling. TLR signaling pathways are well-defined in many studies. In the TLR signaling pathways, there are many shared proteins or factors such as MyD88 and IRAK. TLR2 and TLR9 have a MyD88 dependent signaling, which leads to the assemble IRAK1 and 4. There are some interesting reports about the antagonistic effect of TLR2 and TLR9 by Mycobacterium tuberculosis infection in DCs. [42, 43]. In these reports, research groups indicated that TLR2 signaling depletes IRAK and suppresses cross-presenting and induction of Type Ⅰ IFN by TLR9 signaling. It can be interpreted that their antagonistic effect may need to suppress an excessive inflammatory response that leads to cell death, or some other disadvantages. Moreover, in terms of cytokine production, TNF-α and IL-10, which are derived from TLR2 signaling, may suppress type Ⅰ IFN induced by TLR9 activation in chronic HCV infection [44~45]. Controversially, in herpes simplex virus infection, TLR2 and TLR9 engage together and orchestrate viral clearance [46]. Therefore, the exact modulation mechanisms on how TLR2 and TLR9 co-signaling regulate the expression of MHC Ⅰ molecules should be further investigated.

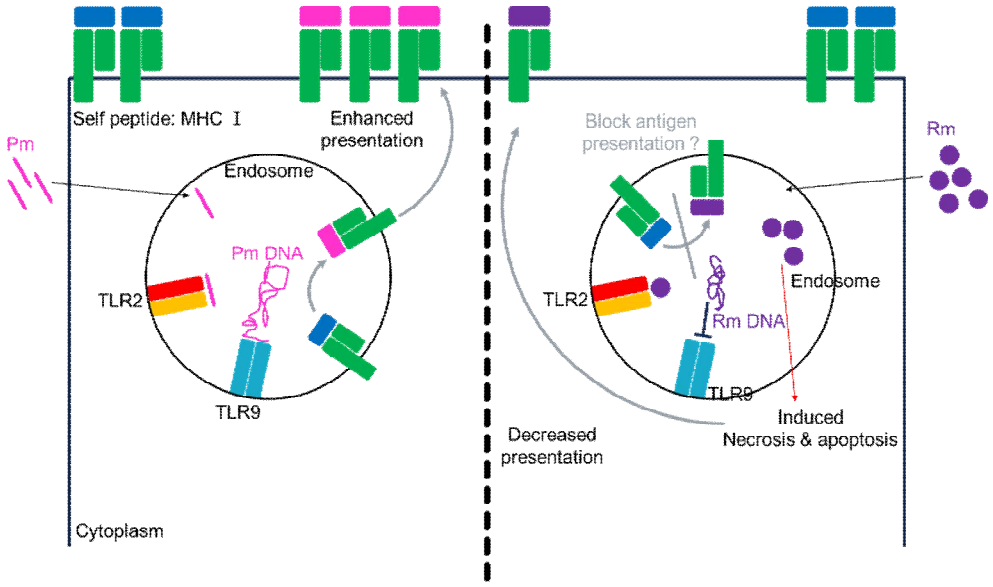


**Fig 9. Proposed mechanisms of dysregulation of HSG cells induced by SS associated bacteria.**

SS-associated bacteria Pm and Rm can dysregulate the expression of MHC Ⅰ molecules in HSG cells. Their differential molecular mechanisms may affect their differential aspects in MHC Ⅰ molecules. Gray words and lines indicated that, not shown in this study, and were proposed mechanisms.

Interestingly, HSG cells expressed TLR2, TLR4, and TLR9 in intracellularly, but not on the surface of the cells. TLR2 and TLR4 are one of the PRRs, which is located in the host cells surface membrane. They recognize cell wall components that are derived from bacteria. In a pilot study, TLR ligands up-regulated the expression of MHC Ⅰ in HSG cells.

However, TLR2 and TLR4 expressions were not observed in our previous study. Because of the TLR ligand up-regulated effects on the expression of MHC Ⅰ in HSG cells, TLR ligands may activate each of their adequate receptors. Finally, intracellular TLRs were detected with intracellular staining by flow cytometry. There are reports on intracellular TLR2 showing it is located in the plasma membrane, endosomes, lysosomes, and Rab-11-positive compartments, however, not in the Golgi of monocytes [47]. There is a report that TLR4 is located in the nucleus and cytoplasm of lung inflammatory cells [48]. To confirm the intracellular expression of TLRs, co-immunostaining of TLRs, the Golgi apparatus, and endoplasmic reticulum (ER) markers can be used to observe the exact location of TLR2 and TLR4. Generally, commensal bacteria do not invade into host cells and protect the host from colonization of pathogens [49, 50]. Therefore, it can be interpreted that the intracellular TLRs in HSG cells have a role in recognizing only invasive pathogen.

The results of this study indicate that SS-associated oral bacteria regulate the expression of MHC Ⅰ molecules in HSG cells as in non-professional APCs. Dysregulated SGECs may present a presumptive peptide and activate the immune cells, or be targeted by immune cells like T cell activation or apoptosis. This mechanism may have an important role in the initiation of autoimmune inflammatory responses such as in SS. Pm up-regulated the expression of MHC Ⅰ molecules, which can lead to the infiltration of $CD8^+$ T cells. However, Rm down-regulated the expression of

MHC Ⅰ molecules and induced cell death of HSG cells. Thus, cell death may affect the dysfunction of salivary glands. In conclusion, these findings suggest that changes in the oral microbiota associated with SS patients and bacterial challenge may potentially induce the dysregulation of SGECs and contribute to the pathogenesis of SS. In addition, the mechanism of infection of SS-associated bacteria and their TLR activation by host cells may explain the etiopathogenesis of SS.

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국문초록

# 구강세균에 의한 주조직적합성복합체 I 조절에 미치는 인간침샘세포의 톨-유사 수용체의 역할

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## 1. 목 적

상피세포가 세균에 감염될 경우, 상피세포의 주조직적합성 복합체에 의해 세균의 항원이 T 림프구에게 제시된다. 본 연구진은 쇼그렌 증후군에 연관된 구강 세균 균주인 *Prevotella melaninogenica* (Pm) 과 *Rothia mucilaginosa* (Rm) 이 인간침샘세포에 효과적으로 침투하고, 주조직적합성 복합체 I 의 발현을 다르게 조절한다는 것을 발견하였다.

본 연구의 목적은 인간침샘세포 내에서 주조직적합성 복합체 I 의 발현 조절의 분자적 기전을 밝히는 것이다.

## 2. 방 법

인간침샘세포에 Pm 과 Rm을 감염시킨 후, 24, 48, 또는 72 시간 후에 주조직적합성복합체 I 의 발현을 유세포분석기를 통해 분석하였다. 그리고 세포의 생존률, 세균의 침투율을 분석하였다. 또한, 인간침샘세포의 톨-유사 수용체 2, 4, 9 의 발현을 측정하였고, 각각의 톨-유사 수용체의 리간드를 처리한 후, 주조직적합성복합체 I 의 발현을 조사하였다. 마지막으로 톨-유사 수용체 리포터 세포를 통해 Pm 과 Rm 의 톨-유사 수용체 활성화 정도를 평가하였다.

### 3. 결 과

세포내 세균 감염 후, 48 시간 뒤의 결과에서 Pm 은 세포의 생존에 영향을 미치지 않았고, 세균의 침투와 주조직적합성복합체 I 의 증가와 약한 양의 상관관계를 보였다 ($r_s$= 0.273, $p$= 0.023). Rm 은 세균의 침투에 따른 세포 사멸을 이끌었으며, 주조직적합성복합체 I 의 발현을 감소시켰다 ($r_s$= -0.358, $p$= 0.003). 또한, Rm 이 감염된 인간침샘세포의 세포 생존률과 주조직적합성복합체 I 의 발현에는 양의 상관관계가 관찰되었다 ($r_s$= 0.496, $p$< 0.001). 세포내 세균 감염 후, 72 시간 뒤의 결과에서도 비슷한 경향이 나타났다 [주조직적합성복합체 I 의 발현 vs 세포 생존률 ($r_s$= 0.496, $p$< 0.001), 세균의 침투 vs 주조직적합성복합체 I 의 발현 ($r_s$= -0.249, $p$= 0.039)]. 인간침샘세포의 내부에 톨-유사 수용체 2, 4, 9 이 발현되었으며, 세 개 수용체의 리간드는 각각 인간침샘세포의 주조직적합성복합체 I 의 발현을 증가시켰다. 또한, 톨-유사 수용체 리포터 세포의 실험 결과 Pm 은 톨-유사 수용체 2 와 9을 활성화 시켰으며, Rm 은 톨-유사 수용체 2 만을 활성화 시켰다.

## 4. 결 론

Pm 에 의해 활성화된 인간침샘세포의 톨-유사 수용체 9 에 의한 신호전달로 인간침샘세포의 주조직적합성복합체 I 의 발현이 증가했을 가능성이 있으며, Rm 에 의해 감소된 인간침샘세포의 주조직적합성복합체 I 의 발현에는 세균 감염에 따른 세포독성이 관련되어 있을 가능성이 있다. 정확한 기전은 더욱 밝혀져야 할 필요가 있다.

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주요어 : 쇼그렌 증후군, 인간침샘세포 (HSG cell), Prevotella melaninogenica, Rothia mucilaginosa, 주조직적합성복합체 I (MHC I)
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