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1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder that result in dysfunction of exocrine glands, particularly the salivary and lacrimal glands, with generalized dryness. Primary SS is not associated with the other autoimmune disease, affecting a 90% of female in approximately 0.1-0.4% of population. Other vital organs including kidneys, liver, and lungs also are involved in SS so that it is considered as systemic disease with organ-specific autoimmunity. Glandular lesions of SS are also associated with infiltration of activated T and B cells. Especially, polyclonal B cell hyperactivity has been shown by intense hypergammaglobulinemia, multiple auto-antibodies (such as antibodies to ribonucleoproteins Ro/SSA and La/SSB), and immune-complex-mediated manifestations. Several pathogenic mechanisms are implicated in the initiation and perpetuation of SS.

The other autoimmune diseases have been frequently observed in families of SS patients, which means SS is associated with genetic predisposition. Alleles within major histocompatibility complex (MHC) class II gene region are involved in the emergence of specific autoantibodies and pathogenesis of SS. The genetic association between interferon regulatory factor 5 (IRF5) allele and development of SS (Odd ratio: 1.93) has been revealed by a genome-wide association study (GWAS). Another Swedish and Norwegian cohort study found a correlation of polymorphisms in IRF5 and STAT4 genes, which participate in IFN signaling for progression of SS.
Cellular and molecular pathways implicated in the pathogenesis of Sjögren’s syndrome

(Re-drawn figure adopted from; Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2010.118)

Glandular viral infection could be the environmental factor, which triggers activation of toll-like receptors (TLRs) in the gland cells. TLR-induced signaling pathways result in the induction of adhesion and costimulatory molecules and production of inflammatory cytokines. Various infectious agents such as human T-lymphotropic virus type 1, hepatitis C virus, and Epstein-Barr virus (EBV) have been suggested as triggering factors for SS. Especially, a high rate of reactivation of EBV that infects salivary glandular epithelial cells and B cells in SS has been
reported. However, it is unclear exactly what makes EBV can be reactivated in the lesions of SS.

The estrogen might be one of the factors responsible for immunologic gender dimorphism. The strong predominance of female, particularly postmenopausal women, and several animal experiments have suggested a role of estrogen deficiency in SS. The estrogen could alleviate lymphocytic infiltration and inhibit cell death of salivary gland in experimental murine models. Aromatase-knock out mice which cannot synthesize estrogen developed a lymphoproliferative disorder similar to SS. Age-dependent autoimmune exocrinopathy that resembles SS was caused by overexpression of retinoblastoma-associated protein 48 (and therefore lead to estrogen-deficiency-dependent apoptosis). Salivary glandular epithelial cells (SGECs) in mice model of estrogen deficiency function as antigen presenting cells (APCs) which upregulated the MHC class II expression and interferon gamma (IFNγ) secretion for activation of CD4+ T cells and development of glandular inflammation.

During the past few years, the importance of SGECs in the development of SS has received much attention. The lymphocytic infiltration to target organs or exocrine glands is the hallmark of SS. The majority of infiltrating lymphocytes are activated T cells in early SS lesions, while B lymphocytes forming ectopic germinal center (GC)-like structure predominate in advanced chronic lesions. Interestingly, the lymphocytic infiltrates are developed around the epithelial structure of the affected tissues or
Cellular and molecular pathways implicated in the pathogenesis of Sjögren’s syndrome

(Re-drawn figure adopted from; Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2010.118)

Organs. As an alternative name for this disease, the term ‘autoimmune epithelitis’ has been suggested, and precisely described the pathophysiology of SS in regard to an interplay between the epithelium and the immune system.

Dysregulated SGECs in SS lesions play an important role in the induction and persistence of the inflammatory process. They seem to possess sufficient features and requirements needed to act like non-professional APCs. Expression of high levels of MHC class I (HLA-ABC) and MHC class II (HLA-DR), as well as CD80/B7.1
and CD86/B7.2 molecules has been shown in the SGECs adjacent to inflammation sites.\textsuperscript{26,27} Cultured SGECs line from SS patients express intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule (VCAM).\textsuperscript{27,28} High expression of functional CD40 and CD40L molecule on the infiltrating lymphocytes has been also detected in the SGECs of SS.\textsuperscript{29} Expression of adhesion and costimulatory molecules could be regulated by various factors. Among them, IFN\(\gamma\) the major cytokine present in pathologic lesions of SS patients has significant effect on upregulation of these molecules.\textsuperscript{30}

Recent findings indicated that SGEC derived from SS patients have higher mRNA levels of TLR2, -3, and -4 than those of controls so that TLR signaling leads to the upregulation of APC-related molecules, adhesion molecules, and the production of proinflammatory cytokines and chemokines.\textsuperscript{31} An increased proinflammatory cytokines such as interleukin (IL)-1, -6, and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) from SGECs in SS have been demonstrated in several studies.\textsuperscript{32} The production of GC-forming and T cell/B cell-attracting chemokines, including CCL3, CCL4, CCL21, CXCL9, CXCL10, CXCL12, and CXCL13 has also been studied in SGECs. Especially, the potent T cell chemoattractants, CXCL9/Mig and CXCL10/IP-10, were expressed by the SGECs in SS pathologic lesions.\textsuperscript{33-35} Most of the periductally infiltrating CD3\(^+\) T lymphocytes express CXCR3 that is receptor for both CXCL9 and CXCL10.\textsuperscript{36-38}

The early appearance of dendritic cells (DCs) producing high levels of type I IFNs
Relative abundance of oral bacteria in phylum and genus level among healthy control, sicca patient control, and SS patients

(This data is previously done by Jehan Alam)

in SGECs is important, as they induce abnormal activation of lymphocytes in these tissue.\textsuperscript{39} Especially, IFN\(\alpha\) is a primary stimulator for production of B cell activating factor (BAFF) by SGECs. BAFF, a cytokine belonging to the TNF superfamily, has been shown to possess a pathogenic role in the aberrant B cell maturation and GC-like structures in the lympho-epithelial lesions of SS patients. It seems to be essential for emergence of autoreactive B cells producing autoantibodies.\textsuperscript{40, 41} Cultured SGECs
Relative abundance of selected oral bacteria in phylum and genus levels among healthy controls, sicca patient controls, and SS patients

(On the basis of microbiota analysis, specific oral bacteria were re-tabulated)

<table>
<thead>
<tr>
<th>Name</th>
<th>Gram</th>
<th>Median (Range)</th>
<th>HC</th>
<th>SC</th>
<th>SS</th>
<th>HC-SC</th>
<th>HC-SS</th>
<th>SC-SS</th>
<th>KW-H</th>
<th>Mann-Whitney U test</th>
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<tr>
<td>Streptococcus salivarius</td>
<td>+</td>
<td>6.78 (4.73) 7.73 (27.26) 12.10 (51.71)</td>
<td>0.411</td>
<td>0.739</td>
<td>0.224</td>
<td>0.381</td>
<td></td>
<td></td>
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<tr>
<td>Streptococcus oralis</td>
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<td>0.56 (3.38) 1.42 (4.59) 1.60 (8.24)</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.88</td>
<td></td>
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</tr>
<tr>
<td>Rothia mucilaginosa</td>
<td>+</td>
<td>0.55 (4.39) 3.86 (11.18) 1.97 (12.98)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.07</td>
<td></td>
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<tr>
<td>Fusobacterium nucleatum</td>
<td>-</td>
<td>0.41 (21.64) 0.30 (1.21) 0.29 (2.27)</td>
<td>0.302</td>
<td>0.202</td>
<td>0.175</td>
<td>0.751</td>
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<tr>
<td>Prevotella melaninogenica</td>
<td>-</td>
<td>0.28 (3.40) 0.11 (1.33) 1.26 (5.39)</td>
<td>0.074</td>
<td>0.096</td>
<td>0.213</td>
<td>0.048</td>
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<tr>
<td>Prevotella histicola</td>
<td>-</td>
<td>0.07 (4.48) 0.05 (3.78) 0.86 (10.46)</td>
<td>0.03</td>
<td>0.84</td>
<td>0.03</td>
<td>0.04</td>
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<table>
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<tr>
<th>Prevotella melaninogenica</th>
<th>Odd ratio (%)</th>
<th>P=Value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>5.4</td>
<td>0.003</td>
<td>1.793 – 16.238</td>
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appear to secret BAFF in response to cytokines produced by T cell, which implicates involvement of SGECs in the erratic B-cell differentiation processes of SS.\(^{42}\)

Previous studies have suggested that bacterial infection probably play a role in the induction autoimmune disease such as granulomatosis with polyangiitis (GPA) and rheumatoid arthritis (RA) through molecular mimicry mechanism.\(^{43}\) Highly conserved proteins or enzymes in the microorganisms, which also have homology with self-antigens in human, could produce cross-reactive autoantibodies during immune response. Autoantibodies to human aquaporin-5 (AQP5) in the sera from SS patients may also present a possibility on pathogenesis of SS.\(^{44}\) However, the exact
roles of change in microbiota in SS patients have never directly shown to trigger SS.

Recently, the communities of oral bacteria collected by mouth rinse from healthy controls (HC, n = 15), patients with dry mouth due to medication as sicca controls (SC, n = 10) and SS patients (SS, n = 25) have been analyzed by pyrosequencing of 16S bacterial RNA gene. Pyrosequencing analysis revealed drastic changes in the bacterial communities associated with the dryness of mouth and subtle differences between the communities of SC and SS. Based on the results of microbiota analysis, specific oral bacterial species such as *Streptococcus salivarius*, *Streptococcus oralis*, *Rothia mucilaginosa*, *Fusobacterium nucleatum*, *Prevotella melaninogenica*, and *P. histicola* were chosen for this study. In particular, an increase in *P. melaninogenica* was associated with SS risk by logistic regression analysis (Odd Ratio 5.4, *p* = 0.003). It has been hypothesized that specific oral bacteria may be responsible for induction of deregulation of SGECs, which manifest APC-like phenotypes and production of cytokines and chemokines.
2. Materials and Methods

2.1. Cell culture

Human salivary gland (HSG) cell line was obtained from Korean cell line bank (KCBL, Seoul, Korea). The HSG cells were cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂. Cells were maintained in DMEM medium with 10% fetal bovine serum (FBS) and 100 unit/ml each penicillin and streptomycin.

2.2. Bacteria culture

All used bacteria for this study were from ATCC (American type culture collection) and KCOM (Korean collection for oral microbiology). *P. melaninogenica* KCTC 5323, *P. histicola* KCTC 15171, and *F. nucleatum* were cultured in recommended KCTC-5457 medium, blood medium (KCOM3), and brain heart infusion (BHI) medium respectively supplemented with 5 μg/ml of hemin (Sigma, St Louis, MO, USA) and 10 μg/ml of vitamin K under anaerobic condition (5% H₂, 10% CO₂, and 85% N₂) at 37°C. *S. salivarius* KCTC 5512, *S. oralis* KCTC 9811, and *R. mucilaginosa* KCTC 19862 were grown in the BHI supplemented with 5 μg/ml of hemin (Sigma, St Louis, MO, USA) and 10 μg/ml of vitamin K under anaerobic condition at 37°C. All bacteria were harvested in log phase and washed more than twice with phosphate buffered saline (PBS) for further experiments.
2.3. Bacterial infection

HSG cells (4 X 10^4 cells/well) were seeded into the 24-well plates one day before infection. Overnight grown cells were infected with specific oral bacterial species at multiplicity of infection (MOI) 50 and 100 for 3 days in the absence or presence of 100 ng/ml IFNγ (Peprotech, USA). To prevent the outgrowth of bacteria, minimum inhibitory concentration (MIC) or more than that of gentamicin was added to the cultured cells 6 h after bacterial infection.

2.4. Stimulation of HSG cell with IFNγ or ligands to pattern recognition receptors

HSG cells were plated at 4 X 10^4 cells per well into 24-well plates one day before use. While changing the culture medium, HSG cells were treated with 10, 25, 50, and 100 ng/ml of IFNγ for 3 days. For stimulation with ligands to pattern recognition receptors (PRRs), HSG cells were also stimulated with 0.1 and 1 μg/ml Pam3csk4 (Invivogen, CA, USA), or with 0.1 and 1 μg/ml MDP (Invivogen, CA, USA), or with 0.1, 1, and 10 μg/ml Tri-DAP (Invivogen, CA, USA), or with 1 and 5 μM CpG-ODN respectively for 3 days.
2.5. Flow cytometry

For staining of APC-related surface molecules, HSG cells were incubated with FITC-conjugated anti-human HLA-DR, DP, DQ monoclonal antibody (mAb) clone IT2.2 (BD Bioscience, CA, USA), PE-conjugated anti-human CD80 mAb clone L307.4 (BD Biosciences CA, USA), PerCP-conjugated anti-human HLA-A, B, C mAb clone W6/32 (BioLegend, Sandiego, CA, USA), and APC-conjugated anti-human CD86 mAb clone IT2.2 (BioLegend, Sandiego, CA, USA) for 20 min on ice. HSG cells were also stained with FITC-conjugated human TLR2 clone TL2.1 (BioLegend, Sandiego, CA, USA) or PE-conjugated human TLR4 clone TF901 (BD Biosciences) for 20 min on ice. In case of TLR9 staining, HSG cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% tritonX-100 for 15 min on ice, washed every step with PBS and then incubated with the PE-conjugated anti-human TLR9 clone eB72-1665 (BD Biosciences) for 30 min on ice. For each isotype control, FITC-conjugated mouse polyclonal Ig (BD Biosciences, CA, USA), PE-conjugated IgG2a clone X39 (BD Biosciences, CA, USA), PerCP-conjugated mouse IgG2a clone MPOC-173 (BioLegend, Sandiego, CA, USA), and APC-conjugated mouse IgG2a clone X39 (BD Biosciences, CA, USA) were stained respectively under same condition. Stained cells were then washed and analyzed using FACSCalibur (BD Biosciences) equipped with CellQuest software for data analysis.
2.6. Immunofluorescent microscopy

HSG cells (4 X 10^4 cells/well) were seeded on cover slides 12-mm diameter in each well of 24-well plate and cultured in the absence or presence of 100 ng/ml of IFNγ for 3 days. After washing with PBS, the cells were fixed with 4% paraformaldehyde and soaked with sodium citrate buffer. The fixed cells were heated at 105°C for 20 min for antigen retrieval. After washing with PBS, the HSG cells were treated with 50 mM ammonium chloride for 10 min to quench the auto-fluorescence, and blocked with 2% BSA for 1 h at room temperature (RT) and then incubated with anti-CD80 mAb (Santa cruz, Dallas, Texas, USA) or anti-CD86 mAb (Santa cruz, Dallas, Texas, USA) in 2% BSA at 4°C. Incubated cells were then stained with Alexa 488 anti-mouse IgG antibody (Invivogen) for 1 h at RT. The HSG cells were washed and incubated with 1 μg/ml Hoechst 33342 for 15 min at RT. After washing with distilled water, stained HSG cells were mounted by ProLongGold antifade reagent (Invitrogen) on the slide glasses. For each cover slides, more than three areas were photographed at 400X magnification under a fluorescent microscope (DM500B Leica, Leica Microsystems, German).

2.7. Cytokine and chemokine enzyme-linked immunosorbent assay (ELISA)

The expression of cytokines and chemokines in the culture supernatant of HSG cells was examined by ELISA. The HSG cells were co-cultured with selected oral bacteria
at MOI 50 and 100 for 3 days in the absence or presence of 100 ng/ml IFNγ. The supernatant of infected cells and control cells was kept at -80°C. The amounts of IL-6, CXCL10 (IP-10), and IFNλ in supernatant were measured using ELISA kit (R&D systems, Minneapolis, MN, USA) according to manufacture instructions.

2.8. Flow cytometric invasion assay and detection of bacteria in late endosomes

Invasion assay with flow cytometry have been described. HSG cells (6 X 10^4 cells/well) were seeded into 24-well plates, and infected with CFSE-labeled bacteria at MOI 50 and 100 for 6 and 24 hours. Fixed cells with 4% paraformaldehyde were also infected with CFSE-labeled bacteria under same condition. Infected HSG cells were washed, detached with 0.05% trypsin-EDTA in PBS, and then transferred to FACS tubes. After quenching the fluorescence of cell-bound bacteria with 0.4% trypan blue, HSG cells were analyzed by FACSCalibur (BD Biosciences). To compare the bacterial invasion ability in different bacterial strain, the invasion index was determined as follows: [mean fluorescent intensity (MFI) of infected cells – MFI of fixed cells as negative control] / MFI of CFSE labeled bacteria. For detection of oral bacteria in late endosomes, HSG cells (2.5 X 10^4 cells/well) were seeded into 24-plates one day before infection. A 10 mM of pHrodo Succinimidyl Ester (Invitrogen) in dimethyl sulfoxide (Sigma) was incubated with the specific bacterial suspension at the final concentration of 0.1 mM for 45 min at RT. After washing with PBS, each
bacteria were stained with CFSE. And then, HSG cells were infected with double-stained bacteria at MOI 50 and 100 for 6 and 24 h. Infected HSG cells were incubated with Hoechst 33342 for 15 min at RT. After washing step with distilled water, HSG cells were mounted with ProLongGold antifade reagent (Invitrogen) on the slide glasses. Bacteria within the endosomes of HSG cells were analyzed by a LSM 700 (Carl Zeiss, Jena, Germany).

2.9. Transepithelial electrical resistance (TER) measurement.

HSG cells (8 X 10^4 cells/well) were plated on the a 3 μm-pore-size polycarbonate filter of a 24-well plate of the transwell two-camber tissue culture system (SPL Life Sciences, Gyeonggi-do, Korea). The HSG cells were cultured for 6 days with medium change every other day until a confluent cell monolayer reached the peak TER value. Monolayer of dense HSG cells was then treated with IFNγ (100 ng/ml), and TNFα (100 ng/ml), or each oral bacterial species (MOI 50 and 100) for 3 days. TER was measured using ERS Volt-Ohm Meter (Millipore Bedford, MA, USA) at 0, 24, 48, and 72 h.

2.10. Statistical analysis

All data are presented as the mean ± standard error of the mean of repeated experiments. The difference between control and experimental groups was analyzed
by t-test. Significance was set at $P < 0.05$. 
3. Results

3.1. Expression of APC markers on HSG cells was upregulated by IFNγ

Previous studies have suggested that SGECs could express the APC-related molecules. Those molecules are regulated by proinflammatory cytokines such as IFNγ, TNFα, and IL-1β. The effect of IFNγ, which is a major cytokine present in lesion of SS, on the expression of APC markers, including MHC class I, class II, and costimulatory molecules in HSG cells was examined by flow cytometry and immunofluorescent microscopy. IFNγ significantly upregulated the expression of MHC class I, class II, and CD86 molecules in a concentration-dependent manner (Fig 1A and 1B). More than 72 h treatment of IFNγ lead to morphological changes and a little detachment of HSG cells. HSG cells expressed very low basal level of CD80 and CD86 molecules compared to MHC class I, and those molecules were slightly upregulated by IFNγ (Fig. 1C).
Fig 1. The effect of IFNγ on expression of APC markers in HSG cells. HSG cells 
(4 X 10⁴ cells/well) were plated into 24-well plates and treated with IFNγ for 72 h. 
The expression of APC-related surface molecules on HSG cells was analyzed by flow 
cytometry and immunofluorescent microscopy. (A) Upregulation of APC markers 
such as MHC class I, class II, and costimulatory molecules on HSG cells was 
analyzed by flow cytometry. Each value represents the mean ± standard error of the 
mean of two experiments in triplicates. *, P < 0.05 versus control. (B) Expression 
levels of APC markers on HSG cells were shown as representative histogram. (C) 
The expression of costimulatory molecules (Green) on HSG cells in the absence or 
presence of IFNγ was confirmed by immunofluorescent microscopy.
3.2. Expression of APC markers on HSG cells was modulated by oral bacteria in the absence or presence of IFNγ

To determine if the bacterial infection can induce deregulation of SGECs, APC-like phenotype in infected HSG cells was examined by flow cytometry. In the absence of IFNγ, *F. nucleatum*, a highly immune stimulatory species used as a positive control, and *P. melaninogenica* upregulated MHC class I and CD86. In contrast, *S. oralis* and *R. mucilaginosa* downregulated CD86 expression in HSG cells (Fig 2A). In the presence of IFNγ, which is artificially similar condition in lesion of SS, *F. nucleatum* and *P. melaninogenica* further upregulated IFNγ–induced expression of MHC class I and CD80. Interestingly, they also upregulated the IFNγ–induced expression of MHC class II. However, *S. salivarius*, the most abundant commensal species used as a negative control downregulated IFNγ–induced upregulation of MHC class I and CD86. *R. mucilaginosa*, a species that increased in both SC and SS compared to HC, also showed suppressive effect on the IFNγ–induced upregulation of MHC class I and CD86 as well (Fig 2B). Taken together, specific oral bacteria can modulate the expression of APC markers on HSG cells, which is characteristic of dysregulated SGECs.
Fig 2. Modulation of APC markers on HSG cells by various oral bacterial species in the absence or presence of IFNγ. HSG cells (4 X 10⁴ cells/well) were plated into
24-well plates and infected with selected oral bacteria at MOI 50 and 100 for 72 h in the absence (A) or presence (B) of IFNγ. The expression of APC markers such as MHC class I, class II, and costimulatory molecules was analyzed by flow cytometry. Each value represents the mean ± standard error of the mean of three experiments in duplicates. *, $P < 0.05$ versus control.
3.3. Production of cytokine and chemokine was modulated by oral bacteria in the absence or presence of IFNγ

Another evidence for dysregulated SGECs is production of inflammatory cytokines and chemokines, inducing recruitment of lymphocytes to the lesions of SS. Therefore, whether or not IFNγ and challenge with specific oral bacterial species can induce production of IL-6 and IP-10 in HSG cells was investigated. Treatment with IFNγ significantly upregulated IL-6 and IP-10 expression in a concentration dependent manner, suggesting that inflammatory process might be compounded by IFNγ-rich environment (Fig 3A). *P. melaninogenica* upregulated and *S. oralis* downregulated IL-6 production only in the absence of IFNγ, whereas *F. nucleatum* upregulated IL-6 expression both in the absence and presence of IFNγ (Fig 3B). In addition, *P. melaninogenica* and *F. nucleatum* upregulated expression of IP-10 both in the absence and presence of IFNγ. In contrast, *S. salivarius* and *R. mucilaginosa* downregulated expression of IP-10 in the absence or presence of IFNγ, respectively (Fig 3C).
Fig 3. Modulation of cytokine and chemokine in HSG cells by various oral bacterial species in the absence or presence of IFNγ. HSG cells (4 X 10⁴ cells/well) were seeded into 24-well plates and infected with selected oral bacteria at MOI 50 and 100 for 72 h in the absence or presence of IFNγ. The expression of cytokine and
chemokine in cell culture supernatant was measured using ELISA kit. (A) The effect of IFNγ treatment on the production of IL-6 and IP-10 in HSG cells was examined. Each value represents the mean ± standard error of the mean of three experiments in duplicates. (B) The expression of IL-6 in HSG cells by bacterial infection was investigated in the absence (left) or presence (right) of IFNγ. Each value represents the mean ± standard error of the mean of three experiments in duplicates. (C) The production of IP-10 in HSG cells by specific bacteria was also measured in the absence (left) or presence (right) of IFNγ as well. Each value represents the mean ± standard error of the mean of three experiments in duplicates. *, $P < 0.05$ versus control.
3.4. Production of type III IFN was modulated by oral bacterial in the absence or presence of IFNγ

Although Type I and type III IFNs bind to different receptors on the cell surface, they induce identical signal transduction pathways, leading to activation of Jak/STAT signaling pathways and transcription of various interferon stimulated genes (ISGs). Both type I and III IFN could be produced by stimulation of PRRs of innate immune system. Signaling pathway events and activation of gene profiles triggered by IFNλ are similar to those of the type I IFN system. Type III IFN have been shown to particularly act on the mucosal tissues or organs, and their functional receptors are mainly expressed on epithelial cells. To examine whether specific oral bacteria can induce type III IFN by SGECs, the production of IFNλ in the culture supernatant of HSG cells in the absence or presence of IFNγ was measured by ELISA. HSG cells stimulated with high concentration of IFNγ decreased the production of IFNλ compared to control cells (Fig 4A). Interestingly, both *P. melaninogenica* and *F. nucleatum* significantly upregulated IFNλ production by HSG cells in the absence of IFNγ. In contrast, *R. mucilaginosa* and *P. histicola* decreased the expression levels of IFNλ in the absence of IFNγ (Fig 4B). In the presence of IFNγ, all bacterial species tended to upregulate IFNλ, but statistical significances were achieved by only *P. melaninogenica* and *F. nucleatum*. (Fig 4C).
Fig 4. Regulation of IFNλ production by various oral bacterial species in the absence or presence of IFNγ. (A) The effect of IFNγ on the production of IFNλ in HSG cells was examined. Each value represents the mean ± standard error of the mean of two experiments in triplicates. HSG cells (4 X 10⁴ cells/well) were seeded into 24-well plates and infected with selected oral bacteria at MOI 50 and 100 for 72 h in the absence (B) or presence (C) of IFNγ. The expression of IFNλ in cell culture supernatant was measured by ELISA. Each value represents the mean ± standard error of the mean of three experiments in duplicates. *, P < 0.05 versus control.
3.5. Invasion ability of oral bacteria into HSG cells varies depending on species.

The relationship between invasion ability of bacteria to epithelium and the onset of disease has been reported. Internalized bacteria can stimulate various pattern recognition receptors (PRRs), leading to production of inflammatory cytokines and upregulation of expression of immunoactive molecules. To investigate the mechanism of deregulation of SGECs caused by oral bacteria, the ability of each species to invade into HSG cells was analyzed. Especially, *P. melaninogenica*, *F. nucleatum*, and *R. mucilaginosa* showing modulation effects seem to have invasion capability compared to *S. salivarius* and *P. histicola*. HSG cells with CFSE-labeled bacteria were investigated to quantify the amount of bacteria using flow cytometry. Even though *F. nucleatum* was not well-quenched, it presented substantial ability to HSG cells. *P. melaninogenica* and *R. mucilaginosa* were also highly invasive to HSG cells (Fig 5). This result suggested that invasion characteristic of specific oral bacterial species might be one of the modulation mechanisms for deregulation of SGECs.
Fig 5. The invasion of oral bacterial species into HSG cells. HSG cells (4 X 10^4 cells/well) were plated into 24-well plates and infected with CFSE-labeled bacteria.
at an MOI 50 and 100 for 6 and 24 h. Ability of bacterial invasion was analyzed by flow cytometry. (A) Fluorescence of bacteria bound on the cell surface was quenched with trypan blue, and then the amount of invasive bacteria within HSG cells was analyzed by measuring the fluorescence of HSG cells containing CFSE-labeled bacteria using flow cytometry. (B) Bacterial invasion ability in different strain was shown as invasion index.
3.6. Invaded bacteria were detected in the late endosomes of HSG cells

In the lumen of endocytic vacuoles, the majority of intracellular bacteria exist and modify endosomes to provide maximal support and protection. These intravacuolar bacteria such as *Salmonella*, *Chlamydia*, and *Brucella* was included in epithelial cells. Many of the bacteria change some strategies that the endosomes do not mature to a point at which they could fuse with lysosomes. To investigate whether the internalized bacteria could undergo the process of endosomal maturation in HSG cells, highly invasive oral bacteria were double-stained with pHrodo dye and CFSE for infection to HSG cells. The labeled bacteria within HSG cells were analyzed by confocal microscopy. Most *P. melaninogenica*, *F. nucleatum*, and *R. mucilaginosa* were stained by both pHrodo dye (Red) and CFSE (Green), which means they were present in the endosomal maturation process in HSG cells (Fig 6A). The colocalization of both red and green signals was significantly confirmed by Mander’s coefficient values (Fig 6B). This result suggested that specific bacteria may stimulate the PRRs located in endosomes and cytosol within HSG cells.
**Fig 6. Internalized bacteria were mostly located in the endosome of HSG cells.**

HSG cells (2.5 X 10^4 cells/well) were plated into 24-well plates one day before infection. Overnight cultured HSG cells were infected with oral bacterial species double stained with pHrodo dye (Red) and CFSE (Green) at MOI 50 and 100 for 6 and 24 h. Nuclei (Blue) in HSG cells was then stained. Internalized bacteria within endosomes of HSG cells were analyzed by co-localization (Yellow) using confocal microscopy. (A) This representative figure showed that invaded oral bacteria were observed within the endosomes of HSG cells. (B) Co-localization of green and red signals was calculated by using Mander’s coefficient. *, P > 0.5.
3.7. HSG cells express bacteria-sensing toll-like receptors (TLRs).

The pathogen-associated molecular patterns (PAMPs) broadly shared by bacteria are recognized by immune system due to presence of TLRs that are expressed in the leukocytes and non-immune cells. TLRs play an important role in the induction and activation of both innate and adaptive immunity. According to previous study, cultured SGECs from SS patients constitutively expressed high levels of TLRs. The expression of TLRs in HSG cells in the absence or presence of IFNγ was examined by flow cytometry. HSG cells expressed low basal level of TLR2 and TLR4, while that of TLR9 was quite high (Fig 7A). The expression of all TLR2, -4 and -9 in HSG cells was significantly upregulated by treatment with IFNγ (Fig 7B). The constitutive expression of TLRs by SGEC in SS suggested that epithelial cells can sense bacterial challenge through these receptors.
Fig 7. Expression of Toll-like receptors in HSG cells in the absence or presence of IFNγ. HSG cells (4 X 10⁴ cells/well) were seeded into 24-well plates and cultured in the absence or presence of 100 ng/ml IFNγ for 72 h. Stimulated HSG cells were stained with anti-human TLR2, -4, and -9 antibody. (A) The representative histogram of TLR-2, -4, and -9 expression in HSG cells. (B) The expression of TLRs on HSG cells in the absence or presence of IFNγ was examined by flow cytometry. Each value of TLR2 and TLR4 staining represents the mean ± standard error of the mean of two
experiments in triplicates. Each value of TLR9 staining represents the mean ± standard error of the mean of three experiments in duplicates. *, $P < 0.05$ versus control.
3.8. Expression of APC markers on HSG cells was regulated by various ligands to PRRs.

In order to elucidate the role of PRRs in modulation of APC markers, HSG cells were challenged with various synthetic ligands such as Pam3csk4 (TLR1/2 ligand), LPS (TLR4 ligand), CPG-ODN (TLR9 ligand), Tri-DAP (NOD1 ligand), and MDP (NOD2 ligand). The expression of APC markers on stimulated HSG cells was analyzed by flow cytometry. The expression of MHC class II and CD80 molecules was upregulated by Pam3csk4. However, most of the PRR ligands downregulated the expression of APC markers in HSG cells (Fig 8). Taken together, each PRR may have distinct roles in modulation of APC markers in HSG cells.
Fig 8. Expression of APC markers on HSG cells by ligands for stimulation of PRRs. HSG cells (4 X 10^4 cells) were plated into 24-well plates and treated with specific concentration of various ligands such as Pam3csk4, LPS, CpG-ODN, Tri-DAP, and MDP for 72 h. The induction of APC markers such as MHC class I, class II, and costimulatory molecules following stimulation with PRR ligands in HSG cells was analyzed by flow cytometry. Each value represents the mean ± standard error of the mean of three experiments in triplicates. *, P < 0.05 versus control.
3.9. Production of cytokine and chemokine was regulated by various ligands to PRRs

To determine PRRs that mediate the bacteria-induced upregulation of IL-6 and IP-10, the culture supernatant of HSG cells stimulated with various ligands to PRRs were analyzed by ELSIA. The expression of IL-6 was upregulated by Pam3csk4, CpG-ODN, and Tri-DAP in a concentration-dependent manner. However, LPS and MDP did not have any effect on production of IL-6 in HSG cells (Fig 9A). In contrast, the production of IP-10 was decreased by Pam3csk4, Tri-DAP, and MDP molecules. Only CpG-ODN induced the expression levels of IP-10 in HSG cells (Fig 9B). This result indicated that unique components of specific oral bacterial species may dysregulate HSG cells by modulating production of cytokine and chemokine.
**Fig 9. The production of IL-6 and IP-10 in HSG cells by ligands for stimulation of PRRs.**

HSG cells (4 X 10^4 cells) were plated into 24-well plates and treated with specific concentration of PRR ligands such as Pam3csk4, LPS, CpG-ODN, Tri-DAP, and MDP for 72 h. After HSG cells were stimulated with those ligands, the expression of IL-6 (A) and IP-10 (B) in culture supernatant was examined by ELISA. Each value represents the mean ± standard error of the mean of three experiments in triplicates. *P < 0.05 versus control.
3.10. Physical barrier of HSG cells was disrupted by inflammatory cytokines and specific bacteria

A layer of epithelium separates microorganisms from the underlying tissues, and thus epithelial barrier function is very important for defense mechanism required to inhibit infection and inflammation.\textsuperscript{54} To investigate whether inflammatory cytokines and specific bacteria affect the barrier function of HSG cells, TER values of HSG cells co-cultured with oral bacteria or inflammatory cytokines were measured using an ERS Volt-Ohm Meter. The TER values of HSG cell monolayer seeded into transwell increased up to 6 days and reached a plateau (Fig 10A). This result indicated that junctional complexes in monolayer HSG cells was formed in 6 days. At that point, tightly compacted HSG cells were treated with IFN\(\gamma\), TNF\(\alpha\), or various oral bacterial species. IFN\(\gamma\) and TNF\(\alpha\) significantly decreased the TER values of HSG cells even one day after treatment. Interestingly, only \textit{P. melaninogenica} significantly reduced the TER levels of HSG cells in 3 days (Fig 10B). Taken together, inflammatory cytokines and a specific oral bacterial species could weaken the physical barrier function of SGECs in SS.
Fig 10. The role of inflammatory cytokines and specific bacteria in barrier function of HSG cells. HSG cells (8 X 10^4 cells/well) were seeded into 24-well plates of transwell culture system. When the TER value reached to peak by using ERS Volt-Ohm Meter, a confluent HSG cells were treated with 100 ng/ml IFNγ and TNFα or infected with selected bacteria at MOI 50 and 100 for 3 d. (A) The TER values of HSG cells were gradually increased over time. (B) The dense monolayer of HSG cells
was affected by inflammatory cytokines and specific oral bacterial species in 3 days.

TER was calculated as a relative percentage of control at day 0 for the baseline.
Discussion

A new immuno-pathogenic concept has recently emerged, which placed the dysregulated SGECs at the center of the pathophysiological process of SS. The dysregulated SGECs in SS are not only the target of the immune system but also seem to be the active participants in the immune responses. In current study, contribution of oral bacteria to deregulation of SGECs has been added. Specific oral bacterial species selected based on the results of microbiota analysis were used to investigate their potential roles in deregulation of SGECs. In the absence or presence of IFNγ, various oral bacterial species modulated the production of APC makers, cytokines, and chemokines in HSG cells. Oral bacteria that invaded into HSG cells underwent endosomal maturation process, suggesting stimulation of TLR9. HSG cells expressed bacteria-sensing TLRs, and various synthetic ligands to PRRs regulated the immunoactive molecules in the HSG cells. These findings suggest that changes in oral microbiota and specific oral bacterial species can dysregulate the SGECs, therefore they have a potential role in the pathogenesis of SS.

Expression of MHC class I with antigenic peptides complex and costimulatory molecules is very important for activation of cytotoxic T cells by providing them with adequate signals needed. Those molecules are usually known to be modulated by virus, but not oral bacteria. The specific oral bacterial species *P. melaninogenica* and *F. nucleatum*, induced expression of MHC class I and CD86 in HSG cells under normal condition. This suggested that dysregulated SGECs may interact and
Fig 11. Expected mechanisms of deregulation of SGECs and pathogenesis of SS by oral bacteria. Specific oral bacterial species can dysregulated HSG cells through modulation of the immunostimulatory molecules including APC markers, cytokines, and chemokine in the absence or presence of IFNγ. They may further activate the immune cells located in the tissues, ultimately leading to chronic inflammatory responses of SS. Solid lines and dotted lines indicate the mechanisms which shown and implicated in this study, respectively.

activate the lymphocytes around the target tissues. Self-derived peptides could be loaded onto the highly expressed MHC class I on HSG cells. Further upregulation of MHC class I by oral bacteria could induce the apoptosis of SGECs, the target of cytotoxic T lymphocytes (CTLs) that recognize specific bacteria (Fig 11, 1). Both *P. melaninogenica* and *F. nucleatum* also upregulated immunostimulatory cytokines and chemokine such as IL-6, IFNλ, and IP-10 in the absence of IFNγ. IL-6 is required in
the induction of T<sub>H</sub>17 cells, a major T cell subset that mediate inflammation in SS.<sup>57,58</sup>

It could also impair the physical barrier through induction of TJ permeability (Fig 11, 2).<sup>59</sup> Interestingly, the expression of IFNλ from SGECs was upregulated by <i>P. melaninogenica</i> and <i>F. nucleatum</i> in the absence or presence of IFN<sub>γ</sub>. Type III IFNs have been known to induce same signal transduction pathways with type I IFNs that cause aberrant retention and subsequent activation of lymphocytes in the tissues (Fig 11, 3).<sup>60,61</sup> IP-10 induced from HSG cells by <i>P. melaninogenica</i> and <i>F. nucleatum</i> can mediate recruitment of T lymphocytes in the early stage. When the lymphocytes recognize the specific antigens in target tissues, they can interact with epithelium through adhesion molecules after migration of lymphocytes by chemokines. These lymphocytic infiltrates lead to apoptosis of salivary glandular cells by Fas and Fas ligand interaction mechanism (Fig 11, 4).<sup>62,63</sup> By disrupting the physical barrier of HSG cells, <i>P. melaninogenica</i> may be able to reach the lamina propria where plasmacytoid DCs (pDCs) and macrophages, major producers of proinflammatory cytokines and chemokines, express those molecules in respond to <i>P. melaninogenica</i> (Fig 11, 5). In addition, <i>P. melaninogenica</i> have porin proteins homologous with AQP5 so that antibody against to <i>P. melaninogenica</i> can cross-reactive with AQP5 in HSG cells (Fig 11, 6).<sup>44</sup> These immune cells may be responsible for induction of excessive inflammatory immune responses and the pathogenesis of SS.

Activated T lymphocytes present around the SGECs could be differentiated into T helper cell type 1 (T<sub>H</sub>1), which mainly produce IFN<sub>γ</sub> for amplifying immune
The high levels of expression of APC markers, cytokines, and chemokines in HSG cells was induced by IFNγ environment, and those were further modulated by specific oral bacterial species. Importantly, *P. melaninogenica* and *F. nucleatum* also upregulated IFNγ-induced expression of MHC class I, MHC class II, CD80 molecules, and production of IFNλ and IP-10 from HSG cells. IFNγ and TNFα also disrupted physical barrier function, providing oral bacteria with more chances to enter inside the tissues (Fig 11, 5). A previous study reported that IFNγ and TNFα disrupted integrity of epithelial barrier by TJ disassembly, which involves activation of JAK and protein kinase D (PKD) signaling cascade. Morphological change and detachment of HSG cells caused by IFNγ may also affect the barrier function. T lymphocytes recruited by bacteria-induced IP-10 could produce IFNγ, which has in turn an important role in secretion of IP-10 from HSG cells. Excessive expression of IP-10 could attract more T lymphocytes and induce formation of lymphocytic infiltrates around the SGECs. Substantially dysregulated SGECs by IFNγ and oral bacterial species participate in severe activation and perpetuation of lymphocytes, causing a vicious cycle of chronic inflammatory process (Fig 11, 7).

Three oral bacterial species *P. melaninogenica*, *F. nucleatum*, and *R. mucilaginosa* showing deregulation effects in HSG cells highly invaded into the cells within few hours. These findings suggested that internalized bacteria can stimulate the endosomal or cytosolic PRRs. A previous study reported that stimulated SGECs from SS with synthetic microbial analogues of TLRs induced upregulation of cell surface
molecules such as MHC class I and CD40 molecules. HSG cells expressed bacteria-sensing TLR-2, -4, and -9 so that signaling transduction events of TLRs would be involved in the upregulation of APC markers and inflammatory responses. The modulation effects of immunoactive molecules in HSG cells also varies depending on the bacterial species. *P. melaninogenica* and *F. nucleatum* tended to dysregulate HSG cells, while *R. mucilaginosa* showed suppressive effects on the HSG cells. Since *P. melaninogenica* and *F. nucleatum* is gram-negative bacteria compared to *R. mucilaginosa*, gram-positive bacteria, HSG cells may be differently modulated by unique microbial components of selected oral bacterial species. HSG cells stimulated with MDP, derived from gram-positive bacteria, downregulated the expression of MHC class I, CD86, and IP-10, which shows similar patterns with those cells infected with *R. mucilaginosa*. Even though gram-negative bacterial component LPS did not show any effects on deregulation of HSG cells, Tri-DAP and CpG-ODN upregulated IL-6 and IP-10 production. This suggested highly invasive bacteria *P. melaninogenica* and *F. nucleatum* may trigger more TLR9 and NOD1 signaling pathways for production of cytokines and chemokines compared to *R. mucilaginosa*. Although internalized bacteria were also found in the late endosomes of HSG cells, CpG-ODN alone do not increase the expression levels of MHC class I and CD86 molecules. Many PRRs present in HSG cells seem to have their distinct roles in modulation of immunostimulatory molecules of HSG cells. There would be also other target molecules modulated by oral bacterial species, such as a NOD-like receptor family CARD domain containing 5 (NLRC5) that has been known to play a positive role in
the regulation of MHC class I expression. However, the exact modulation mechanisms how specific live oral bacteria dysregulate HSG cells should be further investigated.

Dysregulated SGECs acquire features of a non-professional APC, which may present putative peptides and activate the immune cells, thus playing a pivotal role in the initiation and perpetuation of autoimmune inflammatory responses. In conclusion, these findings suggested that changes in the oral microbiota associated with SS patients may potentially induce deregulation of SGECs and contribute to the pathogenesis of SS.
5. Reference


7. Gottenberg, J. E. et al. In primary Sjogren's syndrome, HLA class II is associated exclusively with autoantibody production and spreading of the


Kapsogeorgou, E. K., Dimitriou, I. D., Abu-Helu, R. F., Moutsopoulos, H. M. & Manoussakis, M. N. Activation of epithelial and myoepithelial cells in the salivary glands of patients with Sjogren's syndrome: high expression of...


34 Salomonsson, S. *et al.* Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjogren's


40 Thien, M. *et al.* Excess BAFF rescues self-reactive B cells from peripheral


46 Miyagi, K. *et al.* CpG oligodeoxynucleotides promote the host protective response against infection with Cryptococcus neoformans through induction of interferon-gamma production by CD4+ T cells. *Clin Exp Immunol* **140**, 54


Apostolou, E. *et al.* Expression of type III interferons (IFN lambda s) and their receptor in Sjogren's syndrome. *Clinical and Experimental Immunology*


