이학박사 학위논문

Potential roles of oral microbiota and autoantibodies against either aquaporin-5 or aquaporin-1 in the pathogenesis of Sjögren's syndrome

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Potential roles of oral microbiota and autoantibodies against either aquaporin-5 or aquaporin-1 in the pathogenesis of Sjögren's syndrome

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ABSTRACT

Potential role of oral microbiota and autoantibodies against either aquaporin-5 or aquaporin-1 in the pathogenesis of Sjögren's syndrome

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Background

Sjögren's syndrome (SS) is an autoimmune disorder that primarily targets the salivary and lacrimal glands, leading to the dryness of the mouth and eyes. Aquaporins (AQPs) are small hydrophobic transmembrane water channel proteins that are widely expressed in animals and plants as well as in bacteria and archaea. The expressions of mRNA and proteins for AQP5 and AQP1 have been shown in human salivary glands. AQP5 is expressed at the apical membrane of acinar cells and intercalated ducts in the lacrimal and salivary glands, while AQP1 expression is confined to the capillary endothelial and myoepithelial cells. AQP5 plays an important role in the production of saliva. AQP5-deficient mice secrete hypertonic saliva with

substantially reduced volume. AQPs, porins, or glycerol transport proteins of some oral bacteria have a high degree of homology with human AQP5 and AQP1. B cell epitope analysis showed the presence of evolutionary conserved linear epitopes in the extracellular loops of humans AQP5/1 and the bacterial porins, suggesting a possibility that antibodies produced against the bacterial porins may cross react with the extracellular loops of human AQP5/1 and block the entry of water molecules into the channel.

Growing evidence also suggests that dysbiosis of microbiota is associated with pathogenesis of several autoimmune diseases. However, the role of oral microbiota in the etiopathogenesis of SS in not known. The purpose of this study was to investigate the potential role of autoantibodies against human AQP5 and AQP1 and oral microbiota in the pathogenesis of SS.

Material and methods

Frozen sections of mouse submandibular salivary glands, Chinese hamster ovary (CHO) cells overexpressing green fluorescent protein (GFP) alone or human AQP5-GFP as a fusion protein, and Madin-darby canine kidney cells (MDCK) overexpressing AQP5 or AQP1 were used in the indirect immunofluorescence assay (IIFA) to detect anti-AQP5/1 autoantibodies in the sera from patients with primary SS. The lysates of human embryonic kidney (HEK)-293 cells overexpressing the AQP5/1-GFP fusion protein or

GFP alone were used for immunoprecipitation. Synthetic peptides corresponding to the extracellular loops of human-AQP5 were used to map the epitopes of AQP5. The communities of oral bacteria from healthy control (HC), patients with dry mouth due to medication as sicca control (SC) and SS patients were analyzed by pyrosequencing. Human salivary gland (HSG) cells were infected with selected oral bacteria to examine the bacterial invasion and expression of cytokines and chemokines. C57BL/6 mice were randomly divided into four groups (n=5 per group); Sham, Fn, Pm and Fn-Pm groups. Fn, Pm, Fn-Pm groups were sublingually inoculated six times with $2x10^9$ cells of *F. nucleatum* (Fn), *P. melaninogenica* (Pm), and Fn plus Pm together, respectively, The pilocarpine stimulated salivary flow rate of each mice was measured at two weeks interval. Mouse salivary glands collected for histopathology and mRNA extraction to analyze the expression of cytokines/chemokines. Sera were used to detect anti-AQP5 autoantibodies.

Results

Serum IgG from the SS patients, but not from the control subjects, stained acinar cells in the mouse salivary glands, the signals of which colocalized with those of AQP5-specific antibodies. Serum IgG from the SS patients also selectively stained AQP5-GFP expressed in CHO cells. However, both the control and SS sera immunoprecipitated the AQP5-GFP in protein lysate, suggesting that autoantibodies againstAQP5 were also present in the control

sera. The screening of 53 control and 112 SS serum samples by IIFA using the AQP5-expressing MDCK cells revealed the presence of significantly higher levels of anti-AQP5 IgG in the SS samples than in the control samples with sensitivity of 0.73 and a specificity of 0.68. Anti-AQP1 autoantibodies were also detected only in the 16.07% SS samples. Furthermore, the presence of anti-AQP5 autoantibodies, but not that of anti-AQP1 autoantibodies, was associated with low resting salivary flow in SS patients. Synthetic peptides corresponding to the extracellular loops of AQP5 significantly inhibited the binding of anti-AQP5 autoantibodies in the SS samples to MDCK-AQP5 cells. Pyrosequencing data revealed drastic changes in the bacterial communities associated with the dryness of mouth. Although the bacterial communities of SC and SS were similar to each other, the relative abundance of species/phylotypes were different between SC and SS. Logistic regression analysis revealed that Pm was associated with increased SS risk (OR 5.396 per 1% increase, CI 95% 1.793-16.238, p=0.003). Pm and Fn were chosen for *in-vitro* and *in-vivo* experiments because Pm has a porin with high homology to AQP5 and Fn has high invasive ability among the several oral species with known invasive capabilities. HSG cells produced the high levels of proinflammatory cytokine IL-6 and C-X-C motif chemokine ligand (CXCL)-10 in response to Fn but not to Pm in a dose dependent manner. In addition, sublingual inoculation of

Fn and Pm into C57BL/6 mice produced sicca symptoms with mild recruitment of T cells to the salivary gland tissues in a few mice.

Conclusion

In conclusion, autoantibodies to human AQP5 and altered oral microbiota may contribute to the etiopathogenesis of SS. Anti-AQP5 autoantibodies detected in the sera from SS patients may be a novel biomarker of SS and may provide new insight into the pathogenesis of SS.

Keywords: Sjögren's syndrome, autoantibodies, AQP5, AQP1, Pyrosequencing analysis

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List of Abbreviation

AQPs	Aquaporins
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CFSE	5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
CXCL	C-X-C motif chemokine ligand
ER	Estrogen receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HC	Healthy control
HEK	Human embryonic kidney
HLA	Human leukocytes antigens
HSG	Human salivary gland
ICAM-1	IFN-γ induced intercellular adhesion molecule-1
IFN	Interferon
IIFA	Indirect immunofluorescence assay
IP-10	Interferon gamma-induced protein 10
MDCK	Madin-Darby canine kidney cells
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
MIP	Major intrinsic proteins
NOD	Non-obese diabetic
PBS	Phosphate buffer saline
SC	Sicca control
SS	Sjögren's syndrome
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1

1. Introduction

SS is an autoimmune disorder that primarily targets the salivary and lacrimal glands, leading to dryness of the mouth and eyes (Nikolov & Illei, 2009). In addition to glandular dysfunction, SS patients often present with extraglandular manifestation, such as tubulointerstitial nephritis (Maripuri et al., 2009), primary biliary cirrhosis, autoimmune cholangitis, autoimmune hepatitis (Kaplan & Ike, 2002), interstitial lung disease, development of lymphocytic malignancies (Bournia & Vlachoyiannopoulos, 2012; Parambil et al., 2006; Shen et al., 2012; Tzioufas et al., 2012), and serological abnormalities (Luciano et al., 2015). The disease is named after a Swedish ophthalmologist Heniky Sjögren who first described the clinical features in 1933.

SS is classified into two distinct forms:

- **Primary SS:** characterized by dryness of mouth and eyes without an association with other autoimmune diseases, with population prevalence of 0.1-0.5% and a female preponderance (female to male ratio is 9:1). The primary SS has two age peaks, the first after menarche during 20s to 30s and the second after menopause in the mid-50s.

- **Secondary SS:** characterized by dryness of mouth and eyes associated with other autoimmune disorders e.g. rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and progressive systemic sclerosis (PSS). Ocular symptoms are more prevalent than oral symptoms in secondary SS.

1.1. Revised international diagnosis criteria for SS

For diagnosis of SS the following criteria must be met; (Vitali et al., 2002).

I. Ocular symptoms (at least one)
   - Symptoms of dry eyes (at least 3 months)
   - Foreign body sensation
   - Use of artificial tear (3 or more time per day)

II. Oral symptoms (at least one)
   - Symptoms of dry mouth (at least 3 months)
   - Swollen salivary glands
   - Need of liquid to swallow food

III. Ocular signs (at least one)
   - Abnormal Schirmer's test
   - Positive vital dye staining of the eye surface

IV. Histopathology

- Lip biopsy with focal lymphocytic sialadenitis (focus score $\geq$ 1 per 4 mm^2)

V. Oral signs (at least one)

- Unstimulated whole salivary flow ($\leq$ 1.5 ml in 15min)
- Abnormal parotid sialography
- Abnormal salivary scintigraphy

VI. Autoantibodies (at least one)

- Anti-Ro or Anti-La or both

Revised rules for diagnosis as primary SS

- Any 4 of the 6 criteria, must include either histopathology or autoantibodies
- Any 3 of the 4 objective criteria (III, IV, V, VI)

Revised rules for diagnosis as secondary SS

Patients with well-defined major connective tissue disease, the presence of one symptom (I or II) plus 2 of the 3 objective criteria (III, IV and V) is indicative of secondary SS.

Exclusion criteria:

- Past head and neck radiation treatment
- Hepatitis C virus (HCV) infection

- Acquired immunodeficiency disease (AIDS)
- Pre-existing lymphoma
- Sarcoidosis
- Graft versus host disease
- Use of anticholinergic drugs

1.2. Pathogenesis of SS

SS is a multifactorial autoimmune disease; both environmental and hormonal factors trigger exocrinopathy in individuals with genetic predisposition. These factors are discussed as follows:

- Genetic factors
- Hormonal factors
- Environmental factors
- Role of epithelial cells

1.2.1. Genetic factors

Traditionally, genetic predisposition has been implicated in the development of SS. Family members of SS patients have a higher incidence of SS and other autoimmune diseases than do age and sex match control group (Kuo et al., 2015). Strong association with several human leukocytes antigens (HLA) molecules includes HLA-DR, HLA-DQB1, and HLA-DQA1 as well as several non-HLA regions such as interferon (IFN) regulatory factor 5 (IRF5)

and signal transducer and activator of transcription factor (STAT) 4 has been established (Lessard et al., 2013; Voulgarelis & Tzioufas, 2010). However, none of these associations exceeded the genome wide significance (GWS) threshold of $P=5*10^{-8}$ (Lessard et al., 2013; Luciano et al., 2015; Tzioufas et al., 2012). According to the current literature other genetic factors increasing the risk for developing SS includes; natural cytotoxicity triggering receptor 3 (NCR3), polymorphism in B-lymphocyte kinase (Blk), early B cell factor 1 (EBF1), TNFAIP3-interacting protein 1 (TNIP1), lymphotoxin gene A (LTA), chemokine ligand 11 (Nezos & Mavragani, 2015).

1.2.2. Hormonal factors

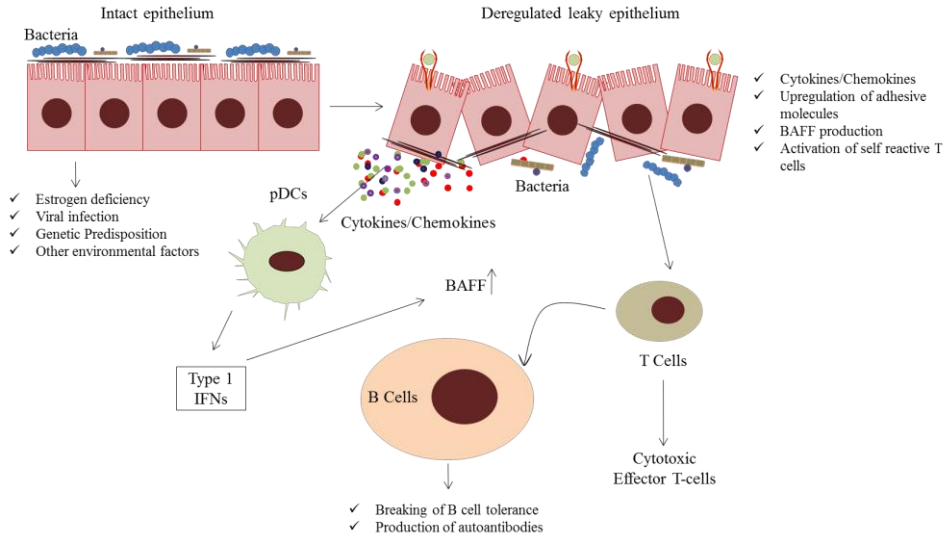
The strong female predominance especially in postmenopausal women and result from many animal models has indicated the role of estrogen deficiency in SS (Konttinen et al., 2012). Studies have shown the presence of estrogen receptor (ER) and ER mRNA in salivary gland tissues (Kassi et al., 2003). In murine experimental model, estrogen can ameliorate T cells recruitment and prevent cell death in salivary gland (Lee et al., 2012). Aromatase (estrogen synthetase) deficient mice develop lymphocytic exocrinopathy resembling SS (Shim et al., 2004). Salivary epithelial cells from estrogen deficient mice function as antigen presenting cells upregulates the expression of major histocompatibility complex (MHC) class II molecules, IFN-γ and interleukin (IL)-18, which suggest that estrogen deficiency stimulates salivary epithelial

cells to present autoantigen to the $CD4^+$ T cells results in the development of SS like lesions in salivary gland (Tzioufas et al., 2012; Voulgarelis & Tzioufas, 2010). On the other hand, estrogen prevents apoptosis of salivary gland epithelial cells as well as inhibits the IFN-γ induced intercellular adhesion molecule (ICAM)-1 expression as adhesive molecule indicative of cellular activation (Mavragani et al., 2012).

1.2.3. Environmental factors

Among the environmental factors, viral infections particularly cytomegalovirus (CMV), Epstein-Barr virus (EBV), HCV, retroviral elements, human herpes virus types 6 (HHV6), and human T lymphotropic virus type I (HTLV-I) has been suspected as triggering factor for SS in patients with genetic predisposition (Triantafyllopoulou & Moutsopoulos, 2007). Although the association between SS and virus infections is still unclear, high incidence rate of EBV reactivation in the salivary glands of SS patients suggests the role of EBV in the initiation or perpetuation of immune response towards the salivary gland (Fox et al., 1991; Toda et al., 1994). The immune responses in the salivary gland that are characterized by type-I IFN signature, including the overexpression of type I IFN inducible genes, such as IFN-stimulated transcription factor-3γ (ISGF3G) and IFN-induced transmembrane protein (IFITM)-1, further support the role of viral infections

in SS (Luciano et al., 2015; Tzioufas et al., 2012; Voulgarelis & Tzioufas, 2010).



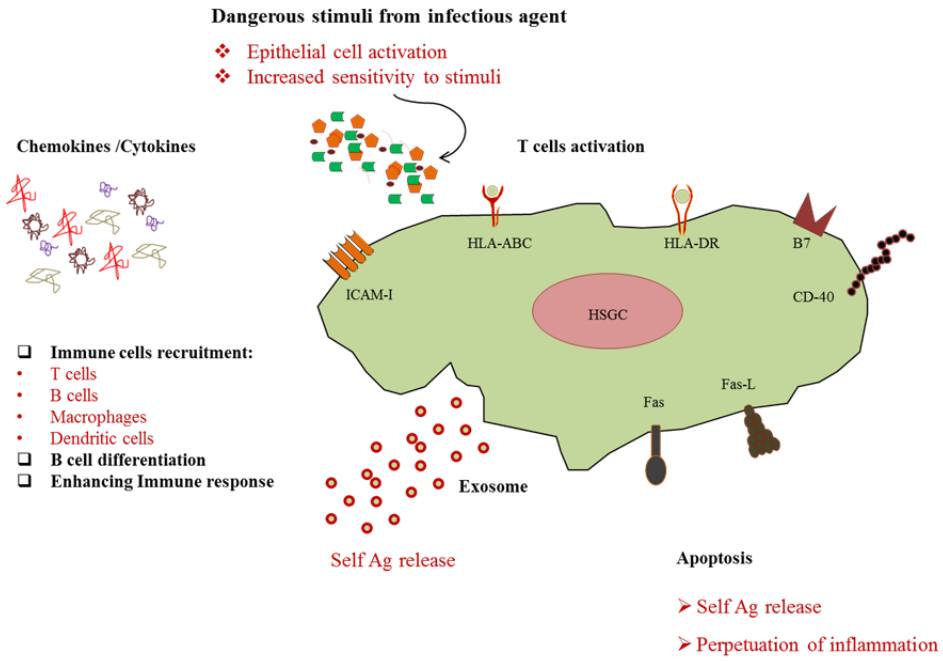
Cellular and molecular pathways implicated in the pathogenesis of SS

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

1.2.4. Role of epithelial cells

Although salivary gland cells are never shown to act as antigen presenting cells, but their enhanced expression of MHC class-I (HLA-ABC) and class-II (HLA-DR) molecules, ICAM-1, vascular cell adhesion molecule (VCAM) 1, E-selectin adhesion molecules, costimulatory molecules such as CD40, CD80 (B7-1) and CD86 (B7-2) (Tsunawaki et al., 2002), increased production of proinflammatory cytokines IL-1, IL-6 and tumor necrosis

factor (TNF) α, several T cell attracting and germinal center forming chemokines (Voulgarelis & Tzioufas, 2010) as well as B cells activating factor (BAFF) show their potential role in the accumulation of dendritic cells (DCs), T cells and B cells in the salivary gland of SS patient and the formation of lymphoid tissue (Manoussakis & Kapsogeorgou, 2007). Recently it has been shown that SS acinar epithelial cells express increased apoptosis regulator BAX and undergoes apoptosis, while infiltrating lymphocytes express Bcl-2 and are resistance to apoptosis which prolonged their survival. Through apoptosis and exosome formation, epithelial cells present intracellular autoantigen which results in loss of immune tolerance (Kapsogeorgou et al., 2005). Epithelial cells not only present the autoantigens but also concomitantly activate B cells by local secretin of BAFF (Luciano et al., 2015; Manoussakis & Kapsogeorgou, 2007; Mavragani & Moutsopoulos, 2014; Voulgarelis & Tzioufas, 2010).



Role of epithelial cells in autoimmune reaction in SS

(Re-drawn diagram adopted from; Clinic Rev Allerg Immunol 2007; 32:225–230)

1.3. Mechanisms for salivary gland secretory dysfunction

Glandular dysfunction had been understood as a result of apoptotic destruction of acinar cells by infiltrated cytotoxic T cells because the salivary and lacrimal glands of SS patients present abundant lymphocytic infiltration and atrophy (Nikolov & Illei, 2009). However, when B cell deficient SS-prone non-obese diabetic (NOD) mice were transferred with IgG from SS patients, the saliva production were altered even in the absence of infiltrated lymphocytes in the salivary gland indicates the role of autoantibodies in the functional impairment of secretory process in SS (Kaleb et al., 2012;

Robinson et al., 1998). Similarly, many SS patients with no glandular function retain substantial amounts of intact acinar tissues in their salivary glands. Furthermore, those acinar tissues regain functionality *in -vitro* (Dawson et al., 2001). Thus the etiology of SS is still unknown. But there are numerous underlying mechanisms suggested to contribute to this loss of secretory dysfunction, though no single mechanism has been identified as the primary cause. Lymphocytic infiltration, autoantibodies targeting muscarinic receptors, proinflammatory cytokines, nitric oxide, and apoptotic cell death of acinar cells all have been implicated as potential cause of secretory dysfunction of salivary gland (Kaleb et al., 2012).

1.3.1. Lymphocytic infiltration

Lymphocytic infiltration and epithelial cells activation mainly in salivary and lacrimal glands is the histopathological hall mark of SS (Chisholm & Mason, 1968). The infiltrated lymphocytes in the salivary and lacrimal glands consist mainly of $CD4^+$ T cells (initiation by Th1 and Th17 cells, progression by Th2 and Tfh cell) (Moriyama et al., 2014), B cells and lesser number of $CD8^+$ T cells (Hamza et al., 2012). The balance between T and B cells depends upon the disease progression. According to the widely accepted American and European criteria and the more recently proposed criteria by American college of rheumatology minor salivary gland pathology is considered compatible with SS diagnosis with an average focus score of >1

(focus score is the number of lymphocytic foci per $4mm^2$ surface in at least four informative lobules; a focus is define as a cluster of at least 50 lymphocytes) (Vitali et al., 2002). T cells are recruited first to the site of infiltration followed by B cells. $CD8^+$ T cells with increased expression of adhesion molecules and cell death surface receptor (Fas/FasL) molecules can directly kill the acinar cells in the salivary gland. Secretory dysfunction has long been considered as a result of glandular destruction mediated by lymphocytic infiltration in these tissues (Kaleb et al., 2012; Mavragani & Moutsopoulos, 2014; Voulgarelis & Tzioufas, 2010).

1.3.2. Role of cytokines in salivary gland dysfunction

Persistant inflammation can lead to chronic tissue damage and salivary gland dysfunction. Salivary glands from SS patients show increased expression of proinflammatory cytokines such as INFα and INFγ, TNFα, IL-6, IL-12, IL-18 and BAFF, in contrast anti-inflammatory cytokines, such as IL-4 and transforming growth factor (TGF) β1 are undetectable or expressed at low level except for IL-10 (Roescher et al., 2009). Baturone et al. reported the significantly increased level of IL-1β, IL-6, IL-10, TNFα and IFNγ in serum of primary SS patients, and reported the increased level of IL-6 with poor quality of life (Baturone et al., 2009). IL-18 and its inducer IL-12 which plays an important role in Th1 driven autoimmune response are involved in the infiltrative injuries and have a correlation with the lymphoma

development in the patient with primary SS (Bombardieri et al., 2004). IFNγ, a major cytokines released from Th1 cells, regulates cell mediated immune responses through activation of natural killer cells, macrophages and $CD8^+$ T cells (Cha et al., 2004). IFNγ or receptor knockout mouse models confirm the role of IFN in the loss of secretory function in SS. Ogawa et al. reported that IFN-γ stimulates the production of interferon gamma-induced protein 10 (IP-10) and monokine induced by gamma interferon (MIG) proteins from the SS ductal epithelium, and that IP-10 and MIG are involved in the recruitment of T cells into the salivary glands of SS patients (Ogawa et al. 2004; Ogawa et al. 2002).

Cytokines released from Th2 cells can play an important role in SS: IL4 knockout NOD mice suggested that IL-4 affects saliva secretion via antibody production (anti-M3R autoantibody) and isotype switching (IgG1), IL-13 facilitates secretory dysfunction possibly through affecting mast cells in the salivary glands (Gao et al., 2006; Jin & Yu, 2013; Reksten et al., 2009). Not only Th1 and Th2 effectors cells but also Th17 cells which release the proinflammatory cytokines IL-17 have been studied for their role in SS pathogenesis (Reksten et al., 2009). Nguyen et al. reported that IL-17 is critical in inducing SS-phenotype in C57BL/6J mice (Nguyen et al., 2010), and reduction of IL-17A level by Ad5-IL17R:Fc blocking vector suppress features of SS in C57BL/6.NOD-*Aec1Aec2* mice (Nguyen et al., 2011).

Recently, Zhang et al. reportd that IL-17 derived from infiltrating lymphocytes impairs the integrity of tight junction through nuclear factor-kB (NF-kB) signaling pathway, and thus contribute to salivary gland dysfunction (Zhang et al., 2016).

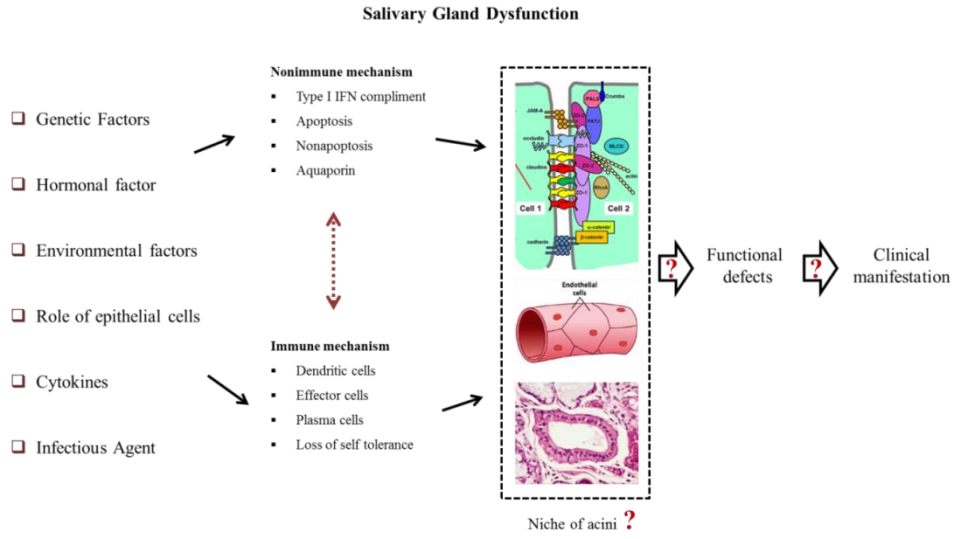
1.3.3. Role of B cell in salivary gland dysfunction

Not only pathogenic T cells and cytokines but also loss of B cell tolerance is critical in development of autoimmune diseases including SS. BAFF can be detected in high levels in patients with autoimmune diseases as compared to HC (Varin et al., 2010). BAFF overexpression causes self-reactive B cell activation and B cell hyperactivity. BAFF in combination with IL-17 influences the survival, proliferation, and differentiation of B cells in SLE (Cheema et al., 2001; Doreau et al., 2009).

1.3.4. Role of autoantibodies in salivary gland dysfunction

Traditionally salivary dysfuction was considered as the result of acinar tissue destruction after lymphocytic infiltration. However, more recently questions arised concerning SS patients who have substancial amounts of intact acinar tissues in their salivary glands but still suffer from xerostomia. These observation suggests the salivary gland functional disruption rather than acinar tissue destruction. In addition to salivary gland lymphocytic infiltration, SS patients exhibit hypergammaglobulinemia with a range of autoantibodies. The presence of anti-Ro/SSA and/or anti-La/SSB

autoantibodies is the hall mark of the disease diagnosis detected in 60-70% of the patients (Hernandez-Molina et al., 2011). However, the levels of anti-Ro/SSA and anti-La/SSB autoantibodies are not associated with the disease activity. A number of other autoantibodies, such as anti-salivary gland protein-1, anti-carbonic anhydrase 6, anti-parotid secretory protein, anti-muscarinic acetylcholine receptor 3 (M3R), anti-tissue kallikrein, and anti-α-fodrin antibodies have been identified in SS (Bournia & Vlachoyiannopoulos, 2012; Lee et al., 2013; Shen et al., 2012). Robinson et al. demostrated that transferring SS patients IgG to NOD.Igμ null mice results in salivary gland dysfuction (Robinson et al., 1998). NOD.Igμ null mice lack functional B lymphocytes and therefore lack the IgG autoantibodies. NOD.Igμ null mice do exhibit lymphocytic infiltration of the salivary and lacrimal galnds but fail to show secretory dysfuction. However transfering IgG from SS patients shows a 54% reduction in saliva production while transfer from HC has no effect (Robinson et al., 1998). Further the transfer of a monoclonal antibody against mouse M3R into NOD-scid mice presented a significant reduction in saliva secretion, while infusion of antibodies to Ro and La or parotid secretory protein had no effect on salivary gland secretory dysfunction. Anti-M3R auto-antibodies have also been shown to affect the autonomic nervous system (Cai et al., 2008; Kaleb et al., 2012; Waterman et al., 2000).



1.4. Morphology of salivary gland and production of saliva

Mammals possess three major (parotid, submandibular and sublingual) and numerous minor salivary glands scattered throughout the oral mucosa (Proctor, 2016). Salivary gland consists of multiple lobes; each lobule consists of a number of secretory units composed of acinar cells either serous or mucous, and ductal cells of several types as well as myoepithelial cells (Proctor, 2016). The acini form a blind end with a lumen surrounded by many secretory cells. The saliva produced in each acinus passes through intercalated, intra-lobular and excretory ducts before finally enters into main excretory duct. Saliva is a watery fluid containing electrolytes and a mixture of proteins. The Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$ cotransporter, located at the basolateral membrane, use the Na^+ gradient to elevate the intracellular Cl^- concentration. The accumulation of ions in the lumen generates a

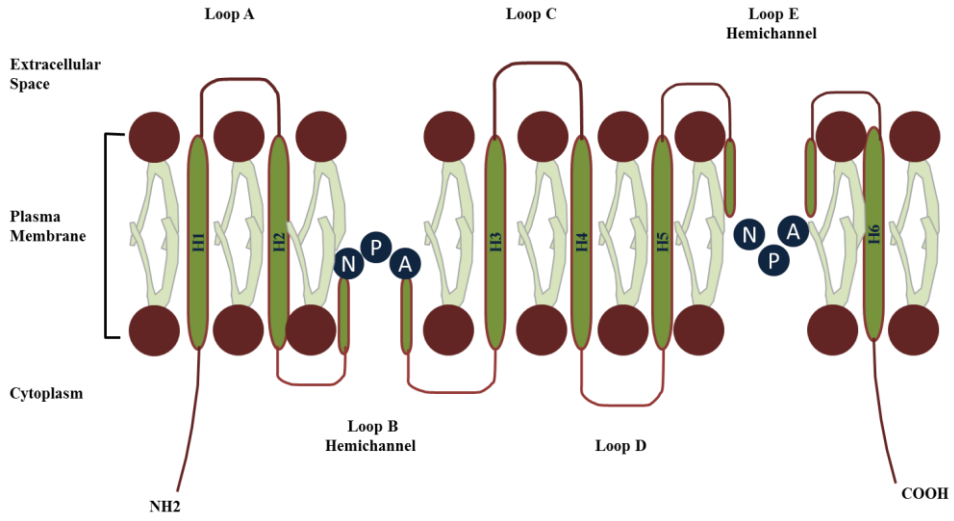
transepithelial osmotic gradient driving water movement through the apical AQP5 channels and paracellular pathways. This leads to the secretion of isotonic plasma like primary fluid (Catalan et al., 2009; Delporte, 2009; Proctor & Carpenter, 2014). The final saliva composition depends on both the origin and the type of the salivary gland stimulated. Typically, salivary glands produce around 1 liter of saliva per day. Saliva has many functions, including protection and hydration of mucosal surface, initiation of digestion, protection from chemical and mechanical stress, and providing antimicrobial defense by production of antimicrobial substances (Delporte, 2013; Delporte & Steinfeld, 2006). Salivary dysfunction is clinically characterized by dysphagia, oral pain, dental caries, and infection from opportunistic microorganisms (Delporte et al., 2016).

1.5. AQPs

The AQPs are small hydrophobic approximately 270 amino acids integral membrane proteins which are widely expressed in animals and plants as well as in bacteria and archaea. AQPs exist as monomers of 28-30 kDa that can associate in tetramers (Delporte, 2009). AQPs structural analyses showed that each monomer consists of six transmembrane helices making three extracellular and two intracellular loops. The signature amino acid sequence motifs of the AQPs are two repeating Asn–Pro–Ala (NPA) sequences present in the first intracellular and the third extracellular loop (Delporte, 2009). To

date, at least 13 members of this channel family have been identified in mammals. Based on their permeability characteristics, the members of the AQPs family can be divided into two subgroups: AQP and aquaglyceroporin (Delporte, 2009).

The AQP (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8) are primarily water channel molecules, whereas the aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) are used for the transportation of glycerol and other small solutes. The transport function of many AQP can be inhibited by mercurial–sulfhydral-reactive compounds such as $HgCl_2$ (Delporte, 2009; Delporte & Steinfeld, 2006).



AQPs contain six transmembrane helices three extracellular and two intracellular loops. The loops B and E, folding in the lipid bilayer, form a single aqueous pore.

1.5.1. AQPs expression and role in saliva production

The expression of mRNA level and detection of proteins of AQP1, AQP5 and AQP3 has been confirmed by many researchers in human salivary glands including parotid, submandibular and sublingual, while the data for the presence of AQP4, AQP6 and AQP7 are still controversial and do not appear to play an important role in the production of saliva (Delporte, 2009). AQP5 seems to be the only AQP which plays a very clear functional role in the production of saliva. AQP5 is expressed at the apical membrane of acinar cells and intercalated ducts in the lacrimal and salivary glands (Takata et al., 2004). AQP5-deficient mice secrete hypertonic saliva with substantially reduced volume (Ma et al., 1999), which is attributed to a decrease in the water permeability of salivary acinar cells (Krane et al., 2001). AQP1 also expressed in the salivary and lacrimal glands, which is confined to the capillary endothelial and myoepithelial cells. Transgenic mice lacking AQP1 shows that AQP1 does not play a major role in saliva secretion (Delporte & Steinfeld, 2006). However, B cell depletion with rituximab increases both AQP1 expression in myoepithelial cells and saliva flow rate in patients with SS (Ring et al., 2006). Many researchers also investigated AQP1 and AQP5 abnormal expression/distribution in salivary gland, and suggested its role in hyposalivation before inflammation (Delporte & Steinfeld, 2006; Hayashi, 2011).

1.5.2 Potential role of AQPs in the pathogenesis of SS

Major intrinsic proteins (MIP) are large superfamily of transmembrane proteins channels that are grouped together on the basis two highly conserved regions called NPA motif and several surrounding amino acids (Benga, 2012). The MIP superfamily includes three sub-families; AQPs, aquaglyceroporins and S-aquaporins (Benga, 2012). AQPs and aquaglyceroporins are fundamentally evolutionary conserved and are widely expressed in animals, plants as well as in bacteria and archaea (Kruse et al., 2006). It has been shown that mouse antibodies raised against *Escherichia coli* (*E. coli*) AQPZ react with human AQP4 (Ren et al., 2012). BLAST (Basic Local Alignment Search Tool)-search of bacterial protein database using human AQP5 and AQP1 amino acid sequence as a query confirmed that AQP-Z, porin or aquaglyceroporins from many human oral bacteria showed a high degree of homology with human AQP5 and AQP1. B cell epitope analysis showed the presence of conserved linear epitope in the extracellular loop E which supports the notion that the loop E may be the target of cross-reacting autoantibodies produced against bacterial proteins. In this context, it was hypothesized that immune response to oral bacteria may produce cross-reacting autoantibodies against AQP5 and AQP1 via molecular mimicry and that anti-AQP1 and anti-AQP5 autoantibodies may interfere in saliva secretion.

1.6. Key research questions

This study will address the following key research questions:

- Are there autoantibodies to AQP5 and AQP1 in the sera from SS patients?
- Are there association between the presence/level of anti-AQP5/AQP1 autoantibodies and the clinical parameters of SS patients?
- Is SS associated with dysbiosis of oral microbiota?
- Can oral bacteria invade into HSG cells and induce inflammation?
- Can selected bacteria produce SS like symptoms in mice?

2. Material and methods

2.1. Human serum and saliva samples collection

This study was done in compliance with the Helsinki declaration after approvals from the institutional review board of Seoul national university hospital (IRB number: 0912-011-302), the institutional review board of Seoul national university school of dentistry (IRB number: S-D20140022), and the institutional review board of Seoul St. Mary's hospital (IRB number: KC13ONMI0646). For this study, serum samples were obtained from two groups of patients: 1) 10 primary SS patients enrolled at the rheumatology clinic, Seoul national university hospital who were diagnosed according to the 2002 American-European consensus group (AECG) classification criteria for primary SS (Vitali et al., 2002) and 2) 102 primary SS patients enrolled at the Korean initiative of primary SS (KISS) who fulfilled the 2002 American-European consensus group (AECG) classification criteria and/or the 2012 American college of rheumatology (ACR) criteria (Shiboski et al., 2012). All samples were obtained before starting treatment. In addition, resting and stimulated whole salivary flow rates were measured by spiting and masticatory stimuli using wax gum (GC America Inc, St. Alsip, IL, USA), respectively, according to the method previously described (Navazesh & Christensen, 1982). All patients were females with ages ranging from 21 to 80 years (mean age 52.5± 10.7 years). Control sera were obtained from 53

healthy female controls who did not show any signs of SS symptoms (mean age 37.1± 7.4 years). Subjects with systemic disease other than hypertension were excluded. Written informed consent was obtained from all the subjects.

2.2. Total bacterial genomic DNA extraction

Bacterial genomic DNA from oral bacteria was extracted using GeneJET genomic DNA purification kit (Thermo Scientific, Inc. Lithuania). Whole mouth wash pellet were first agitated with garnet beads 0.15 mm (MO BIO Laboratories, Carlsbad, CA, USA) for 10 min followed by incubation with gram-positive bacteria lysis buffer (20 mM Tris-Hcl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme before use) for 30 min. The rest of the steps for genomic DNA extraction were according to the manufacturer's instruction.

2.3. PCR amplification and pyrosequencing

PCR amplification was performed using primers targeting from V1 to V3 regions of the 16S rRNA gene with extracted DNA. For bacterial amplification, barcoded primers of 9F (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTCAG-3'; underlining sequence indicates the target region primer) and 541R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-ATTACCGCGGCTGCTGG-3'; 'X' indicates the unique barcode for each

subject) (http://oklbb.ezbiocloud.net/content/1001). The amplifications was carried out under the following conditions: initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 sec, primer annealing at 55 ℃ for 30 sec, and extension at 72 ℃ for 30 sec, with a final elongation at 72 ℃ for 5 min. The PCR product was confirmed by using 2% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Equal concentrations of purified products were pooled together and removed short fragments (non-target products) with Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were conducted emlusion PCR, and then deposited on Pico titer plates. The sequencing was carried out at Chunlab (Chunlab Inc., Seoul, Korea), with GS Junior sequencing system (Roche, Branford, CT, USA) according to the manufacturer's instructions.

2.4. Pyrosequencing data analysis

The basic analysis was conducted according to the previous descriptions in other studies (Chun et al., 2010; Hur et al., 2011; Kim et al., 2012). Obtained reads from the different samples were sorted by unique barcodes of each PCR product. The sequences of the barcode, linker, and primers were

removed from the original sequencing reads. Any reads containing two or more ambiguous nucleotides, low quality score (average score < 25), or reads shorter than 300 bp, were discarded. Potential chimera sequences were detected by the bellerophone method, which is comparing the BLASTN search results between forward half and reverse half sequences (Huber et al., 2004). After removing chimera sequences, the taxonomic classification of each read was assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012), which contains 16S rRNA gene sequence of type strains that have valid published names and representative species level phylotypes of either cultured or uncultured entries in the GenBank database with complete hierarchical taxonomic classification from the phylum to the species. The richness and diversity of samples were determined by Chao1 estimation and Shannon diversity index at the 3% distance. Random subsampling was conducted to equalize read size of samples for comparing different read sizes among samples. The overall phylogenetic distance between communities was estimated using the Fast UniFrac (Hamady et al., 2010) and visualized using principal coordinate analysis (PCoA). To compare OTUs between samples, shared OTUs were obtained with the XOR analysis of CLcommunity program (Chunlab Inc., Seoul, Korea).

2.5. In silico analysis

The human AQP5 and AQP1 amino acid sequence was BLAST-searched as query against bacterial proteins database. The sequences of the selected oral bacterial AQPs or porins were aligned with human AQP5 and AQP1 using *BioEdit* sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The human AQP5/AQP1 amino acid sequences were searched for linear B cell epitope using immune epitope database (http://tools.immuneepitope.org/bcell/).

2.6. Cloning of human AQP5 and AQP1 and transfection of cells

The human AQP5 cDNA was cloned into a pEGFP-N1 vector (Clontech, Mountain View, CA, USA) using XhoI and BamHI sites while both human AQP5 and AQP1 were cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) using BamHI and XhoI restriction sites. Cells were transfected with calcium phosphate precipitation method (Kingston et al., 2003). The following list of primers was used for amplification of DNA as shown in table 1.

Tab. 1 List of primers used in AQP1 and AQP5 cloning

Primer	Orientation	Sequence (5'→3')
pcDNA3.1-AQP1-BamHI	Forward	GGATCCATGGCCAGCGAGTTCAAGAAGAAGC
pCDNA3.1-AQP1-XhoI-R	Reverse	CTCGAGCTATTTGGGCTTCATCTCCACCC

PcDNA3.1-AQP5-BamHI-F1	Forward	GGATCCATGAAGAAGGAGGTGTGCTCC
PcDNA3.1-AQP5-Xho-1-R1	Reverse	CTCGAGTCAGCGGGTGGTCAGC
Human AQP5 XhoI-F1	Forward	CTCGAGATGAAGAAGGAGGTGTGCTCCG
Human AQP-5 Bam HI-R1	Reverse	GGATCCCGGCGGGTGGTCAGCTCCA

2.7. Cell culture

All cell lines were obtained from Korean cell line bank (KCBL, Seoul, Korea). CHO cells were cultured in F-12 medium containing 10% FBS and 100 unit/ml penicillin and streptomycin, while HEK-293, MDCK and HSG were maintained in DMEM medium with 10% FBS and 100 unit/ml each penicillin and streptomycin. Transfected cells were maintained in the presence of G-418 (0.1-o.2 mg/ml).

2.8. IIFA

For IIFA, all tissues and cells were fixed with 4% paraformaldehyde in PBS pH 7.4, subjected to antigen retrieval by incubation in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6) at 105 ℃ for 20 min, before blocking and incubation with primary antibodies, which gave the best result by staining with the commercial anti-AQP5 antibodies.

The use of animals was approved by the Seoul national university animal care and use committee. After stimulating 12 week old C57BL/6 mice (Orient Bio Inc., Seongnam, Gyeonggi, Korea) with 5 μg/g body weight of pilocarpine (Sigma-Aldrich Korea, Seoul, Korea), the submandibular glands were removed and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) pH 7.4 overnight. The fixed tissues were immersed in 30% sucrose in PBS pH 7.4 and embedded in the optimal cutting temperature (OCT) compound. Cryostat sections were mounted on saline coated glass slides (Muto pure chemicals, Tokyo, Japan) and air dried. After antigen retrieval and permeabilization, the sections were blocked with 5% bovine serum albumin (BSA) in PBS and were then incubated with goat anti-AQP5 antibodies (Santa Cruz, Paso Robles, CA, USA) and either the pooled SS or pooled control sera (1:200 dilution) overnight, followed by Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA) and CF^{TM} 594-conjugated rabbit anti-human IgG antibodies (Sigma Aldrich St Louis, MO, USA).

CHO cells cultured on collagen coated glass slides were transfected with either pEGFP-N1-AQP5 or pEGFP-N1. MDCK cells were transfected with pcDNA3.1-AQP5 and subjected to selection with G418 (A. G. Scientific, San Diego, CA, USA). The transfected CHO cells were incubated with rabbit anti-GFP antibodies (Santa Cruz, Paso Robles, CA, USA) and either the

pooled control or the pooled SS sera (1:200 dilution), followed by Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 555-conjugated goat anti-human IgG (Invitrogen, Carlsbad, CA, USA). The transfected MDCK cells were incubated with goat anti-AQP5 and various dilutions of either the control or SS sera, followed by Alexa Fluor 488-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA) and CF^{TM} 594-conjugated rabbit anti human IgG (Sigma Aldrich St Louis, MO, USA). To detect human IgA, Alexa flour 594-conjugated rabbit anti-human IgA antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used. All images were taken with a confocal microscope LSM 700 (Carl Zeiss, Jena, Germany). In the case of the stained MDCK-AQP5 cells, three areas of AQP5-expressing cells were randomly selected and sequentially imaged for the presence of anti-AQP5 IgG/IgA. Because many SS samples had stained nuclei as well as AQP5, the relative intensities of the anti-AQP5 signals were determined by decreasing the brightness of the red signal until the signals of the anti-AQP5 IgG/IgA disappeared. The mean of the Δ brightness obtained in three images was used to express the level of anti-AQP5 IgG/IgA for each sample. The same method was followed for anti-AQP1 antibodies detection and analysis.

2.9. Immunoprecipitation and western blot

HEK-293 cells were transfected with pAQP5-EGFP or pEGFP-N1. Forty eight hours after transfection, cells were lysed with lysis buffer (40 mM

octyl-β-D-1-thioglucopyranoside, 50 mM Tris-HCl, and 150 mM NaCl, pH 7.4). Proteins (250 μg of cell lysates) were incubated with 1 μg anti-AQP5 antibodies, pooled control sera, or pooled SS sera overnight, followed by precipitation with protein A agarose beads (Pierce Biotechnology, Rockford, IL, USA). After washing, the beads were resuspended in Laemmli sample buffer containing β-mercaptoethanol and incubated at 70 °C for 15 min. The proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and immunoblotted with goat anti-AQP5 or rabbit anti-GFP antibodies.

2.10. Bacteria culture and staining

Selected bacteria used in this study were from ATCC (American type culture collection) and KCOM (Korean collection for oral microbiology). Fn ATCC 10953, *Rothia mucilaginosa* (Rm) KCTC19862, *S. salivarius* (Ss) KCTC 5512, *Eikenella corrodens* (Ec) were grown in brain heart infusion (BHI) broth supplemented with hemin 5 μg/ml of hemin (Sigma, St Louis, MO, USA) plus 10 μg/ml of vitamin K under an anaerobic condition (5% H_2, 10% CO_2 and 85% N_2), Ec were supplemented with 16% FBS. Pm KCTC 5323 and *P. histicola* (Ph) KCTC 15171 were grown in KCTC-5457 medium and peptone yeast glucose broth respectively, under anaerobic condition as mentioned above. Bacteria were always harvested in log phase and washed

twice with PBS before doing any experiment. For the fluorescence studies, bacteria were stained with 5 μM of 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probe, Carlsbad, CA, USA).

2.11. Confocal microscopy of internalized bacteria

HSG cells ($2.5x10^4$) were seeded on a cover glass in a 24 well plate one day before infection. Overnight grown cells were infected with CFSE labelled Fn, Pm, Ph, Ss and Ec at MOI 1000 for 6 and 24 hours. Cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Excess aldehyde were quenched with 0.1 M glycine in PBS for 5 min. Cells were then permeabilized with 0.3% Triton-X100 in PBS for 1 min, followed by incubation with Rhodamine-phalloidin (dilution 1:150) ((Invitrogen, Carlsbad, CA, USA) and Hoechst 33342 (1 μg/ml) for 15 min in PBS. In the final step cells were washed three times with distal water and mounted with mounting solution.

2.12. Flow cytometric invasion assay

Flow cytometric invasion assay was performed as previously described (Ji et al., 2010). Briefly, HSG cells were seeded ($6 x10^4$) in 24-well plate in triplicate. For invasion assay, HSG cells were infected with CFSE-labeled bacteria at MOI 1000 for 6 and 24 hours at 37 °C. The cells were detached, washed and then analyzed by flow cytometry after quenching the fluorescence of the bacteria bound on the surface with 300 μl of 0.4% trypan

blue. Non-infected live cells and cells fixed with 4% formaldehyde and exposed to the same amount of CFSE-labeled bacteria served as negative controls. The viability of the HSG cells were determined based on their forward scatter and the FL-3 fluorescence of the trypan blue staining, bacterial invasion was analyzed only for the live cells.

2.13. Cytotoxicity test

The cytotoxic effect of oral bacterial on HSG cells were determined by using a cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoo, Japan). HSG cells (18000 cells/well) were infected with oral bacteria Fn, Pm and Ss with multiplicity of infection (MOI) 50 and 100 for 6 and 24 hours. The cytotoxicity of HSG cells were measured by incubation with 10 µl of CCK-8 reagent per well for 90 min at 37 °C, optical density (OD) value was measured at 450 nm. The viability is based on the principle of conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier. HSG cells viability were expressed as a percentage of control cells.

2.14. Real time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from HSG cells infected with Fn, Pm and Ss at MOI 50 and 100 for 6 and 24 hours or from mouse salivary glands infected with Fn and Pm using easy-BLUE^{TM} total RNA extraction kit (iNtRON Biotechnology, Inc. Korea). Complementary DNA (cDNA) was synthesized from 1µg of total RNA using oligo-dt and M-MLV reverse transcriptase enzymes (Promega corporation, Medison, USA) in a 25 µl reaction mix incubated at 40 ℃ for 10 min and 55 ℃ for 50 min. RT-PCR was performed using SYBR premix *Ex Taq*, ROX passive dye (Takara Bio, Otsu, Japan) in a 20 µl reaction mixture containing 1 µl template cDNA. Amplification was performed in a fluorescence thermocycler (Applied biosystem 7500/7300 real time PCR, Foster City, CA) under the following condition initial denaturation 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 15 sec, annealing 60 ℃ for 15 sec, elongation 72 ℃ for 40 sec. The specificity of the PCR product was verified by melting curve analysis and running on a gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or mouse beta-actin gene was amplified in parallel with the gene of interest. Relative copy number compared to GAPDH was calculated using $2^{-\Delta CT}$. Each experiment was performed in triplicate. List of primers used in real time PCR are shown in table 2.

Tab. 2 List of primers used in real time PCR

Primer	Gene ID	Size (bp)	Forward Primer	Reverse Primer
h-GAPDH	AF261085.1	206	GGAGTCAACGGATTTGGT	GTGATGGGATTTCCATTGAT
h-IL6	NM_000600.3	92	CCACCGGGAACGAAAGAGAA	GAGAAGGCAACTGGACCGAA
h-IFN–α	NM_024013.2	132	CAAAGACTCTCACCCCTGCT	CACAGTGTAAAGGTGCACATGA
h-IFN-β	NM_002176.2	116	TCTCCTGTTGTGCTTCTCCAC	GCCTCCCATTCAATTGCCAC
h-CXCL-10	NM_001565.3	152	CCACGTGTTGAGATCATTGCT	TGCATCGATTTTGCTCCCCT
h-CXCL-13	AJ002211.1	158	GAGGCAGATGGAACTTGAGC	CTGGGGATCTTCGAATGCTA
h-BAFF	NM_006573.4	148	GACCTACGCCATGGGACATC	CAGTTTTGCAATGCCAGCTGAA
m-β-actin	NM_007393.3	218	CTGTCGAGTCGCGTCCAC	TTCCCACCATCACACCCTGG
m-IL6	NM_031168.2	128	TGG TACTCCAGAAGACCAGAGG	AACGATGATGCACTTGCAGA
m-CXCL-10	NM_021274.2	189	GTGCTGCCGTCATTTTCTGC	TCATCGTGGCAATGATCTCAAC
m-CXCL-13	NM_018866.2	195	CTCTCCAGGCCACGGTATTC	TTGGCACGAGGATTCACACA
m-BAFF	NM_033622.1	162	CTGATTGCAGACAGCGACAC	CGTGTATAGAACCTGGCTGTAG
http://www.ncbi.nlm.nih.gov/tools/primer-blast/, https://mouseprimerdepot.nci.nih.gov/				

2.15. Cytokines ELISA

The RT-PCR data was further confirmed by measuring the amount of cytokines and chemokines released into the medium by HSG cells in response of Fn, Pm and Ss infection. The level of human IL-6 and IP-10 in the culture supernatant of HSG cells were measured using DuoSet® ELISA development systems (R & D Systems, Inc. Minneapolis, MN)) according to manufacturer's instruction.

2.16 Peptide synthesis

For epitope mapping synthetic peptides were synthesized in the form of linear or cyclic form corresponding to the three extracellular loops of AQP5 (Peptron Inc. Daejeon, Korea). Amino acid sequences of peptides were selected after B cell epitope analysis. Peptides cover the three extra cellular loops of AQP5 including hemi-channel loop E, which make a pore for water transportation. Irrelevant peptides purchased from Sigma (Sigma-Aldrich Korea, Seoul, Korea) were also included in our study as listed in Table 3.

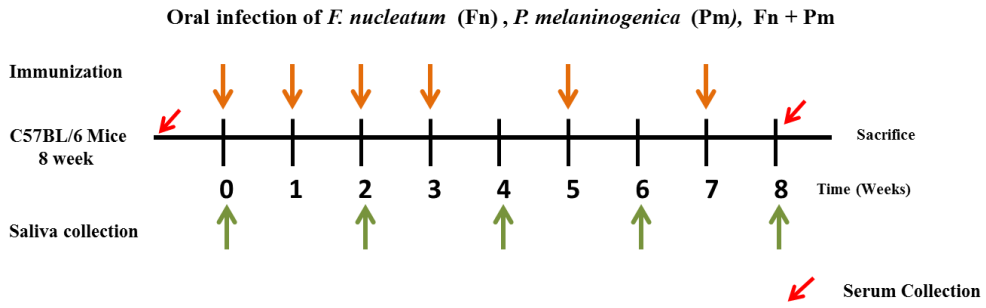
Tab. 3 List of peptides used in epitope mapping

AQP5 Loop A	WPSALPT (Linear)
AQP5 Loop C	CNNNTTQGC (C-C)
AQP5 Loop C2	NNNTTQG (Linear)
AQP5 Loop E1	GCSMNPARSFGC (C-C)
AQP5 Loop E2	CMNPARSFGC (C-C)
IR-Peptides (SCP0156) Sigma	GRADSP

2.17. Animal model

The experimental protocol and animal handling procedures were conducted in accordance with the recommendations in the guide from the institute of laboratory animal resources Seoul national university under the laboratory animals act 9025 of the republic of Korea. Eight-week-old female C57BL/6

mice were purchased from Orient-Bio (Orient-Bio, Seongnam, Korea) and housed under specific pathogen-free conditions in the laboratory animal facility at the school of dentistry, Seoul national university. The mice (n=20) were given randomly divided into four groups: Sham, Fn-group, Pm-group and Fn-Pm-group. The Fn and Pm group were inoculated $2x10^9$ each bacteria per mice while the Fn-Pm group received 10^9 each bacteria in co-infection.



2.18. Histology

Freshly isolated mice salivary gland tissues were fixed with immunohistochemistry (IHC) zinc fixative solution for 48 hours and imbedded in paraffin. Paraffin embedded salivary gland tissues were serial sectioned 5μm each and stained by hematoxylin and eosin (H & E) for histological examination. Cold spring harbor online protocols were used for the whole procedure (http://cshprotocols.cshlp.org).

2.19. Statistical analysis

A receiver operating characteristic (ROC) analysis by a nonparametric method and the Mann-Whitney U test were used to compare the levels of anti-AQP5 autoantibodies between the control and SS groups. Associations between measures of salivary rate and the presence of anti-AQP5 autoantibodies were examined using one-way analysis of variance. Because some groups did not pass the normality test, the difference was also analyzed by Mann-Whitney U test. All statistics were performed using SPSS (SPSS Inc., Chicago, USA). The difference between control and experimental group, two tailed non-paired student's t-test was performed. Logistic regression analysis was used to determine the oral bacteria associated with SS risk.

3. Results

3.1. Homology between human and oral bacterial AQPs suggest immunological cross-reaction

Amino acid sequence of human AQP5 and AQP1 were aligned with AQPs/ porins from eight most abundant species of oral bacteria. AQP5 showed 33-39% identity and 51-57% similarity while AQP1 showed a 35-42% identity and 52-56% similarity with AQPs/porins from selected oral bacteria (Fig. 1 and 2). The homologous sequences were conserved mostly at the transmembrane alpha helixes and the water channel pore forming loops B and E. The extracellular loop E and intracellular loop B insert into the membrane from the opposite site to make a pore for the water transport. Moreover, bepipred linear epitope prediction analysis confirmed the presence of conserved B cell epitope on the extracellular loops including loop E (Tab. 4) which supported the notion that human AQP5 and AQP1 could be the target of cross reacting autoantibodies produced against oral bacteria. In this context, we focused on the role of AQP5 and AQP1 as the culprit autoantigens in the development of SS.

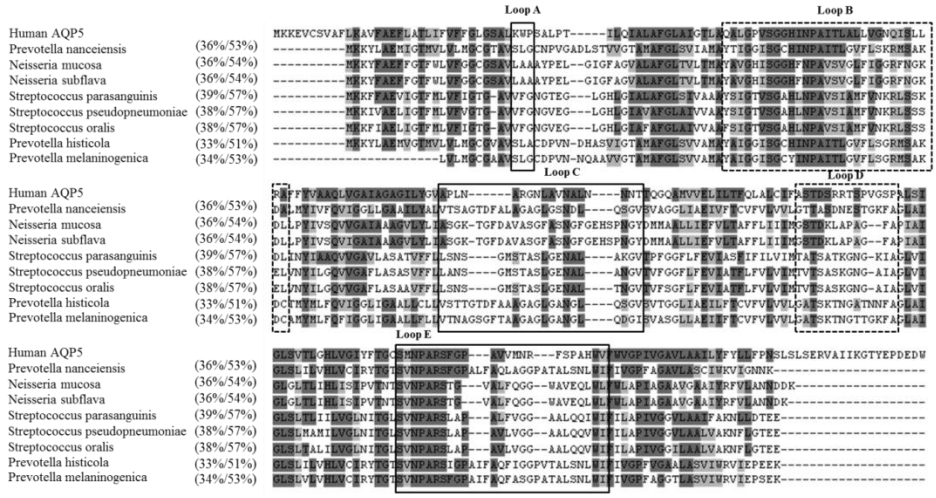


Fig. **1 Multi sequence alignment of human and oral bacterial AQPs/ porins**. The numbers in parentheses indicate percentages of identical and conserved amino acids. Identical and conserved amino acids are highlighted with dark gray and light gray, respectively. The extracellular and cytosolic domains are boxed with solid and dotted lines, respectively**.**

Oral bacterial proteins used in multi sequence alignment

Bacteria	Protein	Accession number
[Prevotella nanceiensis]	aquaporin	|WP_018363007.1|
[Neisseria mucosa]	porin	|WP_036491391.1|
[Neisseria subflava]	porin	|WP_039862497.1|
[Streptococcus parasanguinis]	porin	|WP_031574039.1|
[Streptococcus pseudopneumoniae]	aquaporin	|WP_023937259.1|
[Streptococcus oralis]	aquaporin	|WP_000713413.1|
[Prevotella histicola]	aquaporin	|WP_008822014.1|
[Prevotella melaninogenica]	porin	|WP_036863760.1|

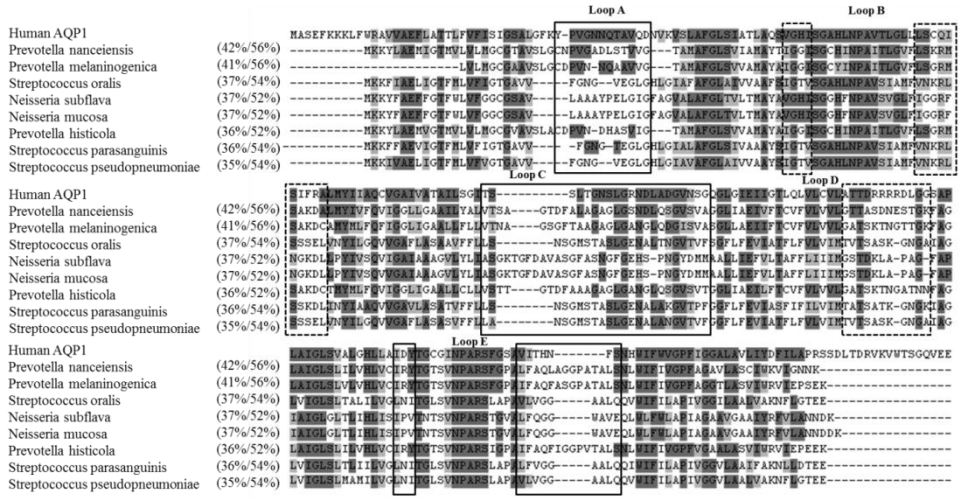


Fig. 2 Multi sequence alignment of human and oral bacterial AQPs/porins. The numbers in parentheses indicate percentages of identical and conserved amino acids. Identical and conserved amino acids are highlighted with dark gray and light gray, respectively. The extracellular and cytosolic domains are boxed with solid and dotted lines, respectively.

Oral bacterial proteins used in multi sequence alignment

Bacteria	Protein	Accession number
[Prevotella nanceiensis]	aquaporin	|WP_018363007.1|
[Prevotella melaninogenica]	porin	|WP_036863760.1|
[Streptococcus oralis]	aquaporin	|WP_000713413.1|
[Neisseria subflava]	porin	|WP_039862497.1|
[Neisseria mucosa]	porin	|WP_036491391.1|
[Prevotella histicola]	aquaporin	|WP_008822014.1|
[Streptococcus parasanguinis]	porin	|WP_031574039.1|
[Streptococcus pseudopneumoniae]	aquaporin	|WP_023937259.1|

Tab. 4 Bepipred Linear Epitope Prediction

AQP5	AQP1
LGPVSGGHIN	PVGNNQTAVQ
NNNTTQG	SLTGNSLGRNDLADGVNSGQG
DSRRTSPVGSPA	RRRRDLGGS
MNPARSFG	CGINPARSF
TYEPDEDWEEQREERKK	SSDLT
	SGQVEEYDLDADDINSR

Underline: B cell epitopes of the extracellular loops of both AQP5 and AQP1

3.2. Serum IgG from SS patients colocalize with AQP5 in mouse salivary glands

To investigate the presence of autoantibodies against AQP5 in the sera of SS patients, sections of mouse salivary glands were dual stained with the pooled sera of four patients and AQP5-specific goat IgG that targets the cytoplasmic tail of murine, rat, and human AQP5. The amino acid sequence of AQP5 is highly conserved between humans and mice with 91% identities and 96% homology. Expression of AQP5 was observed in both the luminal and basolateral membrane of the acinar cells, as previously reported (Larsen et al., 2011). While the pooled control sera barely stained the mouse salivary glands, the SS sera strongly stained the nuclei and plasma membranes of the acinar cells (Fig. 3A). The signals of SS IgG showed a high degree of colocalization with those of AQP5-specific goat IgG, which was confirmed with Mander's overlap coefficient (Fig. 3B).

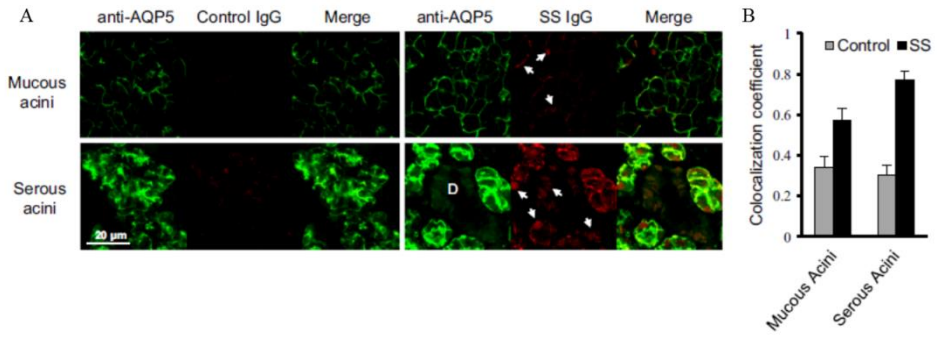


Fig. 3 Serum IgG from SS patients colocalize with AQP5 in the mouse salivary glands. (A) Sections of the mouse submandibular gland were stained with goat anti-AQP5 antibodies and either the pooled control or pooled SS sera followed by Alexa Fluor 488-conjugated anti-goat IgG and CFTM 594-conjugated anti-human IgG. The areas of mucous and serous acini were imaged with confocal microscopy. D: duct; arrows: nuclei. (B) The colocalization of green and red signals in five images was calculated with Mander's coefficient.

3.3. Serum IgG from SS patients selectively stain AQP5-GFP-transfected cells

Because SS patients may have autoantibodies against other proteins expressed in the salivary gland, the specificity of the autoantibodies was further investigated with CHO cells transfected with a transgene encoding GFP alone or a human AQP5-GFP fusion protein. While the GFP was dispersed throughout the cytoplasm and nuclei, AQP5-GFP was localized mostly to the plasma membranes and vesicular organelles. The anti-AQP5 goat IgG stained the CHO cells expressing AQP5-GFP but not those expressing GFP alone. The pooled SS sera stained the nuclei of the CHO cells regardless of the type of transgene but also specifically stained the cells expressing AQP5-GFP. The pooled control sera did not stain either GFP- or AQP5-GFP-expressing cells (Fig. 4A). The signals of both anti-AQP5 goat IgG and SS IgG overlapped with the signal of GFP localized to the plasma membrane and intracellular vesicles in the AQP5-GFP-expressing cells, which was confirmed with Mander's overlap coefficient (Fig. 4B).

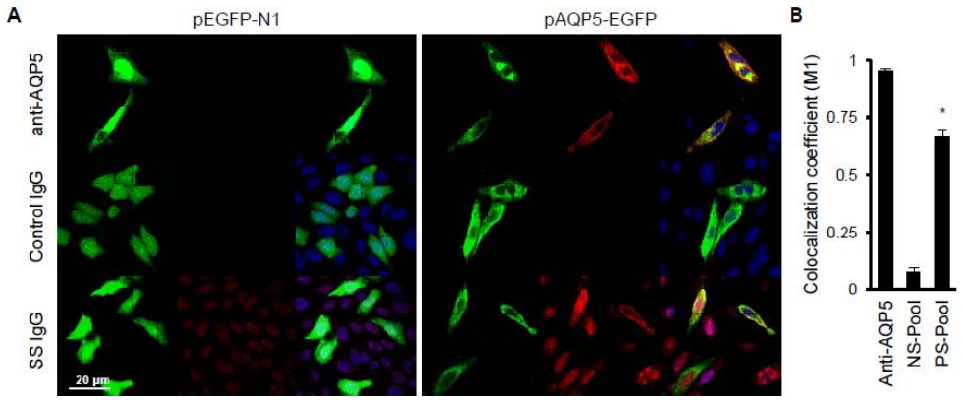


Fig. 4 Serum IgG from SS patients selectively stained AQP5-GFP-transfected cells. (A) CHO cells were transfected with either pEGFP-N1 or pAQP5-EGFP. The transfected cells were stained with goat anti-AQP5 antibodies, control sera, or SS sera (red) together with anti-GFP antibodies (green). (B) The colocalization of green and red signals in 18 images was calculated with Mander's coefficient.

3.4. Both the control and SS sera immunoprecipitate AQP5-GFP

The selective staining of AQP5-GFP but not of GFP by the SS sera suggests that AQP5 acts as an autoantigen in the SS patients. However, colocalization, *i.e.*, co-occurrence of two fluorophores in the same pixel, does not necessarily mean that the two signals target the same molecule. Therefore, the specific binding of SS IgG to AQP5 was further studied by immunoprecipitation. HEK-293 cells were transfected with either pEGFP-N1 or pAQP5-GFP which express the 27 KD GFP and 55 KD AQP5-GFP proteins, respectively (Fig. 5A). The anti-AQP5 IgG precipitated the AQP5-GFP, which was not precipitated from the lysates of the HEK-293 cells expressing GFP alone. Both the control and SS sera precipitated the AQP5-GFP, suggesting the presence of autoantibodies against AQP5 not only in the SS sera but also in the control sera (Fig. 5B left panel). When decreased amounts of sera were used, however, only the SS sera precipitated AQP5-GFP (Fig. 5B right panel). In an additional blinded experiment using several control and SS sera, all samples immunoprecipitated AQP5-GFP (Fig. 6).

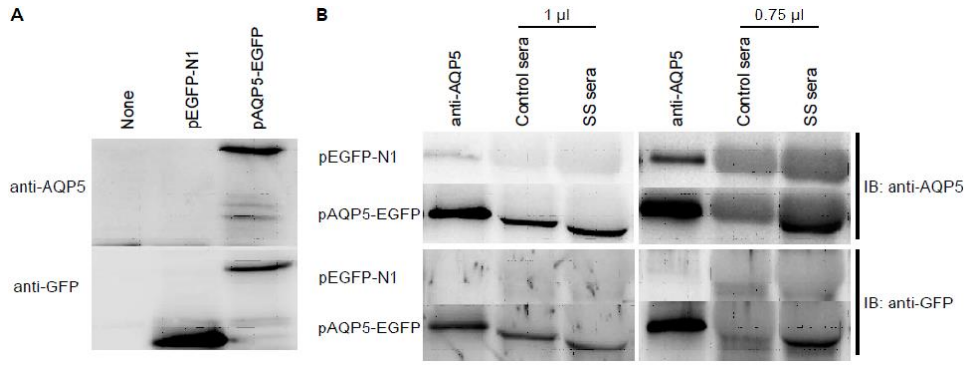


Fig. 5 Both control and SS sera immunoprecipitated AQP5-GFP. HEK-293 cells were transfected with either pEGFP-N1 or pAQP5-EGFP. (A) Lysates of non-transfected and transfected HEK-293 cells were separated by SDS-PAGE and immunoblotted with anti-AQP5 or anti-GFP antibodies. (B) Lysates of HEK-293 cells transfected with either pEGFP-N1 or pAQP5-EGFP were incubated with anti-AQP5 antibodies, control sera, or SS sera. The immune complexes precipitated with protein-A agarose beads were immunoblotted with either anti-AQP5 or anti-GFP antibodies.

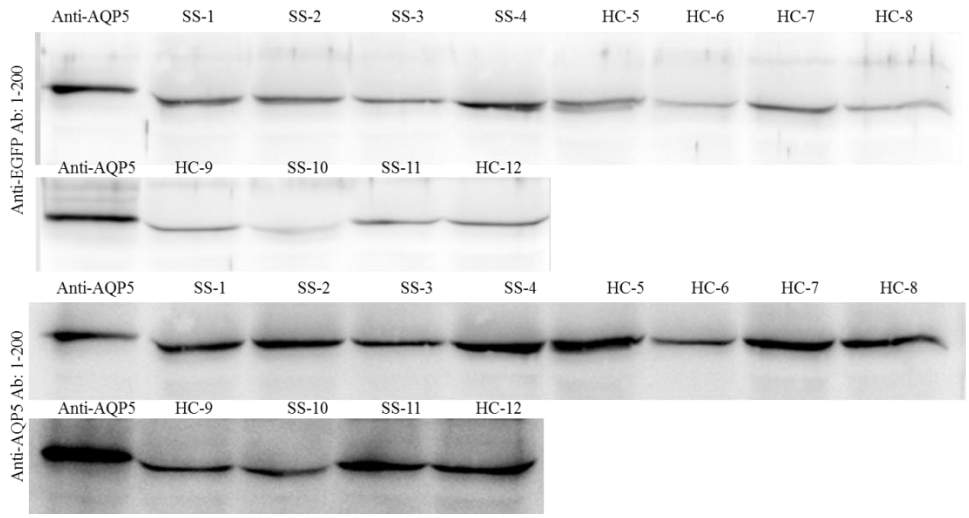


Fig. 6 Immunoprecipitation of AQP5-GFP with blinded sera samples. Lysates of HEK-293 cells transfected with pAQP5-EGFP were incubated with anti-AQP5 antibodies, and blinded sera from HC and SS patients. The immune complexes precipitated with protein-A agarose beads were immunoblotted with either anti-AQP5 or anti-GFP antibodies.

3.5. Higher levels of anti-AQP5 IgG and IgA were detected in the SS sera by IIFA

Currently, the standard method to detect anti-AQP4 antibodies for the diagnosis of neuromyelitis optica is with IIFA (Jarius et al., 2010). Therefore, a similar cell-based IIFA was developed by over-expressing AQP5 in MDCK an epithelial cell which have been widely used in studies on AQP5 function (Kosugi-Tanaka et al., 2006; Wellner et al., 2005). The staining protocol was first optimized with the pooled sera. Both the SS and control sera contained anti-AQP5 IgG; however, a clear difference in their titers was observed (Fig. 7A). The 1:200 dilution was chosen to screen 53 control and 112 SS samples for anti-AQP5 IgG. The anti-AQP5 IgG was detected in 43 control and 103 SS samples with a significant difference in their intensities ($p < 0.0001$). When the cut-off value was set as the mean+2 SD of the control values, 35 (31.3%) SS samples were positive for the anti-AQP5 IgG (Fig. 7B left panel, cut-off 1). When the cut-off value at which the accuracy was the highest was chosen from the ROC curve (Fig. 7B right panel), 17 (32.1%) control and 82 (73.2%) SS samples were positive for the anti-AQP5 IgG, resulting in a sensitivity of 0.73 and a specificity of 0.68 (Fig. 7B left panel, cut-off 2). SS sera samples were also screened for the presence of anti-AQP5 IgA with a 1:20 dilution (Fig. 7C). The anti-AQP5 IgA was detected in only one (1.9%) control and 15 (13.4%) SS samples, where the cut-off was set as the mean+2 SD of the control values (Fig. 7D).

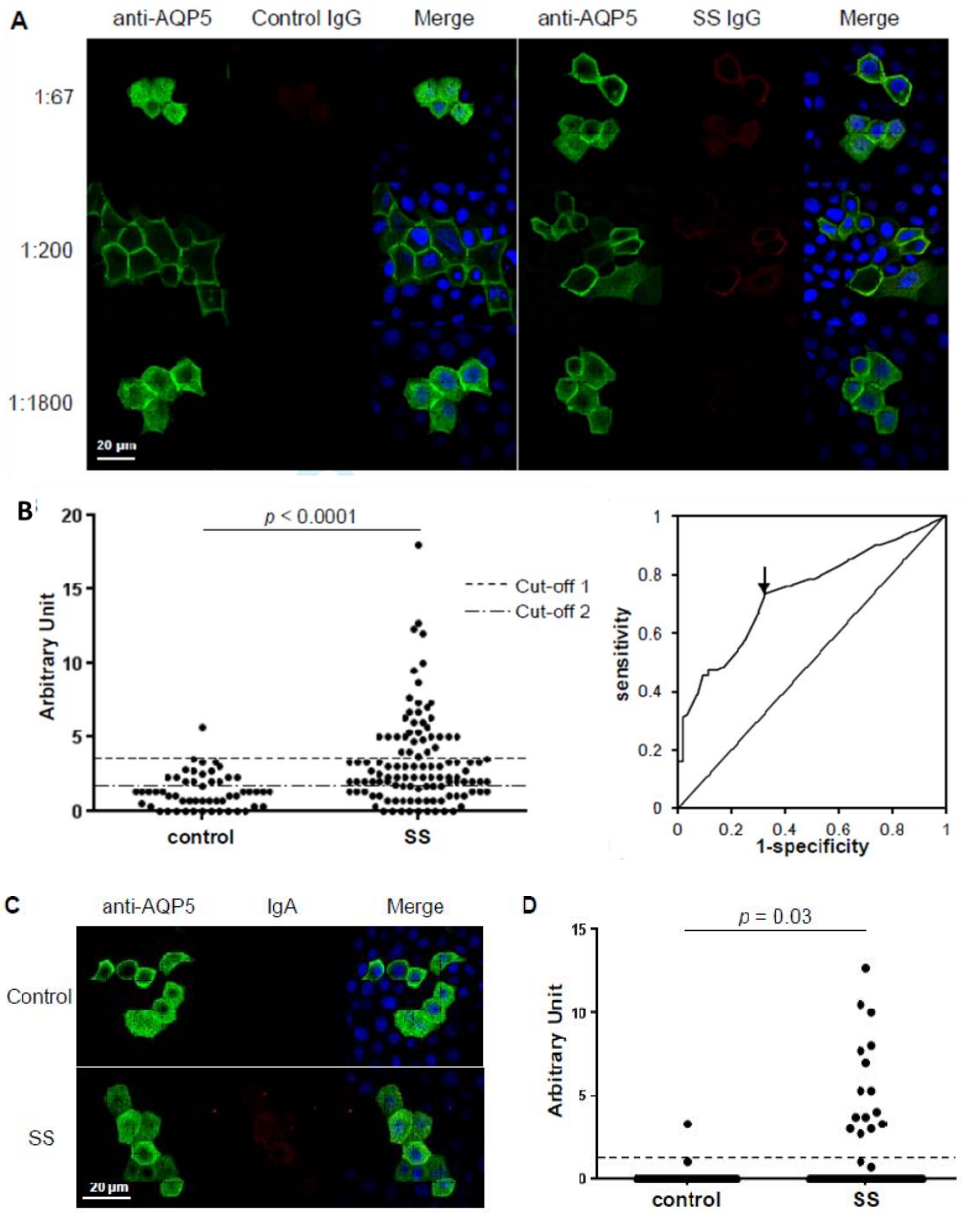


Fig. 7 Higher levels of anti-AQP5 IgG and IgA were detected in the SS sera by IIFA. (A) MDCK cells over-expressing AQP5 were stained with anti-AQP5 antibodies and various dilutions of either the control or SS sera, followed by Alexa Fluor 488-conjugated anti-goat IgG (green) and CF^{TM}

594-conjugated anti-human IgG (red). (B) The intensities of the red signals for anti-AQP5 IgG were expressed by the magnitude of brightness that was reduced until the staining of AQP5 disappeared. A ROC curve for the levels of anti-AQP5 IgG is shown. The arrow indicates the value used for cut-off 2 in the left panel. (C) MDCK cells over-expressing AQP5 were stained with anti-AQP5 antibodies and either the control or SS sera (1:20 dilution), followed by Alexa Fluor 488-conjugated anti-goat IgG (green) and Alexa Fluor 555-conjugated anti-human IgA (red). (D) The intensities of the red signals for anti-AQP5 IgA were expressed by the magnitude of brightness that was reduced until the staining of AQP5 disappeared.

3.6. Anti-AQP1 IgG and IgA were detected in the sera of patients with SS

To detect anti-AQP1 autoantibodies in SS patients sera, MDCK cells overexpressing full-length human AQP1 were double stained with anti-AQP1 antibodies and sera from either control subjects (n=52) or SS patients (n=112). By screening sera diluted to 1:200 for the presence of anti-AQP1 IgG, autoantibodies were detected in 23 patients with SS (20.5%), and the sera with anti-AQP1 IgG showed a high degree of colocalization between the signals of human IgG and those of AQP1-specific rabbit IgG. None of the control sera was positive for anti-AQP1 IgG (Figure 8A and B). Among the anti-AQP1 IgG-positive SS sera, 19 samples were also positive for anti-AQP5 IgG, in the other four samples the anti-AQP5 IgG was also detected but at lower levels than the cut off used for positivity. By screening sera diluted to 1:20 for the presence of anti-AQP1 IgA, autoantibodies were detected in eight SS samples (7.1%) but in none of the control sera (Figure 8C and D). Seven of the anti-AQP1 IgA-positive sera were also positive for anti-AQP5 IgG and IgA, and another sample was positive for anti-AQP5 IgG. However, none of the samples with anti-AQP1 IgA were positive for anti-AQP1 IgG. Altogether, anti-AQP1 autoantibodies were detected in 27.2% of SS patients. When the intensities of anti-AQP1 IgG/IgA were compared with

those of the corresponding anti-AQP5 IgG/IgA, a significant positive correlation ($p = 0.003$) was observed (Figure 8E).

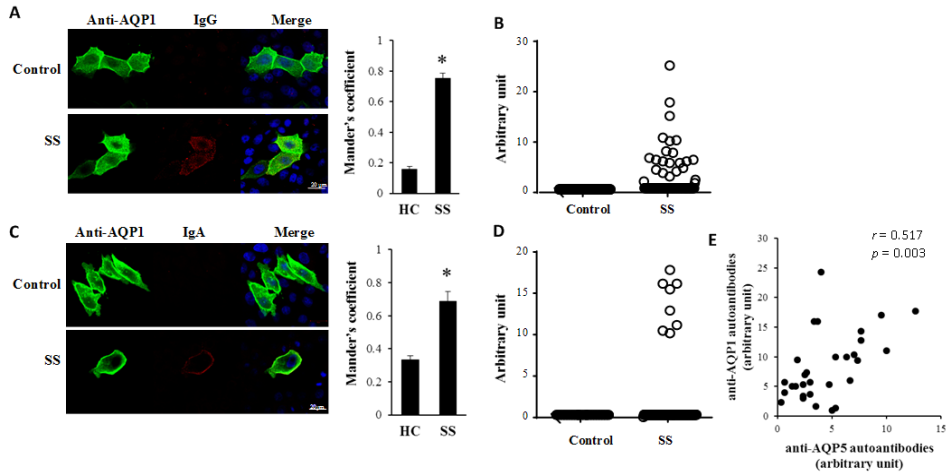


Fig. 8 Detection of anti-AQP1 IgG and IgA in the sera of SS patients

(A) MDCK cells over-expressing AQP1 were stained with anti-AQP1 antibodies and either control or SS sera (1:200 dilution), followed by Alexa Fluor 488-conjugated anti-rabbit IgG (green) and Alexa Flour 555-conjugated anti-human IgG (red). The colocalization of green and red signals in the three images was calculated using Mander's coefficient. *, $p = 0.0001$ (B) The intensities of the red signals for anti-AQP1 IgG were expressed by the magnitude of brightness that was reduced until the AQP1 staining disappeared. (C) MDCK cells over-expressing AQP1 were stained with anti-AQP1 antibodies and either control or SS sera (1:20 dilution), followed by Alexa Fluor 488-conjugated anti-rabbit IgG (green) and Alexa Fluor 555-conjugated anti-human IgA (red). The colocalization of green and red signals in the three images was calculated using Mander's coefficient. *, $p = 0.02$ (D) The intensities of the red signals for anti-AQP5 IgA were expressed by the

magnitude of brightness that was reduced until the AQP1 staining disappeared. (E) The intensities of anti-AQP1 IgG or IgA and of anti-AQP5 IgG or IgA that were identified in each individual were plotted.

3.7 Homology between human AQP1 and AQP5 suggest immunological cross-reactivity

AQP1 is relatively closely related to AQP5 in the phylogenic tree of 13 human AQPs, AQP1 has 44% identity and 62% similarity with the amino acid sequence of human AQP5 (Fig. 9A). In contrast to anti-AQP5 autoantibodies, for which the presence of anti-AQP5 IgA had overlapped with the presence of anti-AQP5 IgG, the presence of anti-AQP1 IgA coincided with the presence of anti-AQP5 IgA, rather than the presence of anti-AQP1 IgG. Similarly, the presence of anti-AQP1 IgG overlapped with the presence of anti-AQP5 IgG. Therefore, anti-AQP1 autoantibodies are likely to be a byproduct of an antibody response to AQP5. A significant positive correlation between the intensities of the two autoantibodies supports it (Fig. 8E). In contrast to AQP5 in which the full lengths of loops B and E are exposed to the cytosolic and extracellular compartments respectively, the middle portion of loops B and E in AQP1 is embedded within the membrane. However, short stretches of conserved sequences were observed in loop B, loop D, and loop E, as well as the C-terminal cytoplasmic tail, which may provide cross-reactive epitopes (Fig. 9B).

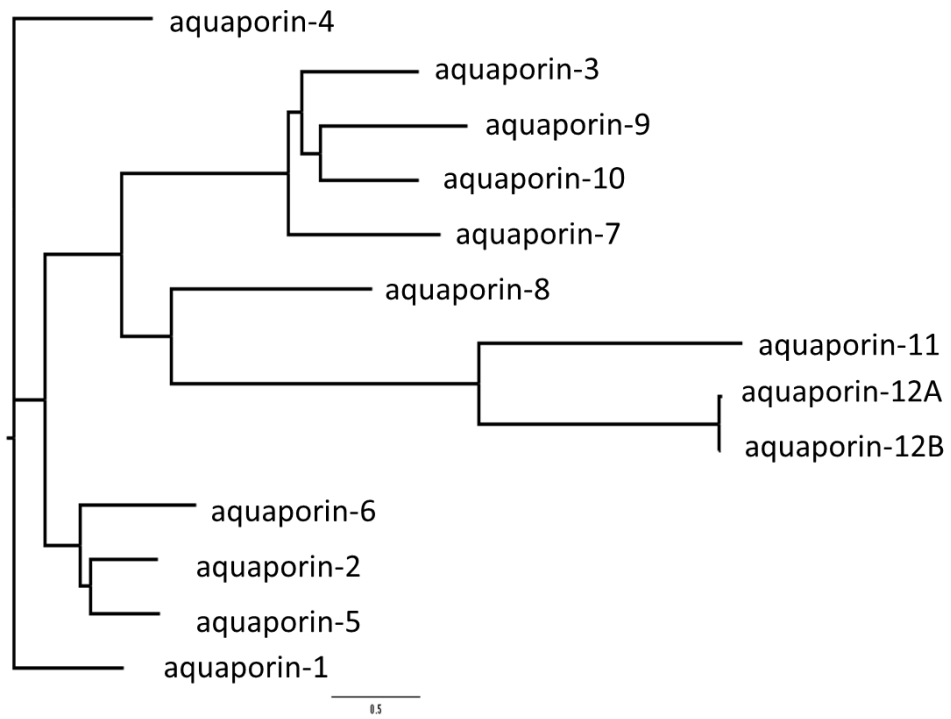


Fig. 9A. The evolutionary relationship of human AQPs. A phylogenic tree for the 13 members of human AQPs was generated by neighbor-joint method.

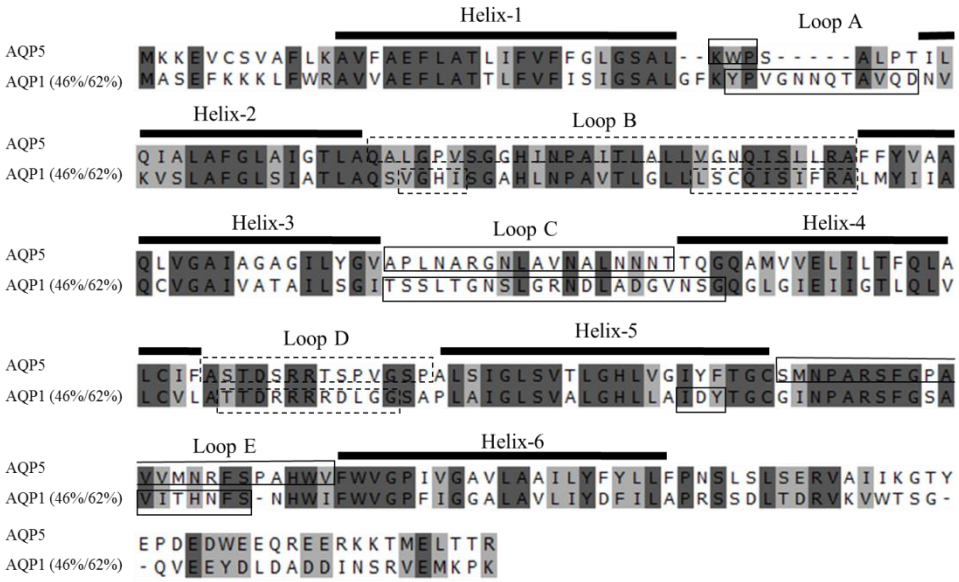


Fig. 9B. **Sequence alignment of human AQP5 and AQP1.** Identical and conserved amino acids are highlighted with dark gray and light gray, respectively. The extracellular and cytosolic domains are boxed with solid and dotted lines, respectively.

3.8. The presence of anti-AQP5 autoantibodies was associated with low resting salivary flow rate

Associations between the presence of autoantibodies to AQP5 and AQP1 and salivary flow rates were examined in patients for whom the salivary flow rates were recorded at the time of sampling. The presence of anti-AQP5 IgG was associated with low resting salivary flow but not with stimulated salivary flow. The patients with anti-AQP5 IgA presented lower levels of both resting and stimulated salivary flow than those without the autoantibodies. However, significance was achieved for only resting salivary flow. In contrast, the presence of neither anti-AQP1 IgG nor IgA had a significant effect on salivary flow. Because more than one-half of the SS patients who negative for anti-AQP1 autoantibodies had anti-AQP5 autoantibodies that were associated with a low resting salivary flow, the effect of anti-AQP1 autoantibodies on salivary flow was also examined in patients with anti-AQP5 autoantibodies. However, no difference in either resting or stimulatory salivary flow was observed by the additional presence of anti-AQP1 autoantibodies. Importantly, the presence of either anti-Ro or anti-La antibodies which was the hall mark of disease diagnosis was not associated with the levels of salivary flow (Tab. 5).

Tab. 5 Association between presence of autoantibodies and salivary flow rates

Presence of autoantibodies	No. of patients	Mean (95% CI)	*P*
anti-AQP5 IgG		Resting salivary flow	
-	28	0.096 (0.044, 0.148)	0.003[a]
+	63	0.049 (0.030, 0.069)	0.147[b]
		Stimulated salivary flow	
-	27	0.554 (0.362, 0.747)	0.697[a]
+	61	0.511 (0.389, 0.633)	0.717[b]
anti-AQP5 IgA		Resting salivary flow	
-	79	0.070 (0.046, 0.093)	0.048[a]
+	12	0.027 (-0.01, 0.064)	0.010[b]
		Stimulated salivary flow	
-	76	0.556 (.444, 0.667)	0.057[a]
+	12	0.326 (0.11, 0.543)	0.089[b]
Anti-AQP1 IgG		Resting salivary flow	
-	74	0.068 (0.041, 0.094)	0.449[a]
+	17	0.047 (0.023, 0.071)	0.838[b]
		Stimulated salivary flow	
-	71	0.518 (0.403, 0.633)	0.821[a]
+	17	0.547 (0.319, 0.776)	0.604[b]
Anti-AQP1 IgA		Resting salivary flow	
-	86	0.064 (0.042, 0.087)	0.814[a]
+	5	0.054 (-0.051, 0.159)	0.674[b]
		Stimulated salivary flow	
-	83	0.534 (0.430, 0.638)	0.394[a]
+	5	0.347 (-0.255, 0.949)	0.194[b]
Anti-AQP1 IgG or IgA in patients who were positive for anti-AQP5 autoantibodies			
		Resting salivary flow	
-	49	0.050 (0.025, 0.074)	0.894[a]
+	19	0.045 (0.020, 0.071)	0.599[b]
		Stimulated salivary flow	
-	49	0.517 (0.374, 0.660)	0.840a
+	19	0.485 (0.275, 0.694)	0.885b
anti-Ro IgG		Resting salivary flow	
-	9	0.040 (0.002, 0.149)	0.445[a]
+	82	0.067 (0.044, 0.090)	0.512[b]
-		Stimulated salivary flow	
+	8	0.482 (0.238, 0.726)	0.793[a]
	80	0.529 (0.419, 0.638)	0.800[b]
anti-La IgG		Resting salivary flow	
-	42	0.066 (0.042, 0.091)	0.851[a]

+	49	0.062 (0.029, 0.096)	0.288[b]
		Stimulated salivary flow	
-	40	0.550 (0.407, 0.692)	0.648[a]
+	48	0.503 (0.357, 0.649)	0.508[b]

a: ANOVA

b: Mann Whitney U test

3.9. Epitope mapping of anti-AQP5 autoantibodies in the sera of patients with SS

Autoantibodies to AQP5 were associated with decreased resting salivary flow rate. Immune recognition of autoantigen is essential for development of Ag-specific immunotherapy. To identify the epitope of AQP5 present on the cell surface, five peptides corresponding to the three extracellular loops of AQP5 were designed after searching for linear-B cell epitope using immune epitope database (Fig. 10A). Based on anti-AQP5 autoantibodies screening results (Fig. 7B), 12 SS and 1 control sera were selected above cut-off 1 and 12 SS and 12 control sera above cut-off 2 for epitope mapping experiment. Purified IgG from SS and HC were also included in this experiment, individual serum samples were pre-incubated with synthetic peptides overnight and then stained MDCK-AQP5 expressing cells, human IgG were detected by IIFA. 76% of the sera from SS reacted with at least one peptide as compared to 21% of the control sera. IgG from 18 SS patients (72%) recognized either linear or cyclic peptides to loop-C, while IgG from 16 SS patients (64%) recognized any of the two cyclic peptide to loop-E, 44% of the sera showed reactivity with the linear peptides corresponding to loop-A (Tab. 6). Interestingly IgG from 6 SS patients (24%) recognized none of the synthetic peptides; the final data were presented as mean of all sera samples (Fig. 10B). Pre-incubation of purified IgG with synthetic peptides significantly blocked the binding of anti-AQP5 autoantibodies to AQP5

overexpressing MDCK cells (Fig. 10C). No significant reactivity was found with irrelevant peptides (IRP).

Tab. 6 Epitope mapping of anti-AQP5 autoantibodies in the sera of patients with SS

		Blocking Peptides					
Sample ID	Cont.	A	C	C2	E1	E2	IRP
005-034 (Cau-P22)	12.8	10.1	11	9.2	5.8	12.1	14
005-043 (Cau-P29)	6.3	10.6	6.3	1.2	5.8	7.3	12.5
005-046 (Cau-P30)	15	5	4.3	6.2	9.5	10.7	15
005-060 (Cau-P42)	10.3	7.8	7.6	5.7	5.6	16.6	9.8
005-063 (Cau-P44)	13.7	6.8	12.1	3.8	6.8	5.7	10.7
005-072 (Cau-P51)	14.9	9.0	10.	7.0	7.8	6.9	11.9
005-073 (Cau-P52)	10.2	7.8	5.8	7.6	9.8	6.7	11.2
005-076 (Cau-P54)	7.4	7.5	8.2	7.5	6.7	5.9	5.4
005-103 (Cau-P73)	16.7	9.9	10.9	12.5	9.6	12.6	13.8
005-105 (Cau-P75)	15.1	9.25	9.1	8.7	4.1	6.7	15.5
005-128 (Cau-P92)	18.1	14.9	16.0	15.2	16.7	15.9	16.9
005-141 (Cau-P101)	10.6	8.1	7.7	3.1	6.2	12	8.1
005-018 (Cau-P6)	15.1	8.1	10.1	9.9	10.6	6.0	15.3
005-020 (Cau-P8)	0.5	5	3.8	2.3	2.2	1.7	7.5
005-021 (Cau-P9)	5	6.1	5.7	3.1	3.7	3.8	7
005-024 (Cau-P12)	3.6	3.6	6.3	7.2	4.2	5	6.5
005-025 (Cau-P13)	4.5	3.6	1.2	6.7	6.2	2.6	3.7
005-029 (Cau-P17)	8.6	5.2	4.3	4.3	3.2	2.8	7
005-031 (Cau-P19)	6.3	5.2	2.7	1.5	2.8	2.2	6.3
005-032 (Cau-P20)	11.1	6.3	4.1	6	3.3	6.8	7.8
005-035 (Cau-P23)	12.6	8.5	8.7	6.8	12.2	10.7	11
005-036 (Cau-P24)	16.2	9	12.3	8.5	6.6	9	11.6
005-039 (Cau-P26)	1.1	5	3.8	4.2	2.1	2.3	2.8
005-041 (Cau-P28)	10.9	12.6	9.8	8.8	9.7	9.2	12.7
SS-purified IgG	21.2	11.1	15.1	12.4	4.5	10.3	19.5
(Cau-C3)	7.1	9.7	7.2	17.3	11.1	9.3	9
(Cau-C4)	8.2	11.3	7.3	6	8	9.2	10
(Cau-C9)	5.3	9.2	10.1	10.3	9	9.5	9.3
(Cau-C11)	5.3	6.8	8.3	8.7	9.8	5.6	6.3
(Cau-C13)	7.8	11.5	8.0	10.2	6.5	7.7	7.9
(Cau-C19)	9.2	9.3	7.7	12.5	9.1	12	10.3
(Cau-C20)	11.5	10.0	14.1	7.11	9.1	10.2	10.5
(Cau-C26)	7.5	8.6	12.8	12.5	9.3	8.5	6.6
(Cau-C35)	5	6.1	6.2	6.8	4.3	2.3	6.3
(SNUH-C2)	10.5	11.9	12.2	9.6	10.3	12.5	7.7
(SNUH-C4)	5.3	4.2	5	9	6.5	7.1	7
(SNUH-C6)	4.8	8.8	8.8	13.1	5.1	9.8	8.2

(SNUH-C3)	7.5	2.5	5.3	8	8.6	8.3	7.3
Sigma-Cont. IgG	19.6	24.6	19.4	20.8	20.5	16	17.4

Anti-AQP5 autoantibodies inhibition by synthetic peptides

20%	30%	40%	50%	60%	70%	80%

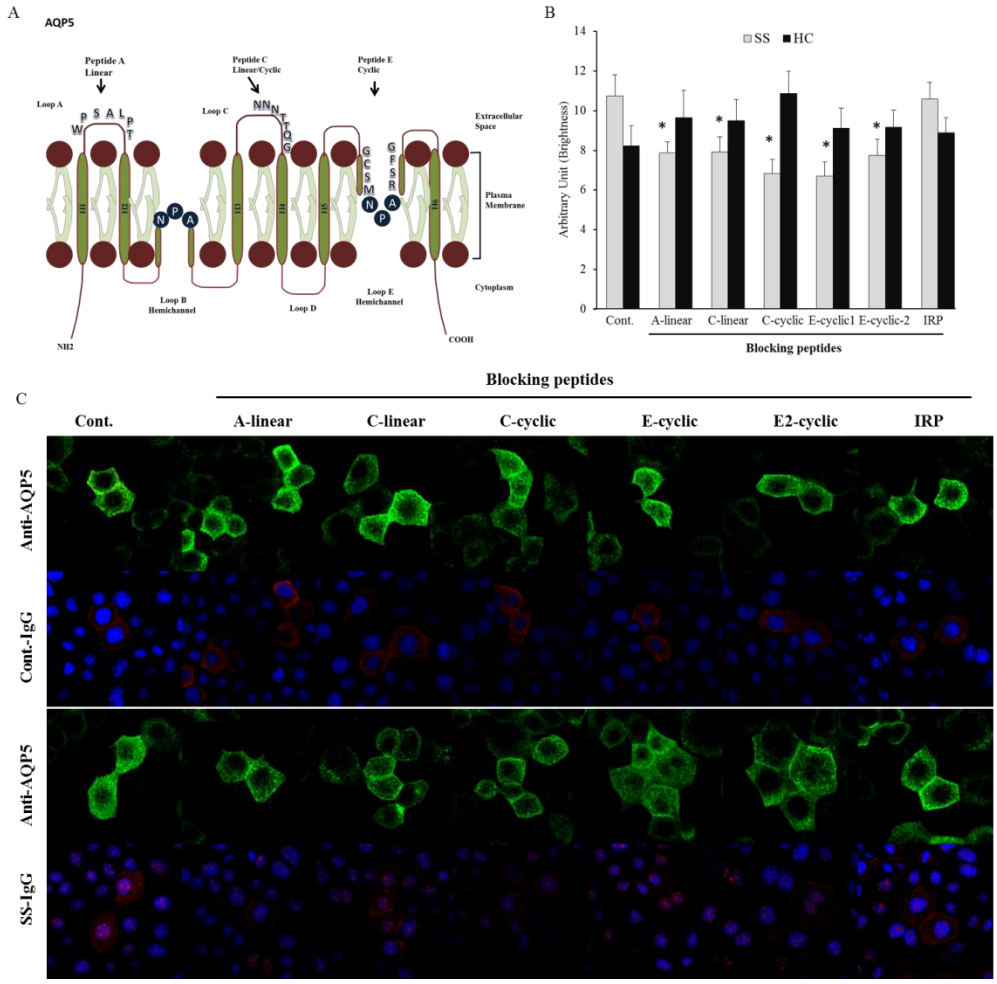


Fig. 10 Synthetic peptides blocked the binding of anti-AQP5 IgG in the sera from SS patients. (A) Synthetic peptides covering the extracellular loops were designed after B cell epitope analysis. (B and C) Purified IgG and

individual serum samples were pre-incubated with synthetic peptides overnight and then stained MDCK-AQP5 expressing cells, the intensities of the red signals for anti-AQP5 IgG were expressed by the magnitude of brightness that was reduced until the staining of AQP5 disappeared the data were presented as mean± SEM, images were taken using confocal microscope.

3.10. Change in oral microbiota was associated with dryness of mouth

Growing evidence suggests that dysbiosis of microbiota is associated with the pathogenesis of several diseases (Carding et al., 2015; Jiang et al., 2015; Li et al., 2014; Ray & Dittel, 2015; Scher et al., 2015; Vujkovic-Cvijin et al., 2013). Therefor a total 50 samples were analyzed for microbiota communities from HC (n=15), SC (n=10) and SS patients (n=25). From 50 samples a total 473869 valid reads were obtained with average 9477 read per sample resulted in 99.15% good's coverage per sample. First the overall bacterial communities of HC, SC and SS were compared using UniFrac, a phylogeny-based distance metric ranging from 0 (complete identical) to 1 (completely different). PCoA clustering reveals clear separation between communities from HC and dry mouth (both SC and SS). Although, there was no separation between SC and SS, SC made a sub-cluster inside SS bacterial communities (Fig. 11A). Further, the intragroup UniFrac distance of HC (0.0546$\pm$ 0.0276) was higher than SS (0.0405$\pm$ 0.0184) and SC (0.0384$\pm$ 0.0234), suggested increased inter-subject variability in HC, followed by SS and SC respectively (Fig. 11B). In addition, the intergroup UniFrac distance of HC (0.0546$\pm$ 0.0276) was significantly smaller than intragroup distance with SC (0.067$\pm$ 0.024) and SS (0.068$\pm$ 0.024) (Fig. 11C) suggesting a significant difference in the oral microbiota of HC and microbiota associated with dryness of mouth (both SC and SS). No significant differences were

observed between intra-UniFrac distance of SC (0.0384± 0.0234) and inter-UniFrac distance with SS (0.0457± 0.0160) suggesting a relatively similar microbiota between these two groups (Fig. 11D).

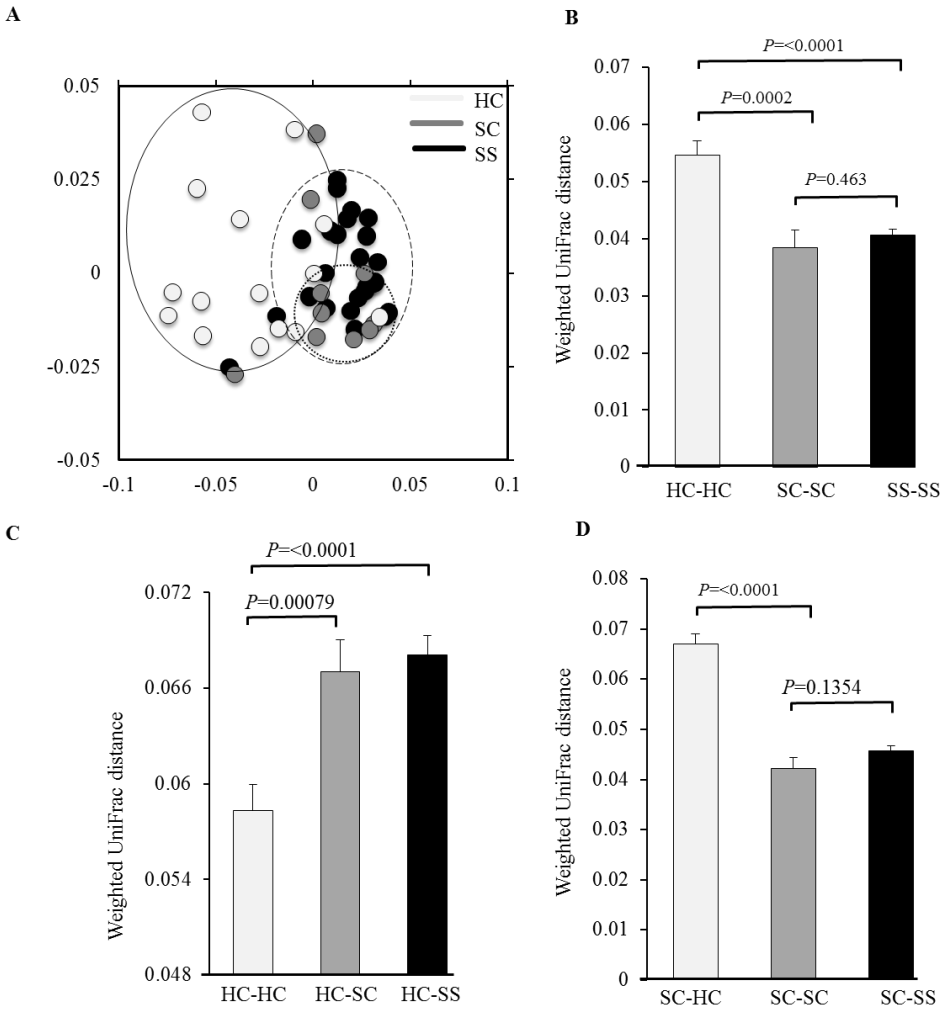


Fig. 11 Microbial biodiversity decreased in dry mouth condition. A) Principal coordinate analysis (PCoA) plot based on weighted UniFrac value showing the similarity among the HC (n=15), SC (n=10) and SS (n=25) patients. B) Variation in bacterial communities' composition with in the same HC group and those between SC and SS patients. C &D, The species richness estimated by Chao1 and Shannon index.

Then, alpha diversity was compared between HC, SC and SS groups. The species richness of HC microbiota estimated by Chao1 (425.51 ± 80.96) was not significantly different from SC (385.91 ± 67.87) and SS (426.59 ± 83.71) (Fig. 12A). Interestingly, the diversities of microbiota determine by Shannon index was significantly changed in SS (4.54 ± 0.44) as compared to the diversity in HC (4.28 ± 0.34) and SC (4.19 ± 0.42) (Fig. 12B).

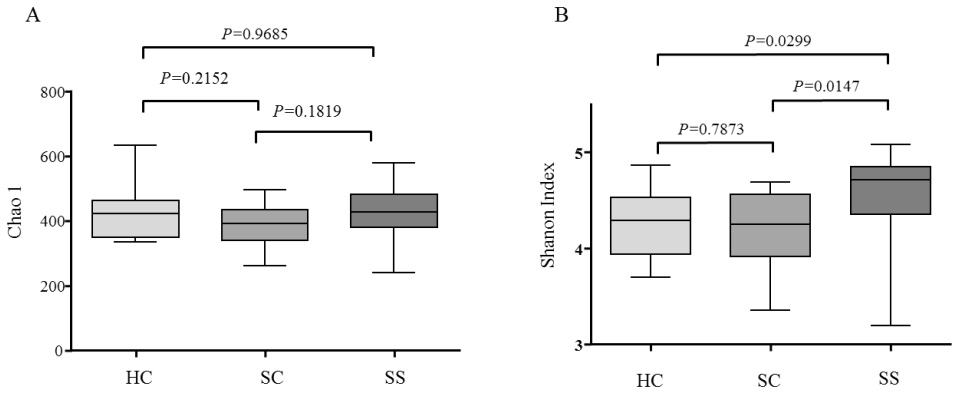


Fig. 12 Bacterial communities richness and diversity in HC and dry mouth condition.

Next, the relative abundance of each taxon among different groups was compared. Although a total 20 different phyla were detected from the saliva samples, Firmicutes, Proteobacteria Actinobacteria, Bacteroidetes and Fusobacteria encompass the majority of the sequences (>95% in HC and >99% in SC and SS group). The relative abundance of major phyla observed in HC was significantly different from that of SC and SS patients (Fig. 13). In comparison to the HC, phylum Firmicutes and Actinobacteria were significantly increased while Proteobacteria and Fusobacteria were significantly decreased in both SC and SS group respectively. The Bacteroids decreased in SC while slightly increased in SS (Tab. 7).

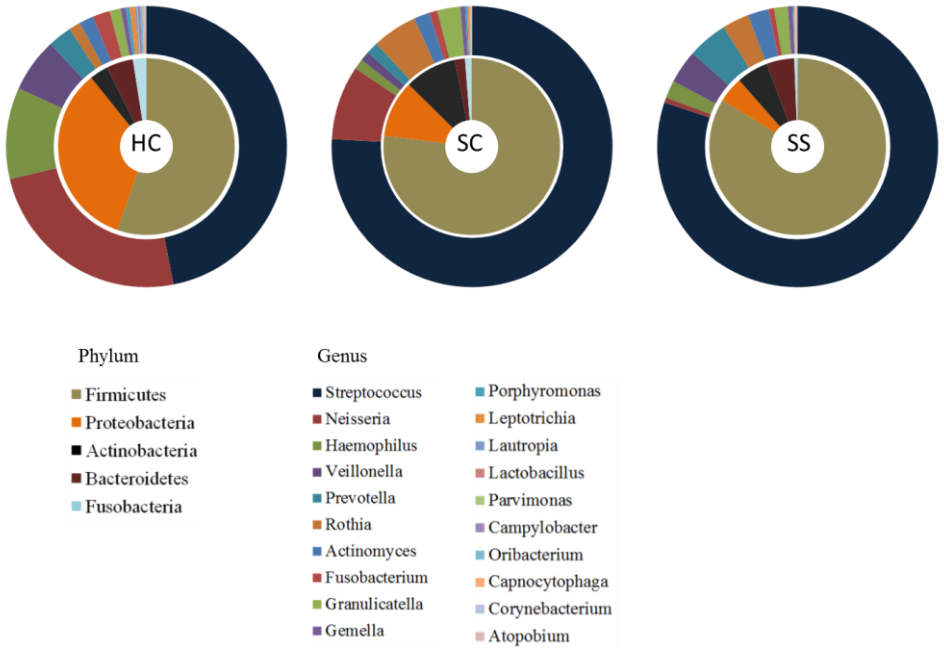


Fig. 13 Relative abundance of major phylum and genus between HC and dry mouth condition. Bacterial DNA extracted from mouth wash samples obtained from HC (n=15), SC (n=10) and primary SS (n=25) were analyzed by pyrosequencing of amplified 16S rRNA fragments. Double pie charts present both Phylum and genera.

At the genus level, *Streptococcus* which constituted up to 38.96 percent of the microbiota from the HC group was increased in both SC (68.56%) and SS group (67.48%), other major genera e.g. *Neisseria, Haemophilus, Veillonella, Fusobacterium* decreases both in SC and SS groups while *Prevotella* selectively increased in SS. Other genera also showed a difference in the relative abundance (Table 7). At the species level, the abundance of most of the bacterial species e.g. *Haemophilus parainfluenzae, Neisseria subflava, Neisseria perflava, Veillonella dispar* was significantly decreased in dry mouth condition. Among the bacterial species only the abundance of *Prevotella histicola* was selectively increased in SS patients (Table 8).

The change in abundance of oral bacterial species and association with SS risk was determined by logistic regression analysis using a forward method, *Prevotella melaninogenica* was found associated with increased SS risk (OR 5.396 per 1% increase, CI 95% 1.793-16.238, p=0.003).

Tab. 7 Relative abundance of major phylum and genus in HC and dry mouth condition

		HC (n=15)	SC (n=10)	SS (n=25)	P=Value
Phylum	Firmicutes	52.16 (71.94)	72.85 (55.13)	77.82 (58.96)	0.0006
	Proteobacteria	31.97 (61.05)	9.84 (52.29)	4.36 (47.15)	0.0001
	Actinobacteria	3.18 (9.00)	9.02 (18.36)	5.38 (17.96)	0.0082
	Bacteroidetes	4.62 (15.03)	1.76 (16.47)	4.72 (20.07)	0.1672
	Fusobacteria	2.33 (23.63)	1.15 (2.03)	0.58 (4.71)	0.0032
Genus	*Streptococcus*	38.96 (82.49)	68.56 (50.00)	67.48 (54.02)	0.0004
	Neisseria	20.24 (45.95)	7.73 (51.09)	0.51 (35.23)	0.0003
	Haemophilus	8.74 (33.47)	1.06 (5.72)	1.70 (13.81)	0.0004
	Veillonella	5.15 (21.49)	1.01 (16.12)	3.24 (15.09)	0.0681
	Prevotella	2.29 (13.47)	1.22 (16.05)	3.89 (19.76)	0.0909
	Rothia	1.11 (5.94)	4.69 (18.07)	2.59 (14.94)	0.0024
	Actinomyces	1.37 (7.98)	1.63 (6.90)	2.01 (8.46)	0.8796
	Fusobacterium	1.64 (22.79)	0.85 (1.67)	0.55 (4.20)	0.0131
	Granulicatella	1.08 (2.89)	2.38 (4.53)	1.33 (6.11)	0.1540
	Gemella	0.47 (3.41)	0.51 (4.77)	0.47 (13.80)	0.9650
	Porphyromonas	0.35 (4.09)	0.21 (1.62)	0.05 (2.31)	0.0449
	Leptotrichia	0.51 (3.62)	0.18 (1.38)	0.07 (1.28)	0.0015
	Lautropia	0.18 (6.45)	0.01 (0.51)	0 (0.24)	0.0000
	Lactobacillus	0 (0.34)	0 (0.02)	0.01 (3.59)	0.0380
	Parvimonas	0.03 (0.60)	0.02 (1.02)	0.02 (5.11)	0.9613
	Campylobacter	0.29 (2.76)	0.04 (0.21)	0.01 (0.24)	0.0000
	Oribacterium	0.18 (1.84)	0.04 (0.85)	0.04 (0.46)	0.0194
	Capnocytophaga	0.19 (2.10)	0.06 (0.51)	0.02 (0.25)	0.0005
	Corynebacterium	0.13 (1.74)	0.03 (0.60)	0.03 (0.65)	0.0289
	Atopobium	0.05 (0.89)	0.06 (0.56)	0.18 (1.22)	0.0843

Tab. 8 Relative abundance of bacterial species in HC and dry mouth condition

	Percentage			KW-H	Mann-Whitney U test		
	HC	SC	SS		HC-SC	HC-SS	SC-SS
Name	Median (Range)			*P*=Value	*P*=Value		
Haemophilus parainfluenzae	6.93 (32.37)	0.98 (5.57)	1.22 (9.75)	0.00	0.00	0.00	0.97
Neisseria subflava	6.55 (33.56)	0.89 (11.65)	0.02 (31.99)	0.00	0.02	0.00	0.21
Neisseria perflava	2.80 (32.16)	0.59 (50.17)	0.00 (11.03)	0.00	0.20	0.00	0.14
Fusobacterium periodonticum	1.07 (2.67)	0.45 (1.59)	0.03 (3.67)	0.00	0.06	0.00	0.26
Streptococcus sanguinis	1.05 (5.84)	0.46 (4.78)	0.35 (2.85)	0.01	0.27	0.00	0.17
Streptococcus oralis	0.56 (3.38)	1.42 (4.59)	1.60 (8.24)	0.03	0.02	0.02	0.88
Rothia mucilaginosa	0.55 (4.39)	3.86 (11.18)	1.97 (12.98)	0.00	0.00	0.01	0.07
Streptococcus parasanguinis	0.55 (1.62)	2.14 (4.60)	1.92 (3.56)	0.00	0.00	0.00	0.58
Veillonella tobetsuensis	0.55 (1.34)	0.04 (0.58)	0.11 (1.13)	0.00	0.00	0.03	0.02
Rothia aeria	0.29 (1.43)	0.32 (3.44)	0.04 (1.09)	0.03	0.82	0.02	0.06
Neisseria elongate	0.29 (2.35)	0.02 (0.36)	0.00 (2.40)	0.00	0.02	0.00	0.27
Lautropia mirabilis	0.19 (6.46)	0.01 (0.52)	0.00 (0.24)	0.00	0.00	0.00	0.15
Haemophilus sputorum	0.16 (4.44)	0.00 (0.19)	0.00 (0.15)	0.00	0.00	0.00	0.18
Veillonella rogosae	0.16 (0.67)	0.01 (0.13)	0.01 (0.22)	0.00	0.00	0.00	0.52
Haemophilus paraphrohaemolyticus	0.13 (2.09)	0.00 (0.06)	0.01 (4.76)	0.00	0.00	0.02	0.07
Campylobacter concisus	0.12 (2.72)	0.03 (0.22)	0.00 (0.21)	0.00	0.00	0.00	0.44
Streptococcus lactarius	0.11 (0.26)	0.29 (1.32)	0.09 (1.37)	0.02	0.00	0.25	0.04
Oribacterium sinus	0.08 (0.72)	0.02 (0.32)	0.03 (0.25)	0.02	0.07	0.00	0.85
Prevotella histicola	0.07 (4.48)	0.05 (3.78)	0.86 (10.46)	0.03	0.64	0.03	0.04
Corynebacterium matruchotii	0.07 (1.47)	0.00 (0.23)	0.00 (0.57)	0.00	0.03	0.00	0.70
Rothia dentocariosa	0.06	0.51	0.23	0.01	0.01	0.03	0.13

	(0.84)	(4.93)	(6.04)				
Capnocytophaga	0.06	0.02	0.00	0.00	0.19	0.00	0.01
gingivalis	(0.48)	(0.12)	(0.04)				
Neisseria oralis	0.05	0.00	0.00	0.03	0.20	0.01	0.31
	(4.81)	(0.69)	(0.31)				
Stomatobaculum longum	0.05	0.03	0.00	0.01	0.16	0.00	0.24
	(0.33)	(0.22)	(0.27)				
Prevotella nigrescens	0.05	0.00	0.00	0.00	0.00	0.00	0.14
	(0.73)	(0.03)	(0.07)				
Oribacterium	0.04	0.02	0.00	0.01	0.10	0.00	0.31
asaccharolyticum	(1.07)	(0.24)	(0.33)				
Capnocytophaga	0.04	0.00	0.00	0.01	0.09	0.00	0.49
leadbetteri	(0.87)	(0.11)	(0.11)				
Kingella oralis	0.04	0.00	0.01	0.01	0.01	0.02	0.28
	(0.77)	(0.04)	(0.17)				
Fusobacterium	0.04	0.00	0.00	0.02	0.10	0.01	0.97
canifelinum	(0.27)	(0.20)	(0.15)				
Leptotrichia wadei	0.03	0.02	0.00	0.00	0.65	0.00	0.03
	(0.19)	(1.02)	(0.11)				
Campylobacter showae	0.03	0.00	0.00	0.00	0.01	0.00	0.14
	(0.31)	(0.02)	(0.04)				
Catonella morbi	0.03	0.00	0.00	0.03	0.07	0.01	0.73
	(0.19)	(0.06)	(0.16)				
Prevotella shahii	0.02	0.00	0.00	0.00	0.04	0.00	0.79
	(0.30)	(0.11)	(0.08)				
Eikenella corrodens	0.02	0.02	0.00	0.00	0.54	0.00	0.02
	(0.19)	(0.07)	(0.09)				
Campylobacter gracilis	0.02	0.00	0.00	0.00	0.01	0.00	0.77
	(0.37)	(0.03)	(0.06)				
Streptococcus anginosus	0.01	0.10	0.11	0.01	0.03	0.00	0.91
	(0.26)	(1.44)	(8.08)				
Veillonella parvula	0.01	0.01	0.08	0.03	0.69	0.02	0.07
	(0.08)	(0.17)	(3.20)				
Porphyromonas	0.01	0.06	0.00	0.04	0.73	0.10	0.01
gingivalis	(2.22)	(0.53)	(0.17)				
Prevotella salivae	0.01	0.01	0.11	0.04	0.50	0.07	0.03
	(1.00)	(0.64)	(1.15)				
Veillonella rodentium	0.01	0.01	0.06	0.01	0.33	0.05	0.01
	(0.22)	(0.06)	(1.28)				
Leptotrichia hofstadii	0.01	0.00	0.00	0.02	0.41	0.00	0.15
	(0.24)	(0.18)	(0.06)				
Alloprevotella tannerae	0.01	0.00	0.00	0.01	0.29	0.00	0.10
	(0.32)	(0.02)	(0.02)				
Capnocytophaga	0.01	0.00	0.00	0.04	0.41	0.01	0.18
granulosa	(0.16)	(0.16)	(0.08)				
Aggregatibacter	0.01	0.00	0.00	0.01	0.03	0.01	0.79
aphrophilus	(0.15)	(0.02)	(0.08)				
Actinomyces johnsonii	0.01	0.00	0.00	0.01	0.72	0.00	0.03
	(0.06)	(0.14)	(0.06)				
Streptococcus mutans	0.00	0.00	0.03	0.01	0.09	0.00	0.31

	(0.07)	(0.19)	(7.14)				
Streptococcus sobrinus	0.00	0.00	0.00	0.04	0.07	0.01	0.33
	(0.00)	(0.03)	(4.55)				
Lactobacillus salivarius	0.00	0.00	0.00	0.04	0.73	0.06	0.05
	(0.06)	(0.02)	(2.44)				
Halomonas hamiltonii	0.00	0.00	0.00	0.00	0.02	0.00	1.00
	(1.99)	(0.00)	(0.00)				
Haemophilus	0.00	0.00	0.00	0.03	0.08	0.17	0.01
haemolyticus	(0.25)	(0.00)	(0.78)				
Lactobacillus fermentum	0.00	0.00	0.00	0.00	1.00	0.01	0.03
	(0.00)	(0.00)	(0.69)				
Selenomonas noxia	0.00	0.00	0.00	0.02	0.18	0.01	0.46
	(0.51)	(0.03)	(0.01)				
Treponema socranskii	0.00	0.00	0.00	0.00	0.04	0.00	0.11
	(0.37)	(0.01)	(0.00)				
Lactobacillus vaginalis	0.00	0.00	0.00	0.04	0.41	0.08	0.04
	(0.12)	(0.00)	(0.24)				
Prevotella dentalis group	0.00	0.00	0.00	0.04	0.17	0.01	0.79
	(0.12)	(0.13)	(0.05)				
Cardiobacterium hominis	0.00	0.00	0.00	0.02	0.08	0.01	0.90
	(0.10)	(0.01)	(0.01)				
Haemophilus pittmaniae	0.00	0.00	0.00	0.02	0.77	0.00	0.04
	(0.10)	(0.06)	(0.08)				
Staphylococcus	0.00	0.00	0.00	0.00	0.20	0.00	0.02
epidermidis	(0.10)	(0.01)	(0.00)				
Selenomonas infelix	0.00	0.00	0.00	0.02	0.04	0.03	0.36
	(0.07)	(0.00)	(0.02)				
Treponema medium	0.00	0.00	0.00	0.04	0.11	0.02	0.91
	(0.10)	(0.01)	(0.03)				
Halomonas johnsoniae	0.00	0.00	0.00	0.00	0.08	0.00	1.00
	(0.13)	(0.00)	(0.00)				
Streptococcus	0.00	0.00	0.00	0.01	0.81	0.01	0.05
oligofermentans	(0.01)	(0.01)	(0.05)				
Eubacterium yurii	0.00	0.00	0.00	0.04	0.18	0.01	0.52
	(0.03)	(0.01)	(0.05)				
Treponema maltophilum	0.00	0.00	0.00	0.02	0.28	0.00	0.11
	(0.03)	(0.01)	(0.00)				
Prevotella enoeca	0.00	0.00	0.00	0.02	0.14	0.02	1.00
	(0.07)	(0.00)	(0.00)				
Cardiobacterium	0.00	0.00	0.00	0.02	0.14	0.02	1.00
valvarum	(0.07)	(0.00)	(0.00)				
Roseburia intestinalis	0.00	0.00	0.00	0.01	0.07	1.00	0.02
	(0.00)	(0.02)	(0.00)				

[a]Kruskal-Wallis H, [b]Mann-Whitney U test

3.11. Bacteria relative abundance was not associated with high serum antibodies level

To investigate the hypothesis of relative abundance and serum antibodies level to oral bacteria. Ss, Rm, Ph, and Pm oral bacteria were selected based on possessing identical or homologous AQPs/porins to human AQP5/1. The antibodies produced against these bacteria may cross react with human AQP5/1. Based on pyrosequencing data, Rm is sicca associated while Ph was significantly increased in SS. Relative abundance of Ss and Pm was not significantly different among the three different groups (Fig. 14A, left panel). Serum samples from SS (n=111) and HC group (n=53) were screened for the presence of antibodies to selected oral bacteria whole cell lysate using ELISA, for data analysis each group was sub-divided into anti-AQP5/1 positive and anti-AQP5/1 negative group (Fig. 14B, right panel). No significant difference was found in serum antibodies level between HC and SS patient to selected oral bacteria except for Rm where the anti-Rm serum antibodies titer was significantly decreased in SS. No association was found between the presence of auto-antibodies to AQP5/1 and high titer of serum antibodies level to selected oral bacteria.

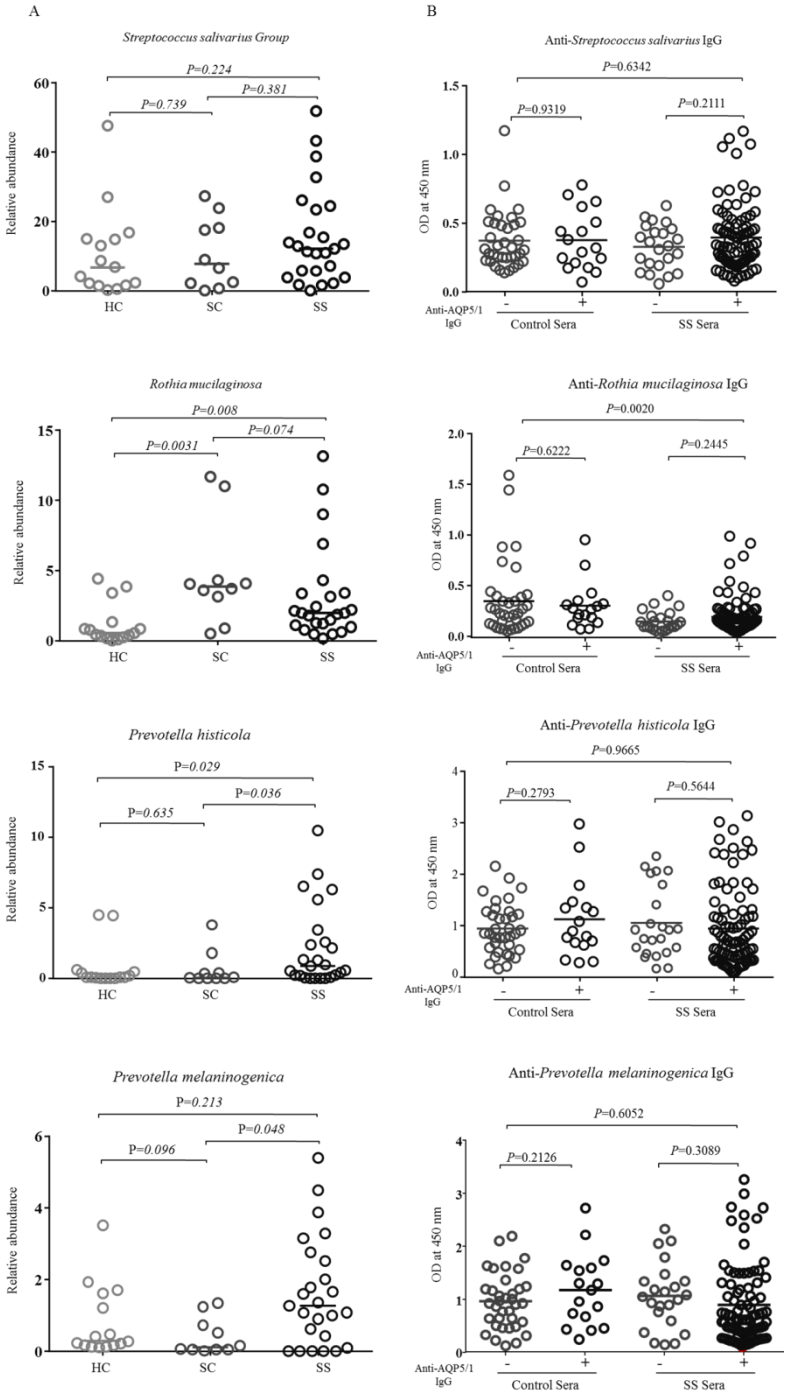


Fig. 14 Oral bacteria relative abundance and serum antibodies level. (A) Based on pyrosequencing data, the relative abundance of selected bacteria

including Ss, Rm, Ph and Pm was determined between HC, SC and SS. (B) Serum antibodies level to whole bacterial cell lysate was determined by ELISA. Significance between two groups was determined by Mann-Whitney U test.

3.12. Selected oral bacteria invade *in-vitro* into the HSG cells

Bacterial invasion has been suggested as the potential primary step to initiate the disease process. To determine the role of oral bacteria in the pathogenesis of SS; Ph a significantly increased bacteria in SS (Tab. 8) and Pm which was associated with increased SS risk were selected to test for their invasiveness to HSG cells. Moreover, Ec and Ss was also included, the former bacteria is negatively associated with SS risk while the latter is the major commensal bacterial specie increased in sicca symptoms. Fn was also chosen because of its high invasive ability among the several oral species with known invasive capabilities. HSG cells were co-cultured with CFSE-labelled bacteria for 6 and 24 hours and the invasion was determined by both flow cytometry, to quantify the internalized bacteria into HSG cells, the fluorescence intensity of adhered CFSE-labeled bacteria to the cell surface was quenched by trypan blue treatment, and the internalized bacteria was quantified by measuring the mean florescence intensity (MFI) using flow cytometry as shown (Fig. 15A). Fn are highly invasive into HSG cells followed by Pm, Ph, Ec and Ss respectively. Prolong incubation up to 24 hours further increased the bacteria invasion into HSG cells. The total numbers of different bacteria's internalized to HSG cells were compared with that of Fn (Fig. 15B). In addition, the internalization of bacteria's was further confirmed by confocal

laser scanning microscopy. CFSE labelled bacteria were found inside the cell boundary as shown (Fig. 15C).

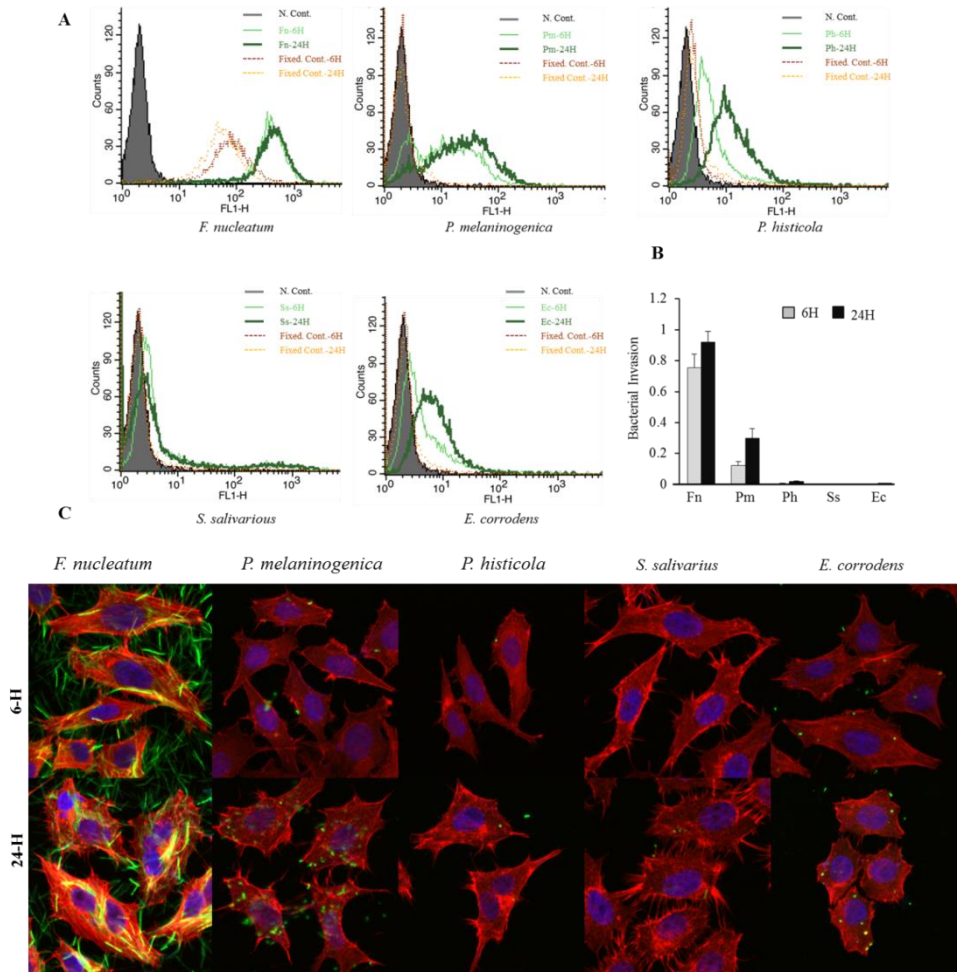


Fig. 15 Assessment of oral bacteria invasion into HSG cells by confocal microscopy and flow cytometry. A) HSG cells were co-cultured with CFSE-labelled bacteria at MOI 100 for 6 and 24 hours and analyzed by flow cytometry. Live HSG cells with internalized bacteria were overlaid over

negative control and fixed cells infected with bacteria. B) Adjusted MFI for the total numbers of different bacteria's internalized to HSG cells were compared with that of Fn. C) HSG cells ($1x10^5$ cells/well) were seeded onto cover slide in a 24-well plate with CFSE-labeled Fn, Pm, Ph, Ss and Ec at MOI 100 per cells for 6 and 24 hours. After fixation, the cells were stained with rhodamine-phalloidin and Hoechst 33342, and observed by confocal microscopy. The representative confocal images are shown (red: Actin fibers, blue: nucleus, and green: Stained bacteria inside the HSG cells) as merged images.

3.13. HSG cells remain viable after infection with selected oral bacteria

Next, the viability of HSG cells was examined after infecting with Fn, Pm and Ss or a co-infection. Fn slightly decreased cells viability at 6 hour but not at 24 hours. Interestingly, Pm slightly decreased the cells viability at 6 hours at MOI 50 in contrast the overall cells viability were increased after 24 hours. Ss slightly decrease cells viability after 24 hours infection only at MOI 100. This result indicates that overall HSG cells remain viable after infection with Fn, Pm and Ss (Fig. 16).

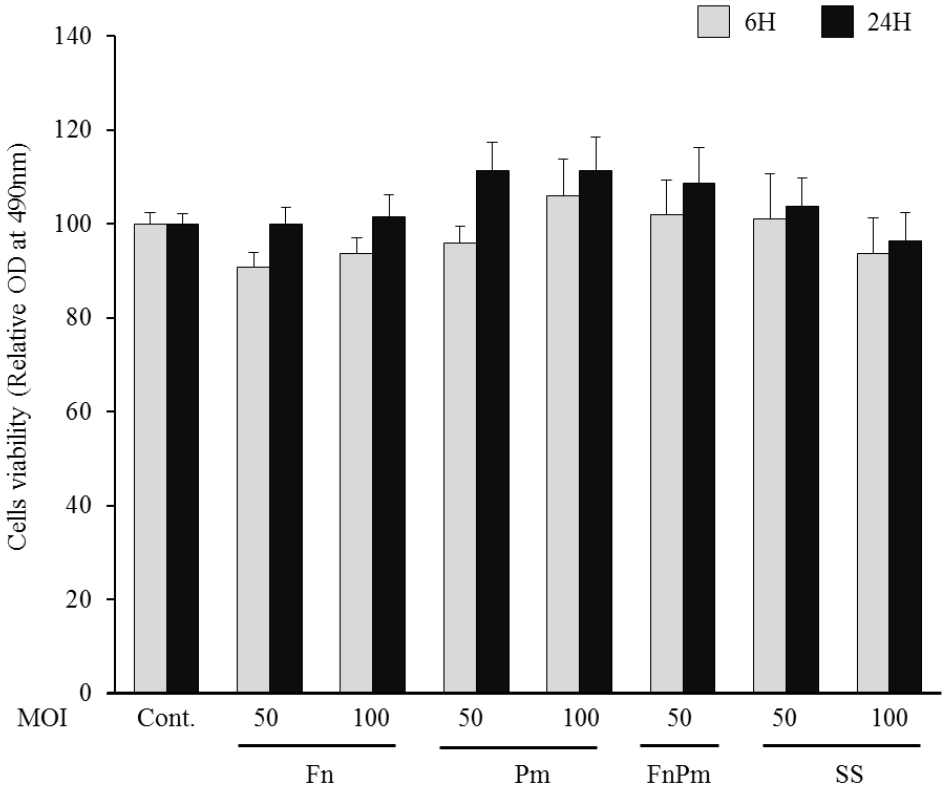


Fig. 16 HSG cells viability did not change after infection with selected oral bacteria. HSG Cells viability was assessed at 6 and 24 hours post-infection using Fn, Pm, Ss or a co-infection at MOI 50 and 100 using CCK-8 assay. The representative data of four independent experiments are shown as the mean ± SEM.

3.14. Induction of chemokines and cytokines by oral bacteria

Lymphocytic infiltration in salivary and lacrimal gland is the histopathological hall mark of SS pathology. Overexpression of proinflammatory cytokine IL-6, BAFF, CXCL-10 and CXCL-13 has been reported in salivary glandular specimens from patients with primary SS. To test whether bacterial invasion into HSG cells effect the expression of cytokines and chemokines, mRNA level of IL-6, BAFF, CXCL-10 and CXCL-13 was examined. Fn significantly up-regulated the mRNA level of IL-6 and CXCL-10 while significance for BAFF mRNA expression was achieved only at early time point of 6 hours at MOI 100. Further, a co-infection with Pm produced a synergistic effect on the production of CXCL-10 mRNA expression. Pm only significantly increased BAFF mRNA level at early time point of 6 hours and MOI 50. The increased mRNA level of IL-6 and CXCL-10 level was further confirmed by detection via ELISA (Fig. 17).

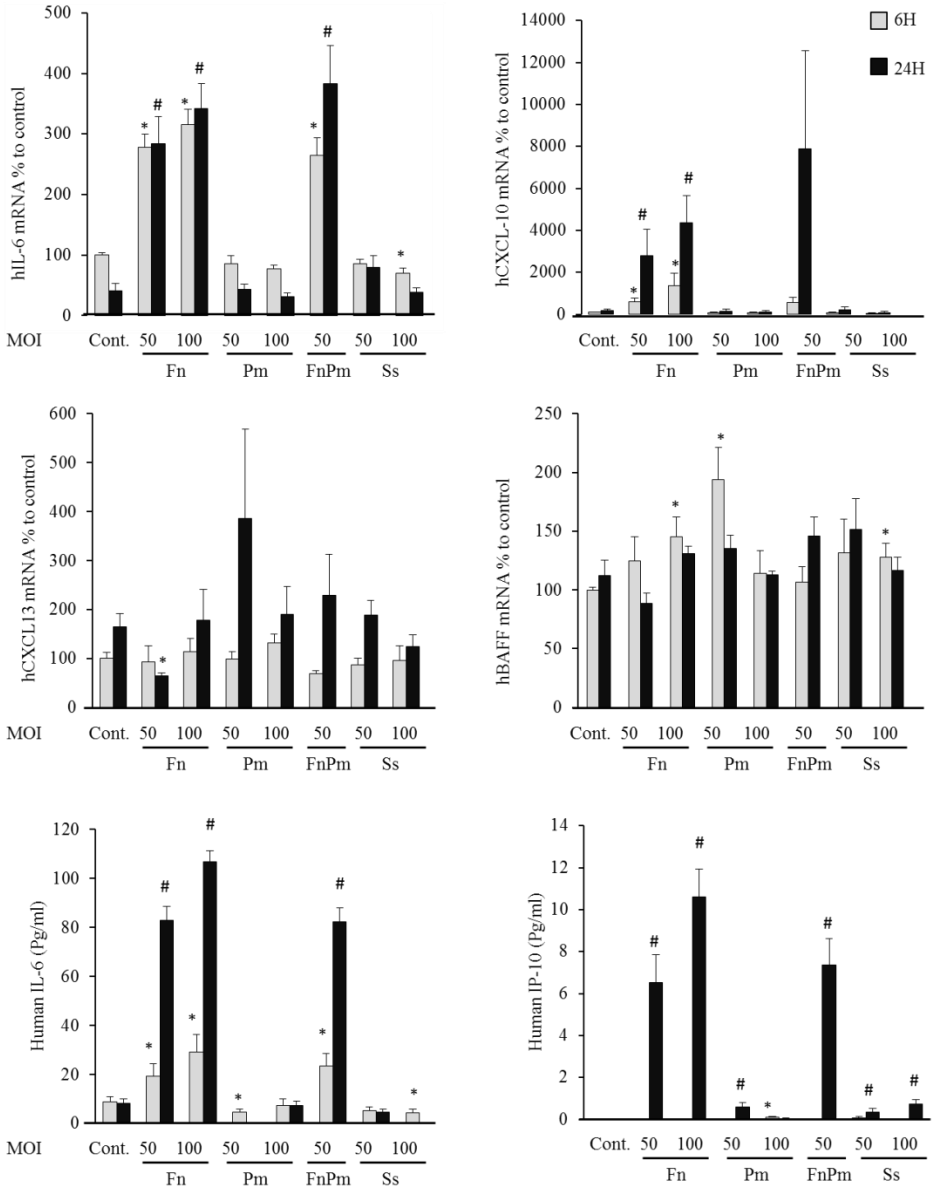


Fig. 17 Cytokines and chemokines were upregulated by selected oral bacteria in HSG cell. The mRNA level of IL-6, CXCL-10, CXCL-13 and BAFF in HSG cells were determined at MOI 50 and 100 after 6 and 24 infection. The mean ± SEM of four independent experiments, IL-6 and CXCL-10 RT-PCR data was further confirmed by ELISA.

3.15. Oral bacteria can produce sicca symptoms in experimental animal model

A number of preliminary animal experiments were performed to optimize the route and inoculum size of oral bacteria using C57BL/6 mice. In the final experiment, twenty mice were randomly divided into four groups; Sham, Fn, Pm and Fn-Pm groups, each mice was sublingually inoculated six times with $2x10^9$ selected oral bacteria (four times at one week interval and two booster doses) and pilocarpine stimulated salivary flow rate was determined after infection at two weeks interval (Fig. 18A). C57BL/6 mice showed progressive decline in salivary flow rate in the first four weeks in response to oral inoculation of Fn, Fn-Pm and Pm respectively. Fn group showed decrease in salivary flow rate in the first four week, a co-inoculation of Fn and Pm showed early and consistent decrease in saliva, while Pm alone showed a mild effect. However once bacterial inoculation was stopped or delayed to 2 weeks, salivary flow rate returned to normal, Interestingly Fn-Pm group showed consistent low salivary flow rate (Fig. 18A). To examine the extent of inflammation in salivary glands in response to oral bacterial inoculation, the mRNA level of proinflammatory cytokines IL6 and chemokines CXCL-10, CXCL-13 and BAFF was quantified by RT-PCR. Unexpectedly, IL-6, CXCL-10, CXCL-13 and BAFF were significantly downregulated by Pm. However, the effect of Fn are insignificant (Fig. 18B). Autoantibodies to AQP5 were also detected by IIFA using MDCK cells

expressing human AQP5. Human AQP5 is 91% identical to mouse AQP5 and autoantibodies can cross react with human AQP5, one mice from Fn group and two mice from Pm group developed cross-reacting autoantibodies to human AQP5 (Fig. 18C). Mouse salivary glands were also stained with hematoxylin and eosin and anti-CD3 antibodies for histo-morphologic changes and lymphocytic infiltration. Only mild lymphocytic infiltrations were seen in all three groups as compared to sham control (Fig. 18D).

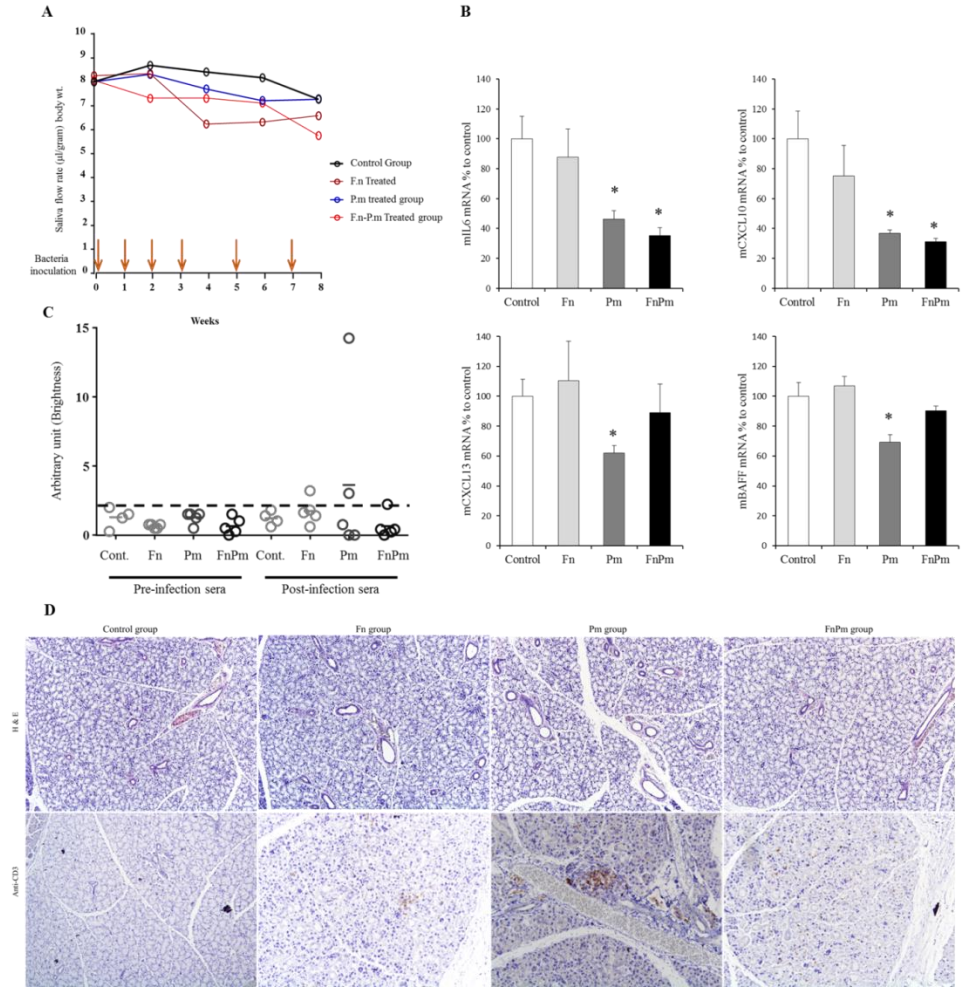


Fig. 18 Evaluation of SS-like character in C57BL/6 mice salivary glands after bacteria inoculation into oral cavity. C57BL/6 mice were inoculated with selected oral bacteria and saliva flow rate was determined by stimulating with intraperitoneal injection of pilocarpine **(**5μg/g) for 3 min, then whole saliva was collected for next 7 min. The saliva flow rate was calculated as microliter per gram body weight (A). The mRNA expression of IL-6, CXCL-10, CXCL-13, and BAFF was quantified in C57BL/6 mice

salivary glands after bacterial inoculation into the oral cavity, quantification was normalized to beta-actin and data were represented as mean ± SEM of the results from five mice per group (B). Detection of anti-AQP5 autoantibodies, cross reacting anti-AQP5 autoantibodies were detected by IIFA using MDCK cells overexpressing human AQP5 (C). C57BL/6 mice salivary glands were histologically examined by staining with hematoxylin and eosin (H & E) and anti-CD3 antibodies (D).

4. Discussion

4.1 Autoantibodies to human AQP5 were detected in Sera from patients with SS

The results of this study showed that autoantibodies against AQP5 are present in the sera of SS patients. Furthermore, patients with anti-AQP5 autoantibodies presented significantly lower resting salivary flow than those without the anti-AQP5 antibodies. This is the first report on the association between anti-AQP5 autoantibodies and SS. Like other AQPs, AQP5 consists of six transmembrane alpha-helixes connected by five loops, and the protein forms a water channel as narrow as 1-4 Å through the plasma membrane (Horsefield et al., 2008). If antibodies bind to the extracellular loops of AQP5, the antibodies may block the passage of water molecules through the channel. AQP5 is mainly localized to the apical membrane of acinar cells and intercalated ducts. AQP5 has also been found at the basolateral membrane of acinar cells in the mouse salivary glands (Larsen et al., 2011). However, the basolateral localization of AQP5 in normal human salivary glands has not been reported yet. Although dyslocalization of AQP5 from the apical to the basolateral sites of acinar cells in SS patients has been reported by two groups, the other two groups reported no difference in the subcellular localization of AQP5 between the normal and SS salivary glands (Beroukas et al., 2001; Gresz et al., 2015; Steinfeld et al., 2001; Xiao et al., 2011).

AQP5 located on the basolateral side can be accessed by anti-AQP5 autoantibodies present in the tissue fluid, while AQP5 present on the apical side of acinar cells can be accessed by anti-AQP5 autoantibodies present in saliva. Unexpectedly, the anti-AQP5 autoantibodies were also detected in the sera of many HC at low levels. Highly conserved AQPs are distributed throughout all the kingdoms of life, including bacteria. It has been shown that mouse immune serum rose against *E. coli* AQPZ react with human AQP4 (Ren et al., 2012).

When the bacterial protein database was BLAST-searched using the human AQP5 sequence as a query, AQPZ or porins from many human-associated bacteria had a high degree of homology with human AQP5 or AQP1. Therefore, there is a possibility that the autoantibodies against human AQP5 were developed during an immune response against bacterial proteins. While IIFA showed a clear difference in the levels of anti-AQP5 autoantibodies between the control and SS sera, and specifically detected anti-AQP1 autoantibodies in sera from patients with SS, immunoprecipitation was not effective in differentiating the control and patient samples. This discrepancy could be attributed to potential differences in the conformations and exposed epitopes present in membrane versus lysates. In this regard, the anti-AQP5 autoantibodies detected in the control and SS samples could have different effects such as inhibiting the function of

AQP5. Unfortunately, the salivary flow rates were available only for 10 control subjects, and the effect of the anti-AQP5 autoantibodies on salivary flow in the control group could not be evaluated. One of the limitations of the current study is a relatively small sample size. Therefore, further studies using samples from larger SS patient cohorts and diverse control subjects, including other autoimmune diseases, are needed.

The presence of anti-Ro/SSA and/or anti-La/SSB autoantibodies in serum is a diagnostic hallmark of SS (Lennon et al., 2004). A number of other autoantibodies such as anti-salivary gland protein 1, anti-carbonic anhydrase 6, anti-parotid secretory protein, anti-α-fodrin, anti-M3R, anti-nuclear and anti-smooth muscle antibodies have been identified in SS (Bournia & Vlachoyiannopoulos, 2012; Lee et al., 2013; Shen et al., 2012). Except for the anti-parotid secretory protein and anti-M3R antibodies, most autoantibodies target antigens that are normally present inside cells. Therefore, those autoantibodies reflect the apoptotic destruction of gland tissues, that is, a result of the disease process rather than the cause of the disease (Smith & Dawson, 2012). The degree of salivary dysfunction in SS patients does not correlate with the degree of glandular tissue destruction (Koo et al., 2008). Indeed, the presence of either anti-Ro or anti-La autoantibodies was not associated with salivary hypofunction in the current study.

In contrast, anti-M3R autoantibodies have the potential to interfere with the secretory process by inhibiting signaling through M3R and AQP5 translocation (Dawson et al., 2006; Koo et al., 2008; Lee et al., 2013). The binding of autoantibodies to M3R also down-regulates the receptors from the plasma membrane by inducing internalization (Jin et al., 2012). However, the functional data have not been reconciled with the sensitivity and specificity of the anti-M3R autoantibodies in screening trials (Smith & Dawson, 2012). Although anti-M3R autoantibodies have been detected in 9 to 100% of SS patients depending on the method and antigens used (Sumida et al., 2014), a recent meta-analysis study concluded that the anti-M3R antibody has high specificity (0.95) but relatively low sensitivity (0.43) to diagnose SS (Deng et al., 2015). In this aspect, the anti-AQP5 autoantibodies identified in the current study could complement anti-M3R autoantibodies.

In conclusion, anti-AQP5 and anti-AQP1 autoantibodies were detected in the sera of SS patients, which could be a novel biomarker of SS and provide new insight into the pathogenesis of SS. In addition, association between the presence of anti-AQP5 autoantibodies and resting salivary flow in SS patients suggests its potential as a biomarker that reflects disease activity.

4.2 The presence of anti-AQP1 autoantibodies was not associated with salivary flow rate

Herein, we reported the presence of serum autoantibodies against AQP1 in some patients with SS. The frequency of anti-AQP1 IgG or IgA (27.4%) detected in SS patients in the current study was higher than that of the anti-AQP1 autoantibodies reported in patients with suspected neuromyelitis optica spectrum disorders (Tzartos et al., 2013). In contrast to anti-AQP5 autoantibodies, for which the presence of anti-AQP5 IgA had overlapped with the presence of anti-AQP5 IgG, the presence of anti-AQP1 IgA coincided with the presence of anti-AQP5 IgA, rather than the presence of anti-AQP1 IgG. Similarly, the presence of anti-AQP1 IgG overlapped with the presence of anti-AQP5 IgG. Therefore, anti-AQP1 autoantibodies are likely to be a byproduct of an antibody response to AQP5. A significant positive correlation between the intensities of the two autoantibodies supports it. The amino acid sequence of AQP1 has 44% identity and 62% similarity with AQP5. In contrast to AQP5 in which the full lengths of loops B and E are exposed to the cytosolic and extracellular compartments, respectively, the middle portion of loops B and E in AQP1 is embedded within the membrane (Figure 9B). However, short stretches of conserved sequences were observed in loop B, loop D, and loop E, as well as the C-terminal cytoplasmic tail, which may provide cross-reactive epitopes.

Interestingly, anti-AQP1 autoantibodies were not detected in any control sera, although 32.1% of them were positive for anti-AQP5 IgG.

Two-thirds of the anti-AQP1 autoantibodies detected in patients with suspected neuromyelitis optica spectrum disorders were bound to the extracellular domain of AQP1 (Tzartos et al., 2013). If the anti-AQP1 autoantibodies detected in SS patients bind to the AQP1 expressed on myoepithelial cells and endothelial cells *in-vivo*, antibodies may block water flow across these cells, interfering with the inflow of water to the acini. However, the presence of anti-AQP1 autoantibodies did not affect either resting or stimulated salivary flow. This supports the notion that AQP1 does not play a major role in salivary secretion, as observed in AQP1-deficient mice (Delporte et al., 2016). The increase in salivary flow observed in SS patients after B-cell depletion may be attributed to the elimination of other autoantibodies, such as anti-M3R or anti-AQP5 autoantibodies.

In conclusion, anti-AQP1 autoantibodies were detected in some SS patients. Although anti-AQP1 autoantibodies are not useful as a diagnostic marker because of their low sensitivity, the presence of autoantibodies to AQP1 may be a major obstacle to AQP1 gene therapy for SS.

4.3 Epitope mapping of anti-AQP5 autoantibodies in the sera of SS patients

The result of this study showed that synthetic peptides corresponding to the extracellular loops of AQP5 neutralized anti-AQP5 autoantibodies in the sera of patients with SS but not those in control sera. Synthetic peptides have been used previously to determine the specificity of autoantibodies found in different autoimmune diseases, including anti-AQP4 (Kampylafka et al., 2011), anti-citrullinated α-enolase (Lundberg et al., 2008), anti-Ro (Frank et al., 1994; Routsias et al., 1996), anti-La (Tzioufas et al., 1997) and anti-T cell receptors (Landsperger et al., 1997). Screening anti-AQP5 IgG positive sera against these peptides revealed that IgG from SS patients were significantly neutralized by synthetic peptides, while control sera showed minimal reactivity. Although anti-AQP5 autoantibodies were present in both SS and control sera, this high reactivity of IgG from SS with synthetic peptides could be attributed to the antibodies in the control sera recognize epitopes in the helix or cytoplasmic loops. The highest inhibition was achieved by epitopes targeting loop-C in 9 samples and peptides targeting loop-E in 9 samples. Interestingly IgG from 6 SS patients (24%) recognized none of the synthetic peptides. No reactivity was found with irrelevant peptides (IRP). Although loop-C was probably the most strong epitope, autoantibodies to loop-E which make the pore for the transportation of water might have functional role in the pathogenesis of SS.

4.4 Dysbiosis of oral microbiota occurs in dry mouth condition

Pyrosequencing data revealed drastic changes in the bacterial communities associated with the dryness of mouth, although the bacterial communities of SC and SS were similar to each other, the relative abundance of species/phylotypes were different between SC and SS. The predominant taxas detected in HC in this study belonged to Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria was consistent with the previous reports (Crielaard et al., 2011; Huang et al., 2011; Zaura et al., 2009). When oral microbiota were compared at the phylum level, Firmicutes and Actinobacteria, two of the predominant phyla, were significantly more abundant in microbiota associated with dryness of mouth, while the proportion of Proteobacteria and Fusobacteria were significantly decreased. At genus level, *Streptococcus*, *Rothia* and *lactobacillus* were significantly increased in both SC and SS, whereas *Neisseria, Haemophillus, Fusobacterium, Porphyromonas*, and *Leptotrichia* along with other minor generas were significantly decreased in both SC and SS (Tab. 7). The decrease in *Fusobacterium* was unexpected in this study; however some previous reports suggested that *Fusobacterium* are more sensitivity to acidic milieu (Almstah et al., 2003; Bradshaw et al., 1989; Kuru et al., 2002; Leung et al., 2007).

At species level, comparison of relative abundance between HC, SC and SS were performed. Among the 70 species significantly changed in relative proportion in dry mouth, members of the normal flora or bacteria associated with oral health was significantly decreased in relative abundance in both SC and SS which include *H. parainfluenzae, N. subflava*, *N. perflava*, *S. sanguinis*, *Lautropia mirabilis*, *Campylobacter concisus* (Takeshita et al., 2009). In contrast a heterogeneous group of bacteria belongs to *streptococcus*, *Rothia mucilaginosa* and *Rothia dentocariosa* was significantly increased in dry mouth, while *Prevotella histicola* was specifically increased in SS (Table 6). The increase in *streptococcus* species in xerostomia could be due to lower pH buffer capacity associated with hyposalivation resulted in increased acid tolerant strain predominating the acid sensitive strains (Hayashi et al., 2015). Dysbiosis of oral microbiota has been characterized by the higher proportion of *Prevotella* and *Veillonella* and lower proportion of *Streptococcus, Neisseria, Haemophilus* and *Gemella* (Said et al., 2014). Current reports proposed a potential link between the *Prevotella* species and autoimmune diseases e.g. transmissible autoinflammation in murine intestine and rheumatic arthritis (Elinav et al., 2011; Scher et al., 2013; Scher et al., 2012). The strong female predominance especially in the postmenopausal women and the results from many animal models suggested the role of estrogen deficiency in SS (Forsblad-d'Elia et

al., 2009; Ishimaru et al., 2003; Ishimaru et al., 1999; Konttinen et al., 2012). Looijer et al. demonstrated reduced estrogen receptor mRNA expression and increased gut permeability (Looijer-van et al., 2011). Defect in epithelial barrier function trigger exocrine gland dysfunction and global immune activation (Yin et al., 2014). AQPS are the major water channel proteins expressed in the lacrimal and salivary glands, and have a critical role in tear and saliva secretion. Highly conserved AQPs are distributed throughout all the kingdoms of life, including bacteria. A multi sequence alignment of AQPs/porins from human and major species of oral microbiota confirmed high degree of homology. Considering changes in the oral microbiota composition and defects in epithelial barrier function, there is a possibility that cross reacting autoantibodies could develop against human AQP5 and AQP1 during an immune response to oral microbiota. However, when control and SS sera were screened for the presence of antibodies to commensal oral bacteria using whole bacteria cell lysate from Ss, Rm, Ph and Pm, no significant difference was found in serum antibodies level to commensal bacteria between HC and SS patient except for Rm where the anti-Rm serum antibodies level was significantly decreased in SS. No association was found between the presence of auto-antibodies to AQP5/1 and high titer of serum antibodies level to commensal bacteria. But in other studies high serum antibodies titer has been reported to *A.*

actinomycetemcomitans, *P. gingivalis* and *P. denticola* in patient with SS (Celenligil et al., 1998; Lugonja et al., 2016). Moreover, It has been shown that mouse immune serum rose against *E. coli* AQPZ react with human AQP4 (Ren et al., 2012). B cell epitope analysis showed the presence of conserved linear epitope in the extracellular loops which supports the notion that the extracellular loops may be the targets of cross reacting autoantibodies produced against bacterial proteins, and AQP5 and AQP1 as the culprit autoantigens in the development of SS.

4.5 Oral bacteria produced sicca symptoms in experimental animal

The present study demonstrated that Fn upregulated proinflammatory cytokine IL-6 and chemokine CXCL-10 from HSG cell and produced sicca symptoms in experimental mice model. SS has been characterized by lymphocytic infiltration and increased level of proinflammatory cytokines and chemokines (Hernandez-Molina et al., 2011; Moriyama et al., 2012; Tishler et al., 1999). Previously, viral DNA has been detected from salivary gland biopsies from patient with SS as a potential triggering agent, the role of oral bacteria in the pathogenesis of SS are not reported (Fox et al., 1986; Jeffers & Webster-Cyriaque, 2011; Saito et al., 1989). Several animal models confirmed the phenomenon of bacterial invasion followed by lymphocytic infiltration during disease process (Van et al., 2002). Defect in epithelial barrier function in SS could allow increased paracellular invasion of oral bacteria into tissues and persistent chronic inflammation. Indeed, bacteria will first encounter underlying tissues and immune cells, the effect of oral bacteria on salivary gland cells cannot be ignored. In this study we found that Fn and Pm efficiently invaded into HSG cells; further, Fn significantly upregulated the IL-6 and CXCL-10 in *in-vitro* condition. This observation suggested that Fn and Pm could effectively attract macrophages, T and B lymphocytes into the salivary glands. In experimental mice model, C57BL/6 mice showed a progressive decline in saliva flow rate in the first four weeks

in response to oral bacterial inoculation, however once the bacterial inoculation was stopped saliva flow return to normal, interestingly Fn-Pm group showed a consistent decrease in salivary flow rate throughout experiment. Salivary glands dysfunction as a result of bacterial infection has been previously reported in acute sialadenitis (Raad et al., 1990). Fn and Pm was isolated from blood culture of patients with acute salivary glands infection (Anthes et al., 1981; Brook, 2003). Sicca symptoms observed in the current study could be due to the fact that both Fn and Pm are gram negative bacteria and capable to invade into HSG cells, releasing bioactive bacterial components such as LPS could induce downregulation of AQP5 mRNA (Yao et al., 2010) and alteration in tight junction (Wang et al., 2016). To investigate the other possible underline mechanisms of salivary gland dysfunction, all mice were killed eight weeks after the first bacterial inoculation and mRNA level of proinflammatory cytokines and chemokines were quantified in salivary glands. Unexpectedly, Pm significantly down regulated IL-6, CXCL-10, CXCL-13 and BAFF. Mild lymphocytic infiltration was seen in all infected groups. Further, anti-AQP5 autoantibodies were detected in two mice from Pm and one mouse from Fn group. However, the presence of autoantibodies to AQP5 did not affect salivary flow rate of those mice.

This study has a limitation of using wild type mice, SS is a multifactorial autoimmune disease; both environmental and hormonal factors trigger exocrinopathy in individuals with genetic predisposition. To include the genetic basis of disease pathogenesis and disrupted epithelial barrier function; further study requires the use of knockout mice e.g. C57BL6 IκBζ-/- and B6.129S4-Id3tm1Zhu.

Although in this study we showed that selected oral bacteria Fn and Pm are capable to invade into HSG cells and produce sicca symptoms in experimental mice model. The exact role of these bacteria in the recruitment of lymphocytes, productions of autoantibodies, and salivary gland dysfunction require further investigation.

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초록

쇼그렌증후군의 병인에서 아쿠아포린 5 및 아쿠아포린 1와 대한 자가항체와 구강세균총의 잠재적인 역할

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배경

쇼그렌 증후군은 침샘과 눈물샘을 주요 표적으로 삼아 눈과 구강의 건조증을 유발하는 자가면역질환이다. 아쿠아포린은 동식물뿐만 아니라 세균과 원시세균에서 발현되는 작은 소수성 막관통 물채널 단백질이다. 인간의 침샘에서 아쿠아포린 5 (AQP5)와 아쿠아포린 1 (AQP1)의 mRNA 및 단백질 발현은 많은 연구에 의해 확인되었다. AQP1의 발현은 모세혈관 내피세피 및 근상피세포에 집중된 반면 AQP5는 눈물샘과 침샘의 포상세포와 개재도관에서 발현된다. 쥐에서 AQP5가 결손 되면 용량이 현저히 감소한 고장성타액을 분비하는 것으로 미루어보아 AQP5

는 타액 생산에 중요한 역할을 한다. 일부 구강세균의 아쿠아포린 또는 포린은 인간의 아쿠아포린과 높은 상동성을 보이고 있다. B 세포 항원결정부 예측 분석에 의하면 인간의 AQP5 및 AQP1과 세균 아쿠아포린의 세포외 루프에 진화적으로 보존된 선형 항원결정부가 존재해 세균 아쿠아포린을 표적으로 생성된 항체가 인간의 AQP5 및 1의 세포외 루프에 교차반응 해 물 분자의 진입을 차단할 가능성을 제시하였다. 또한 세균총의 부조화가 여러 자가면역질환을 유도할 수 있음이 여러 연구에서 보고되었다. 그러나 쇼그렌 증후군의 병인에서 구강세균총의 역할은 잘 알려지지 않았다. 이에 따라 본 연구의 목적은 쇼그렌 증후군의 병인에 있어서 인간 AQP5 및 아쿠아포린 1에 대한 자가항체와 구강 세균총의 잠재적인 역할을 연구하는 것이다.

재료 및 방법

원발성 쇼그렌 증후군 환자 혈청에서 항-AQP5/1 자가항체를 간접 면역형광분석법으로 검출하기 위해 마우스 턱밑 침샘의 동결 절편, 인간 AQP5-GFP 융합 단백질 또는 GFP를 발현하는 CHO 세포, 그리고 AQP5 및 AQP1를 과발현하는 MDCK 세포를 사용하였다. AQP5/1-GFP 융합

단백질 또는 GFP를 과발현하는 HEK-293 세포의 용해물질을 면역 침강에 사용했다. 또한, 인간 AQP5 세포 외 루프에 대응하는 합성 펩티드를 이용해 항-AQP5 자가항체에 대한 AQP5의 에피토프를 결정하였다. 건강한 대조군, 건조증 대조군으로 약물로 인한 구강건조증 환자, 그리고 쇼그렌 증후군 환자에서 구강세균을 수집해 세균 커뮤니티를 파이로시퀀싱으로 분석했다. 인간 침샘 (HSG) 세포를 선택한 구강세균과 함께 배양한 후 세균의 HSG 세포 침투와 HSG 세포에서 사이토카인 및 케모카인 발현을 관찰하였다. C57BL/6 마우스를 무작위로 대조군, Fn, Pm 및 Fn-Pm 4 그룹 (그룹 당 5 마리)으로 나누고, Fn, Pm 및 Fn-Pm 그룹은 각기 6 회에 걸쳐 2×10^9 세포의 *Fusobacterium nucleatum, Prevotella melaninogenica, F. nucleatum+P. melaninogenic* 세균을 설하에 투여 받았다. 각 마우스에 대해 2 주 간격으로 필로카핀에 의한 타액분비량을 측정하였고 8 주 차에 턱밑 침샘과 혈청을 수집해 사이토카인, 케모카인 발현 분석, 조직병리학적 관찰, 그리고 항-AQP5 자가항체 검출을 수행하였다.

결과

마우스 침샘의 포상세포에서 건강한 대조군의 혈청과는 달리 쇼그렌 증후군 환자의 혈청 IgG에 의한 염색이 AQP5을 표적하는 항체에 의한

염색과 중첩함을 확인하였다. AQP5-GFP를 발현하는 CHO에서도 환자 혈청의 IgG에 의한 염색이 GFP와 중첩함을 확인하였다. 그러나 대조군과 쇼그렌 증후군 환자 모두 혈청내 IgG가 AQP5-GFP를 면역침강 하였다. 따라서 대조군의 혈청에도 AQP5에 대한 자가항체가 존재함을 알 수 있었다. AQP5를 과발현 하는 MDCK 세포를 이용한 간접 면역형광 분석법으로 대조군 53 명과 쇼그렌 증후군 환자 112 명의 혈청을 검사했을 때 환자군에서 항-AQP5 IgG 자가항체가 유의하게 높은 수준으로 검출되었고, 검사는 0.73의 감도와 0.68 특이성을 보였다. 항-AQP1 자가항체는 16.07%의 쇼그렌 증후군에서만 발견되었다. 또한 항-AQP5 자가항체의 존재가 쇼그렌 증후군 환자에서 낮은 비자극 타액 분비율과 유의한 연관을 보인 것과 달리 항-AQP1 자가항체의 유무는 타액 분비율과 연관이 없었다. AQP5 세포 외 루프에 대응하는 합성 펩티드는 쇼그렌 증후군 환자 혈청의 항-AQP5 자가항체가 AQP5에 결합하는 것을 크게 억제하였다. 구강세균총 분석 결과 구강건조증과 연관된 것으로 보이는 큰 변화가 관찰되었다. 구강건조증 대조군과 쇼그렌 증후군 환자의 세균 군집이 서로 비슷했지만, ~ 종/phylotypes에서는 두 그룹 간에 상대적인 양의 유의한 차이를 보였다. 로지스틱 회귀 분석 결과 *P. melaninogenica*의 증가가 쇼그렌 증후군 위험 (OR 5.396/1% 증가, 95% CI 1.793 - 16.238, $P = 0.003$)과 연관이 있었다. Pm은 AQP5와 높은 상동

성을 갖는 포린 단백질을 갖고 있고 *F. nucleatum*은 알려진 구강세균 종 중 높은 침투 능력을 가지고 있기 때문에 두 세균종을 세포 실험 및 동물 실험을 위해 선택하였다. HSG 세포는 *F. nucleatum*에 대한 반응으로 용량 의존적인 양식으로 염증성 사이토카인 IL-6와 케모카인 CXCL-10을 생성하였으나 *P. melaninogenica*은 그러한 반응을 유도하지 않았다. 또한 C57BL/6 마우스에 *F. nucleatum*과 *P. melaninogenica*를 설하 접종했을 때 일부 마우스에서 침샘에 T 세포의 약한 침윤을 동반하는 쇼그렌 증후군-유사 증상이 관찰되었다.

결론

결론적으로, AQP5에 대한 자가항체와 구강세균총의 변화가 쇼그렌 증후군의 병인에 기여할 가능성이 있다. 쇼그렌 증후군 환자의 혈청에서 검출된 항-AQP5 자가항체는 쇼그렌 증후군의 신규 생체표지자가 될 수 있으며 질병의 병인에 새로운 통찰을 제공할 것이다.

키워드 : 쇼그렌 증후군, 자가항체, 아쿠아포린 5, 아쿠아포린 1, *Fusobacterium nucleatum*, *Prevotella melaninogenica*, 파이로시퀀싱 분석

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