Aquaporin gene therapy corrects Sjögren's syndrome phenotype in mice

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Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease that is estimated to affect 35 million people worldwide. Currently, no effective treatments exist for Sjögren's syndrome, and there is a limited understanding of the physiological mechanisms associated with xerostomia and hyposalivation. The present work revealed that aquaporin 5 expression, a water channel critical for salivary gland fluid secretion, is regulated by bone morphogenetic protein 6. Increased expression of this cytokine is strongly associated with the most common symptom of primary Sjögren's syndrome, the loss of salivary gland function. This finding led us to develop a therapy in the treatment of Sjögren's syndrome by increasing the water permeability of the gland to restore saliva flow. Our study demonstrates that the targeted increase of gland permeability not only resulted in the restoration of secretory gland function but also resolved the hallmark salivary gland inflammation and systemic inflammation associated with disease. Secretory function also increased in the lacrimal gland, suggesting this local therapy could treat the systemic symptoms associated with primary Sjögren's syndrome.

aquaporin | gene therapy | Sjögren's syndrome

Despite being one of the most frequent autoimmune disorders diagnosed in the United States and a common form of chronic xerostomia, the etiology of primary Sjögren's syndrome (pSS) is still unclear (1–5). Although immunomodulatory treatments have proven effective for other autoimmune diseases, such as rheumatoid arthritis, only some of these biologics, such as rituximab, are reported to result in mild improvement in extraglandular or systemic symptoms in pSS (6). None of the evaluated immunomodulatory drugs resulted in the persistent restoration of salivary gland secretion. Furthermore, there is little understanding of the molecular changes in epithelial cell function associated with pSS.

Elevated proinflammatory cytokine expression is often associated with pSS (7). Although some may be produced by the infiltrating immune cells, much is produced by the epithelial cells of the gland and acts to attract the infiltrating cells. In patients with pSS, B-cell activation factor (BAFF) levels are elevated in the serum, saliva, and exocrine glands (8). Moreover, intracellular therapies targeting BAFF expression in animal models of Sjögren's syndrome have resulted in decreases in immune activation as well as recovery of gland activity (9).

Increased expression of bone morphogenetic protein 6 (BMP6) was also reported in the epithelia of patients with Sjögren's syndrome (10). Overexpression of BMP6 induced salivary gland hypofunction and increased lymphocytic infiltrates within the salivary glands of mice. Unlike other cytokines, such as BAFF, which is primarily thought of as an immune activation protein, BMP6 is linked to a wide variety of effects depending on the tissue in which it is expressed. BMP6 has emerged as a key regulator of iron metabolism in the liver through the expression of hepcidin. BMP6-null mice develop massive iron overload in the liver, similar to the severe childhood-onset forms of human hemochromatosis (11).

A role for BMP6 in autoimmunity is just emerging. Elevated BMP6 expression in the skin of mice has been reported to induce a psoriasis-like condition (12). Increased BMP6 was also reported in 60% of patients with pSS (10). In an animal model of pSS, elevated BMP6 was also detected and linked to the induction of xerostomia and an increase in infiltration in the gland. Interestingly, autoantibody formation and elevated proinflammatory cytokine expression were not associated with BMP6 overexpression (10).

In addition to patients with pSS, chronic xerostomia is associated with patients with head and neck cancer who have undergone ionizing radiation therapy for treatment of their tumors. Like patients with pSS, these individuals experience a marked decline in quality of life (13, 14). Although the ductal cells are still functional and can still generate an osmotic gradient (lumen > interstitium), the acinar cells are highly radiosensitive and represent the only water-permeable portion of the gland. The acinar cells are therefore central to the initiation of fluid movement in secretion. A therapy to restore fluid movement has involved altering the water permeability of the remaining epithelium by expression of the unregulated, polarization-independent water channel aquaporin 1 (AQP1) (15). Several animal models, as well as a phase 1 clinical trial, have demonstrated that gland activity could be restored by expression of AQP1 (16).

Unlike radiation-induced xerostomia, the mechanism associated with the BMP6-induced xerostomia in pSS is unclear. To identify the

Significance

Recent reports of increases in the prevalence and incidence of autoimmune diseases make this disease group a pressing public health concern. Patients suffering from Sjögren's syndrome experience debilitating oral and ocular dryness due to dysfunction within the salivary and lacrimal glands. Due to our lack of knowledge regarding the underlying mechanisms, no effective treatments are available and affected organs gradually degenerate. In this study, we identify the loss of water permeability as a mechanism associated with xerostomia in a subset of patients. We demonstrate that a novel therapy, aquaporin 1 replacement, can increase the water permeability of the gland and restore fluid movement while relieving the dry mouth and eye phenotypes associated with this disease in addition to disease-associated inflammation.

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downstream targets of BMP6, we mapped the BMP6-responsive transcriptome and identified a decrease in aquaporin 5 (AQP5) expression as a critical factor in salivary fluid movement both in patients with Sjögren's syndrome and in mice that overexpress BMP6 in the salivary glands. Expression of a constitutively active water channel, AQP1, in the salivary glands of mice with a Sjögren's-like phenotype restored fluid movement, as well as decreasing the proinflammatory immune response associated with this disease.

Results

Unstimulated Saliva Flow Is Negatively Correlated with BMP6 Expression in Patients with Sjögren's Syndrome. Previous work reported the increase in BMP6 expression in the minor salivary glands (MSGs) of patients with pSS but did not observe any correlation with the extent of lymphocytic infiltration observed in the gland (10). To study the association of BMP6 expression with the clinical symptoms associated with Sjögren's syndrome further, cDNA was prepared from the MSGs of both healthy volunteers (HVs) and patients with pSS (n = 12 and n = 11, respectively) who met the America-European Consensus Group Criteria (Table S1). The expression of BMP6 was quantified relative to GAPDH by ΔΔCT (Delta Delta Ct) and expressed as fold change relative to the average expression in HVs (Fig. S1). In agreement with previous work, Spearman rank correlation did not identify a significant correlation of BMP6 expression with any of the immunological features often associated with pSS, including IgG, Complement (C)3, C4, anti-Ro/SSA (SSA), anti-La/SSB (SSB), and antinuclear antibody (ANA) levels (Spearman r = 0.3363, r = 0.3404, r = 0.3649, r = −0.0966, r = −0.3652, and r = −0.2489, respectively; $P > 0.05$). However, there was a significant correlation between unstimulated saliva flow and BMP6 expression of the patients, both with a linear and nonlinear response (Spearman r = −0.7577, P = 0.0031, R^2 = 0.88). These data suggest a mechanistic link between BMP6 expression in the salivary glands and the reduced salivary secretion in patients with pSS.

BMP6 Inhibits AQP5 Expression. Initiation of fluid secretion in the salivary gland requires movement of water and ions through their respective channels in the acinar cells (reviewed in ref. 17). AQP5 is the main water channel for salivary fluid secretion (reviewed in ref. 18). AQP5 down-regulation in patients with Sjögren's syndrome has been observed by microarray analysis of MSGs from patients with pSS, and altered distribution of the channel has been described by histological studies in patients and mice overexpressing BMP6 (10, 19). In addition, the increased expression of AQP5 has been described in patients with pSS successfully treated with rituximab, suggesting a central role for this protein in gland recovery (20). These findings suggested that BMP6 could inhibit salivary gland function by inhibiting expression of AQP5. A change in AQP5 expression in response to BMP6 was confirmed by treating human salivary gland (HSG) cells with recombinant BMP6. The fluorescent signal of AQP5 protein in the membrane of BMP6-treated HSG cells was reduced 2.5-fold compared with untreated cells (14.9 ± 2.8 vs. 37.7 ± 10.1, respectively; $P < 0.01$) (Fig. 1*A*). No effect on actin expression was detected (Fig. 1*A*). These findings would suggest a connection between BMP6-mediated signaling and AQP5 expression.

AQP5 Is Down-Regulated in Patients with Sjögren's Syndrome Who Overexpress BMP6. The relationship between BMP6 and AQP5 expression in patients was investigated by confocal imaging. MSGs from seven patients with pSS with low saliva flow (unstimulated salivary flow ≤ 1.5 mL over 15 min) and two HVs was examined by immunofluorescent analysis for their relative BMP6 and AQP5 expression following a sample size calculation (www.stat.ubc.ca/~rollin/stats/ssize/n1.html). In the five patients with pSS who overexpressed BMP6, AQP5 was decreased in expression compared with patients who had low levels of BMP6 expression or tissue from healthy controls across the entire gland (Fig. S2) and in

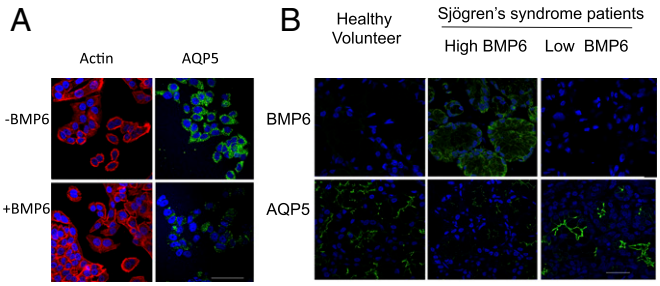

Fig. 1. BMP6 inhibits AQP5 expression in patients with Sjögren's syndrome and HSG cells. (*A*) HSG cells were cultured without (*Upper*) or with (*Lower*) 6 ng/mL BMP6 for 3 d. Actin expression was detected with phalloidin conjugated to TRITC (red fluorescence). AQP5 expression was detected by conjugation of a specific antibody to AQP5 with FITC (green fluorescence). (*B*) Expression of AQP5 in MSGs of patients with pSS with high BMP6 (n = 5) and low BMP6 (n = 2) expression compared with tissue from HVs (n = 2). (Scale bar, 50 μm.)

representative magnified images (Fig. 1*B*). This finding further supports a connection between BMP6 expression and the loss of gland function.

BMP6-Induced Loss of Cellular Water Permeability Is Associated with AQP5 Down-Regulation. AQP5 is a critical protein in regulating water movement across the cellular membrane. To identify if other proteins involved in salivary gland function and membrane water movement were changing in response to BMP6 treatment, we developed an in vitro fluid movement assay in which HSG cells were subjected to hypotonic stress by the addition of hypoosmotic media initiating cell volume increase. The cells respond by triggering a regulatory volume decrease (RVD) to their original volume. Gene expression changes as a result of BMP6 treatment could be identified by isolating mRNA posttreatment and globally analyzing cellular gene expression by microarray hybridization.

As reported for other TGF-β family members, addition of recombinant BMP6 to cells induced a biphasic response in the RVD assay. The recovery rate of RVD (Fig. 2) was significantly inhibited by treatment with 6 ng/mL BMP6 (4.2 ± 2.8%, n = 3; $P < 0.001$) compared with controls. However, little inhibition was observed by treatment with 0.1 ng/mL (57.7 ± 19.2%, n = 3; P = 0.07), and no inhibition was found at the supraphysiological level of 150 ng/mL (82.5 ± 12.3%, n = 3; P = 0.20) compared with controls. mRNA isolated from the treated cells was labeled and used to probe a high-density Agilent 4 × 44,000 microarray. This experiment identified several different patterns of gene expression at the different doses of BMP6. The most significant change in gene expression was observed at a dose of 6 ng/mL (Fig. S3 *A* and *B*). Several of these gene expression changes were verified by quantitative PCR (Fig. S3*C*). Furthermore, these gene expression changes also correlated with differential gene expression observed in pSS (10).

We used the diversity in gene expression patterns associated with BMP6 addition to identify correlations between the physiological RVD data and cellular gene expression. In addition to the three sets of microarray expression data from the BMP6-treated HSG cells, a fourth microarray set obtained by comparing the gene expression patterns from the salivary glands of mice treated with BMP6 in vivo by adeno-associated virus (AAV)-mediated gene transfer was included in the gene expression database to increase the statistical power of the correlative analysis. A total of 12,315 differentially expressed gene patterns were compared with the observed change in RVD, and only the change in AQP5 expression was found to have a statistically significant match to the observed change in RVD (Pearson correlation analysis: coefficient value = 0.97, P = 0.02) (Table S2). Although TNFRSF2, EGR1, and NKRF expression changed in response to BMP6 and have been previously implicated in autoimmune disease, their change in

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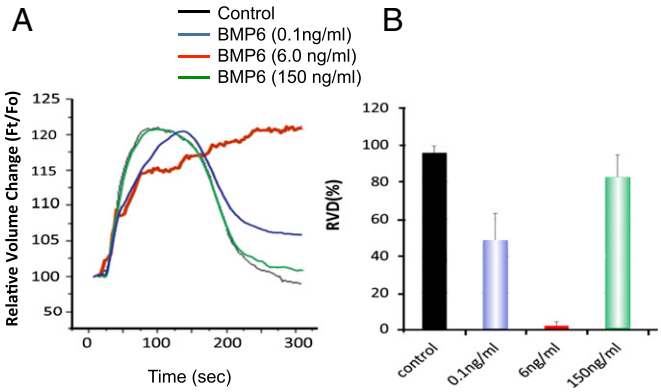

Fig. 2. BMP6 inhibits water permeability of HSG cells. (*A*) Cells were placed in the hypotonic solution following culture without (black line) or with (blue line, 0.1 ng/mL; red line, 6 ng/mL; green line, 150 ng/mL) different concentrations of BMP6. Ft/Fo, cell volume change calculated on fluorescence intensity base on 100% recovery rate of the cell volume. (*B*) Dosage response curve of BMP6 induces cell volume change. The 6-ng/mL dose shows significant inhibition of recovery of cell volume change ($n = 3$). Data are presented as mean ± SEM.

expression was not statistically significant in correlation to the change in RVD ($P > 0.05$).

Rescue of the BMP6-induced loss of RVD in HSG cells was demonstrated by transfection of AQP5 encoding plasmids into HSG cells pretreated with BMP6. Expression of this protein resulted in a dose-dependent increase in RVD. Transfection with 0.1 μg of AQP5 encoding plasmid resulted in a 16.9 ± 9.6% ($n = 3$) recovery of RVD compared with the control group treated with BMP6 alone. Transfection with 0.5, 1.0, or 3 μg of AQP5 resulted in a 40.5 ± 7.4%, 61.6 ± 6.1%, and greater than 90% recovery of RVD, respectively, that was statistically significant ($P < 0.05$) compared with the BMP6 alone treatment control group ($n = 3$). These data support our hypothesis that a critical defect in fluid movement induced by BMP6 is related to a change in membrane water permeability (Fig. 3 *A* and *B*) as a result of a loss of AQP5 expression.

AQP1 Gene Therapy Restores Salivary Gland Fluid Movement in BMP6-Overexpressing Mice. A recent clinical trial demonstrated that expression of AQP1 in the remaining salivary gland cells of patients with radiation-induced xerostomia can bypass the dysfunctional acinar cells and initiate saliva flow by changing water permeability (16). AQP1 is an archetypical water channel with membrane-independent polarity, selected for use in gene therapy due to the channel's ability to induce water flow in a polarity-independent manner. Based on our above findings of a BMP6-induced down-regulation of AQP5 expression and loss of water permeability, we hypothesized that expression of AQP1 (an unregulated water channel that will sort to both the apical and basolateral membranes) via AAV vectors would create a new pathway for water to flow through the cell membrane due to its polarization-independent membrane trafficking. We first confirmed that expression of AQP1 could restore fluid movement in our in vitro assay as was observed with AQP5. Expression of AQP1 in BMP6-treated HSG cells also resulted in a dose-dependent increase in RVD (Fig. 3 *C* and *D*). Transfection with 1.0 or 3 μg of AQP1-encoding plasmid resulted in a statistically significant recovery of 54 ± 7.5% and 94.5 ± 6.4%, respectively ($n = 3$; $P < 0.01$), compared with the BMP6 treatment alone group.

Before testing gene therapy with AAV2-AQP1 in vivo, salivary gland hypofunction was induced in C57BL/6 mice by transducing with AAV vectors encoding BMP6 as previously reported (10). Four weeks postcannulation with the AAV-BMP6 vector, pilocarpine-stimulated salivary gland activity had decreased by over twofold compared with AAV5-GFP–treated control mice [AAV5-GFP: $n = 9$, salivary flow rate (SFR) = 6.456 ± 0.4913 vs. 2.907 ± 0.4598 μL/g body weight in 20 min of AAV5-BMP6, $n = 15$]. To test the ability of AQP1 gene therapy to restore salivary gland activity, the AAV5-BMP6–treated mice were then randomly divided into two groups and treated with an AAV2 vector encoding either AQP1 or GFP. Four weeks after AAV2 transduction, the AAV2-AQP1–treated mice showed a statistically significant increase in SFR compared with the AAV2-GFP control group (AAV2-AQP1: $n = 7$, SFR = 5.186 ± 0.4228 vs. AAV2-GFP: $n = 8$, SFR = 3.063 ± 0.6808 μL/g body weight in 20 min) (Fig. 4*A*). This finding suggests AQP1 gene therapy can restore fluid movement in a murine model of BMP6-induced hypofunction.

AQP1 Gene Therapy Restores Salivary and Lacrimal Gland Fluid Movement in a Murine Model of Sjögren's Syndrome. Previous studies showed that BMP6 expression is increased in the salivary glands of nonobese diabetic (NOD) mice that develop a pSS-like condition (10). To investigate whether AQP1 gene therapy may restore gland activity in a mouse model of pSS, an AAV2-AQP1 vector was delivered via cannulation into the submandibular salivary glands of the NOD-derived C57BL/6.NOD-*Aec1*/*Aec2* mice with established disease (30 wk of age). AQP1 expression by gene transfer was confirmed at the time of tissue collection (Fig. 4*B*).

In AAV2-AQP1–treated mice, salivary gland activity increased by 4 wk postcannulation of the vector compared with control-treated mice that persisted until the end of the study (16 wk postcannulation salivary flow = 147 ± 45 vs. 28 ± 29, respectively; $n = 5$; $P < 0.05$) (Fig. 4*C*). Surprisingly, an increase in lacrimal gland activity was also observed in the mice, suggesting this localized therapy in the salivary gland was able to initiate a systemic effect (Fig. 4*D*). At 6 wk postcannulation, lacrimal gland activity had increased in the AAV2-AQP1–treated mice compared with controls to 20.3 ± 11.0 ($n = 9$) vs. 8.0 ± 10.7 ($n = 7$) μL over 10 min, respectively ($P < 0.05$). This increase in lacrimal gland activity persisted until the end of the study at 16 wk postcannulation.

Aquaporin Gene Therapy in the Salivary Gland Decreases Local and Systemic Inflammation. Like patients with pSS, C57BL/6.NOD-*Aec1*/*Aec2* mice develop an autoimmune phenotype with localized inflammation in the secretory epithelia and increased production of

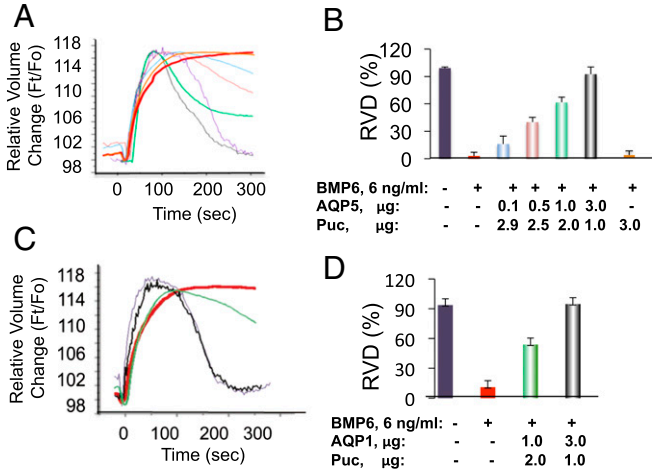

Fig. 3. Aquaporins restore water permeability in BMP6 treated cells. HSG cells were placed in the hypotonic solution following culture without (purple line) or with 6 ng/mL (red line) BMP6 and then transfected with increasing amounts of DNA encoding AQP5 (*A* and *B*) or AQP1 (*C* and *D*), and the recovery of RVD was measured. No increase in recovery of RVD activity was observed with transfection of pUC19 (Puc) alone (*A* and *B*, orange line). Increasing recovery of RVD was observed with increasing amounts of both AQP5 and AQP1. (*B* and *D*) Maximal percent of RVD was determined compared with HSG cells cultured with media alone. Data are presented as mean ± SEM.

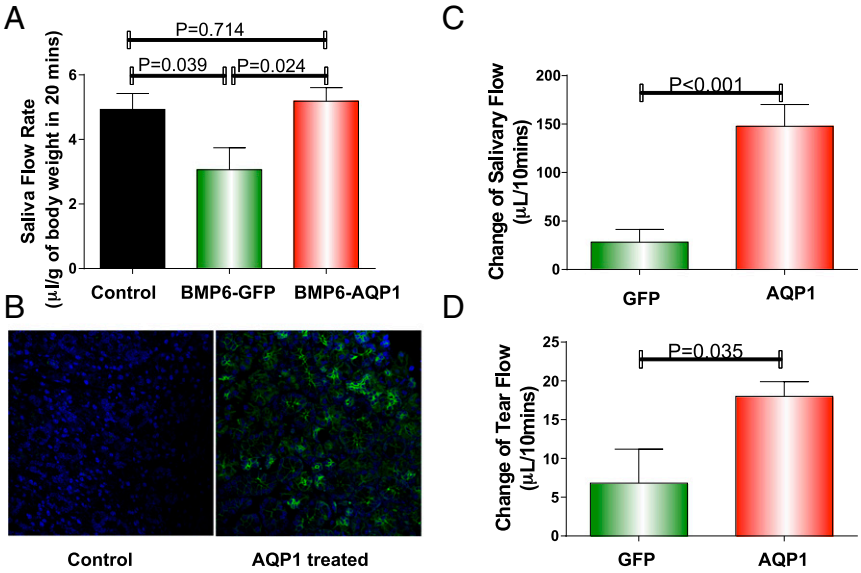

Fig. 4. AQP1 restores fluid secretion in mouse models of Sjögren's syndrome. (*A*) Six- to eight-week-old female C57/B6 mice were locally treated with AAV5-BMP6 vector delivered via cannulation to the submandibular gland. After 4 wk, mice were randomly divided into two groups and treated with either an AAV2 vector encoding GFP ($n = 8$) or AQP1 ($n = 7$). Four weeks after AAV2-GFP or AQP1 transduction, the pilocarpine-stimulated SFR was measured and compared with an age- and gender-matched control group of C57/B6 mice ($n = 9$). SFR is adjusted to body weight due to variability at this young age. (*B*) AQP1 immunofluorescent staining of the submandibular gland tissue obtained from controls or mice treated with AAV2-AQP1 vector. Images are from representative mice. (Magnification, 40×.) (*C*) Forty-six-week-old C57BL/6.NOD-*Aec1*/*Aec2* mice were measured for stimulated salivary gland activity 16 wk after vector delivery of an AAV2 vector encoding either GFP or AQP1 ($n = 4$ per group). (*D*) Change in pilocarpine-stimulated tear flow in C57BL/6.NOD-*Aec1*/*Aec2* mice treated with either AAV2-GFP or AAV2-AQP1 at 18 wk after vector delivery ($n = 4$ per group). C57BL/6.NOD-*Aec1*/*Aec2* mouse saliva and tear volumes are relative to the baseline value of the mouse before treatment with vector. All data are mean ± SEM and analyzed by an unpaired Student *t* test.

autoantibodies and proinflammatory cytokines, such as IFN-gamma (IFN-γ) (21–23). Changes in the local and systemic immune system in the treated C57BL/6.NOD-*Aec1*/*Aec2* mice were investigated by collection of salivary glands, lacrimal glands, and serum at the termination of the study. No significant changes in serum levels of anti-Ro/SSA, anti-La/SSB, or ANA were observed between the AAV2-AQP1– and AAV2-GFP–treated groups. Furthermore, using flow cytometry, we did not detect a change in the $B220^+$ populations within the salivary glands (AAV2-GFP: 1.7 ± 0.2% and AAV2-AQP1: 1.8 ± 0.3%; $P = 0.6250$). In contrast, a significant decrease in $CD3^+CD4^+$ T cells and in $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}17^+$ T cells in the AAV2-AQP1–treated mice was detected compared with the AAV2-GFP–treated mice ($n = 2$ per group). In addition, there was a trend toward an increase in the IL-10–producing $CD4^+CD25^+Foxp3^+$ T regulatory-cell population in the AAV2-AQP1–treated mice compared with the AAV2-GFP–treated mice, but the increase was not statistically significant (AAV2-GFP: 21.65 ± 0.1% and AAV2-AQP1: 23.9 ± 3.6%; $P = 0.4793$). These data suggest that expression of AQP1 in salivary gland cells could decrease the epithelial and T-cell proinflammatory immune response associated with pSS (Fig. 5 and Table S3).

Changes within the systemic immune system were evaluated by multiplex cytokine analysis of the serum from the AAV2-AQP1–treated mice compared with the AAV2-GFP control group. A significant decrease in IFN-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-9, IL-2, IL-13, IL-6, IL-4, and granulocyte macrophage colony-stimulating factor (GM-CSF), as well as chemokines, including keratinocyte chemoattractant, eotaxin, and macrophage inflammatory protein-1 beta (MIP-1β), was detected in the AAV2-AQP1–treated mice compared with the AAV2-GFP control group (Table 1). Similar to the finding within the salivary gland, the production of IFN-γ decreased in the AAV2-AQP1–treated mice compared with the AAV2-GFP group, although the change was not statistically significant (AAV2-GFP: 969 ± 365 pg/mL and AAV2-AQP1: 537 ± 65 pg/mL; $P = 0.0569$). Thus, an overall down-regulation in proinflammatory cytokines and chemokines in the systemic immune system occurs following restoration of fluid movement.

Discussion

Global transcriptome analysis through the use of high-density cDNA microarrays has permitted the investigation of gene expression patterns within a cell or tissue and enabled comparison between populations to identify changes in both broad regulatory networks and specific genes associated with cell function. To test our hypothesis that BMP6-induced loss of cell volume regulation could be linked to a particular target gene within the cell, we used a correlation analysis similar to the program COMPARE, which determines the similarity

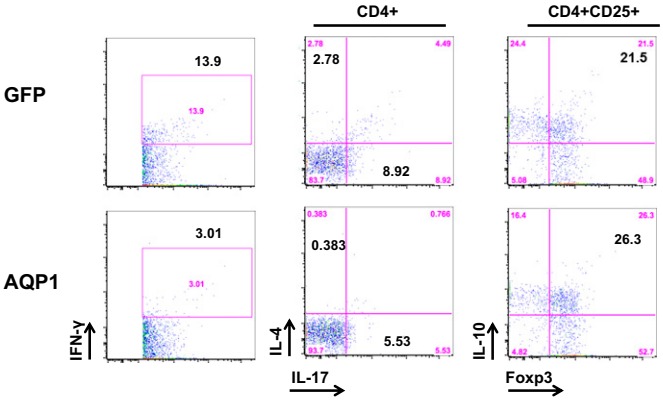

Fig. 5. Aquaporin expression in salivary glands inhibits inflammation. The lymphocytes isolated from submandibular salivary glands of AAV2-AQP1– or AAV2-GFP–treated C57BL/6.NOD-*Aec1*/*Aec2* mice were analyzed by flow cytometry assay for the different populations of cells ($n = 2$ per group). Data show one representative experiment. A statistical data analysis on all of the analyzed mice is provided in Table S3.

Table 1. Cytokine productions in serum from AAV2-GFP and AQP1 mice (pg/mL)

Cytokines	GFP	AQP1	*P* value
IL-1β	3,430 ± 148	2,948 ± 353	0.0475
TNF-α	7,195 ± 1,400	4,116 ± 1,794	0.0396
IL-9	4,016 ± 1,630	1,427 ± 1,331	0.0501
IL-2	1,285 ± 151	731 ± 81	0.0026
IL-13	3,601 ± 790	1,942 ± 319	0.0140
IL-6	209 ± 43	114 ± 4	0.0097
IL-4	251 ± 37	145 ± 28	0.0091
GM-CSF	3,205 ± 330	2,214 ± 243	0.0070
KC	454 ± 21	285 ± 64	0.0100
Eotaxin	8,987 ± 1,093	6,321 ± 394	0.0083
MIP-1β	1,479 ± 172	923 ± 67	0.0033

Serum was collected at the end of the study and then analyzed for levels of the indicated cytokines (pg/mL) by multicytokine assay in duplicate. The data shown are the median ± SD of each group of C57BL/6.NOD-*Aec1*/*Aec2* mice (AAV2-GFP and AAV2-AQP1, $n = 3$ in each group). The unpaired Student's *t* test was used to analyze differences of cytokine production in serum.

of patterns between a given "seed" and "targets" and expresses the result quantitatively as a Pearson correlation coefficient. The approach is very robust and can allow the direct comparison of datasets collected from divergent platforms, such as microarrays, kinase assays, and drug toxicity profiles. Previous work with COMPARE has identified novel correlations between viruses and cellular receptors, multidrug resistance-1 expression, and rhodamine efflux, as well as mutant alleles of the RAS oncogene and sensitivity to cytosine arabinoside and topoisomerase II inhibitors (24–27). Our study provides the first evidence, to our knowledge, of an association between BMP6 and AQP5 expression in both patients with pSS and a mouse model of pSS, and proposes a physiological mechanism for the loss of gland activity associated with this disease. Identification of this mechanism allowed us to propose and test a therapeutic approach for the treatment of pSS.

Salivary gland secretion is a complex process and involves the interaction of many proteins. Knockout mice for AQP5, NKCC1, IP3R types 2 and 3, matriptase, and the M3 and M1 muscarinic receptors are all reported to have a decrease in saliva flow (28–32). The interplay between the epithelia and the immune system adds a further layer of complexity because T-cell knockouts of IP3K, STIM1/STIM2, and ID3 and overexpression of IL-12, BAFF, and sTNFR1 are all also reported to induce loss of gland function and may initiate it by different pathways (33–38). We report an association between elevated BMP6 protein expression and AQP5 expression, but changes in any of these other factors triggered by environmental stimuli could account for the loss of gland activity in the patients with normal BMP6 expression.

We hypothesized that BMP6 up-regulation is related to a change in the transcriptional regulatory state in response to the well-documented inflammatory environment associated with Sjögren's syndrome. Preliminary examination of the BMP6 proximal promoter for transcription factor-binding motifs in combination with a promoter analysis of 670 previously reported differentially expressed genes in patients with Sjögren's syndrome suggested a connection between the previously reported up-regulation of IFN-γ and the inflammation-responsive transcription factor STAT1 and the increase in BMP6 expression. This connection between STAT1 and BMP6 expression offers a direct link between the inflammatory innate immune response common in patients with pSS and BMP6 expression.

Clinically, one mechanism proposed for loss of gland activity is autoantibodies directed against the muscarinic receptor on acinar cells. These receptors are responsible for initiating the cholinergic stimulus to salivate (39). In addition, in vitro and in vivo studies report an inhibitory effect of proinflammatory cytokines on intracellular calcium signaling and fluid movement and loss of the IP3 receptor (39–42). Other work has suggested a loss of polarization within the salivary gland acinar cell, which would contribute to decreased function (19). Our data suggest that decreased AQP5 expression in response to increased BMP6 expression can contribute to the loss of salivary gland activity. Furthermore, our data demonstrate that creation of a facilitated water permeability pathway via expression of AQP1 in the remaining epithelial tissue in the gland can lead to improvements in gland activity and a decrease in inflammation. Thus, despite the differences in insults to the gland (radiation vs. immune-mediated), our findings suggest a common therapeutic treatment for the two conditions by expression of AQP1.

Restoration of fluid secretion exhibited a positive effect on the immune environment of the gland and secretory activity at other distal sites, such as the lacrimal gland. Salivary gland epithelia are reported to play an immune regulatory role as nonprofessional antigen-presenting cells and directly produce proinflammatory cytokines, such as IFN-γ, IL-17, IL-23, and chemokines (43–47). It is possible that the restoration of immune homeostasis may result from the external effect of AQP1 on the restoration of flow. Saliva contains a significant number of antimicrobial peptides and is critical to oral and upper gastrointestinal health. Restoration of flow could increase the release of these peptides and contribute to decreasing innate immune activation. In addition, transepithelial barrier integrity is reported to be decreased in patients with pSS (48, 49), likely leading to exposure to new antigens and inflammation. Restoration of flow could lead to decreased antigen exposure, lowering glandular inflammation and inducing a return to immune homeostasis. Because AQP1 expression could not be detected in the secretory epithelia of the lacrimal glands, it is likely that a return to immune homeostasis contributes to the restoration of lacrimal gland activity.

In this study, we show that AQP1 gene therapy can restore fluid movement in a murine model of Sjögren's syndrome. These findings strongly support the possibility of using gene therapy to correct the defects in salivary glands that occur in patients with pSS. This approach has the potential to improve salivary function and relieve the considerable morbidity experienced by these patients.

Materials and Methods

Patient Samples. All patients fulfilled the American-European Consensus Group criteria for pSS without other confounding autoimmune diseases. The study was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research (NIDCR), NIH, and it is registered at https://clinicaltrials.gov. All subjects provided written informed consent before enrollment. Clinical features of the study subjects are summarized in Table S1. cDNA was prepared and gene expression was quantified as previously described (10).

Study Approval. The MSG samples were obtained from the NIDCR Sjögren's syndrome clinic. The study was approved by the Institutional Review Board of the NIDCR (protocol nos. 94-D-0018, 84-D-0056, and 99-D-0070). Informed consent was obtained in writing from all study subjects before enrollment. All mouse studies were conducted in an American Association for the Accreditation of Laboratory Animal Care-accredited facility under Institutional Animal Care and Use Committee Protocol approval.

Methods. Details on cell culture, BMP6 treatment, and AAV vector administration, as well as serum/saliva/tear collection, microarray preparation and analysis, cell volume measurement, confocal imaging, flow cytometry, and statistical analysis, can be found in *SI Materials and Methods*.

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