Clinical & Translational Immunology

Australian and New Zealand SOCIETY FOR IMMUNOLOGY INC.

*Clinical & Translational Immunology* 2021; e1277. doi: 10.1002/cti2.1277
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ORIGINAL ARTICLE

# IL-17 drives salivary gland dysfunction via inhibiting TRPC1-mediated calcium movement in Sjögren's syndrome

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Received 20 January 2021;
Revised 25 March 2021;
Accepted 25 March 2021

doi: 10.1002/cti2.1277

*Clinical & Translational Immunology*
2021; **10**: e1277

## Abstract

**Objectives.** This study aims to determine a role of interleukin-17A (IL-17) in salivary gland (SG) dysfunction and therapeutic effects of targeting IL-17 in SG for treating autoimmune sialadenitis in primary Sjögren's syndrome (pSS). **Methods.** Salivary IL-17 levels and IL-17-secreting cells in labial glands of pSS patients were examined. Kinetic changes of IL-17-producing cells in SG from mice with experimental Sjögren's syndrome (ESS) were analysed. To determine a role of IL-17 in salivary secretion, IL-17-deficient mice and constructed chimeric mice with IL-17 receptor C (IL-17RC) deficiency in non-hematopoietic and hematopoietic cells were examined for saliva flow rates during ESS development. Both human and murine primary SG epithelial cells were treated with IL-17 for measuring cholinergic activation-induced calcium movement. Moreover, SG functions were assessed in ESS mice with salivary retrograde cannulation of IL-17 neutralisation antibodies. **Results.** Increased salivary IL-17 levels were negatively correlated with saliva flow rates in pSS patients. Both IL-17-deficient mice and chimeric mice with non-hematopoietic cell-restricted IL-17RC deficiency exhibited no obvious salivary reduction while chimeric mice with hematopoietic cell-restricted IL-17RC deficiency showed significantly decreased saliva secretion during ESS development. In SG epithelial cells, IL-17 inhibited acetylcholine-induced calcium movement and downregulated the expression of transient receptor potential canonical 1 via promoting *Nfkbiz* mRNA stabilisation. Moreover, local IL-17 neutralisation in SG markedly attenuated hyposalivation and ameliorated tissue inflammation in ESS mice. **Conclusion.** These findings identify a novel function of IL-17 in driving salivary dysfunction during pSS development and may provide a new therapeutic strategy for targeting SG dysfunction in pSS patients.

**Keywords:** autoimmune sialadenitis, calcium movement, IL-17, primary Sjögren's syndrome, salivary dysfunction

# INTRODUCTION

Primary Sjögren's syndrome (pSS) is a common autoimmune disease characterised by sialadenitis and histopathological changes such as dry mouth and lymphocytic infiltration in salivary glands (SG).[1] Xerostomia caused by hyposalivation may lead to swallowing difficulties and increased risks of oral infections in pSS patients.[2] Available treatments for xerostomia including saliva substitutes and muscarinic agonists are usually symptomatic managements with limited benefits and potential side effects.[2] Currently, effective therapies for treating xerostomia and autoimmune sialadenitis in pSS patients are still lacking.

Interleukin-17A (IL-17) is a pro-inflammatory cytokine with diverse functions. Extensive studies have demonstrated the critical roles of IL-17 in inflammatory immune responses. IL-17 has pleiotropic effects on multiple cell populations. It has been shown that IL-17 plays key roles in host defence against extracellular bacterial and fungal infections at mucosal tissues. The deficiency of IL-17 signalling in epithelial cells leads to impaired bacteria clearance and exacerbated disease development in mice.[3,4] Moreover, IL-17 induces the production of chemokines and inflammatory cytokines by keratinocytes, which drives skin inflammation and tissue damage during psoriasis pathogenesis.[5] We have previously shown that IL-17 promotes pulmonary B cell differentiation and sustains plasma cells survival during influenza virus infection and lupus development, respectively.[6–8] Many studies, including our recent findings, have demonstrated that IL-17 is critically involved in the pathogenesis of pSS.[9–12] Clinical observations have shown elevated IL-17 levels in pSS patients.[11] The expansions of both IL-17-producing T-helper 17 (Th17) cells and $CD4^-CD8^-$ double-negative T cells have been detected in pSS patients.[10,13] Moreover, IL-17-deficient mice are found to be resistant for experimental Sjögren's syndrome (ESS) induction with no salivary secretion reduction.[12] Although both blockade of IL-17 receptor and targeting Th17 cells by proteasome inhibition have been shown to ameliorate disease progression in mice with ESS[14,15], whether IL-17 directly induces SG dysfunction remains unclear.

Lines of evidence have suggested that functional impairment of SG secretory cells is a leading cause of xerostomia during pSS development.[16] Cholinergic activation by neurotransmitters triggers intracellular calcium release from the endoplasmic reticulum (ER). Decreased calcium levels within ER further activates the ion channels located on plasma membrane including transient receptor potential canonical 1 (TRPC1), resulting in robust calcium movement, which is essential for sustained saliva secretion.[17,18] It has been reported that deficiency of TRPC1 results in significantly attenuated agonist-induced calcium movement and 70% loss of saliva production in mice.[19] Notably, the SG epithelial cells from pSS patients exhibited reduced cholinergic activation-induced calcium signalling with dysregulated expression and localisation of the receptors associated with calcium movement, suggesting a defect of neurotransmitter-evoked calcium mobilisation underlying SG hypofunction during pSS development.[20–22]

In this study, we found that elevated levels of salivary IL-17 production negatively correlated with decreased saliva secretion in pSS patients. Moreover, we identified a novel function of IL-17 in regulating calcium movement within SG epithelial cells and inducing SG secretory dysfunction during pSS development. Mechanistically, we demonstrated that IL-17 downregulated TRPC1 expression via promoting *Nfkbiz* mRNA stabilisation. Importantly, we showed that neutralisation of local IL-17 in SG significantly attenuated salivary dysfunction and ameliorated SG tissue inflammation in mice with ESS. Together, these findings suggest that local targeting IL-17 in SG may represent a potential therapeutic strategy for treating xerostomia and autoimmune sialadenitis in pSS patients.

# RESULTS

## Increased salivary IL-17 levels negatively correlate with reduced saliva secretion in pSS patients

To examine local IL-17 production in SG, we measured IL-17 concentrations in saliva samples from pSS patients and non-SS subjects with sicca complaints. Salivary IL-17 levels were markedly elevated in pSS patients. Moreover, the IL-17 levels were negatively correlated with saliva flow rates in pSS patients but not in non-SS subjects (Figure 1a). We further divided the pSS patients into two groups based on the threshold value of unstimulated saliva flow rate (0.1 mL $min^{-1}$). The salivary IL-17 concentrations in pSS patients with low saliva flow rates were significantly higher by approximately threefold when compared with those in patients with high saliva flow rates (Figure 1b). In labial glands of pSS patients, IL-17-producing cells, particularly Th17 cells, were detected by immunofluorescence microscopy (Figure 1c and d). Interestingly, the numbers of IL-17-secreting cells including Th17 cells were significantly increased in pSS patients with low saliva flow rates (Figure 1e). Moreover, we detected the expression of both IL-17 receptor A (IL-17RA) and IL-17RC, two essential receptors for IL-17 signalling transduction, on salivary acinar cells in labial gland biopsies from pSS patients (Figure 1f).

## IL-17 drives SG hypofunction during ESS development

To determine a role of IL-17 in modulating SG secretory dysfunction, we examined IL-17 production in SG from ESS mice. Flow cytometric analysis revealed significantly increased frequencies and total numbers of IL-17-producing cells including Th17 cells in SGs of ESS mice, which were closely associated with the reduced saliva flow rates (Figure 2a). Similar findings were also observed in NOD mice which spontaneously develop autoimmune sialadenitis (Supplementary figure 1). Notably, more than 60% of $AQP5^{+}CD45^{-}$ acinar epithelial cells were found to express IL-17RA and IL-17RC in SG tissues from both naïve and ESS mice as detected by flow cytometric analysis and confocal microscopy, respectively (Figure 2b and c). Upon ESS induction, WT mice exhibited significantly reduced saliva production while both IL-17-deficient and IL-17RC-deficient mice showed no obvious reduction in saliva secretion (Figure 2d), indicating a pivotal role of IL-17 in regulating salivary secretion. To further verify a function of IL-17 in triggering hyposalivation, we successfully generated the chimeric mice with IL-17RC deficiency in non-hematopoietic cells or hematopoietic cells, respectively (Figure 2e). Upon ESS induction, the chimeric mice with non-hematopoietic cell-restricted IL-17RC deficiency exhibited no obvious reduction in saliva flow rates while the chimeric mice with IL-17RC deficiency in hematopoietic cells showed significantly decreased saliva secretion similar to WT controls (Figure 2f). Together, these data demonstrate that IL-17 signalling in non-hematopoietic cells, particularly SG acinar epithelial cells, is critically involved in SG hypofunction during ESS development.

## IL-17 impairs salivary secretion via decreasing calcium movement in SG epithelial cells

To investigate whether IL-17 directly regulates saliva secretion, we examined the effects of IL-17 on acetylcholine-induced calcium movement in cultured primary epithelial cells from human labial glands. Notably, IL-17 treatment dramatically reduced cytosol calcium levels with the presence of calcium in extracellular environment (Figure 3a). Consistently, IL-17 treatment significantly decreased extracellular calcium movement into cytosol in WT but not IL-17RC-deficient SG epithelial cells (Figure 3b–d). Moreover, the SG acini from ESS mice exhibited markedly reduced cholinergic activation-induced calcium movement (Figure 3e). Together, these data suggest that IL-17 could directly trigger SG secretory dysfunction via decreasing cholinergic activation-evoked calcium movement in epithelial cells during SS development.

## IL-17 downregulated TRPC1 expression via *Nfkbiz* mRNA stabilisation in SG epithelial cells

To elucidate the underlying molecular mechanisms, we examined the effects of IL-17 on genes expression profiles in SG epithelial cells. Upon IL-17 treatment, the transcript levels of *TRPC1*, the gene encoding an ion channel critical for calcium mobilisation and saliva secretion, were significantly reduced in epithelial cells from human labial gland and murine SG (Figure 4a and Supplementary figure 2). Moreover, IL-17 downregulated TRPC1 protein expression in human SG cell line A253 (Figure 4b) and primary murine SG epithelial cells (Figure 4c and d). Notably, TRPC1 levels in SG were significantly

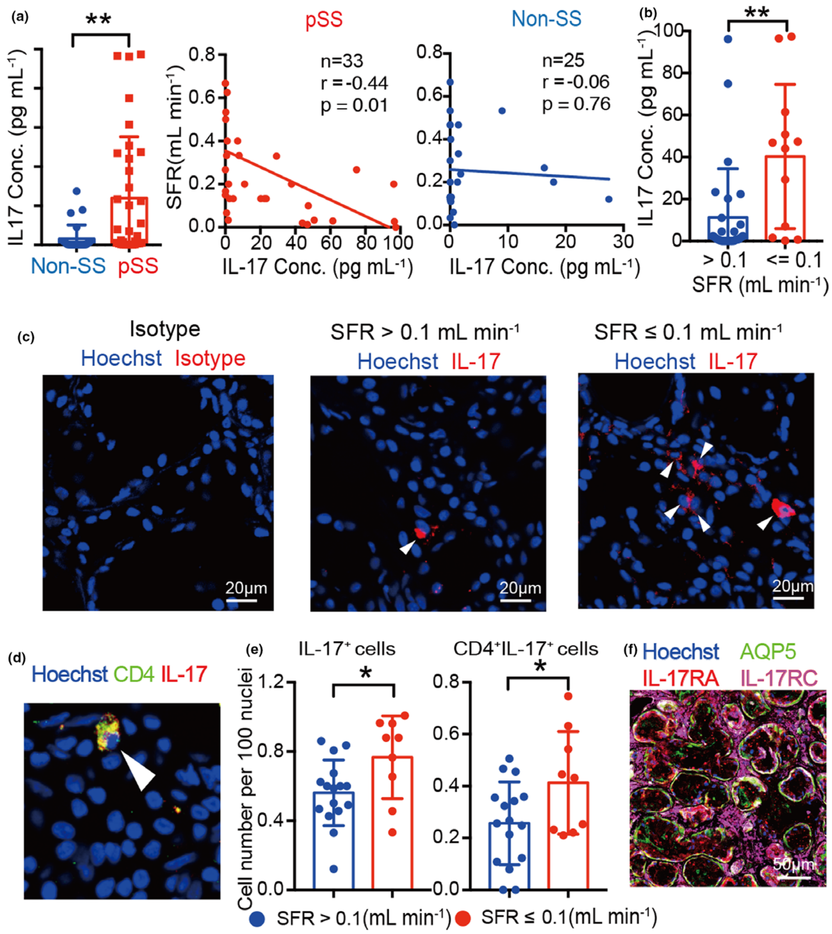


**Figure 1.** Increased salivary IL-17 levels negatively correlate with saliva flow rates (SFR) in primary Sjögren's syndrome (pSS) patients. **(a)** IL-17 concentrations in saliva from pSS patients and non-SS subjects were measured ($n$ = 25 for non-SS, $n$ = 33 for pSS). The plot shows correlation analysis of salivary IL-17 concentrations and SFRs. **(b)** Salivary IL-17 concentrations in pSS patients with SFR values lower and higher than 0.1 mL min$^{-1}$ are analysed ($n$ = 12 for SFR ≤0.1 mL min$^{-1}$, $n$ = 21 for SFR > 0.1 mL min$^{-1}$). **(c)** Representative confocal images showing IL-17-producing cells in labial gland biopsies from pSS patients. **(d)** Confocal images showing CD4$^{+}$IL-17$^{+}$ Th17 cells in labial gland biopsies from pSS patients. **(e)** Numbers of total IL-17$^{+}$ cells and CD4$^{+}$IL-17$^{+}$ Th17 cells in labial gland biopsies from pSS patients were analysed ($n$ = 9 for SFR ≤ 0.1 mL min$^{-1}$, $n$ = 16 for SFR > 0.1 mL min$^{-1}$). **(f)** Confocal images showing the expression of IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC) in labial glands of pSS patients. Data are shown as mean ± SD; **P*-value < 0.05; ***P*-value < 0.01.

downregulated in WT but not IL-17-deficient mice during ESS development (Figure 4e–g).

In cultured SG epithelial cells, the inhibition of NF-κB significantly reduced *Trpc1* expression levels (Figure 5a), suggesting an important role of NF-κB activity for TRPC1 expression. Although previous studies showed that IL-17 activates NF-κB pathway,[23] we found that IL-17 treatment rapidly increased the gene expression levels of *NFKBIZ*, the gene encoding IκB-ζ which is an important inhibitor of NF-κB within nucleus, in epithelial cells from both human labial gland and WT but not IL-17RC deficient murine SGs (Figure 5b and c). Moreover, IL-17 upregulated the IκB-ζ protein expression as detected by Western blotting analysis (Figure 5d). In addition, the gene expression levels of *Nfkbiz* in SG tissues were significantly increased during ESS development (Figure 5e). Notably, we found that silencing of *Nfkbiz* almost completely abrogated IL-17-mediated downregulation of TRPC1 in SG epithelial cells, suggesting that IL-17 regulated TRPC1 expression via IκB-ζ activation (Figure 5f and g). Furthermore, we treated the SG epithelial cells with actinomycin D to block mRNA synthesis prior to IL-17 stimulation. It was found that IL-17 treatment significantly prolonged the mRNA half-life of *Nfkbiz* when compared with control groups (Figure 5h), suggesting that IL-17

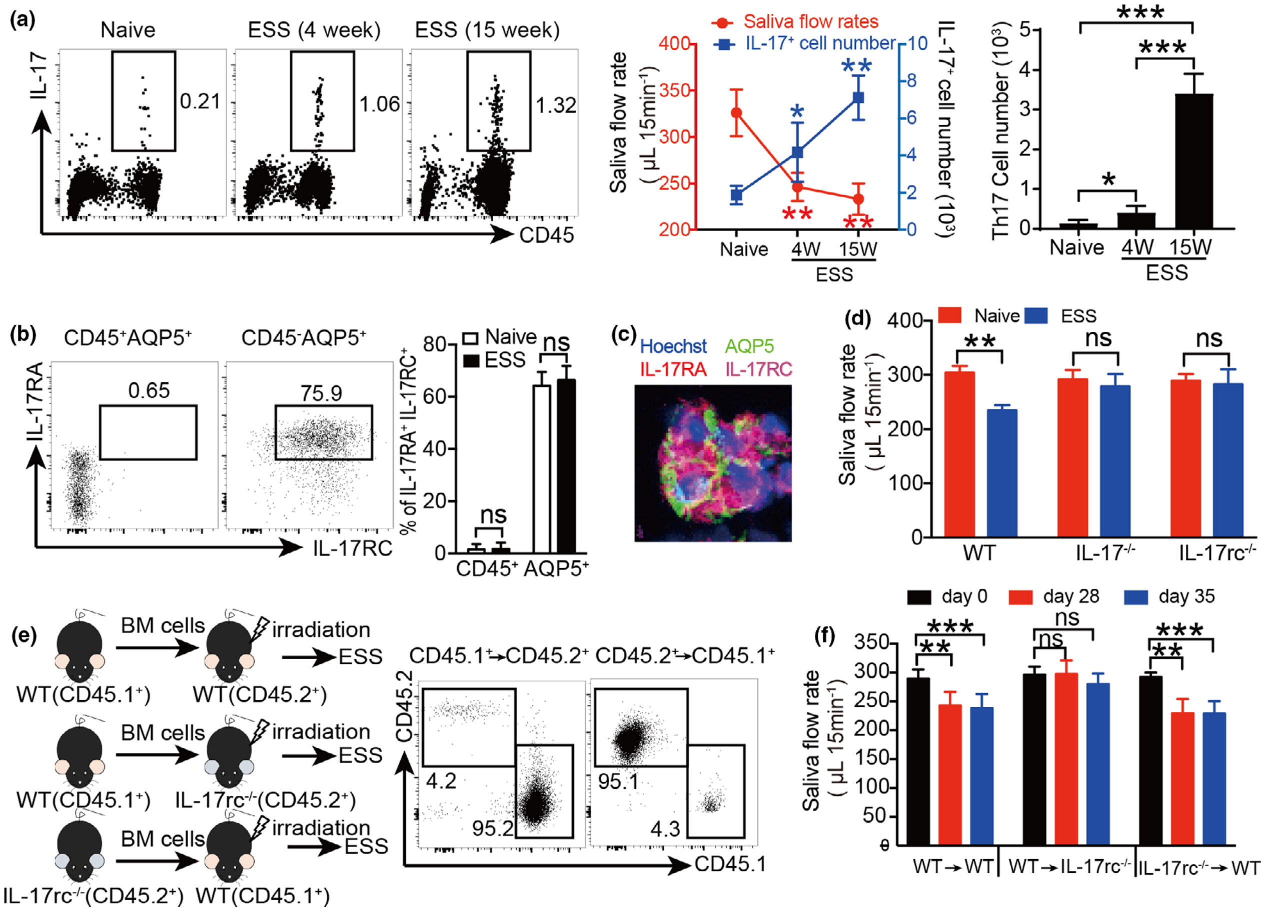

**Figure 2.** IL-17 signalling is critical for salivary gland dysfunction during experimental Sjögren's syndrome (ESS) development. **(a)** IL-17-secreting cells in salivary glands (SGs) from naïve and ESS mice were analysed by flow cytometry. Kinetic changes of saliva flow rates (red line) and numbers of IL-17-secreting cells (blue line) and Th17 cells (histogram) in SGs were analysed during ESS development ($n$ = 6 for each group, asterisks indicate comparisons with naïve mice). **(b)** Flow cytometric profiles showing IL-17RA$^+$IL-17RC$^+$ cells within CD45$^+$ leukocytes and AQP5$^+$ SG epithelial cells in SG tissues are presented. The frequencies of IL-17RA$^+$IL-17RC$^+$ cells in SG from naïve and ESS mice were analysed ($n$ = 5 for each group). **(c)** Representative confocal images showing IL-17RA and IL-17RC expression in AQP5$^+$ SG epithelial cells. **(d)** Saliva flow rates of wild-type (WT), IL-17-deficient and IL-17RC-deficient mice with or without ESS induction were measured ($n$ = 5 for each group). **(e)** The schematic diagram showing the generation of chimeric mice with IL-17RC deficiency in non-hematopoietic and hematopoietic cells is presented. Flow cytometric analysis shows more than 95% of donor-derived leukocytes in recipient chimeric mice after reconstitution. **(f)** Saliva flow rates were measured in the chimeric mice after ESS induction ($n$ = 8 for each group). Data are shown as mean ± SD; ns, not significant; *$P$-value < 0.05; **$P$-value < 0.01; ***$P$-value < 0.001.

downregulated TRPC1 expression via promoting *Nfkbiz* mRNA stabilisation.

## Local IL-17 neutralisation attenuates salivary dysfunction and SG inflammation in ESS mice

To evaluate the therapeutic effect of local IL-17 blockade, we performed salivary retrograde cannulation in ESS mice. Salivary cannulation of recombinant IL-17 triggered a significant reduction of saliva secretion in normal mice (Figure 6a). In contrast, blockade of local IL-17 with neutralisation antibodies via salivary cannulation significantly increased saliva flow rates in ESS mice when compared with control IgG-treated group (Figure 6b). Moreover, local IL-17 neutralisation increased *Trpc1* expression levels (Figure 6c) and markedly ameliorated histological pathology and lymphocytic infiltration in SG tissues of ESS mice (Figure 6d–f). Together, these data demonstrate the therapeutic potential of IL-17 blockade in SG for treating xerostomia and autoimmune sialadenitis in patients with pSS.

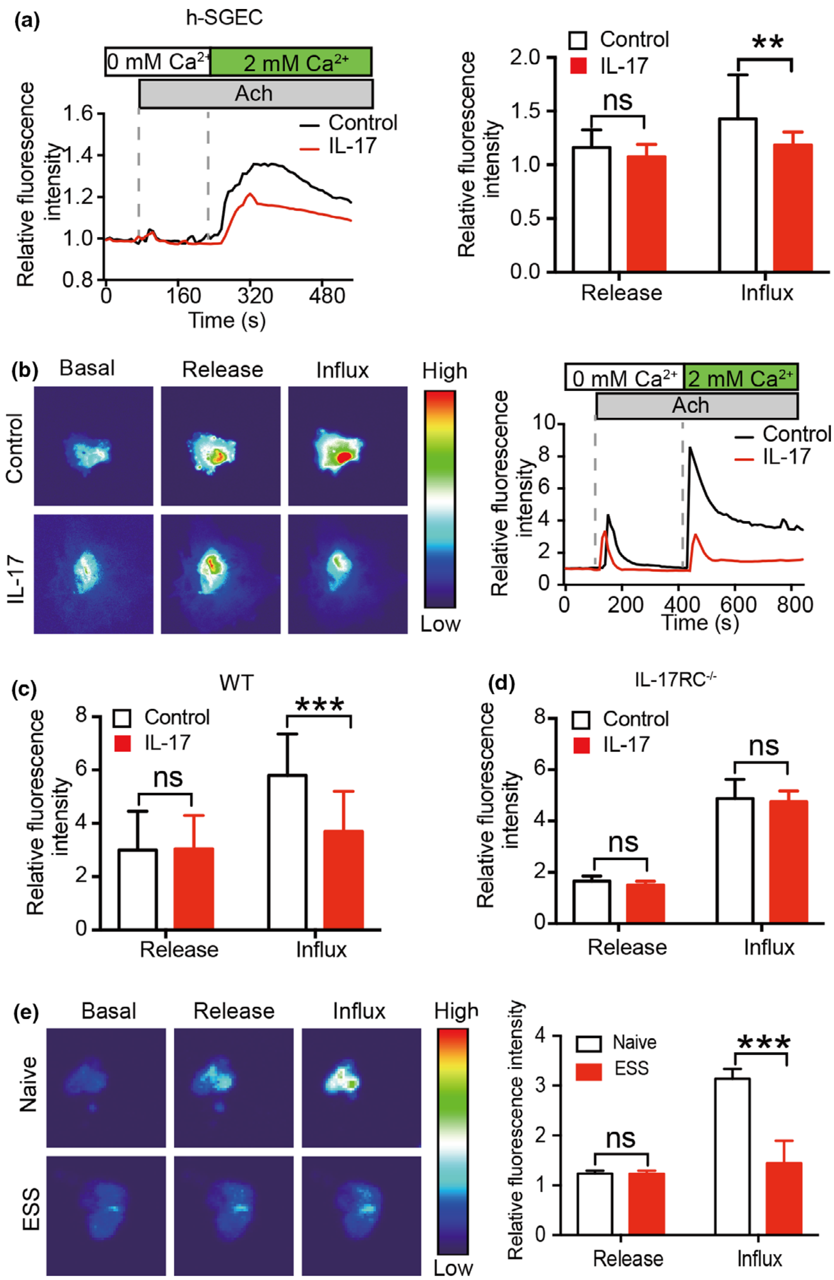


**Figure 3.** IL-17 decreases acetylcholine (Ach)-induced calcium movement in salivary gland (SG) epithelial cells. **(a)** Primary epithelial cells from human labial glands (h-SGEC) were pretreated with or without recombinant IL-17 (25 ng $mL^{-1}$) and labelled with Fluo-4 probe. Calcium levels were determined by time-lapse microscopy. Traces showing kinetics of fluorescence intensities during the indicated treatments are presented. The data show relative fluorescence intensities of the peak after Ach stimulation ('release') and the peak after extra $CaCl_2$ addition ('influx'). **(b, c)** Primary murine SG epithelial cells were pretreated with or without IL-17 (25 ng $mL^{-1}$). Intracellular calcium levels were determined upon indicated treatments. Representative heat map pictures and traces showing relative fluorescence intensities are presented (**b**). The data show relative fluorescence intensities at 'release' and 'influx' stages (**c**). **(d)** Relative fluorescence intensities in IL-17RC-deficient SG epithelial cells with or without IL-17 treatment. **(e)** Calcium levels in SG acini from naïve and experimental Sjögren's syndrome mice were determined. Representative heat map pictures are shown. The data show relative fluorescence intensities at 'release' and 'influx' stages. All experiments were repeated three times. Data are shown as mean ± SD; ns, not significant; ***P*-value < 0.01; ****P*-value < 0.001.

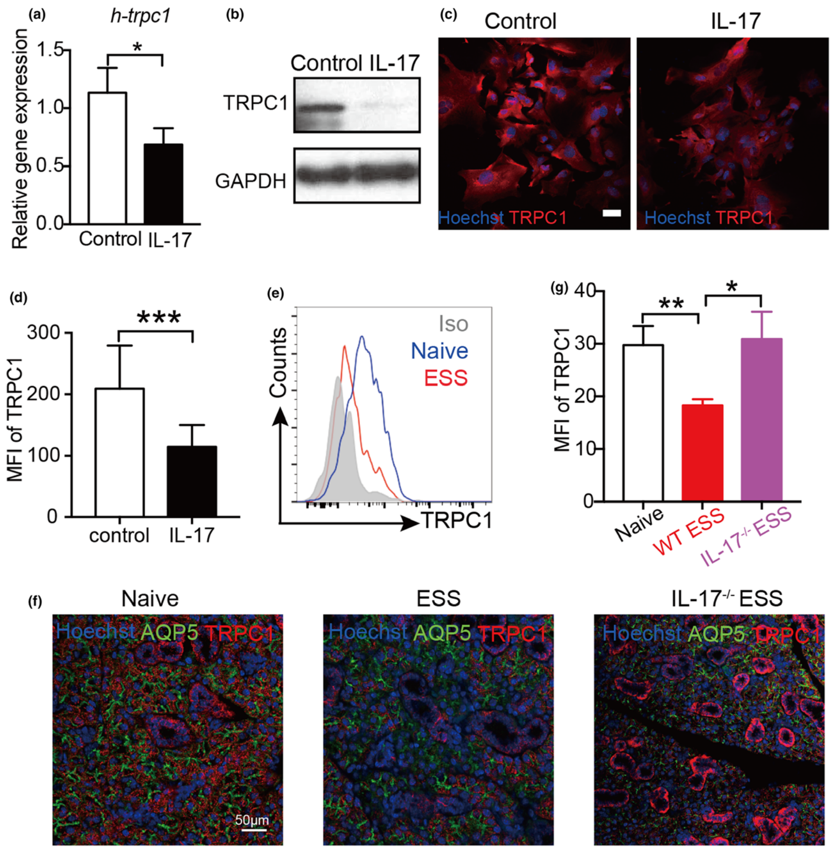


**Figure 4.** IL-17 reduces TRPC1 expression in salivary gland (SG) epithelial cells. **(a)** Primary epithelial cells from human labial glands were stimulated with or without IL-17 (25 ng $mL^{-1}$). Relative gene expression levels of *TRPC1* transcripts were analysed. **(b)** Expression of TRPC1 protein in A253 cells with or without IL-17 treatment (25 ng $mL^{-1}$) was determined by Western blotting. **(c, d)** TRPC1 expression in murine SG epithelial cells with or without IL-17 treatment was detected by immunofluorescence microscopy **(c)**. Mean fluorescence intensity (MFI) values were analysed **(d)**. **(e),** TRPC1 expression in SG tissues of naïve and experimental Sjögren's syndrome (ESS) mice was measured by flow cytometry. **(f, g)** TRPC1 expression in SG sections from naïve, WT ESS and IL-17-deficient ESS mice was detected by immunofluorescence microscopy. MFI values were analysed ($n$ = 5 for each group). Data are shown as mean ± SD; *$P$-value < 0.05; **$P$-value < 0.01; ***$P$-value < 0.001.

## DISCUSSION

In this study, we first revealed that increased salivary IL-17 levels were negatively correlated with saliva flow rates in pSS patients. Moreover, we found that IL-17 signalling in non-hematopoietic cells, particularly SG acinar epithelial cells, was critically involved in salivary dysfunction during ESS development. Furthermore, IL-17 treatment significantly reduced TRPC1 expression via promoting *Nfkbiz* mRNA stabilisation and diminished the acetylcholine-induced calcium movement in primary SG epithelial cells. Importantly, neutralisation of SG local IL-17 markedly improved saliva secretion and ameliorated SG tissue inflammation in ESS mice. Together, these findings identify a novel function of IL-17 in suppressing calcium movement and thereby impairing saliva secretion during pSS development, suggesting that blockade of local IL-17 might be a promising strategy for the treatment of xerostomia in pSS.

Recent studies have detected IL-17-secreting cells in SG from pSS patients and mice with pSS-like disease.[11–13] IL-17 is predominantly derived from inflammatory infiltrates while the expression of IL-17 progressively increases with severe tissue inflammation in labial glands from SS patients.[11] Th17 cells are important IL-17-producing cells identified in SG tissues of pSS patients.[24] Here, we show that the numbers of IL-17-secreting cells including Th17 cells are significantly higher in labial glands of pSS patients with low saliva flow rates than those with high saliva flow rates, which

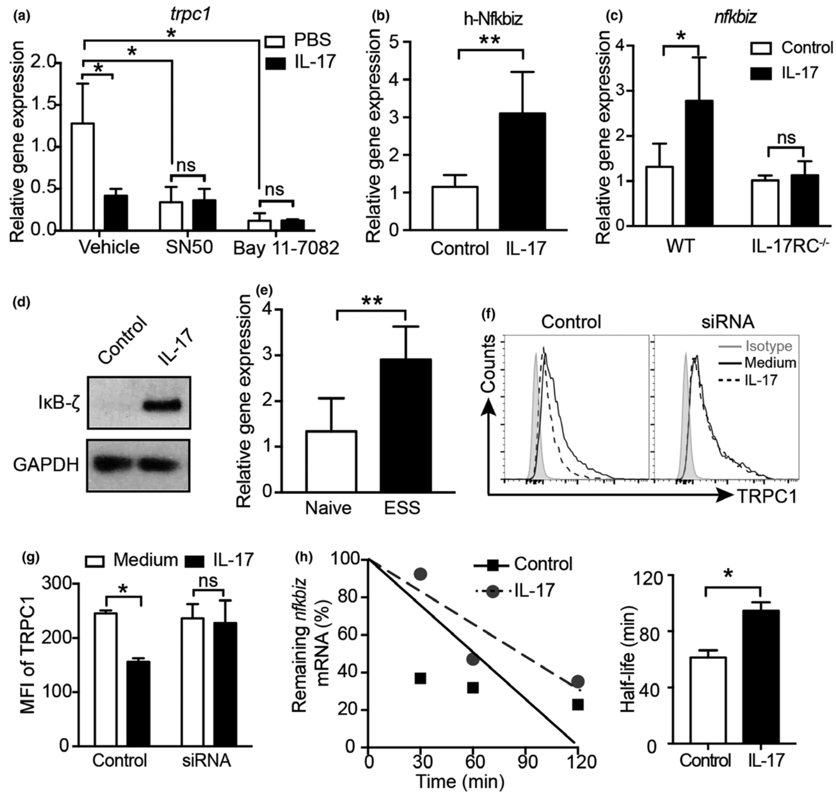

**Figure 5.** IL-17 inhibits TRPC1 expression via promoting *Nfkbiz* mRNA stabilisation. **(a)** Primary salivary gland (SG) epithelial cells were treated with NF-κB inhibitors (50 μg mL$^{-1}$ SN50 and 10 μM Bay 11-7082) for 30 min before IL-17 treatment (25 ng mL$^{-1}$). The relative gene expression levels of *Trpc1* were measured. **(b)** The mRNA levels of NFKBIZ in primary epithelial cells from human labial gland with or without IL-17 treatment (25 ng mL$^{-1}$) were analysed. **(c)** The mRNA levels of *Nfkbiz* in WT and IL-17RC$^{-/-}$ murine primary SG epithelial cells with IL-17 treatment (25 ng mL$^{-1}$) were measured. **(d)** The IκB-ζ protein expression in primary SG epithelial cells with or without IL-17 treatment was examined by Western blotting analysis. **(e)** The mRNA levels of *Nfkbiz* in SGs from naïve and experimental Sjögren's syndrome mice were analysed. **(f, g)** SG epithelial cells transfected with *Nfkbiz* siRNA or control siRNA were stimulated with IL-17 (25 ng mL$^{-1}$). The expression of TRPC1 was determined by flow cytometry (**f**). MFI values of TRPC1 were analysed (**g**). **(h)** Primary SG epithelial cells were incubated with actinomycin D and treated with PBS or IL-17 (25 ng mL$^{-1}$). The remaining mRNA levels of *Nfkbiz* were measured. All experiments were repeated three times. Data are shown as mean ± SD; *$P$-value < 0.05; **$P$-value < 0.01; ***$P$-value < 0.001.

indicates a role of IL-17 in SG secretory dysfunction during pSS development. Lines of evidence have suggested an involvement of IL-17 in the pathogenesis of pSS.[11–13] IL-17 exerts diverse functions on multiple populations, including both immune cells and non-immune cells. Our previous studies reported a critical role of Th17 cells in the development of ESS as revealed by the findings that adoptive transfer of IL-17-producing Th17 cells enhanced peripheral Th1 and B cell responses and SG inflammation in IL-17-deficient mice upon ESS induction.[12] IL-17 has also been shown to modulate Th2 cytokines and autoantibody profiles in a mouse model of pSS.[25] Increasing evidence indicates a critical involvement of IL-17 in the mucosa immunity. The deficiency of IL-17 signalling in lung epithelium resulted in reduced neutrophil recruitment and impaired bacteria clearance in mice.[3] Moreover, IL-17 signalling in oral epithelial cells is required for β-defensin expression and the protection against candidiasis.[4] Recent studies have suggested that IL-17 might be involved in epithelial–mesenchymal transition and fibrosis development in pSS.[26] Here, we find that SG epithelial cells express high levels of IL-17 receptors. Using chimeric mice with IL-17RC deficiency in different populations, we have demonstrated that IL-17 signalling in non-hematopoietic cells, particularly SG acinar epithelial cells, is critical for SG secretory dysfunction during ESS development, which is

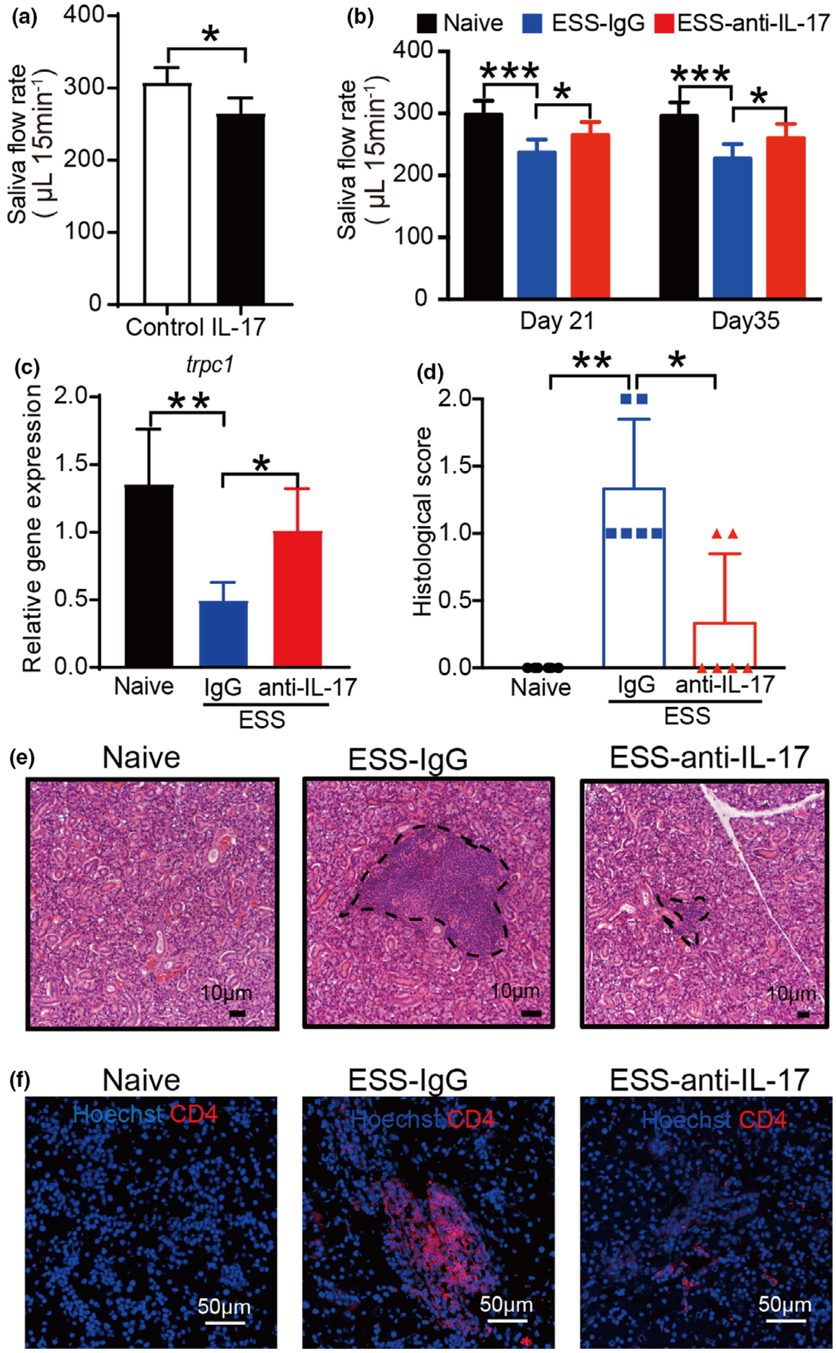


**Figure 6.** Local IL-17 neutralisation attenuates hyposalivation and ameliorates salivary gland (SG) inflammation in experimental Sjögren's syndrome (ESS) mice. **(a)** Saliva flow rates were measured in normal mice with salivary retrograde cannulation of PBS or recombinant IL-17. **(b)** ESS mice received salivary retrograde cannulation of IL-17 neutralisation antibody or control IgG at 14 and 28 days post-immunisation. Saliva flow rates were measured at 21 and 35 days post-immunisation. ($n$ = 8 for each group). **(c)** Relative gene expression of *Trpc1* in SG tissues was measured by q-PCR analysis ($n$ = 5 for each group). **(d)** Histological scores of SGs were analysed ($n$ = 6 for each group). **(e)** Representative H&E staining images showing SG glandular infiltration in the mice. **(f)** Representative confocal images showing infiltrated CD4 T cells in SG tissues. Data are shown as mean ± SD; **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001.

further supported by the findings that IL-17 treatment significantly downregulates the expression of TRPC1 and impairs calcium movement in primary epithelial cells from human labial glands and murine SGs. Previous studies have demonstrated that IL-17RC is an obligate co-receptor with IL-17RA for both IL-17 and IL-17F.[27] Although a potential role of IL-17F in modulating SG function could not be excluded in the present study, our current findings have identified a previously unrecognised function of IL-17 in triggering SG dysfunctions during pSS development. Further studies with large sample sizes are needed to validate the salivary IL-17 level as a biomarker for SG secretory functions in pSS patients. Previous studies have reported the expansion and infiltration of IL-17-producing $CD4^{-}CD8^{-}$ double-negative T cells (DN T cells) in SGs of pSS patients.[13] Consistently, we have detected IL-17-producing DN T cells in draining lymph nodes and SGs of ESS mice, in which other IL-17-producing cells including CD8 T cells and $\gamma\delta$ T cells are detected.[12,28] Thus, other IL-17-producing T-cell subsets, apart from Th17 cells, might also be involved in SS pathogenesis. Recent studies have suggested that many pro-inflammatory cytokines contribute to salivary dysfunction in SS pathogenesis. IFN$\gamma$ has been found to induce SG epithelial cell death,[29] which may lead to SG hypofunction. Moreover, overexpression of TNF$\alpha$ in SGs induces SS-like characteristics including salivary hypofunction in mice.[30] Notably, our previous studies have observed significantly increased IFN$\gamma^{+}$ Th1 cells during ESS development in mice.[12] Although we only examined the function of IL-17 in driving salivary hypofunction in this study, further studies are needed to explore the roles of other cytokines in inducing SG dysfunctions during SS pathogenesis.

Cholinergic activation-induced calcium movement in SG secretory acinar cells is of key significance for sustained saliva fluid secretion.[17,31] Previous studies have identified several key components for calcium mobilisation in SG cells, including muscarinic receptors, regulatory proteins and calcium channels. TRPC1, an ion channel critical for calcium homeostasis, is predominantly involved in neurotransmitter-evoked store-operated calcium entry in SG epithelial cells. Deficiency of TRPC1 results in markedly reduced calcium mobilisation from extracellular environment in SG acini, which leads to a dramatic decrease of saliva secretion in mice.[19] Moreover, it has been suggested that inositol 1,4,5-trisphosphate receptors (IP3R) deficits might be associated with the calcium defect and SG secretory dysfunction in pSS.[21] Here, we have shown that IL-17 inhibits neurotransmitter-induced calcium movement though downregulating TRPC1 expression. In culture, IL-17 treatment significantly reduces TRPC1 expression in primary epithelial cells from human labial glands and murine SGs. Moreover, we detect significantly downregulated TRPC1 levels in SG from WT but not IL-17-deficient mice during ESS development. Furthermore, the SG acini from ESS mice exhibit markedly reduced cholinergic activation-induced calcium movement, suggesting that dysregulated TRPC1 expression is involved in calcium movement defects and salivary dysfunction during pSS development. It has been shown that TRPC1 is regulated by NF-$\kappa$B which could directly bind to the *Trpc1* promoter regions.[32] Consistently, we have observed reduced *Trpc1* expression in SG epithelial cells upon inhibition of NF-$\kappa$B. Moreover, NF-$\kappa$B inhibitors abrogated the suppressive effects of IL-17 on *Trpc1* expression. It has been shown that I$\kappa$B-$\zeta$ inhibits transactivation and DNA-binding of NF-$\kappa$B with tight regulation.[33–35] Overexpression of I$\kappa$B-$\zeta$ increases IL-6 production and inhibits TNF$\alpha$ expression, suggesting dual functions of I$\kappa$B-$\zeta$ in regulating cytokines production.[36] Previous studies have shown that IL-17-activated I$\kappa$B-$\zeta$ signalling in keratinocytes plays a key role during psoriasis development.[37,38] Recently, we have demonstrated a critical role of IL-17 in maintaining long-lived plasma cells survival via promoting *bcl-xl* mRNA stabilisation during lupus pathogenesis.[7] Here, we have found that IL-17 rapidly increases the expression of I$\kappa$B-$\zeta$ via promoting *Nfkbiz* mRNA stabilisation while knock-down of *Nfkbiz* almost completely abrogates the IL-17-mediated downregulation of TRPC1 in SG epithelial cells, suggesting that IL-17 regulates TRPC1 expression in an I$\kappa$B-$\zeta$-dependent manner. Together, these data have revealed a novel function of IL-17 in regulating cholinergic activation-evoked calcium mobilisation by downregulating TRPC1 expression in SG epithelial cells. Further studies are needed to determine the clinical significance of IL-17-mediated TRPC1 expression in SG dysfunction of pSS patients. The blockade of IL-17 has been shown to be effective in the treatment of various autoimmune diseases.[39] Here, our findings that local salivary cannulation of IL-17 neutralising antibodies significantly improves salivary secretion during ESS development further indicate local IL-17 in SG as a promising therapeutic target for treating xerostomia in pSS patients.

Many studies have demonstrated that various pro-inflammatory cytokines including IL-17 play important roles in the development of various autoimmune diseases including those with secondary SS.[40] Although IL-17 has been shown to be critically involved in the pathogenesis of psoriasis, it is currently unclear whether IL-17 contributes to the development of secondary SS with psoriasis. Thus, it remains to be investigated when the levels of IL-17 in saliva or SGs are also increased and whether the increased IL-17 leads to SG hypofunction in patients with secondary SS. It is likely that other pro-inflammatory cytokines may also contribute to the development of secondary SS in patients with autoimmune diseases. In this study, we observed significantly increased IL-17 production in SGs of pSS patients and ESS mice, further supporting the notion that the levels of cytokines in target organs or local microenvironment play a critical role in disease pathology. Currently, the clinical effects of IL-17 antagonists in primary and secondary SS patients remain still unclear. In our previous studies, we observed significantly increased IL-17-producing Th17 cells in draining cervical lymph nodes at an early stage of ESS development while IFN$\gamma^+$ Th1 cells gradually increased and peaked at a chronic stage,[12] which indicates that early intervention with IL-17 blockade might be effective in treating SS patients. Notably, Th17 cytokines have been shortlisted as promising therapeutic targets for SS treatment in the research agenda from European League Against Rheumatism (EULAR) recommendations.[41] Thus, further clinical studies will validate the efficacy of targeting IL-17 for treating SS patients.

In summary, this study identifies a novel function of IL-17 in driving salivary dysfunction by impairing calcium movement during pSS pathogenesis. Mechanistically, IL-17 downregulates TRPC1 expression via promoting *Nfkbiz* mRNA stabilisation. Future studies may provide new insight into understanding the diverse functions of IL-17 in the pathogenesis of pSS.

# METHODS

## Patients

The pSS patients enrolled in this study were recruited from Shenzhen People's Hospital, Shenzhen, China. The pSS diagnosis was based on 2016 American College of Rheumatology (ACR)-European League Against Rheumatism (EULAR) classification criteria for primary Sjögren's syndrome.[42] The study was approved by the Medical Ethical Committee of Shenzhen People's Hospital, Shenzhen, China (Reference Number: LL-KT-2015001 and KY-LL-2020277-01). Informed consents were obtained from the subjects involved in the study. Saliva samples were collected from 33 pSS patients and 25 non-SS subjects with sicca complaints but did not fulfil the classification criteria for pSS. Labial gland biopsies were harvested from the lower lip under local anaesthesia from 25 pSS patients who fulfilled the 2016 ACR-EULAR criteria.[42] Patients with periodontitis and other oral diseases were excluded. The demographic information of the patients was shown in Supplementary tables 1 and 2.

## Mice

Female C57BL/6, B6.SJL-Ptprca Pepcb/BoyJ (BoyJ) and NOD/ShiLtJ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). IL-17-deficient mice were kindly provided by Dr Yoichiro Iwakura at The Institute of Medical Science, The University of Tokyo, Japan. IL-17 receptor C (IL-17RC)-deficient mice were generously provided by Genentech company (South San Francisco, CA, USA). All mice were housed with ad libitum access to food and water in the Laboratory Animal Unit at The University of Hong Kong. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong.

## ESS induction

Experimental Sjögren's syndrome induction was performed as previously described.[15,43,44] Briefly, mice were immunised with 400 μg SG-derived protein antigens emulsified in Freund's complete adjuvant (BD Biosciences, San Jose, CA, USA) on day 0 and boosted with the antigens in Freund's incomplete adjuvant (BD Biosciences) on day 14 via subcutaneous injection. Anesthetised mice were intraperitoneally injected with pilocarpine (Sigma-Aldrich, St. Louis, MO, USA), and saliva was immediately collected for 15 min at room temperature. SG tissue sections were prepared for H&E staining. A widely accepted scoring system based on the size and the degree of lymphocytic infiltration was used to evaluate the severity of SG tissue damage.[45]

## Enzyme-linked immunosorbent assay (ELISA)

Salivary IL-17 concentrations were measured by ELISA with IL-17A Human ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Briefly, saliva samples were added into pre-coated plates and incubated with biotin-conjugated anti-human IL-17A antibodies, Streptavidin-HRP solution and TMB substrate solution sequentially. The absorbance at 450 nm was measured using a Sunrise microplate reader (Tecan, Männedorf, Switzerland).

## Immunofluorescence staining and confocal microscopy

Immunofluorescence staining was performed as previously described.[46] For detection of IL-17, the frozen sections were fixed with 4% Paraformaldehyde (Sigma-Aldrich) for 15 min, permeabilised with 0.25% Triton X-100 (Sigma-Aldrich) for 15 min and blocked with 4% BSA (Sigma-Aldrich) for 30 min at room temperature. Sections were then incubated with fluorochrome-conjugated antibodies overnight at 4°C. For detection of surface antigens, the sections were fixed, blocked and incubated with the antibodies. The following antibodies were used: anti-hCD4 (clone A161A1, Biolegend, San Diego, CA, USA), anti-hIL-17 (clone BL168, Biolegend), anti-hIL-17RA (clone J10MBS, Thermo Fisher Scientific), anti-hIL-17RC (clone 309822, R&D Systems, Minneapolis, MN, USA), anti-mCD4 (clone GK1.5, Biolegend), anti-mIL-17RA (clone PAJ-17R, Thermo Fisher Scientific), anti-mIL-17RC (FAB2270A, R&D Systems), anti-AQP5 (clone H-200; Santa Cruz Biotechnology, Dallas TX, USA) and anti-TRPC1 (clone E-6, Santa Cruz Biotechnology). The samples were counterstained with Hoechst (Thermo Fisher Scientific) and observed under LSM780 confocal microscope (Zeiss, Oberkochen, Germany).

## Flow cytometry

Flow cytometric analysis was performed as previously described.[15] Briefly, single cell suspensions were collected and stained with Zombie Aqua solution (BioLegend) in protein free PBS for 15 min at room temperature in the dark. The samples were then incubated with Fc blocker (anti-CD16/32, BioLegend) for another 15 min to minimise nonspecific binding. After washing, the cells were stained with fluorochrome-conjugated antibodies against surface antigens for 30 min, washed and re-suspended for flow cytometric analysis. For detection of AQP5, the cells were stained with primary antibody (anti-AQP5, clone H-200, Santa Cruz Biotechnology) for 30 min and then incubated with secondary antibody (Donkey anti-rabbit IgG, Poly4064, Biolegend) for another 30 min. For intracellular staining of IL-17, cells were stimulated with PMA (50 ng $mL^{-1}$, Sigma-Aldrich), inomycine (1 μg $mL^{-1}$, Sigma-Aldrich) and monensin (2 μM, Biolegend) for 5 h. Intracellular staining was performed using Cytofix/Cytoperm kit (BD Biosciences), following the manufacturer's instructions. Samples were analysed using BD LSRFortessa (BD Biosciences). The acquired data were analysed with FlowJo software (TreeStar). The following antibodies were used: anti-IL-17 (clone TC11-18H10.1, Biolegend), anti-CD45 (clone 30-F11, Biolegend), anti-CD4 (clone GK1.5), anti-IL-17RA (clone PAJ-17R, Thermo Fisher Scientific), anti-IL-17RC (FAB2270A, R&D Systems), anti-TRPC1 (clone E-6, Santa Cruz Biotechnology), anti-CD45.1 (clone A20, Biolegend) and anti-CD45.2 (clone 104, Biolegend).

## Chimeric mice generation

Chimeric mice were constructed as previously described with modifications.[7] Wild-type (WT) C57BL/6 ($CD45.2^+$), WT BoyJ ($CD45.1^+$) and IL-17RC-deficient mice ($CD45.2^+$) received sub-lethal doses of γ radiation (800 cGy) with head and neck protection by a small lead barrel. Bone marrow cells from congenic mice were suspended in 100% foetal calf serum and adoptively transferred into the recipient mice ($10^7$ cells *per* mouse). Bone marrow cells from donor BoyJ ($CD45.1^+$) mice were transferred into irradiated IL-17RC-deficient mice ($CD45.2^+$) to generate chimeric mice with non-hematopoietic cell-restricted IL-17RC deficiency. Bone marrow cells from IL-17RC-deficient mice ($CD45.2^+$) were transferred into irradiated BoyJ ($CD45.1^+$) mice to generate chimeric mice with hematopoietic cell-restricted IL-17RC deficiency. C57BL/6 ($CD45.2^+$) recipient mice with adoptive transfer of bone marrow cells from BoyJ ($CD45.1^+$) mice served as controls. The chimeric mice were subjected to ESS induction after successful reconstitution at 7 weeks post-bone marrow cell transfer.

## Primary culture of SG epithelial cells

The collected human labial glands were minced into small pieces and digested in RPMI 1640 medium (Thermo Fisher Scientific) with collagenase IV (0.5 mg $mL^{-1}$, Sigma-Aldrich) and DNase I (0.05 mg $mL^{-1}$, Sigma-Aldrich) at 37 °C for 20 min with shaking. The suspensions were washed and passed through a 100 μm mesh nylon cell strainer (BD Biosciences). The cells were collected and seeded into culture plate in McCoy's 5A (Modified) Medium (Thermo Fisher Scientific) with 10% heat-inactivated foetal bovine serum (GE Healthcare Life Sciences, Marlborough, MA, USA), 1000 U $mL^{-1}$ penicillin and 0.1 mg $mL^{-1}$ streptomycin (Sigma-Aldrich). The cells were transferred into a second culture plate 12 h later.[47] Cells in the second plate were used in following experiments. The medium was changed every three days. For murine SG epithelial cell culture, the SGs were isolated in mice after intracardiac perfusion. The collected SGs were minced, digested and seeded into culture plates in a similar way. The purity of $CD45^-AQP5^+$ epithelial cells was routinely above 90% as detected by flow cytometry (Supplementary figure 3). In some experiments, SG epithelial cells were stimulated with recombinant IL-17 (PeproTech, Rocky Hill, NJ, USA) and examined for genes expression and intracellular calcium measurement. Human A253 cell line was purchased from ATCC (Manassas, VA, USA) and cultured in McCoy's 5A (Modified) Medium with 10% heat-inactivated foetal bovine serum, penicillin and streptomycin.

## Intracellular calcium measurement

Salivary gland epithelial cells and acini cells were loaded with 2 μM Fluo-4 (Thermo Fisher Scientific) for 30 min in a 37°C incubator. The cells were then washed with culture medium and incubated for another 30 min in a 37°C incubator. For measurement of calcium movement, basal calcium level was acquired in calcium-free Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific). The cells were then stimulated with acetylcholine (200 μM, Sigma-Aldrich) to induce calcium release from ER. To induce calcium influx, $CaCl_2$ solution (2 mM working concentration, Sigma-Aldrich) was added to allow calcium movement from extracellular environment.[19] Images were captured every 5s under Perkin Elmer UltraView VOX Spinning Disc Confocal microscope

(Perkin Elmer, Waltham, MA, USA) with excitation at 488 nm. The images were analysed using MetaMorph software (San Jose, CA, USA). Regions of interest were selected, and the changes of fluorescence intensities were determined.

### Quantitative PCR analysis

Quantitative PCR (Q-PCR) analysis was performed as previously described.[48] The mRNA was extracted using ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA) following manufacturer's instructions. The cDNA samples were prepared by reverse transcription PCR using PrimeScript™ RT Master Mix kit (Takara, Kyoto, Japan). Q-PCR was performed using TB Green Premix Ex Taq II kit (Takara) according to the manufacturer's instructions. The Ct values of target genes were obtained from the amplification curve. Relative expression of genes was calculated as $2^{\Delta\Delta Ct}$ with normalisation to 18S rRNA. Primers were listed in Supplementary table 3. To detect mRNA stability, epithelial cells were incubated with actinomycin D (5 μg mL$^{-1}$, Sigma-Aldrich) to block mRNA synthesis and treated with IL-17 (25 ng mL$^{-1}$). The remaining mRNA levels of *Nfkbiz* at different time points were measured.

### Western blotting analysis

Western blotting analysis was performed as previously described.[49] Briefly, lysates of epithelial cells were collected with RIPA lysis buffer. The proteins were loaded into 10 % SDS-PAGE gel, fractionated and transferred into polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk, incubated with primary antibodies and HRP-conjugated secondary antibodies. Proteins were detected by chemiluminescence substrates (Thermo Fisher Scientific). The following antibodies were used: anti-TRPC1 (Santa Cruz Biotechnology), anti-IκBζ (Cell Signaling Technology, Danvers, MA, USA), anti-GAPDH (Cell Signaling Technology), HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated anti-mouse IgG (Cell Signaling Technology).

### Transfection of siRNA

Transfection of siRNAs against *Nfkbiz* and control siRNA (Thermo Fisher Scientific) was performed with Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, Lipofectamine RNAiMAX Reagent and siRNAs were diluted and incubated for 5 min to form siRNA-lipid complex. The complex was then added into cell culture for further incubation.

### Salivary retrograde cannulation

Salivary cannulation was performed as previously described.[50] A syringe with a 29-gauge needle was placed inside a polyethylene tube for injection. The polyethylene tube was carefully inserted into Wharton's duct of anesthetised mice with atropine pretreatment (0.5 mg kg$^{-1}$, Sigma-Aldrich) under a dissecting microscope. Solutions were slowly delivered into SGs. Salivary cannulation of trypan blue solution demonstrated successful infusion in the SG (Supplementary figure 4). Recombinant IL-17 (0.05 mg kg$^{-1}$ body weight, PeproTech) or IL-17 neutralisation antibody (1 mg kg$^{-1}$ body weight, R&D Systems) solutions were delivered into SGs in some experiments. Control mice received salivary cannulation of PBS or IgG solution. 40 μL solution was used for each injection. ESS mice received salivary retrograde cannulation of IL-17 neutralisation antibody every two weeks for six weeks, starting from day 14 post-first immunisation.

### Statistics

Results are represented as mean ± standard deviation (SD). Pearson's correlation coefficient was used to analyse the correlation between two parameters. The unpaired Student's *t*-test and the nonparametric Mann–Whitney *U*-test were used for single comparison. One-way ANOVA was used for multiple groups comparison. *P*-value $< 0.05$ was considered statistically significant.

## ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China [82071817 and 82004171], Chongqing International Institute for Immunology [2020YJC10], Hong Kong Research Grants Council [17149716], RGC Theme-based Research Scheme (TRS) [T12-703/19-R], the Fundamental Research Funds for Central Public Welfare Research Institutes (ZZ13-YQ-033-C1), Young Elite Scientist Sponsorship Program by CACM (CACM-2020-QNRC2-05) and HKU Seed Funding for Strategic Interdisciplinary Research Scheme. We thank Mr Otis Ko for technical support and the professional service provided by the Imaging and Flow Cytometry Core of Centre for PanorOmic Sciences (CPOS) and Laboratory Animal Unit at The University of Hong Kong.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

## AUTHOR CONTRIBUTIONS

**Fan Xiao:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing-original draft; Writing-review & editing. **Wenhan Du:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Xiaoxia Zhu:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Yuan Tang:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Lixiong Liu:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Enyu Huang:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Chong Deng:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Cainan Luo:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Man Han:** Formal analysis; Funding acquisition; Writing-review & editing. **Ping Chen:** Project administration; Resources; Writing-review & editing. **Liping Ding:** Data curation; Formal analysis; Project administration; Writing-review & editing. **Xiaoping Hong:** Formal analysis; Resources; Writing-review & editing. **Lijun Wu:** Formal analysis; Writing-review & editing. **Quan Jiang:** Formal analysis; Writing-review & editing. **Hejian Zou:** Formal analysis; Writing-review & editing. **Dongzhou Liu:** Formal analysis; Resources; Writing-review & editing. **Liwei Lu:** Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Writing-review & editing.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.