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## Disruption of CXCR3 function impedes the development of Sjögren's syndrome-like xerostomia in non-obese diabetic mice

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### Abstract

The chemokine receptor CXCR3 plays an important role in T cell recruitment in various immune responses and autoimmune diseases. Expression of CXCR3 ligands, including CXCL9, -10 and -11, is elevated in the salivary glands of patients with Sjögren's syndrome (SS). To elucidate whether interaction between CXCR3 and its ligands is required for the development of SS, we administrated an anti-CXCR3 blocking antibody (CXCR3-173) to the non-obese diabetic (NOD) mice, a well-defined model of SS, during the stage prior to disease onset. Treatment with this anti-CXCR3 antibody significantly improved salivary secretion, indicating a remission of SS clinical manifestation. Anti-CXCR3 treatment did not affect the gross leukocyte infiltration of the submandibular glands (SMGs) as assessed by hematoxylin and eosin staining. However, flow cytometric analysis showed that anti-CXCR3 treatment markedly reduced the percentage of CXCR3<sup>+</sup>CD8 T and CXCR3<sup>+</sup>CD44<sup>+</sup>CD8 T cells, without affecting that of CXCR3<sup>+</sup>CD4 T and CXCR3<sup>+</sup>CD44<sup>+</sup>CD4 T cells in the SMGs and submandibular lymph nodes, suggesting a preferential effect of this anti-CXCR3 treatment on CXCR3-expressing effector CD8 T cells. Meanwhile, SMG expression of inflammatory factor TNF- $\alpha$  was markedly diminished by anti-CXCR3 treatment. In accordance, anti-CXCR3 significantly enhanced SMG expression of tight junction protein claudin-1 and water channel protein aquaporin 5, two molecules that are crucial for normal salivary secretion and can be down-regulated by TNF- $\alpha$ . Taken together, these findings demonstrated that the interaction between the endogenous CXCR3 and its ligands plays a pro-inflammatory and pathogenic role in the development of SS-like xerostomia in the NOD mouse model.

### Introduction

Sjögren's syndrome (SS) is a prevalent autoimmune disease that affects millions of people worldwide, predominantly women<sup>1–3</sup>. A characteristic pathology of this disease is leukocyte infiltration of salivary and lacrimal glands, leading to chronic inflammation, tissue destruction and secretory hypofunction<sup>4–6</sup>. Autoreactive effector T cells play an essential role in the pathogenesis of this disease<sup>7–10</sup> and the migration of these cells into exocrine

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Disclosure

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glands requires the interaction between T cell-expressed chemokine receptors and with their specific ligands<sup>11</sup>.

CXCR3 (C-X-C Motif Chemokine Receptor 3), a G protein coupled chemokine receptor, is absent on resting T cells and preferentially expressed by T helper 1 (Th1) CD4<sup>+</sup> T cell, and T cytotoxic 1 (Tc1) CD8<sup>+</sup> T cells<sup>12</sup>. CXCR3 can bind to and be activated by its ligands CXCL9 (chemokine induced by IFN- $\gamma$ ), CXCL10 (interferon-inducible protein 10) and CXCL11 (interferon-inducible T-cell  $\alpha$  chemoattractant) to orchestrate effector T cell generation in lymphoid tissues and their subsequent trafficking to peripheral inflammatory sites<sup>13</sup>. Expression of CXCL9, -10 and -11 can be up-regulated by pro-inflammatory cytokines, including IFN- $\gamma$  and TNF- $\alpha$ , in a variety of cell types<sup>13-15</sup>. Consistent with this, our recent study demonstrated the down-regulation of CXCL9 in the salivary glands of SS-like mouse model as a result of TNF- $\alpha$  blockade<sup>16</sup>. CXCR3 ligands play important roles in the pathogenesis of various inflammatory and autoimmune diseases<sup>13,17-21</sup>. Deficiency of CXCR3 leads to impaired infiltration of effector T cells into disease target organs and amelioration of autoimmune insulinitis, diabetes and systemic lupus erythematosus (SLE)<sup>22-24</sup>. Inhibition of CXCR3 function with blocking antibodies or small molecule antagonists has shown a therapeutic effect in experimental models of T cell-mediated inflammatory diseases, including rheumatoid arthritis, alopecia areata, adjuvant arthritis and severe sepsis, with the effect mostly associated with inhibition of Th1 and Tc1 cell recruitment to the target organs<sup>25-28</sup>. In addition, a therapeutic regimen that blocks CXCL10-CXCR3 axis using a neutralizing anti-CXCL10 antibody has recently been tested in a randomized and double-blind clinical trial for rheumatoid arthritis and led to promising outcomes<sup>29</sup>. Collectively, CXCR3 and its ligands contribute to the pathogenesis of a variety of T cell-dependent inflammatory and autoimmune diseases.

The recruitment of CXCR3-expressing T cells and elevated CXCR3 ligands CXCL9, -10 and -11 produced by ductal epithelial cells have been detected in the salivary glands of SS patients and experimental mouse models of this disease<sup>30-32</sup>, which suggests that CXCR3 expression on T cells may play a critical role in the migration of these cells into disease-targeting organs and the initiation of local autoimmune responses. Consistent with this potential function of CXCR3, antagonism of CXCL10 activity in MRL/lpr mouse model of SS significantly impedes the progression of SS-like sialadenitis and ameliorates tissue destruction by reducing leukocyte infiltration of the submandibular glands<sup>33</sup>. However, the function of overall CXCR3-dependent pathways constituted by chemokine receptor CXCR3 and its three ligands in the pathogenesis of SS, particularly in the early stage of the development prior to disease onset, has not been characterized.

In the present study, we investigated the particular role of endogenous CXCR3-mediated pathways in the development of SS-like sialadenitis, using a neutralizing antibody targeting the receptor CXCR3, rather than individual ligands, in non-obese diabetic (NOD) mice, a well-defined mouse model of SS. We demonstrated that CXCR3 plays an indispensable role in the development of SS-like xerostomia.

## Materials and methods

### Mice.

Female non-obese diabetic (NOD) mice were purchased from the Jackson Laboratory and were maintained in the specific pathogen-free animal facility at the Forsyth Institution. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Forsyth Institute. All the procedures were performed in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

### Antibodies.

Purified monoclonal anti-mouse CXCR3 (CXCR3-173) and its isotype control hamster IgG used for injection were obtained from BioXCell. For flow cytometry, anti-CD4, anti-CD8, anti-CD44, and anti-CXCR3 antibodies were purchased from BioLegend. For immunohistochemical chemistry, anti-TNF- $\alpha$  and anti-claudin-1 antibodies were purchased from Abcam. For immunofluorescence staining, anti-aquaporin-5 (AQP5) and Alexa Fluor647-conjugated rabbit IgG were purchased from Abcam.

### *In vivo* administration of anti-CXCR3 antibody.

4 week-old female NOD mice received intraperitoneal (*i.p.*) administration of 200  $\mu$ g anti-mouse CXCR3 antibody or control hamster IgG 3 times weekly for 6 weeks. All the analyses were performed 2 days after the last injection.

### Histological, immunohistochemical and immunofluorescence staining.

Harvested SMG tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned to 5  $\mu$ m thickness. The sections were de-paraffinized and subsequently stained with hematoxylin and eosin (H&E) to determine the degree of inflammation. For immunohistochemical staining, the de-paraffinized sections were stained with antibodies against mouse TNF- $\alpha$  and claudin-1 at 4°C overnight using VECTASTAIN Elite ABC Kit (Vector Laboratories) following the manufacturer's manual. For immunofluorescence staining, the de-paraffinized sections were incubated with anti-AQP5 antibody after antigen retrieval. They were then incubated with Alexa Fluor 647-conjugated mouse IgG and imaged using a Leica laser scanning confocal microscope (Zeiss). ImageJ 1.50i software was used to quantify the positively stained areas of immunohistochemical staining and the fluorescence intensity of immunofluorescence staining in the sections.

### Flow cytometry.

Freshly isolated single cells from SMG or submandibular lymph nodes (smLNs) were firstly incubated with anti-CD16/32 antibody to block non-specific binding of immunoglobulin to Fc $\gamma$ III/II receptors. Then, the cells were stained with combinations of fluorescence-conjugated antibodies to CD4, CD8 CD44 and CXCR3 at 4°C for 30 min. After washing with cold PBS containing 1% FBS, all the stained samples were analyzed with FACS Arial II flow cytometer (BD), and the raw data were calculated with FlowJo V10 software.

### Detection of serum antinuclear antibodies (ANA).

ANA levels in mouse sera (1:40 dilution in PBS) were determined using HEp-2 human epithelial cell substrate slides (INOVA Diagnostics), according to the manufacturer's instructions. After staining, the images were acquired on an inverted wide-field fluorescence microscope (Zeiss) at 400× magnification. Images presented were processed using Zeiss software (ZEN blue edition).

### ELISA.

The custom synthesized M3R peptide fragment (Biomatik Corporation) containing 15 amino acids of the second extracellular loop of M3R was dissolved in PBS initially and further diluted to a final concentration of 2 µg/ml in the 1×ELISA coating buffer (BioLegend) immediately before use. The Nunc™ MaxiSorp™ flat-bottom 96 well plates (BioLegend) were coated with the M3R peptide solution at 4°C overnight. Non-specific binding sites on the plates were blocked with ELISA Assay Diluent buffer (BioLegend). Sera (1:6 diluted) were then added to the plate and incubated overnight at 4°C. After washing with 0.05% Tween 20 in PBS, the plates were incubated with 1:300 diluted biotinylated goat anti-mouse IgG antibody (Vector Laboratories) for 1 h. Subsequently, the plates were incubated with avidin-HRP solution for 30 min following removal of excess detection antibody. Finally, the bound antibodies on plates were detected by the TMB substrate and the reaction was stopped with 100 µl 2 N H<sub>2</sub>SO<sub>4</sub> each well. The absorbance at 450 nm was read with a microplate reader (BioTek).

### Measurement of stimulated salivary flow rate.

NOD mice received an *i.p.*- injection of 100 µl PBS-based secretagogue solution containing pilocarpine (0.05 mg/ml) and isoproterenol (0.02 mg/ml). One min after secretagogue administration, saliva was collected continuously for 5 min from the oral cavity of mice with a micropipette. The volume of saliva from each mouse was measured and normalized to the body weight. The measurement was consistently performed in the mid-afternoons to exclude the effect of circadian rhythm on the results.

### Real-time RT-PCR.

Total RNA was extracted using RNeasy Micro kit (Qiagen) and the complementary DNA (cDNA) was synthesized with MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Subsequently, SYBR Green-based real-time PCR amplification (Qiagen) was performed for 40 cycles with annealing and extension temperature at 60°C, on a LightCycler 480 Real-Time PCR System (Roche). Primer sequences are as follows: mouse CXCL9, forward, 5'-CCCTCAAAGACCTCAAACAGT-3'; reverse, 5'-AGTCCGGATCTAGGCAGGTT-3'; CXCL10 forward, 5'-CCAGTGAGAAATGAGGGCCAT-3', reverse, 5'-CCGGATTCAGACATCTCTGC-3'; CXCL11 forward, 5'-GCAGAGATCGAGAAAGCTTCT-3', reverse, 5'-GTCCAGGCACCTTTGTCGTT-3'. All transcript levels were normalized to β-actin.

### Statistical analysis.

The Student's t-test (two-tailed, two sample equal variance) was used to determine all statistical significance. P values smaller than 0.05 were considered as statistically significant.

## Results

### Expression of CXCR3 ligands in the submandibular glands (SMGs) of NOD mice

Elevated expression of CXCR3 ligands, CXCL9, -10 and -11 is often detected in the target tissues of autoimmune diseases<sup>17</sup>, including the SMGs of SS patients<sup>32, 34</sup>. Here, we first determined whether the increased expression of these CXCR3-targeting chemokines in the SMGs accompanies the development of SS disease in female NOD mice. These mice used in our study have the disease initiation around 10 weeks of age as demonstrated by the presence of SMG-infiltrating leukocyte foci and serum ANA, as well as reduced salivary flow rate in majority of these mice compared to 4 week-old female NOD mice as well as 10 week-old control Balb/c mice (data not shown). We thereby examined the mRNA levels of CXCR3 ligands in the SMGs of female NOD mice at 4-, 7-, and 10 weeks of age by real-time PCR analysis. The results showed that the mRNA amounts of all three CXCR3 ligands were significantly increased between 4 and 10 weeks of age (Figure 1A). In contrast, CXCL10 and CXCL11 did not increase with between 4 and 10 weeks of age in the control Balb/c mice (Figure 1B). Although CXCL9 did exhibit an age-dependent increase in the Balb/c mice, the degree of increase (3.6 fold) was significantly smaller than that in the NOD mice (9.1 fold, Figure 1B). Therefore, the increase in CXCR3 ligand levels in the NOD mice is mostly due to the SS disease development but not the aging process per se. Hence, CXCR3 ligand expression in the SMGs is increased accompanying the development of SS in the NOD mice, suggesting the involvement of these CXCR3 ligands in the pathogenesis of this disease.

### ***In vivo* administration of a blocking antibody against CXCR3 impedes the development of hyposalivation without affecting gross leukocyte infiltration of SMGs in NOD mice**

To determine whether CXCR3 ligand-CXCR3 interaction plays an important role in the development of SS, we *i.p.*-injected 200 µg of an anti-CXCR3 antibody, which blocks the interaction between CXCR3 and its ligands<sup>35</sup>, or its isotype control, into 4 week-old female NOD mice 3 times weekly for 6 weeks. Since impaired salivary secretion is one of the signature pathologies of SS, we measured this parameter and demonstrated that anti-CXCR3-treated mice had a higher stimulated salivary flow rate, normalized to the body weight, compared to that of IgG-treated controls (Figure 2A). Hence, blockade of endogenous CXCR3 impedes the development of salivary gland secretory dysfunction in NOD mice.

We further assessed the effect of endogenous CXCR3 on leukocyte infiltration of SMGs, another pathological hallmark of SS. Interestingly, H&E staining showed that the gross amount of leukocytes in the SMGs was not reduced by anti-CXCR3 treatment (Figure 2B).

### **Treatment with anti-CXCR3 reduces the frequency of CXCR3-expressing effector CD8 T cells in the SMGs and submandibular lymph nodes (smLNs)**

Since CXCR3 plays an important role in the trafficking of effector T cells into inflamed peripheral tissues, we further investigated whether CXCR3 blockade could affect the CD4 T- and CD8 T cell subsets. Consistent with the H&E results, flow cytometric analysis revealed that the percentage of CD4 and CD8 T cells in the SMGs was comparable between anti-CXCR3 treated and IgG-treated mice. However, CXCR3 blockade significantly reduced the frequency of CXCR3<sup>+</sup>CD8 T and CXCR3<sup>+</sup>CD44<sup>+</sup>CD8 T cells, whereas it did not alter that of CXCR3<sup>+</sup>CD4 T or CXCR3<sup>+</sup>CD44<sup>+</sup>CD4 T cells in the SMGs (Figure 3A). Consistent with the findings in the SMGs, anti-CXCR3 treatment did not affect the percentage of total CD4 or CD8 T cells, but markedly reduced that of CXCR3<sup>+</sup>CD8 T and CXCR3<sup>+</sup>CD44<sup>+</sup>CD8 T cells without affecting that of CXCR3<sup>+</sup>CD4 T and CXCR3<sup>+</sup>CD44<sup>+</sup>CD4 T cells in the smLNs (Figure 3B). The findings indicate that the accumulation of Tc1 cells, rather than Th1 cells is selectively inhibited in SS disease-targeting organs as a result of CXCR3 blockade. In addition, we also investigated the effect of anti-CXCR3 on B cells, which together with T cells constitute the majority of infiltrating leukocytes in the SMGs<sup>1, 36</sup>. The results demonstrated that the percentage of B cells, defined by CD19 expression, in the SMGs was not changed by anti-CXCR3 treatment in a statistically significant fashion (Figure 3A). Hence, our findings suggest the preferential effect of this anti-CXCR3 antibody treatment on CD8 effector T cell accumulation/expansion in the SMGs and the smLNs, which may contribute to the secretory dysfunction and pathogenesis of SS in NOD mice.

### **Treatment with anti-CXCR3 reduces TNF- $\alpha$ expression and elevates claudin-1 and AQP5 expression in the SMGs**

Excessive pro-inflammatory factor TNF- $\alpha$  has been detected in both salivary glands and blood of SS patients<sup>37,38</sup> and plays a pathogenic role in SS development in the NOD model of SS disease<sup>16</sup>. To assess whether CXCR3 blockade can diminish TNF- $\alpha$  production, we performed immunohistochemical staining of SMG sections, which showed that anti-CXCR3 treatment significantly reduced the TNF- $\alpha$  protein level (Figure 4A). Hence, the results imply that endogenous CXCR3 exerts tissue pro-inflammatory function through exacerbating TNF- $\alpha$  production during the development of SS disease.

Impaired tight junction (TJ) integrity has been documented in the salivary glands of SS patients<sup>39</sup> and contributes to the secretory dysfunction of rat salivary gland epithelial cells<sup>40</sup>. To determine whether CXCR3 blockade affects the tight junction integrity in the SMGs, we examined the protein levels of claudin-1, claudin-2 and occludin in the SMGs by immunohistochemical staining and found that claudin-1 expression was significantly increased, whereas claudin-2 or occludin levels was not altered by anti-CXCR3 treatment (Figure 4B, and data not shown). The results suggest that the improved salivary secretion as a result of CXCR3 blockade may be associated with elevated claudin-1, which is important for TJ structure and function<sup>41</sup>. To define additional potential molecular mechanisms of enhanced salivary secretion caused by anti-CXCR3 treatment, we measured the level of AQP5, a water channel protein that is crucial for normal salivary fluid secretion<sup>42,43, 44</sup>. By immunofluorescence staining of SMG sections, we found a markedly higher level of AQP5 expression in the mice with CXCR3 blockade than IgG treatment controls (Figure 4C).

Thus, the increased APQ5 expression may account for the improvement of salivary secretion induced by CXCR3 blockade.

### Anti-CXCR3 treatment leads to enhanced autoantibody production

To explore whether CXCR3 blockade alters the production of serum autoantibodies, a characteristic pathological change of SS disease, we examined serum antinuclear antibody (ANA) levels by indirect immunofluorescence staining using human epithelial (HEp-2) cells as substrates. The results showed that anti-CXCR3 treatment led to a substantial increase in the level of ANA as indicated by higher fluorescence intensity of the staining compared to IgG-treated control group (Figure 5A). We also measured the level of autoantibodies against M3 muscarinic acetylcholine receptor (M3R), which are important factors contributing to the secretory dysfunction of salivary gland in response to neurotransmitters<sup>2, 45</sup>. Enzyme-linked immunosorbent assay (ELISA) demonstrated a higher level of anti-M3R antibodies in the sera from anti-CXCR3 treated mice than those from IgG-treated mice (Figure 5B). Taken together, these findings showed that CXCR3 blockade leads to exacerbated autoantibody production during SS development, indicating its complex and multifaceted functions in this disease.

### Discussion

This study demonstrated that endogenous CXCR3-mediated pathways play important pro-inflammatory and secretory inhibitory roles in the development of SS-like sialadenitis using NOD mice and provided several potential cellular and molecular mechanisms of CXCR3 function, including promotion of effector CD8 T cell accumulation, enhancement of TNF- $\alpha$  production, and down-regulation of tight junction protein claudin-1 and water channel protein AQP5 in the SMGs, a target tissue of SS.

The antibody clone targeting CXCR3 (CXCR3-173) used in our work has been reported to suppress T cell migration by inhibiting CXCR3-dependent pathways in multiple *in vivo*-studies<sup>26, 46</sup>. An *in vitro* study has demonstrated that CXCR3-173 inhibits the binding of CXCL10 and -11 to CXCR3<sup>35</sup>. It has also shown that the antibody has little inhibitory effect on the binding between CXCL9 and CXCR3, a claim that requires more rigorously designed assays to further confirm. Nevertheless, using this antibody, our study unequivocally demonstrated that the interaction between CXCR3 and its ligands plays a crucial role in the development of SS in the NOD model of this disease.

T cell trafficking into exocrine glands is a critical event for the pathogenesis of SS disease<sup>1</sup>. Studies of SS patients have reported the presence of elevated expression of T cell chemoattractants CXCL9, -10 and -11 in the salivary glands<sup>31, 32</sup>. In accordance with these findings, we showed that the expression of these three CXCR3-binding ligands in the SMGs of NOD mice increases with age accompanying SS development. By inhibiting CXCR3 function with a neutralizing anti-CXCR3 antibody, we demonstrated that CXCR3 is essential for the development of salivary secretory dysfunction in NOD mice. Moreover, anti-CXCR3 treatment preferentially reduces the number of CXCR3-expressing effector CD8 T cells in SMGs and smLNs. Our results are consistent with a recent report showing that administration of the same anti-CXCR3 antibody clone to a mouse model of areata

(AA), another autoimmune disease, prevents the recruitment of a subset of effector CD8 T cells to the target tissue sites and lymph nodes, and restrains the development of this disease<sup>26</sup>. Moreover, CXCR3-deficiency impairs the migration of effector CD8<sup>+</sup> T cells into lung in a lung transplantation rejection model<sup>47</sup>. Thus, CXCR3-mediated pathways have a crucial function in effector CD8 T cell trafficking into the inflamed tissue sites under various disease conditions. In addition, our study did not find a significant effect of CXCR3 blockade on the total SMG-infiltrating mononuclear cells, T cells and Th1 cells, which is different from a previous report showing that CXCL10 antagonism in MRL-lpr model of SS markedly reduces the amount of total mononuclear cells, T cells and CXCR3<sup>+</sup> T cells in the SMGs<sup>33</sup>. The difference may be in part attributed to the different targets of blockade. Compared to the antagonism of CXCL10 activity alone, our study used a neutralizing antibody targeting CXCR3 receptor rather than a single ligand, in order to more comprehensively reveal the function of CXCR3-mediated pathways in the SS development. Additionally, it may also be attributed to the differences in the disease pathogenesis and pathologies resulting from the different genetic and environmental factors in the two mouse models used.

It is also worth noting that our study excluded the interference from the clinical type-1 diabetes, which generally develops after 12–16 weeks of age in female NOD mice, later than the initial onset of SS when the mice were analyzed in this study. Hence, the effect of anti-CXCR3 treatment on SS is not an indirect consequence of altered type-1 diabetes development.

Elevated level of pro-inflammatory factor TNF- $\alpha$  has been detected in the SMGs of SS patients<sup>38</sup>. Our recent report provided evidence that TNF- $\alpha$  is essential for the pathogenesis of SS through promoting CXCR3 ligand expression and facilitating T lymphocyte infiltration of the SMGs of NOD mice<sup>16</sup>. In the present work, we demonstrated that CXCR3 blockade in turn down-regulates TNF- $\alpha$  expression in the SMGs, indicative of a positive feed-forward loop formed between CXCR3 and TNF- $\alpha$  which amplifies salivary gland inflammation. Moreover, both TNF- $\alpha$  neutralization and CXCR3 blockade lead to increased claudin-1 and AQP5 levels in the SMGs<sup>16</sup>, further suggesting a close interplay between TNF- $\alpha$  and CXCR3-mediated pathways. Claudin-1 is an essential component of intercellular tight junction, crucial for a well-regulated diffusion barrier between apical and basal regions of exocrine gland cells and maintenance of water and solute exchange<sup>41</sup>. AQP5 is essential for normal salivary secretion and studies have shown that AQP5 deficiency significantly impairs secretory function due to defective water export in salivary epithelial cells and that local gene therapy with AQP1 overexpression can compensate for AQP5 deficiency<sup>42–44</sup>. Hence, these findings suggest that endogenous CXCR3-mediated pathways promote salivary secretory dysfunction, at least in part, through augmenting TNF- $\alpha$  production to down-regulate the expression of claudin-1 and AQP5.

One intriguing finding in this study is that administration of anti-CXCR3 antibody exacerbates the production of serum ANA and anti-M3R autoantibodies, accompanied by diminished TNF- $\alpha$  expression. In fact, we and other groups have reported the negative correlation between the autoantibody production and TNF- $\alpha$  pathway<sup>16, 48, 49</sup>. We thereby speculate that the appearance of excessive autoantibodies caused by CXCR3 blockade is the



consequence of diminished TNF- $\alpha$  production. Interestingly, anti-TNF- $\alpha$  induced auto-antibodies in patients with lupus erythematosus usually fall back to baseline levels shortly after the termination of anti-TNF- $\alpha$  treatment, and thus only leads to transient and reversible clinical manifestation of this disease.<sup>49–51</sup>. The exact function and potential mechanism of the excessive autoantibody production resulting from the CXCR3 blockade during SS development await to be further investigated.

Finally, the pathogenic functions of CXCR3 and its chemokine ligands have been demonstrated in multiple autoimmune disorders, including those that are frequently associated with SS, such as autoimmune thyroiditis, rheumatoid arthritis and SLE{Antonelli, 2014 #84}. Hence, CXCR3 pathway-mediated enhancement of Th1/Tc1 immune responses in target tissues appears to be a common characteristic and underlying mechanism of SS and its associated autoimmune diseases. A better understanding of the precise contributions of the key players in this process to the disease pathogenesis and persistence could advance the development of new strategies to treat SS and its associated disease conditions.

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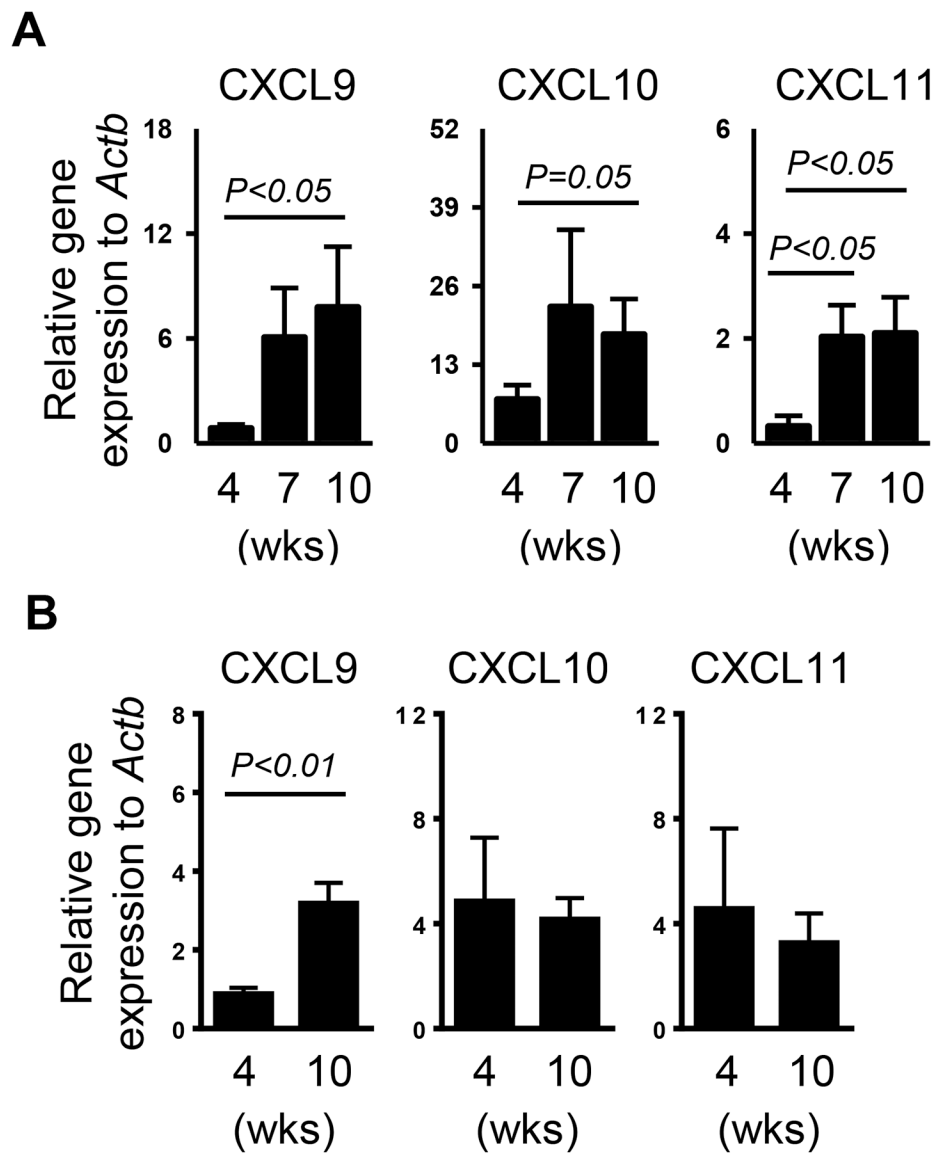
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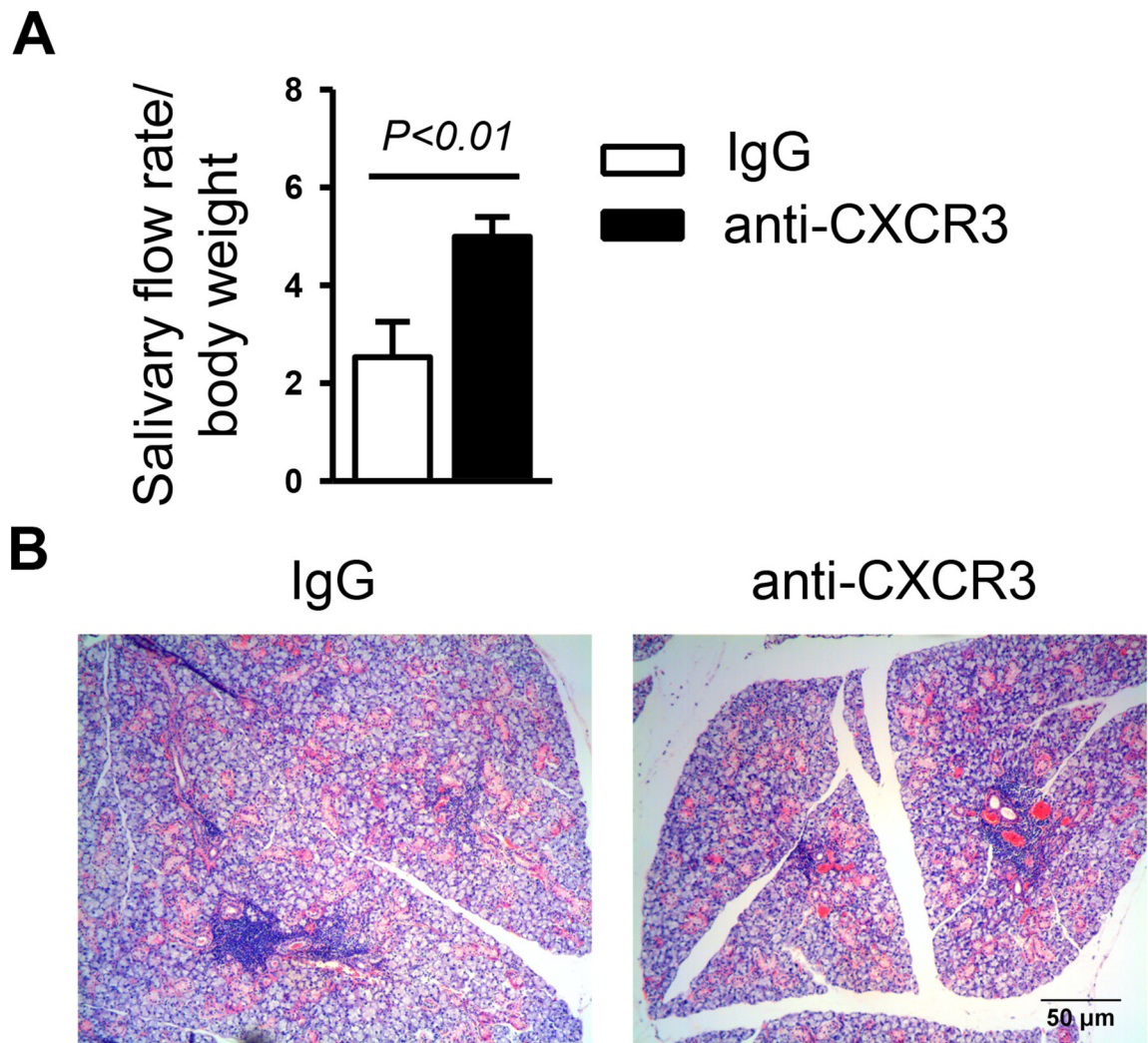
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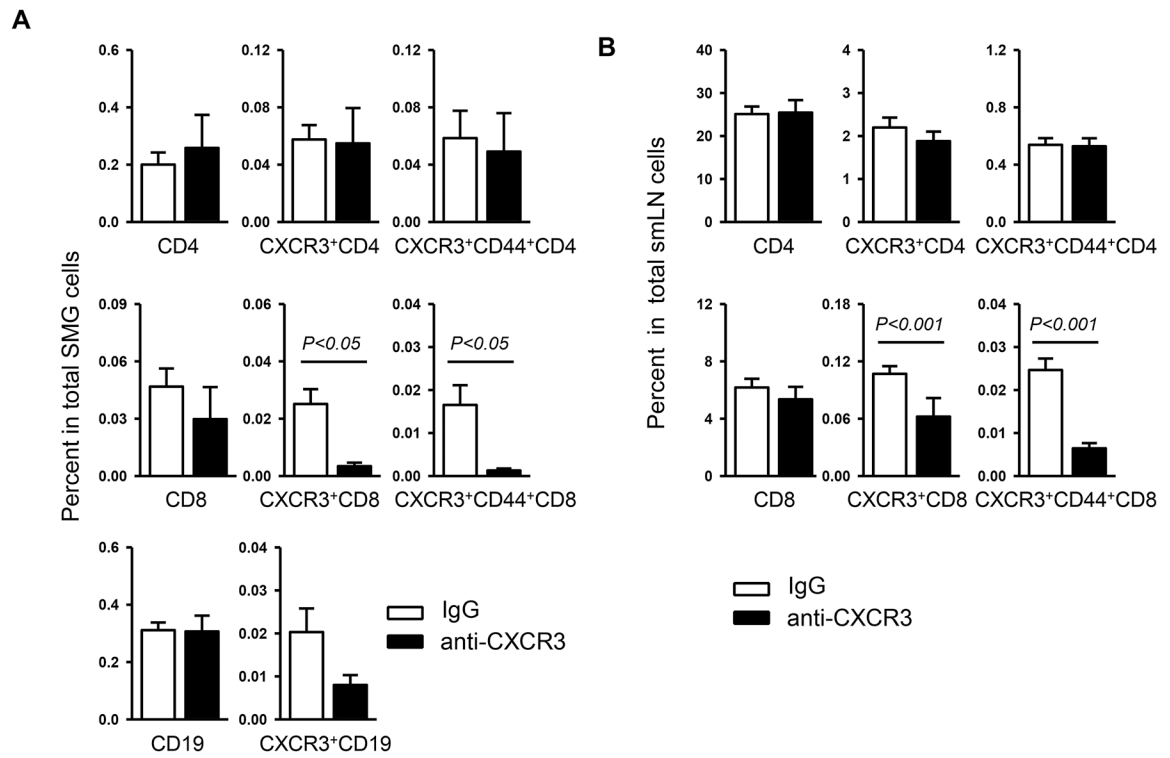


**Figure 1. Expression level of CXCR3 ligands in the SMGs of NOD and Balb/c mice.** Real-time PCR analysis of CXCL9, -10 and -11 levels in the SMGs of NOD mice aged 4, 7 and 10 weeks (A) and those in the SMGs of Balb/c mice aged 4 and 10 weeks (B). The results are presented relative to that of  $\beta$ -actin. Data are the average of analyses of 4–7 mice for each group. Error bars represent the SEM.



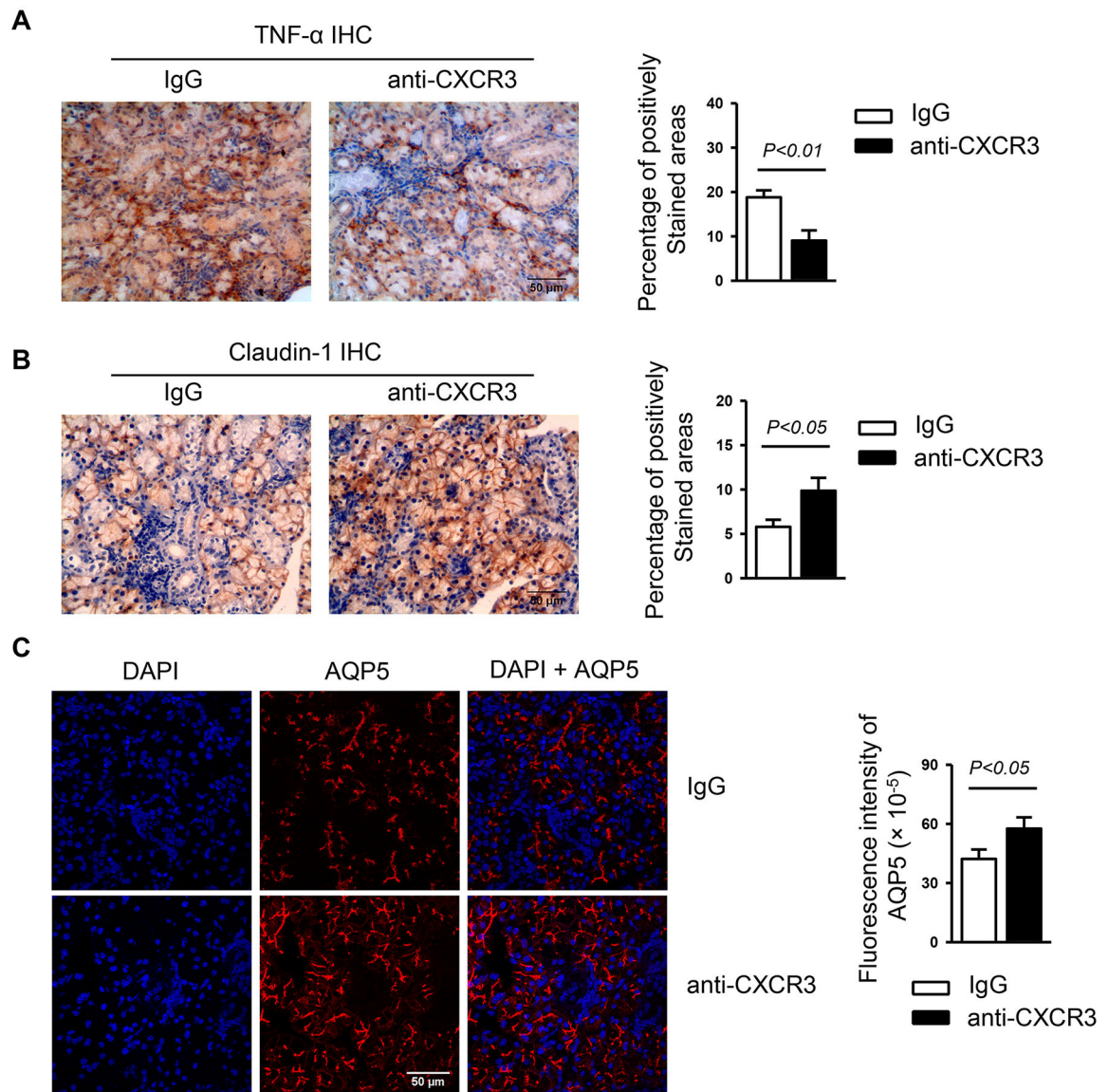
**Figure 2. Anti-CXCR3 treatment impedes the development of hyposalivation without affecting gross leukocyte infiltration of SMG in NOD mice.**

Anti-CXCR3 antibody or IgG was *i.p.*-administered to 4-week-old female NOD mice 3 times weekly for 6 weeks. (A) The volume of saliva that was collected during the 5 min period, normalized to body weight. (B) Images of H&E staining of SMG sections. Original magnification:  $\times 200$ . Data are representative or the average of 6–12 mice for each group.



**Figure 3. Anti-CXCR3 treatment reduces the frequency of CXCR3-expressing effector CD8 T cells in the SMGs and smlNs.**

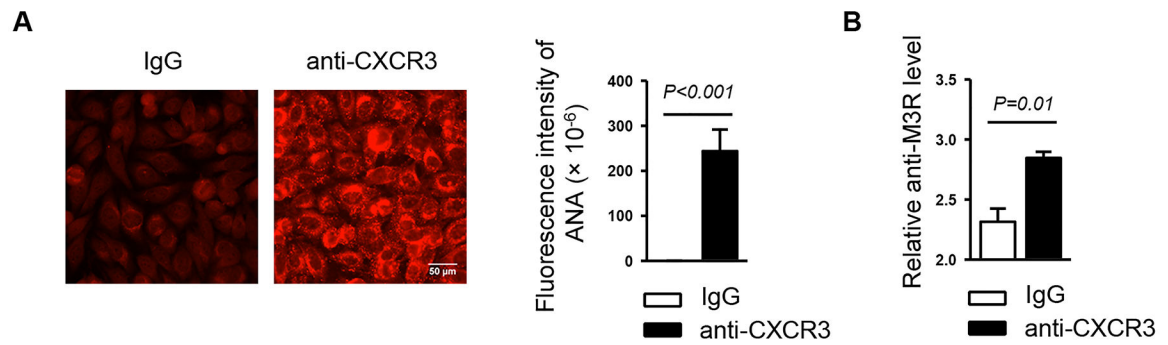
Anti-CXCR3 antibody or IgG was *i.p.*-administered to 4-week-old female NOD mice 3 times weekly for 6 weeks. Flow cytometry of lymphocyte populations in the SMGs (A) and smlNs (B). Data are the average of analyses of 6–12 mice for each group.



**Figure 4. Anti-CXCR3 treatment reduces TNF- $\alpha$  expression and elevates claudin-1 and AQP5 expression in the SMGs.**

Anti-CXCR3 antibody or IgG was *i.p.*-administered to 4-week-old female NOD mice 3 times weekly for 6 weeks. (A) Immunohistochemical staining of TNF- $\alpha$  protein in SMG sections. Bar graph shows the percentage of positively stained areas in the sections. (B) Immunohistochemical staining of claudin-1 protein in SMG sections. Bar graph shows the percentage of positively stained areas in the sections. (C) Immunofluorescence staining of AQP5 protein in SMG sections. Bar graph shows the fluorescence intensity of AQP5 staining. Data are representative or the average of analyses of 12–15 mice for each group. All images were captured with  $\times 400$  original magnification.





**Figure 5. Anti-CXCR3 treatment leads to enhanced autoantibody production.**

Anti-CXCR3 antibody or IgG was *i.p.*-administered to 4-week-old female NOD mice 3 times weekly for 6 weeks. (A) Detection of serum ANA. Original magnification:  $\times 400$ . Bar graph shows the fluorescence intensity of ANA staining. (B) Analyses of serum anti-M3R autoantibody level by ELISA. Data are representative or the average of analyses of 12–15 mice for each group.