Tofacitinib alleviated salivary gland inflammation and reduced the percentages of effector T cells in murine Sjögren's disease

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Abstract

Objective

The Janus kinases-signal transducer and activator of transcription (JAK-STAT) signalling pathway plays a crucial role in autoimmunity and the signalling pathways of many cytokines in Sjögren's disease (SjD). Therefore, the aim of this study was to investigate both the therapeutic and immunomodulatory effects of the oral JAK3/JAK2/JAK1 inhibitor tofacitinib in a murine model of SjD.

Methods

Tofacitinib or vehicle was administered orally to the mice with SjD for 6 weeks. Salivary flow rate was measured every three weeks. Pathological changes of salivary gland were detected by haematoxylin-eosin staining, and the percentages of subsets of CD4+ T cells and B cells in the cervical lymph nodes (cLNs) and spleen was determined by flow cytometry.

Results

Tofacitinib significantly ameliorated submandibular gland inflammation compared to the control group, as evidenced by reduced lymphocytic infiltration. Salivary flow rates improved significantly in tofacitinib treated mice compared to controls, indicating restored salivary gland function. The treatment also led to a substantial decrease in follicular helper T (Tfh) cells and the Tfh/Treg ratio in both the spleen and cLNs. Additionally, the frequencies of T helper 1 (Th1) and T helper 17 (Th17) cells were reduced in the spleen and cLNs.

Conclusion

Our data indicated that tofacitinib reduced percentages of effector T cells in an animal model of SjD. In addition, tofacitinib alleviated salivary gland inflammation and hypofunction, offering new insights into the clinical management of SjD.

Key words

NOD mice, Sjögren's disease, salivary gland, Janus Kinase inhibitors, follicular helper T cells

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Received on September 30, 2024; accepted in revised form on October 21, 2024.

Funding. This work was supported by grants from the National Key R&D Program of China (2022YFE0131700 and 2022YFC3602000), National Natural Science Foundation of China (82071813 and 82271835), and Beijing Nova Program 20220484206.

Competing interests: none declared.

Introduction

Sjögren's disease is a chronic, systemic autoimmune disease characterised by loss of exocrine gland function. Patients with SjD may also show a diversity of extraglandular manifestations, such as lung disease, kidney disease, arthralgia, and fatigue. The aetiology and pathogenesis of SjD are not clear, which may be caused by genetic predisposition, environmental trigger, autoimmunity, and other relevant factors (1, 2). Current treatment strategies mainly improve clinical symptoms and suppress autoimmune responses (3-5). However, medications that improve symptoms achieve limited effectiveness in most patients and have substantial side effects, including increased infection and malignancy risks. Therefore, there is an urgent need to identify new therapeutic drugs.

The pathophysiological features of SjD comprise lymphocytic infiltration, deposition of immunocomplexes, and ectopic germinal centre formation. Mechanistically, activated T cells providing stimulation to B cells are central to these abnormalities. Th17 cells may promote B cell activation by secreting cytokines such as Interleukin (IL)-17 (6, 7), leading to increased levels of autoantibodies. Tfh cells play an essential role in the humoral immune response, as well as, the formation of germinal centre (GC) in secondary lymphoid organs and autoantibodies production (8, 9). Regulatory T (Treg) cells are vital for protecting immune homeostasis via modulation of pathological immune responses and the induction of immune self-tolerance. The imbalance of Teff/Treg has been observed previously and is thought to be the key mechanism for disease initiation and progression in SjD (10). Recent research has systematically confirmed that JAK-STAT signalling is activated in peripheral blood and labial salivary gland (LSG) *in vitro*. Furthermore, an increasing number of studies have provided evidence that the JAK-STAT pathway plays an important role in the pathogenesis of Sjögren's disease (SjD) through the direct or indirect activation of B cells (11, 12).

Tofacitinib inhibits JAK1, JAK2, and JAK3 *in vitro* with functional cellular selectivity for JAK1 and JAK3 over JAK2. It has been reported that tofacitinib mitigated interferon (IFN)-β-induced pSTAT1 proteins in primary salivary gland epithelial cells (pSGECs) and IFNβ induced phosphorylation of multiple STATs in all cell subsets in PBMCs (13). Therefore, therapeutics targeting these signalling pathways may be expected to suppress Sjögren's disease progression. Therefore, the aim of this study was to determine whether tofacitinib could exert anti-inflammatory and immunomodulatory effects, improve the structural and functional damage of the exocrine grands of mice with SjD.

Methods

Mice and experimental protocol

Female NOD mice (NOD/ShiLtJ strain, 7 weeks old, weighing 17-20 g, No. N000235) were procured from the Model Animal Research Centre of Nanjing University (Nanjing, China), housed at the Laboratory Animal Centre in Peking University (Beijing, China) and were adaptively fed for 1 week. The mice, which shared the same genetic background, were randomly allocated into control group (n=5) and experimental group (n=5). From week 8 to week 14, the mice received oral administration of either vehicle or tofacitinib at a dose of 30 mg/kg once daily (14, 15). Saliva flow rates, body weight and the fasting blood glucose were measured every 3 weeks. Then, the mice from each group (n=5) were killed by cervical dislocation at week 14, and submandibular glands (SMGs), cLNs and spleen tissues were collected. The Institutional Ethics Committee of Peking University approved all animal experiments (permit no: 2021PHE026).

JAK inhibitor

Tofacitinib, obtained from Sichuan Kelun Pharmaceutical Research Institute, was suspended in 0.025% Tween-20 (FeiMoBio, Beijing) for *in vivo* studies.

Cell isolation and treatment

Freshly dissected murine spleens and cLNs were prepared for flow cytometry. Spleens were isolated, and the red

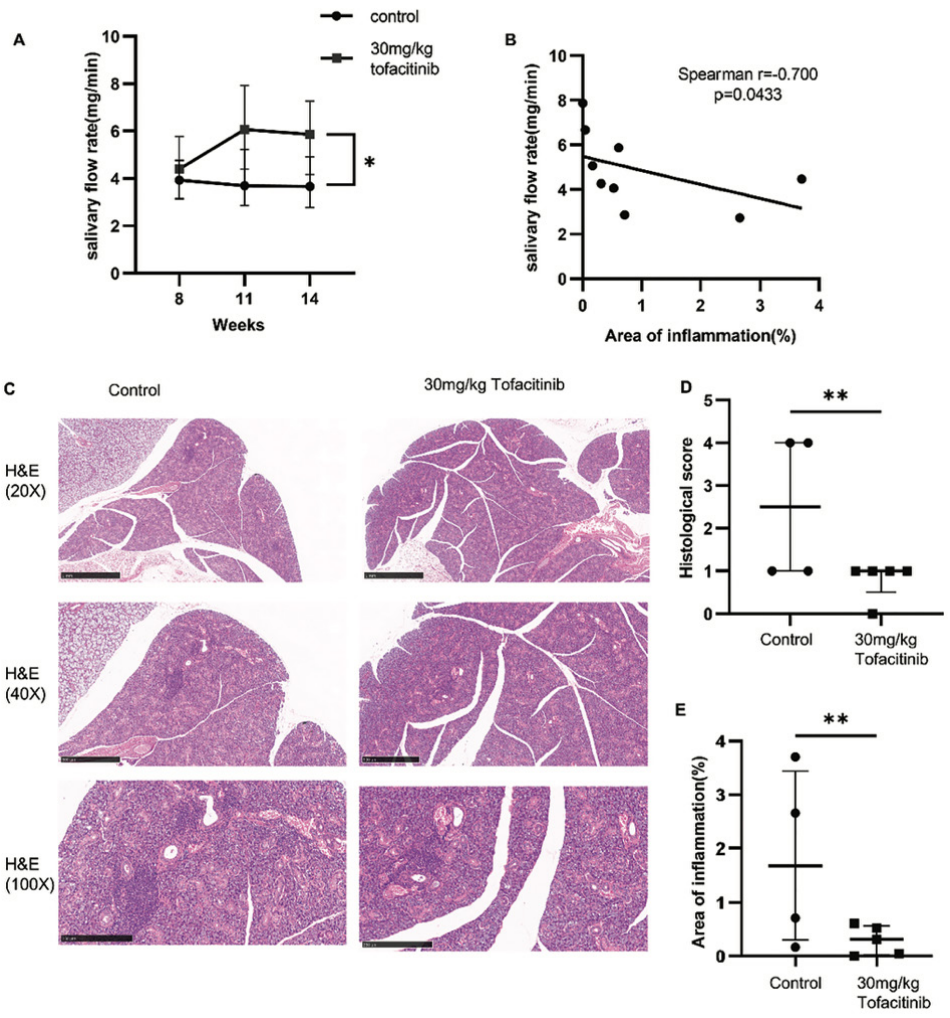

Fig. 1. Effects of tofacitinib on salivary flow rate and lymphocytic infiltration of salivary gland.
A: The salivary flow rate normalised to body weight was collected every 3 weeks after pilocarpine stimulation (n=5 per group).
B: Correlation analysis between the proportion of inflammation area and salivary flow rates.
C, D, E: Mouse SMGs tissues were stained with haematoxylin and eosin (H&E). The lymphocytic foci were circled and visualised under 20× objective, 40× objective, and 100× objective to evaluate the degree of lymphocytic infiltration. The number of lymphocytic foci and the area of lymphocytic infiltration in response to tofacitinib were reduced as compared with control group.
*p<0.05; **p<0.01; ***p<0.001.
SMGs: submandibular glands.

blood cells in splenocyte suspensions were lysed using red cell lysate. Cells were collected and homogenised in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco).

Flow cytometry

Freshly dissected murine spleens and cLN were prepared for flow cytometry. Splenocytes and cLN cells were stained with a combination of fluorescence-conjugated monoclonal antibodies against surface markers CD3, CD4, CD8, PD-1, CD25, CXCR5, CD95, GL-7, and B220 at 4°C for 30 minutes. Subsequently, intracellular and nuclear staining was performed using monoclonal antibodies against Forkhead box P3 (Foxp3), IFN-γ, and IL-17A. Detailed information about all antibodies used in the study can be found in Supplementary Table S1. Stained cells were acquired and analysed using a CytoFLEX flow cytometer (Beckman Coulter, IN, USA) and Kaluza Analysis software (Beckman Coulter, Brea, CA, USA). The flow cytometry gating strategy is available in Supplementary Figures S2-3.

Tissue histology

SMGs were surgically removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Mouse SMG tissues were prepared for sectioning and subjected to H&E staining. Areas of lymphocytic infiltration were captured and evaluated under a photomicroscope. The histological grade was determined based on the following criteria: Grade 1 = 1–5 leukocytic foci (with 50 or more infiltrated lymphocytes per 4 mm^2); Grade 2 = more than 5 foci without significant parenchymal destruction; Grade 3 = multiple confluent foci and moderate degeneration of parenchymal tissue; Grade 4 = extensive lymphocyte infiltration of the gland and parenchymal destruction. The proportion of the inflammatory area was calculated in relation to the total section area, excluding fatty infiltration, to quantify the inflammation.

Saliva measurement

Saliva secretion was stimulated by intraperitoneal injection of pilocarpine (Sigma-Aldrich, St. Louis, MO, United States) at a dose of 0.5 mg/kg body weight after anaesthesia. Stimulated whole saliva was collected gravimetrically using a 20-µL pipette from the oral cavity for 15 minutes at room temperature. Body weight and fasting blood glucose levels were recorded simultaneously.

Statistics

The data were analysed using SPSS

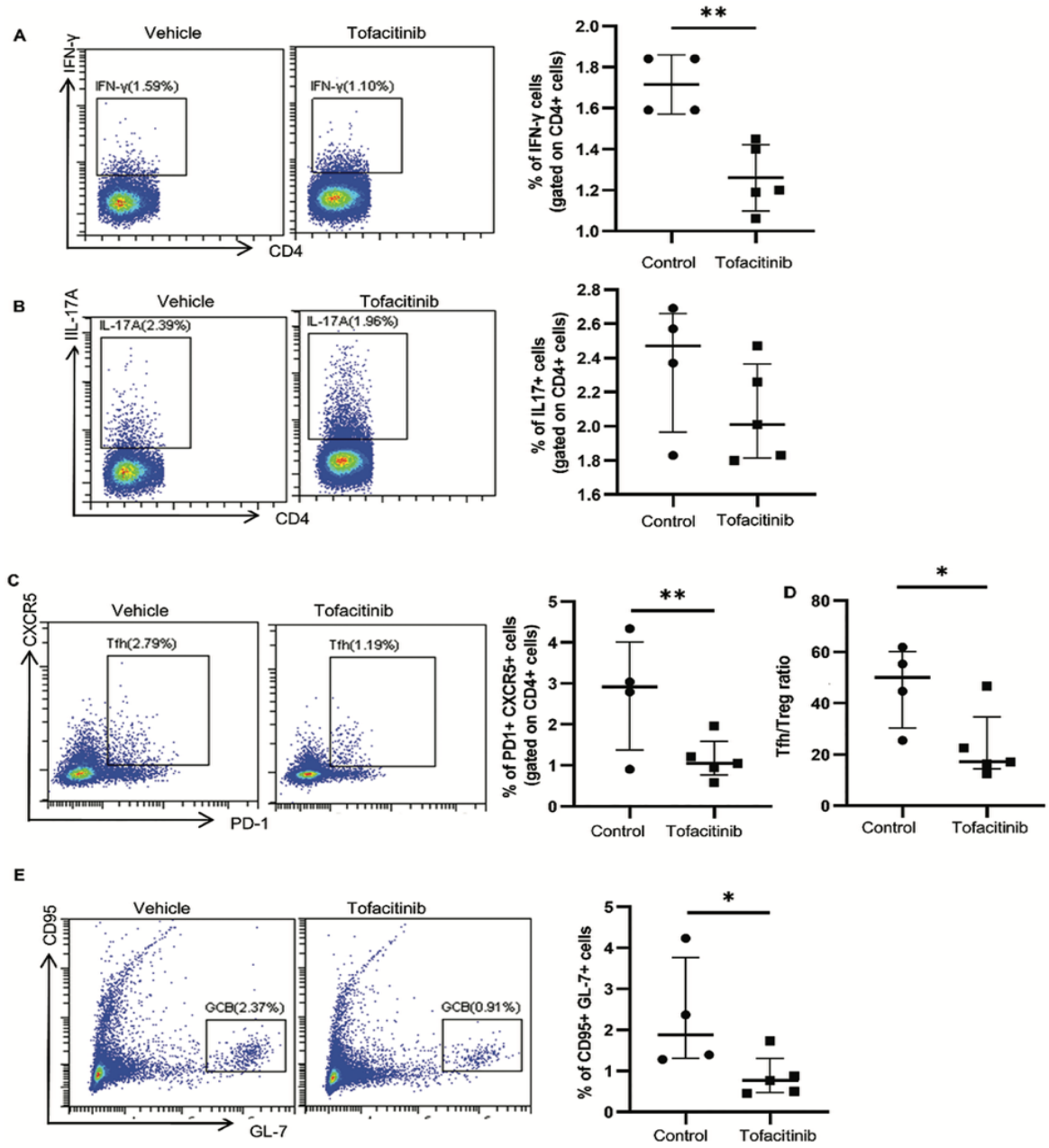

Fig. 2. Tofacitinib inhibits Th1, Tfh and GCB populations in cLNs. cLN cells were obtained from 14 weeks old NOD mice and reacted with antibodies (CD3, CD4, CD8, PD-1, CXCR5, IFN-γ, IL-17, CD25, or Foxp3) and analysed via flow cytometry. The frequencies of Tfh (CD4+Bcl-6+PD-1+CXCR5+), Th1 (CD4+IFN-γ+), Th17 (CD4+IL-17A+), Treg (CD4+CD25+Foxp3+), Tfh to Treg ratio are shown.
A, C: The populations of Th1 and Tfh cells in cervical draining lymph nodes were markedly decreased in tofacitinib treated mice compared with control mice.
B: The population of Th17 cells in cLNs exhibited a decreasing trend in tofacitinib treated mice, although the change was not statistically significant.
D: The Tfh/Treg ratio was significantly decreased in tofacitinib treated groups.
E: The analysis indicated that GC B (B220+GL-7+) cells in cLNs were decreased after tofacitinib treatment.
**p*<0.05; ***p*<0.01; ****p*<0.001. cLNs: cervical lymph nodes.

Statistics 24.0 software (SPSS Inc., Armonk, NY). The results were reported as mean, median, standard deviation (SD), and quartile range. Figures were created using GraphPad Prism (version 8). Continuous variables were analysed using the Student's t-test, the non-parametric Mann-Whitney U-test, and the Kruskal-Wallis test, while categorical variables were analysed using Fisher's exact test. Correlation analysis of the data was performed using the non-parametric Spearman correlation coefficient. Both tests were two-tailed with a 95% confidence interval, and *p*-values ≤0.05 (**p*<0.05, ***p*<0.01, and ****p*<0.001) were considered statistically significant.

Results

Tofacitinib administration ameliorated salivary gland function in the non-obese diabetic mouse Sjögren's disease model

There was a steady increase in body weight following vehicle administration in week 8 and week 14, but there were no statistically significant differ-

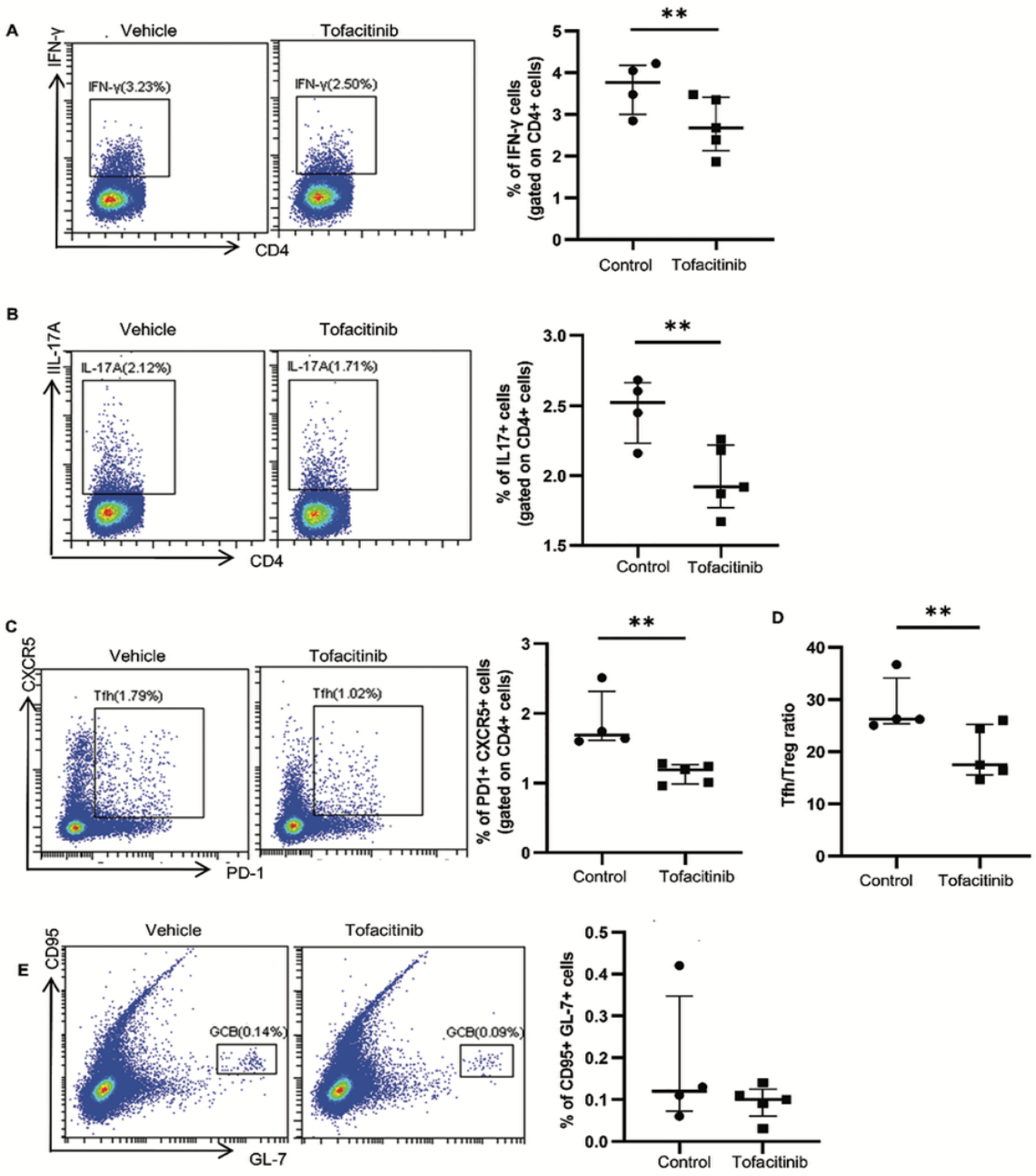

Fig. 3. Tofacitinib modulates CD4+ T cell differentiation in spleen. Spleens were removed at 14 weeks and prepared for flow cytometry. Flow cytometry revealed that the Th1 (**A**), Th17 (**B**) and Tfh (**C**) cell populations in spleens were markedly decreased in tofacitinib treated mice compared with control mice. **D**: The ratio of Tfh toTreg cells was significantly decreased in tofacitinib treated mice. **E**: The population of GCB cells in spleens exhibited a decreasing trend after tofacitinib treatment, although the change was not statistically significant.
*p0.05; **p<0.01; ***p<0.001.

ences (Suppl. Fig. S1A). Throughout the study, we regularly monitored the fasting blood glucose levels in the mice every three weeks. While the group treated with tofacitinib showed slightly lower glucose levels compared to the control group, the differences were not statistically significant (Suppl. Fig. S1B). It is worth noting that one mouse in the control group exhibited blood glucose levels consistent with a diagnosis of diabetes. The follow-up analysis excluded mice that developed hyperglycaemia to evaluate the therapeutic effect of tofacitinib on SjD.

We investigated the potential of tofacitinib in restoring the function of the SMG in a mouse model of SjD (16). Saliva flow rates were measured every 3 weeks to assess salivary gland function. The results demonstrated a significant increase in saliva production in tofacitinib-treated mice compared to the control group, whereas the control group exhibited a noticeable decrease in saliva production from weeks 8 to 14 (p=0.024) (Fig. 1A).

Tofacitinib inhibited lymphocytic infiltration in salivary glands

Histological examination of the SMGs was performed at weeks 14, and the tissue was stained with haematoxylin and

eosin (H&E) for analysis. We applied two histological analytical methodologies (histology score and quantification of the area of inflammation) to evaluate the degree of lymphocytic infiltration by SMGs. Histological score revealed that lymphocytic infiltration was strikingly lower in the tofacitinib treated group compared to the control group (median [IQR], control 2.50 [1.00, 4.00], tofacitinib 1.00 [0.50, 1.00], p=0.003) (Fig. 1D). Similarly, quantification of the area of inflammation showed that the percent of inflammation area was significantly lower in the tofacitinib group (median [IQR], control 1.68 [0.30, 3.44], tofacitinib 0.31 [0.02, 0.57], p=0.002) (Fig. 1E). Interestingly, the severity of inflammation in the submandibular glands showed a significant negative correlation with salivary flow rates (r=-0.700, p=0.0433) (Fig. 1B). These findings suggest that tofacitinib has a protective effect on inflammatory responses in SjD.

Tofacitinib decreased Th1 and Th17 populations in the spleen and cervical lymph nodes

To investigate the effects of tofacitinib on SjD development, with the emphasis on regulatory and effector CD4+ T cell subsets in spleen cells, and cLNs cells were evaluated via flow cytometry. Data showed a marked reduction in the frequencies of Th1 cells in both the cLNs and spleen of tofacitinib-treated mice (median [IQR], cLNs: 1.72 [1.59, 1.84] *vs*. 1.20 [1.12, 1.42], p=0.002; spleen: 3.76[3.01, 4.18] *vs*. 2.61[2.10, 3.30], p=0.009) (Fig. 2A, 3A). The frequency of Th17 cells was significantly decreased in the spleen (median [IQR], 2.52 [2.23, 2.66] *vs*. 1.92 [1.77, 2.22], p=0.002) (Fig. 3B). These results indicate that treatment with tofacitinib modulates the differentiation of CD4+ T cell subsets and attenuates infiltration of a specific SjD-associated T-cell population *in vivo*.

Tofacitinib treatment reduced Tfh cells and restored the balance between Teff and Treg in the spleen and cervical lymph nodes

Given the potent relation of Tfh on GCB cells, we assessed whether tofacitinib inhibits the Tfh differentiation in Non-obese diabetic (NOD) mice. We next analysed the Tfh cells in tofacitinib treated mice. The frequency of Tfh cells was significantly decreased in both the spleen and cLNs (median [IQR], cLNs: 2.92 [1.37, 4.02] *vs*. 1.05 [0.76, 1.58], p=0.002; spleen: 1.69 [1.61, 2.32] *vs*. 1.19 [0.98, 1.26], p=0.002) (Fig. 2C, 3C), consistent with decreased frequencies of GCB cells (median [IQR], cLNs: 1.88 [1.31, 3.76] *vs*. 0.77 [0.48, 1.30], p=0.036) (Fig. 2E) in tofacitinib-treated mice compared to the control group. Thus, tofacitinib treatment may inhibit Tfh and GC responses. Finally, we explored whether tofacitinib can restore Teff/Treg balance. After treatment with tofacitinib, evidently decreased ratios of Tfh to Treg cells were observed in cLNs and spleen of NOD mice compared to controls (median [IQR], cLNs: 50.00 [30.30, 60.22] *vs*. 17.09 [14.36, 34.62], p=0.027; spleen: 26.22 [25.35, 34.09] *vs*. 17.46 [15.49, 25.21], p=0.002) (Fig. 2D, 3D).

Discussion

In this study, our results showed that with the increase of age, the salivary flow rate of NOD mice in the control group decreased gradually, and there were more serious lymphocytic infiltrating lesions in the salivary gland, which was consistent with the previous experimental results (17). Tofacitinib improved the salivary flow rates and relieved the SMGs inflammation, ameliorating the overall the function of the salivary glands and exhibiting a beneficial impact on the SjD disease progression of NOD mice.

Indeed, percentages of Th1, Th17 and Tfh cell populations were significantly increased and percentages and functions of Tregs are impaired in peripheral circulation in patients with Sjögren's disease (18-20). Tfh cells promote B cell activation, differentiation, germinal centre formation, and autoantibody production, and are positively correlated with disease activity (21, 22). JAK-STAT signalling is critical for immune cell activation, proinflammatory cytokine production, and cytokine signalling. Previous studies have shown that tofacitinib mainly acts on CD4 T cells, subsequently suppressing cell proliferation and production of inflammatory cytokines such as IL-17 and IFN-γ and restore the balance between effector and regulatory T cells in immune-mediated disorders, including rheumatoid arthritis, Takayasu's arteritis (23, 24).

To ascertain a potential mechanism underlying the immunoregulatory and anti-inflammatory effects of tofacitinib, we explored the differentiation of subpopulations of T cells affected by tofacitinib administration. Our findings suggest that tofacitinib played a pivotal role in the differentiation of CD4 T cells *in vivo* by reducing the proinflammatory population: Th1, and Th17 cells, consistent with those reported in other studies (24-26). This immunomodulatory effect may be due to tofacitinib inhibition of the JAK2/TYK2/STAT4 and JAK1/JAK2/STAT1 signalling pathways inhibiting th1 cells differentiation, as well as the JAK2/STAT1/3 signalling pathway thereby inhibiting th17 cells differentiation (27-30). Moreover, we proved that tofacitinib potently suppressed the generation of pathogenic Tfh cells, which has not shown in previous studies. This phenomenon is likely a consequence of IFN-α-induced JAK-STAT1 signalling activation, which promotes Tfh cell differentiation by upregulating thymocyte selection-associated high mobility group box protein (31). In light of considerably decreased ratios of Tfh to Treg cells in NOD mice compared to controls, we conclude that tofacitinib maintains the balance between Treg and Teff cells in NOD mice. B cell hyperactivity is the hallmark of SjD, and our study also revealed that tofacitinib suppresses GCB cell formations.

One limitation of our study is the inability to further measure phosphorylated JAKs and STATs in PBMCs due to the complexity limitations of flow cytometry panels. Better measurement of these markers could have provided more insights into the mechanism of tofacitinib treatment for Sjögren's disease. Another limitation of this study is the relatively modest sample sizes mice in each group for analyses. To mitigate this limitation, we conducted three replicates to reduce the uncertainty of the results (32). However, it is crucial to conduct larger and more rigorous controlled experiments to substantiate these findings. Finally, the

short observation period and the mild impairment of salivary gland function in NOD mice limit our ability to study the efficacy of tofacitinib in models of more advanced disease progression and hinder a comprehensive analysis of the drug's effects on the immune response, particularly regarding the different interferon phenotypes at various developmental stages (33). Future studies should prioritise animal experiments with extended intervention and observation periods to comprehensively and reliably confirm the efficacy of tofacitinib in the treatment of SjD.

Utilising a novel human disease model, we identified identify a possible role for oral tofacitinib in restoring immune balance and homeostasis, and consequently, hampering the progression and onset of SjD. More clinical trials of the safety and efficacy of JAK inhibitors in patients with SjD are in progress. Our results suggest tofacitinib as a rational drug selection for SjD patients and serve as the basis of further and larger clinical trials to assess the safety and efficacy of tofacitinib for the treatment of SjD, particularly regarding the improvement of the IFN signature and activation of the JAK-STAT pathway in LSGs and peripheral blood.

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