Aberrant distribution of $CD3^+CD56^+$ NKT-like cells in patients with primary Sjögren's syndrome

L. Zhao[1,2], W. Xu[1,3], Z. Chen[1], H. Zhang[1,4], S. Zhang[1], C. Lian[1,5], J. Sun[1,5], H. Chen[1], F. Zhang[1]

[1]Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing; Key Laboratory of Rheumatology & Clinical Immunology, Ministry of Education, Beijing; [2]Department of Rheumatology and Clinical Immunology, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China; [3]Department of Rheumatology and Clinical Immunology, South Campus, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China; [4]Department of Rheumatology and Clinical Immunology, South Campus, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China; [5]Department of Rheumatology and Clinical Immunology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China.

Abstract

Objective

To elucidate the potential role of $CD3^+CD56^+$ NKT-like cells in the pathogenesis of primary Sjögren's syndrome (pSS).

Methods

We enrolled pSS patients and healthy controls and examined the peripheral population, the surface chemokine receptors and the proinflammatory cytokine production of NKT-like cells by flow cytometry. The infiltration of NKT-like cells in the labial salivary gland (LSG) was examined by immunofluorescence. Serum and tissue levels of CX3CL1 were detected by Cytometric Bead Array and immunohistochemistry, respectively. The chemotaxis of NKT-like cells was examined by transwell migration assay.

Results

Peripheral NKT-like cells from pSS patients were significantly lower than those from HC (3.09±2.35% vs. 5.37±4.06%, p=0.0002), which was negatively correlated with European League Against Rheumatism Sjögren's Syndrome Disease Activity index. NKT-like cells infiltrated into the LSG of pSS patients. Serum and LSG epithelial CX3CL1 levels were higher in pSS patients than those in HC, which promoted the chemotaxis of the NKT-like cells. NKT-like cells from pSS patients expressed a higher level of CD69, and secreted *high level of TNF-α and IFN-γ, which was promoted by CX3CL1 in vitro.*

Conclusion

NKT-like cells decreased in peripheral and infiltrated into the LSG of the pSS patients, which could be driven by CX3CL1-CX3CR1 axis. NKT-like cells might be implicated in the pathogenesis of pSS.

Key words

Sjögren's syndrome, leukocytes, chemokine

*Liling Zhao, MD**
*Wenwen Xu, MD**
Zhilei Chen, PhD
Haoze Zhang, PhD
Shuo Zhang, MD
Chaofeng Lian, PhD
Jinlei Sun, PhD
*Hua Chen, MD***
*Fengchun Zhang, MD***

**These authors contributed equally.*
***Co-corresponding authors.*

The work should be attributed to the Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing; Key Laboratory of Rheumatology & Clinical Immunology, Ministry of Education, Beijing, China.

Please address correspondence to:
Hua Chen,
Department of Rheumatology and Clinical Immunology,
Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College,
No. 1 Shuaifuyuan,
Dongcheng District,
Beijing, China.
E-mail: chenhua@pumch.cn

Fengchun Zhang,
(address as above)
E-mail: zhangfccra@aliyun.com

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Introduction

Primary Sjögren's syndrome (pSS) is a common autoimmune disease characterised by dry eyes and dry mouth. The pathogenesis of pSS remains elusive, however, accumulating evidence suggest that abnormal immune response orchestrated by the genetic factors and environmental factors is the essential mechanism for pSS (1, 2). The hallmark of pSS is the intensive infiltration of mononuclear cells, mainly $CD3^+$T cells and $CD20^+$ B cells, in the exocrine glands such as labial salivary gland (LSG) and lacrimal glands (3, 4). More than 75% salivary gland-infiltrating lymphocytes were T cells, especially $CD4^+$ T cells (5), which are correlated with the labial infiltration grade and biopsy focus score (6). More recently, various T cell subsets (7), including Th1 cells (8, 9), Th17 cells (10, 11), as well as follicular helper T (Tfh) cells and follicular regulatory T (Tfr) cells (12) , have been proved to play pivotal role in the pathogenesis of pSS, mediating B cell hyperactivity. Additionally, $CD16^+CD56^+$ natural killer (NK) cells are involved in the pathogenesis of pSS. Extensive accumulation of NK cells in minor salivary gland is noted in pSS patients and is correlated with the severity of the sialadenitis. Furthermore, NK cells secrete a high level of IFN-γ (13, 14), which implicates that NK cells may promote inflammation in LSG in pSS patients.

$CD3^+CD56^+$ NKT-like cells are a distinct subpopulation of peripheral lymphocytes which express the markers of both $CD3^+$ T cells and $CD56^+$ NK cells (15, 16). NKT-like cells show the potentials of both T cells and NK cells (17), including potent TCR-mediated and NK-like cytotoxicity, and producing abundant IFN-γ and TNF-α (18-20), and serve as a bridge of innate immune and adaptive immune (15). NKT-like cells are implicated in the autoimmune diseases. Matthew *et al*. observed a lower frequency of peripheral NKT-like cells in systemic lupus erythematosus and was inversely correlated with IgG level in the siblings of patients, suggesting NKT-like cells may regulate the immunoglobin production (21, 22). Interestingly, RA patients also have depleted peripheral NKT-like cells (23). Additionally, activated NKT-like cells are accumulated in the liver of patients with primary biliary cholangitis (24).

The potential role of NKT-like cells in the pathogenesis of pSS remains unknown (25-27). To address this, we examined the peripheral and LSG-infiltrating NKT-like cells in pSS and healthy controls (HC). We observed that the NKT-like cells from pSS patients were significantly decreased in peripheral and infiltrated to the LSG, which may be driven by the increased CX3CL1 level. We also found that NKT-like cells from pSS patients were activated and produced a high level of IFN-γ and TNF-α, which were potentially further stimulated by CX3CL1.

Methods

Patients and healthy controls

A total of 110 pSS patients fulfilled the European-American consensus criteria for SS (28) were enrolled from Peking Union Medical College Hospital (PUMCH) between December 2015 and May 2017. Additionally, 83 healthy controls (HC) served as controls. First, NKT-like cell population was examined in 71 patients and 64 controls; second, serum chemokines were detected in 49 pSS patients and 40 controls, including 31 patients whom were previously analysed; third, immune phenotypes of NKT-like cells were measured in 14 patients and 14 controls; finally, chemotaxis and stimulation of CX3CL1 to NKT-like cells were evaluated in 7 patients and controls. Furthermore, labial salivary gland (LSG) samples were collected from 15 of 110 pSS patients and 7 non-pSS sicca controls, who presented with sicca symptoms but did not meet the classification criteria of pSS. Patients with other connective tissue diseases, recent infection or malignancy were excluded.

The study was approved by the Institutional Review Board of PUMCH (S-225), and written informed consent was obtained from all participants. The clinical characteristics of pSS patients were listed in Supplementary Table 1.

Flow cytometry

Fresh PBMCs were incubated with fluorescence-conjugated monoclonal anti-

bodies at 4°C for 30 minutes, then were analysed by BD Accuri C6 flow cytometer. The following antibodies were used: FITC-conjugated anti-CD3 (HIT3a), APC-conjugated anti-CD56 (HCD56), PE-conjugated anti-CX3CR1 (2A9-1), PE-conjugated anti-CD69 (FN50), PE-conjugated anti-CCR7 (3D12) and PE-conjugated anti-CXCR4 (12G5). Data were processed using FlowJo vX (Tree Star).

Intracellular cytokines staining

PBMCs (10^6 cells/ml) were incubated in complete RPMI medium supplemented with 50 ng/ml PMA (Sigma), 1 μg/ml ionomycin (Sigma) and 1 μg/ml Golgiplug (BioLegend) at 37°C in 5% CO_2 for 3 hours. Then cells were washed with PBS and stained with FITC-conjugated anti-CD3 (HIT3a) and PE/APC-conjugated anti-CD56 (HCD56) antibodies, permeabilised with permeabilisation buffer (BD) and subsequently incubated with antibodies against PE-conjugated TNF-α (MAB11), APC-conjugated IFN-γ (B27) and PE-conjugated IL-17A (SCPL1362) at 4°C for 45 minutes. The cells were analysed by flow cytometry within 3 days.

CFSE proliferation assay

Fresh PBMCs were labelled with 1μM CFSE (BD) and were incubated in 96-well plates coated with 1μg/ml anti-CD3 antibodies (OKT3) in complete RPMI-1640 medium supplemented with 1μg/ml CD28 (CD28.2) in the dark. After 4 days, cells were collected, washed with PBS and stained with Percp-cy5.5-conjugated anti-CD3 (OKT3) and APC-conjugated anti-CD56 (HCD56) antibodies. Cells were immediately analysed by flow cytometry.

Apoptosis assay

Fresh PBMC were stained with FITC-conjugated anti-CD3 (HIT3a) and PE-conjugated anti-CD56 (HCD56) antibodies and washed with Annexin V Binding Buffer (BioLegend). Then cells were incubated with APC-conjugated Annexin V (BioLegend) and 7-AAD (BD) for 15 min at room temperature in the dark. Cells were immediately analysed by flow cytometry.

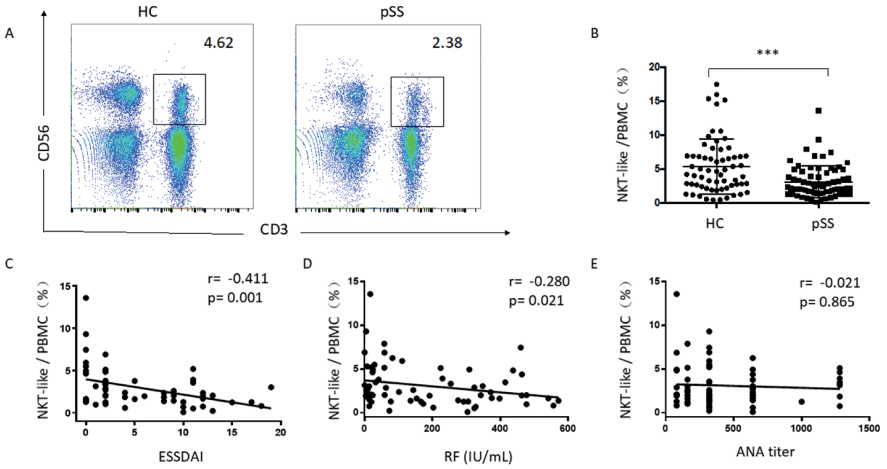

Fig. 1. $CD3^+CD56^+$ NKT-like cells from pSS patients are depleted in peripheral blood.
Fresh PBMCs from pSS patients (pSS) and Healthy Controls (HC) were stained with anti-CD3 and anti-CD56 antibodies and were analysed with flow cytometry.
(A) Representative FACS plots and (B) summary graph demonstrating the peripheral $CD3^+CD56^+$ NKT-like cell population from pSS (n=71) and HC (n=64).
Correlations between peripheral NKT-like cell population and (C) ESSDAI (n=59), (D) RF titre (n=68), (E) ANA titre (n=71), respectively.
Results are presented as mean ±SD.
$^{***}p<0.001$ determined by Student's *t*-test. Correlations are calculated using Spearman correlation analysis.
FACS: fluorescence activated cell sorting; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity index; RF: rheumatoid factor; ANA: antinuclear antibody.

Chemokine measurement

CX3CL1 were measured by Cytometric Bead Array (R&D Systems). CCL21 and CXCL12 were measured by Enzyme-Linked Immunosorbent Assay (ELISA) (RayBiotech). Procedures were performed according to the manufacturer's protocol.

Immunohistochemistry assay

Paraffin-embedded tissues were routinely processed after antigen retrieval. After exhaustion of endogenous peroxidase with 3% H_2O_2 solution, slides were blocked with 3% BSA, incubated with anti-CX3CL1 antibody (10108-2-AP) overnight at 4°C, and then incubated with HRP-conjugated secondary antibody at room temperature for 50 minutes.

Immunofluorescence staining

Paraffin-embedded tissues were routinely processed after antigen retrieval. Non-specific background was blocked with 10% normal goat serum. Rabbit anti-human CD3 (Ab16669) and mouse anti-human CD56 (ab75813) were served as primary antibodies in serial staining, respectively. Goat anti-rabbit/mouse HRP-conjugated secondary antibody was used as secondary antibody. The combined antibodies were detected with different fluorescent TSA reagents. The digital image was acquired with a Zeiss fluorescence microscope (Carl Zeiss, Germany) and mean immunofluorescent intensity was calculated with Image J (National Institutes of Health, USA).

NKT-like cells isolation

NKT-like cells were isolated from fresh PBMCs with human $CD3^+CD56^+$NKT cell isolation kit according to manufacturer's protocol (Miltenyi Biotec). The purity of $CD3^+CD56^+$ NKT-like cells was more than 90% by flow cytometry.

Chemotaxis assays

Chemotaxis assays were performed in triplicate in a 24-well Micro Chemotaxis Boyden chamber (Corning, 5μm) incubated in 5% CO2 at 37°C for 2 hours. Aliquots of 600ul of X-VIVO medium supplemented with rhCX3CL1 (0–50ng/ml) (R&D systems) were loaded in the lower chamber, and 10^5 NKT-like cells were seeded in the upper chamber. Migrated cells adhering to the lower chamber were counted in 5 random-selected high-power fields/well with a light microscope (5hpf; x400).

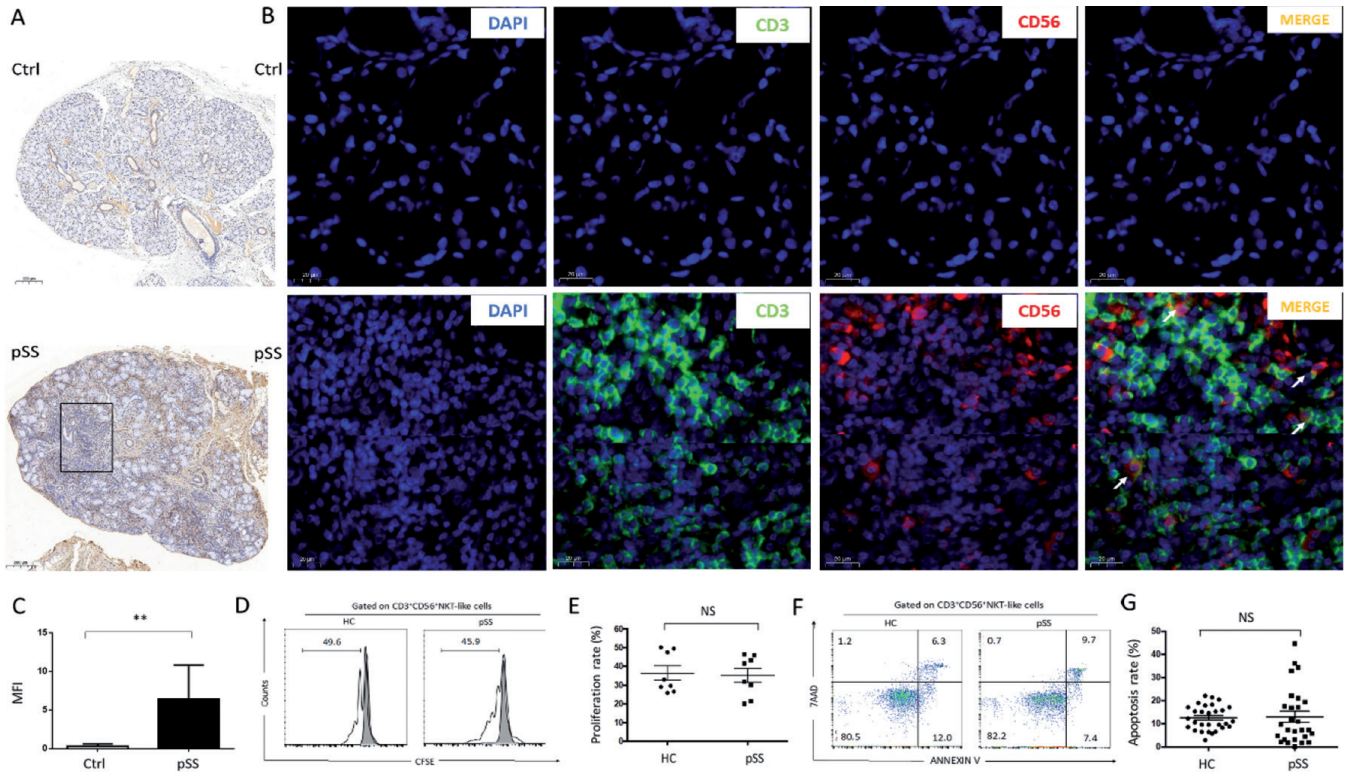

Fig. 2. NKT-like cells infiltrate in the labial gland of pSS patients.
A: Overview of labial salivary gland (LSG) with hematoxylin/eosin staining (X50) from pSS (n=15) and non-sicca control (n=7). Rectangle area depicted the inflammatory infiltrate of LSG from pSS furtherly shown in the subsequent immunofluorescence staining.
B: Representative immunofluorescence staining (X400) of CD3 (green), CD56 (red) and DAPI (blue) of LSG from pSS (n=15) and controls (n=7). Arrows denoted CD3+CD56+NKT-like cells (yellow).
C: Mean immunofluorescent intensity (MFI) of NKT-like cells (yellow) in LSG of pSS (n=15) and control (n=7).
CFSE-labelled fresh PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies for 4 days.
D: Representative FACS plots and (E) summary graph demonstrating the proliferation of CD3+CD56+ NKT-like cells from pSS (n=8) and HC (n=8).
Fresh PBMCs were stained with anti-CD3 antibody, anti-CD56 antibody, Annexin V and 7AAD, and apoptotic cells were defined as Annexin V+ and 7AAD ±.
F: Representative FACS plots and (**G**) summary graph demonstrating the apoptosis of CD3+CD56+ NKT-like cells from pSS (n=27) and HC (n=27).

Statistical analysis

Student's *t*-test and Mann-Whitney test were used to compare differences between the two groups. Correlations were calculated using Spearman correlation analysis. A two-sided *p*-value <0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism v. 6 (GraphPad Software).

Results

Peripheral CD3+CD56+ NKT-like cells from pSS patients are decreased and are inversely correlated with disease activity

We first compared the peripheral NKT-like cells from pSS and HC. We found that the NKT-like cells from pSS patients were significantly decreased in peripheral blood (3.09±2.35% *vs*. 5.37±4.06%, *p*=0.0002) (Fig. 1A-B). Furthermore, the peripheral NKT-like cell population from pSS patients was negatively correlated with ESSDAI (r=-0.411, *p*=0.001) (Fig. 1C) and RF titre (r=-0.280, *p*=0.021) (Fig. 1D), but not correlated with ANA titre (r=-0.021, *p*=0.865) (Fig. 1E), IgG (r=0.124, *p*=0.305), IgM (r=-0.144, *p*=0.234), IgA (r=-0.048, *p*=0.691), CRP (r=-0.064, *p*=0.629), ESR (r=-0.021, *p*=0.860) or age (r=-0.082, *p*=0.497) (Suppl. Fig. 1A-F). Thus, these data implicated that peripheral NKT-like cells were depleted in pSS patients and closely correlated with disease activity, suggesting a potential role in the pathogenesis.

NKT-like cells infiltrate to the labial gland of pSS

We then investigated the underlying mechanism of depleted peripheral NKT-like cells in pSS patients. We examined the NKT-like cells infiltrating into the LSG in pSS and control by immunofluorescence. NKT-like cells were identified in the labial gland in pSS patients but were scarcely observed in those in HC (Fig. 2A-C). Considering NKT-like cell population is relatively the minority of lymphocytes, absence of dramatic accumulation of NKT-like cells in the LSG seems to be reasonable. Furthermore, no significant difference of proliferation rate (35.1±10.69% *vs*. 36.4±10.75%, *p*=0.812) (Fig. 2D-E) or apoptosis rate (13.0±12.15% *vs*. 12.5±5.28%, *p*=0.832) (Fig. 2F-G) were observed between pSS and HC. Therefore, the peripheral depletion of NKT-like cells from pSS did not resulted from aberrant proliferation or apoptosis capacity but may be driven by infiltration into tissues such as the labial gland, as well as other exocrine glands.

Abnormally infiltrated NKT-like cells are driven by elevated CX3CL1 level

We further elucidated the mechanism of

abnormal chemotaxis of NKT-like cells to the labial gland in pSS. We focused on three paired chemokine-chemokine receptors which were reported in pSS (29, 30), *i.e.* CX3CL1-CX3CR1, CCL21-CCR7 and CXCL12-CXCR4 (Suppl. Fig. 2A-D). We found serum CX3CL1 level was significantly higher in pSS patients than that in HC (936.79±108.92 *vs.* 887.80±82.77pg/ml, p=0.015) (Fig. 3A). Furthermore, the CX3CL1 level in the labial gland was also higher in pSS than in HC (Fig. 3B), suggesting elevated serum level and tissue gradient of CX3CL1 potentially attracted peripheral NKT-like cells to the target tissues. Although the expression of CX3CR1, the unique receptor for CX3CL1, were both highly expressed on NKT-like cells from pSS and HC (68.25±22.38% *vs.* 70.06±17.34%, p=0.777) (Fig. 3C), we showed CX3CL1 indeed promoted NKT-like cells chemotaxis in a dose-dependent manner with transwell migration test (Fig. 3D). Therefore, these data suggested that both circulating and tissue level of CX3CL1 were elevated in pSS, which subsequently promoted NKT-like cells to infiltrate into the labial gland.

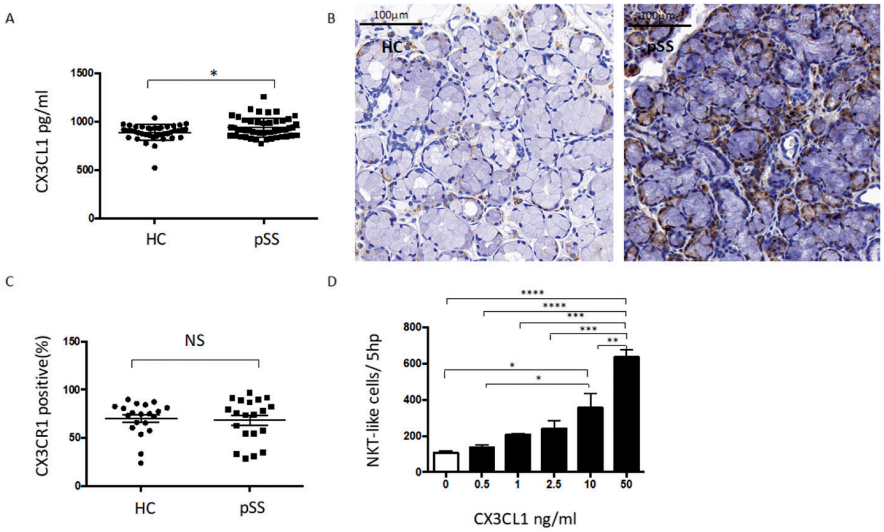

Fig. 3. CX3CL1 promotes NKT-like cells chemotaxis to labial gland.
A: Serum level of CX3CL1 from pSS (n=49) and HC (n=40).
B: Representative immunohistological staining (X400) of CX3CL1 on small labial gland of pSS (n=3) and HC (n=3).
C: The surface expression of chemokine receptor CX3CR1 on NKT-like cells from pSS (n=20) and HC (n=20).
D: Fresh CD3+CD56+ NKT-like cells from pSS (n=3) were loaded into the upper chamber, and X-VIVO supplemented with rhCX3CL1 (0-50ng/ml) were loaded into the lower chamber in transwell invasion model. The number of NKT-like cells in the lower chamber was calculated from five randomly selected high-power fields per well.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 determined by Student's t-test.

NKT-like cells from pSS patients are activated and produce pro-inflammatory cytokines

Finally, we examined the immune phenotype of NKT-like cells from pSS. Comparing with those cells from HC, NKT-like cells from pSS expressed a higher level of T cell activation marker CD69 (10.97±6.75% *vs.* 2.69±1.82%, p<0.0001) (Fig. 4A-B), which implicated NKT-like cells are highly activated *in vivo*. We also examined the proinflammatory cytokines secreted by peripheral NKT-like cells. NKT-like cells from both pSS and HC released a high level of IFN-γ (Fig. 4C-D) and TNF-α (Suppl. Fig. 3A-B), suggesting a Th1 phenotype. Furthermore, a trace amount of IL-17 (Suppl. Fig. 3C-D) was also released by NKT-like cells. However, no significant difference was observed in IFN-γ (62.42±16.66% *vs.* 64.34±14.50%, p=0.759) and TNF-α (70.29±22.74% *vs.* 81.32±10.76%, p=0.137). Given tissue level of CX3CL1 was much higher in pSS patients than those in HC, and CX3CL1 promotes NK cells to produce IFN-γ (31), we also explored whether CX3CL1 stimulated NKT-like cells to produce proinflammatory cytokines. As expected, CX3CL1 indeed promoted NKT-like cells to produce IFN-γ (Fig. 4E-F). Together, we found that NKT-like cells produced a large amount of IFN-γ, which was potentially further promoted by a high concentration of CX3CL1. Thus, the CX3CL1-CX3CR1 - NKT-like cells axis might play a role in the pathogenesis of pSS.

Discussion

In this study, we showed that peripheral NKT-like cells from pSS patients were significantly lower and infiltrated into the labial gland, which was mediated by CX3CL1-CX3CR1 chemotaxis axis. Furthermore, NKT-like cells produced a high level of proinflammatory cytokines and potentially contributed to the pathogenesis of pSS.

We observed that the peripheral NKT-like cells from pSS patients were depleted, which has remained controversial. Szodoray *et al*. have reported that the NKT-like cells from pSS patients were higher than those from HC (pSS 5.2% *vs.* HC 1.6%) (25). However, both Sudzius *et al*. and Richard *et al*. find a significantly lower absolute count of NKT-like cell in pSS patients (27, 32). The discrepancies among these studies were potentially caused by the difference in race, age, disease activity, and background therapy. Notably, the mean percentage of NKT-like cells population from HC in Szodoray's study is much lower than those in the other studies (1.6% *vs.* 6.79%) (27). Furthermore, the prevalence of extraglandular manifestations in Szodoray's study is lower than those in the other studies, suggesting low disease activities in patients and negatively-correlated higher NKT-like cells in circulation. Additionally, all pSS patients were treatment-naive in Szodoray's study, while some patients received hydroxychloroquine and glucocorticoid in the other studies, which potentially regulated NKT-like cells. Nevertheless, we confirmed a smaller population of NKT-like cells from pSS patients (3.09% *vs.* 5.37%). Importantly, we also found that the peripheral NKT-like cell was negatively correlated with pSS disease activity.

We further observed that CD3+

CD56+NKT-like cells infiltrated the salivary gland. Consistently, Awada *et al*. demonstrate that CD56+lymphocytes existed in the labial gland of pSS patients, which are possibly CD16+CD56+ NK cells or CD3+CD56+ NKT-like cells (14). Given most CD56+ cells in LSG were also CD3 positive, the infiltrated CD56+ lymphocytes were likely NKT-like cells. In light of the comparable proliferation and apoptosis potentials between pSS and HC, the lower peripheral NKT-like cells population in pSS was probably attributed to the abnormal infiltration into the salivary gland such as the labial gland.

Chemokines are a family of cytokines inducing chemotaxis of nearby cells. Growing studies have underlined the key role of chemokines in autoimmune diseases (33-36). We found that the serum and tissue level of CX3CL1 were higher in pSS than in HC. Lee *et al*. report that the serum level of CX3CL1 is positively correlated with proinflammatory cytokines, ANA and IgG in pSS patients (30). CX3CL1 is a membrane-bound chemokine promoting the strong adhesion with leukocytes and chemotaxis of NK cells by interacting with the chemokine receptor CX3CR1 (37). CX3CL1 is secreted by endothelial cells (38), epithelial cells (39), smooth muscle cells, dendritic cells, macrophages and neurons (40), and epithelial cells are the principal cells expressing CX3CL1 in the LSG (30, 41). Interestingly, IFN-γ promotes salivary gland epithelial cell line to produce CX3CL1 (30). Furthermore, IFN-γ, IL-1β and TNF-α up-regulate the transcription of CX3CL1 through NF-κB signaling (42). Therefore, the high level of CX-3CL1 in the LSG of pSS patients might be driven by the proinflammatory milieu in LSG. Moreover, NKT-like cells showed a chemotactic response to CX-3CL1, which suggested CX3CL1 did play a role in the pathogenesis of pSS.

In addition to the chemotaxis capacity, Yoneda *et al*. show that CX3CL1 also promote NK cells to produce IFN-γ (42). Similarly, CX3CL1 did promote NKT-like cells to produce IFN-γ. Therefore, positive feedback between CX3CL1 produced by the epithelial cells and IFN-γ produced by NKT-like cells exacerbating the damage of LSG was implicated. In pSS patients, the epithelial cells of LSG might be not only a victim but also an active participant. Breaking this feedback loop is a potential approach to alleviate pSS.

In summary, we demonstrated that NKT-like cells from pSS patients were depleted in peripheral blood and infiltrated in LSG. NKT-like cells from pSS patients were activated and secreted a high level of IFN-γ and TNF-α, which was potentially promoted by elevated CX3CL1 level in LSG. NKT-like cells might play a role in the pathogenesis of pSS. Targeting NKT-like cells might be a potential therapeutic approach for pSS.

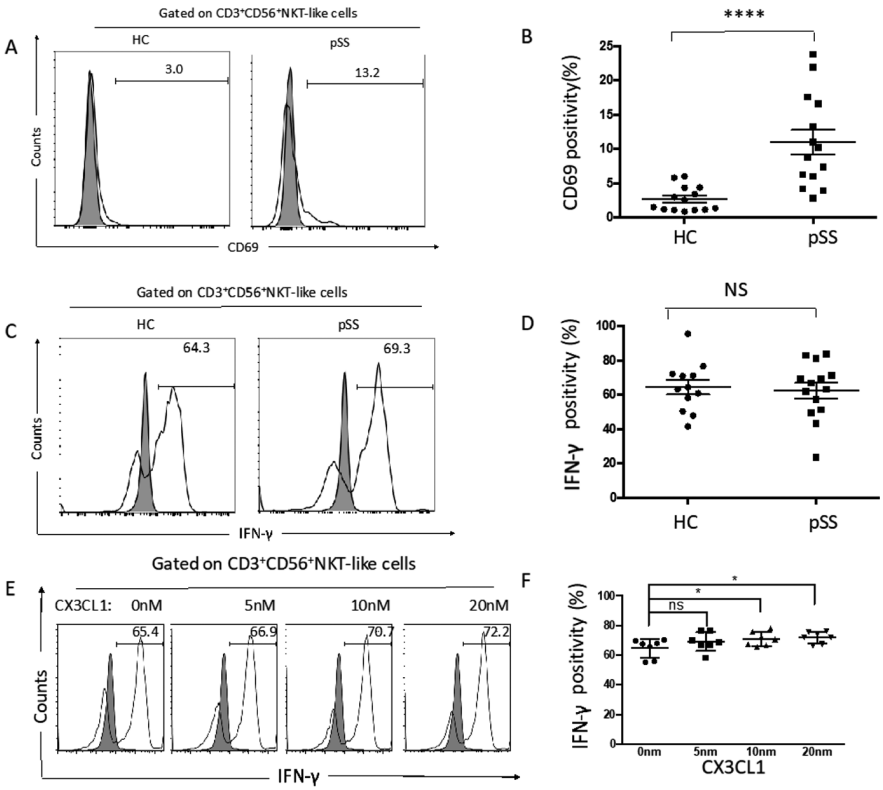

Fig. 4. NKT-like cells from pSS patients are activated and produce proinflammatory cytokines.
Fresh PBMCs from pSS (n=14) and HC (n=14) were stained with anti-CD3, anti-CD56 and anti-CD69 antibodies. (**A**) Representative FACS plots and (**B**) summary graph demonstrating the CD69 expression on CD3+CD56+ NKT-like cells from pSS and HC.
Fresh PBMCs from pSS (n=14) and HC (n=12) were stimulated with PMA (50ng/ml) and ionomycin (1ug/ml) for 3 hours, followed by staining with anti-CD3, anti-CD56 and (**C**-**D**) anti-IFN-γ antibodies. PBMCs from pSS (n=7) were incubated with indicated concentrations of plate-bound CX3CL1 for 60 hours, then were stimulated with PMA (5ng/ml) and ionomycin (1μg/ml) for 4 hours, followed by staining with anti-CD3, anti-CD56 and (**E**-**F**) anti-IFN-γ antibodies.
*$p<0.05$. ****$p<0.0001$ determined by Student's *t*-test.

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