The role of imbalanced CD226/TIGIT on activated peripheral double-negative T cells in the pathogenesis of primary Sjögren's syndrome

C. Deng[1], A. Wang[1], W. Li[2], Y. Chen[1], R. Li[1], L. Zhao[1], J. Zhou[1], W. Zhang[1], M. Li[1], Y. Zhao[1], X. Zeng[1], Y. Fei[1,3]

[1]Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College; National Clinical Research Center for Dermatologic and Immunologic Diseases (NCRC-DID), Ministry of Science & Technology; State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College; Key Laboratory of Rheumatology & Clinical Immunology, Ministry of Education, Beijing; [2]Department of Rheumatology, Key Myositis Laboratories, China-Japan Friendship Hospital, Beijing; [3]Department of Health Medicine, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China.

Abstract

Objective

To explore whether the balance of CD226 and TIGIT is disturbed in $CD3^+CD56^-TCR\alpha\beta^+CD4^-CD8^-$ (DN) T cells and have a better understanding of the potential role of DN T cells in the pathogenesis of primary Sjögren's syndrome (pSS).

Methods

The percentage of DN T cells as well as the expression of CD226 and TIGIT was identified by flowmetry. After in vitro stimulation, we further detected the expression of activation and cytotoxic marker, as well as intracellular cytokines secreted by DN T cells.

Results

DN T cells were found to expand in the peripheral blood of pSS patients (1.77±0.66%) and correlate with IgG ($r=0.451$, $p<0.05$), C3 ($r=-0.438$, $p<0.05$) and C4 ($r=-0.470$, $p<0.05$). Imbalanced CD226/TIGIT was observed on peripheral DN T cells of pSS patients, especially the overexpression of inhibitory immunoreceptor TIGIT. The expression ratio of TIGIT and CD226 on DN T cells was elevated in pSS patients and correlated with ESSDAI scores ≥5 ($r=0.743$, $p<0.05$). Besides, these DN T cells were found to be activated and show strong cytotoxicity.

Conclusion

The balance between CD226 and TIGIT on DN T cells was disturbed and correlated with the disease activity in pSS patients, which may be implicated in the pathogenesis of pSS.

Key word

double-negative T cell, primary Sjögren's syndrome, CD226, TIGIT, CD107a

*Chuiwen Deng, PhD**
*Anqi Wang, PhD**
*Wenli Li, PhD**
Yingying Chen, PhD
Rongli Li, PhD
Lidan Zhao, PhD
Jiaxin Zhou, PhD
Wen Zhang, PhD
Mengtao Li, PhD
Yan Zhao, PhD
Xiaofeng Zeng, MD
Yunyun Fei, PhD

**Contributed equally.*

Please address correspondence to:
Yunyun Fei
Department of Health Medicine, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, 100010 Beijing, China.
E-mail: feiyunyun@pumch.cn

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Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterised by inflammatory infiltrate of salivary and lacrimal glands, usually manifested as oral and ocular mucosal dryness (1). As a complex and heterogeneous disease, although pSS typically affects middle-aged women, it also rarely occurs in male patients and in females during their childhood/adolescence or in the elderly (2). Multiple T cell subsets have been found to contribute to the pathogenesis of pSS by producing pro-inflammatory cytokines and promoting B cell activation (3-7).

DN T cells comprise a very small subpopulation of approximately 1-3% of total $CD3^+$ T lymphocytes in peripheral blood (8). DN T cells were reported to be implicated in the pathophysiology of various autoimmune disorders such as autoimmune lymphoproliferative syndrome, systemic lupus erythematosus (SLE) and psoriasis (9-12). Previous studies also demonstrated the accumulation of DN T cells in peripheral blood and salivary glands of pSS patients (13, 14). Besides, glandular infiltrating DN T cells were associated with extent of glandular involvement and presence of germinal centre-like structures in pSS (14).

TIGIT, T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory (ITIM) domains, is preferentially expressed on activated effector and memory T cells such as $CD8^+$CTL, Tfh and Treg (15). The complementary costimulatory receptor CD226, shares common ligands presented by antigen presented cells with TIGIT. TIGIT could outcompete CD226 for binding these ligands due to higher affinity, thereby exerting inhibitory effects over CD226 to impede transmission of activation signals, as well as inhibit cell proliferation and cytokine production (16, 17). Our previous study revealed that proportional and functional alteration of CD226/TIGIT in $CD4^+$ T cells may be involved in the pathogenesis of pSS (18). Given that current understanding of DN T cells in pSS is not completely clear, we conducted this research to explore whether the balance of CD226 and TIGIT is disturbed on DN T cells, as well as get a better understanding of the potential role of DN T cells in the pathogenesis of pSS.

Methods

Study design

30 patients with a definite diagnosis of pSS were enrolled consecutively from 2022 to 2023 at Peking Union Medical College Hospital. The diagnosis of pSS was established according to the 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) diagnosis criteria (19). EULAR Sjögren's syndrome disease activity index (ESSDAI) was used for disease activity assessment. ESSDAI <5 and ESSDAI ≥5 was defined as low disease activity and moderate-to-high disease activity, respectively (20). Patients with secondary Sjögren's syndrome or combined with other definite autoimmune diseases were excluded.

The gender ratio between females and males was 9:1. Among pSS patients, 19 patients were untreated and 11 patients were under the treatment of hydroxychloroquine (HCQ) at inclusion. Besides, 30 gender/age/ethnicity-matched healthy donors were enrolled as healthy controls (HCs) and 16 patients with rheumatoid arthritis (RA) were taken as disease controls.

This study was approved by the ethics committee of Peking Union Medical College Hospital (no. I-22PJ959), with the written informed consent obtained.

PBMC isolation

Fresh whole blood collected in vacuum anticoagulant tubes was diluted in the same volume of phosphate buffer saline (PBS). The ficoll-paque plus (Cat# 7111011, DAKEWE, China) was added in the bottom of SepMate centrifugation tubes (Cat#85450, Stemcell), and then diluted blood was placed above. After density gradient centrifugation (1200×g, room temperature, 10 minutes), peripheral blood mononuclear cells (PBMCs) were isolated.

Phenotype detection

To identify the percentage of DN T cells in peripheral blood and the distribution of CD226 and TIGIT, isolated PBMCs were washed twice with PBS and pre-

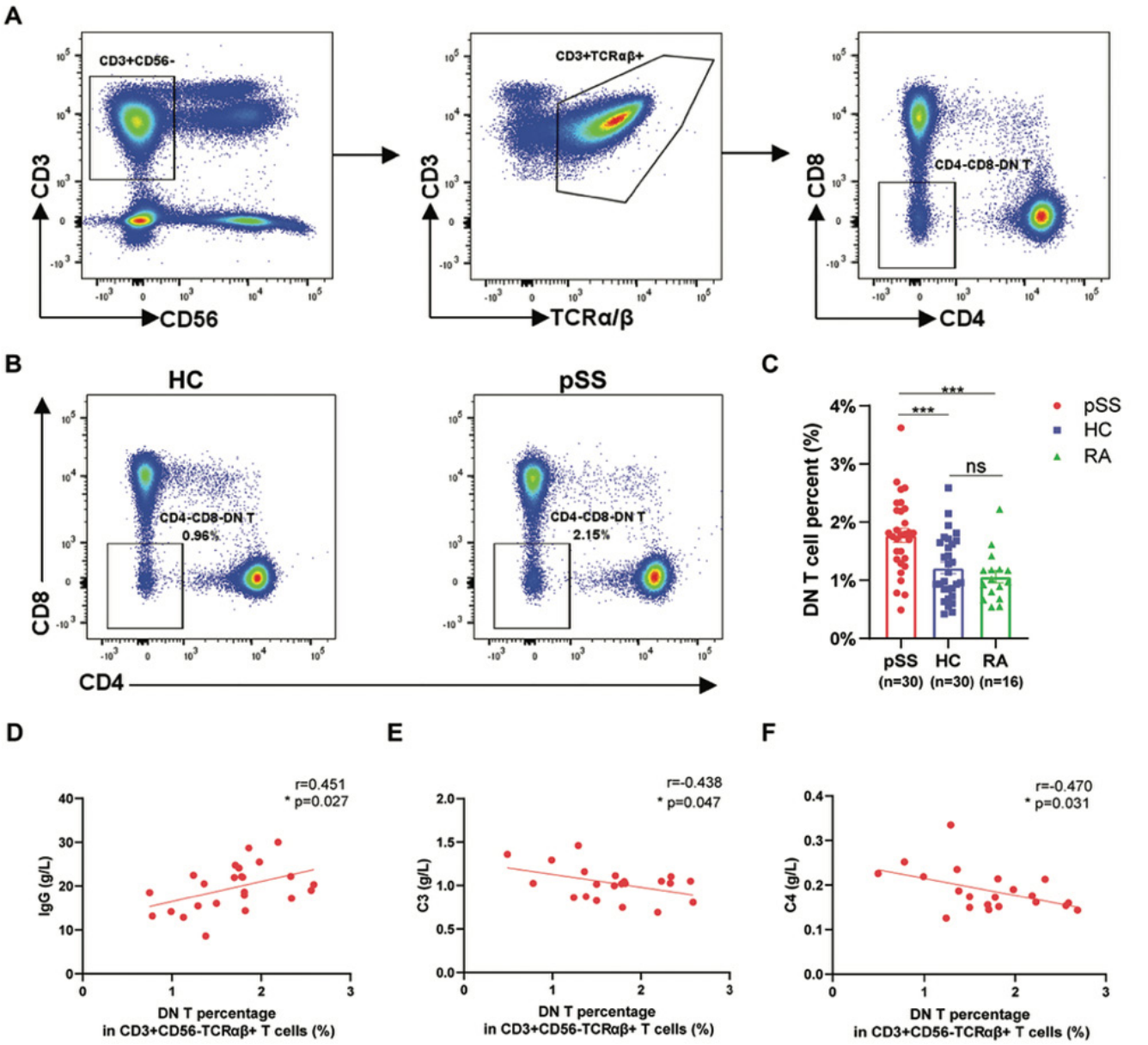

Fig. 1 **A**: The gating strategy for $CD3^+CD56^-TCR\alpha\beta^+CD4^-CD8^-$ (DN T) cells. **B**: Representative flow cytometry images of DN T cells from HCs and pSS patients, respectively. **C**: The percentage of peripheral DN T cells in $CD3^+CD56^-TCR\alpha\beta^+$ T cells in pSS patients (red, n=30), HCs (blue, n=30), and RA patients (green, n=16), respectively. Data are represented as mean ± SEM. **D**: A significantly positive correlation was found between the percentage of DN T cells in $CD3^+CD56^-TCR\alpha\beta^+$ T cells and IgG levels (n=24). **E**: A significantly negative correlation was found between the percentage of DN T cells in $CD3^+CD56^-TCR\alpha\beta^+$ T cells and C3 levels (n=21). **F**: A significantly negative correlation was found between the percentage of DN T cells in $CD3^+CD56^-TCR\alpha\beta^+$ T cells and C4 levels (n=21). *p<0.05.
DN T: $CD3^+CD56^-TCR\alpha\beta^+CD4^-CD8^-$.

pared in aliquots for surface staining. Appropriate amounts of fluorescent conjugated antibodies were added and then incubated on ice for 30 minutes in the dark. Then cells were washed and resuspended in PBS for cytometric analysis on FACS Aria II (BD Biosciences). The immunophenotype of DN T cell was designated as $CD3^+CD56^-TCR\alpha\beta^+CD4^-CD8^-$ and detailed gating strategies are shown in Figure 1A.

Functional assays after in vitro stimulation

For the detection of CD69, CD107a and intracellular cytokines, $CD4^+$ T cells were first depleted from PBMCs using CD4 microbeads (cat no. 130-045-101, Miltenyi Biotec, Cambridge, MA) to obtain $CD4^-$ cells. The purity of $CD4^-$ cells was confirmed to be >95% by flow cytometry. Then $CD4^-$ cells were stimulated with leukocyte activation cocktail (cat. no. 550583-BD Biosciences) in complete culture medium (Glico 1640, 10% foetal calf serum, 1% penicillin and streptomycin) in the 37°C, 5% CO_2 incubator for 6 hours. Then cells were harvested and stained for surface markers, followed by fixation and permeabilisation using the Intracellular Fixation & Permeabilization Buffer Set (cat. no. 88-8824-00, Invitrogen) according to the manufacturer's instructions.

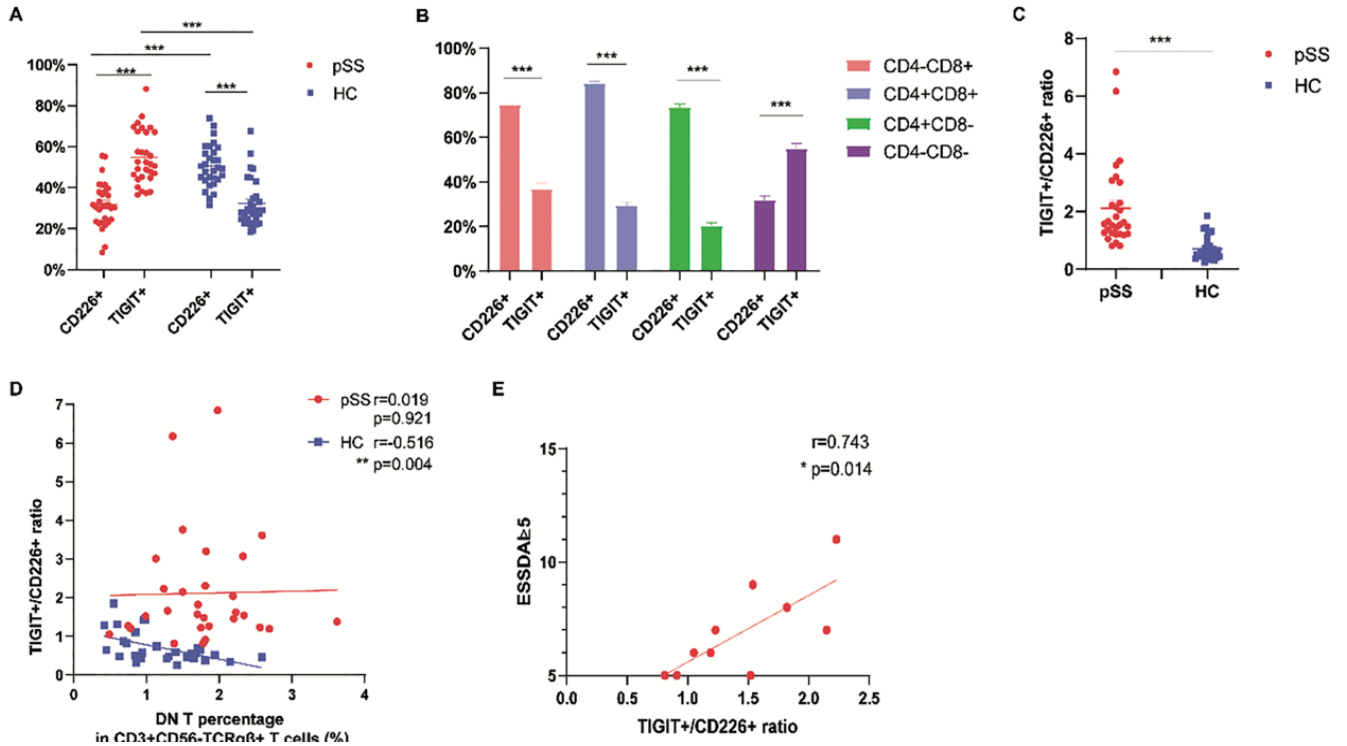

Fig. 2 **A**: The percentage of CD226$^+$ subset and TIGIT$^+$ subset out of DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **B**: The percentage of TIGIT$^+$ subset and CD226$^+$ subset out of CD4$^-$CD8$^+$, CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ and CD4$^-$CD8$^-$ T subsets from pSS patients, respectively. Data are represented as mean ± SEM. **C**: The ratio of TIGIT$^+$ subset and CD226$^+$ subset out of DN T cells from pSS patients (red) and HCs (blue), respectively. Data are represented as mean ± SEM. **D**: Correlations between the percentage of DN T cells in CD3$^+$CD56$^-$TCRαβ$^+$ T cells and the ratio of TIGIT$^+$ subset/CD226$^+$ subset in pSS patients (red) and HCs (blue). **E**: A significantly positive correlation between the ratio of TIGIT$^+$ subset/CD226$^+$ subset and moderate-to-high ESSDAI scores (≥5, n=10).
*p<0.05, **p<0.01 and ***p<0.001.

After intracellular staining in the dark at room temperature for 45 minutes, cells were washed twice and resuspended in PBS for cytometric analysis on FACS Aria II (BD Biosciences). Data analysis was conducted via FlowJo software (v. 10.4, TreeStar, Ashland, OR, USA). Antibodies used for flow cytometry were purchased from BioLegend company and listed as follows: FITC CD3 (cat. no. 300406), PE CD56 (cat. no. 362508), APC/Cyanine7 TCRα/β cat. no.306728)-APC CD4 (cat. no. 300514), PerCP/Cyanine5.5 CD8a (cat. no. 301032), PE/Cyanine7 CD226 (cat. no. 338316), PE/Dazzle™594 TIGIT (cat. no. 372716), PE CD107a (cat. no. 3 28608), APC CD69 (cat. no. 310910), PE IL-17A (cat. no. 512306), APC IFN-γ (cat. no. 506510).

Table I. Demographic and clinical features of enrolled participants.

Parameters	pSS (n=30)	HC (n=30)	RA (n=16)
Demographic features			
Female (n, %)	27, 90.00%	26, 86.67%	11, 68.75%
Age (years)	40.80 ± 12.64	34.90 ± 11.41	53.38 ± 9.94
Clinical features			
Duration (months)	30.00 (18.00-72.00)	NA	12.00 (2.00-138.00)
ESR (mm/h)	21.00 (13.00-33.75)	(-)	37.00 (15.25-68.00)
IgG (g/L)	19.97 ± 5.28	(-)	17.39 ± 6.20
C3 (g/L)	1.03 ± 0.22	(-)	1.24 (1.13, 1.42)
C4 (g/L)	0.18 ± 0.05	(-)	0.26 ± 0.09
RF (IU/ml)	106.30 (32.20, 142.15)	(-)	111.15 (52.20, 409.75)
Anti-SSA antibody positive (n, %)	28, 93.33%	0, 0.00%	2, 12.50%
Anti-SSB antibody positive (n, %)	15, 50.00%	0, 0.00%	0, 0.00%
Anti-Ro52 antibody positive (n, %)	25, 83.33%	0, 0.00%	2, 12.50%
ESSDAI	3.83 ± 2.76	NA	NA
Untreated (n, %)	19, 63.33%	NA	16, 100.00%
DN T (%)	1.77 ± 0.66	1.21 ± 0.54	1.06 ± 0.43

Normally distributed data were presented as mean ± SD; non-normal data were presented as median with the IQR.
pSS: primary Sjögren's syndrome; HCs: healthy controls; RA: rheumatoid arthritis; ESR: erythrocyte sedimentation rate; IgG: immunoglobulin; C3: complement factor 3; C4: complement factor 4; RF: rheumatoid factor; ESSDAI: EULAR Sjögren's syndrome disease activity index; DN T: CD3$^+$CD56$^-$TCRαβ$^+$CD4$^-$CD8$^-$; NA: not applicable.

Statistical analysis

Data analysis was performed using SPSS software (v. 21.0) and GraphPad Prism (v. 8.0). Data were tested for normal distribution. Normally distributed data were presented as mean with the standard deviation (SD) and non-normal data were presented as median with the interquartile range (IQR). Student's *t*-test was used for the comparison between normal distributed variables,

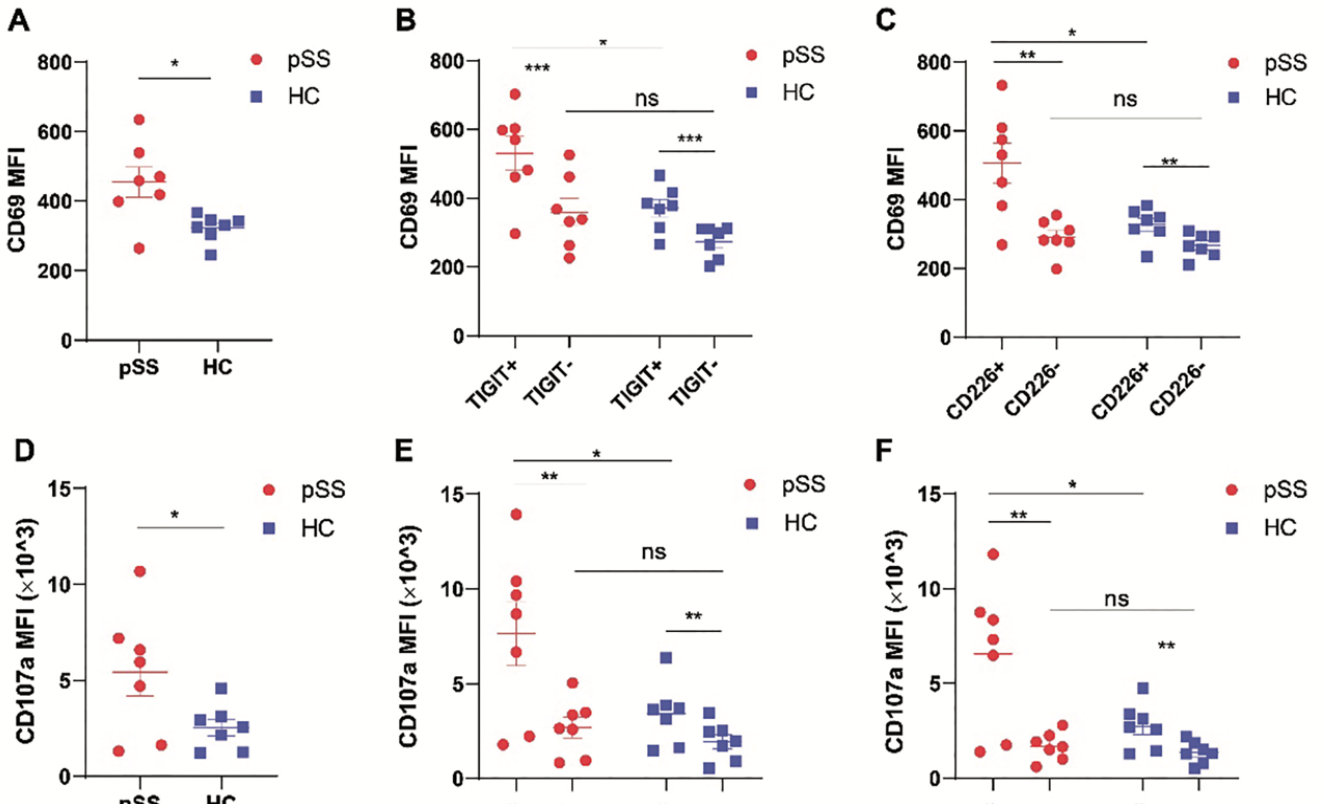

Fig. 3 **A**: The mean fluorescence intensity of CD69 in DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **B**: The mean fluorescence intensity of CD69 in TIGIT$^+$ subset and TIGIT$^-$ subset of DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **C**: The mean fluorescence intensity of CD69 in CD226$^+$ subset and CD226$^-$ subset of DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **D**: The mean fluorescence intensity of CD107a in DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **E**: The mean fluorescence intensity of CD107a in TIGIT$^+$ subset and TIGIT$^-$ subset of DN T cells from pSS patients and HCs, respectively. Data are represented as mean ± SEM. **F**: The mean fluorescence intensity of CD107a in CD226$^+$ subset and CD226$^-$ subset of DN T cells from pSS patients and HCs, respectively.
Data are represented as mean ± SEM. ns: no statistical significance; *p<0.05, **p<0.01 and ***p<0.001.

otherwise the Mann-Whitney test was used. Pearson correlation analysis was performed for describing the correlation between two continuous variables. A p value<0.05 was considered statistically significant.

Results

Expansion of DN T cells in peripheral blood of pSS patients correlates with serum IgG and complement .

The percentage of peripheral DN T cells was detected in 30 pSS patients and 30 gender/age/ethnicity-matched HCs, as well as 16 RA patients, whose demographic and clinical features are shown in Table I. The percentage of CD4$^-$CD8$^-$ DN T cells out of CD3$^+$CD56$^-$TCRαβ$^+$ T cells in peripheral blood from pSS patients was (1.77 ± 0.66) %, compared with (1.21 ± 0.54) % in HCs (Table I, Fig. 1B). As is shown in Figure 1C, the percentage of DN T cells out of CD3$^+$CD56$^-$ TCRαβ$^+$ T cells was significantly higher in pSS patients than that in HCs (p<0.001) or RA patients (p<0.001).

pSS patients were further divided according to their treatment at inclusion to compare the percentages of DN T cells: the untreated group (19) versus the group under HCQ treatment (11), no significance was found (data are not shown). Besides, it was observed that the percentage of DN T cells was positively correlated with the levels of immunoglobulin G (IgG) (r=0.451, p<0.05) (Fig. 1D), negatively correlated with the levels of complement factor 3 (C3) (r=-0.438, p<0.05) (Fig. 1E) and complement factor 4 (C4) (r=-0.470, p<0.05) (Fig. 1F).

Peculiar proportional alteration of CD226 and TIGIT exists in DN T cells of pSS patients

The expression of CD226 and TIGIT was investigated on DN T cells. As is shown in Figure 2A, the percentage of CD226$^+$ subset in DN T cells from pSS patients was significantly lower than that in HCs (p<0.001) while the percentage of TIGIT$^+$ subset significantly increased in DN T cells from pSS patients (p<0.001), indicating a state of over-expressed inhibitory receptors on DN T cells of pSS patients. Interestingly, DN T cells showed a unique proportional alteration of CD226 and TIGIT, distinct from other T subsets which show an overwhelming expression of CD226 (p<0.001) (Fig. 2B). Considering that TIGIT and CD226 act as a pair of functionally antagonistic receptors, the ratio of TIGIT and CD226 was also analysed to represent their relative expression level. Consequently, the ratio of TIGIT$^+$ subset and CD226$^+$ subset (TIGIT/CD226 ratio) significantly increased in pSS patients with a median of 1.56 while the median in HCs was 0.56 (p<0.001) (Fig. 2C). It is intrigu-

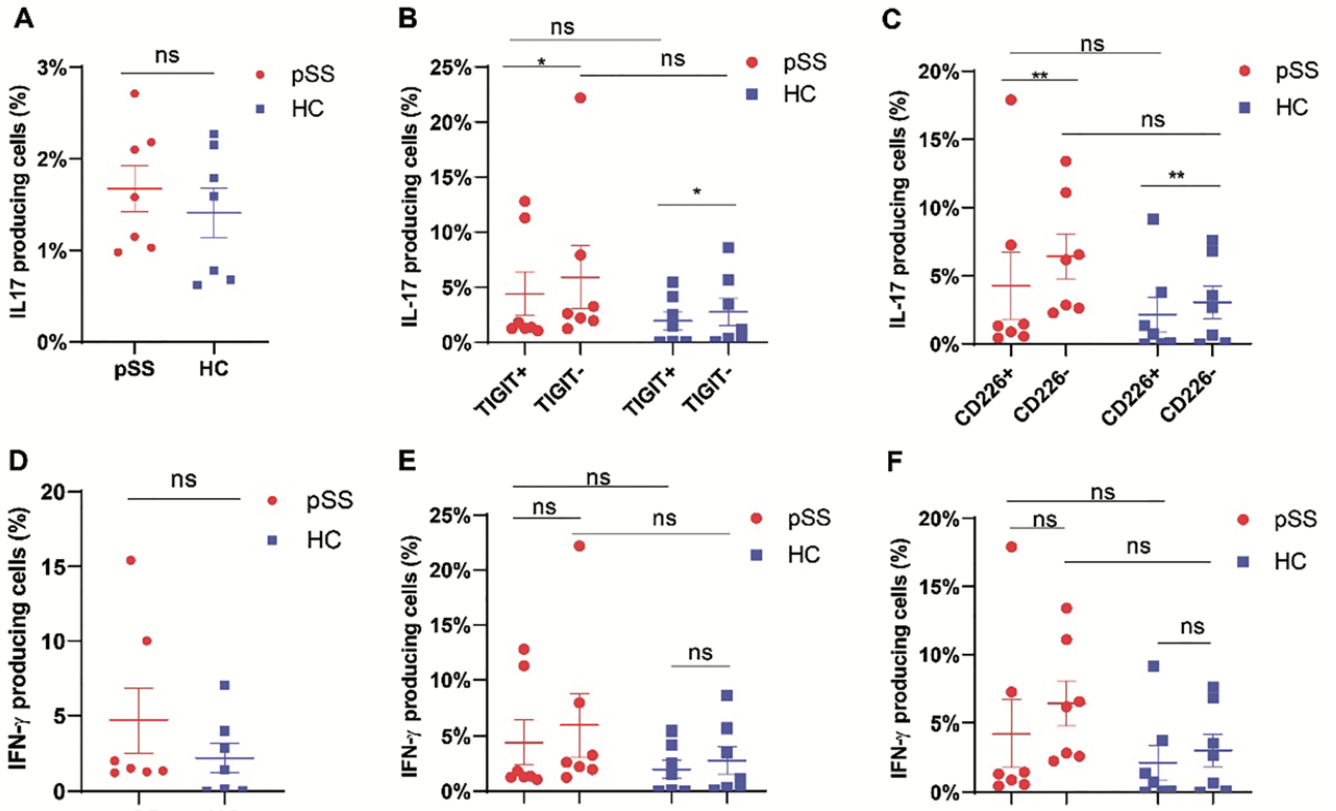

Fig. 4 **A**: The percentage of IL-17 producing cells in DN T cells from pSS patients and HCs, respectively. **B**: The percentage of IL-17 producing cells in TIGIT$^+$ subset and TIGIT$^-$ subset of DN T cells from pSS patients and HCs, respectively. **C**: The percentage of IL-17 producing cells in CD226$^+$ subset and CD226$^-$ subset of DN T cells from pSS patients and HCs, respectively. **D**: The percentage of IFN-γ producing cells in DN T cells from pSS patients and HCs, respectively. **E**: The percentage of IFN-γ producing cells in TIGIT$^+$ subset and TIGIT$^-$ subset of DN T cells from pSS patients and HCs, respectively. **F**: The percentage of IFN-γ producing cells in CD226$^+$ subset and CD226$^-$ subset of DN T cells from pSS patients and HCs, respectively.
Data are represented as mean ± SEM. ns: no significance; *p<0.05 and **p<0.01.

ing to note that the TIGIT/CD226 ratio was found to inversely correlate with the percentage of DN T cells in HCs (r=-0.516, p<0.01) (Fig. 2D). Conversely, such correlation was not observed in pSS patients. In addition, the TIGIT/CD226 ratio had a strong positive correlation with ESSDAI scores (≥5) in those patients with moderate-to-high disease activity (r=0.743, p<0.05) (Fig. 2E).

CD69 and CD107a are elevated in TIGIT-overexpressing DN T cells of pSS patients.

To explore the influence of TIGIT/CD226 alteration on DN T cells, the expression of CD69, a classic T cell activation marker, was detected in DN T cells. It was found that the expression of CD69 significantly increased on DN T cells in pSS patients compared to that in HCs (p<0.05) (Fig. 3A). The TIGIT$^+$ subset showed significantly higher expression of CD69 compared to the TIGIT$^-$ subset in either pSS patients or HCs (p<0.001), with the TIGIT$^+$ subset in pSS patients expressing a higher level of CD69 than the counterpart in HCs (p<0.05) (Fig. 3B). Likewise, the CD226$^+$ subset showed a significantly higher level of CD69 compared to the CD226$^-$ subset in either pSS patients or HCs (p<0.01) (Fig. 3C). CD69 was elevated in the CD226$^+$ subset from pSS patients compared to the counterpart in HCs (p<0.05) (Fig. 3C).

Since previous researches reported the CD8$^+$ T cell-origin of DN T cells (21, 22), we also detected the expression of CD107a (LAMP-1) in DN T cells, a degranulation marker for cytotoxic activity expressed on activated natural killer (NK) cells and CD8$^+$ T cells (23, 24). A higher expression of CD107a was observed in DN T cells from pSS patients (p<0.05) (Fig. 3D). Both of the TIGIT$^+$ subset and CD226$^+$ subset expressed a higher level of CD107a than their negative counterparts (p<0.01) The expression of CD107a was also elevated in both TIGIT$^+$ subset and CD226$^+$ subset from pSS patients compared to their counterparts in HCs (p<0.05) (Fig. 3E, F). These findings indicated an enhanced expression of CD69 and CD107a on TIGIT-overexpressing DN T cells from pSS patients.

DN T cells from pSS patients secrete similar levels of IL-17 and IFN-γ compared to HCs

It is acknowledged that both interleukin 17 (IL-17) and interferon gamma (IFN-γ) are important proinflammatory cytokines in pSS pathogenesis (25-29), so we detected these two cytokines in DN T cells. DN T cells from pSS patients secreted similar levels of IL-17 compared to HCs (Fig. 4A). The TIGIT$^+$ subset in DN T cells from pSS patients produced significantly decreased IL-17 than the TIGIT$^-$ subset (p<0.05) (Fig. 4B). It seems that there was an increased trend of IL-17 secretion in the TIGIT$^+$ subset and TIGIT$^-$ subset from pSS pa-

tients compared to their counterparts from HCs while no significance was found (Fig. 4B). Likewise, the CD226$^+$ subset in DN T cells from pSS patients produced significantly decreased IL-17 than the CD226$^-$ subset (p<0.01) (Fig. 4C) while no significance was found when compared to their counterparts from HCs. Besides, similar levels of IFN-γ were found in DNT cells from pSS patients and HCs (Fig. 4D). No significance was found in IFN-γ secretion between TIGIT$^+$ subset and TIGIT$^-$ subset, or between CD226$^+$ subset and CD226$^-$ subset, as well as between pSS patients and HCs (Fig. 4E, F).

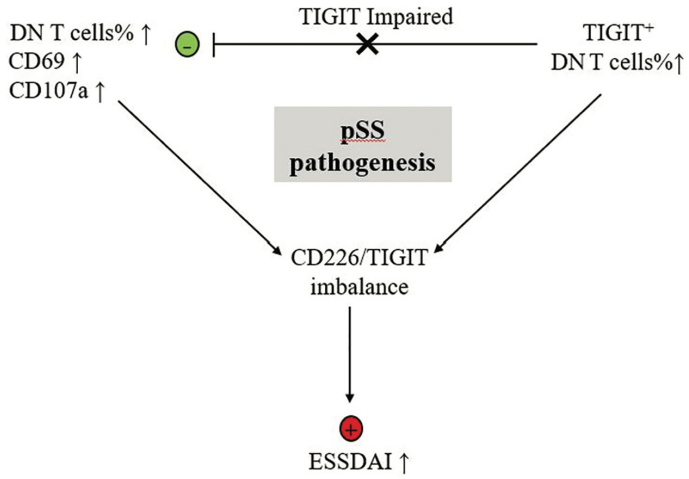

Fig. 5. Hypothesis of the mechanism for CD226/TIGIT imbalance in DN T cells implicated in pSS pathogenesis.

Discussion

This is the first study to explore the alteration of immune checkpoint CD226 and TIGIT in DN T cells from pSS patients. We observed the remarkable expansion of circulating DN T cells in pSS patients, which was consistent with previous researches (13, 14). Besides, we found that the percentage of DN T cells was positively correlated with the level of IgG while inversely correlated with C3 and C4 levels. These correlations between DN T cells and the above inflammatory markers suggest that circulating DN T cells may participate in the pathogenesis, or even correlate with disease activity of pSS.

Our previous study revealed increased expression of the immune checkpoint CD226 and TIGIT on CD4$^+$ T cells from pSS patients (18). These proportionally increased CD4$^+$TIGIT$^+$ and CD4$^+$CD226$^+$ subsets were found to be functionally activated and produce high levels of IFN-γ and TNF-α. It was postulated that over-expressed TIGIT on CD4$^+$ T cells was impaired to exert suppressive effect and the CD226/TIGIT balance in regulation of immune response was disturbed. In this study, comparative analysis of T cell subsets indicated the overwhelming expression of CD226 over TIGIT in CD4$^-$CD8$^+$, CD4$^+$CD8$^+$, and CD4$^+$CD8$^-$ T cell subsets. Conversely, high expression of TIGIT versus lower expression of CD226 was peculiar to DN T cells from pSS patients, as opposed to DN T from HCs. Besides, the TIGIT/CD226 ratio was positively correlated with ESSDAI scores (≥5) representative of a moderate-to-high disease activity. These intriguing findings implied that a disturbed balance between CD226 and TIGIT in DN T cells may be implicated in pSS pathogenesis. The delineation of CD226 and TIGIT on tissue-infiltrating DN T cells will help us better understand the pathological value of this group of cells, which deserves further investigation.

Ferraz *et al*. reported the pathogenic role of DN T cells as the main cytotoxic-related-CD107a$^+$ cells in lesions of an immune-mediated skin disorder (30). In this study, we found the enhanced expression of activation marker CD69 and cytotoxic marker CD107a in DN T cells from pSS patients, which may support that DN T itself could be activated and pathogenic. It is also observed that these expanded TIGIT-overexpressing DN T cells of pSS patients produced low levels of IL-17 and IFN-γ, which were comparable to that of HCs. It is speculated that the suppressive effect of TIGIT over cytotoxicity may be impaired in DN T cells from pSS patients. The role of human DN T cells in regulating immune responses remains controversial. Voelkl *et al*. demonstrated that human DN T cells are highly potent suppressors of both CD4$^+$ and CD8$^+$ T cell responses (31). A study by Fischer *et al*. demonstrated that peripheral DN Treg cells could present acquired alloantigen from APCs to activated CD8$^+$ T cells and lead to apoptosis and suppressive proliferation of antigen-specific CTLs, thus preventing the rejection of skin and heart allografts (8). On the other side, DN T cells have been reported to play a pathogenic role in psoriasis and SLE (12, 32). In this study, the percentage of DN T cells was correlated with serum IgG, C3 and C4, indicating a potential involvement of DN T in the pathogenesis of pSS. Besides, these activated DN T cells had a strong cytotoxicity, which may support its pathogenic role in pSS. In cancer related researches, overexpression of the inhibitory receptor TIGIT on immune cells usually contributed to the down-regulated immune response and immune evasion of tumour cell, leading to restricted antitumor activity and poor prognosis. In this study, the balance between CD226 and TIGIT was disturbed on DN T cells of pSS patients, manifesting as upregulation of TIGIT. Besides, the TIGIT/CD226 ratio was found to inversely correlate with the percentage of DN T cells in HCs while such correlation was not observed in pSS patients. Aberrantly overexpressed TIGIT seemed to be dysfunctional to control the expansion of DN T cells, as well as the cytotoxicity of DN T cells in pSS patients (Fig. 5).

A study by Alunno *et al*. found that freshly isolated peripheral DN T cells produced a very high level of IL-17 (approximately 60%) in pSS patients (13), which is much different from the result of our study. In fact, the percentage of IL-17 producing cells out of freshly isolated DN T cells in our study is quite close to the results of another study (33). One point needs to be paid attention is CD4 endocytosis after *in vitro* stimulation using the leukocyte activation cocktail comprised of PMA,

ionomycin and Brefeldin A (34). Our data indicated that isolated $CD4^+$ T cells produced an obviously higher level of IL-17 and IFN-γ after *in vitro* stimulation with PMA (Supplementary Fig. S1). The high percentage of IL-17 observed in their study was probably attributed to a part of actual $CD4^+$ T cells which were mistaken for DN T cells due to CD4 endocytosis. Considering that pSS is a heterogeneous disease, the majority of pSS patients in our study were at the stage of low disease activity and a better patient stratification may provide more clues.

In conclusion, the balance between CD226 and TIGIT was disturbed on activated DN T cells which were expanded in peripheral blood of pSS patients. The dysfunctional control of overexpressed TIGIT over cytotoxicity may be implicated in the pathogenesis of pSS.

Take home messages

- DN T expansion in peripheral blood of pSS patients correlates with serum IgG, C3 and C4 levels.
- The balance between CD226 and TIGIT is disturbed on activated DN T cells.
- Dysfunctional control of overexpressed TIGIT over cytotoxicity may be implicated in the pathogenesis of pSS.

References

1. FOX RI: Sjögren's syndrome. *Lancet* 2005; 366(9482): 321-31. https://doi.org/10.1016/s0140-6736(05)66990-5
2. FULVIO G, LA ROCCA G, CHATZIS LG *et al*.: Impact of gender and age at onset on Sjögren's syndrome presentation and outcome: state of the art. *Clin Exp Rheumatol* 2023; 41(12): 2547-54. https://doi.org/10.55563/clinexprheumatol/lygrzv
3. YAO Y, MA J-F, CHANG C *et al*.: Immunobiology of T cells in Sjögren's syndrome. *Clin Rev Allergy Immunol* 2021; 60(1): 111-31. https://doi.org/10.1007/s12016-020-08793-7
4. TASAKI S, SUZUKI K, NISHIKAWA A *et al*.: Multiomic disease signatures converge to cytotoxic CD8 T cells in primary Sjögren's syndrome. *Ann Rheum Dis* 2017; 76(8): 1458-66. https://doi.org/10.1136/annrheumdis-2016-210788
5. MINGUENEAU M, BOUDAOUD S, HASKETT S *et al*.: Cytometry by time-of-flight immunophenotyping identifies a blood Sjögren's signature correlating with disease activity and glandular inflammation. *J Allergy Clin Immunol* 2016; 137(6): 1809-21.e12. https://doi.org/10.1016/j.jaci.2016.01.024
6. HONG X, MENG S, TANG *et al*.: Single-cell RNA sequencing reveals the expansion of cytotoxic $CD4^+$ T lymphocytes and a landscape of immune cells in primary Sjögren's syndrome. *Front Immunol* 2020; 11: 594658. https://doi.org/10.3389/fimmu.2020.594658
7. VERSTAPPEN GM, KROESE FGM, BOOTSMA H: T cells in primary Sjögren's syndrome: targets for early intervention. *Rheumatology* (Oxford) 2019; 60(7): 3088-98. https://doi.org/10.1093/rheumatology/kez004
8. FISCHER K, VOELKL S, HEYMANN J *et al*.: Isolation and characterization of human antigen-specific TCR alpha beta+ CD4(-)CD8-double-negative regulatory T cells. *Blood* 2005; 105(7): 2828-35. https://doi.org/10.1182/blood-2004-07-2583
9. BRANDT D, HEDRICH CM: TCRαβ+CD3+ CD4-CD8- (double negative) T cells in autoimmunity. *Autoimmun Rev* 2018; 17(4): 422-30. https://doi.org/10.1016/j.autrev.2018.02.001
10. BRISTEAU-LEPRINCE A, MATEO V, LIM A *et al*.: Human TCR alpha/beta+ CD4-CD8-double-negative T cells in patients with autoimmune lymphoproliferative syndrome express restricted Vbeta TCR diversity and are clonally related to CD8+ T cells. *J Immunol* 2008; 181(1): 440-48. https://doi.org/10.4049/jimmunol.181.1.440
11. CRISPÍN JC, OUKKA M, BAYLISS G *et al*.: Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol* 2008; 181(12): 8761-66. https://doi.org/10.4049/jimmunol.181.12.8761
12. BRANDT D, SERGON M, ABRAHAM S, MÄBERT K, HEDRICH CM: TCR+CD3+CD4-CD8- effector T cells in psoriasis. *Clin Immunol* 2017; 181: 51-59. https://doi.org/10.1016/j.clim.2017.06.002
13. ALUNNO A, BISTONI O, BARTOLONI E *et al*.: IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjögren's syndrome. *Ann Rheum Dis* 2013; 72(2): 286-92. https://doi.org/10.1136/annrheumdis-2012-201511
14. ALUNNO A, CARUBBI F, BISTONI O *et al*.: CD4(-)CD8(-) T-cells in primary Sjögren's syndrome: association with the extent of glandular involvement. *J Autoimmun* 2014; 51: 38-43. https://doi.org/10.1016/j.jaut.2014.01.030
15. CHIANG EY, MELLMAN I: TIGIT-CD226-PVR axis: advancing immune checkpoint blockade for cancer immunotherapy. *J Immunother Cancer* 2022; 10(4): e004711. https://doi.org/10.1136/jitc-2022-004711
16. LOZANO E, DOMINGUEZ-VILLAR M, KUCHROO V, HAFLER DA: The TIGIT/CD226 axis regulates human T cell function. *J Immunol* 2012; 188(8): 3869-75. https://doi.org/10.4049/jimmunol.1103627
17. JOLLER N, HAFLER JP, BRYNEDAL B *et al*.: Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 2011; 186(3): 1338-42. https://doi.org/10.4049/jimmunol.1003081
18. DENG C, CHEN Y, LI W *et al*.: Alteration of CD226/TIGIT immune checkpoint on T cells in the pathogenesis of primary Sjögren's syndrome. *J Autoimmun* 2020; 113: 102485. https://doi.org/10.1016/j.jaut.2020.102485
19. SHIBOSKI CH, SHIBOSKI SC, SEROR R *et al*.: 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for primary Sjögren's syndrome: A consensus and data-driven methodology involving three international patient cohorts. *Ann Rheum Dis* 2017; 76(1): https://doi.org/10.1136/annrheumdis-2016-210571
20. SEROR R, BOOTSMA H, SARAUX A *et al*.: Defining disease activity states and clinically meaningful improvement in primary Sjögren's syndrome with EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). *Ann Rheum Dis* 2016; 75(2): 382-89. https://doi.org/10.1136/annrheumdis-2014-206008
21. CRISPÍN JC, TSOKOS GC: Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype. *J Immunol* 2009; 183(7): 4675-81. https://doi.org/10.4049/jimmunol.0901533
22. HEDRICH CM, RAUEN T, CRISPIN JC *et al*.: cAMP-responsive element modulator α (CREMα) trans-represses the transmembrane glycoprotein CD8 and contributes to the generation of CD3+CD4-CD8- T cells in health and disease. *J Biol Chem* 2013; 288(44): 31880-87. https://doi.org/10.1074/jbc.m113.508655
23. KRZEWSKI K, GIL-KRZEWSKA A, NGUYEN V, PERUZZI G, COLIGAN JE: LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 2013; 121(23): 4672-83. https://doi.org/10.1182/blood-2012-08-453738
24. WIECHMANN A, WILDE B, TYCZYNSKI B *et al*.: CD107a+ (LAMP-1) Cytotoxic CD8+ T-cells in lupus nephritis patients. *Front Med* (Lausanne) 2021; 8: 556776. https://doi.org/10.3389/fmed.2021.556776
25. KWOK S-K, CHO M-L, HER Y-M *et al*.: TLR2 ligation induces the production of IL-23/IL-17 via IL-6, STAT3 and NF-kB pathway in patients with primary Sjögren's syndrome. *Arthritis Res Ther* 2012; 14(2): R64. https://doi.org/10.1186/ar3780
26. LIN X, RUI K, DENG J *et al*.: Th17 cells play a critical role in the development of experimental Sjögren's syndrome. *Ann Rheum Dis* 2015; 74(6): 1302-10. https://doi.org/10.1136/annrheumdis-2013-204584
27. KATSIFIS GE, REKKA S, MOUTSOPOULOS NM, PILLEMER S, WAHL SM: Systemic and local interleukin-17 and linked cytokines associated with Sjögren's syndrome immunopathogenesis. *Am J Pathol* 2009; 175(3): 1167-77. https://doi.org/10.2353/ajpath.2009.090319
28. WILLEKE P, SCHLÜTER B, SCHOTTE H, DOMSCHKE W, GAUBITZ M, BECKER H: Interferon-gamma is increased in patients with primary Sjögren's syndrome and Raynaud's phenomenon. *Semin Arthritis Rheum* 2009; 39(3): 197-202. https://doi.org/10.1016/j.semarthrit.2008.04.002
29. NEZOS A, GRAVANI F, TASSIDOU A *et al*.: Type I and II interferon signatures in

Sjögren's syndrome pathogenesis: Contributions in distinct clinical phenotypes and Sjögren's related lymphomagenesis. *J Autoimmun* 2015; 63: 47-58. https://doi.org/10.1016/j.jaut.2015.07.002

30. FERRAZ R, CUNHA CF, PIMENTEL MIF *et al.*: CD3+CD4negCD8neg (double negative) T lymphocytes and NKT cells as the main cytotoxic-related-CD107a+ cells in lesions of cutaneous leishmaniasis caused by Leishmania (Viannia) braziliensis. *Parasit Vectors* 2017; 10(1): 219. https://doi.org/10.1186/s13071-017-2152-2
31. VOELKL S, GARY R, MACKENSEN A: Characterization of the immunoregulatory function of human TCR-αβ+ CD4- CD8- double-negative T cells. *Eur J Immunol* 2011; 41(3): 739-48. https://doi.org/10.1002/eji.201040982
32. SHIVAKUMAR S, TSOKOS GC, DATTA SK: T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J Immunol* 1989; 143(1): 103-12.
33. ALUNNO A, CARUBBI F, BISTONI O *et al.*: Interleukin (IL)-17-producing pathogenic T lymphocytes co-express CD20 and are depleted by rituximab in primary Sjögren's syndrome: a pilot study. *Clin Exp Immunol* 2016; 184(3): 284-92. https://doi.org/10.1111/cei.12771
34. PELCHEN-MATTHEWS A, PARSONS IJ, MARSH M: Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. *J Exp Med* 1993; 178(4): 1209-22. https://doi.org/10.1084/jem.178.4.1209