Reduced Deltex1 expression in T cells indicates increased disease activity in Sjögren's disease

H.-C. Tsai[1,2], H.-T. Liao[1,2], W.-S. Chen[1,2], C.-C. Lai[1,2], Y.-S. Sun[1,2], T.-S. Hsu[3], M.-H. Chen[1,2]

[1]Faculty of Medicine, National Yang Ming Chiao Tung University, Taipei; [2]Division of Allergy, Immunology and Rheumatology, Department of Medicine, Taipei Veterans General Hospital, Taipei; [3]Institute of Molecular and Cellular Biology, College of Life Science and Medicine, National Tsing Hua University, Hsinchu, Taiwan.

Abstract

Objective

Deltex1 is a transcriptional target of NFAT that promotes T cell anergy. However, whether Deltex1 affects the properties of regulatory T cells (Tregs), which are involved in the pathogenesis of Sjögren's disease (SjD), is unknown.

Methods

T cells were purified from peripheral blood using a negative selection method. Deltex1 mRNA levels were measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR). The mean fluorescent intensity (MFI) of Treg-associated molecules and the cytokine positivity of $CD4_+$ $FoxP3_+$ Tregs were analysed using flow cytometry. The European League against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) and Patient-Reported Index (ESSPRI) were used to evaluate systemic disease activity and symptoms in SjD.

Results

Deltex1 expression in T cells was significantly lower in SjD patients than in age- and sex-matched healthy controls ($p<0.001$). Deltex1 mRNA levels in T cells negatively correlated with visual analog scale scores for fatigue, ESSDAI, and ESSPRI ($r=-0.334$, $p=0.035$; $r=-0.364$, $p=0.021$; and $r=-0.340$, $p=0.032$, respectively). Low Deltex1 levels correlated with some clinical manifestations of SjD, including immune thrombocytopenia, vasculitis, and autoimmune thyroiditis ($p=0.014$, 0.002, and 0.001, respectively). The MFI of PD-1, CTLA-4, TIM-3, LAG-3 on Tregs and the percentage of interferon-γ_+, interleukin (IL)-4_+, IL-$17A_+$ Tregs were significantly higher in the low Deltex1 group (Deltex1/GAPDH ≤0.02) than in the high Deltex1 group (Deltex1/GAPDH > 0.02) ($p<0.05$).

Conclusion

Deltex1 may affect the properties of Tregs; thus, it is a potential biomarker of disease activity in SjD.

Key word

Deltex 1, regulatory T cells, Sjögren's disease, disease activity

Hung-Cheng Tsai, MD
Hsien-Tzung Liao, MD, PhD
Wei-Sheng Chen, MD
Chien-Chih Lai, MD
Yi-Syuan Sun, MD
Tzu-Sheng Hsu, PhD
Ming-Han Chen, MD, PhD

Please address correspondence to:
Ming-Han Chen
Taipei Veteran General Hospital,
201, Sec. 2, Shih-Pai Road,
Taipei 11217, Taiwan.
E-mail: mhchen6@vghtpe.gov.tw

and to:
Tzu-Sheng Hsu
Institute of Molecular and
Cellular Biology,
College of Life Science and Medicine,
National Tsing Hua University,
Hsinchu, Taiwan.
E-mail: tshsu@life.nthu.edu.tw

Received on May 29, 2024; accepted in revised form on November 13, 2024.

Funding: this study was funded by the Ministry of Science and Technology Program, Taiwan (NSTC-1132314-B- 075-024), Yin Shu-Tien Foundation Taipei Veterans General Hospital-National Yang Ming Chiao Tung University Excellent Physician Scientists Cultivation Program (no. 111-V-A-002), and Taipei Veterans General Hospital (V109C-163 and V110C-114). However, these funders were not involved in the research, study design, data collection, analysis, and interpretation of data; writing of the manuscript; or the decision to submit the article for publication.

Competing interests: none declared.

Introduction

Sjögren's disease (SjD) is a common autoimmune disease with a prevalence of 320 per 100,000 in the US and approximately 700 per 100,000 in the Asian population (1). It primarily impairs the function of exocrine glands with focal lymphocytic infiltration, and usually presents clinically as persistent dryness of the mouth and eyes (2). SjD is usually diagnosed in people aged over 40 years, and is more common in women. Systemic manifestations occur in 50-80% of SjD patients and include musculoskeletal, cutaneous, pulmonary, gastrointestinal, renal, and neurological involvement. Additionally, lymphoma is a significant complication associated with SjD (3).

The pathophysiology of SjD involves innate and adaptive immune responses that lead to the destruction of exocrine glands (4, 5). The innate immune system plays an important role in the early stages of SjD. Environmental factors, such as viral infection or hormonal factors, may stimulate innate immunity, resulting in epithelial and dendritic cell (DC) activation. Epithelial cells are further activated by pro-inflammatory cytokines, such as interleukin (IL)-1β, interferon (IFN)-γ, and tumour necrosis factor alpha (TNF-α), which are produced by adjacent T cells. Regulatory T cells (Tregs), which are characterised by the $CD4_+$ $CD25_+$ $FoxP3_+$ phenotype, play a crucial role in maintaining immune tolerance and suppressing the activity of autoreactive T cells in SjD. The malfunction of these suppressive activities, along with the consequent decrease in their levels, significantly contributes to the pathophysiology of SjD (6).

Deltex1 is an E3 ligase involved in Notch signalling in *Drosophila* (7, 8) and is a transcriptional target of nuclear factor of activated T cells (NFAT) in mice known to promote T cell anergy (9). Deltex1 can inhibit T cell activation by E3 ligase-dependent and -independent mechanisms, and regulate the expression of anergy-associated molecules, including growth arrest and DNA-damage-inducible 45 beta (Gadd45 beta) and Cbl-b. The deletion of Deltex1 in T cells in mice significantly increases T cell activation and proliferation as a result of resistance to anergy induction. Furthermore, aged Deltex1 knockout mice exhibit increased inflammation in the lungs and liver, and elevated autoantibody production. These observations indicate that Deltex1 regulates T cell function in a mouse model. Previously, we investigated the role of Deltex1 in human T cell function and its correlation with disease activity in systemic lupus erythematosus (SLE) patients (10). Silencing of Deltex1 expression enhances interferon-γ (IFN-γ) secretion by human T cells after stimulation with anti-CD3 and anti-CD28. Intriguingly, low Deltex1 levels in T cells are associated with increased IFN-γ production in SLE patients and correlated with severe disease activity (10). These findings suggest that Deltex1 regulates IFN-γ secretion and may play an important role in IFN-γ-mediated autoimmune diseases. However, the association between Deltex1 and SjD, particularly the influence of Deltex1 on the properties of Tregs, remains unclear. Deltex1 is critical for maintaining the stability and suppressive function of Tregs in a mouse model by preserving Foxp3 protein levels. Deltex1 antagonises HIF-1α by promoting its degradation through a proline hydroxylation-dependent pathway, thereby preventing HIF-1α-mediated Foxp3 destabilisation under hypoxic or inflammatory conditions (11). Targeting Deltex1 in Tregs offers potential as a therapeutic strategy to restore immune tolerance and treat autoimmune diseases. This study aimed to investigate the role of Deltex1 in SjD and its association with Tregs.

Methods

Patients

Blood samples were collected from SjD patients and healthy controls. Diagnosis of SjD was based on the 2002 American-European Revised Classification Criteria for Sjögren's syndrome (12) or 2016 American College of Rheumatology/European League against Rheumatism classification criteria for Sjögren's syndrome (13). Patients with other autoimmune diseases, such as SLE, rheumatoid arthritis, polymyositis/dermatomyositis,

scleroderma, primary biliary cirrhosis, and mixed connective tissue disease, were defined as having secondary SjD and excluded from this study (14). Additionally, only patients receiving traditional disease-modifying antirheumatic drugs (DMARDs) were included. Patients who had received biologic agents, such as rituximab or steroid pulse therapy, within the past 6 months, as well as those with concurrent malignancies, were also excluded. This study was approved by the Institutional Ethics Committee of Taipei Veterans General Hospital (IRB-no: 2019–05-006CC). Informed consent was obtained from all patients who participated in this study.

Assessment of SjD disease activity

The European League against Rheumatism (EULAR) Sjögren's syndrome outcome measures, the disease activity index (ESSDAI) (range 0-123) (15), and the patient-reported index (ESSPRI) (range 0-10) (16) were used to evaluate systemic activity and patient symptoms in SjD, respectively. Fatigue, pain, and dryness of the eyes and mouth were assessed using a visual analog scale (VAS) (range, 0–10 cm). Clinical and laboratory profiles of the patients were recorded on the same day as blood sampling. Finally, salivary gland ultrasonography was performed, and the disease severity was evaluated using a 0-4 scoring system (17).

Measurement of Deltex1 expression in T cells

Peripheral blood was obtained via venepuncture from SjD patients and from age- and sex-matched healthy controls (HCs). PBMCs (peripheral blood mononuclear cells) were separated from the whole blood by a density gradient centrifugation method using Ficoll Histopaque. T cells were purified from PBMCs by selecting against CD14, CD19, CD235A, and CD11b using immunomagnetic beads (BD Biosciences, San Jose, CA, USA). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to measure Deltex1 messenger RNA (mRNA). The primers used for detection of DTX1 were the forward primer 5´-CAGCCGCCTGGGAAGATGGAGTT-3´ and reverse primer 5´-TGGATGCCTGTGGGGATGTCATAGAC-3'. Total RNA was extracted from separated cells with a 6100 Nucleic Acid PrepStation (Biosystems, Foster City, CA, USA). Next, 500 ng of total RNA was converted to cDNA using the SuperScriptTM III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). cDNA corresponding to 200 ng of reverse-transcribed RNA was amplified by PCR using Maxima SYBR Green qPCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA) in a StepOne PlusTM Real-Time PCR System (Thermo Fisher Scientific). Each reaction was performed in duplicate. DTX1 expression was normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as an endogenous control.

Expression of Treg-associated molecules compared between high and low Deltex1 mRNA levels

Treg-associated molecules encompass a range of surface markers, transcription factors, receptors, and cytokines that are crucial for the identification, function, and regulation of Tregs. These include FoxP3, CD25, cytotoxic T-lymphocyte associated protein 4 (CTLA4), glucocorticoid-induced TNFR-related protein (GITR), inducible T-cell co-stimulator (ICOS), among others (18). For the comparison of Treg-associated molecule expression between subjects with high and low Deltex1 mRNA levels, PBMCs were stained with fluorochrome-conjugated antibodies including APC-Cy7-conjugated anti-CD4 (Biolegend, San Diego, CA, USA), PerCP-Cy5.5-conjugated anti-CTLA-4 (Biolegend), PE-Cy7-conjugated anti-programmed cell death protein 1 (PD-1) (Biolegend), FITC-conjugated anti-T cell immunoglobulin and mucin domain-3 (Tim-3) (Biolegend), AF647-conjugated anti-lymphocyte-activation gene 3 (LAG-3) (Biolegend), and BV510-conjugated anti-T cell immunoglobulin and ITIM domain (TIGHT) antibodies (Biolegend) on ice for 30 min. After washing with PBS, the PBMCs were fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA) following the manufacturer's instructions, then stained with PE-conjugated antibodies against FoxP3 (BD Biosciences, San Jose, CA, USA). The expression of each marker in $CD4^+FoxP3^+$ Tregs was analysed using a FACS LSRII flow cytometer (BD Biosciences). The collected data were further processed and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Production of cytokines in $CD4^+FoxP3^+$ Tregs individuals with high and low Deltex1 mRNA levels

PBMCs were stimulated with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (500 ng/ml; Sigma) for 1 h, followed by the addition of monensin (2 nM) for another 5 h in a cell culture incubator at 37°C. Stimulated PBMCs were stained with APC-Cy7-conjugated anti-CD4 antibody and fixed with the Foxp3/Transcription Factor Staining Buffer Set. The expression of cytokines including IFN-γ (PerCP-Cy5.5-conjugated antibodies, Biolegend), IL-4 (PE-Cy7-conjugated antibodies, Biolegend), IL-17A (BV510-conjugated anti- antibodies, Biolegend), IL-10 (BV421-conjugated antibodies, Biolegend), and FoxP3 was performed as described above.

Treg function assay in vitro

To assess the influence of Deltex1 on Treg function, Tregs were purified from three healthy controls (HCs) using the $CD4_+CD25_+CD127_{dim/-}$ Regulatory T Cell Isolation Kit II, human (Miltenyi Biotec) and expanded in culture following previously established protocols (19). Briefly, the purified Tregs were cultured in X-VIVO 15™ serum-free medium (Lonza, Basel, Switzerland), supplemented with 10% heat-inactivated human pooled AB serum (MP Biomedicals, LLC, Solon, OH, USA) and recombinant human IL-2 (rhIL-2, 50 ng/ml) (Reprotech, Rockyhill, NJ, USA). The cells were initially activated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator and subsequently maintained with periodic IL-2 replenishment (every 2-3 days, medium

Table I. The immunologic profiles of Sjögren's disease patients and healthy controls.

	pSjD n=40	Healthy controls n=20	*p*-value
Age, years, mean (SD)	58.4 (10.7)	56.4 (8.4)	0.097
Age at diagnosis, years, mean (SD)	52.6 (12.2)	-	
Female, n (%)	40 (100.0)	20 (100.0)	-
ANA > 1:80, n (%)	32 (80)	-	
Anti-SSA/Ro positivity, n (%)	39 (97.5)	-	
Anti-SSB/La positivity, n (%)	14 (35)	-	
RF positivity, n (%)	15 (37.5)	-	
Low C3, n (%) [a]	6 (15)	-	
Low C4, n (%) [a]	1 (2.5)	-	
High immunoglobulin, n (%) [b]	13 (32.5)	-	
ESR, mm/hour, mean (SD)	29.1 (25.4)	-	
Mean focus score			
VAS for fatigue (range 0-100), mean (SD)	56.5 (26.5)	-	
VAS for pain (range 0-100), mean (SD)	34.3 (28.3)	-	
VAS for dryness (range 0-100), mean (SD)	63.8 (21.9)	-	
ESSDAI score, mean (SD)	5.8 (4.6)	-	
ESSPRI score, mean (SD)	5.1 (1.8)	-	

[a]level below or within the normal limit; [b]immunoglobulin G, A, or M above upper limit of the normal range (IgG normal range: 700-1600 mg/dl; C3/C4 normal range: 90-180 mg/dl; 10-40 mg/dl).
ANA: antinuclear antibody; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient-reported index; n: number; pSjD, primary Sjögren's disease; RF: rheumatoid factor; VAS: visual analogue scales.

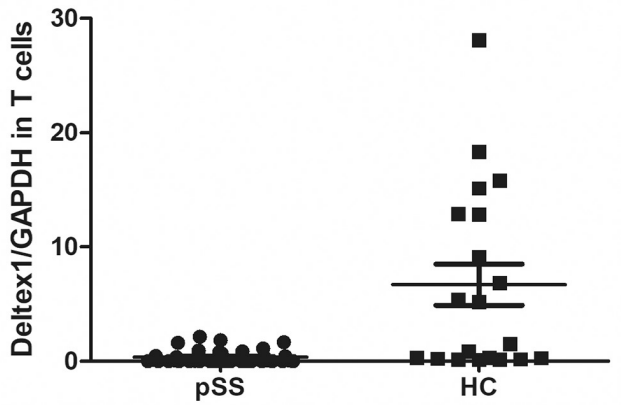

Fig. 1. Deltex1 expression in peripheral T cells is significantly lower in patients with SjD than HCs. Deltex1 expression in peripheral T cells from 40 patients with SjD and 20 healthy controls (HCs) was measured with quantitative reverse transcription polymerase chain reaction (RT-qPCR) and normalised to the GAPDH control. Statistical analysis was performed using the Mann-Whitney U test.

containing IL-2 was added). On day 12, the expanded Tregs were harvested. A Deltex1-targeting short interfering RNA (siDTX) or scramble siRNA (siCtrl) was transfected into the expanded Tregs, following established Nucleofector protocols (Lonza, Germany).

The function of both siDTX1 and siCtrl Tregs was evaluated using *in vitro* suppressive assays. $CD4^+CD25^-$ T cells, used as responder cells (Tresp), were labeled with CellTracker Violet (Thermo Scientific) and stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator in the presence of varying numbers of Tregs, creating different Treg/Tresp ratios (1:1, 1:2, 1:5, and 1:10). The cultures were maintained in *X-VIVO* 15 medium with 10% human pooled AB serum at 37°C. On day 4, the proliferation of Tresp was assessed by flow cytometry, and the percentage of suppression was calculated by comparing the proliferation of Tresp cultured alone to that of Tresp cultured in the presence of Tregs.

Statistical analysis

The Mann-Whitney U-test was used to analyse differences between groups. Correlations between variables were determined using Spearman's rank correlation test. The *p*-value was calculated 2-tailed and values were interpreted as significant when <0.05. All data were analysed using SSJD software (version 22.0; IBM SSJD Statistics for Windows, IBM, Armonk, New York, USA).

Results

Immunologic profiles of patients

Sixty participants, including 40 SjD patients and 20 HCs, were recruited. The mean age at diagnosis was 52.6 years in the SjD group (Table I). All patients were female. The average age at blood draw was 58.4 years for SjD patients and 56.4 years in HCs. The mean age at blood collection did not differ significantly between the two groups (p=0.097). The positivity rates for ANA, anti-SSA/Ro, anti-SSB/La antibodies, and rheumatoid factor (RF) in the SjD group were 80%, 97.5%, 35%, and 37.5%, respectively. Low C3 and C4 levels and high immunoglobulin levels were noted in 15%, 2.5%, and 32.5% of SjD patients, respectively. The mean VAS scores for pain, fatigue, and dryness were 34.3, 56.5, and 63.8, respectively. The mean ESSDAI and ESSPRI scores were 5.8 and 5.1, respectively. Detailed ESSDAI scores are summarised in Supplementary Table S2. The most common involvement domain was articular, with 30% of patients experiencing arthritis, followed by the biological domain at 26.7%. However, considering the weighted scores, the domain with the greatest impact on the ESSDAI was pulmonary (6.25), followed by lymphadenopathy and glandular involvement (4), and then articular (3.33).

Expression of Deltex1 in peripheral T cells in SjD patients and HCs

Because Deltex1 deficiency increases IFN-γ production in human T cells, we hypothesised that Deltex1 is involved in the pathogenesis of SjD. As shown in Figure 1, Deltex1 expression in T cells was significantly lower in the SjD group than in the HCs (Deltex1/GAPDH, 8.04 *vs*. 0.57, p<0.001). These results suggest that low levels of Deltex1 mRNA in T cells may play a role in SjD patients.

Correlation of Deltex1 mRNA levels in T cells with clinical manifestations of SjD

Next, we investigated the association of Deltex1 levels with the clinical manifestations of SjD. As shown in Table II, Deltex1 expression was significantly lower in SjD patients with concomi-

Table II. Deltex1 expression in T cells in relation to clinical and laboratory findings from patients with Sjögren's disease.

	Present		Absent		*p*-value
	n	mean (SD)	n	mean (SD)	
Clinical features					
Age > 60 years	21	0.433 (0.60)	19	0.237 (0.55)	0.46
Musculoskeletal involvement	20	0.40 (0.55)	20	0.34 (0.61)	0.756
Immune thrombocytopenia	6	0.102 (0.15)	34	0.416 (0.61)	0.014*
Vasculitis	5	0.066 (0.74)	35	0.412 (0.61)	0.020*
Autoimmune thyroiditis	4	0.042 (0.45)	36	0.405 (0.59)	0.001*
Lung involvement	4	0.232 (0.19)	36	0.38 (0.60)	0.622
Laboratory profiles					
ANA titre > 1:80	32	0.345 (0.58)	8	0.465 (0.60)	0.514
Anti-SSA/Ro positive	39	0.975 (0.16)	1	0.16	0.714
Anti-SSB/La positive	14	0.412 (0.69)	26	0.345 (0.52)	0.732
Scintigraphy score > 2 [a]	9	0.547 (0.83)	31	0.317 (0.48)	0.297
RF positive	15	0.330 (0.47)	25	0.392 (0.64)	0.741
Low C3 or C4	6	0.213 (0.44)	34	0.40 (0.60)	0.478
CRP > 0.5 mg/dl	4	0.151 (0.15)	36	0.393 (0.60)	0.43
ESR elevation [b]	18	0.217 (0.42)	22	0.493 (0.66)	0.132
Hyper-immunoglobulin G [c]	13	0.206 (0.46)	27	0.447 (0.62)	0.219

ANA: antinuclear antibody; anti-dsDNA: anti-double strand DNA; C3: complement 3; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IgG: immunoglobulin G; RF: rheumatoid factor; SD: standard deviation.

[a] Scintigraphy score: the severity was measured by 0-4 functional score system. 1 represent normal, 2 represent mild to moderated dysfunction, 3 represent moderate to severe dysfunction, 4 represent severe dysfunction.

[b] ESR>20 mm/hour in females under the age of 50 years, while>30 mm/hour in females 50 years of age or older.

[c] IgG>1600 mg/dl.

*p<0.05.

tant haematologic disorders (Deltex1/GAPDH 0.102 *vs*. 0.416, p=0.014); vasculitis (0.066 *vs*. 0.412, p=0.02); and autoimmune thyroiditis (0.042 *vs*. 0.405, p=0.001) than in those without. The prevalence of other clinical features, including older age (>60 years) and musculoskeletal and lung involvement were not significantly different between the groups. In clinical practice, diminished complement C3 or C4 levels and elevated immunoglobulin G, C-reactive protein, or erythrocyte sedimentation rate may implicate active disease in SjD (20, 21). However, these laboratory parameters were not associated with T cells Deltex1 mRNA expression levels.

Correlation of Deltex1 mRNA levels in T cells with disease activity of SjD

As shown in Figure 2, the expression levels of Deltex1 in T cells negatively correlated with visual analog scale (VAS) scores for fatigue, ESSDAI, and ESSPRI (r=-0.457, p=0.03; r=-0.356, p=0.024; and r=-0.379, p=0.016, respectively). However, Deltex1 expres-

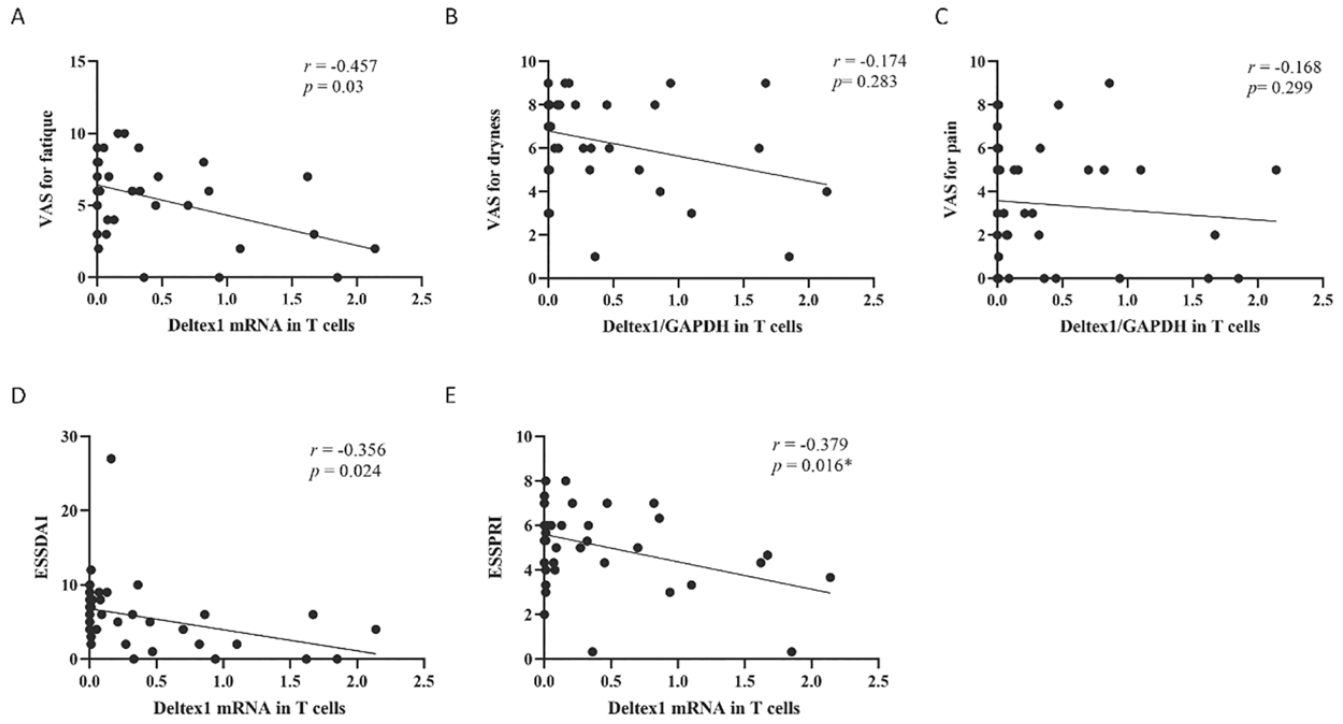

Fig. 2. Correlation between Deltex1 expression in peripheral T cells and clinical manifestations and disease activity in patients with SjD. The association between Deltex1 messenger RNA (mRNA) expression in T cells and fatigue (**A**), dryness (**B**), pain (**C**), European League against Rheumatism (EULAR) score, Sjögren's syndrome disease activity index (ESSDAI) (range 0-123) (**D**), and patient-reported index (ESSPRI) (range 0-10) (**E**) was analysed in SjD patients. Deltex1 expression was measured by quantitative RT-PCR and normalised to that of the GAPDH control. Fatigue, dryness, and pain were evaluated using a visual analogue scale (VAS) (range 0-10 cm). Correlations between variables were determined using Spearman's rank correlation test.

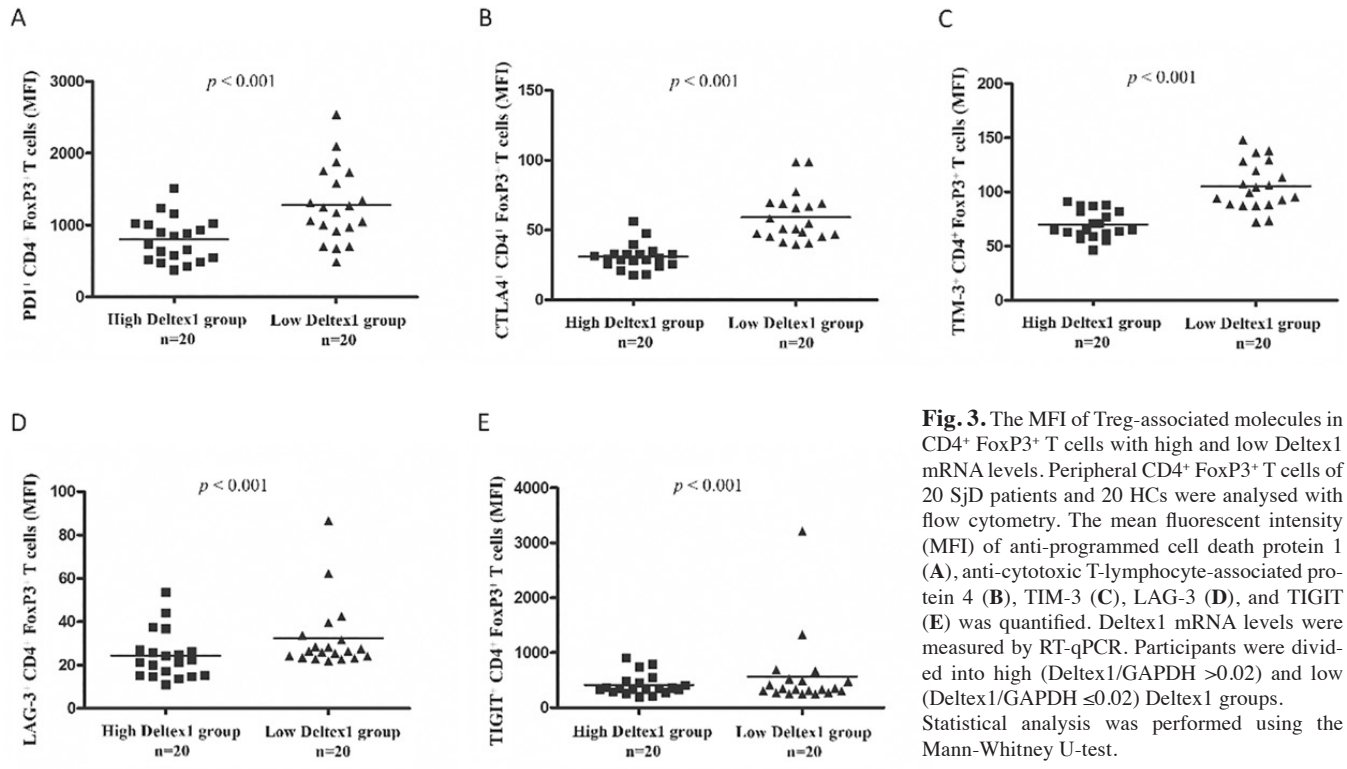

Fig. 3. The MFI of Treg-associated molecules in CD4+ FoxP3+ T cells with high and low Deltex1 mRNA levels. Peripheral CD4+ FoxP3+ T cells of 20 SjD patients and 20 HCs were analysed with flow cytometry. The mean fluorescent intensity (MFI) of anti-programmed cell death protein 1 (**A**), anti-cytotoxic T-lymphocyte-associated protein 4 (**B**), TIM-3 (**C**), LAG-3 (**D**), and TIGIT (**E**) was quantified. Deltex1 mRNA levels were measured by RT-qPCR. Participants were divided into high (Deltex1/GAPDH >0.02) and low (Deltex1/GAPDH ≤0.02) Deltex1 groups. Statistical analysis was performed using the Mann-Whitney U-test.

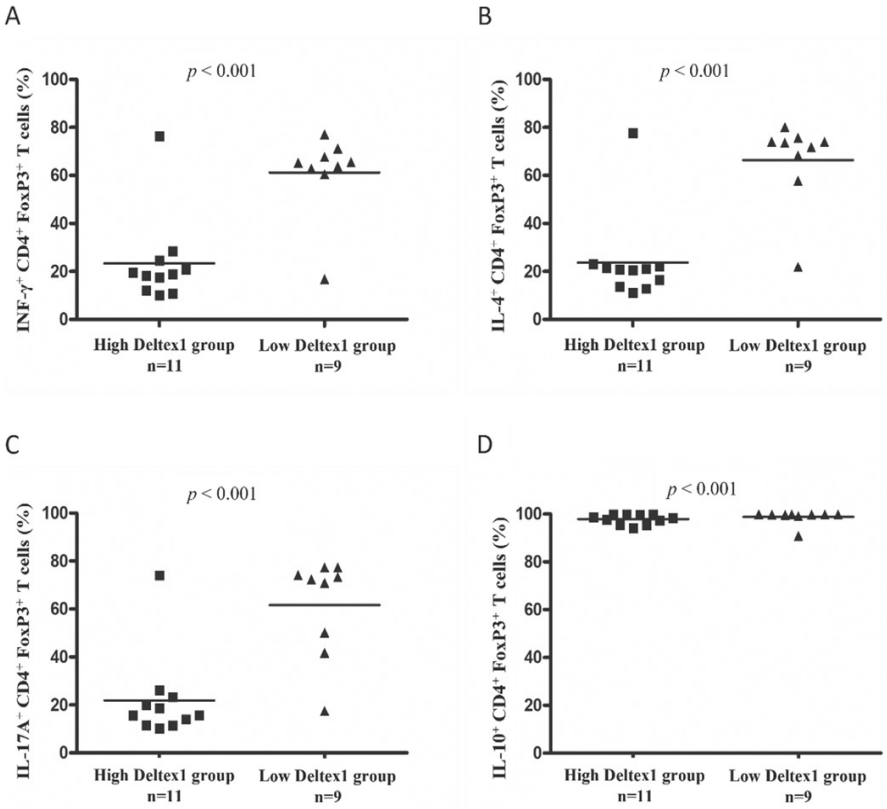

Fig. 4. Quantification of cytokine positive CD4+ FoxP3+ Tregs with high and low Deltex1 mRNA levels following stimulation with PMA and ionomycin. Peripheral blood mononuclear cells of 10 SjD patients and 10 HCs were stimulated *ex vivo* with PMA and ionomycin. The percentages of interferon-γ (IFN-γ)+ (**A**), interleukin (IL)-4+ (**B**), IL-17A+ (**C**), and IL-10+ (**D**) CD4+ FoxP3+ T cells were analysed with flow cytometry. Deltex1 mRNA levels were measured by RT-qPCR. Participants were divided into high (Deltex1/GAPDH >0.02) and low (Deltex1/GAPDH ≤ 0.02) Deltex1 groups. Statistical analyses were performed using the Mann-Whitney U-test.

sion levels did not correlate with VAS score for dryness (r=-0.174, p=0.283); pain (r=-0.168, p=0.299); autoantibody such as RF, ANA, or ant-SSA/SSB positivity; or scintigraphy score (all p>0.05).

Differences between Treg-associated molecules and cytokines in CD4+ FoxP3+ Tregs with high and low Deltex1 mRNA levels

Next, we investigated whether Treg-associated molecules and cytokines differed between $CD4_+$ $FoxP3_+$ Tregs with high and low Deltex1 mRNA levels. Peripheral blood samples from 20 SjD patients and 20 HCs were analysed by flow cytometry. Participants were divided into high (Deltex1/GAPDH >0.02) and low (Deltex1/GAPDH ≤0.02) Deltex1 groups. As shown in Figure 3, the mean fluorescent intensity (MFI) of PD-1, CTLA-4, TIM-3, and LAG-3 on $CD4_+$ $FoxP3_+$ Tregs were significantly higher in the low Deltex1 group than those in the high Deltex1 group (1276.5±523.8 *vs*. 800.3±307.5, p=0.001; 59.2±17.6 *vs*. 31.0±9.2, p<0.001; 105.3±21.7 *vs*. 69.9±12.7, p<0.001; 32.4±15.9 *vs*. 24.4±11.1, p=0.014, respectively). In contrast, the MFI of TIGIT was similar between the two groups (567.3±670.2 *vs*. 414.6±193.4, p=0.947).

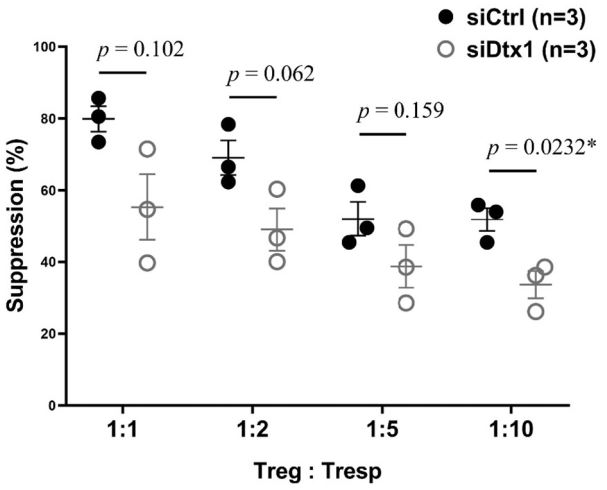

Fig. 5. *In vitro* suppressive activity of expanded human Deltex1-knockdown and wild-type $CD4_+$ $FoxP3_+$ Tregs.
$CD4_+$ $CD25_-$ T cells (Tresp) were stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator in the presence of graded numbers of Tregs transfected by Deltex1 short interfering RNA (siDTX) and control siRNA (siCon).
Proliferation of Tresp was monitored using flow cytometry on day 4 of culture.

The percentages of cytokine positive $CD4^+$ $FoxP3^+$ Tregs were analysed in 10 SjD and patients 10 HCs. Among $CD4_+$ $FoxP3_+$ Tregs stimulated with PMA/ionomycin, the frequency of IFN-γ^+, IL-4^+, IL-$17A^+$ cells, but not IL-10^+ cells, was significantly higher in the low Deltex1 group than the high Deltex1 group (61.2±17.4 *vs*. 23.4±18.4, p=0.007; 66.4±17.8 *vs*. 23.7±18.4, p=0.002; 61.7±20.8 *vs*. 21.9±18.0, p=0.002; 98.8±3.0 *vs*. 97.8±2.1, p=0.175, respectively) (Fig. 4).

The deficiency of deltex1 impaired the function of human Tregs

We also performed an *in vitro* suppression assay to clarify the functional role of Deltex 1.

As illustrated in Figure 5, siDTX1 Tregs inhibited the proliferation of stimulated $CD4_+$ $CD25_-$ T cells (Tresp) less effectively than siCtrl Tregs. At a 1:10 ratio of Tregs to Tresp, siCtrl Tregs were able to suppress 51.8 % of Tresp proliferation, while siDTX1 Tregs exhibited a reduced suppression rate of only 33.6% (p=0.023).

Discussion

To our knowledge, this is the first study investigating the role of Deltex1 in SjD. Deltex1 expression in peripheral T cells was significantly lower in SjD patients than in HCs and negatively correlated with the presence of immune thrombocytopenia (ITP), vasculitis, and autoimmune thyroiditis. Furthermore, Deltex1 expression was inversely associated with disease activity. Interestingly, the levels of Deltex1 were associated with the expression of Treg-associated molecules and production of inflammatory cytokines in $CD4_+$ $FoxP3_+$ Tregs. These results provide evidence that Deltex1 may be involved in the pathogenesis and disease activity of SjD, primarily by modulating inflammatory cytokine expression in Tregs and Treg stability. We also evaluated the impact of Deltex1 on Treg function and found that Deltex1 deficiency significantly attenuates the suppressive ability of human Tregs, making them less effective in regulating immune responses. These findings suggest that reduced expression of Deltex1 in Tregs may partially contribute to the pathogenesis of inflammation-driven autoimmune diseases, including SjD. However, it is important to note that while this study identifies significant associations, further mechanistic studies are required to validate and fully elucidate Deltex1's potential role in disease progression and immune regulation.

T cells play a pivotal role in SjD, with several cytokines, notably IL-17, TNF-α, and especially IFN-γ, which precipitate both local and systemic inflammation. Recent work has elucidated the significance of IFN-γ in the context of SjD (22). Immunohistochemical analyses revealed the presence of IFN-producing cells in SjD patients, but not in controls (23). Similarly, elevated expression of a type II IFN signature has been documented in the minor salivary gland tissues of SjD patients, distinguishing them from healthy individuals and sicca controls (24). Furthermore, the number of INF-γ-secreting PBMCs is significantly higher in SjD patients with Raynaud's phenomenon than in those without Raynaud's phenomenon (25). Elevated IFN-γ and reduced IFN-α levels have been identified in minor salivary gland tissues in SjD patients with lymphoma compared to those observed in patients without lymphoma (26). Given that Deltex1 expression is associated with T cell anergy and reduced IFN-γ production, which may imply a role in the pathogenesis of SjD (9), our findings suggest that Deltex1 could play a role in the pathogenesis of SjD. Furthermore, these results imply that aberrant activation of T cells, leading to heightened INF-γ production, contributes significantly to the progression of this disease. However, more studies should be conducted in the protein level and with functional experiments in order to establish Deltex1 as a therapeutic target. In addition, IFN γ enhances B cell-activating factor (BAFF) secretion via the JAK/STAT pathway in both a time- and dose-dependent manner (26). This promotes aberrant B cell maturation within germinal centre-like structures, culminating in the emergence of self-reactive B cells that produce autoantibodies (27-29).

Aberrations in T cell function extend beyond SjD and encompass a spectrum of pathologies including thrombocytopenia, vasculitis, and autoimmune thyroiditis (30-32). Pro-inflammatory cytokines synthesised by T cells, such as INF-γ, augment vascular inflammation and contribute to the development of vasculitis (33). Autoimmune thyroiditis is characterised by upregulation of INF-γ expression, indicating the involvement of T helper 1 (Th1) cells in its pathogenesis. In addition, Th1/Th2 imbalance with Th1 polarisation was implicated in ITP, as evidenced by the substantial elevation of both INF-γ and IL-18 in these patients (34). In this study, Deltex1 levels in T cells were significantly lower in patients with vasculitis, autoimmune thyroiditis, and ITP. This finding indicates the indispensable role of Deltex1 expression in T cell anergy in the pathogenesis of SjD and its complications.

Interestingly, although prior studies have reported higher autoantibody titres are associated with disease activity and immune dysregulation (35), our study did not find a significant association between autoantibody titres and Deltex1 expression levels. This discrepancy might be attributed to the distinct role Deltex1 plays in modulating Treg function and stability, rather than directly influencing B-cell-mediated autoantibody production. Previous research has shown that decreased Deltex1 expression correlates with a more pronounced inflammatory response driven by T cells, especially through cytokines like IFN-γ, rather than autoantibody-mediated mechanisms. Therefore, the lack of correlation between Deltex1 levels and autoantibody titres in our cohort suggests that Deltex1's regulatory effects may be more directly linked to T-cell-driven inflammation rather than B-cell hyperactivity. Further studies are warranted to explore this differentiation, particularly in examining the cross-talk between T-cell signalling and autoantibody production.

ESSDAI and ESSPRI are considered the gold standards for assessing disease activity and are crucial for drug registration trials (16, 36). However, lack of objectivity is the main limitation of both instruments; the placebo effect might affect both the ESSDAI and ESSPRI domains, which are based on patient-reported data and physicians' evaluation (37). This study highlights the inverse correlation between Deltex1 levels and disease activity. INF-γ mediated pathways serve a pivotal role not only in antiviral defence but also in precipitating fatigue during chronic active Epstein Barr virus infection (38). Moreover, INF-γ levels are closely associated with the severity of chronic fatigue disease (39-41). Furthermore, our study reveals a negative correlation between Deltex1 and fatigue. This finding may be attributable to the deficiency of Deltex1, which results in hyperactive T cells and the consequent secretion of INF-γ.

Tregs are essential for maintaining self-tolerance by suppressing effector T cell proliferation and preventing malfunction. The secretion of inhibitory cytokines such as IL-10, IL-35, and TGF-β1; granzymes; suppression of antigen-presenting cells; adenosine production; and telomerase inhibition represent possible mechanisms employed by Tregs to inhibit autoreactive T cells (42-45). Tregs often exhibit functional impairments, particularly in autoimmune diseases (46). Notably, they can acquire the ability to produce pro-inflammatory cytokines such as IFN-γ, IL-17, and IL-4. This is accompanied by the expression of associated master regulatory transcription factors including T-bet, GATA3 and RORγt. We found that the expression of several inhibitory receptors, including PD-1, CTLA-4, TIM-3, and LAG-3 on Tregs was notably elevated in the low Deltex1 group compared to that in the high Deltex1 group in SjD. Additionally, the percentage of Tregs expressing pro-inflammatory cytokines such as IFN-γ^+, IL-4^+, IL-$17A^+$ was significantly higher in the low Deltex1 group. These observations suggest a potential regulatory role for Deltex1 in modulating Treg function and plasticity. However, this hypothesis requires further validation through targeted mechanistic studies.

This study had several limitations. First, the patient cohort was relatively small, including only 20 patients for analysis. This limitation arose from methodological considerations, where 20 patients were selected to balance statistical power with feasibility, allowing for meaningful comparisons while managing resource constraints. Additionally, the selection criteria were focused on patients with well-characterised and complete clinical data.

Second, all our patients were female; it is unclear whether the results are representative of men. This limitation is associated with the female predilection of the disease and highlights the need for further research in male patients with SjD. Third, the diagnosis of our patients was primarily based on positive SSA results and Schirmer's test. Only one patient underwent a salivary gland biopsy; therefore, the focus score was not included in our analysis. Finally, although Deltex1 expression was notably reduced in SjD T cells and exhibited an inverse correlation with disease activity, the precise role of Deltex1 in Treg function remains unclear, necessitating further in-depth exploration.

In conclusion, Deltex1 expression in T cells is diminished in SjD patients. Furthermore, this reduced expression is inversely associated with disease activity, which is primarily governed through the modulation of inflammatory cytokine expression in Tregs and Treg stability. These findings suggest that Deltex1 may play a role in SjD pathogenesis and could be explored as a potential biomarker for measuring disease activity, but additional research is required to substantiate these initial observations.

Acknowledgments

The authors thank Shi-Tzu Tsai for performing a literature search in support of the background and discussion sections of this study.

References

1. SHAPIRA Y, AGMON-LEVIN N, SHOENFELD Y: Geoepidemiology of autoimmune rheumatic diseases. *Nat Rev Rheumatol* 2010; 6(8): 468-76. https://doi.org/10.1038/nrrheum.2010.86
2. FOX RI: Sjögren's syndrome. *Lancet* 2005; 366(9482): 321-31. https://doi.org/10.1016/S0140-6736(05)66990-5
3. CHATZIS LG, STERGIOU IE, GOULES AV *et al*.: Clinical picture, outcome and predictive factors of lymphoma in primary Sjögren's syndrome: results from a harmonized dataset (1981-2021). *Rheumatology* (Oxford) 2022; 61(9): 3576-85. https://doi.org/10.1093/rheumatology/keab939
4. NOCTURNE G, MARIETTE X: Advances in understanding the pathogenesis of primary Sjögren's syndrome. *Nat Rev Rheumatol* 2013; 9(9): 544-56. https://doi.org/10.1038/nrrheum.2013.110
5. VOULGARELIS M, TZIOUFAS AG: Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome. *Nat Rev Rheumatol* 2010; 6(9): 529-37. https://doi.org/10.1038/nrrheum.2010.118
6. BLINOVA VG, VASILYEV VI, RODIONOVA EB, ZHDANOV DD: The Role of Regulatory T Cells in the Onset and Progression of Primary Sjögren's syndrome. *Cells* 2023; 12(10): 1359. https://doi.org/10.3390/cells12101359
7. FUWA TJ, HORI K, SASAMURA T, HIGGS J, BARON M, MATSUNO K: The first deltex null mutant indicates tissue-specific deltex-dependent Notch signaling in Drosophila. *Mol Genet Genomics* 2006; 275(3): 251-63. https://doi.org/10.1007/s00438-005-0087-3
8. MATSUNO K, EASTMAN D, MITSIADES T *et al*.: Human deltex is a conserved regulator of Notch signalling. *Nat Genet* 1998; 19(1): 74-8. https://doi.org/10.1038/ng0598-74

9. HSIAO HW, LIU WH, WANG CJ *et al*.: Deltex1 is a target of the transcription factor NFAT that promotes T cell anergy. *Immunity* 2009; 31(1): 72-83. https://doi.org/10.1016/j.immuni.2009.04.017
10. LEU CM, HSU TS, KUO YP *et al*.: Deltex1 suppresses T cell function and is a biomarker for diagnosis and disease activity of systemic lupus erythematosus. *Rheumatology* (Oxford) 2019; 58(4): 719-28. https://doi.org/10.1093/rheumatology/key418
11. HSIAO HW, HSU TS, LIU WH *et al*.: Deltex1 antagonizes HIF-1α and sustains the stability of regulatory T cells in vivo. *Nat Commun* 2015; 6: 6353. https://doi.org/10.1038/ncomms7353
12. VITALI C, BOMBARDIERI S, JONSSON R *et al*.: Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; 61(6): 554-8. https://doi.org/10.1136/ard.61.6.554
13. 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. *Arthritis Rheumatol* 2017; 69(1): 35-45. https://doi.org/10.1002/art.39859
14. OXHOLM P: Primary Sjögren's syndrome--clinical and laboratory markers of disease activity. *Semin Arthritis Rheum* 1992; 22(2): 114-26. https://doi.org/10.1016/0049-0172(92)90005-x
15. SEROR R, RAVAUD P, BOWMAN SJ *et al*.: EULAR Sjögren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. *Ann Rheum Dis* 2010; 69(6): 1103-9. https://doi.org/10.1136/ard.2009.110619
16. SEROR R, RAVAUD P, MARIETTE X *et al*.: EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI): development of a consensus patient index for primary Sjogren's syndrome. *Ann Rheum Dis* 2011; 70(6): 968-72. https://doi.org/10.1136/ard.2010.143743
17. DE VITA S, LORENZON G, ROSSI G, SABELLA M, FOSSALUZZA V: Salivary gland echography in primary and secondary Sjögren's syndrome. *Clin Exp Rheumatol* 1992; 10(4): 351-56.
18. OHKURA N, SAKAGUCHI S: Transcriptional and epigenetic basis of Treg cell development and function: its genetic anomalies or variations in autoimmune diseases. *Cell Res* 2020; 30(6): 465-74. https://doi.org/10.1038/s41422-020-0324-7
19. RUITENBERG JJ, BOYCE C, HINGORANI R, PUTNAM A, GHANEKAR SA: Rapid assessment of in vitro expanded human regulatory T cell function. *J Immunol Methods* 2011; 372(1-2): 95-106. https://doi.org/10.1016/j.jim.2011.07.001
20. ZHOU M, YUAN F: Hypocomplementemia in Ppimary Sjögren's syndrome: a retrospective study of 120 treatment-naive Chinese patients. *Int J Gen Med* 2022; 15: 359-66. https://doi.org/10.2147/ijgm.s346188
21. BRITO-ZERÓN P, RAMOS-CASALS M, BOVE A, SENTIS J, FONT J: Predicting adverse outcomes in primary Sjögren's syndrome: identification of prognostic factors. *Rheumatology* (Oxford) 2007; 46(8): 1359-62. https://doi.org/10.1093/rheumatology/kem079
22. YIN H, VOSTERS JL, ROESCHER N *et al*.: Location of immunization and interferon-γ are central to induction of salivary gland dysfunction in Ro60 peptide immunized model of Sjögren's syndrome. *PLoS One* 2011; 6(3): e18003. https://doi:10.1371/journal.pone.0018003
23. GOTTENBERG JE, CAGNARD N, LUCCHESI C *et al*.: Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. *Proc Natl Acad Sci USA* 2006; 103(8): 2770-75. https://doi.org/10.1073/pnas.0510837103
24. 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
25. ITTAH M, MICELI-RICHARD C, ERIC GOTTENBERG J *et al*.: B cell-activating factor of the tumor necrosis factor family (BAFF) is expressed under stimulation by interferon in salivary gland epithelial cells in primary Sjögren's syndrome. *Arthritis Res Ther* 2006; 8(2): R51. https://doi.org/10.1186/ar1912
26. DARIDON C, DEVAUCHELLE V, HUTIN P *et al*.: Aberrant expression of BAFF by B lymphocytes infiltrating the salivary glands of patients with primary Sjögren's syndrome. *Arthritis Rheum* 2007; 56(4): 1134-44. https://doi.org/10.1002/art.22458
27. 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
28. WOO SJ, IM J, JEON JH *et al*.: Induction of BAFF expression by IFN-γ via JAK/STAT signaling pathways in human intestinal epithelial cells. *J Leukoc Biol* 2013; 93(3): 363-68. https://doi:10.1189/jlb.0412210
29. LAVIE F, MICELI-RICHARD C, ITTAH M, SELLAM J, GOTTENBERG JE, MARIETTE X: B-cell activating factor of the tumour necrosis factor family expression in blood monocytes and T cells from patients with primary Sjögren's syndrome. *Scand J Immunol* 2008; 67(2): 185-92. https://doi.org/10.1111/j.1365-3083.2007.02049.x
30. WILDE B, THEWISSEN M, DAMOISEAUX J, VAN PAASSEN P, WITZKE O, TERVAERT JW: T cells in ANCA-associated vasculitis: what can we learn from lesional versus circulating T cells? *Arthritis Res Ther* 2010; 12(1): 204. https://doi.org/10.1186/ar2923
31. RYDZEWSKA M, JAROMIN M, PASIEROWSKA IE, STOŻEK K, BOSSOWSKI A: Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. *Thyroid Res* 2018; 11: 2. https://doi:10.1186/s13044-018-0046-9
32. AN Q, ZHAO J, ZHU X *et al*.: Exploiting the role of T cells in the pathogenesis of Sjögren's syndrome for therapeutic treatment. *Front Immunol* 2022; 13: 995895. https://doi.org/10.3389/fimmu.2022.995895
33. LINTERMANS LL, STEGEMAN CA, HEERINGA P, ABDULAHAD WH: T cells in vascular inflammatory diseases. *Front Immunol* 2014; 5: 504. https://doi.org/10.3389/fimmu.2014.00504
34. JI X, ZHANG L, PENG J, HOU M: T cell immune abnormalities in immune thrombocytopenia. *J Hematol Oncol* 2014; 7: 72. https://doi.org/10.1186/s13045-014-0072-6
35. HSIAO H-W, LIU W-H, WANG C-J *et al*.: Deltex1 Is a target of the transcription factor NFAT that promotes T cell anergy. *Immunity* 2009; 31(1): 72-83. https://doi.org/10.1016/j.immuni.2009.04.017
36. SEROR R, BOWMAN SJ, BRITO-ZERON P *et al*.: EULAR Sjögren's syndrome disease activity index (ESSDAI): a user guide. *RMD Open* 2015; 1(1): e000022. https://doi.org/10.1136/rmdopen-2014-000022
37. DE WOLFF L, ARENDS S, VAN NIMWEGEN JF, BOOTSMA H: Ten years of the ESSDAI: is it fit for purpose? *Clin Exp Rheumatol* 2020; 38 (Suppl. 126): S283-90.
38. BELLMANN-WEILER R, SCHROECKSNADEL K, HOLZER C, LARCHER C, FUCHS D, WEISS G: IFN-gamma mediated pathways in patients with fatigue and chronic active Epstein Barr virus-infection. *J Affect Disord* 2008; 108(1-2): 171-76. https://doi.org/10.1016/j.jad.2007.09.005
39. YANG T, YANG Y, WANG D *et al*.: The clinical value of cytokines in chronic fatigue syndrome. *J Transl Med* 2019; 17(1): 213. https://doi.org/10.1186/s12967-019-1948-6
40. KERR JR, TYRRELL DA: Cytokines in parvovirus B19 infection as an aid to understanding chronic fatigue syndrome. *Curr Pain Headache Rep* 2003; 7(5): 333-41. https://doi:10.1007/s11916-003-0031-3
41. BRENU EW, VAN DRIEL ML, STAINES DR *et al*.: Immunological abnormalities as potential biomarkers in chronic fatigue syndrome/myalgic encephalomyelitis. *J Transl Med* 2011; 9: 81. https://doi.org/10.1186/1479-5876-9-81
42. WANG D, LEI L: Interleukin-35 regulates the balance of Th17 and Treg responses during the pathogenesis of connective tissue diseases. *Int J Rheum Dis* 2021; 24(1): 21-7. https://doi:10.1111/1756-185x.13962
43. WING K, ONISHI Y, PRIETO-MARTIN P *et al*.: CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008; 322(5899): 271-75. https://doi.org/10.1126/science.1160062
44. SCHMITT EG, WILLIAMS CB: Generation and function of induced regulatory T cells. *Front Immunol* 2013; 4: 152. https://doi:10.3389/fimmu.2013.00152
45. ZHDANOV DD, GLADILINA YA, GRISHIN DV *et al*.: Contact-independent suppressive activity of regulatory T cells is associated with telomerase inhibition, telomere shortening and target lymphocyte apoptosis. *Mol Immunol* 2018; 101: 229-44. https://doi:10.1016/j.molimm.2018.07.017
46. LUCCA LE, DOMINGUEZ-VILLAR M: Modulation of regulatory T cell function and stability by co-inhibitory receptors. *Nat Rev Immunol* 2020; 20(11): 680-93. https://doi:10.1038/s41577-020-0296-3