

IL-17-producing double-negative T cells are expanded in the peripheral blood, infiltrate the salivary gland and are partially resistant to corticosteroid therapy in patients with Sjögren's syndrome

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SUMMARY

A small CD3⁺ T-cell population, that lacks both CD4 and CD8 molecules, defined as double negative (DN), is expanded in the peripheral blood of patients with systemic lupus erythematosus, produces IL-17 and accumulates in the kidney during lupus nephritis. Since IL-17 production is enhanced in salivary gland infiltrates of patients with primary Sjögren's syndrome (pSS), we aimed to investigate whether DN T cells may be involved in the pathogenesis of salivary gland damage. Fifteen patients with SS and 15 normal controls (NC) were enrolled. Peripheral blood mononuclear cells were stimulated with anti-CD3 antibody and cultured in presence or absence of dexamethasone (Dex). Phenotypic characterization was performed by flow cytometry in freshly isolated cells and after culture. Minor salivary glands (MSG) from pSS were processed for immunofluorescence staining. Total circulating DN T cells were increased in pSS compared to NC (4.7±0.4% vs 2.6±0.4%). NC and pSS freshly isolated DN T cells produce consistent amounts of IL-17 (67.7±5.6 in NC vs 69.2±3.3 in pSS). Notably, DN T cells were found in the pSS-MSG infiltrate. Dex was able to down-regulate IL-17 *in vitro* production in NC (29±2.6% vs 15.2±1.9% vs 13±1.6%) and pSS (49±4.8% vs 16±3.8% vs 10.2±0.8%) conventional Th17 cells and in DN T cells of NC (80±2.8% vs 3.8±2.1% vs 4.2±1.8%), but not of pSS (81±1.5% vs 85.4±0.8% vs 86.2±1.7%). DN T cells are expanded in pSS PB, produce IL-17 and infiltrate pSS MSG. In pSS, conventional Th17 cells are inhibited by Dex, but DN T cells appear to be resistant to this effect. Taken together, these data suggest a key role of this T-cell subset in the perpetuation of chronic sialoadenitis and eventually in pSS prognosis.

Key words: T helper 17 cells, Sjögren's syndrome, dexamethasone.

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■ INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease mainly affecting exocrine glands that can also target other tissue and organs. Despite pSS pathogenesis is not fully elucidated, chronic inflammation of exocrine gland with massive lymphocyte infiltration culminating in tissue damage and secretory impairment is a hallmark of the disease (1). From a clinical point of view, pSS is mainly characterized by mucosal dryness, typically at eyes and mouth, but extra-

glandular involvement may also occur. The autoimmune etiology of pSS is now well established. However, evidence supporting therapeutic role of corticosteroids (CS) or immune-suppressive (IS) agents in modulating the natural history of chronic inflammation and glandular injury are currently lacking (2).

The long-standing Th1 paradigm, identifying CD4⁺ T cells as major players in the induction of epithelial damage, was recently challenged by the identification of a new Th-cell subset able to produce IL-17 (Th17 cells). In fact, it has been demonstrated that

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Th17 cells play a key role in chronic inflammation and autoimmunity (3-5) and in particular in mediating target organ damage during pSS (6-9).

Th17 cells arise from naïve T lymphocytes in presence of a peculiar cytokine milieu characterized by the presence of both IL-6 and transforming growth factor (TGF) β (10-12). Therefore, they are evolutionally and functionally divergent from Th1 and Th2 cell subsets (10-12).

Furthermore, it has been recently demonstrated that CD4 expression is not mandatory among Th17 cells, since a small T-cell subset lacking both CD4 and CD8 on cell surface has been found to produce IL-17 upon stimulation (13). Such cell population named CD4⁻CD8⁻ double negative (DN), represents less than 5% of total circulating T cells in healthy mice and humans and displays either an $\alpha\beta$ or a $\gamma\delta$ -TCR.

To date, conflicting data have been published concerning DN cell function. In particular, several lines of evidence suggest that they are crucially involved in the pathogenesis of autoimmune disorders, while others support regulatory functions including a suppressive activity exerted by DN cells (14-16).

It has been found that DN T cells are able to promote autoantibody production by B (17, 18) cells in systemic lupus erythematosus (SLE) and to accumulate in kidney during lupus nephritis leading to massive local release of IL-17 (19). The evidence that DN T cells display pro-inflammatory properties and are involved in glandular chronic inflammation occurring during pSS prompted us to investigate the role of this cell subset in IL-17 balance and in consequence in pSS pathogenesis (20).

■ MATERIALS AND METHODS

Patients and normal controls

Fifteen consecutive patients with pSS, classified according to the Euro-American criteria (21) and 10 sex- and age-matched normal controls (NC) were enrolled. All patients were female subjects (age: 54.2 \pm 13 years, disease duration: 7.9 \pm 5 years). At

the time of enrollment all clinical and serological records were collected. None of the patients was taking immunosuppressive drugs or CS but all of them required topic medication for sicca symptoms. The study was approved by the local Ethics Committee (CEAS) and written informed consent was obtained in accordance with the declaration of Helsinki.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient separation. For surface staining Phycoerythrin (PE), Fluorescein isothiocyanate (FITC), Allophycocyanin (APC) or Pe-Cy5 labeled anti-human CD3, CD4, CD8 and respective isotypes were used (BD; eBioscience; BioLegend). When required, cells were permeabilised with 0.1% saponin blocking buffer after 4% paraformaldehyde fixation. Six-hour *in vitro* stimulation with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 mg/mL ionomycin and 0.1 mg/mL brefeldin in complete medium was performed prior to performing intracellular staining. For intracellular staining, APC labelled anti-human IL-17 and the respective isotype were used (BD and R&D). Up to three fluorochromes were used in the same vial and samples were analysed using FACScalibur flow cytometer (BD) and CellQuestPro software (BD).

Cell cultures

Untouched PBMCs from three SS patients and three HD were cultured for 5 days in cross-linked anti-CD3 coated plates in the presence or absence of dexamethasone phosphate (Dex) (Soldesam) (22-24). As negative control, cells were cultured with complete medium alone. Cell proliferation was assessed by ³H thymidine assay. Cell viability was determined by Trypan Blue staining.

Immunofluorescence of minor salivary gland specimens

According to the previously mentioned Euro-american criteria, subjects with sicca syndrome symptoms underwent labial minor salivary gland (MSG) biopsy and 5/6

lobules were analysed. The presence of at least one inflammatory focus within 4 mm² allowed the diagnosis of SS (25, 26). Serial sections of five SS-MSG were cut at 3 mm thickness, deparaffinised and rehydrated. Envision flex target retrieval solution high pH (DAKO) was used for antigen retrieval and unspecific binding was avoided by 30 min incubation with 10% FCS. For double immunofluorescence staining, anti-CD8 (DAKO), anti-CD3 (NeoMarkers) and anti-CD4 (BD) were used diluted in appropriate buffer. Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-mouse were used as secondary antibodies. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI). Slides were mounted with Mowiol 4-88 and analysed

with Olympus AX70 microscope. Normal tonsil specimens were used as controls.

Statistical analysis

Graph-Pad 5.0 software was used and Mann-Whitney U test was calculated. All values are indicated as mean±standard error of the mean (SEM). p Values ≤0.05 were considered significant.

RESULTS

Circulating DN T cells are expanded in the peripheral blood, infiltrate salivary glands and display a Th17 phenotype in pSS patients

As depicted in Figure 1, the percentage of circulating DN T cells is increased in pSS patients with respect to NC ($4.7\pm 0.4\%$ vs $2.6\pm 0.4\%$).

Therefore, we investigated the presence of DN T cells within the mononuclear cell infiltrate that characterizes pSS autoimmune sialoadenitis. It appeared that DN T cells are present among other CD4⁺ and CD8⁺ T lymphocytes in periductal infiltrate and in ductal epithelium (intra-epithelial lymphocytes), thereby confirming an active role played by this T-cell subset in the development of glandular chronic inflammation of pSS.

The phenotypic analysis of freshly isolated T lymphocytes revealed that the proportion of IL-17 producing CD4⁺ cells is rather low ($4.6\pm 0.4\%$ in NC and $5\pm 0.4\%$ in pSS). Conversely, a consistent fraction of DN T cells spontaneously produces IL-17 both in NC and in pSS patients (67.7 ± 5.6 in NC vs 69.2 ± 3.3 in pSS; Fig. 2A)

In summary, our data demonstrate that since CD4⁺ cells represent up to 55% while DN T cells are about 5% of total circulating mononuclear cells, the absolute number of IL-17 producing CD4⁺ and DN cells was overlapping. Finally, we observed that almost all NC and pSS DN T cells (98-100%) express the transcription factor *retinoic acid orphan nuclear receptor* (ROR) γ t which is the hallmark of Th17 cells (data not shown), thereby confirming a Th17 phenotype of DN T cells.

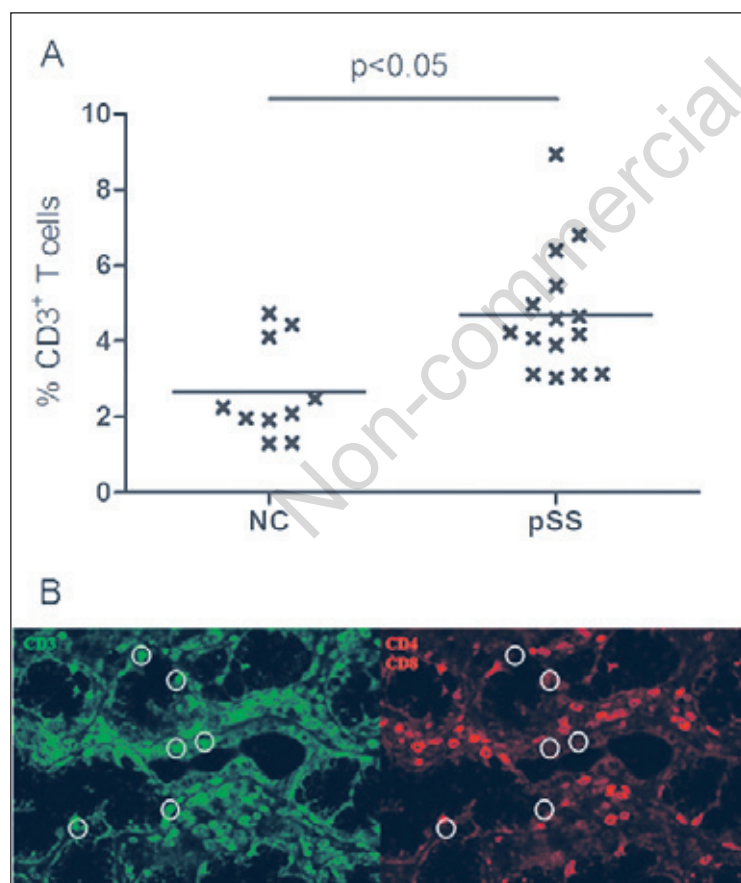


Figure 1 - A) Circulating DN T cell percentage in pSS patients (n°15) compared to normal controls (NC. n°10). Lines indicate mean values. B) Identification of DN T cells in salivary gland infiltrate during pSS. Green indicates anti-CD3+, red indicates anti-CD4+ and anti-CD8+ at 40x magnification.

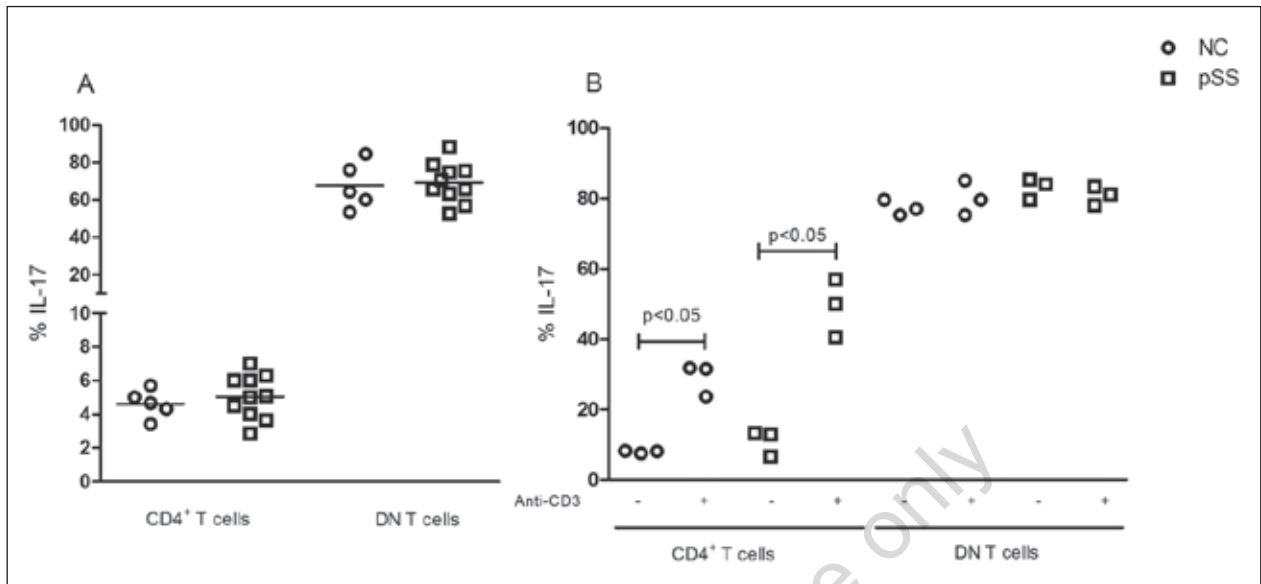


Figure 2 - A) IL-17 expression in CD4+ Th17 cells and in DN T cells isolated from the peripheral blood of 10 pSS patients and 5 NC. B) IL-17 expression in CD4+ Th17 cells and in DN T cells isolated from the peripheral blood of 3 pSS patients and 3 NC and cultured for 5 days in presence or absence of anti-CD3.

Activation effect on IL-17 production by DN T cells

Interestingly, as depicted in Figure 2B, cell stimulation with an anti-CD3 antibody led

to a consistent increase of IL-17 producing CD4+ cells ($8 \pm 0.2\%$ vs $29 \pm 2.6\%$ in NC and $11 \pm 2\%$ vs $49 \pm 4.8\%$ in pSS) but this was not observed in DN T cells ($77.3 \pm 1.2\%$ vs

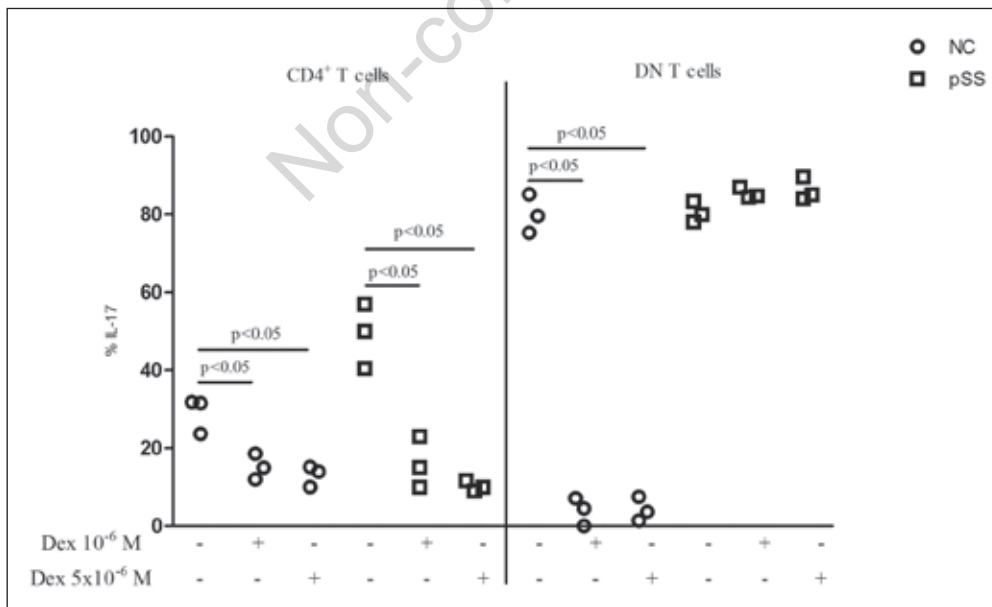


Figure 3 - IL-17 expression in CD4+ Th17 cells and in DN T cells isolated from the peripheral blood of 3 pSS patients and 3 NC and cultured for 5 days in presence or absence of Dex at different concentrations.

80±2.8% in NC and 83±1.7% vs 81±1.5% in pSS) that were characterized by a maximal *in vivo* production of IL-17. Such findings appear to support the hypothesis that CD4⁺ cells include a certain amount of *potential* Th17 cells which require additional stimuli to exert their pro-inflammatory activity. On the other hand, DN T cells are already activated *in vitro* and able to spontaneously produce IL-17.

DN T cells, but not CD4⁺Th17 cells, of pSS are in vitro resistant to dexamethasone (Dex)

Following the results obtained in proliferation assays, we aimed to investigate whether the addition to the culture of an anti-inflammatory compound such as Dex may inhibit IL-17 production by T cells obtained from pSS patients and, therefore, hamper their pathogenic role.

As depicted in Figure 3, we found that Dex was able to reduce IL-17 production by CD4⁺ cells both in pSS patients (49±4.8% vs 16±3.8% vs 10.2±0.8%) and in NC (29±2.6% vs 15.2±1.9% vs 13±1.6%; Figure 3). Similarly, Dex was able to decrease IL-17 production by DN T cells of NC (80±2.8% vs 3.8±2.1% vs 4.2±1.8%), but it was absolutely ineffective towards pSS DN T cells (81±1.5% vs 85.4±0.8% vs 86.2±1.7%; Figure 3).

■ DISCUSSION

IL-17 represents a pro-inflammatory cytokine deeply involved in the pathogenesis of pSS (1,5,6). In fact, high levels of such cytokine in glandular tissue and in consequence its role in epithelial damage has been highlighted in experimental models and in pSS patients (7, 8). The present study demonstrated that T cells lacking of both CD4 and CD8 on their surface are expanded in the peripheral blood and infiltrate salivary glands in patients with pSS. Our results support the idea that DN T cells represent a T-cell subset actively involved in pSS immunopathogenesis and are in line with a number of recent studies suggesting that DN T cells play a key

role in the pathogenesis of systemic autoimmune disorders (27). To note, the ability of quiescent DN T cells to spontaneously produce IL-17 was comparable to that of *in vitro* stimulated CD4⁺ cells. These observations are in agreement with recent data from other groups and suggest a direct link between DN T cells and the development of autoimmune disorders. In this setting an increase of circulating DN T cells has been reported in myasthenia gravis and systemic lupus erythematosus, where this cell subset appear to be involved in immune-regulation and autoantibody production (28, 29). Furthermore, according to their capacity to secrete IL-17, DN T cells might play a key role in several infectious diseases (30-32). Conventional therapeutic approaches for pSS aim to ameliorate sicca symptoms, glandular function and, therefore, patient quality of life. Among commonly employed compounds, CS may represent a tool to interfere with inflammatory infiltrate of exocrine glands occurring during the disease. However, except for a case report describing a reduction of the focus score of glandular infiltrate in a pSS patient following high doses of CS, conclusive data concerning the role of CS in reducing glandular inflammatory infiltrate are currently lacking (2, 33, 34). Therefore, on the basis of our results revealing a strong relationship between IL-17 balance and DN T cells as well as their presence in glandular infiltrate, we aimed to investigate the potential role of CS on IL-17 producing cells. In this setting, we demonstrated that Dex was able to dramatically reduce IL-17 production by activated NC and pSS CD4⁺ cells and by NC DN T cells. Intriguingly, Dex did not affect at all IL-17 production by pSS DN T cells. These observation are of particular interest since CS resistance represents a major therapeutic issue in several chronic inflammatory diseases including chronic obstructive pulmonary disease and acute distress respiratory syndrome (35). A poor clinical response to CS was also observed in patients with rheumatic autoimmune/inflammatory disorders such as rheumatoid arthritis and SLE (36, 37). Furthermore, *in vitro* and *in vivo* Dex resis-

tance of IL-17 producing cells was already described in patients with asthma (38, 39). In conclusion, our results support the concept that DN T cells are actively involved in the pathogenesis of pSS and are resistant to CS effects. It is conceivable that the identification of a CS-resistant pathogenic cell subset in an autoimmune disorder such as pSS may represent an issue of great interest to develop new lines of investigation. Indeed, a better understanding of DN T cell may shed some light on the underlying pathogenic pathways. Such information may be employed to develop specific drugs aimed to hamper DN T cell pro-inflammatory activity and therefore interfere with the natural history of pSS chronic autoimmune sialoadenitis.

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