Angiogenic T cells in primary Sjögren's syndrome: a double-edged sword?

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ABSTRACT

Objective. *The mechanisms underlying increased cardiovascular risk in primary Sjögren's syndrome (pSS) remain unclear. Since the recently discovered angiogenic T cells (Tang) may participate in endothelial repair by cooperating with endothelial progenitor cells (EPC), we aimed to quantify and characterise Tang in the peripheral blood and minor salivary glands (MSG) of pSS patients.*

Methods. *Tang ($CD3^+CD31^+CXCR4^+$) and EPC ($CD34^+CD133^+VEGFR\text{-}2^+$) were quantified by flow cytometry in peripheral blood samples from 36 pSS patients and 20 healthy donors. Tang subsets were assessed on the basis of CD4, CD8 and CD28 expression. Labial MSG sections from 10 pSS patients and 12 non-pSS sicca syndrome controls were subjected to immunofluorescence staining to investigate the presence of Tang and the expression of the CXCR4-ligand stromal cell-derived factor-1 (SDF-1)/CXCL12.*

Results. *Circulating Tang cells were expanded and directly correlated to EPC in pSS. Both Tang and EPC directly correlated with disease activity as calculated with the EULAR Sjögren's syndrome disease activity index (ESSDAI). In pSS, the majority of Tang cells were $CD4^-CD8^-$ double negative (DN) and lacked CD28 revealing a senescent phenotype. A subset of $CD4^+$, $CD8^+$ and DN Tang cells produced interleukin-17. Immunohistology revealed the exclusive presence of periductal and perivascular infiltrating Tang cells along with increased SDF-1/CXCL12 expression in pSS MSG compared to non-pSS sicca syndrome controls.*

Conclusion. *In pSS, Tang cells are expanded in peripheral blood and infiltrate MSG. Tang may be novel actors in pSS-related endothelial dysfunction and glandular neo-angiogenesis and inflammation.*

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease mainly targeting exocrine glands and leading to progressive secretory impairment (1-3). A consistent number of patients also experiences extraglandular manifestations, with lymphoma being the one that mostly worsens the disease prognosis (4). Similar to other systemic autoimmune diseases, pSS is burdened by increased cardiovascular (CV) risk and growing evidence supports a remarkable endothelial dysfunction as well as increased prevalence of CV events compared to the general population in this disease (5-7).

In physiological conditions, endothelial cells may be damaged by several stimuli including shear stress and transmural pressure, but they are promptly replaced thanks to the release from the bone marrow of endothelial progenitor cells (EPC) which migrate to the site of injury and undergo a full maturation process. Endothelial dysfunction is a pathological condition characterised by abnormalities of structure and function of endothelial cells allowing the persistency of arterial wall damage and therefore providing favourable conditions for the formation of atherosclerotic plaques (8). The assessment of circulating EPC along with circulating endothelial microparticles (EMP), which act as surrogate biomarkers of endothelial dysfunction, allowed to verify that this process is occurring in pSS (5). In particular, an increase in EPC in parallel to an increase in EMP may suggest a compensatory mechanism to overcome endothelial cell damage (5).

In recent years, another leading actor in the scenario of endothelial repair has been identified, the so-called angiogenic T cells (Tang) characterised by the co-expression of CD3, CD31 and CXCR4 (9). Tang cells are required for

EPC colony formation and differentiation and secrete consistent amount of proangiogenic factors including vascular endothelial growth factor (VEGF). Therefore, Tang cells also promote endothelial cell proliferation and function and display an angiogenic potential (9). Circulating Tang cells are reduced in rheumatoid arthritis (RA) and comparable to normal controls in systemic lupus erythematosus (SLE) (10, 11). Of interest, a population of immunosenescent $CD28^-$ Tang cells has been observed in SLE but not in RA (12). An expansion of immunosenescent $CD4^+CD28^-$ cells has been described in RA and it has been associated with the presence of endothelial dysfunction and carotid artery wall thickening (13). The lack of CD28 in a proportion of Tang cells may raise the hypothesis that they might be a reliable indicator of endothelial dysfunction.

Since to date no evidence about Tang cells is available in pSS, we aimed to quantify and characterise Tang cells in the peripheral blood and target organs of patients with this disease.

Materials and methods

Patients and healthy donors

Thirty-six female patients with pSS (mean±standard error of the mean (SEM) age, 57±2 years) classified according to the American-European criteria (14) and 20 age-matched healthy female donors (mean±SEM age, 55.3±2.8 years) were enrolled for analyses on peripheral blood samples. Clinical and serological records were collected at the time of enrolment. Disease activity was measured using the EULAR Sjögren's syndrome disease activity index (ESSDAI) (15). All patients were receiving topical medications for sicca symptoms and 19 patients (53%) were on hydroxychloroquine 200 mg/day. None of the patients was taking corticosteroids or immunosuppressive therapies. None of the patients had history of CV events. With regard to CV risk factors, 3 patients were current smokers, 5 patients were former smokers, 10 patients had systemic arterial hypertension and 2 patients had hypercolesterolaemia. No patient was diagnosed with diabetes mellitus. The study was approved by the local ethics committee, and written informed consent was obtained from each participant in accordance with the declaration of Helsinki.

Flow cytometry evaluation

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient from heparinised venous blood samples. In selected experiments, $CD3^+$ cells were magnetically sorted (Human T Lymphocyte Enrichment Set-DM, BD Biosciences, San Jose, CA, USA). Either total PBMC or $CD3^+$ sorted cells were processed for flow cytometry analysis. Surface staining was performed using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy7- or Alexa Fluor-647-labelled anti-human CD3, CD31, CXCR4, CD4, CD8, CD28, CD34, CD133 and VEGFR-2 (all from BD Biosciences, or Miltenyi Biotec, Bergisch Gladbach, Germany). Tang cells were those positive for CD3, CD31 and CXCR4, while EPC were those positive for CD34, CD133 and VEGFR-2. When required, cells were stimulated for 4 hours at 37°C and 5% CO_2 prior to surface staining with 25 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA), 1 μg/ml ionomycin and brefeldin A (BD GolgiPlug™, BD Biosciences). Subsequently, surface staining was performed, cells were fixed with 4% paraformaldehyde and permeabilised with 0.1% saponin blocking buffer. Alexa Fluor-488-labelled anti-human interleukin (IL)-17 and respective isotype were used for intracellular staining. Up to 4 fluorochromes were used in the same vial and samples were acquired with a FACScalibur flow cytometer equipped with the CellQuestPro™ software (BD Biosciences).

Salivary gland specimens and immunofluorescence analysis

Labial minor salivary gland (MSG) biopsies from 10 patients with pSS were collected and compared to samples from 12 age- and sex-matched non-pSS patients with sicca syndrome symptoms and either non-specific chronic sialadenitis (NSCS) or normal parenchyma (n=6 each) as described elsewhere (16). Formalin-fixed and paraffin-embedded MSG sections were subjected to histopathological analyses to assess focus score, Tarpley biopsy score (0–4 scale), T and B cell infiltration and lymphoid organisation according to previously published protocols (17). For immunofluorescence staining, MSG sections (3 μm thick) were deparaffinised, boiled for 10 minutes in 10 mM sodium citrate buffer (pH 6.0) for antigen unmasking, incubated in 2 mg/ml glycine for 10 minutes to quench autofluorescence and then blocked for 1 hour at room temperature with 1% bovine serum albumin in phosphate-buffered saline. The slides were subsequently incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-CD3 (prediluted; catalogue number ab7507, Abcam, Cambridge, UK), rabbit polyclonal anti-CD31 (1:50; catalogue number ab28364, Abcam), rabbit monoclonal anti-CXCR4 (1:100; catalogue number ab124824, Abcam) or mouse monoclonal anti-stromal cell-derived factor-1 (SDF-1)/CXCL12 (1:50; catalogue number MAB350, R&D Systems, Minneapolis, MN, USA) antibodies. The day after, MSG sections were washed and incubated for 45 minutes at room temperature in the dark with Alexa Fluor-488-conjugated goat anti-mouse, Rhodamine Red-X-conjugated goat anti-rabbit or Alexa Fluor-488-conjugated goat anti-rabbit IgG (all 1:200; Invitrogen, San Diego, CA, USA). Double immunofluorescence staining was performed by mixing mouse and rabbit primary antibodies and subsequently mixing fluorochrome-conjugated IgG. Irrelevant isotype-matched and concentration-matched mouse and rabbit IgG (Sigma-Aldrich) were used as negative controls. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Chemicon International, Temecula, CA, USA) and the immunostained sections were photographed under a Leica DM4000 B microscope equipped with a DFC310 FX 1.4-megapixel digital colour camera and LAS V3.8 software (Leica Microsystems, Mannheim, Germany).

Statistical analysis

Data were analysed with SPSS v. 23.0 software (Statistical Package for the

Table I. Demographic and clinical characteristics of pSS patients enrolled for collection of peripheral blood.

Number of patients	36
Age, years*	57 ± 2
Disease duration, years*	11 ± 1
Xerostomia	31 (86)
Xerophthalmia	32 (89)
Salivary gland enlargement	15 (42)
Extraglandular manifestations#	25 (69)
Lymphoma	0
Hypocomplementaemia	24 (67)
Leukopenia	4 (11)
Hypergammaglobulinaemia	22 (61)
Antinuclear antibodies	36 (100)
Autoantibodies	
Neither anti-Ro/SSA nor anti-La/SSB	10 (28)
Anti-Ro/SSA only	12 (33)
Anti-Ro/SSA and anti-La/SSB	14 (39)
Rheumatoid factor	20 (56)
Hydroxychloroquine 200 mg/day	19 (53)
ESSDAI, median (range)	2 (0-12)

*These values are reported as mean ± standard error of the mean. Unless otherwise stated, all other values are reported as number (percentage) of patients. #Twenty patients with articular involvement and five patients with Raynaud's phenomenon. All other extraglandular manifestations were ruled out.
pSS: primary Sjögren's syndrome; ESSDAI: EULAR Sjögren's syndrome disease activity index.

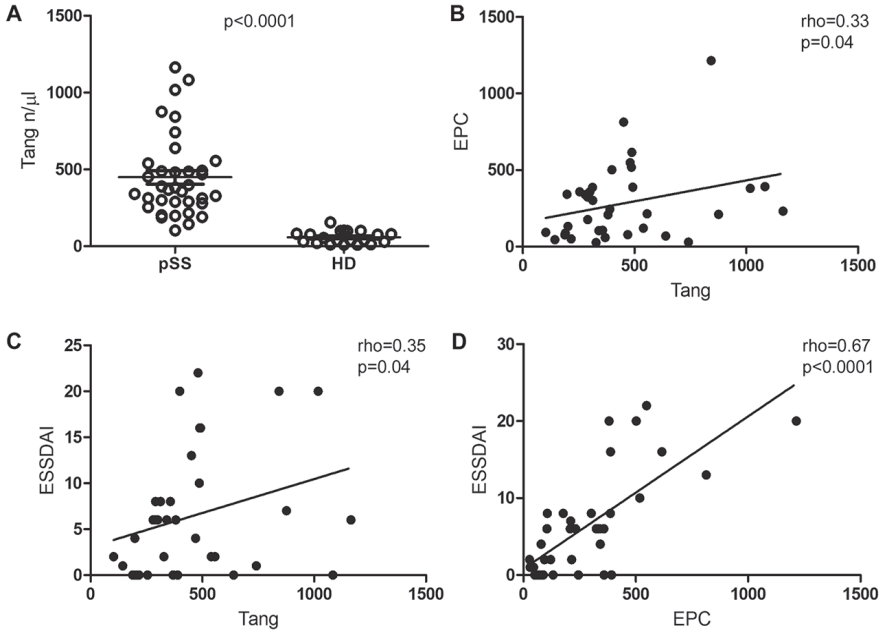

Fig. 1. Circulating angiogenic T cells (Tang), endothelial progenitors cells (EPC) and their relationship with disease activity in primary Sjögren's syndrome (pSS). (**A**) $CD3^+CD31^+CXCR4^+$ Tang cells are expanded in the peripheral blood of patients with pSS (n=36) compared with healthy donors (HD) (n=20). Data are mean ± SEM (p<0.0001 by Mann-Whitney U test). (**B**) In pSS patients, Tang cells are directly correlated with $CD34^+CD133^+VEGFR\text{-}2^+$ EPC (Spearman's rho=0.33, p=0.04). (**C** and **D**) Both Tang cells (**C**) and EPC (**D**) are directly correlated with disease activity as calculated with the EULAR Sjögren's syndrome disease activity index (ESSDAI) (Spearman's rho=0.35, p=0.04 for Tang; Spearman's rho=0.67, p<0.0001 for EPC).

Social Sciences, Chicago, IL, USA). Mann-Whitney U-test, Spearman's correlation coefficient, Kruskal-Wallis test and Dunn's post-hoc test were applied as needed. All tests were two-tailed and values of p<0.05 were considered statistically significant.

Results

Circulating Tang cells are expanded, correlate with EPC and disease activity and are mainly immunosenescent double negative cells in patients with pSS

Demographic and clinical characteristics of pSS patients enrolled in the study are summarised in Table I. Heparinised venous blood samples from 36 pSS patients and 20 age- and sex-matched healthy donors (HD) were subjected to PBMC isolation followed by flow cytometry evaluation of the $CD3^+CD31^+CXCR4^+$ Tang cell population. Since previous studies have shown that Tang cells may be linked to the EPC population (9, 10, 18), the possible relationship between circulating Tang cells and $CD34^+CD133^+VEGFR\text{-}2^+$ EPC was also explored.

As depicted in Figure 1A, the number of circulating Tang cells was significantly higher in pSS patients compared to HD (p<0.0001) and, of interest, it was directly correlated with the number of EPC in the same samples (Spearman's rho=0.33, p=0.04) (Fig. 1B). Moreover, both cell types positively correlated with disease activity as calculated with the ESSDAI (Spearman's rho=0.35, p=0.04 for Tang; Spearman's rho=0.67, p<0.0001 for EPC) (Figs. 1C and D). Neither circulating Tang cells nor circulating EPC correlated with patient age, disease duration and autoantibody titres. In addition, no differences in either Tang cells or EPC were observed when dividing patients according to the serological status (*i.e.* seropositive *vs.* seronegative patients). As far as CV risk factors are concerned, we found no difference in both cell types according to the presence of systemic arterial hypertension, while the possible association with other factors could not be assessed due to the small numbers of patients.

We subsequently explored the phenotype of Tang cells in pSS by evaluating the expression of CD4, CD8 and CD28 based on previous literature (11, 12, 18). All previous studies agree that in normal subjects Tang cells are mainly $CD4^+$ lymphocytes that co-express CD28 (11, 12, 18). We observed that in pSS the majority of Tang cells lacked CD4 and CD8, therefore they were double negative (DN) (Fig. 2A). Furthermore, 50-80% of Tang cells lacked CD28 being consistent with a senescent phenotype (Fig. 2B). Since it has been demonstrated that Tang cells are able to produce IL-17 (9) and we reported that in pSS DN cells are major producers of this cytokine (19), we assessed if and which Tang cell subsets produce IL-17. We studied 3 pSS patients and found that a subset of $CD4^+$, $CD8^+$ and DN Tang cells produce IL-17 (Fig. 3). The proportions of IL-17-producing cells among $CD4^+$, $CD8^+$ and DN Tang did not statistically differ.

Tang cells infiltrate MSG in patients with pSS

Immunohistological analyses were carried out on labial MSG sections from 10 patients with pSS (*i.e.* displaying focal lymphocytic sialadenitis) and 12

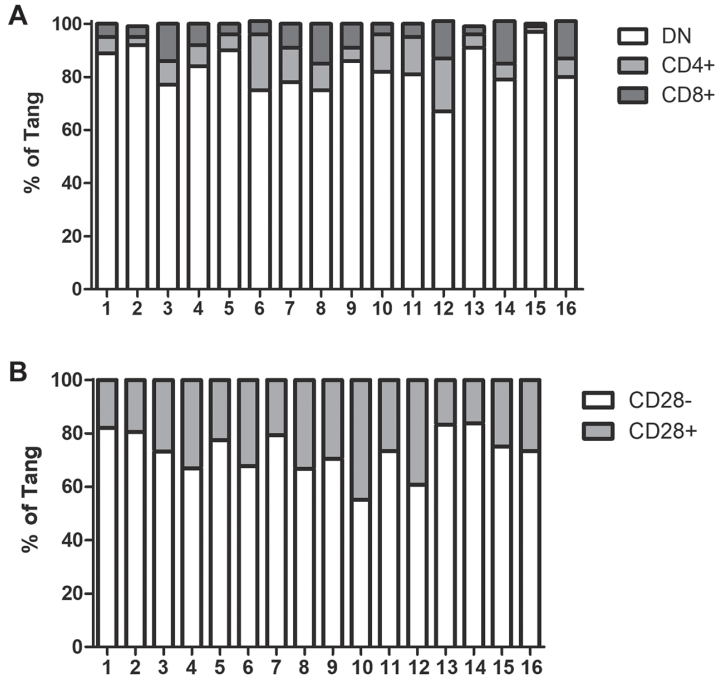

Fig. 2. Phenotype of angiogenic T cells (Tang) in 16 representative primary Sjögren's syndrome (pSS) patients. (**A**) The majority of Tang cells in pSS are $CD4^-CD8^-$ double negative (DN) cells, while only a small fraction expresses either CD4 or CD8 (% $CD4^+$ Tang *vs*. % $CD8^+$ Tang *vs* % DN Tang, $p<0.0001$ by Kruskal-Wallis test; % DN Tang *vs*. % $CD4^+$ Tang, $p<0.0001$; % DN Tang *vs*. % $CD8^+$ Tang, $p<0.0001$; % $CD4^+$ Tang *vs*. % $CD8^+$ Tang, not significant; all comparisons by Kruskal-Wallis test and Dunn's post-hoc test). (**B**) The majority of Tang cells in pSS lack the expression of CD28 being consistent with a senescent phenotype (% $CD28^-$ Tang *vs*. % $CD28^+$ Tang, $p<0.0001$ by Mann-Whitney U-test).

age- and sex-matched non-pSS sicca syndrome controls. Of these, six individuals displayed normal MSG and six displayed a certain degree of MSG inflammation (*i.e*. NSCS), but no evidence of focal lymphocytic sialadenitis (16, 17). Representative images of haematoxylin- and eosin-stained MSG sections are shown in Figures 4A-C.

MSG sections were subjected to immunofluorescence staining to assess the presence of $CD3^+CD31^+CXCR4^+$ Tang cells and the expression of the CXCR4-ligand SDF-1/CXCL12. As displayed in Figs 4D–F, numerous $CD3^+CD31^+$ T lymphocytes were observed in periductal and perivascular inflammatory infiltrates of pSS MSG, while they could not be detected in normal and NSCS MSG. Moreover, CD3/CXCR4 and CD31 immunofluorescence performed on serial tissue sections clearly demonstrated the presence of $CD3^+CD31^+CXCR4^+$ Tang cells in pSS MSG (Figs. 4G-I). As far as SDF-1/CXCL12 expression is concerned, this chemokine was barely detectable in ductal epithelial cells and microvessels of either normal or NSCS MSG (Figs 4J and K). Conversely, in pSS MSG, a strong SDF-1/CXCL12 immunostaining was found in ductal epithelial cells, microvessels and infiltrating inflammatory cells (Fig. 4L).

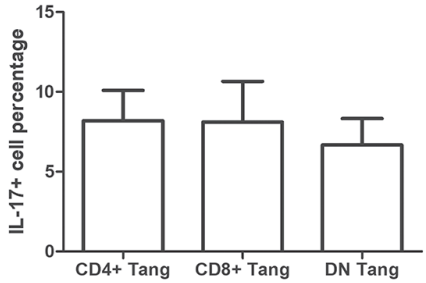

Fig. 3. IL-17^+ cells among $CD4^+$, $CD8^+$ and double negative (DN) angiogenic T cells (Tang) in primary Sjögren's syndrome (pSS) patients. Histograms represent the mean ± SEM of 3 different experiments.

Discussion

Endothelial dysfunction and accelerated atherosclerosis are common features of rheumatic diseases and to the best of our knowledge this is the first study investigating Tang cells, a leading actor in this scenario, in patients with pSS.

We previously demonstrated that although consistent endothelial damage is ongoing in pSS as proven by an increased amount of circulating EMP compared to HD, a counteracting mechanism leading to EPC release from the bone marrow is also taking place (5). The question remains on if and how this mechanism is actually effective since pSS patients still display a higher CV risk compared to the general population (6, 7). The current observation that also circulating Tang cells are raised in pSS, that they are significantly correlated to their partners EPC, and that both Tang and EPC are significantly associated with ESSDAI unmasks another facet of this complex process. However, this makes even more difficult to understand why although the endothelial repair machinery seems to be fully working, still pSS patients display higher CV risk and those with higher disease activity even more than those with a milder disease. A lesson that we learnt in the context of regulatory T cells (Treg) could possibly help to explain this apparent paradox. Indeed, many patients with rheumatic conditions display high proportions of circulating Treg cells that upon isolation effectively suppress effector lymphocytes *in vitro* (20, 21). Nonetheless, the disease is active and therefore a reasonable explanation may be that the local inflammatory microenvironment prevents Treg cells to exert their function. Based on this, one could speculate that although Tang cells and EPC are both expanded in the circulation of pSS patients, their *in vivo* function might be hampered by local stimuli. According to a recent study on SLE (12), the evidence that the majority of Tang cells in pSS are $CD28^-$ might alternatively suggest cytotoxic and pro-inflammatory rather than protective effects of these cells on the endothelium. Furthermore, this and previous studies (9) demonstrated that Tang cells can produce IL-17 and although the pathogenic role of this cytokine in pSS is now well established (19), the actual role of IL-17 in atherosclerosis and cardiovascular disease is still a matter of debate (22).

On a different note, the observation of Tang cells also in the context of MSG

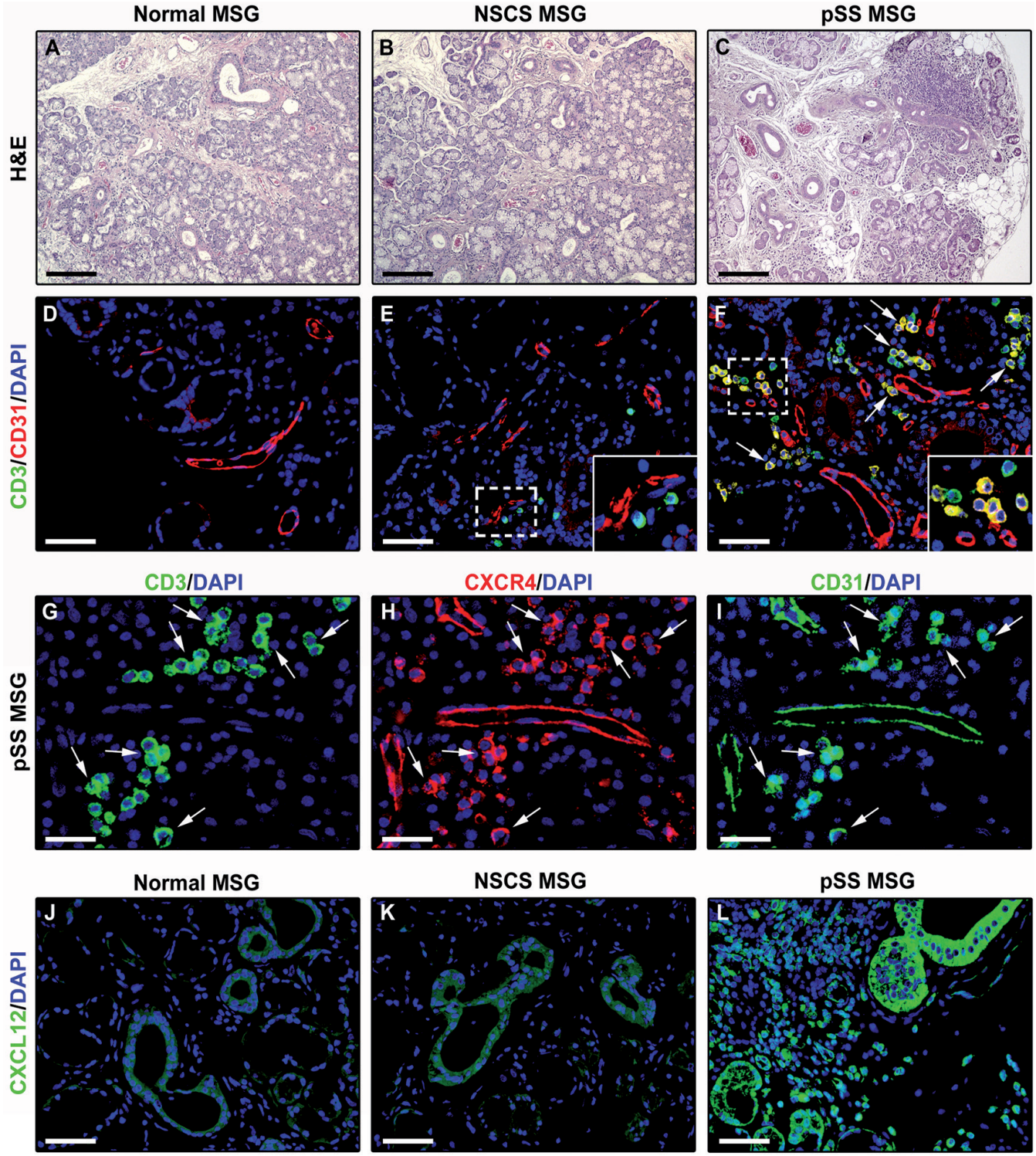

Fig. 4. Infiltrating angiogenic T cells (Tang) and increased expression of the CXCR4-ligand SDF-1/CXCL12 chemokine in minor salivary glands (MSG) from patients with primary Sjögren's syndrome (pSS). (**A–C**) Representative microphotographs of normal, non-specific chronic sialadenitis (NSCS) and pSS MSG sections stained with haematoxylin and eosin (H&E). pSS MSG display periductal inflammatory aggregates (foci) replacing the secretory units. (**D–F**) Representative fluorescence microphotographs of normal, NSCS and pSS MSG sections double immunostained for the pan-T lymphocyte marker CD3 (green) and CD31 (red), and counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue) for nuclei. Microvascular endothelium is $CD31^+$. Arrows indicate periductal and perivascular $CD3^+CD31^+$ T lymphocytes detected in pSS MSG. Insets are higher magnification views of the boxed areas from the respective panels. (**G–I**) Representative fluorescence microphotographs of serial MSG sections from pSS patients subjected to double immunostaining for CD3 (green) and CXCR4 (red) and single immunostaining for CD31 (green). Nuclei are counterstained with DAPI (blue). Arrows indicate $CD3^+CD31^+CXCR4^+$ T lymphocytes (Tang). (**J–L**) Immunofluorescence staining for SDF-1/CXCL12 (green) and DAPI (blue) counterstain for nuclei. Faint expression of SDF-1/CXCL12 is detected in normal and NSCS MSG. In pSS MSG, SDF-1/CXCL12 is strongly expressed in ductal epithelial cells, microvessels and infiltrating inflammatory cells. Scale bar: 200 μm (A–C), 50 μm (D–F and J–L), 25 μm (G–I).

raises intriguing speculations about the possible role of these cells at tissue level. The massive glandular expression of SDF-1/CXCL12, the ligand of CXCR4, in pSS has been extensively described over the last decade (23, 24) but it has been always considered from a 'B-cell perspective', as B lymphocytes express CXCR4 and SDF-1/CXCL12 is a key lymphoid chemokine driving B-cell homing to MSG. Our data, however, raise the hypothesis that glandular SDF-1/CXCL12 may also drive the recruitment of CXCR4-expressing Tang cells to salivary glands as additional players in the scenario of neo-angiogenesis and perpetuation of the inflammatory process. As a matter of fact, Tang cells are absent in normal and NSCS MSG, while they are numerous and close to blood vessels in pSS MSG.

In conclusion, our findings add some insights in the field of endothelial dysfunction in pSS and open a new scenario in the context of glandular neo-angiogenesis and inflammation in this disease, putting Tang cells among the pathogenic cell types worth to be targeted for therapeutic purposes.

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