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-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 (ESS-DAI). 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 females (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 hypercholesterolaemia. 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% CO2 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 formed by mixing mouse and rabbit anti-IL-17 antibodies: mouse monoclonal anti-CD3, CD31, CXCR4, CD4, CD8, CD28, CD34, CD133 and VEGFR-2. When required, cells were magnetically sorted (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% CO2 prior to surface staining with 25

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>36</td>
</tr>
<tr>
<td>Age, years</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>31 (86)</td>
</tr>
<tr>
<td>Xerophthalmia</td>
<td>32 (89)</td>
</tr>
<tr>
<td>Salivary gland enlargement</td>
<td>15 (42)</td>
</tr>
<tr>
<td>Extraglandular manifestations</td>
<td>25 (69)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0</td>
</tr>
<tr>
<td>Hypocomplementaemia</td>
<td>24 (67)</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Hypogammaglobulinaemia</td>
<td>22 (61)</td>
</tr>
<tr>
<td>Antinuclear antibodies</td>
<td>36 (100)</td>
</tr>
</tbody>
</table>

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)

Social Sciences, Chicago, IL, USA). Mann-Whitney U-test, Spearman’s correlation coefficient, Kruksal-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-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).

MRI was also explored.

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-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).

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 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.

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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, p<0.0001 by Kruskal-Wallis test; % DN Tang vs. % CD4+ Tang vs. % CD8+ 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).

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.

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 (focii) 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 bars: 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.

References