Abstract. Aim: To investigate the T-helper (Th1, Th2 and Th17) cell activity in the peripheral blood of patients with primary Sjögren’s syndrome (pSS), non-Sjögren’s sicca syndrome (nSS) and healthy controls. Patients and Methods: Peripheral blood mononuclear cells from 34 pSS, 13 nSS patients and 13 healthy controls were stimulated, labeled for cluster of differentiation-4 (CD4), interferon-γ (IFN-γ), interleukin-4 (IL-4) and IL-17A and analyzed by flow cytometry. Results: The activities of Th1 and Th7 cells in patients with pSS were similar to those of the control group. The percentage of both IFN-γ- and IL-17-producing Th17/Th1-like cells was significantly higher in the pSS, as compared to the control group, whereas that of Th2 cells was lower. A significant correlation was found between all Th-subset activities in the control group. However, in the pSS group, a correlation was found only between Th1 with Th2 and Th17 and Th17 with Th17/Th1-like. Conclusion: The imbalance in Th-subset activities in peripheral blood may play a role in the pathogenesis of pSS.

Among the various types of involved cells in immune responses, cluster of differentiation-4 (CD4)+ T-cells play a central role in immune regulation. Depending on the local balance between particular cytokines, CD4+ T-cells differentiate into three distinct subsets: T-helper-1 (Th1), Th2 and the recently recognized Th17, or one of several regulatory sub-populations. Until recently, autoimmune diseases had been categorized as either Th1- or Th2-mediated diseases. However, the discovery of a novel subset of helper T-cells producing interleukin-17 (IL-17), i.e. Th17 cells, changed this paradigm. Currently, IL-17 and Th17 cells are implicated in many autoimmune diseases, such as rheumatoid arthritis (1), systemic lupus erythematosus (2), psoriasis (3), multiple sclerosis (MS) (4), and inflammatory bowel diseases: Crohn’s disease (5) and ulcerative colitis (6). IL-17 is central to the pathology observed in murine models of these diseases, i.e. collagen-induced arthritis (7) and experimental autoimmune encephalitis (EAE) (8). It was reported that a Th17 cell subset exists which co-expresses interferon-γ (IFN-γ) and IL-17 (5, 9, 10). Such a Th17/Th1 subset (about 40% IL-17-producing T-cells) was identified in the gut of patients with Crohn’s disease. Both Th17 and Th17/Th1 clones exhibited selective expression of IL-23R, chemokine (C-C motif) receptor 6 (CCR6), and the transcription factor retinoic acid-related orphan receptor-γt (RORγt), and they exhibited similar functional features, such as the ability to help B-cells, low cytotoxicity, and poor susceptibility to regulation by autologous regulatory T-cells (5).

Primary Sjögren’s syndrome (pSS) is a systemic chronic inflammatory autoimmune disorder that affects secretory organs. The destruction of the glandular tissue is associated with lymphocytic infiltrates that tend to develop around ducts, whereas the degree of glandular infiltration varies. Total T-cells and their sub-population CD4+ T-cells pre-dominate in mild lesions, whereas B-cells in the severe one (11).

Despite the fact that IL-17 plays great part in the pathogenesis of autoimmune disorders, a role of IFN-γ in autoimmunity cannot be ignored. In addition to the Th17 main cytokine IL-17 as a perpetrator in pSS (12-14), there is evidence for involvement of Th1 cells and their products, such as IFN-γ, in immunopathological glandular disease, considered causative before recognition of the existence of Th17 cells (15, 16). IFN-γ mRNA is overexpressed in minor salivary glands in pSS and this is reflected by the elevated IFN-γ protein in saliva (17-19). The etiology of pSS remains undefined and the pathophysiological mechanism is still poorly-understood despite decades of research.

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Table I. Cytokine expression in cluster of differentiation 4 (CD4)+ T-cells from the control group and patients with primary Sjögren’s syndrome (pSS).

<table>
<thead>
<tr>
<th>T-Cell Subset</th>
<th>Control group (n=26)</th>
<th>pSS (n=37)</th>
<th>p-Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>SD</td>
</tr>
<tr>
<td>Th1 (INF-γ+)</td>
<td>11.90</td>
<td>13.14</td>
<td>6.47</td>
</tr>
<tr>
<td>Th2 (IL-4+)</td>
<td>0.25</td>
<td>0.20</td>
<td>0.25</td>
</tr>
<tr>
<td>Th17 (IL-17A+)</td>
<td>1.06</td>
<td>0.97</td>
<td>0.51</td>
</tr>
<tr>
<td>Th17/Th1 (IL-17A+/IFN-γ+)</td>
<td>0.17</td>
<td>0.15</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Values represent the mean and median of the percentage of CD4+ T-helper cells in peripheral blood mononuclear cells; †Mann–Whitney test; SD, standard deviation.

Immunofluorescent staining and flow cytometric analysis. Stimulated PBMCs were fixed and permeabilized with Fixation/Permeabilization Kit buffers (BD Cytofix and BD Perm/Wash; BD Biosciences, San Diego, CA, USA). Cells were then stained with Human Th1/Th2/Th17 Phenotyping Cocktail (BD Pharmingen, San Diego, CA, USA) containing: anti-human CD4-peridinin chlorophyll protein-cyanine dye (PerCP- Cy5.5), anti-human IL-17A-phycocerythrin (PE), anti-human IFN-γ-fluorescein isothiocyanate (FITC), anti-human IL-4-allophycocyanin (APC). Cells used as negative controls were stained with Three-color Fluorescent Ig Isotype Cocktail (BD Pharmingen) containing: anti-human CD4-PerCP-Cy5.5, and isotype controls FITC-Mouse IgG1, PE-Mouse IgG1 and APC-Rat IgG1. Four-color flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), calibrated with CaliBRITE beads (BD Biosciences) using the CELL-Quest software (BD Biosciences). Data for each sample were acquired until 100,000 lymphocytes were analyzed. Th1 cells were defined as CD4+ IFN-γ+; Th17 cells as CD4+ IL-17A+; Th2 cells as CD4+ IL-4+; and Th17/Th1-like cells as CD4+ IL-17A+ IFN-γ+.

Results

Differences in Th subset activities between patient groups. All three study groups were well-matched for age (pSS=55.8±12.8 years, nSS=52.75±10.40 years and healthy controls=50.4±8.8 years), gender and ethnicity. The majority of the enrolled patients were Caucasian women, as pSS primarily affects women. In the pSS and nSS groups, two Caucasian men were enrolled, one in each group. According to our results, no differences between nSS and healthy controls were found and we, thus, combined the data as one control group for flow cytometry. PBMCs were stained for CD4, IFN-γ, IL-4, IL-17 and the activities of Th subsets were determined by flow cytometry. Upon stimulation, a similar percentage of CD4+ T-cells from pSS patients expressed IFN-γ in comparison with patients from the control group (p=0.3389). The proportion of CD4+ IL-4+...
T-cells from pSS was lower in comparison to that of the control group, but not significantly \((p=0.0683)\). The proportion of CD4+ IL-17+ T-cells from pSS was similar in comparison to that of the control group \((p=1.000)\). Th17/Th1-like cells, defined as CD4+ IL-17+IFN-γ+ were analyzed for expression of both IL-17 and IFN-γ. A clear difference was found in that there was an increased frequency \((p=0.0029)\) of both IFN-γ- and IL-17-producing Th17/Th1-like cells in pSS patients, in comparison to the control group (Figure 1A-C). No correlations were observed between Th subset activities and patient age (data not shown).

We found significantly different results in the pSS group. Th1 cells correlated with Th2 \((r=0.4657, p=0.0048)\) and Th17 \((r=0.3654, p=0.0261)\), but did not correlate with Th17/Th1-like cells. Th2 cells correlated only with Th1 cells. Th17 cells correlated with Th1 and Th17/Th1-like cells \((r=0.7485, p<0.0001)\). Th17/Th1-like cells correlated only with Th17 cells (Figure 2B). Similarly as in the control group, no

**Figure 1. T-Helper subsets in control and primary Sjögren’s syndrome (pSS) groups.** A: Th1; B: Th2; C: Th17; D: Th17/Th1-like. I: Plots indicate the distribution of the four T-helper subsets, each data point represents one individual patient, horizontal lines represent mean values. II: Box and whisker plots indicate the distribution of the four T-helper subsets; data are shown as median values and 5-95 percentiles. III: Representative dot plots of interferon-γ (IFN-γ)-, interleukin-4 (IL-4)-, IL-17- and IL-17/IFN-γ-expressing CD4+ T-cells upon stimulation.
correlations were observed between Th subset activities and patient age. There were also no correlations between Th subsets in PBMCs and ocular symptoms, oral symptoms, Shirmer’s I test, unstimulated salivary flow rate, focus score, anti-Ro/SSA, anti-La/SSB, erythrocyte sedimentation rate, C-reactive protein and rheumatoid factor (data not shown).

Discussion

Possible involvement of Th1 cells in salivary gland dysfunction was suggested by Yin and colleagues (21) in an animal model for pSS. They demonstrated that immunization of Balb/c mice with Ro60-peptide led to the development of xerostomia and correspondingly to consistently higher serum levels of Th1 cytokines (IFN-γ and IL-12), compared with phosphate buffered saline (PBS)-immunized mice. Salivary gland extracts of these mice also contained higher levels of IFN-γ and IL-12 cytokines. These data collectively emphasize the possible role of Th1 cells in salivary gland dysfunction.

In our study, the percentage of IFN-γ-producing CD4+ T-cells from pSS was not significantly different in comparison to that of the control group. Th2 cells appear to play a role in pSS, our results showed a decrease of Th2 cells in the pSS group; however, the differences were not significant. Features of milder pSS are significantly associated with genotypes which contribute to a Th2 disease. A shift towards Th2 differentiation seems, at least clearly, to elicit features of milder pSS (22).

Th17 cells also appear to play a role in the development of pSS. Studies in patients with pSS and animal models of pSS have identified the presence of IL-17 in the lymphocytic infiltrates of the exocrine glands, as well as higher levels of circulating IL-17 in both serum and saliva (14, 23). Importantly, a recent study by Nguyen and colleagues (24) demonstrated that inhibition of IL-17 at early or late disease stages in the SS-susceptible C57BL/6.NODAec1Aec2 mice.

Figure 2. Spearman’s rank correlation between T-helper subsets in peripheral blood mononuclear cells, each data point represents one individual patient. A: Correlation between the proportion of T-helper subsets (%) in the control group. B: Correlation between the proportion of T-helper subsets in the group with primary Sjögren’s syndrome.
model can prevent the onset or even inhibit the development of pSS in these mice. Regarding the fact that the proportion of Th17 cells in the pSS group was very similar to that of the control group, like Abdulahad et al. (25) for Wegener’s granulomatosis, we can argue that circulating Th17 cells should be studied in patients with active disease and not in those with disease in remission.

Some studies revealed that both IL-17 and IFN-γ synergize to trigger severe intestinal inflammation in murine models of inflammatory bowel disease and suggest that both Th1 and Th17 cells may contribute to pathogenesis (26, 27). Sakai and colleagues confirmed the expression of IFN-γ in the salivary glands of patients with pSS and showed that the number of IFN-γ+ cells was smaller than that of IL-17+ cells and that IFN-γ+ cells did not co-express IL-17. These observations suggest that both Th1 and Th17 cells together are involved in the pathogenesis of pSS (23).

However, we found a significantly increased percentage of both IL-17- and IFN-γ-producing Th17/Th1-like cells in pSS patients, as compared to the control group. To our knowledge this is the first description of Th17/Th1-like cells in the peripheral blood of patients with pSS. These findings are in line with current studies supporting the concept that the small Th-cell subset named Th17/Th1-like that co-express both cytokines IL-17 and IFN-γ are involved in the pathogenesis of autoimmunity and chronic inflammatory disorders (28, 29, 10). We observed different correlations between Th subsets in pSS in comparison to the control group. A significantly higher percentage of Th17/Th1-like cells (p=0.0029) and reduced percentage of Th2 cells (p=0.0683) in pSS patients could cause an imbalance among the Th cell subsets. We therefore suppose that such an imbalance in Th subsets plays a role in pSS.

Recently, Boniface and colleagues carried out a study that contributes to new insights into the heterogeneity, functionality, and relationship between human Th17 and Th1 cell subsets. Human T-cells cultured under Th17- (IL-1β, IL-23, PGE2) inducing conditions and secreting both IL-17 and IFN-γ (Th17/Th1), or only IFN-γ+ (Th17/IFN-γ+), were defined as a Th17 cytokine, depending on the milieu and other cytokines produced by T-cell subsets. Furthermore, it seems that cells producing both IL-17 and IFN-γ are more plastic and can become IL-17- and/or IFN-γ-producing cells (28).

In this regard, Kebir et al. recently reported that cells producing both IL-17 and IFN-γ preferentially cross the human blood–brain barrier, and these cells are present in brain lesions of patients with multiple sclerosis (29). A high frequency of IL-17+IFN-γ+ double-positive CD4+ cells was found in the CNS during active EAE (10).

The plasticity of Th17 cells was also recently highlighted in mouse models of ocular inflammation, colitis, and diabetes, showing that after adoptive transfer of IL-17+ cells, these cells converted rapidly into IFN-γ-producing cells that were critical for disease (30, 31). In addition, Th17 cells-only induced diabetes efficiently after conversion into IFN-γ-producing cells in lymphopenic hosts (32). It remains to be determined whether such IFN-γ-producing cells belong purely to the Th1 subset or rather have more the characteristics of the Th17-IFN-γ+ subset.

Despite the fact that Sakai and colleagues did not find T-cells that co-express IFN-γ and IL-17 in the salivary glands of patients with pSS (23), our study showed a significant increase of IL-17+/IFN-γ+ double-positive T-cells in PBMCs of the patients with pSS compared to those from the control group. These findings allow us to speculate that these IL-17+/IFN-γ+ double-producing cells are involved in the pSS pathogenesis. We accept there are imperfections in our study, as we analyzed Th subsets only by their distinctive patterns of cytokine secretion (IFN-γ, IL-4, IL-17), and did not include any other markers. The intention of our study was to determine the Th1/Th2/Th17 balance in PBMCs of patients with pSS compared to the control group, and the finding of a significant increase of IL-17+/IFN-γ+ double-producing cells was unexpected. Further studies should assess whether IFN-γ-producing cells identified ex vivo in inflamed tissues represent a homogeneous population or a mix of Th1, Th17-IFN-γ+ and Th17/Th1-like cells and elucidate their possible pathophysiological role in patients with pSS.

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References


