Salivary gland and peripheral blood T helper 1 and 2 cell activity in Sjögren’s syndrome compared to non-Sjögren’s sicca syndrome.

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Abbreviated title: T helper cell activity in sicca syndrome.
Abstract

Objectives. To investigate whether differences in T helper (Th) 1 and Th2 cell activity in salivary glands (‘local’) or (‘peripheral’) blood could discriminate Sjögren’s syndrome (SS) from non-Sjögren’s sicca syndrome (nSS-sicca). Moreover, relationships of local and peripheral Th activities to each other and to disease activity parameters were studied.

Methods. Sixty-two sicca patients (32 SS and 30 nSS-sicca patients) were studied. Local Th1 (IFNγ) and Th2 (IL-4) activity were determined using immunohistochemistry. T cell production of IFNγ and IL-4 in peripheral blood was determined by ELISA. Erythrocyte Sedimentation Rate (ESR) and serum IgG were considered disease activity parameters.

Results. ESR and serum IgG were higher in SS patients compared to nSS-sicca patients. Local Th1 cell activity was higher whereas peripheral blood Th1 activity was lower in SS compared to nSS-sicca patients. Th2 cell activity was not significantly different between both patients groups. The ratio IFNγ over IL-4 was higher in salivary glands and lower in PB in SS patients compared to nSS-sicca patients. Local and peripheral Th1 and Th2 cell activities correlated with ESR and serum IgG levels. However, neither ESR, serum IgG nor local or peripheral Th1 or Th2 cell activity discriminated between SS and nSS-sicca patients.

Conclusions. This study is the first to describe both local and peripheral Th cell activity in SS compared to nSS-sicca patients. Results demonstrate that an imbalance between Th1 and Th2 activity in sicca patients is clearly related to severity of disease, but cannot be used to discriminate between SS versus nSS-sicca patients.
Introduction
Sjögren’s syndrome (SS) is an auto-immune exocrinopathy primarily affecting lacrimal and salivary glands, characterized by keratoconjunctivitis sicca and xerostomia. SS may occur in the absence (primary SS; pSS) or in the presence of another systemic auto-immune disease including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (secondary SS; sSS).

A distinction between pSS and non-Sjögren’s sicca syndrome (nSS-sicca) is considered to be helpful in defining prognosis, since pSS patients have an increased risk for the development of extraglandular autoimmune manifestations and malignant lymphoma.

In clinical practice, this distinction might be difficult since nSS-sicca patients may present with systemic symptoms, serological abnormalities, systemic characteristics of inflammation (elevated erythrocyte sedimentation rate (ESR) and serum IgG) and unspecific sialadenitis of salivary glands. Moreover, nSS-sicca patients might develop SS later in life. A more detailed evaluation of the disturbed immune system might provide tools to evaluate this apparent overlap between SS and nSS-sicca patients.

Of the lymphocytes infiltrating the salivary glands in SS patients, 45-50% is of T helper/inducer (CD4+) phenotype, 20% of T suppressor/cytotoxic (CD8+) phenotype and about 20% is B cells. T helper (Th) cells can be divided into at least two functionally different subsets based on the cytokines they produce: Th1 cells predominantly produce pro-inflammatory cytokines such as interferon-γ (IFNγ) and interleukin-2 (IL-2), whereas Th2 cells predominantly produce anti-inflammatory and regulatory cytokines such as IL-4, IL-10 and IL-13.

A disturbed Th1/Th2 cell balance has been described to be important in the regulation of many chronic inflammatory disorders. It is suggested that this Th1/Th2 cell imbalance might also play a role in the pathogenesis of SS; although several studies have evaluated cytokine profiles in SS, there is only a limited number of studies (with relatively small numbers of patients included) that have focused on this Th1/Th2 cytokine balance in SS. These studies have focused on either the salivary glands (“local”; 3 6 7) or the blood lymphocyte (“peripheral”; 8 9) compartment. Although some of these studies have included healthy control groups, none of these studies have compared nSS-sicca patients with SS patients. Therefore, we investigated in salivary glands and peripheral blood whether differences in Th1 and Th2 cell activity could discriminate SS from nSS-sicca. Moreover we investigated how local and peripheral Th activities were related, also with respect to disease activity parameters.

Finally, Th1 and Th2 profiles in primary SS patients with or without extraglandular manifestations and serum auto-antibodies were studied; Th profiles were also correlated to the grade of labial salivary gland infiltration.
Patients and Methods

Patients
From 1997-1998, 62 sicca patients referred to our university hospital out-patients clinic were included. Routine diagnostic procedures (including medical history, physical examination, Schirmer-1 test, laboratory investigations and labial salivary gland biopsy) were performed. Thirty two (32) patients met the revised European classification criteria for SS according to the American-European consensus group. 6 of them had sSS (4 patients with RA and 2 with SLE); 30 patients were classified as having nSS-sicca. No relevant differences were found for pSS (n=26) compared to sSS patients (n=6), consequently they were analyzed as 1 group. The study was performed according to the medical ethical regulations of the Utrecht University Medical Center; all patients gave informed consent to use a part of their labial salivary gland tissue for research purposes. Peripheral blood (PB) samples were collected on the same day within 10 minutes before the salivary gland biopsy procedure.

Parameters of disease activity
Serum IgG and erythrocyte sedimentation rate (ESR), determined by standard hospital procedures, were considered representative parameters of disease activity.

Local Th1 and Th2 cell activity
As a measure of Th1 and Th2 cell activity the numbers of IFN\(\gamma\) positive and IL-4 positive cells respectively were determined in labial salivary gland biopsies. Of each labial salivary gland biopsy a part was fixed and embedded in paraffin for routine diagnostic procedures (classification) and the other part was snap frozen in Tissue-Tek\textsuperscript{®} in liquid nitrogen and stored at -70/-80\(^\circ\)C. Seven \(\mu\)m sections were cut on a freezing microtome and mounted on 3-aminopropyl tri-ethoxy silane coated slides. Sections were fixed with dry acetone for 7 minutes, air dried and pre-incubated for 20 minutes in 10\% normal horse serum (NHoS) and 10\% normal human serum (NHuS) in phosphate buffered saline (PBS). Sections were incubated with primary antibodies diluted in PBS with 1\% NHoS/NHuS for 60 minutes. Murine monoclonal antibodies for IFN\(\gamma\) (MD-2; Holland Biotechnology, Rijswijk, the Netherlands) and IL-4 (1-41-1; Novartis, Basel Switzerland) were used. Slides were washed 3 times for 5 minutes with PBS+0.05\% Tween 20, after which a second incubation of biotinilated Horse-anti-Mouse antibody was applied for 30 minutes. After washing with PBS/Tween the sections were incubated with streptavidine conjugated alkaline phophatase for 30 minutes and washed in Tris-HCl. Alkaline phosphatase activity was demonstrated with AS-biphosphate as substrate and new fuchsine as chromogen dissolved in 0.1 M Tris-HCl, pH 8.5, resulting in pink/red staining. Endogenous alkaline phosphatase activity was inhibited by addition of levamisole to the reaction mixture. The entire procedure lacking the first antibodies served as controls and did not reveal any staining. Slides were slightly counterstained thereafter with hematoxilline and embedded in gelatine. Salivary gland INF\(\gamma\) and IL-4 positive cells were counted by 2 independent observers (JM vW and M WvW) in 12 subsequent designated areas of 0.04 mm\(^2\) in each biopsy specimen. Both observers were blinded for the patients’ identity and the monoclonal antibody used.

Peripheral Th1 and Th2 cell activity
From all patients, PB was diluted 1:1 with Dulbeco’s Modified Eagle’s Medium (D-MEM, Gibco 074-01600, Gibco, Paisly, UK). Thereafter, mononuclear cells (MNC) were isolated by density centrifugation using Ficoll-Paque (Pharmacia Biotech, Roosendaal, the Netherlands). Isolated cells (5\( \times \)10\(^5\)/ml) were cultured for 48 h in D-MEM supplemented with penicilline (100 U/ml), streptomycin sulfate (100 \(\mu\)g/ml) and glutamine (2mmol/l) and 10\% human
pooled adult blood group AB$^+$ serum. T cell specific cytokine production was amplified by adding CD3-CD28 monoclonal antibodies (CLB, Amsterdam, The Netherlands 1.5 µg/ml and 2 µg/ml, respectively) $^{12}$. After 48 h of *ex vivo* culture (10), the culture media were harvested, rendered cell-free by centrifugation, frozen in liquid nitrogen and stored at -20°C. IFN$\gamma$ and IL-4 levels were determined by ELISA according to the manufacturer’s instructions (Biosource, Ettenleur, The Netherlands). Detection limits of the assays were 10 pg/ml for both IFN$\gamma$ and IL-4.

**Th1/Th2 cell activity and extraglandular manifestations, auto-antibodies and salivary gland infiltration in the pSS group**

In the pSS patient group (n=26), PB IFN$\gamma$ and IL-4 production as well as SG IL-4+ and IFN$\gamma$+ cell numbers were compared between patients with (n=12) and without (n=14) extraglandular manifestations (cytopenia, arthritis, neuropathy, leukocytoclastic vasculitis, other skin lesions), anti-nuclear antibodies (ANA; n=20 vs. 6, respectively), anti-Ro/SS-A (n=21 vs. 5) and anti-La/SS-B antibodies (n=10 vs. 16). In addition, within the pSS group the grade of salivary gland infiltration expressed by the lymphocytic focus score [LFS] (ranging from 0-6 foci/4 mm$^2$ salivary gland tissue) was correlated to the cytokine profiles in PB and SG.

**Statistical analysis**

All parameters, including ESR, serum IgG, number of cytokine positive cells in salivary glands and cytokine production by PB T cells were not normally distributed. Differences between SS and nSS-sicca patients were therefore expressed as boxplots representing medians and 25% and 75% percentiles and analyzed by the Mann-Whitney-U test (two tailed). For correlations, data were transformed to a logarithmic (ln) scale resulting in a normal distribution and subsequently Pearson analysis was performed. Differences between pSS patients with or without extraglandular manifestations and auto-antibodies were also analyzed by the Mann-Whitney-U test. Spearman correlation coefficients were calculated to relate salivary gland infiltration in the pSS group with cytokine profiles. P values <0.05 were considered statistically significant.
Results

Differences in parameters between SS and nSS-sicca patients

As depicted in figure 1, significant differences in mean ESR and serum IgG levels existed between nSS-sicca and SS patients. Although a clear difference was found, there still was a significant overlap for both parameters between both patients groups (see also figures 3-5). The number of IFNγ+ cells in the salivary gland (figure 2A) was significantly higher in the SS group compared to those in the nSS-sicca group. For the numbers of IL-4+ cells in the salivary gland (figure 2B) no such difference was found. IFNγ production by PB T cells from SS patients (figure 2 C) was significantly lower than that of nSS-sicca patients. In PB, also for Th2 cell activity no such differences were found (figure 2D). In salivary glands, the ratio of IFNγ+ cells over IL-4+ cells was higher in the SS patients compared to nSS-sicca patients (figure 2E). In PB the opposite was found, the IFNγ production over IL-4 production was lower in SS patients compared to nSS-sicca patients (figure 2F).

None of the parameters in either of the two compartments was conclusive to discriminate between SS and nSS-sicca patients on the individual patient level (see also figures 3-5).

Correlations between Th1/Th2 cell activity and disease activity parameters

In the whole sicca population (SS plus nSS-sicca patients), the number of IFNγ+ cells in salivary glands correlated positively with ESR and serum IgG (figure 3A and B, respectively). Moreover, for PB, a negative correlation between IFNγ production and ESR and serum IgG was found (figures 3C and D) in the whole group. For the sub-groups (SS and nSS-sicca) several similar positive and negative correlations were found for the local and peripheral compartment, respectively (table 1 and dotted lines in figure 3). However, because of the relative small numbers in both sub-groups, only in 3 out of 8, correlations neared or reached statistical significance. Importantly, no significant opposite correlations for the sub-groups were found, when compared to the whole group.

Salivary gland IL-4 (figure 4B) correlated positively with serum IgG but not with ESR (figure 4A). PB IL-4 correlated negatively with serum IgG (figure 4D), but again not with ESR (figure 4C). Also for the subgroups (SS and nSS-sicca) several almost identical correlations could be found (figure 4, dotted lines; table 1). No significant opposite correlations were found.

The salivary gland IFNγ+ / IL-4+ cell ratio in the whole sicca population showed a positive correlation with ESR and serum IgG (figures 5A and 5B, respectively) although not statistically significant for ESR. In PB a negative correlation was found for the ratio IFNγ over IL-4 production with ESR as well as with serum IgG (figures 5C and 5D, respectively). Also for the subgroups (SS and nSS-sicca) several almost identical correlations could be found (figure 5, dotted lines; table 1); no significant opposite correlations were found with only one exception: for the nSS-sicca group the ratio IFNγ/IL-4 correlated positively with IgG (corr.coeff. +0.38, p = 0.04, see table 1).
Table 1
Correlations between Th1 (IFNγ) and Th2 (IL-4) cell activity and parameters of disease activity (ESR and serum IgG) in salivary glands (SG) and peripheral blood (PB) in the whole sicca group, the SS and nSS-sicca patients groups (see also figures 3-5).

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<th>ESR</th>
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<td>IFNγ/IL-4</td>
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<tr>
<td>PB</td>
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Correlation coefficients with p values (between brackets) in case significance was neared or reached, i.e. with p values ≤ 0.15. Regression lines in case of significance have been drawn in figures 3, 4 and 5 respectively.

Local Th1/Th2 cell activity related to peripheral Th1/Th2 cell activity
The difference in Th1/Th2 ratio between salivary glands (high for the SS group, low for the nSS-sicca group) and PB (low for the SS group, high for the nSS-sicca group) (see figure 2) suggests a negative relation between local and peripheral Th1 and Th2 cell activity. However, no significant correlations between local and peripheral cytokine profiles were found in the whole sicca population or subgroups, neither for the individual cytokines nor for the ratios (data not shown).

Th1/Th2 cell activity and extraglandular manifestations, auto-antibodies and salivary gland infiltration in the pSS group
In the pSS group, no differences were found between patients with and without extraglandular manifestations, ANA, anti-Ro/SS-A and anti-La/SS-B auto-antibodies in PB IFNγ and IL-4 production or in SG IFNγ+ and IL-4+ cell numbers. Neither did the labial focus score correlate with PB or SG Th1 or Th2 profiles (data not shown).
Discussion

Over the last 10 years there is accumulating evidence that a Th1/Th2 imbalance plays a role in the pathogenesis of SS. The present study is unique in that it analyzed Th1 and Th2 profiles, local versus peripheral (in a paired set-up), in SS versus nSS-sicca patients related to serum IgG as well as ESR using a significant number of patients and using detection of actual protein. We found Th1 cell activity to be high at the site of inflammation (salivary glands) and low in PB when SS patients were compared to nSS-sicca patients. However, neither local nor peripheral Th1 activity discriminated SS from nSS-sicca patients. This is in agreement with a report from Konttinen et al., in which production of classical Th1 cytokines also in normal glands was described. Therefore, there seems to be no rationale for determining local or peripheral Th1 activity in the individual sicca patient for diagnostic purposes. Moreover, although SS patients had higher ESR and serum IgG values than nSS-sicca patients, also for these two parameters both patient groups demonstrated a considerable overlap. From a clinical point of view this is interesting since high serum IgG levels in nSS-sicca patients have been reported to be predictive for development of SS.

In the whole sicca group the number of IFN$\gamma^+$ cells, the ratio IFN$\gamma^+$ over IL-4$^+$ positive cells but also the number of IL-4$^+$ cells in the salivary gland correlated positively with parameters of disease activity. The opposite was found for PB: IFN$\gamma$ and the ratio IFN$\gamma$/IL4, but also IL-4 correlated negatively with disease activity parameters. This demonstrates that the differences between local and peripheral Th1 and Th2 cell activity are most pronounced for Th1 cell activity, but similar, although less outspoken, for Th2 cell activity. High local Th1 activity in SS might be insufficiently counter regulated by a Th2 response. It is tempting to suggest that in SS patients Th cells from PB have migrated to the salivary glands, mostly Th1 cells but also Th2 cells and that this phenomenon depends on or possibly predicts the severity of the disease. In analogy with the present data, in RA patients (compared to healthy controls) low Th1 activity in peripheral blood and high intra-articular Th1 activity were found. However, the lack of correlation between local Th1 profiles and lymphocytic focus scores observed in this study does not support this hypothesis. Moreover, the absence of a (positive or negative) correlation between local and peripheral Th cell activity suggests that peripheral Th cell activity is not simply a reflection of local Th cell activity, but might represent the systemic auto-immune response observed in SS.

Although far from conclusive, the present data strongly suggest that nSS-sicca patients might, at least in some cases, represent SS although not fulfilling classification criteria. On the other hand it could be that a relevant number of patients are unjust classified as SS patients, whereas others that may actually have SS are missed. Whether the latter group only represent a milder form of SS or whether we are dealing with a pre-phase of SS as has been suggested, cannot be confirmed and certainly requires additional study.

The peripheral shortage of Th1 cytokine production might suggest possibilities of therapeutic strategies of systemic Th1 cell cytokine supplementation against disease as suggested by Hagiwara. However, in the light of mechanisms as depicted above and the observed low peripheral IL-4 production correlating with serum IgG levels insist on more study before such approaches should be realized.

In conclusion, neither ESR, serum IgG nor local or peripheral Th1 and Th2 cell activity discriminated between SS and nSS-sicca patients; both populations have at least partly common characteristics in this respect. The elevated local Th1 cell activity correlating with more severe immune dysregulation in sicca patients (ESR and serum IgG) might not be overcome by an adequate local Th2 cell response. This imbalance might be partially responsible for the perpetuation of the immune response in SS. Studies in SS that evaluate
therapeutic agents that shift a balance towards local Th2 cell activity might be considered and studied.
**Figure legends**

Figure 1: ESR (erythrocyte sedimentation rate; mm in the first hour) and serum IgG (gram/liter) of non-Sjögren’s sicca (nSS-sicca) syndrome and Sjögren’s syndrome (SS) patients (n=30 and n=32, respectively). Solid line represents the median value, the boxes stretch from the lower 25th percentile to the upper 75% percentile. P values for differences between SS and non-SS sicca patients groups are given.

Figure 2: Salivary gland and peripheral blood cytokine profile for nSS-sicca and SS patients (n=30 and 32 patients, respectively). Solid line represents the median value, the boxes stretch from the lower 25th percentile to the upper 75% percentile. Left panels A, B and E: salivary gland (SG) number of IFN\(_\gamma\) (A), and IL-4 (B) positive cells per 0.04 mm\(^2\) and the ratio IFN\(_\gamma\) over IL-4 positive T cells (E). Right panels C, D and F: Peripheral blood (PB) \textit{ex vivo} IFN\(_\gamma\) (C), IL-4 (D), and the ratio IFN\(_\gamma\) over IL-4 production (x1000; F) for the SS and non-SS sicca patients groups. P values for differences between SS and non-SS sicca patients groups are given.

Figure 3: Relations between parameters of disease activity and IFN\(_\gamma\) profiles. Top panels (A and B) depict the relation between salivary gland (SG) IFN\(_\gamma\) positive T cells (number per 0.04 mm\(^2\)) and ESR (erythrocyte sedimentation rate; left panel; A) and serum IgG (right panel; B). Lower panels (C and D) depict the relation between peripheral blood (PB) T cell IFN\(_\gamma\) production (pg/ml) and ESR (left panel; C) and serum IgG (right panel; D). Black triangles represent the nSS-sicca patients and the gray squares represent the SS patients. Pearson correlations with logarithmic transformed (ln; to obtain a normal distribution) data have been performed. Regression lines of the correlations in the whole sicca group are given by the (solid) lines. The dotted lines represent regression lines for the correlations in the nSS-sicca groups in figures A and B and the SS group in figure C (see table 1 for details).

Figure 4: Relations between parameters of disease activity and IL-4 profiles. Top panels (A and B) depict the relation between salivary gland (SG) IL-4 positive T cells (number per 0.04 mm\(^2\)) and ESR (erythrocyte sedimentation rate; left panel; A) and serum IgG (right panel; B). Lower panels (C and D) depict the relation between peripheral blood (PB) T cell IL-4 production (pg/ml) and ESR (left panel; C) and serum IgG (right panel; D). Black triangles represent the nSS-sicca patients and the gray squares represent the SS patients. Pearson correlations with logarithmic transformed (ln; to obtain a normal distribution) data have been performed. Regression lines of the correlations in the whole sicca group are given by the (solid) lines. The dotted lines represent regression lines for the correlations in the nSS-sicca group in figures A and B (see table 1 for details).

Figure 5: Cytokine ratio profiles related to parameters of disease activity. Top panels (A and B) depict the relation of salivary gland (SG) IFN\(_\gamma\) over IL-4 positive T cells with ESR (erythrocyte sedimentation rate; left panel; A) and serum IgG (right panel; B). Lower panels (C and D) depict the relation of peripheral blood (PB) T cell IFN\(_\gamma\) over IL-4 production (x1000) with ESR (left panel; C) and serum IgG (right panel; D). Black triangles represent the non-SS sicca (nSS-sicca) patients and the gray squares represent the SS patients. Pearson correlations with logarithmic transformed (ln; to obtain a normal distribution) data have been performed. Regression lines of the correlations in the whole sicca group are given by the (solid) lines. The dotted lines represent regression lines for the correlations in the nSS-sicca group in figures B and D (see table 1 for details).
(solid) lines. The dotted lines represent regression lines for the correlations in the SS group in figures C and D (see table 1 for details).
Reference List


Figure 1
Figure 2

Salivary Gland

A. Box plot showing IFN-γ+ cells / 0.04 mm² with P<0.001.

B. Box plot showing IL-4+ cells / 0.04 mm² with P=0.15.

C. Box plot showing IFN-γ (pg/ml) with P<0.001.

D. Box plot showing IL-4 (pg/ml) with P=0.27.

E. Box plot showing ratio IFN-γ / IL-4 with P<0.02.

F. Box plot showing ratio IFN-γ / IL-4 (x1000) with P<0.001.

Peripheral Blood

P<0.001

Figure 2
Figure 3

(A) SG IFN\(\gamma\) + cells / 0.04 mm² vs. ESR (mm/1st h)

(B) SG IFN\(\gamma\) + cells / 0.04 mm² vs. Serum IgG (g/l)

(C) PB IFN\(\gamma\) (pg/ml) vs. ESR (mm/1st h)

(D) PB IFN\(\gamma\) (pg/ml) vs. Serum IgG (g/l)
Salivary gland and peripheral blood T helper 1 and 2 cell activity in Sjögren's syndrome compared to non- Sjögren's sicca syndrome

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