CX3CL1 and CX3CR1 expression in tertiary lymphoid structures in salivary gland infiltrates: fractalkine contribution to lymphoid neogenesis in Sjögren’s syndrome

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Abstract

Objectives. Primary SS is an autoimmune disease characterized by chronic lymphocytic inflammation and ectopic germinal centre (GC) formation within salivary glands. Fractalkine (CX3CL1), associated with the pathogenesis of RA, is the sole member of the CX3C chemokine (CK) family and acts as an adhesion and chemotactic molecule. The objectives of this work are to determine to what extent CX3CL1 and its receptor CX3CR1 expression might be altered in salivary glands obtained from patients and to establish whether these CKs might be involved in SS ectopic lymphoneogenesis.

Methods. We assessed the presence of CX3CL1 protein in sera by ELISA in 21 patients with primary SS, 11 patients with Sicca syndrome (Sicca), 20 RA patients and 10 blood donors. Histological evaluation was performed on sequential sections of salivary gland tissue. Using TaqMan RT-PCR we studied CX3CL1 and CX3CR1 mRNA expression in salivary gland tissues from a molecular point of view.

Results. Increased serum levels of CX3CL1 protein were observed in SS patients compared with controls ($P<0.0001$) and in RA patients compared with controls ($P<0.0001$), but no difference was found between Sicca patients and controls ($P=0.22$). We identified histologically the cells expressing CX3CL1 and CX3CR1 in salivary glands of SS patients and we localized the molecule within tertiary lymphoid structures. Finally, the mRNA levels of the CK and its receptor were up-regulated in SS salivary glands.

Conclusion. We believe that our findings point to the need for future studies on CX3CL1 and CX3CR1 proteins as contributors to the formation of ectopic GCs and possibly as a new tool in the evaluation and diagnosis of SS.

Key words: Sjögren’s syndrome, fractalkine, salivary glands, germinal centres.

Introduction

Primary SS is an autoimmune disease characterized by chronic focal lymphocytic inflammation and destruction of acinar tissue within the lacrimal and salivary glands [1]. It has been demonstrated that most patients with SS exhibit, within the inflamed salivary glands, lymphoid-like structures characterized by the formation of ectopic germinal centres (GCs) [2]. These follicle-like structures are characterized by the presence of T and B cell segregation, follicular dendritic cell (FDC) networks and the formation of CD21+ GC structures. The functional relevance of ectopic GC in SS is due to the fact that somatic hypermutation and class switch recombination occur within the centres together with the aberrant production of autoantibody-producing plasma cells [3]. Evidence has emerged providing support for the in situ production of autoantibodies and there are data in the literature...
suggesting a pathogenetic link between the formation of ectopic GCs and the expansion of B cell clones related to the development of lymphoproliferative disorders such as the mucosa-associated lymphoid tissue (MALT) type B cell lymphomas [1], known to influence morbidity and mortality in SS patients.

The process of lymphocyte recruitment and organization within the salivary glands is actively regulated by the expression of chemokines (CKs). In particular, CXCL13, CCL21, CXCL12 and CCL19 have been demonstrated as critical for their chemoattractive capacity towards immune cells and for maintenance of the organization [4, 5].

Fractalkine (CX3CL1) is the sole member of the CX3C CK family, and its expression is described in several tissues [6-17]. CX3CL1 has been associated with the pathogenesis of RA after CX3CL1 and CX3CR1 expression was reported to be up-regulated in the synovium of patients with RA [18-20]. Supporting the notion of the disease-modulating activity of fractalkine in RA, administration of anti-CX3CL1 antibodies ameliorated experimental RA [21, 22]. Data in the literature report aberrant proteolytic activity in the salivary glands of non-obese diabetic mice, with generation of a unique CX3CL1 fragment that may contribute to autoimmunity [23, 24]. Studies in human salivary glands show that CX3CL1 is expressed in glandular epithelium as well as ducal structures [17, 25].

Very compelling data further highlight a central role for CX3CL1 in the chemotraction of monocyte precursors of tissue dendritic cells (DCs) that express CX3CR1 [9, 22, 26, 27]. Moreover, it has been demonstrated that this CK is involved in B cell trafficking in human secondary lymphoid organs (SLOs) [28]. It is known that the development of tertiary lymphoid organs (TLOs) in salivary glands of SS patients is a major characteristic of the disease and is strongly dependent upon the expression of several CKs, similar to SLOs [4, 5]. Given the key role played by DCs in the initiation of immune responses and in the development of TSOs, and since their accumulation is one of the early events in SS [1, 2, 5, 29-31], it is possible to hypothesize that CX3CL1 and CX3CR1 could be regulators in the development of TLOs.

The aim of this study is to evaluate the presence of this CK in sera from SS patients, to assess the cellular source(s) in SS salivary glands, to identify the target cells expressing its receptor CX3CR1 and to detect its mRNA expression levels in tissue.

Materials and methods

Patients and samples

This study received Sapienza University of Rome ethics committee approval that covered both blood and biopsy samples, and all patients and healthy controls (HCs) gave written informed consent. Thirty-two consecutive patients were recruited from the SS outpatient clinic of the Rheumatology Division. Twenty-one patients with primary SS (19 satisfying the American-European Consensus Group criteria for SS and 2 satisfying the European Consensus Group criteria) [32, 33] together with 11 patients with Sicca syndrome (Sicca) not satisfying any criteria for SS were enrolled. The control group included 20 age- and sex-matched RA patients and 10 HCs recruited during the same period. RA patients were recruited in the Rheumatology outpatient clinic of the same department. The diagnosis of RA was made according to the 1987 ACR classification criteria [34]. Blood samples were drawn and sera stored for analysis. Table 1 shows the clinical features of subjects enrolled in the study. ANA, anti-SSA/Ro, anti-SSB/La, RF and complete clinical assessments were performed in all patients. An indirect IF procedure using Hep-2 cell substrates was employed to detect the presence and titre of ANA. Titres >1:160 in at least two consecutive determinations were considered positive. RF was assayed using the quantitative immunonephelometry test (Behring, Marburg, Germany) and was considered positive when the concentration was higher than the cut-off value of the kit (15 IU/ml). Anti-SSA/Ro and anti-SSB/La were detected by ELISA. Each assay was performed in duplicate. All SS and Sicca patients were subjected to Schirmer I, tear breakup time (BUT) and ocular dye (fluorescein and lissamine green) tests to assess the presence of keratoconjunctivitis sicca (KCS).

Minor salivary glands were excised through the mucosa of the lower lip in all SS and Sicca patients for a total of 32 biopsies. Samples were matched with sera from each patient. The focus score was determined on the basis of the number of inflammatory cell aggregates containing >50 lymphocytes/4 mm² of salivary gland tissue [4, 35]. Salivary gland biopsies taken for histology and gene analysis were divided in equal parts. One part was embedded in paraffin for immunohistochemistry and the other was frozen in RNALater for RT-PCR analysis.

Measurement of human CX3CL1

The protein was measured using the Quantikine Human CX3CL1/fractalkine assay (R&D Systems), which is a double sandwich polyclonal enzyme immunoassay. Assay diluent RD1-88 was added to each well (100 µl) and within 15 min 100 µl of standard, control or sample were also added and incubated for 3 h at 2-8 °C. Each well was washed with Wash Buffer (400 µl) four times. Cold fractalkine conjugate (200 µl) was then added to each well and incubated for 1 h at 2-8 °C. The aspiration/wash step was repeated, 200 µl of substrate solution were added to each well and an incubation of 30 min was carried out at room temperature, protecting from light. Stop solution (50 µl) was added to each well. To determine the optical density of each well, a microplate reader was set to 450 nm. Each sample was analysed in duplicate.

Histological characterization

Antibodies

A list of primary and secondary antibodies is reported in Table 2.
Histological evaluation of CX3CL1, CX3CR1, CD20, CD3 and CD21

Staining for CX3CL1 and CX3CR1 was performed on sequential sections of salivary glands from SS and Sicca patients in order to evaluate the localization of cells expressing these CKs. Such stainings were performed in tonsil tissue as positive controls. Paraffin-embedded sections measuring 3 μm in thickness were dewaxed and rehydrated. All sections underwent high-temperature antigen retrieval using citrate buffer solution (pH 6.0) for 45 min at 95°C (Dako, Cambridge, UK), except for sections stained with CD21, which was retrieved by proteolytic digestion for 5 min at 37°C using proteinase K (Dako, Cambridge, UK). Peroxidase and protein blocking followed (Dako, Cambridge, UK). Sections were stained using the primary antibodies CX3CL1, CX3CR1 and CD21 overnight. CD20 and CD3 antibodies were incubated for 1 h at room temperature.

Sections were rinsed in PBS and incubated for 1 h at room temperature with the appropriate biotinylated secondary antibody (see Table 2). Slides were then washed and DAPI was incubated for 15 min before finally mounting with Mowiol.

Real-time PCR analysis

Total RNA was extracted from tissue using the PureLink RNA Mini Kit (Ambion, Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. On-column DNase digestion was performed on the bench top for 15 min. One microlitre of RNA was reverse transcribed using the Taqman Reverse Transcription Kit (Applied Biosystems, UK). cDNA was then amplified using real-time PCR using the LightCycler system (Roche Diagnostics, Rotkreuz, Switzerland) with specific primers and probes (see Table 2).

### Table 1: Demographic and laboratory characteristics of the SS, Sicca and RA patients enrolled in this study

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>Sicca</th>
<th>RA</th>
<th>HCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>21</td>
<td>11</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Female gender, n</td>
<td>21</td>
<td>11</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Age, mean (range), years</td>
<td>52.6 (37–69)</td>
<td>52 (21–72)</td>
<td>54.8 (40–68)</td>
<td>47.5 (30–65)</td>
</tr>
<tr>
<td>Xerophthalmia, %</td>
<td>95</td>
<td>90</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Xerostomia, %</td>
<td>95</td>
<td>90</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KCS, %</td>
<td>93.1</td>
<td>54</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RF, %</td>
<td>33.3</td>
<td>0</td>
<td>80.2</td>
<td>—</td>
</tr>
<tr>
<td>ANA, %</td>
<td>85.7</td>
<td>27.2</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Anti-SSA and/or SSB, %</td>
<td>23.8</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fractalkine serum protein, %</td>
<td>66.6</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Anti-fractalkine antibodies, %</td>
<td>23.8</td>
<td>0</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Histology score (0–1–2–3), %</td>
<td>4.7–66.6–19–9.5</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

KCS: presence of keratoconjunctivitis sicca as determined by the ophthalmology and by Schirmer, break-up-time and dye tests.

### Table 2: Material used for immunohistochemistry and RT-PCR

**Antibodies**

<table>
<thead>
<tr>
<th>Clone/name</th>
<th>Specificity</th>
<th>Host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-26</td>
<td>Human CD20</td>
<td>Mouse/rabbit</td>
<td>Dako, Cambridge, UK</td>
</tr>
<tr>
<td></td>
<td>Human CD3</td>
<td>Mouse/rabbit</td>
<td>Dako, Cambridge, UK</td>
</tr>
<tr>
<td>1F8</td>
<td>Human CD21</td>
<td>Mouse</td>
<td>Dako, Cambridge, UK</td>
</tr>
<tr>
<td></td>
<td>Human CX3CL1</td>
<td>Mouse</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td></td>
<td>Human CX3CR1</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

| Goat anti-mouse | Alexa<sup>a</sup> | Goat | Dako Cytomation |
| Goat anti-rabbit| Alexa<sup>a</sup> | Goat | Dako Cytomation |

**Genes, specific primers and probes**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>RefSeq</th>
<th>Gene expression assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CX3CL1</td>
<td>NM_002996.3</td>
<td>Hs00171086_m1</td>
</tr>
<tr>
<td>Human CX3CR1</td>
<td>NM_001171171</td>
<td>Hs0192583_s1</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>NM_001256799</td>
<td>Hs03929097_g1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Both red and green fluorochromes. Primary and secondary antibodies used for immunohistochemistry. Genes, specific primers and probes used for RT-PCR.
transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). For quantitative TaqMan real-time evaluation of mRNA expression levels of human genes, sequence-specific primers and probes from Applied Biosystems were used (Table 2). Gene expression was evaluated for CX3CL1 and CX3CR1. The real-time PCR were run in triplicate using the ABI PRISM 7900HT instrument and results were analysed after 40 cycles of amplification using the ABI Sequence Detection System version 2.1 (SDS 2.1). Relative quantification was measured using the comparative Ct (threshold cycle) method. An endogenous control [human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] was used to normalize for the cDNA of each sample.

Western blot for CX3CL1 antibodies
Western blotting experiments were performed to evaluate the presence of CX3CL1 autoantibodies in the sera of patients and control subjects. For this purpose, for each serum we prepared nitrocellulose filter strips containing 0.5 μg of human recombinant CX3CL1 protein (Abcam 8.5 kDa) and 5 μl of HyperPAGE pre-stained protein marker (BioLine), both eluted with 2× SDS sample buffer, resolved by 15% SDS-PAGE, and then transferred electrophoretically to the filters. After blocking non-specific reactivity, the filter strips were probed overnight with serum from each patient and control subjects or with a primary anti-CX3CL1 antibody used as positive control. Anti-rabbit or anti-mouse IgG ECL antibodies were used to detect immunoreactivity using an enhanced chemiluminescence detection kit (Amersham Biosciences, Italy). If antibodies were present in the sera, a black band was observed on the film at 8.5 kDa.

Statistical analysis
Differences in quantitative variables were analysed by the Mann–Whitney U test when comparing two groups and by the Kruskal–Wallis with Dunn’s post-test when comparing multiple groups. The χ² test with Yates’s correction when required or Fisher’s exact test when appropriate were used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, La Jolla, CA, USA). A P-value < 0.05 was considered statistically significant.

Results
Circulating levels of CX3CL1 protein
We evaluated the expression of CX3CL1 in the sera of SS, Sicca and RA patients and HCs (Fig. 1A). Sixty-six per cent (n = 14) of SS patients showed the presence of CX3CL1 protein (values > 2.5 ng/ml were considered positive). Increased serum levels of CX3CL1 protein were observed in SS patients compared with controls (P < 0.0001) and in RA patients compared with controls (P < 0.0001), but no difference was found between Sicca patients and controls (P = 0.22). CX3CL1 protein levels in Sicca sera were comparable to those found in HCs. CX3CL1 serum levels in SS patients were also significantly higher compared with RA patients (P < 0.0001). Interestingly, the level of CX3CL1 increased in patients...

Fig. 1 CX3CL1 expression in sera
(A) Human CX3CL1/fractalkine assay (R&D Systems). Quantitative measurement of human CX3CL1 protein in serum. Increased levels of CX3CL1 protein were observed in SS patients compared with healthy controls (HCs) (P < 0.0001) and Sicca patients (P < 0.0001). RA patients were considered positive controls and CX3CL1 protein was raised in the serum compared with HCs (P < 0.0001). A significant difference was also found between SS and RA levels of CX3CL1 in the sera (P < 0.0001). No difference was found between Sicca patients and HCs. SICCA: Sicca Syndrome. (B) Correlation between CX3CL1 serum levels and the histology focus score (P < 0.0001, r = 0.92).
with higher histological scores in salivary glands (Fig. 1B). A direct correlation to the infiltrate grade was found ($P < 0.0001$, $r = 0.92$).

**Histological characterization of CX3CL1 and CX3CR1 in salivary glands of SS and Sicca patients**

In order to localize CX3CL1- and CX3CR1-expressing cells, we performed IF staining on the salivary glands of SS and Sicca patients. Tonsil tissue was stained to first localize the CKs within the SLO setting, as shown in Fig. 2A-F. Clearly CX3CL1 could be detected in the tonsil CD20$^+$ B cell area (Fig. 2A and B) but not in the CD3$^+$ T cell area (Fig. 2C). In contrast to the diffuse expression of CX3CL1, CX3CR1 could only be detected in a less expanded area within the CD20$^+$ B cell area (Fig. 2D and E). Cells expressing CX3CR1 were also not found in the CD3$^+$ T cell area of the tonsil (Fig. 2F). The same stainings were performed on salivary gland sections from SS and Sicca patients (Fig. 3). CX3CL1 staining in SS salivary gland infiltrates could be detected diffusely within the infiltrates (Fig. 3A-C). Double staining showed that CX3CL1$^+$ cells were present in the CD20$^+$ B cell area (Fig. 3A and B) and also partially in a CD20$^-$ area of the infiltrate (Fig. 3B). Staining with CD3/CX3CL1 showed no double-stained cells in the T cell portion of the infiltrate (Fig. 3C). Sicca salivary glands were negative for CX3CL1 (Fig. 3D). CX3CR1 was also expressed throughout the infiltrates, but with a diverse distribution (Fig. 3E-G). In contrast to CX3CL1, CX3CR1$^+$ cells were not detected in the CD20$^+$ B cell area of the infiltrate and were seen mostly in the CD3$^+$ T cell area (Fig. 3F and G). Stainings in salivary glands from Sicca samples did not show CX3CR1 staining (Fig. 3H).

**CX3CL1 and CX3CR1 expression within the GCs in SS salivary gland inflammatory infiltrates**

Sequential sections of SS salivary gland samples with GCs were stained for CD20, CD21, CX3CL1 and CX3CR1 (Fig. 3I-L). Consistent with data in the literature, we detected a CD20$^+$ B cell area within the inflammatory infiltrate (Fig. 3I). CD21 is a specific marker for FDCs localized within the GCs. A sequential section stained for CD21 identified a GC within the B cell area of the infiltrate (Fig. 3J). Further sequential sections showed how CD21$^+$ cells within a GC were also CX3CL1$^+$ and CX3CR1$^+$ (Fig. 3J-L). CX3CR1$^+$ cells could be also detected in the CD20$^-$ area of the infiltrate (Fig. 3I and K), consistent with the CX3CR1/CD20 and CX3CR1/CD3 double stainings (Fig. 3F and G) that identified CX3CR1$^+$ cells mostly in the CD3$^+$ area. Interestingly staining on tonsil sections found CX3CR1 only in the CD20$^+$ B cell area, but not in the CD3$^+$ T cell area (Fig. 2E and F).

**Evaluation of CX3CL1 and CX3CR1 gene expression and correlation with the focus score**

In this study we examined whether CX3CL1 and CX3CR1 mRNA gene expression could be detected in tissue from salivary gland biopsy specimens from SS patients. As
shown in Fig. 4A and B, the CK and its receptor were both up-regulated in SS salivary glands as compared with Sicca salivary glands. These data are consistent with the serum protein detection. Interestingly, the highest expression level of CX3CL1 and CX3CR1 mRNA was observed in the salivary glands with higher histological scores (Fig. 4C and D). In particular, CX3CR1 mRNA gene expression was strongly increased in grade 3 samples. This trend parallels the increase in levels of CX3CL1 in serum.

CX3CL1 autoantibodies

Finally, we evaluated the presence of autoantibodies against CX3CL1 (data not shown, but the results are available as supplementary data; see supplementary Fig. S1, available at Rheumatology Online). Anti-CX3CL1 antibodies were detected in five patients with SS (23.8%); conversely, no patient with Sicca nor any HC showed positivity for these autoantibodies as determined by western blot. The five SS patients with anti-CX3CL1 antibodies showed high titre ANA, and four of them were also positive for anti-SSA and/or SSB.

Correlation with clinical and laboratory data

We evaluated a possible correlation between the expression (presence vs absence of the protein) of CX3CL1 in sera and the clinical data of all our patients. No statistically significant difference was observed.

Of the SS fractalkine-positive patients \( (n = 14) \), 85.7% \( (n = 12) \) were positive for ANA, 50% \( (n = 7) \) were positive for RF and 35.7% \( (n = 5) \) were positive for SSA and/or SSB. Significantly, none of the SS fractalkine-negative patients had RF or ANA in their sera. No relevant difference was found in the presence of ANA between the two groups.

Discussion

SS is an autoimmune disorder that affects salivary and lacrimal glands \([1, 4, 5, 36–38]\). Cellular effectors of the immune system gather in the target organs and develop the common features of chronic inflammatory diseases \([39]\). It is known that tissues that harbour the target antigen(s) of chronic immune responses are infiltrated mainly by T cells and macrophages, but also by DCs, B cells and
plasma cells. These cellular elements organize themselves anatomically and functionally as in SLOs, leading to the de novo formation of B cell follicles and T cell areas [39]. This phenomenon has been termed lymphoid neogenesis or TLO formation [2]. B and T cell priming, clonal expansion, antigen retention, somatic hypermutation, affinity maturation, immunoglobulin class switching, B cell-receptor revision and maintenance of peripheral tolerance are crucial processes that take place in SLOs and can also occur in TLOs, contributing to the development and exacerbation of chronic inflammatory diseases. Ectopic lymphoneogenesis has been described in diverse chronic inflammatory conditions such as RA [40, 41], type 1 diabetes [42] and autoimmune thyroiditis [43] and is often related to the development of autoantibodies. It has been proven that in SS, ectopic lymphoid tissues acquire specific features such as the development of FDC networks and the expression of lymphoid CKs (CXCL13, CCL19 and CCL21) [4, 5]. Both CCL21 and CXCL13 are involved in the regulation of the progressive organization and maintenance of periductal foci in salivary gland TLOs [5]. The coordinated signal of CCL21 and CXCL12 through their receptors CCR7 and CXCR4 mediates the ingress of naive lymphocytes through high endothelial venules (HEVs) [4]. CXCL13 is strongly and selectively expressed by FDCs and mediates the homing of B cells carrying the CK receptor CXCR5 [44]. Furthermore, a higher degree of lymphoid organization was significantly related to increased expression of CXCL13 within SS salivary gland infiltrating cells and peripheral node addressins (PNAd+) HEV-associated CCL21-producing cells [4]. Together these data support a role for ectopic lympho-neogenesis and therefore for the ectopic expression of lymphoid CKs in the pathogenic events leading to GC formation and functioning in SS salivary glands [1, 45–47]. Also, specific autoantibodies (SS/Ro and SS/La) have been discovered and are well-known tools in the diagnosis of SS [32, 48]. However, it is likely that these are not the only antigens contributing to the autoimmune process behind SS development.

CX3CL1, the sole member of the CX3C CK family, is known to be involved in B cell trafficking in human SLOs [20]. The CK is responsible for regulating cytokine secretion from macrophages and other inflammatory cells [17, 28], playing a central role in modulating and directing the immune response. CX3CL1 is also expressed on endothelial cells, and this is a further demonstration of its role in recruiting inflammatory cells from blood vessels.
[11, 26, 49]. Given the well-established role played by CKs in the development of TLOs, we hypothesize that CX3CL1 and its receptor CX3CR1 could be regulators in the development of TLOs in salivary gland infiltrates.

In this study we proved that CX3CL1 can be identified in the sera of SS patients and we provide the first in-depth demonstration that the inflammatory infiltrates within salivary glands of SS patients express CX3CL1 and CX3CR1. First, using a specific ELISA, we showed increased serum levels of CX3CL1 protein in SS patients compared with HCs and Sicca patients, who do not have inflammatory infiltrates and GCs in their salivary glands. Furthermore, we found CX3CL1 protein levels to be higher in SS compared with RA patients, who do have an organ-specific autoimmune disease. Consistently CX3CL1 protein levels in Sicca patients were comparable to those found in HCs. The correlation between CX3CL1 and tissue infiltrates was strengthened by the progressive increase of protein levels with higher histological scores.

The importance of these findings was further supported by the evidence of salivary glands from SS patients clearly expressing CX3CL1 and CX3CR1 at tissue level. We first identified both CKs in SLOs (tonsil tissue). At this level, CX3CL1 and CX3CR1 were only clearly detected in the CD20+/CD3- B cell area.

When staining salivary glands, these molecules were evidently localised within the inflammatory infiltrates, and the Sicca samples were negative for both markers. In SS salivary glands, similar to what we demonstrated in the tonsil tissue, CX3CL1 was found in the CD20+ area but not in the CD3+ area. Nevertheless, we could detect a CD20+/CD3-/CX3CL1+ subset of cells (Fig. 3B) that might be identified with dark-zone cells [50]. On the other hand, CX3CR1 staining in SS salivary glands was mostly identified in the CD3+ part of the infiltrate.

Not only could we determine the presence of both CX3CL1 and CX3CR1 in the inflammatory infiltrates from SS salivary glands, but we also demonstrated how the molecules are expressed within GCs. We could in fact show co-localization of CX3CL1+, CX3CR1+ and CD21+ cells. CD21 is a marker for FDCs and it is known that the most important reactions leading to the activation of B cells in GCs are driven by FDCs [1]. We believe that these data prove that CX3CL1 and CX3CR1 are closely involved in GC reactions. Our staining suggests also that there are different subsets of CX3CL1+ cells within SS salivary glands: a CX3CL1+/CD20+/CD21- light-zone cell, a CX3CL1+/CD20-/CD3- dark-zone cell and a CX3CL1+/CD20+/CD21+ FDC network cell. Furthermore, we identified two subsets of CX3CR1+ cells within SS salivary glands: one subset being CX3CR1+/CD3- consistent with T cells and the other being CX3CR1+/CD21+ within the GC [9, 15, 16, 18, 22, 26, 27]. These findings suggest that these CKs are strongly involved in tertiary lymphoid structures in SS salivary glands and in GC reactions.

Importantly, we showed that the increased levels of CX3CL1 in SS sera are paralleled by the in situ overexpression of CX3CL1 and CX3CR1 genes. In particular, we clearly showed that the mRNA expression of these CKs was significantly higher in SS salivary glands compared with Sicca tissues. In keeping with this evidence, we showed that the gene expression clearly follows the same increasing trend as the serum protein levels when correlated with the histological score.

Finally, we detected anti-CX3CL1 antibodies in a small percentage of patients expressing the protein in their sera, causing us to speculate that not only may CX3CL1 be one of the regulators of ectopic lymphoneogenesis in SS, but its up-regulation might also induce an antigenic reaction in the disease autoimmune context.

This is the first demonstration of an involvement of CX3CL1 and CX3CR1 in the ectopic lymphoid tissue of SS salivary glands. In what way CX3CL1 and CX3CR1 may act within SS salivary gland infiltrates remains to be elucidated. Overall, our results provide strong evidence that CX3CL1 and CX3CR1 are involved in SS ectopic lymphoid structures and that future studies on a larger sample of patients may find new diagnostic markers for this disease.

**Rheumatology key messages**

- Circulating CX3CL1 is increased in SS patients with direct correlation to the infiltrate grade.
- CX3CL1 and CX3CR1 expression is found in salivary gland tissue at both a histological and mRNA level in SS patients.

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**Disclosure statement:** The authors have declared no conflicts of interest.

**Supplementary data**

Supplementary data are available at *Rheumatology* Online.

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