# Role of IL-22 pathway in primary Sjogren's Syndrome

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## Introduction

Primary Sjogren's syndrome (pSS) is a chronic inflammatory disorder that affects the exocrine glands, particularly the salivary and lacrimal glands. pSS is a common systemic autoimmune disorder, affecting approximately 0.1–0.4% of the general population with a female to male ratio of 9:1 [1]. Focal, mononuclear cell infiltrates surround the ducts and replace the secretory units leading to xerostomia and xerophthalmia. In addition, it is estimated that people with pSS are 44 times more likely to develop non-Hodgkin's lymphoma than those without pSS [2-4]. During the past 15 years, the importance of epithelial cell in the pathogenesis and evolution of SS has been highlighted and has prompted the use of the term 'autoimmune epithelitis' as an alternative name for the disease [5].

The mechanisms initiating or maintaining the chronic and persistent immunological activation involved in the pathogenesis of SS are poorly understood.

The pathogenesis of pSS is considered to be multifactorial. Although research on pSS has been mainly focused on adaptive immunity, the role of innate immunity has been recently highlighted by several studies.

Among innate immune cells an important role of NK cells in influencing adaptive immune responses has been demonstrated in pSS and immunological interactions between epithelial cells and NK cells seem to provide an intriguing immunological link between innate and adaptive immunity [6].

Also iNKT are considered a hybrid between innate and adoptive immunity that could play a role in the pathogenesis of autoimmune disorders. They may play both protective and harmful roles in the progression of certain autoimmune diseases, such as diabetes, lupus, atherosclerosis, and allergen-induced asthma [7-9].

The histological hallmarks of pSS are focal periductal lymphocytic infiltration of the affected exocrine glands, with up-regulation of Th1 and Th17 cytokine expression as well as B-lymphocyte hyperactivity. Although Th17 cells were thought to be the most important cell type involved in IL-17 and IL-22 responses, several studies indicate that IL-17 in humans is produced by a broad spectrum of cells of the innate and adaptive immune systems with different cell types producing IL-17 and IL-22 at different sites and phases of tissue inflammation [10-13]. IL-23-Th17 pathway plays a central role in autoimmunity and chronic inflammation, once attributed mainly to Th1 cells, and the strong

production of IL-22 by this subset of auto-reactive cells further highlights their role in the pathogenesis of autoimmune disorders [14]. In addition to Th17 cells, a subset of innate lymphoid cells was demonstrated able to strongly produce IL-22 [15].

Salivary gland epithelial cells of patients with pSS are also thought to play an important pathogenetic role in the initiation, regulation, and resolution of immune responses, as suggested by the increased epithelial expression of several inflammatory cytokines and chemokines [16-18]. Recently the IL-22/IL-22R1 pathway has been demonstrated to be critically important at epithelial surfaces performing immune-regulatory properties in infection, chronic inflammatory and autoimmune disease, and cancer [19-22]. The current immunological paradigm of IL-22 contemplates that IL-22 is produced by immune cells, including T-helper (Th) cell subsets and an emerging category of innate lymphoid cells, but it acts only on epithelial cells [23-25]. Interestingly, in chronic inflammatory disorders IL-22 may act either as a protective or pro-inflammatory cytokine depending on the immunologic context in which it is expressed [26]. In pSS IL-22 is up-regulated and produced by innate lymphoid cells as well as by T cells and ductal epithelial cells and is expressed together with other pro-inflammatory cytokines, being

presumably involved in the pathogenesis of salivary gland inflammation [27].

IL-22, a member of the IL-10 cytokine family, is a key player in immune surveillance, inflammation, and tissue homeostasis [28-29]. IL-22 mediates its effects via a heterodimeric trans-membrane receptor complex consisting of two subunits IL-22R1 and IL-10R2. In physiologic conditions IL-22 does not play a role in immune cells cross-talk. In fact, unlike the IL-10R2 which is constitutively expressed in many human tissues such as skin, pancreas, small intestine, colon, liver, lung, and kidney intestinal epithelial cells and hepatocytes, IL-22R1 is not detectable on the surface of immune cells [30]. Binding of IL-22 to its cognate induces STAT3 phosphorylation and to a lesser extent STAT1 and STAT5 phosphorylation, as well as Akt and mitogen-activated protein kinase (MAPK) pathways activation [31-32]. The functional outcome of IL-22 seems to be controlled by the release of the IL-22 binding protein (IL-22BP), also known as cytokine receptor family (CRF) 2-10, CRF2-X, and IL-22RA2 [33]. IL-22BP is a secreted glycoprotein belonging to the type II cytokine receptor family that antagonizes IL-22 activity by specifically binding IL-22 with high affinity and blocking its interaction with the cell surface IL-22R. Although originally thought as a

Th17 cytokine, IL-22 is produced by a broad spectrum of cells both of the adaptive and the innate immune system including CD4+ T cells (Th1, Th17, Th22), $\gamma\delta$ T cells, natural killer T (NKT) cells, lymphoid tissue inducer (LTi) cells, and NK cell subsets [23-25]. Cytokines driving IL-22 expression include IL-23, IL-6, and IL-1$\beta$. At mucosal sites IL-22 is mainly produced by Th1, Th17 and Th22 subsets of CD4^{+}T effector cells and by a subset of mucosal CD3-CD56+NKp44+ (namely NK22 cells) [21-22]. Long considered a subset of NK cells, these IL-23 responsive, IL-22 producing NK22 cells have been recently demonstrated to be developmentally and functionally related to lymphoid tissue inducer cells (LTi) [15]. Both NK and LTi cells are the prototypical innate lymphoid cells (ILC). Recently, several distinct ILC population have been identified that, similarly to NK cells and LTi cells, depend on $\gamma_c$ and ID2 for their development. Type 1 ILCs are defined by their capability to produce interferon-$\gamma$. Type 2 ILCs are able to produce T helper 2 cell-associated cytokines, including IL-5 and IL-13. Type 3 ILCs are capable of producing the Th17 cell-associated cytokines and IL-22 [15].

IL-22 signaling may promote either protective functions at mucosal surfaces or chronic inflammation and dysregulation of IL-22/IL-22R1/IL-22BP pathway seems to play also a role in the pathogenesis of colon

cancer and T and B lymphomas. IL-22 activates pro-inflammatory epithelial defense mechanisms by promoting epithelial proliferation, inducing the expression of antimicrobial and protective proteins (such as β-defensins, RegIIIb and RegIIIc and mucins) [19, 30, 34-37] and the production of pro-inflammatory cytokines and chemokines (e.g IL-6, CXCL1, CXCL5, and CXCL9) [20, 36-37]. These defense mechanisms may cause tissue damage when activation of IL-22 becomes uncontrolled.

While the role of IL-23/IL-17 is well described in pSS, whether or not IL-22 and IL-22-producing cells may be implicated in the local immune response observed in the salivary glands of patients with SS is however still unknown.

The aim of the first part of this thesis was to investigate IL-22 expression and the IL-22 cellular sources in inflamed salivary glands of patients with pSS. Since IL-22 is a downstream effector cytokine of IL-23 and the final outcome of IL-22 seems to be determined by the coexpression of IL-17 [26] I have also evaluated the expression of IL-23 and IL-17. Finally, since studies investigating IL-22-induced signal transduction in cells with endogenous receptor expression demonstrated tyrosine phosphorylation of STAT3 [38], I have also evaluated the expression of phosphorylated STAT3 (p-STAT3).

In light of my findings, I have then studied the expression of IL-22R1 and IL-22BP in the salivary glands and peripheral blood mononuclear cells of p-SS and in p-SS-associated MALT lymphoma. I have also evaluated the expression pattern of IL-22R1 in other autoimmune disease such as systemic lupus eythematosus (SLE) and rheumatoid arthritis (RA) patients.

In the last part of this study I have analysed the effect of anti-CD20 monoclonal antibody (mAb) therapy on lymphocytic infiltrate and cytokines expression, on the grounds, that Rituximab whihch is currently used for the treatment of B-cell lymphoma [39], has also been considered effective in the therapy of pSS.

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## Chapter 1

## Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjögren's syndrome

Several cytokines and chemokines are increased in the peripheral blood and in the inflamed salivary glands [1-3]. A major contribution of B cells in the pathophysiology of pSS through the production of autoantibodies and cytokines [4-6] and the constitution of germinal centres in the salivary glands has been demonstrated [7]. In addition to B lymphocytes, T lymphocytes are also involved in the local immune response observed in patients with pSS, mainly through the activation of the Th1 and Th17 pathways [8-10]. In fact, the presence of Th1- and Th17-related cytokines has been demonstrated in salivary glands from patients with pSS in terms of mRNA and protein expression[8-10]. Interestingly, both Th1 and Th17 cells express interleukin (IL)-22 that is known to play a role in the pathogenesis of T cell-mediated inflammatory disorders [11].

IL-22, a member of the IL-10 cytokine family, is well known as a key player in immune surveillance, inflammation and tissue homeostasis [12-13]. At mucosal sites, IL-22 is mainly produced by CD4$^{+}$ T cells and by a subset of mucosal natural killer (NK) cells expressing the receptor

NKp44 (namely, NKp44^{+} NK cells) [14]. In chronic inflammatory disorders, IL-22 may act either as a protective or as a pro-inflammatory cytokine depending on the coexpression of IL-17 [15]. Interestingly, high levels of IL-22, directly correlated with hyposalivation, anti-SSA and/or anti-SSB antibodies, hypergammaglobulinaemia and rheumatoid factor, have been recently demonstrated in the sera of patients with pSS, suggesting a critical direct role of IL-22 in the development of SS [16]. However, it is still unknown if IL-22 and IL-22-producing cells may be implicated in the local immune response in the salivary glands of patients with SS.

The aim of the present study was to investigate IL-22 expression and the IL-22 cellular sources in inflamed salivary glands of patients with pSS. Since IL-22 is a downstream effector cytokine of IL-23 and the final outcome of IL-22 seems to be determined by the coexpression of IL-17 [15], we have also evaluated the expression of IL-23 and IL-17. Finally, since studies investigating IL-22-induced signal transduction in cells with endogenous receptor expression demonstrated tyrosine phosphorylation of STAT3 [17], we have also evaluated the expression of phosphorylated STAT3 (p-STAT3).

## Materials and methods

### Patients

Thirty-five patients with subjective complaints of dry mouth or dry eyes who were referred to our University Hospital outpatient clinic were considered for this study. Nineteen patients met the American–European Consensus Group criteria for pSS [18]. The remaining 16 patients were classified as having non-specific chronic sialoadenitis (nSS). Minor salivary gland biopsies from the last patients showed various degrees of mononuclear cell infiltration in the absence of focal organisation and did not fulfil the American–European Consensus Group criteria for SS. The presence of other underlying autoimmune diseases or hepatitis C virus infection was carefully excluded. All patients and controls underwent serological evaluations, which included tests for the presence of antinuclear antibodies, anti-SSA/Ro, anti-SSB/La, rheumatoid factor, levels of C reactive protein and erythrocyte sedimentation rate. An unstimulated whole sialometry was also performed. The characteristics of patients and controls are shown in Table 1.

**Table 1**

Baseline characteristics of patients and controls

|  | pSS (n=19) | nSS (n=16) | p Value |
|---|---|---|---|
| Age (years (range)) | 58 (31–70) | 62 (28–76) | NS |
| Female sex (n (%)) | 16 (84) | 12 (75) | NS |
| Disease duration (months (range)) | 80.8 (4–280) | 77 (6–320) | NS |
| Antinuclear antibodies (number of positive patients) | 16 | 2 | <0.001 |
| Anti-SSA and/or anti-SSB antibodies (number of positive patients) | 12 | 1 | <0.001 |
| Rheumatoid factor (number of positive patients) | 11 | 1 | – |
| ESR (mm/h (mean (SD))) | 40 (13) | 11.8 (6) | <0.001 |
| C reactive protein (mg/l (mean (SD))) | 16.6 (6) | 9 (3) | <0.05 |

ESR, erythrocyte sedimentation rate; nSS, non-specific chronic sialoadenitis; pSS, primary Sjögren's syndrome.

**Labial salivary gland biopsy**

Up to 10 labial salivary gland biopsies were obtained after informed consent, during routine diagnostic procedures. Of these biopsies, four were embedded in paraffin and used for histopathological analysis and immunohistochemistry (IHC), and two were used for mRNA isolation and real-time (RT)-PCR analysis. When available, the remaining biopsies were placed into Dulbecco's modified Eagle's medium for cell isolation and flow cytometry analysis.

**Histological evaluation**

Standard paraffin sections at 5 μm thickness were stained with H&E. Histological evaluation of salivary glands for the presence of lymphocytic

infiltrates and/or foci was performed according to Greenspan and coworkers [19]. A focus was defined as an aggregate of ≥50 lymphocytes. The focus score was reported as the number of foci per 4 $mm^2$ of tissue, up to a maximum of 12 foci. All patients with pSS presented a biopsy focus score >1, whereas the control group had a focus score <1. On the basis of the histological evaluation, we identified in patients with pSS a median biopsy focus score of 5.37 (range 2–11), while no foci were observed in patients with nSS.

**RNA isolation and quantitative RT-PCR**

Salivary gland biopsies soon after removal were stored in RNAlater solution (Applied Biosystems, Foster City, California, USA). Each sample was lysed in a tissue homogeniser, and RNA was extracted using the commercially available illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples were stored at −20°C until use. For quantitative TaqMan RT-PCR, master mix and TaqMan gene expression assays for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) control for IL-17 (Hs00174383_m1), IL-22 (Hs01574154_m1), IL-23 (Hs00372324_m1)

and STAT3 (Hs00234174_m1) were obtained from Applied Biosystems. Samples were run in triplicate using the Step-One RT-PCR System (Applied Biosystems). Relative changes in gene expression between nSS and pSS samples were determined using the $\Delta\Delta C_t$ method. Levels of the target transcript were normalised to a GAPDH endogenous control, constantly expressed in both groups ($\Delta C_t$). For $\Delta\Delta C_t$ values, additional subtractions were performed between pSS samples and nSS $\Delta C_t$ values. Final values were expressed as fold of induction.

**Immunohistochemical staining for IL-17, IL-22, IL-23, p-STAT3 and NKp44**

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. IHC for IL-17, IL-22, IL-23, p-STAT3 and NKp44 was performed on 5-μm-thick paraffin-embedded sections from salivary glands and from tonsils and lymph node (used as positive controls) as previously described [20]. Isotype-matched irrelevant antibodies were used as a negative control. Briefly, following rehydration, antigen was unmasked for 45 min in a 95°C microwave using Dako Target Retrieval Solution (pH 6; Dako, Carpinteria, California, USA). Endogenous peroxidase was blocked for 10 min with Dako peroxidase blocking reagent, and non-specific binding was blocked for 20 min with Dako

protein block. The primary antibodies rabbit anti-uman IL-17 (IgG, sc7927, 1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit anti-human IL-22 (IgG, NB100–737, 1:100 dilution) (Novus Biologicals, Littleton, Colorado, USA), mouse anti-human IL-23p19 (IgG1, HLT2736, 1:50 dilution) (BioLegend, San Diego, California, USA), rabbit anti-human pSTAT-3 (Tyr705) (IgG, D3A7, 1:50 dilution) (Cell Signaling Technology, Beverly, Massachusetts, USA) and anti-human NKp44 (Mouse IgG1, P44-8, 1:100 dilution) (BioLegend) were added and incubated for 1 h at room temperature. For control staining, primary antibodies were replaced with irrelevant isotype-matched antibodies (AbCam, Cambridge, UK). Then, the slides were incubated for 30 min with peroxidase-conjugated Dako EnVision polymer, and peroxidase activity was visualised using diaminobenzidine chromogen (Dako). Slides were lightly counterstained with haematoxylin before dehydration and mounting in DePex (VWR International, Oslo, Norway). Quantification of IL-22-expressing cells was assessed by two independent investigators (FC and AR) by manually counting the positive cells on photomicrographs obtained from three random high-power microscopic fields (original magnification, 400×) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera. Among infiltrating cells,

the number of positive cells was expressed as the percentage of IL-22-expressing cells. Ductal and/or acinar IL-22 expression was also graded according to the following criteria: no IL-22 expression, grade 0; IL-22-positive ductal cells <50%, grade 1; IL-22-positive ductal cells >50%, grade 2. Results were reported as the mean±SEM.

**Flow cytometry**

Salivary gland tissues were extensively washed in saline buffer, and enzymatic digestion was performed using collagenase (1.5 mg/ml; Life Technologies, Carlsbad, California, USA) in Dulbecco's modified Eagle's medium containing antibiotics for 2 h. The recovered cells, including epithelial cells, were then cultured with phorbol myristate acetate (50 ng/ml) and ionomycin (0.5 μg/ml) and incubated at 37°C in 5% $CO_2$. Numbers of recovered cells were consistent with visual counts. After 2 h of incubation, Brefeldin A (10 μg/ml; Sigma, St. Louis, Missouri, USA) was added, and after 16 h of incubation, cells were collected and stained with the following m Abs: anti-human CD3-APC (BD Biosciences, San Jose, California, USA), anti-human CD56-PerCP-Cy5.5 (BioLegend), anti-human NKp44-PE (BioLegend), anti-human IL-22-FITC (R&D Systems, Minneapolis, Minnesota, USA), anti-human IFNγ-PE (BD Biosciences), anti-human IL-17-APC (R&D Systems), anti-human CD4-PE-

Cy5.5 (BD Biosciences) and an anti-human cytokeratin (clone MNF116; Dako, Glostrup, Denmark). Isotype-matched irrelevant antibodies were used as a negative control. Total cells were incubated with mAbs for 30 min on ice and washed twice in phosphate-buffered saline, containing 0.1% (w/v) NaN3. After surface staining, the cells were fixed with 1% (w/v) paraformaldehyde (Sigma) for 30 min at 4°C and then were permeabilised with a permeabilisation solution (BD Biosciences) for 10 min at room temperature and stained with antibodies to intracellular antigens for 30 min at 4°C. Four-colour flow cytometry analysis was performed using a FACSCalibur (BD Biosciences). At least 5000 cells (events), gated on lymphocytes region, were acquired for each sample.

**Statistical analysis**

Statistical analysis of quantitative variables was performed using the Mann–Whitney rank-sum test. A $\chi^2$ test with Yates' correction when required was used to evaluate associations of qualitative variables in the different groups. Pearson's correlation analysis was used to quantify the expression associations between the genes of interest. p Values less than 0.05 were considered statistically significant.

**Results**

**IL-22 expression in the salivary glands of patients with pSS**

Previous studies have described the expression of various cytokines in patients with pSS (eg, IL-18, IL-7, IL-17 and IL-23) [10, 15, 21, 22]; however, there are no data on the expression of IL-22 in salivary glands. Minor labial salivary glands were evaluated for IL-22 expression at both mRNA and protein levels to determine whether this cytokine is participates in the inflammatory response in patients with pSS.

Significantly higher levels of IL-22 mRNA were found in patients with pSS when compared with those of patients with nSS ($9.25 \pm 1.32$ vs $0.87 \pm 0.08$, $p < 0.0001$) (Figure 1A) with a positive strong correlation between IL-22 mRNA levels and the focus score (data not shown).

Low levels of IL-22 expression were detected by IHC in all patients with chronic sialoadenitis (Figure 1B). In pSS, the staining appeared localised to lymphocytic infiltrates and ductal epithelial cells. The typical distribution of IL-22 in pSS is shown in figures 1C and D. The relative percentage of positively stained infiltrating mononuclear cells was correlated with the focus score ($r=0.68$, $p < 0.0001$) (Figure 1E). Ductal epithelial cells were also positive for IL-22 in pSS only, and the degree of IL-22 expression was correlated with the grade of inflammation of the

salivary glands (Figure 1F). No evidence of IL-22 staining was detected in acinar epithelial cells, myoepithelial cells or fibroblasts.

**IL-22 expression in salivary glands of patients with pSS**
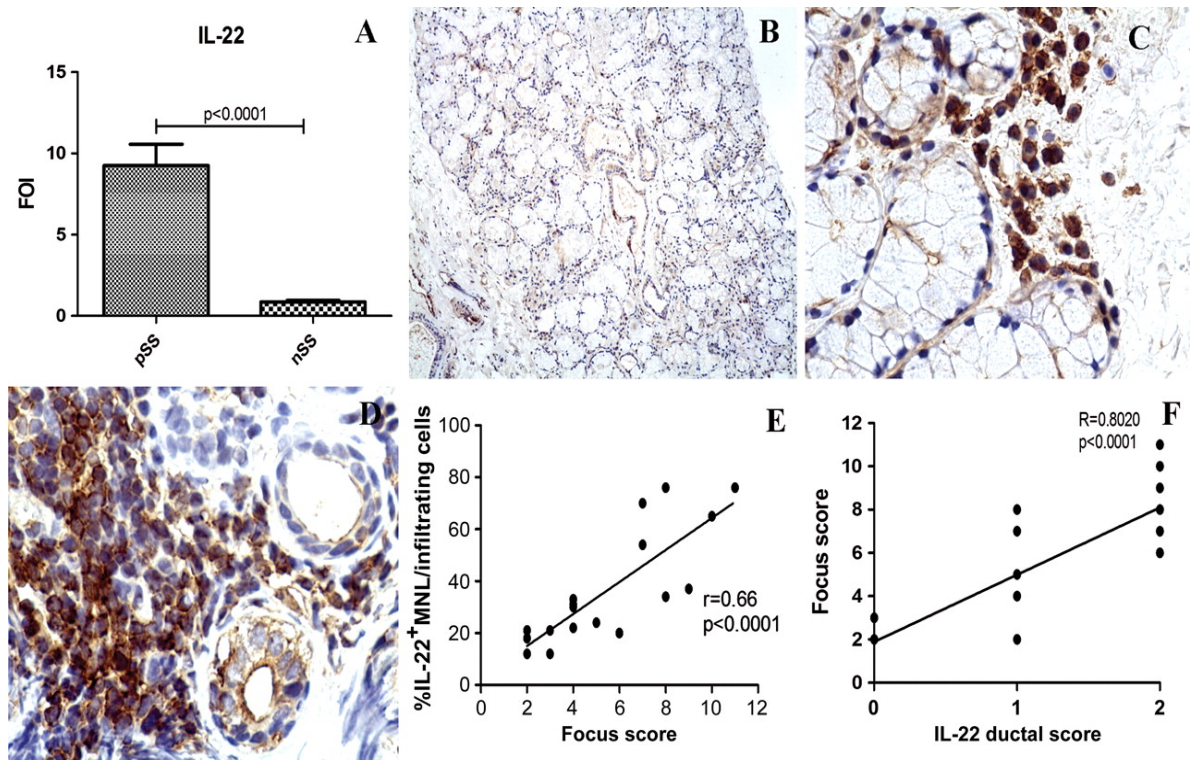


**Figure 1**

IL-22 expression in salivary glands of patients with pSS. (A) Relative expression of IL-22 mRNA in whole minor salivary glands of patients with pSS. Data are normalised for GAPDH and plotted as fold of induction over minor salivary glands obtained from patients with nSS. Results represent the mean±SEM. Representative immunostainings for IL-22 in nSS (B) compared with patients with pSS with different grades of inflammation (C,D). Significant staining of IL-22 was observed only in patients with pSS (n=19) compared with patients with nSS (n=16). Positive stained cells were observed among infiltrating mononuclear cells and ductal epithelial cells (C,D). (E) IL-22^{+} cells were considered as the percentage of the infiltrating mononuclear cells. Correlation of IL-22^{+} mononuclear cells with focus scores. IL-22 expression by IHC was correlated with the focus score of minor salivary glands of pSS. The r (r=0.68) and p (p<0.0001) values were determined with Pearson's correlation

coefficient. (F) Ductal IL-22 expression was shown by the grading scale, and IL-22 expression of ductal cells correlated with focus scores. The r (r=0.80) and p (p<0.0001) values were determined with Pearson's correlation coefficient. (B) Original magnification, 100×. (C,D) Original magnification, 400×. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IHC, immunohistochemistry; IL, interleukin; nSS, non-specific chronic sialoadenitis; pSS, primary Sjögren's syndrome.
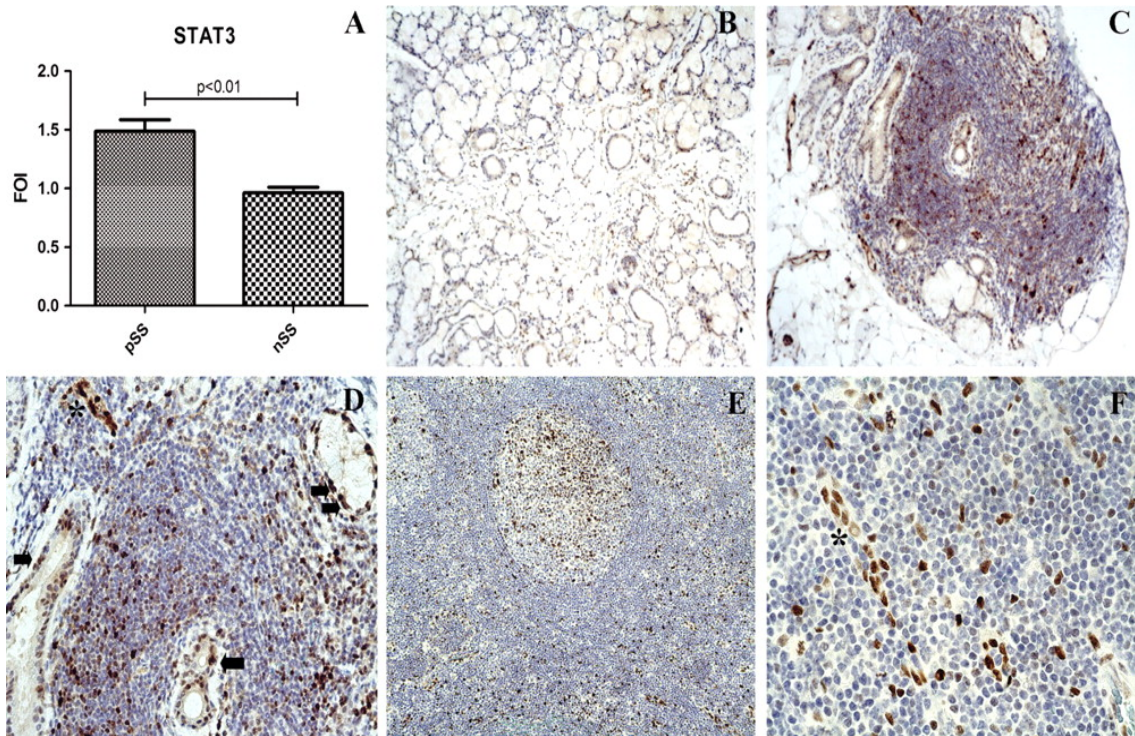
## IL-23, IL-17 and p-STAT3 expression in salivary glands of patients with pSS

Since IL-22 is a downstream effector cytokine of IL-23 [23-24], we next evaluated the expression of IL-23. IL-23 mRNA was significantly increased in patients with pSS but not in patients with nSS (2.72±0.4 vs 0.92±0.1, p=0.0002). Immunohistochemical analysis in all inflamed specimens of patients with pSS confirmed the high expression of IL-23 at the protein level, the intensity of which was particularly increased in those patients displaying a higher focus score (data not shown).

Because the functional outcome of IL-22 (whether protective or pro-inflammatory) seems to be dependent on the coexpression of IL-17 [15], we next evaluated IL-17 expression levels. IL-17 was increased at both mRNA (6.75±1.62 vs 0.9±0.07, p<0.0001) and protein (data not shown) levels in patients with pSS and correlated with the degree of tissue inflammation.

Both IL-23 and IL-22 signal through STAT3 in different cellular targets, the former mainly in lymphoid cells and the latter in epithelial cells. To assess whether IL-22 and IL-23 signals were active, we looked at the activation of downstream signal pathway evaluating STAT3 mRNA and p-STAT3 protein expression. STAT3 mRNA expression was demonstrable both in patients with pSS and those with nSS, being significantly increased in pSS ($1.48\pm0.09$ vs $0.96\pm0.05$, $p<0.01$) (Figure 2A). Since the activation of STAT molecules is indicated by their phosphorylated status [25], we next analysed intracellular p-STAT3. Immune staining of p-STAT3 was observed almost exclusively in the inflamed salivary glands of patients with pSS (Figure 2B, D). As shown in figure 2D , three patterns of positivity were observed. The first pattern was characterised by a large number of p-STAT3-producing cells, presumably dendritic cells, distributed within the lymphomonocytic inflammatory infiltrate in the context of ductal epithelium. The second pattern, mainly epithelial, was characterised by a strong positivity in both acinar and ductal epithelial cells. The third pattern was characterised by the p-STAT3 expression among high endothelial cells. Expression of p-STAT3 in endothelial cells and lymphocytes was also confirmed in lymph node sections used as positive controls (Figures 2E,F).

# p-STAT3 expression in salivary glands of patients with pSS.



**Figure 2**

p-STAT3 expression in salivary glands of patients with pSS. Relative expression of STAT3 at the mRNA level (A) and of p-STAT3 at the protein level (B–D) in whole minor salivary glands of patients with pSS, controls and lymph node (E,F). Immune staining of p-STAT3 was almost exclusively observed in the inflamed salivary glands of pSS (B–D). Infiltrating inflammatory cells, high endothelial cells (asterisk) and ductal (arrows) and acinar (arrows) epithelial cells showed intense p-STAT3 positivity (D). In the context of lymph node, immune staining of p-STAT3 was observed in lymphocytes (E) and high endothelial cells (F) (asterisk). (A) Bars represent the mean±SEM. (B,C and E) Original magnification, 100×. (D,F) Original magnification, 250×. STAT3, signal transducer and activator of transcription; pSS, primary Sjögren's syndrome; p-STAT3, phosphorylated signal transducer and activator of transcription.

# Percentage of IL-22-producing cells in salivary glands with pSS.
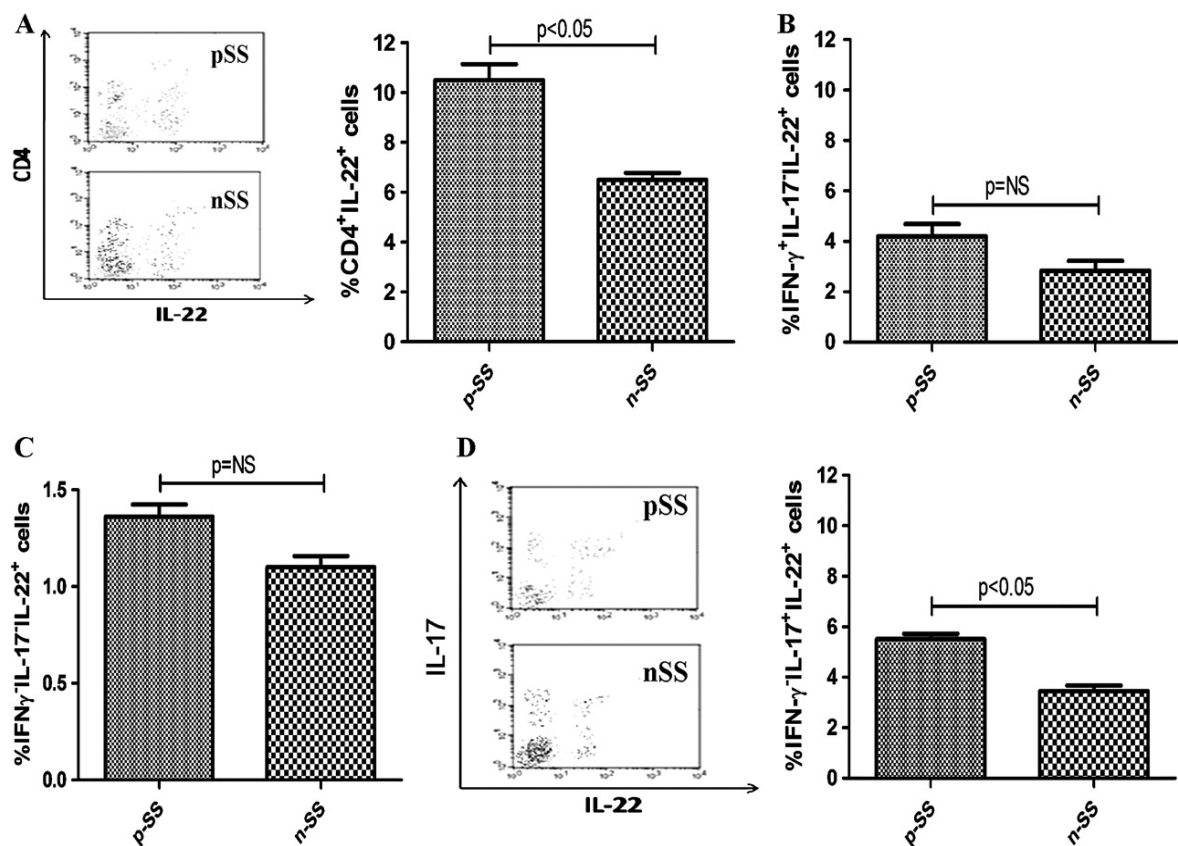


**Figure 3**

Percentage of IL-22-producing cells in salivary glands with pSS. Freshly isolated SGMCs from six patients with pSS and six patients with nSS were stained with CD4-PE-Cy5.5, IFNγ-PE, IL-17-APC and IL-22-FITC and analysed by flow cytometry. (A) Representative dot plot analysis (left panel) of CD4 versus IL-22 among isolated SGMCs and percentages (right panel) of IL-22$^+$ cells among CD4$^+$ T lymphocytes in SGMCs of pSS and nSS. (B) Percentages of IL-22-positive cells among CD4$^+$IFNγ$^+$IL-17$^-$ Th1 lymphocytes. (C) Percentages of IL-22-positive cells among CD4$^+$IFNγ$^-$IL-17$^-$ Th22 lymphocytes in SGMCs of pSS and nSS. (D) Representative dot plot analysis of IL-17 versus IL-22 among isolated SGMCs (left panel) and percentages (right panel) of IL-22-positive cells among CD4$^+$IFNγ$^-$IL-17$^+$Th17 lymphocytes in SGMCs of pSS and nSS. Bars show the mean and SEM. IL, interleukin; nSS, non-specific chronic sialoadenitis; pSS, primary Sjögren's syndrome; SGMC, salivary gland mononuclear cell.

**Identification of IL-22-producing cells in salivary glands**

The expression of IL-22 in the different infiltrating populations was analysed by fluorescence-activated cell sorting analysis on cells isolated from the minor salivary glands of patients with pSS and those with nSS. To date, the best characterised human mucosal cells that secrete IL-22 are CD4^{+} IL-22-expressing T cells (Th1, Th17 and Th22) and NKp44^{+} NK cells [14]. As shown in Figure 3A, IL-22-producing CD4^{+} T cells from patients with pSS were significantly expanded compared to nSS (10.5±0.65 vs 6.5±0.3, p<0.05). Among CD4^{+} T cells, the percentage of IL-22-producing Th1 (4.2±0.51 vs 2.84±0.4, p=NS) (Figure 3B) and Th22 (1.36±0.06 vs 1.1±0.05, p=NS) (Figure 3C) cells was not significantly different when the two groups were compared. Conversely, IL-22-producing Th17 cells (5.5±0.23 vs 3.44±0.3, p<0.05) were significantly expanded in patients with pSS (Figure 3D). IL-22 production was also observed among epithelial cells, with patients with pSS displaying a significantly higher IL-22 expression than the controls (11.2% and 5.8%, respectively, p<0.05) (Figure 4A,B).

**IL-22 expression in salivary gland epithelial cells of patients with pSS.**
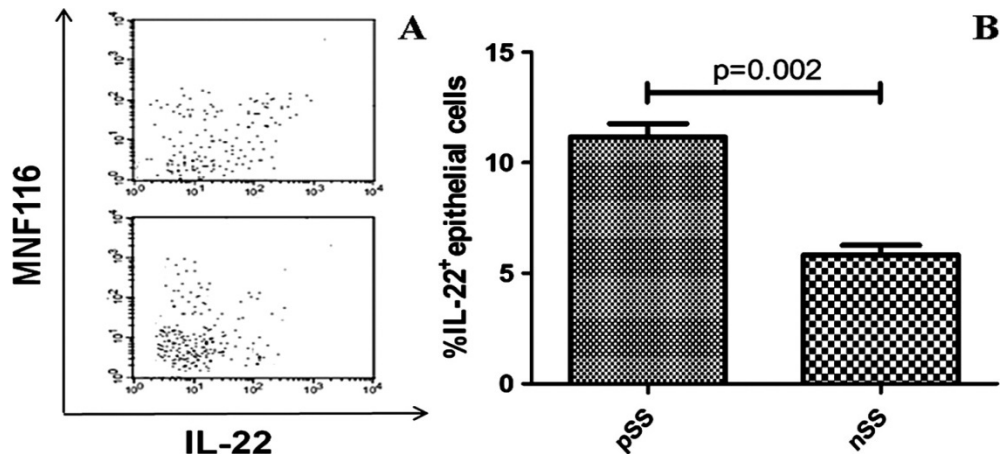


**Figure 4**

IL-22 expression in salivary gland epithelial cells of patients with pSS. (A) Representative dot plot analysis of cytokeratin (MF116) versus IL-22 among isolated salivary gland mononuclear cells. (B) Percentages of IL-22-positive cells among salivary gland epithelial cells. Bars show the mean and SEM. IL, interleukin; pSS, primary Sjögren's syndrome.

## NKp44^{+} NK cells are expanded and produce high levels of IL-22 in salivary glands of pSS

At mucosal sites, IL-22 is also produced by a subset of mucosa-associated NK cells that expresses the NKp44 receptor (namely, NKp44^{+} NK cells) [26]. We investigated IL-22 production by NK cells isolated from the salivary glands of patients with pSS and those with nSS. A significantly increased proportion of NK cells was observed in patients with pSS compared with those with nSS ($3.4\pm0.35$ vs $1.8\pm1.3$, $p<0.05$) (Figure 5A). Among NK cells, CD3^{−}CD56^{+} NKp44^{+} NK cells were significantly expanded ($2\pm0.17$ vs $0.958\pm0.045$, $p<0.05$) and produced

higher amounts of IL-22 in patients with pSS when compared to patients with nSS (0.914±0.07 vs 0.228±0.043, p<0.05) (Figure 5B,C). The percentage of NKp44$^+$ NK cells was directly and strongly correlated with the severity of tissue inflammation in patients with pSS (Figure 5D). Immunohistochemical analysis of salivary gland sections from pSS with anti-NKp44 antibody shows that NKp44$^+$ NK cells reside within the inflammatory infiltrate of pSS (Figure 5E,F).

# FACS analysis of NKp44+ NK cells in salivary glands with pSS.
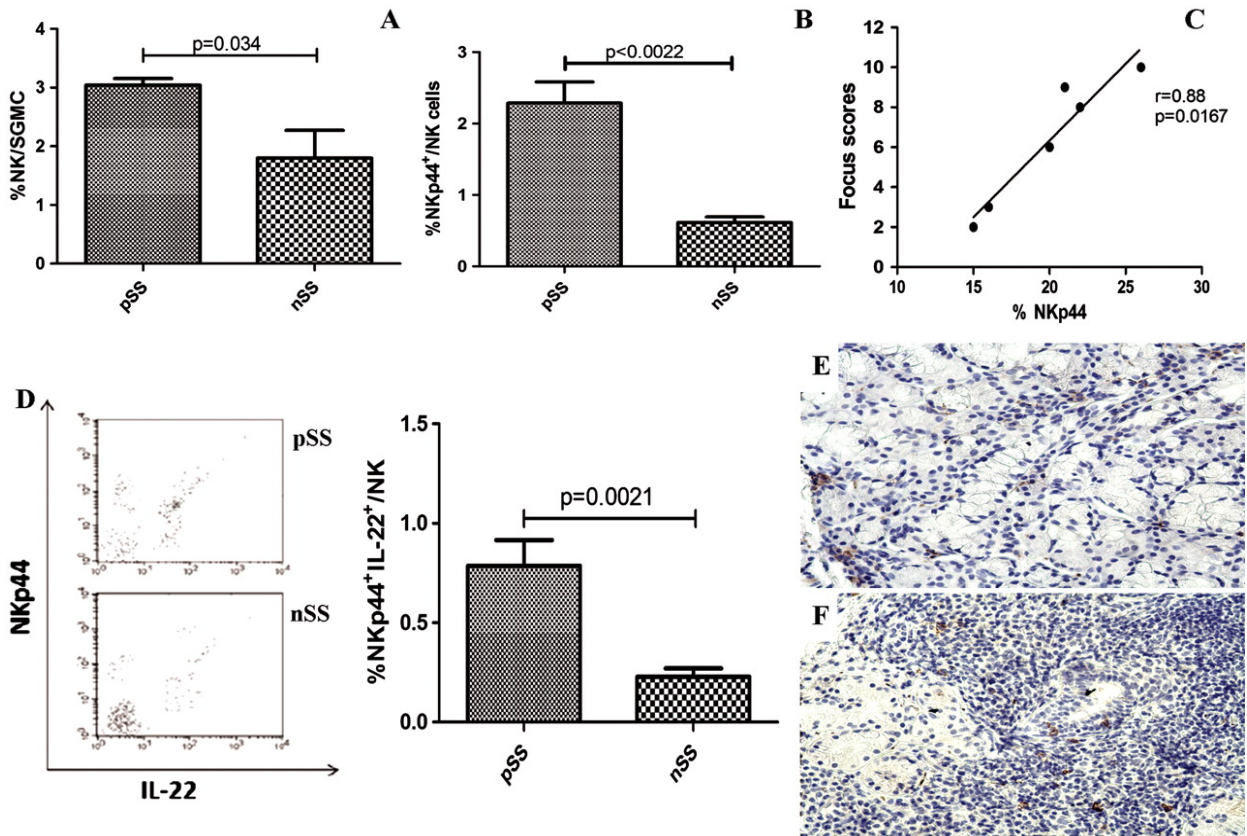


**Figure 5**

FACS analysis of NKp44^{+} NK cells in salivary glands with pSS. Freshly isolated SGMCs from six patients with pSS and six patients with nSS were stained with anti-CD3-APC, CD56-PerCP-Cy5.5, NKp-44-PE and IL-22-FITC and analysed by flow cytometry. Percentages of NK cells among SGMCs of pSS and nSS (A). Percentages of NKp44^{+} cells in SGMCs of pSS and nSS (B). Correlation of percentages of NKp44^{+} NK cells with focus scores (C). Percentages of NKp44^{+} NK cells evaluated by FACS analysis were correlated with the focus scores of minor salivary glands of pSS. The r=0.88 and p=0.0022 values were determined with Pearson's correlation coefficient. Representative dot plot (left panel) of IL-22 versus NKp44 and percentages (right panel) of IL-22-producing NKp44^{+} NK cells in SGMCs of pSS and nSS (D). Representative immunostainings for NKp44 in patients with nSS (E) compared with patients with pSS (F). (E,F) Original magnification, 400×. FACS, fluorescence-activated cell sorting; IL, interleukin; NK, natural killer; nSS, non-specific chronic sialoadenitis; pSS, primary Sjögren's syndrome; SGMC, salivary gland mononuclear cell.

**Discussion**

In this study, we confirm the increased expression of Th17-related cytokines, IL-17 and IL-23 [8, 10-11], and demonstrate, for the first time, that IL-22 is overexpressed at both mRNA and protein levels in the inflamed salivary glands of pSS. Moreover, we also provide evidence that Th17 cells and NKp44^{+} NK cells are the major sources of IL-22 in salivary glands.

IL-22 is a member of the IL-10 cytokine family and plays critical roles in inflammation, immune surveillance and tissue homeostasis at mucosal sites [14]. In autoimmune diseases, however, diverse tissue cytokine milieu and different mechanisms may result in opposing roles of IL-22 in disease pathogenesis [15]. The pro-inflammatory versus tissue-protective functions of IL-22 seem to be regulated by the coexpression of IL-17 [15]. When coexpressed, IL-17 and IL-22 synergistically promote inflammation through chemokine expression and neutrophil recruitment; on the other hand, IL-22 alone seems to act as a protective cytokine [15, 27-30]. Our demonstration of concomitant IL-22 and IL-17 expression seems to suggest that in inflamed salivary glands of patients with pSS, IL-22 should play a predominant pro-inflammatory role.

IL-22 should be considered as a downstream effector cytokine of IL-23 [23-24]. In our pSS samples, both IL-22 and IL-23 were significantly

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increased in pSS, supporting the existence of a functionally relevant IL-23/IL-22 axis in the inflamed salivary glands of patients with pSS.

By IHC, we demonstrate a strong IL-22 positivity in the mononuclear cells infiltrating the inflamed salivary glands and in ductal epithelial cells of patients with pSS, both correlated with the degree of tissue inflammation. Epithelial cells are thought to play an important pathogenetic role in pSS, as suggested by the demonstration that, in the lesions of patients with pSS, ductal and acinar salivary gland epithelial cells display high levels of several immunoactive molecules that are known to mediate lymphoid cell homing, antigen presentation and amplification of epithelial cell–immune cell interactions [31]. In this scenario, the significance of epithelial IL-22 expression in pSS supports the important role of the epithelium in the pathogenesis of pSS.

IL-22 targets mainly tissue epithelial cells but not immune cells [30]. IL-22 mediates signal transduction through a receptor complex consisting of the specific IL-22R1 and the common IL-10R2 subunits [32]. While IL-22R2 is ubiquitously expressed, IL-22R1 is restricted on cells of epithelial origins, allowing for IL-22-mediated regulation of epithelial cell responses to infective or inflammatory stimuli [30-33]. Interaction of IL-22 with its cognate receptor mainly results in tyrosine phosphorylation

of STAT3 [14]. In our study, we observed significant modulation of STAT3 mRNA levels in patients with pSS compared to those with nSS. The immunohistochemical analysis clearly showed that p-STAT3 protein was strongly expressed in periductal mononuclear cells infiltrating the salivary glands of all patients with pSS. Since the p-STAT3 expression in mononuclear cells is mainly induced by IL-6, IL-21 and IL-23, this pattern of positivity could be related to the signalling of the aforementioned cytokines [14]. In addition to mononuclear cells, endothelial cells as well as ductal and acinar epithelial cells appear to represent a major source of p-STAT3. The detection of p-STAT3 in these cells is in accordance with the notion that STAT3 mediates IL-22 signalling in endothelial and epithelial cells [34].

At mucosal sites, IL-22 is mainly produced by different leucocyte subsets, including Th1, Th17 and Th22 cells as well as NKp44^{+} NK cells [14]. Our demonstration that, among the CD4^{+} T cell subset, Th17 cells were the major source of IL-22 in patients with pSS highlights the role of this important subset of effector T cells in the pathogenesis of pSS. In addition, we found that a mucosal subset of NK cells distinct from conventional NK cells (namely, NKp44^{+} NK cells) [26] were expanded and strongly produced IL-22. NKp44^{+} NK cells express RORC, are not

cytotoxic and in human are present in tonsils, intestinal lamina propria and Peyer's patches [26, 35]. Interestingly, regulation of the development and immunological behaviour of NKp44^{+} NK cells is dependent on IL-23 [26]. NKp44^{+} NK through the production of IL-22 seems to play a prevalent protective role in intestinal lamina propria [36]. The strong correlation of the percentage of NKp44^{+} NK cells with the degree of salivary gland inflammation suggests a prevalent pro-inflammatory role in pSS. Nevertheless, the high percentage of IL-22-producing NKp44^{+} NK cells could be the result of a tissue-protective response.

In summary, this is the first study to our knowledge which demonstrates that IL-22 is abundantly expressed in the inflamed salivary glands of patients with pSS. We provide, for the first time, evidence that Th17, NKp44 and epithelial cells are the major cellular sources of IL-22 in pSS. This study confirms and extends previous knowledge on the role of TH17 cells in the pathogenesis of pSS and demonstrates that TH17 cell are capable to induce prevalent tissue pro inflammatory function of IL-22. On the other hand, we demonstrated that NKp44^{+} NK cells are expanded and express a higher amount of IL-22, suggesting an important role of this mucosa-associated NK cell subset in the

modulation of the immunological responses occurring in the inflamed salivary glands of patients with pSS. Finally, we also demonstrated that IL-22 is expressed in the inflamed ductal epithelial cells of patients with pSS, highlighting the role of epithelial cells in the pSS pathogenesis. Further studies are needed to address the direct functional role of IL-22 in the pathogenesis of pSS.

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**Chapter 2**

**Aberrant activation of IL-22/IL-22R pathway in primary Sjogren's Syndrome and Sjogren-associated non-Hodgkin lymphomas**

Primary Sjogren's syndrome (pSS) is a chronic systemic autoimmune disease characterized by dysregulated innate and adaptive immune responses and by a higher rate of non-Hodgkin lymphomas [1, 2]. Recently, we have demonstrated the occurrence in pSS patients of a local over-expression of IL-22 and IL-22-producing cells [3] suggesting a role of IL-22 in the pathogenesis of pSS and indicating IL-22 inhibition as a potential therapeutic strategy in pSS. Interleukin (IL)-22 is a member of the IL-10 cytokine family that mediates its effects via a heterodimeric transmembrane receptor complex consisting of IL-22R1 and IL-10R2 [5, 6]. IL-22 does not play a role in immune cells cross-talk since, unlike the IL-10R2 which is constitutively expressed in many human tissues, IL-22R1 is not detectable in immune cells including monocytes, resting or activated B/T cells, NK cells, macrophages, and dendritic cells [7-9].

Binding of IL-22 to its cognate receptor favours STAT3 phosphorylation and promotes protective functions at mucosal surfaces such as the production of antimicrobial peptides, and proliferation and survival of

epithelial cells (rev. in [5]). On the other hand IL-22 axis participates in the induction of murine and human chronic inflammatory diseases [10-13] and its dysregulation seems to play a role in the pathogenesis of human malignancies. IL-22 axis participates in the pathogenesis of colon cancer [14] and aberrant expression of IL-22R1 has been recently reported in T and B lymphomas [15, 16], suggesting that an autocrine stimulatory loop involving IL-22 and its receptor may contribute to the pathogenesis of lymphoid malignancies.

The aim of this study was to evaluate the expression of IL-22R1 in the salivary glands and in the peripheral blood mononuclear cells (PBMC) of p-SS and in pSS-associated non-Hodgkin lymphoma. The expression pattern of IL-22R1 in patients with other autoimmune disease such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) was also investigated.

Here, we demonstrated that the aberrant expression of IL-22R1 in monocytes specifically occurs in the salivary glands and in the PBMC of p-SS. In vitro IL-22 stimulation of isolated IL-22R1-bearing monocytes resulted in the increased expression of STAT3, IL-17 and IL-22 and in Th17 expansion suggesting that an abnormal IL-22 stimulatory pathway may contribute to the perpetuation of local and systemic inflammation

in p-SS. Lymphomas of pSS patients also aberrantly expressed IL-22R1 on monocytes and neoplastic B-cells, suggesting a putative role of IL-22/IL-22R1 in the pSS associated lymphomagenesis.

**Matherial and Methods**

**Patients**

Thirty patients with subjective complaints of dry mouth or eyes who met the American-European Consensus Group criteria for pSS [17] were consecutively enrolled. Fifteen patients also displaying subjective complaints of dry mouth or eyes who did not meet the American-European Consensus Group criteria for pSS were included as nonSS sicca syndrome (nSS) and used as controls. Patients with RA (n=20) and SLE (n=20), with active disease as defined by a DAS28 >3.2 and a SLEDAI > 6, respectively, were also included as disease controls. Serologic evaluations, which included tests for the presence of antinuclear antibodies (ANAs), anti-SSA/Ro, anti-SSB/La, rheumatoid factor (RF), levels of C-reactive protein and erythrocyte sedimentation rate were also performed. Ten healthy subjects were also enrolled. Five non-Hodgkin lymphoma of pSS patients were retrospectively evaluated. Clinical characteristics of the patients and controls are shown in Table I. All the patients and control gave informed consent and the study was approved by the local ethical committee.

**Labial salivary gland biopsy**

Labial salivary glands biopsies, obtained during routine diagnostic procedures, were placed into formalin fixative, RNA later or RPMI. Paraffin embedded sections of 5-mm thickness were stained with hematoxylin and eosin (H&E) for the histological evaluation of the presence of lymphocytic infiltrates and/or foci. A focus was defined as an aggregate of ≥ 50 lymphocytes and the focus score was reported as the number of foci per 4 mm$^2$ of tissue, up to a maximum of 12 foci.

**RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)**

Salivary gland biopsies soon after removal were stored in RNAlater® solution (Applied Biosystem, Foster City, CA). rt-PCR was performed as previously described [3]. Master mix and Taqman® gene expression assays for GAPDH control for IL-22 (Hs01574154_m1), IL-22R1 (Hs00222035_m1), IL-22BP (Hs00364814_m1), IL-17 (Hs00174383_m1), IL-23p19 (Hs00372324_m1), RORc (Hs01076122_m1),  and STAT3 (Hs00234174_m1) were obtained from Applied Biosystems (Foster City, CA). Final values were expressed as fold of induction (FOI).

**Immunohistochemistry**

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-mm–thick paraffin-embedded sections from salivary glands, from tonsils (used as positive controls) and non-Hodgkin lymphomas from pSS patients as previously described [3]. A list of the primary and secondary antibodies used is provided in table II.

**Flow cytometry**

Salivary gland mononuclear cells and PBMCs were obtained as previously described [3] from 10 pSS patients and 10 nSS subjects. Cell viability (trypan blue dye exclusion) was always >95%. A list of the antibodies used is listed in table II. Four color flow cytometry analysis was performed using a FACSCalibur (BD Biosciences). At least 50.000 cells (events), gated on lymphocytes or monocytes/macrophages region, were acquired for each sample.

**Cell cultures with recombinant IL-22**

PBMCs isolated from heparinized blood samples of 6 p-SS patients and 5 n-SS by centrifugation over Ficoll-Hypaque (Sigma, St Louis, MO) density gradients were cultured in 24 flat bottom plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of $1 \times 10^6$ cells in 1 ml of

RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, 20 nM HEPES and 100 U/ml penicillin/streptomycin with or without recombinant IL-22 (0,1 ng/ml) (R&D Systems, MN, USA). Cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 48 hours and for 5 days; after incubation cells were evaluated for the expression of IL-22 and IL-17 by flow cytometry. RT-PCR for IL-17, IL-22, IL-23, RORc and STAT3 was also performed, after incubation, to evaluate cytokines gene profile.

**Statistical analysis**

Statistical analysis of quantitative variables was performed using the Mann-Whitney rank-sum test. Spearman's correlation analysis was utilized to quantify the expression associations between the genes of interest. Data are expressed as mean ± SEM. *p* values less than 0.05 were considered significant.

**Results**

**IL-22/IL-22R1 axis in the salivary glands of pSS**

Biopsy specimens from pSS patients were characterized by the presence of peri-ductal lymphocytic infiltration accompanied by various degree of atrophy or severe destruction of the acini. Five of the 30 patients with p-SS (16%) showed ectopic germinal centers (GC) formation. The expression levels of IL-22 and STAT3 were studied at both m-RNA and protein level by RT-PCR and immunohistochemistry respectively. Accordingly to our previous study [3], a significant up-regulation of IL-22 and STAT3 m-RNA was observed in the inflamed salivary glands of p-SS patients compared with nSS (Figure 1A-B). The expression of IL-22 and of the phosphorylated form of STAT3 (p-STAT3) was confirmed by immunohistochemistry (Figure 1C-D). Differently from the diffuse IL-22 expression, p-STAT3 was prevalently expressed among mononuclear cells scattered in the inflammatory lymphocytic infiltrates (Figure 1D).
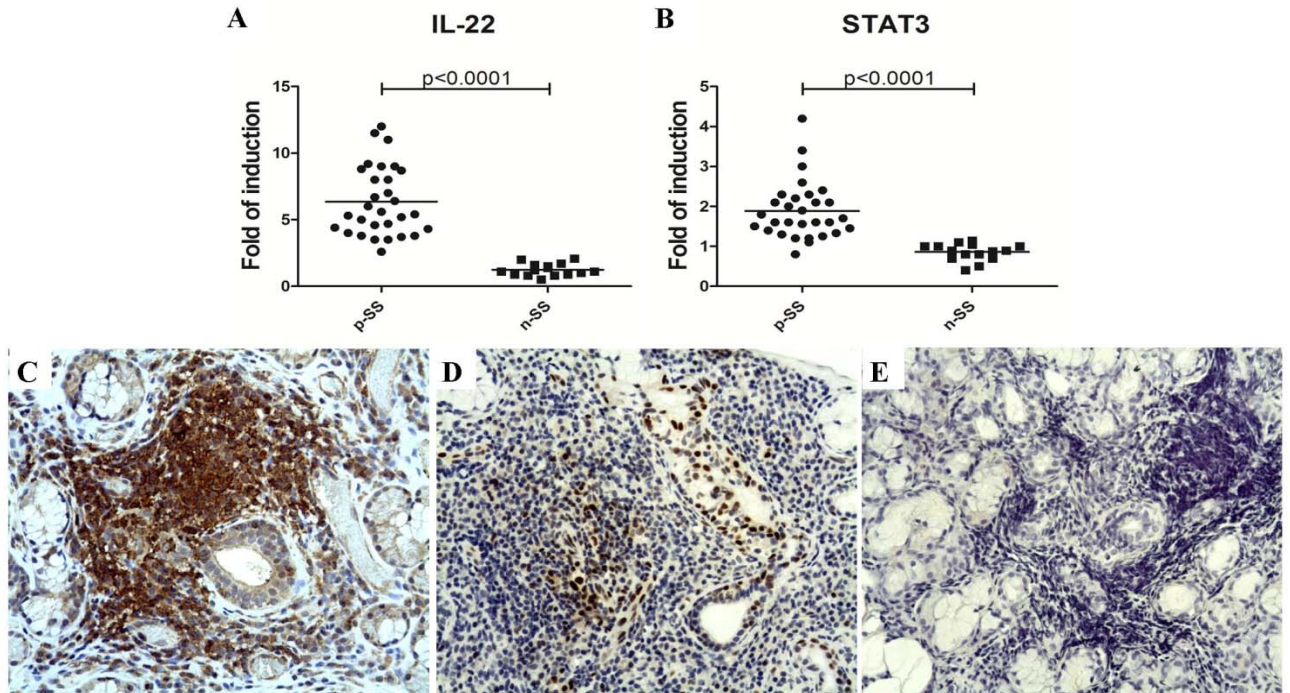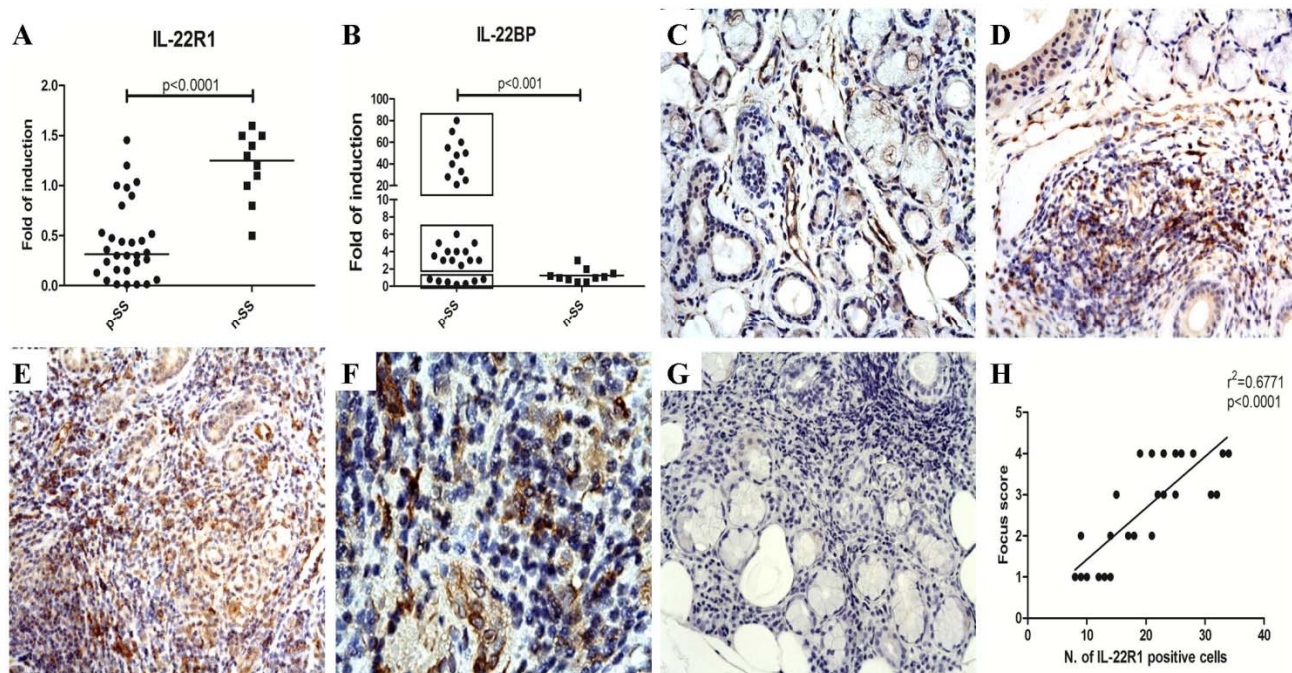
**Figure 1.**

**IL-22 and STAT3 expression in the salivary glands of patients with primary Sjogren's syndrome (p-SS patients).** Relative m-RNA quantification of IL-22 (A) and STAT3 (B) was assessed by quantitative rt-PCR in salivary glands obtained from 30 pSS patients and non Sjogren's syndrome patients (nSS). C-D: Representative microphotographs showing IL-22 (C) and p-STAT3 (D) immunostainings in pSS patients. Diffuse, intense IL-22 expression was observed among infiltrating inflammatory cells and endothelial cells in pSS patients (C). D: p-STAT3 expression was observed in some infiltrating mononuclear cells scattering in the inflammatory infiltrates. E: Representative paraffin sections of salivary glands of pSS patients stained with rabbit immunoglobulins (isotype control). C-E: original magnification x 250

Since the functional outcome of IL-22 seems to depend on the expression of the IL-22R1 inducible subunit and of IL-22BP, their m-RNA expression was next assessed by RT-PCR. Differential expression of IL-22R1 and IL-22BP was observed in the inflamed salivary glands of pSS.

IL-22R1 was, in fact, significantly down-regulated (Figure 2A) whereas IL-22BP was strongly over-expressed (Figure 2B) in the salivary glands of p-SS compared to n-SS. Differently from RT-PCR results, immunohistochemical analysis demonstrated that IL-22R1 was significantly over-expressed in the inflamed salivary glands of pSS compared to nSS. Inflamed salivary glands of pSS showed abundant IL-22R1 expression among infiltrating mononuclear cells (Figure 2D-E) and endothelial cells of vessels scattered through the inflamed salivary glands (Figure 2F). Conversely, IL-22R1 acinar and ductal epithelial positivity was rarely observed in the salivary glands of patients and controls (Figure 2C-F). Interestingly, the number of IL-22R1-expressing cells was significantly correlated with the lymphocytic focus score (Figure 2H).

**Figure 2.**

**IL-22R1 expression in the salivary glands of patients with primary Sjogren's syndrome (pSS patients).** Relative m-RNA quantification of IL-22R1 (A) and IL-22BP (B) was assessed by quantitative rt-PCR in salivary glands obtained from 30 pSS patients and non Sjogren's syndrome patients (nSS). C-G: Representative microphotographs showing IL-22R1 immunostainings in nSS (C), and pSS patients with focus score 3 (D) and 4 (E). IL-22R1 aberrant expression was observed among infiltrating inflammatory cells and endothelial cells in pSS patients (D-E). F: strong IL-22R1 was also observed in microvessels distributed in the inflammatory infiltrates in pSS. G: Representative paraffin sections of salivary glands of pSS patients stained with rabbit immunoglobulins (isotype control). H: Correlation of IL-22R1 with the focus scores. Number of IL-22R1-expressing cells was correlated with the focus score of minor salivary glands of pSS. The r (r=0.6771) and p (p<0.0001) values were determined with the Spearman's correlation coefficient. C-E,G: original magnification x 250; F: original magnification x 630.

In order to clarify which inflammatory cells express IL-22R1 in the salivary glands of pSS patients, double immunostainings by confocal laser scanning microscopy, were performed for IL-22R1/CD3, IL-22R1/CD19 and IL-22R1/CD68. The majority of tissue IL-22R1 expressing cells were CD68^{+} macrophages (Figure 3A-C) whereas only a small percentage of CD3^{+} cells also displayed IL-22R1 positivity (Figure 3D-F). No IL-22R1 expression was found among CD19^{+} cells (Figure 3G-I).
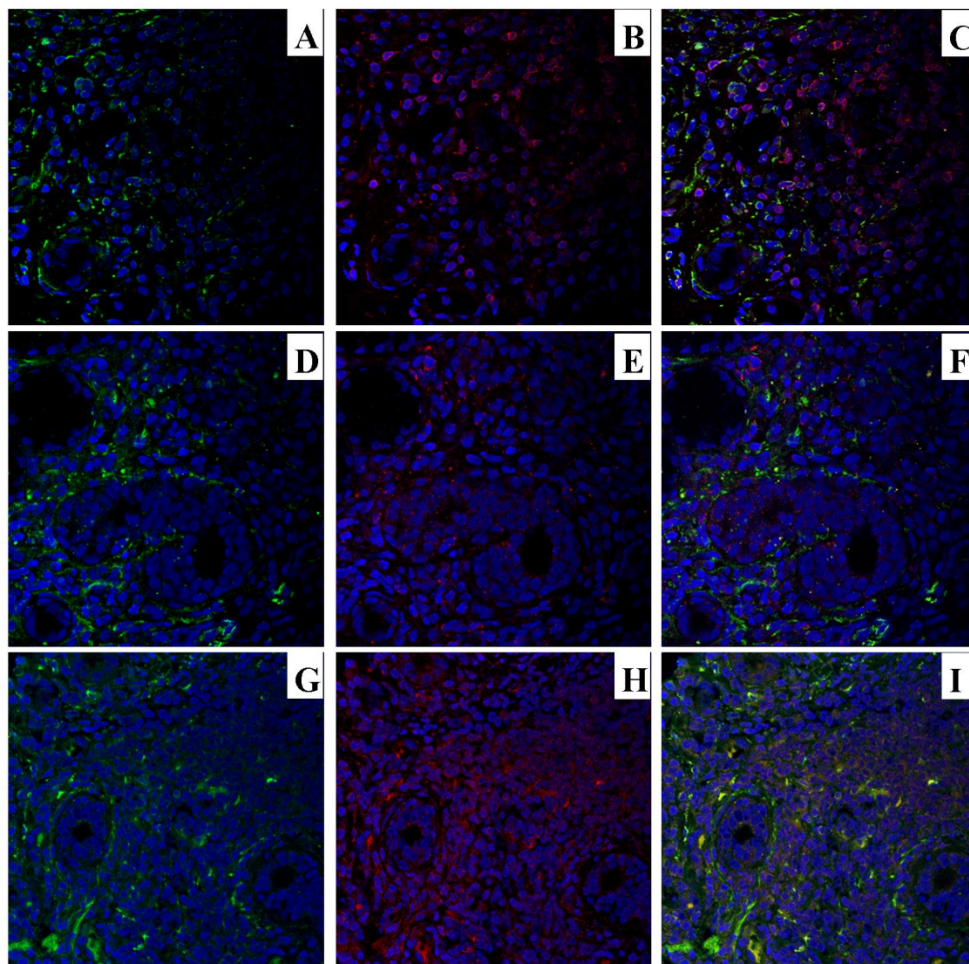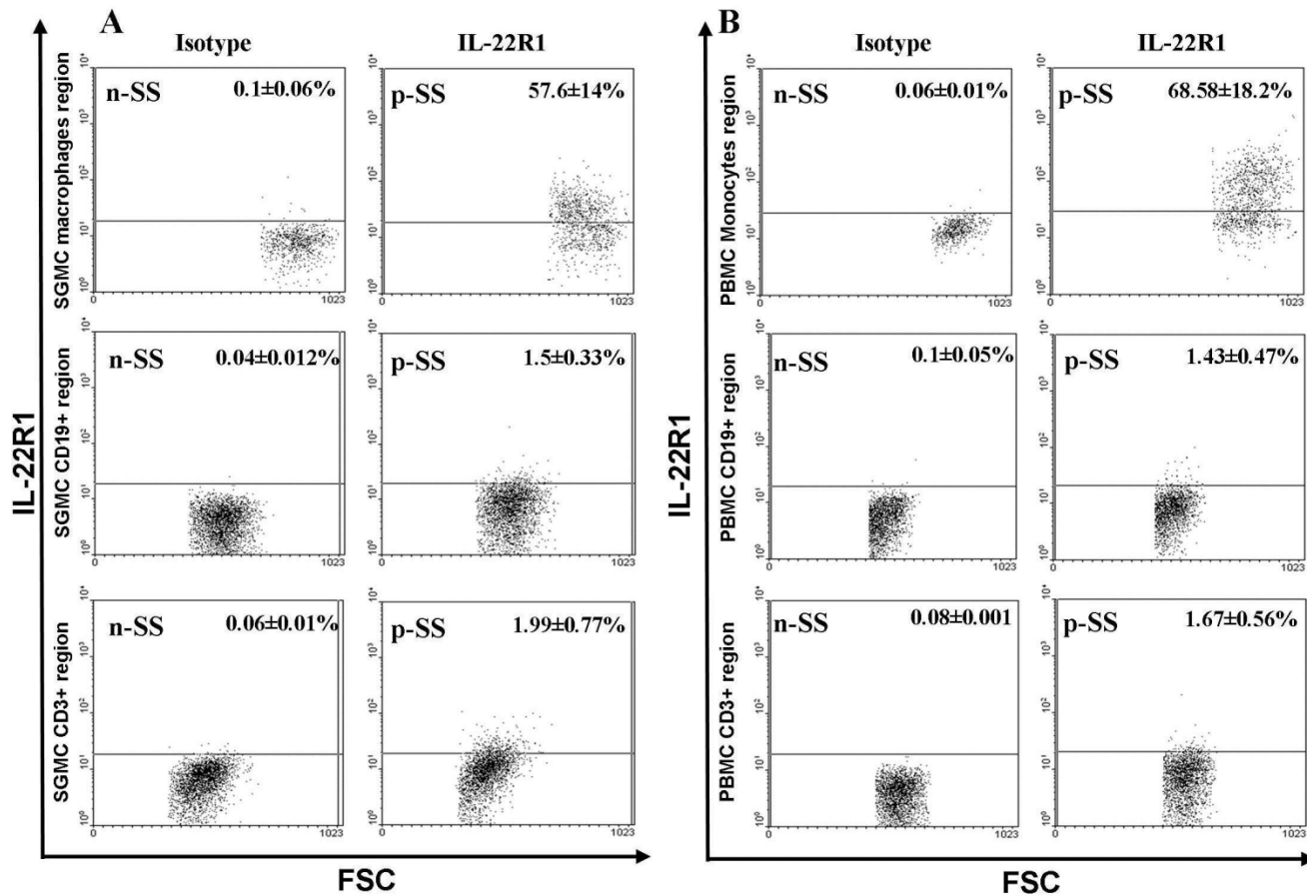
**Figure 3.**

**IL-22R1 is prevalently expressed on CD68+ cells in the inflamed salivary glands of pSS patients.** Representative images of confocal analysis of CD3/CD19/CD68 and IL-22RA1 colocalization in pSS patients. A-B: single staining for CD3 (A) and IL-22RA1 (B). C: merged double staining of CD3 (green) and IL-22RA1 (red). D-E: single staining for CD19 (D) and IL-22RA1 (E). F: merged double staining of CD19 (green) and IL-22RA1 (red). G-I: single staining for CD68 (G) and IL-22RA1 (H). I: merged double staining of CD68 (green) and IL-22RA1 (red). A-I: original magnification x 250

## IL-22R1 expression on isolated SGMC and PBMC of pSS and nSS

To confirm the immunohistochemical data, flow cytometric analysis of IL-22R1 expression was also performed on isolated salivary gland mononuclear cells of pSS and nSS subjects. As shown in figure 4A, among SGMC of pSS patients IL-22R1 was expressed on a significant percentage of macrophages (57.6±14%) and minimally on T and B lymphocytes (1.5±0.33 and 1.99±0.77 respectively), while IL-22R1 was not expressed at all in nSS subjects. We next investigated if peripheral blood isolated from p-SS patients also aberrantly expressed the IL-22R1. Flow cytometric analysis of isolated PBMC from pSS patients demonstrated that a significant percentage of circulating monocytes (61±18%) and a very smallest percentage of B and T lymphocytes (1.3±0.3 and 1.8±0.12, respectively) expressed IL-22R1 in pSS (Figure 4B). Conversely, no expression of IL-22R1 was observed among T cells, B cells and monocytes in the PBMC from nSS, (Figure 4B).
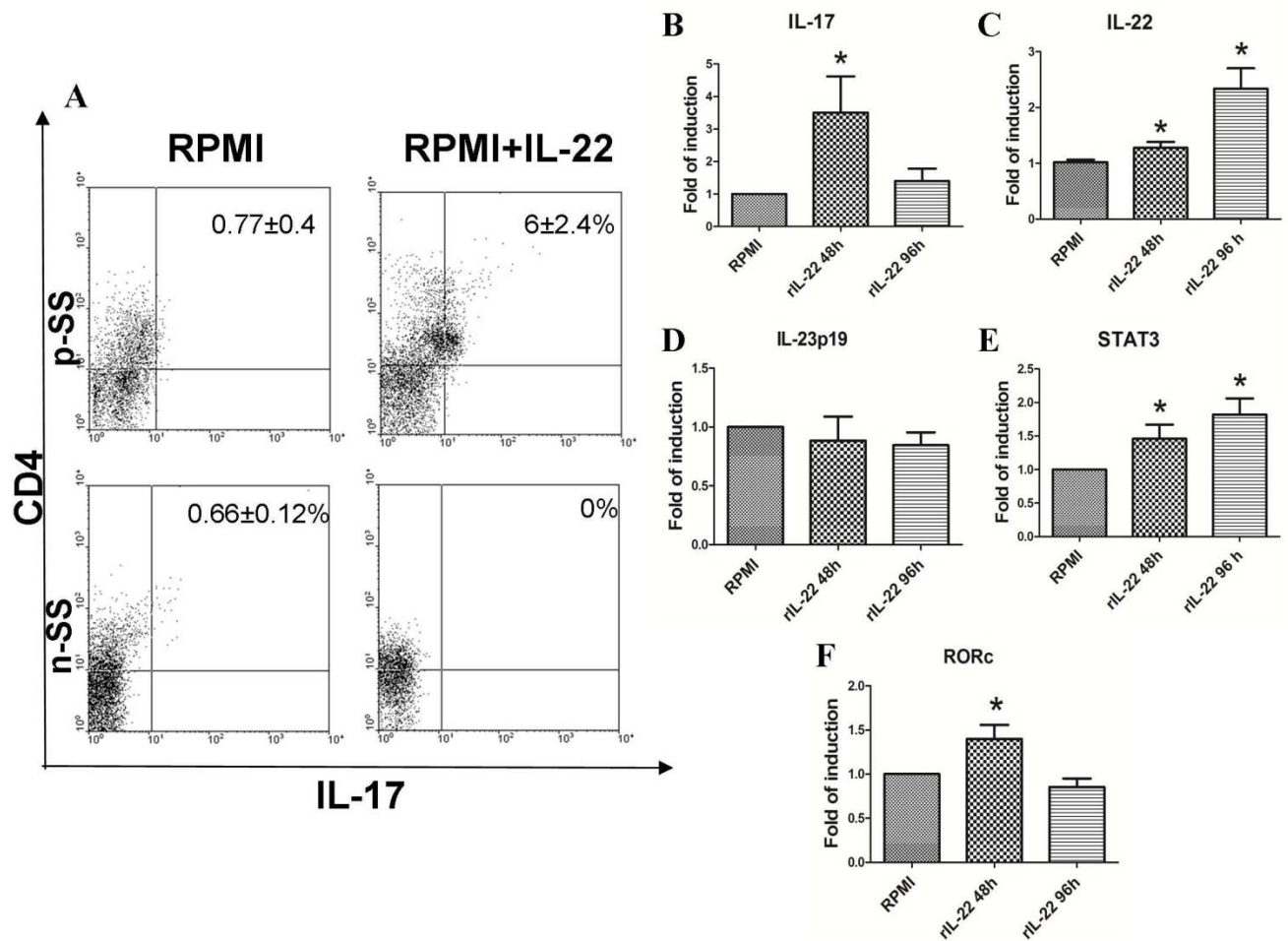
**Figure 4.**

**IL-22R1 expression on isolated mononuclear cells from salivary glands (SGMC) and peripheral blood (PBMC) of pSS and nSS subjects.** IL-22R1 expression was evaluated by flow cytometry on freshly isolated PBMC and SGMC from 10 patients with pSS and 8 nSS subjects. A: representative dot plot of IL-22R1 expression among SGMC from patients and controls. B: representative dot plot of IL-22R1 expression among PBMC from patients and controls. IL-22R1 expression was strongly observed in circulating monocytes and tissue macrophages only in pSS patients. Data are reported as mean ± SD.

In order to investigate if the aberrant expression of IL-22R1 on cells of hematopoietic origin was a specific feature of pSS or a shared immunologic signature with other autoimmune diseases, PBMC were obtained from healthy controls (n=10) and patients with systemic lupus

erythematosus (n=20) and rheumatoid arthritis (n=20) and the expression of IL-22R1 was assessed by flow cytometry. IL-22R1 expression was not detected among circulating B, T and myeloid cells (data not shown), suggesting that aberrant expression of IL-22R1 immunologically characterizes only pSS at both systemic and local levels.

**Stimulation of PBMC of pSS patients with IL-22 strongly up-regulates IL-22 and IL-17 expression**

IL-22 is known to act on epithelial cells but, due to the lack of constitutive expression of IL-22R1, direct evidence of the IL-22 function on hematopoietic cells is missing. We investigated, *in vitro*, the role of IL-22 on PBMC from pSS patients and nSS. *In vitro* stimulation with rIL-22 induced, only in pSS, a significant expansion of Th17 cells (Figure 5A) without effect on Th1, Th2 and Treg cells (data not shown) and was accompanied by significant up-regulation of IL-22, IL-17, RORc and STAT3 m-RNA levels (Figure 5B-F), suggesting a role of IL-22 in inducing IL-22/IL-17 immune responses.

**Figure 5.**

**Effect of recombinant IL-22 on PBMCs of pSS.** The percentage of IL-17 expressing cells and the m-RNA expression levels of IL-17, IL-22, IL-23p19, STAT3 and RORc were assessed by flow cytometry and RT-PCR. A: representative plot showing the percentage of IL-17 expressing cells before and after exposure to recombinant IL-22. B-F: Relative m-RNA quantification of IL-17 (B), IL-22 (C), IL-23p19 (D), STAT3 (E) and RORc (F) was assessed in isolated PBMCs from p-SS before and after incubation with recombinant IL-22. * p<0.05

**IL-22R1 in pSS-associated non-Hodgkin's lymphoma**

Patients with SS have a higher risk to develop non-Hodgkin lymphoma compared to either healthy people or patients with other autoimmune diseases [2]. Given the role of IL-22/IL-22R1 in the pathogenesis of B and T cells lymphoma, we evaluated the expression of IL-22R1 on non-Hodgkin's lymphoma (NHL) developed in 5 pSS patients. The diagnosis of pSS was established 6 to 20 years before the diagnosis of NHL. Three cases were low-grade marginal zone B-cell lymphoma with lymphoepithelial lesions, diffuse lymphoid infiltrates of small lymphocytes or with plasma-cell differentiation and/or centrocyte-like cells. Two were nodal lymphomas with histological features similar to that of low-grade MALT type lymphoma, showing a typical lymphoplasmacytoid infiltration with a variable proportion of blasts. The last neoplasia was a high-grade extra-nodal diffuse large B-cell lymphoma involving the retroperitoneal area. The B phenotype of each lymphoma was confirmed by using the pan-B antibody CD20. IL-22RA1 was significantly over-expressed in the lymphoma tissues of pSS patients and accompanied by significant p-STAT3 expression (Figure 6A and B respectively). As activated STAT3 promotes cell proliferation and survival in B-cell lymphomas [18] we next evaluated whether IL-22R1-

expressing cells also co-express p-STAT3. As shown in figure 6E p-STAT3 and IL-22R1 strongly co-localize suggesting that IL-22R1 could mediate constitutive p-STAT3 in pSS associated NHL. Altogether these results suggest that activation of IL-22R1/p-STAT3 axis may play a role in lymphomagenesis in pSS.
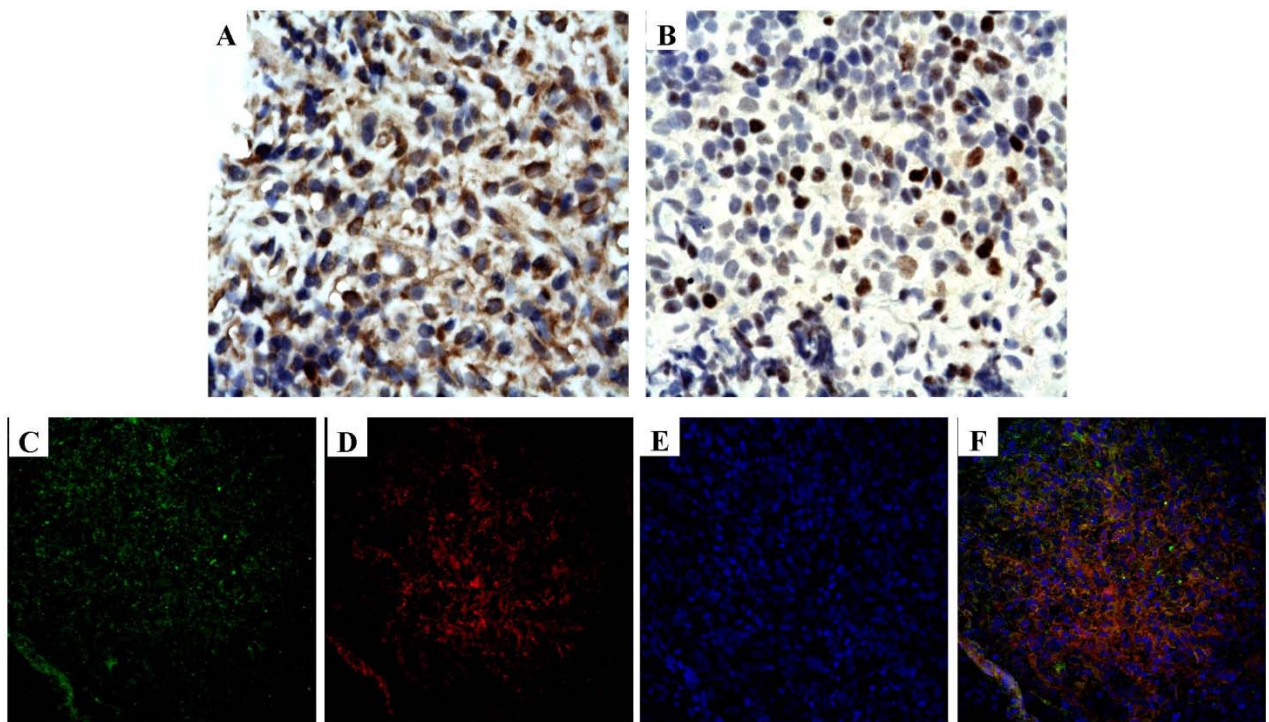


**Figure 6.**

**IL-22R1 and p-STAT3 expression in non-Hodgkin lymphomas (NHL) of pSS patients.** A-B: Representative microphotographs showing IL-22RA1 (A) and p-STAT3 (B) immunostainings in NHL of pSS patients. Intense immunostainings of IL-22RA1 (A) was observed in NHL tissues of pSS and was accompanied by the intense expression of p-STAT3 (B). C-F: Representative images of confocal analysis of IL-22RA1 and p-STAT3 co-localization in NHL tissues of pSS patients. C: single staining for IL-22RA1; D: single staining for p-STAT3; E: nuclei were counterstained with toto-3 (blue). F: merged double staining of IL-22RA1 (green) and p-STAT3 (red). A-B: original magnification x 630; C-F: original magnification x 250.

**Discussion**

In this study in a new set of patients, we confirm IL-22 over-expression in the salivary glands of pSS and provide the first evidence that its functional receptor, IL-22R1, is aberrantly expressed on hematopoietic cells at both systemic and salivary gland level. This immunological behavior seems to be disease-specific since IL-22R1 expression was not found on hematopoietic cells of RA and SLE patients. Since uncontrolled production of IL-22 has been associated with inflammatory disorders [10-13], the aberrant expression of IL-22R1 could provide evidences to support a pivotal role of IL-22 in the pathogenesis of salivary glands inflammation in pSS. We also demonstrate that aberrant expression of IL-22R1 occurs in non-Hodgkin lymphoma tissues of pSS patients suggesting a role for IL-22/IL-22R1 in the pathogenesis of pSS-associated lymphomas.

pSS is a chronic autoimmune disease characterized by altered cytokine networks as testified by the reduced levels of the anti-inflammatory cytokine transforming growth factor (TGF) β and the systemic overexpression of pro-inflammatory cytokines such as Interferon (IFN)α and γ, tumor necrosis factor (TNF) α, interleukin (IL-)12, IL-17, IL-18 and IL-22 [1, 3]. IL-22, in particular, has been recently suggested to play a role in the pathogenesis of pSS. IL-22 is over-expressed in the serum and

salivary glands of pSS patients and correlated with hyposalivation, the presence of autoantibodies and the lymphocytic focus score [3, 19]. Mechanisms through which IL-22 acts in the modulation of cytokine network in p-SS is still lacking however.

IL-22 is a member of the IL-10 family of proteins mainly expressed by activated T, NK and epithelial cells, being its physiological function essentially related to the maintenance of innate immunity [5, 6]. The functional outcome of IL-22 depends on the presence of the inducible IL-22R1 subunit in the epithelial cells of the gastrointestinal tract and skin and on the levels of IL-22 binding protein (IL-22BP). IL-22BP is a soluble IL-22 receptor, which lacks transmembrane and intracellular domains and specifically binds to IL-22 thus preventing its binding to IL-22R1 [20]. IL-22R1 has been reported to be characteristically absent in the cells of the immune system in normal conditions and in auto-immune diseases [9, 13]. Interestingly, the regulation of IL-22 signaling seems to be important in the control of tumorigenesis in humans since IL-22BP seems to be crucial in the control of the intestinal tumorigenic effects of IL-22 [14]. Furthermore, aberrant expression of IL-22R1 has been demonstrated on ALK[+]ALCL leukemia cells [15] and mantle cell lymphoma [16] suggesting that a IL-22 autocrine functional stimulatory

pathway may participate in the pathogenesis of lymphomatous diseases.

Here we show that IL-22R1 is strongly up-regulated at protein but not m-RNA level in pSS salivary glands, being the down-regulation of IL-22R1 transcripts probably related to a negative regulatory feedback loop. By immunohistochemistry, IL-22R1 expression was mainly observed among salivary glands infiltrating macrophages and, albeit in a less number, T lymphocytes. IL-22R1-expressing macrophages were mainly located in close proximity to CD20+ B cells. Since IL-22 seems to be involved in the germinal center formation, probably by driving and maintaining CXCL13 production [21], IL-22R1-expressing macrophages could strategically participate in the organization of immune response in pSS. Interestingly, the abnormal expression of IL-22R1 on hematopoietic cells was accompanied by a concomitant expression of p-STAT3, strongly suggesting the existence of an autocrine IL-22 stimulatory loop in pSS. Notably, IL-22R1 aberrant expression was also observed on peripheral macrophages and T lymphocytes from pSS patients. This immunological behavior appeared to be highly restricted to pSS since IL-22R1 expression was never observed, in our study, in cells of hematopoietic

origin in nSS, RA and SLE peripheral blood and, in a previous paper, in RA synovial biopsies [13].

In the present study, we have also examined the immunological consequences of the atypical expression of IL-22R1 in pSS. In vitro stimulation of PBMC of pSS but not n-SS with IL-22 resulted in a significant up-regulation of the m-RNA levels of IL-17, IL-22, ROR-c and STAT3 accompanied by a significant expansion of IL-17-producing CD3-expressing cells. These results are in agreement with those obtained in previous studies analyzing patients with ALK+ALCL and IL-22R1 transgenic mice. Patients with ALK+ALCL, that are characterized by the abnormal expression of IL-22R1 on lymphomatous T cells, display increased serum levels of IL-22 and IL-17 [15]. Similarly, IL-22R1 transgenic animals, that express IL-22R1 on lymphocytes, developed a systemic inflammatory disease characterized by increased levels of circulating IL-17, granulocyte colony-stimulating factor and IL-22 [22]. Altogether these findings, suggests that the atypical expression of IL-22R1 on hematopoietic cells generates a strong IL-22-polarized immune response in a positive auto-regulatory loop.

SS is a risk factor for the development of salivary glands lymphoma [2]. IL-22/IL-22R1/STAT3 axis has been previously demonstrated to play a

pivotal role in human tumorigenesis [15, 16, 23-26]. Interestingly, it seems that the balance between IL-22 and its soluble antagonist IL-22BP, more than the expression of IL-22 per se, critically regulates tumorigenesis [14]. In the salivary glands of pSS patients IL-22BP m-RNA levels were strongly up-regulated and correlated with the levels of IL-22, suggesting that IL-22/IL-22BP axis is activated and critically involved in the regulation of salivary glands inflammation, presumably counterbalancing the tumorigenic activity of the IL-22/pSTAT3 pathway. As a confirmation of the importance of IL-22/IL-22R1 axis in the pathogenesis of non-Hodgkin lymphomas in p-SS patients, in our study aberrant IL-22R1 expression was observed on lymphoma-infiltrating cells. The abnormal IL-22R1 expression  was also accompanied by co-expression of p-STAT3, suggesting that persistent IL-22 stimulation may be biologically important in the growth of non-Hodgkin lymphoma by constitutively activating the STAT3 signaling.

In summary, we have found evidence of specific aberrant activation of IL-22/IL-22R1/STAT3 pathway in the macrophages of pSS patients providing a potential biologic explanation of the over-expression of IL-22 in pSS. We also provide evidence that the IL-22/IL-22R1 axis is also functional in NHL lymphoma tissues of pSS patients and is accompanied

by the constitutive activation of STAT3, pointing to IL-22 in the pathogenesis of these neoplasms. Hence, blocking the aberrant IL-22 pathway may be a useful therapeutic strategy for p-SS and p-SS-associated MALT lymphoma.

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**Chapter 3**

**Rituximab modulates the expression of IL-22 in the salivary glands of patients with primary Sjogren's syndrome**

We have recently demonstrated that interleukin (IL)-22, mainly produced by Th17 effector cells, NKp44+NK cells and epithelial cells, may be potentially involved in the pathogenesis of primary Sjogren's syndrome (pSS) [1]. The IL-22/IL-22R pathway is known to play a role in the emergence of T and B-cell lymphoma [2-3] and pSS is considered a risk factor for the development of lymphoma [4].

Rituximab (RTX), which has historically been used for the treatment of B-cell lymphoma [5], has also been considered to be effective in the therapy of pSS [6].

Ten consecutive patients with pSS (eight women and two men, with a mean duration disease of 48±18 months), diagnosed according to the American–European Consensus Group criteria for pSS [7], who were treated with two courses of intravenous infusions of 1000 mg RTX (Roche, Woerden, The Netherlands) at days 1 and 15, at baseline and then after 6 months, were considered for this study. After 48 weeks the patients again underwent salivary gland biopsy. The demographic,

clinical and histological characteristics of the patients are shown in Table 1.

**Table 1**
Clinical and histological characteristics of the pSS patients

| | Age/sex | Disease duration (months) | ENA anti-RoSSA/LaSSB | Focus score pre/post-rituximab | N of IL-22^{+} infiltrating cells pre/post-rituximab | N of IL-22^{+} myoepithelial cells pre/post-rituximab | Saliva flow rate ml/min pre/post-rituximab (mean) | Schirmer's test, mm/5 min (mean) |
|---|---|---|---|---|---|---|---|---|
| Patient 1 | 38/F | 44 | +/+ | 3/2 | 41/22 | 30/18 | 0.2/0.4 | 5/10 |
| Patient 2 | 44/F | 22 | +/− | 4/4 | 54/24 | 22/16 | 0.3/0.5 | 6/12 |
| Patient 3 | 52/F | 36 | +/+ | 4/3 | 44/33 | 18/13 | 0.13/0.33 | 5/8 |
| Patient 4 | 43/F | 28 | +/+ | 3/3 | 41/18 | 21/9 | 0.18/0.5 | 4/9 |
| Patient 5 | 45/F | 48 | −/− | 2/2 | 34/19 | 18/7 | 0,25/0.6 | 7/8 |
| Patient 6 | 39/F | 96 | −/− | 3/3 | 28/21 | 24/11 | 0,2/0.3 | 6/6 |
| Patient 7 | 54/F | 60 | +/+ | 2/2 | 23/16 | 13/6 | 0.16/0.33 | 3/7 |
| Patient 8 | 42/F | 96 | +/+ | 3/2 | 22/11 | 18/9 | 0.13/0.45 | 2/9 |
| Patient 9 | 72/M | 60 | −/− | 4/3 | 18/14 | 23/10 | 0.4/0.6 | 4/12 |
| Patient 10 | 52/M | 48 | +/− | 3/3 | 28/21 | 26/14 | 0.5/0.88 | 5/9 |

pSS, primary Sjogren's syndrome; ENA, Extractable Nuclear Antigen.

Ethics approval was granted and written consent was obtained from all patients. Evaluation of the clinical efficacy of RTX treatment was done by measuring the improvement between day 1 and week 48 in the values of salivary and lacrimal gland function. Unstimulated whole, parotid and submandibular/sublingual saliva samples were collected, as described by Meijer *et al* [8]. Lacrimal gland function was evaluated by performing the Schirmer's test. Immunohistochemistry for IL-22 was performed, as previously described, on paraffin-embedded salivary gland biopsies by using a rabbit anti-human IL-22 antibody [1]. The

number of IL-22$^+$ cells was determined by counting, in a blinded fashion before and after rituximab treatment, IL-22 immunoreactive cells on photomicrographs obtained from three random high-power microscopic fields (400× magnification). Intense IL-22 staining was observed among infiltrating mononuclear cells, epithelial and myoepithelial cells. RTX treatment significantly reduced the number of IL-22$^+$ cells infiltrating the salivary glands of pSS patients (Table 1 and Figure 1A,B). The expression of IL-22 by epithelial cells was also reduced in those patients displaying a lower focus score (Table 1 and Figure 1C,D). Interestingly, RTX treatment specifically abolished the production of IL-22 by myoepithelial cells (Figure 1E,F).

# IL-22 expression in salivary glands of patients with primary Sjogren's syndrome (pSS) is modified by rituximab (RTX) treatment.
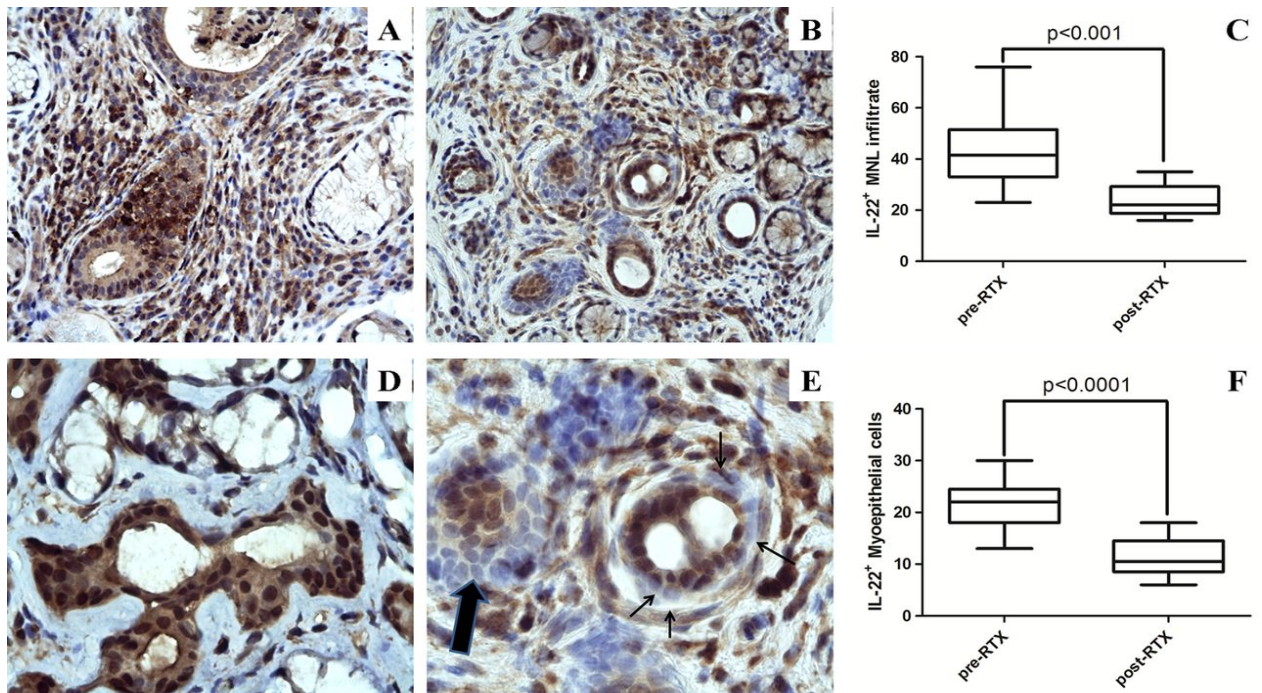


**Figure 1**

IL-22 expression in salivary glands of patients with primary Sjogren's syndrome (pSS) is modified by rituximab (RTX) treatment. Representative immunostaining for IL-22 in the pSS patient number 3 before and after therapy with rituximab. Positive infiltrating mononuclear cells observed before therapy (A) were significantly reduced after rituximab treatment (B) The intense immunostaining observed at baseline in myoepithelial cells (D) was also drastically reduced in rituximab-treated patients (arrows) (E). (C–F) immunohistochemical quantification of IL-22$^+$ infiltrating cells (C) and myoepithelial cells (F) in salivary gland biopsies from pSS before and after rituximab therapy. Results are expressed as the number of positive cells per field. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. (A, B) original magnification × 25; (D, E) original magnification × 63.

The whole saliva flow rate and the lacrimal gland function showed a significant improvement after RTX therapy from 0.24±0.12 ml/min (mean±SD) at baseline to 0.45±0.15 ml/min (mean±SD) at week 48, p=0.0003; Schirmer's test, 4.7±1.5 mm/5 min (mean±SD) at baseline and 8.7±2.11 mm/5 min (mean±SD) at 48 weeks, p=0.00011)). A trend (although not statistically significant) towards a major improvement in salivary and lacrimal glands was detected in those patients with a major reduction in the numbers of IL-22$^+$ cells after RTX therapy.

Although we cannot exclude the possibility that the reduction in IL-22 production may be due to the natural history of the disease, these preliminary results suggest that RTX therapy may modify the immunological micro-environment of inflamed salivary glands of pSS patients reducing the local expression of IL-22, and thus providing an additional immunological explanation for RTX efficacy in pSS. The immunological effects of rituximab therapy beyond B-cell depletion have recently been demonstrated. In this regard, a reduction in the Th17 response in the synovial tissues of patients with rheumatoid arthritis has been observed after RTX therapy [9]. Even if a possible explanation for these immunological changes could reside in the reduction of antigen presentation by B to T cells, the exact mechanisms

by which RTX therapy modifies the IL-22 response in pSS remain to be elucidated. As with the role of IL-22/IL-22RA1 in the pathogenesis of B and T-cell lymphoma [2-3], the RTX-dependent reduction of IL-22 expression may also be of relevance in reducing the risk of the evolution of pSS towards lymphoma.

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## Chapter 4

## Rituximab modulates IL-17 expression in the salivary glands of patients with primary Sjogren's syndrome

Primary Sjogren's syndrome (pSS) is a chronic autoimmune inflammatory disorder in which salivary glands (SGs) inflammation occurs in the presence of altered adaptive and innate immune responses [1] testified by the altered serum and tissue cytokine expression such as the IL-23p19/IL-17 pathway [2-7]. The neutralization of IL-23p19 and/or IL-17 appears therefore to be a promising therapeutic approach in pSS but, despite the demonstration that IL-17 inhibition may directly interfere with the onset of SS-like disease in mice [8], clinical studies involving patients with pSS are missing.

Rituximab (RTX), a monoclonal antibody to CD20, has been used and proved to be effective in reducing symptoms and in ameliorating the extent of tissue inflammation of p-SS patients [9-11]. Beyond its role in depleting B cells, RTX seems to be also able to modulate the T cell responses in autoimmune diseases [12, 13]. Aim of the present work was to clarify whether RTX may be implicated in modulating the IL-23p19/IL-17 immune response in pSS patients.

**Material and Methods**

**Patients**

Fifteen consecutive patients fulfilling the new American-European Consensus Group criteria for primary Sjogren's syndrome [14], were consecutively enrolled at Palermo and l'Aquila University Hospitals (Italy). Disease activity was assessed using the visual analog scales (VAS) for global disease activity. All the patients had active disease with values >50 mm on >2 of 4 100-mm visual analog scales (VAS) (global disease activity including extraglandular manifestations, pain, sicca syndrome, and fatigue) during the last 3 months. Additional inclusion criteria were positivity for either anti-SSA and/or anti-SSB autoantibodies. Ten patients with subjective complaints of dry mouth or eyes who didn't met the American-European Consensus Group criteria for pSS were considered as control group (nSS). Baseline characteristics of patients and controls are summarized in Table I. All the pSS patients were treated with two cycles of intravenous infusions of 1.000 mg RTX (Roche, Woerden, The Netherlands) at days 1 and 15 (at time 0 and then after 6 months). After 48 weeks the patients underwent again to salivary glands biopsy. The study was approved by the ethics committee of local

institutions. Informed consent for the collection and storage of biological material was also obtained.

**Labial salivary gland biopsy**

Labial salivary glands biopsies were obtained and placed into formalin fixative and RNA later. Standard paraffin sections at 5-μm thickness were stained with hematoxylin and eosin (H&E). Histological evaluation of salivary glands for the presence of lymphocytic infiltrates and/or foci was performed according to the classification of Chisholm and Mason. A focus was defined as an aggregate of ≥ 50 lymphocytes. The focus score was reported as the number of foci per 4 mm$^2$ of tissue, up to a maximum of 12 foci. All pSS patients presented a biopsy focus score >1, whereas the control group had a focus score <1.

**Immunohistochemistry**

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-μm–thick paraffin-embedded sections from salivary glands and from tonsils (used as positive controls) as previously described [5, 7]. Isotype-matched irrelevant antibodies were used as a negative control. The primary antibodies anti-human mast cell tryptase (clone AA1) (DAKO, Glostrup, Denmark) (1:100 dilution), rabbit anti-human IL-17 (Santa Cruz

Biotechnology, Santa Cruz, CA) (1:100 dilution), rabbit anti-human IL-17

(R&D Systems) (1:100 dilution), rabbit anti-human IL-23p19 (BioLegend,

San Diego, CA) (1:50 dilution), rabbit anti-human p-STAT3 (Cell Signaling

Technology, Beverly, MA) (1:50 dilution) and mouse anti-human CD3

(DAKO, Glostrup, Denmark) (1:100 dilution) were added and incubated

for 1 hour at room temperature. As a control, primary antibodies were

replaced with irrelevant isotype-matched antibodies (AbCam). The slides

were incubated for 30 minutes with peroxidase-conjugated Dako

EnVision polymer and peroxidase activity was visualized using

diaminobenzidine chromogen (Dako). Slides were lightly counterstained

with hematoxylin before dehydration and mounting in DePex (VWR

International, Oslo, Norway). To characterize IL-17-producing cells a

double staining was performed on paraffin-embedded sections of

human salivary glands. Sections were incubated with unlabeled rabbit

anti-human IL-17 and mouse anti-human tryptase antibodies and then

treated with FITC-conjugated anti-mouse (Alexa fluor, Invitrogen Life

Technologies, Italy) or Rhodamine Red–conjugated (Alexa fluor,

Invitrogen Life Technologies, Italy) anti-rabbit antibodies plus RNasi (200

ng/mL) and counterstained using Toto-3 iodide (642/660; Invitrogen).

Confocal analysis was used to acquire fluorescence staining.

**Flow cytometry**

Salivary gland tissues were extensively washed in saline buffer and enzymatic digestion was performed using collagenase (1.5 mg/ml; Life Technologies, Carlsbad, CA) in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated pooled human AB+ serum, 2 mM l-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, $5 \times 10^{-5}$ M 2-ME, for 2 h. Recovered cells were then cultured with PMA (50 ng/ml) and ionomycin (1 µg/ml) and incubated at 37°C in 5% $CO_2$. After 2 h of incubation Brefeldin A (10 µg/ml; Sigma St. Louis, MO) was added and after 16 h of incubation cells were collected and stained with the following monoclonal antibodies (mAb): anti-human CD4-APC (BD Biosciences, San Josè, CA), anti-human CD117-PE (DAKO, Glostrup, Denmark) and anti-human IL-17-PerCP (BD Biosciences, San Josè, CA). Isotype-matched irrelevant antibodies were used as a negative control. Total cells were incubated with mAbs for 30 min on ice and washed twice in PBS, cointaining 0.1% (w/v) NaN3. After surface staining, the cells were fixed with 1% (w/v) paraformaldehyde (Sigma)  for 30 min at 4°C and then were permeabilized with a permeabilization solution (BD Biosciences) for 10 minutes at room temperature and stained  with antibodies to intracellular antigens for 30

min at 4°C.  Four color flow cytometry analysis was performed using a FACSCalibur (BD Biosciences). At least 50,000 cells (events), were acquired for each sample.

**Isolation of mast cells from peripheral blood mononuclear cells and *in vitro* culture**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples of 10 pSS patients and 10 nSS as previously described [7]. CD117$^+$ cells were purified by positive selection using a mouse anti-human CD117 antibody and anti-mouse IgG microbeads (Miltenyi-Biotec, UK) for the magnetic separation. Next, 1x10$^5$ CD117$^+$ cells from patients and controls, pre-activated with N-Formyl-Met-Leu-Phe (fMLP), were cultured, alone or with heterologous CD117$^-$ cells, in triplicate in 96-well round bottom plates in a volume of 200 $\mu$l per well. After 72h of culture cells were stained with the following antibodies: anti-human-CD4-APC, anti-human-IL17-Percp (from BD, Biosciences) and anti-human-CD117-PE (from BD, Biosciences). For *in vitro* cytotoxicity assays, cells were treated for 2 h with rituximab (10 $\mu$g/ml). Control cells received media only. After treatment, 1x10$^6$ cells were plated in duplicate in 24-well plate in a volume of 1 ml per well and incubated at 37°C in 5% $CO_2$ and 90% humidity for 5 days. Cytotoxicity

assay was performed using FITC-Apoptosis detection kit (from BD, Biosciences). Results are expressed as % of annexin positive apoptotic cells.

## Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad software Inc.,USA). Data are expressed as mean ± SD. *p* values less than 0.05 were considered significant.
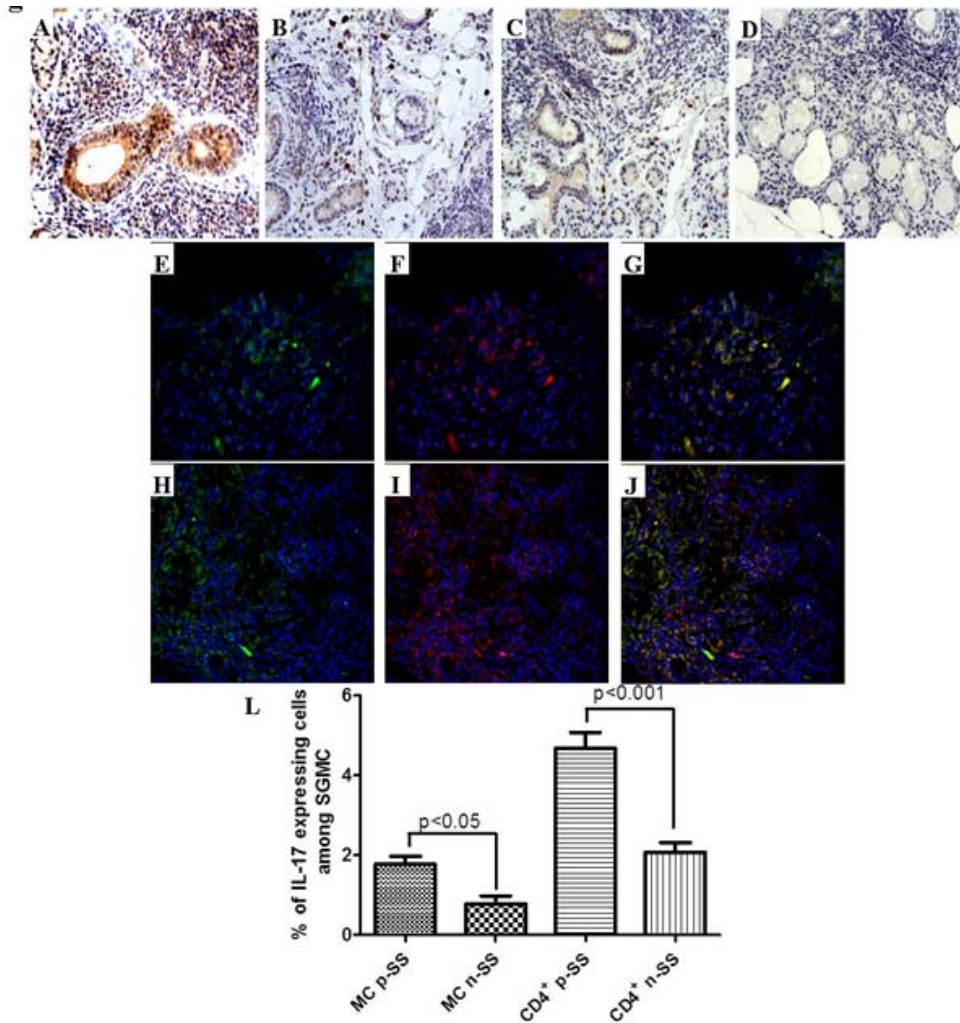
**Results**

**Clinical results**

Fifteen patients (2 men and 13 women) with active pSS were consecutively included between January 2010 and January 2012 in the study. All the patients received 4 infusions of RTX, completed the follow-up visits through week 48, and underwent to labial salivary glands biopsies at base-line and after 48 weeks. RTX demonstrated to be effective in ameliorating the whole saliva flow rate from $0.22 \pm 0.13$ ml/minute (mean ± SD) at baseline to $0.5 \pm 0.2$ ml/minute (mean ± SD) at weeks 48, p=0.0002] and the lacrimal gland function assessed by Schirmer's test from $5.1 \pm 2.1$ mm/5 minutes (mean±SD) at baseline and $9.3 \pm 2.3$ mm/5 minutes (mean±SD) at 48 weeks, p=0.0001].

**Mast cells and T cells produce IL-17 in the salivary glands of pSS**

Since anti-IL-17 antibodies from different suppliers can give conflicting results [15], to evaluate the salivary glands location of IL-17-expressing cells we used two different anti-human IL-17 antibodies. The first antibody, obtained from Santa Cruz, showed a diffuse, intense, cytoplasmic staining of mononuclear, epithelial and vascular endothelial cells (Figure 1A). In contrast, the antibody obtained from R&D Systems

predominantly marked large cells with round-to-oval morphology scattered in the periphery of lymphocytic foci and around blood and lymphatic micro-vessels that highly resembled mast cells (Figure 1B-C; Figure 1D showed isotype control antibody staining). We performed additional double staining with CD3/IL-17A antibody and tryptase/IL-17A antibody in order to clarify the nature of the IL-17A–positive cells, confirming that both T lymphocytes (Figure 1E-G) and mast cells (Figure 1H-J) express IL-17A. To confirm the cellular sources of IL-17 among salivary glands of p-SS patients, salivary glands mononuclear cells were isolated from 5 pSS patients and 5 controls and analyzed for IL-17 production by flow cytometry. As shown in figure 1L, both IL-17-producing CD4+ T cells and mast cells were significantly expanded in the salivary glands of pSS compared to controls.
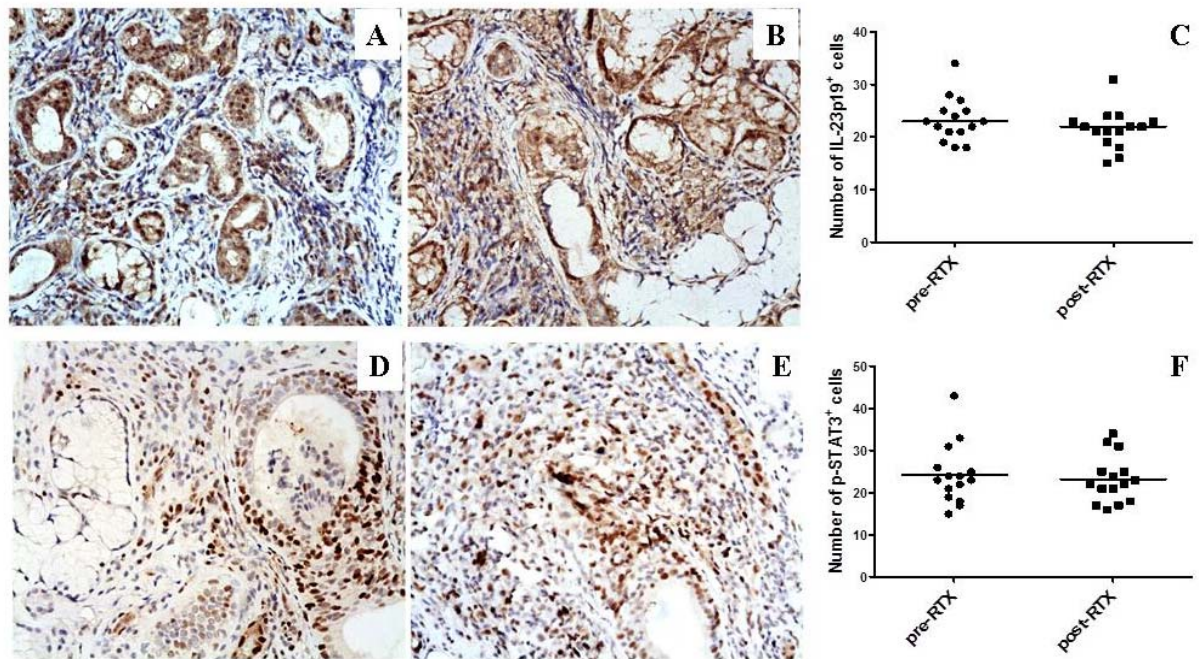
**Figure 1.**

IL-17 is expressed by CD3+ T cells and mast cells in salivary glands (SGs) of patients with primary Sjogren Syndrome (p-SS). Representative immunostaining for IL-17 in p-SS patients. Different results were obtained with two different antibodies. **A**: intense, cytoplasmic staining of mononuclear, epithelial and vascular endothelial cells was observed with the Santa Cruz antibody. **B-C**: the antibody from R&D Systems predominantly marked large cells with round-to-oval morphology scattered in the periphery of lymphocytic foci. **D**: Representative paraffin sections of salivary glands of p-SS patients stained with rabbit immunoglobulins (isotype control). **E-G**: Representative images of confocal analysis of CD3 and IL-17 (R&D) co-localization in SGs tissues of p-SS patients. **E**: single staining for CD3; **F**: single staining for IL-17; **G**: merged double staining of CD3 (green) and IL-17 (red). **H-J**: Representative images of confocal analysis of triptase and IL-17 (Santa Cruz) co-localization in SGs tissues of p-SS patients. **H**: single staining for triptase; **I**: single staining for IL-17; **J**:

merged double staining of triptase (green) and IL-17 (red). **A-J**: original magnification x250. **L**: percentage of IL-17 expressing cells evaluated by flow cytometry among isolated salivary gland mononuclear cells of p-SS patients and controls. Both CD4$^+$ and mast cells isolated from p-SS patients significantly produced more IL-17 than control subjects.
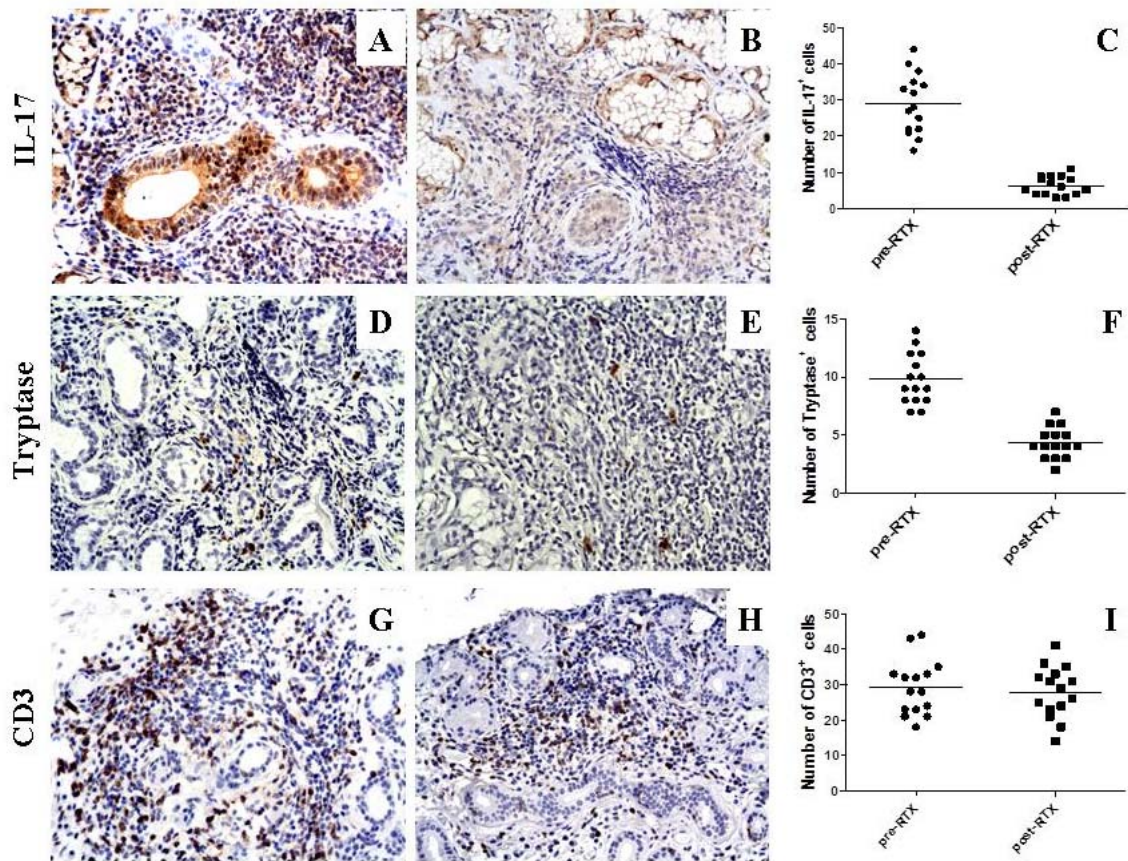


**Figure 2**

IL-23p19 and p-STAT3 expression is not modified by Rituximab (RTX) therapy in in the salivary glands (SGs) of patients with primary Sjogren Syndrome (p-SS). **A-B**: representative immunostaining for IL-23p19 in p-SS SG before (**A**) and after (**B**) RTX therapy. **C**: Number of IL-23p19 positive cells in the SG before and after RTX therapy. **D-E**: representative immunostaining for p-STAT3 in p-SS SG before (**D**) and after (**E**) RTX therapy. **F**: Number of IL-23p19 positive cells in the SG before and after RTX therapy. **A-B, D-E**: original magnification x250.

**Rituximab therapy is accompanied by a reduced expression of IL-17 in the salivary glands of pSS patients**

We next evaluated the effect of RTX on the expression of IL-17, IL-23p19 and p-STAT3 in the pSS patients. After RTX therapy we observed a significant depletion of CD20-positive B cells (data not shown) in the salivary glands without a significant modulation of expression of IL-23p19 (Figure 2A-C) and of p-STAT3 (Figure 2D-F). Conversely, a significant reduction in the expression of IL-17 was clearly detectable (Figure 3A-C) and was accompanied by a pronounced tissue depletion of tryptase-positive cells (Figure 3D-F) but not of tissue infiltrating CD3-positive cells (Figure 3G-I).

**Figure 3**

IL-17 expression is significantly reduced by Rituximab (RTX) therapy in in the salivary glands (SGs) of patients with primary Sjogren Syndrome (p-SS) and is accompanied by a tissue depletion of mast cells. **A-B**: representative immunostaining for IL-17 in p-SS SG before (**A**) and after (**B**) RTX therapy. **C**: Number of IL-17 positive cells in the SG before and after RTX therapy. **D-E**: representative immunostaining for CD3 in p-SS SG before (**D**) and after (**E**) RTX therapy. **F**: Number of CD3 positive cells in the SG before and after RTX therapy. **G-H**: representative immunostaining for tryptase in p-SS SG before (**G**) and after (**H**) RTX therapy. **I**: Number of tryptase positive cells in the SG before and after RTX therapy. **A-B, D-E, G-H**: original magnification x250.

**Rituximab reduces circulating mast cells and induces in vitro mast cell apoptotic death**

We next evaluated the frequency of circulating mast cells in p-SS before and after RTX therapy. Although the percentage of circulating mast cells was not increased in pSS compared to controls (data not shown), a significant depletion of peripheral mast cells was observed after RTX therapy (Figure 4A). We next assessed whether the observed changes in mast cells frequency could be attributable to a direct cytotoxic effect of RTX therapy. PBMC isolated from pSS and nSS were cultured with RTX and the percentage of mast cells was evaluated. An important and significant increase in the percent of apoptotic mast cells, as demonstrated by annexin V labeling, was observed in both patients and controls after incubation with rituximab (Figure 4B-C).

**Mast cells isolated from p-SS patients potently drive Th17 polarization**

We next analyzed the capacity of mast cells isolated from pSS patients and healthy controls to expand human Th17 cells in culture. Isolated PBMC from patients and controls were firstly depleted of mast cells and then incubated with heterologous activated mast cells. As shown in figure 4D, mast cells isolated from pSS patients mediate greater

expansion of Th17 cells than mast cells from healthy controls (figure 4E), suggesting an important role of mast cells in regulating the Th17 response in pSS patients.
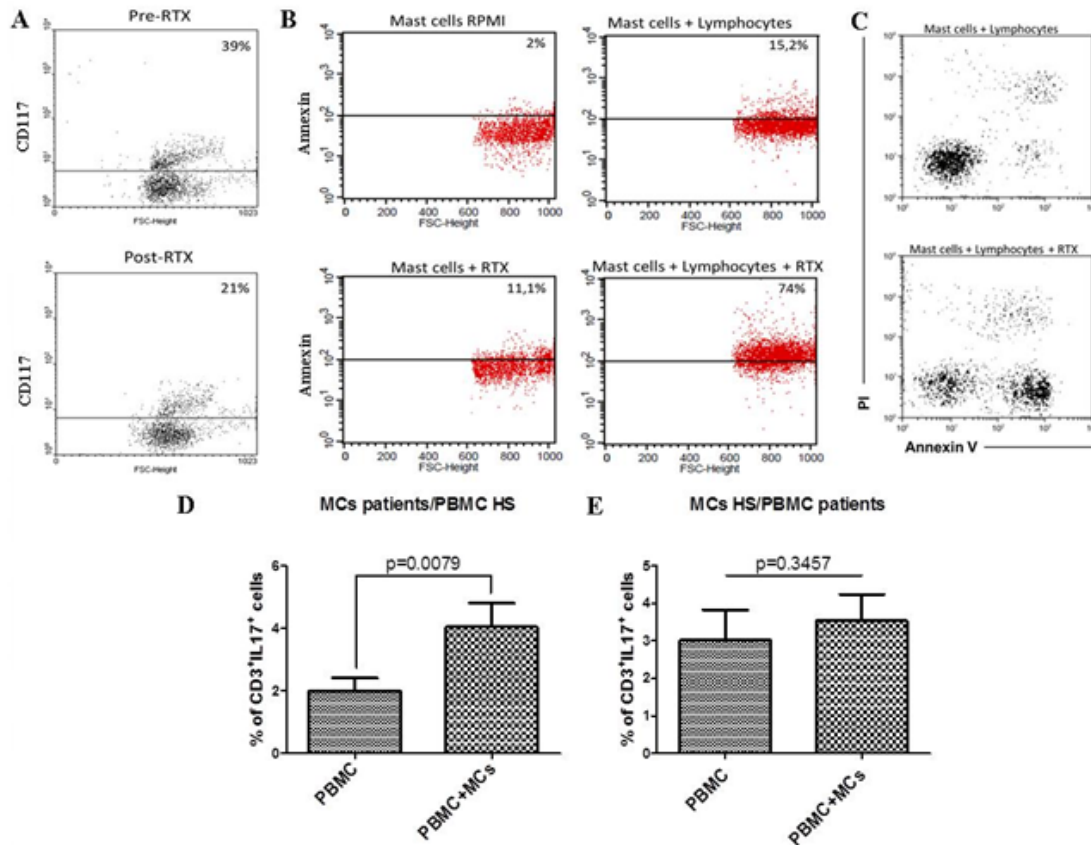


**Figure 4**.

Rituximab acts on mast cells in both *ex vivo* and *in vitro* experiments. PBMCs were isolated from p-SS patients before and after RTX therapy, stained with anti-human CD3 and CD3^{-} cells were evaluated for CD117 expression. **A**: representative dot plot showing the significant depletion of circulating mast cells in a CD117 enriched population after RTX therapy in a patient with p-SS. **B**: Dot plot analysis of annexin expression after co-colture of MCs isolated from p-SS and heterologus lymphocytes in the presence or not of RTX (10 mg/ml). **C**: Dot plot analysis of combined annexin V and PI expression after co-colture of MCs isolated from p-SS and heterologous lymphocytes in the presence of RTX (10 mg/ml). **D-E:** Mean percentage (± SD) of Th17 cells at baseline and after co-colture with isolated MCs from patients and controls.

**Discussion**

This is the first evidence that rituximab treatment in pSS patients is accompanied by a strong reduction of the salivary gland expression of IL-17 but not of IL-23 and p-STAT3 suggesting a specific role of RTX on IL-17-producing cells, that is not mediated by the modulation of IL-23 pathway. A significant reduced IL-17 expression was observed in the salivary glands of RTX treated pSS patients suggesting a direct role of RTX in modulating the IL-17 salivary gland expression.

Although CD4$^+$ T cells were thought to be the most important cell type involved in IL-17 responses, several studies indicate that IL-17 in humans is produced by a broad spectrum of cells of the innate and adaptive immune systems at different sites and phases of tissue inflammation [16]. Conflicting data, however, were obtained from studies analyzing the expression of IL-17 by immunohistochemisty in the inflamed human tissues with T cells and mast cells being indicated, in a mutually exclusive manner, as the main IL-17-producing cells [17-19]. These apparently conflicting results are probably dependent on the type of antibody used rather than on real differences in patients enrolled and/or on different stages of disease [15]. To clarify which cells produce IL-17 in the salivary glands of pSS patients in this study we evaluated

two different antibodies by immunohistochemistry, demonstrating that both CD3+ cells and mast cells are an important cellular sources of IL-17 in salivary glands of pSS patients. The production of IL-17 from salivary glands T lymphocytes and mast cells of pSS patients was also confirmed by flow cytometry analysis performed on isolated salivary glands mononuclear cells.

RTX is a chimeric monoclonal antibody that induces B cells depletion, and is therefore used to treat diseases in which B cells are overactive or dysfunctional [20]. Together with the depletion of B cells, recent evidences indicate that treatment with RTX may reduce the exaggerated Th17 response occurring in RA synovial tissues [12] and the production of IL-22 in salivary glands of pSS patients [13], and suggest a more wide potential role of RTX in the treatment of IL-17/IL-22-related immune diseases.

In our study, RTX treatment globally reduced the amount of IL-17 in the salivary glands but while the number of T cells was not significantly affected, a strong depletion of tissue and circulating mast cells was observed, suggesting a specific immunologic effect of RTX on the mast cell compartment. The reduction of mast cells appears to be specifically induced by rituximab as demonstrated by our *in vitro* experiments

where incubation of mast cells from patients and controls with rituximab was followed by a significant mast cells apoptosis. We actually don't know the exact mechanism of rituximab-dependent apoptosis of mast cells. However, we could speculate that due to the structural homology of CD20 and the high-affinity IgE receptor $\beta$ chain (Fc$\epsilon$RI$\beta$) [21] expressed on mast cells, rituximab may non-specifically bind to mast cells inducing their apoptotic death.

Studies in both human disease and animal models support the idea that mast cells are involved in the initiation of inflammatory response by producing and promoting the release of pro-inflammatory cytokines. In particular mast cells seem to be able to skew the immune reaction toward a Th17 response through mechanisms that require IL-6 and OX40 engagement [22], IL-33/ST2 stimulation [23] and modulation of dendritic cell maturation and function [24]. In our *in vitro* studies, mast cells isolated form pSS patients strongly induced T cells to produce IL-17, confirming the results of previous studies and suggesting a potential role of mast cells in organizing the adaptive IL-17-mediated immune responses in pSS. We do not obviously know if the activation of mast cells preceeds the Th17 response or *vice versa*. However, in view, of the

fact that mast cells are tissue resident cells, one could assume a primary role of mast cells in orchestrating locally the immune Th17 response.

In conclusion, this study suggests that RTX acts on pSS patients by globally reducing the expression of IL-17 and specifically inducing a pronounced apoptotic depletion of mast cells.

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**Conclusion**

The pathogenesis of pSS seems to be multifactorial and both innate and adaptive immunity seem to play important and interconnected roles. IL-22 may be a central cytokine at the bridge between innate and adaptive immunity in pSS. Increased IL-22 expression by unknown stimuli in epithelial cells may be an early step in the pathogenesis of pSS leading to the activation of IL-22 pathway, with IL-22 over-expression and aberrant expression of IL-22R1 on cells of hematopoietic origin. The resulting chronic IL-22 pathway activation may be responsible for the local and systemic inflammation as well as for the long term evolution to non-Hodgkin lymphoma. In this regard, IL-22R1 aberrant expression seems to mark with good specificity the disease at both local and systemic levels and may potentially represent a good candidate for future therapeutic intervention in pSS. Innate immunity and in particular NK cells activity seems also important in the pathogenesis of pSS and may also represent the target for future therapeutic strategies.

**Five years view**

At present one of the greatest challenges of p-SS research is to identify good biomarkers for early diagnosis, prognosis, disease management

and prediction of evolution to lymphoma. To date the absence of clearly effective therapeutic strategies necessitates the identification of new and, until now unexplored, therapeutic targets. Our better understanding of the underlying mechanisms of innate and adaptive will allow us to develop targeted and most likely successful therapeutic approaches. There is much yet to be known, however, particularly in terms of genetic susceptibility. Perhaps large GWAS studies will help us to improve our understanding of the pathogenetic mechanisms of pSS.

**Key issues**

- IL-22 and IL-22R1 over-expression in the inflamed salivary glands of pSS patients suggests the existence of an autocrine pro-inflammatory loop that may be responsible for the self-perpetuation of the salivary glands inflammation.

- The involvement of IL-22/IL-22R1 in the pathogenesis of B and T cell lymphomas may suggest a role for this pathway in the development of Sjogren-associated non-Hodgkin lymphoma.

- Innate immune system through NK cells and in particular through the activating receptor NKp44 may play a key role in the early phase of inflammatory response in pSS patients.