EXTENDED REPORT

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

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ABSTRACT

Objectives In chronic inflammatory disorders, interleukin (IL)-22 may act either as a protective or as a pro-inflammatory cytokine. 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 (NKp44$^+$ NK cells). The aim of this study was to investigate the IL-22 expression in the salivary glands of patients with primary Sjögren's syndrome (pSS).

Methods Minor salivary gland biopsies were obtained from 19 patients with pSS and 16 with non-specific chronic sialoadenitis. Quantitative gene expression analysis by TaqMan real-time PCR and immunohistochemistry for IL-17, IL-22, IL-23 and STAT3 (signal transducer and activator of transcription) was performed on salivary glands from patients and controls. The cellular sources of IL-22 among infiltrating inflammatory cells were also determined by fluorescence-activated cell sorting analysis and immunohistochemistry.

Results IL-22, IL-23 and IL-17 were significantly increased at both protein and mRNA levels in the inflamed salivary glands of patients with pSS. STAT3 mRNA and the tyrosine phosphorylated corresponding protein were also significantly increased in pSS. Th17 and NKp44$^+$ NK cells were the major cellular sources of IL-22 in patients with pSS.

Conclusions Our results suggest that, together with IL-17 and IL-23, IL-22 may play a pro-inflammatory role in the pathogenesis of pSS.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease associated with variable degrees of lymphocytic infiltration of salivary and lacrimal glands leading to xerostomia and xerophthalmia.[1]

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

Several cytokines and chemokines are increased in the peripheral blood and in the inflamed salivary glands.[2 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] The presence of Th1- and Th17-related cytokines has been in fact 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, 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]

Whether 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 purpose of the present study was to investigate the 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 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 signal transducer and activator of transcription (STAT3),[17] we 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.

Labial salivary gland biopsy

Up to 10 labial salivary gland biopsies were obtained, after obtaining informed consent, during routine diagnostic procedures. Of these biopsies, four were embedded in paraffin and used for histopathological analysis and immunohistochemistry (IHC), 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 fluorescence-activated cell sorting 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).

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.

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 (ΔC_t). For $\Delta\Delta C_t$ values, additional subtractions were performed between pSS samples and nSS ΔC_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 antihuman IL-17 (IgG, sc7927, 1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit antihuman IL-22 (IgG, NB100–737, 1:100 dilution) (Novus Biologicals, Littleton, Colorado, USA), mouse antihuman IL-23p19 (IgG1, HLT2736, 1:50 dilution) (BioLegend, San Diego, California, USA), rabbit antihuman pSTAT-3 (Tyr705) (IgG, D3A7, 1:50 dilution) (Cell Signaling Technology, Beverly, Massachusetts, USA) and antihuman 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

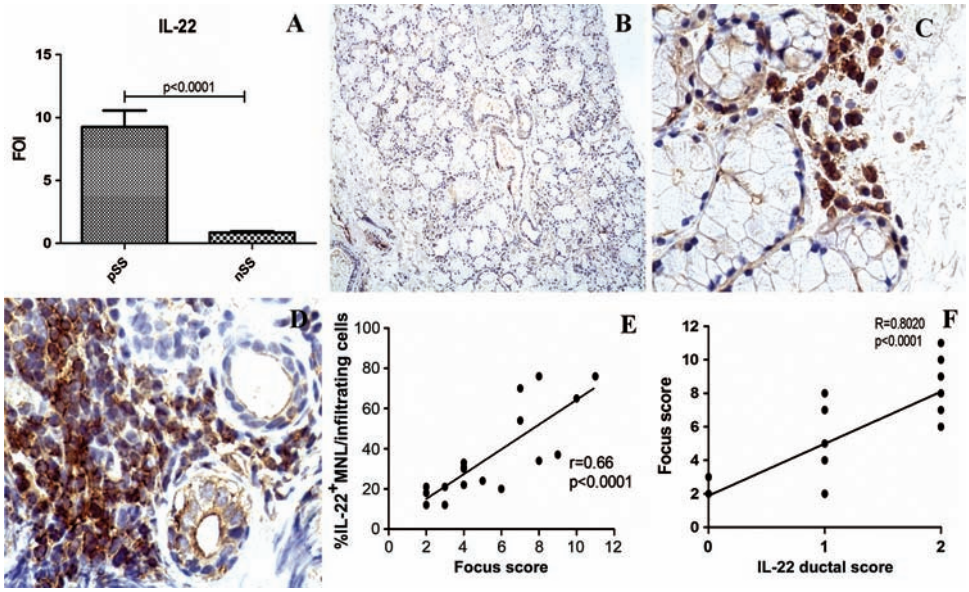

Figure 1 IL-22 expression in salivary glands of patients with pSS. (A) Relative expression of IL-22 mRNA in whole minor salivary glands of patients with pSS. Data are normalised for GAPDH and plotted as fold of induction over minor salivary glands obtained from patients with nSS. Results represent the 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.

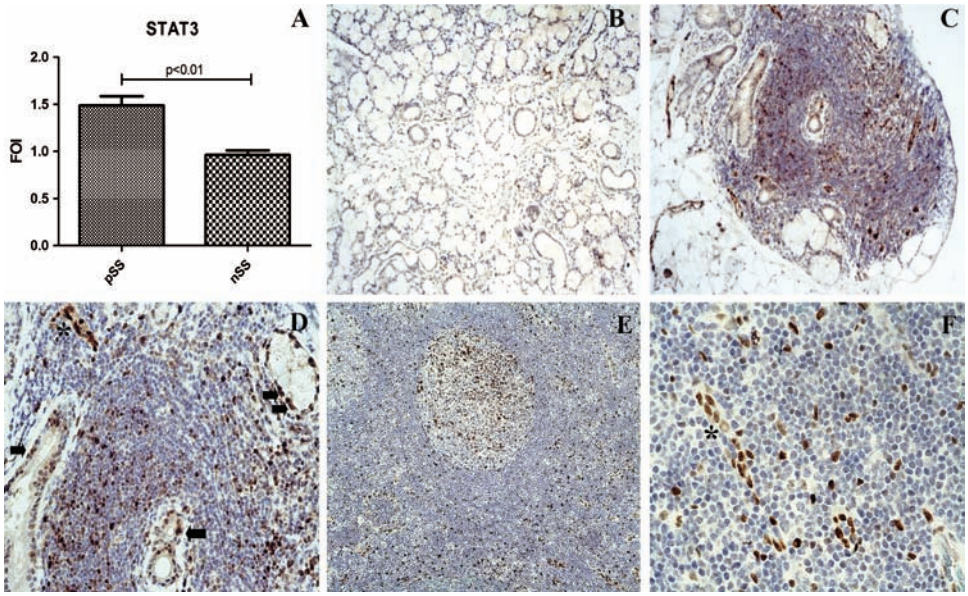

Figure 2 p-STAT3 expression in salivary glands of patients with pSS. Relative expression of STAT3 at the mRNA level (A) and of p-STAT3 at the protein level (B–D) in whole minor salivary glands of patients with pSS, controls and lymph node (E,F). Immune staining of p-STAT3 was almost exclusively observed 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.

(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 monoclonal antibodies: antihuman CD3-APC (BD Biosciences, San Jose, California, USA), antihuman CD56-PerCP-Cy5.5 (BioLegend), antihuman NKp44-PE (BioLegend), antihuman IL-22-FITC (R&D Systems, Minneapolis, Minnesota, USA), antihuman IFNγ-PE (BD Biosciences), antihuman IL-17-APC (R&D Systems), antihuman

CD4-PE-Cy5.5 (BD Biosciences) and an antihuman cytokeratin (clone MNF116; Dako, Glostrup, Denmark). Isotype-matched irrelevant antibodies were used as a negative control. Total cells were incubated with monoclonal antibodies 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 χ^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 part of 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±1.32 vs 0.87±0.08, p<0.0001) (figure 1A) with a positive strong correlation between the IL-22 mRNA levels and the focus score (online supplementary figure 1).

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 figure 1C,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

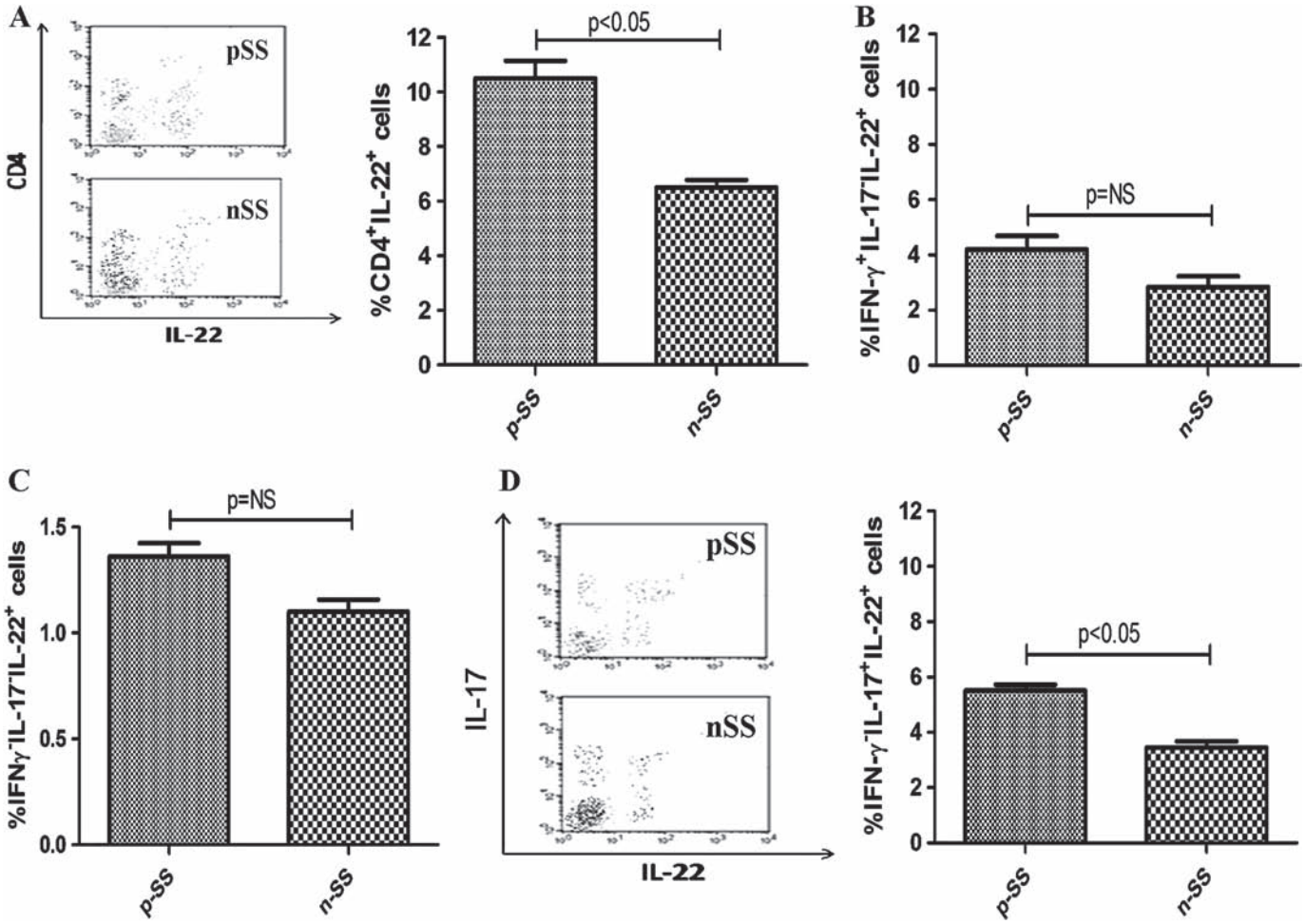

Figure 3 Percentage of IL-22-producing cells in salivary glands with pSS. Freshly isolated SGMCs from six patients with pSS and six patients with nSS were stained with CD4-PE-Cy5.5, IFNγ-PE, IL-17-APC and IL-22-FITC and analysed by flow cytometry. (A) Representative dot plot analysis (left panel) of 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.

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

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) (online supplementary figure 2A). 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 (online supplementary figure 2B).

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) (online supplementary figure 2C) and protein (online supplementary figure 2D) 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, mainly in lymphoid cells in the former and in the epithelial cells in the latter. To assess whether IL-22 and IL-23 signals were active, we looked for the activation of its downstream signal pathway evaluating STAT3 mRNA and p-STAT3 protein expression. STAT3 mRNA expression was demonstrable in both patients with pSS and those with nSS, being significantly increased in pSS (1.48±0.09 vs 0.96±0.05, p<0.01) (figure 2A). Since the activation of STAT molecules is indicated by their phosphorylated[25] status, 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 (figure 2E,F).

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).

$NKp44^+$ 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 receptor NKp44 (namely, $NKp44^+$ NK cells).[26] We investigated the 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±0.35 vs 1.8±1.3, p<0.05) (figure 5A). Among NK cells, $CD3^-CD56^+$ $NKp44^+$ NK cells were significantly expanded (2±0.17 vs 0.958±0.045, 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).

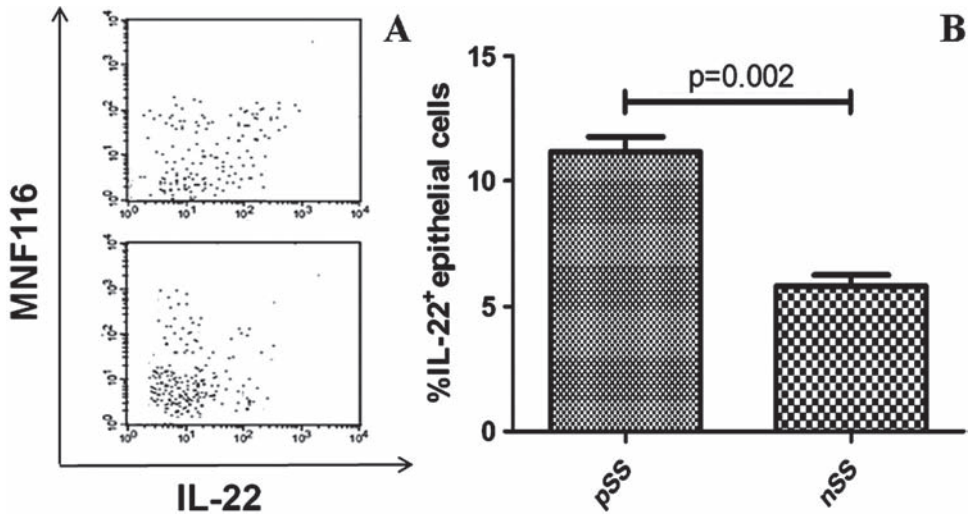

Figure 4 IL-22 expression in salivary gland epithelial cells of patients with pSS. (A) Representative dot plot analysis of cytokeratin (MF116) versus IL-22 among isolated salivary gland mononuclear cells. (B) Percentages of IL-22-positive cells among salivary gland epithelial cells. Bars show the mean and SEM. IL, interleukin; pSS, primary Sjögren's syndrome.

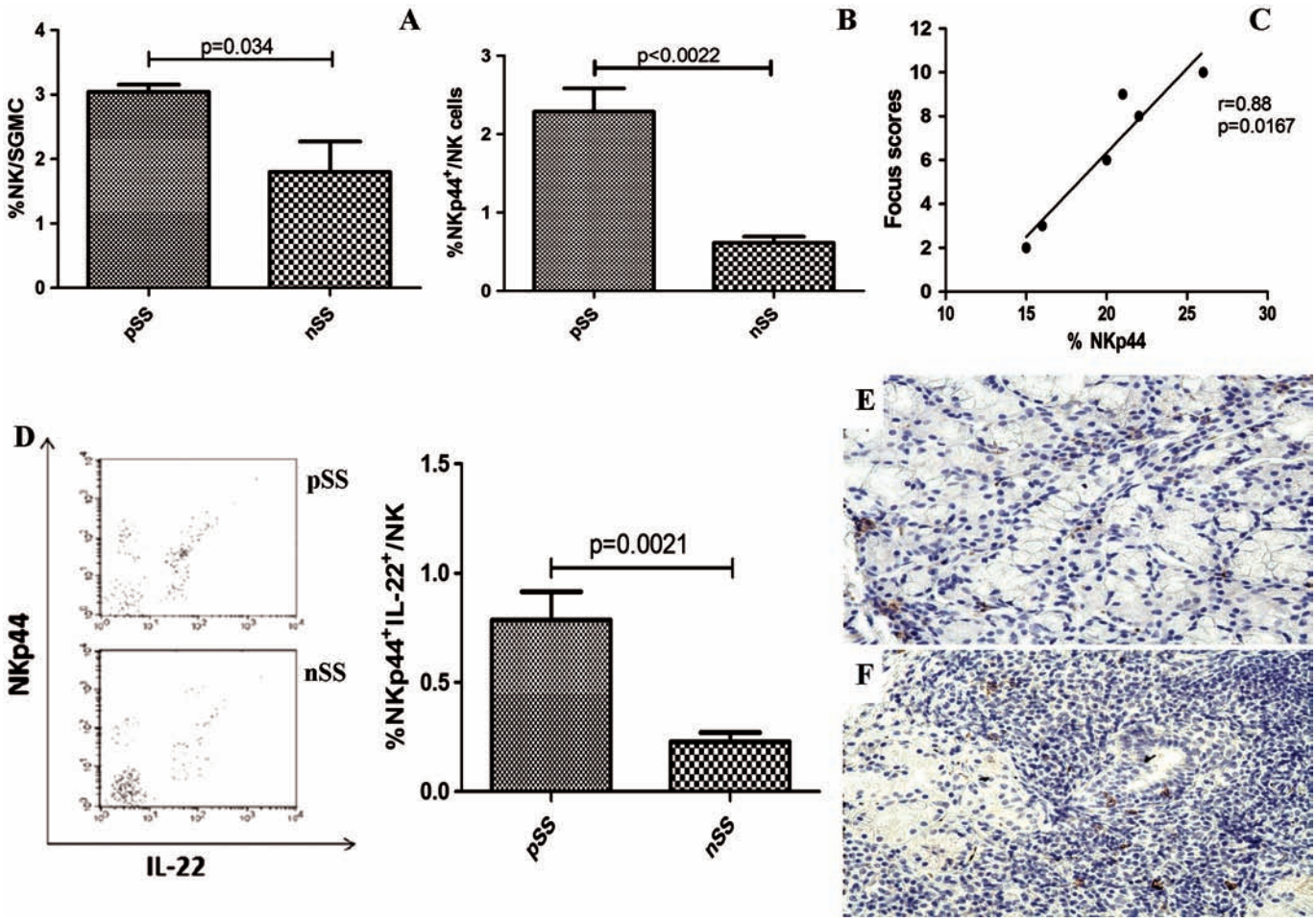

Figure 5 FACS analysis of NKp44$^+$ NK cells in salivary glands with pSS. Freshly isolated SGMCs from six patients with pSS and six patients with nSS were stained with anti-CD3-APC, CD56-PerCP-Cy5.5, NKp-44-PE and IL-22-FITC and analysed by flow cytometry. Percentages of NK cells among SGMCs of pSS and 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. Finally, 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 pathogenic 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 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 demonstrated 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 histopathological lesions of patients with pSS, ductal and acinar salivary gland epithelial cells display high levels of several immunoactive molecules that are known to mediate the lymphoid cell homing, antigen presentation and the 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 appeared 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 are present in human tonsils, human 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 demonstrate 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.

Competing interests None.

Patient consent Obtained.

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

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