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Original article

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

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Abstract

Objective. The aim of this study was to evaluate the role of rituximab (RTX) in modulating the expression of the IL-17/IL-23 pathway in the salivary glands (SGs) of patients with primary SS (pSS).

Methods. Consecutive SG biopsies were obtained from 15 patients with pSS before and after 1 year of RTX therapy. The SG expression of IL-17, IL-23p19 and p-STAT3 was evaluated by immunohistochemistry at baseline and after RTX therapy. The role of mast cells in pSS patients in modulating the Th17 response and the immunologic effect of RTX on mast cells were also studied in *in vitro* experiments.

Results. IL-17 was overexpressed in the SGs of patients with pSS mainly by infiltrating T cells and mast cells. After RTX therapy, the SG expression of IL-17, but not of IL-23p19 and p-STAT3, was significantly reduced and was accompanied by the depletion of tissue mast cells. In *in vitro* experiments with heterologous peripheral lymphocytes RTX significantly induced the apoptosis of isolated mast cells. Finally, mast cells isolated from peripheral blood mononuclear cells of pSS patients *in vitro* significantly increased Th17 lymphocytes.

Conclusion. RTX acts on pSS patients by globally reducing the expression of IL-17 and specifically inducing a pronounced apoptotic depletion of mast cells.

Key words: rituximab, mast cells, IL-17, IL-23, p-STAT3, primary Sjögren's syndrome, T lymphocytes, mast cells.

Introduction

Primary SS (pSS) is a chronic autoimmune inflammatory disorder in which salivary gland (SG) inflammation occurs in the presence of altered adaptive and innate immune responses [1] seen as altered serum and tissue cytokine expression of, for example, the IL-23p19/IL-17 pathway [2–7]. The neutralization of IL-23p19 and/or IL-17 thus appears to be a promising therapeutic approach in pSS. Despite the fact that IL-17 inhibition may directly interfere with the onset of SS-like disease in mice [8], clinical studies involving patients with pSS are currently missing.

Rituximab (RTX), an mAb to CD20, has been used and proved to be effective in reducing symptoms and in ameliorating the degree of tissue inflammation in pSS patients [9–11]. Beyond its role in depleting B cells, RTX seems to be able to modulate the T cell responses in autoimmune diseases [12, 13].

The aim of the present work was to clarify whether RTX modulates the IL-23p19/IL-17 immune response in pSS patients.

Patients and methods

Patients

Fifteen consecutive patients fulfilling the new American-European Consensus Group criteria for pSS [14] were consecutively enrolled at Palermo and L'Aquila

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University Hospitals (Italy). Disease activity was assessed using the visual analogue scales (VASs) for global disease activity. All the patients had active disease with values >50 mm on more than two of four 100-mm VASs (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 did not meet the American–European Consensus Group criteria for pSS were considered as the control group (nSS). Baseline characteristics of patients and controls are summarized in Table 1. All the pSS patients were treated with two cycles of i.v. infusions of 1000 mg RTX (Roche, Woerden, The Netherlands) on days 1 and 15 (at time 0 and then after 6 months). After 48 weeks the patients underwent another SG biopsy. The study was approved by the Ethical Committee of the University of Palermo. Patients signed informed consent for the collection and storage of biological material.

Table 1 Baseline characteristics of patients and controls

	pSS ($n = 15$)	nSS ($n = 10$)	*P*-value
Age, median (range), years	44 (35–72)	55 (30–67)	0.68
Female, *n* (%)	13 (86)	10 (100)	0.77
Disease duration, median (range), months	48 (22–100)	65 (18–120)	0.18
ANAs, % of patients	86	—	—
Anti-Ro and/or anti-LA antibodies, % of patients	66	—	—
RF, % of patients	40	—	—
ESR, mean (S.D.), mm/h	28 (11)	11 (3)	<0.001
CRP, mean (S.D.), mg/l	9 (2)	4 (2)	<0.05

pSS: primary SS; nSS: controls.

Labial SG biopsy

Labial SG biopsies were obtained and placed into formalin fixative and RNA. Standard 5-μm thick paraffin sections were stained with haematoxylin and eosin (H&E). Histological evaluation of SGs 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 SGs 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, Dallas, TX, USA) (1:100 dilution), rabbit anti-human IL-17 (R&D Systems, Minneapolis, MN, USA) (1:100 dilution), rabbit anti-human IL-23p19 (BioLegend, San Diego, CA, USA) (1:50 dilution), rabbit anti-human p-STAT3 (Cell Signaling Technology, Beverly, MA, USA) (1:50 dilution) and mouse anti-human CD3 (Dako) (1:100 dilution) were added and incubated for 1 h at room temperature. For control staining, primary antibodies were replaced with irrelevant isotype-matched antibodies (AbCam). The slides were incubated for 30 min with peroxidase-conjugated Dako EnVision polymer and peroxidase activity was visualized using diaminobenzidine chromogen (Dako). Slides were lightly counterstained with haematoxylin before dehydration and mounting in DePex (VWR International, Oslo, Norway). To characterize IL-17-producing cells, double staining was performed on paraffin-embedded sections of human SGs. Sections were incubated with unlabelled rabbit anti-human IL-17 and mouse anti-human tryptase antibodies and then treated with FITC-conjugated anti-mouse (Alexa fluor, Invitrogen Life Technologies, Monza, Italy) or Rhodamine Red-conjugated (Alexa fluor, Invitrogen) 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

SG tissues were extensively washed in saline buffer and enzymatic digestion was performed using collagenase (1.5 mg/ml; Life Technologies, Carlsbad, CA, USA) in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated pooled human AB-positive serum, 2 mM L-glutamine, 20 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulphonic acid) (HEPES), 100 U/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-ME, for 2 h. Recovered cells were then cultured with phorbol myristate acetate (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, USA) was added and after 16 h of incubation cells were collected and stained with the following mAbs: anti-human CD3-APC (BD Biosciences, San Jose, CA, USA), anti-human tryptase-PE (clone AA1, Dako) and anti-human IL-17- (BD Biosciences). 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, containing 0.1% (w/v) NaN_3. 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 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 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, Bisley, UK) for the magnetic separation. Next, 1×10^5 $CD117^+$ cells from patients and controls, pre-activated with *N*-formyl-Met-Leu-Phe (fMLP), were cultured alone or co-cultured with heterologous $CD117^-$ cells, in triplicate, in 96-well round-bottom plates in a volume of 200 μl/ well. After 72 h of culture the cells were stained with the following antibodies: anti-human-CD4-APC, anti-human-IL17-Percp (BD Biosciences) and anti-human-CD117-PE (BD Biosciences). For *in vitro* cytotoxicity assays, cells were treated for 2 h with RTX (10 μg/ml). Control cells received media only. After treatment, 1×10^6 cells were plated in duplicate in a 24-well plate in a volume of 1 ml/well and incubated at 37°C in 5% CO_2 and 90% humidity for 5 days. A cytotoxicity assay was performed using the FITC-Apoptosis detection kit (BD Biosciences). The results were expressed as a percentage of annexin-positive, propidium iodide (PI)–negative cells.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean (S.D.) and *P*-values <0.05 are 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 four infusions of RTX, completed the follow-up visits through week 48 and underwent labial SG biopsy at baseline and after 48 weeks. RTX was shown to be effective in ameliorating whole saliva flow rate [mean 0.22 ml/min (S.D. 0.13) at baseline and 0.5 (S.D. 0.2) at week 48, $P = 0.0002$] and the SG function assessed by Schirmer's test [mean 5.1 mm/5 min (S.D. 2.1) at baseline and 9.3 (S.D. 2.3) at week 48, $P = 0.0001$].

Mast cells and T cells produce IL-17 in the SGs of pSS

Since anti-IL-17 antibodies from different suppliers can give conflicting results [15], to evaluate the SG location of IL-17-expressing cells we used two different anti-human IL-17 antibodies. The first antibody, obtained from Santa Cruz Biotechnology, showed a diffuse, intense, cytoplasmic staining of mononuclear, epithelial and vascular endothelial cells (Fig. 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 microvessels that highly resembled mast cells (Fig. 1B and C; Fig. 1D shows 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 (Fig. 1E–G) and mast cells (Fig. 1H–J) express IL-17A. To confirm the cellular source of IL-17 among SGs of pSS patients, SG mononuclear cells were isolated from five pSS patients and five controls and analysed for IL-17 production by flow cytometry. As shown in Fig. 1L, both IL-17-producing $CD4^+$ T cells and mast cells were significantly increased in the SGs of pSS patients *vs* controls.

RTX therapy is accompanied by reduced expression of IL-17 in the SGs of pSS patients

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

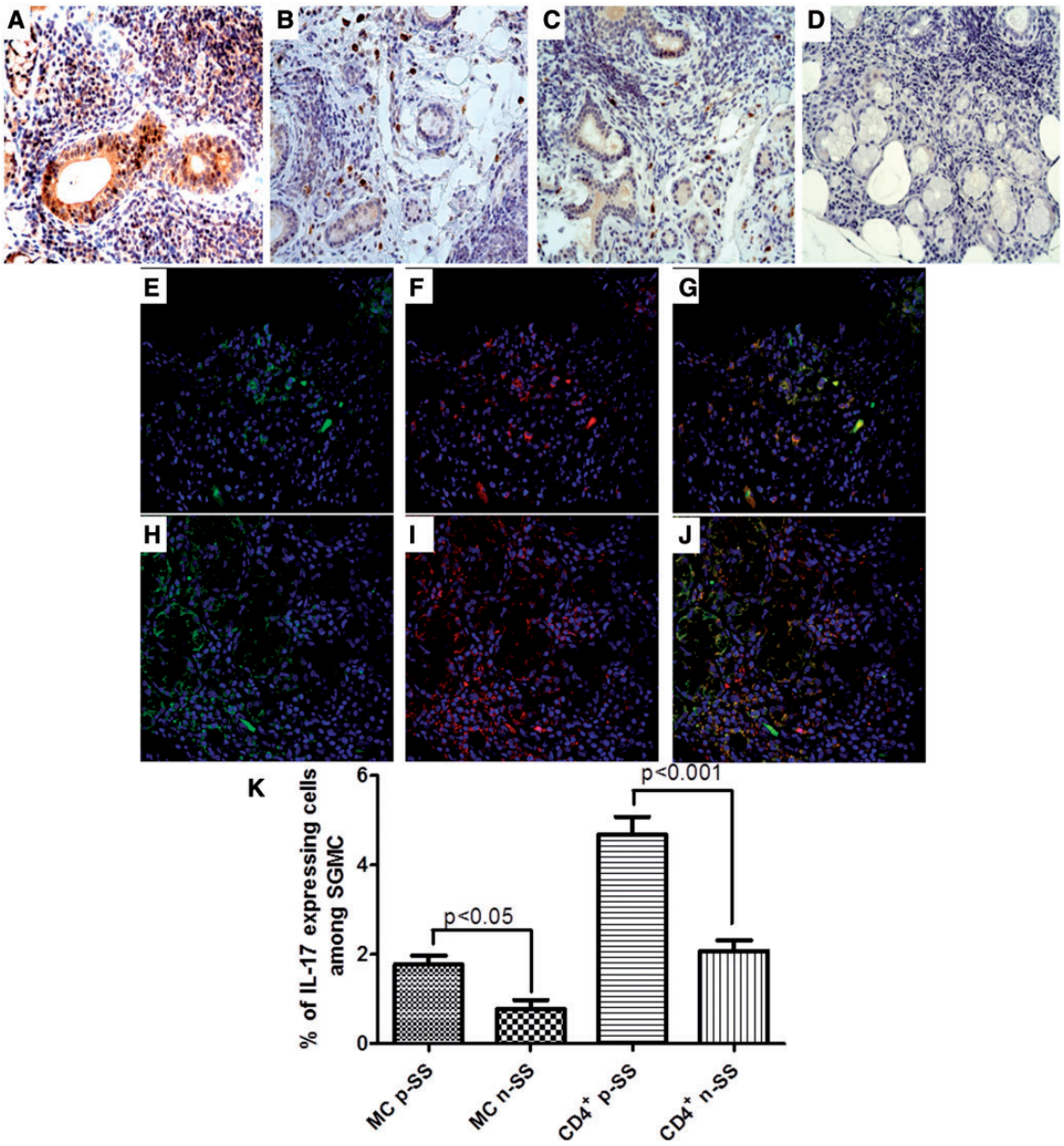
RTX reduces circulating mast cells and induces in vitro mast cell apoptotic death

Since the depletion of tissue mast cells was observed after RTX therapy, we next evaluated the frequency of circulating mast cells in pSS patients before and after RTX therapy. Although the percentage of circulating mast cells was not increased in pSS patients compared with controls (data not shown), a significant depletion of peripheral mast cells was observed after RTX therapy (Fig. 4A). We next assessed whether the observed changes in mast cell density could be attributable to a direct cytotoxic effect of RTX therapy. PBMCs isolated from pSS patients and controls were cultured with RTX and the percentage of mast cells was evaluated. An important and significant increase in the percentage of apoptotic mast cells, shown by annexin V or annexin and PI labelling, was observed in both patients and controls after incubation with RTX (Fig. 4B and C).

Mast cells isolated from pSS patients potently drive Th17 polarization

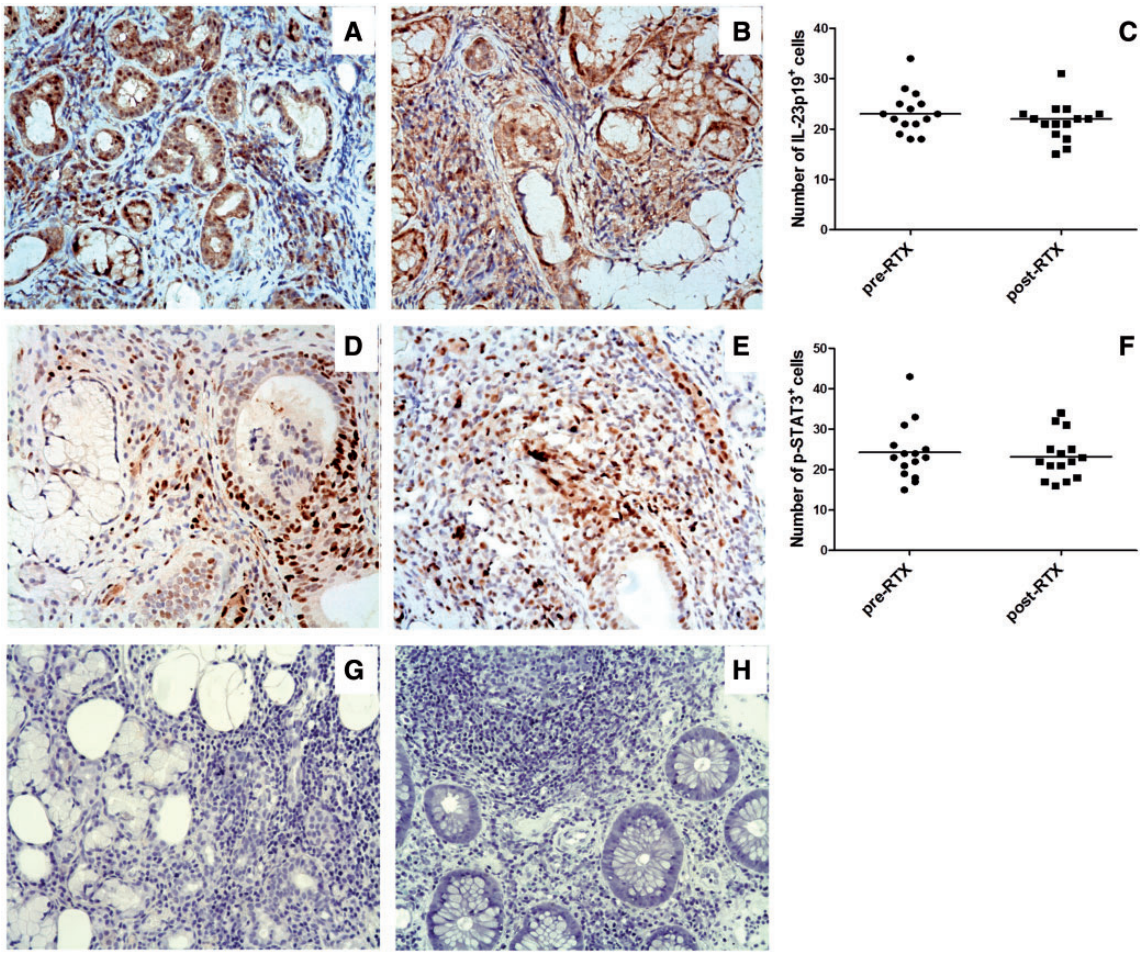
We next analysed the capacity of mast cells isolated from pSS patients and healthy controls to increase human Th17 cells in culture. Isolated PBMCs from patients and controls were first depleted by homologous mast cells and then incubated with heterologous activated mast cells. As shown in Fig. 4D, mast cells isolated from pSS patients produced a greater increase of Th17 cells than mast cells from healthy controls (Fig. 4E), suggesting an important role of mast cells in regulating the Th17 response in pSS patients.

Fig. 1 IL-17 is expressed by CD3$^+$ T cells and mast cells in the salivary glands (SGs) of patients with primary SS (pSS)



Representative immunostaining for IL-17 in pSS 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** and **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 pSS patients stained with rabbit immunoglobulins (isotype control). (**E**–**G**) Representative images of confocal analysis of CD3 and IL-17 (R&D) co-localization in SG tissues of pSS 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 SG tissues of pSS 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 250×. (**K**) Percentage of IL-17-expressing cells evaluated by flow cytometry among isolated SG mononuclear cells of pSS patients and controls. Both CD4$^+$ and mast cells isolated from pSS patients produced significantly more IL-17 than control subjects.

FIG. 2 IL-23p19 and p-STAT3 expression is not modified by RTX therapy in the salivary glands (SGs) of patients with primary SS (pSS)



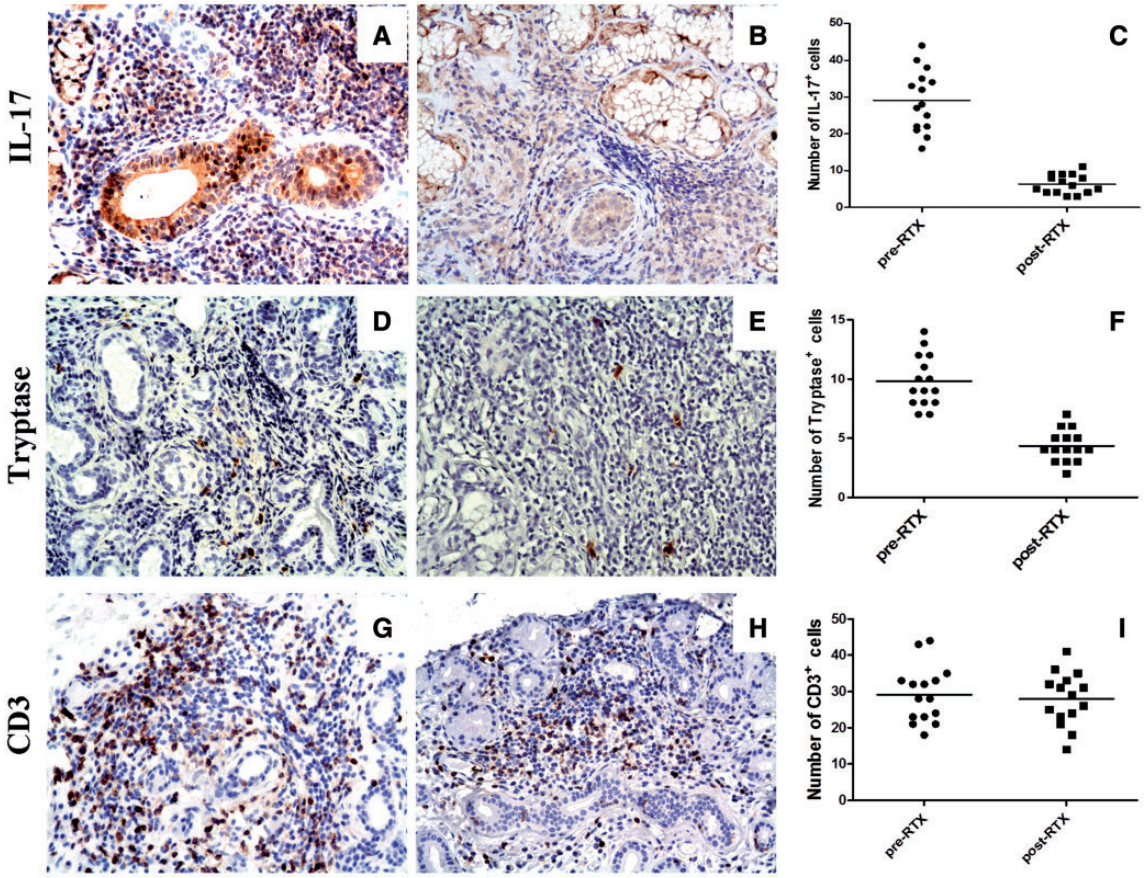
(**A** and **B**) Representative immunostaining for IL-23p19 in pSS SGs (**A**) before and (**B**) after RTX therapy. (**C**) Number of IL-23p19-positive cells in the SGs before and after RTX therapy. (**D**-and **E**) Representative immunostaining for p-STAT3 in pSS SGs (**D**) before and (**E**) after RTX therapy. (**F**) Number of IL-23p19-positive cells in the SGs before and after RTX therapy. Representative image of isotype control staining for (**G**) IL-23p19 and (**H**) p-STAT3. (**A**, **B**, **D**, **E**, **G** and **H**) Original magnification 250×.

Discussion

This is the first study to show that RTX treatment in pSS patients is accompanied by a large reduction of the SG expression of IL-17, but not IL-23 and p-STAT3, suggesting a specific role of RTX on IL-17-producing cells that is not mediated by modulation of the IL-23 pathway. A significant reduced IL-17 expression was observed in the SGs of RTX-treated pSS patients, suggesting a direct role of RTX in modulating SG IL-17 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, with different cell types producing IL-17 at different sites and phases of tissue inflammation [16]. Conflicting data were obtained from studies analysing the expression of IL-17 by immunohistochemistry in inflamed human tissues, with T cells and mast cells being indicated, in a mutually exclusive manner, as the main IL-17 producer cells [17–19]. These results seem to be only apparently conflicting, and are probably dependent more on the type of antibody used rather than real differences in patients enrolled and/or different stages of disease [15]. To clarify which cells produce IL-17 in the SGs of pSS patients in this study we evaluated two different antibodies by immunohistochemistry, demonstrating that both $CD3^+$ cells and mast cells are important cellular sources of IL-17 in the SGs of pSS patients. The production of IL-17 from SG T lymphocytes and mast cells of pSS patients was also confirmed by flow cytometry analysis performed on isolated SG mononuclear cells.

FIG. 3 IL-17 expression is significantly reduced by RTX therapy in the salivary glands (SGs) of patients with primary SS (pSS) and is accompanied by tissue depletion of mast cells



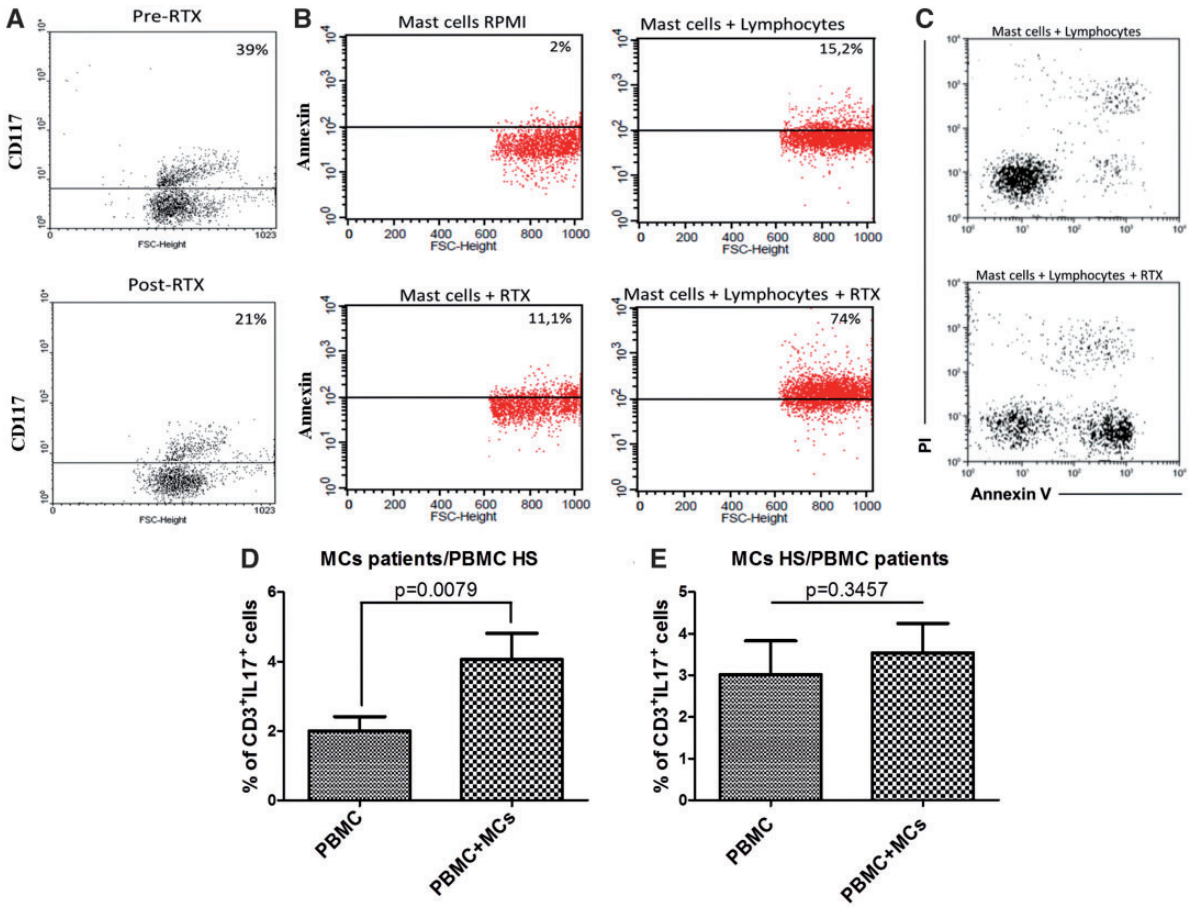
(**A** and **B**) Representative immunostaining for IL-17 in pSS SGs (**A**) before and (**B**) after RTX therapy. (**C**) Number of IL-17-positive cells in the SGs before and after RTX therapy. (**D** and **E**) Representative immunostaining for CD3 in pSS SGs (**D**) before and (**E**) after RTX therapy. (**F**) Number of $CD3^+$ cells in the SGs before and after RTX therapy. (**G** and **H**) Representative immunostaining for tryptase in pSS SGs (**G**) before and (**H**) after RTX therapy. (**I**) Number of tryptase-positive cells in the SGs before and after RTX therapy. (**A**, **B**, **D**, **E**, **G** and **H**) Original magnification 250×.

RTX is a chimeric mAb that induces B cell 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 evidence indicates that treatment with RTX may reduce the exaggerated Th17 response occurring in RA synovial tissues [12] and the production of IL-22 in the SGs of pSS patients [13], and suggests a greater 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 SGs. But while the number of T cells was not significantly affected, a large decrease in 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 RTX, as demonstrated by our *in vitro* experiments where incubation of mast cells from patients and controls with RTX was followed by significant mast cell apoptosis. We actually do not know the mechanism of RTX-dependent apoptosis of mast cells. However, we speculate that, because of the structural homology of CD20 protein and the high-affinity IgE receptor beta chain (FcepsilonRIbeta) [21] expressed on mast cells, RTX 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 proinflammatory 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 from 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 obviously cannot know if the activation of mast cells precedes the Th17 response or vice versa. However, in view of the fact

FIG. 4 RTX acts on mast cells in both *ex vivo* and *in vitro* experiments



Peripheral blood mononuclear cells (PBMCs) were isolated from primary SS (pSS) patients before and after RTX therapy, stained with CD3 and the gate of $CD3^-$ cells was 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 pSS. (**B**) Dot plot analysis of annexin expression after co-culture of mast cells isolated from pSS and heterologous lymphocytes in the presence or absence of RTX (10 mg/ml). (**C**) Dot plot analysis of combined annexin V and propidium iodide (PI) expression after co-culture of mast cells isolated from pSS and heterologous lymphocytes in the presence of RTX (10 mg/ml). (**D** and **E)** Mean percentage (S.D.) of Th17 cells at baseline and after co-culture with isolated mast cells from patients and controls.

that mast cells are resident tissue cells, one could assume a primary role of mast cells in orchestrating 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.

Rheumatology key messages

- T lymphocytes and mast cells are the main cellular source of IL-17 in primary SS (pSS) salivary glands.
- Rituximab (RTX) acts on pSS patients by reducing the expression of IL-17 but not IL-23p19.
- RTX depletes tissue and circulating mast cells in pSS.

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

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