Co-culture system of human salivary gland epithelial cells and immune cells from primary Sjögren’s syndrome patients: an in vitro approach to study the effects of Rituximab on the activation of the Raf-1/ERK1/2 pathway

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Abstract

Primary Sjögren’s syndrome (pSS) is a chronic autoimmune disorder of the exocrine glands with associated lymphocytic infiltrates in the affected glands. Dryness of the mouth and eyes results from involvement of the salivary and lacrimal glands. The efficacy of Rituximab (RTX) in pSS is still open to debate. This study delineates the signaling pathway involved in RTX-mediated down-regulation of pro-inflammatory factors in a co-culture system of pSS salivary gland epithelial cells (SGEC) with syngeneic pSS B-lymphocytes. In addition, the effects of RTX on the activation of the Raf-1/ERK1/2 pathway in pSS SGEC co-cultured with syngeneic pSS T-lymphocytes were also investigated. This study demonstrated that RTX may interfere with the ERK1/2 pathway in a syngeneic co-culture of pSS SGEC with pSS B-lymphocytes, leading to decreased cytokine production by SGEC. These novel findings reveal that syngeneic co-culture of pSS SGEC with pSS B-lymphocytes leads to a down-regulation of Raf-1 in epithelial cells that adversely regulates the activity of the ERK1/2 pathway and determines a subsequent reduction of the release of pro-inflammatory factors.

Keywords: cytokines, ERK, Raf-1, Rituximab, Sjögren’s syndrome

Introduction

Primary Sjögren’s syndrome (pSS) is a relatively common chronic, autoimmune, systemic, inflammatory disorder of unknown cause. It is characterized by dryness of the mouth, eyes, and other mucous membranes because of lymphocytic infiltration of the exocrine gland and secondary gland dysfunction. General symptoms of pSS include fatigue, weight loss and fever (1, 2), and 40–50% of patients develop extraglandular disease, affecting the health-related quality of life (3, 4). Classically, minor salivary glands (MSG) of patients with pSS show infiltrating lymphocytes located in perivascular or periductal areas and B-lymphocyte hyperactivity and MSG infiltration represent the hallmarks of the disease (3). Finally, the risk of non-Hodgkin’s lymphoma in people with pSS is higher compared to the general population (5). Similar to other autoimmune diseases, the understanding of the pathogenesis of pSS and its aetiology is far from complete (6). Most of the patients are females (9:1 female-to-male ratio) and the presence of autoantibodies against SSA (Ro52 and Ro60) and SSB (La) antigens is observed in 50–60% and 30–40% of patients with pSS, respectively (7). The current therapies mostly alleviate the symptoms of sicca and focus on extraglandular manifestations (if present) (8). The results of clinical trials with biological treatments showed minimal or no effect in patients with pSS (9). Therefore, there is an ongoing need for individualized patient treatment. The increasing availability of targeted therapies in the last two decades, mirroring what happened in rheumatoid arthritis (RA), raised the possibility of pSS to selectively interfere with different pathogenic pathways. On this basis, B-cell depletion by Rituximab (RTX) represents an issue of great interest (10–12). RTX is a chimeric human/mouse anti-CD20 mAb approved by the FDA for the treatment of non-Hodgkin’s lymphoma and RA (13, 14). CD20 is expressed on the surface of pre-B-lymphocytes, transitional B-lymphocytes and mature B-lymphocytes, and...
is lost at the plasma cell stage (15). CD20 mediates B-cell activation, proliferation and differentiation (16) and plays an important role in the generation of T-cell-independent antibody responses (17).

Although the direct pathophysiological role of B-cells in glandular tissue destruction in pSS has not been fully elucidated, B-cell depletion therapy with RTX appears to be successful and, importantly, improves salivary flow (18). Regarding the T-cells from pSS patients, they appear to be largely unaffected by RTX treatment (19). However, a recent study by Eggleton et al. (20) demonstrates that a small proportion of circulating Th-17 cells express the surface marker CD20 in healthy individuals and further revealed that the frequency of these cells was significantly higher in patients with RA and were sensitive to the RTX treatment (20). Although the exact function of these CD20+ IL-17 producing cells is not known, it is therefore possible that the positive effect of RTX treatment in pSS patients can be, at least partially, attributed to the depletion of these CD20+ T-cells.

On this basis, particular focus has been placed upon the mechanisms of action responsible for the effects of RTX, and the inhibitory effect of RTX on ERK1/2 pathway activation has been largely documented (21). The purpose of this study was to investigate the role of RTX in the activation of the ERK1/2 pathway in pSS human salivary gland epithelial cells (SGEC) given the central role of salivary epithelium for the pathogenesis of pSS identified as autoimmune epithelitis (22–28). A co-culture system of pSS SGEC with pSS B-lymphocytes was prepared to evaluate whether RTX could be potentially active against activation of the Raf-1/ERK1/2 pathway, where the Raf-1 protein acts by phosphorylating and activating ERK1/2 (29, 30). We further studied the effects of RTX-treatment on the Raf-1/ERK1/2-dependent cytokine/chemokine release by pSS SGEC. In addition, whether RTX inhibits the Raf-1/ERK1/2 pathway in pSS SGEC co-cultured with pSS T-lymphocytes was investigated.

**Methods**

**Patient population**

Twenty pSS patients fulfilling the American-European consensus group criteria for pSS that displayed a salivary gland focus score of at least 3 were enrolled in the study (31). All patients had the clinical symptoms of dry eyes and mouth, a positive Schirmer’s test (less than 5 mm wetting of a strip of filter paper per 5 min), and Rose Bengal staining (increased uptake of the staining agent). All subjects signed the informed consent forms before the biopsy. The patients had all given their written consent, the study was approved by the local Ethical Review Committee and the whole study was carried out in compliance with the ethics principles of the Declaration of Helsinki. Ten healthy volunteers awaiting removal of salivary mucocceles from the lower lip were selected to participate in the study. The healthy subjects had no complaints of oral dryness, any autoimmune disease and normal salivary function. All subjects signed the informed consent forms before participation.

**Positive isolation of B- and T-lymphocytes**

Peripheral blood mononuclear cells were isolated from venous blood of pSS patients and healthy subjects by Ficoll gradient separation. Blood samples were collected in heparinized test tubes by a vacutainer system and diluted (1:1) with Hanks’ balanced salt solution (HBSS, Sigma Aldrich, St. Louis, MO, USA), layered over Ficoll-Paque Plus (Amersham Bioscience) and centrifuged at 1,000 g for 15 min. After a short centrifugation at room temperature lymphocytes, together with monocytes and platelets, were harvested from the interface between the Ficoll-Paque PLUS and sample layers. This material is then centrifuged twice in HBSS to wash the lymphocytes and monocytes and to remove the platelets. The cells were collected and washed in RPMI 1640 supplemented with 10% heat-inactivated (56°C for 30 min) FCS. T- and B-lymphocytes were separated using the human B- and T-lymphocytes positive isolation kits following the recommendations of the manufacturer (Life Technologies). Briefly, for positive isolation of B-lymphocytes, cells were magnetically labeled with anti-human-CD19-conjugated iron microbeads for 15 min at 10°C. The chromatography procedure was performed with a positive selection column placed in a magnetic field. The magnetically labelled CD19+ cells were retained in the column while the unlabelled cells ran through. The column was rinsed with PBS (pH 7.2) supplemented with 0.5% BSA and 5 mM EDTA. After removal of the column from the magnetic field, the magnetically retained CD19+ cells were eluted with 1 ml of release buffer and maintained in complete RPMI-1640 medium supplemented with L-glutamine and 10% heat-inactivated FCS, and 100 U ml⁻¹ of penicillin-streptomycin. The T-cells were isolated with the same procedure, except for using anti-human-CD3-conjugated iron microbeads.

**Culture and co-culture of salivary gland epithelial cells**

MSG biopsy samples were taken from healthy subjects and pSS patients. MSG were harvested from the lower lip under local anaesthesia through normal mucosa, according to the expant outgrowth technique (32). In order to obtain SGEC culture, the cells were isolated from the MSG by microdissection and collagenase (Worthington Diagnostic Division, Millipore, Freehold, NJ, USA) digestion in physiological saline containing 1 mm Ca²⁺. Following dispersal, cells were resuspended in McCoy’s 5a modified medium supplemented with 10% heat-inactivated (56°C for 30 min) FCS, 1% antibiotic solution, 2 mM L-Glutamine, 50 ng ml⁻¹ epidermal growth factor (EGF, Promega, Madison, WI, USA), 0.5 μg ml⁻¹ insulin (Novo, Bagsvaerd, Denmark) and incubated at 37°C, 5% CO₂ in air. Contaminating fibroblasts were selectively removed by treatment of the cultures with 0.02% EDTA. In the co-culture experiments, pSS SGEC were seeded at 5 × 10^⁵ cells into 6-well flat-bottomed culture plates (Nunc, Kamstrup, Denmark). After 4 h allowing cells to attach, the syngeneic pSS B- or T-lymphocytes was co-cultured for 48 h with SGEC. As a control, a co-culture system of healthy SGEC with syngeneic healthy B- or T-lymphocytes was prepared. B- and T-cells were co-cultured with adherent epithelial cells at a B-/T-cell/epithelial-cell ratio of 5:1. To address the implication of RTX in the modulation of the Raf-1/ERK1/2 pathway activity, RTX (20 μg ml⁻¹), Raf-1 inhibitor GW5074 (50 nM)
and MAPK/ERK inhibitor PD98059 (4 μM) (both from Sigma-Aldrich) were added or not in the co-cultures for 48h. After this period, the complete McCoy's 5a medium was replaced and the SGEC were incubated for 24 or 48h for gene and protein expression analysis. The epithelial origin of cultured cells was routinely confirmed by staining with monoclonal antibodies against epithelium-specific markers, including the various cytokeratins and epithelial membrane antigens and the absence of myoepithelial, fibroblastoid and lymphoid markers, using immunocytochemistry as previously described (33) and reported in Supplementary Figure 1, available at International Immunology Online.

Assessment of mRNA expression

RNA was prepared from healthy and pSS SGEC (cultured alone or from the co-culture system with syngeneic healthy and pSS B- and T-lymphocytes) using the Total RNA Purification system (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase I (GIBCO, Life Technologies, Carlsbad, CA, USA) for 15 min at RT followed by the addition of EDTA and heated at 65°C for 10 min to remove degraded genomic DNA. Two micrograms of total RNA was used for cDNA synthesis. The reverse transcription was performed with Moloney murine leukemia virus reverse transcriptase (GIBCO in the presence of RNaseOUT (GIBCO) and used 1/10 of cDNA murine leukemia virus reverse transcriptase (GIBCO) in the synthesis and thereby increase the sensitivity of PCR from cDNA. PCR was performed in a 50-μl reaction mixture composed of 2 μl of each sense and antisense primer, 1 × PCR buffer; 2.4 mM MgCl2, 0.2 mM each dNTP, 10 μl of transcribed cDNA, and 0.04 U μl−1 Taq DNA polymerase. Human Raf-1 primer sequences were as follows: 5′-CAG CCC TGT CCA GTA GC-3′ for the forward primer and 5′-GCC TGA CTT TAC TGT TGC-3′ for the reverse primer. ERK forward primer was 5′-GCT GAC CCT GAG CAC GA-3′ and reverse was 5′-CTG GTT CAT CTG TCG GAT CA-3′. After initial denaturation at 94°C for 5 min, 35 cycles were performed (denaturation at 94°C for 30 s, annealing at 50°C and 58°C, respectively for Raf-1 and ERK, for 30 s and extension at 72°C for 1 min) followed by 10 min at 72°C. Equal amounts of PCR products were run on a 1.5% agarose gel containing ethidium bromide and photographed under UV light. GAPDH was used as the internal control. For real-time PCR, TaqMan expression assays, including fluorescent probes for human Raf-1 and ERK1/2, forward and reverse primers (Assays-On-Demand, Applied Biosystems, Foster City, CA, USA) and the internal control gene β-2 microglobulin (part n°4326319E; β2M) were purchased from Applied Biosystems. Real-time quantitative PCR was performed in a 96-well microtiter plate with an ABI PRISM 7700 (Applied Biosystems). Each reaction contained 5 μl of cDNA template, 2.5 μl of 20x probes and primers mixture, 12.5 μl of TaqMan Universal PCR Master Mix, No AmpEraser UNG (Applied Biosystems), in a total volume of 25 μl. Reactions were amplified for 40 cycles (65°C for 5 min and 95°C for 10 min, followed by 40 denaturation cycles at 95°C for 15 s and annealing/extension at 60°C and 62°C respectively for ERK1/2 and Raf-1 for 30 s). The threshold was determined as 10 times the SD of the baseline fluorescence signal. The cycle number at the threshold was used as the threshold cycle (Ct). The different expression of mRNA was deduced from 2−ΔΔCt.

Western blot

Protein lysates, obtained from untreated and RTX-treated healthy and pSS SGEC (cultured alone or from the co-culture system with syngeneic B- or T-lymphocytes) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were incubated with rabbit anti-human phosphorylated Raf-1 (phospho-Raf-1, p-Raf-1) (Millipore, Billerica, MA, USA) mouse anti-human ERK1/2 monoclonal Ab (mAb), mouse anti-human phosphorylated ERK1/2 (phospho-ERK1/2, p-ERK1/2) (Abcam, Cambridge, MA, USA) anti-human-p-ERK1/2 (R&D systems) as primary antibodies and with secondary antibodies conjugated with Alexa fluor 488 (Invitrogen). The protein expression was analyzed by a Becton Dickinson (BD, Becton Dickinson, Germany) FACSCanto™ II flow cytometer and BD FACS Diva software. The percentages of positive cells were obtained from logarhythmic cytograms of mean fluorescence intensities (MFI), by comparison with the negative isotypic control. Fluorescence intensities were further quantified by using calibrated fluorophores to translate the mean fluorescence of each sample into standardized arbitrary fluorescence units.

Cytokine quantitation with a Multi-Analyte ELISAArray analysis

A human cytokine Multi-Analyte ELISAArray Kit (SA Biosciences Frederick, MD, USA) was used to measure the cytokines production of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GMCSF in supernatants of pSS SGEC after a 48h period of co-culture with pSS B- and T-lymphocytes in presence or not of RTX (20 μg ml−1), GW5074 (50nM) and PD98059 (4 μM), the replacement of the medium and an incubation period of 48h more. The arrays were performed according to the manufacturer’s instructions. The absorbance levels of the cytokines were measured on a plate reader [VERSAmax microplate reader (Molecular Devices Corp, Silicon Valley, CA, USA)] at 450nm. Standard errors (SE) of the mean were calculated from two biological repeats of independent experiments performed.
Statistics
The data were analyzed for normality using the Wilks Shapiro Test. Differences in means for paired observations were analyzed by Student's t-test. In all instances values of $P < 0.05$ were considered statistically significant.

Results
Evaluation of cytokines released by positively isolated B- and T-lymphocytes from pSS patients and controls submitted to RTX exposure
To investigate whether RTX alters the Raf-1/ERK1/2 pathway in pSS SGEC cultured alone, and/or co-cultured with pSS B-lymphocytes and pSS T-lymphocytes, preliminary experiments were performed to evaluate the effect of RTX on inflammatory cytokine production by T- and B-cells before and after RTX treatment. The peripheral blood B- and T-cells were isolated from pSS patients and healthy controls as described above and incubated with the optimal levels of RTX to evaluate cytokine production. pSS and healthy B- and T-cells were cultured for 48 h with or without RTX and the levels of cytokines released into the culture medium were measured using an ELISA multianalyte assay (Fig. 1, panels A, B). As shown and as expected, the expression levels of each cytokine were significantly different among healthy B and T control cells and cells derived from pSS patients ($P < 0.01$). Figure 1 shows that, when RTX was added to the culture medium, compared with their untreated counterparts, pSS B- and T-cells secreted lower levels of inflammation-related cytokines. As expected, the reduction of pro-inflammatory cytokine release after RTX exposure was more prominent in B-lymphocytes in comparison with T-cells. This could be explained considering that a very small proportion of circulating Th-17 cells expressing the surface marker CD20 in healthy and pSS individuals was present (34).

Rituximab-mediated down-regulation of pRaf-1 activity correlates with down-regulation of ERK1/2 activity
In the classical MAPK/ERK cascade, activation of Raf-1 leads to sequential phosphorylation of ERK1/2 that are the last of the serine-threonine kinases that are serially activated in response to extracellular stimulation (35). Before studying the effect of RTX exposure on the activation of the Raf-1/ERK1/2 pathway in pSS SGEC co-cultured with pSS B-lymphocytes and pSS T-lymphocytes, several preliminary experiments were carried out to determine whether pSS SGEC cultured in presence of RTX without the addition of B- or T-lymphocytes showed any changes in Raf-1 and ERK1/2 expression and pro-inflammatory cytokine production. We first evaluated the effects of RTX treatment on the Raf-1 and ERK1/2 phosphorylation in SGEC, from healthy subjects and pSS patients, grown to confluence, as the effects of this biological drug in human SGEC have not yet been analyzed and we were interested in ascertaining whether RTX influences the expression of Raf-1/ERK1/2 signaling in the healthy and diseased epithelial cells. To this end, a series of techniques to identify changes in Raf-1/ERK1/2 mRNA and protein expression induced in healthy and pSS SGEC by RTX was employed. As shown by these control experiments and reported in Figs 2 and 3, there was no significant change in the Raf-1 and ERK1/2 phosphorylation and activation after treatment with RTX. To gain more information on the effect of RTX on inflammatory processes, we screened 12 inflammatory mediators by cytokine array assay. Healthy and pSS SGEC were incubated with medium (control) or RTX. The supernatants were collected after 48h and analyzed for cytokine levels. As demonstrated by previous research in
our laboratory, SGEC from healthy control subjects and from patients with pSS spontaneously secreted several cytokines whose levels are higher in pSS SGEC compared to those observed in SGEC from healthy controls (25–28). As shown in Fig. 4, RTX does not affect cytokine production by healthy and pSS SGEC. Whether and how RTX determines negative regulation of the Raf-1/ERK1/2 pathway in a pSS SGEC and pSS B- or T-lymphocytes co-culture system was then investigated. We established the co-culture system using the SGEC derived from pSS subjects and purified syngeneic pSS B-lymphocytes subsets isolated by positive selection using anti-CD19-conjugated magnetic beads. Experimental controls were represented by co-culture performed associating SGEC and syngeneic B-lymphocytes obtained from healthy donors. As a step toward understanding whether the roles of Raf-1 and its associated proteins are influenced by RTX, we performed a side-by-side analysis of Raf-1 and ERK1/2 both at gene and protein levels. Raf-1 mRNA levels were analyzed by both semi-quantitative (Fig. 5, panels A, B) and quantitative RT-PCR (Fig. 5, panel C) methods. Interestingly, pSS SGEC expressed high levels of Raf-1 mRNA when co-cultured with pSS B-cells without the addition of RTX, which were significantly altered when the co-culture was exposed to RTX ($P < 0.01$). In addition, we found, by quantitative analysis, that RTX caused a 2.7-fold reduction in the amount of Raf-1 mRNA levels in the co-culture system tested ($P < 0.01$). To determine whether this change in mRNA expression also resulted in differential regulation of protein expression, we employed western blotting to control the effect of RTX addition to the co-culture system on Raf-1 expression by pSS SGEC. As demonstrated in Fig. 5, panels D, E, RTX addition in the co-culture system results in a decreased active phosphorylated

![Fig. 2. Raf-1 expression in pSS SGEC cultured alone in the presence or not of RTX. SGEC obtained from healthy volunteer donors treated or not with RTX were used as controls in all the experimental procedures. Panels A, B: semi-quantitative RT-PCR and related densitometric analysis of Raf-1 gene expression in pSS SGEC treated or not with RTX (20 μg ml$^{-1}$) for 24 h (mean ± SE of analyses performed in duplicate). Panel C: quantitative real-time analysis of Raf-1 mRNA expression in pSS SGEC after RTX exposure (20 μg ml$^{-1}$ for 24 h). Data are presented as fold reduction of Raf-1 mRNA levels after normalizing to β-2-microglobulin expression. Results represent the mean ± SE of duplicate samples from independent experiments. Panels D, E: RTX (20 μg ml$^{-1}$ for 48 h) was added to the SGEC culture and total cell lysates (30 μg) were subjected to p-Raf-1 immunoblotting analysis. Western blot and densitometric analysis show the effect of RTX exposure on p-Raf-1 expression in pSS SGEC. Panel F, G: Graphs show the percentage of p-Raf-1 positive SGEC (F) and p-Raf-1 MFI (G) measured by flow cytometry in pSS SGEC after RTX treatment (mean ± SE as determined in duplicate assays).]
form of Raf-1 protein expression by pSS SGEC after 48 h from B-lymphocytes removal, compared with pSS SGEC co-cultured with pSS B-lymphocytes without the addition of RTX ($P < 0.01$). The intracellular expression of Raf-1 was also examined by flow cytometry using a mAb specific for p-Raf-1. As shown in Fig. 5, panel F, the percentage of p-Raf-1-positive pSS SGEC significantly decreased with the addition of RTX to the co-culture system (from 69.67% ± 3.45 (mean ± SE) in the absence of RTX to 34.23 % ± 2.14 in the presence of RTX ($P < 0.01$)). Evaluation of the MFI (Fig. 5, panel G) confirmed the striking decrease of this protein in pSS SGEC after the addition of RTX to the co-culture in comparison with pSS.
SGEC co-cultured with pSS B-cells in the absence of RTX (MFI = 6789 ± 245 and 10 546 ± 314 respectively, P < 0.01). After a 48-h co-culture with pSS B-lymphocytes, the expression of ERK1/2 in pSS SGEC cells was analyzed both at gene and protein levels, to ascertain whether the observed changes in Raf-1 mRNA expression and Raf-1 dephosphorylation also resulted in decreased ERK1/2 gene expression and kinase activity of the ERK1/2 pathway. Furthermore, the expression of ERK1/2 in pSS SGEC 24/48 h after the removal of pSS B-cells in the presence or not of RTX was examined. The analysis of gene expression by semi-quantitative RT-PCR (Fig. 5, panels A, B) demonstrated that the mRNA levels of ERK1/2 in the pSS SGEC co-cultured with pSS B-cells were higher than in the pSS SGEC derived from the co-culture after the addition of RTX (1.98 ± 0.17 versus 0.97 ± 0.23, P < 0.01). These results obtained by RT-PCR were confirmed completely by quantitative real-time RT-PCR (Fig. 5, panel C), showing that direct co-culture of pSS SGEC with pSS B-lymphocytes led to an increase in the expression of ERK1/2 mRNA in pSS SGEC while the expression of ERK1/2 genes was significantly down-regulated (P < 0.01) after the addition of RTX to the co-culture system. These data were corroborated on a protein level by immunoblotting (Fig. 5, panels D, E). Phospho-ERK1/2 proteins appeared to have been up-regulated in the presence of pSS B-lymphocytes while, after the B-cell depletion following the addition of RTX to the co-culture, an evident decrease in the phospho-dependent state of ERK1/2 occurs. These data were further supported by flow cytometric analysis (Fig. 5, panel F).
showing that the binding of RTX to CD20 on B-cells determined a decreased number of positive SGEC for p-ERK1/2 proteins [from 74.7 % ± 4.24 (mean ± SE) in the absence of RTX to 41.34 % ± 5.07 in the presence of RTX (P < 0.01)] and suggesting that exposure of pSS B-cells to RTX in the co-culture system leads to down-modulation of active ERK1/2 in pSS SGEC. To confirm the effect of RTX addition on ERK1/2 in pSS SGEC in the context of phosphorylation-activation, the MFI was evaluated (Fig. 5, panel G), comparing the pSS SGEC from the co-culture system with the same cells derived from the co-culture in the presence of RTX. We found that the p-ERK1/2 MFI levels were significantly decreased (P < 0.01) after the addition of RTX, (MFI = 7654 ± 267 for pSS SGEC from the co-culture with B-cells versus MFI = 3278 ± 245 for pSS SGEC from the co-culture in presence of RTX, P < 0.01) and, then, the decreased phosphorylation of ERK1/2 may be attributed to the pSS B-cell as p-ERK1/2 expression decreased in pSS SGEC after RTX treatment. In the same study we tested whether RTX was responsible for a negative regulation of the Raf-1/ERK1/2 pathway in a co-culture system represented by pSS SGEC co-cultured with syngeneic pSS T-lymphocytes. Results are reported in Fig. 6, panels A–G. Also in this case experimental controls were represented by healthy SGEC co-cultured with syngeneic healthy T-lymphocytes. The same experimental techniques were employed which demonstrated that the administration of RTX to the pSS SGEC with syngeneic pSS T-lymphocyte co-culture system was accompanied with a low but detectable decrease in Raf-1 and ERK gene and protein expression, presumably due to the depletion of the small CD20+ fraction of T-lymphocytes.

**Pharmacological inhibition of ERK1/2 signaling mimics the effects of RTX on the release of pro-inflammatory factors**

The inhibition of the Raf-1/ERK1/2 signaling pathway by RTX prompted us to investigate whether specific inhibition of
this pathway would mimic the consequences of RTX treatment. GW5074 exerts its effects by directly inhibiting Raf-1, thus preventing the ERK1/2 phosphorylation and activation. Optimal concentrations of the inhibitor were determined by pilot studies conducted previously (data not shown). Since the inhibitory effect of RTX on the activity of the Raf-1/ERK1/2 pathway was more pronounced when pSS SGEC were cocultured with pSS B-lymphocytes rather than in the presence of pSS T-lymphocytes, as deduced from previous experiments, the Raf-1 inhibitor GW5074 (50 nM) was added to the pSS SCEG/pSS B-lymphocyte co-culture system followed or not by RTX (20 μg ml⁻¹) addition. After extensive washing to eliminate B-lymphocytes, cytokine profiles were studied to consider the possibility that specific inhibition Raf-1 would mimic RTX. As shown in Fig. 7, panel A, the use of the Raf-1 inhibitor demonstrated a direct role of Raf-1 in the RTX-mediated regulation of the pro-inflammatory cytokine release by pSS SGEC. Using the cytokine Multi-Analyte ELISAArray, we demonstrated that both following RTX and GW5074 treatment, pSS SGEC exhibited a significant decrease of the pro-inflammatory cytokines IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-12, IL-13, IL-17 and GM-CSF and, interestingly, an increase of the anti-inflammatory cytokines IL-4 and IL-10. Since RTX reduced the phosphorylation of the components of the ERK1/2 pathway, we tried to corroborate the RTX-mediated effects on the pro-inflammatory cytokine release by pSS SGEC using the PD98059 inhibitor that exerts its effects by binding to the inactive form of MEK1/2 and prevents MEK1/2 activation by Raf-1, thus inhibiting the activation of ERK1/2 (36). The co-culture was treated with either RTX (20 μg ml⁻¹) or PD98059 (4 μM) for 48h. Then, the B-lymphocytes were removed and the co-culture supernatant was assayed for pro-inflammatory cytokine content. As shown in Fig. 7, panel B either RTX or PD98059 substantially diminished the pro-inflammatory cytokine release by pSS SGEC. These results confirm the ability of RTX to ameliorate the inflammatory condition in pSS through the modulation of the ERK pathway and corroborate the above-mentioned findings demonstrating the regulation of the Raf-1/ERK1/2 pathway by RTX. Collectively, these results support the ability of RTX to negatively regulate activation of the Raf-1/ERK1/2 signaling pathway, modulating the inflammatory response.

**Discussion**

RTX has been proven to be effective against B-cell malignancies (37), with significant clinical benefits (38) and in autoimmune diseases, including autoimmune cytopenia and RA (39). Although few data are present in the literature to date, evidence from clinical studies provides the clue that RTX is effective in reducing various disease manifestations of pSS patients, such as sicca symptoms, extraglandular manifestations and fatigue, and in improving saliva production (11, 12). The patients with residual salivary function and treated in early phases of the disease showed a better response concerning eye dryness and fatigue and consistent clinical improvement was also reported for both glandular enlargement (40) and extraglandular manifestations (41). These data may have

![Fig. 7](image-url)

**Fig. 7.** Specific inhibition of components of the Raf-1/ERK pathway mimics the consequences of RTX treatment. Panels A, B: exponentially growing pSS SGEC were co-cultured with syngeneic pSS B-lymphocytes in the presence of RTX (20 μg ml⁻¹), Raf-1 inhibitor GW5074 (50 nM) and MAPK/ERK kinase (MEK)1/2 inhibitor PD98059 (4 μM) for 48h. After an extensive washing to remove the pSS B-cells, the culture supernatants were collected and the Human Common Cytokines Multi-Analyte ELISAArray Kit that analyzes a panel of 12 pro-inflammatory cytokines was employed to measure IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α and GMCSF production levels. (Data are displayed as the mean ± SE of independent experiments each performed in duplicate; **P < 0.01).**
implications for patient care, since the patients who seem to benefit from RTX treatment are those with early, active disease. An important and recent study of Devauchelle-Pensec et al. (42) represents an advance in the knowledge about the efficacy of RTX in pSS patients; in a randomized, double-blind, placebo-controlled trial, fatigue was the symptom that responded best to RTX therapy in the observational study conducted, whereas effects on dryness were delayed. The data collected do not support the use of RTX therapy in many patients with recent-onset or systemic pSS. Nevertheless, RTX induced several significant improvements in patients with recent-onset or systemic pSS. RTX was well tolerated, with no treatment-related severe or serious adverse events during the observation period of this study (42).

However, the mechanism of RTX-mediated action is not entirely clear. Therefore, characterization of the signaling pathways triggered by RTX is of considerable interest to elucidate its activity and lack of activity in patients refractory to RTX. Depletion of circulating B-cells by RTX can hit the reset button of immune tolerance providing a new chance to an appropriate regulation of autoreactive B-cells. On the other hand, depleting B-cells may also influence the T-cell compartment, since B-cells are effective antigen-presenting cells and can govern autoantigen specific T-cell responses by providing co-stimulatory signals and secretion of cytokines and growth factors (43). This may partially explain the therapeutic efficacy of RTX treatment in autoimmune diseases. Regarding the T-cells from pSS patients, they appear to be largely unaffected by RTX treatment (19). However, a recent and important study by Eggleton et al. (20) provides an additional clue for the impact of RTX treatment on Th-17 cells, revealing that the frequency of a very small proportion of circulating Th-17 cells expressing the surface marker CD20 was significantly higher in patients with RA (20). In addition, Wilk et al. (44) have shown that these CD20+ T-cells were depleted by RTX-treatment in RA-patients. Although the exact function of these CD20+ IL-17 producing cells is not known, it is therefore possible that the positive effect of RTX treatment in pSS patients can be, at least partially, attributed to the depletion of these CD20+ T-cells. To clarify the mechanism involved in the effects mediated by RTX treatment, our laboratory has initiated a series of in vitro studies to examine the signaling pathways mediated by RTX in an experimental disease model constituted by SGEC derived from pSS patients in a co-culture system with pSS B-lymphocytes or pSS T-lymphocytes. This methodological approach derived from knowing that the establishment of non-neoplastic SGEC lines provides a valuable means for the study of the physiology of salivary epithelia, as well as the pathophysiological role of these cells in disorders affecting the salivary glands, such as pSS. During recent years, several lines of evidence have indicated that glandular epithelial cells in pSS lesions are aberrantly activated and play an active role in the induction and perpetuation of the inflammatory processes (45, 46). The epithelial activation by cytokines produced locally by the lymphocytic infiltrates, or due to intrinsically operating processes that may occur in the epithelia of pSS patients, could be responsible for the activation phenomena of pSS SGEC. Evidence from our laboratory had indicated that pSS SGEC represent an important source of cytokines, chemokines and their receptors (23, 47, 48). These concerns prompted us to set up a co-culture system for the functional assessment of the capacity of pSS SGEC to interact with pSS B-lymphoid cells and, eventually with pSS T-lymphocytes, to assess the effect of RTX on the Raf-1/ERK1/2 pathway modulation in pSS SGEC, and to address its implication in the chronic inflammatory lesions of pSS. This study presents evidence that the addition of RTX to the co-culture system results in the disruption of the MAPK cascade involving the Raf-1/ERK1/2 module, leading to the subsequent inhibition of inflammatory factor release by pSS SGEC. RTX represses the phosphorylation and activation of the upstream activator of the pathway, Raf-1, and the decreased Raf-1 activation consequently results in the inhibition of ERK1/2 signaling. In addition, this study demonstrated, for the first time, that RTX, by its binding to pSS B-lymphocytes present in the co-culture, determines a significantly decreased pro-inflammatory cytokine release by pSS SGEC, preventing activation of ERK1/2. In fact, a reduced expression of the pro-inflammatory mediators by pSS SGEC was observed. pSS SGEC exhibited a significantly decreased release of the pro-inflammatory cytokines IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-12, IL-13, IL-17 and GM-CSF. These results suggest that, under chronic inflammatory condition, B-lymphocytes regulate cytokine and chemokine expression by SGEC. pSS SGEC are in close contact with B-lymphocytes within salivary glands and are thus influenced by the local microenvironment formed by the B-cells both under steady state and conditions with increased B-cell influx, such as the chronic inflammation characterizing pSS. In order to clarify the mechanisms suspected to mediate the therapeutic effects of RTX, we used specific chemical inhibitors of the MAPK pathway that mimicked the consequences of RTX treatment, demonstrating a direct role of the Raf-1/ERK1/2 cascade in the RTX-mediated regulation of the pro-inflammatory cytokines released by pSS SGEC. This corroborates the hypothesis of a regulation of the Raf-1/ERK1/2 pathway by RTX and further demonstrates the ability of RTX to modulate the inflammatory response in pSS.

Our findings are consistent with the observations made in other laboratories documenting that RTX triggers a complex network of direct inhibitory signals versus the canonical pathways involving MAPK (49–51). A link between CD20 signaling, p38 MAPK activity, and IL-10 regulation was established recently (51). RTX induces sustained phosphorylation of ERK1/2 and p38 MAPK leading to apoptosis in Ramos and Daudi cells (21) and RTX marks the B-cell lines through the p38 MAPK pathway culminating in the inhibition of IL-10 transcription and secretion by rapid inhibition of the constitutive p38 MAPK activity (51). Although RTX is known to be a typical anti-B-cell agent, recent data suggest its administration to be associated with an increased risk of T-cell-dependent infections (52), raising the question of RTX interference with T-cell activation and function (53). To test this hypothesis in pSS, experiments were performed in this study to evaluate the effects of RTX addition to syngeneic T-cells co-cultured with pSS SGEC demonstrating that exposure to RTX of pSS T-lymphocytes led to an attenuated response, indicated by decreased inflammatory cytokine production. The significant decrease in T-cell function in the presence of RTX demonstrated in our experiments, even in the absence of B-cells or SGEC in the co-culture, implies a potential direct effect of RTX on T-lymphocytes. These results could be explained considering the above cited recent study by Wilk...
et al., suggesting that RTX can directly bind to a small population of CD3\textsuperscript{+}CD20\textsuperscript{+} T-cells (44). The precise role of this ‘novel T-cell population,’ accounting for 3–5% of T-cells in healthy volunteers (54) but not yet quantified in pSS patients, remains obscure, although these cells have been reported to exhibit a marked inflammatory profile (44).

In summary, our results provide the first evidence of the involvement of the Raf-1/ERK1/2 pathway in the regulation of the pro-inflammatory response by RTX in pSS SGEC. One of the limitations with this explanation is that it does not explain how RTX, by B-cell depletion, interferes with the pro-inflammatory activity of pSS SGEC. Although B-cell-depleting therapy with RTX provided promising results, the underlying mechanisms of action are not completely understood and it is still unclear why some pSS patients fail to achieve a good clinical response. More research is needed to better understand the molecular events that follow the binding of RTX to CD20 on the B-cell surface and that transduce signals that lead to the activation of pSS SGEC.

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