




ORIGINAL RESEARCH

Possible role for IL-40 and IL-40-producing cells in the lymphocytic infiltrated salivary glands of patients with primary Sjögren's syndrome

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ABSTRACT

Objectives Aim of this study was to investigate the expression of interleukin (IL)-40, a new cytokine associated with B cells homeostasis and immune response, in primary Sjögren syndrome (pSS) and in pSS-associated lymphomas.

Methods 29 patients with pSS and 24 controls were enrolled. Minor salivary gland (MSG) biopsies from patients, controls and parotid gland biopsies from pSS-associated lymphoma were obtained. Quantitative gene expression analysis by TaqMan real-time PCR and immunohistochemistry for IL-40 were performed on MSG. MSG cellular sources of IL-40 were determined by flow-cytometry and immunofluorescence. Serum concentration of IL-40 was assessed by ELISA and cellular sources of IL-40 were determined by flow-cytometry. An in vitro assay with recombinant IL-40 (rIL-40) was performed to detect the effect on cytokine production from peripheral blood mononuclear cells (PBMCs).

Results IL-40 was significantly increased in the lymphocytic infiltrated MSG of patients with pSS and correlated with focus score and with IL-4 and transforming growth factor- β expression. In addition, IL-40 was increased in the serum of pSS and its levels correlated with the EULAR Sjögren's Syndrome Disease Activity Index score. B cells from patients were shown to be the major source of IL-40 at both tissue and peripheral level. PBMCs from patients, exposed to rIL-40 in vitro, released proinflammatory cytokines, specifically interferon- γ from B cells and T-CD8⁺ and tumour necrosis factor- α and IL-17 from both T-CD4⁺ and T-CD8⁺. IL-40 expression in parotid glands of pSS-associated lymphomas was also increased. Moreover, IL-40-driven NETosis was evidenced in neutrophils obtained from pSS.

Conclusion Our results suggest that IL-40 may play a role in pSS pathogenesis and pSS-associated lymphomas.

INTRODUCTION

Primary Sjögren syndrome (pSS) is an autoimmune disease characterised by chronic

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Interleukin-40 (IL-40) is a novel B-cell associated cytokine whose production is increased by IL-4 and transforming growth factor- β 1. Moreover, human B cells lymphomas are able to constitutively produce and differentially express IL-40. The upregulation of IL-40 has recently been described in rheumatoid arthritis. Up to date, no studies in primary Sjögren syndrome (pSS) have been published.

WHAT THIS STUDY ADDS

⇒ We demonstrated the upregulation of IL-40 in pSS. B cells are the major source of IL-40, but even T cells are able to produce it. In addition, IL-40 promotes the release of proinflammatory cytokines from immune cells and can stimulate NETosis in pSS. Interestingly, we detected an intense expression of IL-40 in pSS-associated non-Hodgkin's lymphoma.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ IL-40 may play a role in the pathogenesis of pSS and pSS-associated lymphomas and may be regarded as a new potential biomarker of disease and treatment target.

inflammation of exocrine, mainly salivary and lachrymal, glands.¹

The pathogenesis of pSS is a multifactorial process, in which environmental factors, most likely viral, in a genetically predisposed individual, determine an aberrant immune response with a complex interplay between innate and adaptive systems which leads to the loss of self-tolerance.^{2,3} B cells, in particular, play a pivotal role in the development of pSS. B cells infiltrate salivary glands and can organise, together with T cells, into ectopic germinal centre-like structures

(GCs-like structures); their chronic aberrant hyperactivation may drive clonal escape and consequent lymphomagenesis.⁴ In fact, up to 5% of patients develop B-cell non-Hodgkin's lymphoma, mainly mucosa-associated lymphoid tissue (MALT) lymphomas.⁵

The knowledge on B cells biology is constantly evolving, and, in the last few years, B cells have emerged as potential effector cells, able to release a wide range of cytokines. B cells can then actively contribute to shaping the microenvironment they act in, thus profiling a more complex scenario in autoimmune diseases, such as pSS.⁶

In 2017, interleukin-40 (IL-40), a novel B-cell associated cytokine encoded by an uncharacterised gene (C17orf99; chromosome 17 open reading frame 99), was described. Naïve B cells, once activated in vitro, can express IL-40 at both tissue and peripheral level. IL-40 levels were found markedly increased when B cells were stimulated with IL-4 and transforming growth factor (TGF)- β 1, cytokines with a pivotal role also in pSS.⁷ Moreover, human B cells lymphomas are able to constitutively produce and differentially express IL-40, thus suggesting a possible role of this cytokine in human disease.^{8,9}

Taking into account the new emerging evidence and considering the well-known role of IL-4 and TGF- β 1 in pSS pathogenesis,^{7,10} we decided to study the expression of downstream cytokine IL-40, in salivary glands and peripheral blood of patients affected by pSS and in pSS-associated non-Hodgkin's lymphoma (NHL). Moreover, we assessed the in vitro effect of IL-40 in activating immune cells obtained from peripheral blood mononuclear cells (PBMCs).

MATERIALS AND METHODS

Patients and controls

Salivary glands (SG) biopsies and blood samples were collected from a total number of 29 patients with pSS, fulfilling the American–European Consensus Group¹¹ criteria for pSS, and 24 patients with non-Sjögren syndrome (nSS), who display subjective issues of dry mouth or eyes but did not meet the American–European Consensus Group criteria for pSS, nor present inflammatory infiltrate in the minor salivary gland (MSG), as control group. Patients were recruited at Policlinico Paolo Giaccone University Hospital, Palermo, Italy. Five patients with pSS with a previous diagnosis of pSS-associated lymphoma were selected and paraffin-embedded samples were obtained from the biopsy bank of the Pathology Unit of the Ospedale Cervello—Palermo, Italy, for immunohistochemistry (IHC) and immunofluorescence staining. Patients' characteristics are presented in [table 1](#).

Paraffin-embedded sections of 5 μ m thickness were stained with H&E for histological evaluation as previously described.¹²

Informed consent was obtained from each patient in accordance with the Helsinki Declaration.

IHC

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. IHC for IL-40 was performed on 5 μ m-thick paraffin-embedded sections from MSG and pSS NHL. Additionally, TGF- β and IL-4 expression was also evaluated in paraffin-embedded

Table 1 Clinical characteristics of patients and controls

| | pSS (n=29) | nSS (n=24) | NHL* (n=5) |
|---|--------------|----------------|--------------|
| Age (years) (range) | 58 (30–77) | 54 (35–68) | 59 (54.8) |
| Female sex, n (%) | 29 (100) | 20 (83) | 5 (100) |
| Disease duration, months (range) | 82 (12–264) | 60 (12–144) | 122 (48–204) |
| Anti-nuclear antibodies, n (%) | 14 (48) | – | 5 (100) |
| Anti-Ro and/or anti-La antibodies n (%) | 17 (59) | – | 4 (80) |
| Rheumatoid factor n (%) | 9 (31) | – | 3 (60) |
| ESR mm/hour, mean (range) | 12.29 (1–34) | 28 (2–107) | 65 (15–100) |
| CRP mg/L, mean (range) | 1.5 (0.12–5) | 4.3 (0.6–14.5) | 29 (5–70) |
| Focus score 0 n (%) | 3 (10.3) | 24 (100) | – |
| Focus score 1 n (%) | 8 (27.6) | – | – |
| Focus score 2 n (%) | 7 (24.1) | – | – |
| Focus score 3 n (%) | 5 (17.2) | – | 5 (100) |
| Focus score 4 n (%) | 6 (20.7) | – | – |
| ESSPRI, mean | 4.3 | – | – |
| ESSDAI, mean | 3.9 | – | – |

*Clinical data of patients with primary Sjögren's syndrome (pSS) who developed non-Hodgkin's lymphoma (NHL) are referred to the time of the onset of lymphoma.

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; nSS, non-Sjögren syndrome.

sections of SG tissue of eight patients, as previously described.¹³ Control staining, without primary antibodies, was used as negative controls.

Evaluation of IL-40/IL-4/TGF- β -expressing cells in SG tissue was assessed by two independent investigators on photomicrographs obtained from three random high-power microscopic fields (original magnification $\times 40$) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera.

Immunofluorescence on paraffin-embedded SG tissue sections

Immunofluorescence (IF) staining of MSG and parotid MALT lymphoma tissues was performed as in the protocol previously described.¹⁴ The monoclonal antibodies used were: mouse anti-human CD3 (PA0553, Leica Bond, Germany), mouse anti-human CD19 (M7296, Dako) and mouse anti-human CD68 (M0876, Dako), diluted 1:100 in phosphate-buffered saline (PBS, Sigma, St Louis, Missouri, USA) containing 3% bovine serum albumin (BSA) and 0.05% Tween 20 (PBS/BSA 0.05 TW20). Secondary rabbit anti-mouse Alexa Fluor 555 antibody was used (A21427, Life Technologies, Carlsbad, California, USA) 1:200 in PBS/BSA 0.05 TW20 at room temperature for 1 hour and 30 min. Then, stained sections were fixed with paraformaldehyde 2% for 30 min and permeabilised with 0.1% Triton X-100 for 10 min. Sections were incubated overnight with polyclonal rabbit anti-human IL-40 (ab122075, Abcam, Cambridge, UK) diluted 1:100 in PBS/BSA 0.05 TW20, and then were incubated with 1:200 secondary goat anti-rabbit Alexa Fluor Plus 488 (A11008, Life Technologies). Sections subjected to rehydration, fixation, permeabilisation and stained with only secondary antibodies were used as negative control. Moreover, nuclei were counterstained with Hoechst 33342 (Cat. H1399, Life Science) for 15 min at room temperature. Lif (Leica image file) files of images were collected by confocal laser-scanning microscope DMI6000 with Leica Application Suite X, at a scan rate of $\times 40$ magnification and $\times 63$ magnification.

Co-localisation analysis was performed using the JACoP plugin available in ImageJ software (Bolte and Cordelières, 2006). Prior to performing calculations, merged colour images were separated into individual channels using the 'Color split' feature, then background correction was done through the math process in the ImageJ. To avoid user bias, both Pearson's coefficient and Mander's coefficients M1 and M2 were calculated by using Costes' threshold within the Jacob plugin.^{15 16}

IF on normal-density neutrophils to detect NETosis

To study NETosis, normal-density neutrophils (NDN) were obtained from blood belonging to three patients with pSS (FS3) and three nSS subjects by density gradient centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Following that, NDNs were aspirated from the interface of Ficoll and red blood cells and washed with 2 mL of PBS. Then, cells were treated with

0.5 molar ammonium chloride for 20 min to lyse any remaining red blood cells and were washed two times with PBS, as previously described.¹⁷

For IF staining, 100 μ L of cell suspension, containing 10^5 cells, was stimulated for 30 min with phorbol myristate acetate (PMA) at the concentration of 1.35 μ M. In addition, cells were stimulated for 30 min and 24 hours with recombinant IL (rIL)-40 (CUSABIO, protein IL-40 (C17orf99), Houston, USA) at the final concentration of 100 ng/mL (rIL-40 concentration was chosen based on a dose-response curve, data not shown). Unstimulated controls were incubated in the same conditions. Afterward, cells were stained with anti-human CD15 FITC (332778, BD Biosciences, California, USA). After 20 min, cells were washed with PBS containing 0.5% BSA and 2 mM EDTA (staining buffer), and fixed using fixation solution (Cat.00-8222, eBioscience, Massachusetts, USA) for 20 min. The cytocentrifuge was used to directly sediment cells on the slides, thus cells were fixed again prior to permeabilisation. For intracellular staining, cells were incubated overnight with rabbit anti-human myeloperoxidase (MPO) polyclonal antibody (PA1-22870, Life Technologies). After washing, we added goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 555 (A-21428) for 1 hour and 30 min. Negative controls for each experiment image were stained with secondary antibodies only. Nuclei were counterstained with Hoechst 33342 for 15 min at room temperature. Lif files of images were collected by confocal laser-scanning microscope DMI6000 with Leica Application Suite X, at a scan rate of $\times 40$ magnification $\times 63$ magnification.

RNA isolation and quantitative real-time reverse transcription-PCR

Reverse transcription PCR was performed as previously described¹⁴ using the following oligonucleotides: human IL-40 (forward 5' CAAGGCACGGGAGGAAGAAA3'; reverse 5' ACAGCAGGTTATGAGCACCC3'). Samples were run in duplicate using the Step One Real-Time PCR system (Applied Biosystems, Foster City, California, USA). Relative changes in gene expression between samples were determined using the $\Delta\Delta C_t$ method. Levels of the target transcript were normalised to the glyceraldehyde-3-phosphate dehydrogenase endogenous control.

Isolation, stimulation and flow cytometry of salivary gland mononuclear cells

Isolation of salivary gland mononuclear cells (SGMCs) was performed as previously described.^{13 14}

For the functional assay, cells were incubated for 24 hours at 37°C in 5% CO₂ in several conditions: (a) with cell culture medium alone, (b) with PMA (150 ng/mL, Sigma) and ionomycin (1 μ g/mL) (CLEARLine, Biosigma, Cona-Verona, Italy) for T cells activation and (c) with 1 μ g/mL of lipopolysaccharides (Sigma) for macrophages activation. To assess cytokine production from B cells, harvested cells were incubated (d) with 5 μ g/mL anti-human pure functional grade CD40 (clone

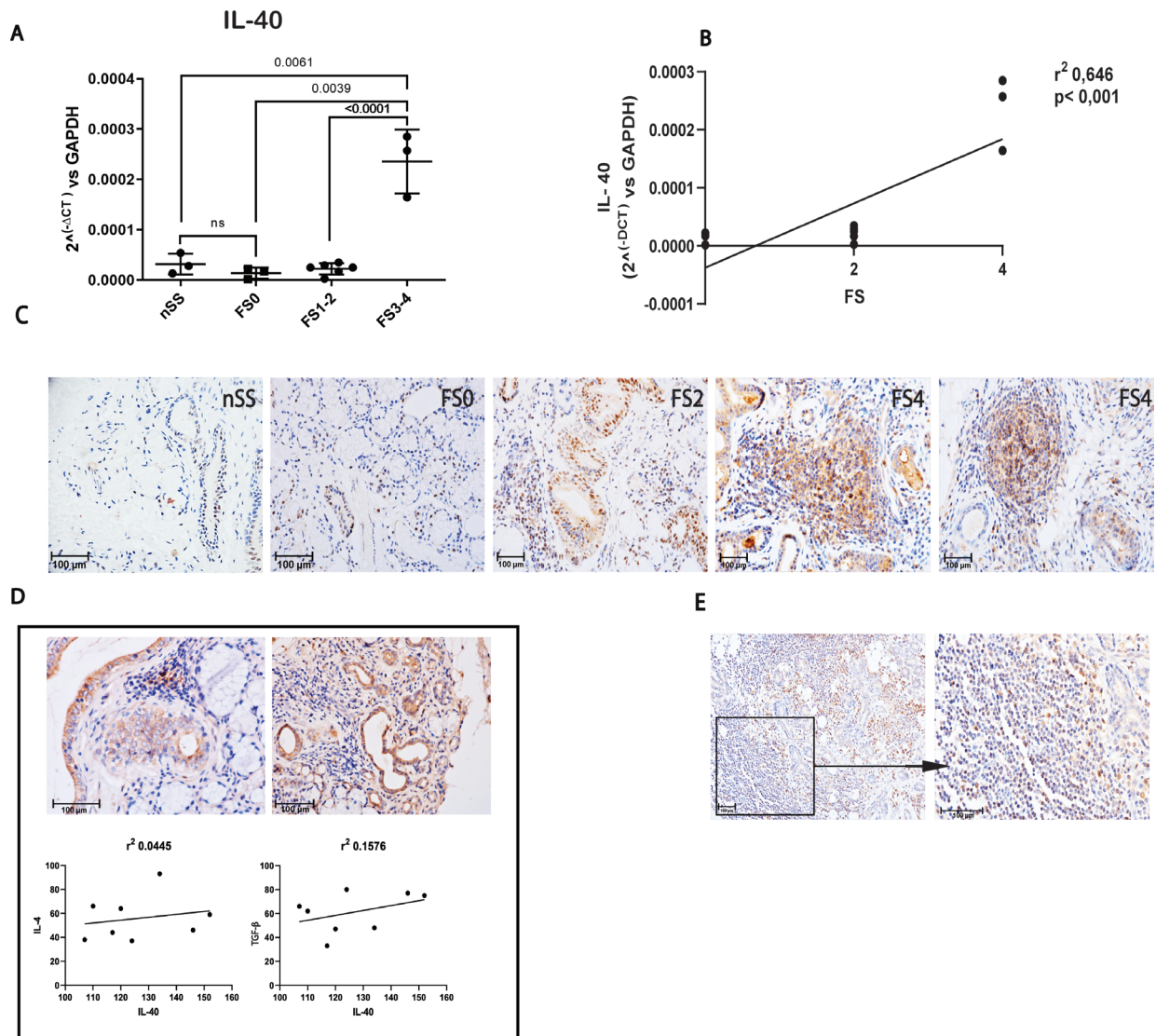


Figure 1 IL-40 overexpression in pSS and pSS-associated lymphoma. (A) Relative mRNA quantification of IL-40 according to FS assessed by quantitative reverse transcription-PCR. (B) Correlation between FS and IL-40 mRNA expression ($r^2=0.65$; $p<0.001$). (C) Salivary gland biopsies stained for IL-40 in nSS and pSS, showing an increase in IL-40 expression in patients with pSS with different FS (FS0, FS2 and FS4 (without and with germinal centre)). (D) pSS salivary gland tissue stained for IL-4 (left) and TGF- β (right) and correlation between their expression and IL-40 (IL-4/IL-40, $r^2=0.0445$; TGF- β /IL-40, $r^2=0.1576$). (E) Parotid gland specimens of pSS-associated lymphoma showing intense staining for IL-40. FS, focus score; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; mRNA, messenger RNA; nSS, non-Sjögren syndrome; pSS, primary Sjögren syndrome; TGF, transforming growth factor.

HB14, Miltenyi Biotec, Bergisch Gladbach, Germany), 5 μ g/mL goat anti-human IgA+IgG+IgM (H+L) (RRID AB_2337548, Jackson Immuno, Pennsylvania, USA) and 10 ng/mL anti-human IL-4 (7A3-3, Miltenyi) for 48 hours. The Golgi blocker monensin (10 mg/mL) (Sigma) was added to each condition after 1 hour.

After appropriate stimulation, cell viability was detected by zombie Violet and zombie Aqua Flexible Viability Kit (BioLegend, San Diego, USA) for 15 min, and then cells were washed with a staining buffer. Then, cells were stained with the following fluorochrome-conjugated monoclonal antibodies (mAbs): anti-human CD45 (REA747, Miltenyi), anti-human CD3 (REA613) and anti-human CD19 (REA675) for 30 min. Then, samples

were washed with a staining buffer. To detect intracellular molecules, samples were incubated for 30 min at room temperature in a fixation solution (eBioscience) followed by permeabilisation (Cat.00-8333, eBioscience) according to the manufacturer's protocol. Thereafter, intracellular staining was performed with anti-human CD68 (REA886) and IL-40 rabbit anti-human polyclonal antibody (C17ORF99, LS-C479150-100, LSBio, Washington, USA). Isotype-matched control mAbs (Miltenyi) were used for cell lineage markers, while we used fluorescence minus one (FMO) tube to detect IL-40 positivity.

To improve the number of acquired cells, SGMCs were pooled (accordingly with focus score (FS), in four pool experiments for pSS and three pool experiments for nSS).

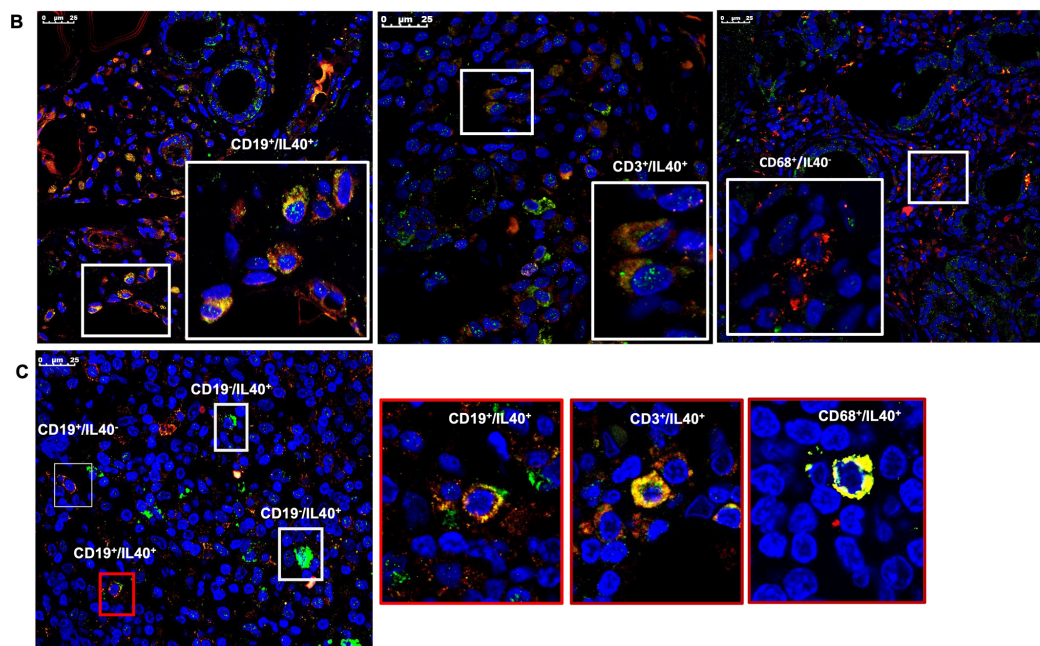
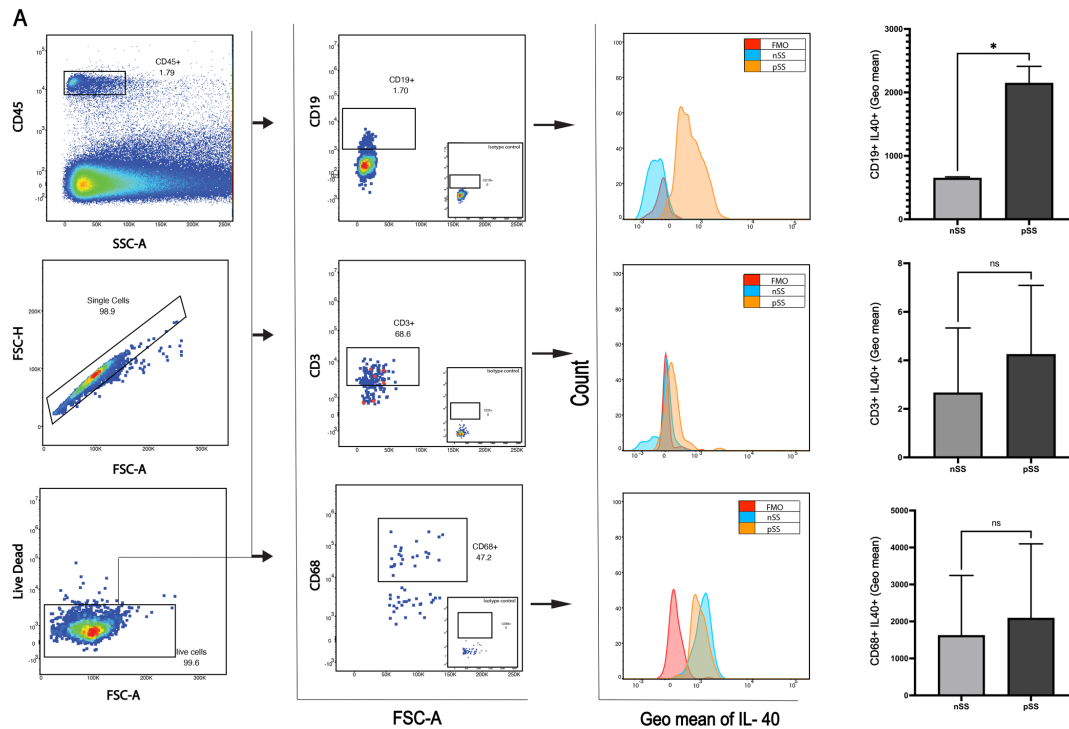


Figure 2 IL-40 is mainly expressed by B cells in SG of pSS and in pSS-associated lymphoma. (A) Gating strategy of patients with a representative pSS used to detect IL-40 expression at tissue level (left panel; isotype control staining is shown within each plot); cells expressing IL-40 among SG CD19⁺, CD3⁺ and CD68⁺ cells, respectively (results are represented as Geo Mean) (right panel). Seven experiments on pooled SG samples were performed. (B) Representative merge panels from pSS SG biopsies. IL-40 positivity (green) was observed in infiltrating cells of pSS SGs, as demonstrated by specific markers (red) for B lymphocytes (CD19) and T lymphocytes (CD3). Macrophage (CD68) do not express IL-40. White boxes highlight double positive cells with their respective magnification. (C) Representative merge panel of pSS-associated lymphoma biopsy showing double positive CD19⁺IL-40⁺ cells with the respective magnification (red box). In the thicker white boxes (right part of C panel) two CD19⁺IL-40⁺ cells are shown; further characterisation of CD19⁺IL-40⁺ identified them as CD3⁺ and CD68⁺ (right small boxes in figure 1C). Nuclei were counterstained by Hoechst 33342 (blue). Representative images of immunofluorescence staining are shown at 40× magnification and 63× magnification. FMO, fluorescence minus one; FSC-A, forward scatter area; FSC-H, forward scatter height; IL, interleukin; nSS, non Sjögren syndrome; pSS, primary Sjögren syndrome; SG, salivary glands; SSC-A, side scatter area.

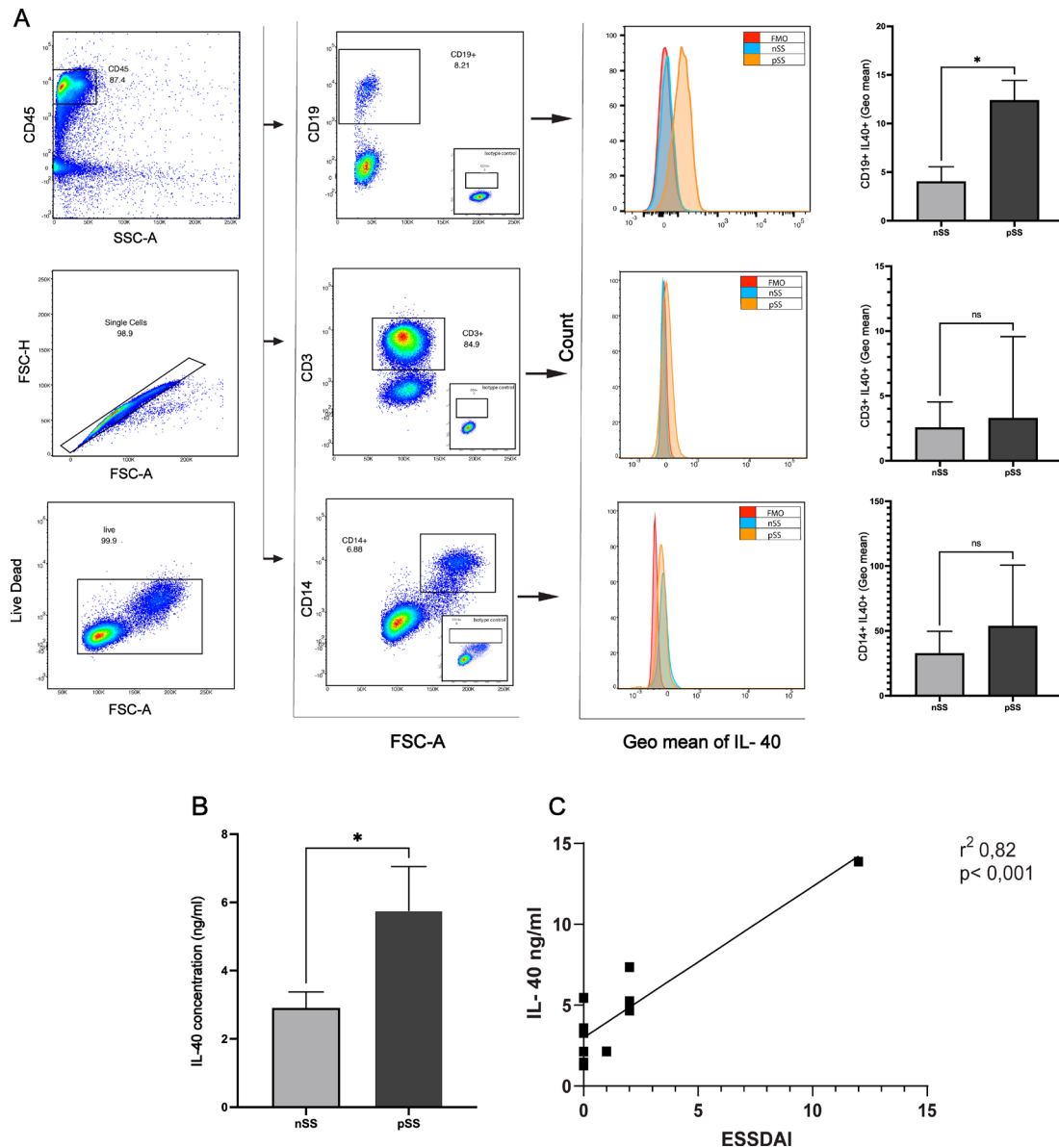


Figure 3 B cells are the major source of IL-40 among PBMCs, its levels are increased in pSS serum and correlate with ESSDAI score. (A) Gating strategy of patients with a representative pSS used to detect IL-40 expression at peripheral level (left panel; isotype control staining is shown within each plot); cells expressing IL-40 among PBMCs CD19⁺, CD3⁺ and CD14⁺ cells, respectively (results are represented as Geo Mean) (right panel). Twelve experiments on PBMCs were performed. (B) Results from ELISA showed statistically significant increase in serum concentration of IL-40 in patients with pSS compared with controls. (C) correlation between IL-40 serum level and ESSDAI score ($r^2=0.82$; $p<0.001$). * p value <0.05 . ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; FSC-A, forward scatter area; FSC-H, forward scatter height; FMO, fluorescence minus one; IL, interleukin; nSS, non-Sjögren syndrome; PBMCs, peripheral blood mononuclear cells; pSS, primary Sjögren syndrome; SSC-A, side scatter area.

Then, cells were acquired on the FACSAria flow cytometer. Data were analysed with FlowJo software (V.10.5.3, Tree Star, Ashland, Oregon, USA). IL-40 expression was calculated using geometric mean values.

Isolation, stimulation and flow cytometry of PBMCs

PBMCs were obtained from peripheral blood, belonging to patients with pSS and nSS subjects, as previously described.¹⁴ Afterwards, PBMCs were incubated with appropriate stimuli to evaluate IL-40 production first and

then with rIL-40 to assess its effect on cytokines production and B cells immunophenotyping.

After PBMCs isolation, cells were cultured with complete medium alone and with stimuli as done for SGMCs stimulation. To evaluate IL-40 production from T cells, B cells and monocytes, cells were stained with zombie Violet and zombie Aqua Flexible Viability Kit (BioLegend) for 15 min, and then were washed with a staining buffer. Then, cells were stained with anti-human CD3 (REA613), anti-human CD19 (REA675) and

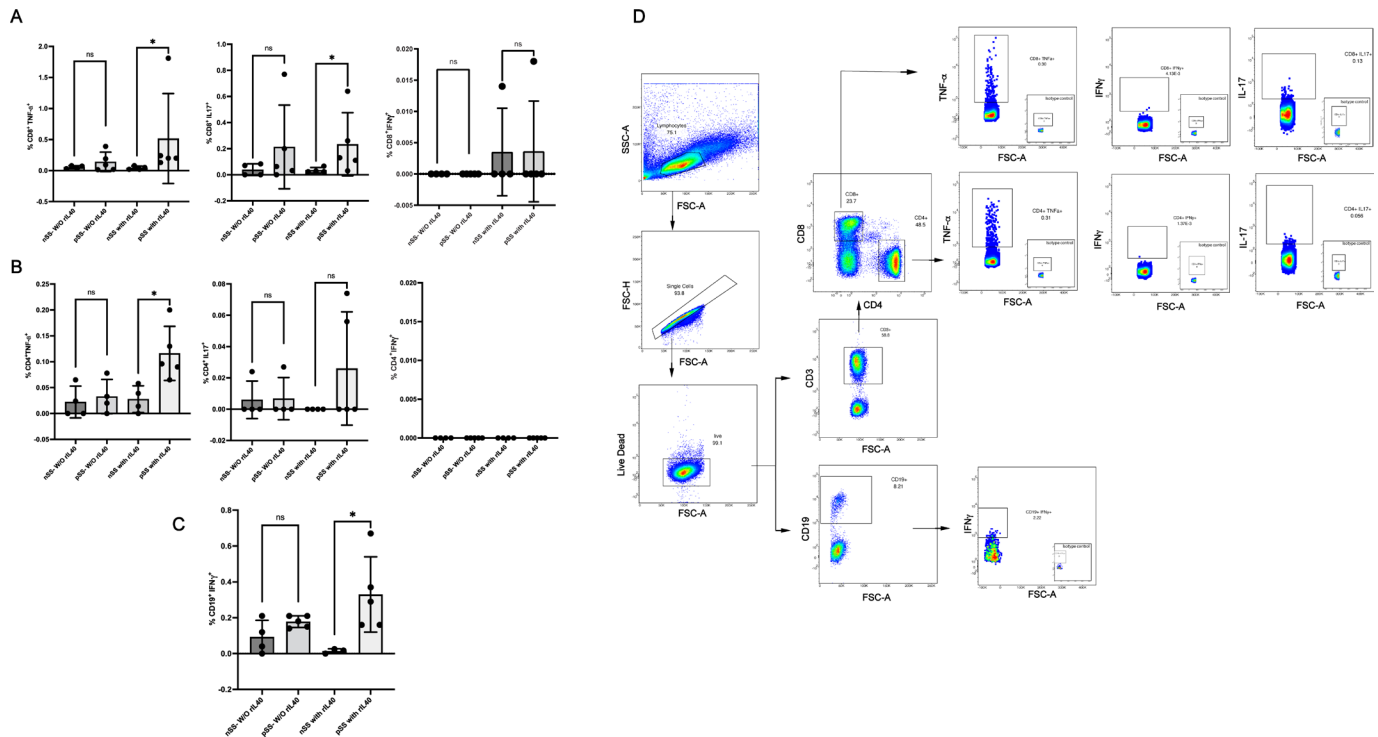


Figure 4 rIL-40 induces cytokine production from T and B lymphocytes in patients with pSS. (A, B) CD8⁺ and CD4⁺ T cells cytokine production, namely TNF- α , IL-17 and IFN- γ , before and after rIL-40 stimulation. (C) CD19⁺ IFN- γ production before and after rIL-40 stimulation in patients with nSS and pSS. (D) Gating strategy of patients with a representative pSS used to detect cytokines production after rIL-40 stimulation in PBMCs (isotype control staining for cytokines are shown within each plot in the gating strategy). *p value<0.05. FSC-A, forward scatter area; FSC-H, forward scatter height; IFN, interferon; nSS, non Sjögren syndrome; PBMCs, peripheral blood mononuclear cells; pSS, primary Sjögren syndrome; rIL-40, recombinant interleukin-40; SSC-A, side scatter area; TNF, tumour necrosis factor; W/O, without.

anti-human CD14 (REA599), conjugated with the appropriate fluorochrome. Following fixation and permeabilisation (eBioscience), cells were incubated with polyclonal rabbit anti-human IL-40 (LS-C479150-100), and as described above, isotype-matched control mAbs (Miltenyi) were used for cell lineage markers, and FMO tube was used to detect IL-40 positivity.

In addition, we stimulated PBMCs with rIL-40 (CUSABIO, protein IL-40 (C17orf99), Houston, USA) to examine its effect on cytokine production. After isolation of pSS and nSS PBMCs, cells were incubated in complete medium alone and with rIL-40 100 ng/mL (concentration chosen on the basis of a dose-response curve, data not shown) for 24 hours in the presence of 10 mg/mL of monensin. Then, live cells were detected with viability dye (BioLegend) for 15 min, then we washed cells with staining buffer, and stained them with anti-human CD3 (REA613), anti-human CD8 (REA734), anti-human CD4 (REA623), anti-human CD14 (REA599), anti-human CD19 (REA675), anti-human IgD (REA740), anti-human CD27 (REA499) and anti-human CD38 (REA616). After 30 min, fixed and permeabilised (eBioscience) cells were stained with intracellular antibodies: anti-human IL-17 (REA1063), anti-human tumour necrosis factor (TNF)- α (REA656), anti-human interferon (IFN)- γ (REA600) and relative isotype-matched control mAbs. Finally, cells were acquired on the FACSaria flow cytometer.

To improve the number of acquired PBMCs, samples were pooled (12 pool experiments for pSS samples and 9 pool experiments for nSS). Stained cells were acquired on the FACSaria flow cytometer. At least 50 000 cells (events), gated on lymphocytes region, were acquired for each sample. FCS (Flow Cytometry Standard) files from FACSaria flow cytometer, were analysed using FlowJo. Additionally, IL-40 expression was calculated using geometric mean values, while cytokines production after rIL-40 stimulation was evaluated as percentage.

ELISA

IL-40 was also detected in sera samples of patients and controls using the human uncharacterised protein C17orf99 ELISA KIT (cat. MBS9337680). All steps were performed following the manufacturer's instruction and colour intensity was measured with a spectrophotometer at 450 nm wavelength.

The OD (Optical Density) value of samples and standards from the spectrophotometer were corrected by subtracting blank average OD and the concentration of cytokine was calculated based on the standard curve.

Gene sets analysis

We subjected messenger RNA (mRNA) microarray of PBMC to a total of 60 sample patients with pSS (n=30) and healthy controls (HCs) (n=30) who did not suffer from

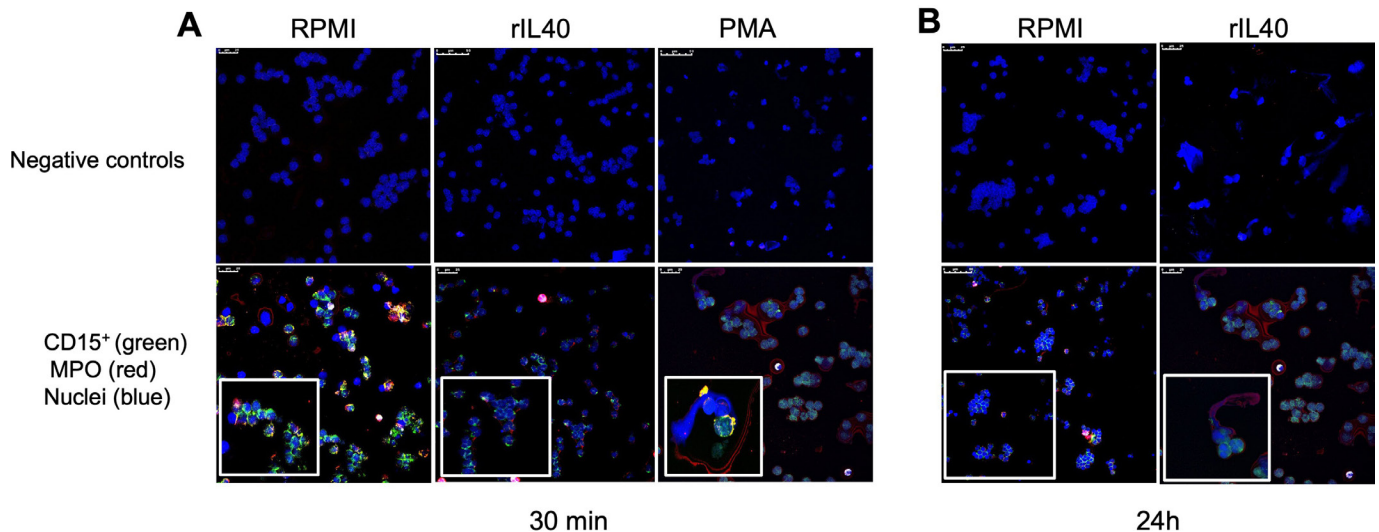


Figure 5 rIL-40 stimulates NETosis in pSS ex vivo NET formation in pSS (FS3). (A) 30 min stimulation. Non-lytic NETosis observed with both RPMI and rIL-40 in vitro stimulation; classic lytic NETosis induced with PMA. (B) 24 hours stimulation with RPMI and r-IL-40. Non-lytic NETosis observed in the absence of any stimulation and classic lytic NETosis found after stimulation with rIL-40. CD15⁺ are stained in green, MPO in red and nuclei are counterstained by Hoechst (blue); to assess NETosis neutrophils were obtained from peripheral blood samples, experiments were repeated three times; negative control staining are shown in the upper part of the figure. MPO, myeloperoxidase; NET, neutrophil extracellular trap; nSS, non- Sjögren syndrome; PMA, phorbol myristate acetate; pSS, primary Sjögren syndrome; RPMI, Roswell Park Memorial Institute medium; rIL-40, recombinant IL-40.

autoimmune diseases or were not receiving any drugs (GSE84844). We also used mRNA microarray of sorted B cell (GSE135809) for a total of patients with pSS (n=6) and HCs (n=6) in the discovery cohort, which was sorted into four subsets: CD38[−]IgD⁺ (Bm1), CD38⁺IgD⁺ (naive B cells), CD38^{high}IgD⁺ (pre-GC B cells) and CD38[±]IgD[−] (memory B cells). R package biomaRt (V.2.48.2) was used for the annotation of GPL570 based on GRCh37/hg19 to cross-map gene symbol identification. Same platforms (GPL570) was considered to have homogeneous data.

Besides genome-wide RNA expression analysis, Gene Set Enrichment Analysis (GSEA) was used to interpret gene expression data that yields insights into several IL-40-related data sets. We used gene sets which contained C17orf99 from the molecular signature database. Those with an enrichment score (ES) were chosen for future investigation as the backbone of this study. Gene expression data was normalised following standard RMA background correction and quantile normalisation using the ‘affy’ package (V.3.14). Log10 normalised gene expression matrix from different study levels was then used for GSEA analysis using GSEA V.4.1.0 software.¹⁸ Association between overlapping genes in two gene sets was predicted using Molecule-Protein interaction modelling through Cytoscape (3.9.1) and string plugin, radial layout (1.7.0). The output of Molecule-Protein interaction modelling has been used for Gene Ontology (GO) and pathway study run by GO Database and pantherDB.

Statistical analysis

Statistical analysis of quantitative variables was performed using the Mann-Whitney rank-sum test and analysis of variance. 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 molecules of interest. P values <0.05 were considered statistically significant. GraphPad Prism V.9 (GraphPad, San Diego, California, USA) software was used to analyse data.

RESULTS

IL-40 is overexpressed in the SGs of patients with pSS and of pSS-associated lymphoma

Significantly higher IL-40 mRNA levels were found in patients with pSS when compared with those of nSS, with a significant increase according to the degree of the inflammatory infiltrate (FS0 vs FS3–4, difference between mean 0.00022±3.71, $p<0.01$; FS1–2 vs FS3–4, difference between mean 0.00021±2.4820.12, $p<0.0001$) (figure 1A) and with a positive strong correlation between the IL-40 mRNA levels and the FS ($r^2=0.65$, $p<0.001$) (figure 1B).

Low levels of IL-40 expression were detected by IHC in all patients with nSS (figure 1C). Conversely, pSS showed stronger expression among lymphocytic infiltrates and ductal epithelial cells. The typical distribution of IL-40 in pSS is shown in figure 1C. Given the role of IL-4 and TGF- β as upstream cytokines of IL-40, we also evaluated the expression of IL-4 and TGF- β in pSS SG tissue. The expression of both cytokines was demonstrated in the cell infiltrate and among epithelial cells in MSG, confirming their presence in pSS as pivotal cytokines in driving inflammatory changes.^{14 19 20} A correlation assay between IL-4, TGF- β and IL-40 production was performed and shown in figure 1D (TGF- β /IL-40, $r^2=0.1576$; IL-4/IL-40,

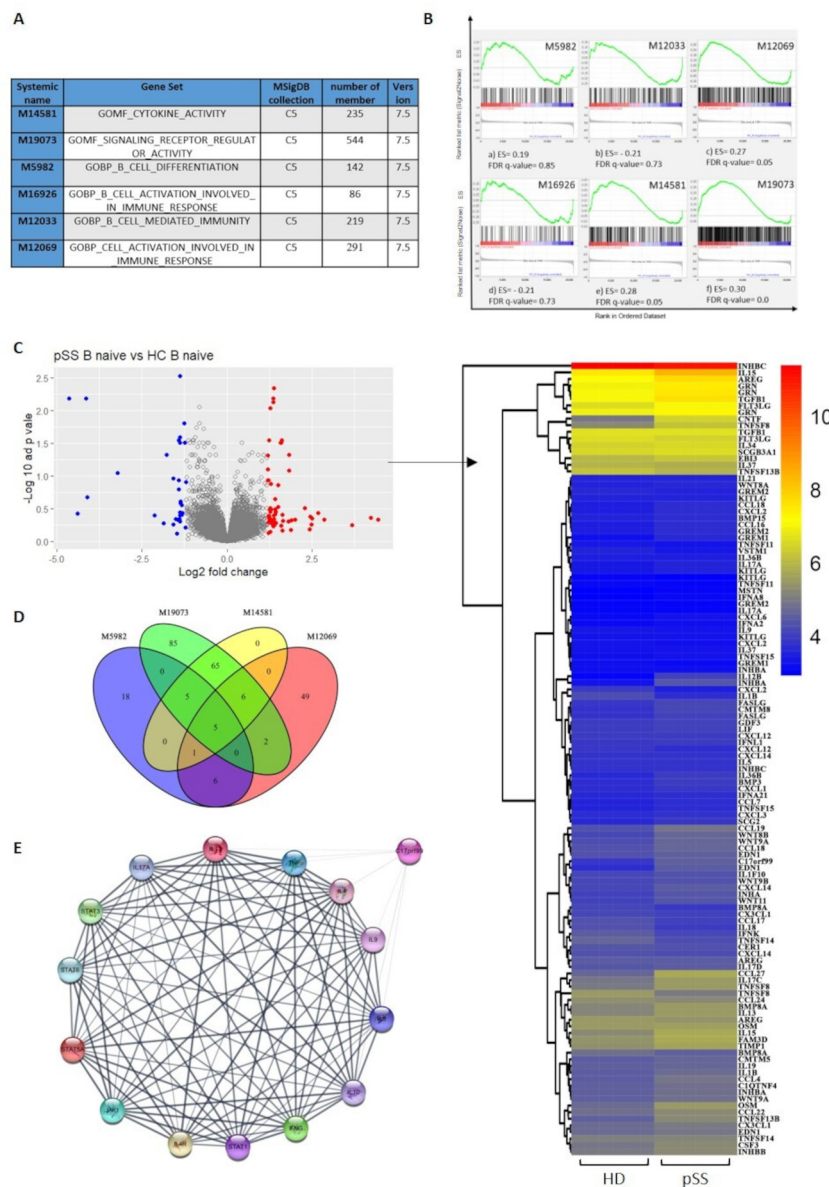


Figure 6 Gene expression Gene Set Enrichment Analysis revealed a potential network between C17orf99 and key cytokines in pSS subjects. (A) Table showing the six genes sets selected for deep analysis in sorted B cells. (B) Six gene sets were investigated in naïve B cells. ES in four gene sets (a, c, e, f) were found to be positive in pSS compared with HC. (C) Gene expression analysis of pSS versus HC showed only 92 genes with significant fold change (adj p value<0.01, 33 genes with negative FC and 59 genes with positive FC but heatmap and gene expression correlation showed signs of a gene expression change in pSS versus HC. (D) DEG analysis of the mentioned four gene sets showed the highest overlap of mutual genes only between two gene sets. (E) String network based on overlapped genes between M19073 and M14581 showed a network of C17orf99 and IL-4, IL-5, IL-9, IL-13, IL-17A, tumour necrosis factor and IL-10. M5982: B-cell differentiation; M19073: signalling receptor regulator activity; M14581: cytokine activity; M12069: cell activation involved in the immune response. DEG, differentially expressed genes; ES, enrichment score; FC, fold change; FDR, false discovery rate; HC, healthy controls; HD, healthy donors; IL, interleukin; pSS, primary Sjögren syndrome.

$r^2=0.0445$). Negative control staining and staining in nSS specimens for both IL-4 and TGF- β are shown in online supplemental figure 1.

Finally, we also carried out an IHC assay on SG tissue obtained from pSS-associated lymphoma (n=5) to assess the expression of IL-40. An intense staining for IL-40 was highlighted in lymphomatous specimens (figure 1E), suggesting a potential role of IL-40 also in pSS-associated lymphoma.

B cells are the major source of IL-40 in pSS SG and among PBMCs

The cellular source of IL-40 among the different infiltrating populations was then analysed by flow cytometry on cells isolated from the SG of pSS and nSS.

Our cytometric analysis confirmed CD19⁺ B cells as the major source of IL-40 in the lymphocytic-infiltrated MSG of patients with pSS compared with nSS (2148±371.7 vs 650.7±25.8, p<0.05) (figure 2A). CD3⁺ T cells were also

able to produce a slight amount of IL-40 but without any significant difference between patients and controls (4.2 ± 5.6 vs 2.6 ± 4.6 , $p>0.05$). An unremarkable IL-40 production from tissue resident CD68⁺ macrophages was detected (2090 ± 4021 vs 1622 ± 2809 , $p>0.05$) (figure 2A).

Double-IF staining confirmed the cytometric data, showing co-localisation of IL-40 with CD19 (B cells) (Pearson's coefficient: $r=0.675$; Manders' coefficients: $M1=0.72$, $M2=0.324$) and, to a lesser extent, CD3 (T cells) (Pearson's coefficient: $r=0.445$; Manders' coefficients: $M1=0.358$, $M2=0.359$) (figure 2B). IF staining on lymphoma biopsies showed that both CD19⁺ (Pearson's coefficient: $r=0.716$; Manders' coefficients: $M1=0.594$, $M2=0.648$) and CD19⁻ cells produced IL-40 (figure 2C). A further characterisation of CD19⁺/IL40⁺ cells in lymphoma sections, using the same markers evaluated in pSS, showed that those cells were mainly CD3⁺ and CD68⁺ cells, thus characterising other non-B IL-40⁺ cells (figure 2C).

Cytometric analysis of PBMCs confirmed at systemic level the expansion of CD19⁺IL-40⁺ B cells in pSS versus controls (12.4 ± 4.5 vs 4.2 ± 4.1 , $p<0.05$) whereas the percentages of CD3⁺IL-40⁺ (3.3 ± 14 vs 2.5 ± 4.4 , $p>0.05$) and CD14⁺IL-40⁺ (53.8 ± 114.9 vs 26.8 ± 32.2 , $p>0.05$) was not significantly different between patients and controls (figure 3A).

Serum levels of IL-40 are correlated with EULAR Sjögren's Syndrome Disease Activity Index in pSS

Serum levels of IL-40 were significantly increased in patients with pSS compared with nSS (5.7 ($13.8-2.1$) vs 2.9 ($4.5-1.2$) ng/mL; $p<0.05$) as demonstrated by ELISA (figure 3B). No correlation between FS and IL-40 serum levels was detected ($r^2=0.0001$, $p>0.9$). Conversely, serum levels of IL-40 in patients with pSS significantly correlated with the disease activity score EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) ($r^2=0.82$; $p<0.001$) (figure 3C). A positive correlation between IL-40 levels and both anti-SSA (Sjögren Syndrome related antigen A) and Ig levels was found, as shown in online supplemental figure 2.

rIL-40 stimulates the expression of proinflammatory cytokines in pSS peripheral T cells

rIL-40 was used to stimulate PBMCs obtained from patients with pSS and controls to reveal a possible role of IL-40 in the inflammatory process related to pSS. To this purpose, the concentration of a panel of cytokines, which play an established role in pSS pathogenesis, namely TNF- α , IFN- γ and IL-17, was assessed at day 0 and after 24 hours of incubation with rIL-40.

After stimulation, both CD4⁺ and CD8⁺ T cells from patients with pSS significantly increased the production of TNF- α (pSS CD4⁺TNF- α ⁺ 0.12 ± 0.05 vs nSS CD4⁺TNF- α ⁺ 0.03 ± 0.03 , $p<0.05$; pSS CD8⁺TNF- α ⁺ 0.52 ± 0.72 vs nSS CD8⁺TNF- α ⁺ 0.04 ± 0.03 , $p<0.05$). IL-17 production was significantly different between pSS and controls only in T CD8⁺ (pSS CD4⁺IL-17⁺ 0.03 ± 0.04 vs nSS CD4⁺

IL-17⁺ 0.0 ± 0.0 , $p>0.05$; pSS CD8⁺IL-17⁺ 0.29 ± 0.25 vs nSS CD8⁺IL-17⁺ 0.04 ± 0.02 , $p<0.05$). On the other hand, IFN- γ was undetectable in the absence of any stimulation but increased after rIL-40 incubation only in T-CD8⁺ without any significant difference between pSS and controls (figure 4A, B and D). Differently from T cells, monocyte cytokine production was not affected by rIL-40 stimulation. Altogether our results suggest that IL-40 may activate T cells driving proinflammatory cytokines' production.

rIL-40 increased IFN- γ production from CD19⁺ B cells in pSS

A significant increase in IFN- γ production among pSS CD19⁺ B cells versus controls was observed after stimulation with rIL-40 (0.33 ± 0.21 vs 0.01 ± 0.01 , $p<0.05$) (figure 4C and D).

The effect of rIL-40 on B cells polarisation was assessed by analysing its impact on different CD19⁺ B cells subtypes, namely naïve (CD27⁻ IgD⁺), switched memory (CD27⁺ IgD⁻), unswitched memory (CD27⁺ IgD⁺) and double negative (CD27⁻ IgD⁻) subsets. No differences in B cells subpopulation frequency between patients and controls were detected after rIL-40 incubation (data not shown). Moreover, we have also assessed the effects of rIL40 on the frequency of plasma cells (CD27⁺CD38⁺), and we did not find any statistically significant difference between pSS and nSS (online supplemental figure 3).

rIL-40 stimulates NETosis in pSS

Recently, a role of IL-40 in modulating NETosis has been described in RA (Rheumatoid Arthritis). Similarly to RA, we observed that, in vitro, IL-40 strongly induces NETosis in pSS after 24 hours (figure 5). Since the increased expression of IL-40 in pSS, we performed IF imaging study to confirm the occurrence of NETosis. We observed that two different forms of this biological process were found in pSS: in the absence of any stimulation or with rIL-40, non-lytic NETosis was found after 30 min while, after stimulation with PMA, the classic lytic form was evidenced (figure 5A). The main difference between lytic and non-lytic NETosis resides in the typical suicidal death occurring in the lytic form, that is, characterised by chromatin decondensation, nuclear swelling, spilling of the nucleoplasm into the cytoplasm and finally membrane perforation with the formation of a web-like structure composed by nucleic acid and granule proteins.²¹ In the non-lytic NETosis, also known as vital NETosis, neutrophils survive being able to release NETs without requiring membrane perforation.²² In nSS no NETosis was observed with or without rIL-40 stimulation (online supplemental figure 4). Our observation underlines that a specific form of neutrophil activity is constantly present in pSS and it is possibly sustained by IL-40.

GSEA reveals a network between C17orf99 and key cytokines in B naïve cells from patients with pSS

To determine which stage of B-cell development could potentially be involved in IL-40 production, we investigated gene set enrichment and network analysis on

different B-cell subsets. Our gene expression analysis between different B-cell subsets failed to detect any C17orf99 significant fold change between patients with pSS and controls. To overcome analytical challenges related to differentially expressed single gene, we performed a GSEA analysis to find gene sets containing C17orf99 with significant ESs. We first selected 19 different gene sets at PBMCs levels which included an enriched core containing C17orf99 (online supplemental table 1). Between 19 gene sets, 6 gene sets had a significantly enriched core containing C17orf99 (ES>0.2, false discovery rate (FDR) q value<0.05) in sorted B cells containing four different B-cell subpopulations (Bm1, PreG B, memory and naïve) (figure 6A). Considering our in vitro results that showed B cells as the main source of IL-40, our GSEA failed to detect any statistically significant ES in the pre-GC, Bm1 and B memory cells of pSS compared with HCs. Interestingly, two gene sets known as 'signaling receptor regulator activity' (FDR q value=0.0, ES=0.30), and 'cytokine activity' (FDR q value=0.051, ES=0.28) were found to be significantly enriched in naïve B cells of patients with pSS (figure 6B), where the pSS hallmark genes expression was not changed significantly (figure 6C). This result illustrated a clue to the existence of a network between C17orf99 and other proteins that are changing during B-cell development in patients with pSS, even if the hallmark gene expression of B cells was not changed significantly.

For finding the network, we focused on the overlap genes between those two gene sets that were highly enriched in naïve B cells of patients with pSS, since those overlapped genes might be involved in a Molecule-Protein network (figure 6D). To address this possibility, we performed a network analysis, and our findings provided first-ever evidence of a possible network between C17orf99 and IL-4, IL-17A, TNF, IL-5, IL-9, IL-13 and IL-10 in B naïve cells from patients with pSS (figure 6E).

DISCUSSION

In this study we demonstrated for the first time that IL-40 expression is increased in pSS SG tissue and peripheral blood, possibly playing a proinflammatory role by polarising proinflammatory cytokine production by B and T lymphocytes.

IL-40, mainly driven by IL-4 and TGF- β 1, was originally described by Catalan-Dibene in 2017 as a cytokine implied in immune response and B-cell homeostasis.⁸ The first link to autoimmune disease dates back to 2012 when the presence of C17orf99 gene was described as a marker of autoimmune hepatitis.²³ Recently, IL-40 was found upregulated in RA, suggesting a role for this molecule in B-cell driven rheumatic diseases.²⁴ As found in RA synovium, IL-40 was strongly upregulated in pSS among lymphocytic infiltrates and ductal epithelial cells and significantly correlated with the FS. Specifically, 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.²⁵ Particularly, IL-40 production from epithelial cells could contribute to the modulation of B-cell functions.

Moreover, IL-4 and TGF- β , cytokines also involved in the development of pSS,^{18–20} were significantly overexpressed in pSS SG, mainly among epithelial cells and directly correlated with SG IL-40 expression. Since the concomitant correlated presence of IL-40, these data might suggest that IL-4 and TGF- β might participate in pSS pathogenesis by modulating IL-40 production. The SG cytokines microenvironment in pSS reflects a complex cellular networking involving both T and B cells that we found to be both a tissue source of IL-40, being however B cells the most relevant IL-40-producing cells. Interestingly, significantly enriched 'signaling receptor regulator activity' and 'cytokine activity' were found in naïve B cells of patients with pSS by in silico analysis that identified several mutual genes overlap with C17orf99. Such overlap revealed a network between C17orf99 and IL-4, IL-5, IL-13, IL-9, IL-10, IL-17A and TNF.

Differentially from other reports, we did not find any significant IL-40 expression by macrophages.

In addition to B and T cells, a further important role of IL-40 could be related to the modulation of neutrophil function within the SG of pSS. In fact, IL-40 was already related to NETosis enhancement in RA²⁴ but, up to date, despite an increase in neutrophils frequency was depicted in pSS, a complete assessment of neutrophils functions is not available.²⁶ The presence of both non-lytic and lytic NETosis was previously described in systemic lupus erythematosus, a systemic disease that shares common pathways with pSS.²⁷ On this literature basis, we performed some preliminary experiments showing an increase in these two forms of NETosis in pSS. We demonstrated an increase in NETosis in pSS SG and a role of IL-40 in activating this biological process and therefore in orchestrating the interaction between adaptive and innate immunity in the development of pSS.

In our study, however, according to the systemic inflammation occurring in pSS, IL-40 did not appear to be a cytokine limited only to the SG of patients with pSS. The increased IL-40 serum levels we found in patients with pSS compared with controls and the significant correlation to pSS disease activity assessed through the ESSDAI, seem to point towards a systemic role of this cytokine in patients with pSS. To further study this systemic role, we checked for the influence of IL-40 on PBMC cytokine production by stimulating PBMCs with rIL-40. Interestingly, in patients with pSS we demonstrated an IL-40-dependent significant increase in both TNF- α and IL-17 from T-CD8⁺; while in T-CD4⁺ we showed only an increased production of TNF- α . Particularly, the activation of T-CD8⁺ mediated by IL-40 can contribute to

their overactivation and abnormal proliferation in pSS which mediates their tissue cytotoxic activity in killing or inducing apoptosis of glandular epithelial cells.²⁸ Moreover, literature dates have pointed out how T-CD8⁺ cells under particular conditions, as described in autoimmune disease,²⁹ acquire the expression of several ILs, including IL-17 and may support Th17 pathogenicity.³⁰

In contrast with what was expected considering pSS pathogenesis, we did not detect a significant increase in IFN- γ from T cells. Conversely, B cells stimulated with rIL-40 markedly increased the production of IFN- γ . These data indicate a strong modulating action of innate and adaptive immunity by IL-40 in patients with pSS and raise the possibility that IL-40 may be considered a biomarker of disease activity in patients with pSS, although further studies are required to confirm this possibility. Therefore, the higher responsiveness of PBMCs from pSS to rIL40 compared with nSS could be related to an activated state of pSS derived cells that come from a proinflammatory microenvironment. So, we can speculate that these cells may express higher levels of IL-40 receptor being more responsive to rIL-40. Our hypothesis needs to be further elucidated through the identification of the downstream pathway related to IL-40 signalling.

IL-40 may play a role even in the development of SG lymphoma that is intimately related to B-cell aberrant hyperactivation.

In this regard, we evidenced a strong staining for IL-40 in parotid MALT associated with pSS. IF analysis demonstrated that B cell, together with other cell types produce IL-40. Previous report in cell lines highlighted the constitutive production of IL-40 from lymphomatous B cells and a differential expression of its gene in different subtypes of lymphomas,^{9 31 32} suggesting its role in promoting B-cell lymphomatous escape in pSS. In addition, consistently with our genes and networking analysis the IL-4/STATs pathway, predictably sustained by IL-40 production from lymphomatous naïve B cells, may be a key contributor to cancer development.

In the era of precision medicine, we suggest that IL-40 can be considered as a possible new marker to assess pSS disease activity and predict the evolution towards MALT.

Some limitations of the present study should be underlined. Up to date, there is no data available on the receptor and the molecular pathways activated by IL-40. The activity of rIL-40 was assessed on PBMCs and not at tissue level, where a specific pro-inflammatory microenvironment is present, so our results only partially mirror what happens at tissue level. Furthermore, we observed a correlation between IL-40 level and FS grading but not with the presence of GCs, that could better reflect local immune system activation and autoantibody production. Regarding lymphoma, we could not assess the serum expression levels of IL-40 before NHL development, as we obtained samples from our tissue biobank. It would be extremely interesting to follow-up patients with pSS and monitor IL-40 prospectively, to identify IL-40 as a potential predictor of MALT occurrence. Similarly, we

could not assess the presence of IL-40 in non-lymphoma parotid specimens, as they are not easily available, to compare the expression of IL-40 between lymphomatous and healthy tissue.

Despite such limitations, our research points out that IL-40 is implicated in pSS pathogenesis and its expression may contribute to shaping tissue inflammation, autoimmunity and possibly lymphoma development. Further research on larger cohorts is needed to clarify the role of this cytokine in pSS.

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