Interleukin-25 Axis Is Involved in the Pathogenesis of Human Primary and Experimental Murine Sjögren’s Syndrome

Giuliana Guggino,1 Xiang Lin,2 Aroldo Rizzo,3 Fan Xiao,2 Laura Saieva,1 Stefania Raimondo,1 Diana Di Liberto,1 Giuseppina Candore,1 Piero Ruscitti,4 Paola Cipriani,4 Roberto Giacomelli,4 Francesco Dieli,1 Riccardo Alessandro,1 Giovanni Triolo,1 Liwei Lu,2 and Francesco Ciccia1

Objective. To investigate the role of the interleukin-25 (IL-25)/IL-17 receptor B (IL-17RB) axis in experimental Sjögren’s syndrome (SS) and in patients with primary SS and primary SS–associated lymphoma.

Methods. Expression of IL-25, IL-17RB, IL-17B, and tumor necrosis factor receptor–associated factor 6 (TRAF6) was analyzed on minor salivary gland (SG) samples from patients with primary SS and on parotid gland samples from patients with primary SS–associated B cell non-Hodgkin’s lymphoma (NHL). IL-17RB expression and the frequencies of natural group 2 innate lymphoid cells (ILC2s), inflammatory ILC2s, and M2-polarized macrophages were assessed by flow cytometry in SG mononuclear cells and peripheral blood mononuclear cells (PBMCs). Tissue distribution of ILC2s was studied by confocal microscopy. The role of recombinant IL-25 and of rituximab in modulating IL-25 expression was investigated in in vitro studies. IL-25/IL-17RB and TRAF6 expression and the role of IL-25 inhibition were also studied in the experimental murine model of SS.

Results. Activation of the IL-25/IL-17RB/TRAF6 axis correlated with the focus score and was observed in patients with primary SS and in patients with primary SS–associated NHL. A significant increase in the frequency of inflammatory ILC2s was observed both in SG mononuclear cells and in PBMCs. IL-25 stimulation of isolated SG mononuclear cells and PBMCs from patients and controls resulted both in inflammatory ILC2 expansion and in increased autoantibody production. Rituximab modulated expression of inflammatory ILC2s and IL-25 in primary SS. SG protein–immunized mice developed overt SS symptoms with increased IL-25 expression and increased frequency of CD4+IL-17RB+TRAF6+ cells. IL-25 neutralization attenuated disease progression and tissue pathology in mice with experimental SS.

Conclusion. IL-25 may promote the inflammatory state in primary SS and may be a potential target for novel disease-modifying therapeutic strategies in patients with primary SS.

Primary Sjögren’s syndrome (SS) is a chronic systemic autoimmune disease characterized by an increased risk of developing lymphomas (1). Primary SS is thought to be essentially driven by a complex interplay between epithelial barrier and adaptive and innate immunity (2). In particular, type 2 adaptive immune responses have been shown in primary SS, mainly dominated by the selective concentration of Th2 cells in the context of germinal centers (GCs) and the increased expression of interleukin-33 (IL-33) in salivary glands (SGs) (3).

Recently, IL-25, a member of the IL-17 cytokine family also known as IL-17E, has been implicated in the regulation of innate and adaptive immune responses, essentially by inducing a type 2 immunity (4); as IL-17E, IL-25 signals through IL-17 receptor B (IL-17RB) expressed on epithelial and immune cells, modulating several activities (5). Specifically, IL-25 regulates the development of autoimmune inflammation mediated by IL-17–producing T cells.
(6). Furthermore, IL-25/IL-17RB activation promotes Th2 responses by increasing the frequency of M2-type macrophages (7) and the differentiation of both natural and inflammatory group 2 innate lymphoid cells (ILC2s) (8,9). Despite these pivotal functions in modulating immune responses, the roles of the IL-25/IL-17RB axis and of ILC2s in the pathogenesis of experimental SS and primary SS, as well as in primary SS-associated lymphoma, have not yet been investigated.

In this study, we demonstrated that the IL-25/IL-17RB axis is activated in experimental SS, primary SS, and primary SS-associated lymphoma and that this is associated, in humans, with the increased frequency of IL-25–responsive inflammatory ILC2s and M2-type macrophages together with an increased production of primary SS–related autoantibodies. Our findings suggest that by interacting locally and systemically with IL-17RB+ cells, IL-25 may promote innate and adaptive immune responses, thus modulating the inflammatory state in primary SS.

**PATIENTS AND METHODS**

*Procedures in experimental SS.* Experimental SS was induced in 8-week-old female C57BL/6 mice by immunization with SG proteins as we have previously described (10). Briefly, proteins extracted from the bilateral SGs of normal mice were emulsified in an equal volume of Freund’s incomplete adjuvant (Sigma-Aldrich) to a concentration of 2 mg/ml. For SS induction, each mouse received multiple subcutaneous injections in the back of 0.1 ml of the emulsion on days 0 and 7. On day 14, a booster injection of 1 mg/ml SG proteins emulsified in Freund’s complete adjuvant (Sigma-Aldrich) was administered. All experiments for animal studies were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.

At various time points in the course of disease, serum samples from mice with experimental SS were collected and enzyme-linked immunosorbent assay kits were used to measure IL-25 (eBioscience), IL-5 (BioLegend), and IL-13 (R&D Systems) in accordance with the manufacturers’ instructions. Since increased IL-25 levels were detectable only at the chronic stage of disease, ~30 weeks postimmunization, mice with experimental SS were injected intraperitoneally with 200 μg of anti-IL-25 monoclonal antibody (35B; BioLegend) or vehicle (phosphate buffered saline [PBS]) 20 weeks postimmunization, before disease entered the chronic stage. Mice were treated twice every week for 4 weeks. The saliva flow rate of mice with experimental SS was assessed before and after anti-IL-25 treatment. SG tissues removed from immunized mice were frozen in OCT compound (Sakura), and sections were cut at 5 μm thickness. For immunofluorescence microscopy, frozen sections were stained with monoclonal antibodies against mouse CD4 (clone RM4-5; BioLegend) and IL-25 (clone 207702; R&D Systems), while nuclei were counterstained with Hoechst 33258 (Calbiochem). Rat IgG antibody was used for control staining.

For the flow cytometry experiments in mice with experimental SS, surface markers were identified with the following anti-mouse monoclonal antibodies: anti-CD4 (clone GK1.5; BioLegend), anti-IL-17RB (clone 752101; R&D Systems), anti-CD45 (30-F11; BioLegend), anti-F4/80 (BM8; BioLegend), anti-IL-7Rα (ATP34; BioLegend), anti-CD206 (C668C2; BioLegend), and antibodies against the lineage markers CD2, CD3, CD4, CD8, CD19, B220, Gr-1 protein, CD11b, CD11c, Fcε receptor type I, and Ter-119 (all from BioLegend). Detection of intracellular IL-25 (R&D Systems) and tumor necrosis factor receptor–associated factor 6 (TRA6) (clone EP591Y; Abcam) was performed using a fixation/permeabilization buffer set (BD Biosciences). Stained cells were analyzed with an LSRFortessa flow cytometer (BD Biosciences), while a Zombie Aqua Live/Dead Cell Discrimination kit (BioLegend) was used to exclude the dead cells. Data were analyzed with FlowJo software (Tree Star).

*Patients.* Peripheral blood samples and labial minor salivary gland (MSG) biopsy samples were obtained from 50 patients (46 women; mean ± SD age 58 ± 12 years, mean ± SD disease duration 34 ± 12 months) who had xerostomia and xerophthalmia and who met the American–European Consensus Group criteria for SS (11). All enrolled patients were negative for hepatitis C virus. Thirty patients reporting dry mouth or dry eyes who did not fulfill the American–European Consensus Group criteria and who showed various degrees of mononuclear cell infiltration in the absence of focal organization were classified as having nonspecific chronic sialadenitis and were considered a control group. Paraffin-embedded samples obtained from 5 patients previously diagnosed as having primary SS–associated mucosa-associated lymphoid tissue (MALT) lymphoma were obtained from the biopsy bank of the Pathology Unit of the Ospedali riuniti Villa Sofia-Cervello. Lymphoma was diagnosed by the demonstration of sheets or halos of mononuclear cells and expression of IgH and/or IgL chain restriction.

Baseline characteristics of the patients and controls are shown in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract. All patients and controls provided informed consent, and the study was approved by the Ethics Committee of the University of Palermo. Multiple labial MSG biopsy samples were obtained from all patients with primary SS and controls and placed into formalin fixative and RNAlater solution (Applied Biosystems) for immunohistochemistry and reverse transcription–polymerase chain reaction (RT-PCR) analyses, respectively. Twenty paired biopsy samples from patients and controls were also placed in RPMI 1640 (Invitrogen Life Technologies) for isolation of SG mononuclear cells and used for flow cytometry analysis.

**Histology and immunohistochemistry**. Paraffin-embedded sections of 5 μm thickness were stained with hematoxylin and eosin for histologic evaluation of the presence of lymphocytic infiltrates, performed as recommended by Greenspan and colleagues (12). A focus was defined as an aggregate of ≥50 lymphocytes. The focus score was reported as the number of foci per 4 mm² of tissue, up to a maximum of 12 foci. All patients with primary SS had a biopsy focus score of ≥1, while controls had a focus score of <1. The presence of GC-like lymphoid structures was determined by the presence of T and B lymphocytes and CD21+ follicular dendritic cell networks on sequentially stained sections. Immunohistochemistry was performed on 5 μm–thick paraffin-embedded sections from SGs as described previously (13). The primary antibodies mouse anti-human IL-25, rabbit anti-human IL-17RB, rabbit anti-human IL-17B (all from Novus Biologicals), and rabbit anti-human TRAF6 (Abcam) were added and incubated for 1
hour at room temperature. Isotype-matched irrelevant antibodies (Abcam) were used as a negative control. The number of positive cells was determined by counting the reactive cells on photomicrographs obtained from 3 randomly selected high-power fields (original magnification × 400).

Confocal microscopy analysis. Triple stainings were performed on paraffin-embedded sections of MSGs for CD3/Thy-1/IL-17RB (for IL2Cs) and CD68/CD163c-Maf (for M2-type macrophages). The sections were treated with fluorescein isothiocyanate−, rhodamine red−, or Cy5-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen) plus RNasin (200 ng/ml) and counterstained using DAPI (Life Technologies). Confocal analysis was used to acquire fluorescence staining.

RNA extraction from SG biopsy samples and quantitative TaqMan RT-PCR. Soon after removal, SG biopsy samples were also stored in RNAlater solution. RT-PCR was performed as described previously (13). Master Mix and TaqMan gene expression assays for GAPDH control (Hs02758991_g1) and the target genes for IL-25 (Hs00344841_m1), IL-17B (Hs00975262_m1), IL-33 (Hs00369211_m1), and arginase 1 (Hs00978791_m1) were obtained from Applied Biosystems. Data were quantified using SDS software version 2.1 and normalized using GAPDH as endogenous control. Relative changes in gene expression between samples from patients with nonspecific chronic sialadenitis and those from patients with primary SS were determined using the ΔΔCt method. Levels of the target transcript were normalized to a GAPDH endogenous control, constantly expressed in both groups (ΔCt). For ΔΔCt values, additional subtractions were performed between ΔCt values for 50 patients with primary SS and ΔCt values for 20 patients with nonspecific chronic sialadenitis. Final values were expressed as fold induction.

Isolation and culture of MSG mononuclear cells and flow cytometry. MSG mononuclear cells were obtained as described previously (13) from 20 patients with primary SS and 20 patients with nonspecific chronic sialadenitis. Cell viability (by trypan blue dye exclusion) was always >95%. Cells were cultured with 0.25 μg/ml recombinant IL-25 (R&D Systems) for 24 hours at 37°C in the presence of 5% CO2. In vitro-cultured cells were stained with the following antibodies: anti-human CD45 (Becton Dickinson), anti-human Lineage Cocktail (anti-human CD3, anti-human CD56, anti-human CD14, anti-human CD19, anti-human T cell receptor γδ, anti-human invariant natural killer T cell (Bio-Legend), anti-human IL-17RB (R&D Systems), anti-human killer cell lectin-like receptor subfamily G member 1 (KLR-G1; BioLegend), anti-human Thy-1 (BioLegend), anti-human GATA-3 (BioLegend), anti-human chemokine receptor-like molecule expressed on Th2 cells (CRT2H2; BD Biosciences), and anti-human CD68 (R&D Systems). Flow cytometry analysis was performed using a FACScanto (BD Biosciences). At least 50,000 cells (events), gated on the lymphocyte region, were acquired for each sample, and data are presented as a percentage of total live cells in the CD45 channel.

Evaluation of anti-Ro/SSA and anti-La/SSB autoantibodies following IL-25 stimulation. The role of IL-25 in modulating the production of anti-Ro/SSA and anti-La/SSB autoantibodies was evaluated as previously described (14). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from anti-Ro/SSA-positive patients with primary SS by density-
gradient centrifugation using Ficoll-Hypaque (Pharmacia). The medium used throughout was RPMI 1640 supplemented with 10% heat-inactivated pooled human AB+ serum, 2 mM l-glutamine, 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 × 10⁻³ M 2-mercaptoethanol. PBMCs were isolated and either left untreated or cocultured with 0.25 μg/ml rIL-25 in U-bottomed 96-well plates for 72 hours at 37°C in the presence of 5% CO₂. After incubation, supernatants were collected to test for production of anti-Ro/SSA and anti-La/SSB by immunoblot (Euroimmune).

Statistical analysis. Parametric and nonparametric statistical analysis was performed calculating the mean ± SEM and median, respectively. For comparison of parametric and nonparametric data, the t-test and Mann-Whitney rank sum test, respectively, were used, as appropriate. Spearman’s correlation analysis was used to quantify associations of gene expression with types of disease. P values less than 0.05 were considered significant. For experiments in mice with experimental SS, results are expressed as the mean ± SD. Data were analyzed using a Mann-Whitney U test or one-way analysis of variance to determine the difference between groups, using SPSS software version 16.0.

RESULTS

Activation of the IL-25 axis in experimental SS. To investigate the pathogenic role of IL-25 during experimental SS development, we first measured serum levels of IL-25 in mice with experimental SS at various time points after disease induction. Interestingly, serum levels of IL-25 were significantly increased 30 weeks postimmunization (Figure 1A), and were found to be further elevated during disease progression. To detect IL-25 production in SGs, harvested SGs were digested for single-cell suspension, followed by stimulation with phorbol myristate acetate (50 ng/ml), ionomycin (1 μg/ml), and monensin for 4 hours before flow cytometric analysis. A significant increase of IL-25-producing CD45⁻ tissue cells was observed in mice with experimental SS with lymphocytic infiltration in SGs compared to mice with experimental SS without glandular infiltration and compared to normal controls (Figure 1B). We further examined the IL-25-expressing cells in situ by

Figure 2. Levels of interleukin-25 (IL-25), IL-17 receptor B (IL-17RB), and IL-17B in salivary glands (SGs) from patients with primary Sjögren’s syndrome (pSS). A–C, Relative quantification of mRNA for IL-25 (A), IL-17RB (B), and IL-17B (C), assessed by quantitative reverse transcription-polymerase chain reaction in SG samples obtained from 50 patients with primary SS and 20 patients with nonspecific chronic sialadenitis (nSS; controls). Significant overexpression of IL-25 and IL-17RB, but not of IL-17B, was observed only in patients with primary SS. D–F, Representative photomicrographs showing IL-25 immunostaining in samples from patients with primary SS (D and E) and a control subject (F). G, IL-25 semiquantitative score in SG samples from patients with primary SS and controls. H–J, Representative photomicrographs showing IL-17RB immunostaining in samples from patients with primary SS (H and I) and a control subject (J). K, IL-17RB semiquantitative score in SG samples from patients with primary SS and controls. L–N, Representative photomicrographs showing IL-17B immunostaining in samples from patients with primary SS (L and M) and a control subject (N). O, IL-17B semiquantitative score in SG samples from patients with primary SS and controls. Significant overexpression of IL-25 and IL-17RB, but not of IL-17B, was detected only in patients with primary SS. In A–C, G, K, and O, symbols represent individual patients; bars show the mean. NS = not significant. Original magnification × 250. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract.
confocal microscopy. IL-25 expression was detected in tissue cells in the submandibular gland, mostly acinar and ductal cells (Figure 1C). Moreover, an increased amount of IL-25-expressing cells was observed surrounding the lymphocytic infiltration in the SGs of mice with experimental SS.

To investigate whether increased IL-25 production results in enhanced IL-17RB expression, we determined the extent of IL-17RB expression on CD4+ T cells obtained from SG lymphoid infiltrates, SG-draining cervical lymph nodes, and spleens. SG-infiltrating CD4+ T cells showed a strong up-regulation of IL-17RB, which was also observed to be significantly up-regulated in cervical lymph nodes of mice with experimental SS compared to those of naive mice (Figure 1D). Since the binding of IL-17RB to TRAF6 is critical for the signaling transduction of IL-25 (15), we further determined the intracellular expression of TRAF6. In mice with experimental SS, enhanced TRAF6 expression was detected in the IL-17RB+ subpopulation of CD4+ T cells in the infiltrates, compared to the IL-17RB− subpopulation (Figure 1E), indicating an IL-25/IL-17RB/TRAF6 axis in autoreactive T cell activation.

**Histologic findings in patients with primary SS.**

On the basis of the histologic evaluation, we identified in patients with primary SS a median biopsy focus score of 6.44 (range 1–11), while no foci were observed in patients with nonspecific chronic sialadenitis. Ectopic lymphoid structures comprising CD3+, CD20+, and CD21+ cells were observed in 17 patients (34%), while the remaining patients had diffuse lymphocytic infiltrates. All the lymphoma cases included in the study were low-grade marginal-zone B cell non-Hodgkin’s lymphomas of the MALT type, thus with lymphoepithelial lesions. The B cell phenotype of each lymphoma was confirmed using the pan-B cell anti-CD20 antibody.

**Activation of the IL-25 axis in patients with primary SS.**

To evaluate how the IL-25/IL-17RB axis relates to local pathology, MSGs obtained from patients and controls were evaluated for expression of IL-25, IL-17RB, and IL-17B. Elevated transcript levels for IL-25 (Figure 2A) were observed in MSGs from patients with primary SS compared to MSGs from controls and were accompanied by significant up-regulation of IL-17RB (Figure 2B) but not IL-17B (Figure 2C). In particular, IL-25 expression was significantly correlated with the lymphocytic focus score ($r^2 = 0.36, \ P < 0.0001$) and the presence of GCs (data not shown). To validate the RT-PCR data, expression of IL-25, IL-17RB, and IL-17B was also assessed by immunohistochemistry.

**Figure 3.** Interleukin-17 receptor B (IL-17RB) is overexpressed among peripheral blood mononuclear cells (PBMCs) and salivary gland mononuclear cells (SGMCs) from patients with primary Sjögren’s syndrome (pSS) and is accompanied by increased expression of tumor necrosis factor receptor-associated factor 6 (TRAF6). A, Representative dot plots showing gating strategy and IL-17RB-expressing cells among PBMCs and SGMCs. APC = allophycocyanin. B and C, Percentages of IL-17RB-expressing cells among PBMCs (B) and SG mononuclear cells (C) from patients with primary SS and patients with nonspecific chronic sialadenitis (nSS; controls). Values are the mean ± SEM. D–F, Representative images showing TRAF6 immunostaining in samples from a control subject (D) and patients with primary SS (E and F). G–I, Confocal microscopy analysis of IL-17RB and TRAF6 in SG samples from patients with primary SS showing perfect colocalization of IL-17RB and TRAF6. G, Single staining for IL-17RB. H, Single staining for TRAF6. I, Double staining for IL-17RB and TRAF6. Original magnification × 250. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract.
Consistent with the messenger RNA data, augmented expression of IL-25 (Figures 2D–G) and IL-17RB (Figures 2H–K) but not IL-17B (Figures 2L–O) was observed in tissue from patients with primary SS compared to control tissue. IL-25 expression was mainly observed among infiltrating mononuclear cells, epithelial cells, and high endothelial venules in close proximity to areas of lymphocytic infiltration (Figures 2D and E). MSGs were also graded histologically, and the number of IL-25+ cells was significantly correlated with the focus score ($r^2 = 0.32, P < 0.0001$) (data not shown), showing that the biopsy samples with ectopic lymphoid structures had the highest number of IL-25+ cells.

Consistent with the tissue distribution of IL-25, IL-17RB expression was largely observed in T cell–rich areas of lymphoid aggregates but not in epithelial cells (Figures 2H and I). In order to characterize IL-17RB+ cells, flow cytometry was performed on isolated PBMCs and SG mononuclear cells. As shown in Figures 3A–C, IL-17RB was expressed on the surface of CD3+, CD19+, and CD68+ cells, among SG mononuclear cells and PBMCs. To demonstrate the functional relevance of IL-25/IL-17RB interaction, we also studied the expression of TRAF6 by immunohistochemistry and confocal microscopy, since activation of TRAF6 is critical for IL-17RB signal transduction (15). TRAF6 was overexpressed in inflamed SGs of patients with primary SS (Figures 3E and F) compared to SGs of patients with non-specific chronic sialadenitis (Figure 3D), mainly among IL-17RB+ cells (Figures 3G–I). These findings suggest that selective activation of IL-17RB on effector immune cells by IL-25 is associated with MSG inflammation.

**Higher frequency of IL-25-responsive inflammatory ILC2s both in SG mononuclear cells and in PBMCs from patients with primary SS.** Since we observed increased expression of IL-25 and IL-17RB, we next evaluated the frequencies of natural and inflammatory ILC2s by flow cytometry both in SG mononuclear cells and in PBMCs. As shown in Supplementary Figure 1 (http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract), natural ILC2s, defined as Lin–CD45+CRTH2+GATA-3+IL-4+ cells, were not significantly expanded in patients with primary SS compared to controls (Supplementary Figures 1A–C). Conversely, a significantly increased frequency of

![Image](http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract)
inflammatory ILC2s, defined as Lin-(ST2–)CD45+IL-25R+KLR-G1+Thy-1+ cells, was observed both in SGs and in peripheral blood of patients with primary SS (Figures 4A–C). SG inflammatory ILC2s were significantly correlated with the focus score (Figure 4D), while circulating inflammatory ILC2s were significantly correlated with disease activity as evaluated by the European League Against Rheumatism Sjögren’s Syndrome Disease Activity Index (ESSDAI) (16) (Figure 4E). Interestingly, the percentages of inflammatory ILC2s were also significantly correlated with the percentage of IL-25–expressing cells ($r^2 = 0.32, P < 0.0001$) (data not shown).

We next used confocal microscopy to evaluate the tissue distribution of ILC2s by analyzing expression of Thy-1, IL-17RB, and CD3 in patients with primary SS and controls. Both diffuse distribution (not shown) and the presence of aggregates of Thy-1+IL-17RB+CD3+ cells (Figures 4F–I) were observed in MSGs from patients with primary SS. Since arginase 1 activity has been demonstrated to profoundly influence the ability of ILC2s to proliferate and exert proinflammatory functions (17), expression of arginase 1 was assessed in inflamed SGs from patients with primary SS and patients with nonspecific chronic sialadenitis. Arginase 1 expression was significantly up-regulated in patients with primary SS compared to patients with nonspecific chronic sialadenitis (Supplementary Figure 1D). Finally, since activated ILC2s have been demonstrated to produce high levels of IL-5 and IL-13 (18), we also evaluated their expression levels in MSGs from patients with primary SS and patients with nonspecific chronic sialadenitis. As shown in Supplementary Figures 1E and F, both IL-5 and IL-13 were significantly overexpressed in MSGs from patients with primary SS.

Expansion of M2-type macrophages in SGs, but not in peripheral blood, of patients with primary SS. IL-25 and ILC2s have been demonstrated to induce alternatively activated (M2-type) macrophages (7,8,19). Thus, we next evaluated the frequency of M2-type macrophages both in MSGs and in peripheral blood. As shown in Figure 5, a significant expansion of CD163+ macrophages was observed in SGs from patients with primary SS (Figures 5A–D). Since M2-type macrophages are better

Figure 5. M2-type macrophages in salivary gland samples from patients with primary Sjögren’s syndrome (pSS) and effects of interleukin-25 (IL-25) on in vitro expansion of inflammatory group 2 innate lymphoid cells (iILC2s) and on levels of anti-Ro/SSA antibodies in patients with primary SS. A–C, Representative photomicrographs showing CD163 immunostaining in cells from patients with primary SS (A and B) and a patient with nonspecific chronic sialadenitis (nSS; control) (C). D, Semiquantitative evaluation of CD163+ cells from patients with primary SS and controls. Symbols represent individual patients; bars show the mean. E, Single staining for CD163. F, Single staining for e-Maf. G, Double staining for CD163 and e-Maf (arrows indicate double-stained cells). Original magnification × 250. H, Representative dot plots showing the frequency of inflammatory ILC2s with or without incubation with recombinant IL-25 (rIL-25). APC = allophycocyanin; FITC = fluorescein isothiocyanate; IL-17RB = IL-17 receptor B; PE = phycoerythrin; KLR-G1 = killer cell lectin-like receptor subfamily G member 1. I, Percentage of inflammatory ILC2s after incubation of peripheral blood mononuclear cells (PBMCs) with rIL-25. J, Titers of anti-Ro/SSA antibodies in isolated PBMCs from patients with primary SS and controls before and after treatment with rIL-25. In I and J, values are the mean ± SEM of 5 different experiments. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract.
defined by the coexpression of different markers such as CD163 and c-Maf (20), double immunostaining was performed and the sections were analyzed by confocal microscopy. As shown in Figures 5E–G, CD163+c-Maf+ cells were expanded in MSGs from patients with primary SS. Interestingly, no expansion of M2-type macrophages was observed in peripheral blood from patients with primary SS compared to peripheral blood from patients with non-specific chronic sialadenitis (data not shown).

**IL-25-induced expansion of inflammatory ILC2s and production of autoantibodies in primary SS.** In order to study the effect of IL-25 on ILCs and macrophage polarization, PBMCs were isolated and either left untreated or cocultured with rIL-25. Recombinant IL-25 induced a significant expansion of inflammatory ILC2s (Figures 5H and I), but not of natural ILC2s and/or M2-type macrophages (data not shown). Since IL-25 has been found to activate signaling pathways in B cells in vitro (21), we also studied the effect of rIL-25 on the production of anti-Ro/SSA and anti-La(SSB) antibodies. As shown in Figure 5J, after stimulation of PBMCs with rIL-25, we documented significantly increased levels of anti-Ro(SSA) in supernatants of cultured PBMCs from patients with primary SS.

**IL-25 neutralization attenuates disease progression and tissue pathology in mice with experimental SS.** To investigate whether IL-25 can be targeted for therapeutic purposes in primary SS, we performed IL-25 neutralization in mice with established experimental SS. Mice with experimental SS at 20 weeks postimmunization (diagnosed as having decreased salivary function and lymphocytic infiltration in the SGs, histologic score of 1) received PBS vehicle or anti–IL-25 monoclonal antibody (200 μg per mouse twice every week) for 4 weeks and were examined 30 weeks postimmunization, while histologic changes of SGs were assessed.

Although severe tissue destruction with multiple foci developed in SGs of vehicle-treated mice with experimental SS, IL-25 neutralization profoundly inhibited the exacerbation of disease pathology (Figure 6A). Notably, blockade of IL-25 resulted in reduced lymphocytic infiltration and numbers of foci during progression of experimental SS. The saliva flow rate in mice with experimental SS after anti–IL-25 treatment was only mildly, and not significantly, improved (P = 0.06) (data not shown). A significant reduction of IL-5, but not IL-13, was also observed in mice with experimental SS treated with anti–IL-25 (see Supplementary Figures 1G and H, http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract). It has been reported that IL-25 suppressed interferon-γ production during colitis (22). Consistent with this, we found that SG-infiltrating Th1 cells were markedly decreased upon IL-25 neutralization (Figure 6B). Similar to the findings in humans with primary SS, we also detected increased CD45+Lin−IL-17RB+IL-7Rα+ ILC2s in SGs of mice with experimental

![Figure 6](http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract)
IL-25 IN PRIMARY SS

SS. However, mice with experimental SS treated with anti-IL-25 showed significantly reduced frequencies and numbers of ILC2s (Figure 6C). In addition, IL-25 neutralization was shown to selectively inhibit the CD206+ F4/80+ M2-type macrophages among infiltrating lymphocytes (Figure 6D). Thus, our findings suggested that IL-25 might serve as a therapeutic target for the treatment of primary SS.

Rituximab modulates SG IL-25 expression and inflammatory ILC2s in patients with primary SS. Rituximab has been demonstrated to modulate local and systemic immune responses in patients with primary SS as well as the function of innate immune cells such as natural killer cells (23,24). We evaluated the local expression of IL-25 and the peripheral frequencies of inflammatory ILC2s and M2-type macrophages in 5 patients with primary SS after rituximab therapy. As shown in Supplementary Figure 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract), rituximab treatment was associated with significantly reduced SG IL-25 expression levels (Supplementary Figures 2A and B) and a significantly reduced percentage of circulating inflammatory ILC2s (Supplementary Figures 2C and D), but not with reductions in M2-type macrophages (data not shown).

Activation of the IL-25/IL-17RB axis in primary SS–associated lymphoma. Since IL-25 regulates hematopoietic and immune functions (25), stimulating the development of B lymphocytes, we next evaluated involvement of the IL-25/IL-17RB axis in primary SS–associated MALT lymphoma in SGs. As shown in Supplementary Figure 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.40500/abstract), significant overexpression of IL-25 was observed in SG samples from patients with lymphoma (Supplementary Figure 2E) accompanied by significant overexpression of IL-17RB (Supplementary Figure 2F) and TRAF6 (Supplementary Figure 2G), indicating activation of the IL-25/IL-17RB axis in primary SS–associated lymphoma as well.

DISCUSSION

In this report we provide, for the first time, evidence that the IL-25/IL-17RB axis is activated in patients with primary SS and in mice with experimental SS and that it is associated with the selective expansion of specific subsets of ILC2s and the occurrence of an M2-type macrophage polarization. We also demonstrate that the neutralization of IL-25 causes a decrease in the progression of disease in a murine model of SS, thus representing a possible successful strategy in the treatment of SS.

IL-25 is an important molecule controlling innate and adaptative immunity (4). It signals through IL-17RB, which specifically binds to IL-17B and IL-25 but not to IL-17A or IL-17C (4). Interaction of IL-17B and IL-25 with IL-17RB induces different biologic activities. IL-17B essentially acts on epithelial cells, inducing the expression of IL-8 and the up-regulation of proinflammatory chemokines such as CXCL1, CCL20, and trefoil factor 1 via the ERK-1/2 pathway (26). Conversely, IL-25/IL-17RB interaction essentially regulates B cell function, induces autoimmune Th17-driven inflammation, and promotes Th2 responses by increasing the frequency of M2-polarized macrophages and the differentiation of ILC2s (6,7,9,21).

In primary SS, IL-17RB was almost exclusively observed on the surface of inflammatory immune cells infiltrating SGs, with very low expression in ductal and/or acinar epithelial cells, indicating a predominant role of this receptor in modulating the function of infiltrating inflammatory cells. In accordance with the expression of IL-17RB on immune cells, we demonstrated that IL-25, but not IL-17B, is overexpressed in SGs in primary SS with a distribution that follows that of IL-17RB. Overexpression of human IL-25 has been demonstrated to result in lymph node medullary expansion due to increases of reactive B cells, plasma cells, and macrophages (27). Consistent with these results, in our study IL-25 expression was correlated with the lymphocytic focus score and with the degree of GC organization. IL-17RB is known to interact with TRAF6 and NF-κB activator protein 1, and TRAF6 in concert with transforming growth factor β-activated kinase 1 activates ERK-MAPK signaling for cell survival (15). In SGs in primary SS, we observed a significant up-regulation of TRAF6, especially among CD3+ cells and ILC2s, indicating that IL-25/IL-17RB signaling is functional in primary SS via coordinated activation of ERK-1/2 and its downstream transcription factors.

In the seminal study reported by Huang et al (8), it was demonstrated that IL-25–dependent inflammatory ILC2s also express high levels of GATA-3 (more than that expressed by natural ILC2s), and that they express an intermediate amount of retinoic acid receptor–related orphan nuclear receptor γt (RORγt) (a smaller amount than that expressed by ILC3s, but significantly distinct from the amount expressed by natural ILC2s). The exact contribution of these different transcription factors in modulating ILC2 expansion needs to be better clarified in future studies. The IL-25/IL-17RB/TRAF6 axis was also studied in SG protein–immunized mice with experimental SS. In experimental SS, serum levels of IL-25 were significantly increased 30 weeks postimmunization, and, similar to findings in primary SS in humans, we detected increased numbers of IL-25–producing cells in SGs of mice with experimental SS that had glandular infiltration. Interestingly, IL-25+ tissue cells were mainly found...
surrounding SG-infiltrating CD4+ T cells. Increased IL-17RB expression was also demonstrated in SG-infiltrating CD4+ T cells, as well as enhanced TRAF6 expression in IL-17RB+CD4+ T cell subpopulations. Taken together, these findings seem to indicate a critical role of the IL-25 axis in the pathogenesis of primary SS and experimental SS.

It has been shown that IL-25/IL-17RB expression induces the expansion of M2-type macrophages and ILC2s (7,8). ILC2s require RORα and GATA-3 for their development and, together with IL-25, IL-33 has also been demonstrated to be the predominant ILC2-inducing cytokine (18). The existence of an inflammatory ILC2 population responsive to IL-25 that complemented IL-33-responsive natural ILC2s was recently reported (8). Inflammatory ILC2s develop into natural ILC2-like cells in vitro and in vivo and acquire IL-17-producing ability (28). In this regard, IL-33 has recently been demonstrated to be overexpressed in patients with primary SS (3). In our study, a strong and significant expansion of inflammatory ILC2s was observed in primary SS at both the peripheral and local levels, and this was significantly correlated with the focus score, disease activity evaluated by the ESSDAI, and the number of infiltrating IL-25+ cells. Conversely, natural ILC2s were not significantly expanded either in the MSGs or in the peripheral blood of patients with primary SS. The role of IL-25 in modulating inflammatory ILC2 expansion in primary SS seems to be confirmed by the demonstration that addition of IL-25 to isolated SG mononuclear cells and PBMCs was able to selectively induce the expansion of inflammatory ILC2s in vitro.

Interestingly, inflammatory ILC2s may be transient progenitors of ILCs mobilized by inflammation and infection, that develop into natural ILC2-like cells or ILC3-like cells contributing to immunity to helminths and fungi (28). Since ILC3s producing IL-22 have been demonstrated to be expanded in patients with primary SS (12), contributing to the pathogenesis of primary SS, we obviously cannot exclude the possibility that a proportion of SG inflammatory ILC2s may become ILC3s.

IL-25-activated ILC2s have been demonstrated to produce Th2 cytokines such as IL-5 and IL-13 (18). In accordance with the presence of ILC2s in the SGs of patients with primary SS, high levels of the Th2 cytokines IL-5 and IL-13 were observed. Th2 cytokines have been demonstrated to elicit specific M2-type macrophage responses (29). Macrophages have essential activities in homeostasis maintenance, being differently polarized, according to various stimuli, into 2 distinct populations of M1- and M2-type macrophages (29). In accordance with IL-25 overexpression and inflammatory ILC2 polarization, we observed a clear expansion of M2-type macrophages both in SGs and in peripheral blood of patients with primary SS. In SGs in primary SS, M2-type macrophages were demonstrated to be CD163+c-Maf+ cells, since the presence of CD163 alone is not sufficient to define M2-type polarization, and their number was correlated with the focus score.

IL-25 regulates hematopoietic and immune functions, specifically stimulating expansion of B lymphocytes in spleen, lymph nodes, and other secondary lymphohematopoietic tissues (21). Our demonstrations that IL-25 is able to induce in vitro the production of primary SS-associated autoantibodies and that the IL-25 axis is also upregulated in patients with primary SS-associated MALT lymphomas seem to support the importance of IL-25 in modulating B cell function. The B cell response observed in our in vitro studies after IL-25 stimulation might be modulated by TRAF6 overexpression, since it has been demonstrated that TRAF6 is required for both T cell-dependent and T cell-independent B cell responses (30). However, other pathways might be involved in autoantibody production, and further functional studies are needed to determine their exact contributions.

Finally, our demonstration that depletion of B cells by rituximab in patients with primary SS significantly reduces the local levels of IL-25 and the frequency of circulating inflammatory ILC2s indirectly suggests that modulation of the IL-25 axis might also be relevant in the treatment of these patients. In this regard, rituximab has been demonstrated to modulate innate and adaptive immune responses beyond B cell depletion in patients with primary SS. In particular, depletion of CD20+ B cells (31) and mast cells (23) and reduction in the expression of different cytokines such as IL-22 (24) and IL-17 (23) have been demonstrated after rituximab therapy. Consistent with the above hypothesis is our demonstration that neutralization of IL-25 resulted in reduced lymphocytic infiltration and numbers of foci during progression of experimental SS, as well as in significant reductions of infiltrating Th1 cells, CD45+Lin−IL-17RB + IL-7Rα+ILC2s, and CD206+F4/80+M2-type macrophages.

In conclusion, in this study we have provided the first demonstration that the IL-25/IL-17RB axis is activated in patients with primary SS, in primary SS-associated MALT lymphomas, and in experimental SS. In patients with primary SS the activation of this axis is associated with the expansion of inflammatory ILC2s and M2-type macrophages. Targeting of this axis might be relevant in treating patients with primary SS.
ACKNOWLEDGMENT

We are deeply grateful to Dr. Francesca Raita (Sezione di Anatomia Patologica, Azienda Ospedaliera Ospedali riuniti Villa Sofia-Cervello) for her technical support in immunohistochemical experiments.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Lu and Ciccia had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Guggino, Lin, Rizzo, Xiao, Saieva, Raimondo, Di Liberto, Candore, Ruscitti, Cipriani, Giacomelli, Dieli, Alessandro, Triolo, Lu, Ciccia.

Acquisition of data. Guggino, Lin, Rizzo, Xiao, Saieva, Raimondo, Di Liberto, Candore, Ruscitti, Cipriani, Giacomelli, Dieli, Alessandro, Triolo, Lu, Ciccia.

Analysis and interpretation of data. Guggino, Lin, Rizzo, Xiao, Saieva, Raimondo, Di Liberto, Candore, Ruscitti, Cipriani, Giacomelli, Dieli, Alessandro, Triolo, Lu, Ciccia.

REFERENCES
