Systemic and Local Interleukin-17 and Linked Cytokines Associated with Sjögren's Syndrome Immunopathogenesis

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Recently recognized as a distinct CD4⁺ T helper (Th) lineage, Th17 cells have been implicated in host responses to infections and in pathogenesis associated with autoimmune diseases. This cytokine is implicated in primary Sjögren's syndrome (pSS) immunopathology because of the increased levels of circulating interleukin (IL)-17 in pSS. Plasma and minor salivary glands (MSGs) from patients with pSS were therefore evaluated for CD4⁺ T cells, T regulatory cells, IL-17, and supporting cytokines by immunohistochemistry, RT-PCR, and microbead assays. MSGs from pSS patients contain IL-17-expressing cells as a dominant population within inflammatory lesions. IL-17 protein expression progressively increased with higher biopsy focus scores ($P < 0.0001$), in parallel with detection by RT-PCR. Transforming growth factor- β , IL-6 and IL-23, which are requisite promoters of Th17 differentiation, were found in abundance compared with the amounts in control tissues. Although transforming growth factor- β is also a pivotal differentiation factor for immunosuppressive Foxp3⁺ T regulatory cells (Tregs), an increase in Foxp3⁺ Tregs was evident in biopsy specimens with mild and moderate inflammation but this increase was disproportionate to escalating pro-inflammatory Th17 populations in advanced disease. Furthermore, the Th17-centric cytokines IL-17, IL-6, IL-23, and IL-12 were significantly elevated in pSS plasma. These data identify a profusion of IL-17-generating cells and supporting cytokines within diseased pSS MSGs without a compensatory increase in immunomodulatory Tregs; this imbalance seems to foster a pathogenic milieu that may be causative and predictive of infiltrative injury and amenable to therapeutic

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Sjögren's syndrome (SS), a complex autoimmune disease that primarily targets lacrimal and salivary glands, results in compromised secretory functions evident by xerostomia and keratoconjunctivitis sicca. SS can also present with multiorgan systemic manifestations and a significant increase in the incidence of malignant lymphoma.¹ Although the etiopathogenesis of SS remains ill-defined, the hallmark of the disease is lymphocytic infiltration of exocrine glands, tissue destruction, and chronic dysfunction. Early periductal infiltration of activated T cells leads to an accumulation of B cells, along with antigen-presenting macrophages and dendritic cells.^{2,3} The prevailing paradigm is that salivary gland (SG) lesions in patients with SS are populated with CD4⁺ T helper type 1 (Th1) lymphocytes and their products, notably interferon- γ (IFN γ), which orchestrate tissue damage and chronicity. Recently, however, additional Th cell populations have been recognized and linked to autoimmune sequelae,⁴ prompting re-evaluation of the cellular constituents of SG lesions. In this regard, based on prior evidence that Th1 cells dominated in the immunopathogenesis of exocrine gland lesions, therapeutic interventions have been targeted for this population and/or their products. However, antagonists of tumor necrosis factor- α (TNF α), successful in other autoimmune diseases,

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have been tested in SS without efficacy,^{5–8} further suggesting that alternative pathways must underlie the evolution of exocrinopathies associated with SS. In our recent studies, we determined that etanercept not only did not diminish the signs and symptoms of SS but also was associated with an unexpected increase in circulating TNF α levels.⁶ Systemically, increased levels of additional cytokines were detected in the plasma of patients with SS compared with plasma of healthy control subjects, including IL-17, a product of a newly recognized population of CD4⁺ Th17 cells with pro-inflammatory pathogenic potential.

To determine whether the systemic levels of IL-17 were a reflection of tissue involvement and glandular pathogenic pathways, we monitored IL-17 and its supportive cytokines systemically and in minor salivary gland (MSG) biopsy specimens in relation to disease parameters. In addition to a striking expression of IL-17, we identified elevated transforming growth factor- β (TGF- β), one of the most influential cytokines in Th17 polarization,⁹ together with IL-6 and IL-23, a heterodimeric p40/p19 member of the IL-12 cytokine family.^{4,10–15} Abundant Th17-positive cells, together with a smaller population of Th1 cells and their collective products, may promote salivary gland pathology in the context of a disproportionate number of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), also developmentally dependent on TGF- β .^{11,16} These findings may suggest new considerations in the quest for intervention targets in this autoimmune disease for which limited therapeutic options exist.

Materials and Methods

Plasma Samples from SS Patient Populations

As detailed in prior studies,^{5,6,17} a pilot study of etanercept (25 mg twice weekly) ($n = 14$; 12 female and 2 male; median age 55.5 years [range, 46 to 59 years] and median biopsy focus score [FS] = 11 [range, 5 to 12]; anti-Ro [SSA] antibodies 86%; anti-La [SSB] antibodies 50%) compared with placebo ($n = 14$; 14 female and 0 male; age 54.5 years [range, 46 to 66 years] and FS = 7 [range 4 to 12]; anti-Ro [SSA] antibodies 86%; anti-La [SSB] antibodies 50%) was performed with SS patients for 12 weeks (approved by the National Institute of Dental and Craniofacial Research institutional review board).

Peripheral blood was collected from healthy control subjects ($n = 11$) and from patients with primary Sjögren's syndrome (pSS) at baseline and at the end of etanercept treatment ($n = 11$ of 14). Plasma was removed from heparinized blood after centrifugation and stored at -80°C until analyzed. Plasma samples obtained from 30 pSS patients (age 54.5 years [range, 46 to 65.2 years]; 28 females and 2 males) not treated with etanercept and 30 age-matched control subjects (age [54.0 years – 47.5 to 63.2 years]; 27 females and 3 males) were used for comparative analyses.^{5,6,17}

Cytokine Assays

Plasma samples were tested using a bead-based immunofluorescence assay (Luminex, Austin, TX) with multiplex cytokine reagents (Biosource International, Camarillo, CA).⁶ Samples and standards were analyzed in duplicate; analyses were repeated twice, and only variation coefficients $<15\%$ were accepted. IL-23 was determined by an enzyme-linked immunosorbent assay (Biosource International).

MSG Histology and Immunohistochemistry

MSG biopsy specimens were obtained with informed consent from 10 individuals undergoing diagnostic evaluation for sicca symptoms indicative of SS³ (median age 48.5 years [range, 39 to 64.5 years]; 10 female) and diagnosed by American-European SS consensus criteria.¹⁸ The control group consisted of 10 gender-matched individuals (median age 57 years [range, 49.5–65.5 years]; 10 female) with subjective complaints of dry mouth or eyes, but who did not fulfill the criteria for SS and had no histopathological evidence of SS. None of the patients had evidence of lymphoma, sarcoidosis, essential mixed cryoglobulinemia, or infection by HIV or hepatitis B or C viruses at time of study. In addition, patients' medical records were evaluated for clinical and serological parameters including anti-Ro (SSA) and anti-La (SSB) antibodies, high erythrocyte sedimentation rate (ESR), and C3/C4 hypocomplementemia.

Biopsy specimens were fixed, embedded, sectioned (5 μm), deparaffinized, rehydrated through alcohol, and stained with H&E. All pSS patients presented a biopsy FS ≥ 1 (focus = aggregate of ~ 50 inflammatory cells) per 4 mm^2 ,¹⁹ whereas the control group had FS < 1 . Histological grading was also categorized based on inflammation as mild (1+), intermediate (2+), and advanced (3+/4+).²⁰ For immunohistochemical analysis, sections were processed for antigen retrieval in a decloaking chamber (Biocare Medical, Concord, CA) with antigen unmasking solution (Vector Laboratories, Burlingame, CA) before blocking by endogenous peroxidase with 3% H_2O_2 in 50% methanol for 15 minutes. Sections were incubated with blocking serum (goat or rabbit) for 30 minutes and incubated overnight at 4°C with the primary antibody: CD4 (clone 1F6, mouse IgG1; Zymed Laboratories, San Francisco, CA), CD8 (clone C8/144B, mouse IgG1; DakoCytomation, Glostrup, Denmark), TGF- $\beta 1$ (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), IL-17A (rabbit polyclonal; Santa Cruz Biotechnology), IL-17F (goat polyclonal; Santa Cruz Biotechnology), IL-17 receptor (IL-17R) (goat polyclonal; Santa Cruz Biotechnology), retinoic acid-related orphan receptor γt (ROR γt) (rabbit polyclonal; GeneTex, Inc., Irvine, CA), phospho-SMAD2 (rabbit polyclonal; Chemicon International Inc., Temecula, CA), IL-6 (rabbit polyclonal; Abcam, Cambridge, MA), Foxp3 (clone 236A/E7, mouse IgG1; Abcam), IFN γ (clone 350B10G6, mouse IgG1; Abcam), IL-23 (p19) (clone HLT2736, mouse IgG1; BioLegend, San Diego, CA), and IL-12p70

(clone 24910, mouse IgG1; R&D Systems, Minneapolis, MN). For control staining, primary antibodies were replaced with irrelevant isotype-matched antibodies (Jackson ImmunoResearch, West Grove, PA). After 30 minutes with biotinylated secondary antibody, staining was developed using Avidin:Biotinylated enzyme Complex (Vectastain Elite Kit; Vector Laboratories) for 30 minutes or streptavidin peroxidase for 10 minutes, followed by 3,3-diaminobenzidine tetrahydrochloride substrate chromogen (Zymed Laboratories) and counterstained with Meyer's hematoxylin. Histopathology, ranking, and immunostaining were evaluated by a cytopathologist (S.R.) blind to diagnosis. To evaluate antigen expression, electronic images of 10 optical fields ($\times 400$ magnification) were taken across sections. The presence and intensity of staining were evaluated and the number of positively stained cells was expressed as a percentage of infiltrating mononuclear cells (MNLs). Mean $\pm$ SEM of 10 fields was calculated per biopsy. Immunostaining of serial sections provided evidence of antigen co-expression.

RNA Extraction and cDNA Synthesis

MSG specimens from SS patients (median age 50.0 years [range, 34 to 52.5 years]; 10 female) were preserved in RNAlater (Applied Biosystems, Foster City, CA) and stored at -80°C . Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany). To eliminate genomic DNA contamination, samples were treated with RNase-free DNase (Qiagen). cDNA was prepared from 0.5 μg of RNA using oligo(dT) primers (MWG Biotechnology, Ebersberg, Germany) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Integrity of RNA was verified by amplification of β -actin mRNA.

Semiquantitative Real-Time PCR

The resulting cDNA was amplified by PCR using an ABI 7500 Sequence Detector (Applied Biosystems). Amplification was performed using TaqMan expression assays for IL-17 (Hs00174383_m1), IL-6 (Hs00174131_m1), IL-23 (Hs00372324_m1), TGF- $\beta 1$ (Hs99999918_m1), Foxp3 (Hs00203958_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) as a normalization control. The data were examined using the $2^{-\Delta\Delta\text{Ct}}$ method, and results are expressed as fold increase.⁶ Each sample was tested in triplicate, and tests were repeated twice.

Statistics

Statistical analyses were performed by Mann-Whitney test for nonparametric data. Correlation of parameters was investigated by Pearson's correlation coefficient, and comparisons between groups were performed using one-way analysis of variance with a Tukey post-test. $P < 0.05$ was considered statistically significant. All analyses were performed using the statistical package SPSS 15.0 (SPSS, Chicago, IL).

Results

IL-17 Expression in Inflamed pSS Salivary Glands

In earlier studies, we demonstrated not only that treatment of pSS with etanercept did not have therapeutic efficacy but also that TNF α levels significantly increased rather than declined during therapy (Figure 1A).⁶ Although systemically elevated levels of several additional chemokines and cytokines, such as IL-17 (Figure 1B) were detected in the plasma of subjects with untreated pSS,⁶ their expression was not examined in parallel in the affected tissues to link them to pathogenesis. Because IL-17 levels were found to be elevated in the circulation of pSS patients and did not decline significantly with etanercept (Figure 1B), nor did systemic clinical parameters, we considered IL-17 a relevant candidate to be examined in salivary gland tissue biopsy specimens of SS patients as a potential contributing mediator of exocrinopathy.

In MSGs from patients with SS, inflammatory cell infiltrates are pronounced (median FS = 7; range, 4.87–9.75; $n = 10$) and contain large numbers of CD3⁺ T cells of which the majority are CD4⁺, with fewer numbers of CD8⁺ T cells (Figure 1, C–F). To determine whether the aberrant blood levels of IL-17 were related to IL-17 in the tissues and lesion development, IL-17 protein was monitored in MSGs by immunohistochemistry. Labial salivary glands from all SS patients with focal accumulation of MNLs localized predominantly around ducts exhibited high numbers of IL-17A-staining cells (Figure 2, A–D). The degree of staining was most intense in those regions enriched in T cells, as judged by CD4⁺ staining in adjacent sections, and corresponded to the degree of MNL infiltration. Ductal epithelial cells were variably positive for IL-17A, even in control tissues, but no evidence of IL-17A staining was detected in acinar cells, myoepithelial cells, or fibroblasts. Immunohistochemical staining for the IL-17R revealed substantial expression on ductal epithelial cells (data not shown), consistent with reports of IL-17R on isolated salivary gland cells by flow cytometry²¹ that may account, in part, for the detection of IL-17 in association with this population, also seen in previous studies,²² but does not exclude their ability to produce this cytokine. Because the widespread distribution of the IL-17R may confound quantification because of an inability to dissociate cells producing IL-17 and those binding/responding to it and multiple cell types may be responsible for IL-17 secretion, the number of IL-17⁺ cells may seem inflated. Nonetheless, isotype control antibody or deletion of the primary antibody resulted in negative staining, indicating that the staining was not nonspecific (Figure 2D). Staining for the IL-17A homolog IL-17F was detected but was weaker than that for IL-17A in the inflammatory lesion (data not shown).

Because of the difficulty in accurately quantifying IL-17-expressing populations by immunohistochemistry (IHC), we assessed IL-17A in patients' inflamed tissues by RT-PCR of amplified mRNA extracted from whole labial salivary glands of mild, intermediate, and advanced

Figure 1. Systemic cytokine levels in pSS before and after etanercept (ETN) and SS tissue infiltrates. Plasma was obtained from healthy control (HC) ($n = 11$) subjects and pSS patients before (pSS) and after (post-ETN, $n = 11$) 12 weeks of treatment with etanercept (25 mg twice weekly). Plasma cytokine levels were measured with a multiplex bead assay. Mean levels $\pm$ SEM (pg/ml) for TNF α (**A**) and IL-17 (**B**) ($*P < 0.05$). **C:** Representative MSG biopsy specimens from etanercept-untreated pSS patients ($n = 10$) stained with H&E and demonstrating periductal mononuclear cell infiltrates. **D:** Immunohistochemical detection of CD3 $^{+}$ T cells in the infiltrate, of which the majority are CD4 $^{+}$ (**E**), with a lesser population of CD8 $^{+}$ T cells (**F**).

inflammatory lesions in comparison with control tissues from individuals with subjective sicca symptoms and normal lip SG biopsy results. We established clear expression of IL-17A RNA in patients' SGs (Figure 2E), with escalating expression in SS patients possessing intermediate ($n = 3$) and advanced lesions ($n = 3$) compared with control MSGs ($n = 3$) ($P < 0.05$). Consonant with the mRNA and protein analyses signifying an abundance of IL-17-expressing Th17 cells was the identification of cellular expression of the Th17 transcription factor, nuclear hormone ROR γ t, as detected by specific staining (Figure 2, F and G).

Correlation of IL-17 with Focus Scores

The intense expression of IL-17A in pSS MSGs as detected by IHC and RT-PCR was suggestive of its participation in disease pathogenesis, but it remained unclear whether this tissue deposition correlated with known markers of SS disease, such as anti-Ro (SSA) and anti-La (SSB) antibodies, high ESR, C3/C4 hypocomplementemia, or FS. Although quantitation of IL-17 staining was difficult because of its widespread distribution and staining intensity, the relative percentage of positively stained MNLs in the tissues, uniformly quantified across all specimens, correlates with increasing biopsy FS, as did increased mRNA levels. Expression of IL-17A in MSG biopsy specimens intensified with disease stage/FS ($n = 10$; $P < 0.0001$) (Figure 2H) when protein staining was evaluated as percentage of infiltrating MNLs, indicative of

a correlation between up-regulated IL-17 and immunopathological manifestations of pSS.

In those patients with MSG tissues assessed for IL-17 mRNA, a significant correlation was evident between IL-17 and ESR ($P = 0.03$) (Figure 2I). Furthermore, in an additional population of 30 SS patients, we monitored IL-17 levels in the periphery and found that, consistent with the increased plasma levels initially detected in the etanercept trial and with the presence of IL-17 in SS MSG, significant increases in circulating IL-17 (163.6 ± 45 pg/ml; $P < 0.0001$) compared with that in a control population (35 ± 4 pg/ml; $n = 30$) clearly indicated evidence of Th17 lineage activation (Figure 2J).

TGF- β in Inflamed Salivary Glands and Systemically

Because TGF- β is required in human naive CD4 $^{+}$ T cell polarization to Th17 in conjunction with additional cytokines,^{13–15} as first shown in murine systems,¹⁰ we examined MSGs for TGF- β by protein and RNA analyses. Immunohistochemical analysis in all inflamed SS specimens revealed high expression of total TGF- β , the intensity of which was increased with advancing stages of disease, in parallel with IL-17 expression (Figure 3, A and B). TGF- β 1 is abundantly and widely expressed and was detected extracellularly and within the cytoplasm of infiltrating inflammatory cells in juxtaposition to the ducts and in ductal cells, whereas acinar expression was variable

Figure 2. Systemic and local IL-17A in pSS. Immunostaining for IL-17A in mild (A) compared with advanced (B) salivary gland lesions in etanercept-untreated SS patients and in control tissues (C). D: Isotype control staining of salivary gland. Expression of IL-17A in tissues with advanced and intermediate lesions was considerably elevated compared with early/mild lesions (original magnification, $\times 400$; $n = 10$). E: Relative expression of IL-17 mRNA in whole MSGs of SS patients with mild ($n = 3$), intermediate ($n = 3$), and advanced lesions ($n = 3$). Data are normalized for GAPDH mRNA content and are plotted as fold change over control (Ct) tissues ($n = 3$). IL-17 mRNA expression was elevated with the progression of lesion severity. Results represent the mean $\pm$ SEM. $*P < 0.05$. Immunohistochemical staining with an antibody directed toward the Th17 transcription factor, ROR γ t (F) and isotype control (G) in advanced MSG lesions. H: Correlation of IL-17A with focus scores. Positive stained cells are presented as a percentage of the infiltrating mononuclear cells. Significant staining of the specific marker was detected in SS patients ($n = 10$) compared with that in control subjects ($n = 10$) ($*P < 0.05$). IL-17 expression by IHC was then correlated with the focus score of MSG biopsy specimens. The r ($r = 0.941$) and P ($P < 0.0001$) values were determined with the Pearson correlation coefficient. I: Correlation of relative IL-17 mRNA expression in whole MSGs of SS patients with plasma ESR ($r = 0.704$, $P = 0.03$ by Pearson correlation coefficient). J: Plasma cytokine levels were measured with a multiplex bead assay in control subjects ($n = 30$) and SS patients ($n = 30$). Levels (pg/ml) for IL-17 are shown for individual patients and lines represent mean levels of expression for each group. $*P < 0.0001$. The dotted horizontal line represents the assay cutoff point (pg/ml).

and of less intensity. In parallel and more definitively, TGF- β 1 mRNA expression increased in SG tissue of patients with intermediate and advanced lesions compared with that of control subjects ($n = 3$ /group, $P < 0.05$) (Figure 3C). Because TGF- β was typically evident both intracellularly and extracellularly, we did not quantify positive cells but rather examined the tissues for an intracellular marker. To assess whether TGF- β was active, we looked for evidence of its downstream signaling pathway with an antibody directed against intracellular phosphorylated Smad2. Greater phosphorylated Smad2 staining was detected in the infiltrated MNLs than in control tissues with few MNLs (Figure 3, E–H), although ductal epithelial cells from control and diseased tissues stained

for phosphorylated Smad2. Collectively, these data implicate TGF- β in its biologically active configuration as a potent mediator of events occurring in the salivary glands of patients with confirmed SS. Moreover, total TGF- β 1 (active and latent) was augmented in the circulation, albeit not significantly ($P = 0.07$) (Figure 3D).

Th17 Supporting Cytokines in Inflamed Salivary Glands and in Circulation

In addition to TGF- β , human Th17 lineage commitment is fostered by IL-6 and IL-23.^{4,10,14} Examination of

Figure 3. Circulating and tissue TGF- β in pSS. **A** and **B:** MSG biopsy specimens from control (**A**), and non-etanercept-treated pSS (**B**) infiltrative lesions were stained with an antibody that detected total TGF- β 1 (latent plus active) (original magnification, $\times 400$). **C:** Relative expression of TGF- β 1 mRNA in whole MSGs of SS patients with mild, intermediate, and advanced lesions, compared with control (Ct) tissues ($n = 3/\text{group}$). Data are normalized for GAPDH mRNA and are plotted as fold change over controls. Results represent the mean $\pm$ SEM. $*P < 0.05$. **D:** Plasma cytokine levels were measured with a multiplex bead assay in control subjects ($n = 30$) and etanercept-untreated SS patients ($n = 30$). Levels (pg/ml) for total TGF- β 1 are shown for individual patients, and lines represent mean levels of expression for each group; $P = 0.07$. **E–H:** Expression of phospho-Smad2 in control MSG (**E**), mild (**F**), and advanced (**G**) disease tissues, indicative of TGF- β signaling, with isotype staining (**H**) in an advanced lesion.

MSG biopsy specimens for evidence of these cytokines, which could establish a Th17 supportive milieu, revealed substantial expression of IL-6 RNA (Figure 4A), which increased with escalating disease as measured by MNL infiltration. In contrast, in control specimens, little or no IL-6 mRNA was detected ($P < 0.05$) (Figure 4A). In addition, in this complex regulatory network, elevated IL-23 (p40/p19), an IL-12 (p40/p35)-related cytokine, is expressed (Figure 4B) and may promote survival of Th17 development through a mechanism linked to IL-6, acting independently or in synergy. Furthermore, SG IL-23 mRNA levels increased in SS patients with intermediate and advanced lesions compared with those in control subjects ($P < 0.05$) (Figure 4B). Beyond expression in the diseased glands, we demonstrated a corresponding significant increase in both IL-6 ($P = 0.001$) and IL-23 ($P = 0.001$) in plasma of SS patients compared with that of control subjects (Figure 4, C and D), possibly underlying the global Th17 lineage activation. To evaluate the potential involvement of these two cytokines in SS pathogenesis, circulating levels of IL-6 and IL-23 were compared with the plasma ESR (Figure 4, E and F). Plasma IL-23 levels significantly correlated with the ESR ($P = 0.01$), whereas IL-6 levels did not ($P = 0.08$).

Th1 Cytokines in pSS Tissues

Although evidence supported a large population of IL-17⁺ cells in the inflamed MSGs, not all CD4⁺ T cells expressed IL-17. Immunohistochemical analyses to detect Th1-related molecules previously implicated in SS revealed staining for Th1-derived IFN γ (Figure 5, A and B), as anticipated. IL-12, recognized as a key cofactor for Th1 lineage development, was also prominently expressed in the MSG biopsy specimens of SS patients as determined by an antibody directed to p70 relative to tissues from non-pSS populations (Figure 5, D and E), but without evident inhibition of Th17-related cytokines, potentially reflecting the newly recognized plasticity of T cell populations.²³ When evaluated in the peripheral blood, IL-12 was consistently elevated in SS patients compared with that in control subjects ($P < 0.0001$) (Figure 5F), whereas blood levels of IFN γ were low and even marginally less in patients than in control subjects ($n = 30$, $P = 0.028$) (Figure 5C). For comparison, circulating IL-2 was elevated in the patients, whereas the representative Th2 cytokine IL-4 was less than 100 pg/ml, although this level was significantly above blood levels in the control population (inset, Figure 5C).

Figure 4. IL-6 and IL-23 in pSS tissues and in the circulation. **A:** Relative expression of IL-6 mRNA in whole MSGs of SS patients with mild, intermediate (Interim), and advanced lesions. Data are normalized for GAPDH mRNA content and are plotted as fold change over controls ($n = 3/\text{group}$). Results represent the mean $\pm$ SEM. $*P < 0.05$. **B:** Increasing IL-23 expression determined by RT-PCR in whole MSGs of SS patients with mild, intermediate, and advanced lesions ($n = 3/\text{group}$). Data are normalized for GAPDH mRNA content and are plotted as fold change over controls ($n = 3$). Results represent the mean $\pm$ SEM. $*P < 0.05$. **C:** Plasma cytokine levels were measured with a multiplex bead assay in control subjects and SS patients ($n = 30$). Levels (pg/ml) for IL-6 are shown for individual patients, and lines represent mean levels of expression for each group. $*P < 0.05$. **D:** Plasma IL-23 levels were measured by enzyme-linked immunosorbent assay. $*P < 0.05$. The dotted horizontal line represents the assay cutoff point (pg/ml). **E:** Plasma IL-6 levels were measured with a multiplex bead assay in control subjects and SS patients and compared with ESR ($P = 0.08$, Spearman nonparametric correlation). **F:** Plasma IL-23 levels, measured with a multiplex bead assay, in control subjects and SS patients were compared with ESR ($P = 0.01$).

TGF- β and Foxp3⁺ Tregs in Salivary Glands

Because TGF- β , besides supporting Th17 lineage commitment, is also linked to generation of Tregs and suppression of Th1 and IFN γ , we next examined the diseased salivary glands for evidence of Foxp3⁺ Tregs as a potential mechanism for disrupting the effector T cell balance in MSG lesions. Foxp3, a key transcription factor for regulatory CD4⁺CD25⁺ T cells, was detected exclusively among infiltrating MNLs of pSS tissue specimens. Increasing expression of Foxp3 was observed in patients with mild and intermediate lesions (Figure 6, A and B) compared with that in control subjects ($P < 0.001$), evident by Foxp3 mRNA expression (Figure 6C) ($P < 0.05$), but in tissues with advanced lesions, no further enhancement of this immunosuppressive population was apparent. The percentage of Foxp3⁺ infiltrating cells plateaued from intermediate to advanced lesions as did levels of Foxp3 mRNA (Figure 6, C and D). Whereas the numbers of Foxp3⁺ cells we report are higher than those reported previously,²⁴ we quantified Foxp3⁺ cells within the CD4⁺-enriched infiltrates in our limited tissue specimens (10 fields, $\times 400$) rather than the entire gland. Nonetheless, all sections were quantified by the same methodology, providing relative distributions within our experimental context. Moreover, the RNA analyses were representative of whole salivary glands and support the IHC data. Thus, the results from the quantitative RT-PCR together with those obtained from IHC, suggest that TGF- β , in the initial stages of inflammation, may induce

Foxp3⁺ Tregs, but, as inflammation persists, along with accumulation of IL-6 and IL-23, the milieu may shift to one favorable for propagation of Th17 cells with correspondingly fewer Foxp3⁺ Tregs, in line with new evidence for a reciprocal relationship between Th17 and Tregs.¹² Collectively, these data support a substantive and potentially unimpeded involvement of Th17 lineage participation in evolution of exocrinopathies characteristic of SS, and probably, in the systemic manifestations of the disease.

Discussion

By multiple parameters, our data support IL-17 as a dominant cytokine linked to immunopathogenesis in MSGs of patients with primary SS. First, circulating levels of IL-17 were elevated in a small population of pSS patients before treatment with etanercept, and neither clinical symptoms nor IL-17 abated with treatment. Second, at the local tissue level, pronounced expression of IL-17 protein in the locale of infiltrating CD3⁺CD4⁺ T lymphocytes was evident by immunohistochemical analyses, similar to recent reports.^{21,22} However, we also demonstrate by detailed quantitative analysis that the number of IL-17⁺ cells escalated as the lymphoepithelial lesions progressed from mild to intermediate and advanced and significantly correlated with focus scores, along with IL-17R-bearing cells. Fourth, levels of glandular tissue IL-17 RNA increased in parallel to protein, being higher in advanced disease than in mild or early stages. Fifth, the

Figure 5. Expression of Th1 cytokines in tissues and blood of SS patients. **A** and **B:** Representative immunostaining for IFN γ in control (**A**) and untreated pSS MSG biopsy specimens, $n = 10$ (**B**) (original magnification, $\times 400$). **D** and **E:** Immunostaining for IL-12 in control (**D**) and SS MSG biopsy specimens (**E**). **C** and **F:** Plasma levels of IFN γ (**C**), IL-2, and IL-4 (**C, inset**) and IL-12 (**F**). Plasma cytokine levels were measured with a multiplex bead assay in healthy control subjects (control, HC) and etanercept-untreated pSS patients ($n = 30$ /group). Mean levels $\pm$ SEM (pg/ml) for IL-12 ($*P < 0.0001$), IL-2 ($*P < 0.0001$), IL-4 ($*P < 0.001$), and IFN γ ($*P < 0.05$) are shown.

Th17 transcription factor ROR γ t in the lesions paralleled the presence of Th17 populations. Sixth, multiple factors known to orchestrate Th17 lineage development were co-expressed in the diseased tissues at high levels. Sev-

enth, Th17 pathogenesis may be enabled by circumvention of Treg accumulation. Finally, in a larger cohort of patients, IL-17 and key lineage commitment cytokines were significantly up-regulated systemically, distinct from

Figure 6. Foxp3⁺ Treg detection in MSG. Representative expression of Foxp3 in control MSGs (**A**) and in pSS salivary gland tissues (**B**) from etanercept-untreated pSS patients. Representative immunostaining of an intermediate lesion is shown. **Inset:** isotype staining (original magnification, $\times 400$). **C:** Relative expression of Foxp3 mRNA in whole MSGs of SS patients with mild, intermediate (Interm), and advanced lesions. Data are normalized for GAPDH mRNA content and are plotted as fold change over controls ($n = 3$ /group). Foxp3 RNA was not higher in advanced lesions compared with intermediate lesions. Results represent mean $\pm$ SEM. $*P < 0.05$; ^{+}NS . **D:** Correlation of Foxp3 with focus scores. Positive stained cells are presented as a percentage of infiltrating MNLs. Significant Foxp3 was detected in SS compared with control tissues ($n = 10$). $*P < 0.05$. Foxp3 expression by IHC was then correlated with MSG biopsy specimen FS. The r ($r = 0.851$) and P ($P < 0.002$) values were determined with the Pearson correlation coefficient. Foxp3 increased from control to mild and intermediate lesions but plateaued as lesions worsened.

recent studies,²² but correlative with clinical manifestations and further indicative of their contribution to exocrinopathy and, potentially, systemic manifestations of pSS, as well as candidate diagnostic and/or prognostic markers.

Whether the amassing numbers of IL-17-expressing cells represent newly recruited and/or cells polarized within the local environs remains to be established, although we have reported increased levels of several relevant chemokines in the periphery of pSS patients⁶ and increased expression of CCR6, linked to Th17 recruitment,²⁵ in advanced SS tissues. Nevertheless, as we demonstrate, the salivary gland milieu is replete with factors known to foster local Th17 lineage polarization. We detected escalating expression of TGF- β , the requisite commitment factor for Th17 lineage development¹⁰ by protein expression and RT-PCR in the tissues, by evidence of signal transduction using phosphorylated Smad2 as a marker and by protein in the circulation. MSG biopsy specimens with advanced disease exhibited not only increased TGF- β 1 by microarray analyses, but also TGF- β receptors I and II (N. Moutsopoulos and S.M. Wahl, manuscript in preparation). Critical to differentiation into the Th17 lineage are ROR γ t and/or ROR α , and their expression in naive T cells is rapidly enhanced and sustained by TGF- β , together with IL-6 and IL-23,^{26,27} both increased in the milieu surrounding the IL-17⁺ cells, along with elevated expression of IL-6 receptor and mirrored by elevations in serum. Among the pro-inflammatory cytokines, TNF α , IL-1, and IL-6, have consistently been detected in pSS MSG biopsy and conjunctiva samples.² Pro-inflammatory Th17 cells are characterized by production of a distinct profile of effector cytokines and have probably evolved to enhance host clearance of a range of pathogens distinct from those targeted by Th1 and Th2 but are also potent inducers of autoimmunity,⁴ and abundant IL-17 in MSG may confer legitimacy to the suspected microbial etiology of this chronic disease in genetically susceptible individuals.²⁸ By triggering macrophage IL-1 and TNF α , IL-17 can act additively or synergistically,²⁹ influencing IL-17R⁺ epithelial cells, endothelial cells, and fibroblasts to generate additional mediators, including IL-6, IL-8, colony-stimulating factor, chemokines, and matrix metalloproteinase^{30,31} and undermine tissue integrity. Collectively, our data favor a SG environment enriched in Th17 and Th17-supportive molecules in the evolution of exocrinopathy and, potentially, systemic disease.

In addition to IL-17 as a perpetrator in SS, our data reflect prior evidence for involvement of Th1 cells and their products, such as IFN γ in immunopathological glandular disease,^{2,3} considered causative before recognition of the existence of Th17 cells. Although we did not focus on cells of the Th2 lineage, low IL-4 levels were evident in the circulation, and earlier data suggested that this population may be most evident during the early stages of inflammation and that the Th2 cytokine pattern, provoking B cell activation and autoantibody generation, may diminish during more advanced phases, but possibly re-emerge in the event of lymphoma development.^{1,32} Correspondingly, anti-B-cell therapy has materialized as

a potential therapy for glandular and extraglandular manifestations of pSS, as well as for management of lymphoma associated with pSS.³³ In comparison, IFN γ mRNA, overexpressed in pSS MSG,^{34–36} is echoed by elevated IFN γ protein in saliva. However, we do not detect heightened levels of IFN γ by microarray, when comparing MSGs with advanced with mild disease (N. Moutsopoulos and S.M. Wahl, manuscript in preparation), nor in pSS plasma, although both IL-12 and IL-18 are increased as described.³ Intriguingly, a subset of Th17 cells reportedly co-express IFN γ with IL-17,³⁷ and such a subset has been identified in the gut in Crohn's disease.³⁸ Whether Th1 and Th17 cells cooperatively induce development of organ-specific autoimmunity in SS, as in infectious diseases, or whether the same CD4⁺ T cells produce both cytokines remains an intriguing question. It has recently been demonstrated that committed Th17 populations, if deprived of TGF- β , are reprogrammable to T-bet⁺Stat4⁺ IFN γ -producing Th1 cells in the presence of IL-12 or, possibly, IL-23, both of which we detected in inflamed MSGs. Under these circumstances, Th17 cells extinguish their ROR γ t/ α and IL-17 production in favor of IFN γ .²³ Whether this phenotype shift, contingent on an altered balance between TGF- β and IL-12/IL-23, occurs in SGs remains to be explored but would be compatible with evidence that both IL-17- and IFN γ -producing cells populate SG lesions and, although highlighting plasticity of the Th17 lineage, would insinuate that this population may represent a moving target.

Under the auspices of TGF- β within the SG, the pro-inflammatory functions of Th17 may spiral pathogenesis out of control. Because TGF- β is also linked to development of Tregs, critical in reigning in uncontrolled immune responses,¹⁶ it might be anticipated that these Tregs would dampen the rampant inflammatory pathogenesis. Tregs specifically express the transcription factor Foxp3 and are initially enriched in inflamed SGs, yet these cells seem incompetent or inadequate in controlling immunopathogenesis. An increased prevalence of circulating Tregs has been observed in patients with pSS,³⁹ but increased Treg recruitment at sites of inflammation compared with peripheral blood has been shown to be stable over time and independent of clinical and laboratory parameters, disease duration, and therapeutic interventions.^{2,40} In this regard, evidence for an increase in Foxp3⁺ Tregs in MSGs is most obvious in early and intermediate stages, but the numbers do not seem to increase proportionately to the escalating disease intensity as reported,²⁴ constructing an imbalance between Tregs and effector T cells.^{41,42} In part, this may stem from the ability of their respective transcriptional factors, ROR γ t and Foxp3, to interact and block each other's function.⁴³ Once the innate immune system is activated in SS,⁴⁴ potentially triggering Toll-like receptor-stimulated dendritic cell production of IL-6 along with IL-23, the generation and/or function of Tregs may be compromised,^{45,46} enabling unchecked recruitment and generation of proinflammatory Th17 cells. In keeping with this scenario, therapy with antibody to IL-6 receptor is showing promise in the treatment of rheumatoid arthritis and juvenile rheumatoid arthritis.^{47,48}

Collectively, our results show that the expression of IL-17 and supporting cytokines in the MSGs of SS patients correlated with degree of inflammation and objective clinical evidence. Their linkage with immunohistopathological features of pSS identifies potential therapeutic intervention targets. Conceivably, dampening more broadly the IL-23/Th17 axis through an upstream activator such as IL-23 may retain the collective regulatory and antitumor/anti-infective properties of the IL-12/Th-1/IFN γ pathway. In this context, encouraging data are emerging from clinical studies in psoriasis and Crohn's disease, with the use of neutralizing antibodies against the IL-12p40 subunit, an approach that, although not initially recognized, is likely to block both the IL-12 (Th1/IFN γ) and IL-23 (Th17) pathways.^{49,50} Although suggestive of an important functional role for IL-17 in the expansion and coordination of infiltrative SG injuries, whether Th17 recruitment and lineage specification are primary or secondary in localized tissue destruction and whether the fine-tuning of Th17 and Th1 cytokines may ultimately account for tissue damage remain to be determined. Nevertheless, a focus on the IL-17-secreting CD4⁺ T cell population will further elucidate their contribution to this paradoxical autoimmune disease.

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References

1. Voulgarelis M, Dafni UG, Isenberg DA, Moutsopoulos HM: Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's Syndrome. *Arthritis Rheum* 1999, 42:1765–1772
2. Katsifis GE, Moutsopoulos NM, Wahl SM: T lymphocytes in Sjögren's syndrome: contributors to and regulators of pathophysiology. *Clin Rev Allergy Immunol* 2007, 32:252–264
3. Manoussakis MN, Boiu S, Korkolopoulou P, Kapsogeorgou EK, Kavantzias N, Ziakas P, Patsouris E, Moutsopoulos HM: Rates of infiltration by macrophages and dendritic cells and expression of interleukin-18 and interleukin-12 in the chronic inflammatory lesions of Sjögren's syndrome: correlation with certain features of immune hyperactivity and factors associated with high risk of lymphoma development. *Arthritis Rheum* 2007, 56:3977–3988
4. Boniface K, Blom B, Liu YJ, de Waal Malefyt R: From interleukin-23 to T-helper 17 cells: human T-helper cell differentiation revisited. *Immunol Rev* 2008, 226:132–146
5. Sankar V, Brennan MT, Kok MR, Leakan RA, Smith JA, Manny J, Baum BJ, Pillemer SR: Etanercept in Sjögren's syndrome: a twelve-week randomized, double-blind, placebo-controlled pilot clinical trial. *Arthritis Rheum* 2004, 50:2240–2245
6. Moutsopoulos NM, Katsifis GE, Angelov N, Leakan RA, Sankar V, Pillemer S, Wahl SM: Lack of efficacy of etanercept in Sjögren syndrome correlates with failed suppression of tumour necrosis factor α and systemic immune activation. *Ann Rheum Dis* 2008, 67:1437–1443
7. Mariette X, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E, Combe B, Puechal X, Pennec Y, Sauvezie B, Perdriger A, Hayem G, Janin A, Sibilia J: Inefficacy of infliximab in primary Sjögren's syndrome: results of the randomized, controlled Trial of Remicade in Primary Sjögren's Syndrome (TRIPSS). *Arthritis Rheum* 2004, 50:1270–1276
8. Moutsopoulos NM, Angelov N, Sankar V, Leakan RA, Pillemer S, Wahl SM: Immunological consequences of thalidomide treatment in Sjögren's syndrome. *Ann Rheum Dis* 2006, 65:112–114
9. Wahl SM: Transforming growth factor- β : innately bipolar. *Curr Opin Immunol* 2007, 19:55–62
10. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT: Transforming growth factor- β induces development of the T_H17 lineage. *Nature* 2006, 441:231–234
11. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B: TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006, 24:179–189
12. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK: Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006, 441:235–238
13. Manel N, Unutmaz D, Littman DR: The differentiation of human T_H-17 cells requires transforming growth factor- β and induction of the nuclear receptor ROR γ t. *Nat Immunol* 2008, 9:641–649
14. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, Soumelis V: A critical function for transforming growth factor- β , interleukin 23 and proinflammatory cytokines in driving and modulating human T_H-17 responses. *Nat Immunol* 2008, 9:650–657
15. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, Kuchroo VK, Hafler DA: IL-21 and TGF- β are required for differentiation of human T_H17 cells. *Nature* 2008, 454:350–352
16. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM: Conversion of peripheral CD4⁺CD25⁺ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J Exp Med* 2003, 198:1875–1886
17. Mavragani CP, Niewold TB, Moutsopoulos NM, Pillemer SR, Wahl SM, Crow MK: Augmented interferon- α pathway activation in patients with Sjögren's syndrome treated with etanercept. *Arthritis Rheum* 2007, 56:3995–4004
18. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH: Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002, 61:554–558
19. Daniels TE, Whitcher JP: Association of patterns of labial salivary gland inflammation with keratoconjunctivitis sicca: analysis of 618 patients with suspected Sjögren's syndrome. *Arthritis Rheum* 1994, 37:869–877
20. Tarpley TM Jr, Anderson LG, White CL: Minor salivary gland involvement in Sjögren's syndrome. *Oral Surg Oral Med Oral Pathol* 1974, 37:64–74
21. Sakai A, Sugawara Y, Kuroishi T, Sasano T, Sugawara S: Identification of IL-18 and Th17 cells in salivary glands of patients with Sjögren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. *J Immunol* 2008, 181:2898–2906
22. Nguyen CQ, Hu MH, Li Y, Stewart C, Peck AB: Salivary gland tissue expression of interleukin-23 and interleukin-17 in Sjögren's syndrome: findings in humans and mice. *Arthritis Rheum* 2008, 58:734–743
23. Lee YK, Turner H, Maynard CL, Oliver J, Chen D, Elson CO, Weaver CT: Late developmental plasticity in the T helper 17 lineage. *Immunity* 2009, 30:92–107
24. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos NM, Moutsopoulos HM: Foxp3⁺ T-regulatory cells in Sjögren's syndrome: correlation with the grade of the autoimmune lesion and certain adverse prognostic factors. *Am J Pathol* 2008, 173:1389–1396
25. Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, Martin-Orozco N, Kang HS, Ma L, Panopoulos AD, Craig S, Watowich SS, Jetten AM, Tian Q, Dong C: CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol* 2008, 181:8391–8401
26. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelletier A, Lafaille JJ, Cua DJ, Littman DR: The orphan nuclear receptor ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* 2006, 126:1121–1133
27. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, Watowich SS, Tian Q, Jetten AM, Dong C: T helper 17 lineage differentiation is programmed

- by orphan nuclear receptors ROR α and ROR γ . *Immunity* 2008, 28:29–39
28. Fox RI: Sjögren's syndrome. *Lancet* 2005, 366:321–331
29. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP: IL-17 stimulates the production and expression of proinflammatory cytokines. IL- β and TNF- α , by human macrophages. *J Immunol* 1998, 160:3513–3521
30. Moseley TA, Haudenschild DR, Rose L, Reddi AH: Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* 2003, 14:155–174
31. Kolls JK, Linden A: Interleukin-17 family members and inflammation. *Immunity* 2004, 21:467–476
32. Ferraccioli GF, De Vita S: Cytokine expression in the salivary glands of Sjögren's syndrome patients in relation to tissue infiltration and lymphoepithelial lesions. *Arthritis Rheum* 1997, 40:987–990
33. Isaksen K, Jonsson R, Omdal R: Anti-CD20 treatment in primary Sjögren's syndrome. *Scand J Immunol* 2008, 68:554–564
34. Mitsias DI, Tzioufas AG, Veiopoulou C, Zintzaras E, Tassios IK, Kogopoulou O, Moutsopoulos HM, Thyphronitis G: The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjögren's syndrome. *Clin Exp Immunol* 2002, 128:562–568
35. Fox RI, Kang HI, Ando D, Abrams J, Pisa E: Cytokine mRNA expression in salivary gland biopsies of Sjögren's syndrome. *J Immunol* 1994, 152:5532–5539
36. Ohshima Y, Nakamura S, Matsuzaki G, Shinohara M, Hiroki A, Fujimura T, Yamada A, Itoh K, Nomoto K: Cytokine messenger RNA expression in the labial salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum* 1996, 39:1376–1384
37. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ: TGF- β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T_H-17 cell-mediated pathology. *Nat Immunol* 2007, 8:1390–1397
38. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S: Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007, 204:1849–1861
39. Gottenberg JE, Lavie F, Abbed K, Gasnault J, Le Nevot E, Delfraissy JF, Taoufik Y, Mariette X: CD4 CD25high regulatory T cells are not impaired in patients with primary Sjögren's syndrome. *J Autoimmun* 2005, 24:235–242
40. DeJaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M: Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* 2006, 117:289–300
41. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank R, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea J: Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 2007, 26:371–381
42. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H: Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 2007, 317:256–260
43. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Vitoria GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR: TGF- β -induced Foxp3 inhibits T_H17 cell differentiation by antagonizing ROR γ t function. *Nature* 2008, 453:236–240
44. Spachidou MP, Bourazopoulou E, Maratheftis CI, Kapsogeorgou EK, Moutsopoulos HM, Tzioufas AG, Manoussakis MN: Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjögren's syndrome. *Clin Exp Immunol* 2007, 147:497–503
45. Pasare C, Medzhitov R: Toll pathway-dependent blockade of CD4⁺CD25⁺ T cell-mediated suppression by dendritic cells. *Science* 2003, 299:1033–1036
46. Kubo T, Hatton RD, Oliver J, Liu X, Elson CO, Weaver CT: Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines produced by TLR-activated dendritic cells. *J Immunol* 2004, 173:7249–7258
47. Woo P, Wilkinson N, Prieur AM, Southwood T, Leone V, Livermore P, Wythe H, Thomson D, Kishimoto T: Open label phase II trial of single, ascending doses of MRA in Caucasian children with severe systemic juvenile idiopathic arthritis: proof of principle of the efficacy of IL-6 receptor blockade in this type of arthritis and demonstration of prolonged clinical improvement. *Arthritis Res Ther* 2005, 7:R1281–R1288
48. Maini RN, Taylor PC, Szechinski J, Pavelka K, Broll J, Balint G, Emery P, Raemen F, Petersen J, Smolen J, Thomson D, Kishimoto T: Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate. *Arthritis Rheum* 2006, 54:2817–2829
49. Krueger GG, Langley RG, Leonardi C, Yeilding N, Guzzo C, Wang Y, Dooley LT, Lebwohl M: A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. *N Engl J Med* 2007, 356:580–592
50. Mannon PJ, Fuss IJ, Mayer L, Elson CO, Sandborn WJ, Present D, Dolin B, Goodman N, Groden C, Hornung RL, Quezada M, Yang Z, Neurath MF, Salfeld J, Veldman GM, Schwertschlag U, Strober W: Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 2004, 351:2069–2079