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Role of salivary gland epithelial cells in the differentiation and activation of T lymphocytes in primary Sjögren's syndrome

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ABBREVIATION LIST

AID	enzyme activation-induced deaminase
ANA	antinuclear antibody
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
autoAb	autoantibody
autoAg	autoantigen
BAFF	B cell-activating factor
Bax	BCL2-associated X protein
Bcl-2	B-cell leukemia/lymphoma-2
BR3	BAFF receptor 3
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CHRM3	muscarinic receptor 3 gene
CIA	collagen-induced arthritis
CTL	cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DAMP	damage-associated molecular pattern
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EC	epithelial cell
ECM	extracellular matrix

FAS	cell surface death receptor
FDC	follicular dendritic cells
FoxP3	forkhead box protein 3
GC	germinal centres
HLA	histocompatibility leukocyte antigen
HSG	human salivary gland
ICAM-1	intercellular Adhesion Molecule-1
ICOS	inducible costimulatory
IFN	interferon
Ig	Immunoglobulin
IRF5	interferon regulatory factor 5
iTreg	induced Treg cells
LFA-1	lymphocyte function-associated antigen 1
LT	lymphotoxin
M3R	Muscarinic acetylcholine receptor
MCP-1	Monocyte chemotactic protein-1
MHC	major histocompatibility complex
MZ B cells	marginal zone B cells
nTreg	natural Treg
OX40L	OX40 ligand
PAMP	pathogen-associated molecular pattern
PD-1	programmed death-1
pDC	plasmacytoid dendritic cells
pSS	primary Sjögren's syndrome
RA	rheumatoid arthritis
RF	rheumatoid factor
SAP	SLAM-associated protein
SG	salivary glands

SHM	somatic hypermutation
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
SS	Sjögren's syndrome
SSA	Anti-Sjogren’s syndrome antigen A
SSB	Anti-Sjogren’s syndrome antigen B
STAT4	signal transducer and activator of transcription 4
T0 B cells	transitional type 0 B cells
T1D	type I diabetes
TCR	T cell receptor
Tfh	Follicular helper T cells
TGF-β	transforming growth factor-β
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFRSF	tumor necrosis factor receptor superfamily
TNFSF	tumor necrosis factor (ligand) superfamily
Tregs	regulatory T cells
VCAM-1	vascular cell adhesion molecule 1
VEGF-A	vascular endothelial growth factor A
VEGFR2	vascular endothelial growth factor receptor 2
VLA-4	very late antigen-4

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PUBLICATION AND COMMUNICATION LIST

Publications

GONG YZ., Nititham J., Taylor K., Miceli C, Sordet C., Wachsmann D., Bahram S., Georgel P., Criswell L. A., Sibilia J., Mariette X., Alsaleh G and Gottenberg J.E., Differentiation of follicular helper T cells by salivary gland epithelial cells and increase of IL-21 correlate with disease activity in primary Sjögren's syndrome. **(Manuscript submitted)**

GONG YZ., LI J.G., LI M., Wachsmann D., Bahram S., Georgel P., Sibilia J., Alsaleh G and Gottenberg J.E., Pathogenic role of the OX40 ligand-OX40 costimulatory pathway in primary Sjögren's syndrome. (**Manuscript submitted)**

Communications

GONG Y., Alsaleh G., Sibilia J., Wachsmann D. and Gottenberg J.E., Salivary gland epithelial cells are capable to directly induce the differentiation of IL-21-secreting follicular helper CD4 T cells in Primary Sjögren's Syndrome. International Symposium on Sjogren Symposia (ISSS). Athens, Greece. September 2011. **(Oral communication)**

GONG Y., Alsaleh G., Sibilia J., Wachsmann D. and Gottenberg J.E., Salivary gland epithelial cells are capable of inducing the differentiation of follicular helper T cells in primary Sjögren's syndrome. American College of Rheumatology (ACR). Chicago, America. November 2011. **(Oral communication)**

GONG Y., Alsaleh G., Sibilia J., Wachsmann D. and Gottenberg J.E., Les cellules épithéliales des glandes salivaires sont capables d'induire la différenciation des lymphocytes T folliculaire au cours du syndrome de Sjögren primitif. Société

Française de Rhumatologie (SFR). Paris, France. December 2011. **(Poster)**

GONG Y., Alsaleh G., Sibilia J. and Gottenberg J.E., A new pathogenic role of salivary gland epithelial cells in the costimulation of T lymphocytes in primary sjögren's syndrome: OX40 ligand expression, T-cell induction of OX40 and promotion of T-cell survival, proliferation and activation. American College of Rheumatology (ACR). Washington, D.C., America. November 2012. **(Poster)**

GONG Y., Alsaleh G., Sibilia J. and Gottenberg J.E., Rôle des cellules épithéliales des glandes salivaires dans l'induction d'OX40, la survie et la prolifération des lymphocytes T au cours du syndrome de Sjögren primitif. Société Française de Rhumatologie (SFR). Paris, France. December 2012. **(Poster)**

I. INTRODUCTION

1 Sjögren's syndrome (SS)

1.1 Definition

1.1.1 Definition and history

Sjögren's syndrome (SS) is a chronic inflammatory systemic autoimmune disease. It was first described by Swedish ophthalmologist Henrik Sjögren in 1933. It is characterized by inflammation and dysfunction of the exocrine glands which are associated with lymphocytic infiltrates and autoantibody secretion. Sjögren's syndrome can arise as a primary disease entity (primary Sjögren's syndrome (pSS)) or be associated with other autoimmune diseases (secondary Sjögren's syndrome), such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), scleroderma, primary biliary cirrhosis etc. About 5-20% of the patients with RA, SLE and scleroderma present a secondary SS [1].

1.1.2 Epidemiology

SS is the second most common systemic autoimmune disease after RA. It has been estimated that SS affects 0.1% of the general population (~0.03% for pSS). Approximately 90% of patients are female. The disease most commonly develops in the fourth and the fifth decade of life, the average age of onset is late 40s. However, males and younger or older subjects can be affected.

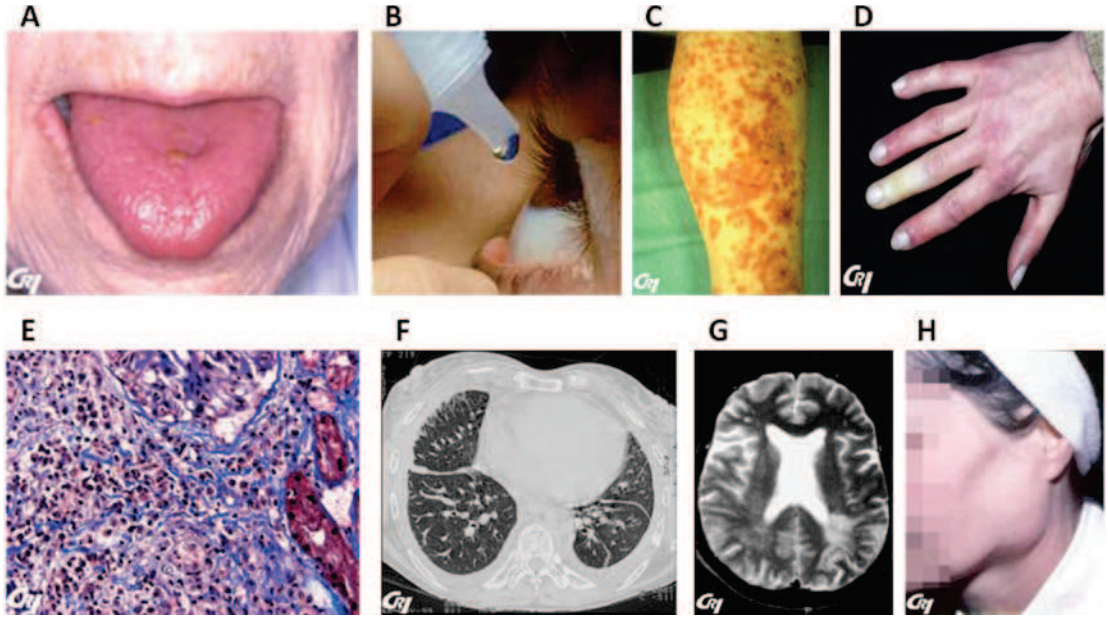
1.1.3 Symptoms and complications

Manifestations of SS are very diverse, but three types of signs are found in most patients: dryness, fatigue and pain. The clinical syndrome is caused by progressive infiltration of the exocrine glands, especially the salivary and lacrimal glands, resulting in dry mouth and dry eyes (Figure 1A and B). Other exocrine glands are also

affected, which often causes skin, nose and vaginal dryness. Asthenia and pain are most frequent. Usually, pain is related to arthromyalgia. The most frequent glandular complications of SS include caris, tooth loss, mycosis of the mouth and keratoconjunctivitis favored by dryness. Approximately one third of the patients with pSS show evidence of extraglandular involvement. Arthritis, myositis, Raynaud's phenomenon, skin vasculitis, lymphadenopathy, renal, pulmonary and central or peripheral nervous system involvement might occur (Figure 1 C-G). Lymphoma represents the most serious systemic complication of pSS.

The development of non-Hodgkin lymphoma is observed in approximately 5% of patients with SS (Figure 1 H). The risk of lymphoma is 16-18 fold higher than the general population. The mucosa associated lymphoid tissue (MALT) type and diffuse large B-cell lymphoma (DLBCL) represent the most common types of pSS-related lymphomas. Some factors have been identified as predictors for lymphoma development in SS. These factors include recurrent or persistent swelling of major salivary glands (SG), lymphadenopathy, cryoglobulinemia, splenomegaly, skin vasculitis or palpable purpura, low levels of complement factor C3 and C4, Monoclonal-component, peripheral neuropathy, glomerulonephritis, $CD4^+$ T-lymphopenia, a low $CD4^+/CD8^+$ T cell ratio and ectopic germinal center-like structures in salivary glands [2, 3].

Figure 1. Manifestations and complications of SS. **Patients with SS hace dry mouth and dry eyes (A and B), purpura (C), Raynaud's phenomenon (D), interstitial nephritis (E), pulmonary (F), central nervous system involvement (G) and non-Hodgkin lymphoma (H). Figures downloaded on the Club Rhumatismes et Inflammation (CRI) website (www.cri-net.com)**



1.1.4 Diagnosis

The diagnosis of Sjögren's syndrome is complicated by the wide range of symptoms that a patient might manifest. Complaints of dry eyes and dry mouth are common and nonspecific. Diagnosis often requires a biopsy of lip minor salivary glands to confirm the lymphocytic infiltration. The detection of rheumatoid factor (RF) and antinuclear antibodies (ANA) is very frequent. About 90% of Sjögren's patients have a positive ANA test result and 60% of patients have a positive RF [4]. Anti-Sjogren's syndrome antigen A (SSA)/Ro and anti-SSB/La are part of the diagnostic criteria. They are present in 60-80% of cases. Cytopenias (especially lymphopenia) can be observed in 20-30% of patients. A monoclonal immunoglobulin (Ig) can be detected in 10-15% of patients. Cryoglobulinemia is observed in 5% of patients. Tear flow reduction may be assessed by the Schirmer's test. The saliva production is mostly measured by the unstimulated salivary flow rate. There is often a several-year delay from the start of symptoms to diagnosis. The American–European Consensus Group criteria (AECC) for Sjögren's syndrome published in 2002 are widely used for the clinical diagnosis of Sjögren's syndrome [5] (Table I, II). Less specific criteria were recently proposed [6].

Table I. Revised international classification criteria for Sjögren's syndrome.

I. Ocular symptoms: a positive response to at least one of the following questions
1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?
II. Oral symptoms: a positive response to at least one of the following questions:
1. Have you had a daily feeling of dry mouth for more than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:
1. Schirmer's I test, performed without anaesthesia (≤5 mm in 5 minutes)
2. Rose bengal score or other ocular dye score (≥4 according to van Bijsterveld's scoring system)
IV. Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue[18]
V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:
1. Unstimulated whole salivary flow (≤1.5 ml in 15 minutes)
2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts[19]
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer[20]
VI. Autoantibodies: presence in the serum of the following autoantibodies:
1. Antibodies to Ro(SSA) or La(SSB) antigens, or both

Table II. Revised rules for classification

For primary SS
In patients without any potentially associated disease, primary SS may be defined as follows:
a. The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive
b. The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
c. The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey
For secondary SS
In patients with a potentially associated disease (for instance, another well defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS
Exclusion criteria:
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft versus host disease
Use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)

1.1.5 Genetic and environmental aspects

1.1.5.1 Genetic aspects

As in many other autoimmune diseases, genetic factors contribute to Sjögren's syndrome. A strong association to specific major histocompatibility complex (MHC)

alleles has been shown. Among the histocompatibility leukocyte antigen (HLA) haplotypes, HLA-DRB1*03, -DQA1*05, and -DQB1*02 have been consistently associated with primary SS [7]. The interferon regulatory factor 5 (*IRF5*) gene on chromosome 7q32.1 and the signal transducer and activator of transcription 4 (*STAT4*) gene on chromosome 2q32,3 are two genes in the type I interferon (IFN) system. Recent replicated studies revealed that they contribute to the genetic predisposition of pSS. The lymphotoxin (LT) system also belongs to the tumor necrosis factor (TNF) superfamily. LT-β is essential for lymphoid organogenesis and the maintenance of tertiary lymphoid tissues. Several single-nucleotide polymorphism (SNP) in the LT-α/LT-β/TNF-α locus have been found associated with pSS [8]. Monocyte chemotactic protein-1 (MCP-1, chemokine (C-C motif) ligand (CCL) 2) is associated with pSS in the Japanese population [9]. Early B-cell factor 1 (EBF1), B lymphoid tyrosine kinase (BLK), genes involved in B-cell development and activation, are associated with pSS [7]. TNFSF4 (OX40 ligand (OX40L), CD252) is also involved in the genetic predisposition to pSS. A NF-κB inhibitory protein, IκB-α, promoter polymorphism is associated with susceptibility to pSS [10]. Polymorphisms of muscarinic receptor 3 Gene (*CHRM3*) are also associated with pSS.

1.1.5.2 Environmental aspects

Virus, hormones and environmental pollutants are suspected to contribute to the pathogenesis of pSS [11, 12]. Glandular viral infection could promote epithelial cells (ECs) to activate the innate immune system through Toll-like receptor (TLRs). ECs of SS patients show high constitutive expression of TLR3. Some retrovirus such as human T cell leukemia virus type 1 (HTLV1) and human immunodeficiency virus (HIV), or hepatitis C virus (HCV) can give manifestations close to pSS, increasing sicca syndrome and lymphoid infiltrates.

Epstein–Barr virus (EBV)

A high incidence of EBV infection was reported in SS patients. EBV is a ubiquitous herpes virus that infects >90% of the general population. EBV mainly infects the salivary gland epithelial cells (SGECs) and B cells. EBV antigen and its DNA have been found in salivary gland tissues of patients with SS. Infectious EBV is present in both the saliva and in the supernatants of B cell lines of patients [13]. EBV reactivation is thought to contribute to the initiation or perpetuation of tissue destruction in SG and lacrimal glands in SS. There is a cross-reactivity between EBV-derived antigens (Eber1 and Eber2) and anti-SSB. These results suggest that EBV might play a role in the pathogenesis of SS. Recently, Inoue and colleagues showed binding of the aryl hydrocarbon receptor (AhR) to environmental pollutants stimulate BZLF1 transactivation which mediates the switch from the latent to the lytic form of EBV infection in the saliva of SS patients [14].

The fact that SS is predominant in postmenopausal women suggests that the lack of estrogen may involve in the etiology of pSS. Functional estrogen receptors (ER) α and β are detected in SGECs. Studies of several estrogen deficiency mouse models shown the development autoimmune exocrinopathy resembling SS. Ishimaru et al., found that estrogen deficiency activates SGECs and $CD4^+$ T cells and induces the release of IFN-γ [15].

1.1.6 Treatment

Up to now, there is no specific treatment which has demonstrated its efficacy in reducing the severity of clinical signs of the disease or in restoring gland secretion. The therapeutic approach is based on symptomatic treatment of glandular manifestations. Artificial tears and saliva substitutes might ease the oral and ocular dryness. Punctual occlusion can help to retain tears on the ocular surface [16]. Pilocarpine is often used to increase salivary secretion. Non-steroidal anti-inflammatory drugs (NSAIDs) may be used to treat musculoskeletal symptoms.

Corticosteroids or immunosuppressive drugs might be used for systemic involvement.

Anti-TNF-α therapy is used to treat other autoimmune disease like RA. A randomized, double-blind, placebo-controlled study of infliximab (Remicade ®), a chimeric monoclonal anti-TNF-α did not show any evidence of efficacy in pSS [17]. Another TNF inhibitor, etanercept, was also ineffective. Actually, TNF blockade results in increased plasma levels of IFN-α and B cell-activating factor (BAFF), which might explain the inefficacy of anti-TNF in pSS [18].

Given the importance of B cells in the pathophysiology of the disease, a therapeutic approach inhibiting B lymphocytes is very attractive. The randomized trial of Rituximab showed a significant improvement of dryness whereas another randomized trial was negative (Saraux A et al., unpublished data). Registry data suggested the interest of Rituximab in patients with systemic involvement [19]. A recent open trial suggested the potential interest of belimumab, a monoclonal antibody targeting BAFF in pSS (Mariette X, et al., unpublished data).

1.2 Physiopathology

The etiology of Sjögren's syndrome remains unclear. Several factors such as genetic predisposition and environmental factors (mainly infectious, hormonal) influence the development of Sjögren's syndrome. The pathogenetic mechanisms of Sjögren's syndrome have not been fully elucidated. A widely accepted model is that in genetically predisposed individuals various environmental factors might drive glandular epithelial cell activation and apoptosis, which triggers autoimmune - mediated tissue injury.

1.2.1 Mice models of SS

There are numerous spontaneous and experimentally induced mouse models of

pSS. Typically, these mouse models show lymphocyte infiltration of the exocrine glands, increased expressions of pro-inflammatory cytokines, present autoantibodies and impair secretory function.

The MRL/*lpr* mouse

MRL/*lpr* mouse is originally a model of SLE. The mice carry a mutated *lpr* gene encoding a defective tumor necrosis factor superfamily, member 6 (TNFSF6) (Fas) protein which results in failure of apoptosis and clonal deletion of lymphocytes in peripheral lymphoid organs. MRL*/lpr* mice also develop inflammation in the lacrimal glands and SG associated with autoantibodies such as anti-SSA, anti-SSB and anti-M3R. MRL/*lpr* mouse is considered as a model of secondary SS.

The NOD mice

The NOD mice is also a model of type I diabetes (T1D). They have the similar manifestations as human SS including the presence of lymphocytic infiltration of the exocrine glands, autoantibodies and decreased secretory function. Its congenic strain NOD.B10-$H2^b$, is also a model for primary SS, without T1D. [20]

The IQI/Jic mouse

IQI/Jic mice develop focal lymphocytic infiltration in both SG and lacrimal glands. Infiltrating lymphocytes consist of T and B cells. Autoantibodies are detected as well. The ductal SGECs have a MCH class II-restricted capacity to present autoantigens [21].

The C57BL/6.NOD-*Aec1Aec2* mouse

The C57BL/6.NOD-*Aec1Aec2* mouse is a C57BL/6 mouse carrying both the *Aec1 (Idd3)* and *Aec2 (Idd5)* genetic regions derived from NOD mice. This model

maintains the NOD SS-like disease profile in the absence of T1D susceptibility [22].

The *Baff* gene knock-in mouse

Transgenic mice for BAFF develop autoimmune-like manifestations, such as increased numbers of mature B cells and effector T cells, the presence of RF, circulating immune complexes, anti-DNA autoantibodies, immunoglobulin deposition in the kidneys [23].

The AdV5-infected C57BL/6 mouse

Bombardieri and his colleagues have recently developed a novel model of pSS. They have delivered a replication-deficient adenoviral vectors (AdV5) in submandibular glands of wild-type (WT) C57B1/6 mice [24]. The mice rapidly developed sialoadenitis characterized by immune cell infiltration evolving into ectopic GC and secrete autoantibodies. The secretion of lymphoid chemokines precedes the development of ectopic GC (CXCL13, CCL19, LT-β).

Poly I:C treated NZB/W F1 mouse

Polyinosinic:polycytidylic acid (Poly I:C) treated NZB/W F1 mouse is one of the inducible mice models of pSS. Poly I:C treatment rapidly up-regulates type I IFN and inflammatory cytokines in the submandibular glands, which results in dryness [25].

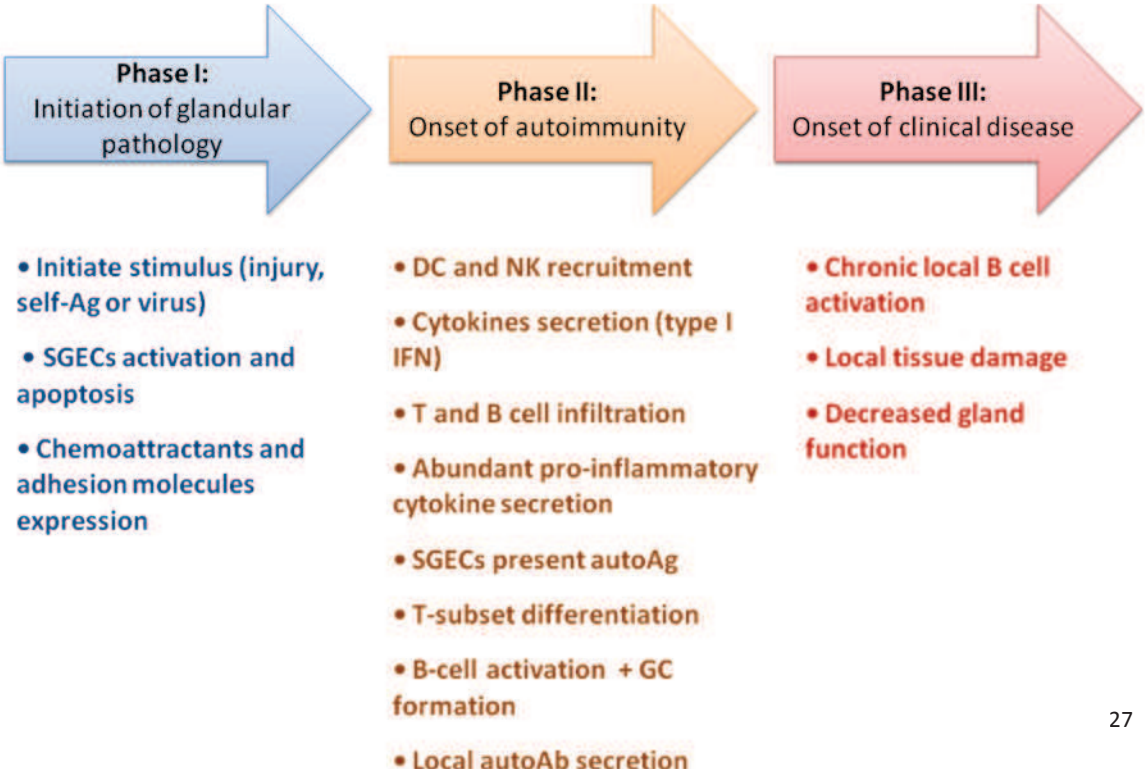
MCMV infected mouse

Murine CMV (MCMV) infected autoimmune-prone NZM2328 mice have acute and chronic glandular disease which resembles human SS. These mice develop severe chronic periductal inflammation in both submandibular and lacrimal glands and have decreased secretory function [26].

1.2.2 Scenario for the pathogenesis of pSS

Based on the results obtained from mouse models of SS or findings observed in patients, it is proposed that SS progresses through three phases (Figure 2). In phase I, in a genetically predisposed individual, a primary infection trigger of SGECs is thought to initiate an immune response. Some chemotactic signals or cytokines contribute to acinar cell apoptosis. In phase II, NK and dendritic cells (DCs) firstly infiltrate the SG and lacrimal glands, secrete IFNs, followed by $CD4^+$ T cells and B cells. Both innate and acquire immune system are then activated. At the same time, epithelial cells function as antigen-presenting cells (APC) to activate T cells. Then, abundant pro-inflammatory cytokines are secreted in the microenvironment. In some patients, ectopic germinal centers develop inside salivary glands. In phase III, B lymphocytes are activated to generate autoantibodies (autoAb). Autoantibodies contribute to local tissue damage and impairment of salivary and lacrimal gland secretory functions [22, 27, 28].

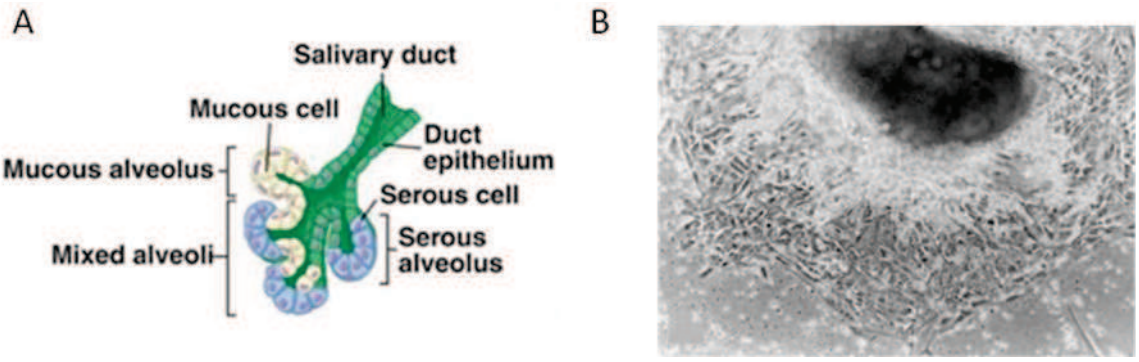
Figure 2. A proposed scenario for the pathogenesis of pSS.



1.2.3 The pathogenic role of epithelial cells

Numerous studies have emphasized the key pathogenic role of SGECs in pSS. The salivary glands are composed of two subsets of epithelial cells: acinar and ductal cells (Figure 3A and B). These two cell types play very different roles in the formation of saliva. Acinar cells are water permeable and salt secreting, whereas ductal cells are relatively water impermeable and salt absorbing. In 2002, Dimitriou *et al*., have published a protocol for the establishment of human non-neoplastic SGEC lines (of ductal type) from a single lobule of labial minor salivary glands for the study of the physiology and pathophysiology of these cells [29] (Figure 3C).

Figure 3. A schema of acinar SGECs and ductal SGECs in salivary glands and human non-neoplastic SGEC lines. **Figures downloaded on Quizlet website (quizlet.com). SGECs outgrowing from a small fragment of labial minor salivary glands after 5 days of culture (B) [29].**



pSS is considered as an autoimmune epithelitis in which salivary gland epithelial cells play a crucial pathogenic role. The immunohistochemical analysis of inflamed salivary gland tissues of patients has indicated that ductal and acinar SGECs display a wide range of MHC molecules, TLRs, costimulatory molecules. These

molecules are known to mediate lymphoid cell homing, antigen presentation, neovascularization and the amplification of epithelial–immune cells interactions. Molecules expressed by salivary gland epithelia cells in SG tissues or in primary culture of SGECs in SS patients, as well as the results obtained from mouse model of pSS, are summarized in table III. This indicates that SGECs are not only victims but also initiators or amplifiers of the autoimmune process.

Table III: Molecules expressed by salivary gland epithelial cells in Sjögren's patients and mice models of SS.

Classification	**Molecules**	**In SG biopsy**	**Cultured SGECs**	**Mouse SG biopsy**	**Ref**
MHC class I	HLA-ABC	+	+		
MHC class II	HLA-DR	+	+		
	HLA-DP				
	HLA-DQ				
TLRs	TLR2	+	+*		[30]
	TLR3				
	TLR4	+			
	TLR6	+			
Costimulatory Molecules	Fas	+			
	FasL	+			
	CD80			+	[31]
	CD86			+	
	CD40				
	OX40L			+	
	PD-1L	+			[32]
	4-1BBL			+	

	GITRL			+	
Cytokines	BAFF			+	
	IL-6		+*		[33]
	TGF-β		-*		
	TNF-α	+	+		
	IL-18		+		
	IL-12	+	+		
	IL-1β			+	
Chemokines	CCL3 (MIP-1α)	+			
	CCL4 (MIP-1β)	+			
	CCL5 (RANTES)	+			
	CXCL1 (GRO-α)	+	+*		[34]
	CCL17 (TARC)	+			[35]
	CCL12 (MDC)	+			
	CXCL9 (Mig)	+			[36]
	CXCL10 (IP-10)	+			
	CXCL13	+			[37]
	CCL21	+			
	CXCR3	+			[38]
	CCL19	+			
	CXCL8 (IL-8)	+			
	CXCL11	+			[39]
	CXCL12	+			
Angiogenesis	VEGF-A	+	+*		[40]
	VEGFR2	+	+*		
Adhesion	α6β4 integrin	-			[41]
	ICAM-1	+	+		

	VCAM-1	+	+		
	E-cadherin	+			
	E-selectin	+			

+/- : up- or downregulated in patients with pSS compared with controls in basal conditions.

+/-*: up- or downregulated in patients with pSS compared with controls after various stimulations

FAS, cell surface death receptor; 4-1BBL, cell surface death receptor 4-1BB ligand; GITRL, glucocorticoid-induced TNF receptor-related protein ligand; TGF-β, transforming growth factor-β; CXCL, chemokine (C-X-C motif) ligand; MIP-1α/CCL3, macrophage inflammatory protein-1α; MIP-1β/CCL4, macrophage inflammatory protein-1β; RANTES/CCL5, regulated upon activation normal T expressed and secreted; GRO-α/CXCL1, growth related oncogene-alpha; TARC/CCL17, thymus and activation-regulated chemokine; MDC/CCL22, macrophage-derived chemokine; MIG/CXCL19, monokine induced by IFN-γ; IP-10/CXCL10, IFN-γ-inducible 10-kd protein; VEGF-A, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2; ICAM-1, Intercellular Adhesion Molecule-1; VCAM-1; vascular cell adhesion molecule 1.

1.2.3.1 SGEC activation by PAMPs and DAMPs

The expression by SGECs of several TLRs indicates their role in the induction of innate immune responses upon the recognition of pathogen-associated molecular patterns (PAMPs). TLR signaling has been shown to result in the production of proinflammatory cytokines and in the upregulation of costimulatory and adhesion molecules resulting in the activation of adaptive immune response. TLRs are considered as a critical link between innate and adaptive immunity. In SG of patients

with pSS, ductal SGECs and infiltrating mononuclear cells express TLR2, TLR4 and TLR6. Cultured SGECs express TLR2, TLR3 and TLR4 when stimulated with ligand of TLR2 (peptidoglycan (PGN)) and TLR3 (poly-inosinic cytidylic acid (poly I:C)) respectively. Stimulations by TLR2, TLR3 and TLR4 ligands up-regulate ICAM-1, CD40, CD86 and MHC-I expressions in cultured SGECs [42]. Treatment of NZB/W F1 mice with poly I:C increases type I IFN and multiple chemokine production, then accelerates sialoadenitis [43]. In pSS, TLR signal also induces apoptosis of SGECs. Only the ligand of TLR3, but not TLR2 or TLR4, induces apoptosis of SGECs from both patients with pSS and normal subjects.

Extracellular ATP can act as a damage-associated molecular pattern (DAMP) under many pathological conditions. High level of extracellular ATP is associated with inflammation and cell apoptosis. Woods et al., suggest that treatment of mouse submandibular gland cell aggregates with ATP or its high affinity agonist BzATP induces membrane blebbing, enhances caspase activity and releases α-fodrin via P2X7R in vitro. In addition, administration in vivo of BzATP in mouse SG enhances immune cell infiltrations and initiates apoptosis of SGECs. These results suggest that DAMPs such as ATP could play a role pathogenic in SS [44].

1.2.3.2 ECM remodeling and homeostatic changes in epithelial tissues

Epithelial tissues rely heavily on the extracellular matrix (ECM) to maintain structure and function. Some studies described that changes in ECM molecules is the first signs of homeostatic changes in epithelial tissues. In human SG biopsies, major ECM laminin 1 and 5 increase their expression before lymphocytic infiltration. With C57BL/6.NOD-*Aec1Aec2* Mice, Peck et al., showed that during the pre-autoimmune phase, molecules associated with interepithelial tight junctions complexes are differently expressed [45]. These complexes are important not only for mechanical adhesions but also for the growth, the differentiation, the morphogenesis, the

migration and the extrusion of apoptotic cells. In patients with ECM alterations, downregulation of adhesion molecule α6β4 integrin makes acinar cells fail to maintain their survival [41]. The homeostasis of epithelial tissues could be altered by some proinflammatory cytokines. TNF-α and IFN-γ could alter the tight junction structure and the function in the rat parotid gland cell line [46].

1.2.3.3 Increased apoptosis and NF-κB activity in epithelial cells

Apoptosis has been proposed as a possible mechanism responsible for the impairment of exocrine gland secretory function associated with SS (Figure 4). Apoptosis is important for tissue remodeling because it is necessary in cell proliferation in the regenerating tissues. A significant reduction of acinar SGECs is observed in patients with pSS. Histopathologic lesions are associated with increased apoptosis. Both ductal and acinar SGECs display elevated *in situ* apoptosis and the ductal SGECs display elevated proliferation. Moreover, cultured SGECs undergo apoptotic process after stimulation with TNF-α and IFN-γ.

Both the Fas/FasL (CD95/CD95L) pathway and the substances (perforin, granzymes) released by cytotoxic T lymphocytes (CTL) are expressed in the SG of patients with pSS. Ductal and acinar SGECs express the Fas and FASL whereas infiltrating T cells express FasL [47]. So apoptotic death of ECs may be caused by autocrine Fas/FasL interaction at the epithelial cell level or by paracrine interaction with infiltrating T cells. The imbalance between the pro-apoptotic proteins BCL2-associated X protein (Bax) and anti-apoptotic B-cell leukemia/lymphoma-2 (Bcl-2) observed in SGECs of patients with pSS might cause increased apoptosis. Converse to SGECs, the infiltrating mononuclear cells present a high Bcl-2/Bax expression ratio. This imbalance may explain their resistance of lymphocytes to apoptosis despite Fas expression. CD4 or CD8 CTL can also induce EC apoptosis by perforin and granzyme B release and the secretion of cytokines, such as TNF-α, IFN-γ

and TGF-β1. At last, direct B cells contact can induce apoptosis of EC line (HSG) in vitro. This B cells-mediated cell death is not dependent on Fas/FasL interactions but requires translocation of protein kinase C delta (PKC δ) into the nucleus of epithelial cells [48].

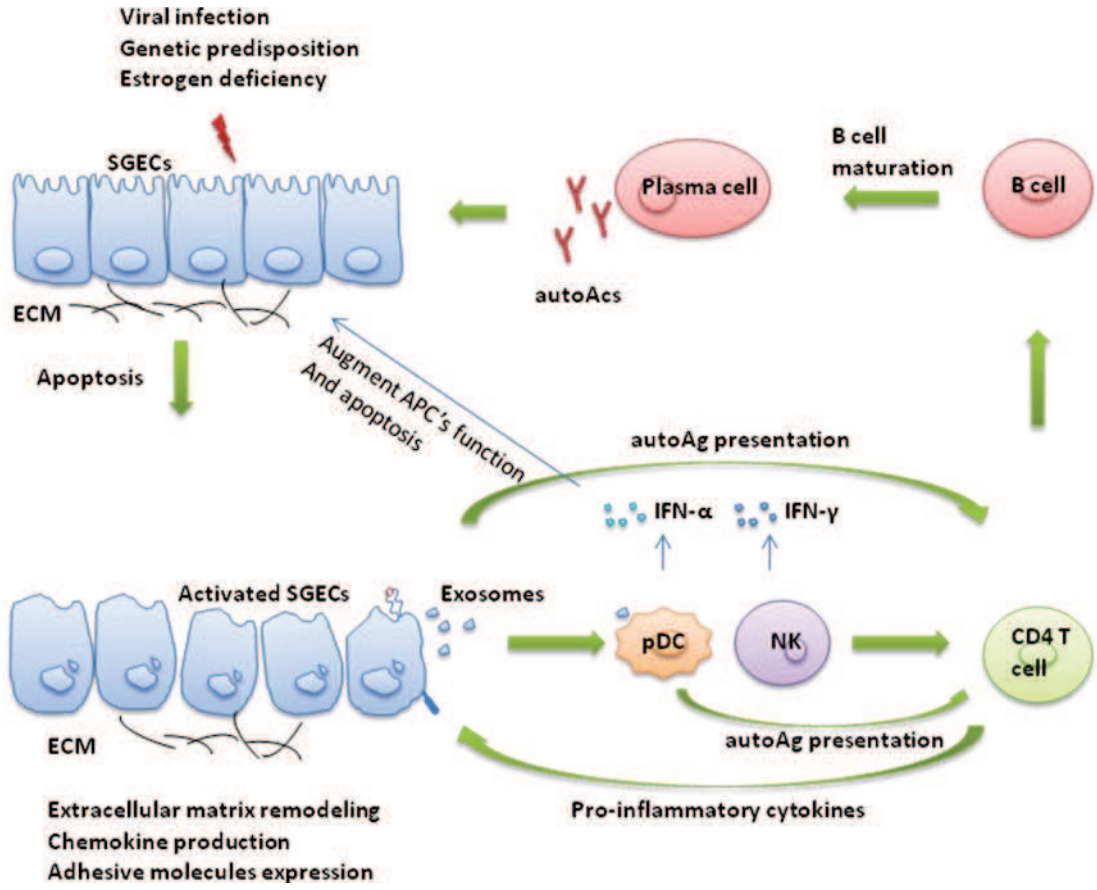
It has been noted that in SS, aberrant apoptosis is not only the consequence but also the trigger of lymphocytic infiltrates. In NOD-*scid* mice, a glandular EC apoptosis occurs in the absence of lymphocytic infiltration. Increased apoptosis of acinar SGECs as well as increased IFN-γ expression occur before infiltration by lymphocytes in NOD mice. In C57BL/6.NOD-*Aec1Aec2* mice, altered glandular homeostasis and elevated apoptotic epithelial cells are observed in the submandibular glands on the 8th week before disease onset [49]. Using neonatal NOD mice, Cha et al., revealed that acinar cell proliferation was reduced while expression of Fas, FasL and bcl-2 were increased in submandibular glands on the 1st day postpartum [50]. Recently, Okuma et al., demonstrated that IκB-ζ-deficient epithelial cells exhibited increased apoptosis. This is sufficient to elicit SS-like pathology in mice, such as lymphocytic inflammatory infiltrates of the lacrimal glands with reduced tear secretion and secretion of anti-SSA and anti-SSB. IκB-ζ regulates NF-κB transcriptional activity, thus being implicated in both cell survival and apoptosis [51]. The expression of another NF-κB inhibitory protein IκB-α is downregulated in SS SGECs [52].

1.2.3.4 Autoantigens presentation by SGECs

Activated SGECs in SS lesions appear to be suitably equipped for the presentation of antigens to T cells and the subsequent crosstalk between T and B cells leading to autoantibody secretion. SGECs express HLA-DR, CD80 and CD86 molecules in close proximity to the lymphocytic infiltrates [53]. Moreover, IFN-γ can induce or upregulate the expression of those molecules in cultured controls or SS

SGECs [54]. The Ro52, Ro60 and La autoAgs are components of human Ro/La ribonucleoprotein (RNP) complex. Normally, the localization of these proteins is mainly nuclear. Under certain physiopathological conditions (e.g. stress, UV radiation or viral infection), these proteins can be found on cell surface. Acinar ECs express autoantigens on their membrane. More specifically, antigen La has been observed in conjunctival epithelial cells of SS patients. In fact, two mechanisms are implicated in the antigen presenting process. Epithelial cells could either express autoantigens on their membrane or release exosomes containing autoantigens (Ro, La and α-fodrin) [4] (Figure 4). The expression of HLA-DR^+ by epithelial cells in SG of patients is closely associated with T-cell infiltrates [55]. In addition, autoAg La-derived peptide contains T cell epitopes and can trigger T-cell proliferation [56]. These data in literature strongly suggest that SGECs are capable to present autoAg to T cells.

Figure 4: A schematic model for early events in the pathogenesis of lymphoepithelial lesions in the salivary glands of SS patients. Environmental and hormonal factors in an appropriate genetic background activate glandular ECs. ECs undergo apoptosis and recruit DCs and NK following T cells via chemokines and specific adhesion molecules productions. Altered extracellular interactions facilitate immune cell migration and amplify the apoptosis of ECs. ECs activate T cells by direct presentation of autoAgs and MHC II and costimulatory molecule expression as well as by the release of autoAg-containing exosomes. The early recruitment of plasmacytoid dendritic cells (pDC) and NK produce high levels of type I and II IFNs that increase the antigen presentation capability of ECs. Activated T cells further activate ECs by secreting proinflammatory cytokines. At last, B cells are recruited into SG leading to plasma cells differentiation and autoAbs production. All the previous step result in local B cells differentiation into autoAb secreting plasma cells.



1.2.3.5 In-situ activation and immunologic functions of SGECs in SS

In patients with pSS, the capacity of SGECs to secrete cytokines and chemokines has been previously reported. Cultured SGECs from SS patients also showed an abnormal cytokine production. Cultured SS SGECs secret more IL-6 and less TGF-β upon IFN-γ stimulation compared with normal controls. This may affect the local balance between Tregs and Th17 cells. The pro-inflammatory cytokine IL-18 is detected in the acinar SGECs of SS patients. IL-18 could amplify the production of IL-6 and IL-8 induced by IL-17 in salivary gland cells (human parotid gland cell line HSY) [57]. Using the autoimmune regulator (Aire)-knockout (KO) mice, Chen et al., have demonstrated that the induction of SS-like lacrimal exocrinopathy mediated by autoreactive $CD4^+$ T cells depends on IL-1 receptor type 1 (IL-1R1) signaling. IL-1R1 is detected only on resident ductal lacrimal gland epithelial cells but not on resident

APCs or infiltrating immune cells. Moreover, IL-1 expression in ocular epithelial cells is significantly correlated with the development of ocular pathological changes. These results indicate that in targeted tissues, the interplay between resident epithelial cells and $CD4^+$ T cells plays a central role in the pathogenesis of SS [58]. SGECs are also capable to secrete BAFF after type I/II IFN stimulations, TLR activation or viral infection [59].

Although T cells proliferate locally in SGs, it seems that much of the infiltration is due to the migration of peripheral blood T cells. In the SG of patients, the ductal SGECs express various chemokines and the adjacent infiltrating T lymphocytes express their cognate receptors (see 1.2.9). This suggests that SGECs are involved in the migration of T cells into salivary glands. CXCL10 (IP-10) antagonist decreases the infiltration of Th1 type $CXCR3^+$ T cells and improves sialoadenitis in MRL*/lpr* mice [60].

SGECs also express costimulatory molecules and adhesion molecules to act as nonprofessional APCs. All these signals as well as molecules mediating APC-T cell adhesion are essential in the formation and stabilization of the immunological synapse which is requested for an effective activation of T cells. In pSS patients with severe sialoadenitis, CD80 and CD86 are strongly expressed on ductal epithelial cells and their ligand CD28 is expressed on some infiltrating cells. In SG biopsies, CD40 is constitutively expressed by lymphocytes, ductal epithelial cells and endothelial cells. CD40 expression is significantly higher in cultured SS SGECs, which could be further enhanced by IFN-γ and IL-1β. CD40L staining is detected in 30-50% of the infiltrating lymphocytes in the biopsies of SS patients [61]. Adhesion molecule ICAM-1, VCAM-1 and E-selectin are detected on duct cells from all patients. These findings indicate SGECs may be involved in the induction and the maintenance of lymphocytic infiltrates.

1.2.4 Salivary gland infiltrates.

Salivary glands are the best-studied organs because they are affected in almost all patients and are readily accessible. The diagnostic of SS often needs a minor salivary gland biopsy. In minor salivary gland, lymphocytic infiltrate mainly consists of activated T and B cells and antigen presenting cells. Poly I:C-treated NZB/W F1 mice showed that dendritic cells and NK cells dominate early cell infiltrates, followed by $CD4^+$ T cells [43]. T lymphocytes (composed of 33% of $CD4^+$ T cells, 15% of $CD8^+$ T cells, 3% of $CD4^+CD8^+$ T cells and 2% of $Foxp3^+$ Tregs) and B lymphocytes represent 90% of infiltrating cells and have variable frequency. T cells predominate in mild lesions, whereas B cells do in advanced lesions. T cells decrease, whereas B cells increase with the extent of infiltration. Macrophages and dendritic cells are also found in up to 5% of infiltrates, mainly in glands with ectopic GCs. NK cells are very rare but were less studied until recently [62]. The presence of macrophages varies according to the severity of lesion. The proportion of major regulators of immune response, $Foxp3^+$ Tregs, is found to decrease with lesion severity. This suggests an inability of regulatory mechanisms to control local immune activation [63].

1.2.5 T-lymphocyte contribution to the pathophysiology of pSS

The predominant cells in SG infiltrates are T cells with a CD4/CD8 ratio of 4:1. Almost all infiltrating T cells express αβ T-cell receptor (TCR), only 1-5% express γδ receptor. These CD4 T cells present an activated (HLA class II^+, $CD25^+$) and primed ($CD45RO^+$) phenotype. On the contrary, T cells in peripheral blood do not to have the same characteristics (absence of expression of HLA class II molecules) [64].

1.2.5.1 T-lymphocyte migration to exocrine tissue

T-cell migration is mediated by chemotactic factors and interactions with

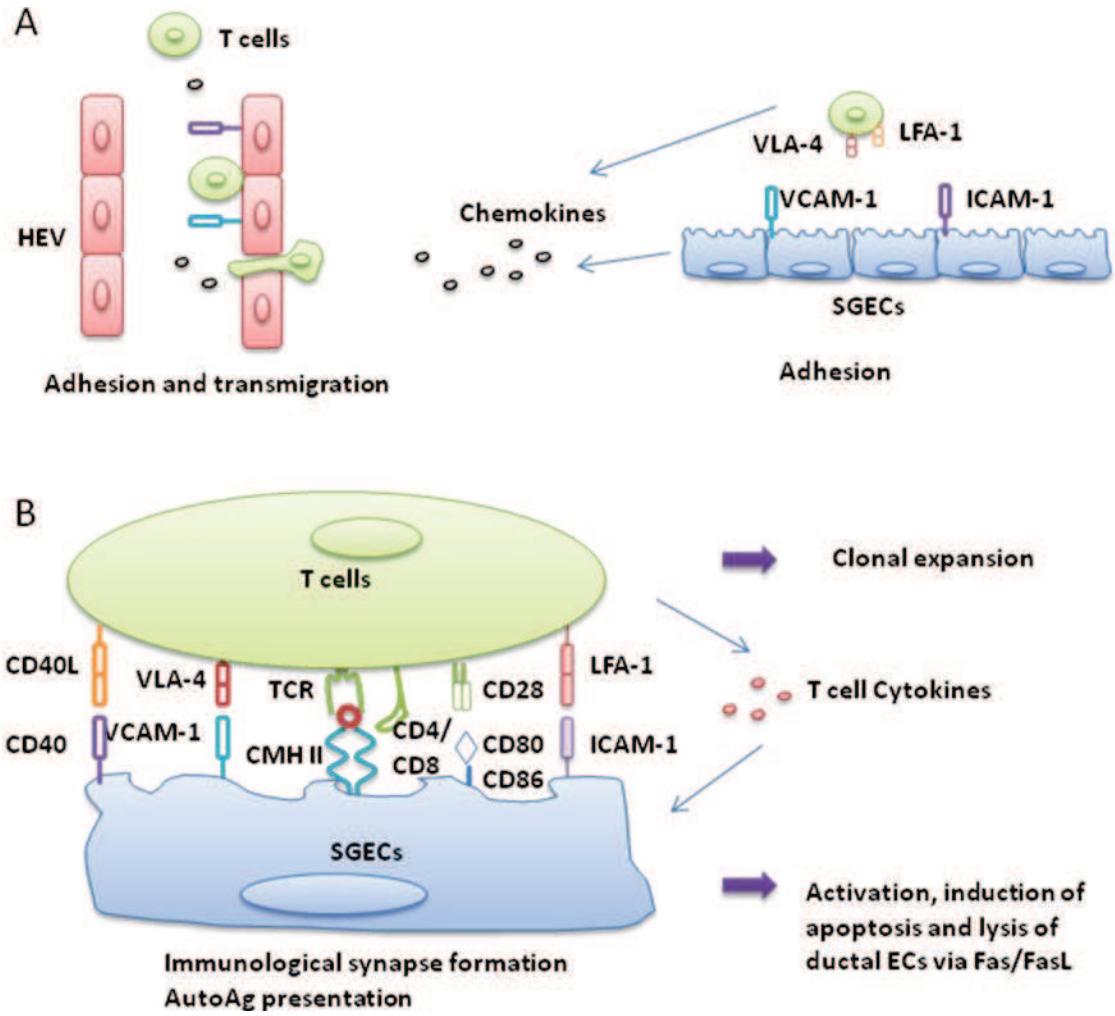
adhesion molecules expressed by vascular endothelium and epithelial tissues. Various chemokines play a role in recruiting T cells. CCL21, CXCL9, CXCL10, CXCR3, CXCL11 and CXCL12 are expressed by SGECs while CXCR4 (ligand of CXCL12) and CXCR3 (ligand of CXCL9, CXCL10) are expressed by activated T cells. SGECs also express ICAM-1 and VCAM-1. These adhesion molecules bind to their ligands expressed on T cells, lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4). These interactions first play a role in T cell-recruitment into salivary gland (Figure 5A) and are also involved in the antigen presentation process and the formation of the immunological synapse (Figure 5B).

1.2.5.2 In situ antigen-driven stimulation of T lymphocytes

Assessment of the T cell receptor repertoire of infiltrating T cells reveals that TCR Vβ gene usage is relatively restricted. This fact suggests an antigen-driven stimulation rather than a superantigen-induced proliferation. In fact, autoantigens (Ro, La and α-fodrin)-specific T cells are detected in patients with SS. DCs, macrophages and activated SGECs function as APCs to activate antigen-specific T cell. Activated SGECs express all the molecules (MHC I and II, CD80, CD86, CD40) needed for antigen presentation and T cell activation. TCR interacts with the MHC–peptide complex in the immunological synapse between SGECs and T cells. The co-stimulatory signals are provided by different pairs of molecules including CD80/CD86 - CD28 and CD40-CD40L. Adhesion molecules stabilize the immunological synapse. After that, engaged T cells undergo proliferation and secrete cytokines. T cells infiltrated in SG are oligoclonal and some proliferating T cells can be detected in the SG of patients [65].

Figure 5. Molecular mechanisms involved in T cell migration into exocrine glands and in the immunological synapse between T cells and SGECs. **A.** Environment triggers lead to the upregulation of adhesion molecules on

endothelial and epithelial cells and to the expression of chemokines. T cells are recruited by chemokines, and interact with adhesion molecules found on vascular endothelium. T cells come across the endothelial vessels and interact with SGECs. B. Two signals are required for T-cell activation that leads to clonal expansion and cytokine secretion. TCR interacts with MHC II-peptide complex as well as CD4 or CD8 to provide the signal-1. Costimulatory molecules CD80/CD86-CD28 and CD40-CD40L are delivered as signal-2. Adhesion molecule interactions such as ICAM-1-LFA-1 and VCAM-1-VLA-4 stabilize the immunological synapse. This interaction leads to clonal expansion of T cells, activation and induction of apoptosis of SGECs and release of T-cell cytokines.



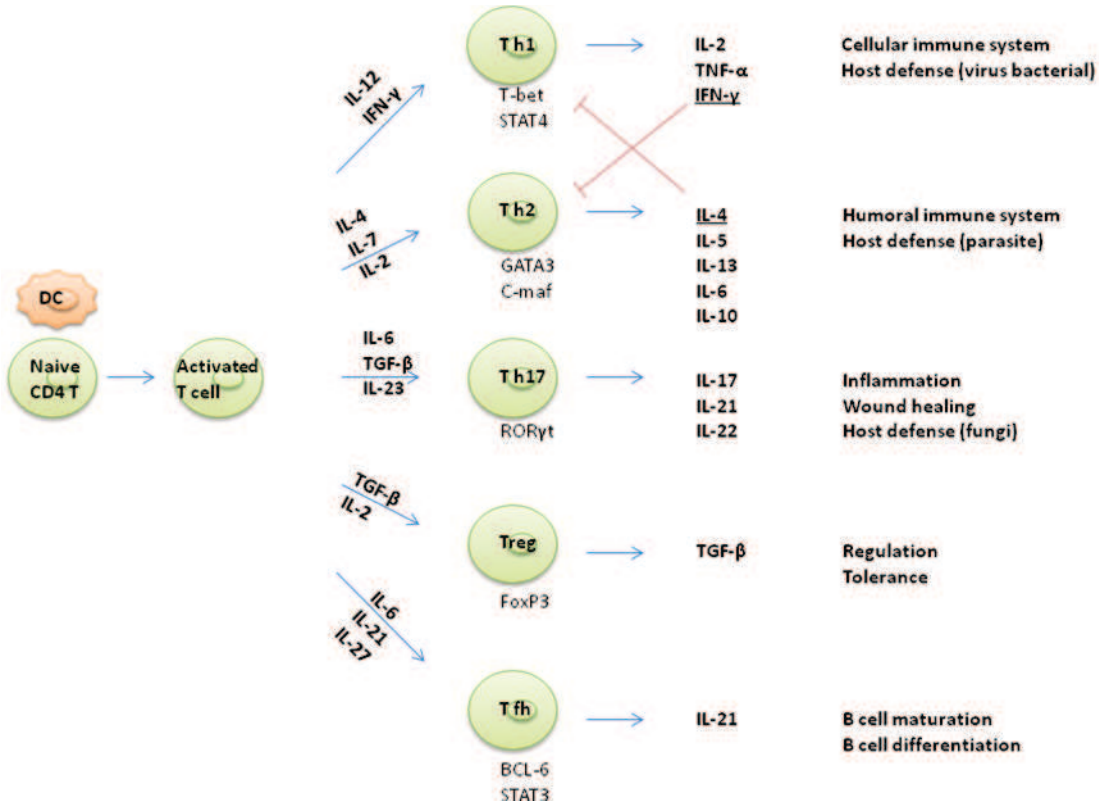
1.2.5.3 T- cell- mediated immunopathogenesis

In pSS, both CD4 helper T cells and CD8 cytotoxic T cells drive tissue lesions in the exocrine glands via different mechanisms. $CD8^+$ T cells are located around the acinar ECs. CD103 (αEβ7 integrin) expressed by CD8 T cells interact with E-cadherin expressed by adjacent SS acinar ECs, and mediates the adhesion between CD8 T cells and acinar ECs. CD8 T cells induce EC apoptosis via both the perforin/granzyme B and Fas/FasL pathways [66]. $CD4^+$ T cells with cytotoxic activity are detected in immunopathologic lesions of minor SG of SS patients. They comprise 20% of the infiltrating lymphocytes and utilize perforin-mediated cell lysis.

1.2.5.4 T helper cell subsets and cytokine profile in pSS.

During TCR activation, naive CD4 T cells differentiate into different lineages of T helper cells, including Th1, Th2, Th17, Tfh and Treg. IL-12 and IFN-γ activates STAT4 and induces T box transcription factor (T-bet) which is specific to Th1 cells. Th1 cells produce Il-2, TNF-α and IFN-γ to promote cellular immune system. Similarly IL-2, IL-7 and IL-4 induces Th2 cells through STAT6, WGATAR nucleotide consensus sequence (Gata-3) and c-maf and promote humoral immune system. The signature cytokines of Th2 are IL-4, IL-5, IL-6, IL-10 and IL-13. IFN-γ inhibits the production of Th2 type cytokines such as IL-4, while IL-10 inhibits a variety of Th1 cytokines including IL-2, IFN-γ and IL-12. Recently, some new cytokine and lineage of CD4 T cells were distinguished. In 2003, Th17 cells were characterized by the production of IL-17A, IL-17F and IL-22 as signature cytokines, as well as retinoid orphan receptor γt (RORγt) as a specific transcription factor. Th17 can be induced by IL-6 and TGF-β. Th17 cells also produce IL-21. It was shown that cells with regulatory function could be induced from naïve CD4 by IL-2 and TGF-β. Distinct from natural regulatory T cells (nTreg), which are not derived in the periphery from naïve CD4 T cells, these cells were designated induced Tregs (iTregs). Induced Treg also express the FoxP3 (Figure 6).

Figure 6. T helper cell lineage development and function. Figure adapted from [67]



In SG from patients with pSS, the expression of cytokines and transcription factors of T helper subsets such as Th1, Th2, Th17 and follicular helper T cells are significantly increased in comparison with controls. The localization of these T helper subsets is associated with disease severity. Th2 and Tfh are closely associated with strong lymphocytic infiltrations, converse to Th1, Th17 and Tregs. Th1 and Th17-related molecules are mostly detected in SG without GC, while Th2 and Tfh-related molecules are more frequently detected in SG with GC. It has been proposed that SS could be initiated by Th1 and Th17 cells, and then be replaced by Th2 and Tfh cells in patients with GC [68].

1.2.5.5 Th1/Th2 balance

It has been proposed that Th1 response is favored in patients with SS and some

Th2 cytokines were also found elevated. Thus, both Th1 (IFN-γ) and Th2 cytokines (IL-4, IL-13) are produced by lymphocytes infiltrating SG of patients [69]. In another report, infiltrating $CD4^+$ T cells mainly secreted IL-2, IFN-γ and IL-10, but neither IL-4 nor IL-5 [70]. Th1 cytokines were associated with the importance of T-cell infiltration whereas Th2 cytokine, such as IL-4 and IL-5, were rather associated with B cell infiltrates [71]. Th1/Th2 balance in salivary glands might also depend on the duration of symptoms. Chemokines involved in the Th1/Th2 balance in pSS are described in chapter 1.2.9.

1.2.5.6 Th17 cells, IL-17 and IL-23

Some studies suggested pathogenic role for Th17 cells in pSS. Cytokines required for the differentiation and the maintenance of Th17, TGF-β, IL-6 and IL-23 are all detected in SGs or in the serum of patients with pSS. High level of IL-17 is found in serum and saliva of pSS patients. IL-17 is predominantly expressed by infiltrating $CD4^+$ T cells rather than by $CD8^+$ T cells [57]. Th17 memory cells are detected within the exocrine glands of C57BL/6.NOD-*Aec1Aec2* mice and human pSS patients. Blockage of IL-17 expression in C57BL/6.NOD-*Aec1Aec2* mice results in decreased SG lymphocytic infiltration and increased saliva secretion.

1.2.5.7 Regulatory T cells

There are two main populations of Tregs: thymus-derived natural Treg (nTreg) cells and peripherally generated induced Treg (iTreg) cells. Both are forkhead/winged helix transcription factor (Foxp3) positive cells. nTreg are critical for the control of immune response, including autoimmunity. Following engagement of their TCR, Tregs suppress the proliferation and the activity of conventional effector $CD4^+$ T cells as well as that of $CD8^+$ T cells.

$Foxp3^+$ Tregs infiltrate SGs of SS patients and their proportion is associated

with importance of the inflammatory infiltrate. In the peripheral blood, the incidence of Tregs is higher in SS patients than in healthy individuals [72]. The migration of Tregs is inversely correlated in minor SG lesions and in peripheral blood [73]. The functionality of Treg in pSS remains unclear. It is reported that Foxp3 positive Tregs can be induced from naive T cells by IL-2 and TGF-β whereas the combination of IL-6 and TGF-β induces Th17. Moreover, IL-6 can convert nTregs to Th17 cells in the presence of TGF-β whereas iTregs are resistant to Th17 conversion by IL-6 [74]. Thus, the imbalance of Tregs and Th17 in pSS patients may be due to the local conversion effect of IL-6 which turns iTreg into Th17 cells.

1.2.5.8 Tfh and IL-21

Characteristics of Tfh

The fifth lineage of CD4 T cells, follicular helper T cells were firstly described by Schaerli P and Breitfeld D in 2000 [75]. They found that a human memory CD4 T cell subpopulation ($CD45RO^{+}$) expressed CXCR5 in tonsils and blood. These $CXCR5^{+}$ T cells migrate in response to CXCL13, which is selectively expressed by reticular cells and blood vessels within B cell follicles. Tfh are localized in the mantle and light zone of germinal centers in the B cell follicles. Ma et al., identified human tonsillar $CD4^{+}CXCR5^{high}$ T cells as Tfh cells. They uniformly displayed the highest levels of ICOS, CD95, PD-1, CD200, cytoplasmic adaptor protein SLAM-associated protein (SAP) and CD57. Tfh cells also express CD126, BTLA (ligand of B7-H4), OX40, CXCR4, CD40L, CD69 and CD126. The transcriptional factor B-cell lymphoma 6 protein (BCL6) but not T-bet, GATA3, Foxp3 or RORγt is highly expressed by Tfh. Tfh also produce high level of B-cell activity cytokines, particularly IL-21, IL-4 and IL-10 [76]. The $ICOS^{+}CXCR5^{+}$ IL-21 producing-Tfh can be induced *in vitro* from naïve T cell by IL-12. Schmitt et al., also described that naive $CD4^{+}$ T cells primed with IL-12 induced B cells to produce Igs in a fashion dependent on

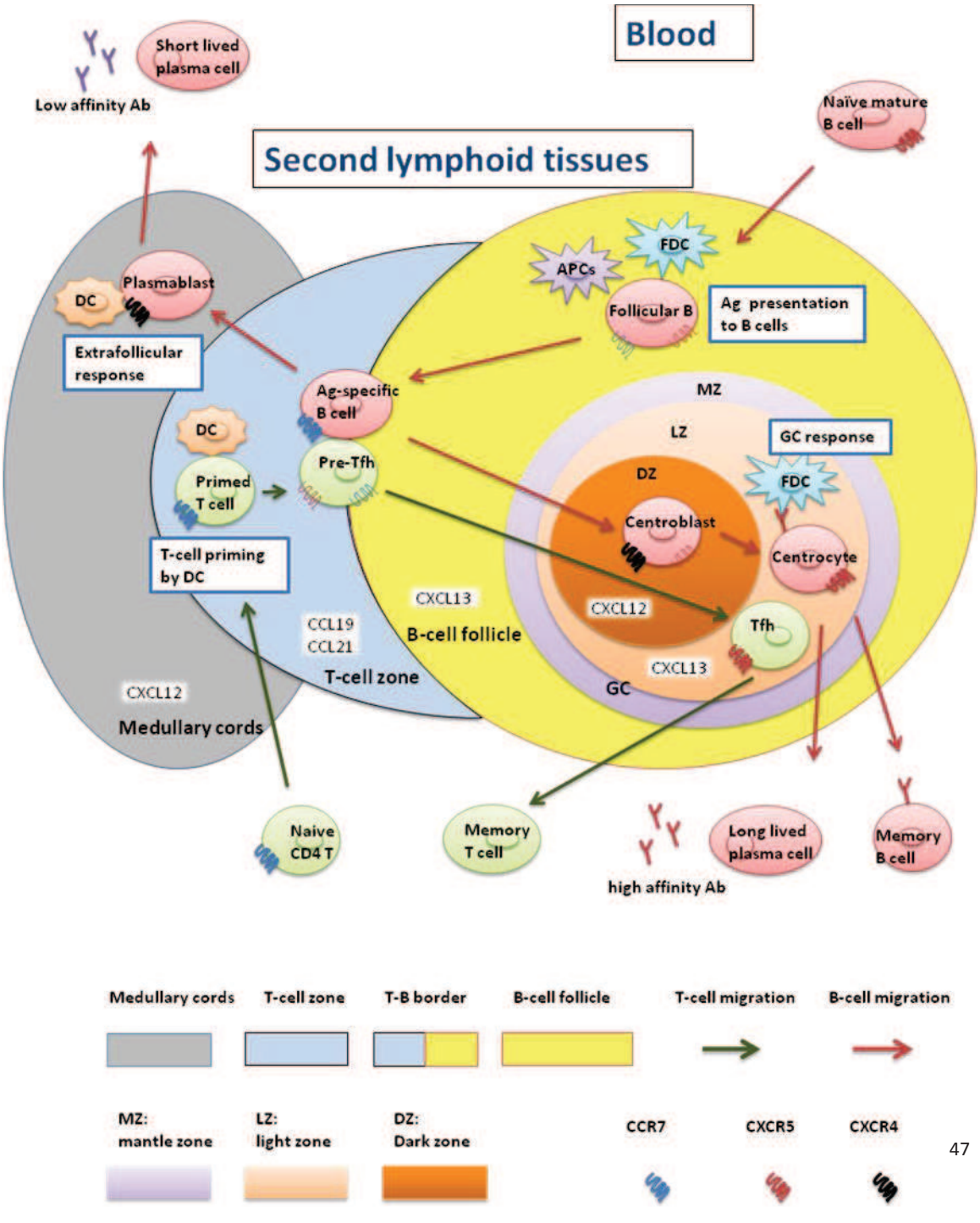
IL-21 and ICOS. There are two different types of IL-21-producing T Cells induced by IL-12: IFN-γ^{+}IL-21^{+} and IFN-γ^{-}IL-21^{+} cells. Furthermore, IFN-γ^{+}IL-21^{+} T cells expressed T-bet, whereas IFN-γ^{-}IL-21^{+} cells did not. Bacteria or CD40L-activated DCs can induce IL-21-producing $CD4^{+}$ Tfh cells through IL-12 [77]. Other studies showed that Tfh also express molecular critical for their development and function, including HLA-DR, CD84 as well as the transcription factors c-Maf. Cytokines such as IL-6, IL-21 and IL-27 are necessary but insufficient for Tfh cell generation and maintenance *in vivo*.

Generation of Tfh

Blood naïve CD4 T cells express CCR7. In respond to CCL19 and CCL21 produced in the T-zone of secondary lymphoid organs: spleen, lymph nodes and mucosal-associated secondary lymphoid tissues, they migrate from the circulation to the T cell zone. T cells recognize Ag and costimulatory signals provided by DCs in the T cell zone. Depending on the costimulatory and cytokine signal provided, T cells differentiate into Th1 or Th2 cells. They downregulate CCR7 and exit lymph node to participate the effector responses. Some primed T cells downregulate CCR7 and upregulate CXCR5 and express BCL-6. The ligand of CXCR5, CXCL13 is expressed by cells in B cell follicle and GC. This results a gradient of CXCL13. In response to CXCL13, these $CXCR5^{+}BCL\text{-}6^{+}$ pre-Tfh cells migrate from T cell zone towards B cell follicle. At the interface between the T and B zones (T-B border), pre-Tfh cells interact with Ag-specific B cells. During this interaction, the help provided by pre-Tfh is essential for the initiation of both germinal center and extrafollicular response of B cells. In turn, depending on the signal provided by B cells, this cognate interaction is important for the complete downregulation of CCR7 which results in pre-Tfh entering the GC and terminally differentiate into Tfh. After this interaction, pre-Tfh cells maintain BCL-6 expression and enter the light zone of GC. There, they fully differentiate into Tfh express PD-1, IL-21, CD84 and ICOS and provide survival

signal to centrocytes. After GC responses, Tfh decrease their expression of signature Tfh molecules. These cells contribute the $CD4^{+}CXCR5^{-}$ memory T cell pool [78] (Figure 7).

Figure 7. Anatomical localization as well as molecules and cellular requirements for Tfh differentiation.

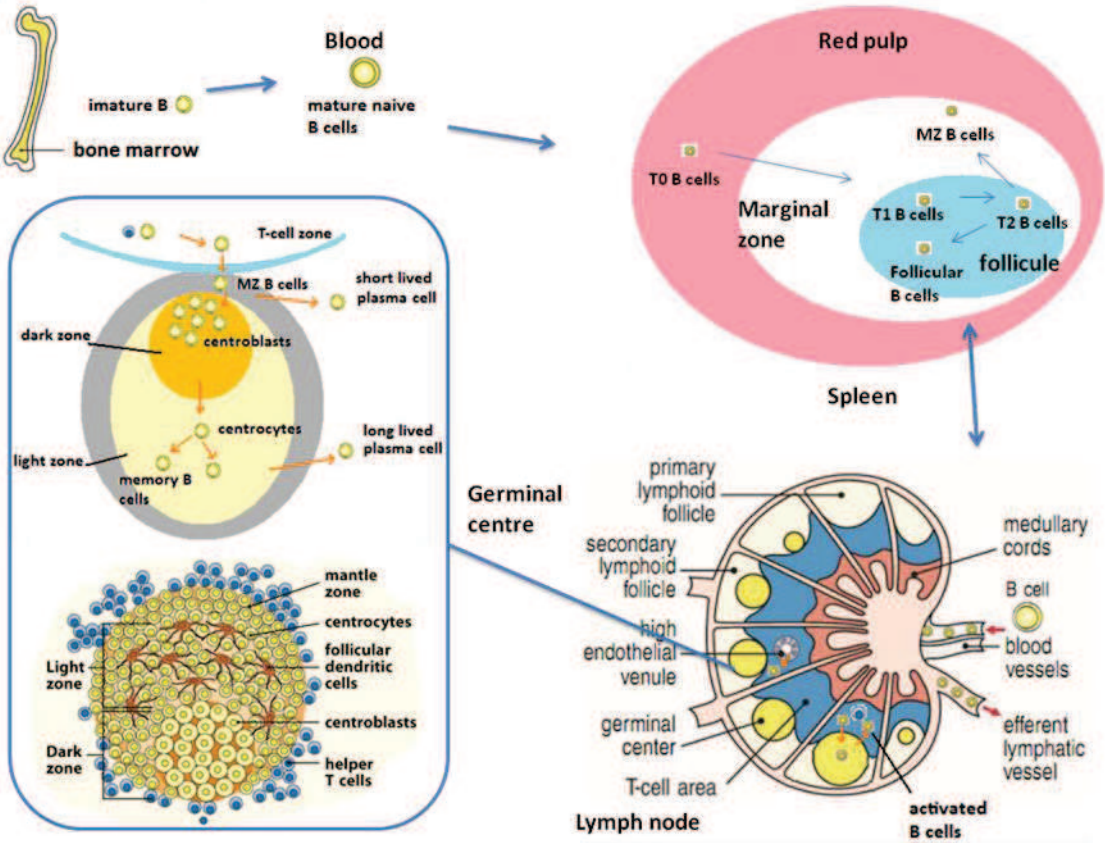


Tfh-cell driven B-cell differentiation in secondary lymphoid tissues.

CD4 helper T cells provide a second signal to drive B-cell maturation, terminal differentiation and isotype switching. Circulating naïve mature B cells migrate into the primary B-cell follicle of the secondary lymphoid tissue of lymph nodes (Figure 8). B-cell follicle is composed of follicular B cells and follicular dendritic cells (FDC). This migration is mediated by the expression of CXCR5 on follicular B cells. At this step, follicular B cells collect the Ag presented by APCs including FDC, DC, macrophages as well as other stromal cells. Ag specific B cells become activated and upregulate CCR7 which allow them to migrate towards T cell zone by a gradient of CCL21. At the T-B border, they present Ag peptide by CMH II to cognate DC-primed T cells. Activated B cells have two distinct fates: an extrafollicular process or affinity maturation in GC (Figure 7).

Figure 8. B cell devellpoment and lymph node stucture. **Immature B cells exit the bone marrow. They circulate as naïve B cells. These circulating B cells then migrate through the circulation to the red pulp of the spleen (transitional type 0 (T0) B cells). These T0 B cells migrate into the white pulp and mature into T1 and then T2 B cells. Finally they differentiate into either follicular or marginal zone (MZ) B cells. Follicular B cells recirculate as mature naïve B cells among secondary lymphoid organs. Once activated, they enter the primary follicles of secondary lymphoid tissues. After being challenged by T-dependent antigen, they move to the T-B border and make cognate interaction with primed T helper cells. Some activated B cells migrate to rapid extrafollicular foci and differentiate into plasma cells with a short lift span which rapidly secrete low-affinity antibodies. Otherwise, a few of activated B cells migrate into the primary lymphoid follicles and form the germinal center (secondary lymphoid follicles). They firstly divide**

as centroblasts and then undergo a SHM. The centroblasts then migrate to the light zone as centrocytes. They come into contact with the FDC following interaction with cognate Tfh which trigger immunoglobulin class switching. B cells which gained improved affinity are positively selected to proliferate whereas the low affinity clone and self-reactive clone undergo a cell death or apoptosis process. Centrocytes then become either plasma cells with a long life span or memory B cells. HEV, high endothelial venules. Figures adapted from the 8th edition of the Janeway's immunobiology.



Extrafollicular process

Some of Ag specific B cells downregulate CCR7 expression and upregulate CXCR4. This leads them to differentiate into plasmablasts and remigrate from B cell follicle to the medullary cords to form extrafollicular foci in response to CXCL12. DC support plasmablasts to survive and differentiate into plasma cells with a short life span (3 days) and produce low and modest affinity Abs. These antibodies may be either switched (e.g. IgG1) or unswitched (IgM). This initial burst of Ig is important in the early control of infection (Figure 7).

Affinity maturation in GC

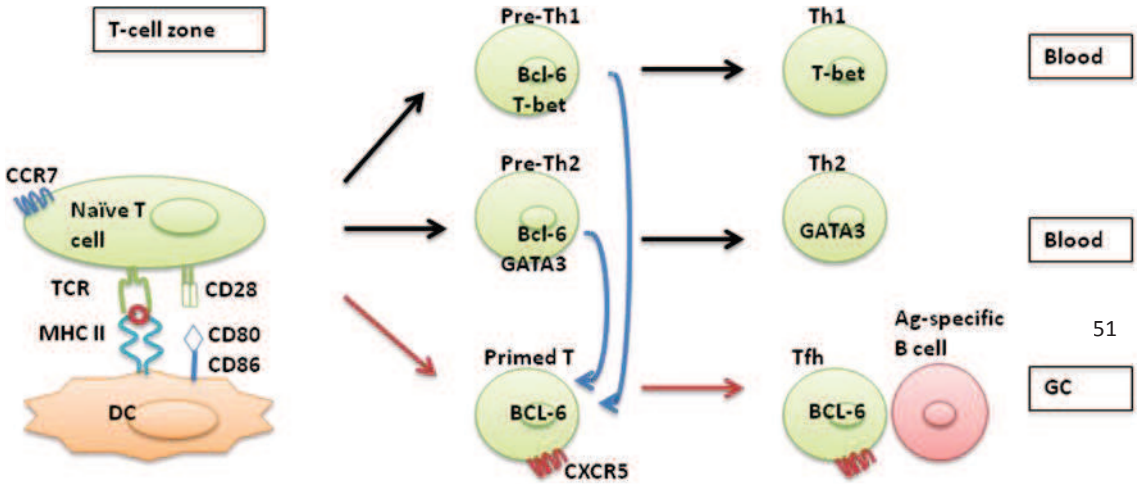
T-cell activated Ag-specific B cells migrate into the B cell follicle in response of CXCL12. There, they continue to divide as centroblasts. The centroblasts form the dark zone of the GC. Thanks to their activation-induced deaminase (AID) expression, they undergo a somatic hypermutation (SHM), on the variable regions of the BCR. The centroblasts stop to proliferate and migrate to the light zone of GC as centrocytes. Centrocytes with mutated BCR come into contact with the FDC. Through interaction with antigen held on FDC, they acquire survival signals. Centrocytes present their Ag to cognate Tfh. At this step, B cells which gained improved BCR affinity during SHM receive positive selection signals from Tfh. They proliferate and either differentiate to plasma cells with a long life span and produce high affinity Ab or become memory B cells. Tfh also trigger Ig class switch recombination to IgG in the GC. At the same time, the low affinity clone and self-reactive clone undergo cell death or apoptosis. [79, 80] (Figure 7).

T-cell priming in the T-cell zone

Depending on the microenvironment and on costimulatory signals, T cells can differentiate into Th1, Th2 or Tfh. Th1 and Th2 will help B cell differentiation in the

extrafollicular pathway. They promote class switching to IgG2a or IgE by secretion of IFN-γ or IL-4 respectively. It is unclear whether Tfh are generated from Th2 and Th1 cells or represent a separate lineage developed from DC primed T cells. After in vitro priming with DCs via IL-12, T cells rapidly upregulate STAT4. STAT4 induces both IL-21, IFN-γ, T-bet and Bcl-6 that contribute to both Tfh and Th1 phenotypes. T-bet can negatively regulate Bcl-6 expression and thus promote full Th1 cell differentiation [81]. Tfh are usually generated under condition of chronic inflammation. Persistent infection or Ag exposure induces or maintains high level of Bcl-6 expression, which in turn suppresses GATA3 or T-bet. (Figure 9)

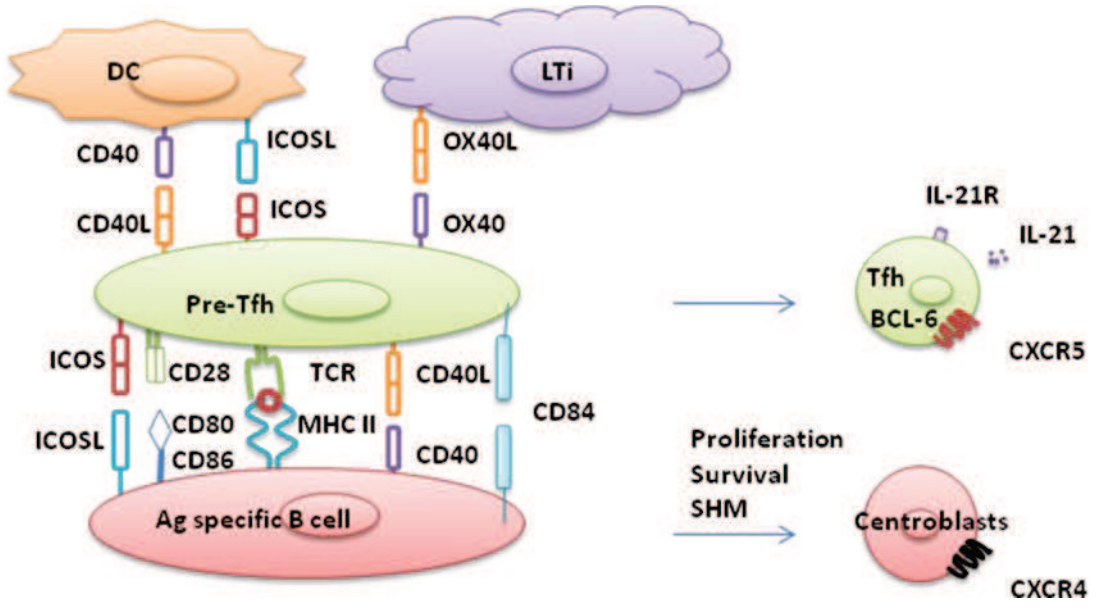
Figure 9. Initial T cell priming by DC and T cell differentiation. Adapted from [82]. **Naïve CD4$^+$ cells interact with Ag-presenting DCs and express Bcl-6. Depending on the nature of Ag, costimulatory signals and the microenvironment, some cells may coexpress T-bet or GATA3. Sufficient level of T-bet or GATA3 can override the effect of Bcl-6, which leads to fully differentiated Th1 or Th2 cells (black arrows). Other conditions result in the downregulation of T-bet or GATA3 and upregulation of Bcl-6 which results in the differentiation into Tfh (blue arrows). Some DC-primed T cells will become GC Tfh after their encounter with cognate Ag-specific B cells (red arrows and see next paragraph and figure 10).**



Interactions with LTi , Dcs and Ag-specific B cell at the T-B border.

The signals received from $CD4^{+}CD3^{-}$ lymphoid tissue inducer cells (LTi) and cognate B cells and DCs at the T-B border are crucial for T-cell fate. LTi express OX40L, and DCs and B cells express CD40 and ICOSL. LTi mainly localize at the T-B border and within the B follicle, and provide survival signals to activated CD4 T cells through OX40L and CD30L. During the interaction with B cells, the primed T cells recognize Ag presented by B cells via TCR MHC II-peptide complex. This Ag presentation is important for both Tfh differentiation and GC differentiation of B cells. The interaction between CD40 on B cells and CD40L on T cells is important for GC maintenance since the blockade of CD40 pathway causes the dissolution of established GCs. ICOS signal provided by DCs or cognate B cells directly controls follicular recruitment of activated T-helper cells. It was shown that ICOS engagement drives coordinated pseudopod formation and promotes persistent T-cell migration at the T-B border *in vivo* [83]. B cells also deliver CD80 costimulatory signal to T cells and provide important Tfh survival and maturation signals which lead to the induction of ICOS, PD-1 and IL-21. After this interaction, pre-Tfh begin to express IL-21 which further upregulates Bcl-6 in an autocrine manner (Figure 10).

Figure 10. Interations with LTi , DCs and Ag-specific B cell at the T-B border.

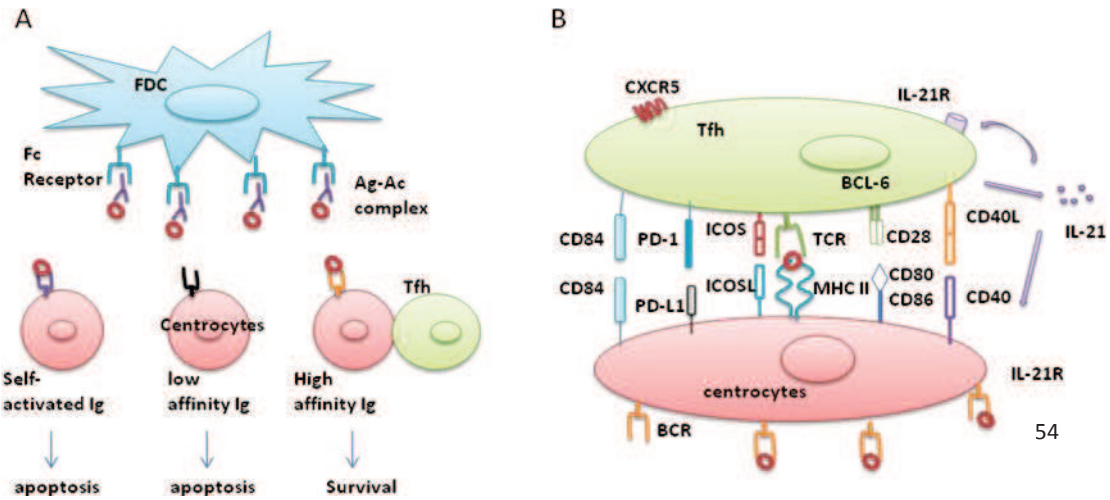


Tfh help: a crucial actor of affinity maturation

When centroblasts stop to proliferate and become centrocytes, they firstly collect Ag from FDCs by the freshly expressed mutated Ig on their surfaces. They also receive survival signals. FDCs do not present Ag in the context of MHC II. They present antigen-antibody complexes via complement receptors and Fc receptors. These complexes are stable for months. It seems that centrocytes expressing higher affinity receptors can take up and express more antigens on their MHC II. Thus they can present Ag to Tfh. Based on the ability to capture and present larger amounts of Ag, centrocytes receive sufficient survival signals from Tfh. Centrocytes compete to receive appropriate costimulation from Tfh, including CD40L, SLAM-associated protein (SAP) and ICOS (Figure 11A). SAP is the adaptor protein of signaling lymphocyte activation molecule (SLAM) family. High affinity centrocytes have a competitive advantage for being selected compared with low affinity centrocytes. Selected centrocytes can differentiate into plasma cells or memory B cells. They can also re-enter the dark zone for another round of SHM. One of the reasons why self-reactive centrocytes undergo cell death via apoptosis is the fact that they cannot not find cognate Tfh. Maintenance of the Tfh cell phenotype requires sustained antigenic stimulation by GC B cells. A strong and prolonged Tfh cell response leads

to GC B-cell activation, hypergammaglobulinemia, and secretion of poly- and self-reactive Abs [84]. Activation of B cells by Tfh depends on costimulatory molecules including ICOS expressed by Tfh and ICOSL by B cells. In mice deficient for the ICOS gene, it is impossible to induce rheumatoid arthritis and myasthenia gravis. The *roquin* mice deficient for ICOS inhibitor have an excess of Tfh, an increased secretion of IL-21. Hyperplasic ectopic germinal centers, hypergammaglobulinemia and develop signs of lupus. PD-1 and CD40L on Tfh interact with PD-1L and CD40 to deliver survival signal to B cells. CD40 signal is implicated in memory B cell differentiation as well. IL-21 has a critical role in promoting B cell SHM, class switching and plasma cell differentiation. Combined with other costimulatory molecules, the SLAM family member CD84 is required for a stable Tfh-B interaction. A stable interaction with B cells is essential for the maintenance of Tfh population Although Tfh can be generated under SAP-deficient conditions, Tfh exhibit compromised function in the absence of SAP costimulation.(Figure 11 B)

Figure 11. Main actors and molecules involved in GC B-cell differentiation. Affinity maturation of centrocytes (A) and cognate interaction between Tfh and centrocytes. Adapted from [85]



Tfh subsets

It is now clear that GC follicular T cells are heterogeneous and have distinct phenotypes and functions. Tfh located in the light zone, but not those located in the mantle zone, express CD57. Light zone Tfh also express CD69, CD45RO but not CD25 (IL-2R). The $CD57^+$ Tfh produce IL-10 and have a B-cell helper activity. They interact with CG B cells via CD40-CD40L to promote their survival and AID expression. $CD57^-$ Tfh located in the mantle zone of GC do not express CD25 or CD69. They do not have a B-cell helper activity for Ab secretion *in vitro*. These cells rapidly express CD40L after TCR activation. Due to the lack of CD25, both $CD57^+$ and $CD57^-$ Tfh do not undergo IL-2 driven proliferation.

Other T subsets share features with Tfh localized in GC. $CD4^+CD25^+CD57^-$ follicular regulatory T cells express molecules associated with both Tfh (Bcl-6, CXCR5, ICOS and PD-1) and Tregs (FoxP3, GITR, CTLA-4 and Blimp-1). Follicular Tregs originate from nTreg. They are different from Tfh cells because they do not express CD40L, IL-4 or IL-21. Follicular Tregs directly suppress GC B cell Ab secretion and Tfh function *in vitro*. Follicular Tregs express a high level of IL-10, which can inhibit the expansion of memory B cells. Follicular Tregs seem to be very important to limit the outgrowth of non-Ag-specific B cells, including autoreactive B cells [86].

Tfh and autoimmunity

Autoreactive B cells represent 5-20% of circulating mature B cells. In the T-cell zone, self-reactive follicular B cells preferentially undergo apoptosis than a GC differentiation. Therefore, they are normally absent from the memory compartment. During GC differentiation, centroblasts might become self-reactive after SHM. Tfh select B cell clones with high affinity toward foreign Ag rather than low affinity B cell

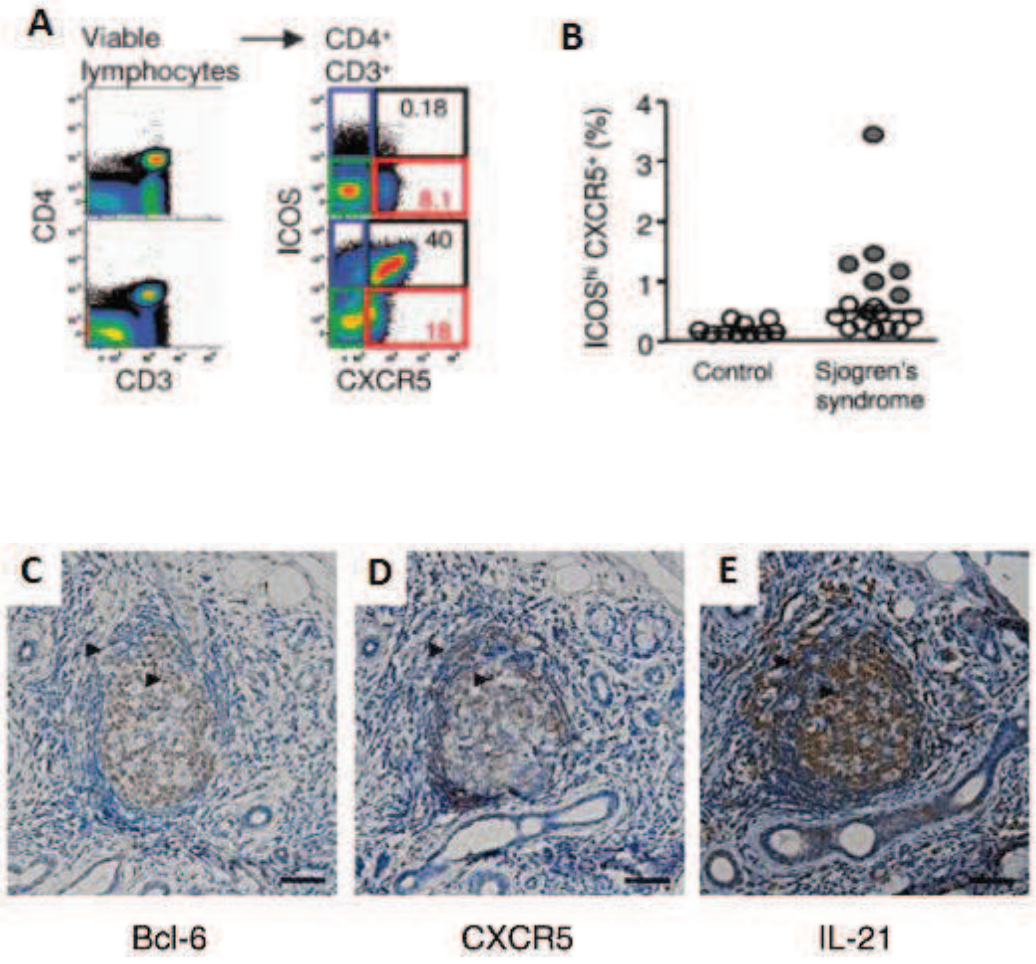
clones or those with self-Ag affinity (Figure 11A). Thus, in physiological conditions, self-reactive centroblasts undergo apoptosis because of the lack of positive selecting signals from Tfh. It is presumed that the uncontrolled generation of Tfh can provide an excessive help to autoreactive B cells by decreasing the competition between B cells for Tfh help. In autoimmune mouse strains, ectopic GCs appear almost at the same time as autoantibodies. In patients with SLE, self-reactive B cells are located in GCs and differentiate into somatically mutated high-affinity Ab producing plasma cells.

An increased proportion of circulating $ICOS^{high}CXCR5^{+}CD4^{+}$ Tfh-like cells was reported in some patients with pSS [87] (Figure 12 A and B). Szabo et al., demonstrated that pSS patients, especially those with extraglandular manifestations, has elevated proportion of peripheral $CD4^{+}CXCR5^{+}ICOS^{+}PD\text{-}1^{+}$ Tfh cells [81]. Meahara et al., have shown that the expression of molecules associated with Tfh, Bcl-6, IL-21 and CXCR5, could be detected in/around the SG GC from SS patients (Figure 12 C-E) [68]. An elevated proportion of other populations of IL-21-producing Tfh-like cells were described in pSS patients. These were $CD4^{+}CXCR5^{+}CCR6^{+}$ Tfh-like cells expressing PD-1, ICOS, CD40L and IL-21 as well as $CD4^{+}CXCR5^{low}CCR9^{+}$ Tfh-like cells expressing ICOS, Bcl-6 and Maf [88, 89].

IL-21 is mainly secreted by Tfh. It can be also secreted by Th17. Patients with SLE have increased levels of intracellular IL-21 in peripheral blood $CD4^{+}$ T cells. in addition, this augmentation is correlated with increased memory B cells. The proportion of circulating $Bcl6^{+}CXCR5^{+}CD4^{+}$ Tfh cells is augmented in patients with SLE and is correlated with increased circulating $Bcl6^{+}CXCR5^{+}$ GC B cells [90]. IL-21 has been detected in the serum and within SGs of SS patients, and its level is correlated with the degree of lymphocytic infiltration, hypergammaglobulinemia and autoantibody levels. $IL\text{-}21^{+}$ infiltrating cells are detected in SGs of patients. IL-21 promotes the survival, proliferation of B cells and differentiation into plasma cells. It

also supports $CD4^+$ T cells activation, proliferation and survival. IL-21 is important for the proliferation of naïve and memory $CD8^+$ T cells as well. IL-21 is also involved in the maintenance of Th17 cells by enhancing IL-23-induced expansion of Th17.

Figure 12: Tfh-like cells in patients with SS. A, circulating and tonsillar Tfh cells. FACS analysis of tonsil (lower) and blood cells (upper), gated on viable $CD3^+CD4^+$ cells. B, FACS analysis of peripheral blood obtained from SS patients (n = 17) and healthy controls (n = 10). Percentage of $ICOS^{high}CXCR5^+$ circulating Tfh cells among circulating $CD4^+CD3^+$ lymphocytes [87]. C-E, expression of Tfh associated molecules (Bcl-6, CXCR5 and IL-21) in the lesions of labial salivary glands from patients with pSS [68].

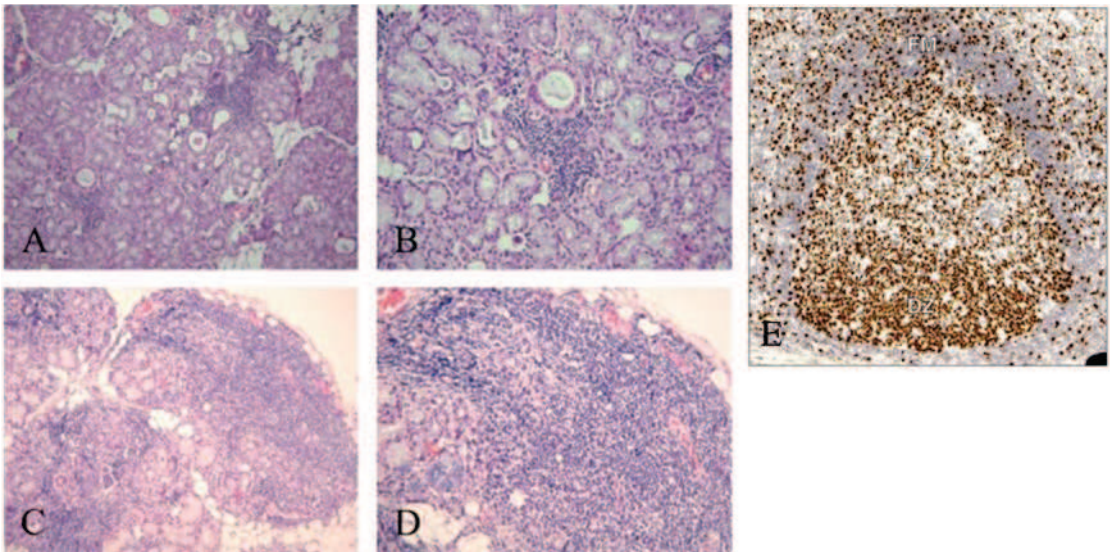


1.2.6 Ectopic germinal center-like structures

Germinal centers are found in secondary lymphoid tissues such as lymph nodes or spleen. Their main function is the maturation of B cells and the secretion of high affinity antibodies. GCs develop in the primary B-cell follicles located at the edge of T-cell rich compartment [91].

Ectopic GCs are found in tertiary lymphoid tissues in autoimmune disease including pSS, RA, SLE, Hashimoto's thyroiditis, multiple sclerosis, etc (Figure 13). They can be observed in some chronic inflammation diseases without apparent autoimmune origin. Ectopic GCs are observed in 17% of minor salivary gland biopsies studied. Morphologically, these GC-like structures are found at the interface of B and T cell areas. They are constituted by a dark zone of proliferating and hypermutating centroblasts surrounded by a light zone with $CD35^+$ FDC network, Tfh and centrocytes (figure 13) [92]. High levels of autoantibody secretion and apoptosis are detected in patients with GCs. In addition, chemokines (CXCL13, CCL21 and CXCL12) and adhesion molecules (ICAM-1, LFA-1, VCAM-1 and VLA-4) implicated in lymphoid neogenesis are detected in patients with GCs. Pottier *et al.,* have studied the characteristics of B cells in GC-like structures of patients with pSS. Autoreactive B cells can be detected in all biopsies. A minority of SG biopsies contain B cells which are similar to those identified in tonsil GC. Signature genes expressed during the GC reaction (AID, transcription factors Pax-5 and Bcl-6) are detected in these biopsies. In the majority of SG biopsies, transitional type 2 and marginal zone-like B cells are detected. These biopsies contain high levels of transcription factors Blimp-1, IRF-4, Notch-2 and BAFF receptor 3 (BR3). The presence of ectopic GC is a risk factor of lymphoma in patients with pSS [2]

Figure 13. **Periductal focal mononuclear cell infiltrates in the minor salivary gland biopsies of patients with pSS (A and B) and salivary gland tissue GC-like structures (C and D). A mature germinal centre from a human tonsil is shown (E) [93]. Follicular mantle zone (FM), the dark zone (DZ) and the light zone (LZ) are indicated [93].**



1.2.7 Salivary gland epithelial cells and lymphoma

Sjögren's syndrome appears to be a crossroad between autoimmunity and malignancy. The main subtypes of B-cell lymphoma associated with SS are mucosa-associated lymphoid tissue lymphoma, diffuse large B cell lymphoma and follicular lymphoma[94]. The presence of ectopic GC is a risk factor of lymphoma in patients with pSS. Moreover, SGECs may involved in the GC formation by expression of 'lymphoid' chemokines especially CXCL13. The development of SS-associated lymphoma, especially the extranodal marginal-zone B cell lymphoma, is associated with uncontrolled GC response [95]. Moreover, increased serum BAFF levels in pSS patients are associated with lymphoma or risk of lymphoma development.

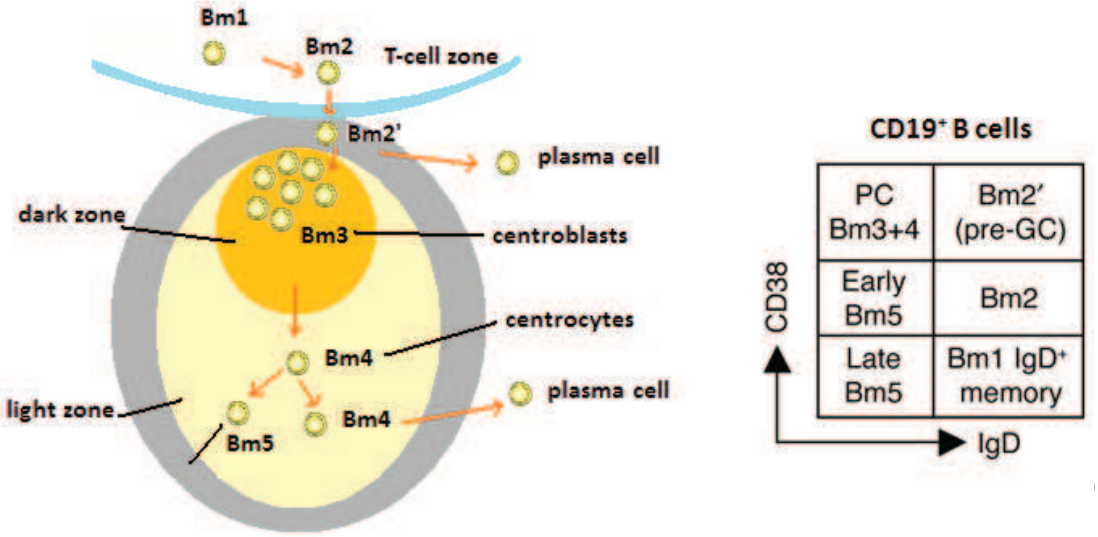
1.2.8 Role of T-cell dependent B-cell activation in pSS

B cells and autoantibodies play an important role in the pSS. B lymphocytes infiltrating SGs are activated. They show oligoclonal or monoclonal B cell expansion [96]. They not only produce autoantibodies, but also synthesize several cytokines and act as APCs. B cell activation in SG is related to the costimulation by T cells, secretion of B-cell activating cytokines by T cells, monocytes, macrophages, DCs and ECs, such as IL-6, IL-10, IL-21 and BAFF.

1.2.8.1 B-cell subsets

Altered homeostasis of peripheral and glandular B cells was reported in pSS [97]. Human mature B cells can be separated into naive Bm1 ($IgD^{+}CD38^{-}CD23^{-}$); Bm2 ($IgD^{+}CD38^{-}$); Bm2′ pre-GC cells ($IgD^{+}CD38^{++}$); germinal center centroblasts Bm3 ($IgD^{-}CD38^{++}CD23^{-}$); centrocytes Bm4 ($IgD^{-}CD38^{++}CD77^{-}$), early/late Bm5 memory ($IgD^{-}CD38^{+}$ and IgD-CD38- respectively) and $CD38^{bright}$ plasma cells subsets (Figure 14). pSS patients have an elevated proportion of activated naïve B cells (Bm2) and a decreased percentage of resting B cells (Bm1, $IgD^{+}CD27^{+}$ memory B cells, early Bm5 and Bm5) in peripheral blood [98, 99].

Figure 14. Main markers of mature B-cells. Adapted from [100].



Patients with pSS and SLE have many clinical and serologic (hyperimmunoglobulinemia, positive anti-SSA and anti-SSB autoantibodies and rheumatoid factor) similarities. One of the differences between pSS patients and SLE patients is the distribution of B cell subsets. Patients with SLE have significantly reduced peripheral B cells, increased circulating $CD27^+$ memory B cells, reduced naive $CD27^-$ B cells and markedly increased $CD27^+$ plasmablast/plasma cells. Patients with pSS have a predominance of $CD27^-$ naive B cells and a reduced frequency of $CD27^+$ $CD27^+IgD^+IgM^+CD5^+$ memory B cells [101, 102]. This might be related to the fact that memory B cells are predominantly recruited and/or differentiated in salivary glands. The plasma cells infiltrating in the SG of pSS predominantly contain IgG and IgM isotypes rather than IgA [103].

1.2.8.2 B-cell polarization

Harris *et al.,* demonstrated that B cell can be primed to differentiate into two cytokine-secreting B-cell subsets after culture with antigen and effector T cells [104]. Two populations of effector B cells have been identified, B effector 1 (Be1) and B effector 2 (Be2). The Be1 produce IFN-γ and IL-2 when stimulated by antigen and Th1 cells, whereas Be2 express IL-4 and IL-6 in the presence of Th2 cells. IFN-γ produced by Be1 cells and IL-4 produced by Be2 cells could subsequently drive the polarization of naïve T cells into Th1 and Th2 cells respectively. In pSS [105], both Be1 and Be2 cells can be detected in SGs. Another study showed that, patients with ectopic GCs have a higher level of IL-4 and other Be2 cytokines than patients without ectopic GCs [106].

1.2.9 Autoantibodies

SS is an autoimmune disease characterized by a strong polyclonal B cell activation. Hypergammaglobulinemia and the presence of high affinity, class switched

autoantibodies are observed in patients with pSS.

Anti-SSA (Ro) and SSB (La) antibodies.

Numerous auto-antibodies are detected in patients with SS. However, no SS-specific pathologic auto-antibody has been found yet. Ro and La are two ribonucleoproteins (RNP). Affected SGs are a major site of autoantibody production in pSS. Anti-Ro and anti-La appear to play a role in the local autoimmune response. IgA and IgG isotypes are found in saliva and sera of patients with SS [107]. B cells infiltrating the SG contain intracytoplasmic immunoglobulins with anti-SSA and anti-SSB activity. Moreover, exosomes released by SGECs from patients contain anti-SSA, anti-SSB and anti-Sm [108]. It is widely accepted that the production of autoAbs is an antigen-driven response. BALB/c mice immunized with Ro peptides develop anti-Ro, salivary gland lymphocyte infiltrates and salivary dysfunction [109]. In SG, the expression of VEGF-A and its receptor VEGFR2 in both acinar and ductal SGECs is higher in patients than in controls. When treated with human anti-SSA Abs, cultured SGECs upregulate the expression of VEGF-A and VEGFR2, secrete pro-angiogenic chemokines/cytokines and stimulate the angiogenic response [40]. Anti-SSA autoantibodies can also induce the secretion of IL-6 and IL-8 by cultured SGECs [110].

Anti-M3R antibodies

Anti-M3R antibodies have raised some interest because of their potential pathogenic role. The muscarinic receptor family is encoded by 5 separate genes (M1-M5). Muscarinic acetylcholine receptor (M3R) is expressed in exocrine glands including salivary glands. M3R plays crucial roles in the control of fluid secretion by salivary acinar cells. Acetylcholine binds to M3R on salivary gland cells and stimulates phospholipase C which generates inositol 1,4,5-trisphosphate (IP_3). IP_3

binds to its receptors on intracellular Ca^{2+} stores which results in the release of Ca^{2+}. Consequently, the rise in intracellular Ca^{2+} activates apical membrane Cl^- channels and induces salivary secretion. Activation of M3R also induces the trafficking of aquaporin 5 (AQP5) to the apical membrane from the cytoplasm, which causes rapid transport of water across the cell membrane [111]. In patients with SS, functional antimuscarinic autoantibodies are detected in 63% of patients [112] and antibodies against M3R are detected in 45.2-54.8% of patients [113]. Presence of several B cell epitopes on M3R are found in SS patients [113]. Anti-M3R antibodies could be associated with decreased salivary secretion in patients with pSS. Autoantibodies from patients with pSS inhibit the function of the human M3R. Due to technical concerns, antibodies against M3R are unfortunately very difficult to detect using conventional techniques such as ELISA.

Anti-α-fodrin

The ubiquitous cytoskeletal protein α-fodrin was firstly identified as an autoantigen in the NFS/sld mouse model of SS [114]. A first study showed a sensitivity and specificity of anti-α-fodrin in pSS of 78.5% and 86.8%, respectively [115]. However, other studies showed a much poorer diagnostic interest [116]. Mice immunized with α-fodrin present lymphocytic infiltration in the salivary glands and autoantibodies such as ANA, anti-alpha-Fodrin and anti-type 2 muscarinic acetylcholine receptor polypeptide (M2RP).

Anti β-fodrin

β-fodrin is a membrane skeleton protein associated with ion channels and pumps. It has no homology with α-fodrin. The sensitivity of anti β-fodrin antibodies in SS was 70% and the specificity was 93% in one study [117].

Anti-CCP antibodies

Anti-cyclic citrullinated peptide (CCP) antibodies are very useful for the diagnosis of RA. The specificity of anti-CCP antibody in the diagnosis of RA has been reported to be about 95%. Several studies have demonstrated that anti-CCP autoantibodies are found in 4-6.9% of pSS patients [118, 119]. Serum level of IgA –anti-CCP is higher in pSS patients than healthy individuals and lower than in patients with RA. [120].

1.2.10 The role of cytokines in the physiopathology of pSS

Cytokines play a central role in the initiation and the perpetuation of autoimmunity in the exocrine glands. The imbalance of pro- and anti- inflammatory cytokines results in tissue damage leading to decreased secretory function. Cytokines also contribute to chronic stimulation of B and/or T cells and formation of ectopic germinal centers, involved in lymphomagenesis. Data regarding cytokines in pSS are summarized in table IV. We will not rediscuss the role of IL-17 and IL-21 that was previously mentioned and decided to focus in this paragraph on some other cytokines.

Table IV. Cytokine expression in patients with pSS.

	In SG	**In serum or plasma**	**Peripheral cytokine producing cells**	**In saliva or tears**	**Reference**
IFN-γ	+	+	-	+	[121]
IL-12		+		+	[69]
IL-2			-		
IL-1β	+	+		+	[122]
BAFF	+		+		[123]
APRIL	-	+			[124]
IFN-α	+		-		[125]
TNF-α	+			+	

LTβ	+				[126]
IL-6	+	+	+	+	[127] [128]
IL-4	+	+		+	
IL-13	+			+	
IL-10	+	+	+	+	
IL-17	+	+		+	[129]
IL-23	+	+		+	[130]
IL-18	+	+			[131]
IL-21	+	+	+		[132]
IL-22	+	+			
IL-27		+			[133]
IL-34	+				[134]
IL-7	+	+			[135]
TGF-β		+		+	
IL-14α			+		

$^{+/-}$ up- or downregulated in patients with pSS compared with controls in the basal conditions.

1.2.10.1 Activation of the type I interferon (IFN) pathway in pSS

Enhanced activity of the type I IFN system has been described in multiple autoimmune diseases including pSS and SLE. Two type I IFN-related genes, *IRF5* and *STAT4* polymorphisms are associated with pSS. Polymorphisms in IRF5 and STAT4 contribute to autoimmunity in general since they are also associated with SLE, RA, systemic sclerosis (SSc). Type I IFNs consist of 13 IFN-α, IFN-β and other less common variants (such as IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ). IFN-α is predominantly produced by pDCs and is mainly induced after viral activation of Toll-like receptors and RNA and DNA cytosolic sensors (DHX9/DHX36) [136].

IFN-β is mainly produced by fibroblasts and epithelial cells. Type-I IFNs signal through its receptor IFNAR induces the expression of hundreds of genes implicated in anti-viral and immune responses. IFNAR is expressed by DC, B cells, T cells, neutrophils as well as NK cells. An increasing number of immunomodulatory effects of type I IFNs has been revealed, such as activation of immature DC through upregulation of MHC I, chemokines and costimulatory molecules; B cell activation and Ig class switching through the induction of BAFF and APRIL; stimulation of Fas ligand expression on NK cells and target cell apoptosis; enhancement of T cell proliferation and survival; Th1 type immune response and triggering of $CD8^+$ memory T cell activation [137].

Several studies of global gene expression profiling of minor SGs or peripheral blood in patients with pSS showed an increased expression of IFN-induced genes, called an 'IFN-signature' [126, 138]. In one study, the expression of 23 genes involved in IFN pathways was significantly different between patients and controls. Most of these genes were significantly up-regulated and belonged both to IFN-I (IFN-α) and IFN-II (IFN-γ) pathways [139]. Plasmacytoid DC is the main cell type to produce type-I IFN cytokines, since they secrete up to 1000 times more IFN-α /IFN-β than other cell types. A greater number of IFNα-producing pDCs was detected in pSS MSG biopsies. In the peripheral blood of patients with pSS, pDCs have a reduced frequency. These circulating pDCs express higher levels of the activation marker CD40, which is correlated with the expression of selected IFN-regulated genes [140]. Expression levels of most interferon-inducible genes in the peripheral blood were positively correlated with titers of anti-SSA and anti-SSB autoantibodies [138]. Brkic *et al.,* showed that the type I IFN signature in monocytes is present in 55% of patients with pSS compared with 4.5% of healthy controls. The presence of IFN type I signature in monocytes of patients was associated with disease activity; higher anti-Ro52, anti-Ro60 and anti-La autoantibodies; higher rheumatoid factor; higher

serum IgG; lower C3, lower absolute lymphocyte and neutrophil counts as well as higher BAFF gene expression in monocytes [141]. Blood MxA levels are strongly correlated with IFN type I signature in monocytes in pSS. So, it is a practical assay for identifying patients with pSS with an IFN signature [142]. In another study, IFN-α plasma activity determined by reporter cell assay was increased in patients with pSS [18].

1.2.10.2 IFN-γ

IFN-γ is the major cytokine involved in the Th1 response. It is mainly secreted by T cells, NK and macrophages. NK is one of the earliest cell types infiltrating SGs. Their number is reduced in the periphery of patients. So IFN-γ appears to play a critical role in the early phase. The production of IFN-γ is directly stimulated by IFN-α and IFN-β. IFN-γ is aberrantly expressed in patients with pSS. T cells isolated from patients continue to produce IFN-γ *in vitro*. In SG, IFN-γ creates a pro-inflammatory environment. It may contribute to the abundant production of many cytokines, including IL-6, TNF-α, IL-12, IL-17 and BAFF. Treatment with IFN-γ induces the expression by salivary gland epithelial cells of HLA class I antigens, HLA-DR antigens, CD80 and ICAM-1. Cultured salivary gland epithelial cells treated with either IFN-gamma or TNF-alpha also induce the proliferation of allogeneic lymphocytes. Moreover, IFN-γ upregulates Fas and caspase-8 expression and therefore increases apoptosis. Furthermore, IFN-γ can induce high levels of IP-10 and Mig proteins from cultured SGECs [36]. IFN-γ upregulate levels of CD80 and CD86 in human salivary duct cell line (HSG) [143]. Compared with SS prone-NOD mice, IFN-γ knockout NOD mice do not exhibit increased acinar apoptosis, lymphocytic infiltration, decreased secretory function as well as secretion of autoAbs. To conclude, IFN-γ appears to play a critical role from the early preimmune phase to later stages of the disease [144].

1.2.10.3 TNF-α

Tumor necrosis factor (TNF)-α plays a role in inflammation and tissue damage of the exocrine glands. It triggers apoptosis in HSG in vitro and leads to the upregulated transcriptional expression of ICAM-1 and CCL20 [145]. TNF-α also induces the redistribution of autoAg (Ro, La and α-fodrin) on cell membrane after apoptosis. TNF-α induces the expression of HLA class I antigens, CD80, CD86 and VCAM in HSG. However, as previously mentioned, blockade of TNF-α does not result in any improvement of pSS [17, 18].

1.2.10.4 BAFF

BAFF is an IFN-induced gene. It has a key role in promoting B-cell activation, proliferation, maturation and survival. Moreover, it emerges as a potent survival factor for B cell malignancies. B cell development is perturbed in BAFF and BAFF Receptor 3-deficient mice with an apparent block at the T1 B cell-stage. BAFF acts on T2 and marginal zone B cells and the effects of BAFF on T1 B cells and naïve peripheral B cells appeared to be minor. BR3 is also found more expressed on T2 and MZ B cells compared with T1 B cells in mice [146].

Aberrant levels of BAFF are detected in the serum/saliva of patients with SS and also in the serum of MRL-*Fas*^{lpr} mice. Moreover, BAFF levels are significantly correlated with disease activity [147], elevated secretion of autoantibodies and lymphocytic infiltrates in patients with pSS. Local BAFF is expressed by infiltrating T cells, some autoreactive B cells [148], and macrophages. A higher level of BAFF is observed in patients with lymphoma or prelymphomatous manifestations and clonal B-cell expansion in the salivary glands [18]. SGECs are also capable to secrete BAFF. At baseline, BAFF expression in cultured SGECs is low in pSS patients and in controls. Stimulation with IFN-alpha, treatment with dsRNA virus and poly I:C

induce high levels of BAFF expression by SS SGECs. The capacity of SGECs to express and secrete BAFF after IFN-α or TLR3 stimulation reflects the pivotal role of SGECs in the pathogenesis of pSS, possibly after stimulation by innate immunity [149]. Blood monocytes are the main cell type secreting BAFF. Blood monocytes in patients with pSS show increased production of BAFF at baseline or after IFN-α or IFN-γ stimulation. IFN-γ stimulated pSS monocytes produce higher levels of IL-6 due to BAFF overexpression [150]. BAFF expression is also increased in blood T cells of patients. In addition, BR3-positive monocytes are significantly higher in patients than in controls. BAFF-R expression is reduced on peripheral B cells of patients with pSS and SLE. This down-regulation occurs through a post-transcriptional mechanism and could be the consequence of BAFF chronic increase [151].

1.2.10.5 IL-6

IL-6 overproduction has been described in the pathogenesis of many autoimmune diseases including pSS and SLE. IL-6 is a cytokine that regulates immune responses, hematopoiesis and bone metabolism. Moreover, IL-6 is critical in the regulation of the Treg/Th17 balance. In patients with pSS, IFN-γ induces IL-6 expression by blood monocytes. IL-6 is augmented in the serum, saliva, tears and salivary glands of patients with pSS [148, 152].

1.2.10.6 IL-7

IL-7 is an IL-2 family member. IL-7 is expressed by stromal cells in primary lymphoid organs and plays a pivotal role in T cell homeostasis. Memory Tfh development requires IL-7 secretion. IL-7 can induce OX40 expression by $CD4^+$ memory T cells [153]. Increased IL-7 in SG and serum is reported in pSS patients and in mice with pSS-like disease and associated with disease parameters [154]. IL-7Rα is overexpressed by T cells in SGs of patients with pSS. Jin and his colleagues

demonstrated that in B6.NOD4*Aec* mice, IL-7 plays a critical role in the development and the onset of pSS by enhancing Th1 responses and CXCR3 expression by glandular epithelial cells [155].

1.2.10.7 IL-12 and IL-18

IL-12 and IL-18 are pro-inflammatory cytokines working together with IFN-γ in order to drive a Th1 response. IL-12 expression is detected in mononuclear cell infiltrates. The level of IL-12 decreases in patients without extra-glandular manifestations [106]. In SG of patients with pSS, IL-18 is mainly secreted by macrophages. It can be also detected in acinar cells and ductal cells. IL-18 can amplify the secretion of IL-6 and IL-8 by human salivary gland HSY and acinar AZA3 cells induced by low amounts of IL-17. IL-12 and IL-18 may be involved in lymphomagenesis since serum levels of IL-18 and IL-12 were reported to be associated with risk factors of lymphoma such as parotid enlargement, or low C4 [156].

1.2.10.8 IL-22

IL-22 is a member of the IL-10 cytokine family. It can be secreted by a broad variety of cells including Th17, Th22, NK, lymphoid tissue inducer (LTi), γδ T cells as well as epithelial cells. In pSS, Th17 and NKp44^{+} NK cells are the major cellular sources of IL-22. IL-6 can induce IL-22 expression by Th17 cells. IL-22 is increased in SG and the serum of patients with pSS. In addition, serum level of IL-22 was significantly inversely correlated with salivary flow, and positively correlated with serum gammaglobulins, anti-SSA/SSB, and rheumatoid factor [129, 157]. In SG of patients, intense IL-22 staining is observed in infiltrating mononuclear cells, epithelial cells and myoepithelial cells. In addition, the number of infiltrating IL-22^{+} cells and the expression of IL-22 by epithelial cells is significantly reduced by rituximab

(anti-CD20) treatment [158]. IL-22 activates STAT3 signaling and has anti-apoptotic and pro-regenerative characteristics. Interestingly, IL-22 is essential in epithelial tissue repair in some infectious models.

1.2.11 Chemokines

The interactions between chemokines and chemokine receptors are crucial autoimmune diseases. In pSS, chemokines are involved in the Th1/Th2 balance, T-cell homing into SG and angiogenesis. The expression of chemokines and chemokine receptors in patients with pSS are summarized in table V.

Table V. Expression of chemokines and chemokine receptors in patients with pSS.

	In SG	**In serum or plasma**	**Peripheral cytokine producing cells**	**In saliva**	**Ref**
CXCL10 (IP-10)	+	+	+	+	[36]
CXCR3 (receptor for CXCL9, CXCL10 and CXCL11)	+	+	+	+	
CCL3 (MIP-1α)	+				[159]
CCL17 (TARC)	+				
CCL22 (MDC)	+	+		+	
CCR4 (receptor for CCL3, CCL4, CCL5 and CCL17)	+				
CXCL8 (IL-8)		+		+	
CCL5 (RANTES)	+				
CXCR2 (receptor for CXCL8 and CXCL1)	+				
CXCL1 (GRO-α)	+				

CXCL9 (Mig)					[36]
CXCL13 (BCA-1)	+	+			[160]
CCL4 (MIP-1β)				+	
CXCL12 (SDF-1)	+				[37]
CXCR5 (BLR-1, receptor for CXCL13)					
CXCR4 (receptor for CXCL12)					
CCL21					
CCL19					
CCR5 (receptor for CCL3, CCL4 and CCL5)	+				[161]
CXCL11	+				

$^{+/-}$ up- or downregulated in patients with pSS compared with controls in the basal conditions

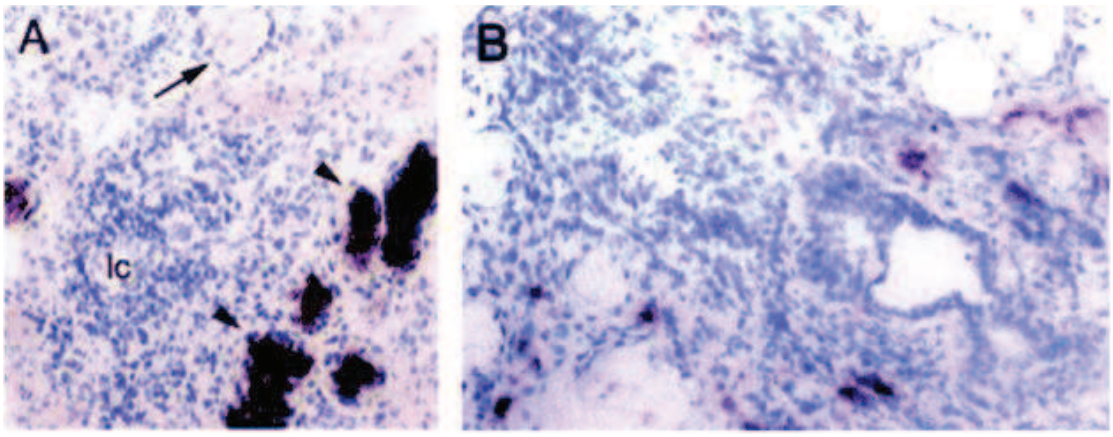
CXCL10, CCL5 and CCL3 are involved in Th1 cell migration, and CXCR3 and CCR5 are specific chemokine receptors expressed by Th1 cells. CCL17, CCL22 and the chemokine receptor CCR4 are involved in Th2 cell migration. All these chemokines and chemokine receptors are detected in SGs from patients. Th1 inducible chemokines CXCL9 and CXCL10 are expressed by infiltrating T cells. CCL12 and CCL17 are detectable in/around the ductal epithelial cells, while CCR4 is expressed by the infiltrating lymphocytes in SGs with strong lymphocytic infiltration. Other chemokines implicated in inflammation and angiogenesis such as CXCL1 (GRO-α) and its receptor CXCR2 are overexpressed in SS biopsies. In addition, cultured SGECs show an increased CXCR2 expression after pro-inflammatory cytokine stimulation (IL-6 and TNF-α) [35]. These chemokines play a role in the neovascularization observed in diseased tissue [40]. Iwamoto et al., demonstrated the expression of CCL2 and its ligand CCR2 in ductal SGECs and infiltrating mononuclear cells of patients with pSS. IFN-γ stimulation can significantly increase

CCL2 production by cultured SGECs *in vitro* from pSS. They suggest that CCL2 interaction with CCR2 perpetuates the infiltration by mononuclear cells [162].

CXCL13 is produced by stromal cells, FDC, mDC and pDC within follicles of secondary lymphoid tissues. Primed T cells can produce CXCL13 as well. CXCL13 can promote follicular homing of B and T cells. LT-α/β is required for CXCL13 expression in lymphoid tissues. In NOD mice, CXCL13 can be inhibited by the blockade of the LT-β receptor (LTBR) pathway [160]. CXCR5 is the receptor of CXCL13. It is expressed by naïve B cells, follicular B cells, Tfh and some memory T cells. The expression of CXCR5 allows them to circulate in secondary lymphoid tissues. It is showed that CXCL13 and its receptor CXCR5 are overexpressed in SG of pSS patients [126, 163]. CXCL13 is expressed by both acinar and ductal SGECs as well as by endothelial cells and infiltrating monocytes (Figure 15). The development of ectopic GC in SS is thought to be regulated by CXCL13, CCL21 and CXCL12. They regulate the recirculation and positioning of $CXCR5^+$, $CCR7^+$ and $CXCR4^+$ cells and are confined to B cell follicle, T cell zone and GC respectively [164]. The ductal SGECs express all these three chemokines. CXCL13 and CCL21 are also detected with in infiltrating cells. In addition, CXCL13 and CCL21 expression within infiltrating cells but not within SGECs is significantly associated with an increased size of periductal inflammatory aggregates and degree of lymphoid organization [165]. Transgenic mice expressing CXCL13 in intestinal epithelial cells have a marked increase of B cells and of the size and number of lymphoid follicles in the small intestine. Moreover, overexpression of CXCL13 in the intestine favors mobilization of LTi and NK cells. LTi accumulated in lymphoid follicles express CXCR5 and produce IL-22 which is implicated in epithelial repair.

Figure 15. Expression of the chemokines CXCL13 and CXCR5 in patients with Sjögren's syndrome. **Salivary gland acinar and ductal epithelial cells express CXCL13 (A) [126]. Positive and negative acini/ducts are observed within the**

same section. Mononuclear cell infiltrates express CXCR5 (B) [163].



1.2.12 Costimulatory molecules

T cell activation requires two signals. Signal 1 is provided by the binding of an Ag peptide to a MHC on the surface of APCs which is recognized by TCR. A number of costimulatory molecules are required to completely active a T cell, referred as signal 2. Signal 2 provides positive signals for maturation, proliferation, survival and cytokine production. There are three groups of costimulatory molecules: B7-CD28 family, integrins and TNF receptor superfamily (TNFRSF) members. B7- CD28 family includes CD80 (B7-1) or CD86 (B7-2) binding to CD28 or CTLA-4, ICOSL (B7-H2, CD275) binding to ICOS (CD278), PD-1L (B7-H1, CD274) or PD-L2 (CD273) binding to PD-1 (CD279), B7-H4 binding to BTLA and B7-H3. They are constitutively expressed or rapidly induced at cell surface of T cells. Integrins interact with adhesion molecules to provide T cell activation signals and mediate T cell migration to the site of inflammation. The TNFRSF include 19 ligands and 30 receptors. Most of them are inducible and are expressed by activated $CD4^+$ and $CD8^+$ T cells. TNFSF and TNFRSF pairs notably include OX40L (TNFSF4, CD252) and OX40 (TNFRSF4, CD134), 4-1BBL (TNFSF9) and 4-1BB (TNFRSF9, CD137), CD70 (TNFSF7) and CD27 (TNFRSF7), TL1A (TNFSF15) and death receptor 3

(DR3, TNFRSF25), CD40L (TNFSF5, CD154) and CD40 (TNFRSF5), GITRL (TNFSF18) and GITR (TNFRSF18), FasL (TNFSF6) and Fas (TNFRSF6, CD95), RANKL (TNFSF11) and RANK (TNFRSF11A), BAFF and its receptors (BR3, TACI, BCMA).

1.2.12.1 CD80/CD86 - CD28 interaction

CD28 provides a strong costimulatory signal. It is needed for nearly all types of T-cell activation. Immature DCs which express low level of MHC II and low to absent levels of CD80/CD86, induce T cell anergy. T cells which receive CD28 signal are driven to proliferate and produce inflammatory cytokines such as IL-6 and IL-1β. The CD28 pathway is not only important for optimal T cell functions, but also for B-cell activation. CD28 deficient mice exhibit impaired GC development and Ab class switching. These mice have reduced T helper cell activity and decreased IL-2 levels.

Cytotoxic T-lymphocyte antigen 4 (CTLA-4), a key negative costimulatory molecule, binds to CD80/CD86 by competing with CD28. CTLA-4 mRNA is significantly increased in the SG of pSS patients. CD86 in patients is not only overexpressed by SGECs, but also displays distinctive binding properties. In cultured SS SGECs, CD86 has higher affinity for CD28 and presents reduced binding activity to CTLA-4. In SG of C57BL/6.NOD-*Aec1Aec2* mice, local expression of recombinant fusion protein CTLA-4-IgG decreases infiltrating T cells, B cell, DC and macrophages, which results in the inhibition of the loss of SG function. In addition, CTLA-4-IgG expression decreases several proinflammatory cytokines and increases TGF-β1 expression [166].

1.2.12.2 ICOS/ICOSL interaction

ICOS is not expressed by naïve T helper cells. It can be rapidly induced after TCR ligation and CD28 costimulation. ICOS is initially found in the light zone of GC.

ICOS binds to its unique ligand ICOSL, which is expressed constitutively on APCs. The function of ICOS involves T-B interaction and Tfh differentiation. Mice lacking ICOS have impaired GC development and impaired Ab class switching and reduced regulatory and effector memory T cell populations. $ICOSL^{-/-}$ mice have the same defects as $ICOS^{-/-}$ mice. ICOS promotes T cell proliferation and Th2 differentiation. ICOS is necessary for effective humoral immunity. Common variable immunodeficiency (CVID) is the most common primary immunodeficiency. It comprises a heterogeneous group of deficiencies including a homozygous deletion in ICOS gene [167]. $ICOS^{-/-}$ T cells have reduced IL-4 production upon activation. T cells activated with $ICOSL^{-/-}$ APCs secrete less IL-4 and IL-13 but not IL-5 or IFN-γ. In fact, depending on the context of the inflammatory response, ICOS can also promote Th1, Th17 and Tfh response. $ICOS^{-/-}$ mice are extremely sensitive to EAE but completely resistant to collagen-induced arthritis. The resistance to CIA could be caused by defective IL-17 secretion [168]. ICOS- deficient patients have a decreased IL-10 and IL-17 secretion.

ROQUIN (Rc3h1) is shown to repress ICOS mRNA posttranscriptionally. The *sanroque* mice have an altered form of Roquin incapable of mediating ICOS mRNA degradation, which results in overexpression of ICOS. The *sanroque* mouse is a lupus- and type 1 diabetes-prone mouse. It has an increased proportion of Tfh and exacerbated GC formation. However, ICOS and T-bet deficiency fail to rescue several autoimmune manifestations. It was recently shown that Ifng mRNA decay is also decreased in these mice. This causes an excessive IFN-γ signaling in T cells and leads to the accumulation of Tfh cells by an increase of Bcl-6 expression. Unlike ICOS and T-bet deficiency, IFN-γR deficiency prevents lupus development by reducing Tfh cells and levels of autoantibodies [169].

1.2.12.3 PD-1/PD-L1 interaction

The interaction between PD-1 and its ligand PD-L1 is important to regulate T cell activation, tolerance and tissue damage induced by immune response. This pathway exerts inhibitory functions in chronic viral infections, tumors and autoimmunity. PD-1 and PD-L1 are expressed by T cells, B cells, macrophages and some types of DCs. PD-1 signaling on T cells can inhibit CD28-mediated activation, thus reduce cytokine secretion and inhibit T cell proliferation and survival [170]. PD-1 ligand 2 (PD-L2) is a second ligand for PD-1. Engagement of PD-1 by PD-L2 dramatically inhibits TCR-mediated proliferation and cytokine production by $CD4^+$ T cells [171]. PD-1 is expressed by T lymphocytes infiltrates from 52% of SS patients. PD-L1 is expressed on ductal and acinar ECs from 68% of SS patients. PD-1-positive SS salivary lymphocytes express IL-10. *In vitro* activation with IFN-γ but not with TNF-α or IL-1β induces PD-L1 expression by HSG cells [32].

1.2.12.4 CD40/CD40L interaction:

CD40 is expressed by numerous cell types including monocytes, dendritic cells, endothelial cells, fibroblasts and epithelial cells. CD40-ligand (CD40L/CD154), a member of the TNF family, is mainly expressed by activated $CD4^+$ T cells. CD40 is involved in the amplification and regulation of inflammatory responses. The interaction between CD40 and CD40L, which is expressed transiently on activated $CD4^+$ T cells, can stimulate B cell proliferation and isotype switching in the presence of appropriate cytokines. Studies in vitro have demonstrated that CD40 activates the proliferation and differentiation of immature or mature B cell subsets, as well as Ig secretion. CD40 activation is needed for B cell differentiation, isotype switching and maturation into memory cells. Most of these processes take place in germinal centers, as previously mentioned. The expression of CD40 by non-professional APCs (endothelial cells, epithelial cells and fibroblasts) is observed under many

pathological conditions as well as *in vitro* after IL-1 or IFN-γ activation.

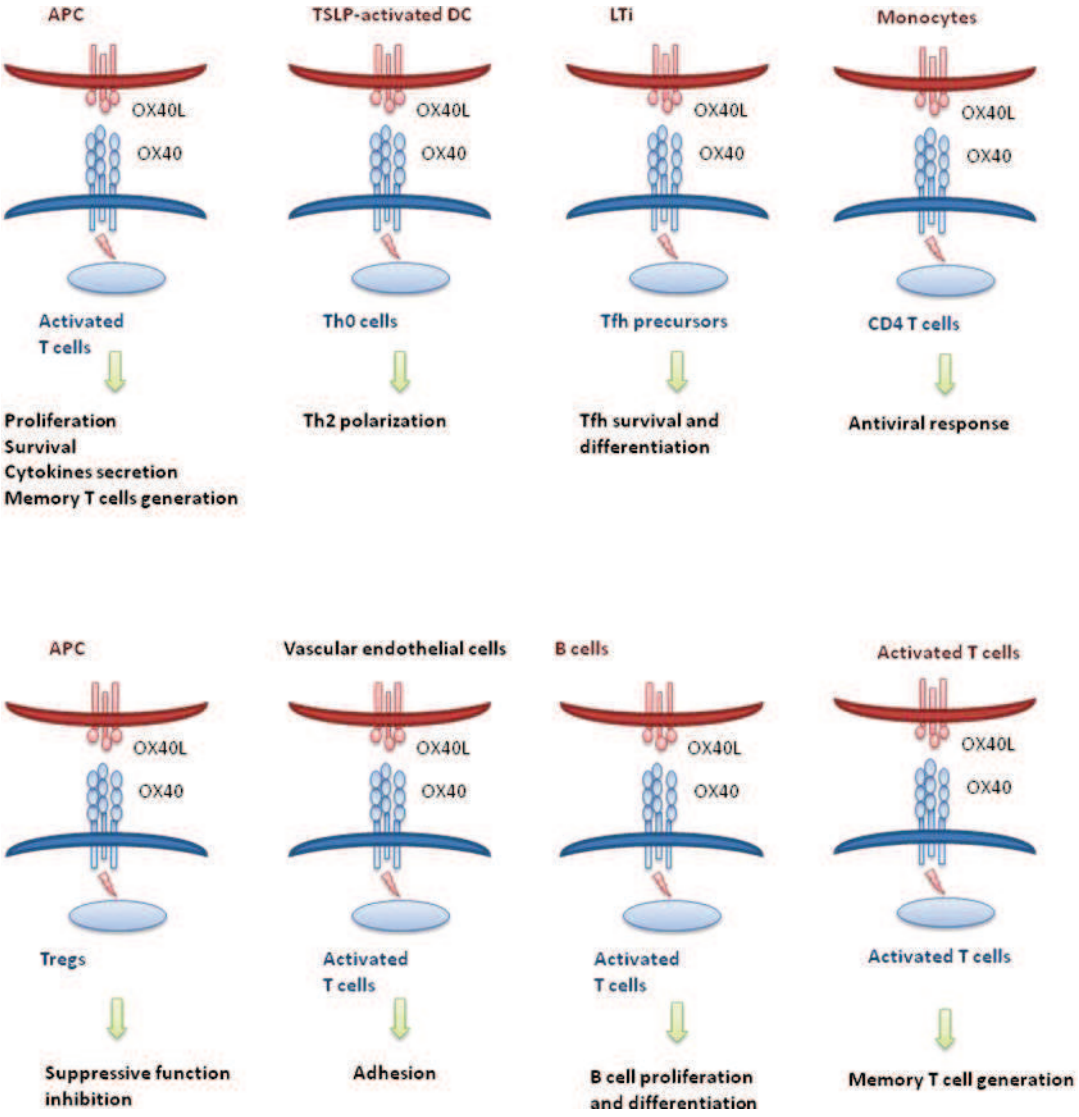
CD40 and CD40L expression is highly expressed by infiltrating mononuclear cells in SG of patients with SS. Elevated CD40L expression is found in SS ductal SGECs [172]. In SG of the pSS patients, interaction between CD40 and CD40L, both expressed by infiltrating T and B cells, might block apoptosis by inducing the expression of apoptosis inhibitors Bcl-2 and Bcl-x expression. CD40 ligation strongly enhances p38MAPK and NF-κB activity in cultured SGECs. The treatment of cells with anti-CD40 mAb results in significant Fas upregulation and induction of Fas-dependent apoptosis [173]. Soluble CD40L enhances ICAM-1 expression in cultured SS SGECs by activation of NF-κB p50 [174].

1.2.12.5 Main immunological consequences of the interaction between OX40/OX40L

OX40 and its unique ligand OX40L are glycoproteins. The crystal structure shows that the OX40L forms a homotrimer and interacts with three OX40 monomers. OX40L is mainly expressed on APCs (mature DCs, Langerhans cells, pDC, activated B cells and macrophages). Many non-professional APCs such as vascular endothelial cells, ECs, smooth muscle cells, LTi and other cell types such as NK and mast cells can be induced to express OX40L. CD40 signal, TLR2 and TLR4 signal and several cytokines (TSLP) are associated with OX40L expression [175-177]. Naïve T cells do not express OX40. OX40 is induced on naïve $CD4^+$ T cells by TCR/CD3 signal and this induction can be further enhanced by CD28 signal. Some cytokines such as IL-2 and TNF can enhance OX40 expression [178]. OX40 is constitutively expressed on mouse nTregs and can be induced on human Tregs at sites of inflammation. Activated $CD8^+$ T cells as well as both central and effector CD4 memory T cells express OX40 [179]. Finally, other cell types including NK T cells (NKT), NK and neutrophils can be induced to express OX40. OX40 on conventional T cells can induce anti-apoptotic

protein Bcl-XL, Bcl-2 and decrease pro-apoptotic protein Bad and Bim, thus enhance T cell survival. OX40 also induces T-cell proliferation. OX40 increases the secretion of IL-2, IL-4, IL-5 and IFN-γ by activated T cells (Figure 16).

Figure 16. Main roles of OX40/OX40L pathway [175, 180, 181].



The role of OX40 in the regulation of $CD4^+$ effector T cells

After clearance of Ag, most Ag specific $CD4^+$ effector T cells undergo cell death and a few effector T cells survive as long-lived memory T cells. They respond rapidly and differentiate into effector cells after Ag encounter. OX40 pathway is important for maintaining T cell memory by providing a late-stage costimulatory signal to sustain the survival of activated T cells. During T cell activation, CD28 signals induce early IL-2 production. Through IL-2R (CD25), IL-2 increases the expression of survival protein Bcl-2. CD28 signal is not sufficient for long-term T cell survival. In the absence of additional signals, the majority of T cells undergo apoptosis. OX40 is induced 24h after TCR engagement. OX40 signal alone does not induce much IL-2, but it strongly enhances T cell expansion and survival together with CD28 signal. Thus, there is a synergy between CD28 and OX40. OX40 deficient T cells cannot maintain anti-apoptotic protein level. So OX40 costimulation prolongs T cell life span and increase the proportion of memory T cells.

Role of OX40 on Treg

Whereas OX40 expression by conventional T cells is inducible, nTregs constitutively express OX40. OX40 signal on these cells makes them resistant to Treg suppression. As for effector and memory T cells, OX40 signal promotes proliferation and survival of nTreg. However, the ligation of OX40 reduces the suppressive activity of nTregs. Moreover, signaling through OX40 blocks the differentiation of $FoxP3^+$ iTreg driven by IL-2 and TGF-β as well as IL-10 producing $FoxP3^-$Tr1 cells. In all these cases, OX40 signal propels the balance in favor of effector activity, away from immunoregulation.

OX40L expression by LTi

Human lymphoid tissue inducer cells are innate lymphoid cells defined as $CD117^+CD3^-CD56^-OX40L^+$ cells located in human secondary lymphoid tissues. They

require transcription factor RORγT for their development and were initially identified for their role in driving lymphoid tissue development during embryogenesis. These cells efficiently express IL-17 and IL-22 [182]. Through IL-22 secretion, they can promote epithelial repair and tissue regeneration [183].

LTi are found to be elevated in the blood of multiple sclerosis patients compared to healthy subjects [184]. LTi are implicated in the formation of both normal and ectopic lymphoid organs. Transgenic mice overexpressing IL-7 have enhanced LTi cell numbers. In these mice, a 5-fold increase in Peyer's patch number as well as the formation of multiple organized ectopic lymph nodes are observed [185]. Signals through IL-7R up-regulate LTα1β2 expression on LTi cells. LTα1β2 expressed by LTi binds to LTβR to stimulate other stromal cell types. LTβR signaling leads to the up-regulation of adhesion molecules (ICAM-1 and VCAM-1) and chemokines associated GC formation (CXCL13, CCL19 and CCL21) [186]. Within GC, LTi cells support $CD4^+$ memory T cell survival in an OX40L-dependent manner [187]. LTi cells can active stromal cells through LTβR, which is important for the recruitment of DCs and B cells and formation of isolated lymphoid follicles in intestine. TNF-α produced by activated LTi cells can enhances IgA production of B cells [188]. It is shown that $CD62L^{high}$ central memory T cells migrate to lymph nodes while $CD62L^{low}$ effector memory T cells migrate to tissues after Ag recall. Both types of memory T cells express OX40 rapidly after Ag encounter. Thus, LTi are suggested to be important for the survival of activated $OX40^+$ central memory T cells. In addition to their involvement in adaptive immune responses, LTi contribute to innate immune responses by producing large amount of IL-22 against microbial infection.

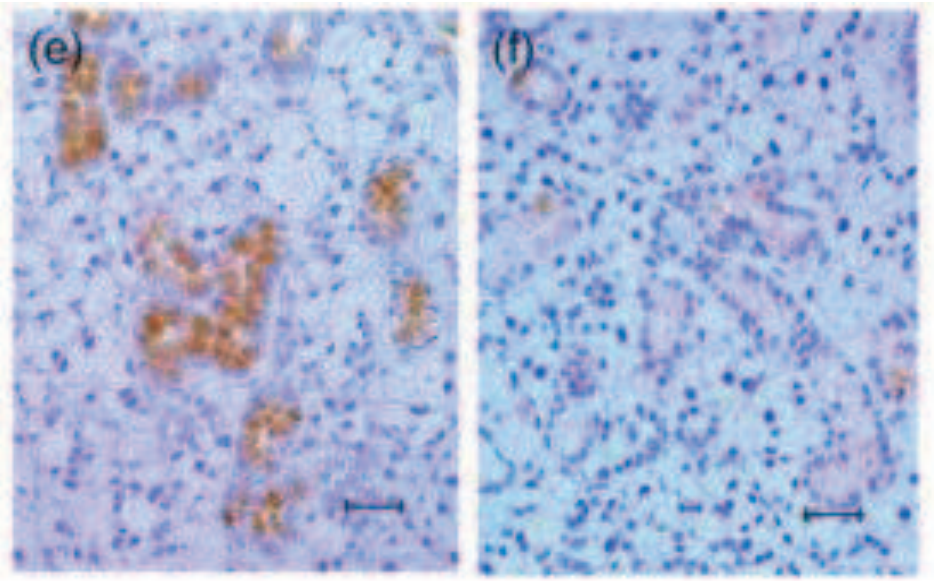
OX40-OX40L in autoimmune diseases

Both OX40 and OX40L are detected in the affected tissues of many autoimmune and inflammatory diseases as well as in their corresponding animal models, including

multiple sclerosis and EAE, RA and collagen-induced arthritis, SLE and $HgCl_2$-induced lupus, diabetes and graft-versus host disease (GVHD). *OX40L* gene is clustered with *FasL* and *GITRL* on human and mouse chromosome 1. Polymorphisms in the TNFSF4 gene region are associated with susceptibility to pSS, SLE and systemic sclerosis [7, 189]. In patients with SLE, both the proportion of conventional $CD4^+$ T cells and of Th17 cells expressing OX40 are significantly higher and are positively correlated with disease activity. Study in EAE mice shows that almost all $OX40^+$ T cells present autoAg-restricted TCR, while only <30% of $OX40^-$ T cells express autoAg-restricted TCR. This suggests that activation by cognate Ag upregulates the expression of OX40. In addition, signaling through OX40-OX40L is bidirectional. The reverse signaling pathway via OX40L can enhance B cell proliferation and contribute to B cell hyperactivity in SLE [190].

OX40L is expressed in SG of MRL-*Fas*^{lpr} mice [31] (Figure 17). However, very few data concerning the expression of OX40/OX40L are available in patients with pSS.

Figure 17. Immunohistochemical staining of OX40L in submandibular glands of MRL-*Fas*^{lpr} and MRL/$^+$ mice [31]. The costimulatory molecules are strongly expressed in duct epithelial cells of tissues from MRL-*Fas*^{lpr} mice but only weakly expressed in tissues from the MRL$^{/+}$ mice.



II. OBJECTIVE 1 AND RESULT 1

1. Objective 1

Recently, a fifth subpopulation of CD4 cell, follicular helper T cell, was identified after the Th1, Th2, Th17 and regulatory T cells. These Tfh have a particularly important role not only in the activation of B cells in the lymphoid organs, but also in extranodal germinal centers. The expression of IL-21 is increased in serum and salivary glands of patients with pSS. However, the relationship between this serum increase and clinical features of the disease, notably systemic disease activity, has not been investigated yet. Tfh cells are increased in blood and salivary glands of patients with pSS. Whether these Tfh cells are recruited from secondary lymphoid organs or locally differentiated from naïve T cells inside salivary glands is not known.

The first objective of this study was to investigate the ability of epithelial cells to induce the differentiation of naïve $CD4^+$ T cells into follicular helper T cells. Secondly, we analyzed the correlation between serum IL-21 and systemic disease activity. Lastly, we analyzed the potential association between 3 SNPs of IL-21 and IL-21R gene and pSS on the one hand, and serum IL-21 on the other hand.

2. Publication No 1:

Differentiation of follicular helper T cells by salivary gland epithelial cells and increase of IL-21 correlate with disease activity in primary Sjögren's syndrome

(Article submitted to the Journal of Autoimmunity)

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Running title: Salivary gland epithelial cells in pSS

Differentiation of follicular helper T cells by salivary gland epithelial cells and increase of IL-21 correlate with disease activity in primary Sjögren's syndrome

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Running title: Salivary gland epithelial cells in pSS

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Abstract

Follicular helper T cells (Tfh), which play a pivotal role in B cell activation and differentiation in lymphoid structures, secrete IL-21 whose augmented secretion is a hallmark of several autoimmune diseases. To decipher the cellular and molecular interactions occurring in salivary glands of patients suffering from primary Sjögren's syndrome (pSS), we investigated whether salivary gland epithelial cells (SGECs) were capable to induce Tfh differentiation. Co-cultures of naïve $CD4^+$ T cells and SGECs from both patients with pSS and controls were performed. Here, we report that IL-6 and ICOSL expression by SGECs contributes to naïve $CD4^+$ T differentiation into Tfh cells, as evidenced by their acquisition of a specific phenotype, characterized by Bcl-6, ICOS and CXCR5 expression and IL-21 secretion, but also but by their main functional feature: the capacity to enhance B lymphocytes survival. We demonstrated an increase of serum IL-21 with systemic activity. Finally, we analyzed the potential occurrence of a genetic association between *IL-21* or *IL-21R* gene polymorphisms and pSS or elevated IL-21 secretion. This study, which demonstrates a direct induction of Tfh differentiation by SGECs, emphasizes a yet unknown pathogenic role of SGECs and suggests that Tfh and IL-21 might be relevant biomarkers and/or therapeutic targets in primary Sjögren's syndrome.

1. Introduction

Primary Sjögren's syndrome (pSS), which is the most common systemic autoimmune disease after rheumatoid arthritis, presents as a disabling sicca syndrome associated with asthenia and pain. One third of the patients develop systemic manifestations [1]. The pathogenesis of pSS involves both genetic and environmental factors, which trigger the activation of innate and adaptive immunity [2,3].

Primary Sjögren's syndrome is an autoimmune epithelitis in which salivary gland epithelial cells (SGECs) play a crucial pathogenic role [4]. This role is particularly well illustrated in the NOD/scid mice model, in which features of epithelial cell activation can be observed in the absence of T, B and NK cells [5]. Likewise, poly I:C stimulated NZB/NZWF1 mice also develop dryness before the occurrence of salivary lymphocytic infiltrates [6]. In patients with pSS, the capacity of SGECs to secrete cytokines (IL-1, IL-6 and B-cell activating factor of the TNF family (BAFF)) and chemokines, as well as to present auto-antigens, was previously reported [7-9]. SGECs also express various molecules implicated in immune response, including TLRs [10], MHC I and MHC II, CD80, CD86 and CD40, Fas and FasL [11, 12]. SGECs also undergo major epigenetic changes that could play a role in the pathogenesis of the disease [13]. However, data regarding the role of SGECs in T cell differentiation and activation are limited and a precise description, at the molecular level, of their contribution to the pathogenesis of Sjögren's syndrome is still lacking.

Presence of different T cell subsets was evidenced in pSS, in peripheral blood and in salivary glands, including Th1, Th2, Th17, and regulatory T cells [3, 14,15]. Follicular helper T cells (Tfh) represent the main T cell subset capable to activate B cells in lymphoid organs. Tfh are notably crucial for the formation and maintenance of germinal center [16]. Tfh might contribute to B cell activation, a hallmark of this disease frequently associated with polyclonal hypergammaglobulinemia and autoantibody secretion. These properties notably result from Tfh-B lymphocytes cell surface interactions through expression of CXC chemokine receptor 5 (CXCR5), inducible T cell co-stimulator (ICOS) and secretion of IL-21. IL-21, which is mainly secreted by Tfh, is a key cytokine involved in B cell activation [17]. An increase in blood Tfh-like cells in pSS was reported, as well as elevated IL-21 and IL-21 receptor expression in salivary glands [18-22] and the pathogenic role of Tfh and IL-21, a potent immunostimulatory cytokine, has been suggested in other autoimmune diseases (AIDs), like systemic lupus erythematosus or rheumatoid arthritis [23-26]. Furthermore, association between gene polymorphisms of *Il-21* and Grave's disease, rheumatoid arthritis, lupus, celiac disease and type I diabetes was reported [27-31] as well as between a gene polymorphism of the *Il-21 receptor* gene and lupus [32].

In the present study, we investigated the capability of SGECs to induce Tfh differentiation and IL-21 secretion. Our work pointed to a prominent role of SGECs in the promotion of follicular helper T cells differentiation and IL-21 secretion through IL-6 and ICOSL expression. We also demonstrated an increase in serum IL-21 with

systemic disease activity. We last analyzed the potential association between 3 single-nucleotide polymorphisms of *IL-21* and *IL-21 receptor* genes and pSS.

2. **Materials and Methods**

2.1. Salivary gland epithelial cells

Primary cultures of SGECs from 8 patients with pSS and 6 controls (sicca syndrome without any feature of autoimmunity: no autoantibody, no salivary gland lymphocytic infiltrate) were established from minor salivary gland biopsies as previously described [33]. All subjects gave their informed consent and the study was approved by the Ethics Committee of Strasbourg University Hospital. The lobule was cut into small fragment and was placed into 75 cm^2 flasks with 3:1 mixture of Ham's F-12 and DMEM supplemented with 2.5% fetal calf serum (FCS) (all purchased from Invitrogen, Cergy-Pontoise, France), epithermal growth factor (Millipore, Molsheim, France) (10 ng/ml), hydrocortisone (0.4 µg/ml), insulin (0.5 µg/ml), penicillin (100 U/ml) and streptomycin (100 U/ml) and were incubated at 37 °C with 5% CO_2 in a humidified incubator. After 4 weeks of culture, cells at 70-80% confluence were dissociated with 0.25% trypsin-EDTA and subcultured on type I collagen coated culture vessels.

2.2 .Naïve $CD4^+$ T cell and B cell purification

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from buffy coats of healthy donors using Ficoll-Paque™. $CD4^+CD45RA^+$ naïve T cells were isolated from PBMCs using a negative selection kit (naïve $CD4^+$ T cell isolation kit II, human, Miltenyi Biotec, Paris, France)

according to the manufacturer's protocol. The purity of $CD4^+CD45RA^+$ Naïve T cells, analyzed by flow cytometry was > 95%. B cells were isolated using a negative selection kit (Negative selection human B cell enrichment kit, Stemcell Technologies, Grenoble, France). The purity of $CD19^+$ B cells, analyzed by flow cytometry was > 95%.

2.3. Cocultures of naïve $CD4^+$ T cells, B cells and SGECs

Naïve $CD4^+$ T cells (4×10^5) were cultured alone or co-cultured with 70%-80% confluence SGECs in a collagen pre-coated 24-well plate in RPMI1640 supplemented with 10% FCS. At day 5, T cells were stimulated for 24 hours by adding MACSiBead Particle loaded with antibodies against human CD2, CD3 and CD28 (bead-to-cell ratio 2:1) (T cell Activation/Expansion kit, Miltenyi Biotec, Paris, France). In some of these co-cultures, 10^5 autologous B cells were added to the culture at day 6. After 72 hours, B cells were stained with APC-conjugated anti-CD19 mAb. B-cell survival was assessed by $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) and Propidium Iodide (PI) staining. T cells were harvested for immunostaining and flow cytometry and culture supernatants were collected for IL-21 (eBioscience, San Diego, USA) and IL-6 (R&D Systems, Lille, France) quantification by ELISA.

For transwell experiments, SGECs (70-80% confluence) and naïve $CD4^+$ T cells (4×10^5) were seeded in the lower and upper chambers, respectively, of a 0.4 μm polycarbonate membrane transwell (Nunc from VWR, Fontenay-sous-Bois, France).

For experiments with supernatants of SGECs cultures, SGECs were incubated for 4 days in RPMI1640 supplemented with 10% FCS and supernatants were harvested and incubated on naïve $CD4^+$ T cells (4×10^5) for 5 days. Then, T cells were stimulated using anti-CD2, anti-CD3 and anti-CD28 beads for 24 h, as described.

For specific inhibitions, anti-IL-6 mAb (20 μg/ml), anti-ICOSL mAb (20 μg/ml) and their corresponding isotype control were added (all purchased from R&D Systems, Lille, France). SGECs were preincubated for 1 h with Abs, before addition of naïve $CD4^+$ T cells.

2.4. Flow cytometry analysis

To study ICOS and CXCR5 expression, T cells were harvested and washed after culture. Then, cells were stained with APC-conjugated anti-CD4 mAb, PE-conjugated anti-ICOS mAb, Alexa 488-conjugated anti-CXCR5 mAb. PE-conjugated Mouse IgG1, κ, Alexa 488-conjugated rat IgG2b, κ and 710®-conjugated Mouse IgG1, κ were used as isotype controls (all purchased from BD Biosciences, Le Pont de Claix, France). Before acquisition, Propidium Iodide (PI) or DAPI were added to stain the dead cells. Cells were acquired with a FACSCalibur™ cytometry using CELLQuest™ software or using a Becton Dickinson SLR II analytical flow cytometer (BD Biosciences, Le Pont de Claix, France).

2.5. RNA extraction and quantitative RT-PCR

Naïve $CD4^+$ T cells (8×10^5) were cultured alone or cocultured with 70%-80% confluence SGECs in a collagen pre-coated 24-well plate. After 4 h, T cells were harvested and total RNA was extracted using the TRIzol (Invitrogen, Cergy-Pontoise, France)/chloroform method, followed by reverse transcription into cDNA using an iScript™ cDNA Synthesis kit (Bio-RAD, Marnes-la-Coquette, france). Quantitative RT-PCR for the detection of Bcl-6 (B-cell CLL/lymphoma 6), T-bet/ TBX21 (T-box expressed in T cells), GATA3 (GATA binding protein 3), RORα/RORA (RAR-related orphan receptor A) and RORγt/RORC (RAR-related orphan receptor C) mRNA was performed using SensiMixPlus SYBR kit (Quantace, Qiagen, Courtaboeuf cedex, France). CD4 expression was used as an endogenous control. Samples were assayed on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Life Science). The primers used were Bcl-6 forward (5'-GTTTCCGGCACCTTCAGACT-3') and reverse (5'-CTGGCTTTTGTGACGGAAAT -3'); T-bet forward (5'-AACATCCTGTAGTGGCTGGTG -3') and reverse (5'-CCACCTGTTGTGGTCCAAGT -3'); GATA3 forward (5'-TTCCTCCTCCAGAGTGTGGT -3') and reverse (5'-AAAATGAACGGACAGAACCG -3'); RORA forward (5'-TCTCCCTGCGCTCTCCGCAC -3') and reverse (5'- TCCACAGATCTTGCATGGA -3'); RORC forward (5'- GGTGATAACCCCGTAGTGGA -3') and reverse (5'-CTGCTGAGAAGGACAGGGAG -3') and CD4 forward (5'-CCACTGGAAAAACTCCAACC -3') and reverse (5'-

GGTCCCAAAGGCTTCTTCTT -3').

2.6. Immunofluorescence experiments

SGECs were cultured in a collagen pre-coated 24-well plate. Cells were fixed with 4% paraformaldehyde for 30 min and then blocked in PBS contained 1% bovine serum albumin (BSA) for 1h. Cells were then incubated with mouse anti-human (ICOS ligand) ICOSL mAbs or Mouse IgG, 2b (R&D Systems, Lille, France) (2h) followed by incubation with DyLight-488 conjugated goat anti mouse IgG pAb (1h) (abcam). Image acquisition was performed under fluorescence microscopy.

2.7. Western Blot

SGECs were cultured alone or cocultured with naïve $CD4^+$ T cells (4×10^5) in a collagen pre-coated 24-well plate. After 24 h, cells were lysed with 100 µL of ice-cold lysis buffer (PBS with 0.05 M Tris-Cl (pH 8.0), 0.5% SDS, 1 mM DTT and protease inhibitors) on ice. Lysates were centrifuged for 10 min at 14,000 g at 4°C and supernatants were subjected to SDS-PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked using 3% BSA in TBS for 1 h at 25°C. The blots were incubated with mouse anti-human ICOSL mAbs (R&D systems, Lille, France) for 2 h at 25°C followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG monoclonal antibodies (1 h at 25°C) and detected by enhanced chemiluminescence (Super Signal West Femto Maximum

Sensitivity Substrate, Pierce, Rockford, USA) according to the manufacturer's instructions. To confirm the presence of equal amounts of proteins, bound antibodies were removed from the membrane by incubation in 62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS for 30 min at 24°C and the blots were reprobed with anti-GAPDH (Millipore, Molsheim, France) mouse monoclonal antibodies.

2.8. Patients

The ASSESS national multi-center prospective cohort (Assessment of Systemic Signs and Evolution in Sjögren's Syndrome) was set up in 2006 with a grant from the French Ministry of Health (Programme Hospitalier de Recherche Clinique 2005 P060228) [34]. Its primary objective was to identify valuable predictive factors of systemic complications and lymphoma in pSS during a 5-years prospective follow-up [35]. The Sjögren's International Collaborative Clinical Alliance (SICCA) cohort is comprised of nine international locations enrolling patients with Sjögren's syndrome or patients displaying symptoms of Sjögren's syndrome [36].

2.9. Quantification of IL-21 and serum markers

DNA, blood RNA and serum samples were obtained at enrollment. All biological samples were immediately frozen, stored and shipped to the Centre de Ressources Biologiques of Bichat Hospital, which has obtained the French Association for Quality Insurance (AFAQ) (certification number 2009/34457) according to the norm NFS 96900. Serum IL-21 of the first 330 pSS patients enrolled in the ASSESS cohort was assessed using ELISA (eBioscience, San Diego, USA), with a detection threshold

of 78 pg/ml. Total Ig levels were assessed by nephelometry as well as kappa and lambda free light chains (FLCs) (Freelite kit (Binding Site)).

2.10. Genotyping

3 SNPs located within *IL-21* locus (rs2221903, rs907715), and within *IL-21 receptor* locus (rs3093301) were genotyped in a cohort of 620 pSS patients and 513 controls of French ancestry. The 3 genotyped SNPs, located within intronic regions of the corresponding genes, were chosen on the basis of previously reported association study in autoimmune diseases in which these SNPs were significantly associated [31.32]. Forty eight additional Ancestry Informative Markers (AIMs) were genotyped in order to identify outliers of non-Caucasian origin. Genotyping was performed by competitive allele specific polymerase chain reaction (PCR) using FRET technology (KASpar genotyping; an improved fluorescence resonance energy transfer method developed by KBiosciences, Hoddeston, UK) (http://www.kbioscience.co.ik/). An association between patients genotypes and IL-21 serum levels was further tested for each studied polymorphism in a subgroup of 330 pSS patients from the ASSESS cohort.

Twenty-nine samples missing more than 15% of genotyping data were removed and one AIMs SNP failed genotyping. Finally, 79 population outliers were removed and case control association tests were performed among the remaining 574 pSS cases and 451 controls using univariate method. A replication study was performed on the SICCA cohort of patients with pSS (n = 227) and controls (n = 292).

2.11. Statistical analysis

Principal components (PC) analysis of 47 AIMs was performed using EIGENSTRAT to determine ancestry outliers and to adjust for population structure. Single SNP logistic regression assuming an additive model and with the first 2 PCs included as covariates were run in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) (Table I). Meta-analysis using a random effects model and including the first PC as a covariate was performed for rs2221903, rs907715 (*IL-21*) and rs3093301 (*IL-21R*) in PLINK to see if there were differences in risk for pSS among the ASSESS, Bicêtre and SICCA cohorts. Heterogeneity p-values were computed using Stata SE 11 (Stata Corporation, College Station, TX).

Statistical analysis of the proportion of cell populations and cytokine secretion in the co-culture experiments was performed by two-tailed unpaired t test with GraphPad 5.0 software.

3. Results

3.1. Salivary gland epithelial cells induce follicular helper T cell differentiation

Given the pivotal role of salivary gland epithelial cells in the pathogenesis of pSS [191-193], we investigated whether these cells could induce the differentiation of naïve $CD4^+$ T cells into follicular helper T cells. SGECs were isolated from minor salivary gland biopsies. To exclude cell dedifferentiation during primary culture, epithelial cells were characterized by immunohistochemistry. All tested samples were positive for cytokeratin 7, cytokeratin 19 and vimentin which confirmed their epithelial origin (Suppl. Fig. 1). Absence of staining with myeloperoxydase, anti-CD20 and anti-CD3 antibodies excluded the possibility of contamination by myeloid cells, B cells or T cells (data not shown). SGECs from controls or from patients with pSS were then co-cultured with purified naïve $CD4^+$ T cells for 4 hours or 6 days and activation markers of $CD4^+$ cells were monitored. As shown in Fig. 1A, $CD4^+$ T cells showed an increased expression of Bcl-6, the specific transcription factor of follicular helper T cells (Tfh), when co-cultured for 4h with SGECs harvested from controls (8.1±1.7 fold) or from pSS patients (10.1±5.1 fold) compared to naïve $CD4^+$ T cells cultured alone. Conversely, no change was observed in the expression of T-bet, GATA3 and RORα/RORA and RORγt/RORC, which are respectively the transcription factors specific for Th1, Th2 and Th17 responses (Figure 1A and data not shown). To gain more insights into the phenotype of T cells co-cultured with SGECs, we performed FACS analysis and labeled $CD4^+$-gated

lymphocytes with CXCR5 and ICOS antibodies (or IgG2b and IgG1 isotype controls) to identify Tfh cells. As illustrated on Fig. 2B, a marked and significant increase in $CXCR5^+$ $ICOS^+$ T cells could be observed when naïve T cells were co-cultured with SGECs from 0.2%±0.2 to 3.6%±2.4 with SGEC from patients with pSS and from 0.2%±0.1 to 3.6%±1.4 with SGEC from controls, Fig. 2C). Moreover, an increased IL-21 secretion was detected in the supernatants of naïve $CD4^+$ T cell co-cultured with SGECs from pSS patients and controls (594.7±265.0 pg/ml, 655.8±302.1 pg/ml, respectively) (Figure 1D). No secretion of IL-21 could be detected in the supernatants from naïve $CD4^+$ T cells or SGECs cultured alone (Figure 1D). No significant difference in the proportion of Tfh or in the amount of secreted IL-21 was observed when naïve $CD4^+$ T cells were co-cultured with SGECs from SS patients compared to SGECs from controls. Co-cultures of SGECs and T cells did not result in increased secretion of Th1, Th2 or Th17 cytokines, such as IL-12, IL-4 and IL-17 (data not shown). These data indicate that SGECs specifically induce the differentiation of naïve $CD4^+$ T cells into Tfh, characterized by the surface expression of Bcl-6, ICOS, CXCR5 and by IL-21 secretion.

A characteristic functional feature of Tfh cells is their ability to enhance B cell survival (12). We therefore performed co-culture of salivary gland epithelial cells (SGECs) and naïve $CD4^+$ T cells for 5 days, activated T cells with CD2/CD3/CD28-coupled beads for 24 hours before adding B cells for 3 days. $CD19^+$-gated cells were labeled with Propidium Iodide (PI) and

3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) to quantify B-cell survival. As shown in Fig. 2, the amount of surviving B cells (PI^- $DiOC_6^+$) was significantly increased after co-culture with T cells and SGECs (93.2%), compared to co-culture with only naïve $CD4^+$ T cells or with only SGECs (30.1% or 45.7%, respectively). Thus, T cells differentiated by SGECs exhibit not only phenotypic (CXCR5, ICOS expression and IL-21 secretion), but also functional characteristics of Tfh cell.

3.2. Induction of Tfh differentiation by SGECs is dependent on soluble factors and cell contact contributes to IL-21 secretion

To determine whether the induction of Tfh differentiation and IL-21 secretion by SGECs was dependent on soluble and/or cell surface factors, we performed transwell experiments. As illustrated in Fig. 3A, the proportion of naïve CD4+ T cell differentiating into $ICOS^+$ $CXCR5^+$ Tfh cells in coculture with SGECs remained similar, whether the two cell types were separated by a transwell (4.9%) or not (5.1%) (Fig. 3B). However, IL-21 secretion was significantly decreased in the experiments with a transwell (140.4±100.6 pg/ml compared to 545.9±260.5 pg/ml without a transwell) (Fig. 3C). To confirm these results, SGECs from SS patients were incubated with medium alone for 4 days, and then naïve $CD4^+$ T cells were stimulated with the supernatant of culture of SGECs, for 6 days. With a ratio similar to that observed in transwell experiments, the supernatants from SS SGECs induced the

differentiation of Tfh but did not result in a significant increase of IL-21 (Fig. 3D and 3E). Thus, the induction of Tfh differentiation by SGECs is dependent on soluble factors, whereas IL-21 secretion requires cell/cell contacts.

3.3 Differentiation of Tfh and IL-21 secretion involves IL-6 and ICOSL

IL-6 and IL-12 are known to play important roles in Tfh differentiation [38-40]. We observed that IL-12 mRNA or protein was not expressed by SGECs alone or in co-culture with naïve $CD4^+$ T cells (data not shown). Conversely, SGECs secreted large amounts of IL-6 (1249±1001 pg/ml) (Fig. 4A). SGECs and naïve $CD4^+$ T cells were then co-cultured in the presence of a blocking anti-IL-6 antibody or a control IgG1. The addition of anti-IL-6 mAb (but not a control IgG1) very effectively neutralized IL-6. Interestingly, the induction of Tfh differentiation (quantified as the proportion of $ICOS^+$ $CXCR5^+$ cells) was partially, but significantly inhibited when a neutralizing anti-IL-6 mAb was added to the culture medium (1.3%±0.8 compared to 3.1%±1.3, with a control IgG1, p= 0.02) (Fig. 4B). No significant change in IL-21 secretion was noted when a anti-IL-6 mAb was added to the SGECs-naïve T cells co-cultures (Fig. 4C). Thus, IL-6 secretion by SGECs contributes to Tfh differentiation.

ICOSL is a co-stimulatory molecule expressed at the cell surface which is pivotal

for the interaction of dendritic cells or B cells with Tfh [41]. We therefore investigated ICOSL expression by SGECs using immunostaining and western blot. Our results showed that, in basal conditions, SGECs expressed ICOSL (Fig. 5A and B). To analyze the contribution of ICOSL to SGEC/T-cell interactions, we selectively blocked ICOSL availability at the cell surface with a monoclonal antibody. Incubation of an anti-ICOSL mAb or a control IgG2b had no effect on the differentiation of $ICOS^+$ $CXCR5^+$ Tfh by SGECs. Interestingly, incubation of anti-ICOSL mAb significantly decreased IL-21 secretion (350.7 ± 243.2 compared to 584.5 ± 261.5 pg/ml with a IgG2b controle isotype (p=0.04) (Fig. 5D). Thus, membrane expression of ICOSL by SGECs contributes to IL-21 secretion by Tfh.

3.4. Association between serum IL-21 and systemic disease activity

As previously mentioned, elevated IL-21 serum level has already been associated to primary Sjögren's syndrome in small population samples [19]. We analyzed the association between serum IL-21 and features of the disease, using the ASSESS cohort. Serum IL-21 was significantly higher in patients with pSS (median: 341 pg/ml, 25^{th}-75^{th} [180-589] than in healthy controls (82 [55-118] pg/ml, $p < 0.0001$). Patients with either anti-SSA or anti-SSA and anti-SSB antibodies had more frequently detectable IL-21 than patients without no anti-SSA or anti-SSB antibodies (94.9% versus 87.0%, p= 0.02). In patients with elevated IgG levels (>

16.0 g/l), serum IL-21 level was significantly higher than in patients with normal IgG levels (391.0 [237.2-861.2] versus 320.0 [166.0-517.7] pg/ml, p= 0.0006). The same association was observed in patients with elevated kappa free light chains of immunoglobulins (371.5 [237.2-693.6] versus 324.0 [129.0-577.7] pg/ml, p= 0.03). Patients with elevated IL-21 levels had a significantly higher score of systemic activity, the international consensus systemic Eular Sjögren's syndrome Disease Activity Index (ESSDAI) [42] (4 [1.0-9.6] versus 2.0 [0-6.0], p= 0.008). The EULAR Sjögren's Syndrome Patient Related Index (ESSPRI), a patient reported outcome multinational consensus score evaluating dryness, fatigue, and pain, was not associated with elevated IL-21. Likewise, IL-21 was not increased in patients with decreased unstimulated salivary flow or abnormal Schirmer's test (data not shown).

3.5. Absence of a genetic association between IL-21 and IL-21R loci with primary Sjögren's syndrome

Since genetic associations between several autoimmune diseases and *Il-21* gene polymorphisms were previously reported [43], we genotyped 2 SNPs (rs907715 and rs2221903) in the *Il-21* gene and one (rs3093301) in the *Il-21 Receptor* gene in the ASSESS cohort of pSS patients and in healthy controls (Table I). No association with *IL-21* or *IL-21R* gene polymorphisms was observed (Table I). A similar analysis in another cohort (SICCA) did not show a statistically significant association between any of the three SNPs and disease status. A meta-analysis of the two cohorts

confirmed this observation. We also performed IL-21 quantification in the serum of the 330 pSS patients of the ASSESS cohort which were genotyped and searched for possible association between serum IL-21 level and polymorphisms in the *Il-21* and *Il-21R* genes. Again, statistical analysis did not reveal any significant association between a given genotype and IL-21 secretion (Table II).

4. Discussion

This study demonstrates the capacity of salivary gland epithelial cells to induce the differentiation of the main T cell subset that secretes IL-21, follicular helper T cells. It also provides important insights at the molecular level regarding the mechanisms by which this essential immune event occurs and participates in the pathogenesis of Sjögren's syndrome.

We frist hypothesized that this feature of pSS could result from the differentiation of naïve $CD4^+$ T cells into follicular helper T cells by salivary gland epithelial cells. We showed that SGECs *per se* do not secrete IL-21, conversely to other cytokines such as BAFF and IL-6. The main result of the present study was to demonstrate the capability of SGECs to directly differentiate naïve $CD4^+$ T cells into Tfh. This follicular subset of T cells was evidenced by the expression of Bcl-6, the specific transcription factor of Tfh [16], the surface expression of ICOS and CXCR5, IL-21 secretion, and enhancement of B-cell survival. These data, however, do not rule out the possibility that other cells present in lymphocytic infiltrates of salivary glands,

such as DC or B cells might also contribute *in vivo* to the differentiation of Tfh [44, 45]. Of note, even in the absence of concomitant stimulation with anti-CD28, an additional stimulating signal provided *in vivo* by antigen presenting cells, SGECs were still capable to induce Tfh differentiation, although to a lower extent (data not shown). Direct differentiation of naïve $CD4^+$ T cells into Tfh by SGECs might contribute to local B cell activation, differentiation in memory B cells, plasma cells, and autoantibody secretion in salivary glands. This capacity of SGECs alone to differentiate naïve $CD4^+$ T cells into Tfh might also contribute to the formation of germinal center-like structures in target organs of autoimmunity, a feature associated with an increased risk of lymphomas in pSS [46, 47]. Interestingly, no difference in terms of Tfh differentiation and IL-21 secretion was observed between patients with pSS and controls with dryness. This might be related to the limited population sample and to the fact that, for obvious ethical reasons, healthy controls could not be studied. The other possibility is that this capacity to induce Tfh differentiation is enhanced by the local environment observed *in vivo* in the disease.

In lymphoid organs, Tfh differentiation is a complex multi-step process depending on both cell-cell interactions and cytokines. SGECs secrete important amounts of IL-6 and the expression level of this cytokine is increased in salivary glands of patients with pSS. Specific inhibition of IL-6 resulted in decreased Tfh differentiation, without any change in IL-21 secretion. In this study, we also also demonstrate that the epithelial expression of ICOSL contributes to IL-21 secretion. ICOSL was previously

known to be expressed at the cell surface by antigen presenting cells, like B cells, monocytes, macrophages, DC and to play a pivotal role in Tfh differentiation [48, 49]. Thus, secretion of IL-6 by SGECs contributes to Tfh differentiation and membrane-bound expression of ICOSL by SGECs enhances IL-21 secretion by Tfh. This role of SGECs in Tfh differentiation and IL-21 secretion adds important new evidence to the active role of salivary gland epithelial cells in the pathogenesis of pSS. Of interest, it was recently shown that local suppression of IL-21 in submandibular glands could delay the development of Sjögren's syndrome in NOD mice [43]. Therefore, our results provide the rationale to evaluate the inhibition of IL-6, IL-21 and follicular helper T cells in primary Sjögren's syndrome.

In the second part of this translational research project, we investigated the interest of IL-21 as a biomarker of pSS. Increased serum IL-21 in pSS was previously reported only in limited population samples [19]. We confirmed this result in a large cohort and showed a significant association between serum IL-21 and the ESSDAI systemic disease activity score.

These results prompted us in the last part of the study to investigate whether the increase of IL-21 could be related to a genetic predisposition. We therefore assessed the genetic association between 3 SNPs located within *IL-21* and *IL-21R* genes and pSS or seum IL-21. No significant association was observed between

these gene polymorphisms of *IL-21* and *IL-21R* and the disease or serum IL-21. Thus, serum IL-21 is increased and associated with systemic disease activity but not with IL-21 gene polymorphisms. The absence of genetic predisposition adds to the potential interest of serum IL-21 as a biomarker candidate for pSS, a disease which definitively lacks predictive factors allowing personalized healthcare. Futher analyses are needed to investigate the relative contribution of Tfh differentiated locally, inside the salivary glands, compared to Tfh differentiated in lymphoid organs, to this systemic increase of IL-21.

In conclusion, this study, which demonstrates a direct induction of Tfh differentiation by SGECs, emphasizes a yet unknown pathogenic role of SGECs and suggests that Tfh and IL-21 might be relevant biomarkers and/or therapeutic targets in primary Sjögren's syndrome.

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Figure Legends

Figure 1. SGECs from both pSS patients or controls induce Tfh differentiation. A. Bcl-6, T-bet, GATA3 and RORα/RORA mRNA expression was determined by RT-qPCR in naïve $CD4^+$ T cells (Tn) after co-culture with or without SGECs from controls (control SGECs) (n = 5) or patients with pSS (SS SGECs) (n = 6) for 4 h. Results were normalized to CD4 expression. **B.** $ICOS^+CXCR5^+$ T cells (top graphs) were quantified by flow cytometry upon gating on $CD4^+$ cells and labeling with isotype controls (bottom graphs). **C.** the proportion of $ICOS^+CXCR5^+$ cells in $CD4^+$ T cells after co-culture with or without SS SGECs (n = 8) or control SGECs (n = 6) are presented. **D**. IL-21 secretion was determined by ELISA in culture supernatants. Data are expressed as the mean of samples ± SD and representative for at least three independent experiments. *: $p < 0.05$; **: $p < 0.005$; ***: $p < 0.0005$.

Figure 2. $CD4^+$ T cells co-cultured with SGECs increase B-cell survival. The proportion of surviving ($PI^-DiOC_6^+$) $CD19^+$ B cells is presented when isolated B cells were cultured for 3 days (B) or co-cultured with naïve T cells (B+Tn) or SGECs (B+SGECs) for 3 days or co-cultured during 3 days with Tn and SGECs that were previously mixed for 5 days followed by a 24h-long CD2/CD3/CD28 activation

(B+Tn+SGECs). Data are representative for at least three independent experiments.

Figure 3. Tfh differentiation by SGECs is dependent on soluble factors. **A.** CD4+-gated cells, $ICOS^+CXCR5^+$ were stained (top graphs) and analyzed by flow cytometry. Bottom graphs illustrate control staining with isotypic antibodies. **B.** The proportion of $ICOS^+CXCR5^+$ cells among $CD4^+$ T cells after co-culture with SS SGECs using or not a transwell (n = 8 in both cases) is presented. **C.** IL-21 release was determined by ELISA in culture supernatants. **D.** Quantification of $ICOS^+CXCR5^+$ cells in $CD4^+$ T cells after addition of medium or supernatant of culture of SGECs (n = 5) is presented. **E.** IL-21 secretion was determined by ELISA in culture supernatants. Data are expressed as the mean of samples ± SD and are representative for at least three independent experiments. ***, $p < 0.0005$.

Figure 4. IL-6 secretion by salivary gland epithelial cells contributes to Tfh differentiation. **A.** IL-6 secretion was determined by ELISA in culture supernatants of SS SGECs cultured alone or with naïve $CD4^+$ T cells with or without neutralizing anti-IL-6 antibody (αIL-6). **B.** The proportion of $ICOS^+CXCR5^+$ cells in naïve $CD4^+$ T cells culture alone or with SS SGECs with or without anti-IL-6 antibody or isotype control (IgG1) is presented (n = 6). **C.** IL-21 secretion was determined by ELISA in

culture supernatants in the same conditions as in B. Data are expressed as the mean of samples ± SD. *: $p < 0.05$; **: $p < 0.005$.

Figure 5. ICOSL surface expression by SGECs contributes to IL-21 secretion. A. Staining with ICOSL with specific mAb (and control labeling with isotype IgG2b) of cultured SS SGECs upon fluorescence microscopy are shown (bottom pictures). Top pictures are the corresponding phase contrast images. **B.** ICOSL expression in three SS SGECs was detected by Western blot with an anti-ICOSL antibody. For protein loading control, membranes were reprobed with anti-GAPDH antibodies. **C.** The proportion of $ICOS^+CXCR5^+$ cells in co-cultures between naïve $CD4^+$ T cells and SS SGECs with or without neutralizing anti-ICOSL antibody (αICOSL) or isotype control (IgG2b) is presented (n = 6). **D.** IL-21 secretion was determined by ELISA in culture supernatants in the same conditions as panel C. Data are expressed as the mean of samples ± SD and are representative for at least three independent experiments. *: $p < 0.05$.

Supplementary figure 1. Immunocytochemical characterization of SGECs. **A.** Positive staining for hematoxylin-eosin (H&E), **B.** cytokeratin 19, **C.** cytokeratin 7 and **D.** vimentin is shown. **E.** Fluorescence analysis indicates the expression of

cytokeratin 19 by SGECs upon specific immune staining (bottom left). Control isotypic Ab shows no signal (bottom right); Top pictures illustrate corresponding phase contrast images. **F.** Negative staining upon cytokeratin 19 labeling in human fibroblast-like synoviocytes (FLS) isolated from synovial tissues from RA patients. Only auto fluorescence is visible in the bottom picture. Experiments were performed between the 3rd and the 9th passage.

Fig. 1

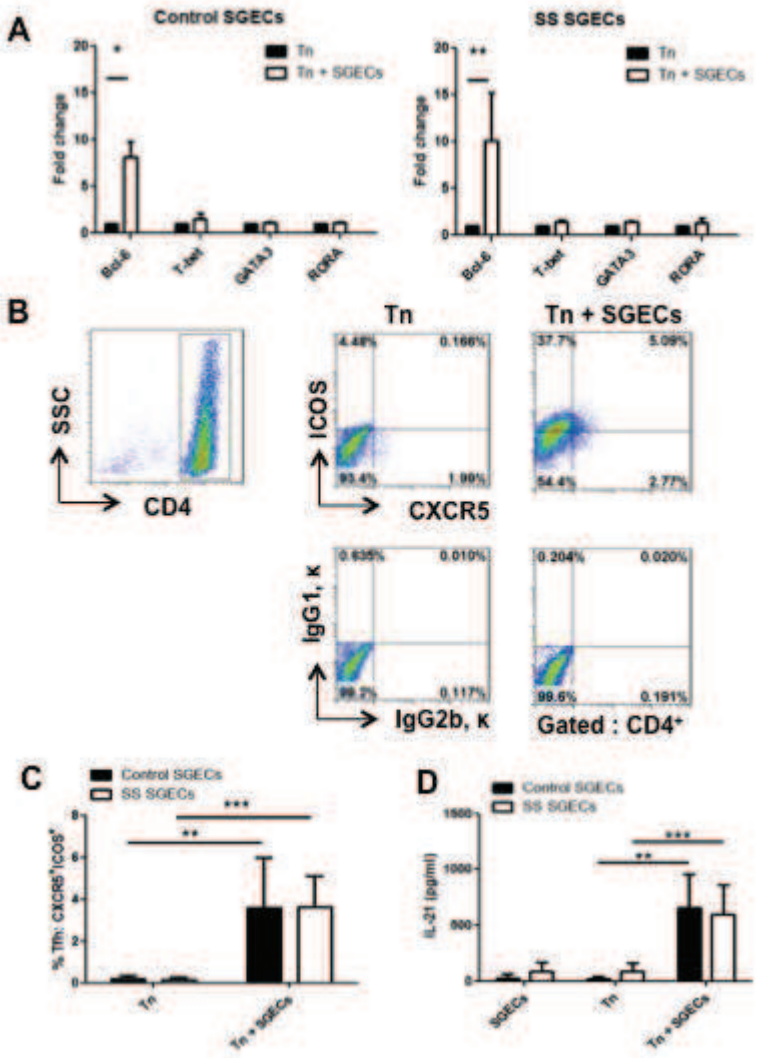

A
Control SGECs
SS SGECs
Tn
Tn + SGECs
Fold change
Bcl-6
T-bet
GATA3
RORA
B
Tn
Tn + SGECs
SSC
CD4
ICOS
CXCR5
IgG1, κ
IgG2b, κ
Gated : CD4+
C
Control SGECs
SS SGECs
% Tfh: CXCR5+ICOS+
D
IL-21 (pg/ml)
SGECs

Fig. 2

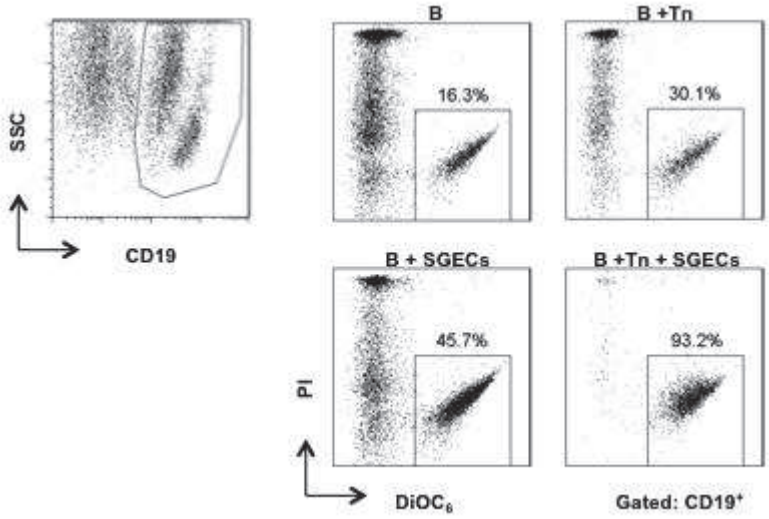

SSC
CD19
B
16.3%
B +Tn
30.1%
B + SGECs
45.7%
B +Tn + SGECs
93.2%
PI
DiOC6
Gated: CD19+

Fig. 3

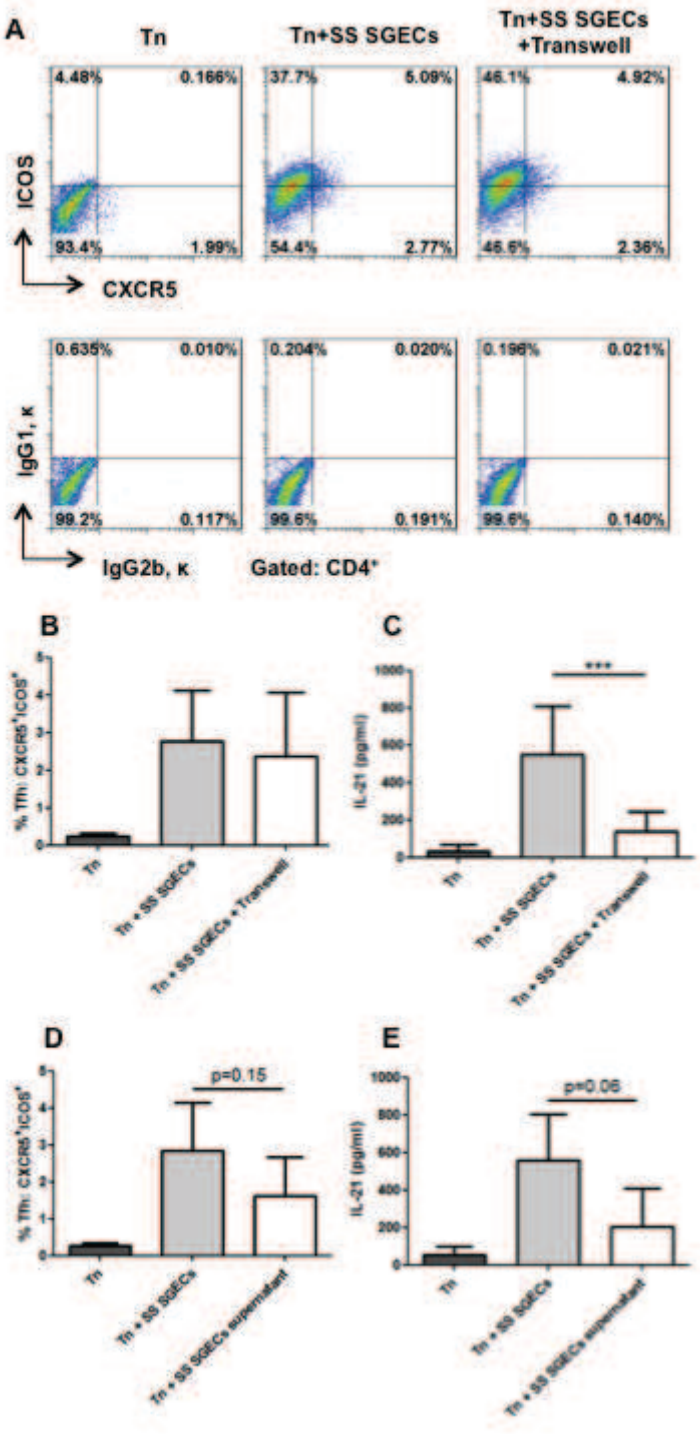

A
Tn
Tn+SS SGECs
Tn+SS SGECs +Transwell
4.48%
0.166%
93.4%
1.99%
37.7%
5.09%
54.4%
2.77%
46.1%
4.92%
46.6%
2.36%
ICOS
CXCR5
0.635%
0.010%
99.2%
0.117%
0.204%
0.020%
99.6%
0.191%
0.196%
0.021%
99.6%
0.140%
IgG1, κ
IgG2b, κ
Gated: CD4+
B
% Th1 CXCR5+ICOS+
Tn
Tn + SS SGECs
Tn + SS SGECs + Transwell
C
IL-21 (pg/ml)
***
D
p=0.15
Tn + SS SGECs supernatant
E
p=0.06

Fig. 4

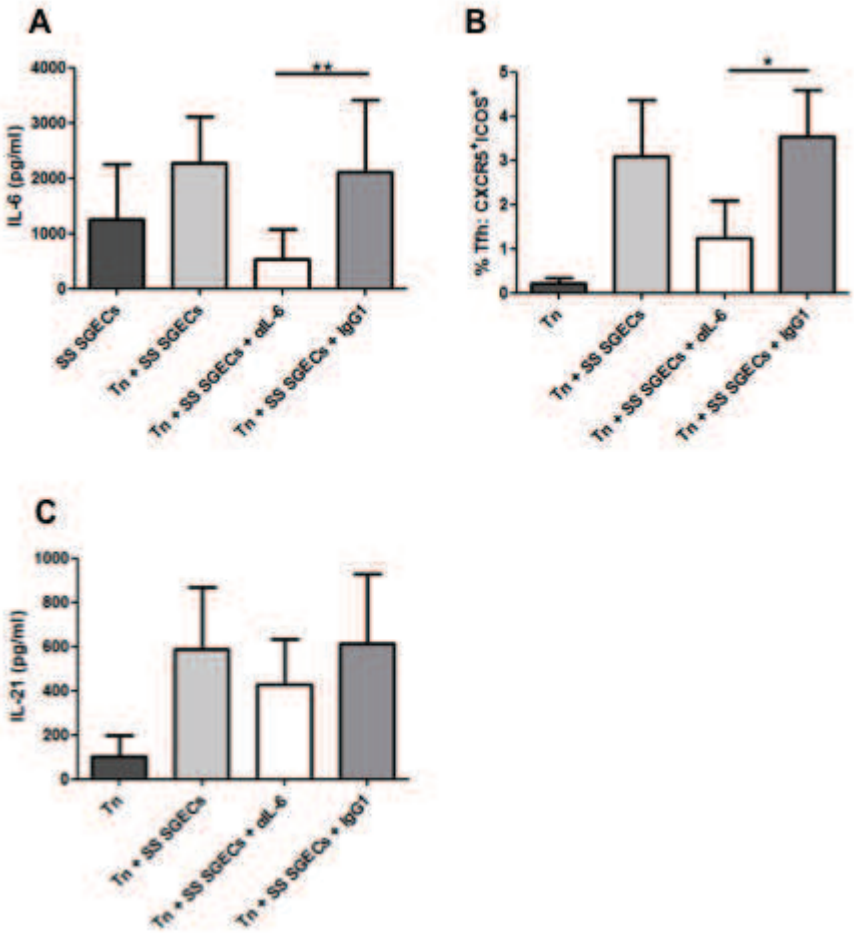

A
IL-6 (pg/ml)
4000
3000
2000
1000
0
**
SS SGECs
Tn + SS SGECs
Tn + SS SGECs + αIL-6
Tn + SS SGECs + IgG1
B
% Tfh: CXCR5+ICOS+
5
4
3
2
1
0
*
Tn
Tn + SS SGECs
Tn + SS SGECs + αIL-6
Tn + SS SGECs + IgG1
C
IL-21 (pg/ml)
1000
800
600
400
200
0
Tn
Tn + SS SGECs
Tn + SS SGECs + αIL-6
Tn + SS SGECs + IgG1

Fig. 5

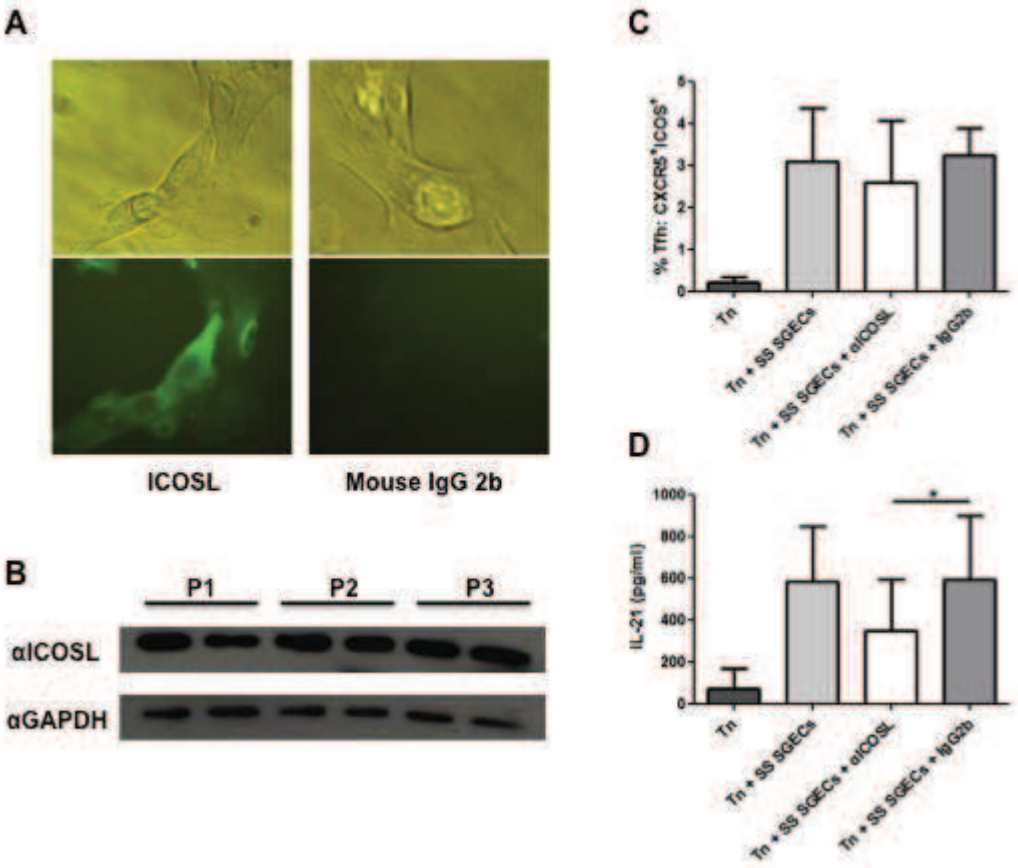
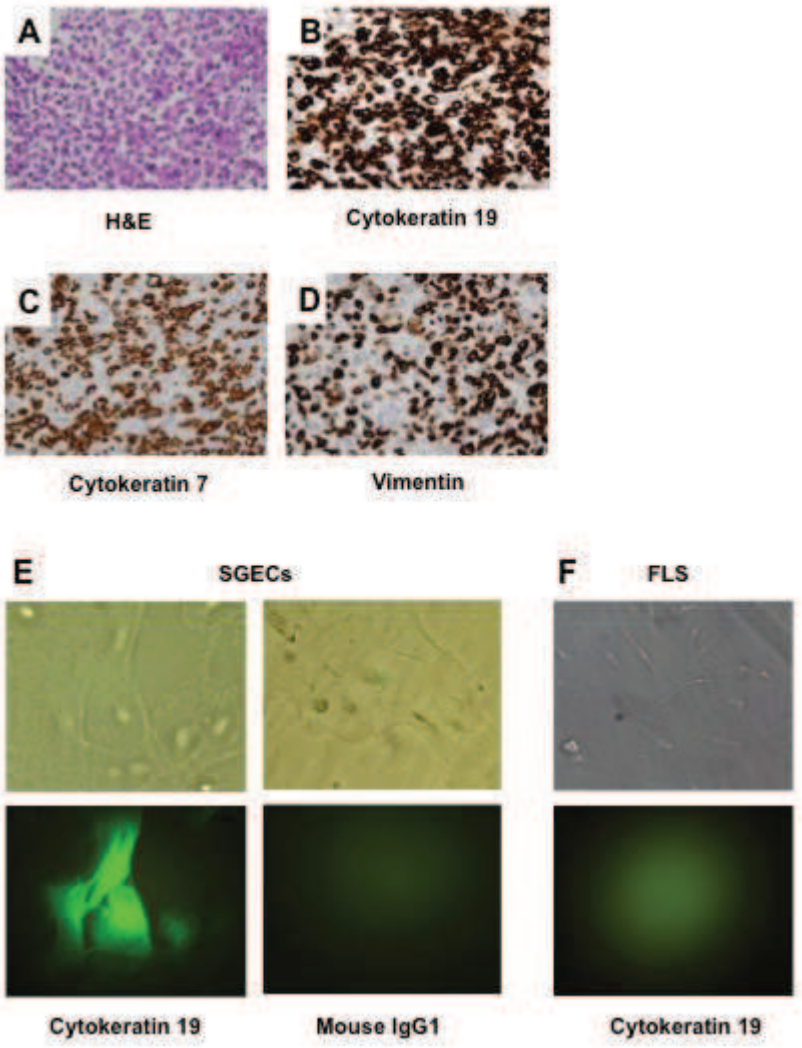

A
ICOSL
Mouse IgG 2b
B
P1
P2
P3
αICOSL
αGAPDH
C
% Tfh: CXCR5+ICOS+
Tn
Tn + SS SGECs
Tn + SS SGECs + αICOSL
Tn + SS SGECs + IgG2b
D
IL-21 (pg/ml)
Tn
Tn + SS SGECs
Tn + SS SGECs + αICOSL
Tn + SS SGECs + IgG2b

Fig. S1


A
B
H&E
Cytokeratin 19
C
D
Cytokeratin 7
Vimentin
E
SGECs
F
FLS
Cytokeratin 19
Mouse IgG1
Cytokeratin 19

	Chr	SNP	Gene	Minor allele	Major allele	OR	95% CI	P	MAF pSS	MAF controls
ASSESS and Bicêtre cohort	4	rs907715	IL-21	A	G	0.8642	(0.71 - 1.05)	0.1379	31 (344)	34 (292)
	4	rs2221903	IL-21	G	A	1.199	(0.98 - 1.46)	0.07188	35 (394)	30 (258)
	16	rs3093301	IL-21R	C	T	1.211	(1 - 1.47)	0.05083	38 (421)	34 (295)
SICCA cohort	4	rs907715	IL-21	A	G	0.848	(0.64 - 1.13)	0.255	29 (132)	32 (185)
	4	rs2221903	IL-21	G	A	1.057	(0.81 - 1.38)	0.6809	33 (151)	32 (188)
	16	rs3093301	IL-21R	C	T	1.003	(0.77 - 1.3)	0.9808	35 (157)	35 (204)
	Chr	SNP	Gene	Minor allele	Major allele	OR	95% CI	P		
Meta-analysis	4	rs907715	IL-21	A	G	0.8696	(0.74 - 1.02)	0.08301		
	4	rs2221903	IL-21	G	A	1.154	(0.99 - 1.35)	0.0719		
	16	rs3093301	IL-21R	C	T	1.135	(0.97 - 1.32)	0.1037		

Table I: Case-control association study of *IL-21* and *IL-21R* SNPs with pSS.
Chr, chromosome; OR, odds ratio; CI, confidence interval; SNP single-nucleotide polymorphism; MAF, minor allele frequency (in %), as well as the number of occurrence (en parentheses) uncounted for the different alleles is indicated for the cohorts of pSS patients and the control group.

	Genotypes			P
rs3093301 (*IL-21R*)	**CC**	**CT**	**TT**	
IL-21 serum levels (pg/ml) +/- SD	361.2 (218.4-660.7)	317.4 (166.0-544.6)	370.3 (210.6-598.9)	0.8
rs907715 (*IL-21*)	**AA**	**AG**	**GG**	
IL-21 serum levels (pg/ml) +/- SD	343.7 (262.2-725.4)	358.8 (234.6-582.7)	275.0 (148.4-536.6)	1
rs2221903 (*IL-21*)	**AA**	**AG**	**GG**	
IL-21 serum levels (pg/ml) +/- SD	337.2 (203.0-645.3)	354.7 (187.2-550.9)	298.9 (148.9-576.4)	0.9

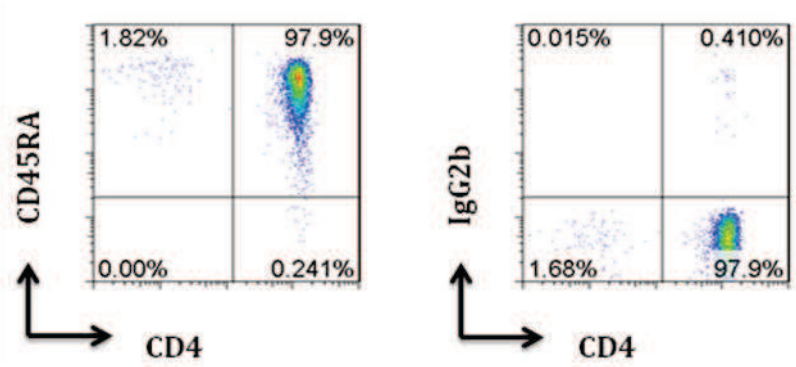
Table II: IL-21 serum levels according *IL-21* and *IL-21R* genotypes among 330 patients with pSS

3. Supplementary data that were not included in the submitted manuscript

3.1 Analysis of cell purification state

Immediately after magnetic isolation, the purity of $CD4^+CD45RA^+$ T cells has been analyzed by flow cytometry. To analyze the purification state of our naïve T cell population, cells have been stained with anti-CD4 and anti-CD45RA mAbs after magnetic separation. As shown in Figure 18, 97.9% of isolated cells coexpressed CD4 and CD45RA. The percentage of naïve T cells expressing both CD4 and CD45RA was 97.9%.

Figure 18. Purity analysis of freshly isolated naïve $CD4^+$ T cells by flow cytometry. **Purified T cells have been stained with APC-conjugated anti-CD4 and PE-conjugated anti-CD45RA mAbs (left) or corresponding isotype control (right). The proportion of each population among living cells is indicated.**

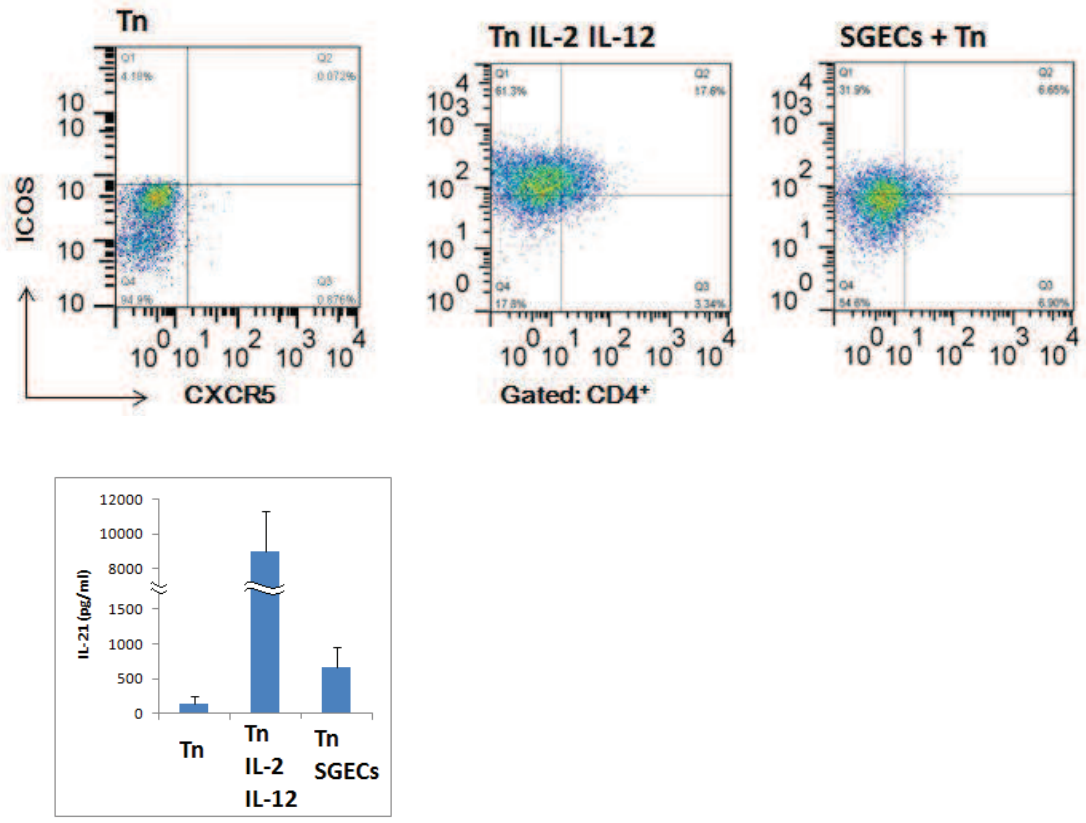


3.2 Using IL-12-induced Tfh as positive control

Ma et al., showed in 2009 that IL-12 can induce the differentiation of naïve T

lymphocytes into Tfh. We have used IL-12 induced Tfh as positive control. Naïve T cells have been subsequently activated with anti-CD2, anti-CD3 and anti-CD28 beads, (cell: beads = 5/1), human recombinant IL-2 (20Uml) and human recombinant IL-12 (20 ng/ml). After 3 days, T cells increased their expression of ICOS and CXCR5 and secreted IL-21 in the supernatant (figure 19). Double positive cells ($ICOS^+CXCR5^+$ T cells) represented 17% of all T cells.

Figure 19. Tfh induction and IL-21 secretion by naïve $CD4^+$ T cells activated by IL-12 or cocultived with SGECs.

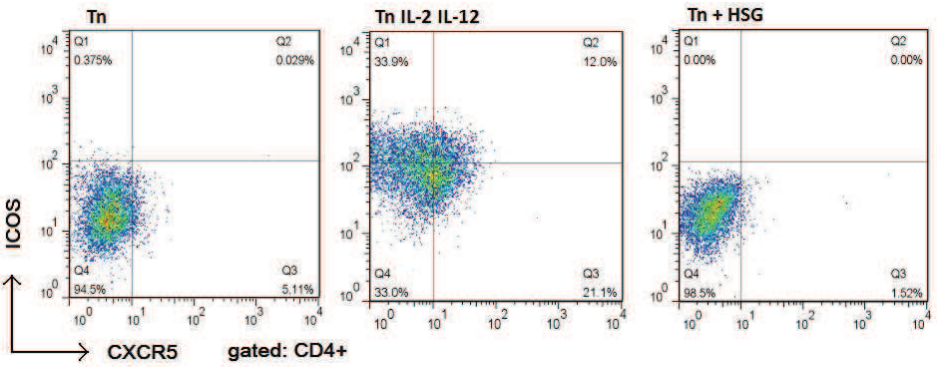


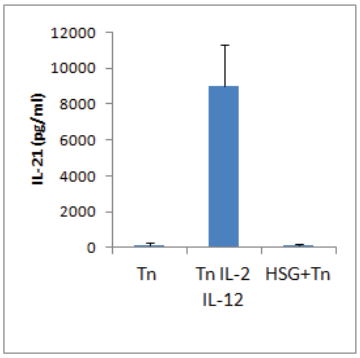
3.3 Epithelial cell line HSG cannot induce the Tfh differentiation

To investigate the effect of epithelial cell line HSG on T cell differentiation, we have cocultured naïve $CD4^+$ T cells with HSG. The naïve T cell-HSG coculture have

been performed under the same conditions as for naïve T cell-SGEC coculture experiments. IL-12 induced Tfh differentiation has been used as positive control. As shown in figure 20, the expression of ICOS, CXCR5 and the synthesis of IL-21 was not increased after coculture. These results forced us to work on primary culture of salivary gland epithelial cells instead of on this HSG cell line.

Figure 20. Expression of ICOS and CXCR5, as well as IL-21 production by naïve $CD4^+$ T cells cocultured with HSG.





III. OBJECTIVE 2 AND RESULT 2

1. Objective 2

OX40/OX40L interaction is a pivotal costimulatory pathway involved in multiple autoimmune diseases. Polymorphisms of OX40L are implicated in the genetic predisposition to many autoimmune diseases such as primary Sjögren's syndrome and systemic lupus erythematosus. The proportion of $OX40^+$ in CD4 T cells especially in IL-17 producing T cells is significantly higher and correlated with disease activity. T cells infiltrating renal biopsies from SLE patients express OX40. Serum OX40L in SLE patients is significantly higher. Therefore, OX40-OX40L interaction plays an important role in the pathogenesis of SLE.

As SLE and pSS share many common physiopathological features, a better understanding of the pathogenic role of OX40/OX40L in pSS could be of interest. Former studies demonstrated that T cells infiltrating salivary glands of patients with pSS undergo clonal expansion and exhibit resistance to apoptosis.. OX40 signalling promotes clonal expansion and survival of effector and memory T cell populations. An additional reason to focus our interest on OX40 after the study of Tfh is the potential synergy between OX40 and ICOS in Tfh differentiation.

The first aim of the present project was to study OX40/OX40L expression in peripheral blood and salivary glands from patients with pSS. The second aim was to detect whether coculture between $CD4^+$ naïve T cells and SGECs could induce OX40 expression by T cells. The last aim was to analyze the influence of OX40/OX40L interaction on T-cell proliferation and survival.

2. Publication No 2:

Salivary gland epithelial cells express OX40L and induce OX40 on $CD4^+$ T cells in primary Sjögren's syndrome

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Running title: OX40L and OX40 in primary Sjögren's syndrome

Salivary gland epithelial cells express OX40L and induce OX40 on $CD4^+$ T cells in primary Sjögren's syndrome

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ABSTRACT

Objectives. OX40/OX40L interaction is a pivotal costimulatory pathway that provides a key signal for T-cell proliferation and differentiation in effector and memory subsets. Polymorphisms of OX40L are involved in the genetic predisposition to primary Sjögren's syndrome (pSS). We therefore investigated the pathogenic role of OX40/OX40L pathway in pSS.

Patients and methods. The expression of OX40 and OX40L in blood and salivary glands from patients with pSS or control subjects was evaluated using flow cytometry and immunohistochemistry. Activated naïve $CD4^+$ T cells were cultured alone or cocultured with salivary gland epithelial cells (SGECs) derived from minor salivary gland biopsies. T-cell expression of OX40, proliferation and survival were analyzed.

Results. The proportion of $CD4^+$ T cells expressing OX40 among peripheral blood CD4+ T cells was elevated in patients with pSS compared with control subjects. Moreover, the proportion of peripheral blood $CD4^+OX40^+$ cells was correlated with systemic disease activity assessed by the Eular Sjögren's Syndrome Disease Activity Index. In salivary glands of patients with pSS, epithelial cells and B cells overexpressed OX40L compared with control biopsies and the expression of OX40 was evidenced on infiltrating T cells in patients with pSS. Coculture of T cells with SGECs increased the expression of OX40 by CD4+T cells compared to T cells

cultured alone and promoted T cell survival and proliferation through OX40/OX40L interaction.

Conclusions. The present study demonstrates an increased expression of OX40 by blood $CD4^+$ T cells associated with systemic disease activity. SGECs express OX40L and are capable to induce the expression of OX40 by T cells, which subsequently promotes T-cell survival and proliferation. These results suggest that the OX40/OX40L pathway might represent a relevant therapeutic target in pSS.

INTRODUCTION

The hallmarks of primary Sjögren's syndrome (pSS), which is the most common systemic autoimmune disease after rheumatoid arthritis, involve a disabling sicca syndrome associated with asthenia and pain. One third of the patients develop systemic manifestations [1].

The pathogenesis of pSS involves both genetic and environmental factors, resulting in the activation of innate and adaptive immunity. pSS is an autoimmune epithelitis in which salivary gland epithelial cells (SGECs) play a crucial pathogenic role. This role is particularly well illustrated in the NOD/scid mice model, where features of epithelial cell activation can be observed in the absence of T, B and NK cells [2]. Likewise, poly I:C stimulated NZB/NZWF1 mice also develop dryness before the occurrence of salivary lymphocytic infiltrates [3]. Okuma et al. recently demonstrated that a deficiency in epithelial tissues of the NF-κB regulator inducible

kinase B (IκB)-ζ is sufficient to elicit SS-like pathology [4]. In patients with pSS, the capacity of SGECs to secrete cytokines (IL-1, IL-6 and B-cell activating factor (BAFF)) and chemokines or present auto-antigens was previously reported [3, 5-7]. SGECs also express various molecules implicated in immune response, including Toll-like receptors, MHC I and MHC II, CD80, CD86 and CD40, Fas and FasL [8, 9]. However, limited data are available regarding the pathogenic role of SGECs in local T-cell activation in salivary glands. The OX40/OX40L pathway plays a crucial role in T-cell activation and differentiation. OX40 (CD134, TNFRSF4) and its ligand OX40L (CD252, TNFSF4) are members of the TNFR and TNF superfamily, respectively [10]. OX40 is mainly expressed by activated CD4 and CD8 T cells and OX40L by professional antigen presenting cells (APCs), such as activated B cells, dendritic cells (DC) and macrophages. OX40 signalling promotes T-cell survival and increases the clonal expansion of effector and memory populations. APCs co-expressing OX40L and CD80 promote naive T- cell proliferation and enhance cytokines secreted by Th1 and Th2 effector cells such as IL-2, IL-4, IL-5, and IFN-γ.

OX40/OX40L interactions have been found to play a critical pathogenic role in multiple autoimmune diseases, including rheumatoid arthritis (RA) [11, 12] systemic lupus erythematosus (SLE) [13] gastrointestinal autoimmune diseases [14], multiple sclerosis [15] and diabetes [16]. OX40L gene polymorphisms are involved in the genetic predisposition to SLE and systemic sclerosis [17, 18]. In pSS, very limited data are available regarding the pathogenic role of OX40/OX40L and are restricted to

the association of OX40L gene polymorphisms and the disease [19]. We therefore analysed the expression of OX40/OX40L in peripheral blood, salivary glands and the functional role of this pathway on T cells in primary Sjögren's syndrome.

PATIENTS AND METHODS

Patients.

Minor salivary gland biopsies or blood samples from patients were obtained from patients attending the Departement de Rhumatologie, Hôpitaux Universitaires de Strasbourg, France and were collected during routine clinical procedures. All subjects gave their informed consent and the study was approved by the Ethics Committee of Strasbourg University Hospital.

For the immunohistochemistry analyses, five patients with pSS and 5 controls (sicca syndrome without any feature of autoimmunity: no autoantibody, no salivary gland lymphocytic infiltrate) were included in. For the analysis of peripheral blood, 18 patients with pSS were included. The systemic disease activity of patients on the day of blood sample was assessed using the EULAR Sjögren's Syndrome Disease Activity Index [20]. Among those patients, 3 were treated with oral prednisone (5 , 5 and 10 mg/d), 2 with hydroxychloroquine, 3 with another immunosuppressant (methotrexate :n= 2, leflunomide : n= 1). Blood samples from 20 age- and sex-matched healthy donors were obtained from EFS (Etablissement Français du Sang, Strasbourg). Ten patients with pSS and 6 controls were included in the coculture analyses.

Immunohistochemistry staining. Minor salivary gland biopsies from 5 patients

with pSS patients and 5 controls were fixed in 4% paraformaldehyde, and embedded in paraffin. 4 μm paraffin sections were treated with citric buffer (10mM citric acid, pH6) to retrieve antigen. Slides were then blocked in 5% normal goat serum (Vector Laboratories) and incubated with primary antibody: rabbit monoclonal anti-CD11c (clone EP1347Y, Abcam); rabbit monoclonal anti-CD19 (clone EPR5906, Abcam); mouse monoclonal anti-CD4 (clone mAbcam67480, Abcam); rabbit polyclonal anti-OX40 (Abcam); mouse monoclonal anti-OX40L (clone 159403, R&D systems), mouse IgG1 isotype control (R&D systems, Lille, France) and rabbit polyclonal anti-cytokeratin 19 (Sigma-Aldrich). After washing, sections were incubated with secondary antibody: DyLight® 650-conjugated goat anti-rabbit IgG H&L (Abcam) or DyLight®-488 conjugated goat anti mouse IgG polyclonal Ab (Abcam) and mounted with Vectashield medium (Vector Laboratories) containing DAPI (Invitrogen). Biopsies of salivary gland were stained simultaneously with two cell markers (OX40 and CD4, OX40L and CD11c (marker of mDC), or CD19 (marker of B cells), or cytokeratin 19 (epithelial marker).

Immunostaining of OX40L. SGECs (5×10^4 cells/well; IbiTreat slides) were fixed and blocked in PBS contained 1% bovine serum albumin (BSA). Cells were then incubated with mouse anti-human OX40L mAbs (clone 159403) or Mouse IgG1 (R&D Systems, Lille, France) followed by incubation with DyLight-488 conjugated goat anti mouse IgG polyclonal Ab (Abcam). Fluorescence was acquired and analyzed using Leica DMRXA2 fluorescence microscopy and ImageJ 1.47q.

Naïve $CD4^+$ T cell purification. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from blood samples of healthy donors using Ficoll-Paque™. $CD4^+CD45RA^+$ naïve T cells were isolated from PBMCs using a negative selection kit (naïve $CD4^+$ T cell isolation kit II, human, Miltenyi Biotec) according to the manufacturer's protocol. The purity of $CD4^+CD45RA^+$ Naïve T cells, analyzed by flow cytometry, was > 95%.

Cocultures of naïve $CD4^+$ T cells and SGECs. Primary cultures of SGECs from 10 patients with pSS and 6 controls were established from minor salivary gland biopsies as previously described [21]. Naïve $CD4^+$ T cells (4×10^5) were cultured alone or cocultured with 70%-80% confluence SGECs in a collagen pre-coated 24-well plate in RPMI1640 supplemented with 10% FCS. At day 5, T cells were stimulated for 24 hours by adding MACSiBead Particle loaded with antibodies against human CD2, CD3 and CD28 (bead-to-cell ratio 2:1) (T cell Activation/Expansion kit, Miltenyi Biotec). For transwell experiments, SGECs and naïve $CD4^+$ T cells were seeded in the lower and upper chambers, respectively, of a 0.4 μm polycarbonate membrane transwell (Nunc from VWR, Fontenay-sous-Bois, France). For specific inhibitions, anti-OX40L mAb (clone MM0505-8S23) (Abcam) and its corresponding isotype control mouse IgG1 were added. T cells were preincubated for 1 h with Abs, before addition of SGECs.

Flow cytometry analysis. For coculture experiments, T cells were stained with

APC-conjugated anti-CD4 mAb, FITC-conjugated anti-OX40 mAb. FITC-conjugated Mouse IgG1, κ was used as isotype controls (all purchased from BD Biosciences). Before acquisition, propidium Iodide (PI) were added to stain dead cells. For peripheral blood cell analysis, whole blood was lysed by a FACS lysing solution (BD Biosciences). Cells were then stained with 1) APC-conjugated anti-CD4 mAb and FITC-conjugated anti-OX40 mAb; 2) PE-conjugated anti-CD8 mAb and FITC-conjugated anti-OX40 mAb; 3) FITC-conjugated lineage markers: CD3 (T cells), CD14 (monocytes), CD20 (B cells), and CD56 (natural killer cells), as well as PerCP-Cy5.5 conjugated HLA-DR, APC-conjugated anti-CD11c mAb, PE-conjugated anti-OX40L and corresponding isotype controls (all purchased from BD Biosciences, Le Pont de Claix, France). Cells were acquired with a FACSCalibur™ cytometry using CELLQuest™ software (BD Biosciences, Le Pont de Claix, France).

T-cell survival and carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assays. T cell survival was assessed after six days of coculture by $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) and PI staining. For T-cell proliferation assays, freshly isolated naïve $CD4^+$ T cells were labeled with 0.5μM CFSE (Invitrogen) and incubated for 10min at 37°C in the dark. Washed CFSE-labeled T cells were then cocultured with SGECs in the presence of anti-CD2, anti-CD3 and anti-CD28 beads. Three days later, cells were stained with an APC-conjugated anti-CD4 mAb. The proliferation of T cells was evidenced by a cell division-dependent decrease in CFSE staining intensity evaluated by flow cytometry.

Western Blot.

SGECs were cultured in a collagen pre-coated 24-well plate. After 24 h, cells were lysed with 100 µL of ice-cold lysis buffer (PBS with 0.05 M Tris-Cl (pH 8.0), 0.5% SDS, 1 mM DTT and protease inhibitors). Lysates supernatants were subjected to SDS-PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked using 3% BSA in TBS. The blots were incubated with mouse anti-human OX40L mAbs (clone: MM0505-8S23, Abcam) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG monoclonal antibodies and detected by enhanced chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, USA) and the blots were reprobed with anti-GAPDH (Millipore, Molsheim, France) mouse monoclonal antibodies.

Statistical analysis. Continuous data were expressed as mean ± SD. Nonparametric tests were used to analyze continuous variables, even when number of patients was large, because the distribution of data was uneven. The Mann-Whitney U test was used to compare continuous data and the chi-squared test to compare nominal data. Correlations were studied with Spearman's rank test. All statistical analyses were performed with Stata SE 9.2 (Stata Corporation, College Station, TX, USA).

RESULTS

In peripheral blood, the expression of OX40 is upregulated by blood $CD4^+$ T cells and correlated with systemic disease activity

First, we analyzed using flow cytometry OX40 expression by peripheral blood $CD4^+$ and $CD8^+$ T cells as well as OX40L expression by circulating mDC. OX40 was detected on $CD4^+$ T cells but not on $CD8^+$ T cells from patients with pSS or healthy controls (Figure 1A). OX40 expression by circulating $CD4^+$ T cells from patients with pSS was significantly increased (15.4±8.9% vs 8.6±5.0% in controls, P = 0.003) (Figure 1B). No significant difference was detected in OX40L expression by mDCs from patients and controls (Figures 1C and 1D). The proportion of $CD4^+OX40^+$ T cells was significantly correlated with systemic disease activity assessed by the recently validated ESSDAI score (Figure 1E, r=0.52, P= 0.03).

In salivary glands, the expression of OX40L by SGECs and B cells is upregulated as well as that of OX40 by infiltrating $CD4^+$ T cells.

Salivary glands are one of the main target organs of autoimmunity in pSS. We therefore analyzed the expression of OX40 and OX40L in salivary glands using immunohistochemistry. The overall expression of both OX40L and OX40 was higher in salivary glands of patients compared with controls (Figure 2A). Double staining

revealed that OX40 was associated with $CD4^+$ helper T cells (Figure 2B). OX40L was expressed by cytokeratin 19^+ SGECs and $CD19^+$ B cells, but not by $CD11c^+$ DC (Figure 2C-E). Converse to SGECs from patients with pSS, SGECs from controls did not express OX40L.

SGECs induce the expression of OX40 by naïve $CD4^+$ T cells and promote T-cell proliferation and survival

Given the pivotal role of salivary gland epithelial cells in the pathogenesis of pSS, we investigated whether these cells could induce the expression of OX40 by naïve $CD4^+$ T cells. SGECs from patients with pSS or controls were co-cultured with purified naïve $CD4^+$ T cells for 6 days and protein expression of OX40 by T cells was analyzed by flow cytometry. Activated $CD4^+$ T cells cultured alone expressed OX40 (Figure 4A) but this expression was highly enhanced after coculture with SGECs (Figure 4B). No difference in terms of OX40 induction was observed between SGECs from patients with pSS (increase of OX40 expression from 29.2%±10.5 by activated T cells cultured alone to 70.0%±9.8 in coculture with SGECs, P= 0.0002) and from controls (from 19.8%±8.0 to 72.7%±18.9, P= 0.005) (Figure 4C) .

A major consequence of OX40L-OX40 interaction between classical APCs and $CD4^+$ T cells is to promote T cell survival and expansion [22, 23]. We first confirmed

the expression of OX40L by SGECs from patients with pSS, previously observed on total salivary gland using immunohistochemistry, by immunostaining and western blotting on primary cultures of SGECs. We then analyzed whether the expression of OX40L by SGECs increased T-cell viability. The survival rate of naïve $CD4^+$ T cells was highly and significantly increased after coculture with SGECs from patients with pSS (18.7%±12.4 versus 57.9%±14.4, *P*= 0.008, Figure 5A). This increase in T-cell lifespan was mediated by OX40-OX40L interaction since the inhibition of OX40L signaling significantly decreased the survival rate of T cells (22.8%±13.9 versus 52.1%±15.6 with control IgG1 isotype, *P*=0.03, Figure 5B). SGECs from patients with pSS also induced an increased proliferation of $CD4^+$ T cells (proliferation rate of 74.2 vs 21.8 % in T cells cultured alone, *P*=0.03). This induction of T-cell proliferation by SGECs was partially inhibited with neutralizing anti-OX40L mAb (43.4% versus 62.4% for isotype IgG1 control, *P*=0.2). These results demonstrate that OX40L costimulatory pathway is involved in the increased survival and proliferation of T cells induced by SGECs.

DISCUSSION

The present results support the implication of OX40/OX40L in the pathogenesis of primary Sjögren's syndrome. The only data in literature concerned the genetic association between OX40L polymorphism and pSS and an increased expression of OX40L by duct salivary gland epithelial cells of MRL-*Fas*^{lpr} mice [24]. First, we report using immunohistochemistry that salivary gland epithelial cells from patients with pSS expressed OX40L, which was not observed in SGECs from controls or in DCs from patients with pSS. Likewise, some renal epithelial cells express OX40L in patients with lupus nephritis [25]. We did not analyse the expression of OX40L by other cell types such as B cells, NK cells, Langerhans cells, endothelial cells, smooth muscle cells, mast cells, or $CD4^+CD3^-$ lymphoid tissue inducer cells [22, 25].

Secondly, the expression of OX40L receptor, OX40, was increased on blood and salivary gland CD4+ T cells in patients with pSS compared to controls. OX40 was not expressed by CD8+ T cells, similarly as reported in SLE [25]. The difference in salivary gland expression of OX40 between pSS and controls was not unexpected, since no lymphocytic infiltrates are present in controls. More interestingly, in pSS, peripheral blood $CD4^+$ T cells also have an increased expression of OX40, compared to controls. In addition, patients with systemic disease according to the ESSDAI had a significantly increased expression of OX40. In patients with SLE also, the proportion of peripheral blood $CD4^+$ T cells expressing OX40 is higher and correlated with

disease activity [13]. We did not analyse the expression of OX40 by other cell types including NK T cells (NKT), NK and neutrophils, which can be induced to express OX40 [26-28].

In addition, we demonstrated that salivary gland epithelial cells were capable to induce OX40 expression by CD4+ T cells, which might play a role in the increased expression observed in salivary glands and, perhaps, in peripheral blood. This capacity of salivary gland epithelial cells might not be conferred by the disease, since no difference in terms of OX40 induction was observed between patients and controls, in whom no T cells infiltrate salivary glands, anyway. The induction of OX40 on T cells by SGECs is independent from cell contact or OX40L, according to transwell assays and cocultures with a monoclonal antibody against OX40L (data not shown). Therefore, cytokines secreted by SGECs, or increased in salivary glands from patients with pSS, and known to induce OX40, such as IL-1, TNF-α, or more interestingly, IFN-α and IL-7 [29-35], could play a role in the capacity of SGECs to induce OX40 expression by T cells and deserve further study. In addition, TCR-driven activation is requested for OX40 expression, as reflected by the experimental conditions using anti-CD2, anti-CD3 and anti-CD28. The increased expression of OX40L by SGECs and of OX40 by CD4+ T cells prompted us to investigate the functional consequence of the binding of OX40L on SGECs to OX40 on T cells. The results showed that OX40L/OX40 interaction between SGECs and T cells can directly extend T-cell life span and proliferation. Interestingly, OX40 signaling can target the canonical NF-κB

(NF-κB1) pathway and promote CD4 T-cell proliferation [36]. In addition, OX40 stimulation promotes anti-apoptotic protein Bcl-xL and B-cell leukemia/lymphoma-2 (Bcl-2) expression by CD4 T cells and prevent these cells from undergoing apoptosis [37]. The interaction between SGECs or B cells and T cells through OX40L/OX40 could also play a role in T-cell differentiation. Thus, OX40L expression by SGECs or salivary gland B cells might contribute to the elevated proportion of memory and follicular helper T cells infiltrating salivary glands of patients with pSS [38, 39]. Of note, the interaction between OX40L on B cells and OX40 on T cells does not only activate T cells but also enhance B-cell proliferation, plasma cell differentiation and IgG secretion, through reverse signaling via OX40L [40]. Thus, the increased expression of OX40 by peripheral blood and salivary gland T cells could contribute to the marked activation of B cells observed in pSS

To conclude, an increased expression of OX40 by CD4+ T cells was demonstrated in blood as well as in salivary glands, and was correlated with systemic disease activity in pSS. In addition, an increased expression by SGECs of OX40L was observed, with a functional effect on T cells. All these results suggest that the increased expression of OX40 and OX40L in pSS contributes to the pathogenesis of the disease. Last, these results add a new pathogenic role to salivary gland epithelial cells, which are not only capable to induce the expression of OX40 on T cells, but

also to increase T-cell survival and proliferation through OX40L surface expression

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LEGENDS

Figure 1. OX40 expression is upregulated in peripheral $CD4^+$ T cells in patients with pSS compared with healthy controls. A Analysis of OX40 expression on circulating $CD4^+$ or $CD8^+$ T lymphocytes in a pSS patient. A gate was previously set as the lymphocytes population based on the forward and sideward scatter plots (FSC and SSC). **B** $OX40^+$ CD4+ T cell proportions in healthy individuals (n=20) and patients with pSS (n=18). **C** Analysis of OX40L expression on circulating myeloïd dendritic cells in pSS patients. mDC are defined as positive for CD11c and HLA-DR but negative for the lineage markers CD3, CD14, CD19 and CD56. A gate was previously set as the lymphocytes and/or mononuclear population. **D** $OX40L^+$ expression on myeloïd dendritic cells in healthy individuals (n=11) and SS patients (n=9). **E** Correlation between the proportion of $CD4^+OX40^+$ T cells and ESSDAI. Statistical analyses were performed with the Pearson test. Individual data and mean ± SD are shown. **, $p < 0.005$.

Figure 2. Increased expression of OX40 by $CD4^+$ T lymphocytes and of OX40L by SGECs in salivary glands from patients with pSS using immunohistochemistry. A Minor salivary gland biopsies from patients with pSS and controls were analysed for OX40 and OX40L expression by immunohistochemistry (bar= 100µm). **B.** Expression of OX40 by $CD4^+$ T lymphocytes (B). **C**. OX40L expression by cytokeratin 19^+ epithelial cells**. D**. OX40L expression by $CD19^+$ B

lymphocytes. **E** Absence of OX40L expression by $CD11c^+$ DC (bar= 5µm). Results are representative for five independent experiments.

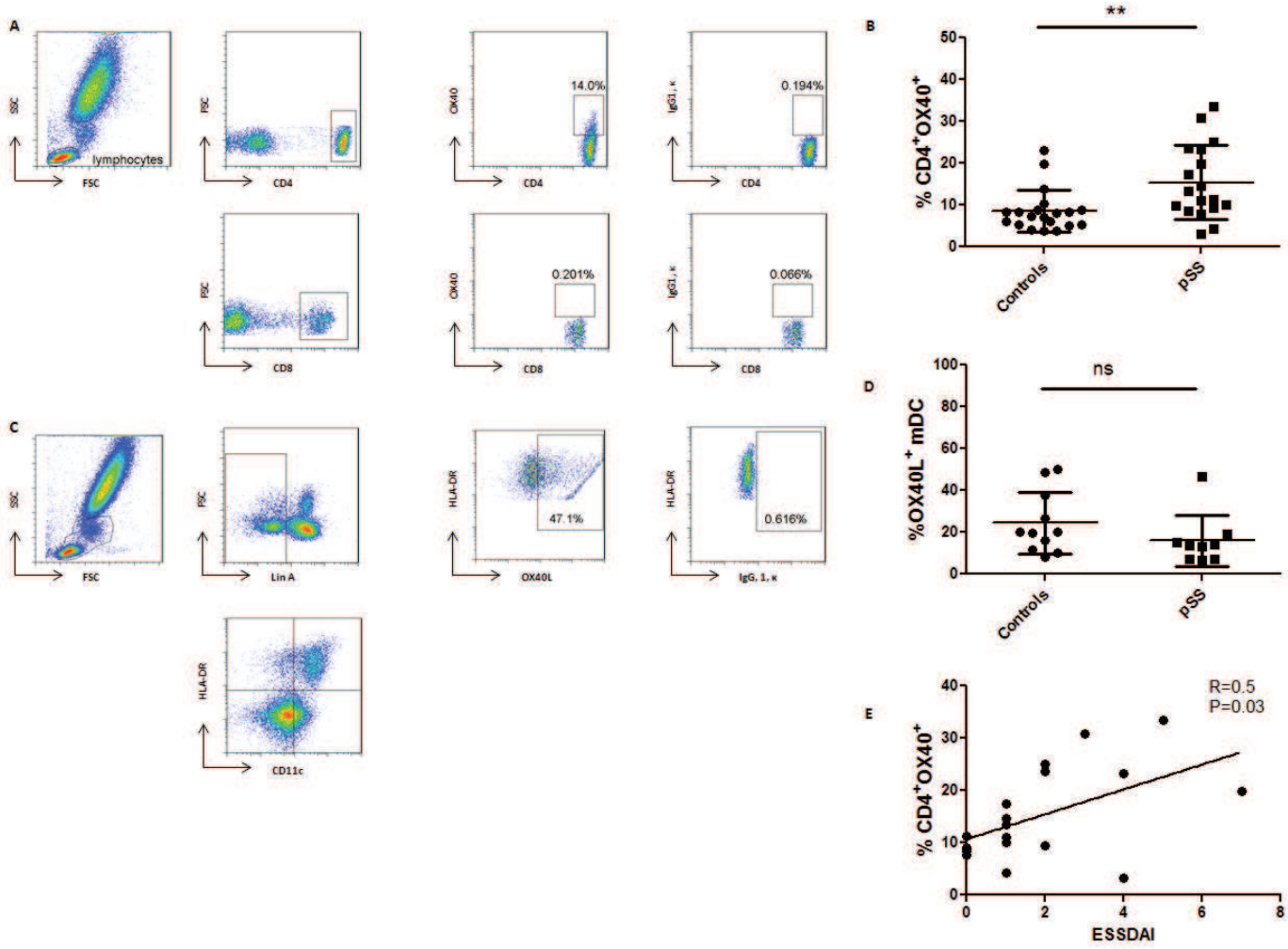
Figure 3. OX40L expression by SGECs in primary culture. A. Staining with a specific OX40L mAb (and control labeling with isotype mouse IgG1) of cultured SGECs from patients with pSS using fluorescence microscopy. Top pictures are the corresponding phase contrast images. **B.** OX40L expression was confirmed using Western blot. For protein loading control, membranes were reprobed with anti-GAPDH antibodies. Results are representative for at least three independent experiments.

Figure 4. SGECs induce OX40 expression by naïve $CD4^+$ T cells. OX40 expression was determined by flow cytometry in naïve $CD4^+$ T cells (Tn) after co-culture with or without SGECs from controls (control SGECs) or patients with pSS (SS SGECs) for 6 days. **A,** $OX40^+$ cells (top graphs) were quantified upon gating on $CD4^+$ cells and labeling with isotype controls (bottom graphs). **B.** Proportion of $OX40^+$ cells in $CD4^+$ T cells after co-culture with or without SS SGECs (n = 10) or control SGECs (n = 6). Data are expressed as mean $\pm$ SD. **: $p < 0.005$; ***: $p < 0.0005$.

Figure 5. OX40L surface expression by SGECS contributes to the increase in

$CD4^+$ T-cell survival and proliferation. A The proportion of surviving ($PI^-DiOC_6^+$) $CD4^+$ T cells after 6 days is presented in the presence or not of SGECs from patients with pSS. 3µg/ml of neutralising anti-OX40L antibody or isotype-matched control antibody were added. **B** The proportion of survival T cells was determined in 5 co-cocultures with or without anti-OX40L antibody. **C** CFSE proliferation assays of $CD4^+$ T cell in the presence or not of SGECs from patients with pSS, 3 days after activation using CD2/CD3/CD28, using a neutralising anti-OX40L or an isotype-matched control antibody. The proportions of proliferating or non-proliferating cells among $CD4^+$ T cells are indicated. Data are expressed as mean ± SD and are representative for at least three independent experiments. *: $p < 0.05$.

Figure 1.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SSC
FSC
lymphocytes
FSC
CD4
OX40
CD4
14.0%
IgG1, κ
CD4
0.194%
FSC
CD8
OX40
CD8
0.201%
IgG1, κ
CD8
0.066%
B
**
% CD4+OX40+
Controls
pSS
C
SSC
FSC
FSC
Lin A
HLA-DR
OX40L
47.1%
HLA-DR
IgG, 1, κ
0.616%
HLA-DR
CD11c
D
ns
%OX40L+ mDC
Controls
pSS
E
R=0.5
P=0.03
% CD4+OX40+
ESSDAI

Figure 2.

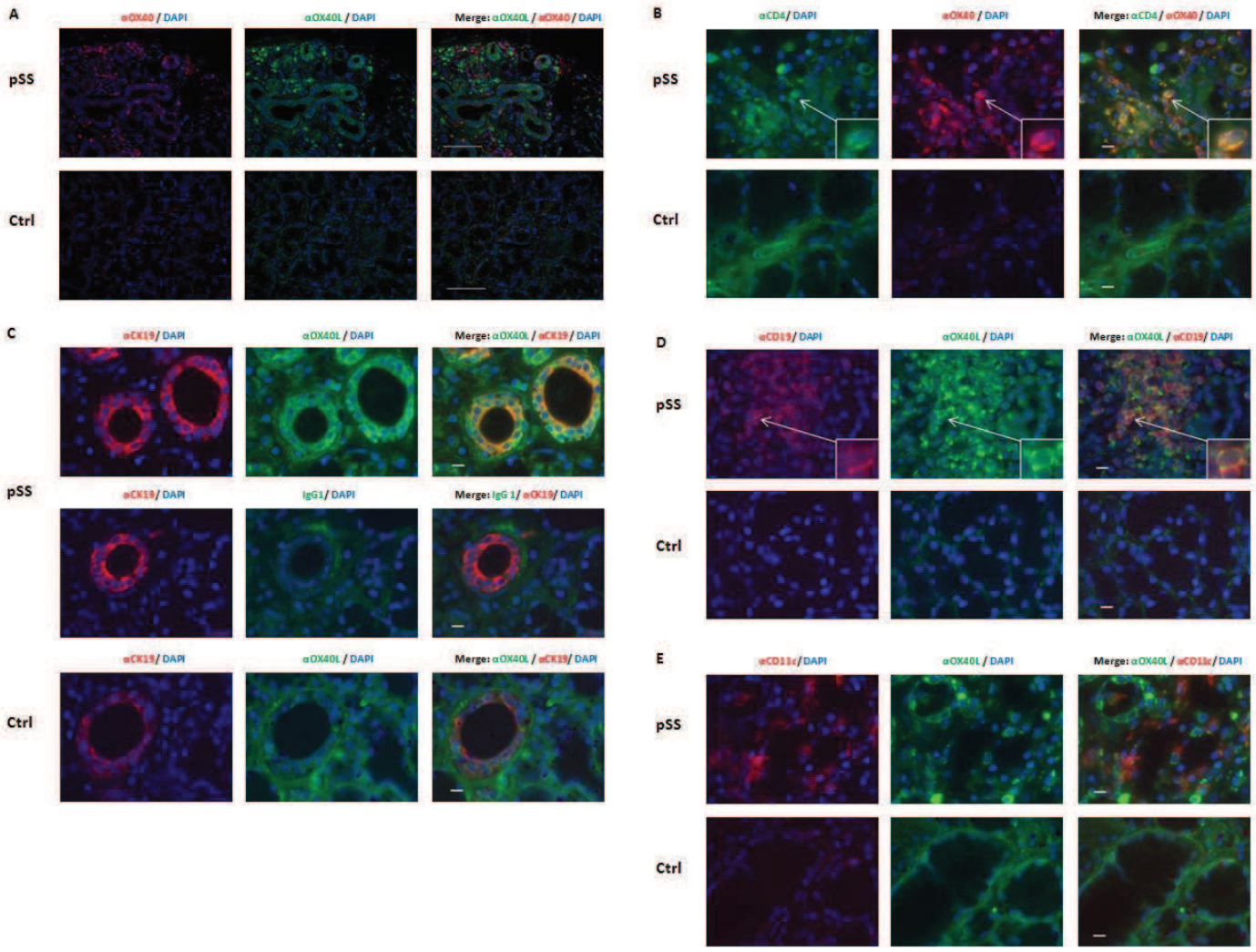
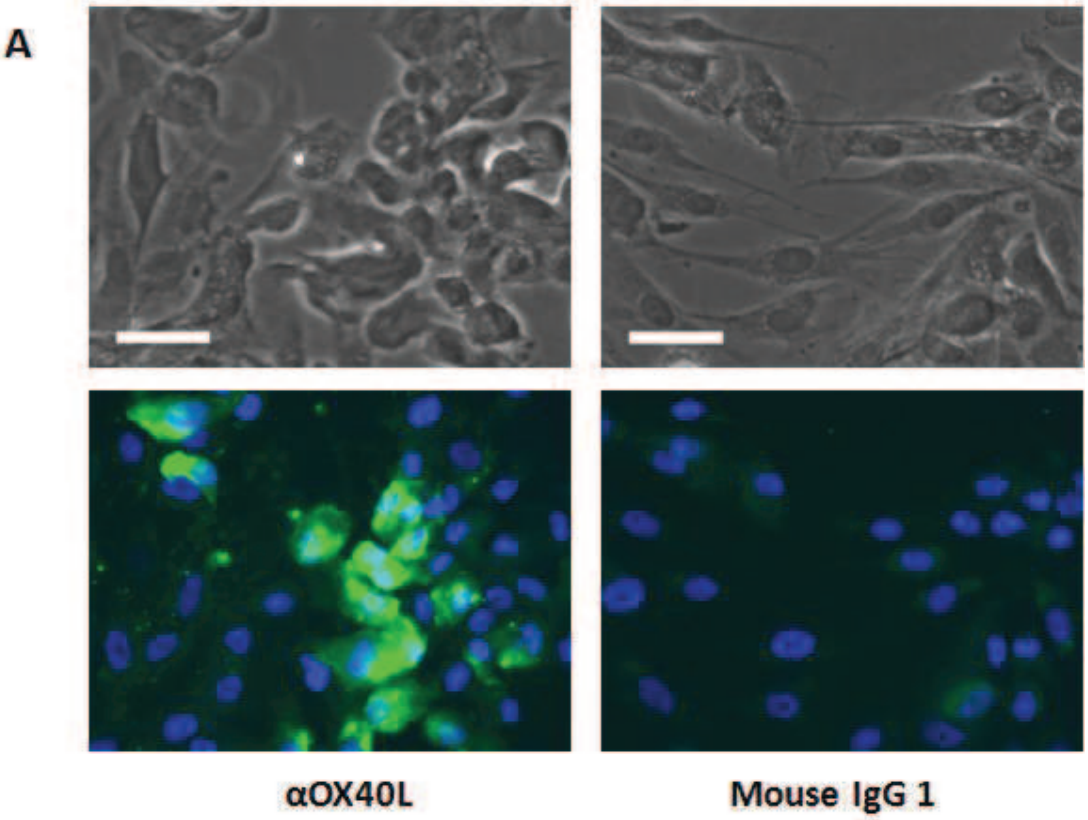

A
αOX40 / DAPI
αOX40L / DAPI
Merge: αOX40L / αOX40 / DAPI
pSS
Ctrl
B
αCD4 / DAPI
αOX40 / DAPI
Merge: αCD4 / αOX40 / DAPI
pSS
Ctrl
C
αCK19 / DAPI
αOX40L / DAPI
Merge: αOX40L / αCK19 / DAPI
pSS
αCK19 / DAPI
IgG1 / DAPI
Merge: IgG 1 / αCK19 / DAPI
Ctrl
αCK19 / DAPI
αOX40L / DAPI
Merge: αOX40L / αCK19 / DAPI
D
αCD19 / DAPI
αOX40L / DAPI
Merge: αOX40L / αCD19 / DAPI
pSS
Ctrl
E
αCD11c / DAPI
αOX40L / DAPI
Merge: αOX40L / αCD11c / DAPI
pSS
Ctrl

Figure 3.



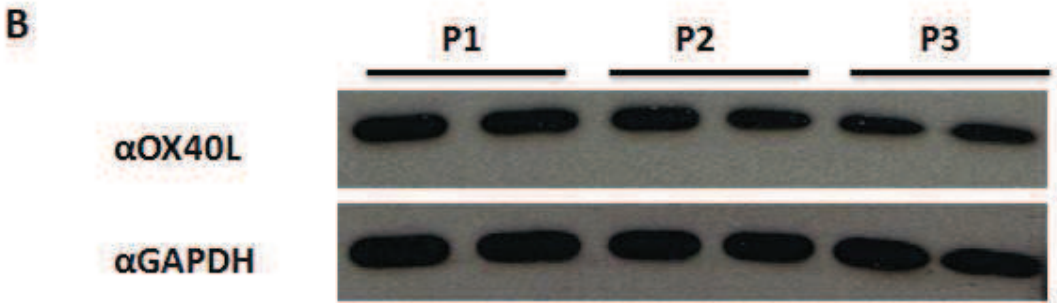


Figure 4

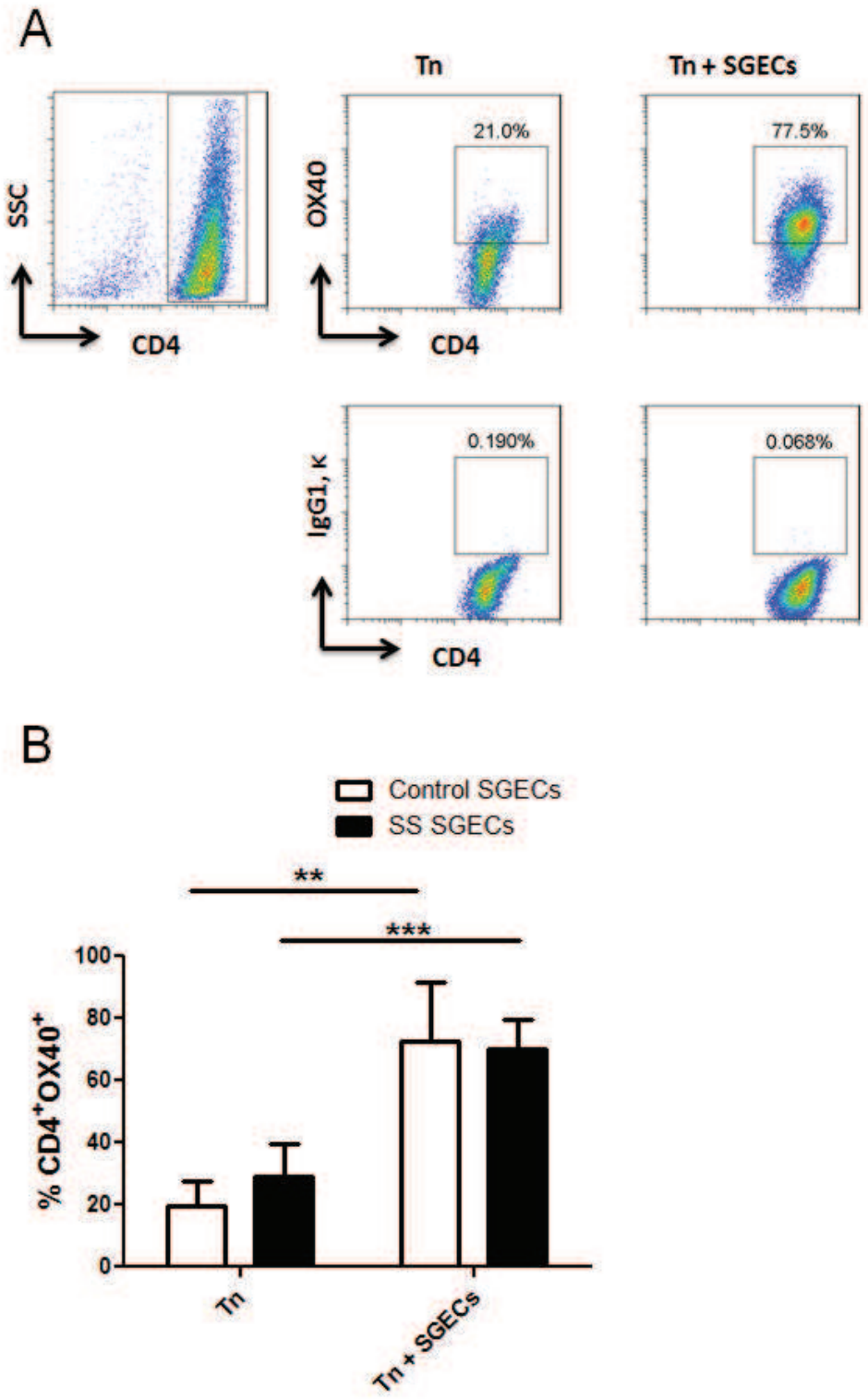
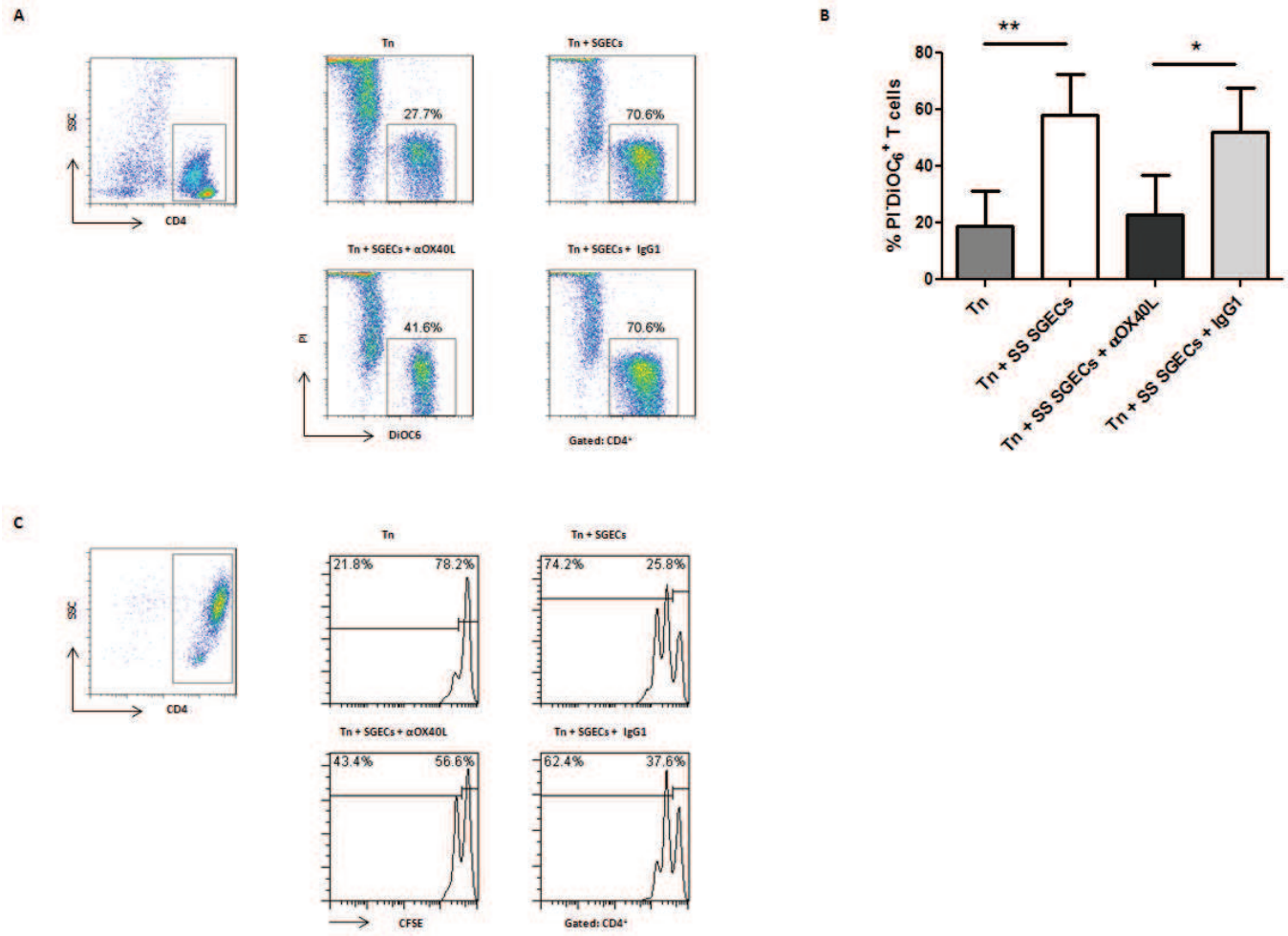

A
Tn
Tn + SGECs
SSC
CD4
OX40
21.0%
77.5%
IgG1, κ
0.190%
0.068%
B
Control SGECs
SS SGECs
**
***
% CD4+OX40+
100
80
60
40
20
0
Tn
Tn + SGECs

Figure 5.

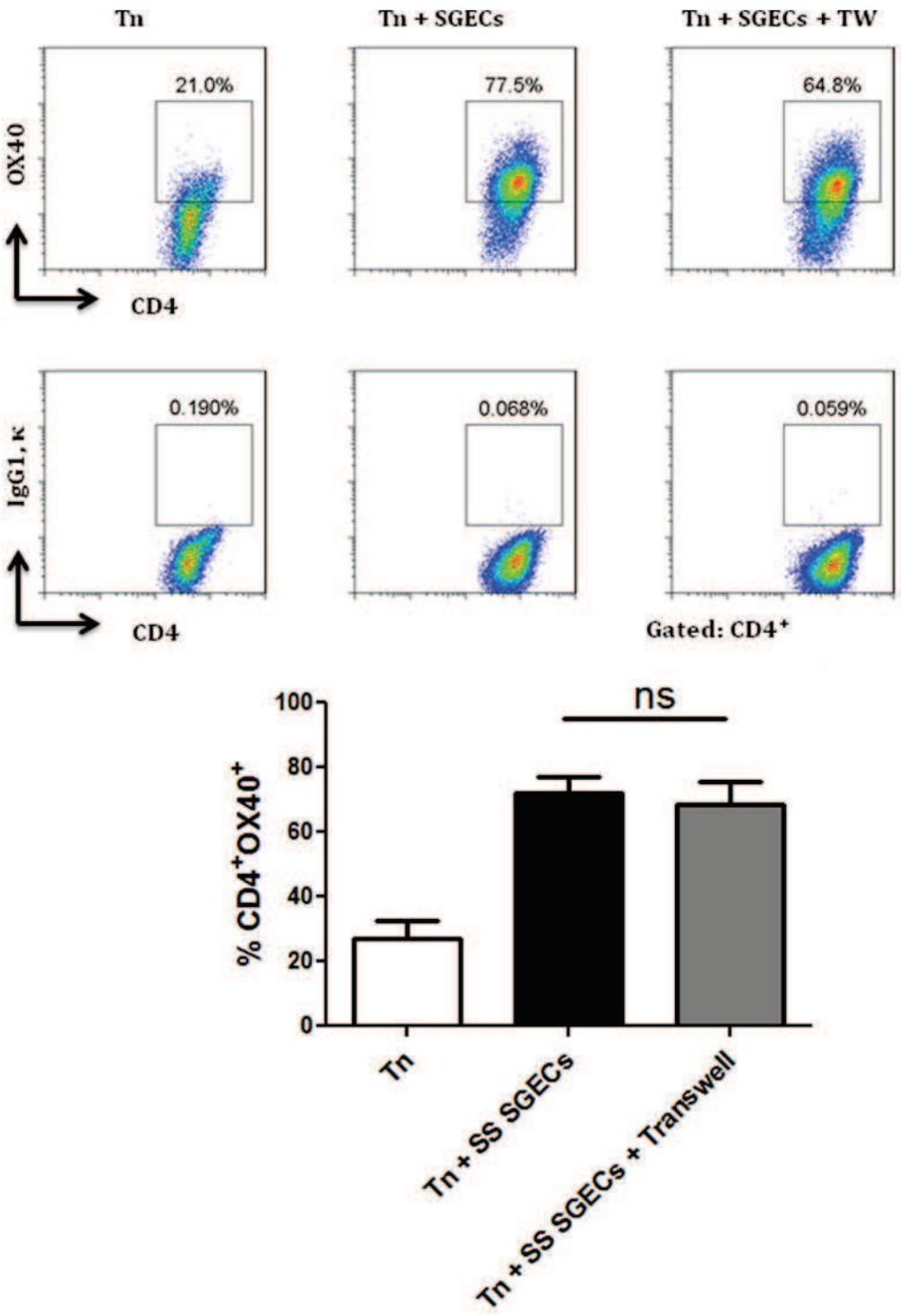

A
SSC
CD4
Tn
27.7%
Tn + SGECs
70.6%
Tn + SGECs + αOX40L
41.6%
Tn + SGECs + IgG1
70.6%
PI
DiOC6
Gated: CD4+
B
**
*
80
60
40
20
0
% PI-DiOC6+ T cells
Tn
Tn + SS SGECs
Tn + SS SGECs + αOX40L
Tn + SS SGECs + IgG1
C
SSC
CD4
Tn
21.8%
78.2%
Tn + SGECs
74.2%
25.8%
Tn + SGECs + αOX40L
43.4%
56.6%
Tn + SGECs + IgG1
62.4%
37.6%
CFSE
Gated: CD4+

3. Supplementary data that were not included in the submitted manuscript

3.1 OX40 induction by SGECs is dependent on soluble factors

To determine whether the induction of OX40 expression by SGECs was dependent on soluble and/or cell surface factors, we performed transwell experiments. As shown in figure 21, no significant change occurred with or without the use of a transwell in the proportion of $CD4^+OX40^+$ T cells in coculture. Thus, the induction of OX40 expression by SGECs is dependent on a soluble factor. The induction of OX40 could not be blocked by neutralizing anti-OX40L Ac (data not shown).

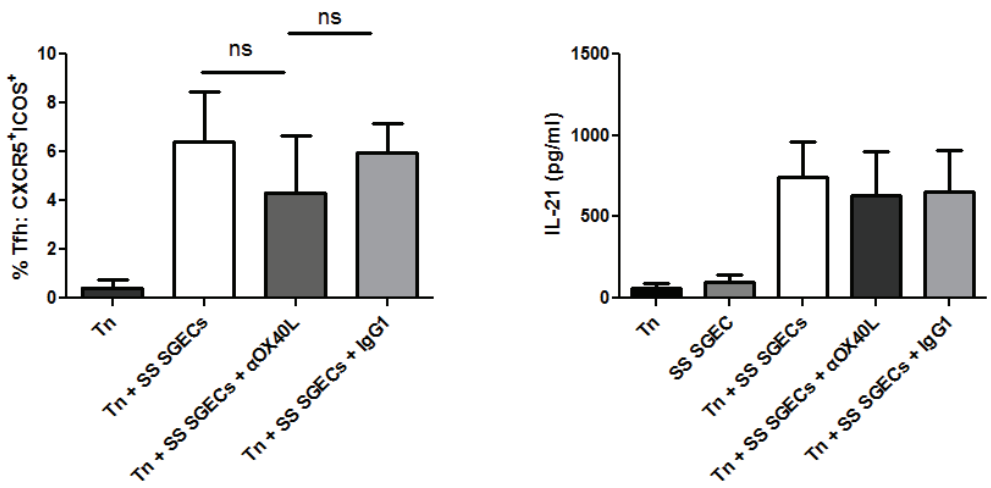
Figure 21. OX40 induction by SGECs is dependent on soluble factors. A. $CD4^+$-gated cells, $OX40^+$ were stained (top graphs) and analyzed by flow cytometry. Bottom graphs illustrate control staining with isotypic antibodies. B. The proportion of $OX40^+$ cells among $CD4^+$ T cells after co-culture with SS SGECs using or not a transwell (n = 3 in both cases) is presented. Data are expressed as the mean of samples ± SD. ns, not significant.


Tn
Tn + SGECs
Tn + SGECs + TW
21.0%
77.5%
64.8%
OX40
CD4
0.190%
0.068%
0.059%
IgG1, κ
CD4
Gated: CD4+
ns
100
80
60
40
20
0
% CD4+OX40+
Tn
Tn + SS SGECs
Tn + SS SGECs + Transwell

3.2 The blockade of OX40L fails to inhibit Tfh differentiation induced by SGECs

OX40L pathway might be involved in Tfh development. We tested whether the blockade of OX40 signaling could inhibit the induction of Tfh by SGECs. Naïve $CD4^+$ T cells were cocultured with SGECs as described in the publication 1. Tfh differentiation was measured by the proportion of $ICOS^+$ $CXCR5^+$ cells in $CD4^+$ cells. The addition of anti-OX40L mAb failed to diminish the Tfh induced in the coculture. The production of IL-21 in the supernatant of coculture did not change either.(Figure 22)

Figure 22. The blockade of OX40L fails to inhibit Tfh differentiation induced by SGECs. Tfh differentiation and IL-21 secretion were determined when naïve $CD4^+$ T cells were cocultured with SS SGECs in the presence of neutralizing anti-OX40L antibody (αOX40L). The proportion of $ICOS^+CXCR5^+$ cells in naïve $CD4^+$ T cells culture alone or with SS SGECs with or without anti-OX40L antibody or isotype control (IgG1) is presented (n = 5, left panel). IL-21 secretion was determined by ELISA in culture supernatants in the same conditions (right panel). Data are expressed as the mean of samples ± SD.



IV. CONCLUSIONS AND PERSPECTIVES

To investigate the physiopathological role of salivary gland epithelial cells in primary Sjögren's syndrome, we first established the experimental procedures of primary culture of SGECs using the protocol developed by Dimitriou and his colleagues (2002). Primary culture of salivary epithelial cells was derived from minor salivary gland biopsies isolated from patients with SS or control subjects (complaining of dry symptoms without any features of autoimmunity). The nature of these cells was verified by the positive staining of SGEC specific markers: cytokeratins 19, cytokeratins 7, vimentin and the absence of CD3, CD20, CD56 and myeloperoxidase. Using immunofluorescence and western blot, we firstly demonstrated that the primary culture of SGECs expressed costimulatory molecule ICOSL and OX40L.

We demonstrated that SGECs were capable of inducing directly the differentiation of IL-21 secreting follicular helper T cell. This might contribute to the marked activation of B lymphocytes in salivary glands. Coculture between T cells and SGECs induced the increased mRNA expression of Bcl-6, the transcription factor directed Tfh differentiation as well as expression of CXCR5 and ICOS, molecules specific to Tfh. Double positivity of CXCR5 and ICOS that characterizes Tfh was observed on approximately 5% of T cells by coculture with SGECs. Secretion of IL-21 was also significantly increased after coculture. In addition to the expression of Tfh associated molecules, we also found that B cell survival was significantly increased after coculture with T cells and SGECs. We then performed cocultures between T cells and SGECs using a transwell to determine whether the differentiation of Tfh was mediated by cell-cell contact or cytokine(s). The inductive effect of SGECs was restricted to Tfh because the transcription factors and cytokines of Th1 (T-bet and IL-12), Th2 (GATA-3 and IL-4) and Th17 (RORA, RORC and IL-17) were not found or did not increase under the same conditions. No significant change was observed in the differentiation of Tfh with or without a transwell, as assessed by ICOS

and CXCR5 expression. However, IL-21 secretion was inhibited when using a transwell. Therefore the differentiation of Tfh is mediated by soluble factor(s) and the secretion of IL-21 needs cell contact. Further analysis demonstrated that the differentiation of Tfh involved IL-6 and that the secretion of IL-21 by Tfh needed ICOSL costimulatory signal.

IL-21 is mainly produced by Tfh and IL-21 serum level is associated to pSS. Our results demonstrated that the elevation of IL-21 was associated with several B cell activation markers such as the presence of autoAbs, elevated IgG levels and kappa free light chains of Ig as well as disease activity assessed by the ESSDAI. Moreover, polymorphisms in the *IL-21* and *IL-21R* genes were not associated with pSS. These results suggest that the elevation of IL-21 is not genetically determined and could be the consequence of the induction of Tfh by SGECs in salivary gland ectopic germinal centers. The absence of genetic determinism also reinforces the interest of serum IL-21 as a potential biomarker of disease activity in pSS.

In the second part of our study, we focused on OX40 and OX40L costimulatory molecules which play a role in Tfh differentiation, effector and memory T cells function and maintenance. We firstly found that the proportion of peripheral blood $CD4^+$ T cells expressing OX40, as well as the expression of OX40 by $CD4^+$ infiltrating T cells were elevated in patients with pSS. Circulating mDC expressed the same level of OX40L in patients and in healthy controls. However, in salivary glands of pSS patients, the epithelial cells and $CD19^+$ B cells, but not $CD11c^+$ DCs overexpressed OX40L compared with control biopsies. Using *in vitro* coculture of naïve $CD4^+$ T cells with SGECs, we then demonstrated that SGECs from pSS patients could significantly increase the expression of OX40 by $CD4^+$ T cells. A similar induction of OX40 expression was observed in coculture of T cells with SGECs from controls. The induction of OX40 expression on T cells was not altered when the coculture was performed using a transwell, which demonstrated that this OX40

induction depended on cytokine(s) and not of cell-cell contacts. In addition, SGECs isolated from pSS patients promoted T cell survival and proliferation via OX40-OX40L interaction.

ICOSL expression on various cell types has been shown to be induced i*n vitro* by IFN-β and IFN-γ [194, 195], whereas DC expression of OX40L is dependent on type I IFN signaling. We have studied the influence of IFNs on the expression of ICOSL and OX40L by cultured SGECs. Our results showed that the activation of IFN-α (2400U/ml), IFN-β (100U/ml) and IFN-γ (5ng/ml) did not significantly change the mRNA expression of OX40L and ICOSL (data not shown). Thus, we were unable to find a relationship between the IFN signature and OX40L or ICOSL expression on SGECs.

In lymphoid organs, T-cell differentiation and induction of OX40 are a complex multi-step process depending on both cell-cell interactions and cytokines. In our somewhat "artificial" coculture system, we added anti-CD2, anti-CD3 and anti-CD28 beads to stimulate T cells. These beads provide strong TCR signaling and a costimulatory signal, thereby mimicking the role of APCs. The differentiation of Tfh requires TCR signaling, CD28 costimulation, ICOS signal, Bcl-6 induction and c-maf as well as cytokines actions, including IL-6, IL-21 and IL-27 [196]. Indeed, in the absence of anti-CD2 anti-CD3 and anti-CD28 beads, SGECs failed to induce Tfh differentiation (data not shown). In the presence of anti-CD2 and anti-CD3, but without CD28 activation, a lower proportion of Tfh was differentiated by SGECs.

In SG of patients with pSS, it is probable that T-cell differentiation involves other cell types, including B cells, DCs and LTi. For example, LTi express OX40L and might contribute 1) to Tfh generation and ectopic GC formation; 2) to the proliferation and survival of OX40-positive T cells. Thus, although SGECs contribute to T-cell differentiation, they certainly do not make the "job" alone and B cells, macrophages and DCs surely also participate. The relative contribution of SGECs

with regards to other cell populations might depend on the stage of the disease. Of note, tissue-infiltrating dendritic cells are predominantly involved in the induction of SS rather than in the maintenance. Thus, in contrast to the early stage of pSS, few mature DCs can be observed in SG of patients with pSS at a later stage [194]. It could be hypothesized that SGECs contribute to the maintenance of Tfh in salivary glands from patients with pSS. Unexpectedly, $CD11c^+$ mDCs do not express OX40L in SGs of patients. OX40-OX40L interaction can therefore only occur between infiltrating T cells and ECs or B cells.

How do we interpret the similar proportions of Tfh and OX40-positive T cells differentiated by SGECS from patients with pSS and controls? This might be related to the limited population sample and to the fact that, for obvious ethical reasons, healthy controls could not be studied. Another possibility is that the SGECs were activated during primary culture. The medium of SGEC contains EGF, insulin and hydrocortisone. Insulin and hydrocortisone enhance the proliferation of mouse submandibular salivary gland cells grown in primary explant culture. Cultured SGECs express epidermal growth factor (EGF) receptor. The EGF can activate the PI3K-Akt pathway and NF-kB in cultured SGECs of pSS patients, which protects SGECs from Fas mediated apoptosis [197]. Both adiponectin, which increases insulin sensitivity and resistin, contributing to insulin resistance, have been shown to be elevated in pSS [198]. Thus, the addition of insulin to the medium of culture might contribute to the activation of SGECs. An intense activation due to the *in vitro* conditions of culture might contribute to mask the differences in terms of of OX40L and ICOSL expression between patients and controls *in vivo*. Agreeing with this hypothesis, our results obtained from patient's biopsies using immunohistochemistry have shown a remarkably higher expression of OX40L by ECs from patients with pSS, which was not the case after 4 weeks of primary culture. The conditions of culture might also contribute, along with OX40L expression by SGECs, to the observed enhanced

survival and proliferation of T cells. Conversely, the capacity of SGECs to induce Tfh differentiation might be increased by the microenvironment observed *in vivo* (and absent in cultured cells) in patients with pSS. For example, IL-6 could be enhanced by locally produced TNF-α and IFN-γ and thus contribute to Tfh differentiation and ICOSL expression could be enhanced by TLR3 ligand, poly I:C (data not shown). Last, other limits of our coculture experiments are that we did neither accomplish the coculture experiments using T cells from patients with pSS (only healthy blood donor T cells were used), nor use T cells and SGECs from the same patient. Anyway, we should also keep in mind that T cells are only recruited in salivary glands in a pathological setting, which limits the conclusions to be drawn on cocultures between control SGECs and control T cells.

Although we demonstrated the capacity of SGECs to differentiate Tfh cells and induce OX40, the increase of Tfh cells and OX40-positive T cells in salivary glands of patients with pSS can also be related, at least in part, to the recruitment of these cells from secondary lymphoid organs and/or peripheral blood. Inversely, the increase in serum IL-21 (and in blood Tfh-like cells according to literature) and in peripheral blood OX40-positive T cells could be related to the local differentiation in salivary glands.

Regarding the mechanisms of Tfh differentiation, the action of IL-6 and IL-21 in Tfh differentiation is controversial in literature. Some studies have reported that early $Bcl6^{+}CXCR5^{+}$ Tfh differentiation was severely impaired in the absence of IL-6 and the IL-6 being absolutely essential for viral control [199, 200]. Others have shown that IL-21 and IL-6 are not absolutely required for the development of $Bcl6^{+}$ Tfh cells *in vivo* [201]. IL-6 and IL-6R double transgenic mice in the lung develop pulmonary lesions with lymphoid tissue-like structures as well as CXCL13 expression in B cell follicles [202]. It seems that the loss of either IL-6 or IL-21 had mild effects on the generation of early Tfh cells and on the formation of GCs during the response

to acute viral infection. However, mice lacking both IL-6 and IL-21 are unable to generate optimal Tfh differentiation [203]. Anyway, IL-6 production by follicular B cells and DCs in the draining lymph node is an important early event during the antiviral response. To be pointed out, using of neutralizing anti-IL-6 Ab in our coculture system only diminished Tfh induction to a half and failed to decrease IL-21 secretion significantly.

Unexpectedly, we found 2 different mechanisms involved in Tfh-differentiation and in IL-21 secretion, which had not been previously reported for other cell types. Again, other mechanisms might be involved, since neither the blockade of IL-6 nor that of ICOSL totally abolished Tfh-differentiation and IL-21 secretion.

We could only demonstrate that soluble mediators, but not soluble OX40L contributed to OX40 expression by T cells. Further analysis is needed to determine the mechanisms involved in the induction of OX40. This unknown soluble factor might involve IL-1, TNF-α, IFN-α, IL-2 and IL-7 [153, 204]. All these cytokines are upregulated in patients with pSS. In addition, cultured SGECs from patients with pSS can secrete IL-1, TNF-α and IFN-α. To test the role of these cytokines in the induction of OX40, we will add a neutralizing Ab targeting each of these different cytokines in our coculture system.

Concerning the function and phenotype of Tfh and OX40-positive T cells, we only showed an effect on B-cell survival, and T – cell proliferation, respectively. Recent papers reported the existence of different subsets of Tfh, and it would be very interesting to determine whether a specific subset of Tfh could be preferentially differentiated by SGECS in patients with pSS. The pattern of cytokine secretion (Th2 cytokines?) and the complete phenotype of OX40-positive T cells (activated T cells, memory T cells?) also deserve further analysis.

There might be some differences regarding the clinical relevance and subsets of patients concerned in our findings on Tfh and OX40-positive T cells. First, the term

"differentiation" seems adequate for Tfh whereas the term "activation" might correspond more adequately to the induction ox OX40. Indeed, OX40 expression is induced on activated $CD4^+$ T cells [205] We did not assess other markers of T cell activation in our experiments. Second, the proportion of Tfh was far lower than that of OX40-positive T cells after coculture with SGECs (Tfh = 5% and OX40-positive T cells = 70%). Secondly, only a minority of patients (between 10 and 20 % of patients with pSS, depending on techniques and studies), has salivary gland GCs, and thus salivary gland Tfh. However, this population represents the patients at risk of lymphoma.

OX40 and ICOS are particularly important for T-cell related B cell activation and our two studies demonstrate the role of SGECs in that matter. Several studies reported the role of OX40 in Tfh development. OX40L transgenic mice express OX40L show accumulation of proliferating $CD62L^{low}$ $CD4^+$ T in B cell areas following immunization. This result indicates that OX40L contributes to CD4 T cell recruitment into B cell follicles and GC formation [206]. Reverse signaling via OX40L enhances B cell proliferation, IgG production and plasma cell differentiation [190]. In addition, OX40 and ICOS work in a cooperative way to induce Tfh differentiation. The systemic knockout of either Roquin-1 or Roquin-2 is lethal in mice. The *sanroque* mice carry a mutated hypomorphic activity $Roquin\text{-}1^{san}$ protein and exhibit increased ICOS levels on naïve and activated T cells. They show an accumulation of Tfh cells and have spontaneous GC formation in the absence of immunization. However, T-cell-specific deficiency of either Roquin-1or Roquin-2 does not increase Icos expression on T cells. The proportion of Tfh or germinal center B cells does not change in these mice. Vogel *et al.,* revealed that Roquin-1 and Roquin-2 control T cell homeostasis because the T-cell-specific deficiency of both Roquin-1and Roquin-2 in mice results in increased $CD62L^{low}CD44^{high}$ phenotype of $CD4^+$ and $CD8^+$ cells and mice develop lymphadenopathy and splenomegaly. In these mice, both Icos and Ox40 in T cells are overexpressed. Thus, they suggested that in T cells, Roquin-1 and

Roquin-2 proteins redundantly restrict T cell activation and costimulation via Icos and Ox40 to prevent inappropriate Tfh cell differentiation [207]. Our results in coculture showed that the blockade of OX40 signaling did not affect Tfh differentiation .This might be due to the limited number of experiments (n =5).

Many factors may cause peripheral self-tolerance breakdown in pSS patients. IL-21 and OX40 might render T cells resistant to Treg-mediated suppression. OX40 signal can inhibit the development or suppressive function of both nTregs and iTregs. The expression of OX40L on SGECs might inhibit the regulatory function of Tregs including follicular Tregs which plays a central role of negative selection in ectopic GCs. The excess in Tfh can result in the positive selection of autoreactive B cells in GCs. In addition, OX40 signaling might break T-cell anergy and allow autoreactive T cells to acquire effector cell functions [208]. The presence of autoreactive T cells in ectopic GCs might provide help to autoreactive B cells, which results in the secretion of high affinity autoAbs.

Regarding the field of biomarkers, we demonstrated that serum level of IL-21 and the proportion of $OX40^{+}CD4^{+}$ T cells are correlated with systemic disease activity assessed by the ESSDAI. A recent paper, only available in abstract form [209] also showed an increase of $OX40^{+}$ T cells in patients with pSS, compared to controls, which was associated with systemic complications. Interestingly, this abstract reported an increase of OX40L expression on blood B cells. The analysis of OX40L expression on blood B cells is currently ongoing in the laboratory Serum IL-21 or circulating $OX40^{+}CD4^{+}$ T cells could therefore be interesting biomarkers of systemic disease activity, which has to be confirmed in further prospective studies.

Regarding the therapeutic perspectives of our studies, some studies suggest that the consequences of ICOS-ICOSL signaling in autoimmune diseases are complex. Blocking ICOS signal can either promote or inhibit T cell activation. It seems that the

impact of ICOS blockade may vary depending on the stages of autoimmune diseases. During the initiation phase, blocking ICOS prevents EAE by decreasing IFN-γ secretion by splenocytes, but the blockade of ICOS during antigen priming effector phase exacerbates disease by enhancing IFN-γ production [210]. Other studies showed that blocking ICOS could inhibit class switching of B cells but could favor Th1 response. ICOS is upregulated and ICOSL is constitutively expressed in nephritic kidneys of MRL-*Fas*^{lpr} mice. Zeller et al., reported that ICOS deficiency in these mice changed serum IgG and autoAb isotypes by markedly reducing IgG1, IgG2a, and IgG2b but not IgG3 and IgM. However, ICOS deficiency increased the frequency of Th1 and $CD8^+$ T cells that secrete IFN-γ and TNF-α while Th2 cells are not altered. In addition, ICOS deficiency in MRL-*Fas*^{lpr} mice does not affect the severity or the progression of lupus nephritis. In addition, ICOS deficiency fails to diminish the extent of lymphocytic infiltration in salivary and lacrimal glands [211]. Other studies have shown that in the New Zealand Black/New Zealand White (NZB/NZW) F1 mouse model of SLE and the collagen-induced arthritis model of RA, treatment with an anti-ICOSL Ab improves disease manifestations and leads to a decrease in Tfh cells and GC B cells [212]. Delivery of ICOS-Ig and CTLA4-Ig fusion protein using adenovirus on experimental autoimmune myocarditis showed a therapeutic effect. Cardiac function and myocardial inflammation significantly improved compared to ICOS-Ig delivery alone [213]. In another study, the combined therapy of adenovirus containing PD-L1 and injection of anti-ICOSL mAb in lupus prone BXSB mice dramatically suppressed lupus-like syndrome compared with inhibiting PD-1 signal alone and resulted in an almost complete improvement of lupus nephritis [214]. Pharmaceutical companies are currently evaluating antibodies against IL-21, CXCR5 and ICOS. The only available drug (marketed for RA) that could target Tfh cells is tocilizumab, a monoclonal antibody against IL-6R. A randomized placebo controlled trial of tocilizumab in pSS has just started in France.

OX40 knockout mice do not show any severe side effects. However, they exhibit reduced $CD4^+$ T cell proliferation, memory T cells and IL-2 levels when infected. Transgenic mice constitutively expressing OX40L on T cells develop a spontaneous autoimmune disease. These mice have hyperproliferative $CD4^+$ T cells and remarkably elevated memory $CD4^+$ T cells. They develop organ-specific autoimmune like disease, namely interstitial pneumonia and inflammatory bowel disease. Administration of anti-OX40L mAb prevents the development of these diseases [215]. Anti-OX40 agonist antibody treatment can ameliorate Ag immune intervention for the prevention of type 1 diabetes. In NOD mice, treatment by autoAg-derived peptide in the early phase of pathogenesis induces an expansion of autoAg-specific $CD4^+CTLA\text{-}4^+FoxP3^+$ Tregs. An OX40 agonist in combination with this Ag-specific therapy prevents type 1 diabetes by additionally expanding Tregs [216]. In fact OX40 agonist administration influences autoimmune severity in opposite directions depending on the timing of administration. During Ag priming, OX40 agonist drives Treg expansion and inhibits disease, whereas later it enhances effector T cell function and exacerbates disease. Thus, the consequences of OX40 signaling in autoimmune diseases are complex. It seems that the impact of OX40 blockade may vary depending on the stages of autoimmune diseases.

To conclude, our results demonstrate various new mechanisms by which salivary gland epithelial cells play a pathogenic role on T cells, B cells and on T-cell related B-cell activation (Figure 23 and 24). These results also suggest that follicular helper T cells, IL-6, ICOS/ICOSL, IL-21 as well as OX40/OX40L might represent relevant therapeutic targets in pSS.

Figure 23. SGECs lead to B-cell activation via different approaches in pSS.

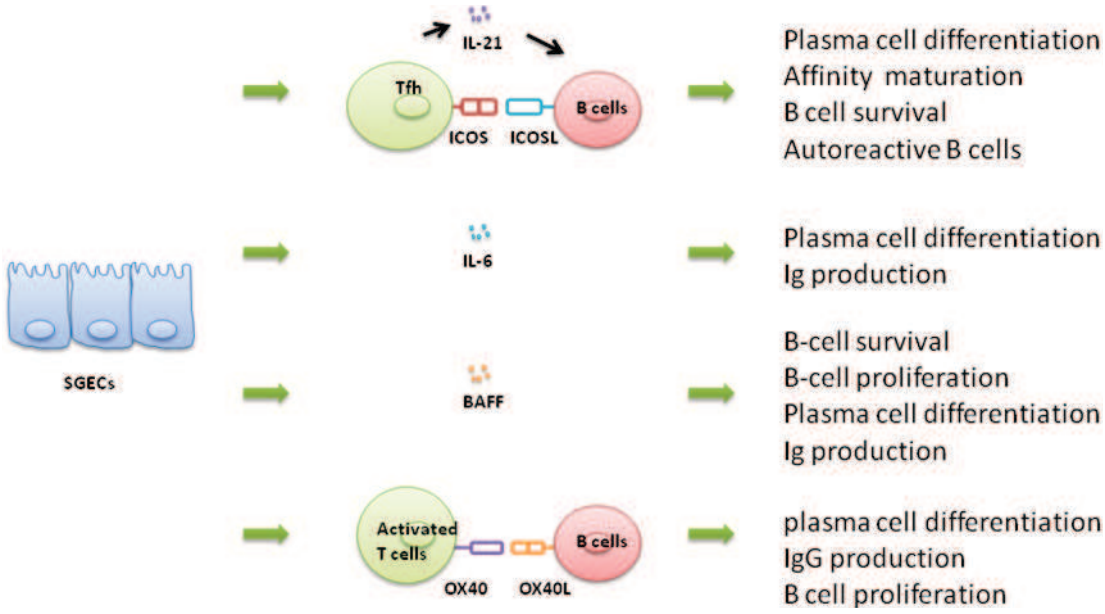
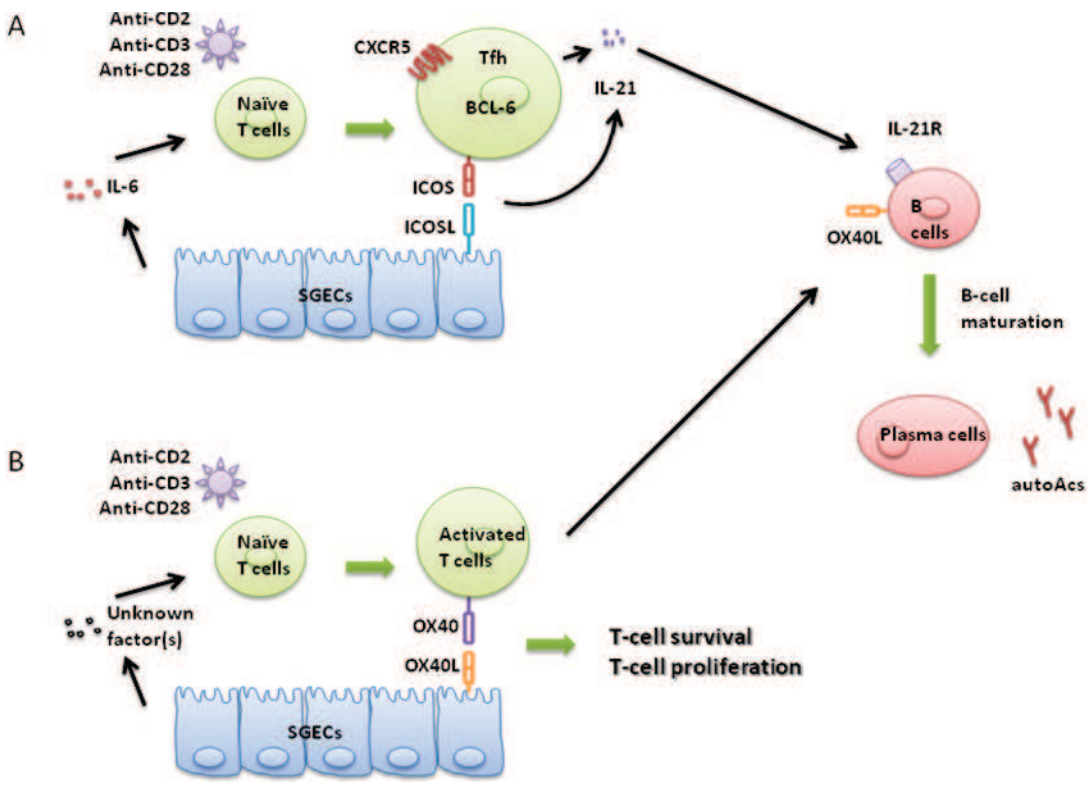


Figure 24. New pathogenic roles of salivary gland epithelial cells in pSS. **A. SGECs are capable to induce the differentiation of IL-21 secreting follicular helper T cells, which might contribute to the marked activation of B lymphocytes in salivary glands. IL-6 is required for the differentiation of follicular helper T cells. The secretion of IL-21 by the Tfh requires cell contact partly involving ICOSL. B. SGECs are capable to induce a dramatic increase of OX40 expression by soluble factor(s) and promote T-cell survival and proliferation via OX40-OX40L interaction.**


A
Anti-CD2
Anti-CD3
Anti-CD28
Naïve
T cells
IL-6
CXCR5
Tfh
BCL-6
IL-21
ICOS
ICOSL
SGECs
IL-21R
B
cells
OX40L
B-cell
maturation
Plasma cells
autoAcs
B
Anti-CD2
Anti-CD3
Anti-CD28
Naïve
T cells
Unknown
factor(s)
Activated
T cells
OX40
OX40L
SGECs
T-cell survival
T-cell proliferation

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VI. Résumé français

Etude du rôle des cellules épithéliales des glandes salivaires dans la différenciation et l'activation des lymphocytes T au cours du Syndrome de Sjögren primitif

Le syndrome de Sjögren primitif (SJp) est une pathologie auto-immune caractérisée par une sécheresse occulo-buccale, un infiltrat lymphocytaire des glandes salivaires et lacrymales, ainsi qu'une sécrétion d'auto-anticorps. Sécheresse, fatigue et douleur constituent les trois éléments principaux de la symptomatologie. Chez les patients atteints de SJp, la capacité des SGEC à sécréter des cytokines (IL-1, IL-6, *B-cell activating factor* (BAFF)) et des chemokines ou à présenter certains auto-antigènes a été rapportée précédemment. En revanche, la capacité des SGEC à différencier les lymphocytes T est mal connue.

Partie I : Différenciation par les cellules épithéliales salivaires des lymphocytes T naïfs en lymphocytes T folliculaires

Introduction :

La population des lymphocytes T folliculaires (LTf) a été identifiée après celle des lymphocytes Th1, Th2, Th17 et T régulateurs. Le développement des Tfh est fortement dépendent du facteur de transcription Bcl-6. Les Tfh expriment le récepteur de chémokine CXCR5, qui leur permet d'aller dans les centres germinatifs des organes lymphoïdes. Ces LTf ont un rôle particulièrement important dans l'activation des lymphocytes B.. Ces LTf sont augmentés dans le sang des patients ayant un SJp. L'activation des lymphocytes B par les lymphocytes T dépend notamment du couple

de molécules de costimulation ICOS/ICOS-ligand (ICOSL). ICOS est exprimé par les Tfh et ICOS-ligand (ICOSL) par les lymphocytes B et les cellules présentatrices d'antigène. Le rôle pathogène des LTf est en grande partie médié par la sécrétion d'IL-21. Cette cytokine a une action anti-virale comme l'IFN de type I, et pourrait jouer un rôle pathogène majeur au cours du SJp. En effet, l'expression de l'IL-21 est augmentée dans le sérum et les glandes salivaires des patients ayant un SJp.

Objectifs :

Les trois objectifs principaux de l'étude sont les suivants:

(1) Etudier la capacité de la cellule épithéliale des glandes salivaire de patients ayant un SJp à induire la différentiation des LTf secrétant l'IL-21 a partir des lymphocytes T naïfs (LTn).

(2) Rechercher une corrélation entre l'augmentation du taux sérique de l'IL-21 avec l'activité de la maladie.

(3) Rechercher l'association entre SJp et les polymorphismes des gènes de l'IL-21 et l'IL-21R

Résultats :

La culture primaire des SGEC a été effectuée selon la technique développée par Dimitriou *et al* (2002). Les cultures primaires des SGEC ont été réalisées à partir de biopsies de glandes salivaires accessoires de patients atteints de SJp ou de contrôles (syndromes secs sans aucun signe d'autoimmunité). La nature des cellules a été vérifiée par des marquages spécifiques des SGEC : cytokératines 19 et 7 et vimentine et l'absence de marquage CD3, CD20, et myéloperoxydase. Nous avons montré que les SGEC expriment ICOSL par immunofluorescence et western blot.

Nous avons ensuite isolé des lymphocytes T $CD4^+CD45RA^+$ naïfs à partir des cellules mononuclées du sang périphérique humain en utilisant un kit de sélection négative. Les SGEC des patients atteints de SJp ont alors été cocultivées avec les LTn purifiés pendant 4 h et 24h. L'expression d'ARNm Bcl-6 par les LTn a été mesurée par RT-qPCR. Pour analyser la différenciation des LTf, les LTn ont été cultivés seuls ou en présence de SGEC. Après 5 jours, les LT ont été restimulés pendant 24 heures par des billes couplées à des anticorps anti-CD2, anti-CD3 et anti-CD28 (bille/cellule = 2/1). Nous avons récupéré les cellules T et mesuré l'expression d'ICOS et de CXCR5 par cytométrie en flux. La sécrétion d'IL-21 a été mesurée par ELISA.

Nous avons observé l'augmentation d'expression des ARNm de Bcl-6, lorsque les LT étaient cocultivés avec les SGEC de patients atteints de SJp ou de contrôles par rapport aux LT cultivés seuls (8,1±1,7 fois avec les SGEC des patients atteints de SJp et 10,1±5,1 fois avec les SGEC des contrôles). Les facteurs de transcription des cellules de type Th1 (T-bet), Th2 (GATA-3) et Th17 (RORγt et RORα) n'étaient pas augmentés en coculture. Nous avons également observé une augmentation de l'expression d'ICOS et de CXCR5 sur les LT après coculture. L'augmentation du double marquage CXCR5/ICOS, qui caractérise les LTf, a été observée (de 0,2%±0,2 à 3,6%±2,4 avec les SGEC des patients atteints de SJp et de 0,2%±0,1 à 3,6%±1,4 avec les SGEC des contrôles). Le surnageant a révélé la présence d'IL-21 (594,7±265.0 pg/ml avec les SGEC des patients atteints de SJp et 655.8±302.1avec les SGEC des contrôles). Les cytokines de type Th1 (IL-12), Th2 (IL-4) et Th17 (IL-17) n'ont pas été détectées dans les surnageants de culture. (Figure 1). Une caractéristique fonctionnelle des Tfh est leur capacité à améliorer la survie des lymphocytes B. Nous avons donc effectué des cocultures des SGEC et les LTn pendant 5 jours avant d'ajouter des lymphocytes B pendant 3 jours. La viabilité des lymphocytes B a été augmentée significativement après coculture LT-SGEC. Ces résultats montrent que les lymphocytes T folliculaires différenciés par les SGEC

présentent non seulement le phénotype (expression de Bcl-6, CXCR5, et ICOS et sécrétion d'IL-21), mais également des caractéristiques fonctionnelles des LTf.

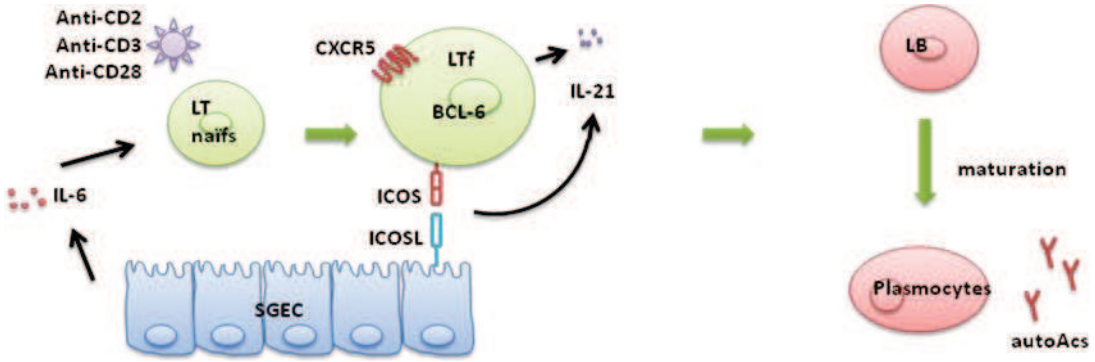
Nous avons ensuite effectué des cocultures entre SGEC et LT en utilisant un filtre (« transwell ») afin de déterminer si la différenciation des LTf dépend du contact cellulaire ou de facteurs solubles. Aucun changement significatif n'a été observé dans la différenciation des LTf en présence ou non de transwell. Cependant, la sécrétion de l'IL-21 a été inhibée en utilisant un transwell (198,6 ± 83,3 avec transwell et 591,4 ± 308,9 pg/ml sans transwell, p = 0,0286). Pour identifier les facteurs solubles impliqués dans la différenciation des LTf, nous avons utilisé des anticorps anti-IL-6 ou anti-ICOSL. Les deux protéines sont connues pour jouer un rôle important dans la différenciation des LTf. L'induction de différenciation des LTf a été partiellement inhibée en utilisent un anticorps neutralisant l'IL-6 (1,3% ± 0,8 avec 20µg/ml anti-IL-6 mAb versus 3,1% ± 1,3 avec contrôle isotypique IgG1, p = 0,02). Le blocage d'ICOSL par des anticorps anti-ICOSL diminue la sécrétion d'IL-21 sans modifier le nombre de LTf (350,7 ± 243,2 pg/ml en présence de 20µg/ml anti-IL-6 mAb versus 584,5 ± 261,5 pg/ ml en présence de contrôle isotype lgG2b, p = 0,04). La sécrétion d'IL-6 par les SGEC contribue donc à la différenciation des LTf et la sécrétion d'IL-21 par les LTf nécessite un contact cellulaire entre les LTf et les SGEC impliquant notamment ICOSL.

En utilisant la cohorte ASSESS, nous avons analysé l'association entre le taux sérique IL-21 et les caractéristiques de la maladie. Nous avons confirmé l'augmentation de l'IL-21 sérique chez les patients atteints de SJp et avons montré une corrélation entre le taux sérique IL-21 et l'activité systémique de la maladie évaluée par le taux ESSDAI.

Conclusion :

Ces résultats montrent que les SGEC sont capables d'induire la différenciation des lymphocytes T naïfs en lymphocytes T folliculaires via des facteurs solubles tels que l'IL-6. La sécrétion d'IL-21 par les LTf nécessite un contact cellulaire impliquant en partie ICOSL. L'induction de LTf par les SGEC pourrait contribuer donc à l'activation des lymphocytes B dans les glandes salivaires de patients. Ces résultats confirment le rôle pathogène important des cellules épithéliales des glandes salivaires et suggèrent que les lymphocytes T folliculaires et l'IL-21 pourraient être des cibles thérapeutiques d'intérêt dans cette maladie (Figure 1).

Figure 1 : les SGEC sont capables d'induire la différenciation des lymphocytes T naïfs en lymphocytes T folliculaires via des facteurs solubles tel que l'IL-6. La sécrétion d'IL-21 par les LTf nécessite un contact cellulaire impliquant en partie ICOSL. L'induction des LTf pourrait contribuer à l'activation des lymphocytes B dans les glandes salivaires.



Partie II : les SGECs induisent l'expression d'OX40 et favorisent la survie et la prolifération des lymphocytes T.

Introduction :

OX40 (CD134, TNFRSF4) et son ligand, OX40L (CD252, TNFSF4), sont

respectivement-membres des superfamilles du TNF et du récepteur du TNF (TNFR). L'expression OX40 peut être induite sur les LT $CD4^+$ ou $CD8^+$ activés, mais n'est pas observée sur les lymphocytes T naïfs (LTn). L'expression d'OX40L peut être induite sur les lymphocytes B, les cellules dendritiques et les macrophages. D'autres types cellulaires ayants potentiellement une fonction de cellules présentatrices d'antigène, par exemple les cellules de Langerhans, les cellules endothéliales, les cellules musculaires lisses, les mastocytes et les cellules NK, peuvent, elles-aussi, exprimer OX40L. La voie OX40/OX40L joue un rôle important dans la prolifération des LT et leur différenciation en LT mémoires et effecteurs. OX40 supprime la différenciation et la fonction suppressive des Treg. La voie OX40/OX40L est aussi impliquée dans la différenciation des LTf.

Le rôle central de la voie de co-stimulation OX40/OX40L a été montré dans le développement de plusieurs maladies inflammatoires et autoimmunes, notamment le lupus érythémateux disséminé. Chez les patients lupiques, l'expression d'OX40 des LT $CD4^+$ est plus élevée et corrélée à l'activité de la maladie. En outre, les biopsies rénales des patients atteints de lupus rénal ont montré une infiltration par des LT $OX40^+$. Dans le SJp, il est rapporté que les polymorphismes d'OX40L sont des facteurs de risque. Une meilleure compréhension du rôle d'OX40/OX40L dans l'étiopathogénie du SJp est nécessaire. L'objectif de l'étude était d'étudier l'expression d'OX40 et d'OX40L au cours du SJp, le rôle des SGECs dans l'induction d'expression d'OX40 par les LT $CD4^+$ et les conséquences fonctionnelles de l'interaction OX40L/OX40.

Objectifs :

Les trois objectifs principaux de l'étude étaient les suivants:

(1) Etudier l'expression d'OX40 et d'OX40L *in vivo* chez les patients atteints de

SJp

(2) Déterminer si les SGEC expriment OX40L et peuvent induire l'expression d'OX40 par les LT

(3) Analyser l'influence de l'interaction entre OX40 et OX40L sur la prolifération et la survie des LT.

Résultats :

L'expression d'OX40 et d'OX40L a été analysée dans les biopsies de glandes salivaires accessoires ou dans le sang de patients atteints de SJp et de contrôles par immunohistochimie et cytométrie de flux. L'expression d'OX40L par les SGEC cultivées de patients atteints de SJp ou de contrôles a été mesurée par Western Blot et immunofluorescence. Les LTn ont été cocultivé avec les SGECs en utilisant la même protocole que dans la première étude et l'expression d'OX40 a été mesurée par cytométrie en flux. La prolifération des lymphocytes T a été évaluée par marquage avec du carboxyfluorescein diacetate succinimidyl ester (CFSE) en présence de billes anti-CD2/anti-CD3/anti-CD28. La survie des lymphocytes T a été évaluée par marquage avec l'iodure de propidium (PI)/DiOC6.

Nos résultats ont permis de montrer que les LT $CD4^+$ sanguins expriment OX40. Le pourcentage de LT exprimant OX40 est augmenté chez les patients atteints de SJp comparé aux sujets sains. Les SGEC et les LB $CD19^+$ présents dans l'infiltrat des glandes salivaires de patients surexpriment OX40L par rapport aux témoins. Les LT $CD4^+$ présents dans l'infiltrat expriment OX40.

Étant donné le rôle central des SGEC dans la pathogénie du SJp, nous avons regardé si ces cellules pouraient induire l'expression d'OX40 par les LTn. Nous avons d'abord montré que les SGEC de patients SS (SS SGECs) ou de témoins expriment

OX40L. La coculture des LTn avec des SGEC de patients ou de témoins augmente significativement l'expression d'OX40 par les LT par rapport aux LTn cultivés seuls (de 29,22%±10,46 à 70,05%±9,77 avec les SGEC des patients atteints de SJp et de 19,75%±8.04 à 72,68%±18,89 avec les SGEC des témoins). Pour déterminer si l'induction de l'expression d'OX40 par les SGECs est dépendante de facteurs solubles et / ou d'un contact cellulaire, nous avons effectué des expériences avec un transwell. Le pourcentage de LT $OX40^+$ induit par les SGECs était similaire avec ou sans transwell. Ces résultats ont montré que l'induction de l'expression d'OX40 par les SGEC dépend de facteurs solubles.

Pour étudier l'impact des SGECs sur la survie et la prolifération des LT, nous avons analysé le taux de viabilité des LT par double marquage IP et DiOC6. Lorsque les LTn sont cultivés seuls pendant 6 jours, la viabilité des LT (IP^-DiOC6^+) était de 18,67%±12,42. La viabilité des LT cocultivés avec des SGEC était de 57,92%±14,44 ($p<0,005$).. Les LT prolifèrent également plus en présence de SGEC. Nous avons analysé l'implication d'OX40L dans l'induction de la survie et de la prolifération des LT. L'anti-OX40 inhibe la survie des LT en coculture avec les SGEC (22,85%± 13,96 versus 52,14%±12,60, avec un contrôle isotypique IgG1, $p<0,05$). Le blocage d'OX40 diminue également la prolifération des LT induite par les SGECs(43,4% versus 62,4% en présence d'IgG1).

Conclusion :

Nos résultats suggèrent l'implication d'OX40 et d'OX40L dans la pathogénie du SJp. Cette étude montre aussi un nouveau rôle pathogène des cellules épithéliales des glandes salivaires au cours du SJp. Elles sont capables d'induire une augmentation de l'expression d'OX40 et de favoriser la survie et la prolifération des lymphocytes T.

Figure 2 : Les SGEC induisent l'expression d'OX40 par les lymphocytes T

$CD4^+$ naïfs. Cette processus est dépendant de facteurs solubles. Les SGEC améliorent la survie et la prolifération des LT via l'expression salivaire d'OX40L.