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Autophagie comme cible thérapeutique potentielle pour le syndrome de Sjögren

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ABBREVIATIONS

3-MA	3-methyladenine
ACR	American College of Rheumatology
AD	Alzheimer disease
AECG	American-European Consensus Group classification criteria
AID	Autoimmune disease
Aly/aly	Alymphoplasia
ANA	Antinuclear antibody
APCs	Antigen presenting cells
ATG	Autophagy related
AVs	Autophagic vacuoles
BafA1	Bafilomycin A1
BAFF	B cell active factor
BAG-1	BCL2-associated athanogene 1
Bax	BCL2 associated X protein
BCL2	B-cell CLL/lymphoma 2
Bif-1	Endophilin B1
BNIP3L	BCL2 interacting protein 3 like
C4	Complement component 4
CA6	Carbonic anhydrase VI
CALCOCO2/NDP52	Calcium binding and coiled-coil domain 2
CCL	C-C motif chemokine ligand
CMA	Chaperone-mediated autophagy
CMV	Cytomegalovirus
CQ	Chloroquine
CTLA-4	T-lymphocyte-associated protein 4
CV	Coxsackie virus
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptors
DC	Dendritic cells
DFCP1	Double FYVE-containing protein 1
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DLBCL	EBV positive diffuse large B cell lymphoma
EAE	Experimental autoimmune encephalomyelitis
EAM	Experimental autoimmune myocarditis
EBV	Epstein Barr virus

EBVEA	Epstein Barr virus early antigen
EM	Electron microscopy
ER	Endoplasmic Reticulum
FAS	TNF receptor superfamily, member 6
FASL	FAS ligand
FcγR	Fc gamma receptors
FDCs	Follicular dendritic cells
FIP200	FAK family kinase-interacting protein of 200kDa
Foxp3	Forkhead box protein P3
FYCO1	FYVE and coiled-coil domain containing 1
GC	Germinal center
GWAS	Genome-wide association study
hAECs	Human amniotic epithelial cells
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIP	HSP70 interacting protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HOP	HSC70/HSP90-organizing protein
HOPS	Homotypic fusion and protein sorting
HSP40	Heat shock 40kDa proteins
HSP90	Heat shock 90kDa proteins
HSPA8	Heat shock protein family A member 8
HTLV-1	Human T-lymphotropic virus Type 1
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IKBKE	Inhibitor of nuclear factor kappa B kinase subunit epsilon
IL	Interleukins
ILD	Interstitial lung disease
IP-10	IFN-γ-induced protein 10
IRFs	Interferon regulatory factors
KIR	Keap1-interacting region
LAMP	Lysosomal membrane protein
LAP	LC3-associated phagocytosis
LD	Lipid droplet
LIMP	Lysosomal integral membrane proteins
LRS	LC3-recognition sequence
LSG	Labial salivary glands
M3R	Muscarinic acetylcholine receptor M3

MAP1LC3/LC3	Microtubule associated protein 1 light chain 3
MRL/lpr	Murphy Roths Large/lymphoproliferation
MS	Multiple sclerosis
MSGs	Minor salivary glands
mTOR	Mammalian target of rapamycin
NBR1	Neighbor of BRCA1 gene 1
NF-κB	Nuclear factor kappa B
NOD	Non-obese diabetic
NZB/W F1	New Zealand Black/White F1 Hybrid
OMM	Outer mitochondrial membrane
OPTN	Optineurin
PAS	Pre-autophagosome structure
PB1	N-terminal Phox and Bem1p region
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PE	Phosphatidylethanolamine
PI3KC3	Class III PI 3-kinase complex
PI3P	Phosphatidylinositol-3-phosphate
PSP	Parotid secretory protein
pSS	Primary Sjögren's syndrome
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RILP	Rab interacting lysosomal protein.
SGEC	Salivary gland epithelial cell
SGP	Salivary gland proteins
SGs	Salivary glands
SHBG	Sex hormone-binding globulin
SICCA	Sjögren’s International Collaborative Clinical Alliance
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SP-1	Salivary glands protein-1
SQSTM1/p62	Sequestosome
SS	Sjögren's syndrome
SSA/Ro	Sjögren's syndrome-related antigen A
SSB/La	Sjögren's syndrome-related antigen B
SSc	Systemic sclerosis
sSS	Secondary Sjögren's syndrome
T1D	Type 1 diabetes
TCR	T cell receptor
TECPR1	Tectonin β-propeller repeat containing 1

Tfh	Follicular helper T cells
Th	Helper T cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFAIP3	TNF-α induced protein 3
TNIP1/ABIN1	TNFAIP3 interacting protein 1
TPC	Two-pore complex
Treg	Regulatory T cells
TRP	Transient receptor potential
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UBDs	Ub-binding domains
ULK1	Unc-51-like autophagy activating kinase 1
VCAM	Vascular cell adhesion molecule
VMP1	Vacuole membrane protein 1
WB	Western blotting
WIPI	WD-repeat domain phosphoinositide-interacting
Zinc	ZZ-type zinc finger domain

INTRODUCTION

A. Sjögren's syndrome

The clinical features of Sjögren's syndrome (SS) were first described in 1888 by Dr. Hadden, who reported a 65-year-old woman with severe ocular dryness to the Clinical Society of London (Hadden, 1888). In 1925, a French ophthalmologist, Dr. Gougerot found that in certain patients, ocular dryness was only a part of systemic dryness affecting mouth, larynx, nasal and vaginal mucosa, and these symptoms might occur with a possible decrease in the function of the thyroid and ovaries (Marchal et al., 1947)

In 1933, Dr. Henrik Sjögren, a Swedish ophthalmologist reported 19 clinical cases suffering from dry eyes and dry mouth. He found that most of those patients were postmenopausal women with arthritis, showing pathological manifestations of the syndrome (Gren, 1933). Since then, SS has been recognized as a chronic inflammatory disease, and numerous investigations and studies in both clinic and laboratory have been performed in this disease.

A.1: Symptoms

A.1.1: Dryness

As a chronic, systemic, inflammatory autoimmune disease, SS is characterized by lymphocytic infiltration in exocrine glands. Lymphocytic infiltration in the salivary glands (SGs) and lacrimal glands (LGs) causes destruction of glandular tissues, leading to symptoms like dry eyes (xerophthalmia) and dry mouth (xerostomia). Patients with dry eyes may feel burn, itch or gritty, and continuous uncomfortable in their eyes. Dry eyes can be found in patients with SS by clinical tests that include tear break-up times, Schirmer’s-I test scores, response rates and cornea fluorescent staining (Lee et al., 2011). Dry mouth is led by reduced saliva secretion. In patients with dry mouth, the oral health is difficult to maintain as the skin in the mouth cannot be moistened, and patients can feel difficulties in speaking and eating. As saliva is the resources of epidermal growth factors that promote tissue growth, differentiation and wound healing, patients with dry mouth are vulnerable for oral

infections and inflammation (Furness et al., 2013). Xerophthalmia and xerostomia are found frequently in old people, and regular pharmacotherapy can be effective. However, as the glandular tissues are attacked by autoimmune responses and damaged in patients with SS, regular treatments must be continuous and relatively long lasting.

Besides SGs and LGs, SS patients also suffer from dryness in other organs or tissues such as nose, respiratory system and skin (Figure 1).

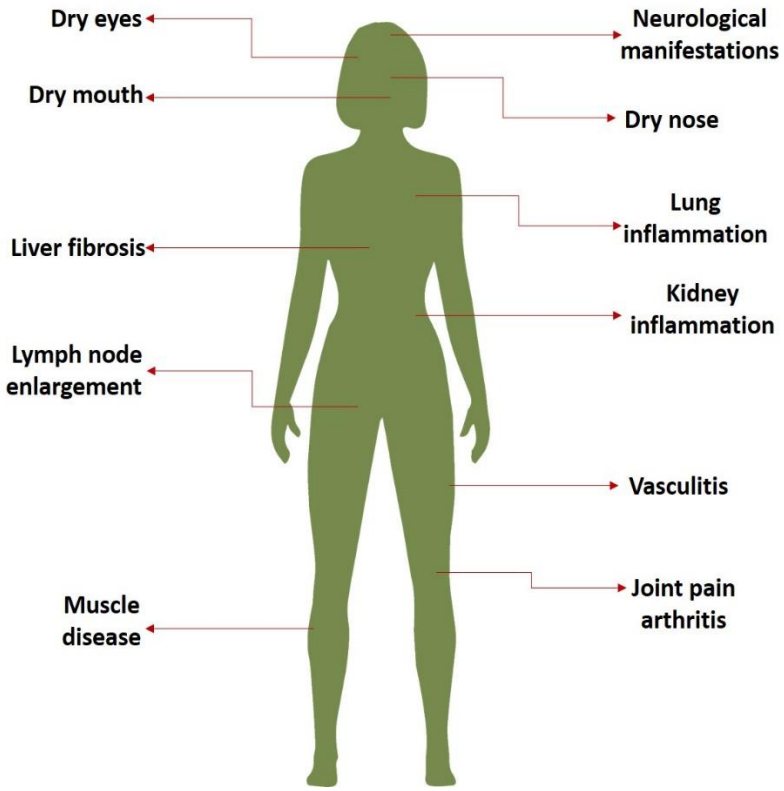

Figure 1. Tissues and organs affected by SS

Patients with SS suffer from various clinical manifestations in multiple organs and tissues. Dryness affects glandular tissues including SGs and LGs. Chronic inflammations are found in lymph nodes, lungs, kidneys, joints and muscles, causing manifestations and dysfunctions in these organs. In addition, central nervous system and respiratory system functions can also be affected by SS.

A.1.2: Chronic inflammation

SS has been recognized as a systemic rheumatic disease. It presents several rheumatic features such as chronic inflammation in kidneys and lungs, as well as

connective tissue arthropathy. Renal function may be affected in two different aspects, which are the tubulointerstitial nephritis caused by epithelial inflammation or glomerulopathy led by immune complex deposition (Evans et al., 2015). Compared with tubulointerstitial nephritis, glomerulonephritis is associated with higher frequency of early mortality (Kidder et al., 2015). Interstitial lung disease (ILD) is one of the major systemic manifestations of SS. Patients with ILD have pulmonary complications that are characterized by various patterns of inflammation and fibrosis in lungs (Vij et al., 2013). Impaired pulmonary functions include reduced carbon monoxide-diffusing capacity, less peak expiratory flow and forced vital capacity, which can result in significant morbidity and mortality (Zhao et al., 2013).

Around 10% of the patients develop cancers, among which B-cell lymphoma is the most frequent type and represents the most severe complications for SS patients (Brito-Zeron et al., 2017). The association between SS and lymphoma was identified as early as 1978, when a much higher incidence of lymphoma (43.8 times of same age general population) was found in SS patients (Kassan et al., 1978).

A study evaluating 25-year outcome of 152 patients with SS found that around 10% of SS patients present vasculitis (Abrol et al., 2014). Vasculitis is inflammation of blood vessels that can affect multiple organs in the body. Vasculitis, which is often caused by immune attack of blood vessels and induces systemic inflammation, leads to symptoms like fever, general aches and pains among patients.

Other organs/tissues including the liver (liver fibrosis) (Lee et al., 2016), the joints (joint pains/arthritis) (Amezcua-Guerra et al., 2013), and muscles (myositis) (Colafrancesco et al., 2015) could also be affected in SS (Figure 1).

A.1.3: Neuronal manifestations

In addition to the symptoms described above, some abnormalities in nervous system have been reported in SS patients. Prevalence of neurological manifestations in SS varies from 10-60%. This wide range is due to the criteria used for SS definition as well as methodology differences, and the methods used for detecting neuropathy (Chai et al., 2010). Several neuropathy subtypes have been found in SS, which

include multiple mononeuropathy, polyradiculopathy, and sensory neuropathy. Patients may have a combination of two or more neuropathies. Both peripheral and central nervous systems are involved, and the frequencies are in 66% and 44%, respectively (Jamilloux et al., 2014). Sensory neuropathies are the most frequent neuropathies among all descriptions. One of the sensory neuropathies is the small fiber sensory neuropathy. SS patients with this form of neuropathy are diagnosed with prominent neuropathic pain, accompanied by loss of pinprick or temperature discrimination and hyperesthesia (Mori et al., 2005).

A.2: Prevalence and criteria

A.2.1: Prevalence

SS is classified into primary and secondary SS. In primary SS (pSS), patients show only symptoms of SS. However, as the disease develops, other autoimmune diseases (AIDs) like rheumatoid arthritis (RA), systemic sclerosis (SSc) or systemic lupus erythematosus (SLE) can occur with SS, named secondary SS (sSS). SS affects 0.01-0.72% of the whole population (Maldini et al., 2014), and 10-30% patients suffer from sSS, in which around 18% patients suffer from SLE (Yao et al., 2012), around 12% patients show SSc associated (Avouac et al., 2010), and 4-22% patients are diagnosed with RA (Haga et al., 2012; Kosrirukvongs et al., 2012) (Figure 2).

SS is considered as the third most prevalent rheumatic autoimmune disorder, following RA and SLE. Generally, the incidence and prevalence of primary pSS can be largely variable according to criteria used and study designed, as well as geographical area chosen. A study in Germany reported a yearly incidence of 4 in 100,000 by using the American-European Consensus Group classification criteria (AECG) criteria (Westhoff et al., 2010). Another five-year study in Karolinska University Hospital reported an incidence of 3.1 in 100,000 inhabitants per year (Kvarnstrom et al., 2015). A recent study using systematic literature search on PubMed and Embase involved 1880 related citations pointed out that the incidence rates for pSS was 6.92 per 100,000 person-years, and the overall prevalence rates was 60.82 per 100,000

(Qin et al., 2015).

Figure 2. Epidemiology of SS

SS is classified into pSS and sSS. Patients show only symptoms of SS in pSS, while patients present combined symptoms of SS and other accompanied AIDs in sSS, which present 70-90% and 10-30% of all SS patients, respectively. In sSS, the most frequent accompanied autoimmune disease is SLE, which affects approximately 18% of sSS patients. Other patients with sSS may develop SSc or RA.

SS could occur in any population and any age, but mostly in middle age and postmenopausal women. A meta-analysis confirmed the peak incidence of pSS occurs in women aged around 56 years (Qin et al., 2015). A study of systemic autoimmune disease geoepidemiology used Google search engine to collect and analyze 394,827 patients with systemic AIDs, found that pSS represents one of the most unbalanced gender ratio, with nearly the M/F ratio at 1:9 (Ramos-Casals et al., 2015).

A.2.2: Criteria

Several criteria were used for disease classification since the first case of SS, aimed to clarify the characteristics of SS as well as to differentiate SS from other symptom-like AIDs.

In 1965, Bloch et al. reported a five-year study of 65 SS patients in which dry eyes, dry mouth and other connective tissue diseases (such as RA, SSc) were used as a definition of SS (Bloch et al., 1965).

In 1968, Chisholm et al. suggested the labial biopsy of SGs as a useful technique to investigate SS. In this technique, the foci per 4 mm^2 salivary tissue were used to standardize the degree of histopathology (Chisholm et al., 1968). The criteria of SS have been developed and modified many times. However, this technique has been inherited and used until now.

In 1986, four classification criteria were presented during the first international symposium on SS, namely the Copenhagen, Japanese, Greek and California criteria (Fox et al., 1986b). These criteria focus on different objective and subjective components, highlighting different diagnosis sets. Although they failed to provide a widely-accepted, complete and precise criterion for SS, they provided a platform for later studies to explore and develop a profound, unified set of criteria.

In 1992, Speight et al. emphasized the value of measuring whole unstimulated salivary flow in SS patients and proposed this simple method as a diagnosis for SS (Speight et al., 1992).

A large-scale study involving 26 clinical centers from 12 countries (11 in Europe, plus Israel) tried to reach a consensus on the diagnostic procedures for SS (Vitali et al., 1993). This study established the preliminary European criteria in 1993 (Figure 3). In this set of criteria, 2 subjective and 4 objective items were proposed:

1> ocular symptom (subjective); defined by a positive response to at least 3 dry eyes related questions.
2> oral symptom (subjective); defined by a positive response to at least 3 dry mouth or swallowing problems related questions.
3> Ocular signs; defined by objective evidence of ocular involvement proved by positive result from either Schirmer's-I test (≤ 5 mm in 5 min) or Rose bengal score (≥4, according to the van Bijsterveld scoring system) (Vitali et al., 1994).
4> Histopathologic features; refer to focus score ≥1 on biopsy of minor salivary glands (MSGs) (focus defined as an accumulation of more than 50 mononuclear cells; focus score defined as the number of focus per 4 mm glandular tissue)
5> SGs involvement; considered as an objective evidence of SGs involvement

determined by positive result from either salivary scintigraphy, parotid sialography or unstimulated salivary flow (≤ 1.5 ml in 15 min).

6> Autoantibodies; in this item, autoantibodies to SS-related antigen A (SSA, also called Ro), SS-related antigen B (SSB, also called La) and antinuclear antibody (ANA), or rheumatoid factors (RF) should be at least one time positive in the serum of patients to provide an objective evidence.

The preliminary European classification criteria led to the first worldwide accepted classification criteria for SS, which have been successfully applied by the community for clinical diagnosis, epidemiologic surveys and scientific research.

However, as the knowledge of SS accumulated, the limitations of this set of criteria started to be realized. For example, the first two items related to dry eyes and dry mouth could be highly subjective and easily influenced by other factors like anxiety or medication (Manthorpe, 2002). To overcome the limitation of these criteria, a revised version of classification criteria was proposed by AECG in 2002, based on the preliminary European criteria (Vitali et al., 2002). In this version (Figure 3), the six items from the preliminary European criteria (4 subjective criteria: ocular symptoms, oral symptoms, ocular signs and oral signs; 2 objective criteria: lymphocytic foci in SGs biopsy and serum autoantibodies) were maintained. However, three revised rules were introduced to make a more accurate diagnosis of:

1> For pSS: pSS is identified by both subjective criteria and objective criteria, namely with the presence of any 4 out of 6 items described above, if either the items of histopathology or serology is positive; or that 3 out of 4 objective criteria were presented.

2> For sSS: presence of ocular symptoms or oral symptoms plus any 2 from 3 items (ocular signs, oral signs and lymphocytic focus in SGs biopsy).

3> Exclusion criteria: past head and neck radiation treatment, hepatitis C infection, use of anticholinergic drugs, etc.

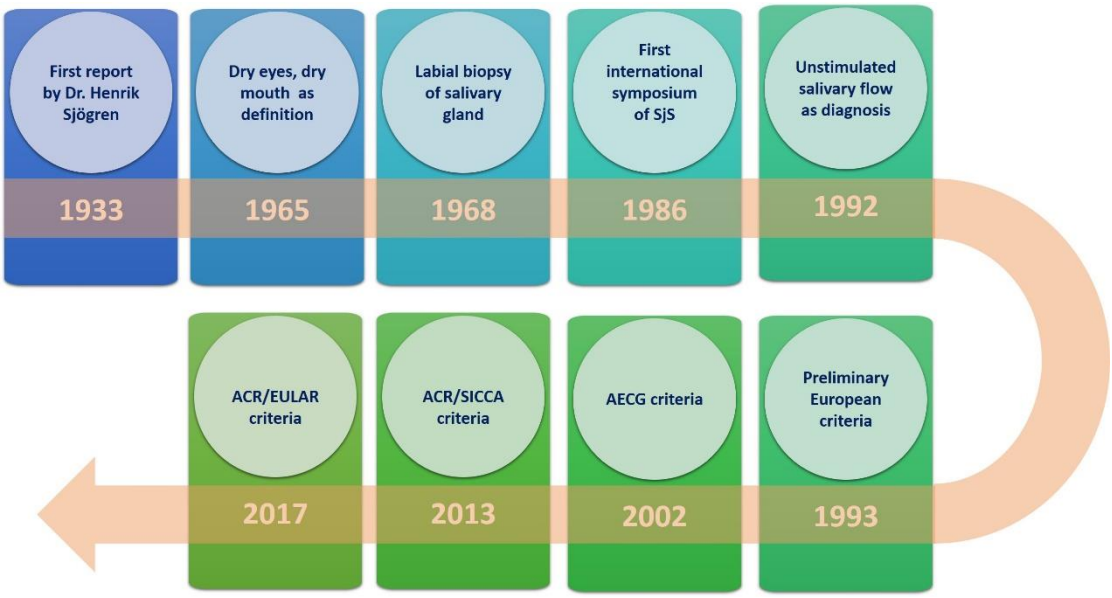

Figure 3. Development of SS criteria

SS was reported for the first time in 1933 by Dr. Henrik Sjögren, who established the disease spectrum. Subsequent studies developed the definition of SS by dry eyes and dry mouth tests as well as labial biopsy of SGs. The first international symposium of SS in 1986 provided a platform for later studies of SS criteria. Unstimulated salivary flow was suggested as a new diagnosis criterion of SS in 1992 and promoted the generation of preliminary European criteria in the following year. At the beginning of the 21st century, AECG criteria were proposed as worldwide criteria and used for more than 10 years. The latest criteria were built in 2017 by ACR/EULAR, based on the earlier ACR/SICCA criteria.

In addition to items listed above, the new criteria modified some items in detail. For example, in ocular signs, it was specified that the Schirmer's-I test should be performed without anaesthesia. The presence of ANA and RF was removed from the list of autoantibodies (Baldini et al., 2012; Goules et al., 2014).

The model from AECG was used for more than 10 years as a "gold standard" for diagnosis of SS, and was refined in 2013 by the American College of Rheumatology (ACR) in association with Sjögren's International Collaborative Clinical Alliance (SICCA) (Shiboski et al., 2012) (Figure 3). These criteria used five items to identify SS, and case definition requires at least 2 of the following 3 items:

1> positive serology of anti-Ro/SSA and/or anti-La/SSB or positive RF in association with ANA titer > 320

2> ocular staining score > 3

3> focus score of SG biopsy >1 focus/4 mm^2

More recently, new classification criteria for SS have been proposed by the ACR in association with the European League Against Rheumatism (EULAR) to use five items as identify for SS (Shiboski et al., 2017):

1> focus score of labial SG ≥1 foci/4 mm^2, scoring 3
2> anti-SSA/Ro antibody positive, scoring 3
3> an abnormal Ocular Staining Score of ≥5 (or van Bijsterveld's score of ≥4), scoring 1
4> Schirmer's-I test result ≤5 mm/5 min, scoring 1
5> Unstimulated whole salivary flow rate ≤0.1 mL/min, scoring 1

When these criteria are used, patients are identified as having SS if the summed score from all five items is ≥4.

These criteria offer different methods and ideas to investigate SS, in both clinic and scientific research (Figure 3). Noteworthy, in most of these criteria, SS is identified by oral and ocular manifestations, while the full spectrum of disease comprises a complex series of systemic symptoms. However, none of these criteria could be able to encompass the whole spectrum of SS, that is also one of the reasons for unachieved worldwide agreement on any criteria above. Other reasons include the acquaintance of this disease is not comprehensive, and all these criteria focus different aspects of the disease which is still controversial in the debating of their importance. For example, a recent study compared the new ACR-EULAR criteria (2016) with three other criteria: the revised Japanese Ministry of Health criteria (JPN, 1999), AECG criteria (2002), ACR classification criteria (2012) in 499 Japanese patients. This study showed that ACR-EULAR criteria display higher sensitivity (95.4%) but lower specificity (72.1%) in diagnosis of pSS, compared to three other sets of criteria.

However, as the knowledge about this disease will further increase with ongoing studies, improved criteria should be proposed and should provide a better understanding of SS, that will also help to develop better therapeutic methods (Seror

et al., 2015).

A.3: Factors and inducers

The inducers of SS could implicate diverse factors. Environmental and genetic factors are the most acknowledged inducers of SS. The triggers for SS could be attributed to genetic factors, hormonal factors and environmental factors (Figure 4). They lead to autoimmune exocrinopathy in which epithelial cells augment the levels of chemokines and pro-inflammatory cytokines, result in the activation of immune cells and finally cause the dysfunction of whole immune system.

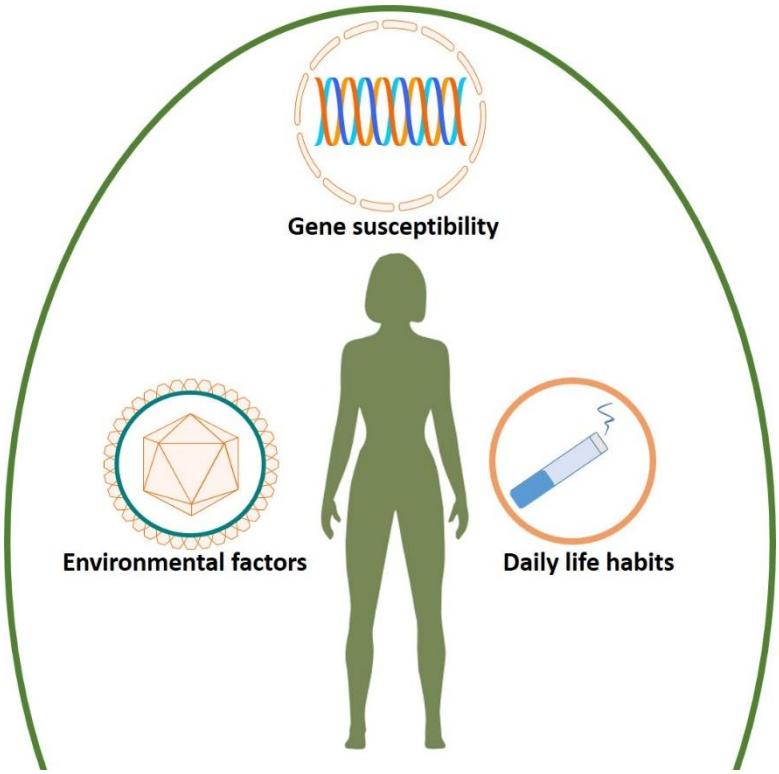

Figure 4. Inducers of SS

SS can be induced by environmental factors like virus infections, genetic factors like genetic risk loci and daily life habits. These factors are found to be associated with SS. However, the mechanisms are largely unclear. An important theory is that SS is initiated by the combained effects of multiple factors but not single inducer.

Daily life habits like smoking are identified as other factors that induce SS. However, the precise role of cigarette smoking, for instance, is still a matter of debates. In

patients with pSS, ANA positivity was significantly associated with smoking (Karabulut et al., 2011). In another study, however, current tobacco smoking was found to be negatively associated with SS, and provide a protection to disease-associated humoral and cellular autoimmunity (Stone et al., 2017). This was further confirmed by a nested case-control study that involved sixty-three patients with pSS, where it was found that current smoking was associated with a reduced risk of pSS, but former smoking was associated with an increased risk (Olsson et al., 2017).

A.3.1: Environmental factors

SS can be triggered by environmental factors. Infections, especially virus infections, have been recognized as important inducers to SS since early times. Infections by hepatitis B virus (HBV), hepatitis C virus (HCV), parvovirus B19, herpesvirus-6 or human immunodeficiency virus (HIV) may trigger symptoms similar with SS (Igoe et al., 2013). Moreover, epidemiological and clinical studies suggest that viral infections, typically Epstein Barr virus (EBV), cytomegalovirus (CMV), HBV, HCV, Coxsackie virus (CV) and Human T-lymphotropic virus Type 1 (HTLV-1) are involved in the pathogenesis of SS (Sipsas et al., 2011) (Figure 5).

It is found that levels of immunoglobulin (Ig) G against above-listed viruses show association with autoantibody levels, and these viruses have been found in biopsy of patients' SGs. The mechanisms and etiology of SS triggered by viruses are multifaceted. In the following paragraphs, we will focus on the role of HCV and EBV in SS development.

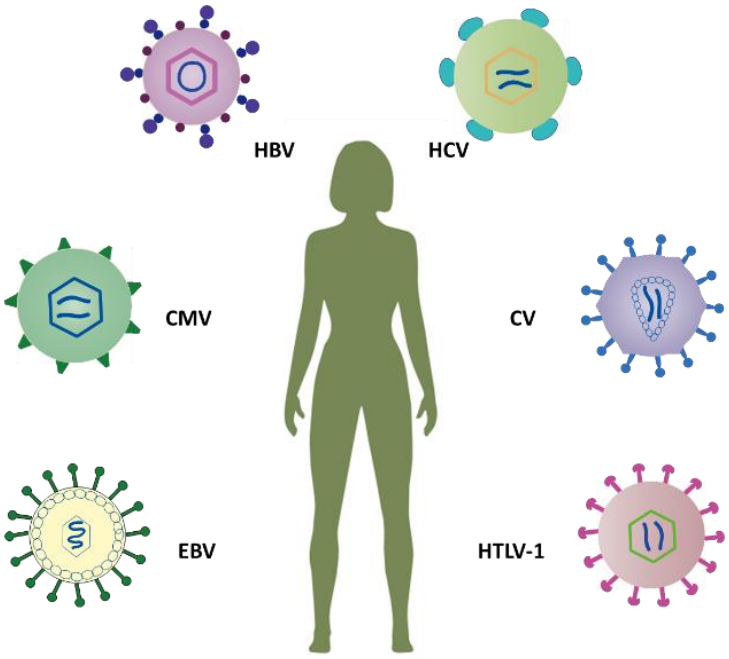

Figure 5. Virus as inducers of SS

Infections by EBV, CMV, HBV, HCV, CV and HTLV may cause symptoms like SS. EBV infection could induce immune responses in SGs and cause inflammation, while CMV infection is rather associated with loss of SG functions. Autoimmune hepatitis in patients with SS is mainly induced by HBV infection. HCV is found to stimulate B cell proliferation in glandular tissues, and CV infections are related to anti-Ro60 autoantibody production. HTLV-I is suggested to affect epithelial cells proliferation and survival.

A.3.1.1: HCV

According to the histological appearance of labial SGs, patients infected with HCV showed higher frequency of focal lymphocytic sialadenitis (57%) compared with controls (5%) (Haddad et al., 1992). This result was deeper developed by Brito-Zeron and colleagues, who found that 13.4% of 783 Spanish patients with SS were infected by HCV. They identified the HCV-driven autoimmune response as a lower frequency of anti-Ro/La antibodies, and an abnormal predominant anti-La antibodies, suggesting that HCV may change the immunological serum pattern of patients with SS (Brito-Zeron et al., 2015).

HCV RNA has been found in the serum and saliva of patients but not of healthy control (Toussirot et al., 2002), and *in situ* hybridization and immunohistochemistry

showed that HCV infects and replicates in the epithelial cells from SGs of patients with SS (Arrieta et al., 2001). These results indicate that HCV may propagate and reside in SG, and may promote HCV-associated sialadenitis.

Interestingly, HCV infection seems to be able to change the cytokines profile in SS patients, with increased circulating levels of interleukins (IL)-6, IL-10, IL-2, and tumor necrosis factor-alpha (TNF-α). This cytokines profile is accompanied by higher frequency of HCV-related cryoglobulinemia and hypocomplementemia (Ramos-Casals et al., 2002). However, the cause-effect relationship between HCV infection and SS still remains unclear.

A.3.1.2: EBV

The association of EBV and SS is in debate since early studies dating from 1980s. At first, it was reported that there are exaggerated immunological responses to virus in patients. This conclusion was challenged by later studies pointing out that false positive antibodies can be detected due to RF (Venables et al., 1985). Infection of EBV was re-confirmed by another study, which revealed two forms of EBV infection in the SGs of patients: the EBER1-positive latency in lymphocytes, and the EBER1-negative latency in epithelial cells (Wen et al., 1996). This suggests that EBV possibly participates in the pathogenesis of SS. Similarly, a 52-year-old woman with pSS developed membranous glomerulonephritis and EBV-positive diffuse large B-cell lymphoma (DLBCL), suggesting the association between EBV infection and SS (Kim et al., 2012).

Recent studies showed more clues to this dispute, as the IgG-mediated immune response against EBV early antigen (EBVEA) was positively associated with SS, and the level of anti-EBVEA IgG was found to be anti-Ro/SSA and anti La/SSB-correlated (Kivity et al., 2014). In addition, it was found that active EBV infection selectively affects ectopic lymphoid structures (ELS) in the SGs of patients with SS. Latent EBV infection and lytic EBV infection were observed exclusively in B cells and plasma cells, respectively. In addition, the persistence of EBV is found to impair CD8-mediated cytotoxicity in ELS-containing SGs (Croia et al., 2014). These studies indicate that EBV

infection may play a causative role in the pathogenesis of SS.

A.3.2: Genetic factors

The pathophysiology of SS involves dysregulation of both innate and adaptive immune pathways, in which multiple processes including cellular immunity and humoral immunity have been proved to play an important role. However, the precise mechanism is largely unknown.

A.3.2.1: Human leukocyte antigen

Human leukocyte antigen (HLA) family contains hundreds of genes that are responsible for antigen presentation in immune response. The HLA class I molecules are expressed on the surface of all kinds of cells, which present intracellular antigens, while the HLA class II molecules only exist on the surface of antigen-presenting cells (APCs) and are responsible for the presenting of extracellular antigens.

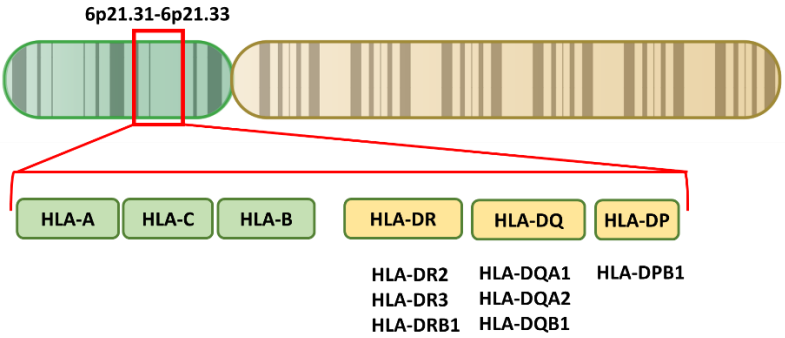

Figure 6. HLA genes associated with SS

The HLA gene complex resides on a 5×10^6 base pairs region within chromosome 6p21. HLA class I molecules are encoded by *HLA-A, B* and *C*, while HLA class II molecules are encoded by *HLA-DR, DQ* and *DP*. *HLA* genes are highly polymorphic and present many different alleles, especially *DRB1*, which contains hundreds of alleles. Some single nucleotide polymorphisms (SNPs) identified in these polymorphisms are considered to be associated with SS. These SNPs are mostly located within HLA class II regions such as *HLA-DR2, DR3, DRB1; HLA-DQA1, DQA2, DQB1* and *HLA-DPB1*.

The HLA family genes seem to have an intimate connection with SS (Figure 6). This family of genes has been emphasized in SS for more than two decades in numerous

studies. The correlation of HLA family genes with SS was first identified by Gershwin et al. in 1975 by comparing the frequency of *HLA8* in healthy control individuals and SS patients (Gershwin et al., 1975). The researchers found that among 25 patients, the frequency of *HLA8* was 58%, which is much higher than the frequency in healthy controls (21%). Thus, they proposed the existence of a linkage between HLA gene family and SS, in addition to the previously known correlation of HLA genes with other AIDs (Fritze et al., 1974; Grumet et al., 1974).

Regarding the HLA signature in SS, other loci like *HLA-Dw3*, *HLA-DR2* and *HLA-DR3* were confirmed to display significant correlations (Chused et al., 1977; Wilson et al., 1984). A large-scale genetic study published in 2013 underscored the importance of HLA genes in innate and adaptive immunity. This study reported several genes of the HLA family, including *HLA-DQA1*0501*, *HLA-DQB1*0201*, and *HLA-DRB*0301* as SS-associated genes and risk factors (Lessard et al., 2013). In addition, a very recent genome-wide association study (GWAS) analyzed a very large cohort of patients from different ethnicities (European and Asian), and found that SNPs in HLA gene family are among the strongest associations with SS. The rs9271573 in *HLA–DRB1, HLA–DQA1* regions associates very strongly ($p=3\times10^{-42}$) with SSA/Ro or SSB/La autoantibodies and focus score status in the European participants (Taylor et al., 2017).

Apart from HLA genes, other genes linked with SS were progressively identified over years. Among all these genes, the most strongly associated genes focus on the nuclear factor kappa B (NF-κB) pathway, interferon (IFN) signaling pathway and lymphocyte signaling pathway (Figure 7).

A.3.2.2: NF-κB pathway-related genes

NF-κB family comprises five members, namely p50, p52, p65 (RelA), c-Rel and RelB. All the member proteins share a highly conserved DNA-binding/dimerization domain called Rel in their N-terminus, and three of them have a transactivation domain in their C-termini. As an evolutionarily conserved signaling pathway, NF-κB has a crucial role in multiple involvements of immune activities, including immune response to

stress and infections, development and activation of lymphocytes, cell differentiation and apoptosis, and inflammatory regulation. Nordmark and colleagues identified the association between SS and polymorphisms of four signaling members from the NF-κB pathway, namely TNF-α induced protein 3 (TNFAIP3, or A20), TNFAIP3 interacting protein 1 (TNIP1, or ABIN1), NF-κB, and inhibitor of nuclear factor kappa B kinase subunit epsilon (IKBKE) (Nordmark et al., 2013). A total of 12 SNPs were genotyped in 1,105 patients and 4,460 controls. This study demonstrated that TNIP1 risk haplotype was associated with pSS and that there were no significant associations of IKBKE, NF-κB or A20 with SS. However, a more recent study involving two cohorts, the UK cohort (308 controls and 590 patients with pSS) and the French cohort (448 controls and 589 patients with pSS) confirmed the role of A20 impairment in pSS-associated lymphoma. Interestingly, the rs2230926 missense polymorphism was not associated with pSS in both cohorts, but the rs2230926G variant was significantly associated with pSS-associated lymphoma in the UK cohort (Nocturne et al., 2016).

A.3.2.3: IFN signaling-related genes

Among all the genes that have been identified to be associated with SS, the strongest one outside HLA family is *IRF5* locus. IRF5 is a member of IFN regulatory factors (IRFs), a group of transcription factors that regulate the expression of target genes like proinflammatory cytokines, including type I IFN, interleukins and TNF. IRFs participate in diverse involvements of immunity such as anti-virus immune response, cell differentiation and apoptosis (Krausgruber et al., 2011). IRF5 was reported to be associated with SS (Figure 7). A 5 base pairs (CGGGG) insertion/deletion polymorphism in the promoter region of IRF5 has been proposed to be a crucial impactor to *IRF5* mRNA levels in peripheral blood mononuclear cells (PBMCs) and SGs epithelial cells, correlating with expression levels of IFN-induced genes *MX1* and *IFITM1* (Miceli-Richard et al., 2009). Miceli-Richard et al. used a genome-wide methylation approach to assess the role of methylation deregulation in pSS, and found that DNA methylation patterns in B cells are important to pSS (Miceli-Richard et al., 2016).

A.3.2.4: Lymphocyte signaling pathway-related genes

Lymphocytes play a crucial role in immunity, especially in autoimmune response. The overactive lymphocytes largely contribute to the dysregulation of immune network and lead to the overproduction of autoantibodies, as well as proinflammatory cytokines and chemokines. As described in previous context, HLA molecules, as antigen presenting molecules in immune system, are correlated to SS (Figure 7). Another key molecular in lymphocytes trafficking, CXCR5, plays a central role in guiding cell movements during immune responses. The onset of adaptive immune response needs antigen specific lymphocytes to transfer efficiently to secondary lymphoid tissues, where they encounter and respond to antigens. The migration of lymphocytes is directed by chemokines including CXCR5. CXCR5 is expressed on different cells, regulating their migration and trafficking (Muller et al., 2003). CXCR5 has been associated with SS by a large scale genotyping in 2013 (Lessard et al., 2013). Another association was identified between disease and risk locus in natural cytotoxicity triggering receptor 3 (NCR3/NKp30). NCR3/NKp30 is a natural killer (NK)-specific activating receptor, responsible for the regulation of type II IFN secretion and the cross talk between NK and dendritic cells (DCs). One of the genetic polymorphisms of the *NCR3/NKp30*, rs11575837 (G>A), is found to be associated with reduced gene transcription and provide a protection against pSS. (Rusakiewicz et al., 2013).

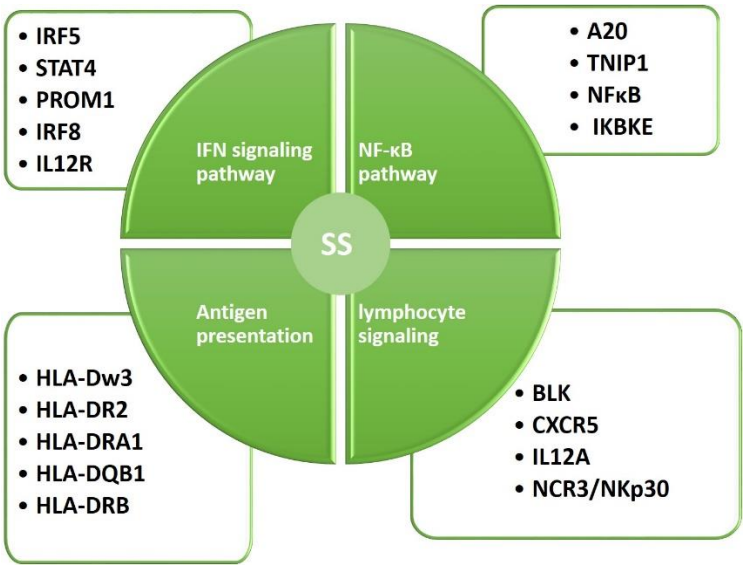

Figure 7. Pathways and genes associates with pathogenesis of SS

SS can be induced by genetic factors. Among the genes identified to be associated with SS, most of the risk loci for SS are found to be related with IFN signaling pathway, NK-κB pathway, *HLA* gene family and lymphocytic signaling-related genes. STAT4, signal transducer and activator of transcription 4; BLK, B lymphoid tyrosine kinase; CXCR5, C-X-C motif chemokine receptor 5; NCR3, natural cytotoxicity triggering receptor 3.

A.3.3: Hormonal factors

The sex bias in SS (female:male around 9:1) strongly suggests the role of sex steroids in the pathogenesis of SS. Compared with healthy controls, SS patients show significantly higher prolactin/progesterone and estrogen/progesterone ratios, suggesting a role of hormones in SS. However, Taiym and colleagues found no significant differences concerning the levels of estrogen and progesterone between patients and healthy individuals in their studies (Taiym et al., 2004). This result was confirmed by another study, which evaluated additional sex hormones including dehydroepiandrosterone (DHEA), DHEA sulfate, androstenedione, testosterone, dihydrotestosterone (DHT), estrone, estradiol, and sex hormone-binding globulin (SHBG) levels. This study was conducted on 39 patients and tried to identify the correlations between sex hormones and autoimmunity in SS. It was found that the concentrations of testosterone were associated with disease activity, but no correlation was found between disease activity and estrogens levels (Brennan et al.,

2003). These results may also suggest the involvement of hormone homeostasis, in addition to hormone levels in the pathogenesis of SS.

Thus, the role of estrogens in SS is still controversial. Aromatase-knockout mice were used as a model of estrogen deficiency. It was found that these mice developed severe spontaneous autoimmune manifestations resembling SS, such as proteinuria and severe leukocyte infiltration in the SGs and kidneys. These results reveal that estrogen might have a crucial role in the initiation of SS (Shim et al., 2004). In addition, upon the removal of main estrogen source by ovariectomy, healthy C57BL/6 mice (non-autoimmune prone) showed a significant increase of apoptotic epithelial cells and alpha-fodrin autoantigen in SGs (Ishimaru et al., 2003). On another hand, SS patients are mostly women in postmenopausal phase, in which estrogen level is much lower compared with other period of women's life. Based on these facts, one of the theories proposed by Konttinen et al. is that secretary acinar cells are primarily targeted by the imbalanced sex steroids, undergo abnormal apoptosis, followed by aberrant handing of apoptotic debris, thus leading to the initiation of SS (Konttinen et al., 2012). This theory highlighted the role of acinar cells and assumed that apoptosis of glandular cells initiates autoimmune responses. Further studies are however necessary to confirm this theory.

Nowadays, the roles of hormones in autoimmune reactions are not well understood. Other pathways beside apoptosis are suggested to be involved in hormones-triggered exocrine gland dysfunction (Mavragani et al., 2012). Hormones might play significant roles in systemic homeostasis, leading to aberrant immune response.

A.4: Pathology

A.4.1: Cytokines

Cytokines act as messengers of immune system, deliver and transduct signals in the immune network. Cytokines orchestrate the full panoply of immune responses and are involved in various immunological effects, such as immune cell recruitment and immune responses activation. Cytokines are functionally divided into two groups,

namely the proinflammatory cytokines that promote inflammation and anti-inflammatory cytokines that decrease inflammation.

A.4.1.1: Th1 and Th2 balance

A major source of cytokines is CD4 T cells, which express a cell surface marker CD4. CD4 T cells are also named helper T cells (Th) according to their capability to help B cells produce antibodies, to active macrophages, and to recruit granulocytes, including neutrophils, eosinophils, and basophils, to infection sites. Notably, these functions of Th cells depend on their production of cytokines and chemokines. Th cells can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1 cytokines and Th2 cytokines (Berger, 2000).

Th1 cytokines tend to be proinflammatory. They facilitate the killing of intracellular invades, and perpetuate chronic inflammation in autoimmune conditions. IFN-γ is a major Th1 cytokine that can promote NK cell activity, induce antibody production of plasma B cells, and increase antigen presentation. Another Th1 cytokine, IL-2, plays a crucial role in T cell differentiation and is involved in immune tolerance generation during the process of T cells maturation in thymus. Other Th1 cytokines include IL-6, TNF-α and lymphotoxin are involved in many inflammation-mediated diseases.

Excessive inflammation can lead to a breakdown of immune tolerance and even tissue damage, thus a counteract mechanism is provided by anti-inflammatory cytokines. The Th2 cytokines include IL-4, IL-5, IL-9 and IL-13, these molecules tend to be anti-inflammatory. For example, IL-4 acts as a regulator of macrophages differentiation, promotes polarization of macrophages into immune suppressive M2 state, and inhibits activation of cytotoxic M1 macrophages (Wang et al., 2014). Further secretion of anti-inflammatory cytokines IL-10 and TGF-β by M2 macrophages helps to attenuate pathological inflammation. Thus, the balance of Th1 and Th2 cytokines is crucial for immunological homeostasis.

The role of Th cells in SS has been recognized since early times when the predominant lymphocytic infiltration in glandular tissues of SS patients was found to be contributed by CD4 T cells (Fox et al., 1983). In recent years, the imbalance of Th

subsets has been suggested to play a role in SS (Moriyama et al., 2014). The percentage of Th1 cells is significantly higher in the PBMC of SS patients compared with healthy people. In coincidence, Th2 cells are found to be less in patients than in control group (Sudzius et al., 2013). This result was further confirmed by another study that included 66 patients with SS. Compared with normal group, the levels of IFN-γ and IL-4 were significantly increased in peripheral blood of patients (Wu et al., 2013). In saliva from SS patients, increased levels of IL-1β, IL-6, TNF-α, and IFN-γ, in association with higher ratio of Th1/Th2 CD4 T cells have been observed, and these enhanced cytokine levels have been found to be related with clinical features of pSS (Kang et al., 2011). These findings imply that a Th1-mediated inflammation may lead to pathology in SGs. Similar findings have been reported in labial SGs (LSG) from SS patients. The mRNAs of both Th1 and Th2 cytokines were found in LSG. However, the balance between Th1/Th2 was broken and shifted to a Th1 profile in patients with high infiltration scores (Mitsias et al., 2002).

A.4.1.2: IL-17, IL-21 and other cytokines

IL-17 protein expression was found to progressively increase with higher biopsy focus scores in MSGs. Th17 differentiation related cytokines such as TGF-β, IL-6 and IL-23, are highly expressed in MSGs of SS patients (Katsifis et al., 2009). In addition, IL-17A mRNA levels in PBMCs from pSS patients are increased (Fei et al., 2014), suggesting that IL-17 may play a role in the process of lymphocytic infiltration in the LSG. The roles of IL-17 and Th17 were further defined in C57BL/6 mice that are non-susceptible to SS. C57BL/6 mice develop a SS-like disease profile after receiving an adenovirus serotype 5-IL17A vector in SGs, showing the appearance of lymphocytic foci, increased cytokine levels and autoantibodies, as well as loss of saliva flow (Nguyen et al., 2010). In good agreement, in another mouse model of SS, which is generated by immunization of homogenized proteins of SGs (SGP) in C57BL/6 mice (Lin et al., 2011), the number of Th17 cells was found to be increased in SGs. In addition, knockout of IL-17A in C57BL/6 led to complete resistance for SS induction by SGP, and adoptive transfer of Th17 cells rapidly restored the onset of SS (Lin et al., 2015). These studies in different mouse models demonstrate that Th17 might be an

important player in SS pathology.

IL-21 is associated with B cell functions like Ig production (Ozaki et al., 2002) and Th17 cells differentiation (Wei et al., 2007). IL-21 thus seems to be involved in the pathogenesis of AIDs in multiple ways. pSS patients are found to have significantly higher serum IL-21 levels compared to control individuals, and the high-level of IL-21 is apparently correlated with autoantibody levels. In addition, lymphocytic foci and the periductal area of the LSG from SS patients showed higher ratio of IL-21/IL-21R than healthy controls. Furthermore, the extent of lymphocytic infiltration was found to be associated with IL-21 expression in LSG (Kang et al., 2011).

In addition to IL-17 and IL-21, the expression of IFN-γ-induced protein 10 (IP-10) and its receptor, CXCR3 was increased in SGs from SS patients, suggesting IP-10 and CXCR3 may contribute to the pathogenesis of SS (Ruffilli, 2014). Apart from cytokines described above, other cytokines such as CXCR2, C-X-C motif chemokine ligand (CXCL) 13 and C-C motif chemokine ligand (CCL) 11 are also found to be linked with disease activity in SS patients (Lisi et al., 2013; Nocturne et al., 2015), thus providing more clues for therapeutic targeting.

A.4.2: B cells

Hyperactivity of B cells is found in SS, and this could be the consequence of the coordinated and integrated effects of B cell activation and cytokines stimulation. It has been found that overexpressed type I IFN and B cell active factor (BAFF) are involved in the enhanced plasma cell formation in pSS patients (Brkic et al., 2013). Then, this enhancement of plasma cells results in secretion of autoantibodies and further promotes autoimmune responses. This has been observed in patients with SS, in which high levels of autoantibodies are linked with the increased frequency of autoreactive B cells and plasma cells (Kroese et al., 2014). Anti-Ro and anti-La autoantibodies-producing plasma cells have been found to reside at sites of infiltration in SGs biopsy tissues from patients with SS (Tengner et al., 1998). These plasma cells showed phenotypic characteristics of the long-lived plasma cell subtype (Szyszko et al., 2011).

A study aimed at characterizing the SSA-specific B cell pattern in SG biopsies showed that SSA-specific memory B cells are lacking in peripheral blood and SG. The authors supposed that this could result from activation of these cells into plasma cells at the site of inflammation (Aqrawi et al., 2012; Aqrawi et al., 2013), suggesting that the B cells differentiation process in local infiltration is imbalanced.

In recent years, lymphoid neogenesis and functional ectopic germinal center (GC) formation have been found in SGs of patients with SS. A microenvironment is required to form GC-like structures in SG, including T and B cell aggregates, follicular DCs (FDCs) and activated endothelial cells, and this microenvironment is found in patients with GC-like structures (Johnsen et al., 2014). SGs microenvironment in SS provides factors vital for plasma cell survival and facilitates the development of long-lived plasma cell subtype (Szyszko et al., 2011). These plasma cells in SG increase the production of anti-Ro/SSA and anti-La/SSB autoantibodies and drive autoimmune responses (Salomonsson et al., 2003). These findings and insights in the role of B cells in the pathogenesis of pSS offer promising targets for successful therapeutic intervention.

A.4.3: T cells

T cells are among the most important cells in the pathogenesis of SS, as they are the predominant infiltrating cells found in glandular tissues. This was noted early in 1983 by Fox and colleagues who found that the phenotype of SGs lymphocytes differs from that of peripheral blood lymphocytes in patients with pSS. They also noticed that CD4 T cells are the majority of infiltrating cells in SGs of patients (Fox et al., 1983). Parallel characterization in mouse models has been confirmed, such as in Murphy Roths Large/lymphoproliferation (MRL/lpr) mice, New Zealand Black/White F1 Hybrid (NZB/W F1) mice and Alymphoplasia (Aly/aly) mice (Soyfoo et al., 2007). The infiltrated T cells present variable activity at different disease stages, tend to be resistant to apoptosis, and lead to interstitial nephritis and peripheral CD4 lymphocytopenia (Manganelli et al., 2003). Moreover, environmental triggers of SS, such as viral infections, are considered to be mediated by CD4 T cells. As introduced

in previous chapters (A.2.1), HTLV-1 can be an inducer of SS. HTLV-1 is a retrovirus that predominately infects CD4 T cells. In SS, HTLV-1 can be transfected from T cells to SGs epithelial cell (SGEC) and induces specific pathological symptoms (Nakamura et al., 2016).

In addition to CD4 T cells, the role of CD8 T cells in the pathogenesis of SS is underlined by its mediation of glandular destruction. The infiltrated CD8 T cells are resistant to apoptosis by a B-cell CLL/lymphoma 2 (BCL2) mediated mechanism, thus present an overactive cytotoxic activity, and induce TNF receptor superfamily, member 6 (FAS)-mediated apoptosis in acinar epithelial cells, leading to glandular injury (Kong et al., 1997).

In recent findings, the impairment of regulatory T cells (Treg) is linked with SS. It has been found that the frequency of forkhead box protein P3 (Foxp3) expressed Treg in MSGs of SS patients is correlated with glandular inflammation grade and lymphoma risk factors such as complement component 4 (C4) hypocomplementemia and MSGs enlargement (Christodoulou et al., 2008; Sarigul et al., 2010). These findings suggest that Tregs play an important role in the pathogenesis of pSS. However, further studies are needed to explore the mechanisms that mediate the relationship between Tregs and SS.

A.4.4: Central role of epithelial cells

AIDs are recognized as the deregulation of systemic immune network, in which immune cells are found to lose self-tolerance and show features of autoreactivity. As described above, other players like inflammatory cytokines, chemokines and regulators are involved in the catastrophe of immune homeostasis. Studies of SS pathology have lasted for over one hundred years since the first case had been reported in 1888. Studies revealed large amount of evidence related to immune cells deregulation in SS. B cells were found to be overactive and produce autoantibodies, which is a key feature of SS. However, except for the formation of immune complex, which might lead to overload burden for immune clearance, the autoantibodies seem not to play direct pathogenetic role in SS. In contrast, the inducers of

overactive B cells are notable. It has been well recognized that several B-cell-activating cytokines promote and mediate the activation of B cells. These cytokines include BAFF, B cell growth factor IL-14, B cell promoting cytokines IL-6 and IL-10. All these cytokines are demonstrated to be produced by epithelial cells in glandular tissues (Kawanami et al., 2012; Martel et al., 2014). Crucially, autoantigens, the targets of autoantibodies, are largely produce by epithelial cells in SS (Katsiougiannis et al., 2015). Therefore, epithelial cells are thought to play a pivotal pathogenetic role in SS, highlighted by occurrence of infiltrating pathology in various epithelial tissues and the increased inflammatory cytokines produced by epithelial cells in patients (Figure 8).

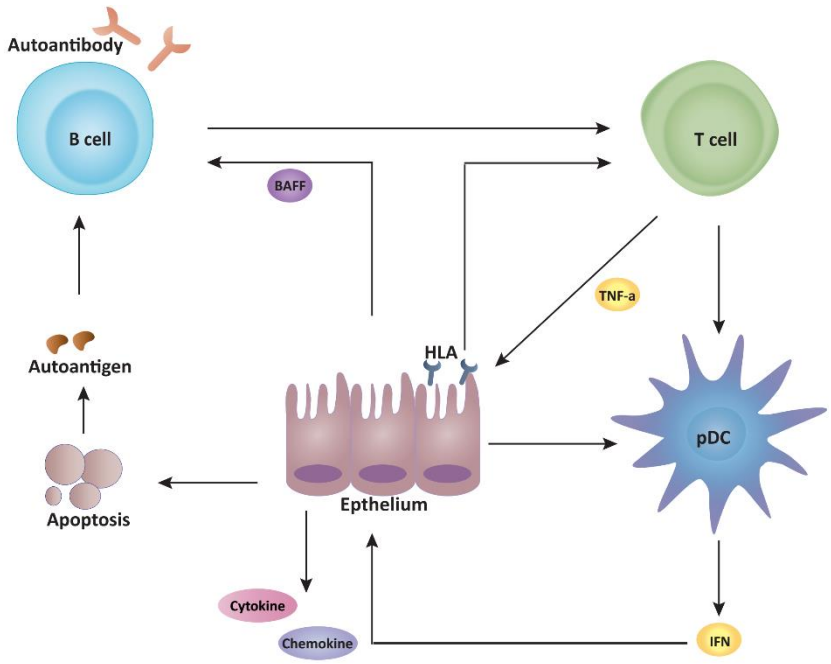

Figure 8. Essential roles of epithelial cells in SS

SGs epithelial cells play central roles in SS pathogenesis. Immune-competent molecules, such as TLRs and cytokines are constitutively expressed by epthelial cells of the salivary or lacrimal glands. Activation of TLR signalling in gland epithelium facilitates production of cytokines and chemokines, including those can active B cells and T cells. Epthelial cells are recognized as targets of activated immune cells. Apoptosis and hypofunctions are then induced in epithelial cells, leading to the production of autoantigens. Autoantigens can be released from SGECs and presented to immune cells. HLA class II molecules expressed by epithelial cells enhance the autoantigen presentation and activation of T cells.

A.4.4.1: Autoantigen Production

The epithelial cells of the SGs differentiate into acinar cells, duct cells, and myoepithelial cells, and these cells form the secretory unites (Figure 9).

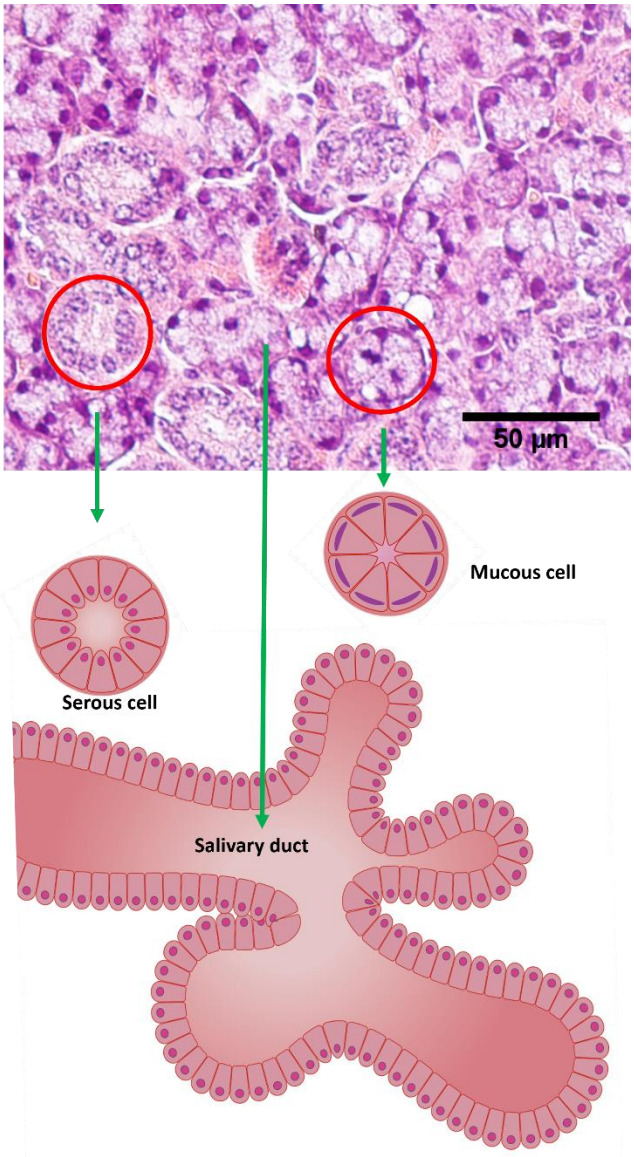

Figure 9. Structure of SGs and secretory units

Basically, two types of acinar epithelial cells exist in the secretory end of SGs: the serous cells and the mucous cells. The serous cells secrete a watery fluid, which is essential for saliva. A serous cell is pyramidal in shape, and its nucleus is round and close to the base of the cell. The mucous cells produce mucins, enzymes and other proteins, and their productions are slightly viscous. Mucous cells are pyramidal in shape (similar with serous cells), and the nucleus of mucous cells are usually very flat and lie against the basal end of the cell. Histology of SGs is represented by H&E staining of SG from a 29-week-old C57BL/6 mouse.

As suffering from systemic autoimmune disease, patients with SS present large amounts of various autoantigens, which trigger autoimmune responses and induce the hyperreactivity of immune system, leading to the final exhaustion of host homeostasis. In fact, our normal constituents become “autoantigen” in autoimmune patients whose immune system differentiate self from non-self less well. The most known autoantigens of SS are the SSA/Ro (includes Ro52/TRIM21 and Ro60/TROVE2), SSB/La, nuclear antigens, and RFs. Glandular epithelial cells are demonstrated to be a factory for autoantigens production. Twenty-six non-neoplastic SGEC lines established from patients with rheumatic disorders were found to release significant amounts of exosomes. These exosomes were found to contain autoantigenic Ro/SSA, La/SSB, and Sm RNPs, as well as epithelial-specific autoantigen cytokeratins (Kapsogeorgou et al., 2011).

The traditional biomarkers, SSA and SSB are widely used in diagnosis, but this specificity is doubtful since they are not always positive, especially in the initiation stage of SS and can be found in other related diseases. In recent studies, new autoantigens for SS have been identified, especially proteins specific to the salivary and lacrimal glands, like SGs protein-1 (SP-1), parotid secretory protein (PSP), carbonic anhydrase VI (CA6) (Suresh et al., 2015) and cleaved alpha-fodrin (Wang et al., 2006). On one hand, as these novel biomarkers may be detected in early stages of disease, they can be recognized as a complement to traditional biomarkers; on another hand, increased level of autoantigens identified in glandular tissues revealed the autoantigenic property of epithelial cells.

FAS and FAS ligand (FASL) were emphasized in SS, and it has been illustrated that increased apoptosis in epithelial cells leads to autoantigen production, as well as collapse clearance of apoptotic debris. It was reported that FAS and FASL levels in non-neoplastic SGEC line established from SS patients were significantly higher than in SGEC line derived from healthy individuals (Abu-Helu et al., 2001; Ping et al., 2005). In SS conditions, the increased apoptosis of epithelial cells may result from various pathways. Firstly, the imbalance between the apoptosis-inhibitor BCL2 and apoptosis-inducer BCL2 associated X protein (Bax) can lead to abnormal apoptosis.

Secondly, the interaction of FASL (expressed by T lymphocytes) with FAS (expressed by epithelial cells) results in the apoptosis of epithelial cells. (Manganelli et al., 2003). The consequences of apoptosis in epithelial cells could be lesion of tissues and impaired secretory functions, which cause dryness. Moreover, apoptosis acts as a perpetuation of autoantigen production, which in turn enhances autoantibody and autoimmune responses, leading to SS like inflammatory lesions (Okuma et al., 2013).

A.4.4.2: Non-professional antigen-presentation

Ductal and acinal glandular epithelial cells were demonstrated to express HLA-DR antigens inappropriately, whereas rare HLA-DR molecules could be found in normal epithelial cells (Moutsopoulos et al., 1986). The expression of HLA-DR in epithelial cells seems to depend on the local production of IFN-γ by immune cells (Fox et al., 1986a). Consequently, SGs epithelial cells were suggested to act as non-professional APCs (Nagai et al., 2015), as evidenced by the expression of HLA-I antigens and HLA-II antigens after the stimulations of TNF-α (Tsunawaki et al., 2002).

Not only HLA molecules, but also costimulatory molecules like B7 and adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM), and E-selectin, are found to be expressed by SGs epithelial cells, and facilitate the non-professional antigen presenting activity (Manoussakis et al., 1999; Tsunawaki et al., 2002).

A.4.4.3: Stimulation of T cells and B cells

T cells activation involves two signals: the antigen specific signal and the co-stimulatory signal. The antigen-specific signal is mediated by the interaction of TCR and peptide-MHC molecules on the membrane of APCs. The co-stimulatory signal is provided by the interaction between T cell membrane bond receptors and co-stimulatory molecules expressed by APCs. This process is nonspecific to antigens and crucial for T cell homeostasis. It is required for T cell proliferation, differentiation and survival.

As showed in Figure 10, the most studied and first identified co-stimulatory signals

are CD80 (B7-1) and CD86 (B7-2) expressed on the membrane of APCs which interact with CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) displayed on the membrane of T cells. Activation of T cells without co-stimulation may lead to T cell anergy and even cell death, and also the development of improper immune tolerance.

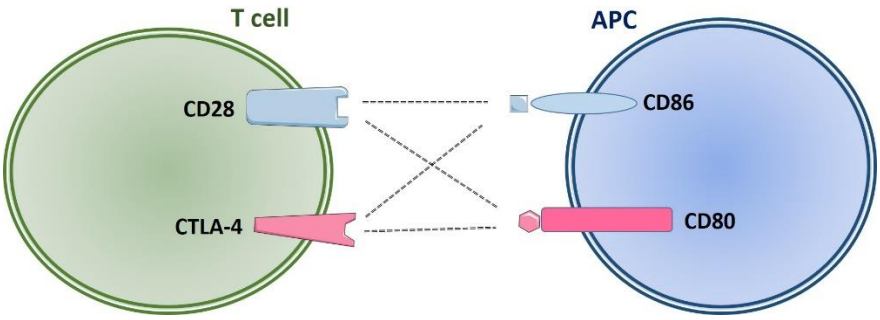

Figure 10. Co-stimulatory signals between T cell and APC

CD28 co-stimulatory receptors can be ligated by CD80 or CD86. By interaction with CD80/CD86, CD28 offers co-stimulatory signals that are required for naive T cell activation. The CTLA-4 co-inhibitor competes with CD28 for binding to CD80 and CD86. The interaction between CD80/CD86 and CTLA-4 results in inhibition of T cell activation.

The mRNA levels of B7 costimulatory molecules CD80 and CD86 in glandular epthelial cells were found to be increased in patients with SS, indicating the active status of epithelial cells and their potential to active T cells (Manoussakis et al., 1999). CD80 and CD86 molecules were found to be efficiently expressed by salivary ductal cells in SS patients after the stimulation of IFN-γ, suggesting that these cells could have antigen presentation to T cells and cause a cascade of immune response *in situ* (Matsumura et al., 2001). In addition, IFN-γ stimulation increased the production of IP-10 (CXCR10) and Mig (CXCR9) in SS patient ductal epithelium, and these chemokines might act as recruiters of infiltrated T cells in SGs (Ogawa et al., 2002).

As has been described in the previous chapter (A.4.2), overexpressed BAFF is associated with the increased plasma cells and autoantibodies in SS patients. Recently, SGEC was found to be a source of BAFF. Enhanced level of BAFF was found

in both infiltrated lymphocytes and ductal epithelial cells in SS patients, which indicate epithelial cells could act as activator of self-antigen-driven autoimmune B cells (Lavie et al., 2004). The correlation of clinical disease activity with BAFF mRNA expression levels suggests the role of BAFF in the development of SS (Brkic et al., 2013).

A.4.4.4: Cytokines

Cytokines are messengers which deliver signals in immune system and play an important role in cell signaling. Cytokines mediate and regulate maturation and differentiation of immune cells, and modulate gene expression and signals transduction (Siebert et al., 2015). In addition, cytokines connect innate immunity to adaptive immunity. Various cytokines are involved in the initiation, perpetuation, and deterioration of SS (Mavragani et al., 2014).

Type I IFN

Patients with SS have a deregulated type I IFN system. Although the circulating IFN-α level in patient is lower than in healthy controls, enhanced IFN-α inducing capacity of sera from patients has been demonstrated. Moreover, increased IFN-α-secreting cells were found in SGs (Nordmark et al., 2012), indicating the role of IFN-α in local pathogenesis.

The up-regulation of type I IFN-induced genes was found in PBMCs and MSGs from pSS patients (Figure 11). As professional IFN-α producing cells, plasmacytoid DCs (pDCs) show a reduced frequency in the peripheral blood, which might be an explanation for decreased IFN-α levels in sera. Another mechanism elucidated in pDC suggests RNA-containing immune complexes as an inducer of IFN-α. RNA interacts with Fc gamma receptors (FcγR) IIa, therefore activating pDCs and prolonging IFN-α production in SGs (Bave et al., 2005).

Genetic polymorphisms of *IRF5* and *STAT4* are associated with increased risks for pSS, and these genes are both members of type I IFN system, further supporting the pivotal role of type I IFN in SS (Fang et al., 2015).

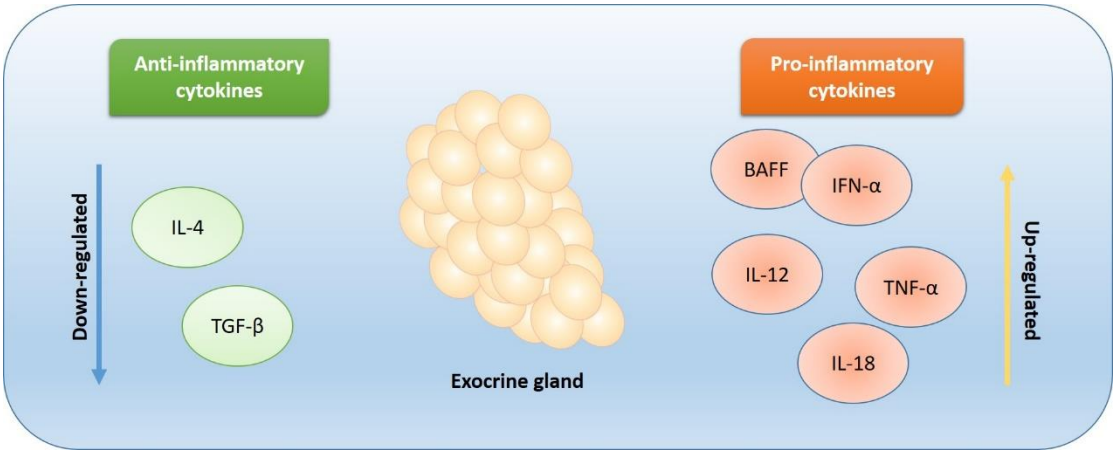

Figure 11. Cytokines deregulation in exocrine glands in SS

In exocrine glands of patients with SS, chronic inflammations are found to cause tissues damage, leading to the loss of secretory functions in acinar cells. Cytokine levels are imbalanced in exocrine glands of SS: anti-inflammatory cytokine levels are decreased, while pro-inflammatory cytokine levels are increased.

IL-7

Increased IL-7 levels were found in both the saliva and labial SGs of SS patients as compared with healthy controls (Bikker et al., 2010; Kabeerdoss et al., 2016). The enhanced IL-7 levels were correlated with disease activity, and IL-7 seemed to play a central role in immunopathology of SS as it expanded the immunological signals by stimulating other cytokines and activating multichannel immune responses (Bikker et al., 2012). It was found that IL-7 drive T cells activation and differentiation by stimulating related cytokines (monokine, IL-12, and IL-15). IL-7 up-regulates the production of inflammatory cytokines that include IFNγ (Th1), IL-4 (Th2), IL-17A (Th17), proinflammatory (tumor necrosis factor alpha and IL-1α), and regulatory (IL-10 and IL-13) cytokines by PBMCs from patients with SS (Bikker et al., 2010). By using the B6.NOD-Aec mouse model to study SS, Jin and colleagues found that IL-7 regulates IFNγ-producing Th1 and CD8 T cells in the SGs and enhances the expression of CXCR3 ligands that mediates lymphocyte infiltration of target organs (Jin et al., 2013).

Other studies provided evidence that IL-7 can also contribute to regulate regulatory

cytokines and immune cells (Bikker et al., 2012).

BAFF

BAFF is a member of the TNF family that can be produced by many cell types, including innate immune cells like activated T lymphocytes, neutrophils, APCs (B cells, macrophages and DCs). BAFF can also be expressed by epithelial cells (Woodland et al., 2008). It has been shown that BAFF is overexpressed in SS patients as well as in mouse models, and a correlation between BAFF levels and the titers of autoantibodies has been identified (Mariette et al., 2003; Pers et al., 2005).

Upon stimulation by type I IFN or viral infection, SGEC of SS patients up-regulates BAFF to active B cells and increase the survival of B cells by reducing B cell apoptosis (Ittah et al., 2006). Meanwhile, BAFF stimulates several pathways associated with cell growth and survival, through the protein kinase mTOR (Woodland et al., 2008).

Thus, BAFF is considered as a crucial molecule in the immunopathology of SS. Clinical trials attempted to develop inhibitors targeting BAFF. A BAFF-specific monoclonal antibody called Belimumab was tested on SS patients and showed both efficacy and safety in an open-label phase II study (Mariette et al., 2015).

A.4.4.5: Toll-like receptors

Toll-like receptors (TLRs) are a group of receptors, which recognize antigens and stress signals. Upon activation, TLRs recrute adapters and transduct immune signals to downstream signaling pathways (Hoffmann, 2003). TLRs are expressd by both immune cells (DCs, macrophages, NK cells, T cells and B cells) and non immune cells (epithelial and endothelial cells, and fibroblasts). TLRs are considered to have significant roles in immune network (Imler et al., 2001). By comparing non-neoplastic SGEC cell lines established from SS patients and healthy individuals, higher mRNA levels of TLR1, TLR2 and TLR4 were observed in SGEC from SS patients (Spachidou et al., 2007).

Moreover, another member of TLRs family that deserves attention is TLR3. TLR3 is highly expressed by SGECs, and localized on the cell-surface by contrast with the

cytoplasmic localization in resting DCs, suggesting an activation of TLR3 pathway in SGECs (Kyriakidis et al., 2014). TLR3 ligation by Poly I:C (a synthetic analogue of viral dsRNA) induces anoikis (detachment-induced apoptosis) in SGEC, and this is associated with the upregulation of pro-apoptotic molecules such as Bax, together with the down-regulation of the pro-survival BCL2 molecule. It was found that SGEC from patients with SS are particularly susceptible to TLR-3-induced anoikis compared to healthy individuals (Manoussakis et al., 2010). In addition, TLR3 regulates autoantigens localization. It was found that Poly I:C stimulation up-regulates Ro52/TRIM21 mRNA levels in SGs epithelial cells, accompanied by protein redistribution from nucleolar-like pattern to multiple coarse dots spanning throughout the nucleus (Kyriakidis et al., 2014).

A.5: Therapeutic strategy

As described in previous chapters (A.2), SS is classified into pSS and sSS. However, this mode of classification contributes little in clinical practice because there is no difference in the management of pSS and sSS patients.

Until now, there is no cure for SS. Clinically used treatments could relieve symptoms like dry eyes and dry mouth, which are the hallmarks of SS.

A.5.1: Dryness

In human, saliva is mainly secreted by major SGs that includes parotid, submandibular, and sublingual glands. Around 70% of saliva is produced by submandibular glands, and the rest of saliva is mainly secreted by parotid glands. Apart from major SGs, there are approximately 1,000 MSGs located in the oral cavity and surrounded by connective tissues. They cover the oral cavity with saliva and make minor contribution to mucous production. In SS, dysfunction of SGs caused by autoimmune response leads to dryness in mouth, which is named “xerostomia” in clinics. The most used approach to relieve dry mouth is to promote non-pharmacological treatments like drinking water and using salivary substitutes or stimulators (such as citrus juice and sugar-free gum). Avoidance of diuretics,

antidepressants, beta blockers, anxiolytics or anti-histamine agents should be taken into consideration as they are able to exacerbate dryness symptoms. Daily life habits like alcohol drinking and smoking should be avoided.

A.5.2: Organs regeneration

However, these measures are limited to palliative dryness symptoms, without recovering the secretory functions of SGs. Recently, some novel strategies aimed to reconstitute functional organs highlighted SGs regeneration. Zhang et al. reported an investigation that uses human amniotic epithelial cells (hAECs) transplantation to restore functional SGs (Zhang et al., 2013). Ogawa and colleagues developed a three-dimensional cell manipulation method, the "organ germ method" that can reproduce organogenesis through the epithelial-mesenchymal interaction (Nakao et al., 2007). It was found that the bioengineered SGs germ can regenerate a functionally complete and structurally correct SGs totally *in vitro*. More importantly, the bioengineered SGs can not only secrete saliva into the oral cavity successfully, but also reestablish the responsivity to afferent and efferent nervous stimulation. In addition, the bioengineered SGs can improve symptoms of xerostomia, including bacterial infection and swallowing dysfunction (Ogawa et al., 2015).

A.5.3: Chronic inflammation

Apart from dryness in various organs, chronic inflammation is another pathological character underscored in SS. Chronic inflammation is induced by loss of immune homeostasis. It in turn facilitates the autoimmune responses and leads to a cascade of autoimmunity. Chronic inflammation has been found in kidneys and lungs of SS patients, and leads to connective tissue arthropathy, liver fibrosis, joint arthritis, lymph node enlargement and vasculitis. Thus, another therapeutic strategy focuses on remission of the systemic chronic inflammation. Anti-inflammatory therapies by immunosuppressant like corticosteroids are used in severe systemic involvement (Brito-Zeron et al., 2013), aiming to decrease the chronic inflammation caused symptoms.

New approaches regarding diagnosis and therapeutics of SS are in development

(Table 1). These novel approaches involve biological tools such as monoclonal antibodies. A monoclonal antibody targeting B cell surface marker CD20, Rituximab, was used in recent investigations and presented as a promising therapy for SS (van Vollenhoven et al., 2013). Rituximab binds directly to the membrane-bond CD20 in B cells and causes a depletion of CD20-positive B cells. The mechanisms by which Rituximab deletes B cells are not totally understood but antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and apoptosis are considered to be involved (Abulayha et al., 2014). The deletion of B cells by Rituximab is transient, as it would not target to pre/pro-B cells and plasma cells since these cells do not express CD20, therefore providing a mechanism for the regeneration of B cells and reconstitution of B cell immune responses (Faurschou et al., 2014). The influence of Rituximab treatment to SGs echostructure (size, parenchymal echogenicity and inhomogeneity reflected by ultrasonography) and vascularization was evaluated in patients with pSS, and it was found that SGs echostructure in patients was improved after receiving Rituximab (Jousse-Joulin et al., 2015).

Title	Patients	Interventions	Type	Study phase	NCT number
PD of VAY736 in Patients With pSS	pSS	VAY736	Drug	Phase 2	NCT02149420
Safety, Pharmacokinetics and Preliminary Efficacy Study of CFZ533 in Patients With pSS	pSS	CFZ533 active - Cohort 1	Drug	Phase 2	NCT02291029
Evaluation of the Efficacy and the Mechanism of Chinese Herbal Formula SS-1 for SS	SS	SS-1	Drug	Phase 2	NCT02110446
Acupuncture for pSS	pSS	Acupuncture group	Device	Phase 2	NCT02691377
Efficacy and Safety of Abatacept in Patients With pSS	pSS	Abatacept SC	Drug	Phase 3	NCT02067910
Modulation of Immunity-related Gene Expression Under the Chinese Herbal Formula SS-1 Treatment for SS	SS	Chinese Herbal Formula SS-1	Drug	Phase 2	NCT02855658
Safety and Efficacy Study of Subcutaneous Belimumab and Intravenous Rituximab Co-administration in Subjects With pSS	pSS	Belimumab, Rituximab	Drug	Phase 2	NCT02631538
A Study to Assess the Efficacy of RO5459072 in Participants With pSS	pSS	RO5459072	Drug	Phase 2	NCT02701985
Safety and Efficacy Study of Filgotinib, GS-9876 and Tirabrutinib in Adults With Active SS	SS	Filgotinib, GS-9876, Tirabrutinib	Drug	Phase 2	NCT03100942
Safety and Efficacy of VAY736 in Patients With pSS	pSS	VAY736	Biological	Phase 2	NCT02962895
A Phase 2a, Randomized, Placebo Controlled, Study to Evaluate the Safety and Efficacy of AMG 557/MEDI5872 in pSS	pSS	AMG 557/MEDI5872	Biological	Phase 2	NCT02334306
UCB Proof of Concept Study in Patients With pSS	pSS	UCB5857	Drug	Phase 2	NCT02610543
A Long-term Study of KCT-0809 in Dry Eye Patients With SS	Dry Eye of SS	KCT-0809 ophthalmic solution	Drug	Phase 3	NCT02503163
Efficacy and Safety of Mycophenolate Mofetil in subjects with SS	SS	Mycophenolate mofetil	Drug	Phase 2	NCT02691949
Laser Therapy to Treat the Dry Mouth of SS	Dry Mouth of SS	Lasertherapy	Device	Phase 3	NCT02066896
Study of Iguratimod in SS	SS	Iguratimod	Drug	Phase 1/2	NCT03023592
A Study to Assess the Efficacy and Safety of Abatacept in Adults With Active Primary Sjögrens Syndrome	pSS	Abatacept	Biological	Phase 3	NCT02915159
Lacripep™ in Subjects With Dry Eye Associated With pSS	Dry Eye of SS	Lacripep	Drug	Phase 1/2	NCT03226444
Treating Dry Eyes and Corneal Ulcers With Fingerprick Autologous Blood	Dry Eye of SS	Your own (autologous) finger prick of blood produced with a diabetic lancet	Other	Phase 3	NCT02153515
DNase Treatment for Dry Eyes	Dry Eye	DNase	Drug	Phase 1/2	NCT02193490

Table1. Ongoing clinical trials of SS (data from ClinicalTrials.gov, accessed in July 2017)

B. Autophagy

Autophagy is a conserved degradation pathway in eukaryotic cells, and an important mechanism for cellular homeostasis. Autophagy removes and recycles harmful or unneeded materials including bulk cytoplasm, protein aggregates, damaged organelles [mitochondria, peroxisomes, ribosomes, lipid droplets (LP) etc.] and intracellular pathogens. A vital function of autophagy is to overcome cellular stress-like starvation or nutrient deprivation. However, other functions of autophagy have been highlighted in recent years. In Parkinson's disease and Huntington's disease, autophagy was found to degrade aggregated pathological proteins, α-synuclein and mutant huntingtin respectively, and alleviate neurodegenerative symptoms (Nixon, 2013). In infectious diseases, autophagy clears pathogens (*Mycobacterium tuberculosis*, *Salmonella typhimurium*) and protects the host from pathogens invasion (Deretic et al., 2013). In addition, autophagy shows a regulatory function of inflammation and contributes to immune responses.

Around forty autophagy related (ATG) proteins have been identified so far in yeast (or mammalian). These proteins participate in the dynamic process of autophagy and maintain the machinery of autophagic pathway. Some of the core ATG proteins are listed in Table 2.

Protein in Yeast	Function	Protein in Mammalian
Atg1	A serine/threonine protein kinase	ULK1
Atg2	A protein that acts along with Atg18 to mediate the retrograde movement of Atg9	ATG2A, ATG2B
Atg3	A ubiquitin conjugating enzyme (E2) analog	ATG3
Atg4	A cysteine protease	ATG4A, ATG4B, ATG4C, ATG4D
Atg5	A protein acts as an E3 ligase	ATG5
Atg6	A component of the PI3KC3 complex	BECN1
Atg7	A ubiquitin conjugating enzyme (E1) analog	ATG7
Atg8	A ubiquitin-like protein that is conjugated to PE	LC3A, LC3B, LC3C, GABARAP, GATE16
Atg9	A transmembrane protein that act as a lipid carrier for expansion of the phagophore	ATG9
Atg10	A ubiquitin conjugating (E2) enzyme analog that conjugates Atg12 to Atg5.27	ATG10
Atg11	A specific cargo recognition	FIP200
Atg12	A ubiquitin-like protein that modifies an internal lysine of Atg5	ATG12
Atg13	A component of the ULK1 complex that is needed for ULK1 kinase activity	ATG13
Atg14	A component of the PI3KC3 complex	ATG14L
Atg15	A vacuolar protein that contains a lipase/esterase active site motif	—
Atg16	A component of the Atg12–Atg5-Atg16 complex	ATG16L1, ATG16L2
Atg17	A yeast protein that is part of the ULK1 complex	FIP200
Atg18	A protein that binds to PI3P	WIPI1, WIPI2, WIPI3, WIPI4
Atg19	A receptor for the Cvt pathway	—
Atg20	A PI3P binding protein that is part of the ULK1complex	—
Atg21	A PI3P binding protein that is a homologue of Atg18	ATG21
Atg22	A vacuolar amino acid permease that is required for efflux after autophagic breakdown of proteins	—
Atg23	A yeast protein that transits with Atg9	—
Atg24	A PI3P binding protein that is part of the Atg1 kinase complex	ATG24A, ATG24B
Atg25	A coiled-coil protein required for macropexophagy	—
Atg26	A sterol glucosyltransferase that is required for micro- and macropexophagy	—
Atg27	An integral membrane protein that is required for the movement of Atg9 to the PAS	—
Atg28	A coiled-coil protein involved in micro- and macropexophagy	—
Atg29	A protein required for efficient nonspecific macroautophagy in fungi	—
Atg30	A protein required for the recognition of peroxisomes during micro- and macropexophagy	—
Atg31	A protein required for nonspecific macroautophagy in fungi	—
Atg32	A mitochondrial outer membrane protein that is required for mitophagy in fungi	—
Atg33	A mitochondrial outer membrane protein that is required for mitophagy in fungi	—
Atg101	An Atg13-binding protein required forULK1 complex	ATG101

Table 2. ATG proteins in yeast and mammalian

B.1: Autophagy pathways

There are at least three major forms of autophagy pathways which have been defined, namely macroautophagy (MaA), microautophagy and chaperone-mediated autophagy (CMA).

B.1.1: Macroautophagy

MaA, as the most studied pathway, has been proved to participate in various immune activities, including pathogen infection-induced pathophysiology, autoimmune inflammation, neural systemic dysfunctions and cancers. Briefly, this pathway starts from an isolated membrane, which forms an "Ω"-like structure named omegasome (in yeast, this structure is named PAS for pre-autophagosome structure). With the participation of several protein complexes, this membrane structure elongates and encapsulates substrates, including aggregated proteins and dysfunctional organelles. After the formation of a double-membrane structure named autophagosome, lysosome fuse with autophagosome to give autolysosome and the substrates are degraded by lysosomal hydrolytic enzymes (Galluzzi et al., 2017).

B.1.2: Microautophagy

Microautophagy is mediated by direct uptake of the substrate by lysosomes for degradation. Microautophagy has been demonstrated to participate in anti-pathogen response (Gudelsky et al., 1989). However, the role of microautophagy, as well as the mechanism of this pathway, are largely unknown.

B.1.3: Chaperone-mediated autophagy

CMA is a process relying on the recognition and direct targeting of substrates to lysosomes by chaperone molecules heat shock protein family A member 8 (HSPA8/HSC70) (Figure 12). The recognition of substrates is mediated by the CMA-targeting motif known as KFERQ, which was first identified in the amino acid sequence of RNase A (Dice, 1988). The typical motif of CMA substrates consists of:

1> an invariant glutamine (Q) residue, which presents at the beginning or end of the structure;

2> one positively charged residue (R or K);

3> one hydrophobic (I, F, L or V) residue;

4> one negatively charged (E or D) residue.

Substrates with this motif exist abundantly in cytosolic proteins (Wing et al., 1991). However, only a few of them have been identified as substrates that are degraded *via* the CMA pathway. This could be attributed to modifications of proteins, for example by acetylation or ubiquitination of certain residues, which may block the interaction between chaperones and substrates (Xilouri et al., 2016).

After the recognition by chaperones, substrates can be targeted to lysosomes with the assistance of HSPA8 and co-chaperones (heat shock 90kDa proteins/HSP90, heat shock 40kDa proteins/HSP40, Hsp70 interacting protein/HIP, Hsc70/Hsp90-organizing protein/HOP, and BCL2-associated athanogene 1/BAG-1) (Agarraberes et al., 2001). Lysosomal associated membrane protein (LAMP)-2A is identified as an adapter of chaperone-substrate complex. As a single-span membrane protein, LAMP-2A interacts with the chaperone-substrate complex through its cytosolic tail, which varies in the three distinct LAMP-2 isoforms (Cuervo et al., 2000). LAMP-2A assembles from monomeric protein to multimeric complex and forms a trafficking channel for the translocation of substrate, thus enables substrates to transfer into the lysosomal lumen. The lysosomal forms of the chaperones HSPA8 (lys-HSPA8) and HSP90 (lys-HSP90) promote unfolding and translocation of substrates by LAMP-2A in coordinated ways: lys-HSP90 interacts with LAMP-2A to maintain the stability of its multimer, while lys-HSPA8 facilitates LAMP-2A multimeric form to disassemble into monomers or smaller complexes after substrate translocation (Bandyopadhyay et al., 2008).

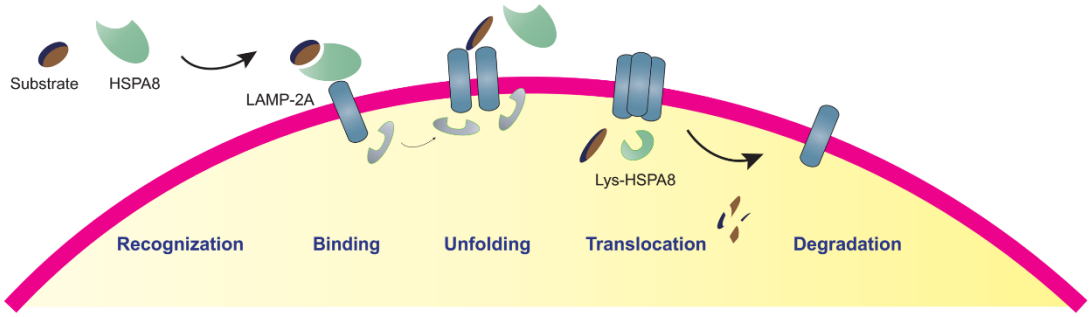

Figure 12. The CMA pathway

The CMA pathway can be recognized as 5 steps: 1, recognition of substrates characterized by KFERQ motif and formation of substrate-chaperon complex; 2, binding of the substrate-chaperon complex and LAMP-2A monomer; 3, unfolding of substrates and release of HSPA8 chaperon; 4, translocation of substrates into lysosome by the multimerized LAMP-2A; 5, disassembly of LAMP-2A multimerization and degradation of substrates by lysosomal enzymes.

CMA pathway can be activated by stimulus such as oxidative stress or trophic deprivation. It regulates the recycling of amino acid residues and provides a survival mechanism for cells. Moreover, it was found that overexpression of LAMP-2A or HSPA8 could enhance cytoplasmic peptide presentation by MHC II molecules. Therefore, CMA has been suggested to play a role in facilitating antigen presentation in APCs.

These two pathways of autophagy, CMA and MaA, are suggested to crosstalk with each other, since starvation and several other stimuli can be inducers of both pathways. Typically, CMA is activated much later than MaA (Backer et al., 1986). However, the signal transduction existing between these two pathways still remains to be fully elucidated.

B.1.4: Mitophagy

Mitochondria supply the cell with large amount of energy, which is crucial for cell survival. Important metabolic processes such as fatty acid oxidation and oxidative phosphorylation take place in mitochondria, making mitochondria the pivotal organelle in cellular activities. Damaged mitochondria release cell death-inducing factors such as cytochrome *c*, decrease the efficiency of ATP generation, leading to a

failure of cellular renewal and metabolism. They were found to play an important role in various diseases, especially in neurological disorders, such as Parkinson's disease (Anderson et al., 2014). In addition, harmful cellular reactive oxygen species (ROS) are mainly produced by mitochondria. Thus, the equilibrium of mitochondria could be crucial for cell survival.

Mitophagy was found, in both yeast and mammalian cells, to be a regulatory mechanism for mitochondria quality control (Figure 13). Firstly, mitophagy regulates the number of mitochondria by inhibiting their biogenesis (Palikaras et al., 2014). Secondly, mitophagy recognizes and clears the damaged mitochondria by encapsulating dysfunctional mitochondria into autophagosomes for degradation (Campello et al., 2014).

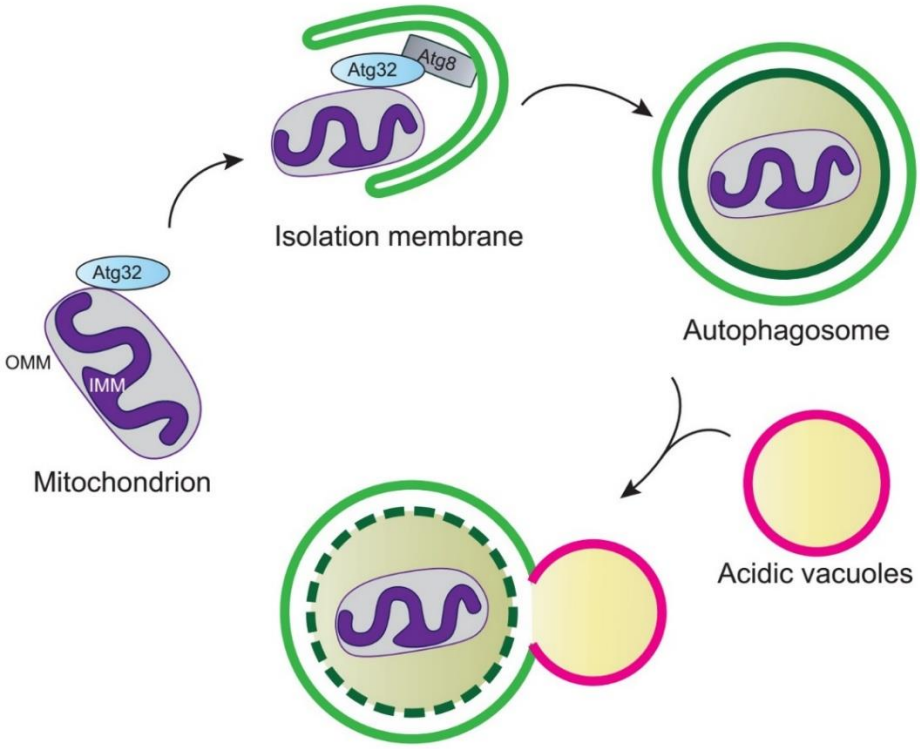

Figure 13. Mitophagy in yeast

In yeast, damaged mitochondria can be targeted to the initiation machinery of mitophagy Atg32. Atg32 is an outer mitochondrial membrane (OMM) protein, which can directly bind to the autophagosome protein Atg8 (or through the mediation by an adapter protein Atg11). After the encapsulation of mitochondria, double-membrane autophagosome is formed and fuses with acidic vacuoles (lysosomes in mammalian cells) and the entrapped mitochondria can be degraded.

B.1.5: Other autophagy pathways

Other autophagy pathways, such as lipophagy, ribophagy and xenophagy have been identified in recent studies. They are named according to their functions and have been found to present as degradation pathways, which target specific substrates.

Lipophagy is a pathway involved in the sequestration of LD into autophagosomes in which LD can be degraded by acid lipases in lysosomes (Liu et al., 2013), to provide energy by generating free fatty acids that are β-oxidized (Martinez-Lopez et al., 2016).

Ribophagy was identified since ribosomes were observed in autophagosomes by electronic microscopy (EM). The ribophagy pathway provides a quality control mechanism for ribosomes in eukaryotes, as ribophagy can target and eliminate translationally defective ribosomes (LaRiviere et al., 2006).

Xenophagy is described as a mechanism to eliminate intracellular pathogens by capturing them into autophagosomes, followed by subsequent killing of pathogens in lysosomes (Levine, 2005). Xenophagy does not only kill pathogens as a host defense mechanism, but also enhance the immune response of infection by processing antigen related peptides (Paludan et al., 2005).

Other types of autophagy have also been described in recent studies, such as microtubule associated protein 1 light chain 3 (MAP1LC3/LC3)-associated phagocytosis (LAP), pexophagy which degrades peroxisomes and nucleophagy which targets nuclei (Nikoletopoulou et al., 2015).

B.2: Machinery of autophagy

The autophagic machinery includes the network of various proteins and highly organized membrane trafficking events. It is a multi-step process, including initiation, elongation, fusion and degradation (Figure 14).

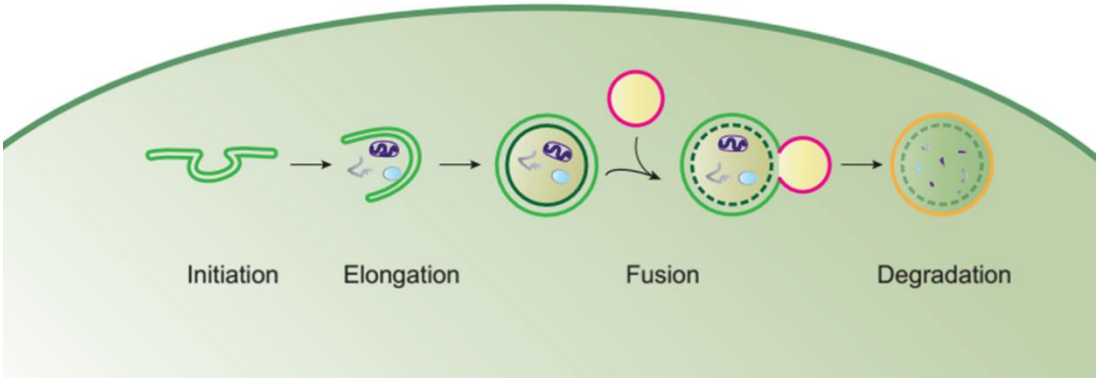

Figure 14. Processes of autophagy

The autophagy pathway consists of several main steps, namely 1, initiation, an isolation membrane forms an "Ω"-like structure to recruit other proteins or complexes; 2, elongation, the membrane elongates and encapsulates substrates to form a double-membrane structure named autophagosome; 3, fusion, autophagosomes fuse with lysosomes and 4, degradation, lysosome enzymes degrade the substrates.

B.2.1: Biogenesis and Initiation

B.2.1.1: Membrane source

The isolation membrane (omegasome in mammalian and pre-autophagosomal structure/PAS in yeast) can be generated from multiple origins, including endoplasmic reticulum (ER), plasma membrane, Golgi, mitochondria, endosomes and nuclear membrane (Lamb et al., 2013) (Figure 15).

Here in this manuscript, we would like to focus on the better characterized and also the first identified membrane resource of autophagosome, which is ER. It was found in 2008 by Axe and colleagues that some autophagosomes are formed from a PI3P-enriched membrane compartment connected with ER structure (Axe et al., 2008), suggesting ER may provide essential components for autophagosome formation. One year later, a direct observation by electron microscopy (EM) was reported by Hayashi-Nishino and collaborators who showed that the ER membrane directly associates with early autophagic structures in mammalian culture cells (Hayashi-Nishino et al., 2009). Hereafter, increasing number of studies confirmed ER as a major membrane source of autophagosome (Goder, 2017).

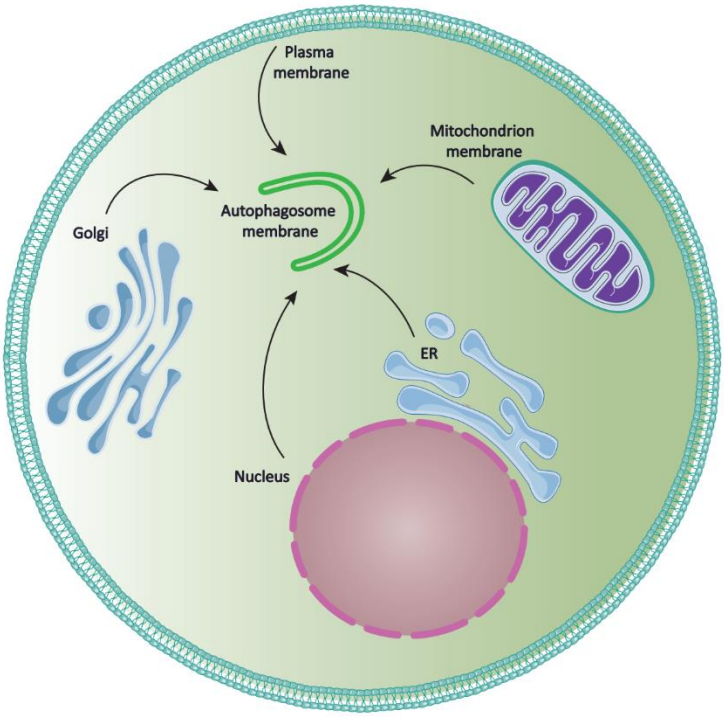

Figure 15. Membrane sources of autophagosome

The membrane of autophagosomes can be generated from various organelles, including the Golgi, the plasma membrane, mitochondria, ER and nucleus. Several protein complexes including ULK complex, PI3KC3 complex and ATG8 conjugation systems are found to be crucial players in the generation and elongation of autophagic structures.

B.2.1.2: The initiation machinery

Upon stress conditions like nutrient starvation, autophagy pathway can be induced by the mediation of the mammalian target of rapamycin (mTOR). This whole process is mediated by several protein complexes and can be characterized by the following steps (Figure 16).

1) In fed condition, unc-51-like autophagy activating kinase 1 (ULK1) complex, which consists of ULK1, ATG13, ATG101 and FAK family kinase-interacting protein of 200kDa (FIP200), binds mTOR and thus the kinase activity of ULK1 is inhibited. Upon starvation, mTOR dissociates with ULK1 complex, and the Ser/Thr kinase activity of ULK1 can be activated.

2) The activated ULK1 phosphorylates substrate proteins ATG13 and FIP200, leading to the translocation of ULK1 complex from cytosol to ER structures. Vacuole membrane protein 1 (VMP1), a multi-membrane spanning protein, was found to locate in ER or Golgi structures, which functions as a target for ULK1 and is required for ULK1 locating on ER.

3) After the location of ULK1 complex, class III PI 3-kinase complex (PI3KC3) is recruited, which consists of VPS15, Beclin 1, ATG14, AMBRA1 and VPS34. VPS34 can be phosphorylated by activated ULK1 and enhance the activity of PI3KC3 complex.

4) PI3KC3 complex produces an activator phosphatidylinositol-3-phosphate (PI3P), and the latter recruits double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins.

5) DFCP1 and WIPI translocate from ER to PI3P-enriched membrane compartment to generate an “Ω”-like structure named omegasome (Cottam et al., 2011). ATG14L, another protein localized in ER, was found to interact with DFCP1 and promote the formation of omegasome.

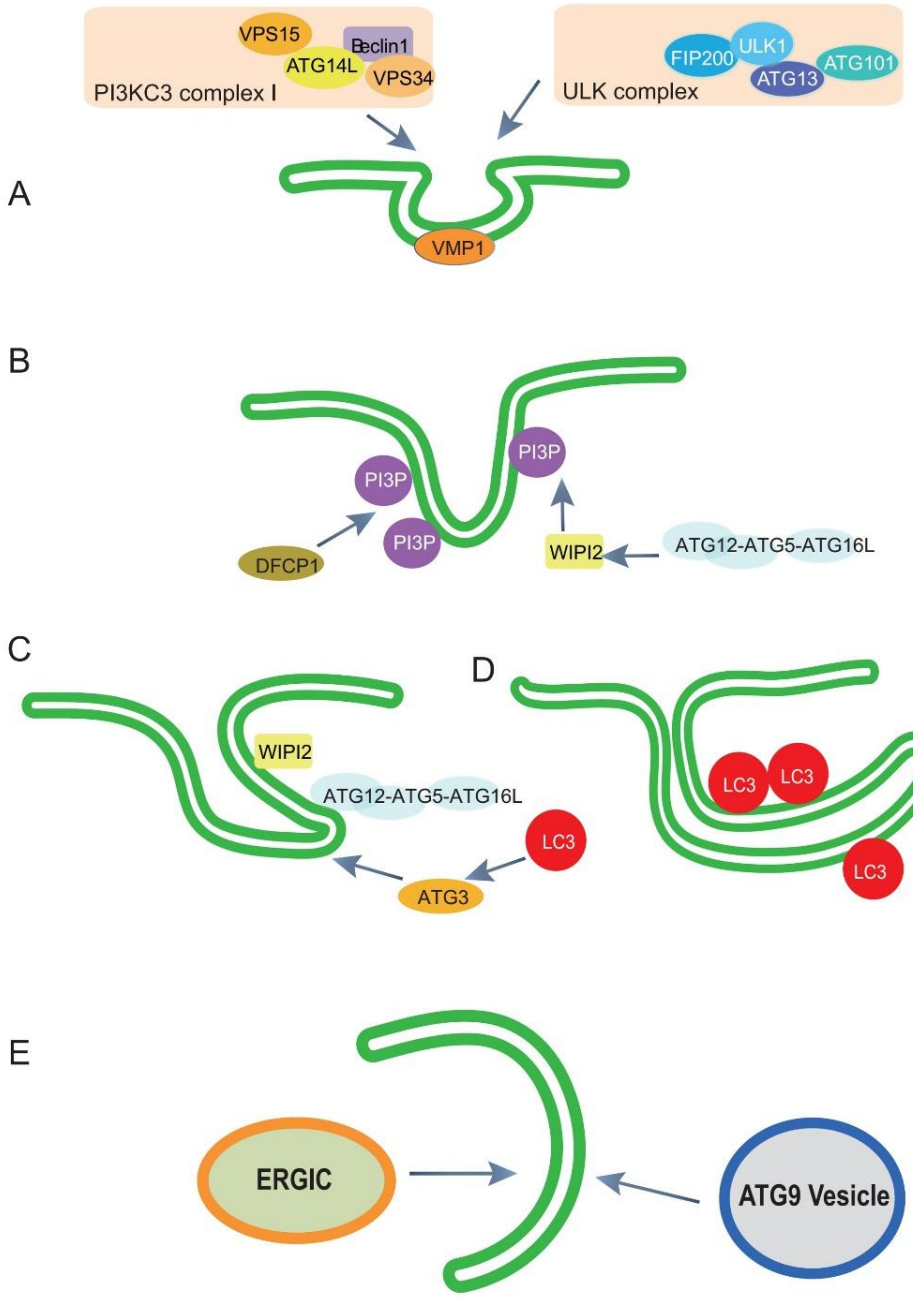

Figure 16. Initiation of autophagosome biogenesis

(A) Upon activation by starvation, the ULK1 complex is recruited to isolation membrane containing VMP1. The localization of ULK1 onto isolation membrane is followed by recruitment and activation of PI3KC3 complex by ULK. (B) PI3P activator produced by PI3KC3 further enhances membrane bending and forms a platform for the recruitment of DFCP1 and WIPI, leading to the translocation of these proteins from ER to PI3P-enriched membrane compartment. (C) WIPI2 recruits ATG12–ATG5–ATG16Ll which activates LC3, resulting in the lipidation of LC3 at the curved membrane and generating an "Ω"-like structure which is named omegasome (D). (E) Vesicles originating from the ER-Golgi intermediate compartment (ERGIC) and ATG9 vesicles are added to the omegasome to elongate and expand the membrane.

Recent studies have increased the knowledge about the processes of autophagy initiation. However, the precise molecular mechanisms regarding each step above is largely unknown, especially for the formation of omegasome. Proteins like ATG2 and ATG4 have been found to be required for omegasome formation (Hirata et al., 2017). Silencing of ATG2A and ATG2B blocks autophagic flux, resulting in accumulation of unclosed autophagic structures containing ATG proteins (Velikkakath et al., 2012), thus suggesting that omegasome formation is an ATG2-dependent process. ATG2 appears to be recruited by WIPI through a direct interaction (Bakula et al., 2017). However, the role of ATG2 is not understood yet and further studies are in need.

B.2.2: Elongation and formation of autophagosome

B.2.2.1: ATG8 modification by two ubiquitin systems

Ubiquitin-like pathways are involved in the elongation of autophagic vesicles from omegasome (Zens et al., 2015). ATG8 proteins are synthesized in an inactive pro-form that contains a conserved glycine residue which is localized near the C-terminus. There are several additional amino acid residues following this conserved region, which can be cleaved. ATG4 is a cysteine protease that regulates autophagy by cleaving the C-terminus of pro-ATG8 after the conserved glycine (Woo et al., 2014), which makes this glycine residue available for modification by the phosphatidylethanolamine (PE) conjugation reaction (Figure 17).

There are two subgroups of Atg8 homologs have been identified in mammalian cells, namely LC3 and GABARAP (Weidberg et al., 2010). LC3 has arginine residues on the C-terminal, which can be removed by the cysteine protease ATG4, leaving a glycine residue at the C-terminus, which is the LC3-I. Subsequently, the C-terminal glycine residue can be activated by E1-like enzyme ATG7, followed by the modification of LC3-I by E2-like enzyme ATG3 (Nath et al., 2014). Afterwards, the ATG5-ATG12-ATG16L complex acts as E3-like enzyme to conjugate PE with LC3-I, leading to the formation of LC3-II and its association with membranes. The formation of E3 like ATG5-ATG12-ATG16L needs at least 6 proteins and several reactions (Metlagel et al., 2014). ATG12 is an ubiquitin-like protein, which can bind and be activated by an E1-

like enzyme ATG7 by the C-terminal glycine residue. The activated ATG12 is then transferred to the E2-like enzyme ATG10, to form a ATG12-ATG5 conjugation. With the participation of ATG16L1, the final supramolecular complex ATG5-ATG12-ATG16L is generated and acts as an E3-like enzyme for the PE conjugation of LC3-I (Tanida et al., 2004; Walczak et al., 2013).

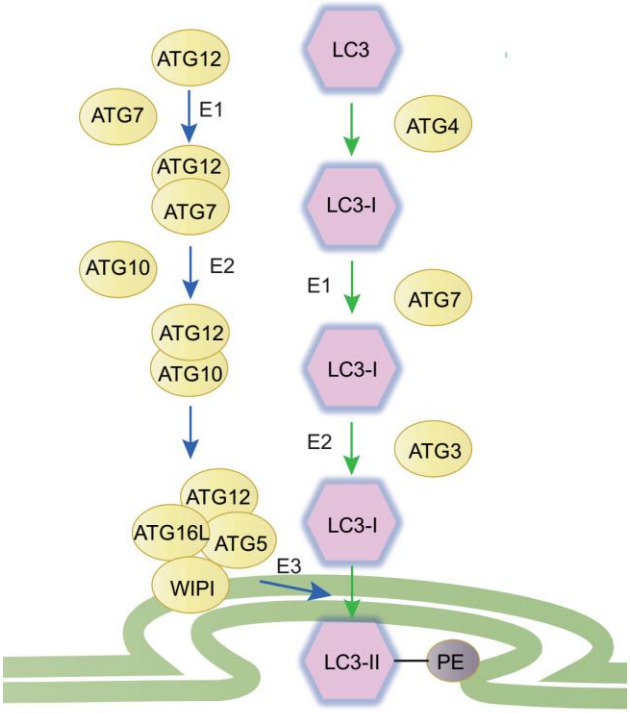

Figure 17. Two ubiquitin systems involved in LC3 modification

LC3 are synthesized in a pro-form that can be cleaved by cysteine protease ATG4. After the cleavage by ATG4, LC3-I with an active glycine residue at C-terminus is generated. Subsequently, the LC3-I can be activated by E1-like enzyme ATG7 and E2-like enzyme ATG3, followed by conjugation of PE by E3-like ATG5-ATG12-ATG16L complex. These modifications result in the formation of LC3-II and its association with membranes. The formation of E3-like ATG5-ATG12-ATG16L complex needs several reactions and at least 6 proteins.

B.2.2.2: Membrane expansion

ATG9 is the first identified transmembrane ATG protein, which is required during multiple stages of autophagy. ATG9 exists at the surface of specific cytoplasmic vesicles (ATG9 vesicles) which are developed from Golgi vesicles. ATG9 is a

membrane-elongating protein, which delivers lipids and membrane structure-like vesicles to omegasome to expand the membrane structure. It has been found that ATG9 is recruited to the PAS location *via* the interaction with ATG13, thus enabling ATG9 vesicles to become part of the autophagosomal membrane (Suzuki et al., 2015).

VMP1 is a multimembrane-spanning protein localized to ER and Golgi, and it was suggested to be another autophagy-related transmembrane protein (Vaccaro et al., 2008). VMP1 interacts with Beclin 1 by its hydrophilic C-terminal region (also known as ATG domain), to recruit Beclin 1 proteins to the initiation site and to promote the elongation of autophagosome. It was found that VMP1 expression can trigger autophagy in nutrient replete conditions, suggesting the existence of alternative mechanisms for autophagosome formation.

ATG14L, a protein localized on ER membrane has been demonstrated to play an essential role in the formation of autophagosome. It is not only identified as a component of PI3KC3 complex but also demonstrated to direct the transportation of PI3KC3 to omegasome structure. It was found that knockdown or deletion of ATG14L can lead to defective autophagy induction (Matsunaga et al., 2010).

B.2.2.3: Cargo Receptors

As introduced in previous sections, MaA is a pathway during which substrates are sequestered and encapsulated by autophagic membranes. This process was proved to be selective and not random, and the selectivity is mediated by autophagy receptors. Autophagy receptors are identified by the capability of recognizing both autophagosomal membrane and the degradation signals on the substrates (Rogov et al., 2014). Autophagy cargo receptors recognize substrates by ubiquitin (Ub)-binding domains (UBDs) and interact with autophagosome by LC3-recognition sequence (LRS). In this way, cargo receptors build bridges between substrates and autophagosome, mediating the engulfment of substrates by autophagy. The most frequent degradation signal for autophagic pathway is ubiquitination that modifies the cargos with Ub (Varshavsky, 2017). In mammalian cells, the best studied autophagy receptors are sequestosome (SQSTM1/p62), BCL2 interacting protein 3

like (BNIP3L), calcium binding and coiled-coil domain 2 (CALCOCO2/NDP52), neighbor of BRCA1 gene 1 (NBR1) and optineurin (OPTN) (figure 18).

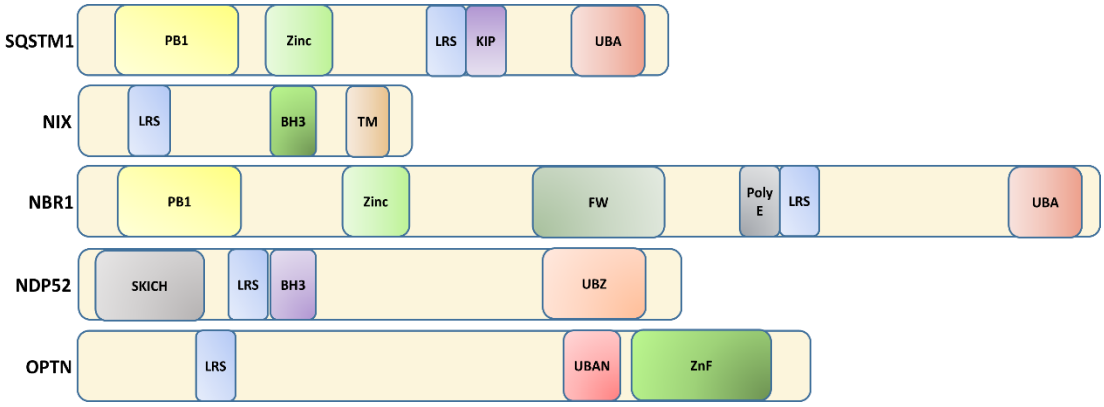

Figure 18. Cargo receptors of autophagy in mammalian cells

Autophagy cargo receptors recognize substrates by UBD and interact with autophagosome by LRS. These two domains are conserved in different cargo receptors, especially the LRS domain. LRS is a motif consisting of 11 amino acids (Ser334-Ser344) including the conserved acidic and hydrophobic residues across species. PB1, N-terminal Phox and Bem1p region; Zinc, ZZ-type zinc finger domain KIR, Keap1-interacting region; UBA, ubiquitin-associated domain.

SQSTM1 is the most understood cargo receptor. It encompasses five functional regions that are N-terminal Phox and Bem1p region (PB1), ZZ-type zinc finger domain (Zinc), LRS, Keap1-interacting region (KIR) and ubiquitin-associated domain (UBA) (Figure 19).

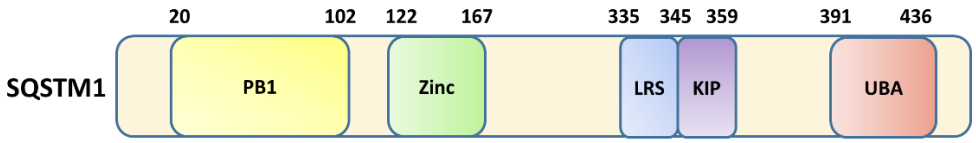

Figure 19. Structure of SQSTM1

The cargo receptor SQSTM1 contains an N-terminal PB1 (residues 20–102), which is required for self-oligomerization and binding to other PB1-domain proteins, Zinc domain (residues 122–167), LRS region (335–345), KIR region (346–359), and C-terminal domain UBA (residues 391–436). Trp340 and Leu343 in LRS are crucial for SQSTM1 as they interact with hydrophobic pockets on the ubiquitin fold of LC3.

Ichimura and colleagues showed that the direct interaction between SQSTM1 and LC3 is mediated by the LRS region, which is responsible for the recognition of LC3. Deletion of the LRS leads to SQSTM1 accumulation without being entrapped into autophagosomes (Ichimura et al., 2008). SQSTM1 is considered as a marker for autophagy since its level could be decreased by functional autophagic degradation. In contrary, defective autophagy may lead to accumulation of SQSTM1 (Dikic, 2017).

In addition to the interaction with LC3, the self-oligomerization of SQSTM1 is required for its degradation by autophagy (Wurzer et al., 2015). It was reported that SQSTM1 self-oligomerization is mediated by the PB1 domain, and deletion of PB1 region leads to delay of its degradation in MEFs cells (Ichimura et al., 2008; Walczak et al., 2013; Wurzer et al., 2015).

B.2.3: Fusion

B.2.3.1: Maturation

The autophagosome-lysosome fusion is a crucial step in autophagy pathway, as the disruption of fusion can lead to a blockage of the whole pathway. Some bacteria and viruses can make use of this character to avoid being degraded by autophagy (Ding et al., 2014; Hu et al., 2015).

The fusion of autophagosomes with lysosomes could take place in different manners. Autophagosomes can fuse with early or late endosomes to form a structure named amphisome, and then fuse with lysosomes to form the final autolysosomes. In other conditions, autophagosomes can directly fuse with lysosomes to form autolysosomes. Notably, in either conditions, only matured and enclosed autophagosomes can fuse with other structures. If the fusion machinery is recruited and activated before the mature/enclosure of autophagosomes, the intravesicular substrates would be released into cytosolic or simply attached to the outer membrane of lysosomes, and thus could not be degraded. Hence, the mature signal is a checkpoint and strictly required for functional fusion. An important mature signal is the loss of LC3 on the outer membrane of autophagosome, which is recognized as a sign to remove the initiation machinery. Once the initiation-related proteins and

complexes are detached, the double-membrane structures are successfully formed and equipped to fuse with other structures like endosomes or lysosomes. In this way, the fusion of autophagosomes and other structures can be strictly controlled and well organized for the next process.

B.2.3.2: Delivery to lysosomes

In fed cells, lysosomes are homogeneously distributed in the cytosol (Mellman, 1989). Upon nutrient starvation, lysosomes are redistributed to the perinuclear regions, where autophagosome-lysosome fusion primarily takes place (Korolchuk et al., 2011).

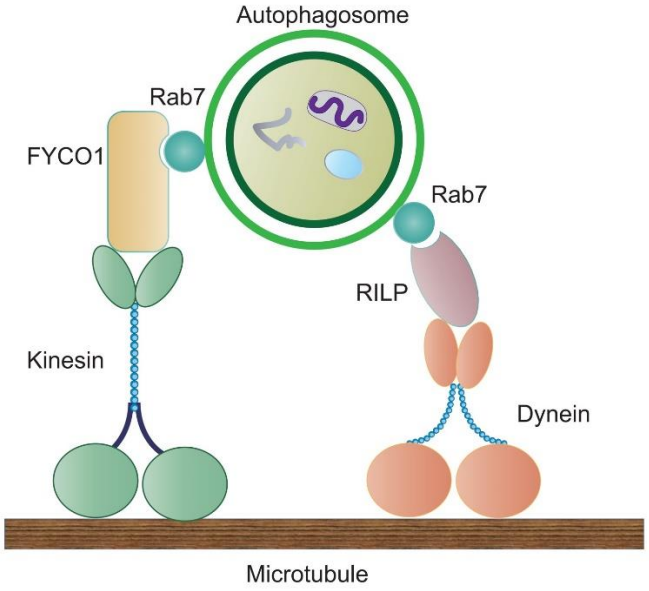

Figure 20. Dynein and kinesin in the transportation of autophagosomes

Dynein and kinesin can be recruited by small GTPase Rab7 that is localized on the membrane of late endosomes or autophagosomes. Kinesin can be recruited by Rab7 by the mediation of FYCO1, which directly binds to Rab7 and acts as an adaptor of kinesin. Similarly, dynein binds to Rab7 by another mediator named RILP. Dynein and kinesin are powered by ATP hydrolysis and move along the microtubules. Dynein moves towards centrosome and delivers autophagosomes to perinuclear localized lysosomes. In contrary, kinesin moves towards the periphery to prevent over moving of autophagosomes towards nuclear. FYCO1, FYVE and coiled-coil domain containing 1; RILP, Rab interacting lysosomal protein.

After the maturation of autophagosomes, a delivery system is required to transport autophagosomes to lysosomes for fusion. In this delivering system, microtubules

were found to play a crucial role. Microtubules are components of cytoskeleton, which are characterized by polar structures with two ends: the “plus” end stretches into the periphery of the whole cell and the “minus” end, which often connects to centrosome that is associated with the nuclear membrane. Motor proteins move along microtubules and carry vesicular structures in cytosol. In autophagosome trafficking, two motor proteins, dynein and kinesin, are required for the movement of autophagosomes to lysosomes along microtubules (Figure 20). Dynein and kinesin are recruited by small GTPase Rab7 that is localized in late endosomes or autophagosomes. Dynein is “minus”-end directed and moves towards centrosome, thus delivering autophagosomes to perinuclear localized lysosomes. In contrary, kinesins are “plus”-end directed and moves towards the “plus” end of microtubules, acts as a machinery to prevent over moving towards nucleus, which may cause perinuclear clustering of autophagosomes and blockage of fusion (Cardoso et al., 2009). Mutations of the dynein machinery impair autophagic clearance, which can lead to aggregate-prone protein-related disease, like Huntington’s disease (Ravikumar et al., 2005).

B.2.3.3: The final fusion

Once autophagosomes are close enough to fuse with lysosomes, tethering factors are required to mediate the fusion. The homotypic fusion and protein sorting (HOPS) complex serves as a connection factor to link autophagosomes and lysosomes (Figure 21). HOPS mediates the mechanism of fusion by interacting with both the autophagosome protein UVRAG (through VPS16 subunit) and Rab7 protein on the membrane of lysosome (through VPS39 subunit) (Brocker et al., 2010).

Tectonin β-propeller repeat containing 1 (TECPR1), another tethering complex, recognizes autophagosomes through ATG12-ATG5 conjugate and PI3P, promoting autophagosome-lysosome fusion. The deletion of TECPR1 leads to accumulation of autophagosomes and blockage of degradation (Chen et al., 2012).

Besides HOPS and TECPR1, LC3 has also been suggested to play a role in autophagosome-lysosome fusion. It was found that knockout of LC3/GABARAPs

leads to the failure of autophagosome-lysosome fusion (Padman et al., 2017).

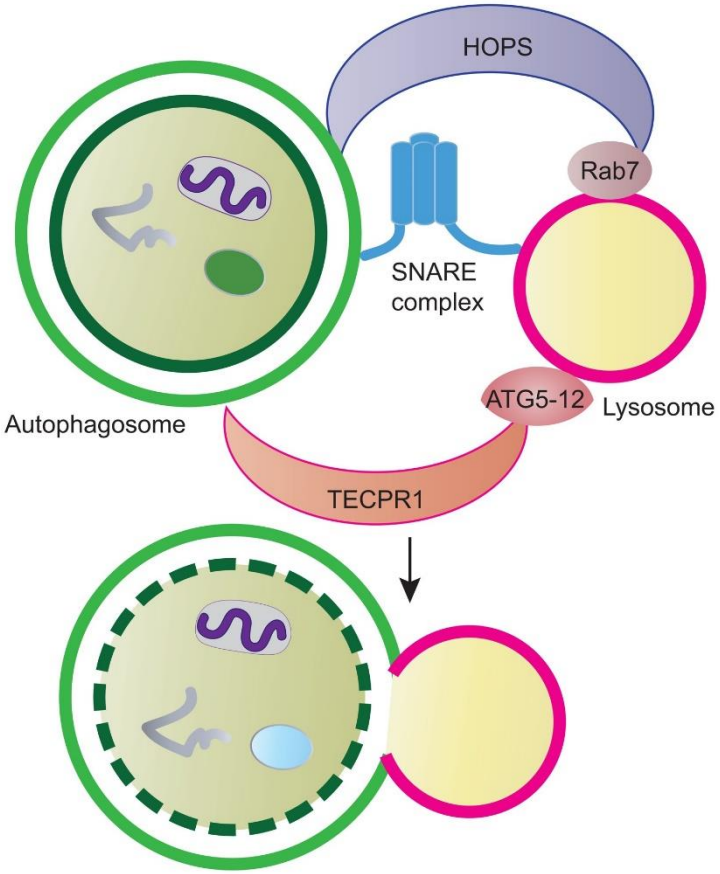

Figure 21. Autophagosome-lysosome fusion

Autophagosomes fuse with endosomes/lysosomes through the tethering complex HOPS and TECPR1. First, a long-range docking interaction occurs between HOPS and Rab7. This long-range interaction allows the SNARE complex (in blue) to be formed, which drives the actual fusion. TECPR1, another tethering complex, recognizes autophagosomes through ATG12-ATG5 conjugate and PI3P, promoting autophagosome-lysosome fusion.

B.2.4: Degradation

B.2.4.1: Lysosome: the organelle for degradation

The final degradation of contents delivered to lysosome depends on the function of lysosome. Lysosomes are generated from endosome pathway. Vesicles include endocytosed materials develop into early endosomes (pH around 6.5) labeled by Rab5, then progressively maturate into late endosome (pH around 6.0). After, late

endosome fuses with lipid vesicles from Golgi, to form the final lysosome (pH around 5.0).

In mammalian cells like metazoan cells, there are hundreds of lysosomes, and their diameters range from 0.1-1µm. Lysosome contains a single phospholipid bilayer of around 7-10nm, which harbored 3 families of transmembrane proteins, namely LAMP, lysosomal integral membrane proteins (LIMP) and CD63. LAMP protein family represents 80% of total membrane proteins. All these proteins are glycosylated at the inner membrane side to form a glycocalyx. In this way, these proteins can be protected from auto-digestion by the luminal enzymes in lysosome.

In general conditions, lysosomes are heterogeneous in size, morphology, and are distributed at the cell periphery (Mellman, 1989). Upon nutrient starvation, lysosomal number is dramatically reduced, coordinating with lysosomal membrane fusion which leads the increase of size (Bandyopadhyay et al., 2014). In addition, lysosomes are redistributed to the perinuclear region (Korolchuk et al., 2011). These features favor the adaptation of lysosomes to cellular environmental changes and serve as a mechanism for lysosomal homeostasis.

B.2.4.2: V-ATPases

The acidic luminal pH is a crucial feature of lysosomes. Normally it ranges from 4.5-5.5, and this low pH can be maintained by V-ATPase and ions like Cl^-, Na^+, K^+.

The acidic pH is regulated by V-ATPase, a type of proton pump, which uses the energy generated from ATP hydrolysis to drive protons into the lysosome. The ability of the V-ATPase to generate proton gradient depends on the efficacy of ATP hydrolysis. The structure of V-ATPase is similar to that of the F_0F_1 ATPase in mitochondria. The difference is that V-ATPase can only hydrolyze ATP, while F_0F_1 ATPase can either synthesize or hydrolyze ATP.

V-ATPases form a multisubunit complex composed of a soluble V_1 subcomplex and a membrane-embedded V_0 subcomplex. The V_1 complex consists of at least 8 subunits including subunit A-H, and the V_0 complex contains 6 subunits (a, c, c', c'', d and e).

These two complexes are connected by a central stalk, which consists of the D and F subunits (Figure 22).

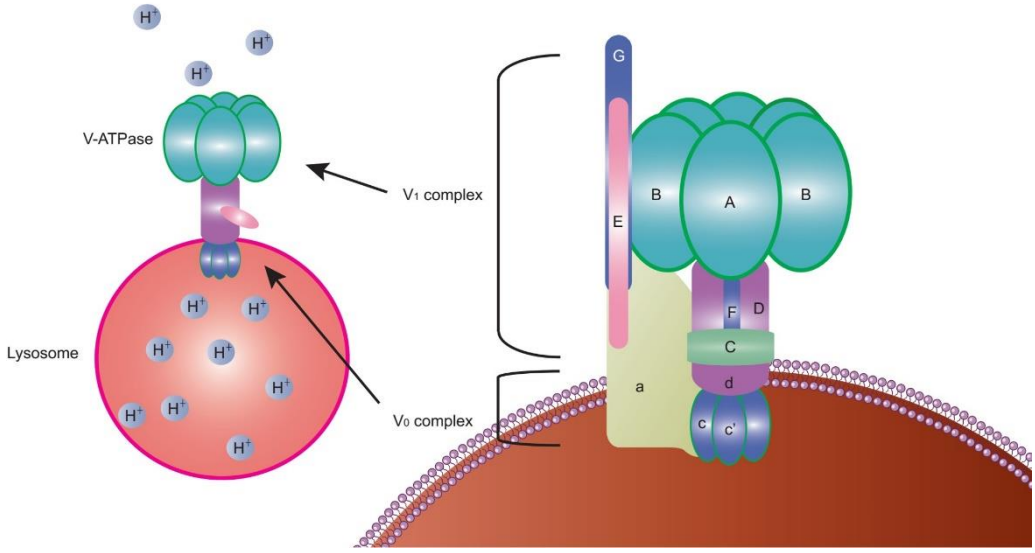

Figure 22. Structure of V-ATPase

The V-ATPase consists of V_1 and V_0 complexes. The V1 complex contains at least 8 subunits including subunit A-H, and the V_0 complex contains 6 subunits (a, c, c', c'', d and e). These two complexes are connected by a central stalk, which consists of the D and F subunits. Different from F_0F_1 ATPase in mitochondria, the V-ATPase can only hydrolyze ATP.

B.2.4.3: Counterion pathway

Besides V-ATPase, the lumen homeostasis is maintained largely by lysosomal membrane potential (ΔΨ, defined as $V_{cytosol} - V_{lumen}$; V_{lumen} is set to 0 mV) (Kolter et al., 2005). The membrane potential homeostasis promotes proton pumping and maintains the luminal acidic pH, thus allowing the lysosomal hydrolases to be active and functional.

V-ATPase is a proton pump that drives in a single direction, allowing protons only to come from cytoplasm and enter the channel, and then release into the lysosomal lumen. This leads to a luminal-positive transmembrane voltage, which is the decrease of ΔΨ ($V_{cytosol} - V_{lumen}$). In order to balance transmembrane voltage, the

counterion pathway is required to either carry cations out of lysosome, or transport anions into the lysosomal lumen (Mindell, 2012). Transient receptor potential (TRP) channels are a group of calcium-permeable cation channels, which are similar to K^+ and Na^+ channels, as well as other Ca^{2+} channels (Ramsey et al., 2006), allowing cations to be transported outside of lysosomes. The anions involved in $\Delta\Psi$ maintainability are mainly Cl^-, which is mainly transported by a Cl^- channel named cystic fibrosis transmembrane regulator (CFTR), which can be activated by ATP or phosphorylation. In addition to CFTR, another group of Cl^- channels is the CLC family, which participates in the balance of transmembrane voltage (Jentsch, 2008),

Two-pore complex 1 and 2 (TPC1 and TPC2) function as cation-releasing channels, hence regulate lysosomal membrane potential. It was shown that TPC1 and TPC2 channel complexes detect nutrient status, are activated upon starvation and control the membrane potential of lysosome (Cang et al., 2013).

B.2.4.4: Enzymes in lysosome

There are around 60 acid hydrolases in the lysosome to digest proteins, LDs, nucleic acids and carbohydrates. Briefly, all the lysosomal enzymes can be classified into three groups: phosphatase, glycolytic enzymes and proteolytic enzymes. Among them, more than 20 hydrolytic enzymes are peptide-bond cleaving proteolytic enzymes. These proteases can be classified into serine-, aspartic- and cysteine-peptidases according to their structures and catalytic types. In addition, all these hydrolases optimally function at acidic pH condition, which relies on the lysosomal homeostasis.

B.2.4.5: The regulator of lysosome: transcription factor EB

Transcription factor EB (TFEB) has been identified as a transcription factor for lysosome-related genes. Its expression is related to both morphology and function of lysosome. Overexpression of TFEB in cultured cells leads to increased lysosomal biogenesis and degradation of complex molecules such as aggregate-prone proteins (Sardiello et al., 2009).

In fed conditions, TFEB is phosphorylated and retained in cytoplasm. Cellular stress or stimulations like starvation can inhibit mTORC1, which is a regulator of TFEB, leading to the dephosphorylation of TFEB and its translocation to nucleus. Hence, the transcription of target genes is enhanced and the levels of lysosomal-related protein are enhanced (Efeyan et al., 2012). In this way, measuring the phosphorylation of TFEB and the expression of related genes serves as a suitable method to evaluate lysosomal activity.

B.3: Autophagy and autoimmunity

Essential immunological functions have been suggested to be related to autophagy, such as antigen uptake and presentation, phagocytosis, removal of pathogens, immune cells survival and cytokines network modulation (Doulatov et al., 2017). Dysregulation of autophagy can have various destructive consequences, including failure of clearance of invasions, immune response dysregulation and even cell death (Papandreou et al., 2017; Ho et al., 2017).

Autophagy has been demonstrated to be involved in various AIDs by GWAS, biochemical and cell biological approaches (Diaz et al., 2015; Liu et al., 2016). Autophagy is implicated in both the induction and prevention of autoimmunity, and it plays various roles in pathogenesis of autoimmune disorders (Wu et al., 2017). Therefore, modulators of autophagy hold high potential for the treatment of AIDs.

B.3.1: Autophagy in cellular homeostasis

Autophagy participates in cell metabolism and homeostasis, which are essential for immune responses and activities, and its deregulation can lead to AIDs. In autoimmune conditions, dysregulation of autophagy has been observed in various cell types including both immune cells and non-immune cells.

B.3.1.1: Autophagy in T cells

T cells are one of the most studied immune cells in autoimmune context (Pierdominici et al., 2012). It has been demonstrated by numerous studies that

autophagy is deregulated in T cells of AIDs (Botbol et al., 2016). The levels of autophagy-related proteins (including Akt, mTOR, ULK1, and LC3) in T cells of oral lichen planus (OLP) lesions have been shown to be significantly increased, suggesting the role of autophagy in T cell-mediated immunoregulation of OLP (Zhang et al., 2017). In addition, T lymphocytes from patients with SLE are resistant to autophagy induction, and up-regulate genes negatively regulating autophagy (Alessandri et al., 2012). In our laboratory, a pioneering study by Gros and colleagues showed that autophagy was significantly up-regulated in T cells from both SLE patients and two distinct lupus-prone mouse models (Gros et al., 2012), suggesting that autophagy could have an impact on autoreactive T cell during lupus. This has been further confirmed by another study, which found that autophagy is overactivated in T cells of SLE patients compared with that of healthy individuals. A positive correlation was found between autophagic activity and apoptotic rate in activated T cells (Chen et al., 2016). Besides these direct changes of autophagic activity in T cells, it has been proposed that in thymic epithelial cells, autophagy contributes to CD4 T cells education, and regulates positive and negative selections (Nedjic et al., 2008). These studies indicate that aberrant autophagy could be involved in AIDs by interfering T cell activities in distinct ways.

B.3.1.2: Autophagy in B cells

Autoantibodies are produced by autoreactive B cells, which act as crucial players in autoimmune responses. Thus, the breakdown of B cells tolerance is a major event which leads to AIDs.

In a MyHC-α-induced experimental autoimmune myocarditis (EAM) mouse model, autophagy was found to participate in IL-17-mediated plasma cell differentiation. The level of autophagy was positively correlated with plasmablast percentage and autoantibody levels. Moreover, by regulating the expression levels of Beclin 1 and SQSTM1, elevated autophagy enhanced B cell anti-apoptotic ability (Yuan et al., 2014), suggesting autophagy as a crucial pathway in the differentiation of B cells in EAM.

The role of autophagy in SLE has been underscored by recent studies. It was found that autophagy is overactivated in B cell of SLE patients. Similarly, in the lupus mouse model NZB/W, autophagy was found to be overactivated in early stages B cells and progressively increase with age (Clarke et al., 2015). In both human and mice, the differentiation of B cells into plasmablasts could be blocked by inhibition of autophagy, suggesting that autophagy pathway might be involved in the autoreactivity and differentiation of B cells.

Altogether, these results strongly suggest the possibility to introduce novel therapies by inhibiting B cell autophagy. This was confirmed by a study using TLR7-mediated mouse model of autoimmunity, in which the hallmarks of SLE (ANA production and inflammation) were weakened by B cell-specific ablation of autophagy (Weindel et al., 2015). In addition, a 21-mer peptide P140 was found to exhibit therapeutic effects in SLE by modulating CMA pathway in B lymphocytes (Macri et al., 2015). These results strongly suggest that B cell autophagy is crucial for the development of AIDs, making B cell autophagy a potential therapeutic target for AIDs (Figure 23).

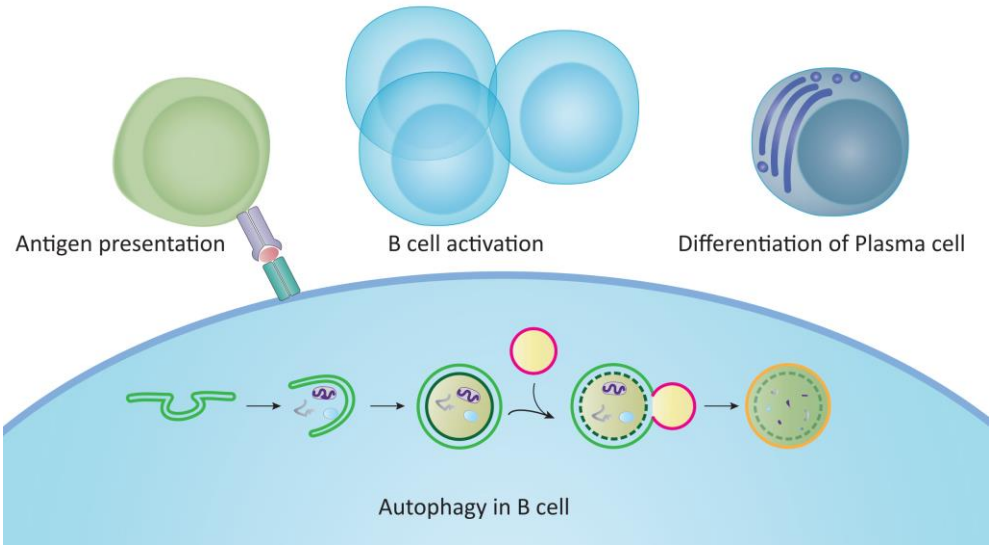

Figure 23. Roles of autophagy in B cells

Autophagy is found to be a crucial pathway in B cell activities. In AIDs, autophagy participates in autoantigen presentation of B cells, and regulates the B cell activation. In addition, autophagy is associated with the differentiation of plasma cells which produce autoantibodies, leading to chronic inflammation in AIDs.

B.3.1.3: Non-immune cells

Not only immune cells are related to autophagy, but also non-immune cell features could be affected by autophagy and thus play a role in the pathogenesis of AIDs. Autophagic activity is found to be inhibited in type 1 diabetic hearts, which shows impaired diastolic function (Xu et al., 2013), and treatment with an autophagy inhibitor, chloroquine, worsened cardiac performance in type 1 diabetes (T1D) (Kanamori et al., 2015). This was further confirmed by another study using Fenofibrate to treat T1D, showing that Fenofibrate prevents T1D heart abnormalities by up-regulating Sirt1-mediated autophagy (Zhang et al., 2016). The protective effects of autophagy to diabetic hearts suggest induction of autophagy might alleviate T1D.

A study aimed to examine the interplay between ER stress-induced autophagy and apoptosis in SGs epithelial cells showed that autophagy and apoptosis were enhanced after thapsigargin (ER stress inducer) treatment. Moreover, Ro/SSA and La/SSB autoantigens were redistributed from the cytoplasm to cell membrane and blebs in the apoptotic cells (Katsiougiannis et al., 2015). This study confirmed that epithelial cells are a source of autoantigens and provide a non-professional antigen presentation mechanism, which is crucial for the initiation of autoimmunity. Moreover, this study provides the first clue of autophagy involvement in SS. However, the precise role of autophagy is not studied. It is possible that there is a crosstalk between ER stress, autophagy and apoptosis, which cooperate to decide the cell fate. Another possibility is that autophagy contributes to the release and presentation of autoantigenic materials by epithelial cells. As it will be discussed later, autophagy is an important process which regulates and participates in antigen presentation, in both professional and non-professional conditions (Munz, 2016).

B.3.2: Autophagy and cytokines

Cytokines, the main players for inflammation, interact with autophagy pathway. They are small, soluble molecules produced by various cell types, and they respond to stimulus and transduce signals by binding to specific receptors. They can act in

autocrine, paracrine and endocrine manners, affecting cell differentiation, maturation, and activation. Cytokines generate and develop a net-working signaling system that transduces signals between immune cells. The abnormalities of cytokines lead to the failure of homeostasis, causing chronic inflammation, which acts as a crucial characteristic for AIDs. This is one of the reasons that most of the AIDs are characterized by chronic inflammation, leading to the possibility of developing effective therapeutics by targeting cytokines (Ronnblom et al., 2010).

Autophagy can be regulated by cytokines, including those that can activate autophagy, like IFN-γ, TNF-α and CCL2, and those that can inhibit autophagy, like IL-4, IL-13 and CXCL12 (Levine et al., 2011).

Within the autoimmune context, the relationship between autophagy and autoinflammation can be diverse and complex. On one hand, autophagy can participate in AIDs by directly regulating cytokines expression. It was found that aberrantly activated autophagy in macrophages enhanced the production of proinflammatory cytokines TNF-α and IL-6, which facilitated inflammation and promote the pathogenesis of murine lupus (Li et al., 2014). On the other hand, autophagy can be induced by cytokines and participate in autoimmune response. RA, a chronic inflammatory disease, is characterized by severe destruction of articular cartilage and bone driven by TNF-α. Autophagy was found to be activated by TNF-α, and promote osteoclast differentiation as well as osteoclast-mediated bone resorption. These indications suggest that autophagy could be elevated by the pro-inflammatory cytokine TNF-α and promotes the induction of bone disease (Lin et al., 2013).

In other conditions, autophagy was found to coordinate with cytokines in autoimmunity. Mutation of *atg5* was identified as a risk factor for SLE, which is linked with the high level of IL-10 in patients. It was found that Atg5 mutation is associated with SLE only in patients that had high expression of IL-10, but not in patients with low IL-10 levels (Lopez et al., 2013). This suggests that the coordination of cytokines and autophagy might be a mechanism mediating autoimmune

responses.

The relationship between inflammation and autophagy can be more complex than a simple direct coordination. In fibrosis, a fibrotic disorder that is mainly triggered by cytokines, chemokines and growth factors are released by immune cells. Defective autophagy was found to participate in the perpetuation of chronic inflammation, amplifies autoimmune responses, and promotes the progression to fibrosis (Del et al., 2011).

These studies suggest that the crosstalk between autophagy pathway and inflammation could adopt multiple modes. Besides the situation described above, there could be other possible ways through which autophagy affects inflammations (Joven et al., 2014). Autophagy is known as a pathway which maintains the balance of production and degradation of cellular components, monitoring the intracellular homeostasis. In this way, not only the gene expression of cytokines could be modulated due to stress stimulation, but also the vacuolar transportation and secretion of cytokines can be related to autophagy (Jiang et al., 2013). In addition, as a checkpoint for malfunctional proteins, autophagy could degrade wrongly synthesized or modified cytokines (Giegerich et al., 2014). These hypotheses need to be confirmed by further studies. However, the importance of autophagy in inflammation conditions, especially in chronic inflammation-induced AIDs is noticeable.

B.3.3: Autophagy and antigen presentation

During antigen presentation, antigenic proteins need to be degraded and processed to generate corresponding antigens for further presentation. The MHC I presentation is known to deal with intracellular antigens. Processed ligands are generated by proteasomes and then imported into the ER for loading onto MHC I molecules. The extracellular substrates are processed to generate antigenic peptides that can be presented by MHC II molecules, for CD4 T cells activation (Romao et al., 2013).

As introduced in previous chapters, autophagy is a degradative process, which encapsulates constituents and delivers them to lysosomes for degradation. In this

way, autophagy is suggested to contribute to the antigen processing, which is crucial for antigen presentation (Deretic et al., 2013; Munz, 2016) (Figure 24).

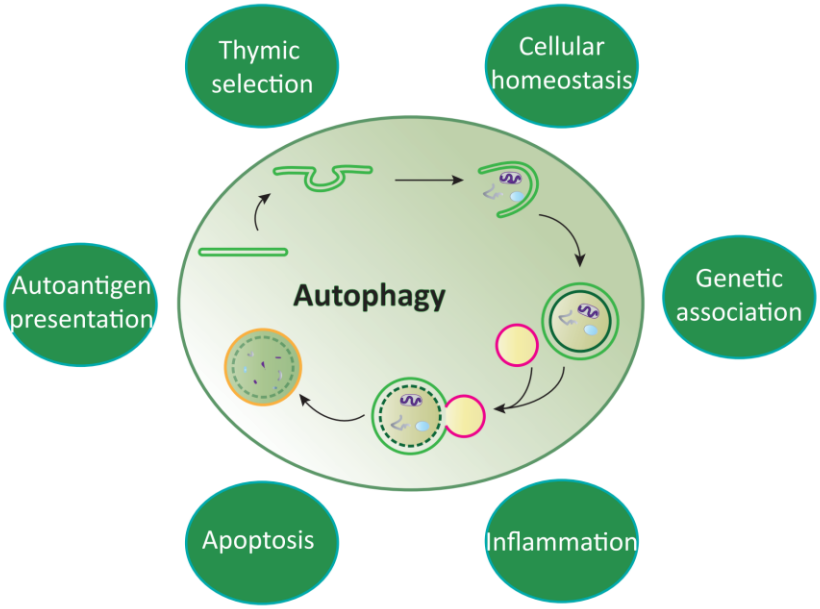

Figure 24. Roles of autophagy in AIDs

Autophagy has been found to participate in both innate and adaptive immune responses in AIDs. Crucial activities like thymic selection, apoptosis and autoantigen presentation are found to be associated with autophagy pathway, highlighting the important roles of autophagy in AIDs.

This has been confirmed by studies that identified autophagy as a pathway, which modulates intracellular antigens processing and presentation in MHC I presentation (Oliveira et al., 2015). In herps simplex virus type 1 (HSV-1) infected macrophages, distinct forms of autophagy facilitate the presentation of HSV-1 antigens on MHC I molecules (English et al., 2009), which suggests a role of autophagy in MHC I presentation.

Not only MHC I pathway, but also MHC II molecules-mediated presentation is found to be related to autophagy. By decreasing the activity of lysosomal cathepsins, autophagy alters the antigen processing and promotes the presentation of intracellular peptides by MHC II molecules in DCs (Dengjel et al., 2005).

In addition, autophagy has been found to play a role in antigen cross-presentation (Mintern et al., 2015), implying the influence of autophagy in both classical and non-

classical antigen presentations. The roles of autophagy in various antigen presentation pathways are well identified in pathogen-host interactions, which shed light on possible mechanisms of autophagy in autoantigen presentation.

One hypothesis for AIDs development is that post-translational modifications of self-peptides generate neo-antigens, which can be recognized by the immune system and form the target of autoantibodies, thus leading to autoimmune responses and further possible development of AIDs. In recent studies, the self-epitopes that generated by abnormal citrullination are underscored. In RA, citrullinated peptides have shown high affinity to arthritis-susceptible HLA-II molecules (Reyes-Castillo et al., 2015). In addition, citrullinated epitopes are found to play important roles in other AIDs, including T1D and MS (Nguyen et al., 2016), indicating the autoantigenicity of citrullination in AIDs.

In hen egg-white lysozyme (HEL) transgenic mice, the processing of self-protein HEL has been found to result in citrullination of peptides. These modified peptides are recognized as antigenic ones and presented to MHC II molecules. Surprisingly, this process can be blocked upon autophagy inhibition (Ireland et al., 2012), suggesting that autophagy is crucial for the citrullination of self-peptides.

Another study described an autophagy inhibitor, the phosphopeptide P140, which can directly bind MHC II molecules and affects antigen presentation. P140 peptide decreases the overexpression of MHC chaperone HSPA8 and impairs its refolding properties, leading to a decrease of CMA, which is abnormally increased in MRL/lpr B cells. Moreover, P140 decreases MHCII dimers in MRL/lpr antigen-presenting B cells, and down-regulates autoreactive T-cell priming by modulating autophagic pathway (Monneaux et al., 2007; Page et al., 2011b; Macri et al., 2015).

Experimental autoimmune encephalomyelitis (EAE) is a mouse model of human multiple sclerosis (MS). This disease is mainly driven by autoreactive CD4 T cells. It was reported by Bhattacharya et al. that deletion of *atg7* in DCs reduced T cells priming and ameliorated EAE, and that administration of the autophagy-lysosomal inhibitor chloroquine could prevent disease (Bhattacharya et al., 2014). These

studies suggest the versatile roles of autophagy in autoantigen presentation, thus providing more indications for the etiology of AIDs and also valuable information for therapies development.

B.4: Autophagy and other diseases

B.4.1: Autophagy in infection

The studies of autophagy in pathogen-host responses reveal that autophagy machinery interacts with bacteria and virus during the processes of infection (Espert et al., 2008). The selective autophagic pathway that targets intracellular pathogens, xenophagy, protects against infections by degrading intracellular bacteria (such as *Streptococcus pyogenes*, *Mycobacterium tuberculosis, Shigella flexneri, Salmonella enterica*), viruses (such as HSV-1, EBV, HCV) and protozoan pathogen (Toxoplasma gondii) (Paulus et al., 2015).

The mechanism of xenophagy in defending against group A Streptococcus (GAS) infection has been well defined. GAS invades host cells *via* the endocytic pathway, and then escapes from endosomes to the cytoplasm by secreting streptolysin O. After escaping into the cytoplasm, GAS is captured by autophagosome-like vacuoles and then degraded after fusion of these vacuoles with lysosomes. In autophagy-deficient cells, GAS can survive, proliferate, and be released from the cells (Nakagawa et al., 2004). Interestingly, the autophagosome-like vacuoles that engulf intracytoplasmic bacteria share some similarity with autophagosomes, but there are some distinctions. One apparent difference is the vacuole size. The diameter of GAS containing autophagosome-like vacuoles can be as large as 10μm, whereas the diameter of autophagosomes is normally 0.5-1μm (Yoshimori et al., 2009). Another distinction is that these large vacuoles are generated by small isolation membranes in a Rab7-dependent manner (Yamaguchi et al., 2009). Rab7 is an adaptor that mediates the autophagosome–lysosome fusion process in autophagy (as introduced in the previous chapter B.2.3), which suggests the escaping of GAS might be attributed to the blockage of autophagosome–lysosome fusion process.

With the capability of eliminating invading pathogens, autophagy is recognized as a protective mechanism against infections (Espert et al., 2007). However, autophagy-mediated responses vary depending on the type of invaders as well as host cell type. In some conditions, the intracellular microbes develop strategies to compromise, inhibit, and even make use of host cell autophagy systems to promote their own survival, replication, and pathogenesis.

Taking Legionella pneumophila as an example, the intracellular pathogen could interfere with autophagy by RavZ, which acts as a cysteine protease that specifically targets LC3-II. As introduced in a previous chapter (B.2.2.1), LC3 contains a C-terminal glycine residue, which can be modified by E1, E2 and E3-like enzymes to generate LC3-II. RavZ hydrolyzes the amide bond between tyrosine residue and the C-terminal glycine residue, generating a LC3 protein without active glycine, which therefore cannot be reconjugated by ATG7 and ATG3. In addition, RavZ can prevent LC3 accumulation on the structure of phagophores and inhibit the formation of autophagosomes (Choy et al., 2012).

B.4.2: Autophagy and neurodegenerative diseases

Autophagosomes and other prelysosomal autophagic vacuoles (AVs) are abnormally abundant in neocortical biopsies from Alzheimer disease (AD) brain. Large numbers of autophagosomes, multivesicular bodies, multilamellar bodies, and cathepsin-containing autophagolysosomes are found to present within synaptic terminals and even exist as the predominant organelles of dystrophic neurites. The accumulations of immature forms of autophagosomes suggest that the machineries of autophagosome formation and autophagosomes-lysosomes fusion may be impaired (Nixon et al., 2005).

Not only in human, autophagy is also found to be involved in the development of neurodegenerative diseases in animal models (Muller et al., 2017). Numerous double- or multiple-membrane autophagosomes, autophagolysosomes and large multivesicular bodies are observed by EM in two different neuronal degeneration models caused by prion, the Echigo-1 strain of Creutzfeldt-Jakob disease (CJD) in

hamsters and the Fujisaki strain of Gerstmann-Sträussler-Scheinker (GSS) syndrome. In both models, autophagic vacuoles were observed in several cellular compartments including neuronal cell bodies, cytoplasm of neurons, and especially dystrophic neurites, suggesting that autophagy is an important mechanism of neuropathology in prion-induced neurodegenerative diseases (Liberski et al., 2011).

Another neurodegenerative disorder, Parkinson's disease is also linked with deregulation of autophagy (Dehay et al., 2013; Bourdenx et al., 2016). Parkinson's disease is characterized by damaged mitochondria and intracellular inclusions containing α-Synuclein named Lewy bodies. Overexpression of mutant A53T α-synuclein leads to massive mitochondrial dysfunction, which causes bioenergetic deficit and neuronal degeneration. This process can be blocked by silencing autophagy-related genes, suggesting the role of autophagy in α-synuclein accumulation and mitochondrial dysfunction in Parkinson's disease (Choubey et al., 2011).

B.4.3: Autophagy and cancer

Autophagy is claimed to be involved in cancer development, and modulation of autophagy has been suggested as a mechanism for cancer treatment. Several studies evidenced the association of Beclin 1 with cancers (White et al., 2015). Autophagy is identified as a protective tumor-suppression mechanism in hepatitis B virus-induced premalignant lesions, and heterozygous disruption of Beclin 1 results in increased cellular proliferation and accelerates the development of spontaneous malignancies (Qu et al., 2003). In another study, endophilin B1 (Bif-1) regulates cell survival *via* an Beclin 1-dependent mechanism, and knockout of Bif-1 significantly enhances the development of spontaneous tumors in mice (Takahashi et al., 2007).

The PI3K pathway is an evolutionarily conserved mechanism that regulates diverse cellular processes, including growth, survival, proliferation, and apoptosis (Hawkins et al., 2015). *PI3K* and its negative regulator *PTEN* are two of the most frequently mutated genes in human cancers. These mutations result in unregulated growth which promotes tumorigenesis (Ortega-Molina et al., 2013). The oncogenic property

of *PI3K* mutants is associated with its role in autophagy. In coincidence, natural products that display anti-tumor activities are found to be able to induce autophagy-related cell death *via* the PI3K/Akt/mTOR pathway in cancer cells (Sun et al., 2013; Lien et al., 2017).

Based on the known roles of autophagy in tumors, modulators targeting autophagy provide new possibilities to cancers therapies (Katheder et al., 2017). In a Myc-induced model of lymphoma, tumor cells are resistant to apoptosis due to a lack of nuclear p53. Administration of p53 activator results in tumor regression followed by tumor recurrence. It was found that the activation of p53 was associated with the induction of autophagy in surviving cells, and autophagy serves as a survival pathway for tumor cells after treatment with apoptosis activators (Amaravadi et al., 2007). Inhibition of autophagy combined with therapies designed to induce apoptosis might be a good strategy in cancer therapy (Sanderson et al., 2017).

AIM OF THE STUDY

As it is described in the introduction of this manuscript, SS is one of the most common systemic AIDs, which affects 0.5-3% of the population (Kvarnstrom et al., 2015). The hallmark of this chronic inflammatory disease is the lymphocytic infiltration in exocrine glandular tissues. Clinically, lymphocytic infiltration in the salivary and lacrimal glands causes destruction of glandular tissues, and leads to symptoms like dry eyes and dry mouth (Haga et al., 2012). The traditional therapeutics for SS could relieve dryness or inflammation, but are not efficient at treating systemic disorders.

Prior to this study, our team found a 21-mer linear peptide P140, which has been shown to be an efficient therapeutic tool to ameliorate clinical and biological manifestations in both SLE patients and lupus-prone mice (Monneaux et al., 2003; Page et al., 2011a; Page et al., 2011b; Schall et al., 2012; Zimmer et al., 2013). Upon intravenous administration of P140 peptide, lupus-prone mice were able to resist infectious viral challenge, with an equal efficacy compared to non-treated MRL/lpr mice (Monneaux et al., 2007). Moreover, P140 exerted prominent protection against lupus, as it dramatically decreased proteinuria and increased mice survival (Page et al., 2011b). As SS shares several similarities with SLE, such as a strong sex bias of female, increased autoantibodies, and systemic abnormalities of immune responses, our first aim was to test if P140 peptide could provide therapeutic effects in SS (Figure 25).

MRL/lpr is a well-established mouse model that mimics several characteristics of patients with SS, including lymphocytic infiltrations in SGs and lacrimal glands as well as anti-SSA/Ro and anti-SSB/La autoantibodies. Moreover, the pathologic progression in MRL/lpr mice can be used to study different stages of SS in patients. In addition, the short life span of MRL/lpr mice compared to other models can be a useful parameter to evaluate the survival effect of therapeutics. Therefore, we used MRL/lpr mice as mouse model to examine the effects of P140 in SS. Two approaches were used to administrate P140 in MRL/lpr mice, a so-called “short-term” treatment that involved one injection of P140 in 11-13 weeks old mice and SGs were collected on the 5th day; and a so-called “long-term” treatment that included 4 injections of

P140 into young mice during a period of 18 weeks (SGs examined at week 23). After the administration, we evaluated the pathological features of SS in MRL/lpr mice, including the lymphocytic infiltration in SGs, histological profile of SGs, and serological autoantibody levels. By comparing the effects of P140 with control groups of mice (administration with saline of "scramble" peptide), our aim was to elaborate some conclusions about the possible therapeutic effects of P140 peptide.

The second aim of our study was to determine the role of autophagy in SS (Figure 25). As largely mentioned above in the Introduction chapters, autophagy has been demonstrated to be a regulatory mechanism in autoimmunity, participating in the autoreactive immune cells activation, autoantigen presentation and proinflammatory cytokines production. The role of autophagy has been described in AIDs like RA, MS and SLE. However, the role of autophagy in SS has not yet been described. Our purpose was therefore to study autophagy in SGs cells of MRL/lpr mice and to find out whether autophagy pathway is involved in the pathogenesis of SS.

The third aim of our project was to decipher the possible role of P140 in autophagy in SS settings (Figure 25). The previous data generated in my team have shown that CMA pathway is a target of P140 (Macri et al., 2015). The beneficial effects of P140 in lupus have been suggested to be linked to its inhibition effect on HSPA8 chaperone protein and CMA, as it was found to be able to down-regulate HSPA8 and LAMP-2A in B cells of lupus prone mice (Page et al., 2011b). However, the role of P140 in autophagy in SS settings has not yet been described. Here in this study, our aim was to find out the influence of P140 on autophagy in SGs cells of MRL/lpr mice.

In these studies, we used a combination of immunological and biochemical techniques to evaluate therapeutic effects of P140 in SS. In the first article, we describe these methods that are complex and require a careful validation to ascertain the validity of results. In the second paper, we describe our results generated in MRL/lpr mice. In this article, we present experimental arguments leading to our conclusion that autophagy plays an important role in SS. It is the first

study that reveals the involvement of autophagy in SS pathology. This study therefore provides useful information on the pathogenesis of SS and suggests autophagy as a potential therapeutic target for SS.

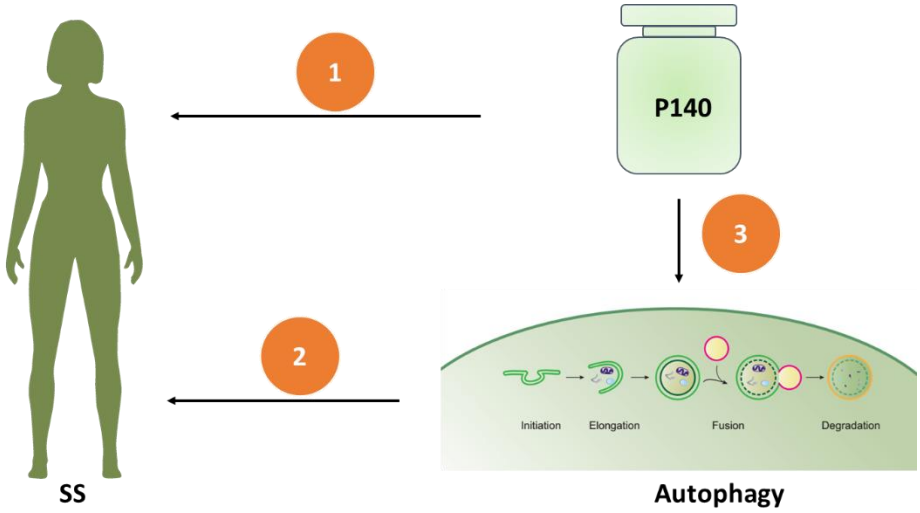

Figure 25. Schematic summary: the aims of this study

In this project, we would like to 1, test if P140 peptide could offer therapeutic effects in SS; 2, determine the role of autophagy in SS; 3, discover the role of P140 on autophagy in SS settings.

RESULTS

PUBLICATION 1

Fengjuan Wang, Baihui Li, Nicolas Schall, Maud Wilhelm and Sylviane Muller

Assessing Autophagy in Mouse Models and Patients with Systemic Autoimmune Diseases

Introduction

Autophagy is a crucial mechanism for cell renewal and survival. It degrades the misfolded or aggregated proteins, as well as damaged organelles. The autophagy pathway therefore acts as a sentinel in cellular metabolisms. Several roles of autophagy have been identified in certain immune functions, such as inflammation, antigen presentation, and pathogens clearance. Recent studies highlighted the role of autophagy in the context of immunological abnormalities including AIDs. AIDs are recognized to be complex in both etiology and pathology. The roles of autophagy unveiled shed light on the mechanisms of AIDs induction and perpetuation, thus making autophagy to be a crucial pathway in AIDs studies. In addition, understanding of autophagy in AIDs may provide new possibilities for diagnosis and therapeutics design and development.

However, to study the processes of autophagy or the effects of autophagy in AIDs conditions can be difficult. Autophagy has been recognized as a dynamic process, which involves various organelles and around 40 proteins and probably more. The autophagic machinery is constructed with many complexes, which contain diverse activators, adaptors, inhibitors and recruiters. Each step of autophagy depends on the cooperation of multiple effectors and efficient signaling transduction. For these reasons, the methods applied to study autophagy can be miscellaneous and should be extremely well calibrated and designed.

The studies of autophagy mainly focus on two aspects. One of them is to identify the proteins involved in each step and their corresponding roles. Autophagy related proteins are named as ATG proteins, which indicate their specific and professional roles in autophagy pathway. Crucial proteins can be used as markers of autophagy such as LC3, SQSTM1 and other proteins largely described in the Introduction chapters.

The other aspect is to consider autophagy as a whole process which can be regulated experimentally. Autophagic inducers and inhibitors like rapamycin, 3-methyladenine (3-MA), chloroquine (CQ), bafilomycinA1 (BafA1), pepstatin A and E64D are widely

used in autophagy studies. Application of these modulators would be helpful for autophagy-manipulating studies in certain conditions.

The aim of our first paper was to describe the methods we applied in our laboratory to investigate autophagy in autoimmune conditions. Our study involved both autoimmune mouse model and patients. MRL/lpr lupus-prone mice were used as a mouse model for SLE and sSS in these studies. We analyzed autophagic pathways in autoimmune symptoms-related organs and tissues including spleen, lymph nodes and SGs. In addition, we described the techniques we used to analyze autophagy in PBMCs of patients with SLE. The methods regarding cell collection and purification, as well as techniques applied to investigate autophagy in these cells were also described in detail.

This study aims to provide some technical information for autophagy (both MaA and CMA) studies in AIDs. These methods described in our studies might be adapted to other studies in which autophagy is involved, and may be able to improve our understanding of the implication of autophagy in AIDs.

Result: Assessing Autophagy in Mouse Models and Patients with Systemic Autoimmune Diseases

cells

MDPI

Review

Assessing Autophagy in Mouse Models and Patients with Systemic Autoimmune Diseases

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Abstract: Autophagy is a tightly regulated mechanism that allows cells to renew themselves through the lysosomal degradation of proteins, which are misfolded or produced in excess, and of damaged organelles. In the context of immunity, recent research has specially attempted to clarify its roles in infection, inflammation and autoimmunity. Autophagy has emerged as a spotlight in several molecular pathways and trafficking events that participate to innate and adaptive immunity. Deregulation of autophagy has been associated to several autoimmune diseases, in particular to systemic lupus erythematosus. Nowadays, however, experimental data on the implication of autophagy in animal models of autoimmunity or patients remain limited. In our investigations, we use Murphy Roths Large (MRL)/lymphoproliferation (lpr) lupus-prone mice as a mouse model for lupus and secondary Sjögren's syndrome, and, herein, we describe methods applied routinely to analyze different autophagic pathways in different lymphoid organs and tissues (spleen, lymph nodes, salivary glands). We also depict some techniques used to analyze autophagy in lupus patient's blood samples. These methods can be adapted to the analysis of autophagy in other mouse models of autoinflammatory diseases. The understanding of autophagy implication in autoimmune diseases could prove to be very useful for developing novel immunomodulatory strategies. Our attention should be focused on the fact that autophagy processes are interconnected and that distinct pathways can be independently hyper-activated or downregulated in distinct organs and tissues of the same individual.

Keywords: macroautophagy; chaperone-mediated autophagy; systemic lupus erythematous; Sjögren's syndrome; autoimmunity; MRL/lpr mice; autophagy markers; salivary glands

1. Autophagy in Immunity and Autoimmune Diseases

Autophagy is a cellular process, which removes unwanted cytoplasmic content, such as long-lived proteins, damaged organelles or invading microorganisms, via lysosomal degradation. Key features of this cytoprotecting process lead to cellular generation of energy and recycling of metabolic precursors. Three major pathways characterize bulk autophagy: macroautophagy (MaA), chaperone-mediated autophagy (CMA) and microautophagy (Figure 1). Other forms of autophagy exist that are more specific, e.g., mitophagy, which involves the selective degradation of mitochondria, lipophagy (degradation of lipids), and xenophagy, a type of selective autophagy that is used for eliminating invading pathogens. In MaA, a double membrane structure is formed to capture parts of cytosolic content to form the autophagosome, which further fuses with lysosomes to form the autolysosome in which the sequestered content is degraded [1] (see [2] for the nomenclature of autophagic vesicles). In CMA, specific substrates that contain the KFERQ-like motif are recognized by heat shock 70 kDa

protein 8 (HSPA8/HSC70) chaperone protein, which targets the substrates to the surface of lysosomes and facilitates the binding of substrates to lysosome-associated membrane protein type 2A (LAMP-2A). The binding of substrates to LAMP-2A leads to the multimerization of the latter, and the translocation of substrates into lysosomal lumen, followed by their degradation by lysosomal proteases [3]. In microautophagy, substrates are trapped in vesicles formed by the invagination of lysosomal membrane, which are later pinched off from lysosomal membrane and degraded by lysosomal proteases [4].

Autophagy plays a broad spectrum of physiological roles, and acts decisively and in a coordinated, interconnected manner in various cellular processes, including immune processes. The role of autophagy in innate and adaptive immunity includes direct elimination of microorganisms, control of inflammation, secretion of immune mediators, control of homeostasis of immune cells and antigen presentation, which have been detailed in several excellent reviews [5–12] and summarized in Figure 1. The role of autophagy in major histocompatibility complex class II (MHCII) antigen presentation is illustrated in Figure 1. Using "loss- and gain- of-function" mutation-based experiments, both MaA and CMA have been demonstrated to play a role in MHCII antigen presentation. Deletion of proteins implicated in MaA, such as phosphoinositide 3-kinase (PI3K), autophagy-related 12 (Atg 12), autophagy-related 5 (Atg 5), compromises antigen presentation through MHCII and $CD4^+$ T cell responses, while targeting antigens to autophagosomes through coupling microtubule associated protein 1 light chain 3B (MAP1LC3B; a MaA protein) to antigens dramatically increases their MHCII presentation [11–15]. Furthermore, pathogens, which inhibit MaA, are capable to escape from MHCII presentation [16,17]. Overexpression of LAMP-2A (CMA protein) has been found to be in favor of MHCII presentation of autoantigens [18]. Beside its role in MHCII antigen presentation, MaA has been reported to contribute to major histocompatibility complex class I (MHCI) presentation as well [11,12].

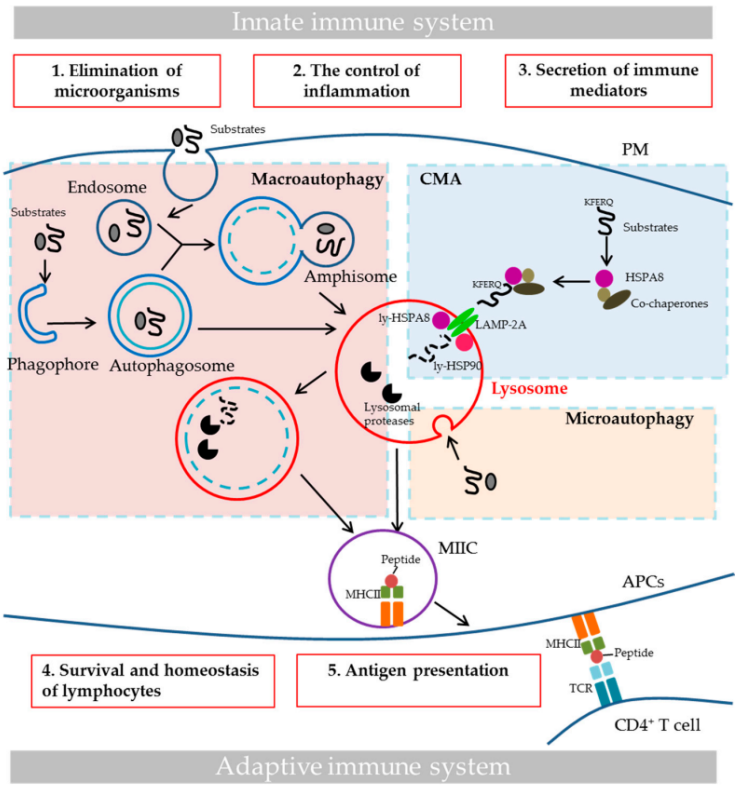

Figure 1. The three major autophagy pathways and the role of autophagy in immunity. The three principal bulk autophagy pathways are illustrated here: macroautophagy in the pink area of the figure,

CMA in the blue area and microautophagy in the yellow area. The roles of autophagy in innate and adaptive immunity are highlighted in the red boxes. (**1**) Autophagy can directly eliminate invading microorganisms through xenophagy, MAP1LC3/LC3-associated phagocytosis (LAP), sequestosome-like receptors recruitement and still other scenarios [6]. (**2**) Autophagy controls inflammation, notably by affecting TLR signaling and suppressing inflammasome activation [19]. (**3**) Autophagy also controls inflammation through regulatory interactions with innate immune signaling pathways, via the removal of endogenous inflammasome agonists and through effects on the secretion of immune mediators, such as cathepsin K, lysozyme, IL-6, IL-8, damage-associated molecular patterns, etc. (**4**) Autophagy plays important role in T cell repertoire selection, maturation activation and polarization. Moreover, it is essential for the survival and function of B1 cells and plasma cells [20]. (**5**) As is illustrated here, autophagy participates in MHCII antigen presentation, and it could impact MHCI presentation as well [21]. APCs, antigen-presenting cells; CMA, chaperone-mediated autophagy; LAMP-2A, lysosomal-associated membrane protein type 2A; MAP1LC3/LC3, microtubule associated protein 1 light chain 3; ly-HSPA8 and ly-HSP90, lysosomal luminal HSPA8 and HSP90; MIIC, late endosomal MHC class II compartment; MHCI and II, major histocompatibility complex class I and II; IL-6 and -8, interleukin-6 and -8; PM, plasma membrane; TCR, T-cell receptor; TLR, toll-like receptor.

Autoimmune diseases represent a group of more than 100 illnesses in which the immune system shows breakdown of tolerance and consequently targets self-tissues and organs. It can be organ specific, such as in type 1-diabetes in which pancreas is attacked, or systemic when a variety of organs and tissues are affected, such as in systemic lupus erythematosus (SLE) in which skin, muscle, heart, joints, skin, lungs, blood vessels, liver, kidneys and nervous system can be damaged [22–24]. Based on epidemiology studies it is estimated that between 25 and 50 million Americans have an autoimmune disease.

Deregulation of autophagy has been suggested to be implicated in autoimmune diseases. Genome-wide association studies on SLE have identified several autophagy-related susceptibility genes, including *ATG5*, *ATG7*, *PRDM1*, *DRAM1* and *IRGM* (Table 1) [25–28]. A few papers have described aberrant autophagy in B and T lymphocytes collected from peripheral blood mononuclear cells (PBMCs) from SLE patients, and from lupus mice models [29–32]. Accumulated autophagosomes and increased MaA flux have been observed in T cells from both SLE patients and Murphy Roths Large (MRL)/lymphoproliferation (lpr) or MRL/MpJ-Fas^{lpr}, henceforth referred to as MRL/lpr, and the F1 hybrid of New Zeeland black (NZB) and New Zeeland white (NZW), or (NZB/W)F1 lupus mouse models [29]. These dysfunctions could be closely related to well-documented T-cell autoreactivity and abnormal TCR signaling in lupus [33]. Similarly, the increase of autophagosomes and MaA flux has been observed in B cells from PBMCs of SLE patients and NZB/W lupus mice [31]. CMA has also appears to be upregulated in MRL/lpr B splenocytes [30]. B cells are important antigen-presenting cells (APCs) in lupus. They contribute to the abnormal (auto)antigen presentation [34,35]. As summarized above, both MaA and CMA have been suggested to play an important role in antigen presentation. We have proposed that the hyperactivity of MaA and CMA, found notably in lupus B cells, contribute in a decisive manner to the aberrant (auto)antigen presentation in lupus [30,36]. It is possible that autoantigens can be substrates of both MaA and CMA. However, experimental details directly linking the irregular autophagy and altered antigen presentation in autoimmune diseases are still not available. Furthermore, one needs to take into consideration that lysosomes are dysfunctional, at least in some organs [30], which also contributes to the abnormal (auto)antigen presentation in lupus [36]. MaA in B cells has been shown to mediate autoimmunity in *Tlr7* transgenic mouse strains [37]. These findings and other data strongly suggest that the abnormalities of both autophagic pathways in immune cells are directly or indirectly linked to the autoimmune pathology of lupus.

The status of autophagy in other autoimmune diseases is less well known, likely due to the difficulty of analyzing autophagy in patient's samples and the fact that pertinent animal models are lacking or imperfectly mimic the human disease. In this recently growing area of research, hereby we

update available information summarized previously regarding autophagy in various autoimmune diseases [23]. The model systems or the type of patients' samples tested, the methods used and the data obtained are highlighted (Table 1). Other information centered on neurological autoimmune diseases is compiled elsewhere [38].

Table 1. List of autoimmune diseases with autophagy abnormalities and of the type of animal model organs/tissues or patient's samples tested.

Autoimmune Diseases	Autophagy Abnormalities	Methods	Model Systems or Patient Samples Tested	Ref.
Systemic lupus erythematosus	Associated genes: *ATG5*, *ATG7*, *PRDM1*, *DRAM1*, *IRGM*	N/A	N/A	[25–28]
	Accumulation of autophagosomes and increased MaA flux in T splenocytes	WB, EM	MRL/lpr and (NZB/W)F1 mice (thymus, spleen)	[29]
	Increased amount of autophagosomes in T cells	EM	Patients (blood)	
	Increased MAP1LC3 puncta, decreased SQSTM1/p62, and increased MaA flux in B cells	MIFC, FC	NZB/W F1 mice (spleen, bone marrow)	[31]
	Increased MAP1LC3 puncta and increased MaA flux in B cells	MIFC	Patients (blood)	
	Increased mRNA of Beclin-1, MAP1LC3 and SQSTM1 in PBMCs	qPCR	Patients (blood)	[39]
	Increased expression of *ATG5*, *ATG12* and *BECN1* in macrophages	pPCR	Induced lupus mice (spleen, kidneys) and patients (blood)	[40]
	Increased HSPA8 expression in B cells	WB, FC, qPCR	MRL/lpr mice (spleen)	[41]
	Increased LAMP-2A and CTSD expression in B cells; defective lysosomes in B cells	WB, FC	MRL/lpr mice (spleen)	[21]
	Increased MAP1LC3-II protein level	FC	MRL/lpr mice (spleen)	[42]
Secondary Sjögren's syndrome	Defective autophagy in salivary glands	WB, EM	MRL/lpr mice (salivary glands)	Li & Muller, unpublished
Crohn's disease	Associated genes: *ATG16L1*, *IRGM*	N/A	N/A	[43–46]
Rheumatoid arthritis	Associated genes: *ATG5*, *ATG7*, *BECN1*	N/A	N/A	[47,48]
	Increased protein expression of ATG7 and BECN1	WB, IHC	Patients (bones)	[47]
	Increased BECN1, ATG5, MAP1LC3 mRNA expression; increased MAP1LC3-II protein level	qPCR, IH, WB	Patients (synovial tissues)	[49]
	Decreased SQSTM1 protein expression	WB	Patients (synovial tissues)	[50]
Polymyositis	Increased levels of MAP1LC3-II and decreased level of p70S6 kinase	WB	Patients (muscle)	[51]
Multiple sclerosis	Associated gene: *ATG5*	N/A	N/A	[52]
	Increased mRNA and protein level of ATG5	qPCR, WB	EAE mice (blood) and patient (blood and brain)	[52]
	Decreased expression of *ATG16L2* and *ATG9A* genes and increased expression of *ULK1* gene	qPCR	Patient (blood)	[53]
Type 1 diabetes	Decreased MAP1LC3 and ATG5/12 protein level	WB	Induced diabetic mice (heart)	[54]

ATG, autophagy related; BECN1, beclin-1; CTSD, cathepsin D; EAE, experimental autoimmune encephalomyelitis; EM, electron microscopy; FC, flow cytometry; IHC, immunohistochemistry; MAP1LC3/LC3, microtubule associated protein 1 light chain 3; MaA, macroautophagy; MIFC, multispectral imaging flow cytometry; N/A: not applicable; qPCR: quantitative polymerase chain reaction; SQSTM1/p62, sequestosome-1; ULK1, Unc-51 like-autophagy activating kinase 1; WB, Western blot.

2. MRL/lpr Mice as a Model for SLE

Numerous murine models have been developed to understand the cellular and genetic requirement for SLE induction, development and recurrence after asymptomatic periods. There are spontaneous lupus models, including the (NZB/W)F1 and MRL/lpr [55] mice, which display

different MHC haplotypes (H-$2^{d/z}$ and H-2^{k}, respectively). Both models have been used for autophagy studies. There are also induced models, such as the pristane-induced model and the chronic graft-versus-host-disease models [56], and genetically-modified mouse models [57,58]. Several reviews have described these different models of lupus [59,60].

The MRL/lpr strain is one of the best characterized models for SLE. It develops many SLE-like features, including increased levels of autoantibodies (antinuclear, anti-double stranded (ds)DNA, and anti-Sm antibodies) and circulating immune complexes that can be pathogenic and at the origin, at least in part, of the glomerulonephritis classically visualized in these mice. The MRL background plays an important role in the development of lupus phenotype in these mice. $MRL^{+/+}$ mice, which develop a milder and slower disease than MRL/lpr mice, while also sick, are sometimes used as mouse control for MRL/lpr mice. The recessive autosomal mutation *lpr* is responsible for the nonfunctional transcription of Fas (CD95), a member of the TNF receptor super family, leading to defects in apoptosis and an impressive amplification of the disease [59,61]. This mutation that spontaneously arose on the MRL genetic background aggravates the lupus-like symptoms in these mice, due in particular to abnormally surviving autoreactive $CD4^+$ T cells and B cells in MRL/lpr. Unlike human SLE disease that mainly affects women (9:1 female to male ratio of disease incidence in human), both males and females develop SLE-like phenotype in MRL/lpr mice. In our laboratory, however, we principally work with female mice to better mimic the human (hormonal) conditions. We have privileged the MRL/lpr mouse model because with regard to (female) (NZB/W)F1 mice, lupus symptoms appear much earlier in these mice (10–14 vs. 40 weeks), and because they are much more severe than in (NZB/W)F1 mice. We should note, however, that MRL/lpr mice display some clinical and biological features that are not typically found in lupus patients or in (NZB/W)F1 mice. These are the presence of serum circulating rheumatoid factors, for example, salivary gland (SG) involvement, and peripheral cell lymphoproliferation. MRL/lpr mice also show progressive and lymphadenopathy due to the accumulation of an unusual population of $CD4^-CD8^-CD3^+CD45^+/B220^+\alpha\beta^+$ double negative (DN) T cells [62,63]. On the other hand, they develop a syndrome that in addition to be serologically similar to SLE, is pathologically close of the human disease in terms of kidney and brain involvement [64]. They also develop dermatitis and vasculitis.

We have previously reported a hyperactivity of MaA in T cells from both thymus and spleen from MRL/lpr mice [29]. Using electron microscopy, we have quantified the amount of autophagic vacuoles in splenic T cells from both MRL/lpr mice and control CBA/J mice (inbred strain from a cross of a Bagg albino female and a DBA male), and found a much higher amount of autophagosomes in MRL/lpr spleen T cells. Analyses of the MAP1LC3-II levels measured by Western blotting in the presence and absence of lysosomal inhibitors (pepstatin A and E64d) indicated that there is a higher MaA flux in MRL/lpr T cells compared to CBA/J T cells. Together, these data argue for a higher MaA activity in splenic T cells from MRL/lpr vs. control mice. In the thymus, there was no significant change regarding the amount of autophagosomes, but the MaA flux, as measured by Western blotting, was found to be increased compared to control mice [29]. Although not observed in our earlier studies, MaA activity has also been reported to be over-activated in splenic B cells of (NZB/W)F1 mice [31]. In addition to the above results related to MaA, we also identified an overexpression, in MRL/lpr mice, of CMA-specific lysosomal receptor LAMP-2A, suggesting a higher CMA activity in splenic B cells in these mice [30]. Besides the abnormality of autophagic pathways, biochemical studies showed an increase of cathepsin D protein expression, an elevated lysosomal volume and a significant raise of average lysosomal pH in the MRL/lpr B cells [30,36], strongly suggesting the existence of lysosomal dysfunction in those cells, which might be related to the aberrant autophagy activity that was observed.

As mentioned above, besides being a pertinent animal model for SLE, MRL/lpr mice can be used to mimic other autoimmune diseases. For example, the SGs of MRL/lpr mice have been used to study secondary Sjögren's syndrome [65]. The brain of MRL/lpr mice is an excellent model to investigate elements of neuropsychiatric lupus, a severe form of SLE featured by various brain malfunctions [64,66].

3. Methods and Notes

In this short technical review, first we will explain how to adequately collect and purify immune cells from organs and tissues from lupus individuals (mice and patients), and then we will shortly describe the various techniques currently applied in our laboratory to measure autophagy activity in these cells (Table 2).

Table 2. Techniques currently used in routine in our laboratory to evaluate MaA activity in different organs and tissues from autoimmune mice and patients.

	Mice			Human
	Spleen	**Lymph Node**	**Salivary Gland**	**Blood**
EM	Yes	No	Yes	Yes
WB	Yes	Yes	Yes	Yes
FC	Yes	No	No	Yes

EM, electron microscopy; FC, flow cytometry; WB, Western blot.

B and T cells display very limited cytosolic space, precluding extensive immunofluorescence studies in pooled lymphocytes and even less at the single cell level. Likely for the same inherent reasons, fluorescence microscopy approaches have seldom been used for studying autophagy in autoimmune diseases. Multispectral imaging flow cytometry (MIFC) only has been applied for studying MaA in B cells stained with anti-MAP1LC3 antibodies. MIFC is a combination of flow cytometry (FC) and fluorescence microscopy, which allows differentiating the punctate pattern of MAP1LC3-II and diffused fluorescence of MAP1LC3-I.

General notes:

- We highly recommend, whenever possible, to analyze specific cell subtypes rather than studying whole organ homogenates or unfractionated peripheral blood samples that contain mixed cell subsets, as the latter may exhibit very different autophagy activation status that can affect the detection of events.
- As recommended in authoritative reviews on autophagy, several different autophagic assays need to be applied to make reliable conclusions [67–70]. One single assay is by far not sufficient to determine whether the autophagy activity is abnormally increased or decreased. The number of individual samples analyzed also has to be sufficient to allow robust statistic interpretation of data.

3.1. Obtaining Cell Homogenates from Organs

3.1.1. Obtaining Homogenates from the Spleen

Splenocytes are obtained according to standard procedures, as described for example in the series of *Current Protocols in Immunology* [71]. Briefly, spleens collected from control mice (e.g., CBA/J, C57BL/6 mice or $MRL^{+/+}$ mice) and MRL/lpr mice are placed in a cell strainer (40 or 70 µm), mashed using the plunger end of a syringe and rinsed with Roswell Park Memorial Institute (RPMI) culture medium supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and antibiotics. Cells are centrifuged and then treated with 1–2 mL ACK [150 mM NH4Cl, 10 mM KHCO3, 0.1 mM ethylene diamine tetra acetic acid (Na2EDTA), pH 7.2–7.4] lysis buffer to obtain a single spleen cell suspension (around 10 million from CBA/J or C57BL/6 control mice and 10 to 50 million cells from MRL/lpr mice) free of red cells for further tests.

The immune cell subpopulations in the total spleen cell fraction vary in MRL/lpr mice and control mice. For example, at 12 weeks of age, the spleen cells of C57BL/6 mice consist of 60% B cells ($CD3^{-}B220^{+}$) and 30% T cells ($CD3^{+}B220^{-}$), whereas the MRL/lpr splenocyte fraction is composed of 30% B cells ($CD3^{-}B220^{+}$), 20% T cells ($CD3^{+}B220^{-}$), and 10–40% DN T cells.

B and T cells can then be isolated from the total splenocytes using standard B and T cell isolation kits that are commercially available. We have chosen kits from Miltenyi Biotec (Bergisch Gladbach, Germany) (Pan B Cell Isolation Kit II, mouse, 130-104-443; Pan T Cell Isolation Kit II, mouse, 130-092-130), as they allowed better enrichment and higher purity of sub-populations of interest in our experiences. The purity of B cells or T cells isolated from MRL/lpr mice is around 90%, while that of B or T cells purified from CBA/J or C57BL/6 mice, is usually higher than 95%.

Notes:

- Some fat tissues are commonly found in the splenic cell suspension prepared from MRL/lpr mice (rarely observed in the case of control CBA/J or C57BL/6 mice). They can be removed by filtering cell fraction through a 40-µm cell strainer, according to our experiences.
- The spleen of MRL/lpr mice is usually 4–6 times the size of that of CBA/J mice at the same age (Figure 2A). It is important to take into consideration this huge difference, as for certain experiments pooling 2–3 spleens from CBA/J or C57BL/6 control mice will be required to have enough control splenocytes.
- The final purity of B or T cells should always be checked as it has been sometimes observed, for example, that depending on the isolation kits, 5–10% DN T cells can remain in the MRL/lpr B cell fraction in our experiences.

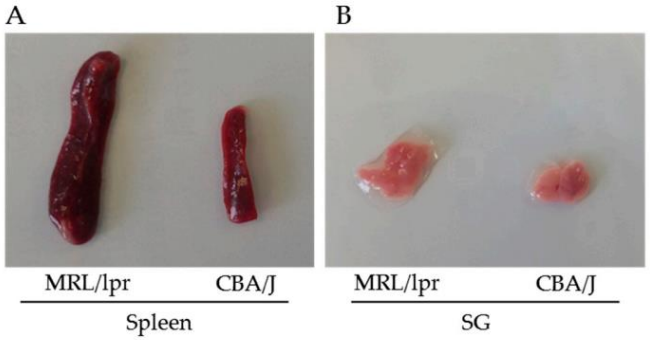

Figure 2. Images of spleens (**A**) and SGs (**B**) from MRL/lpr lupus mice and CBA/J control mice. The MRL/lpr and CBA/J mice were 26 and 28 weeks old, respectively.

3.1.2. Obtaining Homogenates from Lymph Nodes

The method developed to obtain cell suspension from lymph nodes (LNs) is identical to the one used above to prepare splenocytes from spleens [72]. Enzyme digestion is not required in order to obtain the immune cells from LNs. The LNs that are easier to collect are the mesenteric, brachial and inguinal nodes. The other LNs are generally much smaller. In order to obtain as many cells as possible, we routinely obtain all LNs to prepare the cell suspension. The amount of cells that can be obtained from all LNs is comparable to that obtained from spleens. However, depending on the purpose of study, one particular type of LNs can be isolated. Similar to spleen, in comparison to control mice, there is a dramatic increase of LN size in MRL/lpr mice. The number of B and T cells in LNs varies importantly among studies. This notably relies to the degree of immune cell activation. In a past study [73], we studied the activated state in LNs of $CD4^+$ T cells from MRL/lpr and CBA/J control mice. This was tested by analyzing the expression of CD44 and CD62L molecules at the surface of LN $CD4^+$ cells by flow cytometry. In unprimed seven-week-old MRL/lpr mice, the frequency of activated $CD4^+$ T cells ($CD44^{hi}/CD62L^{hi}$) and memory $CD4^+$ T cells ($CD44^{hi}/CD62L^{lo}$) was significantly increased compared to the corresponding cell subsets in CBA/J mice (13% vs. 3%, and 20% vs. 5%, respectively).

The isolation of B and T cells from LNs can be done using commercial T cell isolation kit (such as Pan T Cell Isolation Kit II, mouse, 130-092-130, Miltenyi Biotec,). Several reviews have been published which describe the conditions for isolating dendritic cells [70,74] and stroma cells [75].

3.1.3. Obtaining Homogenates from Salivary Glands

Isolation of SGs should be performed with a fine touch for avoiding their alteration and also collecting unwanted tissues. There are three major paired SGs in the mouse (as also in many other species), which are submaxillary (submandibular), parotid, and sublingual SGs, and minor SGs [76]. The minor SGs are located in the oral submucosa and tongue, and are missed, in general. The three other major SGs are closely associated and interconnected. They are located in the subcutaneous tissue of the ventral neck area. The lobulated submaxillary (submandibular) are the largest SGs. Submaxillary SGs of healthy male mice are larger and more opaque than those of females. A single excretory duct from the anterior dorsal surface of each gland opens on the floor of the oral cavity posterior to the incisor teeth.

Once collected, each SG is immediately digested by 2 mL RPMI medium supplemented with 2% (v/v) FBS, collagenase D (1 mg/mL; Roche, Basel, Switzerland, 11088866001) and DNase I (50 µg/mL; Roche, 10104159001) at 37 °C under gentle agitation. The tissues that remain after 1 h of treatment are mashed using the plunger end of a syringe on a cell strainer (70 µm). Two milliliters ACK buffer is then added to the resulting cell suspension of each gland to lyse red blood cells. Eight Milliliters RPMI medium supplemented with 10% (v/v) FBS is then added to stop the lysing of ACK and cells are centrifuged at 320× g for 5 min. Around 0.13 million cells can be obtained from 1 mg of SG.

In CBA/J SGs, there are no immune cells, whereas in MRL/lpr SGs, there is a characteristic infiltration of immune cells, which is composed of about 4% $CD4^+$ T cells, less than 4% of $CD8^+$ T cells and less than 0.1% of $CD19^+$ B cells (Li and Muller, unpublished data).

Notes:

- The size of SGs from MRL/lpr mice is usually 1.5–2 times larger compared to that of SGs from CBA/J or C57BL/6 mice at the same age, depending on the severity of the disease (Figure 2B).
- The concentration of collagenase D and DNase needs to be optimized, as an excessive enzyme concentration can induce a loss of cell viability and a too low enzyme dose will obviously lead to insufficient digestion.
- When SG cell suspensions are prepared for FC measurement, it is essential to add EDTA in the FC buffer (such as PBS supplemented with 2% v/v FBS) to avoid cell aggregation and ensure single cell suspension. We have determined that 0.3 mM EDTA is the optimal concentration as higher concentration causes toxicity and a lower concentration is insufficient to separate cells.

3.1.4. Isolating Peripheral Blood Mononuclear Cells from Patient's Blood

PBMCs can be obtained from human blood by standard Ficoll or Ficoll–Paque methods using density gradient centrifugation [77]. Briefly, blood is first diluted 1:1 in PBS; then, at room temperature, 30 mL diluted blood is carefully layered over 10 mL of Ficoll, e.g., Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA, 10771) and tubes are centrifuged at 1300× g for 20 min at room temperature in a swining-bucket rotor (Eppendorf, Hamburg, Germany, 5810R) without brake. The opaque interface that contains PBMCs is then carefully collected using a Pasteur pipette. The last step consists to wash PBMCs with PBS or RPMI medium to eliminate remaining Ficoll reagent.

The percentage of different sub-populations in the PBMC fraction varies somewhat among blood donors. In general, the PBMC fraction is composed of 40–50% $CD4^+$ T cells, 20–30% $CD8^+$T cells and 5–15% B cells. It is possible to isolate these populations using kits, such as B cell isolation kit II, human (Miltenyi Biotec, 130-091-151) and Pan T cell isolation kit II, human (Miltenyi Biotec, 130-091-156). The purity of cells obtained using these kits, is greater than 90% as determined by FC.

Notes:

- In the case of blood samples taken from lupus patients, we have occasionally observed a large amount of red blood cells remaining in the PBMC layer after centrifugation in Ficoll. In this case, an additional step of ACK lysis has been included after the Ficoll step for lysing these red blood cells.
- In the case of $CD4^+$ T cell isolation from blood, instead of performing a Ficoll density gradient centrifugation, RosetteSep^{TM} Human $CD4^+$ T Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, Canada, 15062) can be added directly to the whole blood to isolate $CD4^+$ T cells by negative selection.
- Note that the number of B cells recovered from the blood of lupus patients is usually very low (2–5 $\times$ 10^6 cells from around 40 mL of blood). This is probably related to the immunosuppressive treatments given to patients with lupus. This considerably reduces the number of assays that can be performed. Therefore, it is important to miniaturize the assays to maximum (without losing too much sensitivity and specificity) and prioritize the tests that will be carried out for the autophagy analysis.

3.2. Measurement of Macroautophagy by Electron Microscopy

It is widely accepted that electron microcopy represents one of the most accurate method for detecting autophagy and quantifying accumulation of autophagic vesicles [70]. The general procedure consists to fix cells with glutaraldehyde and post-fix them in osmium tetroxide, followed by ethanol dehydration, embedding in the resin, and cutting of ultrathin sections. For the detailed experimental conditions, see [78]. Vacuoles (usually 0.5–1 µm in diameter) can be identified as autophagosomes when meeting at least two of the following criteria: double membrane, absence of ribosomes at the cytosolic sides of the vacuole, similar luminal density as that of cytosol, visible organelles or parts of organelles in their lumen [29,79]. Vacuoles of similar size but with single membrane containing dense or clear amorphous material can be considered as autolysosomes. Autophagosomes present in the SG cells collected from MRL/lpr mice are shown in Figure 3 as an example (Li and Muller, unpublished). The occurrence of "autophagic vacuoles" or "autophagic compartments" can be quantified as previously described [29].

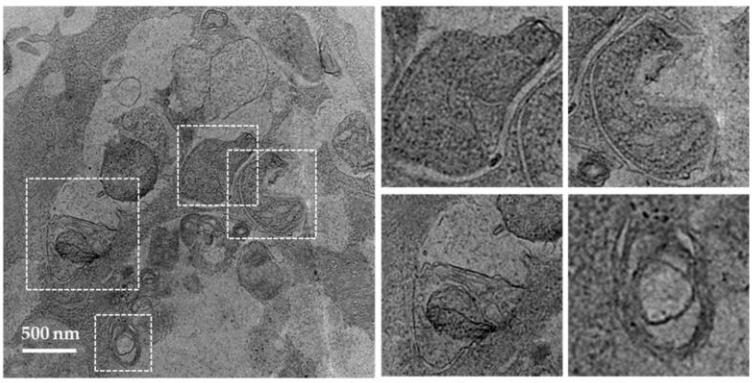

Figure 3. Transmission electron microscopic images of autophagosomes in SG cells. SG cells were isolated from MRL/lpr mice and treated with pepstatin A and E64d to block the degradation of autophagosomes. Autophagosomes with double membrane structures can be identified in the white dashed squares in image on the left panel, and the zoomed images of individual autophagosomes are presented on the right panel.

Notes:

- For statistical reasons, a minimum of 50 cell sections per condition should be examined.
- It is recommended to examine grids prepared from different resin blocks to avoid counting the same cells several times.

3.3. Measurement of Macroautophagy by Western Blot

The mammalian protein MAP1LC3 is a widely accepted marker for characterizing autophagosomes. The detailed usage, caution and pitfalls of MAP1LC3 as a MaA marker have been extensively reviewed elsewhere [70,80,81]. MAP1LC3 presents in two forms, namely MAP1LC3-I, which is cytoplasmic, and MAP1LC3-II, which is associated to the membrane of autophagosomes. Therefore the amount of MAP1LC3-II correlates to the number of autophagosomes, while only the turnover of MAP1LC3-II corresponds to the activity of MaA, also called "MaA flux". The turnover of MAP1LC3-II can be assessed by comparing the difference of the MAP1LC3-II in the presence and absence of lysosomal inhibitors (pepstatin A and E64d, chloroquine and others). In the absence of lysosomal inhibitors, the MAP1LC3-II on autophagosomes will be quickly degraded once inside lysosomes. By adding lysosomal inhibitors, the degradation of MAP1LC3-II is inhibited and therefore the amount of MAP1LC3-II delivered to lysosomes via autophagy can be monitored. As illustrated below in the images of MAP1LC3 blots, the MAP1LC3-II level is increased in SG cells of C57BL/6 mice incubated with lysosomal inhibitors (Figure 4A). In contrast, in the SGs of MRL/lpr mice, there is no accumulation of MAP1LC3-II in the presence of lysosomal inhibitors, indicating defective autophagy in the SGs of MRL/lpr mice (Li and Muller, unpublished).

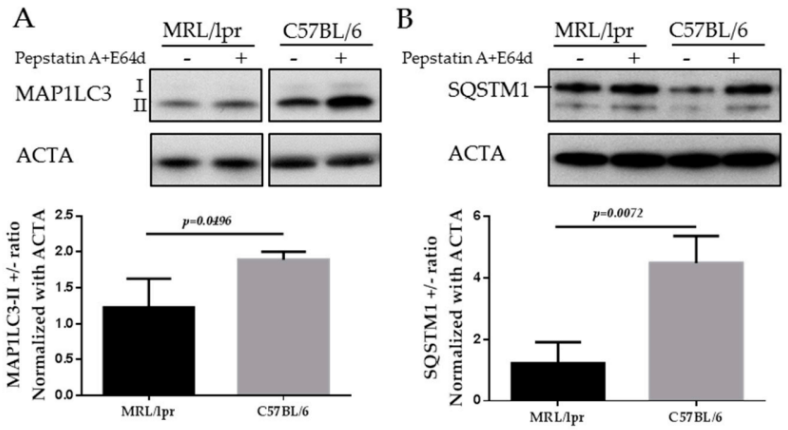

Figure 4. Western blots of MAP1LC3 and SQSTM1 in the SGs of MRL/lpr mice and C57BL/6 control mice. SG cells were isolated from MRL/lpr and C57BL/6 female mice, followed by starvation for 12 h in the presence or absence of lysosomal inhibitors (pepstatin A+E64d). Cells were then subjected to SDS-PAGE and Western blot. Two autophagy markers are shown in the figure: MAP1LC3 (**A**); and SQSTM1 (**B**). Actin-α (ACTA) was used as a loading control. Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SQSTM1/p62 is selectively incorporated into autophagosomes through binding to MAP1LC3 and is degraded through MaA. Therefore, the cellular expression level of SQSTM1 negatively correlates with MaA activity. As shown in Figure 4B, the level of SQSTM1 is lower in C57BL/6 SGs cells compared with MRL/lpr SGs (Li and Muller, unpublished). This result reinforces the MAP1LC3 data since an accumulation of SQSTM1 is suggestive of a defective MaA.

In practice, specific procedures have been detailed in numerous articles and technical reviews [82,83]. The protocols routinely used in our own studies to visualize and quantify MAP1LC3-II

conversion and SQSTM1 accumulation have been described elsewhere [29]. The antibodies we selected are listed in Table 3.

Table 3. References of antibodies used in our settings to analyze autophagy activity by WB and FC in various organs or blood from mice and patients with lupus.

	Antibodies	Company, References	Organs or Tissues Tested
WB	MAP1LC3	MBL International Corporation, M186-3	Mice (spleen, LN, SG), human (blood)
	SQSTM1	Abcam, ab109012	Mice (spleen, SG), human (blood)
	ATG5/ATG12	Abcam, ab155589	Mice (spleen, SG), human (blood)
	LAMP-2A	Abcam, ab18528, polyclonal; Abcam, ab125068 monoclonal	Mice (spleen, LN, SG), human (blood)
	HSPA8	Abcam, ab51052	Mice (spleen, SG), human (blood)
	HSP90	ENZO, ADI-SPA-831	Mice (spleen, SG), human (blood)
	Actin-β HRP	Abcam, ab49900	Mice (spleen), human (blood)
	Actin-α HRP	Abcam, ab203696	Mice (SG)
FC	MAP1LC3-FITC (FlowCellect^{TM})	Millipore, FCCH10071	Mice (spleen), human (blood)
	SQSTM1 AlexaFluor 647	MBL International Corporation, M162-A64	Mice (spleen), human (blood)
	HSPA8-PE	Abcam, ab65170	Mice (spleen), human (blood)
	HSP90-PE	Abcam, ab65171	Mice (spleen), human (blood)

FC, Flow cytometry; FITC, fluorescein isothiocyanate; PE, phycoerythrin; WB, Western blotting. Informations of companies: Abcam, Cambridge, United Kingdom; ENZO life sciences, Lausen, Switzerland; MBL International Corporation, Woburn, MA, United States; Millipore, Billerica, MA, United States.

Notes:

- Serum deprivation is widely used as a stimulus to induce autophagy. It is important to keep in mind that B cells are much more sensitive to serum withdrawal compared to T cells and many other cells. Therefore, we do not recommend incubating B cells in serum free media for more than 4 h, while more than 12 h serum withdrawal can be used for T cells and SG cells.
- The limitation of using Western blotting for B cells isolated from the blood of lupus patients relies to the number of recovered cells, which is generally too low to perform several conditions (including the controls run in the presence or absence of lysosomal inhibitors). In our experience, it is optimal to use one million cells (or around 20 μg protein) per condition/lane to obtain good signals, while commonly only 2–5 million B cells are recovered from lupus patients. It is therefore more feasible to use alternative approaches such as FC-based methods, as in the latter, much fewer cells are needed per condition.
- Loading controls are essential for proper interpretation of Western blots. They are important to assess the total proteins that have been loaded in each lane across the gel, thus allowing a more accurate blot calibration. Loading controls also permit gel conditions to be checked and compared from gel to gel.
- Regarding the type of proteins used as loading controls, we noticed that actin-α, but not actin-β, can be readily used as a loading control of SG extracts, whereas actin-β, but not actin-α, can be used when splenocytes are studied. Among usual loading controls, attention should be paid not to use a marker that is affected by autophagy alteration; for example, in certain cases, glial fibrillary acidic protein (GFAP) is not an appropriate control.

3.4. Measurement of Macroautophagy by Flow Cytometry

3.4.1. Measurement of MAP1LC3 by Flow Cytometry

Traditional FC can only measure the total cellular fluorescence of MAP1LC3 antibody staining, leaving MAP1LC3-I and MAP1LC3-II undistinguished. This can be overcome by using a MIFC, such

as Amnis ImageStream^X instrument (EMD Millipore, Seattle, MA, USA), which can discriminate the punctate MAP1LC3-II from the diffused cytosolic MAP1LC3-I [31,84]. The number of MAP1LC3$^+$ punctates per cell is calculated to represent the number of autophagosomes. However, there are limitations for this method. Firstly, not every laboratory is equipped with this instrument. Secondly, five million cells are required for each sample, and this number could be too high for conditions where cell number is limited.

We have obtained rather good results using FlowCellect^{TM} Autophagy MAP1LC3 antibody-based assay kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions (see below). This method overcomes the problem that classical FC measurement cannot distinguish MAP1LC3-I from MAP1LC3-II, by adding a permeabilization solution that extracts the soluble cytosolic MAP1LC3-I while protecting the MAP1LC3-II form, which is sequestered in the autophagosomes. In this method, unsorted PBMCs collected from blood can be used, as surface staining required to distinguish certain cell populations can be included to measure the number of autophagosomes in multiple cell populations. We have calibrated this assay using total splenocytes from CBA/J and MRL/lpr mice. Only 0.1 million cells were used per condition, and we included samples with and without lysosomal inhibitors (pepstatin A and E64d). This method has been used to measure the activity of MAP1LC3-II level in splenic macrophages in MRL/lpr as well [42].

CYTO-ID Autophagy detection kit (Enzo Life Sciences, Lausen, Switzerland) is another commercially available tool to measure autophagy by FC. The probe used in this kit is a cationic amphiphilic tracer dye that primarily stains autophagosomes, while staining lysosomes minimally. This dye has been successfully used in both cell lines and primary cells from human and mouse origin (summarized in the company's website). It can be used in conjunction with surface markers that discriminate different cell populations [85,86].

We deliberately do not describe here other modes of flow cytometric measurement, such as those exploiting fluorescence probes GFP-MAP1LC3 [67,87] or mCherry-GFP-MAP1LC3 [88]. These methods require transfection or transduction to introduce the fluorescence proteins, which is usually inapplicable in our autoimmunity projects that mostly examine primary cells isolated from organs and rarely cell lines that are easily tranfectable.

Notes:

- The first advantage of measuring autophagosomes by MAP1LC3 staining with FC is that a limited number of cells is needed (around 0.1 million per condition, except in the imaging cytometer), compared with the number of cells required for Western blot (0.5–1 million cells per condition).
- Secondly, surface staining to distinguish cell populations (Figure 5A) can be done before permeabilization and immunostaining of MAP1LC3; therefore, no prior cell isolation is needed. We highly recommend FC measurement of MAP1LC3 staining in combination of surface cell markers when the number of cells that are available is low, such as it is the case when B cells from lupus patients are analyzed.
- It is very important to avoid high background staining, which we have observed when some MAP1LC3 staining kits are used. We strongly advise the users to include the following controls when calibrating the MAP1LC3 assay:

    - positive controls: cells treated with lysosomal inhibitors that block degradation of autophagosomes and therefore increase the amount of MAP1LC3-II;
    - negative controls: cells in which the initiation of autophagy has been blocked, e.g., cells from autophagy deficient mice, and unstained cells.

The appropriate autophagy-detecting reagent is the one that allows separation of these controls. As illustrated in Figure 5B, the level of MAP1LC3-II (stained using the FlowSellect^{TM} kit) is much higher in CD4$^+$ T cells incubated in the presence of lysosomal protease inhibitors, compared to that in the absence of lysosomal protease inhibitors, while the MAP1LC3-II level in the CD4$^+$ T cells

from autophagy-deficient mice is as low as cells that were left unstained, indicating the staining of FlowSellect^{TM} kit is successful.

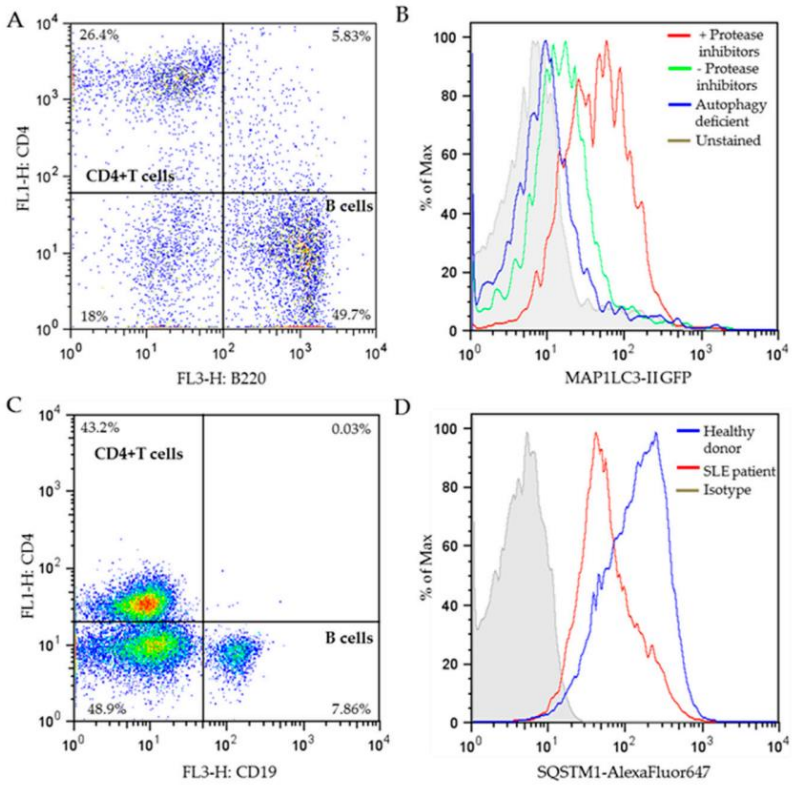

Figure 5. Flow cytometry measurement of autophagy markers. (**A**) Dot plot of MRL/lpr spleen cells after surface staining of CD4-FITC/B220-APC. The percentages of individual cell populations are indicated. (**B**) Representative histograms of MAP1LC3-II GFP fluorescence in stimulated CD4$^+$ T cells from MRL/lpr spleen cells (gated from the dot plot of A) in the presence or absence of protease inhibitors (pepstatin A+E64d), or stimulated CD4$^+$ T cells from autophagy-deficient mice (Atg5f/f dLck-cre mice; unpublished). Cells were stained using the FlowCellect^{TM} Autophagy MAP1LC3 antibody-based assay kit according to manufacturer's instructions, or left unstained as indicated. (**C**) Dot plot of PBMCs from SLE patients after surface staining of CD4-FITC/CD19-APC. The percentages of separate cell populations are indicated. (**D**) Representative histograms of SQSTM1-AlexaFluor^{647} fluorescence in stimulated CD4$^+$ T cells (gated from the dot plot of C) from the PBMCs of a healthy donor and a patient with SLE stained with SQSTM1 antibody or control isotype. The selected patient displayed a relatively high SLEDAI severity score of 20 on a scale of 0–105. CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies. APC, allophycocyanin; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PBMCs, peripheral blood mononuclear cells; SLEDAI, systemic lupus erythematosus disease activity index.

3.4.2. Measurement of SQSTM1 with Flow Cytometry

Various specific antibodies have been developed for measuring SQSTM1 level by FC. In line with their observation that the number of autophagosomes was increased in (NZB/W)F1 B cells compared to normal B cells, Clarke et al. observed a decrease of SQSTM1 expression in B cells of lupus NZB/W mice in comparison to B cells collected from control C57BL/6 mice, using FC measurement of SQSTM1 antibody staining [31].

In our laboratory, we have successfully carried out FC measurement with SQSTM1 antibody in stimulated PBMCs from healthy donors and lupus patients. PBMCs were first stained with

surface markers for B cells and T cells to differentiate these cell subtypes (Figure 5C), followed by permeabilization and staining of SQSTM1 antibody. $CD4^+$ T cells were gated from the dot plot of Figure 5C, and the SQSTM1 expression level of $CD4^+$ T cells was analyzed and illustrated in Figure 5D. The results show much lower SQSTM1 expression in SLE patient's $CD4^+$ T cells compared to healthy donors, supporting that in average, T cells from lupus patients display higher MaA activity. This result is highly consistent with the $CD4^+$ T cells "hyperautophagic" phenotype reported previously in both lupus prone mice and patients [29].

Notes:

- Autophagic flux can also be evaluated by measuring the difference of SQSTM1 level in the presence and absence of lysosomal inhibitors [31].

3.5. Measurement of CMA by Western Blot

There are relatively few relevant assays currently designed to readily study and analyze CMA and, nowadays, no commercial quantitative assay is available. An important contribution was made by Cuervo and colleagues who developed several sophisticated functional assays and steady-state measurement to evaluate CMA activity [89–91].

A first functional assay set up to follow CMA is based on a "pulse and chase" experiment in which cells are pulsed with a radiolabeled amino acid residue to preferentially label long-lived proteins in the presence of lysosomal inhibitors or inhibitors for MaA. The release of free radiolabeled amino acids in the culture media during the chase time can be measured as the rate of proteolysis. The difference of proteolysis measured between untreated cells and cells treated with lysosomal inhibitors corresponds to the total lysosomal degradation; the difference of proteolysis monitored between untreated cells and cells treated with MaA inhibitors represents the lysosomal degradation through MaA; the difference of the total lysosomal degradation and the degradation through MaA is the degradation through CMA (and also microautophagy) [89]. A second functional assay is based on photoconvertible CMA reporters, which are fluorescence proteins with an addition of the KFERQ motif, allowing its degradation through CMA. The advantage of using photoconvertible probes is that they could distinguish a subset of reporter converted to a different fluorescence from the constantly newly synthesized reporters (original fluorescence). Upon CMA activation, the former forms lysosomal puncta while the latter shows diffused cytosolic pattern [92]. Using this method, we have demonstrated that a therapeutic phosphopeptide currently evaluated to treat lupus patients (P140/Lupuzor^{TM}) exhibits inhibition effect to CMA [30,36]. This method has been better established in cell lines [30,92], however transfections of plasmid carrying the CMA reporter in primary cells are often challenging. The third and ultimate functional assay that was described consists to reconstitute CMA in vitro using isolated lysosomes (see methods of isolation of lysosomes adapted for CMA measurement in [89,91,93]). The differences of CMA substrates, such as GAPDH, recovered after incubation with lysosomes in the presence or absence of lysosomal protease inhibitors or protease K (that digests the substrate at lysosomal surface) could represent the binding, association and uptake steps of CMA [89]. Technical details have been described in [89–91]. This method requires a large amount of cells from tissue/organ samples, which could be difficult to obtain in certain settings, in particular when examining blood samples or purified cell subsets.

The steady-state CMA measurement includes examining the amount of key CMA components, which is however an indirect way to measure CMA and should be complemented with functional assays as those described above. The lysosomal amount of LAMP-2A and HSPA8 usually correlates with CMA activity and can be measured with Western blot as indirect indicators of CMA activity. The perinuclear localization of HSPA8-positive lysosomes can also be used as an indirect indication of CMA activation. In the context of autoimmune diseases, measuring the amount of LAMP-2A has been used to indirectly measure CMA activity in lupus-prone and healthy mice [30]. An unexpected

overexpression of LAMP-2A was repeatedly detected in spleen lupus B cells as the disease progresses, indicating hyperactivity of CMA in these mice [30].

Notes:

- It should be kept in mind that three variants of LAMP-2 exist and share the same lysosomal lumen region [90]. Therefore, for detecting LAMP-2A only, it is important to use antibodies that specifically detect the cytosolic tail of LAMP-2A. Specific antibodies can be raised as the 12 amino acid residues on the cytosolic side of LAMP-2A significantly differ from those encompassed in its variants LAMP-2B and C.
- We have experienced that sometimes LAMP-2A cannot be detected using whole cell lysate (homogenate). In this case, for studying LAMP-2A, we recommend preparing a membranous cell fraction with enriched lysosomes and mitochondria through classical step-wise centrifugation. This membrane cell fraction can be prepared by following the protocol published in Kaushik and Cuervo, 2008 (Section 3.1, Step 1–4) [91], however without going through the long and challenging process of preparing the highly purified lysosomes. Figure 6 illustrates our own data: LAMP-2A level is not detectable in cell homogenates (HOM), while there is a strong signal in the membrane fraction (MEM).

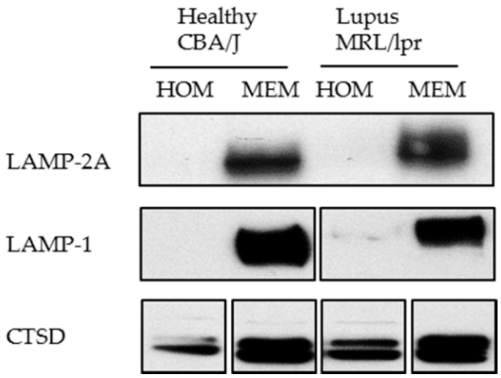

Figure 6. Western blotting of LAMP-2A in the homogenate (HOM) and membrane (MEM) fractions prepared from CBA/J and MRL/lpr spleen cells. Twenty microgram of protein was loaded per lane. The result shows a good detection of LAMP-2A in the MEM fraction in which lysosomes are enriched, but no visible signal in HOM fraction. LAMP-1 (lysosomal membrane associated protein type 1) and CTSD (cathepsin D) are lysosomal markers showing enrichment of lysosomes in the MEM fractions.

4. Conclusions

This review aims at providing some technical information for studying autophagy (both MaA and CMA) in lupus setting using MRL/lpr mice as a model. We also describe some methods we use in routine with human cells. As it is detailed above and summarized in Table 4, all the techniques have their pros and cons. Therefore, it is essential to combine several techniques to make robust conclusions. We and others have already obtained important information on the activity of MaA in both B and T cells in autoimmune mouse models and patients. The next step will be to identify the precise links between aberrant MaA and autoimmune responses both in vitro and in vivo. In contrast to what is known in MaA, data made available on the CMA activities in various immune cell types are still scarce. Pertinent probes that could detect directly CMA activity in vivo and specific inhibitors or activators of CMA are still lacking [10]. For both MaA and CMA, new mouse models that carry features of SLE and with deletion of genes involved in these pathways would be useful in order to better understand the role of these two major autophagic pathways in SLE and hopefully also in other autoinflammatory diseases.

Table 4. The pros and cons of the techniques described in this review.

	Pros	Cons
EM	- It is the best method to visualize the double membrane structure of autophagosomes.	- Quantification of autophagic vesicles through EM is time/sample-consuming. - It is prone to be subjective.
WB	- It is the best way to distinguish the two forms of MAP1LC3 and semi-quantify the MaA flux.	- Prior cell isolation is required in order to study cell subsets in the organs or blood. - 0.5–1 million cells per condition are needed.
FC	- Immunostaining of surface markers can be carried out at the same time, in order to examine autophagy on cell subsets. - A small number of cells (0.1 million cells) is needed. - Time saving.	- Some kits are not able to distinguish the two forms of MAP1LC3.
MIFC	- It can distinguish the punctate MAP1LC3-II from the diffused MAP1LC3-I. - It is both quantitative and qualitative.	- A large number of cells (5 million cells per condition) are needed.

EM, electron microscopy; FC, flow cytometry; MIFC, multispectral flow cytometry; WB, Western blot.

We hope that this review and the many technical details we list herein will be useful more widely, to researchers who would like to assess the status of autophagy in chronic and acute inflammatory diseases, both in animal models and patient's samples. All kinds of information on the implication of autophagy in this context would be of great importance for the development of novel, specific and safe therapies that target autophagy [10,23,36].

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Conflicts of Interest: M.W. was supported in part by an ImmuPharma France doctoral fellowship; N.S. is a salaried employee of ImmuPharma France; and S.M. is consultant of ImmuPharma. The remaining authors declare no conflicting financial interests.

Abbreviations

ATG, autophagy-related; APC, allophycocyanin; APCs, antigen-presenting cells; BECN1, beclin-1; CMA, chaperone-mediated autophagy; CTSD, cathepsin D; DN, double negative; EAE, experimental autoimmune encephalomyelitis; EM, electron microscopy; FC, flow cytometry; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HSPA8, Heat Shock Protein Family A (Hsp70) Member 8, HSC70; HOM, homogenate; IHC, immunohistochemistry; IL-6 and -8, interleukin-6 and -8; LAMP-2A, lysosome-associated membrane protein type 2A; LN, lymph node; ly-HSPA8 and ly- HSP90, lysosomal luminal HSPA8 and HSP90; MIIC, late endosomal MHC class II compartment; MaA, macroautophagy; MAP1LC3, microtubule associated protein 1 light chain 3; MEM, membrane; MIFC, multispectral imaging flow cytometry; MHCI and II, major histocompatibility complex class I and II; MRL/lpr, Murphy Roths Large/lymphoproliferation; N/A: not applicable ; NZB/NZW, New Zeeland black/New Zeeland white; PBMCs, peripheral blood mononuclear cells; PI3K, phosphoinositide 3-kinase; PM, plasma membrane; qPCR: quantitative polymerase chain reaction ; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; SG, salivary gland; SQSTM1/p62, sequestosome-1; TCR, T-cell receptor; TLR, toll-like receptor; ULK1, Unc-51 like-autophagy activating kinase 1; WB, Western blot.

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Comments

In this study, we described the methods we used in our laboratory to study autophagy in MRL/lpr mice and patients with lupus. We compared different methods in diverse organs/tissues, and summarized the pros and cons of each technique (Table 4 of the article) applied to investigate autophagy.

These methods can be useful for the researches of autophagy pathway, in both disease and naive conditions. Our illustrations regarding samples preparation and autophagy detection can be suggestive for other investigations related to rare samples and tissues.

Besides the technical information provided in this article, the original data showed in this study can be useful for understanding the role of autophagy in AIDs. For example, the observation of autophagosomes in SG cells of MRL/lpr mice and the hyperautophagic phenotype in CD4 T cells of SLE patients are helpful for understanding the role of autophagy in AIDs. In addition, these studies of autophagy in AIDs provide new clues for the development of therapeutics by modulating autophagy.

Perspectives

1. Multiple organs/tissues deliver different messages.

We observed that even within the same mouse model, autophagy profiles can be different in diverse organs/tissues. Increased autophagy was found in spleen, LNs and thymus of MRL/lpr mice (Gros et al., 2014), whereas defective autophagy has been shown in SG cells of same mice strain (Figure 4 of the article). These results indicate that the role of autophagy differs in different organs/tissues, which is reasonable since the functions of each organ/tissue are different from the others. In addition, the homeostasis needed for each functional organ/tissue is also specific. For these reasons, more organs/tissues should be tested to better elucidate the roles of autophagy in AIDs. Moreover, the interpretation of results from different organs/tissues should refer to the functions of target organs/tissues.

2. New technologies, effective markers and corresponding interpretations are required.

We described some routine technical information for studying MaA and CMA in MRL/lpr mice and human cells, and these techniques are well applied in the studies of autophagy. However, new technologies are required for better understanding this pathway and its roles.

EM, as a method providing the most direct information of autophagic structures, is quite powerful and highly recommended. However, this technique is time-consuming and expensive. The proper identification of autophagic structures is the cornerstone of this technique, which appears to be the most common problem for researchers (Klionsky et al., 2016), and this can be more difficult within cells containing abundant vascular structures. As summarized in the article (Table 4), each technique shows pros and cons. Therefore, it is necessary to combine several techniques such as EM, western blotting (WB), fluorescence microscopy and other methods in autophagy studies (Klionsky et al., 2016).

Reliable markers of autophagy are required to illustrate autophagy-related results. It is crucial to ensure the specificity of autophagy markers, as unspecific markers can give misleading results. To combine the information from several markers can be more persuasive. As introduced above, in contrast to what has been known for MaA, pertinent markers that could be used to detect CMA activity *in vivo* are still lacking (Cuervo, 2010; Wang et al., 2017).

The interpretation of autophagy, which is a dynamic process, can be incomplete. A well-acknowledged example is the enhanced level of LC3, which may indicate increased autophagy or blocked autophagy, as the level of LC3 can be enhanced in both conditions. Therefore, it is important to include lysosomal inhibitors to assess autophagic flux, which provides valuable indications for autophagy studies. A robust interpretation of autophagy relies on the use of appropriate techniques and markers.

3. Autoimmune conditions need to be considered.

To study the role of autophagy in AID, the autoimmune conditions are not negligible. In autoimmune settings, the functions of immune cells and non-immune cells are different from naive conditions. For example, autophagy is found to be involved in autoantigen presentation (chapter B.3.3). Autophagy might be linked with autoimmune activities and mediate autoimmune responses in AIDs. We have identified the profile of autophagy in autoimmune mouse models and patients. However, how autophagy plays its roles in the pathogenesis of SLE and SS is largely unknown. In the future, further studies will be performed to study the precise links between aberrant autophagy pathway and autoimmune responses.

PUBLICATION 2

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Critical role of autophagy processes in Sjögren's syndrome

Introduction

SS is one of the most common rheumatic diseases that affects 0.01-0.72% of the whole population. The characteristics of SS are the lymphocytic infiltrations in SG and LG, causing dry mouth and dry eyes in patients. SS has been recognized as a chronic, systemic autoimmune disorder that can be induced by both environmental and genetic factors. However, the etiology and pathology of SS are not clearly understood.

In this study, we used the MRL/lpr mouse model to investigate SS in two directions, namely some aspects of SS pathological mechanism and the introduction of a possible therapeutic option.

With regard to our pathological mechanism study, we would like to evaluate the role of autophagy in SS. The autophagic markers LC3 and SQSTM1 were followed in SG cells of MRL/lpr mice and healthy mice, to evaluate the autophagic activity in SG cells. We found that autophagy may be defective in SG cells of MRL/lpr mice, as the autophagic inhibitors E64D and Pepstatin A failed to accumulate autophagic markers. As described in previous chapters, autophagy is a dynamic process which involves multiple machineries, and the defected accumulation can be resulted from various reasons. To explain these results, we used other methods (starvation, BafA1, and EM) to study autophagy initiation, autophagosome formation and autophagosome-lysosome fusion. We further discovered that the defective autophagy in SGs of MRL/lpr mice could be attributed to impaired lysosomal functions.

For the therapeutic study, we used the peptide P140 described in our team and which has been demonstrated to be an efficient therapeutic tool to ameliorate clinical and biological manifestations in both SLE patients and MRL/lpr mice (Monneaux et al., 2003; Page et al., 2011a; Page et al., 2011b; Schall et al., 2012; Zimmer et al., 2013). Our aim was to test whether P140 peptide could provide any therapeutic effects for SS with potential applications in human. As introduced in the chapter “Aim of the study”, two therapeutic approaches were used to administrate P140 in MRL/lpr mice, the “short-term” treatment and the “long-term” treatment.

SS-related pathological features in MRL/lpr mice were evaluated after P140 administration. The lymphocytic infiltration in SG, histological profile of SG, and serological SS-related autoantibody levels were compared in mice that received P140 or vehicle/ScP140 only. In these studies, we found that P140 acts as a potential therapeutic peptide for SS.

Our second paper contains strong arguments supporting the involvement of autophagy in SS pathology. The findings provide novel clues to the pathogenesis of SS and suggest autophagy as a potential therapeutic target for SS. In addition, we identified the therapeutic effects of P140 in SS. These data may shed lights on the therapeutic applications of P140 in SS, and possibly in other autoimmune inflammatory diseases.

Result: Critical role of autophagy processes in Sjögren's syndrome

Critical role of autophagy processes in Sjögren's syndrome

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Abbreviations: ACTA, actin-alpha; APC, antigen-presenting cell; ATG, autophagy related ; CMA, chaperone-mediated autophagy; B6, C57BL/6; CSTD, cathepsin D; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HSPA8/HSC70, heat shock 70-kDa protein 8; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LAMP2A, lysosomal-associated membrane protein 2A; *lpr*, lymphoproliferation; MAP1LC3, microtubule-associated protein light chain 3; mAb, monoclonal antibody; MHCII, major histocompatibility complex class II; MRL, Murphy Roths large; NOD, non-obese diabetic; PBS, phosphate-buffered saline; SD, standard deviation; SEM, standard error of the mean; ScP140, scrambled peptide P140; SG, salivary glands; SLE, systemic lupus erythematosus; SQSTM1/p62, sequestosome; SS, Sjögren's syndrome; TRIM, tripartite motif.

Abstract

Sjögren's syndrome is a systemic autoimmune disorder that affects the whole body. Along with common symptoms of extensive dry eyes and dry mouth resulting from mucous membranes and moisture-secreting glands dysfunctions, other serious complications include profound fatigue, chronic pain, major organ involvement, neuropathies and lymphomas. Today, as many as 4 million Americans are living with this disease, with over 90% of patients being female. Current treatments only focus on relieving symptoms and do not target to the origin of the disease, which is largely unknown. Here, we evaluated the extent of alterations of autophagy processes occurring in salivary glands of a recognized murine model of secondary Sjögren's syndrome. We identified hitherto unknown molecular alterations of autophagy occurring in salivary glands of MRL/lpr mice, and discovered that opposite (up- or down-regulated) autophagy events can arise in distinct organs of the same mouse strain, here in lymphoid organs and salivary glands. Impressively, the phosphopeptide P140 known to directly act on chaperone-mediated autophagy rescued MRL/lpr mice from cellular infiltration and autophagy defects occurring in salivary glands. P140 that showed effective benefit in patients with systemic lupus might therefore also offer a great potential as future therapeutic agent in Sjögren's syndrome.

Introduction

Macroautophagy, a catabolic process allowing degradation of cytosolic material, displays central physiological roles in the immune system. It participates in antigen presentation, differentiation and balance regulating survival/death and activation of lymphocytes, and several aspects of inflammation (1-5). A growing body of evidence from across genetic and cellular studies considers autophagy a core pathogenic contributor to abnormal immunity in autoimmune disorders such as inflammatory bowel diseases, systemic lupus erythematosus (SLE) and autoimmune diseases affecting the central or peripheral nervous system (6-9). Dysregulation of autophagy may have decisive implications both on innate and adaptive immunity in affecting, for example, the normal removal of dead cells that represent a highly diversified source of autoantigens, the scavenging of intracellular DNA and RNA, the regulation of pro-/anti-inflammatory cytokines balance, and the processing and presentation of endogenous antigenic peptides by major histocompatibility complex (MHC) molecules in the late endosomal MHC class II compartment (10).

Autophagy machinery positively or negatively regulates inflammation in different settings. Thus, according to the type of autophagy process that is considered and the organs or tissues, which are examined, autophagy might turn out to display positive or negative effects on inflammation in the same individual. We addressed this question using the MRL/Mp-Fas^{lpr} (thereafter named MRL/lpr) mouse model in which we evaluated the autophagy defects in salivary glands (SGs), in comparison to lymphoid organs wherein both macroautophagy and chaperone-mediated autophagy (CMA) dysfunctions have been previously identified (11-12).

The MRL/lpr mouse spontaneously develops a systemic autoimmune disease mimicking human lupus and secondary Sjögren's syndrome (sSS; 13-19). It is characterized by inflammation of multiple tissues (e.g. skin, joints, glands, lungs, heart, kidneys), massive lymphadenopathy and splenomegaly that progress in an age-dependent manner. Destructive

mononuclear infiltrates in the lacrimal and SGs of MRL/lpr mice are hallmarks of the SS disease. Most individuals with SS present with sicca symptoms, such as xerophthalmia (dry eyes), xerostomia (dry mouth), and parotid gland enlargement. Very little information is available regarding possible autophagy defects in this pathology. In an experimental setting it was shown that TRIM21 (also known as Ro52/SSA) binds several key autophagy regulators and that together with other TRIM proteins, it participates to the organization of active complexes of the autophagic machinery (20-21). Among these autophagy factors and regulators are serine/threonine-protein kinase ULK1 (orthologue of yeast Atg1), Beclin-1 (orthologue of Atg6), a subset of mammalian Atg8s containing MAP1LC3A, B and C, GABARAP, GAPARAPL1, and GABARAPL2, as well as sequestosome 1 (SQSTM1)/p62. TRIM21 also recognizes the activated, dimeric form of interferon (IFN) regulatory factor 3 inducing type I IFN gene expression. It is not known if autoantibodies to TRIM21/Ro52 that circulate in the blood of SS and SLE patients influence these interactions and if possible perturbations of IFN-γ-TRIM21 autophagy axis may be involved in the hyperactivation of type I IFN signaling found in these autoimmune conditions.

In the present study, we evaluated the extent of possible alterations of autophagy processes specifically occurring in SGs of MRL/lpr mice. We identified hitherto unknown molecular defects of autophagy arising in these glands and discovered thus that they radically differ from those revealed previously in lymphoid organs (i.e. thymus and spleen) of the same mouse strain. When we treated MRL/lpr mice with the therapeutic peptide P140, which acts on CMA and reduces excessive autophagy in MRL/lpr spleen B cells (12), we found that it rescued MRL/lpr mice from the various defects identified in SGs, especially in the lysosomes of SG cells. The comparison of autophagy defects discovered in different organs of MRL/lpr mice highlights the complexity of self-regulating processes of homeostasis. Furthermore, observing the extent to which these failures can be corrected by the therapeutic peptide P140

led us to refine the mechanism of action of this unique peptide and precise its regulatory functions on autophagy processes occurring in secondary lymphoid organs of autoimmune individuals. These findings may have important therapeutic consequences in the personalized management of patients with SS for whom targeted treatments are scarce today, and potentially also of individuals with other autoimmune diseases.

Results

The cell content and distribution are altered in MRL/lpr salivary glands and corrected upon treatment with P140 peptide

Compared to C57BL/6 (thereafter called B6) control mice of the same age, SGs from MRL/lpr mice, the size of which is increased 1.5-2 times (**Fig. 1A**), contained 6.5 times more $CD45^+$ cells (n=4 B6 *vs.* n=10 MRL/lpr mice; p=0.0071; **Fig. 1B**) in % of the total cell population, and 56.6 and 23.7 times more $CD4^+$ and $CD8^+$ T cells, respectively (n=4 B6 *vs.* n=4 MRL/lpr mice; p=0.0012 and 0.005, respectively; **Fig. 1C**). Lymphocytic infiltrates contained virtually no $CD19^+$ B cells (<0.04% of the total gland cell population in MRL/lpr mice aged of 15 and 29 weeks). Histology and immunofluorescence studies of SGs confirmed cell infiltration (**Fig. 1D,E**) and also confirmed that after 25 weeks of age, as previously shown by others (13,14), the SG tissue was markedly destroyed in MRL/lpr mice, preventing any further exploration to be performed in these glands.

Previous studies have shown that treating MRL/lpr mice with P140 peptide dramatically reduced kidney inflammation, proteinuria, dermatitis and serum levels of double-stranded (ds) DNA IgG antibodies; P140 also significantly enhanced MRL/lpr life span (22-24). To examine the possible effect of P140 peptide on the cell content of SGs of this mouse strain, MRL/lpr female mice were treated with P140 peptide (100µg/mouse) administrated intravenously either once (protocol 1: mice aged of 11-13 weeks at the start of the experiment; sacrifice 5 days later; ref. 24) or four times (protocol 2: mice aged of 5 weeks at the start of the experiment; injections at weeks 5, 7, 9, and 13; sacrifice at week 23; ref. 22) (**Fig. 2A**). As expected, the short-term P140 treatment (protocol 1) had no effect on the mean weight of SGs or on the total gland cell content of treated versus untreated MRL/lpr mice. P140 peptide had no effect either on the percentage of $CD45^+$ cells or on $CD8^+$ T cells and $CD19^+$ B cells subsets (**Fig. 2B**). However, compared to untreated MRL/lpr mice, we noticed a significant

decrease of $CD4^+$ T cells percentage in the SG cell fraction upon P140 treatment (p=0.003; **Fig. 2B**). The percentage of TCR $beta^+$ T cells in the total cell population was also significantly diminished (p=0.042, compared to untreated MRL/lpr mice; not shown). No effect was observed when the scrambled (Sc) peptide P140 was administrated to MRL/lpr mice (**Fig. 2B**). These data were confirmed by immunofluorescence analysis (**Fig. 2C**). The foci visualized in the SGs of MRL/lpr mice contained $CD45^+$ cells that were mostly stained with CD4 but not with CD8 marker. As shown, fluorescent CD45 and CD4 labeling was markedly attenuated 5 days after P140 administration. This effect was not related to the number of foci that remained unchanged at day 5 (**Fig. 2D,E**). Inflammation, however, appeared weaker post-P140 treatment as seen by histochemistry after hematoxylin and eosin (H&E) staining (**Fig. 2E**) and reflected by the focus score determined as described (25) (**Fig. 2F**; p=0.0091, compared to untreated MRL/lpr mice). Again no significant effect was seen with the ScP140 control peptide. The extent of C3 complement deposits was also profoundly diminished in P140-treated mice (**Fig. 2G**).

The long-term protocol 2 was the procedure used in our earlier experiments to demonstrate that P140-treated MRL/lpr mice exhibit a much longer life span (22; **Fig. 3A**). This multi-injection design was implemented in a preventive setting including 5-week-old mice showing no clinical signs (10 per group), and the readout was measured 10 weeks after the last peptide injection i.e. at sacrifice, on week 23. In this setting option, as above, P140 peptide had no effect on the mean weight of SGs or on the total cell content of treated versus untreated MRL/lpr mice. However, it induced a significant decrease of the percentage of $CD45^+$, $CD4^+$ and $CD8^+$ T cell subsets (**Fig. 3B**). The $CD19^+$ B cell compartment was not affected. The ScP140 peptide had no effect on $CD45^+$, $CD4^+$ and $CD19^+$ cells. Immunofluorescence analysis (**Fig. 3C**) confirmed the decrease of cell percentages evaluated by flow cytometry. The foci visualized in the SGs of MRL/lpr mice contained less $CD45^+$ and

$CD4^+$ T cells, an observation supported by the histochemistry images (**Fig. 3D**) and by the post-treatment measure of inflammatory grades (**Fig. 3E**; p=0.0124 *vs.* untreated MRL/lpr mice). No effect could be visualized post-ScP140 peptide treatment (**Fig. 3 C-E**). The level of some typical autoantibodies was measured in the serum of mice of the three groups. In good agreement with our earlier results (22), the levels of anti-dsDNA IgG antibodies were significantly decreased in P140-treated mice compared to untreated mice (**Fig. 3F;** p=0.0293 *vs.* untreated MRL/lpr mice; ScP140, ns). At 23 weeks of age, anti-TRIM/Ro52 IgG antibody levels were also significantly decreased after P140 treatment (**Fig. 3G;** p=0.0285 *vs.* untreated MRL/lpr mice). Anti-Ro60 and anti-La IgG antibody levels were not significantly changed (not shown). The average weight of mice remains also unchanged (not shown). Although we noted remarkable differences when we considered the first mouse that died in the study groups (10 weeks in the saline group *vs.* 19 weeks in the P140 group), statistically, at 23 weeks when we sacrificed the mice for studying their SGs, there was no significant effect of P140 on their survival rate (the lengthening of mouse survival upon treatment is seen at longer time; **Fig. 3A**). Proteinuria, however, was already lower at week 23 in the P140-treated group (**Fig. 3H**; p=0.0421 *vs.* untreated MRL/lpr mice).

Autophagy processes are defective in SGs of MRL/lpr mice and corrected by the treatment with P140 peptide

We previously showed that the P140 peptide binds HSPA8/HSC70 chaperone protein (26) and reduces autophagic flux in B lymphocytes of peptide-treated MRL/lpr lupus-prone mice (23). *In vitro*, we found that P140 peptide but not the ScP140 control peptide, readily interferes with CMA, presumably leading to lowering overexpression of MHCII molecules at the surface of antigen-presenting B cells (12,27) and consequently modifying the peptide presentation to autoreactive T cells demonstrated earlier *in vivo* (28). Our past work was

essentially performed using thymocytes, splenocytes and peripheral cells of MRL/lpr mice (11,12). Here we examined the extent of autophagy activity in SGs of MRL/lpr and the possible effect of P140 on this pathway. The results presented below show that in contrast to what was seen in the organs and tissues we studied so far, the autophagic activity is reduced in MRL/lpr SGs compared to control SGs, and that P140 treatment reestablishes the basal autophagy activity in affected glands.

Several independent assays, the experimental conditions of which were previously described (11,27,29), were carried out to investigate the autophagy alterations in SGs of MRL/lpr. Western immunoblotting methods were used to evaluate the macroautophagy flux by comparing the expression of MAP1LC3B-II protein in the presence or not of lysosomal proteases inhibitors E64D and pepstatin A. As shown in **Fig. 4A**, in the SGs of MRL/lpr mice there was a reduced accumulation of MAP1LC3B-II in the presence of lysosomal inhibitors, suggestive of a defective autophagic flux in these glands (p=0.0074 *vs.* B6 SGs). The macroautophagy activity in SGs was also quantified by measuring the accumulation of SQSTM1 protein, the expression level of which generally negatively correlates with macroautophagy activity (30). Compared to B6 SGs, the level of SQSTM1 was effectively found to be statistically higher in MRL/lpr SGs (**Fig. 4B**; p=0.0281).

To understand where the defects lie in the macroautophagy pathway, several studies were conducted in SGs of MRL/lpr mice and healthy counterparts. We first discovered that in response to nutrient starvation that induces autophagy through the upstream mTOR signaling pathways (31,32), or upon incubation with Bafilomycin A1, an endosomal acidification inhibitor that prevents maturation of autophagic vacuoles by inhibiting autophagosomes-lysosomes fusion (33), cells from MRL/lpr and B6 SGs were equally able to induce MAP1LC3B-II conversion (**Fig. 4C,D**), suggesting that autophagy initiation was not impaired. Double-membrane autophagic vesicles were also visualized in SGs of MRL/lpr

mice by transmission electron microscopy (**Supplementary Fig. 1**) indicating further that the upstream, initiation steps of autophagosome formation readily occurred. The expression of ATG12, a partner protein of the ATG12-ATG5 complex that after its association with ATG16L1 is involved in the autophagosome elongation by promoting the conjugation of ATG8 to phosphatidyl ethanolamine (34,35) appeared non-affected either in MRL/lpr SGs as compared to B6 glands (**Fig. 4E**). All together these findings support the idea that downstream rather than upstream failures are implicated in autophagy defects observed in SGs of MRL/lpr mice.

CMA activity was also investigated in MRL/lpr SGs. Among its diverse lysosomal functions, LAMP2A, an integral membrane protein with a heavily glycosylated luminal region, a single transmembrane region and a short cytosolic tail, acts as a receptor for the cytosolic proteins that undergo degradation *via* CMA and also for the cytoplasmic antigens presented by the MHCII (36-38). LAMP2A is considered as the best CMA marker available today. Western immunoblotting analyses of SGs cells revealed a marked decrease in the amount of LAMP2A in the MRL/lpr SGs extracts compared to B6 SGs (**Fig. 4F**; p=0.0006). All together, these findings suggest that autophagy defects observed in MRL/lpr SGs might result from a blockade in completion of basal autophagy rather than from impairment in the whole (macro)autophagy pathway involving early *atg* genes activation (34,39,40). Our results also strongly support that CMA is particularly affected in the MRL/lpr SGs.

To gain more insight into the relevance of our results, we examined further the functional status of lysosomes in the MRL/lpr SGs. Lysosomes were found to display altered functions in the MRL/lpr glands. Flow cytometry analysis after LysoSensor Green DND-189 staining revealed that the mean pH of acidic vesicles in MRL/lpr SGs was significantly higher with regard to B6 control glands (**Fig. 5A**; p=0.068; the lysoSensor Green DND-189 dye, the pKa of which is ~5.2, becomes more fluorescent in acidic environment). We also found that the

ATP content was significantly diminished in MRL/lpr SGs cells (**Fig. 5B**; *p*=0.0013 *vs.* B6 SGs cells).

Our findings generated using several independent approaches strongly argue for macroautophagy and CMA alterations as well as lysosomal functional failures affecting at least a pool of cells present in the SGs of MRL/lpr. At this point, it is not known which SG cell subset(s) is/are affected and if the failures we observed are directly linked to infiltrating (immune) cells that invade SGs during the course of the disease (**Fig. 1**). Some of the autophagic and lysosomal alterations we discovered in SGs have been described to similarly occur in primary and secondary lymphoid organs of MRL/lpr mice (11,12). However, the macroautophagic flux that is over-activated in MRL/lpr splenocytes and thymocytes seems to be reduced in MRL/lpr SGs of these autoimmune mice (**Fig. 4A**). CMA activity is also reduced in MRL/lpr SGs (**Fig. 4F**). We thus examined whether P140 peptide could influence these defaults.

To determine if P140 may affect macroautophagy, as above, we evaluated the expression in western immunoblotting of MAP1LC3B-II, in the presence or not of E64D and pepstatin A protease inhibitors. In the SGs of MRL/lpr mice that were treated using the procedure 2 (four injections and study at sacrifice occurring 10 weeks after the last injection), accumulation of MAP1LC3B-II in the presence of lysosomal inhibitors was observed, indicating that autophagic flux was regained in the glands of P140-treated mice (exemplified in **Fig. 6A** with 3 mice; *p*=0.022 *vs.* non-treated mice that received saline only). It should be noticed that this remarkable effect was observed as soon as 5 days after a single administration of P140 (protocol 1; **Supplementary Fig. 2**). Remarkably, the CMA marker LAMP2A was again expressed **(Fig. 6B;** *p*=0.0003 *vs.* control mice). The acidity of lysosomes, as revealed by LysoSensor Green DND-189 staining, also showed a recovery, at least in part, of expected pH of SG cells (**Fig. 6C**; *p*=0.0063 *vs.* control mice that received saline only). Finally the ATP

content was significantly raised in the SG cells of P140-treated MRL/lpr mice (**Fig. 6D**; p=0.0272 *vs.* control mice). In most of all these conditions, the control ScP140 peptide analogue had no effect.

Our previous results supported a beneficial effect of P140 peptide in MRL/lpr mice as visualized by the prolonged lifespan of these mice upon treatment, and the diminution of several clinical and biological signs (22-24). Here we show further that P140-treated MRL/lpr mice of 23 weeks display less immune cell infiltrates, less complement C3 deposition and weaker inflammation in their SGs. The serum levels of anti-TRIM21/Ro52 IgG antibodies were also decreased in the peripheral blood of treated mice. Moreover, our data indicate that the autophagy activity, which seems to be decreased in MRL/lpr SGs in contrast to what occurs in lymphoid organs (11,12), returns to its basal rate upon P140 treatment and that lysosome defects are, at least in part, corrected. Collectively these data reinforce our assumption for a role of autophagy in the mechanism of action of P140. They underpin a model in which, likely due to its mode of systemic (i.v. route) delivery, P140 primarily targets the autophagy pathway in immune cells present in lymphoid organs and *via* a bystander effect, exerts only secondarily its influence on the autophagy pathway of other cells, including cells of target organs, such as SG cells in SS-like mouse models.

Discussion

This work was done using a mouse model of secondary SS, recognized throughout the Community, with an emphasis on SG pathophysiology in MRL/lpr mice. Regarding lymphocytic infiltrates, it has been established previously by independent groups that they are mostly constituted by TCR$\alpha\beta^+$ and $CD4^+$ T cells within submandibular glands of MRL/lpr mice and patients with SS, whereas $CD8^+$ T cells and B cells are less frequent in number (14,15). This composition of lymphocytic infiltrates differs from the one revealed in non-obese diabetic (NOD) mice that develop type I diabetes and SS-like autoimmune exocrinopathy (19). B cell counts were found to increase with age in submandibular glands of NOD mice while the number of T cells remained rather constant (15). By comparison to B6 mice included as controls in our own experimental settings, the percentages of both $CD4^+$ and $CD8^+$ T cell subsets were found to be increased in the total SG cell population, while effectively the percentage of B cells was extremely low. In contrast to what we know in MRL/lpr mice regarding the spleen, lymph nodes and kidneys in which there is an important organ remodeling and enlargement likely due to the *lpr* mutation (29,41), in the present study we found that the volume of infiltrated SGs (as also in the case of liver; ref. 42) remains unaffected during the course of the disease.

Our experimental data demonstrate, for the first time, that both macroautophagy and CMA are compromised in the SGs of MRL/lpr mice. Information linking possible autophagy defects and SS are scarce and indirect in the existing literature (43,44). In this study, impaired autophagy occurring in MRL/lpr SGs was demonstrated by studying classical autophagy markers MAP1LC3, SQSTM1, ATG12/5, and LAMP2A. We found that compared to B6 SGs, used as healthy control, the macroautophagy flux is significantly diminished in MRL/lpr SGs and the expression of the CMA marker LAMP2A is significantly reduced. Some defects were also measured in the MRL/lpr SG lysosomes. Their pH appears to be appreciably

increased with possible dramatic consequences on lysosomal proteases (e.g. cathepsins) activity and also some effects on the completion of autophagic flux that closely depends on the pH of acidic compartments (45). Interestingly, notable changes have been reported in various lysosomal enzymes in patients with Sjögren's syndrome (46,47). We also observed a sharp drop of ATP intracellular content in MRL/lpr SGs, which could be related to a higher consumption by ATP-dependent proteases and/or mitochondrial deficiencies. The molecular mechanisms underlying this observation as well as kinetic studies are currently under investigation as it bears important implications. At this stage of our investigation, the possible impact of autophagy activity in SGs of MRL/lpr mice is not known. It is not known either if the same defaults exist in patients with SS, and also if the secretory impairment of lacrimal and salivary glands may be related to any autophagy dysfunctions. However, it is tempting to propose that any failure of autophagy pathways will let unprocessed some proteins that are abundant in saliva (and tears) and can be misfolded or secreted in excess. Accumulation of such unwanted proteins could be pathogenic.

An initially unexpected finding that has emerged from our studies is the observation that the defects we observed in MRL/lpr SGs cells are in marked contrast to those described in lymphoid organs, namely the spleen, lymph nodes and thymus, of the same mouse strain (11,12,23,27,48). In T and B cells collected from secondary lymphoid organs, autophagy activity was rather hyperactivated (confirmed by us and others in lupus-mouse models and human; 49-51). These observations led us to conclude that aberrant autophagy activity - impairment or hyperactivation - occurring in pathologic settings, is not a general feature that affects equally all organs or tissues of any individual. On the contrary, dysregulation can highly differ from an organ to another one, a conclusion also underpinned in other autoimmune or non-autoimmune indications, such as neurological inflammatory diseases (9). Without solid experimental arguments, it is hazardous to propose a valid interpretation of

these findings. We can only theorize that these apparently opposite phenomena relate in part from compensation systems, which restore normal levels of homeostasis and eliminate excessive proteins rendered pathogenic because misfolded or denatured, that can be different and more efficient in individual organs or tissues.

Another major result is the beneficial effect of P140 in MRL/lpr mice. In MRL/lpr mice, intravenous P140 administration induces a reduction of clinical signs such as dermatitis, proteinuria, and renal vasculitis; it also decreases pathognomonic biological features, such as serum anti-dsDNA antibodies, and reestablishes the basal level of leukocytes in the peripheral blood of treated mice (22-24). Mice that have received the P140 peptide display a delayed onset of the disease and a significantly increased lifespan. We showed previously that P140 binds both the HSPA8 chaperone (26) and MHCII molecules, and co-localizes with these two molecules intracellularly and at the cell surface in splenic MRL/lpr B cells (23). After peptide administration to MRL/lpr mice, the levels of both HSPA8 and MHCII molecules that are overexpressed in these cells are reduced. Besides these significant effects that contribute at improving the status of MRL/lpr mice by reducing the activation of autoreactive T and B cells, we show here in the same mouse strain that P140 significantly reduces lymphocytic infiltration in SGs, decreases inflammation in these glands, and reduces C3 deposition. At this point it is not known if salivary flow rate and glandular dysfunctions are improved in treated *versus* non-treated MRL/lpr mice.

Taken as a whole, these data led us to conclude that autophagy plays a decisive role in the mechanism of action of P140, and that the primary targeting sites where it regulates autophagy are very probably in lymphoid organs. In the case of MRL/lpr mice in which autophagy is hyperactivated in lymphoid organs, the P140 effect is highly beneficial. Note in this context that in biodistribution experiments carried out previously in 6 and 12 week-old MRL/lpr mice (23), fluorescent-labeled peptide P140 was found to accumulate in the lungs

and spleen as early as 10-15 min after P140 injection and remain on site for at least 90-120 min. In the spleen, labeled P140 peptide co-localized essentially with B cells and resident macrophages, which act as potential antigen-presenting cells. Additional experiments based on refined methodology are however required to determine whether P140 readily accumulates in SGs.

A central question that remains open is why P140 does not affect autophagy in certain organs. As mentioned above, P140 binds to HSPA8 that plays an important role in its mode of action (12,26). It is noteworthy that in human the expression level of HSPA8, which is high to very high in certain organs such as the brain, kidneys, lymph nodes, spleen and intestine, for example, is particularly low in SGs and pancreas (52,53). This may explain why autophagy, and CMA in particular, in which several active steps are mediated by the HSPA8 content (54), can be weak in certain organs and why, *de facto*, the P140 peptide does not exert significant direct effects.

Thus, when the autophagy processes are abnormally elevated in lymphoid organs, the P140 peptide seems to readily correct the defects. Hereby, we thus propose using the P140 peptide for disrupting the SS-occurring patho-biological cascade leading to SG infiltration, dysfunction, and ultimately destruction. Our data argue that a convergence point proximal to SGs where lymphocytic infiltration occurs is not absolutely necessary and that impressive effects can be potentially generated in SGs while the primary target of the peptide drug is distant (or distal) from the affected organ. This very novel information is of great significance as we show here that autophagy processes can be abnormally elevated in some organs or tissues while they are impaired in others.

Methods

Mice and treatments. Female MRL/lpr mice and MHC-matched B6 mice were purchased from Harlan-France or Charles River-France. Two procedures were used for treating mice. In the procedure 1 (24), 11-13 week-old female MRL/lpr mice received a single intravenous administration of P140 or ScP140 in saline or saline alone. Mice were sacrificed 5 days later for study. In the procedure 2 (22), 5 week-old mice received the same preparations at weeks 5, 7, 9, and 13 by the intravenous route and were sacrificed at week 23 for study. Peptides were injected at a rate of 100µg in 100µL saline per mouse. Proteinuria was measured in fresh urine using Albustix (Bayer Diagnostics) and was semi-quantitatively estimated according to a 0-4 scale recommended by the manufacturer.

Synthetic peptides. P140 and ScP140 peptides were synthesized using classical Fmoc (N-[9-fluorenyl] methoxycarbonyl) solid-phase chemistry and purified by reversed-phase high-performance liquid chromatography (HPLC). Their homogeneity was checked by analytical HPLC, and their identity was assessed by LC/MS on a Finnigan LCQ Advantage Max system (Thermo Fischer Scientific).

Preparation of salivary glands homogenates. SG homogenates were prepared as described (29). In brief, SGs collected from mice were let to incubate in RPMI 1640 medium (Lonza BioWhittaker) supplemented with 2% (v/v) fetal calf serum (FCS), collagenase D (1 mg/mL; Roche, 11088866001) and DNase I (50 µg/mL; Roche, 10104159001) at 37°C under agitation (1,500 rpm/min) for 1h. Cells were passed through 70-µm and 40-µm cell strainers and ammonium-chloride-potassium (ACK) lysing buffer was used to lyse red blood cells.

Flow cytometry. SG cells were incubated with fluorochrome-conjugated antibodies for 40 min at 4°C in phosphate-buffered saline (PBS) containing 2% (v/v) FCS and 0.3 mM EDTA, pH = 7.4. For each staining, mouse FcR Blocking Reagent (Miltenyi Biotec, 130-092-575) was used to block Fc receptors. Antibodies used in these studies include fluorescein isothiocyanate (FITC)-labeled anti-mouse CD45 (553080), phycoerythrin (PE)-labeled anti-mouse CD19 (553786), peridinin chlorophyll protein (PerCP)-Cy5.5-labeled anti-mouse CD8a (551162), allophycocyanin (APC)-labeled anti-mouse B220 (553092), FITC-labeled anti-mouse CD3ε (553062), FITC-labeled anti-mouse CD4 (553729), PE-labeled anti-mouse CD3e (553064), PerCP-Cy5.5-labeled anti-mouse CD45 (550994), all from BD Bioscience.

Data were collected on a BD FACSCalibur™ flow cytometer and analyzed using FlowJo software.

Immunohistochemistry. SGs were collected from B6 and MRL/lpr mice and fixed with 4% (v/v) paraformaldehyde (PFA) for at least 48h before use. Tissues were washed with PBS to remove PFA before being transferred into series of dehydration solutions, namely 70%(v/v) ethanol/3h, 95% ethanol/ 3-4h, 100% ethanol/overnight, and fresh 100% ethanol/4h. Tissues were then transferred into butanol followed by paraffin incubation at 60°C overnight. Finally, tissues were embedded into paraffin blocks and cooled overnight. Paraffin sections of 6-7 μm were cut and mounted onto slides and dried for at least 10 minutes. Before H&E staining, slides were heated at 62°C overnight. Staining and fixation were performed as follows: toluene/10 min, 100% ethanol/2 min, 95% ethanol/1 min, MilliQ H_2O/5 min, hematoxyline/ 3 min, running water to wash away the color, MilliQ H_2O/2x2 min, 1% eosin/3 min, MilliQ H_2O/1 min, 100% ethanol/1 min, and toluene/10 min. Slides were fixed with Eukitt® quick-hardening mounting medium (Sigma-Aldrich, 03989). Pictures were taken with Nikon DXM-1200C Digital Camera (Nikon, Japan).

The number of foci was determined for each mouse, in which one focus is defined as an aggregate of 50 or more lymphocytes (55). We scored SG inflammation on a scale of 0 to 3 (25). Basically, grade 0: no inflammatory cells; grade 1: few perivascular and periductal inflammatory infiltrates (<100 cells); grade 2: moderate number of perivascular and periductal inflammatory infiltrates (100 to 500 cells); grade 3: extensive inflammation with large inflammatory foci (>500 cells).

Immunofluorescence. SG tissues were collected and embedded into Optimal Cutting Temperature (OCT) embedding matrix (CellPath, KMA-0100-00A). Sections of 5-7 μm were cut using a Cryostat CM 3050S (Leica Biosystems, Germany) and dried overnight at room temperature (RT), followed by fixation with pre-cooled 100% (v/v) acetone for 30 min and stored at -80°C until use. Before staining, slides were defreeze at RT for 5 min and rehydrated in PBS containing 2% (w/v) bovine serum albumin, used as blocking reagent. FITC-labeled anti-mouse CD4 (557307), APC-labeled anti mouse CD45 (559864), and PerCP-Cy5.5-labeled anti-mouse CD8a (551162) antibodies (all from BD Bioscience and diluted 1:200) were led to incubate at 4°C overnight. Diaminido phenyl indol (DAPI; Thermo Fisher Scientific, D1306; 1:5,000) was used to labeled nuclei. Slides were washed with PBS and fixed with 4% PFA for 20 min, followed by assembly with DAKO Fluorescence Mounting

Medium (DAKO, S3023). Pictures were observed on Spinning Disk Microscopy (Carl Zeiss, Germany).

Measurement of macroautophagy and CMA by western blotting. The methods used in these studies were recently fully detailed (29). The primary antibodies used here were the following: ACTA (ab203096), SQSTM1 (ab109012), ATG12 (ab155589), LAMP2A (ab125068), all from Abcam, and MAP1LC3B (M186-3) from MBL. The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (Jackson ImmunoResearch, 115-035-008 and 111-035-008). Signal was detected using Clarity^{TM} Western ECL Blotting Substrate (Biorad, 1705061). The expression levels of LC3-II, SQSTM1, ATG12, CTSD, and LAMP2A were normalized by densitometry on ACTA level using ImageJ or Image Lab softwares.

Transmission electron microscopy. The procedure used was as described (56). Briefly, the samples were fixed with 2.5% (v/v) glutaraldehyde for one week and followed by post-fixation in 0.5% (v/v) osmium tetroxide for 1h. Serial dehydration was as follows: 25% ethanol 10 min, 50 % ethanol 10 min, 70% ethanol 10 min, 95% ethanol 10 min, 100% ethanol 10 min repeat 3 times, 100% ethanol/propylene oxide (1:1) 10 min, 100% propylene oxide 10 min (twice). Tissues were then transferred into EPON for inclusion at 60°C for 2 days. 70nm-sections were cut by Ultra-microtome (Leica Biosystems, Germany) and stained with uranyl acetate.

Measurement of serum autoantibodies by ELISA. The presence of TRIM21/Ro52 and dsDNA antibodies in the mouse sera were studied by indirect ELISA as described earlier (57,58). In short, TRIM21/Ro52 (20 ng/mL in 0.05M carbonate buffer, pH 9.6) or dsDNA (Sigma; 100 ng/mL in 25 mM citrate buffer, pH 5.4) were directly adsorbed onto plastic of ELISA microtiter plates (Thermo 2801). After incubation overnight at 4°C and saturation of free sites using 1% (w/v) bovine serum albumin in Tween 20-containing PBS (PBS-T), diluted sera (1:500 in PBS-T) were incubated at 37°C in the wells of coated plates. After completion and washing of plates, horseradish peroxidase-conjugated goat anti-mouse IgG second antibody (diluted 1:10,000 and 1:20,000 in PBS-T in the case of TRIM21/Ro52 and dsDNA, respectively) was added to reveal the binding of mouse antibodies. The final reaction was visualized by adding H_2O_2 as peroxidase substrate and 3,3',5,5'-tetramethyl benzidine as chromogen for 15 min at 37°C, and after blocking the reaction with 1M HCl, absorbance was

measured at 450nm using an ELISA reader. Normal mouse sera were used to calibrate the ELISA conditions and as internal controls.

Measurement of pH and ATP levels in total salivary glands cells. The evaluation of pH was performed using LysoSensor Green DND-189 fluorescence cell staining (Macri et al., 2015). The ATP level was measured using the Luminescent ATP Detection Assay Kit (abcam, ab113849). Following the manufacturer's instructions, cells cultured into 96-well plates were successively led to incubate with 50 µL detergent (5 min) and 50 µL substrate (5 min). Luminescence was measured and ATP levels were calculated according to a standard curve.

Statistics. Statistical analyses were performed using Wilcoxon Rank Sum tests (comparison of diversity between two groups), nonparametric Mann-Whitney test, paired or unpaired t-tests when sample distribution followed a Gaussian distribution. Differences in life span were analyzed using both the Gehan-Breslow-Wilcoxon method that gives more weight to deaths at early time points, and the log-rank test that gives equal weight to all time points. p value <0.05 were considered statistically significant.

Study approval. Animal protocols were carried out with the approval of the local Institutional Animal Care and Use Committee (CREMEAS, Strasbourg, France) and the French Ministère de l'Enseignement Supérieur de la recherche et de l'innovation (procédure APAFiS, autorisation de projet utilisant des animaux à des fins scientifiques). According to our agreement, and taking into account the best European practices in the field, we took the necessary measures to avoid pain and minimize the distress and useless suffering of mice during the time of experiment and killing process.

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Author contributions

B.L. performed laboratory experiments and participated to the interpretation of results; H.D., performed some laboratory experiments; J.-D.F. performed imaging experiments, participated to the interpretation of results and contributed to the preparation of figures; F.W. contributed to the interpretation of results. S.M. conceived and coordinated the study, contributed to the interpretation of results and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Legend to Figures

Figure 1. **Lymphocytic infiltration in salivary glands of MRL/lpr mice**. (A) Image of SGs collected from 15 week-old MRL/lpr mice compared to those of B6 control mice of the same age; (B) Frequency of total lymphocytes in SGs from B6 and MRL/lpr mice (15-29 weeks; n= 4 and 10, respectively) as measured by flow cytometry; (C) Frequency of T $CD4^+$ and $CD8^+$ cells and $CD19^+$ B cells in SGs from B6 and MRL/lpr mice (15-29 weeks; n= 4 in each group) as measured by flow cytometry. The data presented in (B,C) are means ± SD; *p* values are from two-tailed Student's *t* tests. (D) Histology of SGs by H&E staining (top panels, original magnification ×2.5; bottom panels, original magnification ×20; bar: 500µm). (E) Immunofluorescence of $CD45^+$ and $CD4^+$ cells in SGs of 15 week-old B6 and MRL/lpr mice (original magnification ×20; bar: 100µm). Diaminido phenyl indol (DAPI) staining (blue) was used to detect nuclei. Data are representative of analyses/staining from at least 3 mice per group.

Figure 2. **Therapeutic effects of short-term P140 peptide in MRL/lpr mice**. (A) Schematic schedules of short-term (protocol 1) and long-term (protocol 2) strategies. Green arrows represent injections time points; the red arrow indicates the sacrifice point. (B) Frequency in SGs of immune cell subsets in MRL/lpr mice, as quantified by flow cytometry (10 mice per group). (C) CD45 and CD4 immunofluorescent staining in SGs of MRL/lpr mice (original magnification ×20; bar: 100µm). The nuclei were stained with DAPI (blue color). (D) Analysis of foci numbers in SGs of MRL/lpr mice that were either kept untreated (saline) or treated with P140 or ScP140 peptides (7 mice/group). (E) Histology of SGs of MRL/lpr mice as visualized by H&E staining (left panels, original magnification ×2.5; right panels, original magnification ×20. Bar: 500µm). (F) Analysis of inflammatory grade in SGs of MRL/lpr mice (7 mice/group) that were either kept untreated (saline) or treated with P140 or ScP140 peptides. (G) Analysis of C3 deposits in SGs of MRL/lpr mice that were either kept untreated (saline) or treated with P140 or ScP140 peptides. The nuclei were stained with DAPI. MRL/lpr mice were all females aged of 11-13 weeks at the start of experiments (sacrificed at day 5). Data in (B), (D), (F) are expressed as means ± SD. Representative of analyses/staining from 7-10 mice per group are shown. *p* values are from two-tailed Student's *t* tests.

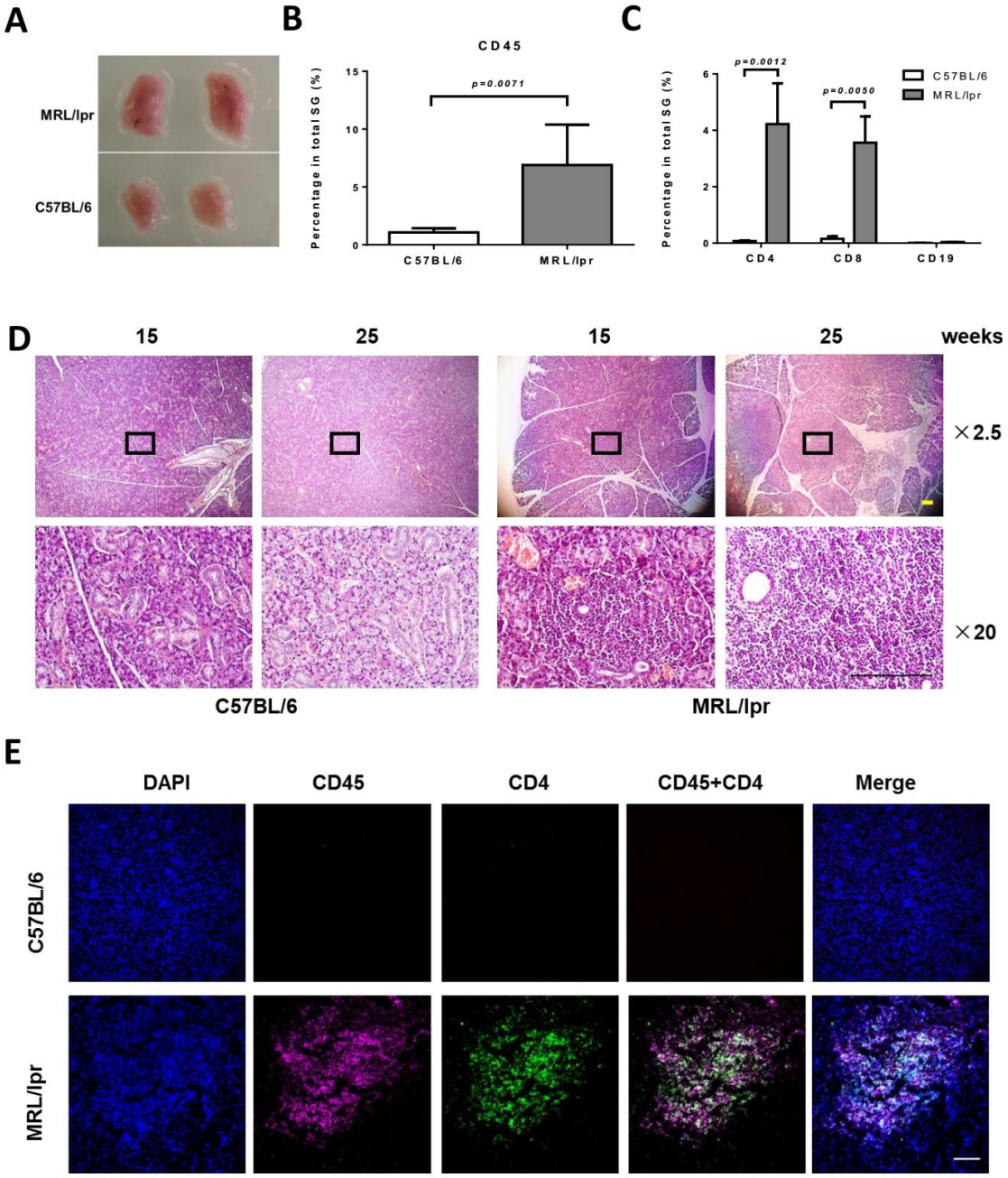
Figure 3. **Therapeutic effects of long-term P140 treatment in MRL/lpr mice**. (A) Effect of P140 peptide on the survival of MRL/lpr mice (69 female mice per group corresponding to 8 different experiments performed over 4 years). The arrows represent injections time points. The median lifespan was 25 weeks in the non-treated group *vs.* 35 weeks in the P140-treated group ($p<0.0001$ using both the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test). (B) Frequency of $CD45^+$ lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells in SGs from 23 week-old female MRL/lpr mice that were either kept untreated (saline) or treated with P140 or ScP140 peptides. Cell subsets were quantified by flow cytometry. (C) CD45, CD4 and CD8 staining as visualized by immunofluorescence (original magnification ×20; bar: 100µm). The nuclei were stained with DAPI. (D) Histology of SGs by H&E staining (top panels, original magnification ×2.5; bottom panels, original magnification ×20; bar: 500µm). (E) Focus scores in SGs of 23 week-old female MRL/lpr mice that were either kept untreated (saline) or treated with P140 or ScP140 peptides. (F-H) Serum autoantibodies levels (ELISA) to dsDNA and TRIM21/Ro52 antigen and proteinuria levels of mice, as monitored during the course of the treatment. Green arrows in G represent injections time points. The cut-off points for ELISA positivity were determined with a series of sera from naïve control mice. Results were considered positive when OD values were higher than the mean OD +2 SD).

MRL/lpr mice (10 per group except in panel B/CD8 that includes 7 mice) were all females aged of 5 weeks at the start of experiments. The number of remaining mice at each testing point is indicated in G. Data shown are means ± SD (panels B and E) or means ± SEM (panels F-H). *p* values are from two-tailed Student's *t* tests.

Figure 4. Autophagic flux in salivary glands from MRL/lpr mice. (A, B) SG cells were starved for 12h in serum-deprived RPMI-1640 medium and followed by 4h treatment with or without inhibitors (5 µg/mL E64D and PepA). MAP1LC3-II (A) and SQSTM1 (B) levels were analyzed by western blotting and quantified. (C and D) Accumulation of MAP1LC3-II after 12h of starvation (C) or after adding 100nM bafilomycin (Baf) A1 for 12h (D). (E-F) Levels of ATG12-ATG5 conjugate (E) and LAMP2A (F) measured by western blotting in B6 and MRL/lpr SG cells treated as in (A-B). Data shown in this figure are means ± SD (3-5 female mice per group). *p* values are from two-tailed Student's *t* tests. α-Actin (ACTA; 42 kDa) was used as a loading control and for normalizing western blots.

Figure 5. **Impaired Lysosome function in salivary glands of MRL/lpr mice.** (A) Intensity of LysoSensor Green DND-189 fluorescence in SG cells as acquired by flow cytometry. The fold fluorescence increase corresponds to the ratio of specific fluorescence (red lines) measured in cells incubated with LysoSensor Green) on autofluorescence measured in control cells (blue lines) incubated with the vehicle only (n= 8 mice per group). (B) Measurement of ATP content in SG cells collected from B6 and MRL/lpr mice (n= 4 per group). ATP levels were determined using the ATP lite^{TM} kit. Data shown in this figure are mean ± SD. *p* values are from two-tailed Student's *t* tests.

Figure 6. **Restoration upon P140 treatment of defective autophagic flux and lysosomal functions in salivary glands of MRL/lpr mice.** SG cells were isolated from MRL/lpr mice included in the long-term therapeutic procedure 2. They were starved 12h followed, or not, by incubation with anti-protease E64D and PepA. (A) Autophagic marker MAP1LC3-II levels as analyzed by western blotting and quantified. (B) Levels of LAMP2A measured by western blotting in SG cells collected from MRL/lpr mice that received saline only, P140 peptide or ScP140 control peptide. (C) Fold increase of LysoSensor Green in SG cells from MRL/lpr mice that received saline, P140 or ScP140. (D) Measurement of ATP content in SG cells collected from MRL/lpr mice that received saline only, P140 peptide or ScP140 control peptide. ATP levels were determined using the ATP lite^{TM} kit. Data shown in this figure are mean ± SD (3-8 female mice per group). *p* values are from two-tailed Student's *t* tests.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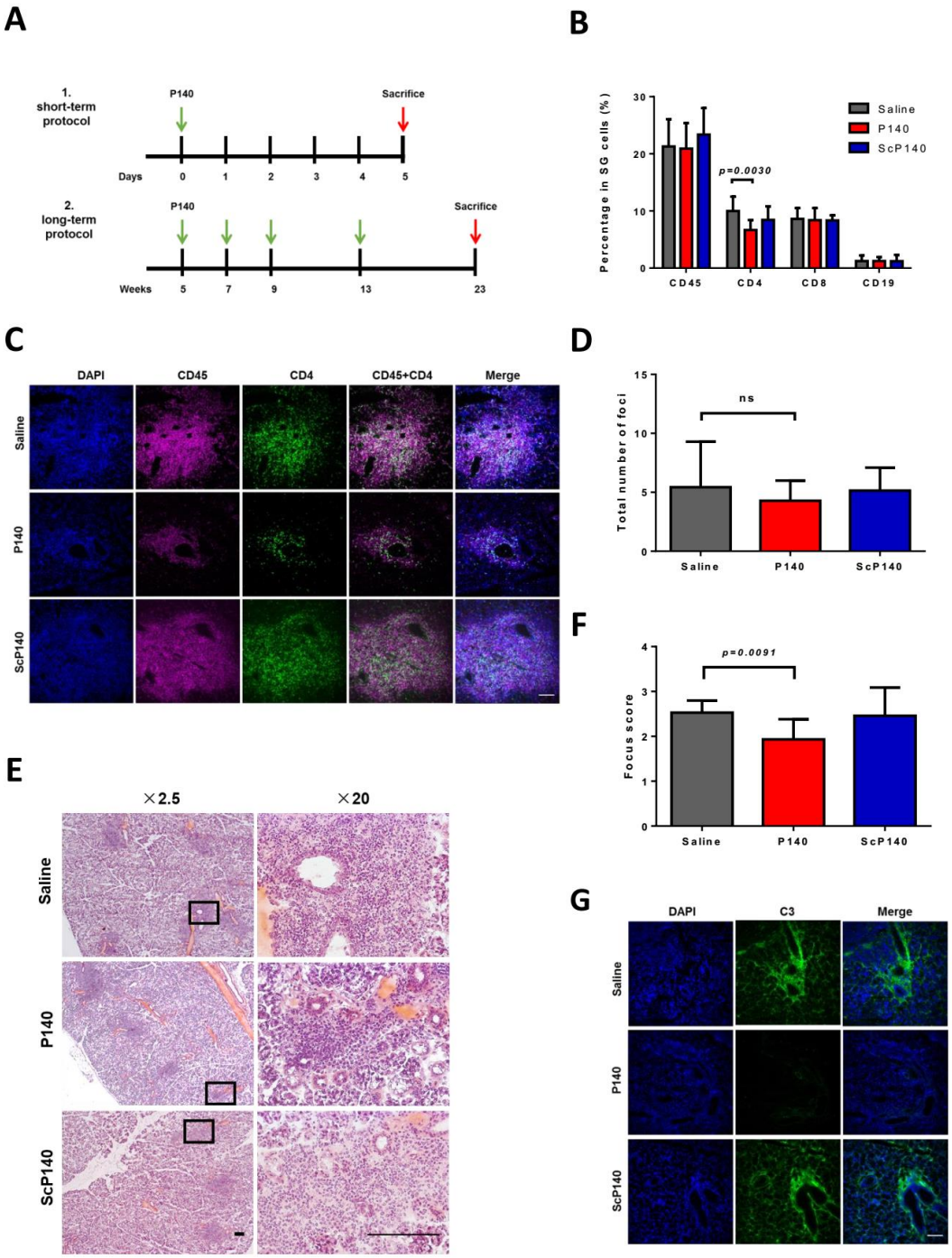
MRL/lpr
C57BL/6
B
CD45
p=0.0071
Percentage in total SG (%)
C57BL/6
MRL/lpr
C
p=0.0012
p=0.0050
C57BL/6
MRL/lpr
Percentage in total SG (%)
CD4
CD8
CD19
D
15
25
15
25
weeks
×2.5
×20
C57BL/6
MRL/lpr
E
DAPI
CD45
CD4
CD45+CD4
Merge
C57BL/6
MRL/lpr

Figure 1


A
1.
short-term
protocol
P140
Sacrifice
Days
0
1
2
3
4
5
2.
long-term
protocol
P140
Sacrifice
Weeks
5
7
9
13
23
B
Percentage in SG cells (%)
Saline
P140
ScP140
p=0.0030
CD45
CD4
CD8
CD19
C
DAPI
CD45
CD4
CD45+CD4
Merge
Saline
P140
ScP140
D
Total number of foci
ns
Saline
P140
ScP140
E
×2.5
×20
Saline
P140
ScP140
F
Focus score
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P140
ScP140
G
DAPI
C3
Merge
Saline
P140
ScP140

Figure 2

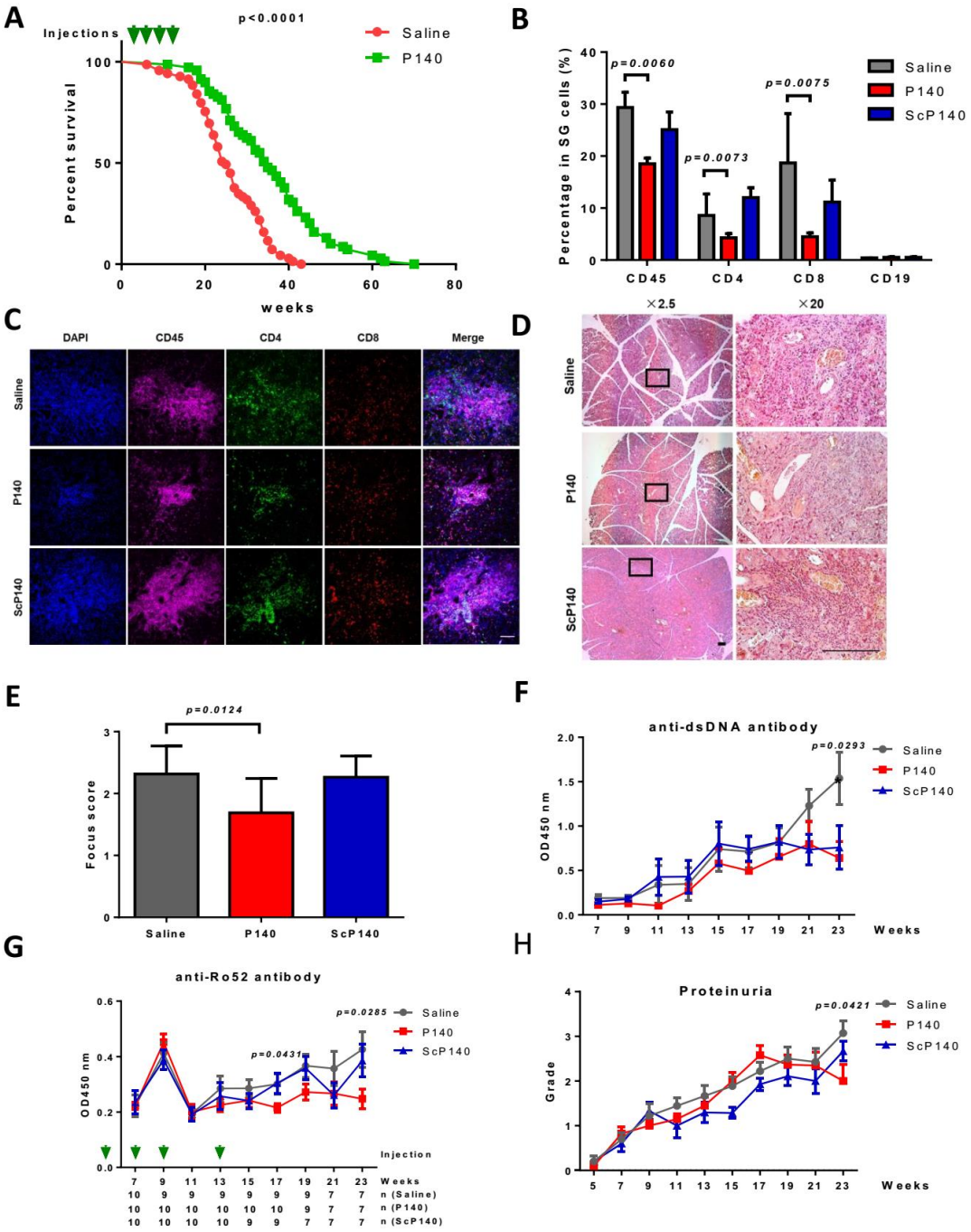
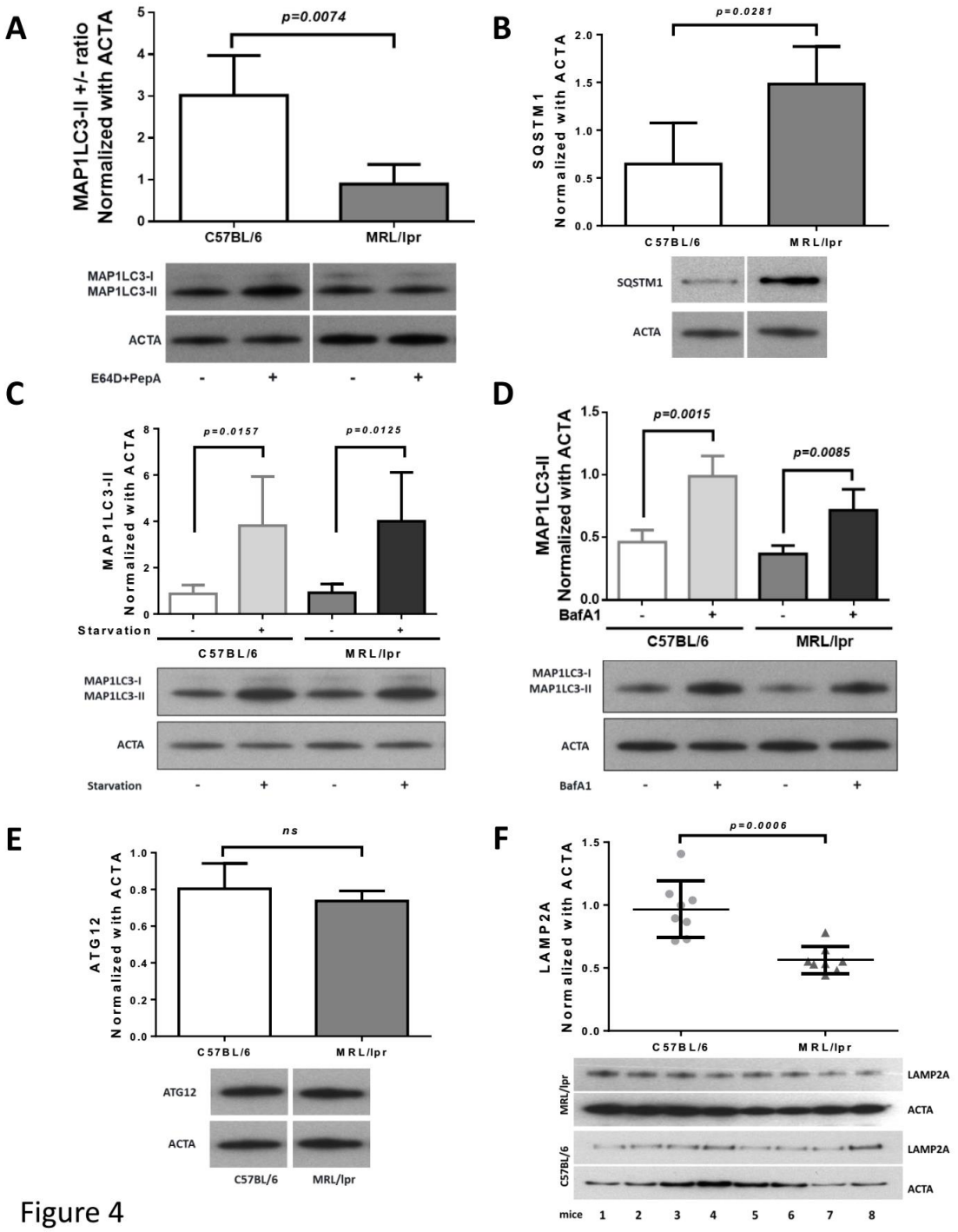

A
Injections
p<0.0001
Saline
P140
Percent survival
weeks
B
Percentage in SG cells (%)
p=0.0060
p=0.0073
p=0.0075
Saline
P140
ScP140
CD45
CD4
CD8
CD19
C
DAPI
CD45
CD4
CD8
Merge
Saline
P140
ScP140
D
×2.5
×20
Saline
P140
ScP140
E
p=0.0124
Focus score
Saline
P140
ScP140
F
anti-dsDNA antibody
p=0.0293
Saline
P140
ScP140
OD450 nm
Weeks
G
anti-Ro52 antibody
p=0.0285
p=0.0431
Saline
P140
ScP140
OD450 nm
Injection
Weeks
n (Saline)
n (P140)
n (ScP140)
H
Proteinuria
p=0.0421
Saline
P140
ScP140
Grade
Weeks

Figure 3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Normalized with ACTA
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C57BL/6
MRL/lpr
MAP1LC3-I
MAP1LC3-II
ACTA
E64D+PepA
B
SQSTM1
Normalized with ACTA
p=0.0281
C57BL/6
MRL/lpr
SQSTM1
ACTA
C
MAP1LC3-II
Normalized with ACTA
p=0.0157
p=0.0125
Starvation
C57BL/6
MRL/lpr
MAP1LC3-I
MAP1LC3-II
ACTA
Starvation
D
MAP1LC3-II
Normalized with ACTA
p=0.0015
p=0.0085
BafA1
C57BL/6
MRL/lpr
MAP1LC3-I
MAP1LC3-II
ACTA
BafA1
E
ATG12
Normalized with ACTA
ns
C57BL/6
MRL/lpr
ATG12
ACTA
C57BL/6
MRL/lpr
F
LAMP2A
Normalized with ACTA
p=0.0006
C57BL/6
MRL/lpr
MRL/lpr
LAMP2A
ACTA
C57BL/6
LAMP2A
ACTA
mice 1 2 3 4 5 6 7 8

Figure 4

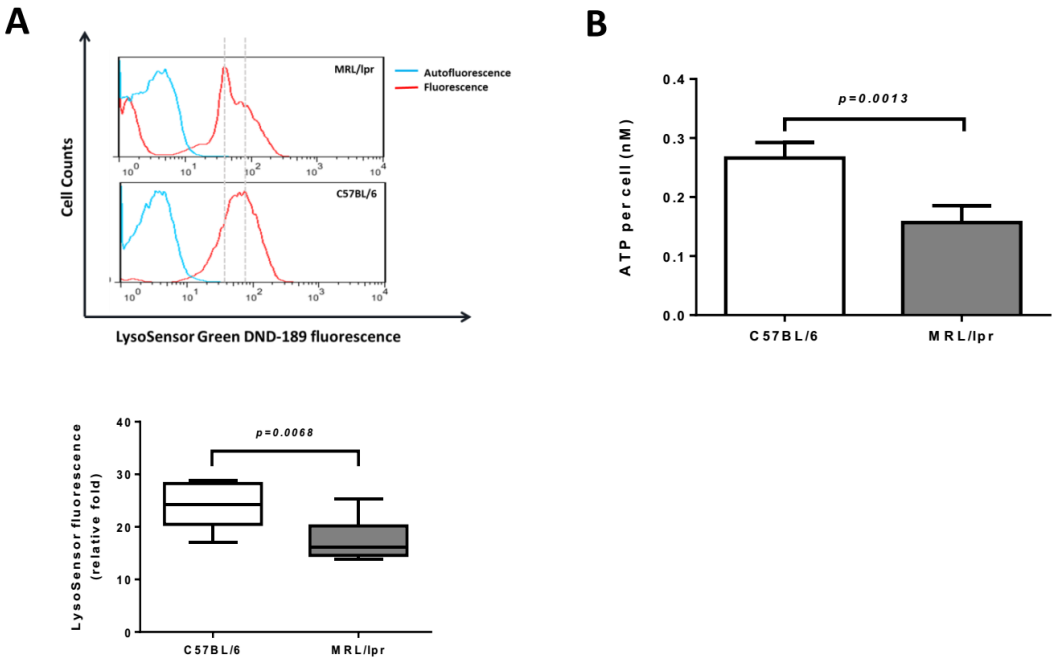

A
MRL/lpr
C57BL/6
Autofluorescence
Fluorescence
Cell Counts
LysoSensor Green DND-189 fluorescence
B
p=0.0013
ATP per cell (nM)
0.4
0.3
0.2
0.1
0.0
C57BL/6
MRL/lpr
p=0.0068
LysoSensor fluorescence (relative fold)
40
30
20
10
0
C57BL/6
MRL/lpr

Figure 5

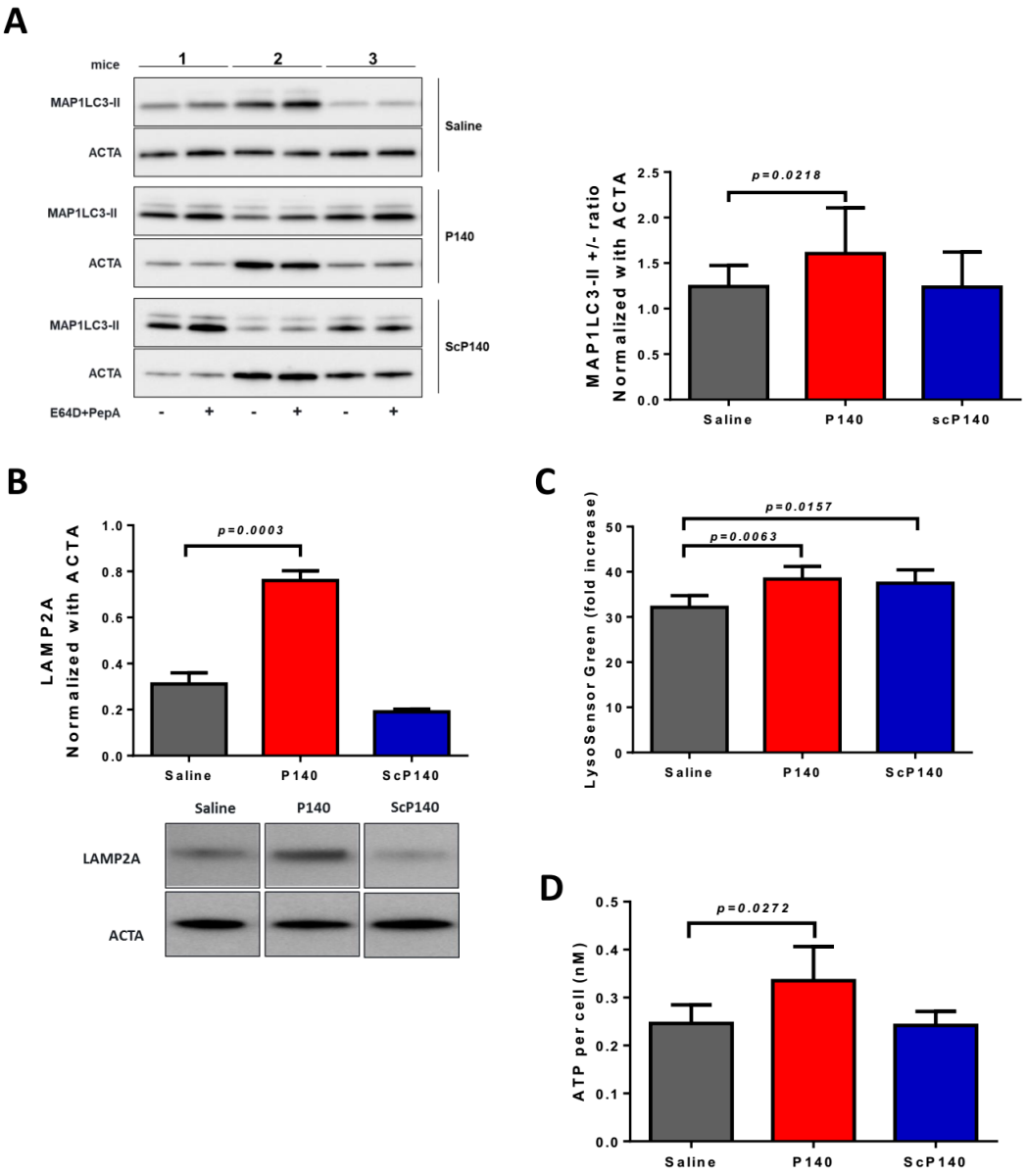
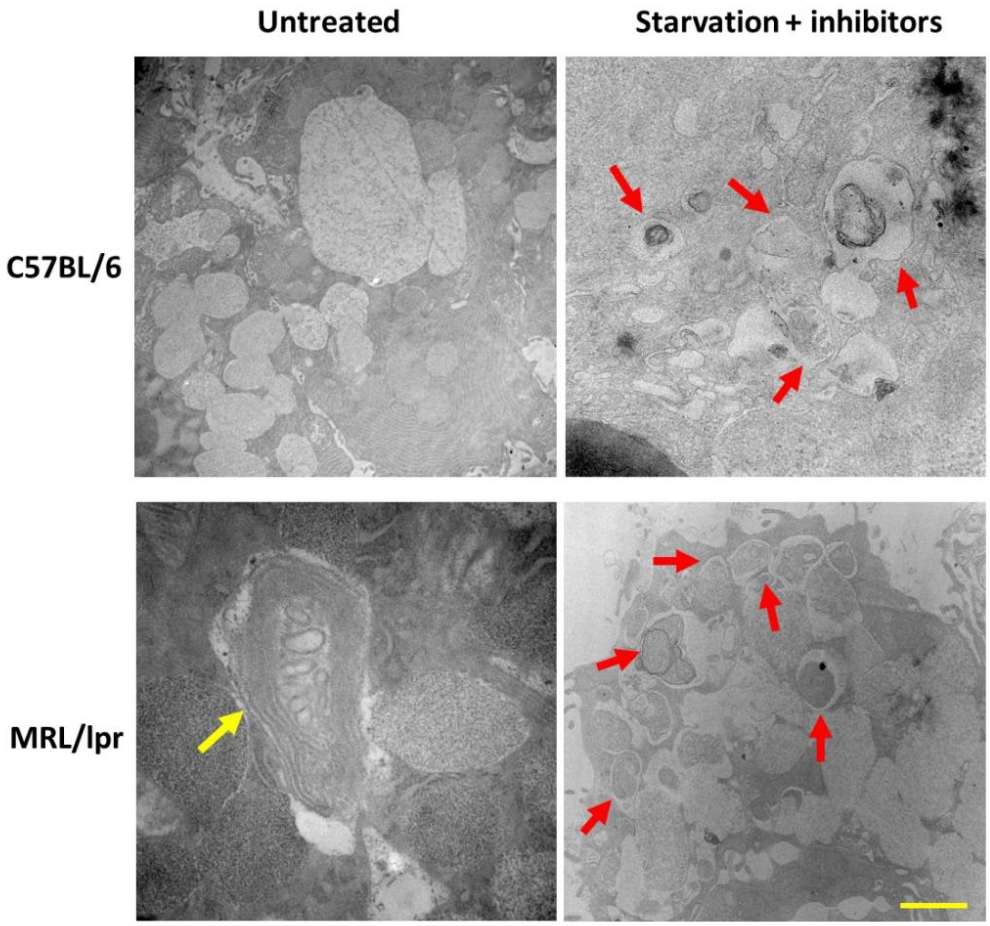
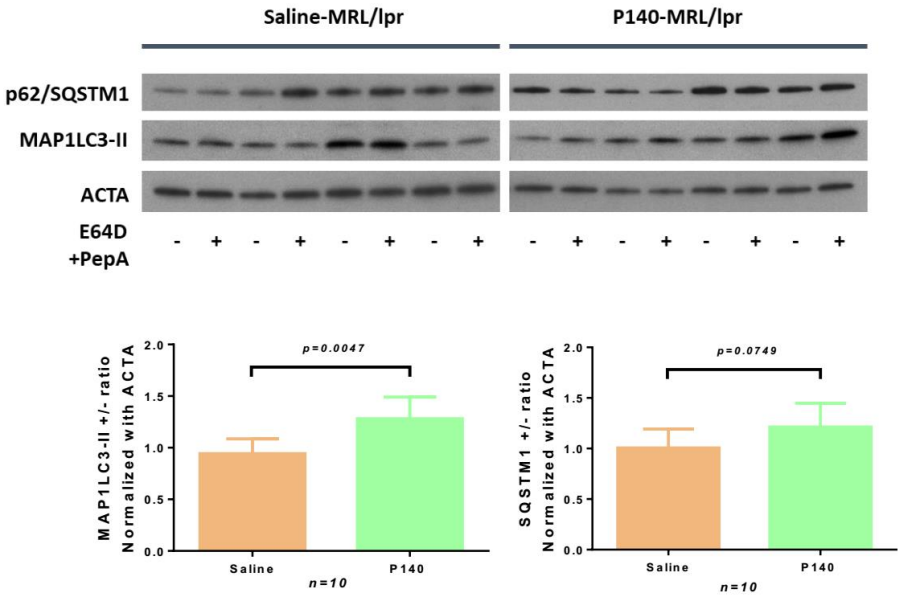

A
mice
1
2
3
MAP1LC3-II
ACTA
Saline
P140
ScP140
E64D+PepA
- + - + - +
MAP1LC3-II +/- ratio
Normalized with ACTA
p=0.0218
Saline
P140
scP140
B
LAMP2A
Normalized with ACTA
p=0.0003
Saline
P140
ScP140
LAMP2A
ACTA
C
LysoSensor Green (fold increase)
p=0.0157
p=0.0063
Saline
P140
ScP140
D
ATP per cell (nM)
p=0.0272
Saline
P140
ScP140

Figure 6



Supplementary Figure 1. Autophagosomes in SG cells from C57BL/6 and MRL/lpr mice. SG cells were isolated from C57BL/6 and MRL/lpr mice and prepared for transmission electron microscopy. Autophagosomes were identified in SG cells that were left untreated (left panels) or starved and incubated with inhibitors (right panels). Yellow arrow: large size multi-membrane structures in untreated MRL/lpr SG cells. Red arrows: autophagic vacuoles in SG cells starved and incubated with inhibitors. Bar:500nm.



Supplementary Figure 2. Restoration upon P140 treatment of defective autophagy processes (short treatment). SG cells were starved for 12 hours in serum deprived RPMI-1640 medium and followed by 4 hours treatment with/without inhibitors (5 µg/ml E64D and PepA). MAP1LC3-II and SQSTM1 levels were analyzed by Western blotting and quantified. Data shown are mean ± SD and representative of 10 mice. *p* values are from two-tailed Student's *t* test.

Comments

In this study, we uncovered that autophagy pathway is defective in SG cells of MRL/lpr mice. Autophagy has been demonstrated to be a regulatory mechanism in autoimmunity and participates in the pathogenesis of AIDs like RA, MS and SLE. However, the roles of autophagy in SS have not yet been described. This is the first study that reveals the involvement of autophagy in SS pathology, and it would be useful for further investigations of SS. The involvement of autophagy in SS also suggests autophagy as a potential therapeutic target.

In addition, our study used immunological and biochemical techniques to evaluate therapeutic functions of P140 in SS and identified the protective effects of P140 peptide in MRL/lpr mice. The beneficial effects of P140 on the SG of SS mouse model may shield lights on the promising application of P140 in SS, and possibly also in other AIDs.

Perspectives

1. Studies need to be performed in different cell types.

As introduced in the previous chapter (result 1), the autophagy profile can be different between organs/tissues of the same individual. Similarly, the activity of autophagy can be different in diverse cell types. In this study, we examined the autophagy activity in whole SG cells, which indicates an imprecise profile of autophagy in all cell types of SG. However, in each cell population, the activity of autophagy is unknown. It would be decisive to inspect autophagy in different cell populations and identify the specific role of autophagy in certain cell type. The function of autophagy in mucous cells and acinar cells can be very interesting, as these cells are mainly responsible for saliva secretion and consist most of the cells in SG. As observed by TEM, these cells are filled with vacuoles to transport and deliver saliva products. Whether autophagy is involved in the production or secretion of saliva in these cells is unknown. Notably, loss of secretion function is a typical feature in SG of patients with SS, and causes the symptom of dry mouth. Thus, further studies on this topic should be performed for elucidating secreting dysfunctions in

SS.

2. The role of lysosome need to be more deeply studied.

We obtained data suggesting that defective autophagy might result from impaired lysosomal functions, as the acidity of lysosomes was changed in SG cells of MRL/lpr mice. However, the exact pH change was not measured. In the future, we would like to more precisely determine the pH of lysosomes in SG cells (and even better in particular cell types). Moreover, to investigate the size and localization of lysosomes could be interesting, as these lysosomal features are determining in their activity and likely change in stress conditions. It was reported that in starved conditions, lysosomes translocate to perinuclear locations and tend to display increased size. Moreover, the activities of hydrolytic enzymes (cathepsins) in lysosomes should be tested as they reflect the lysosomal function. As the activity of cathepsins strictly depends on the acidic environment in lysosomal lumen, the change of acidity may lead to cathepsins inactivation.

3. Other organelles are involved in the SG lesions.

We observed large amount of damaged mitochondria in SG cells of MRL/lpr mice, indicating the metabolism dysregulation in SS. Combined with our findings of decreased ATP level in MRL/lpr SG cells, these results suggested that mitochondria dysfunction plays a role in SS. The decreased ATP level might be linked to other cellular activities and organelle functions. For example, ATP is the energy resource to maintain acidic lysosomal pH. In addition, ATP is required for the delivery and transportation of matured autophagosomes to lysosomes.

In another aspect, damaged mitochondria can be degraded by autophagy pathway. The abundantly existed damaged mitochondria in SG cells of MRL/lpr might be due to defective autophagy, which need to be examined by further studies.

4. The role of P140

P140 has been found to have impressive therapeutic functions in patients with SLE (Muller et al., 2008; Zimmer et al., 2013). However, the precise mechanisms in human are not clear. The data generated previously in our team have shown that

CMA pathway is a target of P140 in B cells (Macri et al., 2015). However, the target of P140 in SS has not yet been identified. In our study, P140 was intravenously injected to MRL/lpr mice for both “short-term” and “long-term” administrations. Following the first studies of the team regarding the biodistribution of P140 *in vivo* (Page et al., 2011b), more precise investigation should be performed, especially with regard to SGs.

For these questions remaining to be answered, further studies will be performed gradually to discover the roles of autophagy in SS, as well as the mechanism of P140 in AIDs.

CONCLUSION AND GENERAL DISCCUSION

As one of the most prevalent rheumatic diseases, SS affects numerous patients. Unfortunately, there is no effective and systemic treatment for SS nowadays. The medical need to treat SS is thus highly unmet, which justifies the necessity for more efforts on the elucidation of the mechanism of SS disease.

Mouse models of Sjögren's syndrome

Direct clinical studies on SS are often restricted, mainly due to the limited access to patient samples and the lack of preclinical signs for SS. Mouse models, however, provide us an alternative way to study the etiology and the pathology of SS. Moreover, the development of mouse models also enables us to discover and validate potential therapeutic targets of SS, as well as to predict the therapeutic effect of potential treatments.

An ideal mouse model of SS disease should fulfill several conditions. Firstly, the mouse model should be able to fully recapitulate the symptoms observed in SS patients. Secondly, the mouse model should share the same etiology of disease with SS patients. This criterion is crucial for both the mechanism studies of the disease and the development of novel therapeutics. Thirdly, the mouse model is expected to be able to predict the therapeutic effects of new treatments.

Mouse models of SS can be classified into primary and secondary models, referring to the clinical features of primary SS and secondary SS. Mouse models can also be classified into spontaneous models and induced models, based on the difference of their etiologies. Spontaneous mouse models mainly consist of gene-deficient mice with susceptibility loci or transgene/knockout (Tg/KO) mice, while induced mouse models use specific antigens or sometimes some chemicals to induce SS in non-autoimmune prone mice.

In our study, we have used the MRL/lpr mouse as a mouse model for SS. MRL/lpr mouse has been suggested as a mouse model of SS since 1988, when Jabs and colleagues described several clinical features and immunologic abnormalities of three strains of autoimmune mice, including MRL/lpr mice (Jabs et al., 1988). Th cells

are found to be the predominant infiltrated lymphocytes in LGs of MRL/lpr mice, which cause LG lesions. Later, similar features were found in SGs of MRL/lpr mice, where local cytokines production including IL-1, IL-6 and TNF was increased (Hayashi et al., 1994). SS typical anti-Ro and anti-La autoantibodies have been detected by ELISA in the sera of MRL/lpr mice (Wahren et al., 1994), and the secretion of saliva was found to be impaired in MRL/lpr mice compared with normal C57BL/6 mice (Iwabuchi et al., 1994). These studies suggest the usefulness of MRL/lpr mouse as a model for SS. However, the MRL/lpr mice also develop clinical and biological signs of other AIDs such as lupus and RA. Hence, this mouse model is recognized as a mouse model of sSS.

Another well acknowledged mouse model of SS is the non-obese diabetic (NOD) mice. Lymphocytic infiltrations in SG and LG of these mice occur at 12-16 weeks (Yamano et al., 1999). As observed in SS patients, CD4 T cells are the predominant infiltrated lymphocytes (Skarstein et al., 1995). However, NOD mice are not only used as mouse model for SS, but also commonly used as a model for T1D (Pearson et al., 2016).

A C57BL/6 derived mouse model, the C57BL/6.NOD-Aec1Aec2 mice, presents the SS disease phenotype without developing diabetes. This mouse strain with C57BL/6 background incorporates double congenic of *Idd3* and *Idd5* susceptibility loci, which are the most important loci for SS development in NOD mice (Lee et al., 2012). Compared with NOD mice, C57BL/6.NOD-Aec1Aec2 mice tend to show more rapid SS progression. The onset of autoimmune responses starts from 0-8 weeks, and the lymphocytes infiltrate in exocrine glands at 8-16 weeks. Importantly, the predominant infiltration is driven by CD4 T cells, which is similar with that in SS patients (You et al., 2015). After 16 weeks, secretory dysfunction is observed in glandular tissues, which is one of the hallmarks of SS (Park et al., 2015).

Mouse models of SS can also be induced by virus (MCMV, for example) or autoantigens such as Ro60 and muscarinic acetylcholine receptor M3 (M3R) peptide in mice with healthy background. The main features of different mouse models are

summarized in Table 3.

Mouse models enable us to study the biological and immunological mechanisms of SS. There are several advantages of mouse models: 1, the genetic similarity between mice and human makes mouse models a powerful tool for etiopathogenetic studies of the disease; 2, mice are amenable to genetic modifications and experimental administration, which may facilitate strictly controlled experiments that are highly valuable for translational studies; 3, the disease progression in mouse models provides useful information about the pathological development of SS; 4, SS tends to develop in relatively short phase in mouse models, which decreased the time needed for SS studies; 5, mouse models are useful in preclinical evaluation of potential therapies. In recent years, the use of mouse models has greatly facilitated the studies of SS.

However, it is well acknowledged that discrepancies exist between human and mice, such as susceptibility to diseases, clinical symptoms and immune responses (Delaleu et al., 2011). Thus, it is not unexpected to realize that none of the mouse models is able to reproduce all the clinical characters observed in SS patients (Table 3). Moreover, the genetic background of mice may contribute to the pathogenesis of SS in mouse models. For instance, the primary SS mouse model C57BL/6.NOD-Aec1Aec2 mice are generated with *Idd3* and *Idd5* loci, which play key roles in SS development in mice, but their involvement in SS patients has not been reported. Therefore, the SS-related characters in the C57BL/6.NOD-Aec1Aec2 mice should be interpreted within the context of this genetic background. For these reasons, current mouse models are generally useful for pathological studies and for therapeutic investigations, but are not totally convincing for studying the etiology of SS in patients. Not only for SS, there is no ideal mouse model for other AIDs such as MS and RA, and the development of new mouse models is eagerly needed.

Mouse models of SS

		Name	Strain Backgrounds	Gene/virus/Ag	Lymphocytic Infiltration				Other Organs	Autoantibody			Onset	Cytokines	Other diseases	References
					SG	LG	Secretion loss	Primary infiltration		Ro	La	Others				
Gene deficient mice	susceptibility loci	MRL/lpr	MRL	*lpr*	**Y**	**Y**	**Y**	CD4 T cells	spleen, kidney	Y	Y	ANA	8-10wk	IL-10, IL-6, TNF	SLE	(Hayashi et al., 1994)
		NOD	NOD	*Idd*	**Y**	**Y**	**Y**	CD4 T cells	pancreas	Y	Y	α-fodrin; M3R	12-16wk	IL-10, IL-7, IFN-γ	T1D	(Yamano et al., 1999)
		C57BL/6.NOD-*Aec1Aec2*	C57BL/6	*Idd3*, *Idd5*	**Y**	**Y**	**Y**	CD4 T cells	N	Y	Y	ANA	0-8wk	IL-7; IL-17	_	(You et al., 2015)
		NOD.B10-H^{2b} mice	NOD	*MHC I-A* ^{b}	**Y**	**Y**	**Y**	B cells	N	Y	Y	ANA	8wk	IL-4	_	(Gao et al., 2006)
		IQI/*Jic* mice	ICR	*Jic*	**Y**	**Y**	**Y**	B cells	kidneys; lungs	N	N	ANA	8wk	B7-2, IL-12, IFN-γ	dermatitis, colitis	(Konno et al., 2003)
	Tg/KO	*BAFF* Tg mice	C57BL/6	*BAFF*	**Y**	**N**	**Y**	B cells	kidneys	N	N	ANA	13mom	LT	SLE	(Srivastava et al., 2010)
		Id3 KO mice	C57BL/6	*Id3*	**Y**	**Y**	**Y**	CD4 T cells	_	Y	Y	_	6-12mon	_	_	(Guo et al., 2011)
		PI3K KO mice	C57BL/6	*Pik3r*	**Y**	**Y**	**Y**	CD4 T cells	lungs, liver, intestines	Y	N	ANA	12mon	IL-4,IL-10,IFN-γ	_	(Oak et al., 2006)
		Ar KO mice	C57BL/6	*Ar*	**Y**	_	_	B cells	marrow, kidneys	_	_	α-fodrin	12mon	IFN-γ	_	(Shim et al., 2004)
Induced mouse models	virus	MCMV induced SS	C57BL/6	MCMV	**Y**	_	**Y**	CD4 T cells	_	Y	Y	_	4wk	_	**SLE**	(Fleck et al., 1998)
	autoantigens	Ro-induced SS	BALB/C	Ro60	**Y**	_	_	CD8 T cells	_	Y	Y	_	16wk	_	_	(Scofield et al., 2005)
		M3R peptide induced SS	NOD/LtJ	M3R	Y	_	Y	CD4 T cells	_	_	_	_	8wk	IL-17, IFN-γ	_	(Yang et al., 2013)
	chemic	Alum induced SS	NZM2758	Alum	Y	_	Y	_	_	N	N	_	8wk	IL-1β, IL-18	_	(Bagavant et al., 2014)

Table 3. Mouse models of SS

Autophagy in autoimmune diseases

Various roles of autophagy in autoimmune response

Recent studies have highlighted the various roles of autophagy in autoimmune responses. As described above, autophagy participates in the homeostasis regulation of immune and non-immune cells, inflammation and autoantigens presentation. The versatile roles of autophagy revealed in AIDs underscore the importance of this pathway (Ma et al., 2013). The implication of this crucial pathway in various cellular events of AIDs may be partially explained by two facts.

Firstly, the immunometabolism activities in cells are interconnected. Autophagy, as a degradation pathway, is connected with protein modification (Klionsky et al., 2014), cell proliferation (Phadwal et al., 2013), apoptosis (Kaminskyy et al., 2014), ATP production, receptor recognition (Weindel et al., 2015) and other cell events. Hence, the roles played by autophagy in AIDs might result from interactions between various central cellular pathways and activities.

Secondly, the role played by autophagy in different types of cells may vary, due to different cell functions and cellular circumstances. For instance, in vascular smooth muscle cells, autophagy is involved in contractile-to-synthetic phenotype transition and plays a critical role in vascular remodeling (Salabei et al., 2015). In macrophages, autophagy is found to have an influence on macrophage migration, differentiation and polarization (Chen et al., 2014). Autophagy can therefore play various roles in systemic disorders like AIDs in which various types of cells are involved (Figure 26).

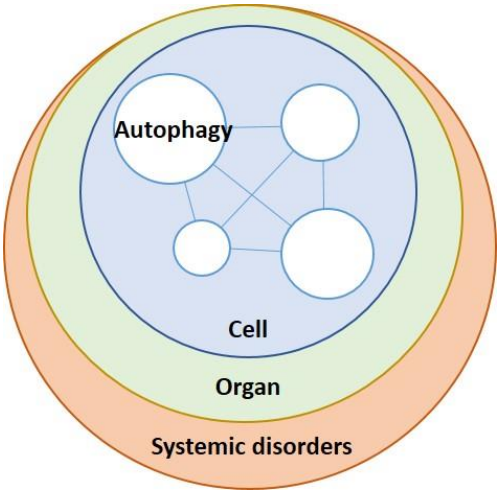


Figure 26. Autophagy in AIDs

The roles of autophagy in AIDs can be due to its interactions with other cellular pathways, as well as its functional variation in different cell types. Aberrant autophagy in certain cell types results in the lesion of organs, and finally leads to systemic dysregulation, which is AID.

The roles of autophagy in Sjögren's syndrome

Autophagy has been demonstrated to be a regulatory mechanism in autoimmunity, participating in the pathogenesis of AIDs like RA, MS and SLE (Cadwell, 2016). However, whether autophagy plays a role in the pathogenesis of SS was not known before our own studies. As discussed above, the functions of autophagy can differ in distinct cell types. Therefore, the roles of autophagy in SS might be related to its functions in particular cell types.

Nowadays, epithelial cells are considered to play a central role in the pathogenesis of SS. Epithelial cells in glandular tissues are mainly composed of acinar cells that produce saliva components. Loss of functional secretion leads to dryness in patients, which is the hallmark of SS. Ca^{2+} signaling has been identified as a crucial regulator of fluid secretion in SG acinar cells. It is found that the key event in activation of fluid secretion is the increase of intracellular Ca^{2+}, which regulates the ion fluxes required to drive fluid secretion (Ambudkar, 2014). Whether autophagy is involved in Ca^{2+} signaling in SG acinar cells is unknown. However, lysosomes are recognized as a Ca^{2+} source that release Ca^{2+} into the cytosol, thereby regulating the Ca^{2+} signaling (La

Rovere et al., 2016). In our studies, we found that lysosomal function is impaired in SG cells of MRL/lpr mice. It is likely that the Ca^{2+} signaling is also affected by lysosomal dysfunction in SS. At this stage, further studies are needed to test this hypothesis.

Apart from Ca^{2+} signaling, epithelial cells apoptosis might also be associated with autophagy. Apoptosis of epithelial cells generates autoantigens that induce the production of autoantibodies, leading to autoimmune responses in SS. The relationship between autophagy and apoptosis has been illustrated in numerous studies (Marino et al., 2014). However, the interplay between autophagy and apoptosis in the circumstance of SS is still poorly understood. Autophagic cell death was found to be involved in IL-1-induced injury of LGs, and shown to trigger tissue repairment after IL-1 injection (Zoukhri et al., 2008). Whether a similar mechanism exists in glandular tissues of SS is unknown. Further studies may shed light on this question.

Therapeutics of Sjögren's syndrome

The therapeutics in use for SS and those that are in development have been discussed above (chapter A.5). These approaches aim at targeting various pathways or particular types of cells that are involved in SS. However, very few of them have shown systemic effects. This can be attributed to the complexity of etiopathology and manifestations of SS and other AIDs. Although large amount of evidence was found to link virus, genes, and daily life habits with SS (Brito-Zeron et al., 2016), the precise mechanism of SS pathogenesis is not clear, which increases the difficulty to develop therapeutics of this disease.

The involvement of different organs and systemic disorders are hallmarks of both SS and other AIDs. The etiopathology and therapies of AIDs, including SS, are discussed below.

Autoimmune diseases

AIDs can be considered as diseases caused by overactivated immune responses. In

healthy conditions, the immune responses aim to aid individuals to defend against invaders and eliminate these pathogens. Immune recognition, defense and tolerance are three major mechanisms and events of immunity. These three mechanisms corporate to maintain the immune homeostasis, forming an efficient immune system. However, when autoimmune responses are triggered in this system, AID can be induced.

In AIDs, the immune tolerance is broken to some extend and the immune system loses its capability to distinguish “self” from “non-self”. The relationships between the three events of immunity in the context of AIDs can be briefly described as follows (Figure 27):

Firstly, the breakdown of immune tolerance leads to self-antigen recognition.

Secondly, self-antigen recognition enhances the immune defence, which further increases the breakdown of immune tolerance.

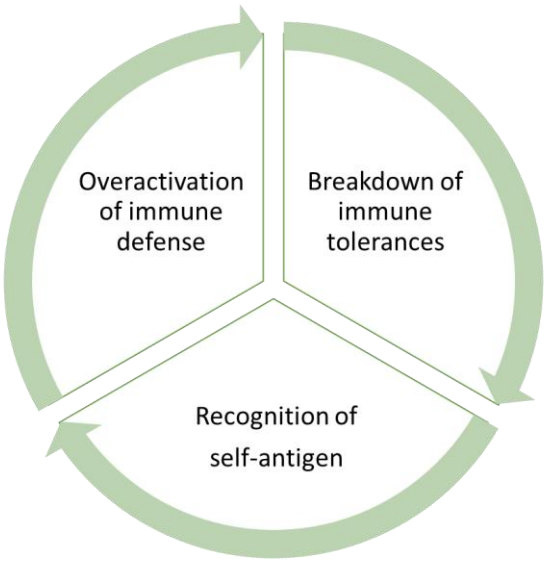


Figure 27. The interaction between immune defence, immune tolerance and self-antigen recognition in AID

In patients with AIDs, the relationship between these three mechanisms can be more

interactive and complicated. For example, the recognition of self-antigens may initiate the breakdown of immune tolerance, and the loss of tolerance can in turn promotes immune defence. In general, these three factors connect with each other, contributing to the development of AID.

As AIDs are chronic inflammatory diseases, inflammation is found to play crucial roles in the pathogenesis of AID. Cytokine dysregulation has been found in RA, MS, T1D and many other AIDs including SLE and SS (Cadwell, 2016). Fundamental studies revealed different pathways involved in the regulation of inflammation and proposed various approaches to treat AID by targeting inflammation. However, whether inflammation is an ideal therapeutic target is worth to deliberate. It is well acknowledged that inflammation is driven by immune responses to protect individuals. In addition, the chronic inflammation is more complicated than its classical form. In general, inflammation indicates the local reaction of redness, swelling, heat and pain. In chronic inflammation, it represents the activation of various immune cells and the dysregulated network of cytokines, host defense and immune tolerance. For these reasons, it would be difficult to target inflammation in a precise way.

Therapeutics of autoimmune diseases

As AIDs are usually systemic diseases, developing methods, which regulate the systemic immune responses would be an ideal solution. However, the most widely applied systemic therapy is to use non-steroidal anti-inflammatory drugs combined with immunosuppressants. Apart from the alleviation of symptoms induced by inflammation, another consequence of the suppression of immune system is that patients are more vulnerable to infections and suffer from functional tissue impairments.

B cells have shown great potential as a therapeutic target in SLE. In recent years, a monoclonal antibody targeting BAFF, called Belimumab, achieves its success in SLE. In more recent studies, Belimumab was suggested to also apply to other AIDs such as autoimmune hemolytic anemia, RA and MS (Faurschou et al., 2014; Stohl et al.,

2017). Rituximab, another monoclonal antibody, which transiently depletes CD20+ B cells, has shown promising effects in RA (van Vollenhoven et al., 2013; Collison, 2017). The precise mechanism of action of these B cells-targeting therapies is not fully understood. The predominant hypothesis is that B cells inhibition decreases the production of autoantibodies. In autoimmune settings, autoantibodies production is aberrantly increased, which leads to the activation of other immune cells (*via* Fc receptor) and the deposition of immune complexes that cannot be efficiently cleared. Thus, depletion of autoantibody-producing cells can represent a promising approach in treating AIDs. Moreover, since B cells can also function as professional APCs, B cells depletion/inhibition can not only reduce autoantibody level, but also decrease autoantigen presentation. Hence, targeting B cells is acknowledged as a breakthrough for AIDs therapies, as evidenced in past by clinical trials (Faurschou et al., 2014). Although very promising, some B cell targeting therapies effectively failed to provide significant clinical benefits due to inadequate responses and insufficient activity (Genovese et al., 2011). This prompts researchers to further investigate on novel therapies.

Therapeutic peptide P140

Compared with the classical pharmacologic treatments, therapeutic peptides have shown numerous advantages such as higher activity, selectivity and specificity, as well as weaker immunogenicity and better organ penetration (Schall et al., 2012). Functional peptides have been developed in experimental autoimmune encephalomyelitis (EAE) mouse model to act as a dual blockade of the costimulatory CD28:B7 and CD40:CD40L pathways. The blocking peptides were designed to mimic the functional binding area of costimulatory molecules, thus the peptides are capable to block signals transduction (Allen et al., 2005). In addition to preclinical studies on mouse model, clinical trials based on immunotherapeutic peptides are currently ongoing (Zonneveld-Huijssoon et al., 2013). The therapeutic peptide P140 is currently on clinical III trail in US, Europe and countries of western India Ocean, emphasizing the potential of therapeutic peptides for AID treatment.

The P140 peptide was spotted in our laboratory in a study designed to identify epitopes of the small nuclear ribonucleoprotein U1-70K, which is a well-known autoantigen of lupus (Kattah et al., 2010). A peptide sequence encompassing the residues 131-151 (RIHMVYSKRSGKPRGYAFIEY) of the U1-70K protein was identified as been recognized by MRL/lpr mouse CD4 T cells (Monneaux et al., 2000). It was found later that an analogue of the peptide modified by phosphorylation on Ser140, hence P140, was strongly recognized by CD4 T cells (Monneaux et al., 2003). In later studies, P140 has been demonstrated to be an efficient therapeutic peptide for lupus in both mouse models and patients (Page et al., 2011b; Zimmer et al., 2013).

In the studies presented in my manuscript, we observed the protective effects of P140 in SG of MRL/lpr mice, suggesting P140 could also be a promising treatment for SS. Our results tend to link the therapeutic effects of P140 with autophagy pathway and lysosomal functions. However, whether P140 directly targets lysosome *in vivo* is still unknown. Further studies are necessary to decipher the molecular mechanisms of action of P140 in SS both *in vitro* and *in vivo*.

In our current study, we found that both autophagy and lysosomes are defective in the SGs of MRL/lpr mice, and that P140 restored both autophagic flux and lysosomal acidity. These results suggest that defective autophagy and lysosomes may contribute to the pathogenesis of SS, a very novel finding important to understand the physiopathology of SS. Interestingly, the restoring effect of P140 on autophagy is contrary to what we have observed in splenocytes of lupus, where P140 has an inhibitory effect on autophagy that is hyperactive (Macri et al., 2015). We therefore suspect that the beneficial effects of P140 on AID might be due to its capability to maintain cellular homeostasis, rather than a direct effect to up/down regulate autophagy. Moreover, the roles of P140 are not clearly understood, and the target of P140 is not totally defined. Whether P140 is involved in other pathways or cellular activities is unknown. As previously discussed, the role of autophagy can be different in diverse cell types, indicating that the particular cellular context may contribute to the function of autophagy pathway. Hence, the roles of P140 might be different in distinct cell types. Further studies are needed to test this hypothesis.

P140 has shown its potential as therapies for both lupus and SS. Whether P140 could be used in other AIDs is an interesting and valuable question. To understand the mechanism of action of P140 is an important task. Too many other questions remain to be answered concerning the P140 peptide. For instance, what is the biological distribution of P140 *in vivo*? Is P140 recognized by any immune receptor? Is P140 modified *in vivo*? Which cells types are targeted by P140?

AIDs present as a clinical challenge to our society for centuries and significant efforts have been carried out in attempts to understand the pathogenesis of the diseases as well as to find adapted therapeutic solutions. Despite the substantial increase of our knowledge on AIDs in recent years, the medical needs for treating AIDs remain unmet nowadays. Further studies armed with innovative technologies and interdisciplinary approaches are still in need.

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Résumé de thèse

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Titre

Autophagie comme cible thérapeutique potentielle pour le syndrome de Sjögren

Contexte

Le syndrome de Sjögren (SjS) est l'une des maladies autoimmunes (MAI) systémiques les plus fréquentes qui affectent 0,5 à 3% de la population [1]. La caractéristique de cette maladie inflammatoire chronique est l'infiltration lymphocytaire dans les glandes exocrines. Cliniquement, cette infiltration cellulaire dans les glandes salivaires et lacrymales provoque leur destruction et conduit à des symptômes dits de « yeux secs » et à la bouche sèche [1]. Les traitements actuels ne peuvent que soulager la sécheresse ; il n'existe aucun médicament qui traite les causes de la maladie.

Le peptide P140 correspond à une séquence linéaire de 21 résidus qui a été démontrée comme un outil thérapeutique efficace pour améliorer les manifestations cliniques et biologiques chez les patients atteints d'un lupus érythémateux disséminé (LED) et des souris modèles développant un lupus [3-6]. Ce peptide protège les individus contre le lupus ; la protéinurie des souris est considérablement diminuée et leur survie très augmentée [7]. Il a été démontré que suite à l'administration intraveineuse du peptide P140, les souris résistent à un challenge viral infectieux, suggérant qu'il n'exerce pas d'activité immunosuppressive globale du système immunitaire comme les molécules actuellement administrées aux patients (corticoïdes et immunosuppresseurs). Comme le SjS partage plusieurs similitudes avec le LED, avec un biais fort de pénétrance chez les femmes et certaines anomalies systémiques des réponses immunitaires, il a été décidé dans l'équipe d'étudier l'efficacité d'action du P140 dans le SjS.

L'autophagie est une voie intracellulaire conservée qui notamment dégrade les organites et protéines altérés (dénaturées, mal repliées) et les agrégats ; elle joue un rôle central dans le maintien de l'homéostasie cellulaire [8]. En outre, l'autophagie a été démontrée comme un mécanisme de régulation de l'autoimmunité ; elle est centrale dans l'activation des cellules immunitaires autoréactive et la présentation des autoantigènes par les cellules présentatrices d'antigènes.

Il existe trois formes majeures d'autophagie, à savoir la macroautophagie, la

microautophagie et l'autophagie médiée par les chaperonnes (CMA). Lors de la macroautophagie, des structures à double membrane nommées autophagosomes, sont générées pour encapsuler les composants cytoplasmiques. Ces structures fusionnent avec les lysosomes pour donner des autolysosomes dans lesquels se déroule leur dégradation par des protéases et hydrolases lysosomales [9] (Figure 1).

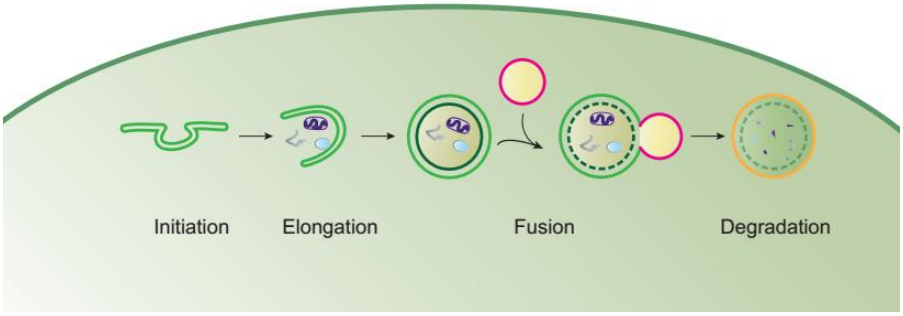

Figure 1. Processus dynamiques d'autophagie

La microautophagie survient lorsque le lysosome engloutit et dégrade les granules du cytoplasme directement. La principale fonction de la microautophagie est de maintenir la taille des organelles, l'homéostasie membranaire et la survie cellulaire, notamment en conditions d'anaérobie [10]. La voie CMA est médiée par un complexe composé de chaperonnes et co-chaperonnes, notamment d'HSPA8/HSC70 et de la protéine marqueur LAMP2A. Avec les actions coordonnées des chaperonnes situées sur les deux faces de la membrane lysosomale, les substrats destinés à être traités par la voie CMA sont sélectivement transportés vers le lysosome et passent dans la lumière lysosomale où ils sont dégradés [11]. Dans le mécanisme d'action du peptide P140 tel qu'il se dessine aujourd'hui, les effets bénéfiques du peptide dans le lupus semblent étroitement liés à sa liaison à HSPA8 et par voie de conséquence à son effet d'inhibition de la CMA qui est hyper-activée dans le lupus. Il a été démontré en effet qu'après administration du peptide l'expression de HSPA8 et LAMP2A retrouve son niveau basal dans les cellules B de souris MRL/lpr qui développent un lupus [12]. A ce jour, très peu de données sont connues concernant l'autophagie dans le SjS humain ou murin.

But de la recherche

Le sujet de ma thèse porte i. sur l'étude et la compréhension des phénomènes de macroautophagie et CMA dans le SjS (niveau moléculaire et cellulaire), notamment dans les glandes salivaires (SG) et ii. Sur l'effet éventuel du peptide P140 dans cette MAI.

Résultats

1. Effets thérapeutiques de P140

Dans cette étude, deux modes d'administration différents ont été adoptés pour tester les effets du P140 dans les SG des souris MRL/lpr, un modèle de souris établi pour le SjS secondaire. Le traitement court implique une injection unique de P140 et le sacrifice des souris pour prélever les SG à jour 5. Le traitement long comprend 4 injections de P140 pendant une période de 18 semaines et le sacrifice des souris à la semaine 23 (Figure 2). Toutes ces expériences ont été réalisées après avoir obtenu des comités *ad hoc* les autorisations d'expérimenter sur animaux.

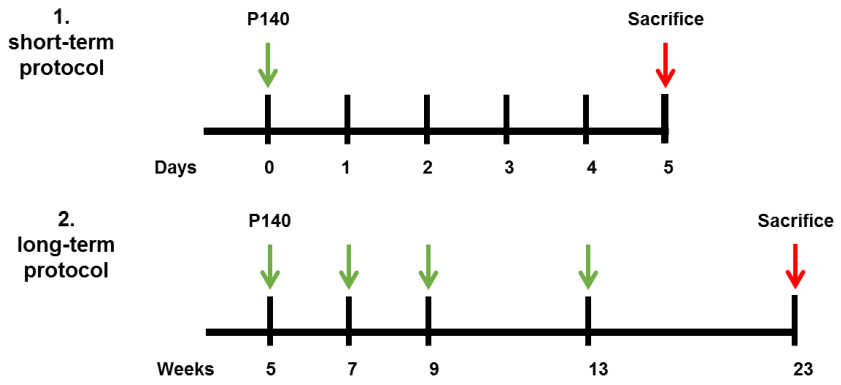

Figure 2. Protocoles courts et longs appliqués pour traiter les souris MRL/lpr

Nous avons constaté des effets protecteurs importants de P140 contre le SjS avec les deux méthodes d'administration, comme en témoignent l'amélioration de l'inflammation, une diminution de l'infiltration lymphocytaire dans les SG de souris MRL/lpr (Figure 3). En outre, nous avons observé une baisse des niveaux d'autoanticorps circulants. Ces résultats soutiennent un effet protecteur du P140.

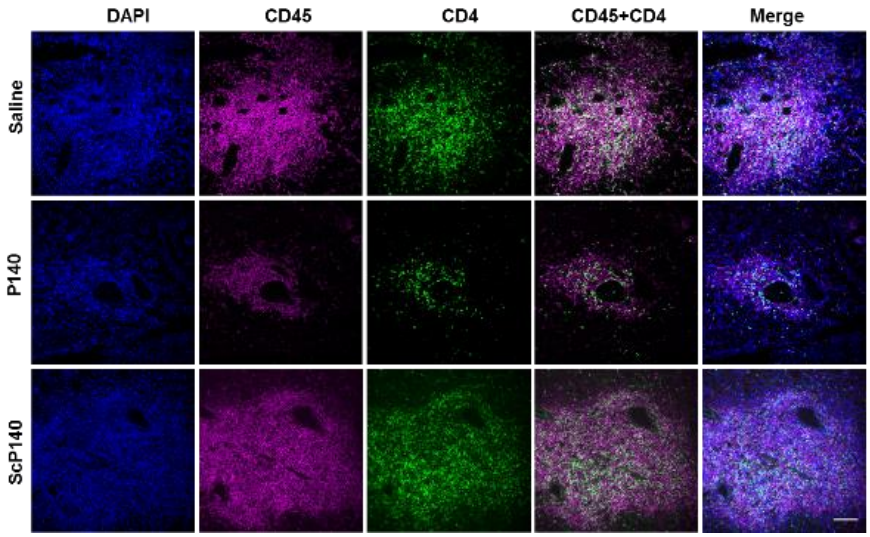

Figure 3. Le peptide P140 diminue la taille des infiltrats de cellules CD45 $^{+}$ et T CD4 $^{+}$ dans les glandes salivaires de souris MRL/lpr traitées

2. L'autophagie est défectueuse dans les souris MRL/lpr.

Par les travaux de mon équipe, confirmés depuis par d'autres, nous savons que l'autophagie est hyperactivée dans le lupus humain et murin. Nous examinons ici le statut de l'autophagie dans les SG de souris atteintes de SjS. En comparaison de souris saines C57BL/6, l'autophagie apparait comme défectueuse (ralentie) dans les SG de souris MRL/lpr. Ceci a notamment pu être démontré en suivant l'expression de la protéine MAP1LC3-II (marqueur des autophagosomes) en présence ou absence de E64D et pepstatine A qui sont des inhibiteurs de protéases lysosomales (Figure 4). L'autophagie étant reconnue comme un processus lysosome-dépendant, nous avons aussi testé la fonction lysosomale et avons constaté que l'acidité lysosomale est diminuée dans les SG de souris MRL/lpr, ce qui suggère que l'autophagie défectueuse pourrait résulter de la fonction lysosomale altérée.

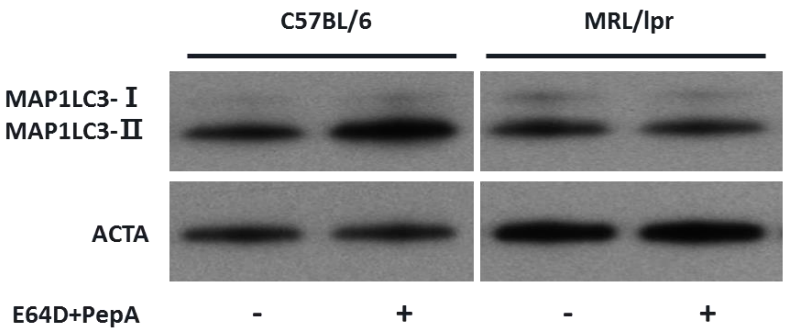

Figure 4. L'autophagie est défectueuse dans les SG de souris MR/lpr

3. <u>L'autophagie défectueuse chez la souris MRL/lpr est corrigée par le peptide P140</u>

L'effet de P140 sur l'autophagie a également été testé. De manière significative, nous avons constaté que le niveau bas d'autophagie a été restauré par le P140 dans les deux protocoles, court et long (Figure 5). En outre, la fonction lysosomale a été récupérée, témoignant de l'augmentation de l'acidité lysosomale.

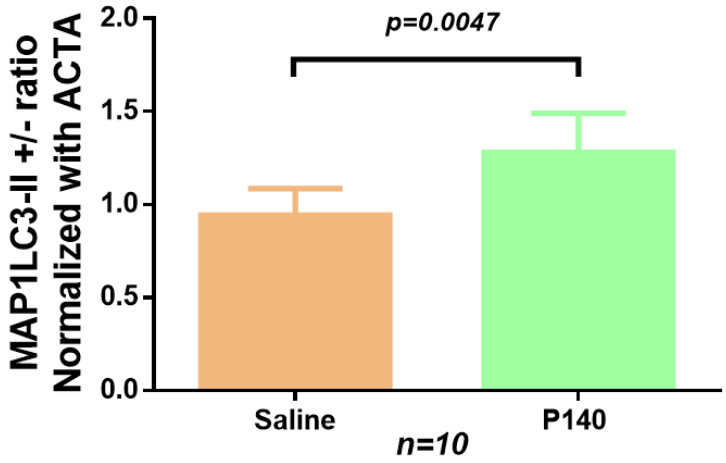

Figure 5. Le flux autophagique a été restauré après le traitement P140 (Students 't test)

Discussion

Des études récentes ont mis en évidence les différents rôles de l'autophagie dans les réponses autoimmunes. Ici, nous avons identifié une voie autophagique défectueuse dans les SG de souris MRL/lpr et avons constaté que le peptide thérapeutique P140 corrigeait la maladie ainsi que le niveau d'autophagie.

A ce jour, il est suggéré que l'autophagie joue des rôles multiples dans les MAIs. Diverses associations génétiques ont été identifiées liant des gènes de l'autophagie (ATG) et des MAIs, comme le LED et la maladie de Crohn, par exemple [13, 15]. Il n'existe aucune association génétique actuellement connue avec le SjS, en dehors de données fondamentales décrivant la présence de cellules acinaires agrandies et de granules sécrétés qui s'accumulent dans les SG de souris mutées pour ATG5. Ces résultats évoquent que l'autophagie pourrait agir comme régulateur de l'homéostasie dans les SG [14, 15].

Dans l'étude actuelle, nous avons constaté que l'autophagie et les lysosomes étaient défectueux dans les SG des souris MRL/lpr et que le P140 rétabli le flux autophagique et l'acidité lysosomale. Ces résultats suggèrent que l'autophagie défectueuse et des défauts des lysosomes pourraient contribuer à la pathologie du SjS et que les effets thérapeutiques de P140 pourraient être liés à son effet sur l'autophagie et les lysosomes. Fait intéressant, l'effet de restauration de P140 sur l'autophagie est contraire à ce que nous avons observé dans les splénocytes de MRL/lpr, où le P140 possède un effet inhibiteur sur l'autophagie qui est hyperactivée [12]. Nous pensons donc que l'effet de P140 sur le SG pourrait être dû à son effet global de protection sur le système immunitaire, plutôt qu'un effet direct de P140 sur l'autophagie dans les SG. D'autres études seront nécessaires pour élucider pleinement ce point.

Dans cette étude, nous avons utilisé un ensemble de techniques immunologiques et biochimiques pour découvrir le rôle de l'autophagie dans SjS. C'est la première étude qui révèle une autophagie défectueuse dans les SG. Ces résultats nous offrent des informations décisives sur la pathogenèse de SjS et suggèrent que l'autophagie

pourrait être une cible thérapeutique de choix dans le SjS. Au-delà, les effets protecteurs de P140 dans le modèle de souris SjS nous éclairent sur l'application thérapeutique du P140 dans d'autres MAIs.

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Baihui Li

Autophagie comme cible thérapeutique potentielle pour le syndrome de Sjögren

Résumé

Mots-clés : syndrome de Sjögren, maladies autoimmunes, P140, autophagie, souris MRL/lpr

Le syndrome de Sjögren (SS) est l'une des maladies autoimmunes (MAI) systémiques les plus fréquentes chez l'Homme. Cette maladie est caractérisée par une infiltration lymphocytaire dans les glandes exocrines conduisant à des symptômes dits de « yeux secs » et à la bouche sèche. Il n'existe actuellement aucun traitement curatif pour cette maladie.
Le peptide P140 a été démontrée comme un outil thérapeutique efficace chez les patients atteints d'un lupus érythémateux disséminé (LED) et des souris modèles développant un lupus.
L'autophagie est une voie intracellulaire conservée qui joue un rôle central dans le maintien de l'homéostasie cellulaire. En outre, l'autophagie a été démontrée comme un mécanisme de régulation de l'autoimmunité.
Les effets bénéfiques du peptide P140 dans le lupus semblent étroitement liés à son effet d'inhibition de l'autophagie qui est hyper-activée dans le lupus. A ce jour, très peu de données sont connues concernant l'autophagie dans le SS.
Le sujet de ma thèse porte i. sur l'étude et la compréhension des phénomènes de l'autophagie dans le SS et ii. sur l'effet éventuel du peptide P140 dans cette maladie.
Dans cette étude, nous avons d'abord montré des effets protecteurs du P140 contre le SS dans les souris modèle MRL/lpr, comme en témoignent l'amélioration de l'inflammation, une diminution de l'infiltration lymphocytaire dans les SG de souris et une baisse des niveaux d'autoanticorps circulants.
Nous avons également constaté que l'autophagie et les lysosomes étaient défectueux dans les SG des souris MRL/lpr et que le P140 rétabli le flux autophagique et l'acidité lysosomale. Ces résultats suggèrent que l'autophagie défectueuse et des défauts des lysosomes pourraient contribuer à la pathologie du SS et que les effets thérapeutiques de P140 pourraient être liés à son effet sur l'autophagie et les lysosomes.
Les résultats obtenus dans le cadre de cette thèse nous offrent des informations décisives sur la pathogenèse de SS et suggèrent que l'autophagie pourrait être une cible thérapeutique de choix dans le SS. Au-delà, les effets protecteurs de P140 dans le modèle de souris SS nous éclairent sur l'application thérapeutique du P140 dans d'autres MAIs.

Résumé en anglais

Keywords: Sjögren's syndrome, autoimmune diseases, P140, autophagy, MRL/lpr mice

Sjögren's syndrome (SS) is one of the most common systemic autoimmune diseases (AIDs) in human. This disease is characterized by lymphocytic infiltration in the exocrine glands which leads to symptoms named dry eyes and dry mouth. There is no cure for SS currently.
P140 peptide has been demonstrated to be an effective therapeutic tool in patients with systemic lupus erythematosus (SLE) and lupus mouse model.
Autophagy is a conserved intracellular pathway that plays a central role in maintaining cellular homeostasis. In addition, autophagy has been demonstrated to be a mechanism of autoimmune regulation.
The beneficial effects of P140 peptide in lupus seem to be closely related to its inhibitory effect on autophagy which is overactivated in lupus. However, the role of autophagy in SS is largely unknown.
The aim of my thesis is: 1, to study the role of autophagy in SS and 2, to examine the possible effect of the P140 peptide in this disease.
In this study, we first demonstrated the protective effects of P140 against SS in MRL/lpr mouse model, as evidenced by ameliorated inflammation, decreased lymphocyte infiltration in mouse salivary glands (SGs), as well as decreased levels of circulating autoantibodies.
We also found that autophagy and lysosomes were defective in SGs of MRL/lpr mice and that P140 restored the autophagic flux and lysosomal acidity. These results suggest that defective autophagy and lysosome dysfunction may contribute to the pathology of SS and that the therapeutic effects of P140 may be related to its effect on autophagy and lysosomes.
The results obtained in this study provide us valuable information about the pathogenesis of SS, and suggest that autophagy could be a potential therapeutic target in SS. Moreover, the protective effects of P140 in SS mouse model shed light on the therapeutic application of P140 in other AIDs.