John G. Routsias and Athanasios G. Tzioufas

Η αυτοάνοση απόκριση και τα αυτοαντιγόνα στόχοι στο σύνδρομο Sjogren.

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REVIEW

Autoimmune response and target autoantigens in Sjogren's syndrome

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ABSTRACT

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Keywords Anti-La, anti-Ro52, anti-Ro60, autoantigens, autoimmunity, Sjogren's syndrome.

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Introduction

Sjogren's syndrome is a chronic autoimmune disease of unknown aetiology, characterized by the presence of a variety autoantibodies directed against organ- and non-organ-specific autoantigens. Antinuclear antibodies (ANA) are present in the sera of 90% of patients. Among them, two antibodies known as Ro or Sjogren's syndrome antigen A (SSA) and La or Sjogren's syndrome antigen B (SSB) are directed against two ribonucleoprotein (RNP) antigens. Although these autoantibodies are included in the European–American Diagnostic Criteria for Sjogren's Syndrome [1], they are also found in other autoimmune diseases, particularly systemic lupus erythematosus (SLE).

Over the last decade, studies have delineated the pathogenetic mechanisms and the clinical utility of autoantibodies in Primary Sjogren's syndrome (pSS) [2]. It is now appreciated that the production of autoantibodies is an antigen-driven immune response, as (i) certain autoantibodies are disease specific, (ii) contain multiple epitopes and (iii) the autoimmune response is perpetuated and augmented via intra- and intermolecular spreading against the same or other autoantigens. It remains unknown whether any of the autoantibodies has a direct pathogenic potential or they merely participate in a secondary response to salivary glands that are already damaged by another process. Nevertheless, anti-Ro and anti-La antibodies appear to play a role in the local autoimmune response located in the affected exocrine glands because (i) autoantibodies to Ro and La are found in the saliva of patients [3,4], (ii) B cells infiltrating the salivary glands contain intracytoplasmic immunoglobulins with anti-Ro and anti-La activity [5–7]; in this case, ectopic lymphoid germinal centres that contain antigen-presenting dendritic cells, T cells and B lymphocytes are found,

providing a conducive microenvironment for the propagation of the autoimmune response [6], (iii) an increased mRNA production of La in acinar epithelial cells has been observed [8] and (iv) translocation and membrane localization of the La protein has been demonstrated in conjunctival epithelial cells of Sjogren's syndrome patients [9]. Other studies have shown that cultured epithelial cells from patients with Sjogren's syndrome constitutively secrete exosomes that contain the major autoantigens Ro and La [10]. This mechanism may represent a pathway whereby intracellular autoantigens are presented to the immune system.

In this review, we focus primarily on candidate mechanisms for autoantigen presentation and perpetuation of the autoimmune response within the autoimmune tissue lesion of pSS.

Characteristics of the intracellular autoantigens related to hYRNPs

Structurally, human Ro/La RNP is composed of one of the four small, uridine-rich human cYtoplasmic RNAs (hY RNAs) noncovalently associated with at least three proteins, the Ro52, La and Ro60 autoantigens [11,12]. Additional components of the complex have been identified and include the proteins calreticulin [13] and nucleolin [14]. The localization of these complexes is mainly cytoplasmic, although their protein components are also found in the nucleus. After the assembly in the nucleus, the Ro/La RNP complex is rapidly and quantitatively transported to the cytoplasm [15,16]. Under certain circumstances (e.g. stress, UV radiation or viral infection), some of the protein components Ro/La RNP can be also found on the cell surface.

Ro52 belongs to the tripartite motif (TRIM) or RING-B-box-coiled-coil protein family, thus comprising an N-terminal RING, followed by a B-box and a coiled-coil region. The RING is a cysteine-rich Zn^2-binding motif of the form C_3HC_4 [17], which binds two Zn^2 ions in a tetrahedral manner [18]. The RING is predominantly a protein–protein interaction motif, which also acts as a ubiquitin-protein isopeptide (E3) ligase in the ubiquitination pathway [19]. The B-box is the second Zn^2-binding motif of Ro52 and has the form CHC_3H_2 [20]. Ro52 possesses also a leucine zipper domain, which may be utilized for its homodimerization [21]. In line with many other RING-containing proteins, Ro52 is involved in ubiquitination pathway [22]. In fact, additional findings suggest that Ro52 autoantigen is a RING-dependent E3 ligase that is overexpressed in patients. Thus, Ro52 may be directly involved in the reduced cellular proliferation and increased apoptotic cell death that is observed in pSS and SLE [23].

The Ro60 antigen is found in virtually all vertebrate cells as well as the nematode *Caenorhabditis elegans* [24]. Its function is related to the quality control or discard pathway for nascent transcripts synthesized by RNA polymerase III (e.g. 5S rRNA precursors). Thus, Ro60 binds misfolded small RNAs (e.g. 5S RNA) leading them to degradation [24]. The structure of the *Xenopus laevis* Ro60, that is 78% identical to human Ro60, has been solved and found to consist of two distinct domains [25]. One domain resembles the von Willebrand Factor A domain, which is found in extracellular matrix proteins and proteins that operate in cell adhesion. The other domain consists of a series of alpha-helical repeats (HEAT repeats) that are arranged orbicularly around an inner hole of 10–15 Angstroms ('doughnut'-like structure). This hole most probably holds the 3' ends of misfolded RNAs, while the YRNAs bind conserved residues to the outside of the 'doughnut'. Another conserved role of Ro60 in facilitating cell survival after ultraviolet irradiation has been emerged from studies in the radiation-resistant eubacterium *Deinococcus radiodurans* [26] and mammalian cells lacking Ro60 [27]. Studies of mice lacking the Ro60 protein suggest also that the normal function of Ro may be important for the prevention of autoimmune disease [27]. In these studies, mice lacking Ro60 were found to develop autoantibodies and membrano-proliferative glomerulonephritis.

The La antigen is a phosphoprotein that associates with a variety of small RNAs, including 5S cellular RNA, tRNA, 7S RNA and hY RNAs, all transcribed by RNA polymerase III [28]. At the molecular level, it binds a short polyuridylate sequence (poly-U) that exists at the 3' end of almost all nascent pol III transcripts. Moreover, La binds viral RNAs (e.g.VA RNA, Adenovirus Viral Associated RNA; EBER RNA, Epstein–Barr virus-encoded small RNA), viral and human RNAs possessing internal ribosomal entry elements (IRES) and RNA components of the telomerase complex [29]. Structurally, human La is a multi-domain protein that contains the La motif in its N-terminal region, a typical RNA recognition motif (RRM) in its central part and an unusual RRM, encompassing residues 229–326. The latter is followed by a long, flexible polypeptide that contains a short basic motif (SBM), a regulatory phosphorylation site on Ser366 and a nuclear localization signal (NLS). The three-dimensional structure of the La motif, along with the central RRM and the carboxyl-terminal RNA recognition domain of the autoantigen, has been solved [30,31]. The La motif folds into a winged-helix motif elaborated by the insertion of three helices. The central RRM consists of a four-strand β-sheet attached to two α-helices, while the C-terminal domain folds to generate a five-stranded, antiparallel β-sheet surface that is terminated by a long α-helix. It seems that both the La motif and the adjacent central RRM are required for high-affinity poly-U RNA binding, and that the C-terminal RRM, in conjunction with the SBM downstream, contributes to La interactions with non-poly-U RNA targets such as viral RNAs and terminal oligopyrimidine tract (TOP) mRNAs [32]. The specific binding of La to precursor RNA molecules protects them from exonu-

clease digestion and thereby regulates their downstream processing. La also serves to retain precursor RNA molecules in the nucleus. Other cellular functions of La/SS-B autoantigen include an ATP-dependent helicase activity that melts RNA–DNA hybrids, association with telomerase and influencing the telomere homeostasis, an RNA chaperone activity performed by transient bipartite (5′- and 3′- end) binding of nascent transcripts synthesized by polymerase III (e.g. tRNA precursors) and induction of cap-independent translation (La binds IRES to elements and promotes the internal, cap-independent initiation of translation).

Pathological lesion in pSS and routes for presentation of autoantigens

The common feature of all organs affected in Sjogren's syndrome patients is a periepithelial lymphocytic infiltration that leads to functional disability with various clinical manifestations. Salivary glands are the best-studied organs because they are affected in almost all patients and are readily accessible. The histopathological characteristics of minor salivary gland (MSG) biopsy include: focal aggregates of at least 50 lymphocytes, plasma cells and macrophages adjacent to and replacing the normal acini; and the consistent presence of these foci in all or most of the glands in the specimen. T cells predominate in mild lesions, whereas B cells in more severe lesions [33]. T regulatory cells have also been detected [34]. Macrophages and dendritic cells are also found in up to 5% of the infiltrate, mainly in those glands with ectopic germinal centre-like formation [35]. Both T and B lymphocytes are activated, as attested by the membrane expression of HLA class II molecules, interleukin-2 receptor (IL-2R), lymphocyte function-associated antigen 1 (LFA-1) Fas (CD95) molecule and interleukin (IL-2) production [36]. Evaluation of the isotypes of intracytoplasmic immunoglobulins of the plasma cells infiltrating the salivary glands of Sjogren's syndrome patients showed that the IgG and IgM isotypes predominate in contrast to the plasma cells of the normal salivary glands, where the IgA isotype is dominant [37].

Several studies performed over recent years have suggested that the glandular or acinar epithelial cells play a key role in the pathogenesis of the disease. The epithelial cell is activated and is armed with the armamentarium of an antigen-presenting cell [33]. Histopathological studies in newly diagnosed cases of Sjogren's syndrome demonstrated that the focal lymphocytic infiltrates start around the ducts. Staining of the labial salivary glands (LSG) with anti-class II HLA monoclonal antibodies has shown that the ductal and acinar epithelial cells express inappropriately these molecules [33]. In addition, the salivary gland epithelial cells (SGEC) express B7 molecules [38]. These molecules are expressed on classic antigen-presenting cells and play a critical role in the regulation of immune responses by providing activation or inhibitory signals to T cells through the ligation with CD28 or cytotoxic T-lymphocyte antigen 4 (CTLA4) receptors respectively. B7 molecules expressed in the epithelial cells of primary Sjogren's syndrome patients are functional, inducing co-stimulation signals in $CD4^+$ T cells [33]. Finally, the expression of adhesion molecules such as ICAM-1 has been demonstrated [39].

Local priming of the autoimmune response

La/SSB mRNA expression was studied by *in situ* hybridization in biopsies of pSS patients [8]. Serum levels of anti-La/SSB autoantibodies were correlated with the presence and the intensity of La/SSB mRNA labelling predominantly in acinar cells of the salivary glands. Moreover, it was found that MSGs from patients with positive La/SSB mRNA expression had a higher intensity of staining in the cytoplasm of both the ductal and acinar epithelial cells, compared with patients without La/SSB mRNA expression. These results suggest that La protein expression is up-regulated in the site of the immunopathological lesion in pSS and this active synthesis of La autoantigen is correlated with the anti-La/SSB antibody response. In another study, mRNA expression of La, Ro60 and Ro52 antigens was analysed in MSG from patients with primary Sjogren's syndrome[40]. La and Ro60 RNA expression was higher in MSGs from patients with pSS compared with controls ($P < 0{\cdot}05$). On the other hand, the Ro52 expression pattern was similar in patients and controls. These data suggest that these Ro60 and La autoantigens, but not Ro 52, are probably involved in triggering and maintaining the tissue-specific autoimmune response in pSS MSG. Taken together, the above results indicate that Ro60 and La autoantigens may contribute to the antigen-driven immune response and local autoantibody production in pSS.

In fact, local anti-Ro60 and anti-La autoantibody production within the LSG of pSS patients has been documented a decade ago [5]. In subsequent studies [6], it was demonstrated that the infiltrating lymphocytes in pSS salivary glands are organized in ectopic germinal centres, which are characterized by the anti-Ro60, anti-Ro52 and anti-La autoantibody production. The ectopic germinal centre formation and lymphoid neogenesis that takes place in the salivary glands of patients with pSS argue in favour of the antigen-driven immune response hypothesis that has been raised for pSS.

Alternative isoform expression of autoantigens in salivary glands is a mechanism that could potentially explain their antigenicity and eventually targeting by autoimmune response in pSS. In fact, a novel La mRNA isoform (La1′) was found to be expressed in salivary gland tissue of patients with pSS [41,42]. In the La mRNA isoform La1′, the exon 1 was replaced (Fig. 1). The alternative exon was termed exon 1′. Genomic analysis

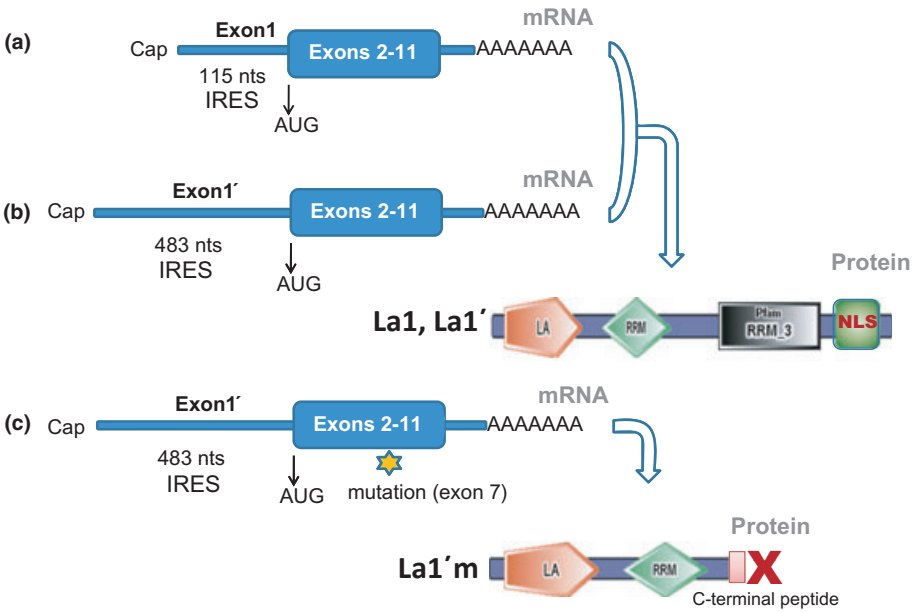

Figure 1 At least three different La mRNAs have been described in patients with pSS. La1 is the wild-type mRNA (a) that translates to the full-length protein product that contains La, recognition motif (RRM), RRM_3 and nuclear location signal (NLS) motifs (Pfam: Protein families database, http://pfam.sanger.ac.uk/). In the mRNA isoform La1′, (b) the exon 1 was replaced by the alternative exon 1′. Although the final protein product is the same with La1 mRNA, the 5′ untranslated region of the La1 and La1′ transcripts possess internal ribosomal entry elements (IRES) with different capacities to mediate IRES-mediated translation. On the other hand, La1′m mRNA (c) contains a frameshift mutation at exon 7 that results in premature stop codon, 11 amino acids downstream of the frameshift mutation. Consequently, the La protein translation end prematurely with a novel 12 aa C-terminal peptide. Moreover, C-terminally truncated La1′m protein lacks the NLS and cannot move to the nucleus, thus remaining in the cytoplasm.

showed that the exon 1′ La mRNA was the result of a promoter-switch in combination with alternative splicing. In more detail, when the promoter upstream of exon 1 is used, the complete intron between exons 1 and 2 (including the exon 1′ sequence located within the intron) is removed during splicing. If the promoter upstream of exon 1′ but downstream of the exon 1 is used, then (i) the exon 1 is not transcribed and (ii) an alternative donor splice site within the intron is spliced to the same acceptor splice site at the exon 2. At the mRNA level, this results in a complete exchange of the exon 1 by the exon 1′. Both La1 and La1′ mRNA isoforms are finally processed cytoplasmic abundant La mRNAs that are expressed and regulated in parallel at ratios between 5 : 1 and 1 : 1. In a subsequent study [42], a mutated La cDNA was identified that contains a frameshift mutation in a hot spot region of the La gene (Fig. 1c). This mutation resulted in premature stop codon, which is located 11 amino acids downstream of the frameshift mutation. Consequently, only the sequence of the 12-amino acid La peptide (193–204aa: MKKENKIKWKLN) encoded by the patient's La cDNA markedly differed from the corresponding La protein sequence. However, translation of the patient's mutant La mRNA in transfected mouse cells resulted in a C-terminally truncated La protein, which because of the lack of the nuclear location signal remained in the cytoplasm. Antibodies specific for the mutant La form are found in approximately 30% of sera from anti-La-positive patients [43], while mice transgenic for the mutant La form show that mutant La mRNAs are not repressed in these animals and are translated to mutant La protein. Interestingly, expression of the mutant La results in a lupus-like disease in mutant La-transgenic mice, accompanied by proteinuria, immune complex deposits in glomeruli and development of autoantibodies against La, Ro, and dsDNA. Interestingly, in some of the animals, anti-Ro60 antibodies were detected as the initial specificity with subsequent spreading of the response to La [43]. However, future studies are required to clarify the physiological function of the alternative La mRNA in human autoimmunity.

The differential expression of La1 and La1′ transcripts was also studied [44]. It was found that La1 transcripts are expressed constitutively in various tissues with the highest levels in the spleen, prostate, testis, ovary, heart and brain and lowest levels in the thymus, peripheral blood leucocytes, lung and liver. On the other hand, La1′ transcripts demonstrated a tissue-specific expression profile with predominant expression

in peripheral blood leucocytes, especially in B, T and natural killer cells.

More interestingly, the 5′ untranslated region of both La1 and La1′ transcripts possess IRES elements that can mediate translation initiation by internal initiation [44]. In this regard, La protein synthesis can be assured under conditions when cap-dependent translation is compromised, such as inflammation, apoptosis or certain viral infections. The latter observation is of particular interest because autoantigen La stimulates IRES-mediated translation of virally encoded RNAs in cells infected by viruses including hepatitis C virus (HCV), poliovirus, coxsackievirus B3 and human immunodeficiency virus (HIV) [45–48] but truncated La (by granzyme H) or La peptides 11–28aa and 174–197aa (derived from its RRM) are capable of suppressing IRES-mediated translation of HCV [49–51].

Inappropriate expression of autoantigens. Alterations of autoantigens within the tissue

Type 1 interferon and autoantigen expression

Enhanced activity of the type 1 interferon (IFN) system has been linked to multiple autoimmune diseases including Sjogren's syndrome. Increased expression of IFN-regulated genes has been described in the salivary glands [52,53]. The plasmacytoid dendritic cells (pDCs) were identified as the main source of IFNα [52]. Recently, it was shown that in Sjogren's syndrome, circulating pDCs express higher levels of the activation marker CD40, which is correlated with the expression of selected IFN-regulated genes. In fact, more than half of the genes overexpressed in peripheral blood monocytes (PBMCs) were IFN inducible. The presence of IFN signature was confirmed in both PBMCs and whole blood in a study [54] that analysed peripheral blood gene expression profiles in Sjogren's syndrome. A large proportion of the 197 overexpressed transcripts correlated with anti-Ro and anti-La (45 and 39% of transcripts, respectively) [54]. Taken together, these data suggest that similar to the local level, pDCs may be a major source of IFNα in the systemic circulation [55]. IFN-α up-regulates Toll-like receptor (TLR) expression [56], and as a result, B cells remain sensitive to inflammatory signals and are more responsive to the adjuvant effect of TLR-binding nucleic acids. Other cytokines induced by interferons, including IL-6, TNF, IL-12/IL–23p40 and ultimately IL-17 [57], can subsequently amplify autoreactivity inducing T-cell activation, germinal centre expansion, B-cell survival, neutrophil infiltration and TLR up-regulation [56,58–60]. Among the IFN-inducible factors that provide negative feedback regulation in inflammation are some members of the TRIM family of proteins, which are important components of antiviral defence [61]. TRIM21 is the autoantigen Ro52 [62]. The study of Ro52-deficient mice reported by Espinosa *et al.* [63] connects Ro52 to an IFN-associated negative feedback loop that prevents unrestrained inflammation. Ro52 is an E3 ligase that ubiquitinates various members of the IRF family and translocates to the nucleus upon IFN-α stimulation [64,65]. Espinosa *et al.* [63] showed that Ro52-deficient mice develop uncontrolled inflammation and systemic autoimmunity as a consequence of minor tissue injury caused by ear tagging. These data demonstrate that the ubiquitin ligase Ro52 is induced by IFN activation of immune cells, where it acts as a negative regulator of IFN signalling. A recent study by Yoshimi *et al.* [66] supports these conclusions and also shows that Ro52 is a negative regulator of IFN I *in vivo*. In addition, RNP complexes contain Y-RNAs, which have been shown to promote dendritic cell maturation and IFN production [67]. These effects are dependent on the TLR adaptor protein MyD88 and endosome acidification, suggesting that an endogenous adjuvant effect through TLR7 activation is operated. The potential association of Ro52 with RNA-binding molecules in IFN-induced supramolecular complexes could promote the presentation of Ro52 epitopes upon translocation to the nucleus or other cellular compartments.

Post-translational modifications of autoantigens

Many post-translational modifications appear to play a role in autoimmune disorders and are increasingly considered as possible triggering factors for the breakdown of immune tolerance against self-proteins. Previous studies have shown that adenovirus infection increases the extent of phosphorylation of La/SSB as well as its antigenicity, and results in the translocation of the protein to the cell membrane [68–70]. Affected SGEC from pSS patients bear an activated phenotype compatible with a virus infection [39]. In this regard, a virus infection of salivary glands might trigger phosphorylation of La/SSB protein, increasing its antigenicity by presenting the molecule to the immune system on the surface of the glandular epithelium.

In a previous study from our laboratory, immunoblot analysis on two-dimensional gels using purified anti-La/SSB antibodies revealed that pSS salivary glands contain high levels of post-translationally modified La/SSB autoantigen, while the native form of the protein is recognized faintly, in contrast to normal controls [71]. Moreover, salivary glands of pSS patients contain post-translationally modified actin that becomes immunogenic in the microenvironment of the affected tissue. Phosphorylation of the major linear B-cell epitope of La/SSB 349–368aa was found to increase its recognition by anti-La positive sera in terms of both prevalence and antibody avidity [72]. On the other hand, phosphorylation (or citrullination) of the major linear B-cell epitope of Ro/SSA reduced its recognition by autoantibodies [73].

Routes for autoantigen presentation

Apoptosis

Apoptosis is an important homeostatic mechanism that mediates elimination of damaged cells during organogenesis and throughout life [74]. Several studies have demonstrated increased apoptosis of the glandular ductal and acinar epithelia of pSS patients. [75–78]. Epithelial cell apoptosis apparently contributes in the glandular destructive lesions of pSS and apoptotic cell death may also function as a specialized mechanism whereby nuclear antigens, such as autoantigenic Ro/SSA and La/SSB RNPs, gain access to the immune system in an immunogenic fashion [79]. During early apoptosis, La/SSB protein, among other autoantigens, has been shown to redistribute diffusely to the cytoplasm. In later phases, both Ro/SSA and La/SSB autoantigens are located mainly in the surface apoptotic blebs and bodies [80]. In this regard, apoptosis can be a route, providing autoantigens for presentation to autoreactive T cells (Fig. 2). In addition, it was recently reported that anti-Ro and anti-La antibodies are able to trigger the extrinsic pathway of apoptosis in cultured human SGEC accompanied by a transcriptional up-regulation and activation of caspase-8 [81].

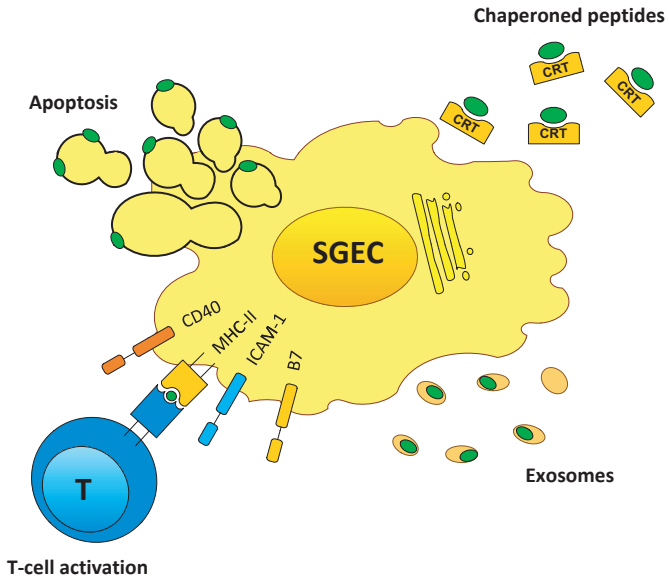

Figure 2 The epithelial cell as a conductor of the autoimmune response in pSS. The epithelial cell in labial salivary glands of patients with Sjogren's syndrome is activated, bearing characteristics of an antigen-presenting cell, inappropriately expressing class II HLA, B7 and ICAM-1 molecules. Epithelial cell exhibits also obscured autoantigen presentation via apoptotic blebs and bodies, exosomes and HSP-mediated cross-priming.

Exosomes

A novel cell-free mechanism of antigen presentation has been recognized [82] involving small membrane vesicles (30–100 nm), which are termed exosomes and are distinct from apoptotic bodies. Exosomal vesicles are secreted following the fusion of multivesicular late endosome/lysosomes with the plasma membrane. Exosomes have been shown to be produced by a variety of cell types, including reticulocytes, platelets, cytotoxic T lymphocytes and B lymphocytes, dendritic cells, and neoplastic intestinal epithelial cells. Several physiological roles have been assigned to exosomes, including expulsion of obsolete membrane constituents, exchange of cellular material and intercellular communication [83]. In addition, several lines of evidence implicate exosomes in antigen presentation [82,83]. In this regard, exosomes, equipped with functional surface proteins involved in antigen presentation, are secreted from antigen-presenting cells (APCs), directly stimulating T cells in an antigen-dependent manner, hence inducing immunogenic or tolerogenic responses. Kapsogeorgou *et al.* [10] found that human non-neoplastic epithelial cells, derived from MSG biopsies, constitutively secrete exosomes that contain epithelial-specific cytoskeletal proteins and the intracellular RNP autoantigens Ro/SSA, La/SSB and Sm. Thus, loading of professional APCs by RNP-containing exosomes appears to be a plausible mechanism for the transfer and efficient antigen presentation to autoantigen-specific T cells (Fig. 2).

Cross-priming and CD91-mediated presentation

Heat shock or stress proteins (HSPs) are intracellular molecules that expressed under cellular stress and have cytoprotective functions. Many of them act also as molecular chaperones assisting the correct folding, stabilization and translocation of proteins. In pathological situations, such as necrotic cell death, they can be released into the extracellular environment complexed with intact proteins or peptides. HSPs such as HSP70, HSP90, gp96, HSP110, grp170 and calreticulin can associate with a broad array of peptides [84,85]. These peptides include normal self-peptides as well as antigenic peptides derived from tumour, [86], bacterial antigens, or viral antigens [87]. In the cross-presentation process, complexes of HSP-chaperoned peptides released from the cells, after stress or cell death, are taken up by the APCs, resulting in representation (cross-presentation) of the peptides by major histocompatibility complex (MHC) molecules of the APCs [85]. Internalized HSP–peptide complexes can enter the MHC class I- and class II-enriched compartments by a receptor-mediated uptake and be presented to CD8 and CD4 T cells, eliciting a peptide-specific response to HSP-chaperoned peptides. One of the best-studied examples is the endoplasmic reticulum (ER)-resident chaperone calreticulin. Calreticulin is up-regulated in response to various types

of ER stress and has the ability to bind to glycoproteins containing monoglucosylated core glycans as well as to several non-glycosylated peptides. In our laboratory, it has been demonstrated that calreticulin can be complexed with specific epitope-analogues of Ro60 autoantigen, inducing conformation-dependent recognition by autoantibodies from autoimmune human sera [88]. In this study, calreticulin was isolated from the human spleen, using a multistep purification method, and allowed to interact with seven biotinylated epitopes of Ro60, La and Sm autoantigens. Among the synthetic peptides tested, only the two epitope-analogues of Ro60 autoantigen, spanning the sequences 175–184aa and 216–232aa, exhibited a substantial binding to calreticulin. Our results indicated that calreticulin–Ro60 peptide interaction was favoured at a temperature of 40 °C, and in the presence of ATP and optimum concentrations of divalent ions. These conditions not only increased the interaction of calreticulin with Ro60 epitope-analogues, but also the antigenicity of the complex that exhibited a stronger anti-Ro60 reactivity compared with that observed when calreticulin and peptides were tested individually. In fact, all anti-Ro60-positive sera of patients with autoimmune rheumatic diseases recognized the complex calreticulin–peptide, while the same sera displayed very low reactivity when tested individually with calreticulin or the Ro epitope-analogues alone. It was, therefore, proposed that calreticulin may play a more active role in the generation of autoimmune response against Ro60. According to our hypothesis, calreticulin can be released into the extracellular space, together with Ro fragments, after necrosis or cell lysis by cytotoxic T cells (Fig. 2) [89,90]. Under certain physicochemical conditions favoured by the microenvironment, calreticulin can bind Ro peptides, eventually increasing their antigenicity. Thus, after induction of apoptosis or after a viral infection, which is accompanied by ER remodelling, calreticulin can interact with intact or fragmented Ro autoantigen, thus enhancing

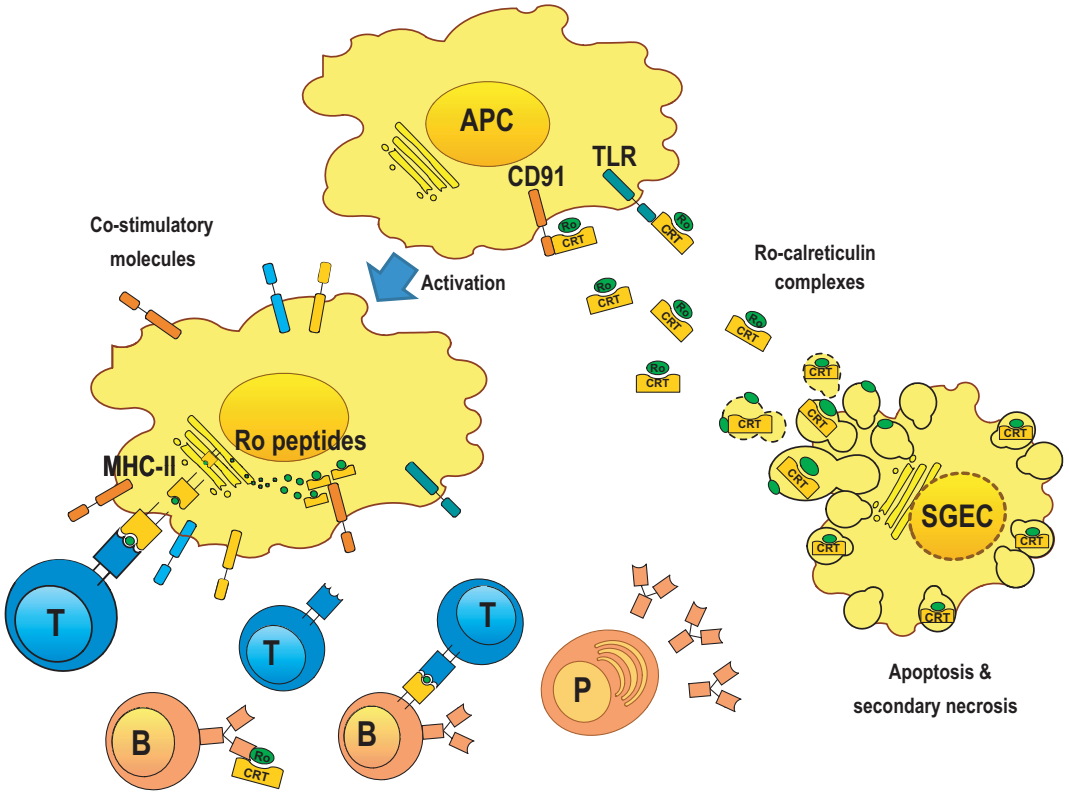

Figure 3 Calreticulin in the pathological lesion of pSS can be used as a vehicle to deliver peptides in the immune system, augmenting the specific autoimmune response. In the proposed mechanism, calreticulin is released into the extracellular space, together with Ro fragments, after necrosis or cell lysis by cytotoxic T cells. The complex is then transported to professional antigen-presenting cells (APCs) where it is endocytosed in a CD91-mediated fashion. The same complex can act also as a 'danger signal' activating Toll-like receptors (TLRs) on the APC. In this regard, APC is activated and presents Ro peptides to autoreactive Th cells. In turn, Th cells receive an activation signal that is accompanied by clonal expansion, activation of autoreactive B cells and differentiation of the latter to plasma cells that are capable to produce autoantibodies.

its antigenicity [88,91]. The complex is then transported to professional APCs where it is endocytosed in a receptor-mediated fashion and finally the peptides are presented to autoreactive Th cells, thus activating them (Fig. 3). In this regard, calreticulin is used as a vehicle to deliver peptides in the immune system, augmenting the specific autoimmune response.

A major receptor for capture of HSP–peptide complexes and presentation of their peptide cargo is CD91. CD91 is a multifunctional molecule, acting as a receptor for alpha 2-macroglobulin, HSPs and calreticulin [92]. CD91 has also been implicated in cross-presentation of peptides chaperoned by these proteins to MHC molecules, thus eliciting antigen-specific immune responses [92,93]. In addition, CD91 receptor/calreticulin pathway has been considered to play a key role in the removal of apoptotic cells [94,95]. These functions of CD91 are of particular interest, as in our recent work, we found that CD91 receptor was constitutively expressed in human SGEC [96]. More specifically, the expression of CD91 mRNA was readily detected in the cultured non-neoplastic SGEC lines (derived from patients MSGs biopsies) by RT-PCR, flow cytometry and confocal microscopy [96]. In addition, we evaluated the capacity of SGEC to bind and internalize one of the major CD91 ligands, the alpha 2-macroglobulin. In this regard, FITC-labelled alpha 2-macroglobulin was found to bind to CD91-expressing SGEC cells and to be internalized in a CD91-dependent manner, as the latter process was inhibited by an inhibitory anti-CD91 mAb or unlabelled alpha 2-macroglobulin. Therefore, CD91 receptor was found to be functionally expressed on SGEC cells, suggesting that this pathway is available for the presentation of exogenous autoantigens in pSS pathological lesion.

Concluding remarks

The epithelial cell in LSG of patients with Sjogren's syndrome is activated, bearing characteristics of an antigen-presenting cell, inappropriately expressing, class II HLA, B7 and ICAM-1 molecules. Increased expression of IFN-regulated genes has also been described in the salivary glands. Among the IFN-inducible factors, the autoantigen Ro52 translocates to the nucleus upon IFN-α stimulation and provides negative feedback regulation in inflammation. The other main targets of autoantibodies in patients with Sjogren's syndrome, Ro60 and La autoantigens also appear to play a major role in the local autoimmune response in Sjogren's syndrome because (i) La and Ro60 mRNA expression is up-regulated at the site of immunopathological lesion in pSS and (ii) different isoforms of La autoantigen mRNA are expressed in patients with pSS, with different capacity for IRES-mediated translation. Among them is a frameshift-mutated La mRNA that is translated to a truncated protein product that lacks its NLS signal, (iv) at the protein level La/SSB in pSS salivary glands is post-translationally modified and (v) in the salivary glands there is increased autoantigen presentation via apoptotic blebs and bodies, via exosomes and via HSP-mediated cross-priming. Taken together, autoantigen alterations in a microenvironment of local inflammation with increased *in situ* apoptosis, TLR signalling and antigen presentation may drive the autoimmune response and local autoantibody production in pSS. Consistent with this mechanism, infiltrating lymphocytes in pSS salivary glands have been found to organize in ectopic germinal centres and actively produce anti-Ro60 and anti-La autoantibodies locally.

Further studies regarding autoantigen recognition at the very early stages of the disease, before the occurrence of epitope spreading, are required to unravel the mechanisms involved in the initiation of autoimmunity in pSS. Understanding of these mechanisms is the key for the development of novel disease-specific therapies.

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