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Rescue of autophagy and lysosome defects in salivary glands of MRL/lpr mice by a therapeutic phosphopeptide

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ABSTRACT

Sjögren's syndrome is a multifactorial systemic autoimmune disorder characterized by lymphocytic infiltrates in exocrine organs. Patients present with sicca symptoms, such as extensive dry eyes and dry mouth, and parotid enlargement. Other serious complications include profound fatigue, chronic pain, major organ involvement, neuropathies and lymphomas. Current treatments only focus on relieving symptoms and do not target the origin of the disease, which is largely unknown. The question we addressed here was whether some defects exist in autophagy processes in Sjögren's syndrome and if they can be corrected or minimized using an appropriate mechanism-driven treatment targeting this central survival pathway. Using a recognized murine model of secondary Sjögren's syndrome, we identified molecular alterations of autophagy occurring in the 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. We showed further that the therapeutic P140 peptide, known to directly act on chaperone-mediated autophagy, rescued MRL/lpr mice from cellular infiltration and autophagy defects occurring in salivary glands. Our findings provide a proof-of-concept that targeting autophagy might represent a promising therapeutic strategy for treating patients with Sjögren's syndrome.

1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder that affects the whole body. Along with common symptoms of extensive dry eyes (xerophthalmia), dry mouth (xerostomia), vaginal dryness and chronic bronchitis resulting from mucous membranes and moisture-secreting glands dysfunctions, other serious complications include profound fatigue, chronic pain, major organ involvement, neuropathies and lymphomas. It is one of the most common inflammatory rheumatological diseases. Today, as many as 4 million Americans are living with this disease, with over 90% of patients being female. Neurological involvements mostly linked to the peripheral nervous system also occur in 20–25% of cases of SS, generally at a late stage in the course of the disease. As with most autoimmune diseases, the etiology of SS is not yet fully understood, which complicates the design and development of disease-specific drugs with minimal unwanted side effects. It is unknown, for example, if as in certain neurodegenerative and autoimmune diseases there is a failure of proteolytic systems to adequately eliminate aggregated or misfolded proteins that might represent a source of possible autoantigens. Two recent studies showed an increase in the macroautophagy levels in salivary gland (SG) T lymphocytes, and in tears and conjunctival epithelial cells, respectively, of patients with primary SS [1,2]. It has not been addressed however if chaperone-mediated autophagy (CMA), a central clearance system delivering soluble cytosolic damaged

Abbreviations: ATG, autophagy related; CMA, chaperone-mediated autophagy; B6, C57BL/6; 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; ScP140, scrambled peptide P140; SG, salivary glands; SLE, systemic lupus erythematosus; SQSTM1/p62, sequestosome; SS, Sjögren's syndrome; TRIM21, tripartite motif-containing protein 21.

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components to lysosomes, is affected in SS.

Autophagy, especially macroautophagy and CMA, 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 [3–7]. A growing body of evidence from across genetic and cellular studies considers autophagy as 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 [8–11]. 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 [12].

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 SGs, in comparison to lymphoid organs wherein both macroautophagy and CMA dysfunctions have been previously identified [13,14].

The MRL/lpr mouse spontaneously develops a systemic autoimmune disease mimicking human lupus and secondary SS (sSS) [15–21]. 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. Very little information is available regarding possible autophagy defects in this pathology [1,2]. 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 [22,23]. 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 the glands of these mice and discovered 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 21-residues long phosphopeptide P140, which acts on CMA and reduces excessive autophagy in MRL/lpr spleen B cells [14], we found that it rescued MRL/lpr mice from the various defects identified in SGs, especially in the lysosomes of SG cells. In a multicenter, randomized, placebo-controlled phase IIb study for SLE, the P140 peptide showed no adverse safety signals and met its primary efficacy end points. Phase III-clinical trials are currently on-going for this indication. 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 compound 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.

2. Results

2.1. 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 and the weight of both of which are increased (Fig. 1A, Supplementary Fig. 1) 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; 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). Similar results were obtained when we analyzed 10–19 versus 20-29 week-old mice (Supplementary Fig. 2). Histology and immunofluorescence studies of SGs confirmed cell infiltration (Fig. 1D and E) and also confirmed that after 25 weeks of age, as previously shown by others [15,16], 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 [24–26]. 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 (100 μg/mouse) administrated intravenously either once (protocol 1: mice aged of 11–13 weeks at the start of the experiment; sacrifice 5 days later) [26] 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) [24] (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 ($p = 0.0034$; Fig. 2B). The percentage of T cell receptor β$^+$ T cells in the total cell population was also significantly diminished ($p = 0.0425$, compared to untreated MRL/lpr mice). No effect was observed when the scrambled (Sc) peptide P140 was administrated to MRL/lpr mice (Fig. 2B). The foci visualized by immunofluorescence analysis in the SGs of MRL/lpr mice contained CD45$^+$ cells that were mostly stained with CD4 but, probably for operational reasons linked to the detection method used, not with CD8 marker (Fig. 2C). 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 and E). Inflammation, however, appeared weaker post-

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