Chapter 9
Citrullination and Autophagy

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9.1 Introduction

During evolution, the eukaryotic cells develop different processes in order to adapt themselves to environmental changes, as well as to die or to survive. These processes include apoptosis, NETosis, and autophagy, and citrullination is implicated in all of these physiological mechanisms (Mohammed et al. 2013), as summarized in Fig. 9.1.

This chapter will focus on the role of autophagy and citrullination in the pathogenesis of autoimmune diseases.

9.2 Autophagy: General Aspects

The term "autophagy" derives from the ancient Greek words "αὐτός φαγεῖν," which means "self-eating." It was first coined by Christian de Duve over 40 years ago and was largely based on the observed degradation of mitochondria and other intracellular structures within lysosomes of rat liver perfused with the pancreatic hormone glucagon (Glick et al. 2010). In recent years, the scientific world has reevaluated autophagy, in order to better understand the molecular mechanisms of its regulation. In this sense, many molecular studies in delineating how autophagy is regulated and

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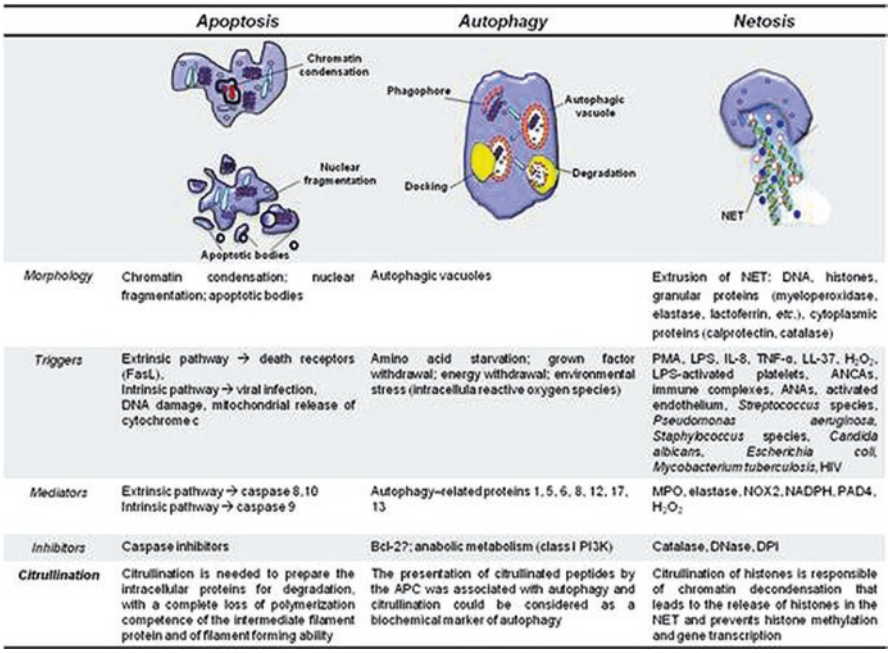

	Apoptosis	Autophagy	Netosis
Morphology	Chromatin condensation; nuclear fragmentation; apoptotic bodies	Autophagic vacuoles	Extrusion of NET: DNA, histones, granular proteins (myeloperoxidase, elastase, lactoferrin, etc.), cytoplasmic proteins (calprotectin, catalase)
Triggers	Extrinsic pathway → death receptors (FasL). Intrinsic pathway → viral infection, DNA damage, mitochondrial release of cytochrome c	Amino acid starvation; grown factor withdrawal; energy withdrawal; environmental stress (intracellula reactive oxygen species)	PMA, LPS, IL-8, TNF-α, LL-37, H_2O_2, LPS-activated platelets, ANCAs, immune complexes, ANAs, activated endothelium, *Streptococcus* species, *Pseudomonas aeruginosa*, *Staphylococcus* species, *Candida albicans*, *Escherichia coli*, *Mycobacterium tuberculosis*, HIV
Mediators	Extrinsic pathway → caspase 8,10 Intrinsic pathway → caspase 9	Autophagy–related proteins 1, 5, 6, 8, 12, 17, 13	MPO, elastase, NOX2, NADPH, PAD4, H_2O_2
Inhibitors	Caspase inhibitors	Bcl-2?; anabolic metabolism (class I PI3K)	Catalase, DNase, DPI
Citrullination	Citrullination is needed to prepare the intracellular proteins for degradation, with a complete loss of polymerization competence of the intermediate filament protein and of filament forming ability	The presentation of citrullinated peptides by the APC was associated with autophagy and citrullination could be considered as a biochemical marker of autophagy	Citrullination of histones is responsible of chromatin decondensation that leads to the release of histones in the NET and prevents histone methylation and gene transcription

Fig. 9.1 The role of citrullination in cell death modalities. *ANAs* anti-neutrophil antibodies, *ANCAs* anti-neutrophil cytoplasmic antibodies, *APC* antigen-presenting cell, *Bcl-2* B cell lymphoma 2, *DPI* diphenyleneiodonium, *HIV* human immunodeficiency virus, *IL* interleukin, *LL-37* cathelicidin LL-37, *LPS* lipopolysaccharide, *NET* neutrophil extracellular trap, *MPO* myeloperoxidase, *NAPDH* nicotinamide adenine dinucleotide phosphate, *NOX2* NADPH oxidase 2, *PAD4* peptidylarginine deiminase 4, *PI3K* phosphatidylinositol 3 kinase, *PMA* phorbol 12-myristate 13-acetate, *TNF-α* tumor necrosis factor-α (Modified from Valesini et al., Autoimmun Rev., 2015)

executed have been made in yeast (*S. cerevisiae*), but the process is highly conserved, so that the importance of autophagy is well recognized in mammalian systems. At present, about 32 autophagy-related genes (*atg*) in yeast have been characterized, and their mammalian equivalents are now known (Glick et al. 2010).

Autophagy can be considered a cellular surveillance process, which in physiological conditions works to remove misfolded or aggregated proteins, damaged organelles (such as mitochondria or endoplasmic reticulum (ER), and intracellular pathogens. Moreover, autophagy is involved in cellular senescence and antigen presentation, playing a role in many diseases such as cancer (Durrant et al. 2016), neurodegeneration, autoimmune diseases, and infections. When autophagy is deregulated or altered, such as in pathological conditions, the process may be linked to non-apoptotic cell death (Glick et al. 2010).

9.2.1 Primary Autophagy Mechanisms

Generally, three main types of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy. They share the principal outlines, with some differences.

During macroautophagy (hereafter referred to as autophagy), parts of the cytoplasm and intracellular organelles are sequestered within characteristic double- or multi-membraned autophagic vacuoles (named autophagosomes) and are finally delivered to lysosomes to form what is known as the autophagolysosome for bulk degradation. Autophagy is a highly regulated process that can either be involved in the turnover of long-lived proteins and whole organelles in a generalized fashion or can specifically target distinct organelles, thereby eliminating supernumerary or damaged organelles. Thus, apoptosis and autophagy constitute the two processes through which superfluous, damaged, or aged cells or organelles are eliminated. Beyond this homeostatic function, autophagy is also a process by which cells adapt their metabolism to starvation, imposed by decreased extracellular nutrients or by decreased intracellular metabolite concentrations that result from the loss of growth factor signaling, and which often governs the uptake of nutrients. By the catabolism of macromolecules, autophagy generates metabolic substrates that meet the bioenergetic needs of cells and thereby allows for adaptive protein synthesis (Maiuri et al. 2007).

In microautophagy, by contrast, cytosolic components are directly taken up by the lysosome itself through invagination of the lysosomal membrane. Both macro- and microautophagy are able to engulf large structures through both selective and nonselective mechanisms (Glick et al. 2010).

In chaperone-mediated autophagy, target proteins are translocated across the lysosomal membrane in a complex with chaperone proteins, such as heat shock protein-70 (Glick et al. 2010). These complexes are then recognized by the lysosomal membrane receptor lysosome-associated membrane protein-2A (LAMP-2A), resulting in their unfolding and degradation (Glick et al. 2010). On the other hand, as reported in a recent review on autophagy monitoring (Klionsky et al. 2016), several other selective autophagy mechanisms have been described, which involve a huge heterogeneous amount of organelles.

The cytoplasm-to-vacuole targeting (Cvt) pathway is a biosynthetic route that utilizes the autophagy-related protein machinery, whereas other types of selective autophagy are degradative. The latter include pexophagy, mitophagy, reticulophagy, ribophagy, and xenophagy, and each of these processes has its own marker proteins. To note, the Cvt pathway has been demonstrated to occur only in yeast (Klionsky et al. 2016).

9.2.2 Selective Autophagy Mechanisms

As previously mentioned, several types of selective autophagy have also been described. For example, aggrephagy is the selective removal of aggregates by macroautophagy. This process can be followed by monitoring the levels of an aggregate-prone protein such as mutant α-synuclein, whose role in autophagy regulation (i.e., in lymphocytes) was the object of some intensive studies (Colasanti et al. 2014; Klionsky et al. 2016).

In *C. elegans*, mitochondria and hence mitochondrial DNA from sperm, are eliminated by an autophagic process. This method of allogeneic (nonself) organelle autophagy is termed allophagy. During allophagy in *C. elegans*, both paternal mitochondria and membranous organelles (a sperm-specific membrane compartment) are eliminated by the 16-cell stage (100–120 min postfertilization). The degradation process can be monitored in living embryos using ubiquitin labeled with green fluorescent protein (Klionsky et al. 2016).

Regarding selective degradation of mitochondria (mitophagy), as with any organelle-specific form of autophagy, it is necessary to demonstrate (a) increased levels of autophagosomes containing mitochondria; (b) maturation of these autophagosomes that culminates with mitochondrial degradation, which can be blocked by specific inhibitors of autophagy or of lysosomal degradation; and (c) whether the changes are due to selective mitophagy or increased mitochondrial degradation during nonselective autophagy. Recently, studies of mitophagy in yeast identified a mitochondrial membrane protein, Atg32, which plays an essential role in this organelle's turnover (Ohsumi 2014).

Although the process of pexophagy is prominent, relatively little work has been done in the area of selective mammalian peroxisome degradation by autophagy. Typically, peroxisomes are induced by treatment with hypolipidemic drugs, such as clofibrate or dioctyl phthalate, which bind to a subfamily of nuclear receptors, referred to as peroxisome proliferator-activated receptors. Indeed, the selective degradation of peroxisomes in methylotrophic yeasts, such as *P. pastoris* and *H. polymorpha*, had been well studied. The peroxisomes of these yeasts proliferate tremendously when the microorganisms are cultured in medium containing methanol as the sole carbon source. When these cells are shifted to ethanol or glucose, proliferated peroxisomes are degraded by autophagy. Tuttle (Tuttle and Dunn 1995) showed that *P. pastoris* utilizes different modes of autophagy, macroautophagy, and microautophagy, depending upon the carbon source.

Besides functioning as the primary energy source for plants, chloroplasts represent a major reservoir of fixed carbon and nitrogen to be remobilized from senescing leaves to storage organs. The turnover of these organelles has long been considered to occur via an autophagy mechanism, called chlorophagy. So, while the detection of chloroplasts within autophagic body-like vesicles or within vacuole-like compartments has been observed for decades, only recent studies described a direct link between chloroplast turnover and autophagy, through the analysis of *atg* mutants (Ohsumi 2014; Klionsky et al. 2016).

Starvation in yeast induces a type of selective macroautophagy of the ER, which depends on the autophagy receptors Atg39 and Atg40. ER stress also triggers an autophagic response, which includes the formation of multi-lamellar ER whorls and their degradation by a microautophagic mechanism. ER-selective autophagy has been termed ER-phagy or reticulophagy. Selective autophagy of the ER has also been observed in mammalian cells. Since reticulophagy is selective, it should be able to act in ER quality control, sequester parts of the ER that are damaged, and eliminate protein aggregates that cannot be removed in other ways. It may also serve to limit stress-induced ER expansion, for example, by reducing the ER to a normal level after a particular stress condition has ended (Klionsky et al. 2016).

9.2.3 Macroautophagy and Immune Mechanisms

The macroautophagy pathway has emerged as an important cellular factor in both innate and adaptive immunity. Many in vitro and in vivo studies have demonstrated that genes encoding macroautophagy components are required for host defense against infection by bacteria, parasites, and viruses.

Xenophagy is often used as a term to describe autophagy of microbial pathogens, mediating their capture and delivery to lysosomes for degradation. Since xenophagy presents an immune defense, it is not surprising that microbial pathogens have evolved strategies to overcome it. The interactions of such pathogens with the autophagy system of host cells are complex and have been the subject of several excellent reviews (Klionsky et al. 2016). Here, we will make note of a few key considerations when studying interactions of microbial pathogens with the autophagy system. Importantly, autophagy should no longer be considered as strictly antibacterial, and several studies have described the fact that autophagy may serve to either restrict or promote bacterial replication both in vivo and in vitro (Klionsky et al. 2016). Minor components of the autophagic process also include zimophagy, lipophagy, ferritinophagy, and some others (He and Klionsky 2009).

9.3 Autophagy and Autoimmune Diseases

Growing evidence supports the importance of autophagy in physiology and pathophysiology, such as aging, infectious diseases, cancer, and neurodegenerative diseases (Choi et al. 2013; Durrant et al. 2016). According to an emerging hypothesis, perturbations in autophagy have also been implicated in autoimmune diseases.

Autophagy participates in several aspects of immunity, affecting both innate and adaptive immunity processes. Indeed, autophagy is known to have a role in thymic selection of T cells, survival of B cells, immune tolerance, and antigen presentation (Pierdominici et al. 2012). A crosstalk between autophagy and inflammatory mechanisms has been recently suggested (Netea-Maier et al. 2016), in part since autophagy

displays also a pivotal role in the processing of antigen. Moreover, autophagy can also mediate processing of both extracellular and nonconventional intracellular antigens (Dengjel et al. 2005). Like apoptosis, autophagy is a genetically programmed process that requires the activity of Atg proteins. Genome-wide association studies have linked polymorphisms in genes codifying Atg proteins with autoimmune diseases, such as systemic lupus erythematosus (SLE), inflammatory bowel disease (see Chap. 23), and multiple sclerosis (see Chap. 18).

The involvement of the lysosomal compartment in autoimmunity was suggested for the first time in 1964, by a pioneering study that associated lysosomal functions with SLE (Weissmann 1964). In a recent study, we described a significant disparity in the autophagic propensity between T lymphocytes from healthy donors and patients with SLE, the latter being resistant to autophagy induction (Alessandri et al. 2012). Indeed, whereas no significant differences were seen in spontaneous autophagy of T lymphocytes from patients with SLE compared to healthy donors, autophagic resistance in SLE T cells may result in increased apoptosis and could be associated with the defective removal of apoptotic bodies favoring the persistence of autoimmune phenomena (Alessandri et al. 2012). Of note, a deregulation of autophagy has also been described in T cells from lupus-prone mice. More recently, Clarke and colleagues (Clarke et al. 2015) demonstrate enhanced autophagy in murine and human lupus B cells. Requirement for autophagy in B cell survival and differentiation during early B cell development is therefore an immunological checkpoint for the formation of plasmablasts (immature antibody-secreting plasma cells). Although the precise mechanisms leading to autophagic dysregulation in SLE are still not understood, this pathway has been implicated in promoting survival of autoimmune T and B cells.

It is well known that the majority of patients with SLE develop autoantibodies to lymphocyte surface antigens able to inhibit T cell activation and proliferation. We added further insights in this scenario, since we discovered that serum IgG from patients with SLE were able to induce autophagy in T lymphocytes from healthy donors, suggesting a role for anti-lymphocyte antibodies as autophagy inducers. We also identified the small GTPase family inhibitor D4GDI as a possible key antigenic determinant of anti-lymphocyte antibodies implicated in the pathogenesis of SLE disease (Barbati et al. 2015). These autoantibodies, once bound to D4GDI at the cell surface, can "unlock" Rho small GTPases and activate actin network remodeling. Furthermore, anti-D4GDI autoantibodies could contribute to the selection of SLE T cell clones that are resistant to autophagy. Interestingly, we found a significant association between the presence of anti-D4GDI antibodies and hematologic manifestations (i.e., leukopenia and thrombocytopenia) occurring in SLE patients (Barbati et al. 2015).

Actually, genetic studies have linked some mutations of autophagic regulators with SLE disease (International Consortium for Systemic Lupus Erythematosus Genetics SLEGEN et al. 2008). T lymphocytes from patients with SLE showed overexpression of genes negatively regulating autophagy, such as α-synuclein and single-nucleotide polymorphisms (SNPs) of *atg*5 (Colasanti et al. 2014). In addition, activation of the mammalian target of rapamycin (mTOR), a key player in

autophagy regulation, has been demonstrated in SLE (Pierdominici et al. 2012), and blockade of mTOR with rapamycin improved the clinical conditions of SLE patients (Fernandez et al. 2006).

Finally, modulation of autophagy may represent a promising therapeutic approach for a wide range of autoimmune diseases. Several drugs that have been demonstrated to act as autophagy modulators are already used or are under preclinical development in SLE, as well as in other similar autoimmune disorders. Thus, new perspectives in the development of therapeutic strategies aimed at modulation of autophagic pathways would be mandatory in autoimmune disease research.

For example, in the last few years, autophagy has been shown to play a role in the pathogenesis of rheumatoid arthritis (RA), one of the most common autoimmune disorders in humans (Ireland and Unanue 2012; Valesini et al. 2015; Dai and Hu 2016). This includes several observations supporting the hypothesis that continued removal of unfolded and misfolded proteins by the proteasome pathway and by autophagy is more active in RA synovial fibroblasts, as compared to normal cells (Clausen et al. 2010). In addition, RA synovium exhibits a highly increased ER stress, and TNF-α has been shown to increase the expression of ER stress markers in RA synovial fibroblasts (Connor et al. 2012). Indeed, autophagy induction by either proteasome inhibition or ER stress is higher in RA synovial fibroblasts than in those from control patients with osteoarthritis. Thus, a dual role of autophagy in the regulation of stress-induced cell death in RA synovial fibroblasts has been reported (Kato et al. 2014).

Autophagy activation exhibited a protective role in MG132-induced apoptosis and contributed to the apoptosis-resistant phenotype (Kato et al. 2014). In contrast, fibroblasts were hypersensitive to autophagy under conditions of severe ER stress induced by thapsigargin, which was associated with imbalance in p62/sequestosome (also known as the ubiquitin-binding protein p62, an autophagosome cargo protein that targets other proteins that bind to it for selective autophagy) and autophagy-linked FYVE protein (ALFY) expression, leading to the formation of polyubiquitinated protein aggregates and non-apoptotic cell death (Clausen et al. 2010; Kato et al. 2014).

9.4 Autophagy and Citrullination

A central role for autophagy in citrullination of peptides by antigen-presenting cells (APCs) has been hypothesized. Ireland and Unanue (2011) were the first to demonstrate that autophagy was involved in the generation of citrullinated peptides by APCs, with increased peptidyl arginine deiminase (PAD) activity detected in purified autophagosomes.

In fact, citrullination has been shown to alter the structure of immunogenic peptides that can lead to increased accessibility to proteolysis and expand the repertoire of presented peptides in a process known as epitope spreading (Hanyecz et al. 2014). For example, Ireland and Unanue (2011) demonstrated that APC presentation

of citrullinated peptides, but not of similar unmodified peptides, was associated with autophagy.

As a result, it can be theorized that self-peptides posttranslationally modified by a process such as citrullination would possibly form neo-antigens that are recognized by APCs of the immune system and thus represent a target for autoimmunity. The presentation of citrullinated peptides then would be included as a biochemical marker of an autophagy response in APCs.

Supporting this idea, our recent results (Sorice et al. 2016) revealed a role for autophagy in the citrullination process in vitro. Human synoviocytes treated with a potent ER stress inducer such as tunicamycin (Sakaki et al. 2008; Matarrese et al. 2014), as well as with the mTOR activator rapamycin (Fleming et al. 2011), revealed an activation of PAD4, with consequent generation of citrullinated proteins (Fig. 9.2). PADs are a family of enzymes that mediate posttranslational modifications of protein arginine residues by deimination or demethylimination to produce peptidyl-citrulline.

Although the exact processes for regulation of PAD activity in vivo remain largely elusive, there is growing evidence that the deregulation of PADs is involved in the etiology of multiple human diseases, including RA (Suzuki et al. 2003; Yamada et al. 2005). To further support the role of autophagy in the citrullination process, we investigated whether PAD4 may be present in autophagy vesicles. We observed that, during the autophagic process, the autophagy marker microtubule-associated protein light chain 3 (LC3)-II (one of the mammalian homologues of Atg8 that undergo lipid conjugation, leading to the conversion of the soluble form of LC3, named LC3-I, to the autophagic vesicle-associated form LC3-II) is recruited into autophagosomes, where it strictly interacts with PAD4. These findings are in agreement with previous data reporting that PAD activity can be detected in isolated autophagosomes with LC3-II enrichment (Ireland and Unanue 2011), supporting the view that citrullination may occur in these compartments. This concurs with our observations that generation of citrullinated proteins was a consequence of PAD4 activation following autophagic stimuli (Fig. 9.2). Interestingly, the protein citrullination was significantly increased in fibroblast-like synoviocytes from RA patients, as compared to control cells from osteoarthritis, a noninflammatory arthritis without anti-citrullinated protein antibodies. In particular, we demonstrated that the main citrullinated RA candidate antigens (Snir et al. 2010), including vimentin, α-enolase, filaggrin, and fibrinogen β, were processed, following autophagic stimulus. In vivo, a significant association between levels of autophagy and anti-citrullinated protein antibodies (ACPA) was observed in "naïve" RA patients with early active disease. These findings support the view that processing of proteins in autophagy generates citrullinated peptides recognized by the immune system in RA (Fig. 9.3), prompting a hypothesis that this similarly also occurs in vivo. As a result, it can be suggested that in RA patients who display a significant increase of both protein citrullination and LC3-II expression in synovial fibroblasts, autophagy may be able to trigger biochemical pathway(s) leading to PAD activation, with consequent processing of defined RA-associated citrullinated antigens, which in turn may be responsible for the presence of ACPA.

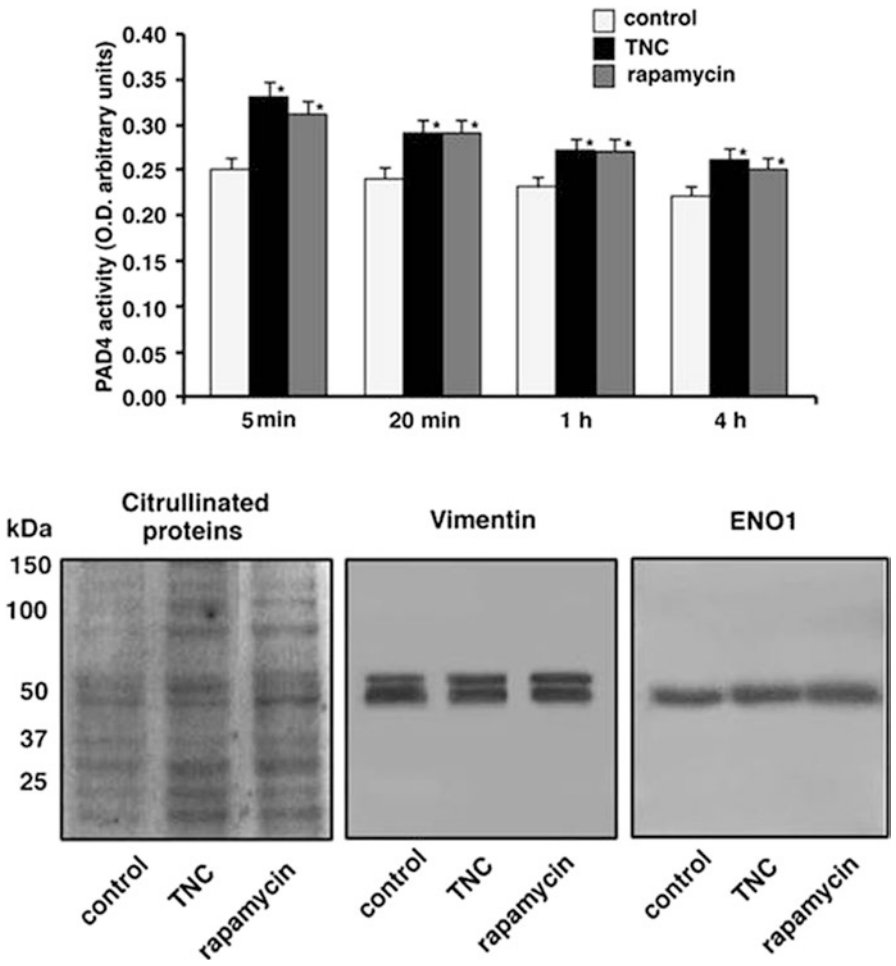

Fig. 9.2 Role of autophagy in activation of PAD. *Upper panel*: fibroblast-like synoviocytes from RA patients were treated with either tunicamycin or rapamycin. The results showed that the enzyme was active in a time-dependent manner, even after just 5 min. Statistical analysis: $*P < 0.01$ *vs*. control, as determined using a *t*-test. Results are expressed as mean ± SD of three independent experiments. *Lower panel*: the analysis of fibroblast-like synoviocytes from anti-citrullinated protein antibodies (ACPA)-positive RA patients by Western blot showed the appearance of numerous bands (corresponding to citrullinated proteins) following autophagic stimulus. To better characterize these bands, each polyvinylidene difluoride membrane was stripped and re-probed with specific anti-vimentin or anti-α-enolase antibodies. As expected, the main citrullinated bands were also stained by these antibodies, indicating that autophagic stimuli were able to induce citrullination of vimentin and α-enolase [Modified from Sorice et al., Rheumatology (Oxford), 2016]

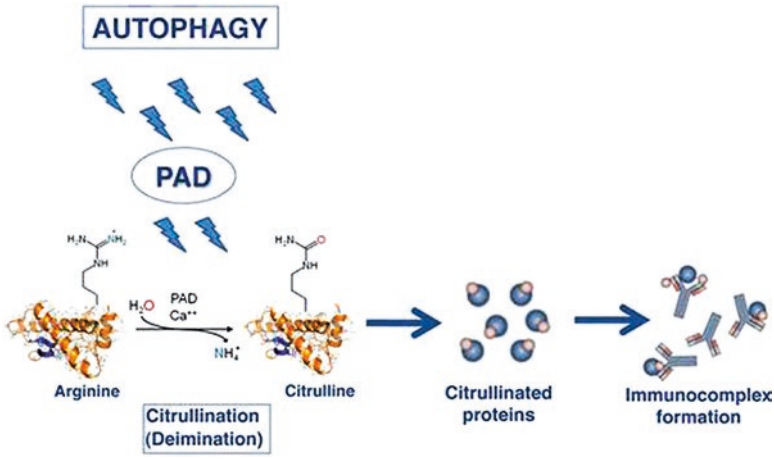

Fig. 9.3 Autophagy-induced PAD activation and protein citrullination. In RA patients, processing of autophagic proteins induces PAD activation and consequently may generate citrullinated peptides recognized by the immune system. The resultant complexes would then have an important role in the pathogenesis of the disease

Citrullination may be involved in the formation of neo-antigens as a result of a posttranslational protein modification where protein-bound arginine is converted to citrulline in antigen-presenting cells (Romero et al. 2013). These neo-antigens resulting from citrullination may be recognized by the immune system, eliciting a specific T cell response (Lundberg et al. 2005). In fact, autophagy is a key cellular event which may be the common feature of several factors, including stress conditions such as smoking, joint injury, and infection, that may be involved in the break of tolerance (Pierdominici et al. 2012), with an adaptive response to citrullinated self-proteins, triggering ACPA (Ireland and Unanue 2011; Ireland and Unanue 2012). Whether citrullination really occurs in APCs, is not completely understood; however, autophagy may play a role in the generation and presentation of citrullinated peptides by APCs. Indeed, autophagy was associated with the presentation of citrullinated peptides, but not unmodified peptides, and PAD activity was also detected in autophagosomes. Dendritic cells and macrophages show constitutive levels of autophagy; thus, these cells are able to present citrullinated peptides via autophagy. Presentation of these peptides can also be inhibited by autophagy-blocking compounds, such as 3-methyladenine (3-MA). As a result, B cells may present citrullinated peptides following autophagy induction through serum starvation or B cell antigen receptor engagement, and this presentation was shown to be blocked by 3-MA or by Atg5 inhibition (Romero et al. 2013).

In conclusion, these collective observations support the view that processing of proteins in autophagy may generate citrullinated peptides recognized by the immune system in RA patients and thus provide evidence that autophagy may also play a role in the pathogenesis of this and other autoimmune diseases.

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