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## Review

Specific functions of lysosomal proteases in endocytic and autophagic pathways<sup>☆</sup>Sabrina Müller<sup>a,b,1</sup>, Julia Dennemärker<sup>a,c,1</sup>, Thomas Reinheckel<sup>a,d,\*</sup><sup>a</sup> Institute of Molecular Medicine and Cell Research, Albert-Ludwigs-University Freiburg, Freiburg, D-79104, Germany<sup>b</sup> Faculty of Biology, Albert-Ludwigs-University Freiburg, Freiburg, Germany<sup>c</sup> Dept. of Visceral Surgery, Albert-Ludwigs-University Freiburg, Freiburg, Germany<sup>d</sup> BIOS Centre for Biological Signalling Studies, Albert-Ludwigs-Universität Freiburg, Freiburg, Germany

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## ABSTRACT

Endolysosomal vesicles form a highly dynamic multifunctional cellular compartment that contains multiple highly potent proteolytic enzymes. Originally these proteases have been assigned to cooperate solely in executing the unselective 'bulk proteolysis' within the acidic milieu of the lysosome. Although to some degree this notion still holds true, evidence is accumulating for specific and regulatory functions of individual 'acidic' proteases in many cellular processes linked to the endosomal/lysosomal compartment. Here we summarize and discuss the functions of individual endolysosomal proteases in such diverse processes as the termination of growth factor signaling, lipoprotein particle degradation, infection, antigen presentation, and autophagy. This article is part of a Special Issue entitled: Proteolysis 50 years after the discovery of lysosome.

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## 1. Introduction

The concept of the lysosome as a cellular compartment for the degradation of biological macromolecules, as set in place by the seminal work of De Duve, represents a foundation of modern cell biology [1]. In fact, the lysosomal compartment provides the perfect 'home' for more than 50 hydrolytic enzymes that are most stable and show their greatest activity within the acidic interior of the lysosome. On the other hand, the lysosomal membranes lock the destructive enzymes within the lysosomal vesicles, thus providing protection for the other cellular compartments. Among the hydrolases of the acidic cellular compartment are multiple peptide-bond cleaving proteases. A so-called 'catheptic activity' (derived from the Greek Kathépsin, meaning to digest or to boil down) was initially discovered in the acidic gastric juice during the 1920s [2]. Following this tradition many of the primarily intracellular proteases that exhibit optimal proteolytic activity in acidified vesicles, i.e. 'classical' lysosomes, endosomes, multivesicular bodies, and acidic secretory vesicles, have been named 'cathepsins'. However, today cathepsins are classified based on their structure and catalytic type into serine (cathepsins A and G), aspartic (cathepsins D and E), and cysteine cathepsins. The latter constitute

the largest cathepsin family, with 11 proteases in humans annotated as clan CA, family C1a: cathepsins B, C/DPP1, F, H, K, L, O, S, W, V, and Z/X [3]. To complicate matters more, the lysosomal compartment contains additional proteases that are not named cathepsins, such as the aspartic protease Napsin, which is related to cathepsins D and E, and the cysteine protease asparagine endopeptidase (AEP, also named legumain; clan CD, family C13). Recently, interesting 'non-canonical' physiological and pathological functions in the cytosol, the nucleus, and even mitochondria have been established for almost all of these lysosomal proteases [4–8]. Cathepsins have also been found secreted to the extracellular milieu where these proteases are often contributing to the pathogenesis of diseases such as arthritis and cancer [9–18]. However, with regard to De Duve we will focus this review on recent work concerning specific functions of individual serine-, cysteine-, and aspartate-type 'acidic' proteases and also their combined 'networking' actions. The 'lysosome', however, is no longer a stand alone organelle; complex mechanisms regulate the biogenesis of lysosomes and the delivery of degradation-prone substrates into the lumen of this organelle [19]. Hence, we organize our discussion of cathepsin functions along the main delivery routes into lysosomes, i.e. endo- and phagocytosis as well as the autophagy pathways.

## 2. Involvement of endolysosomal proteases in endocytosis

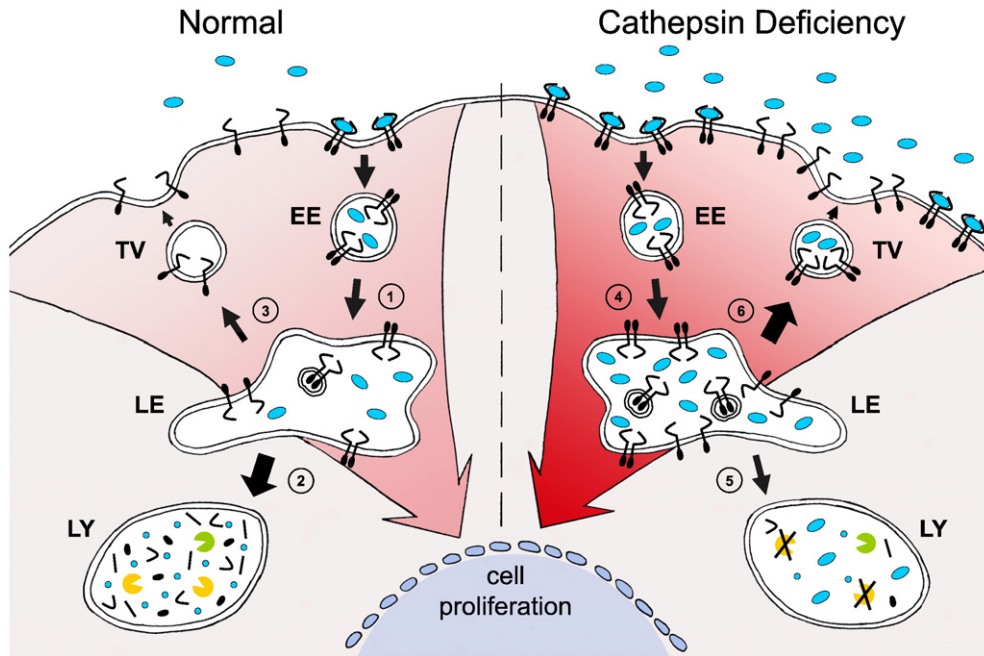
The endocytic pathway, by providing a set of dynamic and biochemically specialized endomembrane structures that physically communicate with the plasma membrane, is a highly flexible scaffold for mediating precise spatiotemporal control and transport of diverse biological signals (Fig. 1). Early endosomes (pH 6.5–6.0) are the first

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**Fig. 1.** Cathepsins in growth factor signaling—the EGF example. After binding of epidermal growth factor (EGF) to the EGF-receptor at the cell surface, receptor–ligand complexes are internalized. The complexes are then transported by vesicles termed early endosomes (EE), where EGF dissociates from its receptor, to late endosomes (LE) (1). Under physiological conditions some receptors are recycled to the plasma membrane (3) by transporting vesicles (TV), however, the majority of the receptors as well as EGF are terminally transported to lysosomes (LY), where they are degraded to shut off the signaling cascade (2). In cathepsin L deficient keratinocytes the receptor–ligand complexes are taken up normally and are transported by EE to the LE compartment (4). The loss of the protease, however, leads to reduced degradation of the receptor and its ligand in LY (5), and consequently to enhanced recycling of the EGF receptor, and notably, of intact EGF by TV to the plasma membrane (6). This results in increased EGF signal transduction (indicated by the red arrow) and hence in increased cell proliferation. ● Active endolysosomal protease, ✕ deficient endolysosomal protease, Y dimerized EGF receptors, ● two EGF molecules.

station along the endocytic pathway receiving most of the vesicle types which are derived from the cell surface and containing, for instance, internalized plasma membrane receptors. In early endosomes (pH 5.5–6.8) many ligands dissociate from their receptors (e.g. EGF from EGFR) that are subsequently recycled to the cell surface [20,21]. Late endosomes have a more acidic pH (pH 5.0–5.5) and receive internalized material from early endosomes, phagosomes or newly synthesized molecules from the trans-Golgi network and transport those substances to their final destination in the lysosomes (pH 4.0–5.0) [22]. The proteases contained in this acidic cellular compartment play multiple and essential roles in diverse tasks of endolysosomal protein degradation and protein processing.

### 2.1. Cathepsins in receptor-mediated endocytosis and growth factor signaling

A main function of the endolysosomal compartment is the modulation and termination of signal transduction pathways by dissociating growth factors from their receptors and then subsequently degrading them and/or recycling them to the plasma membrane [23]. It was reported that cathepsin B is responsible for the degradation of epidermal growth factor (EGF) in the liver as well as for the degradation of internalized EGF receptor complexes [24]. In further studies, insulin-like growth factor-I (IGF-I) was identified as another target of endolysosomal degradation. Furthermore, the inhibition of cysteine proteases by the compounds E64 and the cell permeable Ca074Me, which is more specific for cathepsin B, altered the receptor trafficking and signaling of several tumor cell lines [25]. Treatment with these lysosomal protease inhibitors also led to inhibition of lysosomal degradation of IGF-I and to abrogated IGF-I receptor signaling by reduction of IGF-I receptor levels on the cell surface [25]. Earlier, the same group had shown that treatment of cancer cells with the general cysteine cathepsin inhibitor E64 blocked their invasiveness and decreased liver metastasis [26]. It was later demonstrated that the protease responsible for IGF-I degradation was

cathepsin B [27]. However, cathepsin B is not the only protease implicated in selective growth factor degradation. For example, the aspartic protease cathepsin D was identified as being responsible for the endosomal degradation of insulin into two major primary end products [28], while in cathepsin L deficient fibroblasts the insulin-like growth factor binding protein 3 (IGFBP-3) accumulates in the culture medium due to decreased intracellular degradation [29]. Furthermore, the proliferation of primary keratinocytes from cathepsin L null mice is hyper-responsive to EGF stimulation [30]. Tracing the fate of radioactively labeled EGF in keratinocytes revealed that cathepsin L deficient cells exhibit enhanced recycling of intact growth factor to the medium and to the plasma membrane (Fig. 1). In addition, it has been shown that treatment with medium conditioned by cathepsin L deficient keratinocytes results in higher keratinocyte proliferation than with medium from cultured wild-type keratinocytes [30]. Further studies revealed the activation of a number of positive regulators and down-regulation of inhibitors in growth factor signaling pathways in cathepsin L deficient keratinocytes [31]. In fact, it was shown *in vivo* that levels of active Ras were significantly elevated in the skin of cathepsin L null mice [31]. Hyperactive Ras is central in many cancer-promoting signaling processes, such as the MAP-kinase and Akt/PKB pathways [32]. In agreement with this, both pathways have been found to be activated in cathepsin L deficient keratinocytes as well. The activation of Akt by phosphorylation was further enhanced upon incubation of the cathepsin L deficient cells in medium conditioned by cathepsin L deficient keratinocytes, supporting autocrine Akt stimulation caused by the absence of the protease [31]. These findings fit the observation made in the K14-HPV16 mouse model of skin cancer, where the loss of cathepsin L surprisingly leads to an enhanced tumor phenotype. As summarized in Fig. 1, these data suggest that impaired degradation and enhanced recycling of plasma membrane receptors and their ligands in the absence of cathepsin L result in increased proliferation of basal keratinocytes. This in turn leads to epidermal hyperproliferation and periodic hair loss in cathepsin L deficient mice. In addition, the sustained oncogenic

signaling in keratinocytes of cathepsin L deficient K14-HPV16 skin cancer mice results in an overall aggravated progression of the tumor [31,33,34]. Taken together, lysosomal cathepsins play an important role not only in bulk protein degradation but, by degrading growth factors and their receptors, are also involved in receptor-trafficking and -signaling.

## 2.2. Cathepsins in low density lipoprotein (LDL) degradation and atherosclerosis

The LDL receptor binds apolipoprotein B-100 of the cholesterol-rich LDL particle, resulting in endocytosis of the LDL/receptor complex and its delivery to the endolysosome, where LDL dissociates from the receptor and cholesterol is released into the cell for membrane synthesis, steroidogenesis and bile acid synthesis. The LDL receptor is recycled to the plasma membrane for subsequent cycles of endocytosis, resulting in a decrease in cholesterol levels in the blood [35]. LDL receptor expression is regulated by intracellular cholesterol levels. Mutations of either the LDL receptor or its ligand, the apolipoprotein B-100, results in decreased ability of the cell to import cholesterol, causing hypercholesterolemia and, subsequently, cardiovascular disease [36,37]. Early work suggested that the proteases responsible for lysosomal LDL degradation might be the endolysosomal cathepsins B and D [38,39]. Van Lenten et al. showed that it is primarily cathepsin D which cleaves the apolipoprotein B under normal conditions, and proposed that the accumulation of cholesteryl esters in macrophages in atherosclerosis might be due to the fact that at high concentrations of LDL the number of LDL-receptor complexes reaching the compartment for hydrolysis is rate-limiting [39]. Cathepsin D has also been identified as the relevant protease for the degradation of another complex of the LDL-receptor family, namely the t-PA/PAI-1 (tissue-type plasminogen activator/plasminogen activator inhibitor-1) complex which is highly important for the regulation of fibrinolysis [40]. It was demonstrated that the t-PA/PAI-1 complex is internalized by human monocytes via a member of the LDL receptor family, followed by degradation through cathepsin D after delivery to the lysosomes.

It was later shown that macrophages as well as intimal smooth muscle cells in atheroma contained abundant immunoreactive cysteine cathepsins K and S contributing to ECM remodeling due to their elastinolytic and collagenolytic activity [41]. Both proteases were upregulated upon stimulation with the atheroma-associated inflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) or interferon- $\gamma$  [41]. In a subsequent study the involvement of cathepsin S in atherosclerosis was demonstrated in mice with a double deficiency in cathepsin S and the LDL receptor [42]. In this mouse model the lack of cathepsin S reduced atherosclerosis by more than 50% after 8 and 12 weeks of an atherogenic diet, and by an additional 30% after 26 weeks. The authors of this work proposed several mechanisms to explain these results such as the contribution of secreted cathepsin S to the proteolysis of the elastic lamina, ECM and basement membrane of blood vessels, thereby disturbing the vessels' structural integrity. An additional suggestion was an altered immune response due to defective antigen presentation of CD1-positive 'foamy' macrophages [42]. Recently, a mechanism linking macrophage cathepsins B and L to atherosclerosis was proposed [43]. These experiments revealed impairment in the inflammatory response after intraperitoneal injection of cholesterol crystals in mice deficient in either components of the NLRP3 inflammasome, cathepsin B, cathepsin L or IL-1 $\beta$ . The proposed pathway involves lysosomal damage after uptake of cholesterol crystals by macrophages. In consequence, the cathepsins are released to the cytosol where they activate the NLRP3 inflammasome by an as yet unknown mechanism. The caspase 1 activity of the activated NLRP3 inflammasome selectively processes the pro-inflammatory IL-1 $\beta$ , which is a hallmark of the inflammatory microenvironment that propagates atherosclerotic lesions.

## 3. Role of endosomal proteolysis in antigen presentation and infection

The acidic cellular compartment is crucial for major histocompatibility complex (MHC) class II mediated antigen presentation. In antigen presenting cells (APC) proteins taken up from the extracellular space are processed into antigenic peptides by endosomal proteases within the acidic environment of the late endosomal compartment, which is also called the MHC class II compartment. Furthermore, the MHC class II protein complex is directed from the endoplasmic reticulum (ER) to the late endosomal compartment, where it proteolytically matures in order to present the newly generated, antigenic peptides on the cell surface of APCs.

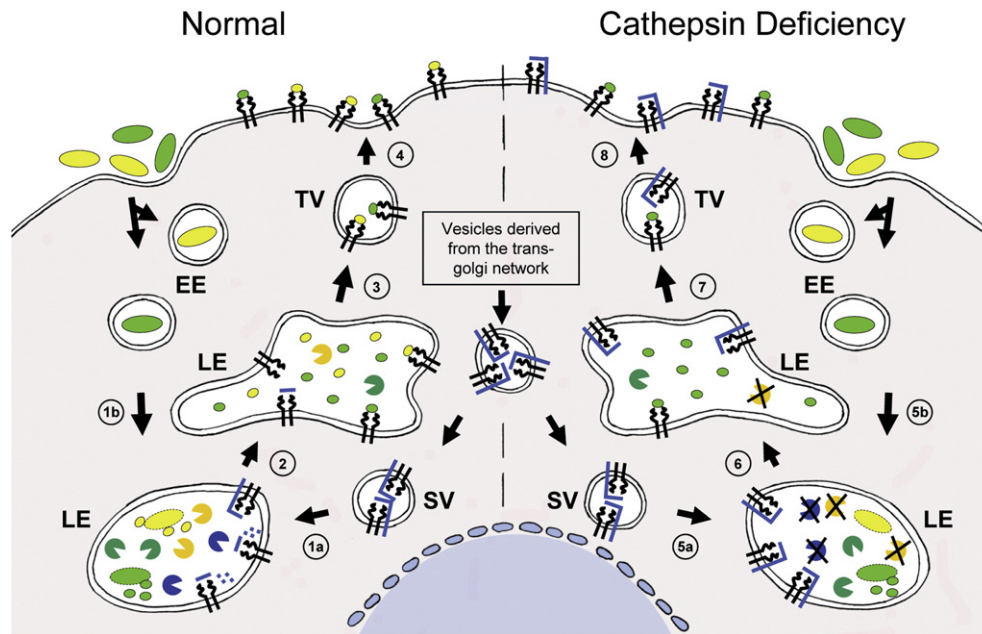
### 3.1. 'Acidic' proteases generate or destroy antigenic peptides

The presentation of self and antigenic peptides by MHC class II molecules is an exclusive feature of APCs such as dendritic cells (DCs), macrophages, and B cells. APCs can efficiently take up material from the extracellular space by phagocytosis, pinocytosis or receptor-mediated endocytosis. As shown in Fig. 2, ingested material is directed to the endosomal compartment where proteins get processed and degraded by 'acidic' proteases, e.g. cathepsins. Peptides generated by these enzymes are subsequently presented at the cell surface by MHC class II molecules, activating CD4 positive (CD4<sup>+</sup>) T helper cells in the case of exogenous pathogens.

DCs are the most potent APCs because of their ability to migrate to the lymph nodes after activation to foster an adaptive immune response [44]. It is therefore important that antigenic peptides remain preserved in DCs rather than becoming destroyed. In agreement with this it was demonstrated that DCs can regulate endosomal pH through the activity of NADPH oxidase 2, whose products alkalinize the pH and therefore limit antigen degradation by 'acidic' proteases [45]. Furthermore, the immunogenicity of proteins is highly dependent on their susceptibility to endolysosomal proteases as enhanced protein degradation leads to strong attenuation of the adaptive immune response [46].

The identification of antigenic peptides specifically generated or destroyed by one particular protease is, however, difficult as a result of functional redundancy between endolysosomal proteases. Asparagine endopeptidase (AEP, also called legumain), for example, has been proposed to degrade the autoantigen myelin basic protein (MBP) peptide 85–99 in a human Epstein Barr Virus-transformed (EBV) B cell line, thereby removing it from the peptide repertoire which induces tolerance to self antigens in the thymus [47]. In contrast, in another study which used the same cell line but a different AEP inhibitor there was no change in the T cell response to MBP peptide 85–99 detected [48]. However, it was claimed that in these cells the neutrophil serine protease cathepsin G, which is taken up by human B lymphoblastic cells via endocytosis, degrades MBP [49]. In contrast to peptide degradation, AEP specifically cleaves the microbial tetanus toxin antigen for presentation via MHC class II molecules in both a murine as well as in a human B cell line. In line with this, inhibition of AEP activity or mutation of AEP cleavage sites in the tetanus toxin antigen led to a reduction in the T cell response to the antigenic peptide [48,50,51]. Recently, however, immunization of AEP deficient mice with tetanus toxin antigen showed no differences in endpoint analyses of the immune response in comparison to WT mice [52]. Hence, AEP appears to be dispensable for the presentation of antigenic tetanus toxin peptides by murine DCs, although its activity might accelerate the kinetics of the presentation of tetanus toxin antigen [52]. Besides AEP, other endolysosomal cathepsins such as the aspartic cathepsins E and D, as well as the cysteine type cathepsins, have been under intense scrutiny with regard to peptide generation for MHC class II mediated antigen presentation. It has been shown that exogenous material is selectively targeted to cathepsin S in





**Fig. 2.** Cathepsins in MHC class II antigen presentation. In APCs, newly synthesized MHC class II molecules are transported by sorting vesicles (SV) from the trans-Golgi network to the late endosomes (LE) (1a) where the invariant chain is proteolytically processed by endolysosomal proteases. Extracellular bacteria, which are taken up by phagocytosis, are transported by early endosomes (EE; also named phagosomes) to the late endosomal compartment (1b). Within this compartment antigenic protein complexes are degraded by various endolysosomal proteases to antigenic peptides. These newly generated antigenic peptides are loaded into the binding groove of MHC class II molecules in LE (2). These complexes are routed to the cell surface by transporting vesicles (TV) (3) and are subsequently presented to  $CD4^+$  T helper cells to initiate an adaptive immune response. In cathepsin S deficient APCs, the MHC class II molecules reach the LE from the trans-Golgi network as previously described (5a). However, the invariant chain is incompletely processed within the LE, leading to a blocked peptide binding groove. Because of this, antigenic peptides are insufficiently loaded onto MHC class II molecules (6), which in turn results, after transport to the cell surface (7), in reduced presentation of antigenic peptides to  $CD4^+$  T helper cells (8). Furthermore, the deficiency for a single endolysosomal protease, i.e. AEP, leads to a reduction in the repertoire of antigenic peptides generated in LE (5b;6), which can be presented by MHC class II molecules (7;8). This finally results in an impaired immune response, normally induced by the particular antigenic peptides. ● Active endolysosomal protease, ✗ deficient endolysosomal protease, †† MHC class II molecule, | invariant chain, ● antigenic peptide, ● bacterium/pathogen/antigenic protein.

human DCs [53]. This might be due to an early fusion of phagosomes with vesicles of the MHC class II compartment which predominantly contain proteolytically active cathepsin S as has been shown for primary murine APCs [54]. However, in terms of peptide generation for MHC class II presentation the evidence for a non-redundant role of cathepsin S in APCs is quite limited [55]. Thus, deciphering the contribution of a single endolysosomal protease to the generation or destruction of antigenic peptides might be elusive as it varies according to the combination of proteases expressed within the particular cell type studied [52,54,56,57].

Using protease deficient mouse models, it was shown that cathepsin D and the cysteine protease cathepsin B are dispensable for antigen presentation [58]. Consistent with these findings, transplantation of cathepsin D deficient bone marrow into irradiated mice caused no impaired immune response of these chimera to two different antigens in comparison to WT mice [59]. However, chemical inhibition or genetic ablation of cathepsin D actually results in enhanced presentation of antigenic peptides derived from myoglobin, a known cathepsin D substrate. In this setting myoglobin is processed by residual aspartic proteolytic activity, which was assigned to cathepsin E. Hence, a picture is emerging in which cathepsin D degrades protein substrates completely, while the structurally related cathepsin E generates peptides sufficient for MHC class II presentation [60]. The primarily destructive role of cathepsin D in antigen presentation is further demonstrated by its ability to initiate degradation of the renal Goodpasture autoantigen, which is a specific isoform of type IV collagen and causes Goodpasture disease [61]. During degradation of the Goodpasture autoantigen major self epitopes are destroyed, providing a reasonable explanation as to how 'Goodpasture' autoreactive T cells escape the negative T cell selection process within the thymus [61]. Hence, a subtle balance between peptide generation and protein degradation in the MHC class

II compartment is of utmost importance for proper antigen presentation.

The activation of cytotoxic T cells depends on the presentation of endogenous peptides bound to MHC class I molecules by almost all cell types of the body. These peptides are mainly generated by the proteasome in the cytosol and are transported by TAP (transporter associated with antigen processing) into the ER. APCs, however, are able to present 'foreign' exogenous peptides on MHC class I molecules via both TAP-dependent and TAP-independent pathways, a process generally termed cross presentation [62]. Although the TAP-independent 'vacuolar' cross presentation mechanisms are still insufficiently understood, it is reasonable to assume that endolysosomal proteases play a role [63]. For instance, the extracellular portions of transmembrane proteins are turned over in the lumen of the endolysosome while aspartic cathepsins can contribute to the MHC class I presentation of antigenic peptides derived from these transmembrane proteins [64]. In addition, cathepsin S processes ovalbumin and influenza virus antigens for subsequent peptide presentation on MHC class I molecules *in vivo*, which is restricted to TAP-independent MHC class I cross presentation [65,66]. Recent experiments on the presentation of endocytosed hen egg white lysozyme derived peptides revealed that cathepsin S dependent cross presentation on MHC class I occurs in early endosomal compartments, while the late endosomal compartment targets peptides solely to MHC class II complexes [67].

### 3.2. Endolysosomal proteases process the invariant chain of MHC class II molecules

Fig. 2 illustrates that endolysosomal proteases are not only responsible for the generation of antigenic peptides during the process of antigen presentation, but are also involved in the

maturation of MHC class II molecules with respect to the processing of the MHC class II invariant chain (Ii) [68]. Ii serves as a chaperone for the MHC class II molecule, preventing undesired peptide binding to the binding groove and targeting MHC class II molecules to the endolysosomal compartment, where Ii gets processed (reviewed in [46,69]). Complete removal of Ii from MHC class II molecules, allowing access of an antigenic peptide to the binding groove, is essential for proper antigen presentation to CD4<sup>+</sup> T helper cells.

The initial cleavage of Ii was suggested to be executed by a leupeptin-insensitive protease [70]. In this regard, AEP was proposed to be the initiator protease in Ii-processing [71]. However, AEP-deficient mice show no impairment in Ii-processing [52]. Combining leupeptin and a selective inhibitor for AEP (AEPi) only leads to a slight delay in the formation of an early processed Ii intermediate in human EBV-transformed B cell lines [48]. This delay could not be observed when using AEPi alone, indicating that AEP performs an initial cleavage step in the Ii processing cascade when the proteolytic activity of other endolysosomal serine and cysteine proteases is inhibited. Moreover, AEP activity differed between cell lines and cell types leading to the hypothesis that the first cleavage events in Ii processing depend on the repertoire of active proteases present within a cell [48,52]. Although an involvement of aspartic proteases in initial Ii processing was claimed [72,73], use of the recently discovered aspartic protease inhibitor grassystatine A, pepstatin A or cathepsin D deficient cells revealed no change in T cell responses to distinct antigens [48,58,74]. Therefore, the precise definition of the proteolytic network that initiates Ii processing in a particular cell type needs to be investigated further.

The additional sequential processing of Ii, leaving only a small Ii-derived peptide named CLIP (class II associated invariant chain peptide) bound to the MHC class II binding groove, is mainly performed by cathepsin S, which is predominantly expressed in APCs [75,76]. Mice deficient for cathepsin S show an accumulation of Ii degradation products in APCs, which is dependent on a high affinity of MHC class II molecules to the CLIP fragment [77]. The endoprotease cathepsin L performs the Ii processing steps in cortical thymic epithelial cells (CTECs), leading to reduced CD4<sup>+</sup> T helper cell numbers in cathepsin L deficient mice due to a disturbance in positive selection in the thymus [78–80]. Considering its expression pattern and the results of functional analyses in mice, it is likely that cathepsin V, the human orthologue of murine cathepsin L, is important for this process in the human thymus [81,82]. Interestingly, the p41 splice variant of Ii (p41) is able to bind to the active site of cathepsin L. Having isolated p41 as well as cathepsin L from a human kidney homogenate, Bevec et al. showed that p41 dose-dependently inhibits the proteolytic activity of cathepsin L [83]. Subsequently, it became clear that p41 acts as a chaperone for cathepsin L in APCs, thereby stabilizing its active form within endocytic compartments as well as in the extracellular space [84,85]. A comparison of the human and the murine system revealed similar interaction characteristics for p41 and cathepsin L in these species [86].

Taken together, our current understanding of MHC class II invariant chain proteolysis reveals extensive cooperation between endolysosomal proteases in this crucial immunological process.

### 3.3. Role of the endolysosomal proteases in infection

The endolysosomal compartment is perfectly equipped to efficiently sense the presence of phagocytosed pathogens to set up an inflammatory response. So-called pattern recognition receptors (PRR) of the toll like receptor (TLR) family, namely TLR3, TLR7, TLR8 and TLR9 (and also presumably TLR13), are expressed intracellularly in the endolysosomal compartment. TLR3 recognizes double stranded (ds) RNA encoding for the genomes of some viruses [87]. TLR7, and TLR8 in humans, bind to single stranded (ss) RNA [88], whereas the ligands for TLR9 are comprised of DNA pieces such as unmethylated

CpG motifs, which are characteristic for bacteria and DNA viruses [89]. Activation of these nucleic acid-specific TLRs results in activation of the prominent inflammatory transcription factor NF- $\kappa$ B and consequently in secretion of proinflammatory cytokines (reviewed in [90]). However, intracellular TLRs can also be activated by host nucleic acids, because vertebrate DNA packed into liposomes activates TLR9 after entering the endolysosomal compartment [91]. Hence, compartmentalization is necessary to separate 'self' stimuli from the TLR receptors in order to avoid autoimmunity, while 'non-self' TLR stimuli should reliably induce a cellular defense response. The activation of TLR3, TLR7, TLR8 and TLR9 is restricted to the endolysosomal compartment as reagents blocking acidification shut down receptor signaling [92]. In addition, it has been shown that TLR9 and TLR7 are processed by proteases after delivery to the endolysosomal compartment [93,94]. Both the full length and the processed form of the receptor are able to bind their ligand, but only the processed form of the receptor recruits the adapter protein MyD88, which is essential for down-stream signaling. Ewald et al. were unable to block TLR9 processing using broad spectrum inhibitors of cathepsins, i.e. E64d, pepstatin A and leupeptin, in the RAW mouse macrophage cell line [93]. However, Park et al. inhibited receptor processing of TLR 9 in the same cell line using the cysteine cathepsin inhibitor z-FA-fmk [94]. Simultaneous targeting of cathepsins L and S with more selective inhibitors, i.e. Clk 148 and LHVS, respectively, significantly decreased the TNF- $\alpha$  response to the TLR9 ligand CpG, suggesting a major role for the prominent immune proteases cathepsin L and S in TLR9 processing [94]. Because of the discrepancy between these two reports it is conceivable that TLR9 processing cannot be assigned to the action of a single protease. Rather, several endolysosomal proteases may be able to execute TLR9 processing in a context specific manner. Consistent with this hypothesis, TLR9 signaling is also impaired in cathepsin K deficient DCs [95], and reduced levels of the processed form of TLR9 were found in AEP deficient cells [96].

However, while many viruses are sensed through intracellular TLRs, some viruses, probably through coevolution with the endolysosomal compartment, use the host proteolytic machinery of this compartment to escape into the cytosol via fusion of the viral envelope with the endosomal membrane. It has been shown that the endolysosomal processing of the glycoprotein of Ebola virus (EboV), which is critical for the delivery of viral material into the cytosol, occurs predominantly through the action of cathepsin B and to a minor by extent cathepsin L [97]. In addition, chemical inhibition and genetic ablation of cathepsin B revealed that proteolytic processing of EboV glycoprotein by cathepsin B is a prerequisite for the infection of Vero cells by vesicular stomatitis virus expressing the EboV glycoprotein [98]. Furthermore, the endoprotease cathepsin L functions as an uncoating factor for reovirus disassembly converting the virion to the infectious subvirion particle in murine fibroblasts [99]. Cathepsin L is also essential for the infection of cells with SARS (severe acute respiratory syndrome) coronavirus, cleaving the S glycoprotein of the virus and thereby mediating membrane fusion [100,101].

In summary, endolysosomal proteases play contradictory roles in infection. On the good side, they are involved in sensing infection and strengthening the cellular defense. However, these proteases also support several virus types in carrying out their rather deathly actions.

## 4. Lysosomal proteases in cellular homeostasis and autophagy

The catabolic reactions occurring in the endolysosomal compartment are critical for maintaining cellular homeostasis. Failure to degrade the macromolecules contained in this compartment results in their accumulation, causing first malfunction of the compartment and subsequently failure of cell and tissue functions. This is most clearly evidenced by the lysosomal storage disorders, a group of rare inherited diseases in which single deficiencies for lysosomal enzymes

cause severe defects in skeletal development, myopathy, movement disorders, dementia, and very often premature death [102].

Complementary to the degradation of extracellular- and plasma membrane-derived macromolecules by the endocytic pathways already described in the previous sections, cells also employ a conserved and regulated turnover of long-lived intracellular proteins and organelles through lysosomal degradation [103]. Generally known as autophagy, this pathway has been subdivided into three distinct processes in mammalian cells, namely macroautophagy (Fig. 3), microautophagy, and chaperone-mediated autophagy [104]. In eukaryotic cells, autophagy at a basal level is constitutively present in all cells to perform housekeeping functions such as degradation of proteins and destruction of dysfunctional organelles. The fusion of the autophagic vacuole with the lysosome and the subsequent degradation of its content by lysosomal enzymes are crucial for the correct execution of macroautophagy. It is well conceivable that endolysosomal proteases play important roles in the degradation and regulation events of autophagic processes. An excellent review on the role of lysosomes and lysosomal proteases in apoptotic and autophagic cell death has been recently published, therefore this aspect of lysosomal cathepsins will not be further discussed in this article [105].

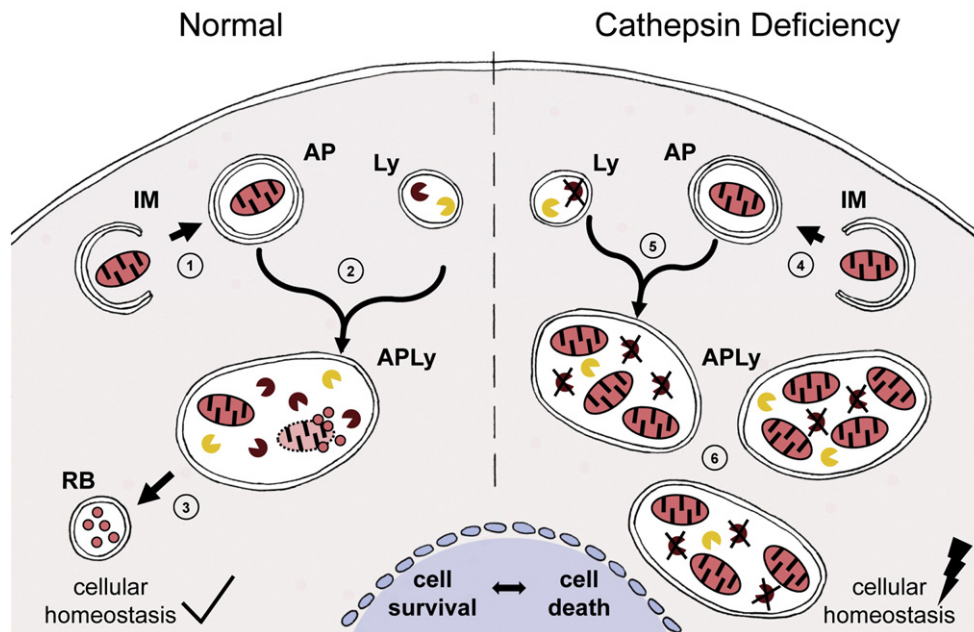
#### 4.1. Cathepsin A as regulator of chaperone-mediated autophagy

One protease shown to be critical for chaperone mediated autophagy (CMA) is the protective protein/cathepsin A (PPCA), a serine carboxypeptidase. Cuervo and colleagues provided evidence for the lysosome-associated membrane protein type 2a (Lamp2a) as a substrate of PPCA [106]. Lamp2a functions as a receptor of chaperone mediated autophagy [107]. It is one of three splice variants of the *lamp2* gene, which are all single-span lysosomal membrane proteins with a highly N-glycosylated luminal domain [108]. Proteins prone to CMA specifically bind to the cytoplasmic domain of Lamp2a leading to a multimerization of Lamp2a and triggering the translocation of the substrates into the lysosome [107]. The subsequent disassembly of

Lamp2a from the translocation complex is mediated by chaperones [109]. In the whole CMA process the binding of CMA substrate proteins to Lamp2a is the limiting step. Lamp2a levels are regulated mainly by de novo synthesis and by changes in its stepwise degradation within discrete microdomains [110]. In the course of this process the cleavage of the cytosolic portion of Lamp2a by an as yet unidentified metalloprotease is followed by the cleavage by PPCA on the luminal side, leading to the release of a truncated form of Lamp2a which is then rapidly degraded by other lysosomal proteases [106,110]. Cells defective in PPCA show reduced rates of Lamp2a degradation, higher levels of Lamp2a, and higher rates of CMA, while restoration of PPCA protease activity reduces both Lamp2a levels and CMA rates [106]. This clearly indicates a crucial role for PPCA in CMA and thereby in many physiological processes where CMA is implicated, such as the cellular response to oxidative stress, aging, the immune response and general cell homeostasis [111].

#### 4.2. Cathepsin D in macroautophagy and human neuronal ceroid-lipofuscinosis

Congenital neuronal ceroid-lipofuscinosis (NCL) is a neurodegenerative disease characterized by progressive psychomotor retardation, blindness, and premature death due to the accumulation of lipopigments/lipofuscin in neuronal cells and many organs, including the liver, spleen, myocardium, and kidneys. In the year 2000, Tyynelä et al. discovered that a genetic form of NCL in sheep was caused by a deficiency in the lysosomal aspartic protease cathepsin D [112]. A point mutation led to the conversion of the active site aspartate to asparagine resulting in a stable, yet enzymatically inactive protein. Later, the screening of patients with NCL revealed a mutation in the cathepsin D gene resulting in an inactive enzyme [113]. Mutations in the cathepsin D gene leading to a protein with reduced proteolytic activity and lower levels of cathepsin D in patient fibroblasts have also been linked to an early onset NCL disorder characterized by early blindness and progressive psychomotor impairment [114]. This



**Fig. 3.** Cathepsins in macroautophagy and cellular homeostasis. In the process of macroautophagy, dysfunctional organelles, such as mitochondria, are enclosed by a double membrane, the so-called isolation membrane (IM), and subsequently an autophagosome (AP) is formed (1). AP fusion with lysosomes (LY) results in autophagolysosomes (APLY) in which AP cargo is degraded (2). In healthy cells, only small residual bodies (RB) remain (3). Lack of critical protease activities in the lysosome, i.e. cathepsin D or cathepsin L deficiency, does not primarily impair IM (4) nor APLY (5) formation. However, in the case of protease deficiency material enclosed by autophagolysosomes cannot be degraded, resulting in an accumulation of unusually large vesicles, i.e. APLY (6), defective in termination of the autophagic process. This pathomechanism of lysosomal storage may impair tissue function and eventually cause cell death which in turn results in a clinically relevant lysosomal storage disorder. ● Active endolysosomal protease, ✕ deficient endolysosomal protease, Ⓜ mitochondrion.



deleterious effect of an insufficiency in cathepsin D has also been observed in cathepsin D deficient mice. These display a phenotype characterized by severe general hypotrophy and neurodegeneration, including intracellular accumulation of autophagic vesicles very similar to ceroid-lipofuscinosis [115–117], and die shortly after birth [118]. Neuronal cell death in this model is not mediated by the mitochondria, i.e. it is independent of the proapoptotic Bcl2 family member Bax [119]. However, levels of the autophagosomal marker LC3-II were increased, suggesting that cell death and neurodegeneration in cathepsin D deficiency are mediated by an alteration in the macroautophagy-lysosomal degradation pathway [119]. These results demonstrate the importance of lysosomal proteases in cellular homeostasis, especially in tissues with a low regeneration rate such as heart, muscle or neurons. Because of this, it is not surprising that deficiency for cathepsins other than cathepsin D is also linked to defects in macroautophagy (Fig. 3). For example, cathepsin B/L double deficient mice develop a lethal neurodegeneration with accumulation of autophagosomal vesicles in brain tissue, which is similar to the phenotype observed in cathepsin D deficient animals [117,120,121].

#### 4.3. Cathepsin L in the termination of the macroautophagy process

Mice deficient for the cysteine endoprotease cathepsin L show multiple complex phenotypes [122,123]. Strikingly, these phenotypes, such as cardiomyopathy, hair loss, and impaired generation of thyroid hormones, are frequently associated with unusually large vesicles in the various cell types and tissues affected by the loss of the protease [34,124,125]. For instance, an increased amount of low density vesicles with high levels of the lysosomal marker Lamp1 was detected in the myocardium of cathepsin L deficient mice. Cardiomyocyte specific re-expression of cathepsin L in the otherwise cathepsin L deficient animals was sufficient to normalize the vesicular organization in the cells and significantly improved myocardial function [126]. Furthermore, proteomic analysis of cathepsin L deficient hearts revealed increased levels of many mitochondrial proteins together with impaired mitochondrial respiration [127]. Macroautophagy represents the major pathway for removal of 'aged' dysfunctional organelles, such as mitochondria [103]. It was therefore hypothesized that cells defective for cathepsin L may also have a defect in the autophagic removal of mitochondria and in autophagy in general. This was recently addressed using cathepsin L deficient mouse embryonic fibroblasts as a model system [128]. However, the initiation of autophagy and formation of autophagosomes in response to nutrient deprivation or rapamycin treatment was not significantly altered in cathepsin L deficient cells. In contrast, cathepsin L deficiency caused the accumulation of large autophagolysosomes indicative of impaired degradation of autophagocytosed material, thus prohibiting correct termination of autophagy [128]. Interestingly, other proteases appear to compensate for the loss of cathepsin L, shifting the endolysosomal proteolytic system to a new homeostatic balance with just enough functionality to be compatible with life and reproduction. Previous studies provided evidence for increased protein levels of the lysosomal aspartic protease cathepsin D upon cathepsin L down-regulation in thyroid and epithelial cells [124,129]. This finding was confirmed in cathepsin L deficient fibroblasts [128]. An elevation in the level of cathepsin D has also been detected in the brains of cathepsin B/L double deficient mice [120]. As discussed in the previous section, the cathepsin D as well as the cathepsin B/L double deficient mouse models develop severe neurodegenerative disorders [117]. Thus, it appears that cathepsin L together with cathepsins B and D are the major proteases involved in the turnover of intracellular organelles, i.e. autophagolysosomes, in various tissues (Fig. 3). This also seems to be true for human cathepsin L, because transgenic expression of the human protease in a cathepsin B/L double deficient background rescues neurodegeneration and lethality of these mice [121].

## 5. Conclusions and further directions

In the previous sections we were able to present only part of the evidence in support of endolysosomal proteases executing individual, specific and decisive functions in many important cellular processes. Much of this current knowledge was obtained by genome-wide protease gene discovery in combination with genetic studies employing 'knockdown', 'knockout', or transgenic overexpression strategies. Less progress has been achieved in the biochemical analysis of proteolytic events within the endolysosomal compartment. This is due to the fact that this compartment consists of a multitude of intracellular vesicles which dynamically change with the actual state of cells and which are, therefore, very difficult to isolate at a purity which is satisfactory. In many instances this situation prevents the identification of specific protease substrates and hinders the understanding of the compensatory networking of proteases in the acidic cellular compartment. Since novel technologies are at hand [130–134], temporally and spatially resolved monitoring of endolysosomal protease activities and the detection of specific protease cleavage sites within substrates might be the next addressable challenges for protease investigations in de Duve's lysosomes.

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