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A review of the potential roles for phagosomal NADPH oxidase and redox sensitive cysteine cathepsins during adaptive immune responses in EAE.

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Abstract:
This review discusses the role of redox-sensitive cysteine cathepsins during MHC-II- restricted MOG antigen processing, and MOG- induced experimental autoimmune encephalomyelitis (EAE). The phagosomal redox environment can modify the activity of multiple cysteine cathepsins and these proteases can, in turn, perturb antigen processing and presentation, particularly of MOG. Mice deficient in NOX2 exhibit protection from EAE, which is likely due to inefficient MOG- antigen processing and presentation, and less likely to be due to other T cell mediated effects. NOX2 controlled redox-sensitive cysteine cathepsins B, S, and L are redundant for the processing and presentation of MOG antigens and EAE, despite older inhibitor studies suggesting otherwise, but mice simultaneously deficient in multiple cathepsins (via genetic or pharmacological inhibition) have been shown to be protected from EAE, and have a MHC-II processing deficiency. Collectively the bulk of published work definitively indicates that these enzymes have intricate roles in antigen processing and autoimmunity, with particular relevance to MHC-II-restricted adaptive immune responses during MOG- induced EAE.

Keywords: EAE, cathepsin, MS, antigen presentation, NOX2

Introduction

Phagocytosis is essential for maintaining homeostasis and for efficient immune responses. Phagocytosed apoptotic cells and endogenous materials are degraded, leading to the recycling and clearance of these materials from all tissues. Phagocytic proteolytic processing of microbes and endogenous tissue is also critical for the adaptive immune response. Protein-based antigens are degraded into the linear peptides, which are required for the initiation of adaptive immunity. Importantly, a diverse group of phagocytic cells have to consistently process proteins in a reproducible manner, despite having differential protease activity. Variation in this phagosomal processing can lead to mismatched peptide epitopes being produced, and allow for the escape of self-reactive immune cells. These auto-reactive cells can damage endogenous tissue and, if left unchecked, be lead to autoimmune pathogenesis.

Autoimmune diseases, such as multiple sclerosis (MS), are thought to be the result of a dysfunction in the adaptive immune response, and have been implicated with phagosomal proteases and the processes that restrict protease activity [1-12]. Interestingly, some phagosomal processing is regulated by intraphagosomal redox conditions, but the extent to which these processes modulate autoimmunity is unknown. The predominant objective of this review is to highlight the role of redox-sensitive cysteine cathepsins in central nervous system (CNS) autoimmune susceptibility and pathogenesis. Understanding the roles of redox-sensitive cysteine cathepsins, and the factors controlling intraphagosomal redox states (primarily NADPH oxidase 2, NOX2), may provide important evidence for targeted autoimmune therapies that do not

modulate autoimmunity through the generalized inhibition of the immune system [13]. To understand phagosomal proteases, such as cysteine and aspartic cathepsins, phagosomal maturation must be considered.

Phagosomal Maturation

Phagosomal maturation is a complex process by which newly formed phagosomes go through a dynamic series of sequential fusion-fission events with early endosomes, late endosomes and lysosomes to form late stage phagolysosomes [14, 15]. These fission-fusion events result in the exchange of contents between early-intermediate phagosomes and endosomes, and allow for the recycling or degradation of lumenal and membrane bound components of the phagosome. The abundance/activity of phagosomal proteases (especially cathepsins) increases as the pH of the phagosome steadily decreases to a pH <5, because many of these phagosomal proteases have acidic pH optima. This highly degradative environment is characteristic of the late phagosome's maturation into a phagolysosome, and the primary compartment for microbial killing and macromolecule degradation [13, 16].

MHC-II antigen processing and presentation

Before processed exogenous antigens can be presented to CD4+ T cells, MHC-II complexes must be proteolytically processed in the endosomes/lysosomes of APCs. Dendritic cells (DCs) and classically activated macrophages have elevated cathepsin and MHC-II expression [17]. MHC-II molecules require phagolysosomal cysteine cathepsins to process Class II-associated invariant chain (Ii) peptide (CLIP) in their peptide groove before the dissociation of CLIP from the MHC-II (initiated by human leukocyte antigen DM [HLA-DM; H-2M in mice]) and the subsequent binding of exogenous antigens to a free MHC-II can occur [17, 18]. MHC-II molecules, and their bound antigenic peptides, are then transported to the cell surface for presentation to CD4+ T cells. An immune response is perpetuated by these cells if the CD4+ T cell's TCR recognises its cognate antigen on MHC-II, and the APC has been primed by PAMPs to express co-stimulatory molecules (B7) which provide a second signal to the T cell (CD28) to induce activation [13, 19].

While MHC-II is being transported and Ii is being processed, exogenous antigens in the recipient phagosome are also being produced by the activity of a plethora of phagosomal proteases. Full antigens must be digested by intra-lumenal proteases, because only linear peptide epitopes of 11-24 amino acids can replace CLIP on MHC-II molecules [20]. Furthermore, disulfide bonds must be reduced to allow proteases access to cleavage sites within these antigenic proteins. Phagosomal proteases and reductases are not only responsible for degrading microbes and macromolecules, but also for the generation of these exogenous antigens for presentation on MHC-II [13]. Of particular interest are the redox-sensitive cysteine cathepsins, because NOX2 can post-transcriptionally modify their activity by altering the redox state in the phagosome [16].

NOX2 and the oxidative burst

NOX enzymes are transmembrane proteins that reduce oxygen to superoxide by transferring electrons across biological membranes [21]. The resulting superoxide causes a build-up of ROS on one side of the membrane. The prototype NOX enzyme is the NOX2 complex, which is the primary contributor of superoxide and the resulting ROS in the phagosome, and the facilitator of the intra-phagosomal oxidative burst in all professional phagocytes [15, 21, 22]. The NOX2 complex is first assembled on the early phagosomal membrane of professional phagocytes and is composed of 6 proteins: 2 membrane, 3 cytosolic, and 1 GTP-binding [22]. During NOX2 recruitment, the $p47^{phox}$ subunit (Ncf gene) brings the recently associated catalytic membrane bound NADPH (Cybb gene, NOX2 on the phagosomal membrane) subunit and $p67^{phox}$ activating subunit together at the

phagosomal membrane (Figure 1) [23]. p67 ^{phox}

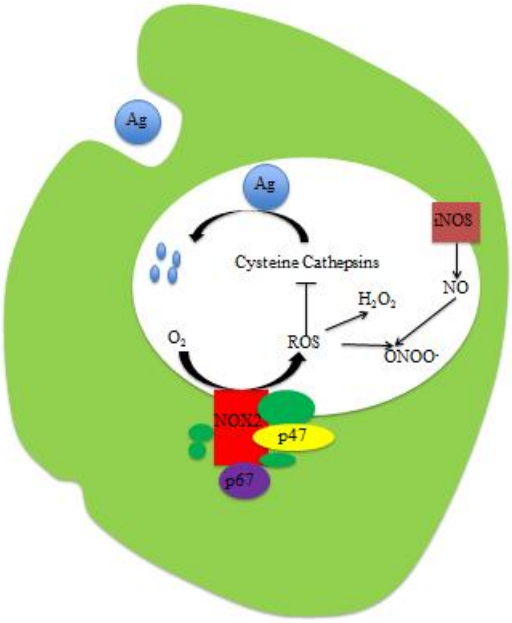

Figure 1. Phagosomal NOX2 activity. ROS produced as a result of NOX2 activity inactivates phagosomal cysteine cathepsins causing decreased levels of phagosomal proteolysis.

then regulates the transfer of electrons from NADPH down the transmembrane redox chain [24]. The process causes cytosolic NADPH to be oxidized by NOX2, and the energy that is released is used to convert molecular oxygen to superoxide in the phagosome [25]. The resulting superoxide and its products, particularly H_2O_2 and hydroxyl radicals, are considered to be the main microbicidal effectors in the phagosome [26]. These products also cause cellular damage to bystander tissue, and if unchecked can result in disease pathology. Interestingly, in addition to its microbicidal activity, it has been shown that NOX2 has roles in the regulation of proteolysis and antigen processing [13].

Redox (NOX2) - controlled MHC-II antigen processing

It has been very well established that the production of ROS are crucial to successful innate immunity, and microbial killing is the central role for ROS in phagocytes. It has recently been shown that an important peripheral role for NOX2-generated intraphagosomal superoxide, is the control of phagosomal proteolysis though the attenuation of disulfide reduction and the oxidative inactivation of cysteine cathepsins [25, 27].

Disulfide bonds, contributing to the secondary structure of antigens, must be intraphagosomally reduced before many proteases can efficiently access their cleavage sites. Phagosomal disulfide reduction is generally mediated by gamma-interferon-inducible lysosomal thiol reductase (GILT), but may involve other systems [28]. Nucleophilic attack of a target disulfide requires the thiol group, of the glutathione/thioredoxin enzymes, to be in the thiolate (S-) state. Reduced NADPH is believed to provide the electrons necessary for this reduced thiolate state, and a reducing environment in the phagosomal lumen is required for this to happen efficiently [28]. GILT is the only fully described phagolysosomal disulfide reductase, and also requires a reducing environment to be activated. This enzyme is thought to require processing by lysosomal proteases to become activated, and may also have roles in re-activating oxidized cysteine cathepsins [25, 29]. Modifications to the levels of these reductases in the phagosome can compromise the efficiency with which antigens are processed, and thus the ability of APCs to present these antigens to CD4+ T cells [13]. It has been shown that GILT deficient mice have less efficient MHC-II antigen presentation of myelin oligodendrocyte glycoprotein (MOG) and hen egg lysozyme (HEL) because they have inefficient processing of these antigens [30, 31]. Sustained phagosomal ROS, generated by NOX2, can compromise proteolysis by inhibiting of disulfide reduction and preventing denaturation of antigens in the phagosome. This inhibition limits the cleavage sites that are available to active proteases, in addition to the

direct effects ROS have on lysosomal proteases, particularly cysteine cathepsins rather than other active phagosomal proteases [25].

Cysteine Cathepsins

Mammalian cells have two major protein degradation systems: the ubiquitin-proteasome, and the endosomal-lysosomal hydrolase system [32]. With regards to MHC-II antigen processing and presentation, the endosomal-lysosomal processing system is by far the most important process for degrading exogenous proteins. As mentioned above, numerous hydrolases are responsible for degrading macromolecules but the production of immunogenic antigens is particularly dependent upon serine, aspartic, and cysteine proteases. Of these proteases, cysteine proteases (particularly cysteine cathepsins) have been strongly implicated in the production of viable MHC-II molecules and immunodominant peptides for presentation to CD4+ T cells.

Cysteine cathepsins have a huge diversity of physiological roles and, in general, are ubiquitously expressed at a tissue level. One notable exception to this ubiquitous expression is cathepsin S, which is thought to be exclusively expressed in APCs [33, 34]. Specifically, APCs tend to express high levels of active cathepsin B, S, and L [4].

Generally, cysteine cathepsins are believed to have conserved, papain-like, structural domains consisting of a monomer that is composed of heavy and light chains connected by disulfides, and a V-Shaped active site with the left cleft containing three α-helices and a β-barrel on the right [35]. The active site has a vital catalytic cysteine residue (Cys25) which must be in its reduced- thiol state to be active (requires a reducing environment), and generally requires an acidic pH to accommodate the Cys25's low pK_a. The low pK_a of Cys25 facilitates interactions with His159, which is the other catalytic residue on all cathepsins. Cathepsin substrates undergo nucleophilic attack of their carbonyl carbon by the reduced-thiol of the active site [13]. Cysteine cathepsins can have very specific optimal cleavage sites, but exhibit flexibility in the substrates that they cleave [35]. Because of their diverse range of substrates and broad expression, cathepsins are tightly regulated [13].

Cysteine cathepsins are regulated on multiple levels including: transcriptional, endosomal trafficking, cystatin regulatory proteins, pro-domain removal, and compartmental pH [36]. These enzymes are synthesised as inactive zymogens (propeptide-enzyme) in the endoplasmic reticulum, and must be in the presence of an acidic pH so that the tertiary structure loosens, exposing the propeptide to proteases (such as other cathepsins or elastase) for cleavage. These active enzymes are further regulated by endogenous inhibitors, the most notable being cystatins and thyropins. Cystatins are non-selective reversible competitive inhibitors that block the catalytic center indirectly; whereas thyropins selectively block the active site from the enzyme [35]. More recently, it has been shown that NOX2-derived ROS oxidatively inactivate cysteine cathepsins (particularly cathepsins B, S, and L), due to the requirement of a reducing environment for optimal catalytic activity of their Cys25 and direct oxidation of the catalytic cysteine (Figure 1) [25, 35, 37].

Only two of the 11 described human cysteine cathepsins have been shown to have non-redundant roles in MHC-II antigen processing and presentation. Cathepsins S and L have been shown to be involved in Ii processing, the processing of some MHC-II restricted antigens, and thymic selection of CD4+ T cells [38, 39]. It is well established that cathepsin L is highly expressed and active in cortical thymic epithelial cells (TEC). TEC are responsible for positive selection of thymocytes by low avidity binding to self MHC-bound peptides presented by the TEC. Cathepsin L is responsible for Ii cleavage in TEC and, if removed, results in failure of CD4+ T cells to undergo positive

selection and an organism wide CD4+ T cell deficiency [4]. Other cathepsin L deficient APCs have functional Ii and MHC-II molecules, and show normal responses to some antigens. Cathepsin S, on the other hand, has been shown to be important for the presentation of both exogenous and endogenous antigens in both DCs and macrophages, although the association is much stronger in DCs [40]. Specifically, cathepsin S deficient DCs showed some evidence of impaired Ii degradation and haplotype specific β-microglobulin, rab5, HEL, and OVA antigen presentation efficiencies [17, 39-41]. Macrophages that are deficient in cathepsin S, have been shown to have some Ii and antigen processing deficiencies, but these defects are not thought to have a strong effect on the presentation abilities of these cells [17, 42]. The roles of individual cathepsins on MHC-II restricted antigen processing and presentation are debated; but it is likely that their roles are contextually dependent on the cell type and antigen in question [13].

The ability of NOX2-derived superoxide to selectively compromise proteolytic activity by inactivating cathepsins and inhibiting disulfide reduction, could result in fundamental changes to the processing of any given antigen [13]. The functional implications of these specific changes to antigen processing, in combination with the idea that invariant chain processing could be altered, could be that the efficiency of MHC-II presentation and the epitope repertoire in the phagosome is under significant influence by NOX2 [25, 39]. These potential redox-generated perturbations to MHC-II presentation, and peptide processing, could have drastic effects on the immune response and may have unknown effects in individual tissues. Tissues such as the CNS are believed to be particularly sensitive to redox and MHC-II processing changes because of their unique structure, complex function, and relative immune isolation [13].

CNS Autoimmunity

CNS autoimmunity is generally associated with a broad group of diseases known as idiopathic inflammatory demyelinating diseases (IIDDs) [43]. These diseases vary extensively in their immunopathology, clinical course, and location; but most of them are believed to be the result of some autoimmune insult on myelinated tissue. The most common, and one of the most debilitating, IIDD is MS [44, 45]. This disease affects millions world-wide, and is of particularly high prevalence in young adult North American and European populations. It took the better part of 80 years before the discoveries of CNS inflammation, demyelination, familial risk, and CNS autoantibodies were synthesized into a single hypothesis regarding MS pathogenesis: the clinical MS symptoms are result of an autoimmune response to CNS tissue, most likely myelin and the cells that produce it [13]. Most of what is understood about the immunology behind MS has been elucidated though the use of animal models. Experimental autoimmune encephalomyelitis (EAE) is the oldest, most studied, and most clinically powerful animal model of MS to date [46-48]. ROS, redox sensitive proteases, and phagocytosis are all thought to be strongly implicated with MS and its models.

Experimental Autoimmune Encephalomyelitis

Human diseases are inherently difficult to study for variety of logistical, economic, ethical, and temporal reasons [13]. Disease of the CNS, like MS, have the additional investigative limitation of being in a tissue that is difficult to image and dangerous biopsy when the patient is living. Animal models have been developed to help to illuminate as much as possible about the mechanism behind the damage and inflammation that occurs during MS, and have been indispensable in the pre-clinical trial stage of drug development [49]. These models are responsible for generating nearly all the current hypotheses regarding the initiation and maintenance of inflammation in the CNS during CNS autoimmune conditions [13].

There are numerous toxin-, mechanical-, viral-, and peptide-induced models for MS in mammals. EAE, to date, has been the most commonly used and clinically relevant MS model [19, 50]. It can be induced in many mammals, from rodents to primates, but inbred mouse strains are most widely used model. Active EAE is induced by immunization of an animal with myelin antigens (such as MOG) and adjuvant, which stimulates an immune response that is targeted against the CNS [19, 50]. The ensuing CNS inflammation, lesion formation, cellular infiltration, cytokine environment, demyelination, and clinical disability of these models resembles MS symptoms/pathology [51].

Canonically, EAE in initiated by peripheral APCs that encounter PAMPs present in complete Freund's adjuvant (CFA), and myelin antigens (such as the proto-CNS antigen MOG) at the injection sites [13]. These APCs upregulate costimulatory molecules (induced by exposure to PAMPs) and MHC-II on their surface, and migrate to the lymph nodes where they present MOG antigens (MOG^{35-55}, in the case of C57Bl/6 mice on $I\text{-}A^{b}$) to naive CD4+ T cells [45, 47]. Autoreactive myelin-specific CD4+ T cells become activated, during the initial antigen challenge, and migrate to the CNS during the asymptomatic initiation period of EAE. They then infiltrate the CNS via the blood brain barrier (BBB) and are reactivated by resident APCs of the CNS, or CNS associated tissue [13]. CD4+T cells enter the meninges, where they are exposed to meningeal macrophages and DCs, and infiltrate through tight junctions into the CNS via the perivascular and subarachnoid spaces [50]. Resident macrophages and microglia of these areas present endogenous myelin components to these autoreactive cells and are responsible for their reactivation. This reactivation causes pro-inflammatory cytokine/chemokine release, local APC activation, further immune cell infiltration (both autoreactive lymphocytes and inflammatory macrophages), and extensive CNS demyelination [13]. This CNS inflammation and demyelination are clinically exhibited by the formation of extensive spinal lesions and ascending paralysis in murine models [19].

The effects of NOX2 and redox sensitive cysteine cathepsins on EAE

ROS have been known to be a source of damage during EAE. Oxidative stress in the CNS can induce cell death of oligodendrocytes, which are particularly sensitive cells, and destabilize myelin sheaths of neurons. The role of phagosomal ROS on CNS damage has been shown to affect the susceptibility of inbred mice to EAE [52-55]. Spontaneous mutations to the Ncf gene, which codes for the $p47^{phox}$ subunit and effectively eliminates a detectable oxidative burst, afford mice full or partial protection from MHC haplotypes- specific peptide- induced EAE [52, 53]. MOG^{79-96} and MOG^{35-55} induce active EAE in $H2^{q}$, and $H2^{b}$ genetic backgrounds, respectively. $Ncf^{-/-}$ mutant $H2^{b}$ mice were found to be completely protected from MOG^{35-55-} stimulated EAE as a result of excessive activity of nitric oxide synthase**,** even though this peptide could stimulate proliferation of splenocytes *in vitro* indicating that there were probably additional mechanisms at work [53]. Similarly, $Ncf^{-/-}$ mutant $H2^{q}$ mice showed a reduced clinical score, but similar initiation and duration of disease, when immunized with MOG^{79-96} compared to wild type (WT) mice [52]. Intriguingly, $Ncf^{-/-}$ $H2^{q}$ mice exhibited an exacerbated clinical score, although similar initiation and duration of EAE, when they were immunized with native recombinant MOG protein (rMOG); but these findings were not explained [52].

More recently, it has been shown that NOX2 significantly alters the proteolytic environment of the phagosome in APCs by decreasing the activity of cysteine cathepsins, and inhibiting disulfide reduction [25, 38]. The cysteine cathepsins, that NOX2 affects, have been shown to be involved with inflammation-induced neurodegeneration and neuronal death in many

neurological disorders [12]; but most importantly, their activity is believed to have a controlling effect on MHC-II antigen processing and the induction of EAE. Additionally, specific inhibitors of redox-sensitive cysteine cathepsins have been used to attenuate EAE and other autoimmune models, by modifying Ii processing [39, 56, 57].

NOX2 activity modifies the patterns of epitopes that are produced when a given antigen is processed by perturbing the reductive environment and causing the inhibition of cysteine cathepsins [54]. Importantly, MOG antigen processing (to the immunodominant MOG^{35-55} peptide) has been shown to be attenuated when NOX2 activity is abolished in macrophages, which can lead to a deficiency in MOG antigen presentation *in vitro* and *in vivo,* and clinical protection from EAE presumably resulting from inefficient processing of endogenous myelin [54]. This inefficient processing due to excessive activity of cathepsins L and S, which can efficiently cleave and destroy the MOG^{35-55} epitope *in vitro,* has been shown to be protective in the context of MOG induce EAE [54]. These findings indicate that NOX2 activity inhibits multiple cysteine cathepsins, and may prevent the excessive proteolytic destruction of MOG^{35-55}; further implying that NOX2 may have a central role in controlling the levels of activated cysteine cathepsins in the phagosome to prevent over-digestion of key MHC-II- restricted epitopes (Figure 2). It has been suggested that because DCs have lower activity of many cathepsins [36], it is possible that NOX2 activity in macrophages helps to recapitulate the antigenic repertories produced in other APCs by limiting proteolysis to similar levels observed in DCs [13, 54].

As previously mentioned, cysteine cathepsins B, S, and L have been strongly associated with autoimmune, and MS, pathology and treatment; but the mechanism and extent of their involvement in autoimmunity is complicated and contested [1, 2, 5, 11, 57-64]. Cathepsins S and L, in particular, are believed to be non-redundant for efficient MHC-II-restricted antigen processing and presentation of many antigens but surprisingly, all three cathepsins, are redundant in MOG antigen processing and presentation [36].

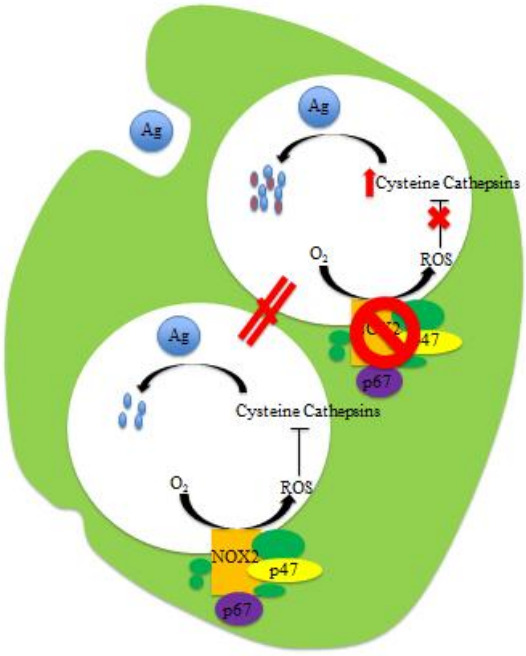

Figure 2. NOX2 prevents excessive antigen processing by inactivating multiple redox sensitive cysteine cathepsins. The amount and pattern of antigen processing is modified by perturbations in NOX2 activity.

Cathepsin S- and B- deficient mice have been shown to be fully susceptible to EAE, despite studies showing that inhibitors of cathepsin S could attenuate EAE (Cathepsin L- deficient mice have a well characterized thymic CD4+ T cell deficiency and are not susceptible to T cell driven autoimmune models [4]). When inhibitor studies with LHVS were repeated by a different group, it was found that one such cathepsin inhibitor was non-specific *in vivo*, and inhibited multiple cysteine cathepsins *in vitro* [55]. Simultaneous-genetic removal of cathepsins S and B have been shown to result in

reduced MHC-II expression in macrophages, and these animals are not susceptible to EAE. These findings indicate that there are functional redundancies between cysteine cathepsins during MHC-II/MOG processing and EAE; and that, in this context, multiple cathepsins must be modified to alter the adaptive immune response and circumvent compensation for the loss of a single cathepsin (Figure 3).

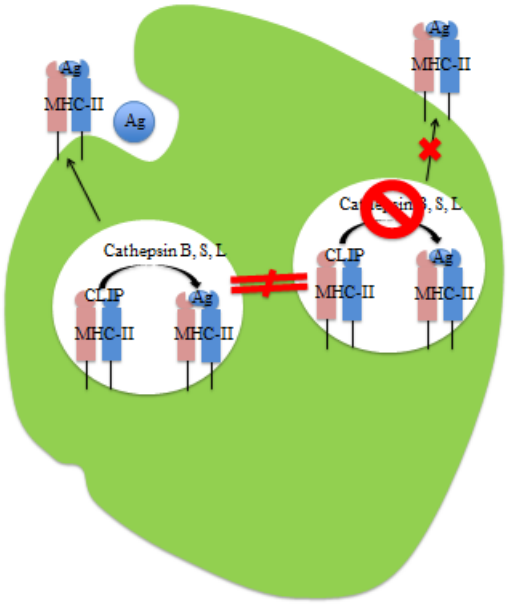

Figure 3. Cathepsin deficiencies. Inhibition of multiple phagosomal redox sensitive cysteine cathepsins is necessary for modifying MHC-II restricted antigen processing and presentation in many APCs.

This mechanism of protection is in concordance other work showing that that the effects of NOX2 are likely the result of overactivity of these same cathepsins [13]. Together, these studies reveal that there may be fine balance in redox-sensitive cysteine cathepsin activity required to consistently produce the same abundance of a given antigen. Though these cathepsins show redundancy, too much cathepsin activity results in the destruction of key epitopes, while excessive inhibition of multiple cathepsins can impede mature MHC-II generation in macrophages (Figures 2 and 3) [13]. It is interesting that antigen processing and presentation, though robust to changes in the presence of a single protease, can be so diversely modified by simple changes in the redox conditions of the phagosome. Future studies should address the interplay between NOX2 and the cathespins by crossing NOX2-deficient mice with cathepsin- deficient mice (in different combinations and individually), in an attempt to prevent the destruction of MOG^{35-55} and rescue efficient antigen presentation of MOG and EAE, this could help to elucidate which cathepsin may be physically cleaving this peptide *in vivo*. Elaborating on the mechanism that underlies EAE attenuation in NOX2-deficient mice also requires exploration of the effects of NOX2 in models that do not rely solely on an autoimmune initiation of CNS damage [13].

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