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Immunoproteasomes at the interface of innate and adaptive immune responses: two faces of one enzyme

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The immunoproteasome is a specific proteasome isoform induced by interferons. Its proteolytic function has been almost exclusively connected with the adaptive immune response and improved MHC class I antigen presentation. However, IFN-signaling also exposes cells to oxidative stress with concomitant production of nascent-oxidant damaged poly-ubiquitylated proteins. Here we discuss how immunoproteasomes protect cells against accumulation of toxic protein-aggregates and how i-proteasomes dysfunction associates with different diseases. We propose that the immunoproteasome has a central function at the interface between the innate and adaptive immune response and that its predominant protective innate function determines its favorable role in the adaptive immune response.

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General introduction

Degradation of misfolded proteins, oxidant-damaged proteins, and short-lived regulatory proteins requires the activity of the ubiquitin–proteasome-system (UPS) [1]. Similarly, antigenic peptides bound by major histocompatibility complex (MHC) class I molecules to be recognized by cytotoxic T-lymphocytes at the cell surface are generated in a proteasome dependent manner. The UPS is the major ATP-dependent proteolytic system in eukaryotes [2,3], in which degradation of proteins demands in most cases the conjugation of lysine 48 (K48) linked poly-ubiquitin (ub)-chains via a thioester-cascade of E1, E2 and E3 enzymes on selected protein substrates [2,3].

Poly-ubiquitylated proteins are degraded by the 26S proteasome, which consists of a catalytic core complex,

that is, the multi-subunit 20S proteasome associated with two 19S regulator complexes governing the access to its catalytic cavity. The barrel shaped standard 20S proteasome (s-proteasome) is formed by four stacked rings of seven subunits each with the arrangement of $\alpha 1-\alpha 7(\beta 1-\beta 7)_2\alpha 1-\alpha 7$. With the $\beta 1$, $\beta 2$ and $\beta 5$ subunits the two inner β -rings harbor the six catalytically active sites [4]. Although the proteasome prefers cleavage behind hydrophobic or basic amino acid residues, almost every residue within a protein can serve as a cleavage site. These almost unrestricted catalytic properties guarantee the proteasome-dependent generation of the vast majority and large variety of MHC class I bound peptides, which are mostly derived from poly-ubiquitylated defective ribosomal products, that is, DRiPs [5] (Box 1).

Importantly, mammalian proteasomes comprise a heterogeneous population of enzyme complexes, with different subunit compositions, regulator affinities and hence also diverse functional properties [6,7]. Predominantly in response to cytokine induction, new catalytically active immuno β -subunits, that is, $\beta 1i$ /LMP2, $\beta 2i$ /MECL1 and $\beta 5i$ /LMP7, are incorporated into an alternative isoform, that is, immuno (i)-proteasomes[8], thereby replacing the standard- β subunits $\beta 1$, $\beta 2$, and $\beta 5$ within the 20S proteasome. Consequently, mammalian cells can harbor 20S s-proteasomes and i-proteasomes exhibiting different ratios of active site standard and immunosubunits. Because $\beta 1i$ /LMP2 and $\beta 5i$ /LMP7 are encoded within the MHC class II region [1] the terms immunosubunits (i-subunits) and i-proteasome were introduced for designation of this specific proteasome isoform.

S-proteasomes are constitutively expressed in almost all cells of an organism, whereas i-proteasomes are constitutively expressed only in immune relevant cells or tissues and are induced by cytokines in almost all other cell types. Importantly, like s-proteasomes, i-proteasomes generate peptide ligands for MHC class I presentation as part of the adaptive immune response, albeit in many cases at a considerably higher rate [9,10].

This latter observation previously connected i-proteasome function almost exclusively with a central role in improved antigen presentation; however, only surprisingly modest effects on the T-cell repertoire were observed [9,10]. Notably, in tissues affected by pathogens, high levels of pro-inflammatory cytokines also induce i-proteasome formation in non-infected cells without any requirement for improved antigen processing. It

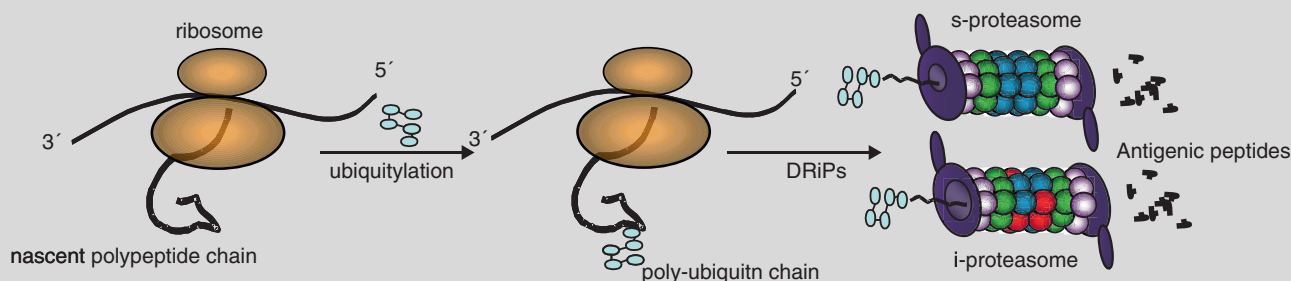
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Box 1 DRiPs and antigen presentation

For MHC class I antigen processing and presentation the prevalent view is that cellular proteins that are targeted for degradation by the UPS as part of normal cellular protein turnover are the main source of antigenic peptides. However, two major questions arose. How does the UPS gain access to proteins that are compartmentalized or metabolically stable and how is a rapid antiviral CD8⁺ T cell activation achieved from viral proteins whose half-life is as least as long as that of typical cellular proteins?

The DRiP (defective ribosomal products) hypothesis offers a possible solution to these questions, explaining how antigen processing and presentation is guaranteed for the majority of MHC class I ligands. The DRiP model is based on several observations. First of all a considerable percentage of newly synthesized nascent proteins are poly-ubiquitylated and rapidly degraded ($t_{1/2} \approx 1\text{--}5\text{ min}$) by proteasomes suggesting that they represent a pool of proteins that never

reach a functional state owing to translational errors, misfolding or other defects [32^{••}]. Secondly, transport of antigenic peptides into the ER via TAPs (transporter associated with antigen processing) is tightly coupled to continuous protein synthesis, implying that MHC class I ligands are predominantly derived from newly synthesized proteins [33^{••}]. Third, proteasomes are found in association with polyribosomes providing a means for coordinated translational processing of proteins. Nevertheless, the physical nature of DRiPs is still not well understood. Incidents, which may result in dysfunctional proteins include unfolded and misfolded proteins of the proper length, proteins with sequence errors or errors in posttranslational modification, prematurely terminated proteins or false open reading frames. Oxidative stress also can influence translational fidelity by misacylation of methionyl-tRNA [30] or by directly modifying mRNA [31], representing additional reactions that potentially contribute to an increase in the number of DRiPs.



was therefore suggested that i-proteasomes must have important functions apart from antigen-processing [11,12[•],13].

Here we review recent data connecting i-proteasome functions with the protection of cells and tissues exposed to cytokine-induced and inflammation-induced oxidative stress and discuss new concepts that position i-proteasome function at the interface of the innate and adaptive immune response.

IFNs and the UPS

Proinflammatory cytokines like type I or type II interferons (IFNs) are important triggers for the immune response. As part of the innate response many cell types produce type I IFNs, whereas activated T-lymphocytes and natural killer secrete both type I and type II IFNs. Through the cytokine dependent induction of i-proteasome formation the immune relevant function of proteasomes is significantly improved [10]. Owing to the concomitant upregulation of almost all essential components of the MHC class I antigen-processing machinery, the presentation of foreign and self-antigenic peptides is also strongly enhanced. In addition, the UPS is consecutively regulated at different levels by IFN signaling exerting profound effects also on the immune adaptation of the UPS. Thus, IFN-signaling not only results in the accelerated *de novo* formation of i-proteasomes [14], but also specifically induces many components of the ub-conjugation system including E1, E2 and E3 enzymes [9^{••},15].

Lymphoid tissues and especially immature dendritic cells (DCs) constitutively express i-proteasomes at high levels [10] and their synthesis is only slightly upregulated during DC maturation [15,16]. High i-proteasome expression is also found in macrophages, T cells and B cells [10]. Compared to lymphoid tissues, i-proteasome expression in non-lymphoid peripheral tissues is, however, rather low and requires induction by IFNs, TNF α , or LPS [10[•]]. Nevertheless, many cells and tissues express low amounts of mainly $\beta 5i$ /LMP7 also in the absence of cytokine stimulation [9^{••},14,17[•],18].

Innate immunity and oxidative stress

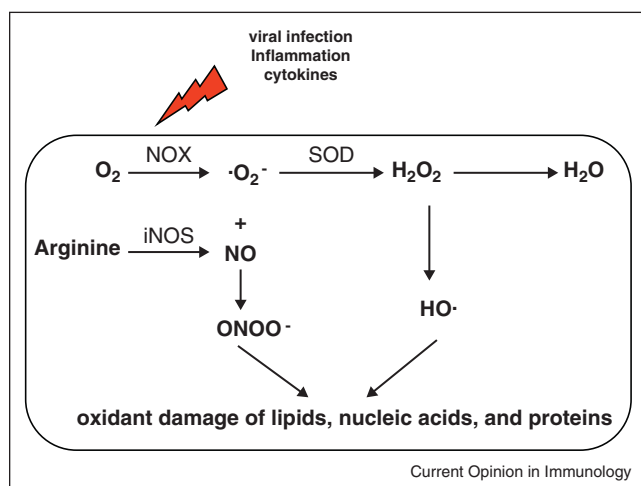
Immune cells produce large amounts of microbiocidal reactive oxygen (ROS) and reactive nitrogen species (RNS) during the innate immune response. The production of free radicals in phagocytes by NADPH-oxidases (Nox) and by the inducible nitric oxide synthetase (iNOS) during phagocytosis is well established. Similar to activated immune cells, non-immune cells that are exposed to cytokines, whether they are infected or not, also will produce ROS and RNS [19[•],20^{••}].

Nox2 is the prototypical host defense oxidase of phagocytes, whereas Nox1 and Nox4 are also active in other cell types and tissues. Several lines of evidence suggest that these oxidases located on apical cell surfaces are involved in host defense. Fibroblasts as well as epithelial cells induce Nox1 by IFN signaling, and almost all cells express Nox4 [20^{••},21].

Being an essential arm of the innate immune response, ROS and RNS play an important protective role against invaders in the first line of defense. It therefore appears that endogenous production of radicals is innate to non-immune cells during inflammation [20^{••},22,23]. However, ROS and RNS do not selectively attack only molecules of invading pathogens and consequently their induction is also hazardous for any cytokine-exposed cell by oxidant-damage of vital nucleic acids, lipids, or proteins (Figure 1).

Production of radicals represents a normal physiological outcome of metabolic reactions in all heterotrophic organisms where ROS and RNS also participate in signaling pathways. However, when present in high amounts their high chemical reactivity can cause oxidative stress. Cells affected by radicals induce a battery of cytoprotective systems, including antioxidative enzymes and enzymes responsible for the production of antioxidants, reducing equivalents and cofactors [24]. It is therefore conceivable that cells that are exposed to such 'pathophysiological' oxidative stress conditions with profound consequences for the functional integrity of proteins require effective proteolytic degradation machinery such as the UPS or autophagy to eliminate irreversibly oxidant-damaged and potentially toxic proteins [9^{••},25[•],26,27] to preserve cell viability and basic cellular functions.

Figure 1



Inflammation and production of ROS and RNS. Danger signals as an irritant or infection provoke inflammation, which is among other events associated with the massive release of inflammatory cytokines and the production of free radicals. Reactive oxygen and nitrogen species are produced by a variety of sources. ROS like superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) are mainly produced by NADPH-oxidases (NOx) and superoxide dismutases (SOD). The inducible nitric oxide synthase (iNOS) is the major producer of RNS like nitric oxide (NO) or peroxynitrite ($ONOO^{\bullet}$). Perturbation of the cellular redox balance leads to direct cellular injury by oxidant damage of proteins at the side chains of certain amino acids, lipids and nucleic acids.

DRiPs and the role of oxidative stress

The MHC class I-dependent CD8⁺ T cell triggered adaptive immune response strongly depends on the unrestricted availability of antigenic peptides provided by proteasome dependent degradation of proteins [28[•]]. Owing to errors in translation, folding, or oxidant damage, a relatively large percentage of nascent proteins never seem to reach a functional state [29–31]. These defective ribosomal products (DRiPs) [32^{••},33^{••}] are polyubiquitylated either during or shortly after translation and rapidly degraded by 26S proteasomes, giving rise to a peptide pool from which MHC class I ligands are primarily selected (Box 1). Despite its attractiveness, the DRiP hypothesis did not explain how the amount of generated peptides can be adapted to the demands of an upregulated antigen presentation machinery upon cytokine induction. In addition, the definition of the type of defect encountered by the proteins remained largely unanswered.

Both IFNs and viral infections trigger the production of radicals, affect translation, and induce i-proteasome formation. Because newly synthesized or nascent proteins are particularly sensitive to oxidant-damage, IFNs unavoidably will enhance the formation of nascent, oxidant-damaged, unfolded polyubiquitylated proteins. This production of DRiPs is supported by upregulation of UBE2L6 as the major E2 enzyme mediating ub-conjugation in response to IFNs and by other components of the ubiquitylation machinery [9^{••},15]. Therefore IFN-induction combines enhanced translational activity with an increase in DRiPs, the rapid increase in the pool of polyubiquitylated substrates that require processing by 26S proteasomes and secure the demands of a strongly upregulated antigen presentation pathway.

Immunoproteasomes and oxidative stress

Given the above scenario, it does not need much imagination to assume that the IFN-induced formation of i-proteasomes represents the physiological adaptation to the cytokine-induced oxidative stress situation and the concomitant enhanced formation of poly-Ub-conjugates in form of DRiPs. Indeed, the functional importance of i-proteasomes for coping with cytokine induced oxidative stress and DRiP formation becomes most evident in LMP7/β5i-deficient mice being devoid of i-proteasomes.

Upon proinflammatory stimuli, i-proteasome deficient cells and tissues are not only more sensitive to apoptosis but also reveal a strongly delayed activation of NF-κB after TNF-α stimulation both *in vitro* and *in vivo* [9^{••},34[•],35], highlighting a previously controversial discussion of the role of i-proteasomes in the timely processing of regulatory proteins.

IFN-stimulated cells in the absence of i-proteasomes strongly accumulate nascent-oxidant damaged poly-ubiquitylated proteins forming aggresome-like induced

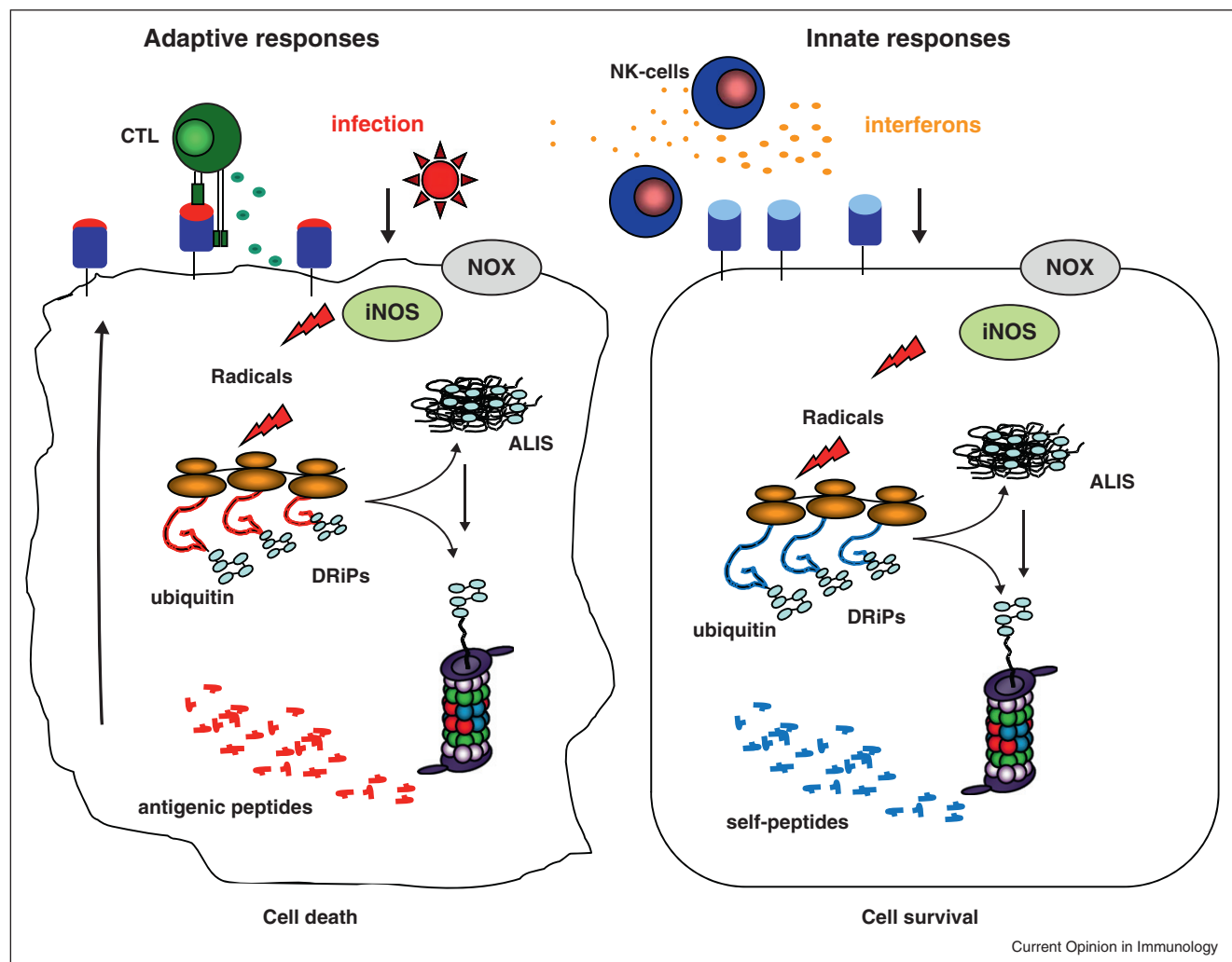
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structure (ALIS). ALIS to which DRiPs are transiently deposited in response to different forms of stress are large and dynamic ub-rich cellular inclusions [25[•]]. Consequently, we also observed an accumulation of ub-conjugates and oxidant-damaged proteins in inflammation-challenged liver and brain tissues of i-proteasome-deficient mice [9^{••}]. Moreover, i-proteasome deficiency contributed to disease progression in experimental autoimmune encephalomyelitis and upon Coxsackie virus B3 infection [9,36]. Aggregation of misfolded proteins forming ub-rich inclusions is also a common

feature of many neurodegenerative diseases. Because neuroinflammation is a common characteristic of many of these disorders [37], one might hypothesize that i-proteasomes also play important roles in the brain and may contribute to or prevent disease progression in general. Indeed, there is increasing evidence that i-proteasomes are important to prevent auto-inflammatory diseases [38–41].

Our recent experiments demonstrate that i-proteasomes are essential for the efficient elimination of nascent oxidant damaged proteins and for protection of toxic

Figure 2



Adaptive and innate functions of i-proteasomes. In an infected tissue, diverse immune cells like NK-cells (blue) are recruited that produce cytokines like IFNs (yellow). Infection and IFN-signaling trigger the production of radicals by NOX or iNOS in infected as well as in IFN-challenged cells. Concomitantly, accelerated i-proteasome formation is induced. ROS and RNS damage newly translated polypeptides, which mostly derive from pathogenic sources in infected cells (red) and in cytokine-challenged cells exclusively from endogenous sources. These oxidant-damaged nascent polypeptides are subsequently ubiquitinated and form DRiPs. DRiPs tend to aggregate and prompt the arrangement of DRiPs in ALIS. This process is dynamic, since the enhanced poly-ubiquitin substrate turnover activity of i-proteasomes results in clearance of DRiPs and ALIS as part of the innate function of i-proteasomes independent of infection. In infected cells, antigenic peptides generated by i-proteasomes from pathogenic DRiP sources are loaded onto MHC class I molecules and presented on the cell surface to CTLs as part of the adaptive responses. The interaction of the CTL (green) with the loaded MHC class I molecule will force the infected cell into cell death, whereas i-proteasome action in clearance of DRiPs and ALIS preserves survival of cytokine-challenged non-infected cells.

aggregate formation under inflammatory conditions, a function that apparently cannot be sufficiently fulfilled by 26S s-proteasomes [9^{••}]. But why are i-proteasomes able to cope with a sudden wave of poly-ubiquitylated substrate whereas 26S s-proteasomes are not? The reason is that in comparison to 26S s-proteasomes, 26S i-proteasomes exhibit a significantly increased poly-ub-substrate turnover capacity that is two-fold to three-fold higher than that measured for s-proteasomes. Therefore, i-proteasomes are perfectly suited to handle extreme physiological stress conditions such as those occurring in connection with cytokine-induced oxidative stress as part of the innate response.

To date it is completely unclear why i-proteasomes possess a higher proteolytic activity compared to their s-proteasome counterparts. From digestion experiments with synthetic polypeptide substrates encompassing MHC class I epitopes, it is known that incorporation of i-subunits may confer structural changes within the 20S proteasome complex [42], resulting in improved accessibility of substrates to the active sites. Such conclusions, however, still demand the support of careful structural investigations of i-proteasome complexes.

Induction of i-proteasome formation can also be the result of heat stress [43], arsenic trioxide [44,45], nitric oxide [46], and in neurodegenerative diseases, as a result of aging [47–49]. Almost all of these described stresses or dysfunctions result in enhanced poly-ubiquitylation of proteins and are physiological attendees of inflammatory processes and/or are characterized by tissue damage. Thus, i-proteasome expression appears to be initiated by various physiologically or environmentally induced stress-associated signaling events, supporting the view that the higher proteolytic capacity of i-proteasomes and possibly the timely proteolytic processing of regulatory proteins are required to maintain cell viability in physiologically adverse situations.

Conclusions

Inflammation and IFN signaling represent a potent contribution to innate responses against pathogens. By producing radicals they also confer severe stress into cells and tissues. In infected tissues, signaling cascades of the innate response rapidly induce the massive release of proinflammatory cytokines to recruit lymphocytes, thereby also triggering the production of ROS and RNS in lymphocytes and target cells. These radicals affect infected cells and proteins derived from pathogens, but at the same time also proteins of non-infected cells that are also exposed to cytokines. Thus, in non-infected cytokine exposed cells or tissues, i-proteasomes preserve cell viability and tissue integrity by efficiently degrading nascent oxidant-damaged poly-ubiquitylated proteins and preventing the accumulation of potentially toxic protein aggregates. On the contrary, infected cells must

rapidly signal their infectious state to the adaptive immune system by presenting epitopes on MHC class I molecules at the cell surface, a mechanism that is strongly improved by i-proteasome function.

These two non-excluding functions locate i-proteasomes with their superior proteolytic capacity at the crossroad of the innate and the adaptive immune response (Figure 2). As part of the innate response associated with oxidative stress, i-proteasomes possess a more general protective role by maintaining cellular proteostasis of both immune and non-immune cells [9^{••}]. At the same time, i-proteasomes also allow the timely availability of regulators controlling cytokine production and proliferation [34[•],50,51[•],52,53].

As part of the adaptive immune response i-proteasomes process nascent-oxidant damaged proteins from pathogenic sources thereby increasing the peptide supply for MHC class I antigen presentation. Although our recent findings and those of others have released the blockade from a restricted view on i-proteasome function and have given i-proteasome research a new direction, i-proteasome function(s) is unfortunately still far from being fully understood and a number of questions remain to be answered. One important issue certainly concerns the identification of the E3 ubiquitin ligase(s), conferring specificity to the poly-ubiquitylation of oxidant damaged proteins in response to IFNs. Also, the role of the IFN inducible proteasome activator PA28, binding to 20S i-proteasomes is in this context still unresolved.

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