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# The ongoing saga of the mechanism(s) of MHC class I-restricted cross-presentation

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## Abstract

Cross-presentation is an MHC-I antigen processing pathway that results in the presentation of peptides from exogenous viral, bacterial, parasitic, and tumor antigens and ultimately leads to priming of naïve $CD8^+$ T cells. This process involves several cellular compartments and multiple components. Successful generation of MHC-I-peptide complexes requires that these components act together in a coordinated fashion. We discuss recent findings on the source of MHC-I, the role of the TAP transporter, the importance of intracellular trafficking events, mechanisms of antigens access the cytosol, and how innate immune signals can affect presentation, with an emphasis on how these pathways compare to conventional antigen presentation and how they correlate with existing data.

## Introduction

Processing of antigens and presentation of peptides on Major Histocompatibility Complex Class I (MHC-I) molecules is an important immunological event leading to $CD8^+$ T cell recognition of tumor cells as well as cells infected with viruses, bacteria, and parasites. Most of the peptides bound by MHC-I are derived from cytosolic proteins that have been degraded by proteasomes, translocated by the Transporter associated with Antigen Processing (TAP) into the endoplasmic reticulum (ER) lumen, where they are loaded onto MHC-I with the aid of members of the peptide loading complex (PLC), which include TAP, tapasin, calreticulin, and ERp57 [1]. Although MHC-I processing and presentation of peptides from endogenous proteins is a highly coordinated and complex process requiring numerous accessory molecules, the general process is quite well characterized and understood. This is primarily because most of the players involved have been identified and their functions ascertained. In addition, a logical series of events culminates in peptide loading, beginning with digestion of

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cytosolic proteins by cytosolic proteasomes and ending with peptide loading in the ER and peptide editing facilitated by ER-localized PLC components.

In contrast, cross-presentation describes mechanisms by which antigens derived from extracellular sources are processed and loaded onto MHC-I for presentation to $CD8^+$ T cells. It can occur in many cell types, but the most immunologically relevant is the dendritic cell (DC) and specialized subsets thereof [2,3]. Functionally, cross-presentation is the major mechanism by which naïve $CD8^+$ T cells are primed, and it is essential for priming them to tumor antigens and antigens derived from pathogens that do not directly infect DC. To date, many of the proteins involved in cross-presentation remain unidentified and/or differ in professional APCs versus other cross-presenting cells. Furthermore, cross-presentation pathways can vary depending on the antigen, route of uptake, ligation of innate signaling pathways, and cell type. Overall, there does not appear to be one straightforward model for cross-presentation that incorporates all of the data published to date (Figure 1). In fact, recent advances have added complexity to an already ill-defined process. This review will focus on how findings in the last several years fit with existing data. We apologize in advance to readers whose contributions are not included due to length restrictions.

## Formation of the antigen processing and loading compartment

In order to mount an effective immune response, the antigen cross-presentation pathway should mimic the conventional antigen presentation pathway in terms of the peptides generated and loaded onto MHC-I (Figure 2). This raises a fundamental question: for a given antigen how is the peptide-MHC-I complex generated by the cross-presentation machinery replicated by the conventional antigen presentation pathway? Two aspects of cross-presentation provide explanations for how cross-presentation and conventional antigen presentation pathways can overlap. First, the proteolytic machinery that generates the peptides, and second, the identity and functional properties of the intracellular compartment were the peptide is loaded.

### Generation of Peptides for MHC-I Loading

Proteasomal processing plays a central role in MHC-I presentation. Constitutive proteasomes and immunoproteasomes are multi-catalytic and multi-subunit protein degradation machines that degrade cytosolic and nuclear proteins [4,5]. Antigen degradation by proteasomes generates peptides that are translocated into the ER and loaded onto MHC-I [1]. It has been shown that cross-presentation also depends on proteasomal activity for many antigens. Indeed, the finding that proteasomes are involved in cross-presentation was the first indication that cross-presented antigens can access the cytosol [6]. While most of the data supporting a proteasome requirement for cross-presentation has relied on inhibitors, it has also been shown that an antigenic peptide specifically generated by immunoproteasomes for conventional MHC-I presentation is also cross-presented in an immunoproteasome-dependent manner when delivered exogenously, supporting the role of cytosolic proteasomes in the cross-presentation pathway [7].

Apart from the cytosolic proteasomal machinery, the activity of cytosolic and ER localized peptidases impacts MHC-I presentation. Requirements for these enzymes are largely antigen

dependent: presentation of some peptides requires the activity of certain peptidases, whereas the presentation of different epitopes is inhibited by the same peptidases. These findings have been reviewed elsewhere [8]. In addition to cytosolic peptidases, ER resident aminopeptidases have been identified, ERAP1, or ERAAP in mice, and ERAP2, that trim the amino termini of peptides generated by proteasomes post ER import to generate the appropriate peptide for MHC-I binding [9,10]. Interestingly, ERAAP knockout DCs efficiently cross-present a model soluble antigen (OVA), whereas they fail to cross-present OVA when given as immune complexes [11]. The variable requirement of ERAAP/ERAP1 adds complexity to the relationship of cross-presentation and conventional antigen presentation.

Lysosomal proteases can also play a role in the processing of antigens that are either completely processed/loaded in the endocytic system ([12–15], vacuolar pathway) or gain access to the cytosol for further proteasomal processing ([16] cytosolic pathway). A key lysosomal protease, cathepsin S, can process exogenous antigens to generate antigenic peptides [17]. Cell free experiments have shown that cathepsin S can process a model antigen (OVA) to generate antigenic peptides that can stimulate T-cells. However, it is not clear whether this is restricted to OVA or whether other antigenic peptides can be generated by cathepsin S. In addition to cathepsin S, endosome localized Insulin Regulated Aminopeptidase (IRAP), which is closely related to ERAP1 and ERAP2, has been show to play a role in cross-presentation. Interestingly, IRAP co-localizes with MHC-I in phagosomes [18], and it has been hypothesized that IRAP, in a manner analogous ERAP function in the ER, facilitates the formation of peptide-MHC-I complexes by trimming antigenic peptides generated within the endocytic compartment. Overall, the extent of overlap of peptides generated by lysosomal proteases and those generated by proteasomes is seriously in need of critical evaluation.

## Intersection of Peptide-receptive MHC-I with Peptide and Loading Components

Four key functional attributes are required for effective MHC-I loading: an appropriate environment within the compartment, a mechanism to deliver peptide into the compartment, access of MHC-I molecules to the compartment, and the presence of critical accessory proteins. For conventional antigen presentation, the ER provides the proper environment. It contains newly synthesized MHC-I-β2m dimers, peptides (provided via TAP transport from the cytosol), and PLC components that interact with MHC-I and facilitate loading (reviewed in [1]). In the context of cross-presentation, antigen processed in the cytosol can be translocated into the ER and follow the conventional antigen presentation pathway [6]. However, multiple studies have shown that phagosomes and endosomes can form an MHC-I loading compartment for exogenous antigens [19–23]. In this situation, what is the origin of MHC-I and which components of the PLC, if any, participate in cross-presentation?

As phagosomes/endosomes mature in DCs after uptake of particulate or soluble antigens, they receive membrane from at least three distinct intracellular organelles that can influence cross-presentation, namely ER [19–26], lysosome related organelles [27] and recycling endosomes [28*,29**, 30]. Input from each of these organelles brings various functional attributes to phagosomes that make them competent for antigen survival and loading.

Lysosome-related organelles deliver the NADPH oxidase complex NOX2 to phagosomes [27]. The NOX2 complex generates free radicals within the phagosomal lumen resulting in an increased pH [31]. Thus the luminal environment of DC phagosomes remains close to neutral and less proteolytic compared to macrophage phagosomes [31,32]. This preserves internalized antigens and allows their delivery to the cytosol for proteasomal processing. It is also likely that the maintenance of neutrality within phagosomes contributes to an appropriate environment for peptide loading of MHC-I, although, to our knowledge, the precise pH range compatible with effective peptide loading has not been investigated.

Multiple studies have shown ER derived membranes along with ER proteins are recruited to the phagosomes and endosomes [19–26]. Perhaps the strongest functional evidence demonstrating ER-phagosome membrane fusion is that an appropriate peptide substrate undergoes N-linked glycosylation in phagosomes [25,33]. ER-derived membranes traffic through the ER-Golgi intermediate compartment (ERGIC), facilitated by an ER-Golgi SNARE Sec22b and possibly plasma membrane SNARE Stx4 [25]. The ER-derived membranes deliver TAP, other PLC components, and potentially MHC-I molecule to the phagosomes [25], creating an ER-phagosome hybrid compartment likely to have the proper environment for MHC-I loading.

A requirement for functional TAP has been identified in many cross-presentation systems. While TAP likely does play a major role in peptide transport during cross-presentation, old and recent data involving TAP deficient cells [14,34] should be interpreted with caution. Because MHC-I assembly is defective in the absence of TAP, post-ER MHC-I is drastically reduced. This means that any system requiring MHC-I from a post-ER source will have a limited pool available for loading. Therefore, decreased cross-presentation seen in TAP deficient cells could be due either to a requirement for TAP-dependent peptides or to a lack of available MHC-I. Furthermore, normal cross-presentation in TAP-deficient cells cannot necessarily be interpreted to mean a lack of antigen access to the cytosol. A proteasome-dependent, but TAP-independent pathway has been described recently, suggesting than cross-presented peptides generated in the cytosol may use an alternate peptide transporter [34]. Consistent with this, it was recently demonstrated that the SIINFEKL peptide, the classical $K^b$-restricted epitope derived from OVA, can be imported into purified phagosomes in an ATP-dependent but TAP-independent manner [35**]. A similar TAP-independent but proteasome-dependent pathway has also been characterized in our laboratory that functions under certain conditions (D. Sengupta, unpublished results).

Sec22b-mediated delivery of ER membranes can potentially deliver MHC-I molecules to phagosomes. However, endosomal recycling compartments regulated by, and containing, the small GTPases Rab11 and Rab22 may be a major source of MHC-I for cross-presentation [28*,29**]. When either of these Rab species is depleted by knockdown, both MHC-I trafficking to phagosomes and cross-presentation are decreased [28*,29**], suggesting that recycling MHC-I may acquire antigenic peptides in phagosomes/endosomes. Determining the source of MHC-I for cross-presentation is important for evaluating the role of accessory components in this form of peptide loading. Apart from the likely requirement for TAP-mediated peptide transport, there is little to no evidence supporting a role for the PLC during cross-presentation. Two PLC components directly interact with MHC-I, tapasin and

calreticulin, the latter of which only interacts with MHC-I via its N-linked glycan, and then only when this is in the monoglucosylated form, characteristic of glycoproteins undergoing folding in the ER. One study demonstrated that most, if not all, of the phagosomal MHC-I pool contains glycans resistant to removal by endoglycosidase H, which therefore cannot be monoglucosylated [19]. Hence, it is unlikely that recycling MHC-I is able to functionally interact with the PLC in phagosomes as it does in the ER, leaving open the possibility that alternate accessory molecules may play a role. The tapasin homologue TAPBPR, which interacts with MHC-I independently of the PLC [36,37] and can mediate peptide exchange [38*,39*], is a strong candidate for an accessory role in cross-presentation.

## How do antigens get into the cytosol?

Two pieces of evidence have been used to argue that a majority of cross-presented antigens need to access the cytosol, namely the requirements for both proteasomal processing and TAP transport (both discussed above). The process of antigen dislocation into the cytosol has been studied extensively. Indeed, many translocation assays have been developed using model proteins (OVA [23,40]), enzymes (cytochrome c [41], β-lactamase [25], luciferase [33,42]), and toxins (gelonin [6], exotoxin A [33], saporin [43]) that demonstrate the cytosolic appearance of exogenously added proteins. However, the proteins and mechanisms involved in cytosolic translocation remain ill defined.

Extensive studies on phagosomes have revealed that a specific set of ER components are recruited to endosomes/phagosomes during cross-presentation mediated by Sec22b and ER-phagosome fusion [19–26,33,44,45]. Importantly, knock down of Sec22b in DCs by shRNA results in less efficient antigen dislocation into the cytosol [25], suggesting that an ER-derived component functions in or regulates antigen translocation. These findings illuminated a important and intriguing question: does the ER-associated degradation (ERAD) machinery, which translocates ER-localized misfolded proteins to the cytosol, also translocate exogenous antigens for cross-presentation? Initial results pointed to yes, as a dominant negative version of the cytosolic ERAD factor p97, which facilitates extraction of misfolded proteins as they enter the cytosol, was shown to inhibit cross-presentation, while addition of recombinant p97 to purified phagosomes enhanced the export of trapped luciferase [33]. While others have reported a role of p97 in cross-presentation [46–48], there does not appear to be a role for additional ERAD factors tested, such as Hrd1, gp78, HERP, and Derlin-1 ([46,49**], reviewed in [50]). As these membrane proteins mediate ERAD, together, separately, or perhaps in cooperation with an as yet identified translocon, it appears unlikely that protein complexes that function in ERAD also mediate antigen translocation from phagosomes.

Secretory proteins are co-translationally translocated into the ER lumen by the Sec61 translocon, which consists of Sec61α, β, and γ, with Sec61α forming the core translocon channel [51]. Sec61 has long been proposed as a potential translocation channel for ERAD and cross-presented antigens. Because of its importance in translation from membrane-associated ribosomes as well as ER import, testing this hypothesis has been a technical nightmare. Recently, it was demonstrated that siRNA-mediated downregulation of Sec61 inhibited cross-presentation at a timepoint when conventional MHC-I and MHC-II

processing was intact [49**]. Moreover, when Sec61 trafficking to endosomes was inhibited using an ER-targeted antibody (intrabody) designed to retain Sec61 in the ER, both cross-presentation and antigen dislocation were inhibited. While it remains possible that both methods of Sec61 inhibition could prevent proper translation or trafficking of a specific component of a translocation channel or a critical regulatory element, these findings are consistent with the hypothesis that Sec61 is the Sec22b-delivered ER protein responsible for antigen translocation to the cytosol.

Rather than a translocon-dependent mechanism for cytosolic access, recent evidence suggests that a different mechanism can release internalized antigens into the cytosol. NOX2-produced ROS can induce endosomal lipid peroxidation, membrane damage, and release of antigen from leaky endosomes into the cytosol of DCs [52**]. Inhibition of NOX2 activity by siRNA knockdown or scavenging free radicals significantly reduced release of endosomal antigen into the cytosol and cross-presentation [52**]. While endosomal leakiness or rupture may account for cytosolic delivery of antigens under certain conditions, there is substantial direct and indirect evidence suggesting that active transport is important in cross-presentation. The requirement of disulfide bond reduction by a lysosomal disulfide isomerase suggests unfolding is necessary for translocation [53], while a requirement for factors such as p97 [33] and Sec61 [49**] fit with a translocon model. Furthermore, antigens derived from bacteria or parasites can access the cytosol even at times when the membrane of the phagosome or parasitophorous vacuole is intact [22,26].

## Regulation of cross-presentation pathways

Mouse bone marrow-derived DCs can cross-present *in vitro* in the absence of any additional stimuli, indicating that the ability can be constitutive. However, different DC subclasses exhibit different molecular requirements. For example, $CD8\alpha^+$ versus inflammatory DCs have different requirements for IRAP and Rab43 [54*,55]. Nevertheless, many studies have shown that, as well as regulating the migratory properties of DC, their phagocytic capacity, and their expression of co-stimulatory molecules, innate immune signaling alters their capacity for cross-presentation. LPS is the primary experimental stimulus used but variation in the quality of the LPS as well as in concentration and timing have left the literature somewhat confused (reviewed in [56,57]). A number of recent studies have provided some clarification, although different authors have attributed alterations in cross-presentation to different primary causes.

Prolonged LPS stimulation of DCs (24hrs) results in increased synthesis of the transcription factor TFEB and its translocation into the nucleus [58**]. TFEB is a component of the lysosomal nutrition sensing mechanism, and its upregulation and subsequent nuclear localization activates transcription of lysosomal proteases and increased lysosomal acidification [59]. Consistent with previous observations that increased lysosomal proteolysis inhibits cross-presentation, prolonged stimulation of bone marrow-derived DCs with LPS induces an increase in lysosomal proteolytic activity, which significantly decreases their capacity for cross-presentation [58**]. shRNA-mediated TFEB knock down reversed this decrease, as well as the concomitant enhancement of MHC-II-restricted antigen presentation observed [58**].

Addition of LPS to DCs concomitant with the addition of a particulate antigen causes endosomal compartments to undergo reorganization. A pool of MHC-I localized to a perinuclear recycling compartment is redistributed to phagosomes and the cross-presentation efficiency of the stimulated DC is enhanced, consistent with a role for recycling MHC-I in cross-presentation [29**]. Short term LPS stimulation also leads to NOX2 activation, lipid peroxidation, and leaky endosomes [52**], leading to increased access of internalized antigens to the cytosol and enhanced cross-presentation, as described above. DCs lacking TRIF signaling fail to recruit sec61 to endosomes and show reduced cytosolic translocation and cross-presentation [49**]. Others have found that cross-presentation by DCs is increased with longer (16hr) LPS treatment, but this was attributed to reduced phago-lysosomal fusion and antigen preservation in phagosomes with no effect on antigen translocation to the cytosol [60**]. Overall, while it is clear that innate signaling dramatically alters intracellular trafficking and the overall phagosomal/endosomal proteome, exactly how these processes coordinate to regulate cross-presentation *in vivo* remains obscure. Perhaps the variety of mechanisms proposed genuinely reflects a complex reality.

## Conclusions

As discussed throughout this manuscript and illustrated in Figure 1, cross-presentation requires an elaborate series of steps that in the end result in MHC-I at the cell surface loaded with relevant peptides for stimulation of $CD8^+$ T cells. Many different pathways have been identified to explain requisite cross-presentation events, such as reduced vacuolar proteolysis, cytosolic antigen access, peptide generation and transport, MHC-I trafficking/loading, and LPS-induced alterations. It may be that multiple pathways working concurrently give rise to the same or similar final result, and the relative importance of the different pathways varies with the antigen or cell type. Determining the relative importance of these pathways for cross-priming *in vivo* is an important next step.

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## Highlights

- Requirements for epitope matching in conventional MHC-I presentation and cross-presentation;
- Vacuolar and cytosolic pathways;
- Proteasomal versus lysosomal proteolysis;
- Regulation of trafficking;
- Source of the relevant MHC-I molecules

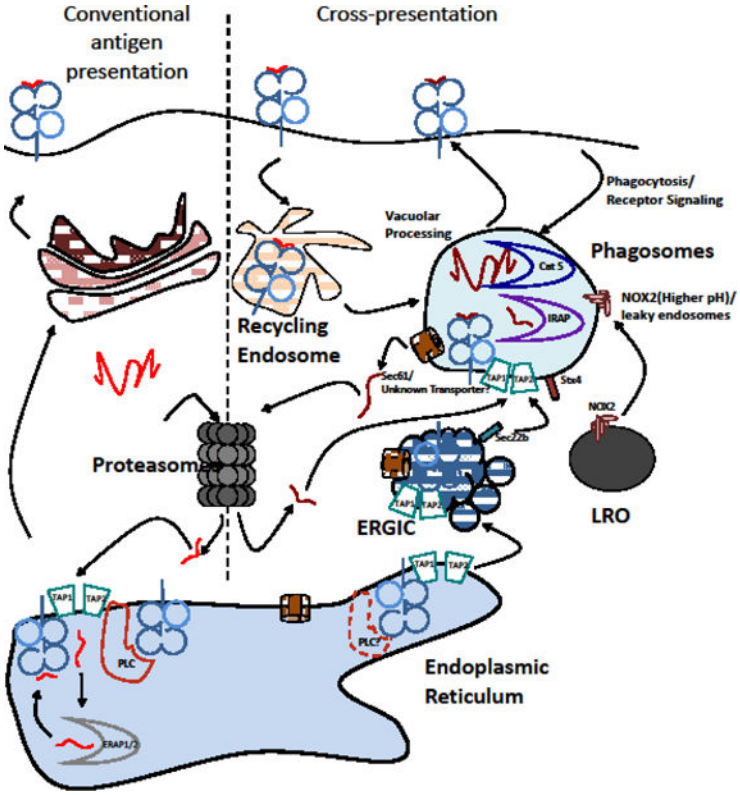


**Figure 1. Cross-presentation of antigens depend on well-orchestrated delivery of factors to phagosomes**

Conventional as well as cross-presentation of most antigens depend on proteasomal activity. In the model shown, phagocytosed antigens may be translocated into the cytosol via a transporter (possibly involving Sec61 and/or other processes), potentially delivered to the phagosome via the ER-Golgi Intermediate Compartment (ERGIC), and degraded by cytosolic proteasomes. Processed peptides are transported back into the phagosomes for loading via TAP or an unidentified transporter, also likely acquired from the ERGIC. Antigenic peptides may be further processed by IRAP and loaded onto MHC-I, acquired from recycling endosomes. Maintenance of a near neutral pH and reduced proteolysis is achieved by delivery of NOX2 machinery from Lysosome-Related Organelles (LRO), which may also facilitate antigen entry into the cytosol.

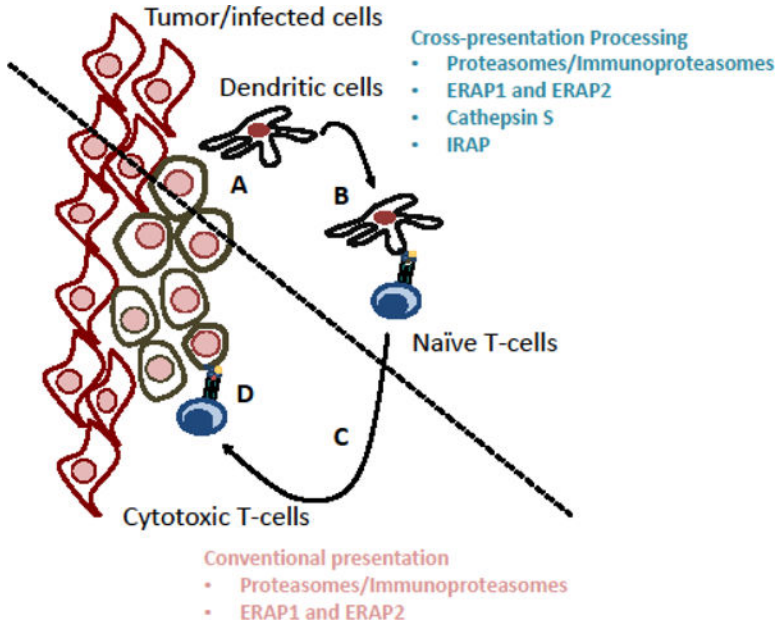


**Figure 2. To mount an effective immune response, peptide-MHC-I complexes generated by cross-presentation need to match those generated by the conventional MHC-I presentation pathway**

**(A)** Dendritic cells phagocytose/endocytose antigens from infected tissue. **(B)** These dendritic cells process and present antigenic peptides, generated by cross-presentation and loaded onto MHC-I, to naïve CD8-positive T-cells, priming them. **(C)** The primed T-cells migrate to the infected tissue and, **(D)**, recognize peptide-MHC-I presented by infected cells and eliminate them. An effective response requires presentation of the same peptides by DCs (involving cross-presentation) and infected/tumor cells (involving conventional MHC-I antigen presentation). Processing of antigens by the conventional pathway depends on proteasomes and in some cases ER aminopeptidases. Along with proteasomes and ERAPs, cross-presentation can also depend on IRAP and cathepsin S.