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Into the intracellular logistics of cross-presentation

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The induction of cytotoxic $CD8^+$ T cell responses requires the presentation of antigenic peptides by MHC class I molecules (MHC I). MHC I usually present peptides derived from endogenous proteins. However, some subtypes of dendritic cells have developed the ability to efficiently present peptides derived from exogenous antigens on MHC I via a process called cross-presentation. Cross-presentation is intimately linked to the induction of anti-viral, -bacterial, and -tumor cytotoxic T cell (CTL) responses, as well as a wide variety of CTL-mediated diseases and transplant rejections. The molecular and cellular mechanisms underlying cross-presentation have been studied intensively since its original description, yet understanding of this process is incomplete and on the forefront of immunological research. Numerous pathways and models, some of them conflicting, have been described so far. Here, we review the various pathways reported as involved in cross-presentation, highlighting the complexity of this process. We also discuss in detail the different intracellular steps required, from antigen capture and routing, to processing, and finally peptide loading, emphasizing the need for a better understanding of the cell biology of this phenomenon.

Keywords: antigen, cross-presentation, dendritic cell, MHC class I, phagosome, vaccination, cross-priming, gap junctions

INTRODUCTION

The mammalian adaptive immune response is crucial in the clearance of many infections. Classically, immune cells present exogenous antigens on MHC Class II (MHC II) to "helper" $CD4^+$ T cells, and endogenous antigens via MHC Class I (MHC I) to "cytotoxic" $CD8^+$ T cells. In this review, we will focus on the $CD8^+$ T cells that are key in the elimination of infected or cancer cells. $CD8^+$ T cells are first activated when their unique T cell receptor (TCR) is triggered by fragments of microbial or tumor antigens in association with MHC I. All nucleated cells express MHC I, however dendritic cells (DCs) also express a range of co-stimulatory molecules, uptake receptors, and other key immune molecules to specifically initiate a cytotoxic $CD8^+$ T cells response.

In cases where DCs are not infected directly, but a cytotoxic T cell (CTL) response is required, DCs have the capacity to sample antigens from their environment by a process called cross-presentation, as it differs from the normal pathway of MHC I antigen presentation where the antigens are merely cytosolic or nuclear. For cross-presentation, exogenous antigens (e.g., from an infected cell) are taken up by DCs and rerouted to the MHC I pathway for presentation to and activation of $CD8^+$ T cells.

The cell biology of cross-presentation is clearly different from the classical MHC I antigen presentation. A myriad of studies have interrogated various cell biological pathways including the antigen uptake pathway, antigen translocation from endosome to cytosol, ER–phagosome fusion, the proteasome, the endosomal pH, the TAP transporter, and gap junctions (reviewed in Neefjes et al., 2011; Segura and Villadangos, 2011). Based on these findings, various pathways and mechanisms have been proposed that may all be correct or mutually exclusive. Here, we present a critical evaluation of the various models and observations reported on cross-presentation.

WHAT IS "TRUE" IN CROSS-PRESENTATION?

The classical pathway of MHC I antigen presentation is nowadays understood in detail (Neefjes et al., 2011). Cytosolic and nuclear antigens are degraded into peptide fragments by the proteasome, further trimmed by peptidases, and transported from the cytosol into the ER lumen by the peptide transporter TAP. Peptides are then loaded on newly synthesized MHC I and these complexes are released from the ER and transported to the cell surface via the Golgi.

As expected, the components of the classical MHC I antigen presentation have been tested for their involvement in the process of cross-presentation. In several studies, the proteasome has been implicated in cross-presentation in experiments where cells were treated with proteasome inhibitors for long periods of time (Brossart and Bevan, 1997; Rodriguez et al., 1999). Here an involvement of the proteasome in cross-presentation was implied due to its role in generation of peptides. However, proteasome inhibitors are known to alter the ubiquitin profile in cells by accumulating polyubiquitinated proteins and inducing alterations in transcription due to histone deubiquitination (Dantuma et al., 2006). Therefore, the inhibition of cross-presentation by proteasome inhibitors might be the direct consequence of a defect in peptide generation, or a subsequent event to transcriptome alterations or to any other process involving ubiquitin such as endosome formation.

Whether cross-presentation requires the peptide transporter TAP was also tested. If involved, this would imply that antigenic peptides originate from the cytosol, most likely after trimming by

the proteasome. DCs isolated from TAP-deficient mice were found unable to cross-present (Brossart and Bevan, 1997). However, these observations have been contradicted by others (Dantuma et al., 2006). Moreover, a recent model proposed by Merzougui et al. implies a dissociation of TAP and proteasome dependence. In particular, a role for TAP has been implicated in the recycling of MHC I necessary for cross-presentation of particulate antigens (Merzougui et al., 2011).

Finally, the involvement of newly synthesized MHC I in cross-presentation was addressed using the chemical inhibitor Brefeldin A which blocks ER–golgi transport (Brossart and Bevan, 1997). Moreover, as it also affects the entire endosomal pathway, it is conceivable that the inhibition of cross-presentation does not reflect that peptide loading occurs on newly synthesized MHC I in the ER.

To conclude, some well accepted concepts concerning cross-presentation can be contested, and it is important to reconsider and take into account the different options.

CROSS-PRESENTATION, WHO IS ALLOCATED TO DO IT?

Cross-presentation is considered an exclusive feature to DCs. Amongst the many subsets of DCs, the CD8α$^+$ DCs in mouse and their human equivalent, the BDCA3$^+$ DCs, the monocytes-derived DCs (Mo-DCs), and the migratory CD103$^+$ DCs are presumed to be unique in their capacity to cross-present the antigen (*in vitro* and *in vivo*; den Haan et al., 2000; del Rio et al., 2007; Hildner et al., 2008; Bedoui et al., 2009; Bachem et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). As our main focus in this review is the cell biological aspect of cross-presentation, and as there has been none performed with the CD103$^+$ DCs, we will focus mainly on the CD8α$^+$ DCs and the Mo-DCs.

The obvious question is what distinguishes the cross-presenting DC subsets from the others. Although the DC types have been profiled by a series of techniques including microarray and mass spectrometry, a clear "cross-presentation signature" has not been defined (Robbins et al., 2008; Luber et al., 2010; Segura et al., 2010). One option is that all cells (including non-immune cells) are able to cross-present but the cross-presenting DCs are just remarkable because they display the perfect arsenal of the required characteristics for this process, from the surface expression of specific uptake receptors, a low endosomal protease activity, an extremely high expression of MHC I and finally a series of co-stimulatory molecules to allow cross-priming. These DCs may therefore be better equipped, without being unique. This would explain why cross-presentation has also been observed, but to a lesser extent, in other immune cells like CD8α$^-$ DCs (den Haan and Bevan, 2002), Langerhans cells (Oh et al., 2011), B cells (de Wit et al., 2010), macrophages (Asano et al., 2011), as well as in non-immune cells (Gromme et al., 1999; Neijssen et al., 2005; Pang et al., 2009). Alternatively, the cross-presenting DCs may have a cell biological secret that is yet to be revealed.

RECEPTOR-MEDIATED ANTIGEN UPTAKE AND ROUTING TO THE MHC I PATHWAY

One issue that is not contested is that exogenous antigens have to be captured by DCs for cross-presentation (**Figure 1**). Antigens can be taken up by fluid phase or by receptor-mediated endocytosis. A number of endocytosis/phagocytosis receptors have long been thought to be critical for antigen uptake as they concentrate antigens in the endocytic pathway. However, many of them have a more important function in rerouting and targeting the antigen to defined endosomal compartments: efficient uptake in combination with cargo delivery to the appropriate compartments is a decisive event for antigen cross-presentation (Burgdorf et al., 2007; Caminschi et al., 2008; Sancho et al., 2009; Tacken et al., 2011).

A series of receptors have been implicated including Fcγ receptors (den Haan and Bevan, 2002; Schuurhuis et al., 2002) and lectin receptors such as the mannose receptor that mediates the uptake of soluble but not cell-associated antigens (Burgdorf et al., 2006), Dectin-1 that is involved in uptake and cross-presentation of cellular antigen (Weck et al., 2008), Clec9A that mediates the capture and cross-presentation of antigens derived from necrotic cells (Caminschi et al., 2008; Sancho et al., 2009), and others.

Collectively, these studies illustrate that different antigens can use multiple uptake mechanisms and pathways leading to cross-presentation of antigenic peptides on MHC I.

ARE ALL ENDOSOMES EQUAL FOR CROSS-PRESENTATION?

As written in classical textbooks, captured antigens enter the endocytic pathway. Antigens first enter early endosomes, then late endosomes, and finally lysosomes. In the case of cell-associated antigens or bacteria, the endosomes are enlarged and called phagosomes. Each of these compartments has specific markers and pH. Early endosomes are positive for Rab5 and are mildly acidic without many proteases while late endosomes and lysosomes are positive for Rab7 and more acidic (around pH 5.0) with a substantial proteolytic activity. However, this concept of a simple endosomal pathway, where material moves from early to late endosomes then to lysosomes as their inescapable fate, has been challenged for many years especially through the description of lysosome-related organelles such as cytotoxic granules, melanosomes, and MHC II loading compartments (MIIC). The endosomal pathway can therefore undergo specialization, and this may be applied in antigen cross-presentation (Lakadamyali et al., 2006; Burgdorf et al., 2007; Saveanu et al., 2009; Tacken et al., 2011).

In this regard, Lakadamyali et al. showed that internalized proteins can be sorted into two different categories of early endosomes, "dynamic" or "static." The dynamic population matured rapidly toward late endosomes and subsequently fused with lysosomes yielding material for MHC II antigen presentation. The more "static" population displayed a slower maturation rate (Lakadamyali et al., 2006), that would favor cross-presentation, as it displayed a low proteolytic activity, which is believed to protect antigens from excessive destruction (Savina et al., 2006; Jancic et al., 2007). Interestingly, the mannose receptor (Burgdorf et al., 2007) and DC-SIGN (Tacken et al., 2011) are surface receptors that target antigens to these low maturating compartments and that mediate cross-presentation.

But what specifies these endosomes involved in cross-presentation? They are marked by the GTPase Rab14, and contain MHC I and the trimming peptidase IRAP (Saveanu et al., 2009). Of note, *Salmonella*-containing phagosomes are also marked by Rab14, a GTPase that controls phagosomal fusion with lysosomes (Kuijl et al., 2007).

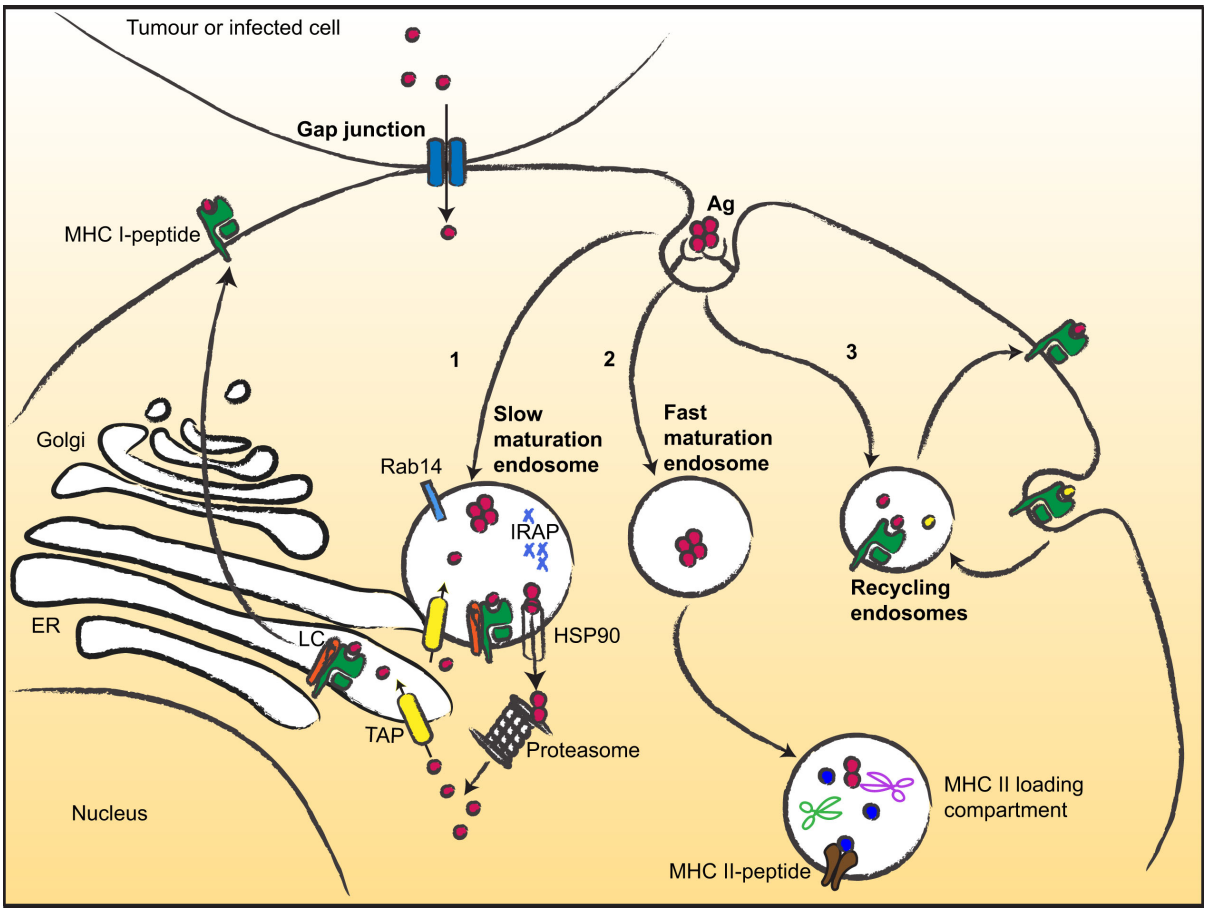

FIGURE 1 | Multiple ways for MHC I cross-presentation. After uptake, antigens can follow different routes and be targeted to (1) slow maturating endosomes (or phagosome) displaying low proteolytic activity. Then, antigens can escape to the cytosol (via HSP90) and are degraded by the proteasome into peptides that are translocated to the ER or back to the endocytic compartment for loading; (2) Fast maturating endosomes, addressing the antigens to low pH- and high proteolytic- compartments suited for MHC II antigen presentation; (3) Recycling endosomes, where loading occurs on recycling MHC I molecules. Gap junctions mediate transfer of small peptides between neighboring cells. LC, loading complex.

Some studies have provided functional evidence for the presence of endosomal compartments in DCs dedicated for long-term storage of antigen, therefore providing material for cross-presentation by MHC I over long periods of time after uptake (Faure et al., 2009; van Montfoort et al., 2009). Whether the "static Rab14 endosome" corresponds to the long-term antigen storage organelle is, however, unclear.

ANTIGEN PROCESSING FOR CROSS-PRESENTATION

As MHC I classically presents fragments of antigens in the form of 8–10 amino acid long peptides, antigens have to be processed for cross-presentation (Neefjes et al., 2011). A low proteolytic endosomal activity, characteristic of the $CD8^+$ DCs, is believed to be decisive to avoid excessive antigen destruction and allow its processing for cross-presentation (Delamarre et al., 2005; Savina et al., 2006; Jancic et al., 2007). Endocytosed antigens can be processed for cross-presentation by different pathways most likely displaying distinct proteolytic specificities: (1) by endosomal proteases such as cathepsin S and D (Fonteneau et al., 2003; Shen et al., 2004) and (2) by the cytosolic proteasomal machinery used in the classical MHC I antigen presentation pathway (Fonteneau et al., 2003; Shen et al., 2004; Neefjes et al., 2011) which implies that exogenous antigens find a way to enter the cytosol for degradation by the proteasome. Various reports present different solutions to this topological problem.

Antigen translocation from the endosome to the cytosol has been proposed to be specific to internalized antigen and dependent on the size (Rodriguez et al., 1999). As most proteins do not spontaneously pass lipid bilayers, this would require a dedicated – unknown – transporter. Most models imply the ER retrotranslocation machinery (ERAD) involved in the translocation of misfolded ER proteins into the cytosol for degradation by the proteasome (Haug et al., 2003; Taylor et al., 2010; Imai et al., 2011; Oura et al., 2011). Two recent studies demonstrate a role for the chaperone HSP90 in this process (Imai et al., 2011; Oura et al., 2011). An independent study of the group of Cresswell showed that some antigens might be transported from endosomes back to the ER and delivered to the cytosol by the ERAD machinery followed by degradation by the proteasome (Ackerman et al., 2005). This mechanism is used by some toxins to enter the cytosol, but

is poorly – if at all – used by most proteins (Neefjes et al., 1988). It is therefore unlikely to be the major route. A role for the mannose receptor in antigen translocation to the cytosol has also been suggested (Burgdorf et al., 2008; Zehner et al., 2011). The underlying mechanisms remain unclear but it is unlikely that the receptor itself performs the translocation step.

An alternative model implicating a fusion of the ER with phagosomes has been suggested by several groups (Gagnon et al., 2002; Guermonprez et al., 2003; Houde et al., 2003). This process may involve a Sec22b-dependent fusion of ER–golgi intermediate compartments with the phagosome (Cebrian et al., 2011), an event that would allow a recruitment of the ERAD machinery to the phagosome, and therefore antigen translocation to the cytosol where proteasomal degradation finally occurs. Peptides can then either be transported by TAP into the newly formed ER–phagosome hybrid organelle or to the ER for loading (Gagnon et al., 2002; Guermonprez et al., 2003; Houde et al., 2003). These models are fairly unique in cell biology and make assumptions that (1) the energy for ERAD is available in the ER–phagosome hybrid organelle, and (2) the chaperones involved in directing antigens to ERAD recognize and consider the antigens sufficiently misfolded for degradation. These considerations complicate the involvement of the ERAD system in cross-presentation and reinforces the fact that the antigen export machinery and especially the actual retrotranslocon pore remains to be defined.

PEPTIDE LOADING

Although there may be various pathways for cross-presentation and different locations for antigen processing, peptides and MHC I finally have to meet in the same compartment or organelle. These meeting point options suggested so far are:

- In the ER. This pathway is the most evident from the MHC I point of view as it represents the classical pathway for efficient peptide loading in the presence of the complete MHC I machinery. This pathway has been extensively studied and is nowadays understood in detail (Neefjes et al., 2011). The major issue is how exogenous antigens enter this pathway in an efficient manner to compete for loading with the myriad of peptides that are in the ER.
- In endosomes. This pathway would be simpler as it does not require (unknown) mechanisms for delivering antigens to the cytosol. Antigens would simply be degraded by endosomal proteases and peptides loaded on recycling MHC I. Peptide release and exchange can occur efficiently under mild acidic conditions, without the support of chaperones (Gromme et al., 1999; Burgdorf et al., 2006; Di Pucchio et al., 2008; Zou et al., 2009; Win et al., 2011).

ALTERNATIVE MECHANISMS FOR CROSS-PRESENTATION

Cross-presentation may also result from the transfer of MHC I-peptide complexes from infected cells or tumor to DCs via exosomes or trogocytosis (Wolfers et al., 2001; Herrera et al., 2004; Wakim and Bevan, 2011). Whether these processes are relevant *in vivo* is unclear.

An alternative that would solve the topological problem of exogenous antigen delivery to the cytosol of DCs is peptide transfer via gap junctions (Neijssen et al., 2005). Gap junctions are small channels directly connecting the cytosol of two adjacent cells (Neijssen et al., 2007). The proteins constituting the gap junctions are called connexins and these are upregulated on monocytes and DCs upon exposure to danger signals (Pang and Neefjes, 2010; Saccheri et al., 2010). Gap junctions are able to mediate the transfer of small peptides from apoptotic and tumor cells to DCs (Pang et al., 2009; Saccheri et al., 2010). The transferred peptides have been shown to be efficiently presented by MHC I at the cell surface to trigger activation of specific $CD8^+$ T cells (Neijssen et al., 2005; Pang and Neefjes, 2010; Saccheri et al., 2010).

THE FINAL STEP: CELL SURFACE DELIVERY FOR CROSS-PRESENTATION

Ultimately, MHC I molecules have to present antigenic information at the cell surface. Depending on the site of peptide loading, the route of the MHC I-peptides complexes to the cell surface differs. If peptide loading occurs in the ER, complexes simply follow the standard secretory pathway (Neefjes et al., 2011). If MHC I molecules acquire antigenic peptides in endosomal compartments, they may use the pathway also used by MHC II molecules after peptide loading in the MIIC (Gromme et al., 1999).

CONCLUDING REMARKS AND BEYOND

The last few decades have uncovered profound insights into the biology of antigen presentation, but many components still have to be defined to unequivocally understand the complex cross-presentation logistics from antigen uptake and routing, processing, and peptide loading. The many conflicting opinions in the field might be partially explained by the fact that the process has different requirements depending on the cell type (e.g., $CD8\alpha^+$ vs $CD8\alpha^-$ DC or Mo-DCs), on the antigen form (soluble, antibody-, or cell- associated), and source (necrotic, apoptotic, infected, or tumor cell), and on the uptake route (gap junctions, receptor-mediated endocytosis). Also, we cannot exclude the option that multiple pathways may be active in the cross-presentation of a specific antigen. Moreover, a study on the cell biology of cross-presentation usually focuses on one step of the process, without relating it to the upstream and downstream events. For the above reasons, drawing a general consensual scheme of cross-presentation based on independent studies is akin to putting together a picture puzzle using a collection of pieces originating from different sources.

Another consideration is the difference in kinetics between the different mechanisms. The endosomal cross-presentation pathway may be faster that the cytosolic one, allowing rapid expression of significant levels of MHC I with cross-presented peptides at the cell surface (Burgdorf et al., 2008; Di Pucchio et al., 2008). Moreover, the pathways engaged for cross-presentation appear to be cell type dependent (Segura et al., 2009). While inflammatory Mo-DCs may be relying mainly on the fast endosomal pathway, the ability of "steady state" $CD8\alpha^+$ DCs to cross-present seems to depend more on the cytosolic pathway. All these variations should have major implications when the cell biology of cross-presentation is translated into the design of new therapies aiming to target cross-presentation by DCs to stimulate specific CTL to control infections and cancer.

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